The mRNA level of basic helix-loop-helix transcription factor DEC1 (BHLHB2)/Stra13/Sharp2 was up-regulated during chondrocyte differentiation in cultures of ATDC5 cells and growth plate chondrocytes, and in growth plate cartilage in vivo. Forced expression of DEC1 in ATDC5 cells induced chondrogenic differentiation, and insulin increased this effect of DEC1 overexpression. Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) suppressed DEC1 expression and the differentiation of ATDC5 cells, but DEC1 overexpression antagonized this inhibitory action of PTH/PTHrP. Transforming growth factor-β or bone morphogenetic protein-2, as well as insulin, induced DEC1 expression in ATDC5 cultures where it induced chondrogenic differentiation. In pellet cultures of bone marrow mesenchymal stem cells exposed to transforming growth factor-β and insulin, DEC1 was induced at the earliest stage of chondrocyte differentiation and also at the hypertrophic stage. Overexpression of DEC1 in the mesenchymal cells induced the mRNA expressions of type II collagen, Indian hedgehog, and Runx2, as well as cartilage matrix accumulation; overexpression of DEC1 in growth plate chondrocytes at the prehypertrophic stage increased the mRNA levels of Indian hedgehog, Runx2, and type X collagen, and also increased alkaline phosphatase activity and mineralization. To our knowledge, DEC1 is the first transcription factor that can promote both chondrogenic differentiation and terminal differentiation.

The development of the vertebrate long bones occurs through the process of endochondral ossification, which is initiated in the embryo with the condensation of mesenchymal cells and then progresses with their commitment and differentiation into chondrogenic cells. By the late embryonic stage, the epiphysal growth plate has developed with distinguishable, well organized and spatially distinct zones of resting, proliferating, and post-proliferative hypertrophic chondrocytes. The hypertrophic cartilage calcifies and is invaded by cartilages, and is subsequently replaced by new bone (1). Recent studies have identified several transcription factors involved in endochondral ossification. Among these, Sox9 is required for the condensation of prechondrogenic mesenchymal cells, and Sox5 and Sox6, as well as Sox9, are required for the activation of type II collagen expression during chondrogenesis (2). In addition, different sets of Smads are involved in stimulation or inhibition of chondrocyte hypertrophy by transforming growth factor-β superfamily members (3, 4). Runx2/Cbfa1/AML3/Pebp2a-A is essential for intramembranous ossification, and mutations in this gene are responsible for cleidocranial dysplasia, a syndrome characterized by open fontanelles and hypoplastic clavicles (5–8). Furthermore, Runx2 plays a crucial role in endochondral ossification. In Runx2-deficient mice, chondrocyte hypertrophy, mineralization, and vascular invasion are suppressed in most parts of the skeleton (9–13). On the other hand, little is known about the role of the basic helix-loop-helix (bHLH) transcription factor family in endochondral ossification, although many bHLH proteins play a critical role in neurogenesis, myogenesis, and hematopoiesis (14–19).

DEC1(BHLHB2), a novel bHLH transcription factor, was identified in human chondrocytes by the subtraction method (20). A mouse ortholog (Stra13) and a rat ortholog (Sharp2) of DEC1 were cloned from P19 embryonic carcinoma cells and rat brain, respectively (21, 22). DEC1/Stra13 works as a transcriptional repressor, decreasing its own transcription, as well as that of c-myc, through the histone deacetylase-dependent and general transcription factor-dependent mechanisms, respectively (23). In P19 cells, DEC1/Stra13 overexpression promoted neuronal differentiation when the cells were exposed to retinoic acid in monolayer culture, or promoted it after aggregation in the absence of retinoic acid (21). In NIH3T3 cells, DEC1/Stra13 expression was associated with growth arrest, and overexpression suppressed proliferation (23). Recently, DEC1/Stra13-deficient mice have shown defects in several phases of T cell activation, resulting in lymphoid organ hyperplasia and chronic systemic lupus-like autoimmune disease (24).

To explore the role of DEC1 in chondrocyte differentiation, we overexpressed human DEC1 in mouse ATDC5 cells, rabbit mesenchymal stem cells (MSC), and rabbit chondrocytes. ATDC5 cells can mimic chondrocyte differentiation processes...
Chondrocyte Differentiation Induced by DEC1

from chondroprogenitors to fully differentiated hypertrophic chondrocytes in response to insulin or insulin-like growth factor-1 (25, 26). Bone marrow MSC can differentiate into chondrocytes, osteoblasts, tenocytes, adipocytes, muscle cells, and nerve cells in vitro and/or in vivo (27–29). MSC in pellet, but not in monolayer cultures, undergo chondrogenic differentiation in response to insulin and TGF-β. We show here that forced expression of DEC1 promotes chondrogenic differentiation, hypertrophy, and/or mineralization in the cultures of ATDC5 cells, MSC, and chondrocytes. Furthermore, TGF-β, bone morphogenetic protein-2 (BMP-2), and insulin all induced DEC1 expression, whereas PTH/PTHrP suppressed this expression. DEC1 may play an important role in the control of chondrocyte differentiation from the early to the terminal stage.

