DEC2 is a negative regulator for the proliferation and differentiation of chondrocyte lineage-committed mesenchymal stem cells

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Abstract. Differentiated embryo chondrocyte 2 (DEC2) is a basic helix-loop-helix-Orange transcription factor that regulates cell differentiation in various mammalian tissues. DEC2 has been shown to suppress the differentiation of mesenchymal stem cells (MSCs) into myocytes and adipocytes. In the present study, we examined the role of DEC2 in the chondrogenic differentiation of human MSCs. The overexpression of DEC2 exerted minimal effects on the proliferation of MSCs in monolayer cultures with the growth medium under undifferentiating conditions, whereas it suppressed increases in DNA content, glycosaminoglycan content, and the expression of several chondrocyte-related genes, including aggrecan and type X collagen alpha 1, in MSC pellets in centrifuge tubes under chondrogenic conditions. In the pellets exposed to chondrogenesis induction medium, DEC2 overexpression downregulated the mRNA expression of fibroblast growth factor 18, which is involved in the proliferation and differentiation of chondrocytes, and upregulated the expression of *p16INK4*, which is a cell cycle inhibitor. These findings suggest that DEC2 is a negative regulator of the proliferation and differentiation of chondrocyte lineage-committed mesenchymal cells.

Introduction

Differentiated embryo chondrocyte 2 (DEC2; also known as SHARP-1 or BHLHE41) is a member of the basic helix-loop-helix-Orange (bHLH-O) family, characterized by a basic DNA binding domain, a helix-loop-helix (HLH) dimerization domain, and Orange extended dimerization domain (1,2). DEC2 is a transcriptional repressor similar to other bHLH-O proteins such as HES and HESR (3,4). The DEC2 homodimer directly binds to the E-box sequence (CACGTG) and represses the transcription of target genes through the recruitment of a corepressor, histone deacetylase 1 (3,5). DEC2 also antagonizes the transcriptional activity of other transcription factors, including BMAL1 and SREBP-1, by direct protein-protein interactions and/or by competing for binding to the E-box (5,6). Furthermore, DEC2 modulates various biochemical processes, including circadian rhythm (5,7-9), cancer metastasis (10,11), proliferation (12-16) and differentiation (3,4,17,18).

DEC2 is ubiquitously expressed in varying amounts in both embryonic and adult tissues (1,19). The expression level of *DEC2* is high in developing limbs, muscle and brain. In our preliminary experiments, *DEC2* was expressed at higher levels in mesenchymal stem cells (MSCs) than in fibroblasts (data not shown). MSCs have the capacity to differentiate into a number of types of cells including adipocytes, chondrocytes, osteoblasts and smooth muscle cells (20). Previous studies have shown that DEC2 suppresses myogenesis and adipogenesis of MSCs by inhibiting the transcriptional activities of MyoD and C/EBP, respectively (3,4,17). These observations suggest that DEC2 may be involved in maintaining MSCs in an undifferentiated state. However, whether DEC2 modulates differentiation of MSCs into osteoblasts or chondrocytes remains unclear.

In preliminary experiments, the overexpression of DEC2 in human MSCs suppressed chondrogenic differentiation, whereas

it had a marginal effect on osteogenic differentiation (data not shown). Therefore, we focused on the role of DEC2 in chondrogenic differentiation of MSCs. The results revealed that DEC2 is a negative regulator for the proliferation and differentiation of chondrocyte lineage-committed MSCs, although it has little effect on the proliferation of undifferentiated MSCs. We also examined the effects of DEC2 overexpression on the expressions of fibroblast growth factors (FGFs) and cell cycle-related genes, as these genes play important roles in the proliferation and/or differentiation of chondrogenic cells (21,22).

Materials and methods

Cells. Human iliac bone marrow MSCs were obtained from RIKEN (Tsukuba, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1 ng/ml FGF2 (Kaken Pharmaceutical, Tokyo, Japan) and 1% antibiotic-antimycotic solution (Sigma) at 37°C in 5% CO_2. MSCs harvested at the 4th-6th passage were used in the experiments.

Chondrogenic differentiation in pellet cultures. The MSCs were centrifuged at 500 x g for 5 min in 15-ml conical polypropylene tubes to form pellets (2.5×10^5 cells/pellet). The pellets were cultured at 37°C in a humidified atmosphere of 5% CO_2 for 3 days, with 0.5 ml chondrogenesis induction medium consisting of α-minimum essential medium (α-MEM) supplemented with 100 nM dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, 4.5 mg/ml D-glucose (all from Sigma), 100 µg/ml sodium pyruvate (Wako Pure Chemical, Osaka, Japan), 1% ITS+ Premix (Thermo Fisher Scientific, Waltham, MA, USA), and 10 ng/ml TGF-β3 (Peprotech, Rocky Hill, NJ, USA). Thereafter, the cells were fed every other day with 1 ml fresh medium.

Low-density monolayer cultures. The MSCs were seeded at a low density (700 cells/cm²) on 6-well plates for RNA isolation or 96-well plates for the proliferation assay and incubated with DMEM supplemented with 10% FBS at 37°C in a CO_2 incubator.

