

# Ethyl-3,4-Dihydroxybenzoate with a Dual Function of Induction of Osteogenic Differentiation and Inhibition of Osteoclast Differentiation for Bone Tissue Engineering

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The current approach in biomaterial design of bone implants is to induce *in situ* regeneration of bone tissue, thus improving integration of the implants and reducing their failure. Therefore, ethyl-3,4-dihydroxybenzoate (EDHB), which stimulates differentiation of osteoblasts and the resultant bone formation, should be studied. In this study, the osteoinductive ability of EDHB in preosteoblasts and human mesenchymal stem cells was examined. EDHB for future use in bone tissue engineering was evaluated by examination of early markers of differentiation (such as alkaline phosphatase [ALP] activity and collagen type I expression) and late markers of osteoblast differentiation (bone nodule formation). As bone remodeling and implant osteointegration depend not only on osteoblast response but also on interaction of the biomaterial with bone-resorbing osteoclasts, differentiation of osteoclasts in response to the compounds was also observed. For *in vivo* study, alginate gel comprised of EDHB and cells was transplanted into the back subcutis of mice. Our results show that EDHB might have beneficial effects through regulation of both osteoblast and osteoclast differentiation. Therefore, we suggest that EDHB could be a strong candidate for dual regulation to increase osteoblast differentiation and decrease osteoclast differentiation.

## Introduction

ONE OF THE MAIN REASONS bone implants fail is their inability to induce osteoinduction, osteoconduction, or the growth of bone tissue into the structure of graft. Therefore, a new approach in biomaterial design is to create materials that can induce *in situ* regeneration of bone. To stimulate local bone tissue repair, new composites combining materials and growth factors have recently been studied.<sup>1</sup> In particular, the ability of bone morphogenetic proteins to induce ectopic bone formation has been discovered, and these proteins are considered promising therapeutics for bone tissue engineering. However, recombinant proteins are, in general, less active than their natural forms and require very high doses to be effective. Other problems include their instability, short lifetime in the body, high production cost, and difficult handling.<sup>2,3</sup> Natural compounds may be new possible candidates. Natural compounds, such as antioxidants (e.g., epigallocatechin-3-gallate derived from green tea) and other flavonoids (e.g., resveratrol and quercetin), induce differentiation of mesenchymal stem cells (MSCs) to osteoblasts and enhance formation of bone nodules.<sup>4–7</sup> Additionally, histone deacetylase inhibitors, such as valproic acid, trichostatin A, and sodium butyrate, alter transcription of genes related to osteogenic

differentiation by modifying chromatin structure and may also be promising candidates.<sup>8,9</sup>

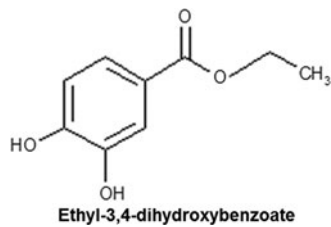
Ethyl-3,4-dihydroxybenzoate (EDHB) is a component of the *Rubus coreanus* extract (Fig. 1), and the extract has recently been shown to have a bone protecting-effect on postmenopausal osteoporosis in ovariectomized rats.<sup>10</sup> EDHB reduces collagen production in keloid fibroblasts<sup>11</sup> and smooth muscle cells,<sup>12</sup> and it inhibits myoblast differentiation.<sup>13</sup> Also, EDHB regulates heme oxygenase-1 mRNA expression through hypoxia-inducible factor-1 $\alpha$ -independent mechanisms<sup>14</sup>; however, the dual function of EDHB of inducing osteoblastic differentiation and inhibiting osteoclast differentiation has not been previously evaluated and warrants further evaluation.<sup>15–17</sup>

Alginates have proven to be a biodegradable delivery vehicle for tissue engineering application.<sup>18</sup> They have favorable properties, such as good biocompatibility, a high porosity, and interconnected porous structures for nutrient and oxygen diffusion.<sup>19</sup> The most common method of incorporating bioactive molecules or cells into alginate matrices is via extrusion, in which an alginate suspension is extruded through a needle to form droplets that fall into a solution that contains divalent cations, causing alginate cross-linking.<sup>20</sup>

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**FIG. 1.** Chemical structure of ethyl-3,4-dihydroxybenzoate (EDHB).

