Cytokine-like 1 (CYTL1) Regulates the Chondrogenesis of Mesenchymal Cells

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To identify novel molecules regulating chondrogenesis and cartilage development, we screened a cartilage-specific expressed sequence tag data base. Cytokine-like 1 (Cyt1), a possible cytokine candidate with unknown function that was originally identified in bone marrow-derived CD34-positive cells, was selected for functional characterization. In view of the initial observation that Cyt1 is predominantly expressed in chondrocytes and cartilage, we investigated its possible role in chondrogenesis and hypertrophic maturation of chondrocytes. Cyt1 expression was very low in mesenchymal cells, dramatically increased during chondrogenesis, and decreased during hypertrophic maturation, both in vivo and in vitro. The role of Cyt1 in chondrogenesis and hypertrophic maturation was examined by treating chondrifying mesenchymal cells with exogenous Cyt1 or ectopic expression of Cyt1. Notably, exogenous Cyt1 caused chondrogenic differentiation of mouse limb bud mesenchymal cells during micromass culture. Lentivirus-mediated overexpression of Cyt1 additionally induced chondrogenic differentiation of mesenchymal cells. However, Cyt1 did not affect the hypertrophic maturation of chondrocytes. Cyt1 exerted its chondrogenic effect via stimulation of Sox9 transcriptional activity. In addition, Cyt1 caused expression of insulin-like growth factor 1, which has a capacity to induce chondrogenesis. Thus, our results collectively suggest that chondrocyte-specific Cyt1 regulates chondrogenesis as a novel autocrine factor, but not hypertrophic maturation of chondrocytes during cartilage development.

Cartilage formation during embryonic development begins with the aggregation of mesenchymal cells, which ultimately differentiate into chondrocytes. Differentiated chondrocytes proliferate rapidly and secrete a cartilage-specific extracellular matrix such as type II collagen and sulfated proteoglycan to form cartilage. The cartilage serves as a template for endochondral ossification, which requires the maturation of hypertrophic chondrocytes (1–4). These sequential events during chondrogenesis and cartilage formation are precisely regulated by various growth factors released from cartilage elements and perichondrium. Secreted growth factors exert their effects by modulating intracellular signaling (1, 2). Although several regulatory growth factors have been identified, including bone morphogenetic proteins, fibroblast growth factors, insulin-like growth factor-1 (IGF-1),2 transforming growth factor-β, and parathyroid hormone-related peptide, the precise mechanisms of regulation of chondrogenesis and cartilage development remain to be elucidated. In this study, we analyze a cartilage-specific expressed sequence tag (EST) data base in an attempt to identify novel molecules that modulate chondrogenesis and cartilage development.

The EST data base provides important information on novel genes displaying tissue-specific expression profiles (5–7). We analyzed the human cartilage UniGene library (8), and selected Cyt1 as a possible candidate regulator of chondrogenesis. Cyt1 is a functionally unknown cytokine candidate originally cloned from bone marrow and cord blood mononuclear cells that bear the CD34 surface marker (9). Previous studies report Cyt1 mRNA and protein expression in cartilaginous tissues, including mouse inner ear and human articular cartilage, respectively (10, 11). Alignment of the predicted primary amino acid sequences of Cyt1 proteins from multiple species (human, mouse, chicken, and puffer) reveals an N-terminal secretory signal peptide and four α-helices, a common characteristic of cytokines. Accordingly, Cyt1 is categorized as a novel cytokine, despite no functional evidence (9–11). In this study, we investigated the function of Cyt1 in chondrogenesis and cartilage development. Our findings clearly indicate that Cyt1 regulates chondrogenesis, but not hypertrophic maturation of chondrocytes during cartilage development.

EXPERIMENTAL PROCEDURES

Tissue Preparation and Cell Culture—Mouse tissues and rib cartilage were obtained from 4-week-old male ICR mice and

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.
3 The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) EF108311.
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2 The abbreviations used are: IGF-1, insulin-like growth factor 1; DMEM, Dulbecco’s modified Eagle’s medium; dpc, days post-conception; ERK, extracellular signal-regulated protein kinase; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP-13, matrix metalloproteinase-13; p38 MAP kinase, p38 mitogen-activated protein kinase; PKC, Protein kinase C; qRT, quantitative real time; SAGE, serial analysis of gene expression; RT, reverse transcription; shRNA, short hairpin RNA; siRNA, short interfering RNA.
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TABLE 1
PCR primers and conditions