Construction of an adenoviral expression vector. The recombinant adenovirus containing *Dec2* (Ad-Dec2) was constructed using the Adeno-X Expression System (Clontech, Mountain View, CA, USA). Full-length mouse *Dec2* (mDec2) cDNA (1) was inserted into the *Nhe*I and *Xba*I sites of the pShuttle vector to generate the expression cassette under the regulation of the cytomegalovirus promoter. The expression cassette was ligated to Adeno-X viral DNA. The adenoviral vector pAd-Dec2 was digested with *Pac*I and then transfected into 293 cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA). The resulting adenoviruses were further propagated in 293 cells and purified using the Adeno-X™ purification kit (Clontech). Viral titers were determined using the Adeno-X™ rapid titer kit (Clontech). The recombinant adenovirus containing lacZ (Ad-LacZ) was prepared as a negative control (23). MSCs were mock-infected or infected with Ad-Dec2 or Ad-LacZ at the indicated multiplicity of infection (MOI) for 2 h.

Western blot analysis. MSCs were infected with Ad-Dec2 at the indicated MOI (control: MOI =0), and then incubated in DMEM supplemented with 10% FBS. The cells were lysed with Laemmli buffer, and the lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated overnight with rabbit antibodies specific for DEC2 (1:2,000 dilution at 4°C). After washes with TBST buffer (25 mM TBS, 0.05% Tween-20, pH 7.4), the membrane was incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (1:2,000 dilution at room temperature; Dako, Carpinteria, CA, USA). The antibody-bound bands were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Nacalai Tesque, Kyoto, Japan). The anti-DEC2 antibodies were produced by immunizing synthetic peptide fragment Cys-Asn-Pro-Glu-Ser-Ser-Gln-Glu-Asp-Ala-Thr-Gln-Pro-Ala. The obtained antibodies were purified by affinity column chromatography.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. MSCs were cultured for 2 days after infection with Ad-LacZ at the indicated MOI. The transduction efficiency of MSCs with the adenovirus was estimated by staining with X-gal (Wako Pure Chemical). The cells were fixed with phosphate-buffered saline (PBS) containing 2% formaldehyde and 0.2% glutaraldehyde (both from Wako Pure Chemical) for 5 min, washed twice with PBS and incubated in X-gal staining solution overnight at 37°C. After rinsing in water, images of the cells were captured using an Olympus IX70 microscope and an Olympus DP20 camera (Olympus, Tokyo, Japan).

Proliferation in monolayer culture. Following mock infection or infection with Ad-Dec2 or Ad-LacZ at the indicated MOI, MSCs were seeded at a low density (700 cells/cm²) on 96-well plates, and incubated with DMEM supplemented with 10% FBS at 37°C in a CO_2 incubator for 4 days. Cell proliferation was estimated with a Cell Counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) using WST-8 according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Chondrocyte pellets were frozen in liquid nitrogen and crushed using an SK-Mill (Tokken, Chiba, Japan). Total RNA was prepared using an RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed with ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The synthesized First-Strand cDNA was used for PCR amplification with specific primers and TaqMan probe sets (Table I). Quantitative PCR analyses were performed using an ABI PRISM 7900HT Sequence Detection System instrument and software (Applied Biosystems Inc., Foster City, CA, USA) based on the ΔΔCq method (24). Data were normalized against 18S rRNA levels. The PCR cycling conditions included an incubation of 50°C for 2 min and a denaturation of 95°C for 10 min, which was followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min (25).

Toluidine blue staining. In order to estimate the extent of proteoglycan accumulation in the pellets, the diameter of the pellets was measured using a ruler, and toluidine blue staining was performed. The pellets were fixed in 10% buffered formalin, and embedded in paraffin. Sections of 5 mm were prepared and stained with toluidine blue (26). The sections

Table I. Primer and probe sequences used for RT-qPCR.

Gene	Primer (5'→3')	Probe (5'→3')
ACAN	F: AGTATCATCAGTCCCAGAATCTAGCA R: GGAATGCAGAGGTGGTTTCAC	AGACGTCCGCCTATCCTGAAGCTG
ALPL	F: AGACGTCCGCCTATCCTGAAGCTG R: GCCATACAGGATGGCAGTGA	CCCCATGCTGAGTGACACAGACAAGAA
COL2A1	F: GAGACAGCATGACGCCGAG R: GGCTGCGGATGCTCTCAAT	ATGCCACACTCAAGTCCCTCAACAACCA
COL10A1	F: CTAGTATCCTTGAACTTGGTTCATGGA R: ACTGTGTCTTGGTGTTGGGTAGTG	CGCTGAACGATACCAAACGCCCAC
OSX	F: ATGAGCTGGAGCGTCATGTG R: AGGTGGTCGCTTCGGGTAA	TCACCTGCCTGCTCTGCTCCAAGC
hDEC2	F: GCATCAGAAGATAATTGCTTTACAGAA R: TCTCAAACCGGGAGAGGTATTG	CGTTCCACTCGGGATTTCAAACATGC
mDec2	F: ATTGCTTTACAGAATGGGGAGCG R: AAAGCGCGCGAGGTATTGCAAGAC	CGACTTGGATGCGTTCCACTCGG
FGF2	F: TTCTTCCTGCGCATCCAC R: TGCTTGAAGTTGTAGCTTGATGT	Roche Universal Probe #7
FGF7	F: GCAAAGTAAAAGGGACCCAAG R: TCACTTTCCACCCCTTTGAT	Roche Universal Probe #59
FGF18	F: ATGAACCGCAAAGGCAAG R: GAACACACACTCCTTGCTGGT	Roche Universal Probe #60
Cyclin D1	F: GAAGATCGTCGCCACCTG R: GACCTCCTCCTCGCACTTCT	Roche Universal Probe #67
p21	F: TCACTGTCTTGTACCCTTGTGC R: GGCGTTTGGAGTGGTAGAAA	Roche Universal Probe #32
p16INK4	F: GTGGACCTGGCTGAGGAG R: CTTTCAATCGGGGATGTCTG	Roche Universal Probe #34

