Effects of overexpression of basic helix-loop-helix transcription factor Dec1 on osteogenic and adipogenic differentiation of mesenchymal stem cells

Tomoyuki Iwata[a,b], Takeshi Kawamoto[a,*], Eri Sasabe[a], Kazuko Miyazaki[a], Katsumi Fujimoto[a], Mitsuhide Noshiro[a], Hidemi Kurihara[b] and Yukio Kato[a]

[a]*Department of Dental and Medical Biochemistry, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Hiroshima 734-8553, Japan*

[b]*Department of Periodontal Medicine, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Hiroshima 734-8553, Japan*

*Corresponding author. Tel: +81-82-257-5629, fax: +81-82-257-5629.

E-mail address: tkawamo@hiroshima-u.ac.jp

Abstract

We recently reported that forced expression of basic helix-loop-helix transcription factor Dec1 accelerated chondrogenic differentiation of mesenchymal stem cells (MSC) in pellet cultures (M. Shen, E. Yoshida, W. Yan, T. Kawamoto, K. Suardita, Y. Koyano, K. Fujimoto, M. Noshiro and Y. Kato, 2002. J. Biol. Chem. 277:50112-50120). Since MSC have multilineage differentiation potential, we investigated the roles of Dec1 in osteogenic and adipogenic differentiation of human bone marrow-derived MSC. After osteogenic induction of MSC in medium containing dexamethasone, β-glycerophosphate, and ascorbic acid, *Dec1* expression gradually increased from day 5 to day 14, while expression levels of *Dec1* mRNA markedly decreased on days 3 and 7 after adipogenic induction. Infection with adenovirus expressing Dec1 raised mRNA levels of several bone characteristic molecules such as osteopontin, PTH receptor and alkaline phosphatase, even in the absence of the osteogenic-induction medium, although it had little effect on Runx2 expression or calcification. In the osteogenic-induction medium, Dec1 overexpression enhanced the expression of osteopontin and alkaline phosphatase and induced matrix calcification. Knockdown of *Dec1* with siRNA

suppressed the expression of osteoblastic phenotype by the induced MSC. Using MSC cultures, we also confirmed that forced expression of Dec1 suppressed adipogenic differentiation. These findings suggest that Dec1 modulates osteogenic differentiation of MSC by inducing the expression of several, but not all, bone-related genes.

Key words: Dec1; Stra13; Bhlhb2; DEC2; mesenchymal stem cell; osteogenesis; adipogenesis.

Introduction

Basic helix-loop-helix transcription factor Dec1 (Stra13/Bhlhb2), which was previously identified as a differentially expressed transcript in chondrocytes (Shen et al., 1997), can bind to the CACGTG E-boxes and repress transcription from the target genes (Hamaguchi et al., 2004; Honma et al., 2002; Kawamoto et al., 2004; Li et al., 2003; Sato et al., 2004; St-Pierre et al., 2002; Zawel et al., 2002). Overexpression of Dec1 enhanced chondrogenic differentiation of mouse ATDC5 cells and rabbit bone marrow mesenchymal stem cells (MSC) (Shen et al., 2002), but inhibited adipogenic differentiation of mouse 3T3-L1 cells (Yun et al., 2002); stable expression of DEC2 in mouse C2C12 cells inhibited myogenic differentiation by interacting with MyoD (Azmi et al., 2004). Dec1 also plays a part in circadian rhythm regulation by repressing CLOCK/BMAL1-induced promoters (Butler et al., 2004; Hamaguchi et al., 2004; Honma et al., 2002; Kawamoto et al., 2004; Li et al., 2004; Noshiro et al., 2004; Sato et al., 2004). During endochondral bone development in mouse tibia, *Dec1* mRNA expression was observed from E15.5 in post-mitotic hypertrophic chondrocytes, co-localizing with collagen X mRNA (Maclean and Kronenberg, 2004). At E15.5-E18.5,

Dec1 was also expressed in the primary spongiosa, where Dec1 may be involved in primary bone formation.