EXPERIMENTAL PROCEDURES

Cell Cultures—Chondrocytes were isolated from growth plates of the rib cartilage of 4-week-old male Japanese white rabbits, as previously described (30). The experimental procedures on animal care and treatment were performed with permission, and following the rules and guidelines of Hiroshima University. Chondrocytes were seeded at a density of 3 × 10⁴ or 7 × 10⁴ cells in a 16- or 22-mm plastic tissue culture dish, respectively, and maintained in α-minimal essential medium (Sanko Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 32 units/ml penicillin, 60 µg/ml kanamycin (Meiji Seika Co., Tokyo, Japan), and 250 ng/ml amphotericin B (Dainippon Pharmaceutical Co., Osaka, Japan) at 37 °C in a humidified atmosphere of 5% CO₂ in air.

ATDC5 cells (Riken, Tsukuba, Japan) were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (Flow Laboratories) containing 5% FBS, 10 µg/ml human transferrin (Roche Molecular Biochemicals, Mannheim, Germany), 3 × 10⁻⁵ M sodium selenite, 32 units/ml penicillin, 60 µg/ml kanamycin, and 250 ng/ml amphotericin B (in the absence of sodium a) or presence of 10 µg/ml bovine insulin (Sigma) (26). Inoculum density of the cells was 3 × 10⁴ cells/23 mm in 12-multwell plates or 6 × 10⁴ cells/36 mm in 6-multwell plates (Corning, New York, NY). The medium was replaced every other day. In some studies, ATDC5 cells were incubated with a medium containing 0.5% FBS for 3 days. Insulin (10 µg/ml) was added at 72 h, and BMP-2 (100 ng/ml), TGF-β1 (5 ng/ml), or bone extracts (2.5 µg/ml) were added 48 h before the end of the incubation. Bone extracts (Sangi BMP Mixture) were purchased from Wako Pure Chemical Industries, Ltd., Osaka Japan. BMP mixture induces alkaline phosphatase activity in osteogenic cells in vitro and induces bone formation in vivo.

Marrow aspirates were obtained from three 4-week-old male Japan White rabbits. The cells were seeded at 2 × 10⁴ cells per 100-mm tissue culture dish and maintained in 10 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 32 units/ml penicillin, and 60 µg/ml kanamycin at 37 °C under 5% CO₂ in air (29). Three days after seeding, floating cells were removed and the medium was replaced by fresh medium. Thereafter, attached cells were fed with fresh medium every 3 days and used as MSC. Passages were performed when cells were reaching confluence. Cells were seeded at 5 × 10⁵ cells/cm² in 100-mm dishes. For chondrogenic differentiation, cells were seeded at 2 × 10⁶ cells/15-ml plastic centrifuge tube, and maintained in 0.5 ml of serum-free, α-minimal essential medium (high glucose) supplemented with 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenite, 5.33 µg/ml linolate, 1.25 mg/ml bovine serum albumin, 10 ng/ml TGF-β1, 100 µg/dexamethasone, and 50 µg/ml ascorbic acid-2-phosphate (Wako). The cultures were fed with 0.5 ml of the medium for 4 days after seeding. Thereafter, the cultures were fed with 1 ml of medium every other day (22).

Plasmid Construct and Transfection—Full-length human DEC1 cDNA was cloned into the HindIII-XbaI site of the expression vector pcDNA3.1/Zeo (+) (Invitrogen) to yield pcDNA3.1/Zeo-DEC1. Stable transfection for pcDNA3.1/Zeo-DEC1 or pcDNA3.1/Zeo (+) was carried out using SuperFect transfection reagent (Qiagen, Crawley, UK). After transfection, the cells were incubated in medium A containing 0.15 µg/ml Zeo (+) (Invitrogen), and several individual clones were selected using 5 µg/ml zeocin (Invitrogen). pcDNA3.1/Zeo-DEC1 or pcDNA3.1/Zeo (+) were cultured in 1 ml of medium A/23-mm dish in 12-multwell plates for 20 days. The cells were exposed to [³⁵S]sulfate (0.5 µCi/culture) for 8 h before the end of the incubation. We estimated the level of proteoglycan synthesis by measuring incorporation of [³⁵S]sulfate into material precipitated with cetlylpyridinium chloride after digestion with 2 mg/ml Pronase E (31). The glycosaminoglycan content was determined as described previously (32). In some experiments, the amount of proteoglycan accumulation in the cell layer was estimated by toluidine blue staining. DNA was determined using bicinechazol (Hoechst 33258) (33).