ACAN, aggrecan; ALPL, alkaline phosphatase liver/bone/kidney; COL2A1, type II collagen alpha 1; COL10A1, type X collagen alpha 1; OSX, osterix; hDEC2, human differentiated embryo chondrocyte 2; mDec2, mouse differentiated embryo chondrocyte 2; FGF, fibroblast growth factor; F, forward; R, reverse.

were then examined with an Olympus IX70 microscope and an Olympus DP20 camera.

Quantification of glycosaminoglycan (GAG) and DNA content. The pellets were washed twice with phosphate-buffered saline (PBS) and digested with 0.3 mg/ml papain (Wako Pure Chemical) in 50 mM phosphate buffer, pH 6.5, containing 2 mM EDTA and 2 mM N-acetyl-cysteine (Wako Pure Chemical) at 60°C. The papain-digested extracts were assayed for GAG and DNA content. Sulfated GAG content was quantified using the Blyscan™ Sulfated Glycosaminoglycan assay kit (Biocolor, Newtownabbey, UK) according to the manufacturer's instructions. The DNA content of the pellets was determined using the Quant-iT™ PicoGreen dsDNA assay kit (Thermo Fisher Scientific) with lambda DNA as a standard.

Statistical analysis. All experiments were performed at least in triplicate. Data are expressed as the means ± standard devia-tion (SD). One-way ANOVA followed by Dunnett's post hoc test was performed to determine the significance of differences in multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Endogenous expression of DEC2 during chondrogenic differentiation of MSCs. To examine the role of DEC2 in chondrocyte differentiation, we examined the changes in the expression levels of *DEC2* and the main chondrocyte marker genes, namely aggrecan (*ACAN*), alkaline phosphatase liver/bone/kidney (*ALPL*), type II collagen alpha 1 (*COL2A1*), type X collagen alpha 1 (*COL10A1*) and osterix (*OSX*) in MSC pellet cultures after exposure to chondrogenesis induc-tion medium (Fig. 1). The mRNA levels of *COL2A1*, *OSX* and *ACAN* started to increase on day 7, and then those of *ALPL* and *COL10A1* started to increase on day 14. The expression
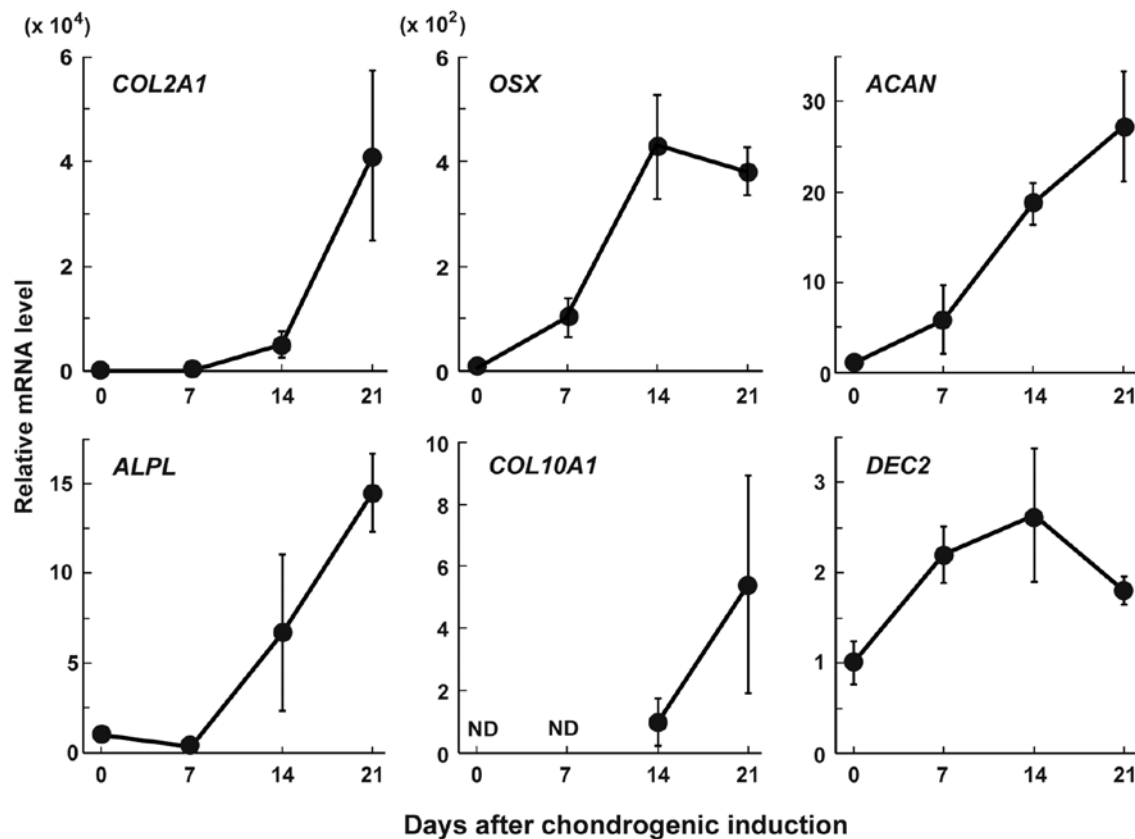
Figure 1. Time course of gene expression of chondrocyte marker genes and differentiated embryo chondrocyte 2 (*DEC2*) during chondrogenic differentiation of mesenchymal stem cells (MSCs). MSCs in pellet cultures were exposed to chondrogenesis induction medium. On the indicated days after the induction of differentiation, total RNA was extracted from the MSC pellets, and the mRNA levels of chondrocyte marker genes (*COL2A1*, *OSX*, *ACAN*, *ALPL* and *COL10A1*) and *DEC2* were determined by RT-qPCR. Data represent the means ± SD of fold-changes relative to expression of each mRNA on day 0, except for *COL10A1* (n=3). The expression level of *COL10A1* mRNA is relative to that on day 14 because no mRNA was detected on days 0 and 7. ND, not detected.

