

Expression Analysis of LacZ Gene Placed in the Locus of Cnot7 Exhibits Its Activity in Osteoblasts In Vivo and in Mineralized Nodules In Vitro

Kaoru Washio-Oikawa,^{1,2,3} Takahisa Nakamura,⁶ Michihiko Usui,^{1,2} Mitsuhiro Yoneda,⁶ Youichi Ezura,¹ Isao Ishikawa,^{2,3} Kazuhisa Nakashima,^{1,3} Tadashi Yamamoto,⁶ and Masaki Noda^{1,3,4,5*}

¹Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 101-0062, Japan

²Department of Periodontology, Tokyo Medical and Dental University, Tokyo 101-0062, Japan

³21st Century Center of Excellence (COE) Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo 101-0062, Japan

⁴Integrated Action Initiative, Core-to-Core Program, Tokyo Medical and Dental University, Tokyo 101-0062, Japan

⁵Hard Tissue Genome Research Center, Tokyo Medical and Dental University, Tokyo 101-0062, Japan

⁶Department of Oncology, Institute of Medical Sciences, Tokyo 108-8639, Japan

Abstract CCR4-NOT complex 7 (Cnot7) was identified as a regulator of gene expression in yeast and evolutionally conserved in mammals. Cnot7 deficient male mice exhibit abnormality in spermatogenesis. As these mice contained construct to express *LacZ*, we followed the expression patterning in these animals. *LacZ* was expressed in osteoblasts located in the primary spongiosa in adult mice. Cellular analysis indicated that *LacZ* is expressed in osteoblasts but not in osteoclasts. In the mineralized nodules formed in the culture of bone marrow cells obtained from *Cnot7* +/- mice, *LacZ* expression was mainly observed in the cells forming mineralized nodules but not in un-mineralized area scattered around the periphery of the nodules. *LacZ* blue positive cells were gradually depositing minerals along its time course of the in vitro mineralization assay. *Cnot7* expression was enhanced by the treatment with BMP. These data suggest that Cnot7 is expressed in osteoblasts and is associated with mineralization. *J. Cell. Biochem.* 99: 538–544, 2006. © 2006 Wiley-Liss, Inc.

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Bone metabolism is controlled by many cytokines and hormones, which coordinately regulate the differentiation, and proliferation of mesenchymal cells to become bone forming cells, namely osteoblastic cells [Manolagas,

2000]. However, the mechanisms underlying such osteoblastic differentiation are not fully understood yet.

Osteoblastic differentiation is under the control of a set of transcription factors [Burdan, 2005; Dusso et al., 2005; Kobayashi and Kronenberg, 2005; Komori, 2005]. Among those, two of the critical transcription factors are Runx2 and Osterix whose deletion resulted in the total loss of osteoblastic cells in vivo [Komori, 2002; Nakashima et al., 2002; Komori, 2003; Komori, 2005]. In addition to these core transcription factors, osteoblasts also express transcription factors whose function have been either identified or yet to be identified. These transcription factors include ATF4 and AP-1 as well as nuclear receptors for vitamin D and PPAR-gamma [Haussler et al., 1997; Steinmul-

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*Correspondence to: Masaki Noda, Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, 3-10 Kanda-Surugadai, 2-chome Chiyoda-ku, Tokyo 101-0062, Japan.
E-mail: noda.mph@mri.tmd.ac.jp

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ler et al., 2001; Kawaguchi, 2004; Yang et al., 2004; Dusso et al., 2005]. As to these categories of nuclear receptors, RXR has been observed to be expressed in osteoblastic cells. RXRs form heterodimers with nuclear receptors known to bind to specific ligands as described above (vitamin D, retinoic acid) and bind to response elements in the regulatory regions of genes encoding osteoblastic phenotype-related molecules. RXRs have also been known to bind to gene expression regulators, which are not classified as nuclear receptors [MacDonald et al., 1993; Ferrara et al., 1994; Haussler et al., 1997].