Since *Dec1* is expressed in various tissues and induced by various growth factors or hypoxia (Boudjelal et al., 1997; Fujimoto et al., 2001; Miyazaki et al., 2002; Rossner et al., 1997; Shen et al., 2001; Shen et al., 1997), it may be involved in the control of differentiation in numerous tissues. Since MSC can give rise to osteoblasts, chondrocytes, myocytes, and adipocytes (Muraglia et al., 2000; Pittenger et al., 1999), these cells may be useful for analysis of lineage determination and the differentiation steps (Matsubara et al., 2004; Pittenger et al., 1999; Tsutsumi et al., 2001). Among various transcription factors involved in osteogenic differentiation, Runx2 plays a central role in bone formation: In Runx2 null mice, osteoblast differentiation was arrested in both the endochondral and intramembranous skeleton (Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). For adipogenic differentiation, the nuclear hormone receptor PPARγ plays a crucial role in the function of many fat cell-specific genes (Rosen and Spiegelman, 2001). However, numerous other regulatory molecules are likely to be involved in the complex processes of

osteogenic and adipogenic differentiation, stage-dependently. To clarify the roles of Dec1 in differentiation of mesenchymal cells, we examined the effects of Dec1 overexpression on osteogenic and adipogenic differentiation of bone marrow MSC: Forced expression of Dec1 up-regulated bone-related gene expression and enhanced calcification, and it suppressed adipogenic differentiation of MSC.

Materials and methods

Cell culture and adenovirus infection

Human MSC were purchased from BioWhittaker (Walkersville, MD) and cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in 5% CO_2 atmosphere. For osteogenic induction, MSC were plated at a density of 1×10^4 cells/cm^2 in 16-mm wells coated with type I collagen. For adipogenic induction, MSC were plated at a density of 2×10^4 cells/cm^2 in 16-mm wells.

Twenty-four hours after seeding the cells, infection of these cells with adenovirus

expressing human Dec1 (ad-Dec1) or adenovirus expressing LacZ (ad-LacZ) was performed at a multiplicity of infection (MOI) of 50 or 100 p.f.u/cell, as described previously (Shen et al., 2002). Adenovirus carrying the LacZ gene was generously supplied by Dr. Kohei Miyazono (The University of Tokyo) (Fujii et al., 1999). Six hours later, the culture medium was replaced with an induction medium suitable for osteogenic or adipogenic differentiation.

Osteogenic induction

Thirty hours after plating, MSC were transferred to the osteogenic induction medium (Os-medium), an α-minimal essential medium (αMEM) supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate (Pittenger et al., 1999). As a control, MSC were incubated in αMEM supplemented with 10% FBS alone (medium-A).

Adipogenic induction

For adipogenic differentiation, MSC were transferred to DMEM supplemented with

10% FBS, 0.1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methyl-xanthine (the adipogenic induction medium) (Pittenger et al., 1999). After 3 days, this medium was replaced with DMEM supplemented with 10% FBS and 10 μg/ml insulin (the adipogenic maintenance medium), and the cells were cultured for 24 h in the new medium. Then, the induction/maintenance cycle for adipogenic induction was repeated.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and subjected to real-time quantitative RT-PCR analysis using ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) with TaqMan probes and primers shown in Table 1. TaqMan probe and primers for PTH receptor and GAPDH were obtained from Applied Biosystems. The values for mRNA levels, relative to internal control GAPDH, represent the mean ± SEM for three wells. The experiments were repeated 2 to 4 times and similar results were obtained each time.

RNA interference

Twenty-four hours after MSC were seeded at a density of 5×10^3 cells/cm^2 in 12-well plates, the cells were transfected with Dec1 siRNA (DHARMACON, CO) or non-silencing control siRNA (QIAGEN, CA) using Trans-IT TKO transfection reagent (Mirus, WI). After 48 h incubation, the culture medium was replaced with Os-medium for osteogenic induction.

ALPase staining

Cells were fixed with 3.5% formaldehyde and stained using ALPase staining kit (Muto pure chemical, Tokyo, Japan). The stained cells were rinsed with deionized water and dried overnight.

Alizarin red and von Kossa staining

Fixed cells were incubated with 1% Alizarin red-S (Sigma, St. Louis, MO) for 1 h at room temperature. Alternatively, fixed cells were exposed to sunlight for 30 min in the presence of 5% silver nitrate, and then incubated with 5% sodium thiosulfate for 5

min (von Kossa staining). The stained cells were rinsed with deionized water and dried overnight.

Calcium content

After MSC were rinsed with PBS, calcium in the cell cultures was dissolved in 0.1 M HCl at 4°C overnight. Calcium content of each sample was determined using Calcium C kit (Wako pure chemical, Osaka, Japan).

Oil red-O staining

MSC fixed with 3.5% formaldehyde were incubated with 0.3% Oil red-O (Sigma) for 1 h at room temperature. The stained cells were rinsed with deionized water and dried overnight. Lipid drop areas stained with Oil red-O were measured using NIH image program (RSB, NIMH/NIH, Bethesda, MD).