levels of these mRNAs, with the exception of that of *OSX*, continued to increase at least until day 21. *DEC2* mRNA expression started to increase on day 7 and then decreased on day 21.

Effect of DEC2 overexpression on MSC proliferation in monolayer cultures. MSC monolayer cultures were infected with Ad-Dec2. The DEC2 protein levels successfully increased in an MOI-dependent manner up to an MOI of 500 in MSCs infected with Ad-Dec2 (Fig. 2A), and a high level of DEC2 expression in MSCs was maintained until at least 7 days after infection with Ad-Dec2 (data not shown). In similar cultures, the transduction efficiency of the adenovirus was determined with Ad-LacZ. It increased the intensity of β-galactosidase staining in an MOI-dependent manner, and >90% of the cells showed positive staining for β-galactosidase at an MOI of 500 (Fig. 2B), suggesting that this method allows the over-expression of DEC2 in most MSCs.

To examine the effect of DEC2 overexpression on cell proliferation, MSCs were infected with either Ad-Dec2 or Ad-LacZ at various MOI levels and incubated at a low density with DMEM supplemented with 10% FBS. On day 4, there was no significant difference with regard to proliferation between Ad-Dec2- and Ad-LacZ-infected cells, and infection with Ad-Dec2 or Ad-LacZ did not show cytotoxicity up to an MOI of 500 (Fig. 2C).

Effects of DEC2 overexpression on extracellular matrix formation during chondrogenesis. MSCs were infected with either Ad-Dec2 or Ad-LacZ at an MOI of 500 and then exposed to chondrogenesis induction medium in pellet cultures. The size of these pellets gradually increased after the onset of differentiation (Fig. 3A). However, pellets of Ad-Dec2-infected cells were smaller than those of Ad-LacZ- or mock-infected cells. Further, the diameter of Ad-Dec2-infected cell pellets was significantly smaller than that of Ad-LacZ- or mock-infected cell pellets on day 28 (Fig. 3B). There were no differences between the diameters of Ad-LacZ- and mock-infected cell pellets.

To estimate the extent of proteoglycan accumulation in the pellets, we performed toluidine blue staining. On days 21 and 28, proteoglycan deposition (characterized by a red-purple color) was observed at high levels in the Ad-LacZ- and mock-infected pellets; however, it was scarcely detected in the Ad-Dec2-infected pellets (Fig. 3C). Microscopic analysis also indicated the accumulation of abundant proteoglycans and typical lacunae structures around chondrocytes in both the Ad-LacZ- and mock-infected pellets on days 21 and 28, whereas the proteoglycan accumulation and lacunae structures were scarcely observed in the Ad-Dec2-infected pellets.