Northern Blot Analysis—Total RNA was extracted by the guanidinium thiocyanate/cesium trifluoracetate method (34). Poly(A)+ RNA (2 µg) that had been enriched using Oligo(dT)₃⁺(Nippon Roche Ltd., Tokyo, Japan) was electrophoresed on 1% agarose gel containing 2.2 M formamide, and transferred to Nytran nylon membrane (Schleicher & Schuell). Hybridization was carried out with 3P-labeled specific cDNA probes. The membranes were washed at 65 °C for 30 min with 0.1x SSC containing 0.5% SDS, and exposed to BioMax x-ray film (Eastman Kodak Co.) at −70 °C with an intensifying screen.

RT-PCR and Southern Blot Analysis—The first-strand cDNA was synthesized from 1 µg of total RNA using the SuperScript II preamplification system (Invitrogen). Pairs of oligonucleotides: 5’-AGAGGCTT- GACCCTGATTAA-3’ and 5’-CATATGGACTTCGTTGCTG-3’ for rabbit DEC1; 5’-AGAGAGCTGTCGGACTTAAA-3’ and 5’-CGGTATCTTGTTCTGGTCTCA-3’ for mouse DEC1; 5’-ATGATCCGATTCCTGGGCTCT-3’ and 5’-TCTGGGGACACACGCCTTCTC-3’ for rabbit type II collagen; 5’-CGGCAAATCCGTGACAGCAG-3’ and 5’-ACCTTGAGGACCACTGGT-3’ for mouse type X collagen; 5’-GCTTGGATCCCAAACTGAAAC-3’ and 5’-AAACCTTA-3’ and 5’-AAAAAGGCGACTTGTGGA-3’ for Runx2; and 5’-CAAGAGTTCGCCACCCAG-3’ and 5’-AGCTGGCCCTGTCAGCTC-3’ for Indian hedgehog (Ihh), were used as amplification primers. Other gene-specific primers were as previously described (35–37). PCR reactions were performed using an aliquot of the first-strand cDNA as a template, under standard conditions with KlenTaq polymerase (Clontech Laboratories Inc.) for 22 cycles, which proved optimal for comparison of the amplified products. The amplified products were separated on 1% agarose gels and stained with ethidium bromide, or blotted onto the pGEM-T-easy vector (Promega, Madison, WI) to determine the cDNA sequences. Hybridizations were performed with 32P-labeled specific cDNA probes under the same conditions as above described.

Quantitative Real Time PCR Analysis—Quantitative real time PCR analysis was performed using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc., Foster City, CA). First-strand cDNA prepared by RT-PCR reaction was amplified using 5’-GCAAGGAAACTTCAAACGTC-3’ and 5’-CATTGACATCCTGGTGCTG-3’ for mouse DEC1, 5’-GAAAGATGGCCGTGAAATCAA-3’ and 5’-CATCATTGGGAAGCTGCTC-3’ for human DEC1, 5’-ACGGCCGATGTACATCTACATATTG-3’ and 5’-CAAGAAGGAGGCTGGAAAAAGA-3’ for β-actin, and 5’-AATCCATGCTCTTGAGCCT-3’ and 5’-GCTTCCACACCTCTTGTAG-3’ for GAPDH. The amplified cDNAs were quantified using 6FAM-CACCGCGTATTGAGAAAAGA-3’ and 5’-FAM-CACCCTGAGCGGGGAAACCTC-3’ for mouse TMRA-3’, 5’-VIC-CAACGAGGCTGCAAGCTC-3’ and 5’-VIC-TGGCGGCCTGAGAAGCCTG-3’ for GAPDH.

Proliferation Assay—DEC1-overexpressing, empty vector integrated and wild-type ATDC5 cells were maintained in 36-mm dishes in the presence or absence of 10 µg/ml insulin. On days 4, 7, and 10, the growth was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, using CellTiter 96 Aqueous One solution cell proliferation assay (Promega).

Construction of DEC1 expression Adenovirus—The recombinant adenovirus was constructed as described (38). Briefly, human DEC1 cDNA was subcloned into a TA-11 vector and produced seeds of pAdeno-X (Clontech), which is defective in adenovirus E1A, E1B, and E3 regions. Each cosmid bearing the expression unit and adenovirus DNA-terminal protein complex was cotransfected into the E1 transcomplementing cell line HEK293. Adenovirus carrying human DEC1 was grown in HEK293 cells and purified. Infection of the recombinant adenoviruses was performed at a multiplicity of infection (m.o.i.) of 100. Adenovirus carrying LacZ was generously supplied by Dr. Kohei Miyazono (The University of Tokyo) (3). Alkaline Phosphatase Activity and Calcium—Alkaline phosphatase activity was determined by the method of Bessey et al. (39), and calcium content was determined by the method of Gitelman (40).