Results

Changes in *Dec1* mRNA levels during osteogenic differentiation of MSC

Osteogenic differentiation of human MSC was induced by Os-medium. After osteogenic induction, *Dec1* mRNA expression began to increase on day 5 and reached a peak on day 14 (Fig. 1A). In these cultures, mRNA levels of ALPase and PTH receptor started to increase on day 2, reaching a peak on day 14 (Fig. 1B and C); osteopontin mRNA level increased on days 14 and 21 (Fig. 1D).

Effect of forced expression of and *Dec1* siRNA on osteogenic differentiation of MSC

To test the effect of Dec1 overexpression in the absence of Os-medium, MSC infected with Dec1-expressing adenovirus (ad-Dec1) were maintained in medium-A. Forced expression of Dec1 elevated osteopontin mRNA levels at MOI 25 or 50 (Fig. 2A) and the increase of osteopontin mRNA levels was significant from day 2 to day 14 compared with infection with LacZ-expressing adenovirus (ad-LacZ) (Fig. 2B). Expression of PTH receptor mRNA was also enhanced from day 5 to day 14, while

Runx2 expression was not significantly changed. In addition, the Dec1 overexpression raised the activity and mRNA levels of ALPase (Fig. 2C). However, calcification was not induced by Dec1 overexpression until day 28 (Fig. 2D).

In further studies, the effect of Dec1 overexpression on bone-related gene expression in MSC cultured in Os-medium was examined. Infection of MSC with ad-Dec1 up-regulated osteopontin mRNA levels from day 2 to day 14 in the presence of Os-medium (Fig. 3A). The Dec1 overexpression enhanced the activity and mRNA expression of ALPase (Fig. 3B). The effect of Dec1 overexprssion on mRNA levels of osteopontin was much greater than that of Os-medium on day 7, and Dec1 overexpression and Os-medium showed a synergistic or additive effect on day 7 or 14 (Fig. 3C). Furthermore, Dec1 overexpression promoted calcification of MSC cultures in Os-medium: The calcium level in the cultures overexpressing Dec1 on day 25 was 5 times as high as that in control cultures expressing LacZ (Fig. 4A). The accelerated calcification was confirmed by Alizarin red or von Kossa staining (Fig. 4B). Moreover, PTH receptor expression induced by Os-medium was attenuated by *Dec1* siRNA on days 5 and 7 (Fig 4), showing the involvement of Dec1 in osteogenic differentiation

process of MSC.

Suppression of adipogenic differentiation of MSC by Dec1 overexpression

To explore the role of Dec1 in adipogenesis, expression levels of *Dec1* during adipogenic differentiation of MSC were examined: After adipogenic induction, mRNA levels for Dec1 markedly decreased on days 3 and 7 (Fig. 5A), but not on day 14, which suggested that the expression of Dec1 at high levels may suppress adipogenic differentiation. To test this hypothesis, we looked at whether Dec1 overexpression would inhibit adipogenic differentiation of MSC: Infection with ad-Dec1 transiently lowered mRNA levels of PPARγ until day 7 (Fig. 5B), and the Dec1 overexpression also suppressed lipid accumulation on days 3 and 7, but not on day 14 (Fig. 5C), although Dec1 mRNA levels remained high on day 14 (Fig. 5D). These results indicate that Dec1 lowers the rate of adipogenic differentiation only in the early stage.

Discussion

Osteogenic differentiation of MSC is inducible in Os-medium containing dexamethasone, β-glycerophosphate, and ascorbic acid (Matsubara et al., 2004; Pittenger et al., 1999; Tsutsumi et al., 2001): ALPase activity and calcium levels increase a week after osteogenic induction, and the differentiation continues to progress at least until day 21. In the present study, we demonstrated that expression of Dec1 – as well as bone-related genes – was up-regulated in the induced MSC. Furthermore, forced expression of Dec1 in uninduced MSC up-regulated the expression of some bone-related proteins – such as osteopontin, PTH receptor and ALPase – and accelerated the osteogenic differentiation and calcification in MSC cultures under osteogenic conditions. Decrease in the Dec1 mRNA level by about 60% in the presence of siRNA resulted in the suppression of PTH receptor, but it did not decrease the level of osteopontin or ALPase under these culture conditions (data not shown). Furthermore, Dec1 overexpression alone did not induce calcification in MSC cultures. Taken together, these observations suggest that Dec1 is not essential for osteogenesis, but is involved in some aspects of the osteogenic differentiation process.