| Genes            | Primer sequence* | Expected size | $A_g$ °C |
|------------------|------------------|---------------|----------|
| Cyt1 (cDNA)      | Sense 5'–atg tca cca aag aca cta cct–3' | 417          |         |
|                  | Antisense 5'–gcc tgt cca gtc tgg agg–3' | 309          | 50       |
| Cyt1 (RT-PCR)    | Sense 5'–ccg cct gct act ctc gca tgt–3' | 484          | 50       |
|                  | Antisense 5'–cct cgg gaa tta gtt ctt c–3' |             |          |
| Cyt1 (lentivirus) | Sense 5'–gcc tgt att ttc aga ttc tcc tcc–3' | 584 (Coll-IIA) | 55       |
|                  | Antisense 5'–ggg tct cct gtc tcc tcc tgg gc–3' | 380 (Coll-IIB) | 55       |
| Collagen-IIA/B (RT-PCR) | Sense 5'–cct ctt ctc ggg ttc ctt cgg ccc taa ttt ctc ggg–3' | 204          | 62       |
|                  | Antisense 5'–cgg aat ccc ggc tct cga cca cgg ggt–3' | 402          | 55       |
| Collagen-IIB (in situ probe) | Sense 5'–gcc aat ccc ggc tct cga cca cgg ggt–3' |             |          |
|                   | Antisense 5'–gaa gcc gac gac atc acc atc cag–3' | 581          | 56       |
| Aggrecan         | Antisense 5'–ggg aag cct cca ggc aga c–3' | 325          | 50       |
| Collagen-X (RT-PCR) | Sense 5'–aag ccc ggg gct ccc gca gta gaga–3' | 347          | 50       |
|                  | Antisense 5'–ccc cag ctt ggg aac gcc gta ccc ggt gca–3' | 473          | 50       |
| MMP-13           | Antisense 5'–cat cca cat gtt tgg gaa ggt ctt–3' | 473          | 50       |
| Sox9             | Sense 5'–aaa gct ttc cca cca gca gta g–3' | 392          | 50       |
|                  | Antisense 5'–cag tact caa gac gac tag gac–3' | 385          | 50       |
| GAPDH            | Sense 5'–tca ctc cca ccc cca cag ata–3' | 476          | 50       |
|                  | Antisense 5'–ttg agg ccc tga gtt cca ccc–3' |             |          |

*Primers are designed from known mouse sequences.

$A_g$ means annealing temperature.

3-day-old newborn mice, respectively. Mouse limb buds were prepared from forelimb buds of mouse embryos at the indicated embryonic day. Mouse rib chondrocytes were prepared from 3-day-old newborn ICR mice, as described previously (12). Briefly, cartilaginous rib cages were preincubated for 45 min in 0.2% type II collagenase (381 units/mg solid; Sigma) and rinsed with PBS. Tissues were further dissociated enzymatically for 4 h in 0.2% type II collagenase. Isolated chondrocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin. Mesenchymal cells were isolated from the limb buds of 11.5 dpc ICR mouse embryos and maintained as micromass culture to induce chondrogenesis and hypertrophic maturation (13, 14). Briefly, 4.0 × 10⁷ cells/ml in Dulbecco’s modified Eagle’s medium/F-12 medium (2:3) containing 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin were spotted as 15-μl drops on culture dishes to induce chondrogenesis for 6 days. The culture medium was altered to DMEM/F-12 (2:3) supplemented with 50 μg/ml ascorbic acid, 5 mM β-glycerol phosphate, to induce hypertrophic maturation up to 15 days. Chondrogenesis was determined by examining the expression of collagen IIB and aggrecan by RT-PCR, as described below, or Alcian blue staining to detect accumulation of sulfated proteoglycans (15). Hypertrophic maturation of chondrocytes was determined by examining the expression of markers, such as collagen X and matrix metalloproteinase (MMP)-13, by reverse transcription-PCR (RT-PCR) or Alizarin Red staining to detect mineralization (13, 14).

Expression Vectors and Reporter Gene Assay—Cyt1 cDNA (GenBank™ accession number EF108311) was subjected to RT-PCR amplification from mouse rib chondrocytes using specific primers (Table 1) designed to introduce EcoRI and XhoI restriction sequences at the 5’ and 3’ ends of the amplified fragment, respectively. The resulting cDNA was cloned into the corresponding restriction sites of pcDNA3.1(+) Myc/His vector (Invitrogen). A reporter gene containing the 48-bp Sox9-binding site in the first intron of human COL2A1 was constructed by inserting the chemically synthesized sequence in the pGL3-promoter luciferase reporter vector (Promega, Madison, WI), as described previously (16). The Sox9 reporter gene was transfected into a suspension of isolated mesenchymal cells using the Lipofectamine 2000 reagent (Invitrogen). After transfection with pGL3-control or pGL3-Sox9 reporter gene for 2 h, mesenchymal cells were spotted and maintained as micromass culture in complete medium for 12 h, and treated with IGF-1 or Cyt1-conditioned medium in serum-free conditions, as described for each experiment. For the reporter gene assay, the Sox-9 reporter gene (1 μg) was co-transfected with pCMV-β-galactosidase expression vector (0.3 μg) as an internal control to ensure transfection efficiency. Luciferase activity was normalized against β-galactosidase activity, as described previously (16).