RESULTS

Expression of DEC1 mRNA During Differentiation of Growth Plate Chondrocytes and ATDC5 Cells—To examine the changes
were obtained in repeated studies. Total RNA was extracted from the cells on the days indicated and subjected to RT-PCR/Southern blot analysis for DEC1 and GAPDH or Northern blot analysis for collagen types II and X. B, total RNA was extracted from the slices of growth plate cartilage (S1, the proliferating zone; S2, the matrix forming zone; S3, the hypertrophic zone) and subjected to RT-PCR/Southern blot analysis. C, after ATDC5 cultures in 36-mm dishes became confluent (5 days after seeding), 10 μg/ml insulin was added to the culture medium. RNA was extracted from the cells on the indicated days after the addition of insulin, and RNA was then subjected to RT-PCR/Southern blot analysis. Similar results were obtained in repeated studies.

in the DEC1 mRNA level during chondrocyte differentiation, we incubated rabbit growth plate chondrocytes in high density cultures. The chondrocytes underwent proliferation (day 6), cartilage-matrix synthesis (days 10–14), prehypertrophy (day 18), and hypertrophy (day 22) (41). Chondrocyte differentiation was associated with the sequential expressions of types II and X collagen mRNA, which are the markers of cartilage-matrix synthesis and hypertrophy, respectively (Fig. 1C). In this system, PTHrP receptor mRNA was expressed at the highest level on day 18 (42). The DEC1 mRNA level was low during the proliferating stage, increased during the matrix-forming/prehypertrophic stages, and reached a maximum in the hypertrophic (terminal) stage. The mRNA level of GAPDH was consistent throughout the culture period (Fig. 1A).

We also sliced the growth plate cartilage to identify the expression of DEC1 mRNA in vivo (S1, the proliferating zone; S2, the matrix forming zone; and S3, the hypertrophic zone) (49). Aggrecan mRNA was expressed in all zones, with the level decreasing in the hypertrophic zone. Type X collagen mRNA was undetectable in the proliferating zone, whereas it was abundant in the hypertrophic zone. DEC1 mRNA was expressed in the proliferating, matrix-forming and hypertrophic zones at low, moderate, and high levels, respectively (Fig. 1B).

Mouse embryo cell line ATDC5 cells in confluent cultures exposed to a high concentration of insulin undergo chondrogenic differentiation. In ATDC5 cultures, DEC1 mRNA was barely detectable before the addition of insulin, increased during the earliest stage of chondrogenic differentiation in response to insulin, when the expressions of type II collagen and aggrecan were initiated (6 days after adding insulin), and further increased during the hypertrophic stage (14–18 days after adding insulin) (Fig. 1C). These findings obtained with primary chondrocytes, growth plate slices, and ATDC5 cells suggest that DEC1 expression starts at the early stage of chondrocyte differentiation and reaches a maximum at the hypertrophic stage.

** Forced Expression of DEC1 Promotes Chondrogenic Differentiation of ATDC5 Cells in the Absence of Insulin—**To determine whether the bHLH protein DEC1 is functionally involved in chondrogenic differentiation, we isolated several zeocin-resistant clones (D1–D7) expressing human DEC1. Northern blot analysis showed that human DEC1 mRNA was expressed in D1, D2, and D7 cultures at high levels, and in D3, D4, D5, and D6 cultures at low or moderate levels (Fig. 2). The endogenous mouse DEC1 mRNA level in undifferentiated wild-type ATDC5 cultures was very low compared with the human DEC1 mRNA levels in D1, D2, and D7 cultures on day 5 (Fig. 2), but it markedly increased during chondrogenic differentiation (Fig. 1C). Thus, the human DEC1 mRNA level in mouse D1, D2, and D7 cultures may be comparable with the endogenous DEC1 level in differentiated chondrocytes.

In pilot studies, D1, D2, and D7 cells showed prominent chondrogenic differentiation even in the absence of insulin, estimated under a phase-contrast microscope, whereas D3, D4, D5, and D6 cells showed low or moderate levels of differentiation. The degree of chondrogenic differentiation correlated with the human DEC1 mRNA levels. Thus, subsequent studies used the D1, D2, and D7 clones as DEC1-overexpressing cells unless otherwise specified.

