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# Cystatin C stimulates the differentiation of mouse osteoblastic cells and bone formation

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**Abstract**

Cystatin C (CysC) is a natural cysteine proteinase inhibitor that suppresses the differentiation and bone-resorptive function of osteoclasts. By contrast, the effect of CysC on the differentiation and bone-formative function of osteoblasts has not been elucidated thoroughly. We examined the effects of CysC on mouse osteoblastic cells using *in vitro* cultures from bone marrow and calvaria and *ex vivo* calvarial cultures. CysC-stimulated cells showed increased alkaline phosphatase (ALP) activity, mineralization of the new bone matrix, and calvarial bone formation. The cells treated with CysC immunodepleted by anti-CysC antibody (iCysC) and a chemical papain-like cysteine proteinase inhibitor, E-64, did not induce mineralization. Elevated mRNA levels of bone morphogenetic protein (BMP)-2, the differentiation marker osteocalcin, and a master osteogenic transcription factor, Runx2, were observed in CysC-treated cells. These results suggest that CysC affects the BMP signaling cascades in osteoblastic cells and then promotes osteoblast differentiation, mineralization, and bone formation.


*Keywords:* Cystatin C; Osteoblast differentiation; Mineralization; Bone formation; BMP-2; Runx2; Osteocalcin; E-64; *Ex vivo* calvarial culture

In the development and physiological homeostasis of skeletal tissue, osteoblasts, which originate from mesenchymal tissue, play a central role in bone matrix formation and in osteoclast recruitment and activation [1]. Osteoblasts synthesize and secrete bone-specific proteins such as osteocalcin (OCN), as well as the enzyme alkaline phosphatase (ALP) [2].

Bone morphogenetic protein-2 (BMP-2), a member of the TGF-β superfamily, plays important roles in the differentiation of bone marrow-derived precursor cells into osteoblasts and the mineralization of bone matrix [3]. Runx2 is a specific transcriptional factor that is essential for osteoblast differentiation and bone formation [4]. BMP signaling is required for the Runx2-dependent induction of the osteoblast phenotype [5].

Cystatins are endogenous inhibitors of cysteine proteinases and constitute a large superfamily that is divided into three groups: stefins (family I), cystatins (family II), and kininogens (family III) [6]. CysC, a family II cystatin, is synthesized intracellularly in several cell types and is secreted into the extracellular space [7,8]. It is abundant in cerebrospinal fluid, saliva, and milk. CysC is produced in osteoclasts and osteoblasts, and is secreted into the bone resorption lacunae under the ruffled border of osteoclasts

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[9]. CysC has also been reported to regulate osteoclastic bone resorption and osteoclast differentiation [10,11], although it is not clear whether CysC acts as a proteinase inhibitor in osteoclastogenesis [12]. Little is known about osteoblast differentiation and bone formation related to CysC. One report indicated that the CysC gene is expressed in mature osteoblast-like cells [13], and one cDNA microarray analysis has shown that the CysC gene is expressed during both the differentiation and mineralization phases of osteoblastogenesis in MC3T3-E1 cells [14].

Here, we investigated the effects of CysC on the differentiation and mineralization of new bone matrix in a mouse bone marrow-derived osteoblast culture system, using cytochemical, biochemical, and gene-expression analyses. To confirm the effects of CysC, we performed an immunodepletion assay using antibodies against CysC and a synthetic papain-like cysteine proteinase inhibitor, E-64. Finally, the influence of CysC on calvarial bone formation was examined in *ex vivo* and *in vitro* culture systems.

## Materials and methods

*Cell culture.* Six-week-old C57BL/6J mice (Clea Japan, Tokyo, Japan) were treated in strict accordance with the Guidelines for Animal Experiments of Kyushu University. Bone marrow cells were flushed from the femurs and tibias of young male mice. A single-cell suspension was obtained using a 100-μm cell strainer. The suspension was seeded at $2.0 \times 10^6$ cells per 100-mm culture dish in α-minimum essential medium (αMEM; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 50 μg/ml gentamicin, and cultured until sub-confluent, with medium changes twice a week.