Since Dec1 overexpression had little effect on expression of Runx2 in MSC, the Dec1-induced osteogenesis could not be attributed to the induction of Runx2. In fact, in vivo studies using Runx2-transgenic mice showed that overexpression of Runx2 in osteoblasts inhibited their maturation (Liu et al., 2001), while Runx2 is essential for osteogenic differentiation in the early stage. These findings suggest that Runx2 stimulates or inhibits osteogenic differentiation of MSC stage-dependently. Some other transcriptional regulators must therefore be involved in the differentiation process of MSC: Dec1 could be one of these transcription factors, although Dec1 alone cannot induce the whole osteogenic differentiation program.

A previous study reported that Dec1 functions as an effector for hypoxia-mediated inhibition of adipogenesis via PPARγ suppression: Stable expression of Dec1 resulted in nearly complete inhibition of adipocyte differentiation of a mouse adipogenic cell line – 3T3-L1 cells (Yun et al., 2002). In the present study, we found that expression levels of Dec1 decreased only in the initial stage of adipogenic differentiation of MSC, and that Dec1 overexpression suppressed PPARγ expression only in the initial stage. This result, obtained with the primary MSC, revealed the stage-dependent suppression of

adipogenesis by Dec1.

Since Dec1 stimulates both osteogenesis and chondrogenesis (Shen et al., 2002), it is not involved in lineage determination, but Dec1 may increase or decrease the rate of differentiation when triggered by other transcription factors: Once the lineage is determined, increased Dec1 possibly enhances the differentiation of MSC into osteoblasts or chondrocytes while simultaneously inhibiting their differentiation into adipocytes, while decreased Dec1 may facilitate the onset of adipogenic differentiation in the presence of adipogenic induction factors. Thus, Dec1 may work in co-operation with several transcription factors to regulate the rate of osteogenic, adipogenic or chondrogenic differentiation.

Acknowledgments

This work was supported by grants-in-aid for science from the Ministry of Education, Culture, Sport, Science and Technology of Japan.

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Figure legends

Fig. 1. Changes in mRNA levels of Dec1, ALPase, PTH receptor and osteopontin during osteogenic differentiation of human MSC. Osteogenic differentiation was induced by Os-medium as described in Materials and methods. Total RNA was isolated from the cells on the indicated days after osteogenic induction, and subjected to real-time quantitative RT-PCR analysis. The values represent mRNA levels (mean ± SEM) for Dec1 (A), ALPase (B), PTH receptor (C) or osteopontin (D) relative to GAPDH mRNA levels. P-values were calculated by using the Student's t-test (**P < 0.01, *P < 0.05).

Fig. 2. Effect of Dec1 overexpression on osteopontin, PTH receptor and ALPase expression in MSC in medium-A in non-osteogenic status. (A) Six hours after MSC were infected with ad-Dec1 at MOI of 0, 5, 10, 25, or 50, the culture medium was replaced with Os-medium. Expression levels (mean ± SEM) of osteopontin mRNA were determined after 2-day incubation. (B) After MSC infected with ad-Dec1 or ad-LacZ at

MOI of 50 was cultured in medium-A, mRNA levels (mean ± SEM) for osteopontin, PTH receptor, and Runx2 were measured on the indicated days. (C) ALPase activity on day 14 was demonstrated by staining the cells with ALPase staining kit. Expression levels of ALPase mRNA (mean ± SEM) were also determined. (D) Matrix calcification on day 28 was examined using von Kossa staining. **$P < 0.01$, *$P < 0.05$ (Student's t-test).

Fig. 3. Effect of Dec1 overexpression on osteopontin and ALPase expression in MSC in Os-medium. Six hours after MSC were infected with ad-Dec1 or control ad-LacZ at MOI of 50, the culture medium was replaced with Os-medium. (A) Expression levels of osteopontin mRNA (mean ± SEM) on the indicated days were examined by real-time quantitative RT-PCR analysis. (B) ALPase activity on day 21 was examined by staining the cells with ALPase staining kit. Expression levels of ALPase mRNA (mean ± SEM) were also determined. (C) Comparison of osteopontin mRNA levels in DEC1-overexpressing MSC, MSC induced by Os-medium, and DEC1-overexpressing MSC induced by Os-medium. **$P < 0.01$, *$P < 0.05$ (Student's t-test).

Fig. 4. Effect of Dec1 overexpression on matrix calcification in MSC cultures in Os-medium. (A) After MSC infected with ad-Dec1 or ad-LacZ were cultured in Os-medium for the indicated days, calcium content (mean ± SEM) in the cell layers was determined. (B) Matrix calcification in MSC cultures was shown by Alizarin red or von Kossa staining on day 7 and 28. **P < 0.01, *P < 0.05 (Student's t-test).