CCR4-NOT complex 7 (*Cnot7*) is a component of CCR4-NOT complex [Bai et al., 1999]. Within this complex, *Cnot7* is involved in gene expression in yeast and is conserved from yeast to human [Albert et al., 2000]. In mammals, *Cnot7* appears to be involved in the spermatogenesis as *Cnot7* deficient male mice were found to be sterile [Berthet et al., 2004; Nakamura et al., 2004]. In these animals, *Cnot7* is shown to interact with RXR-beta and it has been proposed that *Cnot7* would function as a coregulator of the transcriptional factor [Nakamura et al., 2004]. As RXRs act as a partner for vitamin D receptor to regulate osteoblastic function [Haussler et al., 1997], we examined the expression of *LacZ* gene, which is replaced with the exon 2 of *Cnot7* gene in the process of producing *Cnot7* deficient mice. We observed that *LacZ* expression under the control of the regulatory system to express *Cnot7* gene could be found in osteoblastic cells and the expression was associated with the formation of mineralized the nodules in culture.

MATERIALS AND METHODS

Animals

Cnot7 deficient mice were produced as described previously [Nakamura et al., 2004]. The construct used for the homologous recombination contained an nls-*LacZ* + neomycin cassette inserted into exon 2 of the *Cnot7* gene. Genotyping of the offspring used polymerase chain reaction (PCR) of tail DNA with the appropriate primers to identify either the intact exon 2 or the *LacZ* insertion as described [Nakamura et al., 2004]. The *Cnot7* deficient mice and wild-type mice were subjected to the following experiments. All the animal experiments were appro-

ved by the animal welfare committee of our institute.

LacZ Staining

Bone tissues were fixed in solution (0.2% glutaraldehyde, 0.1 M NaPB, 5 mM EGTA, 2 mM MgCl₂, and 0.8% formaldehyde) for 45 min at room temperature. After rinsing twice for 30 min at room temperature in rinse buffer (0.1 M NaPB, 2 mM MgCl₂, 0.1% sodium deoxycholate, and 0.2% NP40), they were incubated overnight at 37°C in X-gal staining solution (Rinse buffer, 1 mg/ml X-gal, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆), washed twice for 30 min in cold PBS, postfixed in 4% paraformaldehyde in PBS overnight at 4°C and embedded in paraffin. Histological sections of paraffin-embedded stained tissues were counterstained with nuclear fast red. Wild-type mice were used as negative control.

β-Galactosidase activity was measured by using chemiluminescent substrate Galacto-PLUS kit according to the manufacturer's instructions (Tropix). β-Galactosidase activity was normalized by its total amount of protein.

Bone Marrow Cell Culture

Bone marrow cells were flushed out from the long bones and plated in 4-well plates at the density of 1 × 10⁶ cells/ml. For mineralized nodule assay, the bone marrow cells were cultured in alpha minimal essential medium supplemented with 10% fetal bovine serum and antibiotics-antimycotics in the presence of ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM). Medium was changed every 2–3 days. After 5, 10, or 15 days cultures, the cells were fixed in fix solution for 5 min, stained with X-gal solution for 3–4 h and rinsed with PBS. The cells were re-fixed with PFA and stained with alizarin red to visualize mineralization of nodules.

For osteoclast formation assay, the bone marrow cells were cultured for 7 days in alpha minimal essential medium supplemented with 10% fetal bovine serum, antibiotics-antimycotics, vitamin D₃ (10⁻⁸ M), and dexamethasone (10⁻⁷ M). Medium was changed every 3 days. After the fixation and X-gal staining as described above, the cells were re-fixed with PFA and stained with TRAP staining buffer containing 100 mM sodium acetate, 50 mM sodium tartrate,

AS-MX, FRV-LB. We recognized the cells containing more than three nuclei and TRAP activities as osteoclasts.

Isolation of Total RNA and RT-PCR Analysis

For RT-PCR analysis, we used osteoblastic cell line, MC3T3E1 cells (obtained from Hiroaki Kodama, Oh-u University, Koriyama, Japan). MC3T3 cells were maintained in alpha-MEM supplemented with 10% FBS in 10 cm² dish. The cells in sub-confluency were treated with TGF-beta (10 ng/ml), BMP2 (200 ng/ml), or vehicle (control), and cultured for 3 days. Total RNA was extracted from the cells, according to acid guanidium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Reverse transcription was carried out using 1 µg of total RNA, 0.2 µg of oligo (dT) primer, dNTP mix (10 mM/L), and MMLV-RT (100 units) in a final volume of 20 µl. RNA and primers were incubated together at 65°C for 10 min and then cooled rapidly on ice before addition of other reagents. RT was carried out at 37°C for 1 h. Complementary DNA was amplified by PCR according to the "hot-start" method in a 25-µl reaction volume containing 2.5 mM dNTP mix, 10 µM *Cnot7* primers, and rTaq DNA polymerase (1 unit). After an initial denaturation at 94°C for 5 min in a Gene Amp PCR System 9700 (PE Biosystems), amplifications were performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Reaction products were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide. The *Cnot7* primers used for our RT-PCR experiments are 5'-CTCGG-