During the bone regeneration process, progenitor cells play a role in receiving multiple signals, particularly from proteins in extracellular matrix (ECM). Following these signals, the cells initiate chemotaxis to the site of injury and are subsequently differentiated to mature osteoblasts. This is followed by secretion of ECM proteins and bone mineralization.<sup>21</sup> Therefore, a successful bioactive compound candidate for use in bone tissue engineering should be able to stimulate and enhance the above-mentioned processes, and we have analyzed them in the present study.

When bone integrity is damaged, under normal circumstances, MSCs play an important role in bone healing. MSCs are multipotent cells of mesodermal origin, capable of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, and myoblasts.<sup>22–27</sup> These cells are identified as marrow stromal cells because they are relatively abundant in the bone marrow, which is their typical source. Apart from bone marrow, they are found in the endosteum of the trabecular bone and in the periosteum.<sup>28</sup> Limited sources of MSCs include fat tissue, funicle blood, muscle, and synovial membrane.<sup>29,30</sup> MSCs are found in small quantities in peripheral blood and other tissues<sup>31</sup> and have been isolated from the liver, brain, and pancreas.<sup>32</sup>

Successful bone remodeling should be balanced between bone formation and bone resorption. Upon implantation, biomaterial interacts with both osteoblasts and osteoclasts. Bone mass is controlled by continuous bone remodeling through bone formation and bone resorption of osteoclasts. Abnormalities in bone remodeling can produce a variety of bone-decreasing disorders such as osteoporosis, hypercalcemia, rheumatoid arthritis, tumor metastasis into bone, and periodontitis.<sup>33</sup>

The purpose of this study was to evaluate the osteoinductive effect of EDHB on preosteoblasts and human MSCs (hMSCs). Using preosteoblast cells, the ability of the EDHB to induce osteogenic differentiation and bone formation was examined by studying both early and late markers of differentiation, inducing matrix mineralization, and analyzing bone nodule formation. Also, the effect of EDHB on osteoclast differentiation was evaluated. Finally, the osteoinductive effect of EDHB was evaluated *in vivo*.

## Materials and Methods

### Cell culture and materials

RAW264.7 mouse monocyte cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Wel-GENE) containing 4.0 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

MC3T3-E1 mouse preosteoblast cells (Riken Cell Bank) were maintained in minimum essential medium  $\alpha$ -modification ( $\alpha$ -MEM; WelGENE) without ascorbic acid and supplemented with 10% FBS and 1% antibiotic-antimycotic solution.

hMSCs (Lonza) were maintained in MSC basal media (Lonza) supplemented with MSC growth medium Single-Quots Kit (Lonza). Osteogenic media was composed of dexamethasone (10 nM), beta-glycerolphosphate (10 mM), and ascorbic acid (50  $\mu$ g/mL).

EDHB was kindly supplied by CellSafe. The compound was dissolved in ethanol, followed by filtering with syringe. Sodium alginate was purchased from Sigma.

### Osteoclast differentiation and tartrate-resistant acid phosphatase assay

RAW264.7 is a murine monocyte cell line that can be induced to differentiate into osteoclast-like cells by treatment with receptor activator of nuclear factor- $\kappa$ B ligand (RANKL).<sup>34</sup> RAW264.7 cells were seeded at  $2.5 \times 10^3$  cells/cm<sup>2</sup> and incubated with RANKL (50 ng/mL) for 6 days to obtain osteoclasts. Inhibition of differentiation was examined by co-treatment with the EDHB at 1, 2, and 4  $\mu$ g/mL. Mature osteoclasts were visualized by tartrate-resistant acid phosphatase (TRAP) staining using the leukocyte acid phosphatase kit (Sigma), according to manufacturer's instructions. Multinucleated, TRAP-positive cells were counted under inverted phase-contrast microscope (IX-50; Olympus), and multinucleated cells containing three or more nuclei were scored as osteoclasts.

For measuring TRAP activity, multinucleated osteoclasts were fixed with 10% formalin for 10 min, followed by 95% ethanol for 1 min. The cells were incubated with 100  $\mu$ L of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM  $\rho$ -nitrophenylphosphate (pNPP; Sigma) for 1 h. The reaction was stopped by adding 0.1 N NaOH. The absorbance of the resulting solution was measured using an ELISA reader (Spectra MAX3340; Molecular Devices Corp.) at a wavelength of 410 nm.