Preparation of Conditioned Medium for Cyt1—Stable control and mouse fibroblast L929 cell lines expressing mouse Cyt1 were generated by transfection with empty vector and Cyt1 cDNA, respectively. One day after transfection, cells were subcultured and selected by adding 0.8 mg/ml G418. Cyt1 expression was confirmed by RT-PCR and Western blotting. Following growth to 90% confluency, control and Cyt1-expressing L929 cells were washed and maintained in serum-free DMEM for 48 h. Conditioned media were clarified by centrifugation at 10,000 × g for 5 min, followed by filtration (0.2 μm pore size), and concentrated 30 times by ultrafiltration in Amicon stirred cells (Millipore) using a YM membrane with a 10-kDa molecular mass cutoff.

Construction and Infection of Lentivirus—Lentivirus bearing mouse Cyt1 was constructed by the Macrogen LentiVector
Institute (Seoul, Korea). Briefly, the Cytl1 gene fragment was amplified from pcDNA3.1-Cyt1-myc/his vector by PCR. A Cyt1-specific primer set (Table 1) was designed to tag Myc sequences at the C terminus of the gene. The amplified product was digested with EcoRI-ClaI and inserted into a lentiviral vector (Lenti-mCMV-IRES-puro) containing the mouse cytomegalovirus promoter and a puromycin-resistant gene, using the internal ribosome entry site system. The constructed transfer vector, a vesicular stomatitis virus-G expression vector, and a gag-pol expression vector were co-transfected into 293T cells at a 1:1:1 molar ratio using Lipofectamine Plus (Invitrogen). The culture supernatant containing viral particles was harvested at 48 h after transfection, clarified with a 0.45-μm membrane filter (Nalgene, New York, NY), and stored at −70 °C. The titer obtained (~1–5 × 10^7 transduction units) was used without further concentration. Titer were determined using human immunodeficiency virus, type 1, p24 enzyme-linked immunosorbent assay. Mesenchymal cells were maintained as micromass culture for 24 h and infected with mock lentivirus or Cyt1 lentivirus for 18 h. Infected cells were cultured for up to 5 days in serum-free DMEM/F-12 medium.

siRNA and shRNA—Five siRNA and two shRNA were used to knock down Cyt1 expression in condensing mesenchymal cells during micromass culture. Briefly, siRNAs were designed based on the coding sequence of mouse Cyt1 (supplemental Fig. 3), and siRNA oligonucleotides were synthesized by Samchully Pharm (Daejeon, Korea) as follows: siRNA1, 5′-aua cca uga acu ccu utt-3′ (sense) and 5′-aag gag uuc aug uga utt-3′ (antisense); siRNA2, 5′-ggc uuu acc ugg aca ucc att-3′ (sense) and 5′-ugg aug ucc ugg uaa aga ctt-3′ (antisense); siRNA3, 5′-uga cug cag ucu aga att-3′ (sense) and 5′-uuc ugg gcu agc gca gtt-3′ (antisense); siRNA4, 5′-gaa cgu aau ccc ucu ccc att-3′ (sense) and 5′-uga gaa gaa uca gga cct-3′ (antisense); and siRNA5, 5′-gac gug aag uca ugg cag att-3′ (sense) and 5′-ucu ggc ugc aug uca cgg ctt-3′ (antisense). After heating the siRNA oligonucleotides for 2 min at 90 °C to denature secondary structures within single strands, the sense and antisense digoxigenin-labeled riboprobes. Next, sections were hybridized with digoxigenin RNA labeling mix (Roche Diagnostics). Briefly, the cDNA fragments of mouse collagen IIB and collagen X were amplified by PCR using specific primers designed to introduce HindIII (sense) and EcoRI (antisense) restriction sequences at the 5′ end (Table 1). The cDNA sequences were digested with HindIII and EcoRI. The cDNA sequences of mouse Cyt1 inserted in the pcDNA3.0 vector (Invitrogen) were digested with PstI and SalI. Digested collagen IIB and collagen X and Cyt1 cDNA sequences were cloned into the pSPT-18 vector (Roche Diagnostics), linearized with EcoRI or HindIII digestion, and transcribed with SP6 and T7 RNA polymerase to generate sense and antisense probes, respectively.