ACTGACCTTTATGA-3' and 5'-AGCAACTT-CCTGTAATCCAC-3'. The *GAPDH* primers are 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3'. The bands were quantified based on densitometry (ONE-D scan, Scanalytics Inc.).

Statistical Analyses

The data were analyzed based on analyses of variance. *P* values less than 0.05 were regarded as statistically significant.

RESULTS

Expression Pattern of *LacZ* in Bone Tissues of *Cnot7* Heterozygous Mice

To address the activity of regulatory region of *Cnot7* in bone tissue, tibia of *Cnot7* heterozygous mice (*Cnot7* +/- mice), which exhibited similar phenotype to wild-type mice, was subjected to *LacZ* staining followed by section. The results indicated that *LacZ* positive signals were observed in osteoblastic cells localized in epiphyseal trabecular bone region (Fig. 1a). Higher magnification of the histological section exhibited that osteoblastic cells sitting on the bone matrix were positive for the *LacZ* staining (Fig. 1b).

In order to follow the expression of *Cnot7* in osteoblast, we obtained bone marrow cells from the femur of *Cnot7* +/- mice. The cells were cultured in the presence of ascorbic acid and beta-glycerophosphate to induce differentiation of osteoblastic cells, which could form mineralized nodules. These cultures were subjected to

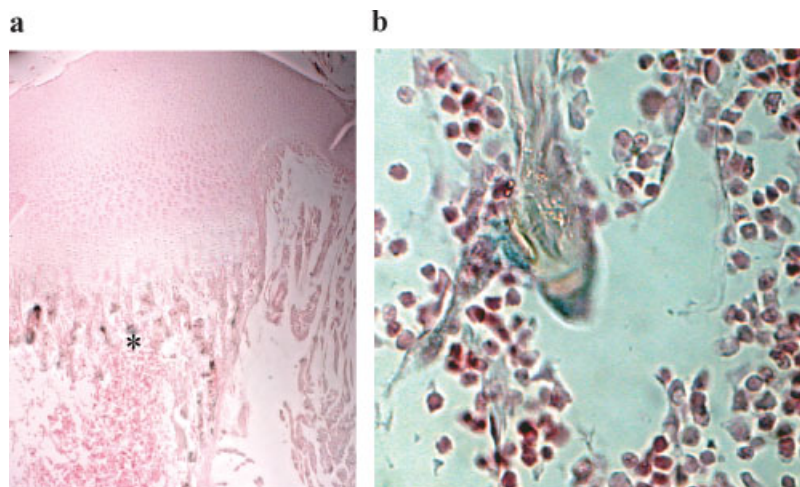


Fig. 1. *LacZ* expression in osteoblasts in vivo. The microscopic images of sections of tibiae from 1-week-old mice subjected to *LacZ* staining. *a: Proximal end of tibia, b: primary spongiosa.

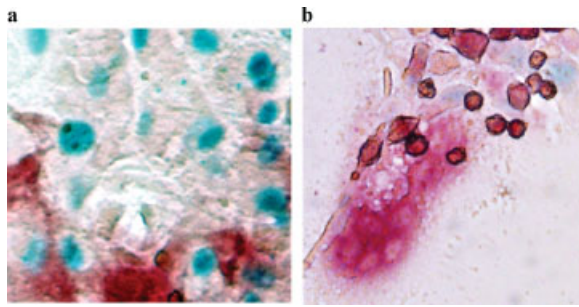


Fig. 2. LacZ expression in osteoblasts and osteoclasts in culture. **a:** Osteoblasts developed in the bone marrow cell culture in osteogenic medium expressed LacZ signals. **b:** Osteoclasts developed in bone marrow cells cultured in the presence of vitamin D and dexamethasone. These cells did not express LacZ signals.