*Osteoinduction culture and in vitro treatment with CysC.* The bone marrow cells were re-seeded at $1.5 \times 10^4$ cells/cm$^2$ (for the osteoinductive effects of CysC) or $5.0 \times 10^4$ cells/cm$^2$ (for the initial effects of CysC) in growth medium. At day 1, the same cells were stimulated with or without 0.1–100 nM CysC (BioVender Laboratory Medicine, Brno, Czech Republic), immunodepleted CysC (iCysC, prepared as described below), anti-CysC antibody (R&D Systems, Minneapolis, MN), or *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64; Sigma, St. Louis, MO) for comparative experiments in an osteoinduction medium consisting of 10% FBS, 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate, and 10 nM dexamethasone in αMEM for bone marrow cells. The medium was changed every 3 days. Cultured cells were stained with alizarin red and von Kossa stain; ALP was assayed and the presence of calcium ions was examined at days 7, 14, 21, and 28. The total positive area was measured using Scion Image (Scion, Frederick, MD).

*Immunodepletion experiments.* CysC (10 μM) was immunodepleted with anti-CysC antibody (7 μM) by incubation for 10 min at 37 °C, followed by storage at 4 °C for 16 h. After centrifugation at 25,000*g* for 10 min, the supernatant was diluted (1:1000) and added to the culture under osteoinduction conditions.

*Enzyme assays.* Mouse spleen extracts were used to examine the inhibitory effect of CysC on cysteine proteinase activity, with benzyloxycarbonyl phenylarginine-4-methyl-7-coumarylamide (Z-Phe-Arg-MCA) as the substrate, according to a described method [15].

*RT-PCR analysis.* Total RNA was extracted from the cultured bone marrow cells using an SV Total RNA Isolation Kit (Promega, Madison, WI), and reverse transcription was performed using ReverTra Ace (Toyobo, Tokyo, Japan). The primer sequences are available on request. PCR was performed with a GeneAmp 9700 (Applied Biosystems, Foster City, CA). The PCR products were electrophoresed in 2% agarose gels, and the transcript abundance was assessed quantitatively relative to G3PDH expression, using Scion Image software.

*Ex vivo and in vitro culture of mouse calvaria and morphometric analyses.* Whole calvaria from neonatal mice were cultured in osteoinduction medium with or without CysC (0.1 nM), iCysC (CysC:αCysC, 0.1:0.07 nM), anti-CysC antibody (0.07 nM), or E-64 (0.1 nM) for 7 days. Calvarial explants were fixed with 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 7-μm-thick serial sagittal sections before hematoxylin and eosin staining. Bone thickness was measured for each of five serial sagittal sections cut 1.2 (p1) and 2.4 (p2) mm from the midline, according to a previous study [16] (Fig. 4A). The calvaria of newborn mice were treated sequentially with 0.1% collagenase and 0.2% dispase, five times for 10 min at 37 °C, and the cells were collected from the final digested solution (fraction V). The single-cell suspensions were cultured as described above and re-seeded at $1.0 \times 10^4$ cells/cm$^2$ in osteoinductive medium without dexamethasone.

*Statistics.* All data are expressed as means ± SD of triplicate determinations. Means of groups were compared using analysis of variance (ANOVA), and the significance of differences was determined by *post hoc* testing using the Bonferroni method.