In the absence of insulin, D1, D2, and D7 cells mimicked the insulin-inducible cell changes, including cellular condensation, cartilage nodule formation, and the growth of cartilage nodules, after the cultures became confluent. The cell shape changes in D1, D2, and D7 cultures were observed from day 10, and most D1, D2, and D7 cells were morphologically altered from fibroblast-like cells to spherical cells with a refractile extracellular matrix by day 20. In the absence of insulin, such cell shape changes were rarely observed with parental ATDC5 cells (AT) or vector-integrated cells (Pc1 and Pc2) throughout the culture period (Fig. 3A). Accordingly, the intensity with which toluidine blue stained cartilage proteoglycan was much greater in D1, D2, and D7 cultures than in AT, Pc1, and Pc2 cultures (Fig. 3B).

Proteoglycan synthesis by these cells was estimated by measuring the incorporation of [35S]sulfate into glycosamignoglycans precipitated with the cetylpyridinium chloride on day 20. The level of proteoglycan synthesis was higher in D1, D2, and D7 cultures than in AT, Pc1, and Pc2 cultures (Fig. 3C).

Northern blot analysis showed that DEC1 overexpression
markedly increased the expression of aggregan, type II collagen, and type X collagen mRNAs, whereas these mRNAs were barely detectable in the control cultures (Fig. 3D). These findings indicate that the expression of DEC1 initiates chondrogenic differentiation from the early to the terminal stage.

In other studies, D1, Pc1, and AT cells were seeded and maintained in 36-mm dishes in the presence or absence of insulin, and on days 4, 7, and 10, the growth was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. The cells proliferated at similar growth rates under these conditions (Fig. 3E). In addition, we repeatedly observed, using a phase-contrast microscope, that DEC1 overexpression had little effect on the proliferation of ATDC5 cells.

Synergism between the Effects of Insulin and DEC1 Overexpression on Chondrogenesis, and the Effect of PTH/PTHrP on Chondrogenic Differentiation—Because insulin also induces chondrogenic differentiation, the effect of DEC1 overexpression was compared with that of insulin. The intensity with which toluidine blue stained the cell-matrix layer in human DEC1-expressing cultures (D1 and D2) without insulin was similar to that in the control cultures (Pc1 and Pc2) maintained with insulin on days 10 and 18 (Fig. 4B, left and middle lanes). Furthermore, DEC1 overexpression plus insulin elicited a synergistic enhancement in proteoglycan accumulation on days 10 and 18 (Fig. 4B, middle lanes).

Because PTH/PTHrP suppresses the cellular condensation...
process and subsequent chondrogenic differentiation of ATDC5 cells (26), we examined the effect of PTH/PTHrP on chondrogenic differentiation in DEC1-overexpressing cells. PTH abolished both the cell shape changes, from fibroblast-like cells to spherical cells (Fig. 4A, right lane), and proteoglycan accumulation (Fig. 4B, right lanes) in insulin-exposed Pc1 and Pc2 cultures. In contrast, PTH had little, if any, effect on chondrogenic differentiation or proteoglycan accumulation in insulin-exposed D1 and D2 cultures (Fig. 4A and B, right lanes). Thus, forced expression of DEC1 at high levels circumvented the inhibitory effect of PTH on differentiation.

D3 cells expressing human DEC1 at a lower level (Figs. 2 and 4C) also showed increased proteoglycan accumulation in the absence or presence of insulin (Fig. 4B1). However, D3 cells, unlike D1 or D2 cells, showed decreased proteoglycan accumulation in the presence of PTH (Fig. 4B, right lane). Hence, the low level of human DEC1 expression in D3 cultures did not influence the inhibitory effect of PTH on chondrogenesis.

Changes in the DEC1 mRNA Level in PTH/PTHrP-exposed Cultures—To examine the changes in DEC1 mRNA level in PTH/PTHrP-exposed cultures, we determined the mouse DEC1 mRNA level in wild-type ATDC5 cultures using the real time PCR method. In wild-type ATDC5 cultures, the DEC1 mRNA level gradually increased in response to insulin by day 20, but PTH virtually abolished the insulin induction of mouse DEC1 expression (Fig. 5A). These observations suggest that PTH inhibits insulin-induced chondrogenic differentiation partly through the down-regulation of DEC1 expression.

We also estimated the endogenous mouse DEC1 mRNA levels in Pc1, Pc2, D1, and D2 cultures on day 18 (Fig. 5B). In these cultures, insulin increased the mouse DEC1 mRNA level, whereas PTH or PTHrP (not shown) suppressed the up-regulation of mouse DEC1 by insulin. Although PTH abolished the insulin-induced mouse DEC1 expression in D1 and D2 cells, PTH had little effect on the cytomegalovirus promoter-mediated human DEC1 expression in these cells. Thus, PTH did not abolish chondrogenesis in D1 and D2 cultures.