the LacZ staining. As shown in Figure 2a, the LacZ staining positive cells were observed in and around the nodules. These cells could create mineralized matrix, which was stained positive for alizarin red. Thus, these cells were regarded as the cells in osteoblastic cell lineage. We also examined *Cnot7* expression by using LacZ staining method in osteoclasts, which were formed in bone marrow cells cultured in the

presence of vitamin D and dexamethasone. As shown in Figure 2b, multinucleated TRAP positive cells were developed in cultures. However, these osteoclast-like cells were negative for LacZ staining. These data showed that LacZ positive cells were specifically observed in the cells of osteoblastic lineage but not in those of osteoclastic lineage indicating certain specificity of *Cnot7* expression in osteoblasts.

In the culture of osteoblastic cells, one of the functional evidences is to form mineralized nodules in vitro. Therefore, we cultured bone marrow cells obtained from the *Cnot7* +/- mice to form the mineralized nodules in the cultures using the osteogenic medium. When the cells were cultured in the presence of beta-glycerophosphate and ascorbic acid, nodules were observed under the phase-contrast microscope as shadowed materials. Alizarin red staining conformed the deposition of mineral in the shadowed materials. Positive LacZ staining was detected in the cells, which surrounded well-mineralized matrix in the nodules produced in the culture (Fig. 3 lower panels). In these cultures, some cells did not indicate deposition of mineralized materials even in the

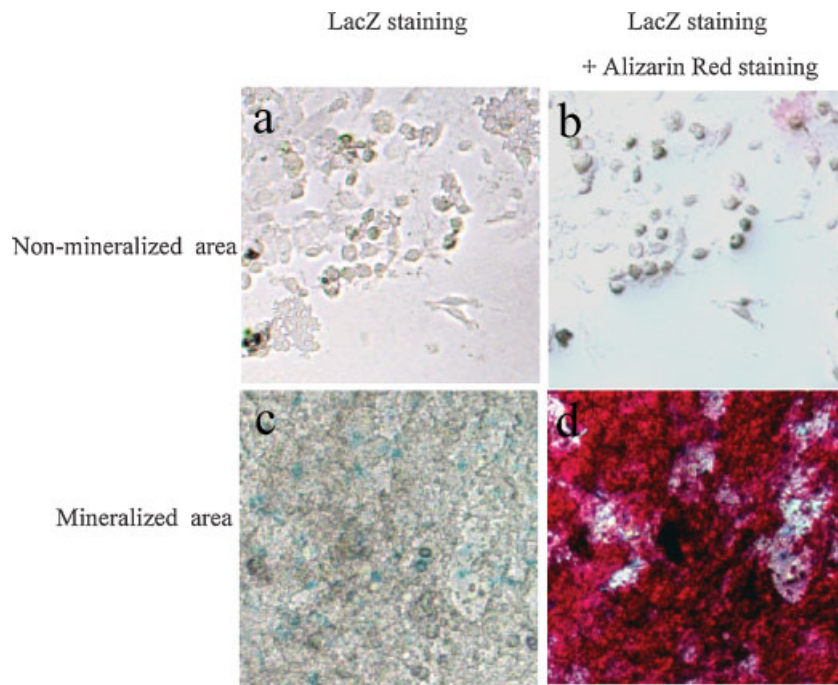


Fig. 3. LacZ expression in the cells subjected to mineralized nodule formation assay. Nodules were formed in the bone marrow cells of *Cnot7* +/- mice cultured in the presence of ascorbic acid and beta-glycerophosphate. *Cnot7* expression was monitored by LacZ staining. **a:** LacZ expression was hardly observed in non-mineralized area. **b:** Cultures shown in (a) were subjected to Alizarin red staining. **c:** LacZ expression was observed in mineralized area. **d:** Mineralization in (c) was visualized by alizarin red staining.

same well and most of these cells did not exhibit *LacZ* positive blue signals (Fig. 3 upper panels).

Time course experiments provided evidence that *LacZ* positive cells were associated with the mineralized nodule formation. In earlier cultures such as day 5, *LacZ* positive cells were scattered all over the culture dish without association with mineralized materials (Fig. 4a). By day 10 in culture, nodule formation with mineralized materials was observed in the culture of bone marrow cells. When the cells were stained with both alizarin red and X-gal, the red and blue staining were associated together. The result indicated that the *LacZ* positive cells were depositing mineralizing matrix. By day 15, *LacZ* positive cells were totally surrounded by the highly mineralized materials (Fig. 4a). The levels of the formation of miner-

alized nodules were similar in these *Cnot7* heterozygous cells and wild-type cells (Fig. 4a).