Fig. 5. Effect of RNA interference with Dec1 in MSC cultures in Os-medium. After MSC were transfected with Dec1 siRNA or non-silencing control siRNA, the cells were cultured in Os-medium for the indicated days. The values represent mRNA levels (mean ± SEM) for Dec1 and PTH receptor relative to GAPDH mRNA levels. **P < 0.01, *P < 0.05 (Student's t-test).

Fig. 6. Effect of Dec1 overexpression on adipogenic differentiation of MSC. MSC were cultured in the adipogenic induction/maintenance medium as described in Materials and methods. (A) Total RNA was extracted from the cells on the indicated days and

subjected to real-time quantitative RT-PCR analysis to determine endogenous Dec1 mRNA level. (B) Before adipogenic differentiation was induced, MSC were infected with ad-Dec1 or ad-LacZ at MOI of 100. Relative mRNA levels (mean ± SEM) for PPARγ on the indicated days were determined. (C) Lipid accumulation was analyzed using Oil red-O staining. Representative data on day 7 are shown. Percent (mean ± SEM) of lipid drop areas stained with Oil red-O were also determined. (D) To confirm overexpression of Dec1 by ad-Dec1 infection, Dec1 mRNA levels (mean ± SEM) in MSC infected with ad-Dec1 or ad-LacZ were determined after the cells were cultured in the adipogenic medium for the indicated days. $^{**}P < 0.01$, $^{*}P < 0.05$ (Student's t-test).

Table 1. Sequence of TaqMan probes and primers used for quantitative real-time RT-PCR analysis.

Dec1	
	TaqMan probe: 5’-CAAGAGTCCGAAGAACCCCCCACAAAA-3’
	Forward primer: 5’-GAAAGGATCGGCGCAATTAA-3’
	Reverse primer: 5’-CATCATCCGAAAGCTGCATC-3’
ALPase	
	TaqMan probe: 5’-CCCCATGCTGAGTGACACAGACAAGAA-3’
	Forward primer: 5’-CCGTGGCAACTCTATCTTTGG-3’
	Reverse primer: 5’-GCCATACAGGATGGCAGTGA-3’
Osteopontin	
	TaqMan probe: 5’-CCTCCTAGGCATCACCTGTGCCATACC-3’
	Forward primer: 5’-ATGAGAATTGCAGTGATTTGCTTTT-3’
	Reverse primer: 5’-AGAACTTCCAGAATCAGCCTGTTT-3’
PPARγ	
	TaqMan probe: 5’-TCAGGGCTGCCAGTTTCGCTCC-3’
	Forward primer: 5’-GGTGGCCATCCGCATCT-3’
	Reverse primer: 5’-GCTTTTGGCATACTCTGTGATCTC-3’

Fig. 1. Iwata et al.