### Alkaline phosphatase assay

To determine alkaline phosphatase (ALP) activity, MC3T3-E1 cells were seeded at an initial density of  $7 \times 10^3$  cells/cm<sup>2</sup>. Osteogenic media was supplemented after 24 h incubation and exchanged every 3 days. The cells were cultured in the presence or absence of EDHB at 5  $\mu$ g/mL. At specific culture times (i.e., 3, 7, and 10 days after seeding), the cells were rinsed with phosphate-buffered saline (PBS) and lysed with 1% Triton X-100 in Tris/NaHCO<sub>3</sub> buffer (12.5 mM each, pH 6.8). ALP activity in the cell lysate was determined by a colorimetric assay using phosphatase substrate (pNPP; Sigma), which is converted to a yellow, soluble end product. Cell lysates were mixed with 10 mM pNPP solution in glycine buffer (0.1 M glycine with 1 mM MgCl<sub>2</sub> and 1 mM ZnCl<sub>2</sub>, pH 10.4) and incubated at 37°C for 30 min. The absorbance was measured at 405 nm. At the same time, cell lysate protein concentrations were determined by bicinchoninic acid assay (Pierce Biotechnology), according to the manufacturer's protocol.

### Collagen assay

Collagen is specifically bound by Sirius Red stain, which was used as described previously.<sup>35</sup> Briefly, collagen deposited

in the ECM on days 7, 10, and 14 after treatment with EDHB (5 µg/mL) was visualized with 0.1% Sirius Red stain (Sircol; Biocolor Ltd.). The cells were fixed with 3% paraformaldehyde and stained with Sirius Red solution for 30 min. For quantitative analysis, the collagen-bound stain was eluted by washing with 0.1 M NaOH, and its absorbance at 540 nm was measured using an ELISA reader.

#### *Mineralization test: Alizarin Red staining*

Mineralization of collagenous matrix was observed by Alizarin Red S (ARS) staining of calcium deposits. MC3T3-E1 was cultured in the presence or absence of EDHB (5 µg/mL), and mineralization was detected after 3, 4, and 5 weeks of culture. Cell monolayers were rinsed with PBS, fixed in 70% ethanol, and stained with a 40 mM solution of ARS (pH 4.2) for 30 min. After washing, the plate was photographed. For quantitative analysis, the staining was extracted by incubation in 1 mL of 10% (w/v) cetylpyridinium chloride (Sigma) with shaking for 30 min. Optical density of the extracted stain was read at 570 nm.

#### *Western blot analysis*

To conduct western blot analysis of the runt-related transcription factor 2 (Runx2) expression, MC3T3-E1 cells were seeded at an initial density of  $2 \times 10^5$  cells/well in six-well plates, and incubated for 5 days in the presence or absence of EDHB at 1 and 5 µg/mL. On given days, the cells were rinsed with ice-cold PBS and lysed with ice-cold RIPA buffer. The cell lysate was collected and centrifuged at 14,000 g for 15 min at 4°C. Protein concentrations in the solution were determined by DC Bio-Rad assay according to the manufacturer's protocol (BioRad Laboratories, Inc.). Protein samples were separated using 12.5% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were blocked with blocking buffer (5% nonfat dry milk and 1% Tween-20 in 20 mM TBS, pH 7.6) for 1 h at room temperature, and then probed overnight at 4°C with rabbit monoclonal anti-Runx2 antibody (1:2000 dilution; Cell Signaling Technology) or mouse monoclonal anti-GAPDH antibody (1:5000 dilution; Chemicon International). Finally, the membranes were incubated with secondary antibody (i.e., anti-mouse IgG [1:2000], anti-rabbit IgG [1:2000] from Santa Cruz Biotechnology, Inc.), horseradish peroxidase-conjugated. Protein expression was detected by ECL (Amersham Biosciences). Densitometry of bands was evaluated using Image J software.