Such *LacZ* expression was also monitored by based on the biochemical assay. On day 5 and day 10, the levels of the *LacZ* activity were similar. However, by day 15, due to the increase in the protein content per culture dish, total *LacZ* activity normalized against protein was reduced (Fig. 4b).

Since matrix protein was known to contain BMP and such BMP signal may be related to nodule formation in culture, we treated the osteoblastic cell line, MC3T3E1 cells, with BMP. The BMP treatment enhanced the expression of *Cnot7* mRNA (Fig. 5). Such increase was not observed when TGF-beta was treated with the cells suggesting the specificity of BMP to modulate the expression levels of *Cnot7*.

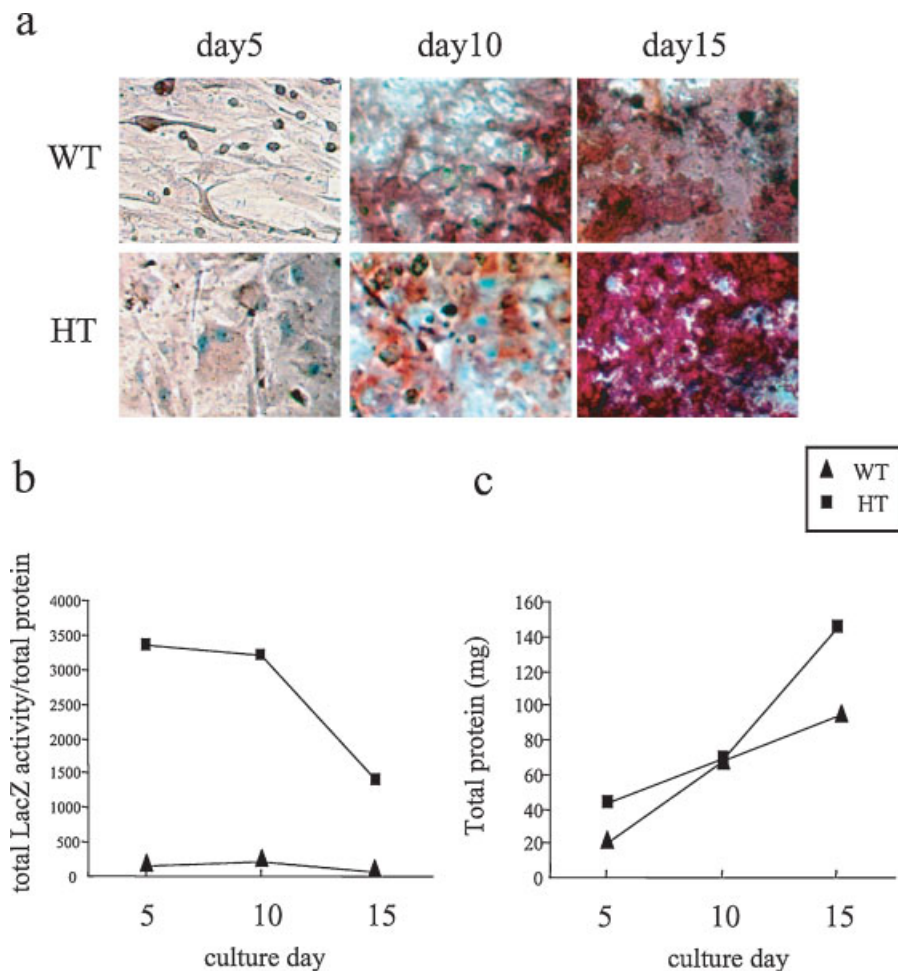


Fig. 4. Time course of the formation of mineralized nodule and expression of *LacZ*. **a:** Nodules were formed in the bone marrow cells obtained from *Cnot7* $+/+$ (WT) and $+/-$ (HT) mice. These nodules were stained with X-gal and alizarin red after 5, 10, and 15 days culture. **b:** *LacZ* activities per protein **c:** total protein content per well. *Cnot7* $+/+$ (WT); triangle and *Cnot7* $+/-$ (HT); square.