We subsequently quantified the total GAG content per pellet (Fig. 4A), along with the GAG content per microgram DNA on day 21 (Fig. 4B and C). The total GAG content and GAG/DNA of the Ad-Dec2-infected pellets were lower than that
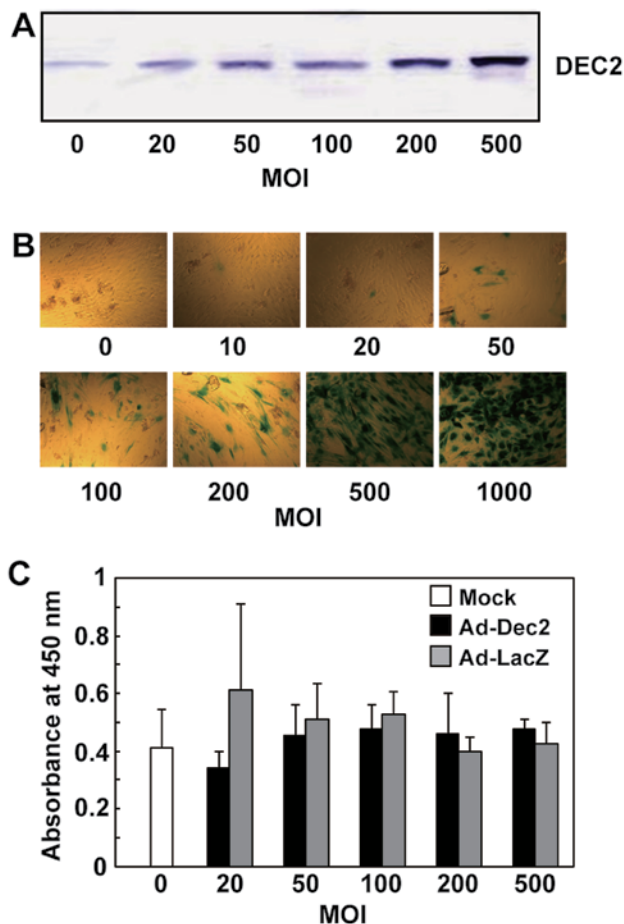
Figure 2. Effect of differentiated embryo chondrocyte 2 (DEC2) overexpression on the proliferation of mesenchymal stem cells (MSCs) in monolayer culture. (A) Confluent monolayers of MSCs were infected with Ad-Dec2 at the indicated multiplicity of infection (MOI) and the expression levels of DEC2 were determined by western blot analysis using anti-DEC2 antibody. (B) Confluent monolayers of MSCs were infected with adenovirus containing lacZ (Ad-LacZ) at the indicated MOI and the expression levels of LacZ were determined by staining with X-gal (magnification, x200). (C) MSCs were mock-treated, or infected with either Ad-Dec2 or Ad-LacZ at the indicated MOI, and then seeded at a low density (700 cells/cm^2) on 96-well plates. After 4 days of culture, cell proliferation was measured using the WST-8 assay. Data represent the means ± SD (n=3).

in the Ad-LacZ- and mock-infected pellets (Fig. 4A and C). In addition, a significant decrease in the DNA content per pellet was observed in the Ad-Dec2-infected pellets in comparison with the Ad-LacZ-infected pellets, indicating the suppression of cell growth by DEC2 (Fig. 4B). By contrast, there were no significant differences between the Ad-LacZ- and mock-infected pellets with respect to the GAG content, DNA content and GAG/DNA.

Effects of DEC2 overexpression on the expression of chondrocyte-related genes. We examined the effects of DEC2 overexpression on the expression of chondrocyte-related genes in the pellet cultures. DEC2 overexpression suppressed the expression of *ACAN* and *COL10A1*, but not the expression of *OSX* and *ALPL*, on day 28 (Fig. 5A). The inhibitory effect of DEC2 on *ACAN* and *COL10A1* expression was not observed on day 7, suggesting that DEC2 may indirectly suppress the expression of the two chondrocyte genes. We also examined the expression levels of *Dec2* in DEC2-overexpressing MSC

pellets. The *mDec2* mRNA level was ~80-fold higher than the endogenous human expression on day 7, and the high-level expression of *mDec2* decreased to less than one tenth on day 28 (Fig. 5A), suggesting that the infection with Ad-Dec2 affects the chondrogenic potential of MSCs in the early stage.

Because FGFs play an important role in the control of proliferation and differentiation of chondrocytes (21,22,27), we also examined whether DEC2 affected the expression of some FGFs. DEC2 overexpression strongly suppressed the expression of *FGF18*, but not that of *FGF2* and *FGF7*, on days 1 and 7 (Fig. 5B).

Effects of DEC2 overexpression on the expression of cell cycle-related genes. The regulation of cell cycle progression is closely associated with cell proliferation and differentiation (28). Therefore, we examined whether DEC2 overexpression in MSCs altered the expression levels of the cell cycle-related genes, namely cyclin D1, p21 and p16INK4. Cyclin D1 acts as a positive regulator of cell cycle progression, whereas p21 and p16INK4 negatively regulate the cell cycle by inhibiting cyclin-dependent kinases (CDKs) (28). The mRNA expression of p16INK4 was upregulated on day 1 by DEC2 overexpression in the MSC pellets exposed to chondrogenesis induction medium but not in the monolayer cultures exposed to growth medium (Fig. 5C and D). In the pellets, DEC2 overexpression also increased the average level of p21 and decreased cyclin D1 expression levels, although the observed differences were not statistically significant (Fig. 5C). These findings suggest that DEC2 suppresses cell cycle progression of the mesenchymal cells in pellet cultures exposed to the differentiation induction medium by regulating the expression of cell cycle-related genes in a stage-dependent manner.

Discussion

In this study, we demonstrated that DEC2 acts as a negative regulator of chondrogenic differentiation. The forced expression of DEC2 resulted in the inhibition of both cell proliferation and GAG accumulation during chondrogenesis in MSC pellet cultures, whereas in repeated experiments, it did not suppress the proliferation of MSC monolayer cultures. These findings indicate that DEC2 inhibits the proliferation of chondrocyte lineage-committed MSCs, but not undifferentiated MSCs. In pellet cultures of MSCs, the expression of *DEC2* began to increase earlier than that of chondrocyte markers, such as *ALPL* and *COL10A1*, and then decreased in the late differentiation stage, suggesting that DEC2 inhibits chondrogenic differentiation of MSCs in premature stage, and may delay the maturation of chondrocytes.