## Results and discussion

### *CysC stimulates mineralized nodule formation in mouse bone marrow-derived cells*

Mouse bone marrow-derived cells were cultured in osteoinductive medium, and the ALP activity and mineralized nodule formation were examined (Fig. 1). Although CysC is expressed in osteoblasts and osteoclasts in bone tissue [9,17], CysC is reported to inhibit osteoclastic bone resorption and osteoclastogenesis. CysC treatment at concentrations of 0.1, 1, and 10 nM induced ALP activity at day 14 and mineralization at day 21 (1 and 10 nM, data not shown). At day 7, the ALP activity assay did not differ significantly between non-treated (control) and CysC-treated cultures, whereas at day 14, there was a significant increase in ALP activity, especially in the group treated with 0.1 nM CysC compared with the control. Mineralization was not seen at day 7 or 14 (data not shown). The deposition of calcium ions into the extracellular matrix was examined using alizarin red staining and a calcium C test at days 21 and 28 (Fig. 1B). On both days, the calcium content was significantly higher in the group stimulated with 0.1 nM CysC than in the control, but not in the 100-nM group. Remarkable phosphate ion-deposition was also found with von Kossa staining of the cells treated with 0.1, 1, and 10 nM CysC (Fig. 1C). Concentrations of 0.1, 1, and 10 nM CysC enhanced the differentiation of osteoblasts and mineralized tissue formation, whereas CysC concentrations higher than 100 nM had little effect. Lerner et al. [17] found that 1–50 μg/ml CysC (0.1–5.0 mM) suppressed bone resorption by osteoclasts and that 50 μg/ml CysC (5.0 mM) did not affect osteoblastic differentiation markers (ALP and OCN). The different results might be due to the concentration of CysC used. CysC is a ubiquitously expressed secretory protein with a plasma level of around 80 nM [18,19]. As the concentrations we used were within the physiological range, CysC might positively affect the mineralization system of *in vitro* mouse bone marrow-derived osteoblasts.

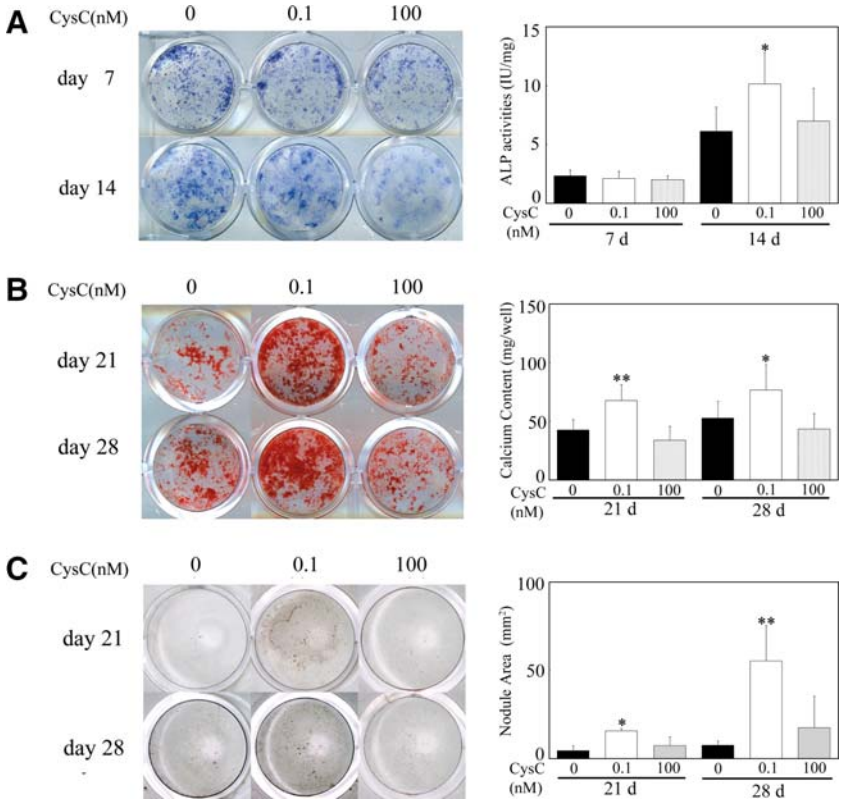


Fig. 1. **CysC stimulates mineralization in bone marrow-derived cells.** Bone marrow-derived osteoblastic cells cultured with or without CysC (0.1 and 100 nM). (A) CysC induces ALP. Left panel, ALP staining; right panel, ALP enzyme activity. (B) CysC induces calcium deposits in *de novo* nodules. Left panel, alizarin red staining; right panel, calcium content in the nodules. (C) CysC induces phosphate deposits in *de novo* nodules. Left panel, von Kossa staining; right panel, total area of von Kossa-positive nodules. $^{*,**}p < 0.05$ or 0.01, respectively, compared with control cells. Black column, control cells (CTRL); white column, 0.1 nM CysC-treated group; gray column, 100 nM CysC-treated group.