RT-PCR Analysis and Quantitative Real Time PCR (qRT-PCR)—Total RNA was isolated using RNA STAT-60 (Tel-Test, Woodlands, TX) and reverse-transcribed with ImProm-IITM reverse transcriptase (Promega). The cDNA generated was amplified by PCR with Taq polymerase (Takara Bio, Shiga, Japan). Primers and conditions for amplification are summarized in Table 1, and qRT-PCR was performed using a chromo 4 cycler (Bio-Rad) and SYBR Premix Ex TaqTM (Takara Bio). qRT-PCRs were performed in duplicate, and the amplification signal from the target gene was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same reaction.

Northern and Western Blot Analyses—Total RNA was isolated with a single-step guanidine thiocyanate-phenol-chloroform method using RNA STAT-60 (Tel-Test), according to the manufacturer’s protocol. Next, total RNA (3 μg) was fractionated on formaldehyde/agarose gels. The probe (417 bp) for Cyt1 transcripts was generated by RT-PCR using a sense primer corresponding to nucleotides +1 to +21 and an antisense primer corresponding to nucleotides +399 to +417 of Cyt1. For Western blotting, whole cell lysates were prepared as described previously (16, 18) were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Anti-type II collagen (Chemicon, Temecula, CA), anti-exacellular signal-regulated protein kinase (ERK)-1 (BD Biosciences), anti-Sox9 (Chemicon, Temecula, CA), anti-extracellular signal-regulated protein kinase (ERK)-1 (BD Biosciences), anti-Sox9 (Chemicon, Temecula, CA), and anti-Myc-tagged (Cell Signaling Technology, Beverly, MA) antibodies were employed to detect proteins.

Micro Liquid Chromatography-Tandem Mass Spectrometry Analysis of Cyt1—Mouse rib chondrocytes were cultured in DMEM. After growth to 90% confluency, chondrocytes were washed and maintained in serum-free DMEM for 48 h. Supernatant fractions prepared from chondrocytes were precipitated using the trichloroacetic acid/acetone method. Proteins from the precipitated supernatant were reduced, alkylated, and trypsin-digested, as described previously (19). Briefly, digested proteins were loaded onto fused silica capillary columns, and placed in line with an Agilent HP1100 quaternary LC pump. Separated peptides were directly electrosprayed into an LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, Palo Alto,
CA). A data-dependent scan consisting of one full MS scan and three data-dependent MS scans was used to generate MS/MS spectra of eluted peptides. MS/MS spectra were searched against an in-house protein data base containing mouse Cyt1 sequences using TurboSequest. Search results were filtered with Bioworks version 3.1.

RESULTS

Cyt1 Is Predominantly Expressed in Chondrocytes and Cartilage—To identify cartilage-specific regulatory genes, we analyzed the human normal cartilage library (Library 8940) deposited in the UniGene data base at the NCBI (www.wcbi.nlm.nih.gov). Briefly, functionally unknown and novel cartilage-specific genes in the library were classified on the basis of reference criteria (6, 7). A gene was categorized as “cartilage-specific” if the number of cartilage ESTs was significantly higher than that of non-cartilage ESTs analyzed using monochromatic serial analysis of gene expression (SAGE)/cDNA virtual Northern (cgap.nci.nih.gov/SAGE). Among these, Cyt1 was selected for functional characterization during chondrogenesis and hypertrophic maturation of chondrocytes. Alignment of Cyt1 amino acid sequences from multiple species (human, mouse, chicken, and puffer) revealed four α-helices and six conserved cysteine residues, which are common characteristics of cytokines and interleukins (supplemental Fig. 1).

We initially examined the expression patterns of Cyt1 in various mouse tissues. RT-PCR, and quantitative RT-PCR analyses disclosed that Cyt1 is predominantly expressed in cartilage (Fig. 1, A and B). Among the cell types examined, Cyt1 was detected in primary culture chondrocytes but not HTB-94 chondrosarcoma or ATDC5 chondroprogenitor cells (Fig. 1C). Upon ectopic expression in chondrocytes, Cyt1 was detected as an ∼1.0-kb single transcript in primary culture mouse rib chondrocytes (Fig. 2A), and protein was identified in both cell lysates and culture supernatant (Fig. 2B). The secreted form of Cyt1 displayed increased molecular weight (approximately ∼6.4 kDa), indicative of post-translational modifications during secretion. Secretion of endogenous Cyt1 was further confirmed by micro-liquid chromatography-tandem mass spectrometry analysis of the culture supernatant of chondrocytes. Specifically, trypsin-digested protein from the culture supernatant fractions was reduced, alkylated, and analyzed by micro liquid chromatography-tandem mass spectrometry. As shown in Fig. 2C, two peptides corresponding to mouse

![Figure 1](#) Expression of Cyt1 in mouse tissues. Cyt1 expression was determined by RT-PCR (A and C) and qRT-PCR (B) in mouse tissues or cell lines. The relative levels of Cyt1 mRNA were normalized using GAPDH as an internal control. The data in A-C represent typical results from three independent experiments.