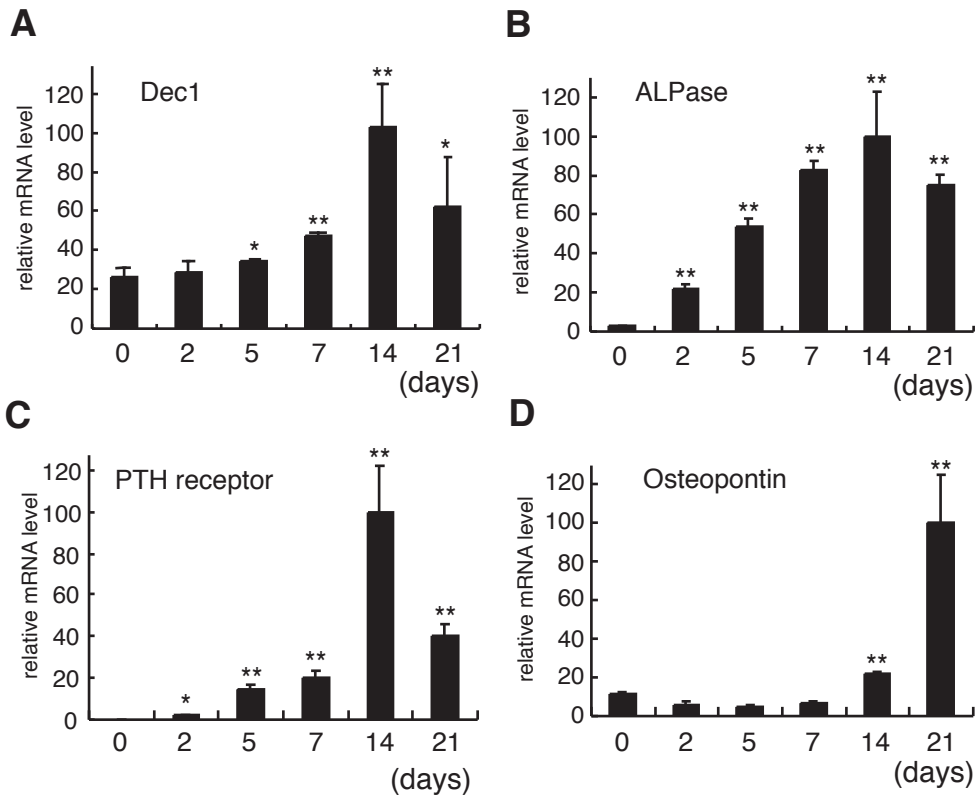
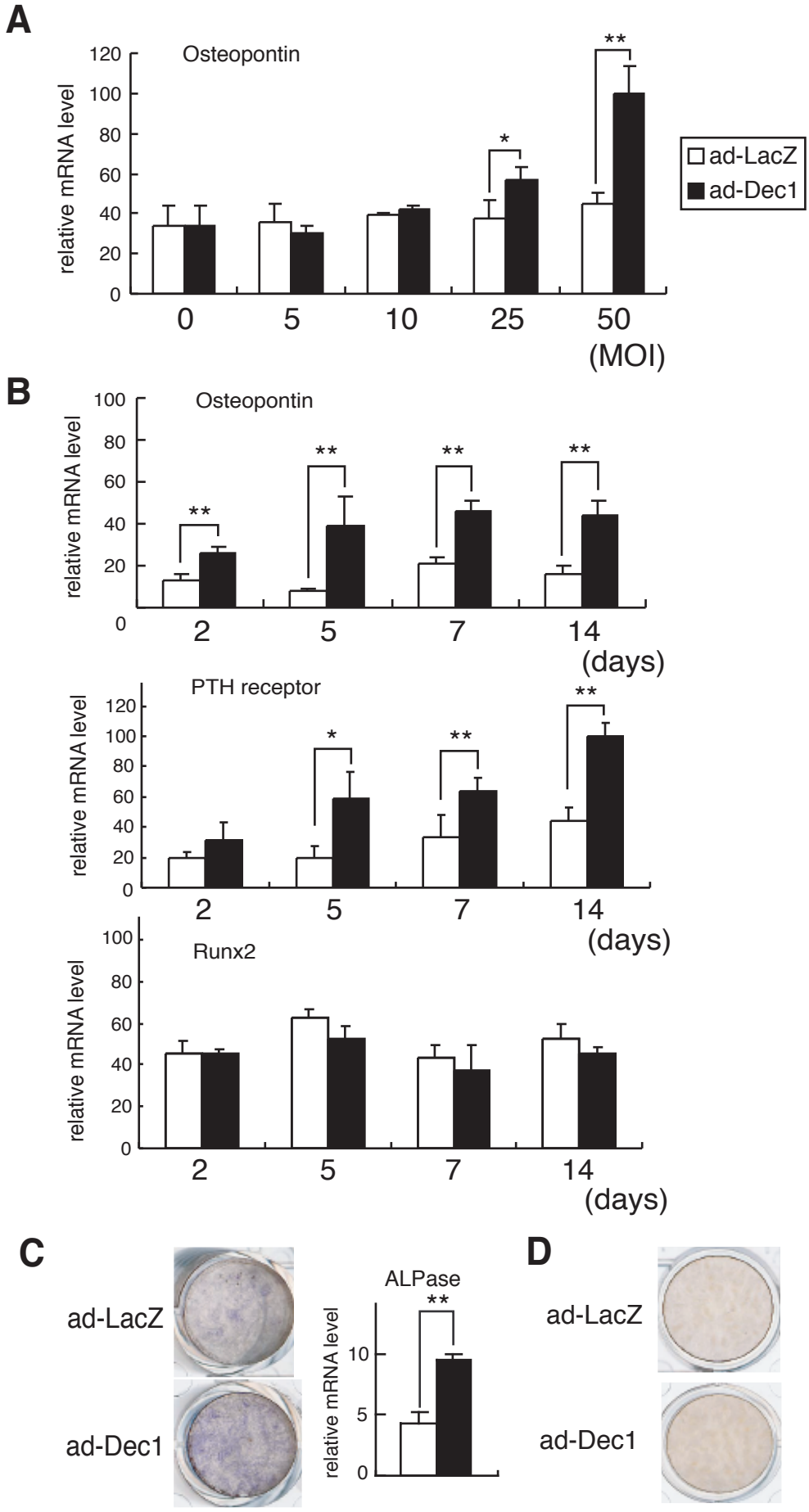


Fig. 2. Iwata et al.