### *Immunodepletion of CysC diminishes mineralized nodule formation in mouse bone marrow-derived cells*

To confirm the effects of CysC (0.1 nM) on mineralization, immunodepletion experiments were performed (Fig. 2). The degree of immunodepletion was determined by the inhibitory effect of CysC on the proteinase activity of mouse spleen extracts using Z-Phe-Arg-MCA, which is a synthetic substrate for cathepsins B and L. A specific antibody against CysC removed the inhibitory effect of CysC on the cysteine proteinase activity in a dose-dependent manner (Fig. 2A). A higher concentration of anti-CysC antibody removed up to 83% of the CysC inhibitory effect but did not completely eliminate it (Fig. 2A).

After immunodepletion, the supernatant (iCysC) reduced the induction of mineralized nodule formation by CysC (Fig. 2B–D). Treatment with anti-CysC antibody did not affect the mineralization in bone marrow-derived cells (Fig. 2B–D). These findings support the proposal that exogenous CysC induced osteoblastic differentiation and bone formation. CysC is a high-affinity inhibitor of papain family proteinases such as cathepsins B, H, K, L, and S, and the legumain family. Brage et al. [12] reported that mutated CysC, which has no or only a minor effect on the activity of papain-like cysteine proteinases, inhibited osteoclast formation to the same degree as wild-type CysC. Conversely, legumain inhibited osteoclast formation [20]. Based on these confusing reports, the mechanisms of CysC cannot be explained by the inhibition of proteolysis alone. E64, an extracellular papain-like cysteine proteinase inhibitor [21], had little effect on the calcium content and von Kossa-positive area in the mineralized nodules (Fig. 2B–D), although CysC had inductive effects on osteogenesis. These findings suggest novel function(s) of cystatins in addition to the inhibition of cysteine proteinases.

### *Expression of BMP-2, Runx2, and OCN mRNA in osteoblastic cells after CysC treatment*

Semi-quantitative PCR analysis was used to examine the effects of CysC on the expression of genes related to osteoblast differentiation in bone marrow-derived cells stimulated by 0.1 nM CysC or iCysC (Fig. 3). BMP-2 is a potent osteoinductive signal, inducing osteoblast differentiation and bone formation [3]. Runx2, the downstream target of BMP-2, is a critical transcriptional regulator of osteoblast differentiation [5]. At day 7 (Fig. 3A), bone marrow-derived cells with CysC showed upregulation of BMP-2 and Runx-2 mRNA compared with the control and iCysC cells. At day 14, the BMP-2 mRNA level was almost equal in control and CysC-treated cells. These findings suggest that CysC stimulates the BMP-2/Runx2 cascade,