![Figure 2](#) Cyt1 is a secretory protein in chondrocytes. Cyt1 mRNA was detected as a single transcript in mouse rib chondrocytes (A). Rib chondrocytes were transfected with ectopic myc-tagged Cyt1. After 48 h incubation in serum-free medium, recombinant Cyt1 was detected with an anti-Myc antibody in both supernatant and cell lysates (B). Culture supernatant prepared from rib chondrocytes was digested with trypsin, and analyzed by micro-liquid chromatography-tandem mass spectrometry. Two detected peptides corresponding to the mouse Cyt1 sequence are specified in boldface (C). The data represent typical results from three independent experiments.
Cyt1 (77LRDFVASPQCWK88 and 89MAWVDTLKDR98) were detected, confirming its role as a secretory protein in chondrocytes.

Expression of Cyt1 during Cartilage Development—To elucidate the in vivo expression pattern of Cyt1 during cartilage development, we performed RT-PCR analysis using mouse forelimb buds isolated from embryos at 11.5–15.5 dpc displaying limb development and cartilage formation (4, 20). Collagen IIB was detected at 14.5 and 15.5 dpc limb buds in which individual fingers are visible and longitudinal growth of cartilage occurs (Fig. 3A). Cyt1 expression occurs prior to collagen IIB expression with a peak level at 14.5 dpc limb buds (Fig. 3A).

In situ hybridization in developing limb buds at 14.5 dpc led to the detection of significant Cyt1 transcript levels in the cartilage region, which was determined by collagen IIB transcript, respectively. PC, proliferating chondrocytes; HC, hypertrophic chondrocytes.

FIGURE 3. Chondrocyte-specific expression patterns of Cyt1 during cartilage development. A, mouse forelimb buds were prepared from 11.5 to 15.5 dpc embryos. Expression of Cyt1 and collagen IIB was determined by RT-PCR (upper panel) and qRT-PCR (lower panel). B and C, Cyt1 and collagen IIB (Coll-IIB) transcripts were detected by in situ hybridization from forelimb buds of 14.5 dpc embryo (B). Expression pattern of Cyt1 and collagen IIB in the cartilaginous primordia of developing metacarpal region of forelimb buds was compared with Alcian blue staining, which detects sulfated proteoglycans (C, D), transcripts of Cyt1 and collagen IIB, and collagen X (Coll-X) were detected in the developing ulna region of forelimb buds (D). Sense riboprobes were used as a negative control. Differentiated/proliferating chondrocytes and hypertrophic chondrocytes were determined by analyzing the expression pattern of collagen IIB and collagen X transcript, respectively. PC, proliferating chondrocytes; HC, hypertrophic chondrocytes.
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A

B

FIGURE 4. Chondrocyte-specific expression patterns of Cyt1 during chondrogenesis of mesenchymal cells and hypertrophic maturation. A and B, mesenchymal cells obtained from 11.5 dpc mouse embryos were maintained as micromass culture for 5 days to induce chondrogenesis. Hypertrophic maturation of chondrocytes was induced by adding 50 μg/ml ascorbic acid, 5 mM β-glycerol phosphate on day 6. Expression levels of Cyt1, chondrocyte markers (collagen (Col) IIα/β, collagen IIβ, and aggrecan) and hypertrophic markers (collagen X and MMP-13) were determined by RT-PCR (A) and quantified by qRT-PCR (B). C, cells expressing Cyt1 and collagen IIβ during micromass culture were detected by in situ hybridization from sections of a micromass culture spot. The data represent a typical result (A and C) and mean values with standard deviation (B) from four independent experiments.

FIGURE 5. Chondrogenic effects of exogenous Cyt1. A, mesenchymal cells were maintained as micromass culture for 1.5 or 4 days in the presence of control conditioned medium (60 μl), IGF-1 (100 ng/ml), or Cyt1 conditioned medium (60 μl) under serum-free conditions. Cells were stained with Alcian blue to determine chondrogenesis at day 4 or peanut agglutinin (PNA) to determine precartilage condensation at day 1.5. B, Alcian blue-stained cells were extracted and quantified by measuring absorbance. C, mesenchymal cells were maintained as micromass culture for 4 days in the presence of control (Con) conditioned medium (40 μl) or IGF-1 (100 ng/ml) or the indicated amounts (μl) of Cyt1 conditioned medium. Expression levels of collagen IIβ mRNA and collagen IIβ protein were determined by RT-PCR and Western blotting (WB), respectively. GAPDH and ERK were employed as the loading controls. Cyt1 secreted into conditioned medium from L929 cells was detected by Western blotting using the anti-Myc antibody (D). The data represent a typical result from five independent experiments.

developing ulna, Cyt1 transcript was detected in the region where collagen IIβ is expressed, but it was not detected in the region where collagen X is expressed (Fig. 3D), indicating that Cyt1 is expressed in differentiated and proliferating chondrocytes but not in hypertrophic chondrocytes.