DEC2 suppresses the proliferation of various types of cells, including human lung cancer cell lines (A549, NCI-H520 and NCI-H596 cells), human epidermoid carcinoma cells (HEp3), human mammary epithelial cells (HMECs), and NIH3T3 cells (12-15), although DEC2 has little effect on the proliferation of HepG2 cells (13). DEC2-induced cell growth arrest is reportedly associated with decreased *cyclin D1* expression in C2C12 and NCI-H520 cells as well as HMECs (4,13,14). Consistent with these findings, the DEC2-mediated inhibition of proliferation in MSC pellet cultures was associated with a decrease in the average *cyclin D1* levels and an increase in the average *p16INK4* and *p21* levels. However, DEC2 exerted
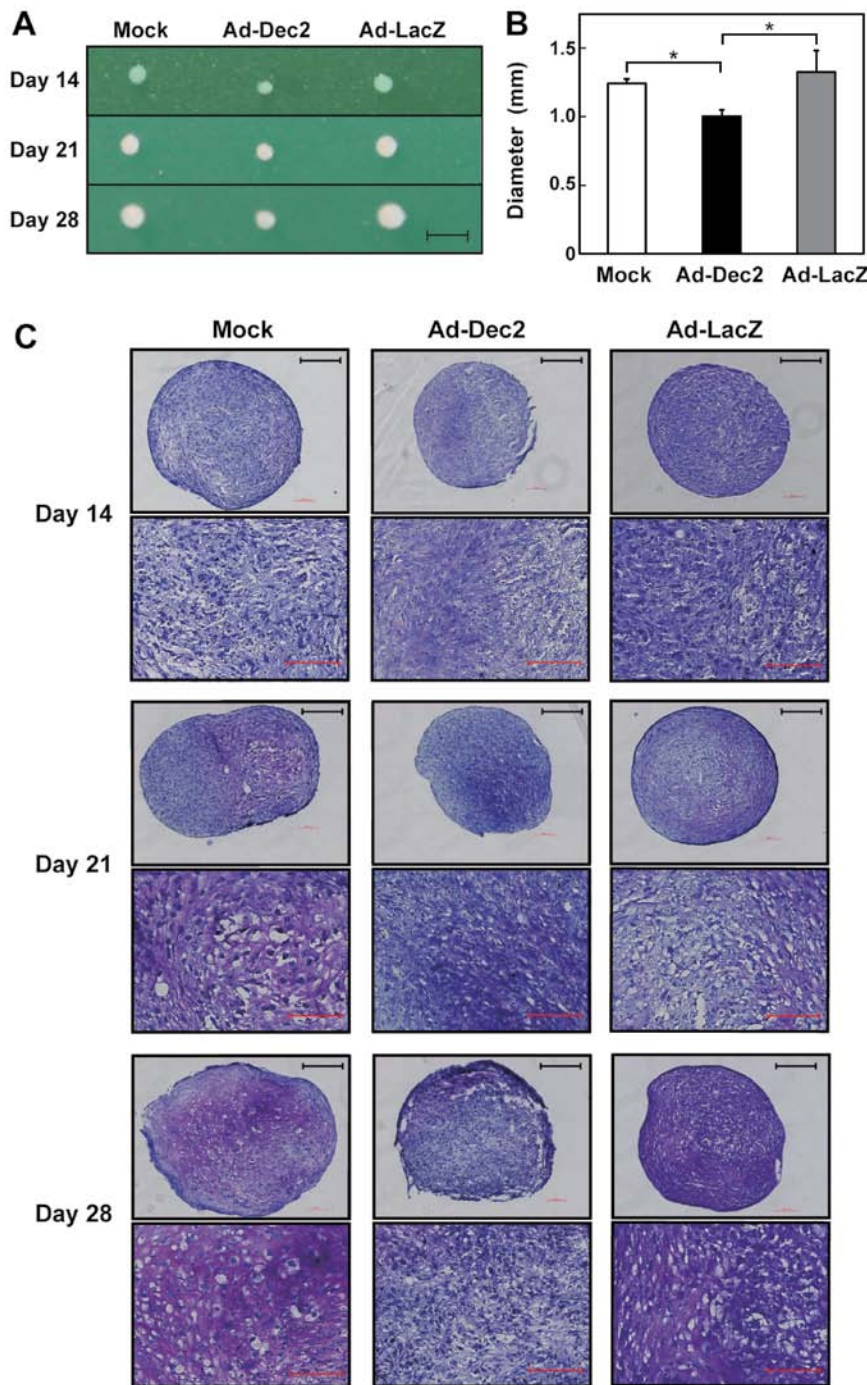
Figure 3. Morphological and histological analysis of chondrogenic pellet cultures of mesenchymal stem cells (MSCs) overexpressing differentiated embryo chondrocyte 2 (DEC2). Following mock infection or infection with Ad-Dec2 [multiplicity of infection (MOI) of 500] or adenovirus containing lacZ (Ad-LacZ) (MOI of 500), MSCs were induced to differentiate into chondrocytes. The extent of chondrogenic differentiation was evaluated by the following morphological and histological analysis. (A) Gross observation of MSC pellets on days 14, 21 and 28 after chondrogenic induction. Scale bars, 2 mm. (B) Size measurement (diameter) of MSC pellets on day 28 after chondrogenic induction. Data represent the means ± SD (n=3). *P<0.05. (C) Toluidine blue staining of MSC pellets on days 14, 21 and 28 after chondrogenic induction. Overview (upper panels) and magnified (lower panels) images of the pellets are shown. Upper black scale bars, 200 μm; lower red scale bars, 100 μm. The results presented are typical of three independent experiments.