#### *hMSC culture in an alginate bead three-dimensional culture system*

hMSCs were suspended at  $1 \times 10^6$  cells/mL in sterile 4% (w/v) sodium alginate solution. The cell-gel solution was dropped through a 20-gauge needle into a stirred solution of 100 mM calcium chloride (Sigma). Alginate beads were allowed to stabilize for 10 min followed by culture in a HARV bioreactor (Synthecon, Inc.) for 21 days. The vessel was rotated at 20 rpm. The control media consisted of dexamethasone (10 nM), beta-glycerolphosphate (10 mM), ascorbic acid (50 µg/mL), and CaCl<sub>2</sub> (200 mg/L). Ethanol alone was used as a control solution because EDHB was dissolved in ethanol. The EDHB media was treated with EDHB (10 µg/mL) that was exchanged every 2 days.

#### *In vivo study: subcutaneous injection of nude mouse with alginate-EDHB beads*

Six-week-old Balb/c nude mice were purchased from Orient Bio. All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit No. 2011-0107). All mice were maintained in the specific pathogen-free facility of the YLARC. A total volume 200 µL of alginate gel with cells was subcutaneously transplanted into the back of the mice. The mice were divided into the following groups: the control group was transplanted with alginate gel with hMSCs; the second group received alginate gel with dexamethasone at (10 nM) and hMSCs; and the third group received alginate gel with EDHB (5 µg/bead, 50 µg/bead) and hMSCs. The animals were sacrificed at 5 or 8 weeks following transplantations, and the transplanted site was isolated for histological and immunohistochemical examinations.

#### *In vitro release test of EDHB from EDHB-loaded alginate bead*

To evaluate the *in vitro* release profiles of EDHB from EDHB (25 µg/mL)/alginate beads and EDHB (250 µg/mL)/alginate beads, each sample was immersed in PBS buffer (pH 7.4) at 37°C. At predetermined time intervals of 1, 2, 3, 4, 5, 24, 72, 120, and 168 h, the supernatant was collected and replaced with fresh PBS solution. The amount of EDHB released in the collected solution was determined spectrophotometrically at a wavelength of 214 nm.

#### *Histology and immunohistochemistry*

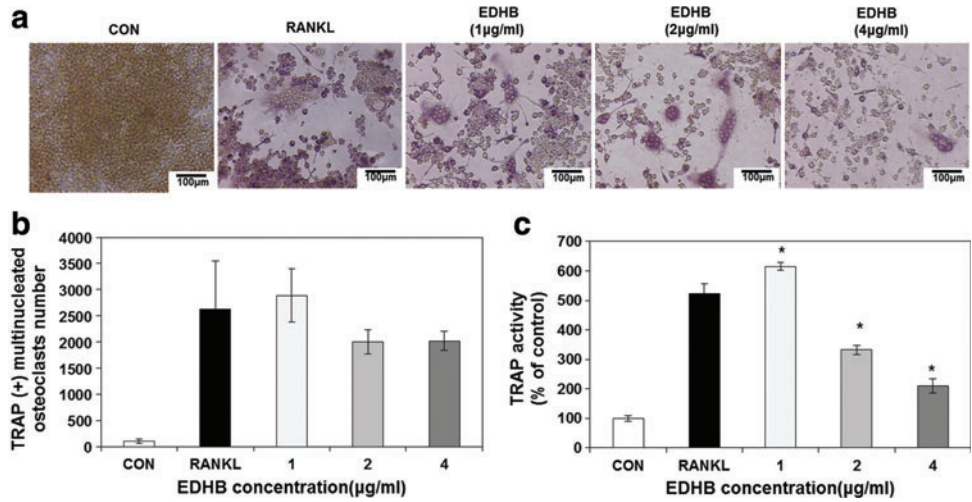
The injection site was completely excised and processed for classical histology. hMSCs seeded in the alginate bead were fixed in neutral buffered formaldehyde solution. Specimens were cut into 4-µm-thick sections, after which the nucleus and cytoplasm were stained with hematoxylin and eosin (H&E). For von Kossa staining, sectioned alginate gel slides were incubated in 1% silver nitrate solution under UV lamp for 1 h. The reaction was stopped by incubation in 5% sodium thiosulfate solution for 5 min. As mentioned above, ARS staining was also carried out.