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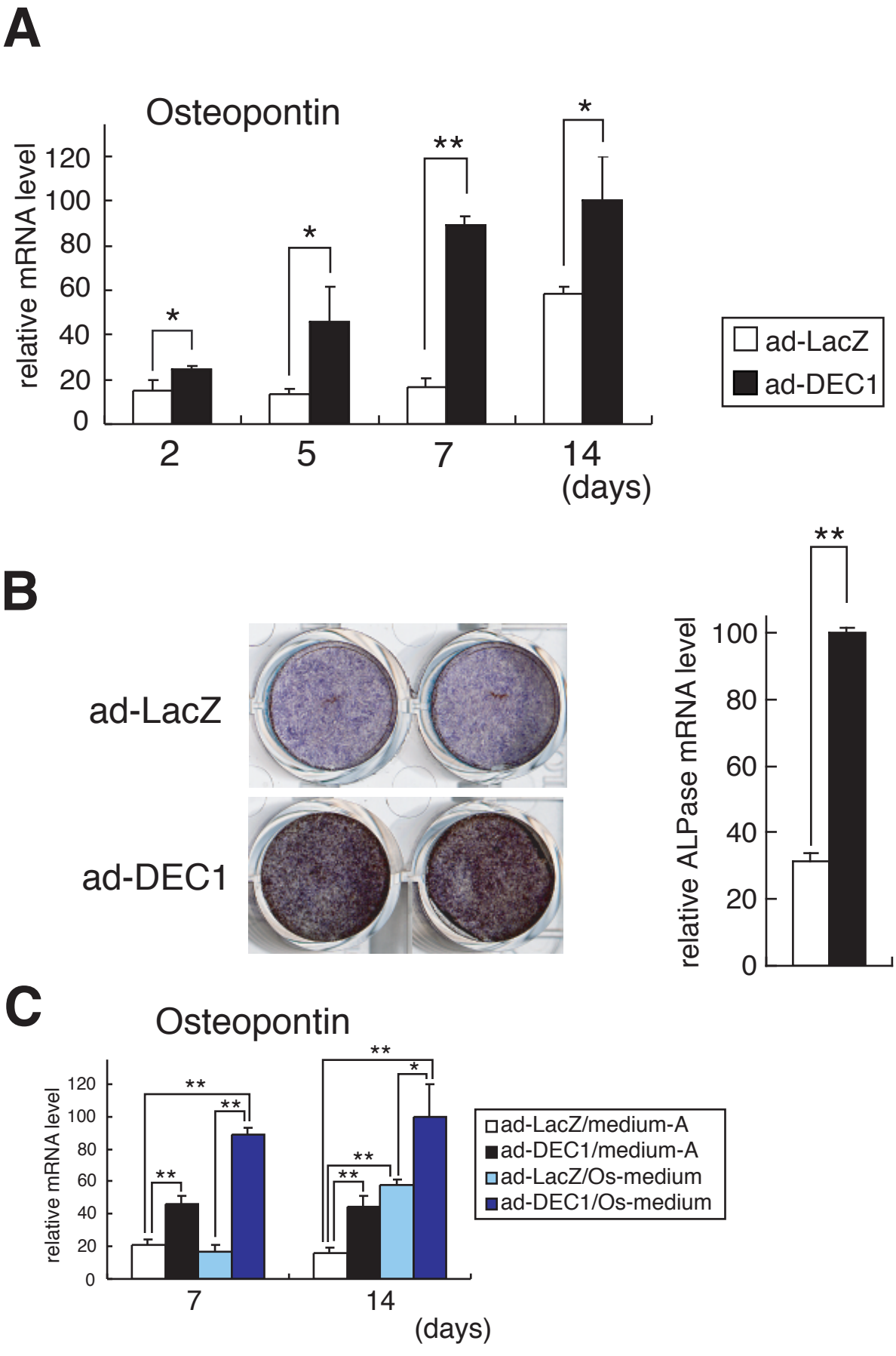