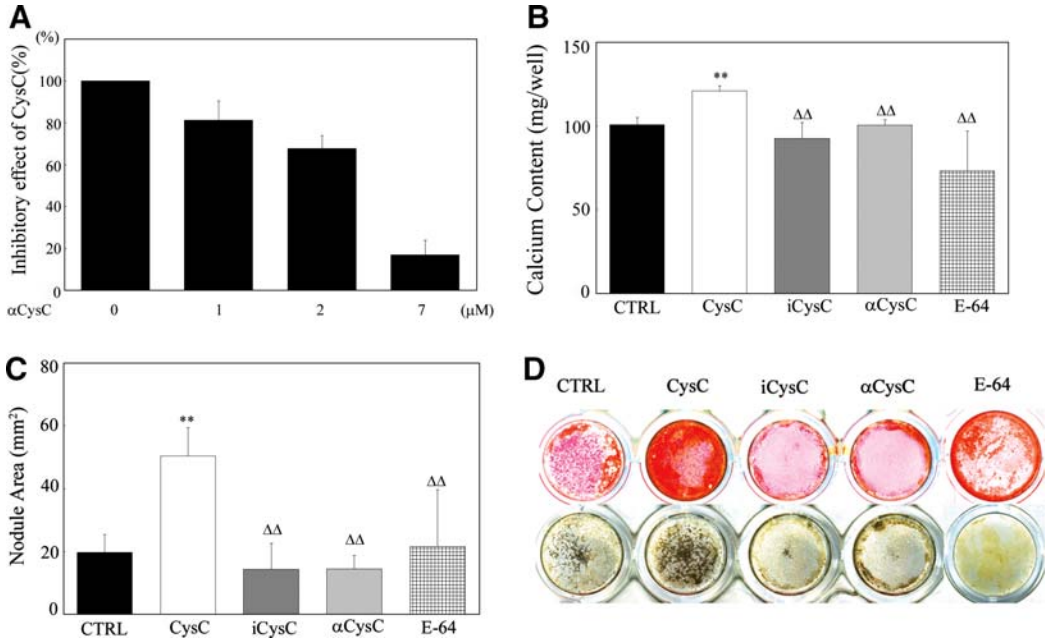


Fig. 2. Immunodepleted CysC or E64 does not stimulate mineralization in bone marrow-derived osteoblastic cells. (A) Changes in cathepsin activity with increased anti-CysC antibody (αCysC). Immunodepletion with anti-CysC antibody (0–7 μM) to examine the inhibitory effect of CysC (10 μM) on the cysteine proteinase activity of mouse spleen extracts with Z-Phe-Arg-MCA. The data are means ± SD of values from three independent experiments. (B,C) Effects of CysC, iCysC, anti-CysC antibody, or E-64 on calcium and phosphate deposits in *de novo* nodules after 28 days. (B) Calcium content in the nodules. (C) Total area of von Kossa-positive nodules. $^{**}p < 0.01$ compared with control (non-treated) cells. $^{\Delta\Delta}p < 0.01$ compared with CysC-treated cells. Black column, control cells (CTRL); white column, CysC-treated cells (CysC); dark-gray column, iCysC-treated cells; light-gray column, anti-CysC antibody-treated cells; checked column, E-64-treated cells (E-64). Each value is mean ± SD of triplicate determinations. Error bars show the SD. (D) Effects of CysC, iCysC, anti-CysC antibody, or E-64 on calcium and phosphate deposits in *de novo* nodules after 28 days. Upper panel, alizarin red staining; lower panel, von Kossa staining.