Next, we examined Cyt1 expression during in vitro chondrogenesis and hypertrophic maturation of chondrocytes caused by micromass culture of mesenchymal cells isolated from 11.5 dpc mouse limb buds (13, 14). For this purpose, mesenchymal cells were maintained as micromass culture to induce chondrogenesis up to day 6, and hypertrophic maturation was induced by switching chondrogenic medium to hypertrophic medium up to day 15. As expected, the chondrocyte-specific markers collagen IIβ and aggrecan (1, 21) were expressed at day 3, reached peak levels at day 6, and decreased during hypertrophic maturation (Fig. 4, A and B). Markers of hypertrophic chondrocytes, collagen X and MMP-13, were detected at day 9 (Fig. 4, A and B). Cyt1 expression was very low in undifferentiated mesenchymal cells (day 1), dramatically increased during chondrogenesis, and decreased during hypertrophic maturation (Fig. 4, A and B), similar to collagen IIβ. Cells expressing Cyt1 and collagen IIβ were identified from sections of day 6 micromass culture spots by in situ hybridization. Collagen IIβ expression analyses supported the presence of significant amounts of the Cyt1 transcript in cells located in cartilage nodules composed of differentiated chondrocytes (Fig. 4C).

Analogous to the expression pattern of Cyt1 during chondrogenesis and hypertrophic maturation of mesenchymal cells, Cyt1 expression level in undifferentiated ATDC5 cells (a chondroprogenitor cell line) was very low, elevated during chondrogenesis, and decreased during hypertrophic maturation (supplemental Fig. 2). The above results clearly demonstrate chondrocyte-specific expression of Cyt1 during cartilage development, both in vivo and in vitro.

Cyt1 Induces Chondrogenesis of Mesenchymal Cells without Effects on Hypertrophic Maturation—Because the chondrocyte-specific expression of Cyt1 suggests a possible function in chondrogenesis and/or hypertrophic maturation, we examined its role in chondrogenic differentiation of mesenchymal cells and hypertrophic maturation of chondrocytes. For this purpose, recombinant Cyt1 was prepared from serum-free conditioned medium of L929 cells expressing ectopic Cyt1 and applied to mesenchymal cells maintained as micromass culture in serum-free medium. IGF-1 (100 ng/ml) was used as the positive control for induction of chondrogenic differentiation (22). Exogenous Cyt1 did not modulate precartilage condensation, as determined by peanut agglutinin staining (Fig. 5A), but caused chondrogenic differentiation of mouse limb bud mesenchymal cells, as evident from the increased syn-
thesis of sulfated proteoglycan (Fig. 5, A and B) and expression of collagen IIB (Fig. 5C). The role of Cylt1 in chondrogenesis was further confirmed by overexpression in chondrifying mesenchymal cells using lentivirus prepared in serum-free medium. To increase cell viability, mesenchymal cells were maintained as micromass culture in complete medium for 24 h, followed by exposure to lentivirus in serum-free conditions. Under these conditions, mesenchymal cells underwent slight chondrogenic differentiation in control culture (Fig. 6A). Lentivirus-mediated overexpression of Cylt1 led to increased synthesis of sulfated proteoglycan (Fig. 6, A and B) and expression of collagen IIB (Fig. 6C), confirming enhancement of chondrogenesis.

We next examined a role of Cylt1 in hypertrophic maturation of chondrocytes. Exogenous Cylt1 was added to hypertrophic medium from days 5 to 13 (the time period of hypertrophic maturation). Contrasting the effects on chondrogenesis, exogenous Cylt1 did not affect hypertrophic maturation of chondrocytes (Fig. 7A). Additionally, lentivirus-mediated ectopic expression of Cylt1 did not affect hypertrophic maturation of chondrocytes (Fig. 7B). The results provide conclusive evidence that Cylt1 induces chondrogenesis of mesenchymal cells without significant effects on hypertrophic maturation of differentiated chondrocytes.