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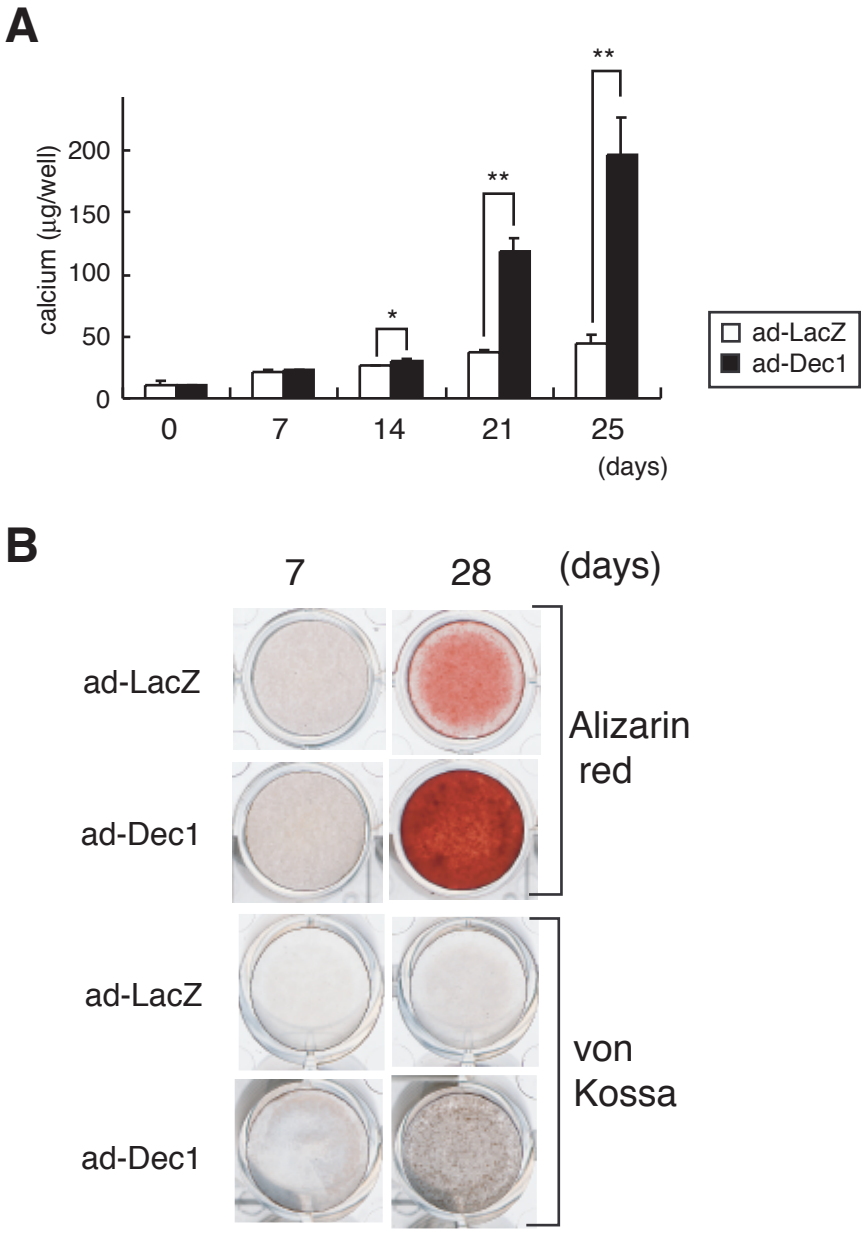

Fig. 5. Iwata et al.

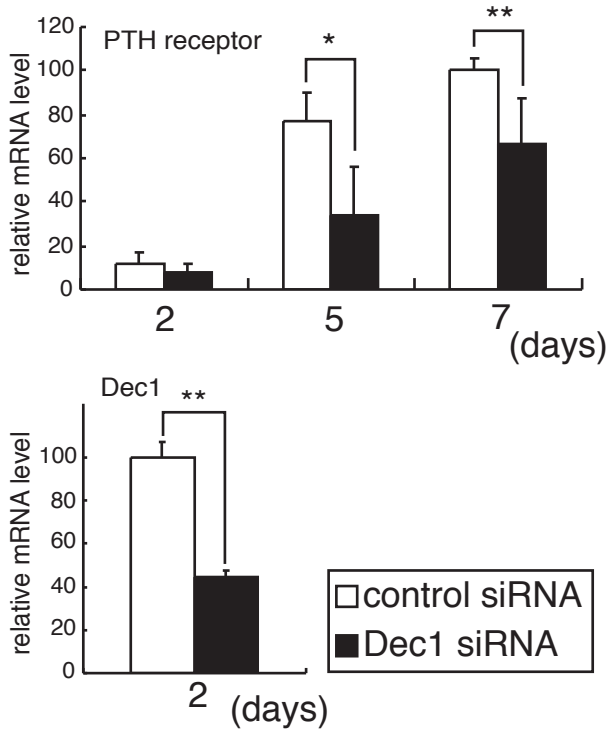

Fig. 6. Iwata et al.