**DEC1 Is Inducible by TGF-β and BMP**—To examine whether DEC1 is involved in the TGF-β and BMP signaling pathways, wild-type ATDC5 cells in confluent cultures were exposed to BMP-2 or TGF-β1 for 48 h or insulin for 72 h. BMP-2 and TGF-β1 markedly increased the expressions of DEC1, type II collagen, and aggrecan mRNAs within 48 h, and insulin increased the expression of DEC1 at 72 h (Fig. 6),
free medium in the presence or absence of mRNA levels of DEC1 (results). Three independent experiments were performed with similar results. Total RNAs were isolated from the pellet cultures on the indicated days, and subjected to RT-PCR/Southern blot analysis. The values are average ± S.D. for three cultures. B, total RNAs were isolated from the pellet cultures on the indicated days, and subjected to RT-PCR/Southern blot analysis. C, the relative mRNA levels of DEC1 (☐) and Runx2 (■), compared with GAPDH, were quantified by BAS2000. Three independent experiments were performed with similar results. D, cells were exposed to the serum-free medium in the presence or absence of 10 ng/ml TGF-β1, in the presence of insulin (10 µg/ml) for 48 h after aggregation. Total RNAs were isolated from the pellet cultures and subjected to RT-PCR/Southern blot analysis.

**DEC1 Is Induced during Chondrogenesis of MSC in Pellet Cultures**—In subsequent studies, we used rabbit bone marrow-derived MSC to confirm the observations obtained with ATDC5 cells. MSC were maintained in pellet cultures in serum-free medium containing TGF-β and insulin, because these cells do not undergo chondrogenic differentiation in monolayer cultures even in the presence of these growth factors. Previous studies had shown that in pellet cultures, spherical cells (chondrocytes) appeared on day 6 near the surface of aggregates, and that the morphology of aggregates was entirely cartilaginous by days 14–20 (29). The glycosaminoglycan level started to increase on day 10 and reached a plateau on day 14 (Fig. 7A). The DNA content did not change during the chondrogenesis of MSC under these conditions (46).

In pellet cultures, type II collagen mRNA was expressed before histological appearance of chondrocytes (Fig. 7B). The type II collagen mRNA level started to increase on day 2 and reached a plateau on day 6; the aggrecan mRNA level started to increase on day 10, reaching a plateau on day 14; the Ihh mRNA level started to increase on day 14, with the maximal level on day 18; and type X collagen mRNA was expressed during the late stage of the culture (days 14–22). The Runx2 mRNA level showed two peaks on days 2 and 14, at the earliest stage and the hypertrophic stage, respectively, which was similar to the Runx2 expression in vivo (9, 11–13). Runx2 expression is suppressed once chondrogenic differentiation starts, and thereafter Runx2 is up-regulated in prehypertrophic/hypertrophic chondrocytes. Interestingly enough, the DEC1 mRNA level in MSC pellet cultures also showed two peaks on days 2 and 14, with an expression pattern very similar to that of Runx2 (Fig. 7C). The first peak of the DEC1 mRNA expression was not observed in ATDC5 cells, possibly because, as chondroprogenitor cells, they are already committed to the chondrocyte lineage.

When MSC were maintained in pellet cultures in the presence of insulin and in the absence of TGF-β, no chondrocyte differentiation took place (46). However, the addition of TGF-β to the cultures increased DEC1, Runx2, and type II collagen mRNA levels within 48 h (Fig. 7D).

**Effects of DEC1 Overexpression on Morphology of MSC Aggregates and Gene Expression**—To explore the role of DEC1 at the earliest stage of chondrocyte differentiation, we mock-infected or infected MSC in monolayer cultures with adenovirus carrying human DEC1, and then maintained these cells in pellet cultures in the presence of insulin and TGF-β1. The infection had little effect on the growth of MSC (data not shown). In control cultures of mock infected MSC, spherical cells were observed only near the surface of aggregates on day 6 as described previously (29), but in cultures overexpressing DEC1, most cells were spherical by day 6. These cells were surrounded by cartilage-characteristic proteoglycans that stained metachromatically with toluidine blue (Fig. 8A), and the glycosaminoglycan level also increased with the infection of DEC1-expression adenovirus (Fig. 8B). The infection of MSC with DEC1-expression adenovirus increased the human DEC1 mRNA level (Fig. 9A) and the mRNA levels of type II collagen,
Runx2, and Ihh on day 6 multiplicity of infection dependently (Fig. 9B), whereas the infection decreased the endogenous rabbit DEC1 mRNA level and had little effect on the GAPDH mRNA level. Fig. 9C shows that enhancement of type II collagen and Runx2 and the suppression of rabbit DEC1 were also observed on days 12 and 18 in DEC1-overexpressing cultures at m.o.i. of 50, but not in control cultures of mock infected cells or cells infected with adenovirus carrying LacZ (Fig. 9C). Previous studies had shown that infection of mesenchymal cells with the LacZ-expression adenovirus had little effect on chondrogenic or osteogenic differentiation even at a high m.o.i. of 300 (3).