minimal effects on MSC proliferation, and the expression of *p16INK4* in the undifferentiated state in low-density mono-layer cultures. Taken together, these findings suggest that the growth inhibitory effect of DEC2 depends upon cell type, cell density, and/or differentiation stage. However, we have not examined the effect of DEC2 on the proliferation and differentiation of other tissue-derived MSCs, including adipose tissue-derived MSCs and umbilical cord-derived MSCs as

bone marrow-derived MSCs have been most widely used in studies of chondrogenesis. This issue warrants further investigation in the future using the other tissues-derived MSCs.

FGF signaling has been implicated in the regulation of endochondral bone growth (21,22). Even though FGF3 receptor (FGF3R) activation suppresses the terminal differentiation of growth plate chondrocytes, FGF18, a ligand of FGF3R, may exert a positive effect on the proliferation and
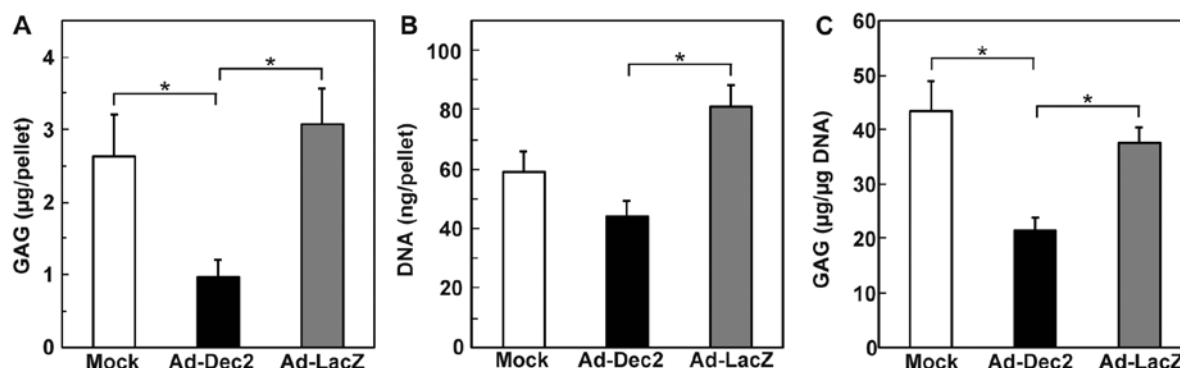
Figure 4. Inhibition of glycosaminoglycan (GAG) synthesis and proliferation of mesenchymal stem cells (MSCs) by the overexpression of differentiated embryo chondrocyte 2 (DEC2). Following mock infection or infection with Ad-Dec2 [multiplicity of infection (MOI) of 500] or adenovirus containing lacZ (Ad-LacZ) (MOI of 500), MSCs were induced to differentiate into chondrocytes. Pellets cultured for 21 days were analyzed for (A) GAG content per pellet, (B) DNA content per pellet and (C) GAG content per microgram of DNA. Data represent the means ± SD (n=3). *P<0.05.
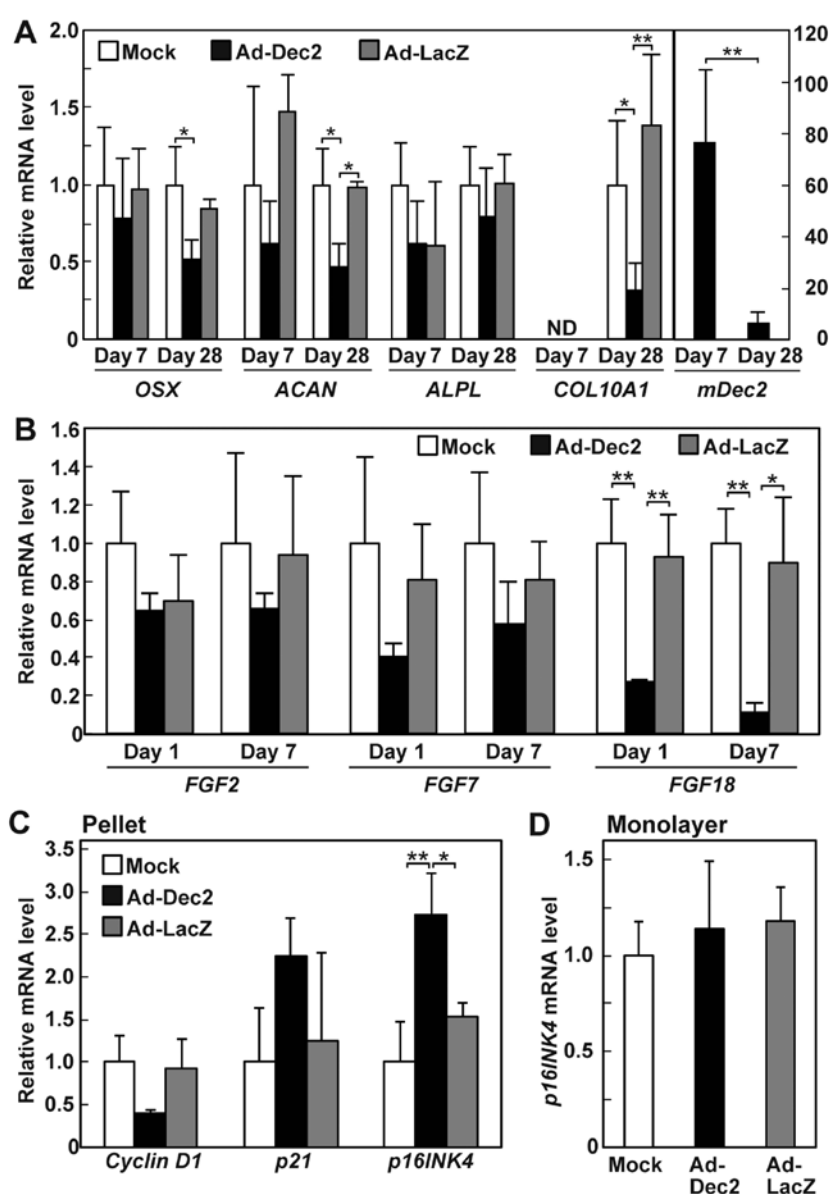