For immunohistochemical analysis, unstained paraffin section slides were rehydrated with a series of ethanol. In sequence, these slides were exposed to 0.3% hydrogen peroxide, incubated in citrate buffer (pH 6.0) at 95°C for 20 min, and blocked in 1% BSA solution for 10 min. The slides were then incubated overnight at 4°C with primary antibodies to collagen type I (1:500 dilution; R&D System) or osteocalcin (1:20 dilution; Abcam) and staining with LSAB + System-HRP kit (Dako) following the manufacturer's protocol. Sections were dehydrated and mounted. The samples were observed using an optical microscopy (BH-2; Olympus).

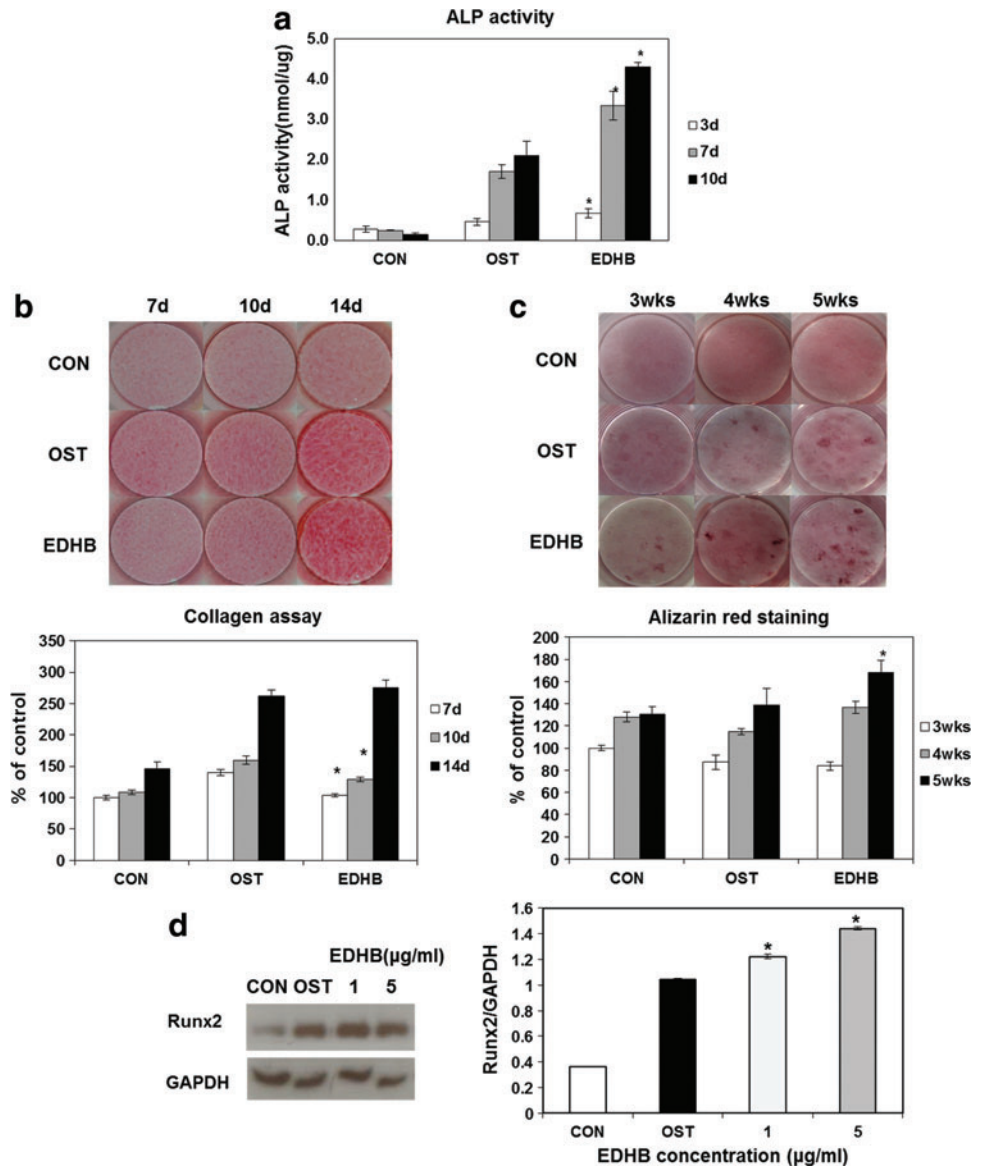
#### *Statistical analysis*

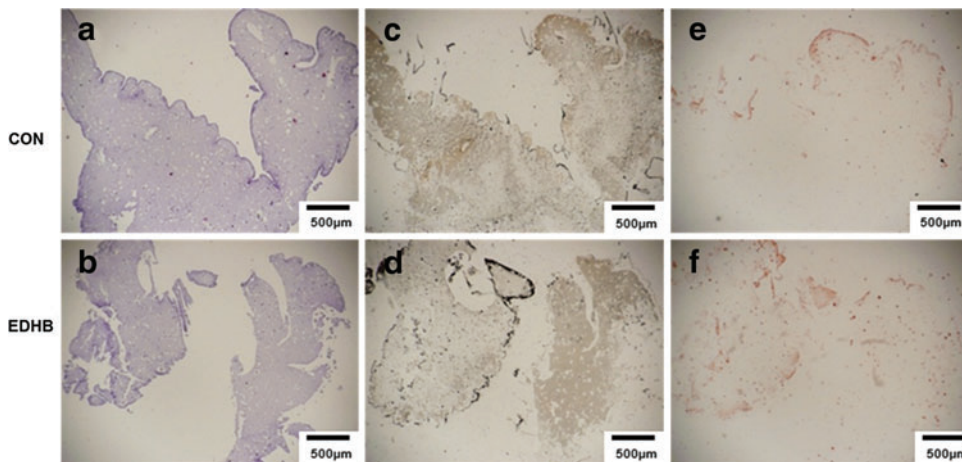
All results are expressed as mean ± standard deviation for the three determinations and analyzed by Student's *t*-test. Statistical significance was defined as  $p < 0.05$ .

**FIG. 2.** EDHB decreased osteoclast formation. (a) RAW264.7 cells were co-treated with receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and EDHB (1–4  $\mu$ g/mL) for 6 days. (b) Mature multinucleated osteoclasts were counted following staining to detect tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells. (c) EDHB treatment significantly decreased TRAP activity compared with treatment with RANKL alone (\*  $p < 0.05$ ). Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)



**FIG. 3.** Osteogenic differentiation of MC3T3-E1 cells cultured *in vitro*. Analysis of alkaline phosphatase (ALP) activity (a), collagen assay (b), Alizarin Red S (ARS) staining (c), and western blot analysis for the runt-related transcription factor 2 (Runx2) (d). Each error bar represents the mean  $\pm$  standard deviation (SD) ( $n = 3$ ). \*Statistically significant differences ( $p < 0.05$ ). Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)





**FIG. 4.** Histological images of alginate beads cultured in a bioreactor for 21 days. (a, b) hematoxylin and eosin (H&E) staining, (c, d) von Kossa staining, and (e-f) ARS staining. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

## Results

### *Inhibitory effect of EDHB on osteoclast differentiation*

To evaluate the effect of EDHB on osteoclast differentiation, RAW264.7 cells were cultured in the presence of RANKL with EDHB (Fig. 2a). Osteoclast differentiation was confirmed by counting large multinucleate osteoclasts following 6 days of culture in the presence or absence of EDHB (Fig. 2b). RAW264.7 cells treated with RANKL showed multinucleated osteoclasts after 6 days of treatment. The number of multinucleated osteoclasts was decreased in the EDHB-treated group, but it was not significantly different.

The effect was further determined by quantification of TRAP activity (Fig. 2c). EDHB at low concentrations led to an increase in TRAP activity. EDHB induced a significant decrease in osteoclast differentiation, starting at concentration of 2 µg/mL. EDHB at concentration of 2 and 4 µg/mL steadily reduced TRAP activity by 54% and 77%, respectively, in comparison with RANKL-only treated cells. These results suggest that osteoclast formation significantly correlated with the amount of EDHB in the culture medium and EDHB has an inhibitory effect of osteoclast formation. Also, EDHB induced a biphasic effect on osteoclast differentiation, inducing differentiation at lower concentrations and inhibiting differentiation at higher concentrations.