DEC1 negatively regulates transcription from its own promoter in luciferase reporter gene assays (23). The reduction of rabbit DEC1 mRNA level in MSC expressing human DEC1 at a high m.o.i. of 50 or 100 (Fig. 9, A and C) may be because of the feedback regulation of DEC1 expression.

Effects of DEC1 Overexpression on Expression of Mineralization-related Phenotype by Chondrocytes—We examined the effect of DEC1 overexpression on the expression of the mineralization-related phenotype using rabbit growth plate chondrocytes, because most (>95%) of these cells can undergo hypertrophy even on plastic dishes. When hypertrophying chondrocytes were infected with adenovirus carrying DEC1 on day 14, the mRNA levels of Runx2, Ihh, and type X collagen increased the multiplicity of infection and human DEC1 mRNA levels dependently by day 21 (Fig. 10A). The chondrocytes overexpressing DEC1 showed higher levels of calcium deposition and alkaline phosphatase activity than did control cells (Fig. 10, B–D).

**FIG. 8.** Effects of overexpression of DEC1 on the morphology of MSC pellets. MSC were mock-infected or infected with the DEC1-expression adenoviruses 24 h before aggregation. The aggregates of these cells were maintained in pellet cultures in a serum-free medium containing TGF-β and insulin. A, for histological evaluation, the aggregates in the cultures on day 6 were fixed in 10% formalin. After fixing, tissues were embedded in paraffin, sectioned (6 μm thick), and stained with toluidine blue. B, the glycosaminoglycan level in these cultures was determined on day 6. The values are averages ± S.D. for three or five cultures.

**FIG. 9.** Effects of overexpression of human DEC1 on mRNA expressions of type II collagen, Runx2, Ihh, and rabbit DEC1. Rabbit MSC were mock-infected or infected with adenovirus carrying human DEC1 or LacZ 24 h before aggregation, and total RNA was isolated on days 6, 12, or 18. A, the mRNA level of human DEC1 was determined on day 6 by real time quantitative RT-PCR analysis. B, the expressions of type II collagen, Runx2, and Ihh mRNA were determined on day 6 by RT-PCR/Southern blot analysis. Three independent experiments were performed with similar results. C, rabbit MSC were mock infected or infected with adenovirus carrying human DEC1 or LacZ (m.o.i. 50) 24 h before aggregation. Type II collagen, Runx2, rabbit DEC1, and GAPDH mRNA levels were determined on days 12 or 18 by RT-PCR/Southern blot analysis. Three independent experiments were performed with similar results.

**DISCUSSION**

Previous studies had shown that DEC1 mRNA is expressed in numerous tissues (20, 22). However, in situ hybridization studies with embryonic mice revealed that the level of the DEC1 transcript was much higher in developing cartilage than in surrounding tissues (21). Thus, DEC1 expression appears to be up-regulated as a development-related event in chondrogenic cells both in vitro and in vivo.

Several growth factors and hormones, including BMP, TGF-β, insulin and PTH/PTHrP, are necessary for the control of chondrocyte differentiation in developing cartilage. The DEC1 mRNA expression was induced by TGF-β, BMP, and insulin, all of which stimulate chondrogenesis in vitro and in vivo. In contrast, PTH/PTHrP, which suppressed chondrogenic differentiation, did not increase DEC1 mRNA expression during the whole culture period (20 days), except for a transient increase in the DEC1 mRNA level at 1 h in ATDC5 cultures (47). The growth factor regulation of DEC1 expression was closely associated with chondrocyte differentiation.

PTH/PTHrP abolished the chondrogenesis of ATDC5 cells. It is unlikely that the inhibition of differentiation by PTH/PTHrP is because of stimulation of cell division, because insulin enhances both proliferation and differentiation of ATDC5 cells. In addition, the mitogenic effect of PTH/PTHrP is far less than that of other growth factors (48). The PTH/PTHrP suppression of chondrogenesis at the earliest stage may be relevant to the chondrodysplasia, which is because of the mutation of the PTHR1 receptor gene that produces a consistently active PTHR1 receptor (49).

Because chondrocyte maturation can occur only after proliferation ceases, DEC1 overexpression may enhance differentiation by suppression of growth. DEC1/Strai13 overexpression in NIH3T3 cells has been shown to suppress cell growth (23, 50). However, DEC1 overexpression had little effect on the proliferation of MSC and ATDC5 cells. On the other hand, DEC1...
overexpression enhanced proliferation of T-lymphocytes in some situations (24), and the DEC1 mRNA level decreased after the cessation of proliferation at the terminal differentiation stage of B-lymphocytes (51). These findings suggest that DEC1 stimulation of chondrocyte differentiation is not because of inhibition of proliferation.