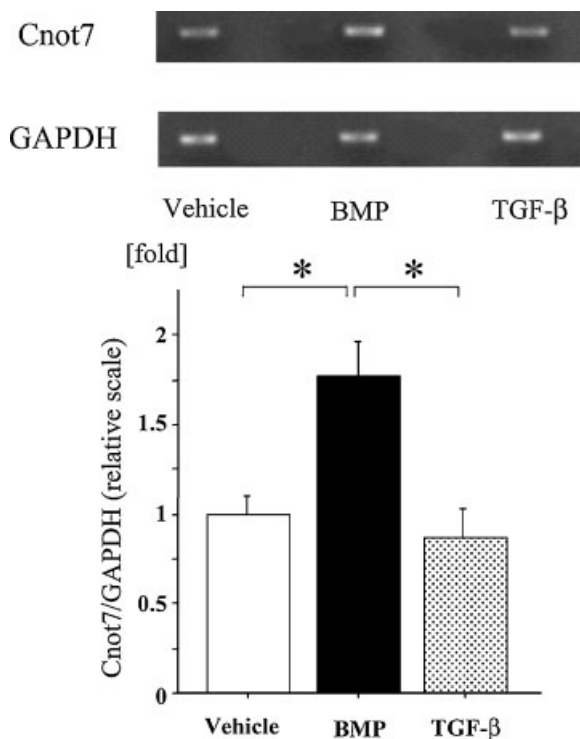


Fig. 5. BMP treatment enhances *CNOT7* mRNA expression. Osteoblastic MC3T3E1 cells were cultured in the presence of vehicle, 200 ng/ml BMP or TGF-beta for 3 days. Total RNA was extracted from these cells and the expression levels of *Cnot7* were examined based on RT-PCR. *Cnot7* mRNA expression in osteoblasts was enhanced by BMP treatment. Asterisks indicate that the difference is statistically significant.

DISCUSSION

We have shown that the expression of *LacZ* gene placed in *Cnot7* locus was observed in the osteoblastic cells located in mineralized nodule formed in culture. From the histological examination, we observed that *LacZ* expression was detectable in osteoblastic cells sitting on the trabecular bone. Furthermore, in vivo analysis on the histology also indicated that the *LacZ* activity was not associated with osteoclasts. These observations suggested that *Cnot7* activity would be specifically confined in osteoblastic cells.

When bone marrow cells obtained from *Cnot7* +/- mice were cultured in the presence of osteogenic medium, *LacZ* expression was observed in bone forming cells. In contrast, when the bone marrow cells were cultured in the presence of vitamin D and dexamethasone to produce multinucleated osteoclast like cells, *LacZ* expression was not observed to associate with the expression of TRAP, a maker for

osteoclastic cells. These observations clearly point to the specific expression of *LacZ* in the cells of the osteoblastic lineage in both in vitro and in vivo.

Time course study of the nodule formation in culture also supported the association of the expression of *LacZ* gene with the formation of alizarin red positive nodule in culture. This is corresponding to the observation on the association of *LacZ* gene activity in osteoblasts in vivo. When we followed a long term culture for the mineralization (15 days), *LacZ* expression was kept to be high enough to be detectable in the cells embedded in highly mineralized nodule.

Osteoblastic cells are considered to differentiate from immature cells, which are present in bone marrow environment. In the bone marrow environment, it has been claimed that stem cell type population could exist even in adult animals. Expression of *LacZ* gene placed in *Cnot7* locus was also clearly observed in the cells, which were not positive for the alkaline phosphatase at least the beginning of the bone marrow cell culture. These observations suggest that the *Cnot7* expression may be commonly observed in the relatively early stages of osteoblastic cells as well as the mature osteoblasts, which are involved in the formation of mineralized nodules. Since BMP treatment enhanced the expression of *Cnot7*, it is possible that *Cnot7* may be expressed as a common marker of immature and mature osteoblastic cells during the differentiation though this point needs further elucidation.

In conclusion, we identify that expression of *Cnot7* was detected in osteoblastic cells in vivo as well as in culture. Culture experiments revealed that *Cnot7* was specifically observed in the osteoblastic cells and such *Cnot7* activity was clearly associated with the relatively young osteoblastic cells as well as mature osteoblastic cells producing mineralized nodule.

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