Figure 5. Effects of overexpression of differentiated embryo chondrocyte 2 (DEC2) on the expression of chondrocyte marker genes and cell cycle-related genes in mesenchymal stem cells (MSCs). Following mock infection or infection with Ad-Dec2 [multiplicity of infection (MOI) of 500] or adenovirus containing lacZ (Ad-LacZ) (MOI of 500), MSCs were (A-C) maintained in pellet or (D) monolayer cultures. (B-D) On days 1, (A and B) day 7 and (A) day 28, total RNA was extracted from MSCs, and the mRNA levels of (A) chondrocyte marker genes and mouse $Dec2$ ($mDec2$), (B) fibroblast growth factors (FGFs), (C) cell cycle-related genes and (D) p16INK4 were determined by RT-qPCR. Data represent the means ± SD fold-changes relative to expression in mock-infected cells, except for m$Dec2$ (n=3). The mRNA expression of m$Dec2$ is relative to that of endogenous human $DEC2$ on day 7. ND, not detected. *P<0.05 and **P<0.01.

differentiation of immature committed chondrocytes (29-32). *FGF18* knockout (*FGF18*⁻/⁻) mice exhibited decreased chondrocyte proliferation activity and a delay in the initiation of chondrocyte hypertrophy at the embryonic stage (29). We found that DEC2 overexpression strongly represses the expression of FGF18. Although the role of FGF18 in cartilage development remains controversial, the signaling may be associated with DEC2-mediated suppression of proliferation and/or differentiation of chondrocyte-lineage committed MSCs.

FGF18 is also involved in cartilage angiogenesis through the upregulation of vascular endothelial growth factor (VEGF) (29). By contrast, DEC2 represses the expression of *Vegf*, resulting in the reciprocal expression of *Dec2* and *Vegf* in mouse rib cartilage (33). Therefore, DEC2 and FGF18 appear to exert opposite effects on vasculogenesis as well as chondrogenesis.

DEC1 and DEC2 are structurally similar, but they often have different functions. For example, DEC1 has pro-apoptotic effects, whereas DEC2 has anti-apoptotic effects on TNF-α-induced apoptosis in MCF-7 cells (34). Furthermore, DEC2, but not DEC1, suppresses the expression of *Vegf* through binding with HIF1α in NIH3T3 cells (33). Previous findings have shown that the overexpression of DEC1 promotes chondrogenic differentiation of rabbit MSCs and mouse ATDC5 cells (23). Therefore, DEC1 and DEC2 seem to have opposite actions on chondrogenesis. Besides direct DNA binding, DEC1 and DEC2 modulate gene expression through protein-protein interactions with common and distinct transcription factors (3,5,6,8,10,17,33). Identifying the partners of DEC2 will clarify how DEC2 regulates the chondrogenic differentiation of MSCs.

In conclusion, we have shown that DEC2 is involved in the suppression of proliferation and differentiation of chondrocyte lineage-committed MSCs. DEC2 inhibits the proliferation of MSCs by regulating the expression of cell cycle-related genes under chondrogenic conditions. The decreased proliferation of MSCs may decrease their subsequent differentiation into chondrocytes. An alternative possibility is that DEC2 suppresses chondrogenic differentiation by downregulating FGF18 independently of MSC proliferation. Besides inhibition of chondrocyte differentiation, DEC2 suppresses the differentiation of MSCs into adipocytes (17) and myoblasts (3,4), suggesting that DEC2 may be implicated in maintaining MSCs in the undifferentiated state.

Acknowledgements

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