Hypoxia induces DEC1 expression in ATDC5 cells, HeLa cells, 3T3-L1 cells, and U-87 glioblastoma cells (52–54). This is noteworthy because chondrogenesis takes place in low oxygen conditions, and the cartilage is avascular tissue. In contrast, hypoxia suppresses adipogenesis of mesenchymal cells (52), and DEC1 overexpression in 3T3-L1 cell cultures repressed PPARγ2 expression and adipogenesis (52). Thus, DEC1 induced by hypoxia may promote chondrogenesis by inhibiting MSC from moving to the adipogenic lineage.

In the presence of 5% serum and in the absence of added growth factors, forced expression of DEC1 was sufficient to induce chondrogenic differentiation of ATDC5 cells, allowing differentiation through the matrix-forming stage (aggrecan mRNA expression) to the hypertrophic stage (type X collagen mRNA expression). Insulin increased this effect of DEC1 overexpression. However, forced expression of DEC1 induced chondrogenesis in MSC cultures in serum-free medium only in the presence of TGF-β. Thus, the induction of DEC1 alone is insufficient for chondrogenesis. Additional signals induced by TGF-β or other growth factors are required.

Overexpression of MyoD or PPARγ2 in mesenchymal cells induces myogenic or adipogenic differentiation, respectively (18, 19, 55). However, overexpression of Smad proteins that transmitted BMP or TGF-β signal to nucleus did not induce chondrogenic differentiation of ATDC5 cells (5). Although Sox9-deficient mice have defects in chondrogenesis (4), the expression of Sox9 alone seems to be insufficient for chondrogenesis. Sox9 was expressed at high levels even in undifferentiated ATDC5 cells. Therefore, post-translational modifications of Smads and Sox9 may be crucial for chondrogenesis. Alternatively, these transcription factors may require DEC1 to induce chondrogenesis at the maximal level.

The mechanism by which DEC1 overexpression induces the expression of so many cartilage-specific genes remains unknown. It is clear that DEC1 is not a master gene for chondrogenesis, because it is expressed in both chondrogenic and non-chondrogenic cells (20–22). DEC1 may enhance transcription of type II collagen, aggrecan, and type X collagen genes directly or indirectly only in the presence of tissue-specific transcription factors such as Sox9 and other transcription factors activated by TGF-β or BMP. In addition, DEC1 may repress the synthesis of negative regulators for cartilage-specific gene expression, such as aA crystalline-binding protein 1 (56). DEC1/Stra13 has been shown to interact with several transcription factors including DEC1, Mash1, E47, and upstream stimulatory factor (a bHLH-leucine zipper protein) (21, 57, 58). In addition, it interacts with histone deacetylase and general transcription factors (TATA-binding protein, transcription factors IID, IIB, and AII-100) (21, 23). Furthermore, DEC1 can bind to E-box sequences, at least in some cases (50). Thus, the identification of transcription factors and regulators that bind to DEC1 in chondrogenic cells will aid in the understanding of DEC1 actions.

We observed a close relationship between DEC1 and Runx2 during chondrocyte differentiation. The overexpression of DEC1 enhanced the expression of Ihh and type X collagen, alkaline phosphatase activity, and mineralization, effects very similar to those of Runx2 overexpression in vitro and in vivo (9–13). Also, both DEC1 and Runx2 were expressed in a biphasic fashion at the early stage and the hypertrophic stage, and DEC1 overexpression enhanced Runx2 expression. These findings suggest that at least some part of DEC1 actions is mediated by induction of Runx2. Because Runx2 enhances the expression of the hypertrophy related genes including Ihh, alkaline phosphatase, and type X collagen (9–13), the induction of Runx2 by DEC1 may be important at the hypertrophic stage. However, even in the presence of DEC1 and Runx2, Ihh, alkaline phosphatase, and type X collagen were not expressed at the early stage in MSC cultures and in vivo. This suggests that, besides DEC1 and Runx2, another regulator(s) is required for transcriptional regulation of hypertrophy.

The DEC family consists of DEC1 and DEC2 (58). DEC2 is similar to Sharp1 (22), which is a minor frameshift mutant of DEC2 (59). DEC2 may also be involved in the regulation of chondrocyte differentiation, because DEC2 expression is markedly induced in limb buds and prevertebra in vivo (45). In conclusion, the findings in this study strongly suggest that DEC1 is involved in the onset of both chondrogenic differentiation and terminal differentiation.

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3 E. Yoshida and Y. Kato, unpublished data.