Mechanisms of Cylt1 Regulation of Chondrogenesis—To clarify the regulatory mechanisms of Cylt1 in chondrogenesis, we investigated whether the protein modulates the expression and/or transcriptional activity of Sox9, a master transcription factor for chondrogenic differentiation (15, 24). A reporter gene containing the 48-bp Sox9-binding site in the first intron of human COL2A1 (16, 24) was employed to examine transcriptional activity. Exogenous Cylt1 did not affect the Sox9 protein level (Fig. 8A) but enhanced its transcriptional activity (Fig. 8B). These results strongly support our theory that Cylt1 is a soluble factor that induces chondrogenic differentiation of mesenchymal cells by stimulating Sox9 transcriptional activity. The regulatory mechanisms of Sox9 transcriptional activity were further elucidated by examining signaling pathways. Because our previous results indicated that chondrogenesis is enhanced by

the inhibition of ERK (25) whereas differentiation is blocked by the inhibition of p38 mitogen-activated protein (MAP) kinase (15) or protein kinase C (PKC) α (25), a role for these signaling pathways in Cylt1-induced chondrogenesis was examined by using specific inhibitor. Consistent with our previous reports (15, 25), Cylt1-induced collagen II expression and accumulation of sulfated proteoglycan were enhanced by the inhibition of ERK with PD98059, whereas inhibition of p38 MAP kinase with SB203580 or PKCα with Go6976 blocked Cylt1-induced collagen IIB expression and proteoglycan accumulation (Fig. 8, C and E). Similar to the effects on collagen II expression, ERK inhibition enhanced Sox9 activity, whereas Sox9 activity was blocked by the inhibition of p38 MAP kinase or PKCα (Fig. 8D). Interestingly, Sox9 expression was not affected by the inhibition of ERK or p38 MAP kinase but blocked by the inhibition of PKCα (Fig. 8C).

Finally, we examined whether there is a cross-talk between Cylt1 and IGF-1 in chondrogenesis of mesenchymal cells. As shown in Fig. 9, A and B, Cylt1 conditioned medium or lentivirus-mediated overexpression of Cylt1 caused enhanced expression of Cylt1, indicating a positive feedback regulation of Cylt1 expression. In addition, IGF-1 expression was significantly increased by Cylt1 conditioned medium or lentivirus-mediated overexpression of Cylt1 with a concomitant expression of collagen II. In contrast, IGF-1 did not modulate Cylt1 expression whereas collagen II expression was induced by IGF-1 (Fig. 9C). Taken together, the above results suggest that Cylt1 causes IGF-1 expression which induces chondrogenesis of mesenchymal cells during micromass culture.

DISCUSSION

We demonstrate in this study that Cylt1 expression is strongly correlated with chondrocyte differentiation, both in
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FIGURE 8. Activation of Sox9 transcriptional activity by exogenous Cytl1 in mesenchymal cells. A and B, mesenchymal cells were maintained in micromass culture up to 4 days in the presence of the indicated amounts (µl) of control conditioned medium (CM) (Control) or indicated amount (µl) of Cytl1 conditioned medium under serum-free conditions (CM). Expression levels of collagen (Col) II and Sox9 were determined by Western blotting. ERK was employed as the loading control (A). Cells were transfected with the Sox9 reporter gene and maintained as micromass culture up to 4 days in the presence of control (Con) or indicated amount (µl) of Cytl1 conditioned medium. Sox9 transcriptional activity was determined with the reporter gene assay (B). C–E, mesenchymal cells were maintained in micromass culture up to 4 days in the absence (None) or presence of IGF-1 (100 ng/ml) or 40 µl of Cytl1 conditioned medium. Thirty min prior to Cytl1 treatment, the cells were exposed to 10 µM of PD98059 (PD) to inhibit ERK, 10 µM of SB203580 (SB) to inhibit p38 MAP kinase, or 1 µM Go6976 (Go) to inhibit PKC. Expression levels of collagen II and Sox9 were determined by Western blotting (C). Sox9 transcriptional activity was determined with the reporter gene assay (D). Accumulation of sulfated proteoglycans was detected by Alcian blue staining (E). The data represent a typical result (A, C, and E) and mean values with standard deviation (B and D) from four independent experiments.

FIGURE 9. Cytl1 causes induction of IGF-1 expression. Mesenchymal cells were maintained in micromass culture for 4 days in the presence of 40 µl of control conditioned medium (CM) (Control) or indicated amount (µl) of Cytl1 conditioned medium under serum-free conditions (A). Mesenchymal cells from mouse embryos were maintained as micromass culture for 24 h. Cells were infected for 18 h with the supernatant fractions of mock (350 µl) or Cytl1 (150 µl and 350 µl)-containing lentivirus, and cultured up to 5 days in serum-free medium (B). Mesenchymal cells were maintained as micromass culture for 4 days in the absence or presence of indicated amount (ng/ml) of IGF-1 (C). mRNA expressions of Cytl1, IGF-1, Sox9, and collagen IIB (Coll-IIB) were determined by RT-PCR. GAPDH was employed as the loading controls. The data represent a typical result from five independent experiments.

vitro and in vivo. Moreover, Cytl1 is a chondrocyte-specific secreted protein with the capacity to induce chondrogenesis of mesenchymal cells. Cytl1 was selected from a human cartilage UniGene library in an attempt to identify novel regulatory genes involved in cartilage development. The protein contains four α-helices and six conserved cysteine residues, which may form intra-disulfide bonds to give a globular structure. These structural characteristics are common in cytokines (26, 27), leading to the assumption that Cytl1 is a cytokine-like protein. SDS-PAGE analyses additionally disclose that secreted Cytl1 displays slower mobility, compared with its cellular form. Therefore, it is likely that Cytl1 undergoes post-translational modifications, although the nature of these modifications remains to be established. Similar sequences to Cytl1 have not been detected in invertebrates, such as Drosophila or Caenorhabditis elegans, but are present in several other vertebrates, such as human, chicken, and Fugu rubripes, as revealed by comparative protein sequence analyses with mouse Cytl1 (supplemental Fig. 1).