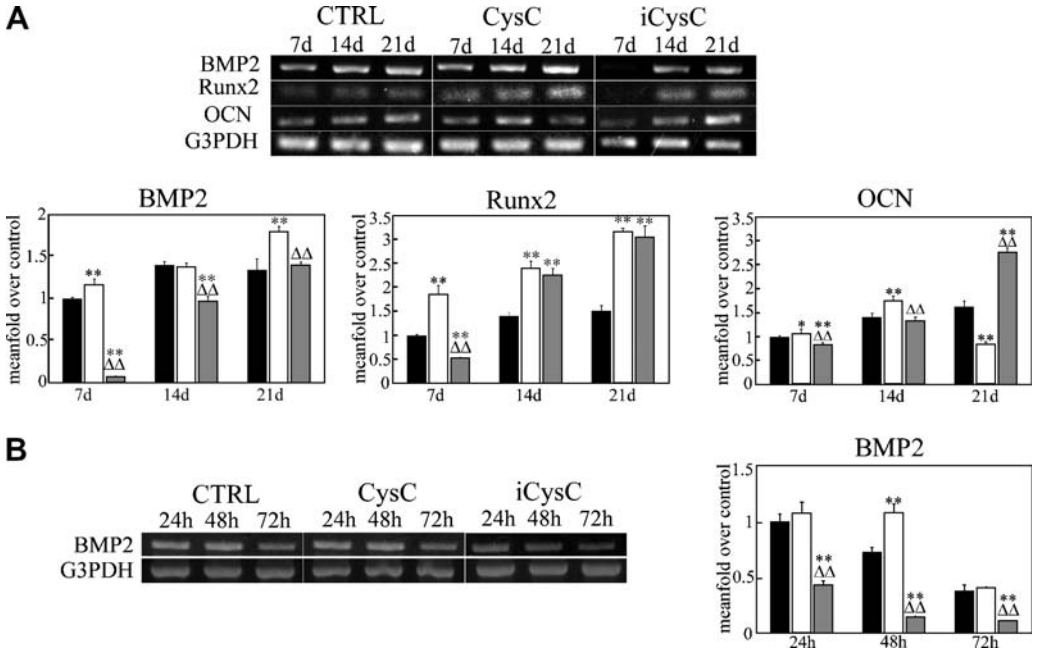


Fig. 3. **Involvement of BMP-2-Runx2 signaling in CysC-treated cells.** RT-PCR results showing the mRNA levels for BMP-2, Runx2, and OCN in bone marrow-derived osteoblastic cells treated with 0.1 nM CysC or iCysC. (A) Runx2 and OCN are analyzed at 7, 14, and 21 days. (B) BMP-2 is analyzed after 24, 48, and 72 h. CTRL, control (non-treated) group; CysC, CysC-treated group. The results show the expressions of BMP-2, Runx2, and OCN to G3PDH (internal standard) for the control cells. Black column, control cells (CTRL); white column, CysC-treated group (CysC); dark-gray column, iCysC-treated cells. $^{*,**}p < 0.05$ or 0.01, respectively, compared with non-treated osteoblastic cells. $^{\Delta\Delta}p < 0.01$ compared with CysC-treated cells. Each value is mean ± SD of triplicate determinations. Error bars show the SD.

inducing bone marrow-derived cells to become osteogenic cells in the early differentiation phase. To examine the initial effects of CysC on osteoblastic cells, the expression of BMP-2 was analyzed under CysC stimulation after 24, 48, and 72 h (Fig. 3B). At 24 h, the BMP-2 mRNA expression in the CysC-treated cells was almost equal to that in the control cells but was higher than that in the iCysC-treated cells. At 48 h, the BMP-2 mRNA expression in the CysC-treated cells was greater than that in the control or iCysC-treated cells. Thus, a high expression level of BMP-2 mRNA was maintained for 24–48 h in the cells with CysC, despite decreased BMP-2 mRNA expression in the control and iCysC-treated cells. These results indicate that CysC itself enhances the production of BMP-2 as its initial effect. Further study is needed to reveal the molecular mechanism of CysC and BMP-2 in osteoblastic cells.

Although the level of OCN mRNA was low in all of the groups at day 7, the level of OCN mRNA in the CysC-treated cells reached a peak at day 14, whereas the control and iCysC-treated cells reached a peak at day 21 (Fig. 3A). Given that OCN is a marker of late-stage osteoblast differentiation, the bone marrow-derived cells under CysC were thought to differentiate into osteogenic cells at day 14. OCN is the downstream target of Runx2 [4]. At day 21, in CysC-treated cells, OCN was decreased, while Runx2 was as high as in iCysC-treated cells. BMP-2 mRNA was higher with CysC expression than with iCysC (at day 21). BMP-2 antagonizes Runx2 activity, which enhances the OCN promoter in C2C12 cells [22]. As OCN is a negative regulator of bone formation [23], CysC treatment might lead to a low level of OCN and then cause increased bone formation. This hypothesis is supported by the effect of CysC on mineralization at day 21 (Fig. 1B and C).

### *CysC stimulates the mineralization of bone in ex vivo calvarial bone culture*

To confirm the effects of CysC on osteoblastic bone formation, calvarias from neonatal mice were cultured under stimulation with CysC, iCysC, anti-CysC antibody, or E-64 for 7 days (Fig. 4A–C). In the groups treated with 0.1 nM CysC, the thickness of the parietal bones increased significantly compared with that in the control (Fig. 4B and C). The thickness of parietal bone with E64 was similar to that of the control. With the iCysC or anti-CysC antibody treatments, the bone was thinner than or similar to that in the control. Interestingly, the osteoblastic cells in the CysC-treated group were rounder than those in the other groups. The bone thickness patterns from p1 and p2 were similar.

To confirm the effects of CysC on calvarial bone formation *ex vivo*, osteoblastic cells from calvaria were cultured under CysC stimulation, and the mineralized tissue forma-

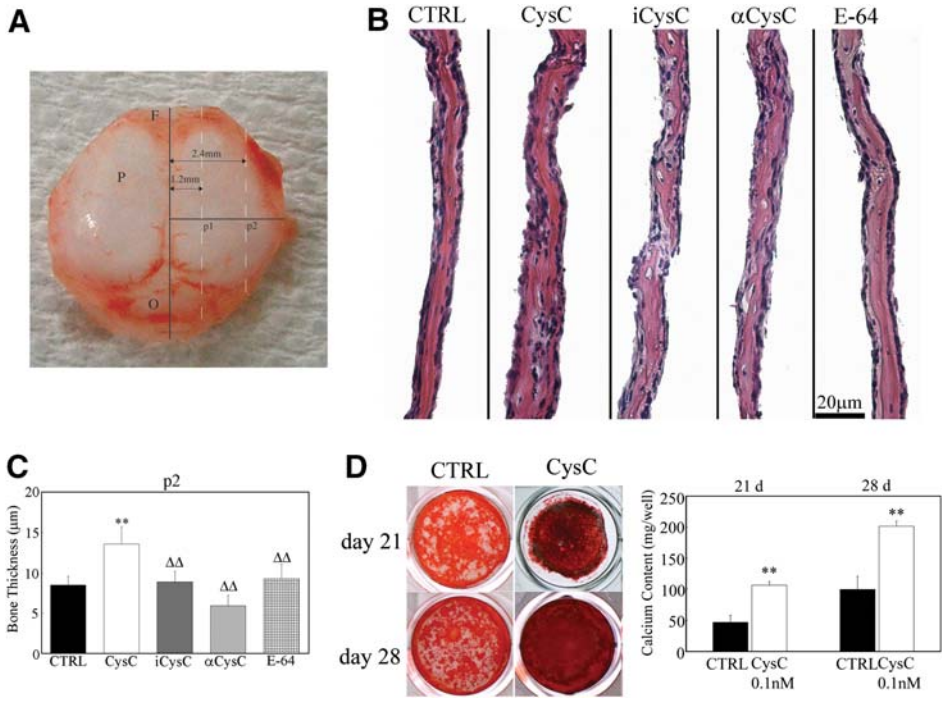


Fig. 4. **CysC stimulates mineralization in calvaria-derived osteoblasts and *ex vivo* calvarial bone culture.** Calvarial explants cultured with or without CysC, iCysC, anti-CysC, or E-64 for 7 days. (A) A typical calvarial explant showing the frontal (F), occipital (O), and parietal (P) bones. Observation points are indicated as p1 and p2. Sagittal sections taken at p1 and p2 are depicted with dotted lines. (B) Photomicrographs showing the bone thickness in the sagittal sections of parietal bone at p1 for the five groups, with hematoxylin and eosin staining. (C) Measurement of the parietal bone thickness at site p2 in the sagittal sections. Black column, control cells (CTRL); white column, CysC-treated cells (CysC); dark-gray column, iCysC-treated cells; light-gray column, anti-CysC antibody treated cells; checked column, E-64-treated cells (E-64). $^{**}p < 0.01$ compared with non-treated calvarial explant. $^{\Delta\Delta}p < 0.01$ compared with CysC-treated calvarial explants. Each value is mean ± SD of triplicate determinations. Error bars show the SD. These results are representative of three independent experiments. (D) Calvaria-derived osteoblastic cells cultured with or without CysC. Left panel, alizarin red staining; right panel, calcium content in the nodules. Black column, control cells (CTRL); white column, CysC-treated groups (CysC). $^{**}p < 0.01$ compared with control cells.

tion was analyzed (Fig. 4D). Based on the results of alizarin red staining and the calcium test, stimulation with 0.1 nM CysC significantly accelerated the formation of mineralized tissue compared with the control. The calcium content in the mineralized nodules was similar between the E-64-treated and control groups (data not shown).

In conclusion, this study found that CysC promotes the differentiation of osteoblasts and the mineralization of new bone matrix in bone marrow-derived cells *in vitro* and in *ex vivo* calvarial cultures. Furthermore, the effect of CysC on osteoblastic cells is associated with BMP signaling cascades. Milk basic protein (MBP), whose main ingredient is bovine CysC [24], is effective in preventing *in vivo* bone loss in ovariectomized rats [25]. We propose that CysC has potential for bone therapy in osteolytic diseases such as osteoporosis and periodontal disease.

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