Thus, Cytl1 appears to originate early in vertebrate evolution (28). Cytl1 is specifically expressed in differentiated chondrocytes and cartilage. In our experiments, Cytl1 expression was low in undifferentiated mesenchymal cells, dramatically elevated in differentiated chondrocytes, and decreased during hypertrophic maturation. In view of this chondrocyte-specific expression pattern, we initially hypothesized that the protein regulates chondrogenesis, maintenance of differentiated phenotype of chondrocytes, and/or hypertrophic maturation of chondrocytes. Experiments with exogenous Cytl1 and lentivirus-mediated overexpression of Cytl1 clearly support a capacity of the protein to induce chondrogenesis without affecting hypertrophic maturation. The role of Cytl1 in chondrogenesis was further confirmed with a loss-of-function study. We prepared five types of siRNA oligonucleotides and two types of vector-based shRNAs (supplemental Fig. 3). Although control experiments clearly indicated efficient transfection of siRNA oligonucleotides and shRNA vectors, the examined siRNAs and shRNAs did not induce knockdown of Cytl1 expression during micromass culture of mesenchymal cells for unknown reasons (supplemental Fig. 3). Nevertheless, based on the facts that Cytl1 is expressed in chondrocytes with a capacity to induce chondrogenesis of mesenchymal cells and that Cytl1 expression is regulated by a positive feedback mechanism, Cytl1 may act as an autocrine factor that regulates chondrogenesis and cartilage development.

Because the levels of chondrocyte-specific markers, such as Col2A1, Col19A2, Col11A2, and Age, are modulated by Sox9, a key transcriptional regulator of chondrogenesis (29), we investigated the effects of Cytl1 on Sox9 expression and/or transcriptional activity. Cytl1 clearly enhanced Sox9 transcriptional activity without affecting its expression. This stimulation of Sox9 transcriptional activity by Cytl1 possibly contributes to its chondrogenic effect. Moreover, Sox9 transcriptional activity is
controlled by protein-protein interactions, for instance, positive modulation by factors such as cAMP-response element-binding protein-binding protein (CREB/p300) and peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) (30, 31). Protein modification, such as phosphorylation, is another mechanism for regulating Sox9 transcriptional activity. Indeed, our current results indicated that Cytl1-induced activation of Sox9 transcriptional activity is regulated protein kinase signaling pathways, including ERK, p38 MAP kinase, and PKC. However, Western blotting to determine whether Sox9 is phosphorylated at Ser-211 revealed that Cytl1 does not affect the phosphorylation status. Accordingly, we conclude that modulation of Sox9 phosphorylation is not related to Cytl1 regulation of transcriptional activity and that the regulatory role of ERK, p38 MAP kinase, and PKCa is not because of direct modification of Sox9 by these protein kinases. It is likely that Cytl1 controls Sox9 transcriptional activity via regulatory binding proteins or modifications, such as phosphorylation at unidentified sites. For example, L-Sox5 and Sox6 cooperatively regulate chondrocyte-specific genes, such as Col2A1 and aggrecan, with Sox9 (32). In addition to the modulation of Sox9 activity, our results indicated a cross-talk between Cytl1 and IGF-1 in chondrogenesis of mesenchymal cells. Because Cytl1 significantly enhances IGF-1 expression and IGF-1 has a capacity to induce chondrogenesis, our results strongly suggest that Cytl1-induced IGF-1 expression might contribute to the induction of chondrogenesis.

Although the chondrogenic effects of Cytl1 are evident, our current results demonstrate that the protein does not regulate hypertrophic maturation of chondrocytes. Because the Cytl1 level in chondrocytes undergoes a dramatic decrease during hypertrophic maturation, we initially assumed that exogenous or sustained Cytl1 expression inhibits or delays hypertrophic maturation of chondrocytes. However, our results suggest that down-regulation of Cytl1 is not a critical factor in the hypertrophic maturation process. Our findings provide collective evidence that Cytl1 is a novel regulatory factor predominantly expressed in chondrocytes and developing cartilage, which induces chondrogenesis of mesenchymal cells as an autocrine factor.

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