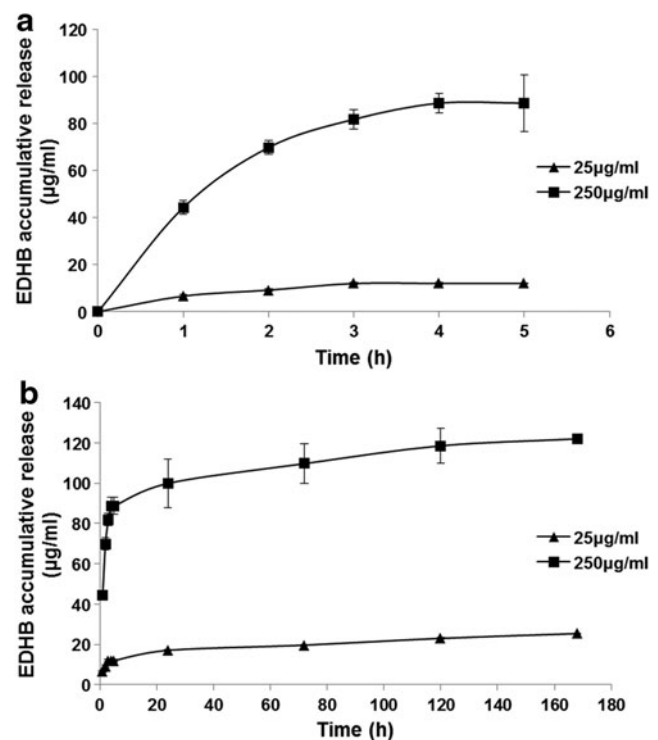
### *Osteoblast differentiation effect of EDHB on MC3T3-E1*

To determine whether osteoblastic differentiation was stimulated by EDHB treatment, preosteoblasts were treated with EDHB (5 µg/mL). ALP activity as an early marker of osteoblast differentiation was examined. This activity was evaluated over 10 days of culture of MC3T3-E1 in control media or osteogenic media with or without EDHB (Fig. 3a). The ALP activity of cells in osteogenic media only and EDHB-treated cells continuously increased even after day 7 but did not reach their peak until the end of the assay (day 10). The ALP activity of treated EDHB was increased significantly for 10 days, and the increase over this time period was more than twofolds compared with cells in only osteogenic media.

Collagen assay was measured over 14 days as a middle marker of osteoblast differentiation in MC3T3-E1 cells stimulated by EDHB in osteogenic media (Fig. 3b). In MC3T3-E1 cells treated with EDHB, collagen deposition

steadily increased up to day 14 when it was reached its peak. Because type I collagen is the major matrix component of bone and plays a critical role in mineralization, we compared the quantity of collagen deposited by the EDHB-treated and non-treated MC3T3-E1 cells. At days 7 and 10, the collagen deposition was decreased by 37% and 31% respectively, in the group of MC3T3-E1 treated with EDHB, compared with cells treated with osteogenic media only. However, as shown in Figure 3b, the quantity of collagen deposited in the ECM was increased in the group of cells treated with EDHB on day 14 compared with cells treated with osteogenic media only.

To evaluate bone nodule formation, MC3T3-E1 cells were cultured in osteogenic medium with EDHB (5 µg/mL)



**FIG. 5.** Accumulative release profiles of EDHB ((a) up to 5 h, (b) up to 168 h) from alginate beads. The error bars represent mean  $\pm$  SD ( $n=4$ ).

for 3, 4, or 5 weeks (Fig. 3c). The levels of bone nodule formation were visualized by staining with ARS and quantified by measuring the absorbance of the red dye eluted. As shown in Figure 3c, the stained region of red indicates bone nodule-like mineralized matrix accumulation. ARS staining showed large mineralized areas (in red) in the presence of EDHB, whereas little staining was visible in the untreated group. Quantitatively, the amount of calcium deposition at 5 weeks was 34% higher in the EDHB-treated group compared with the untreated group, and this difference was statistically significant ( $*p < 0.05$ ). Overall, MC3T3-E1 cells treated with EDHB showed markedly increased calcification of ECM compared with that in the non-treated cells.

We determined the expression of the bone-associated transcription factor Runx2, which is essential for osteogenesis, especially for the development of osteoblasts.<sup>36</sup> Quantitative analysis of this transcript in MC3T3-E1 cells demonstrated a significant increase in Runx2 activity at day 5 compared with untreated cells (Fig. 3d).

These results indicate that EDHB may play a role in the stimulatory effect on osteoblast differentiation in preosteoblasts.

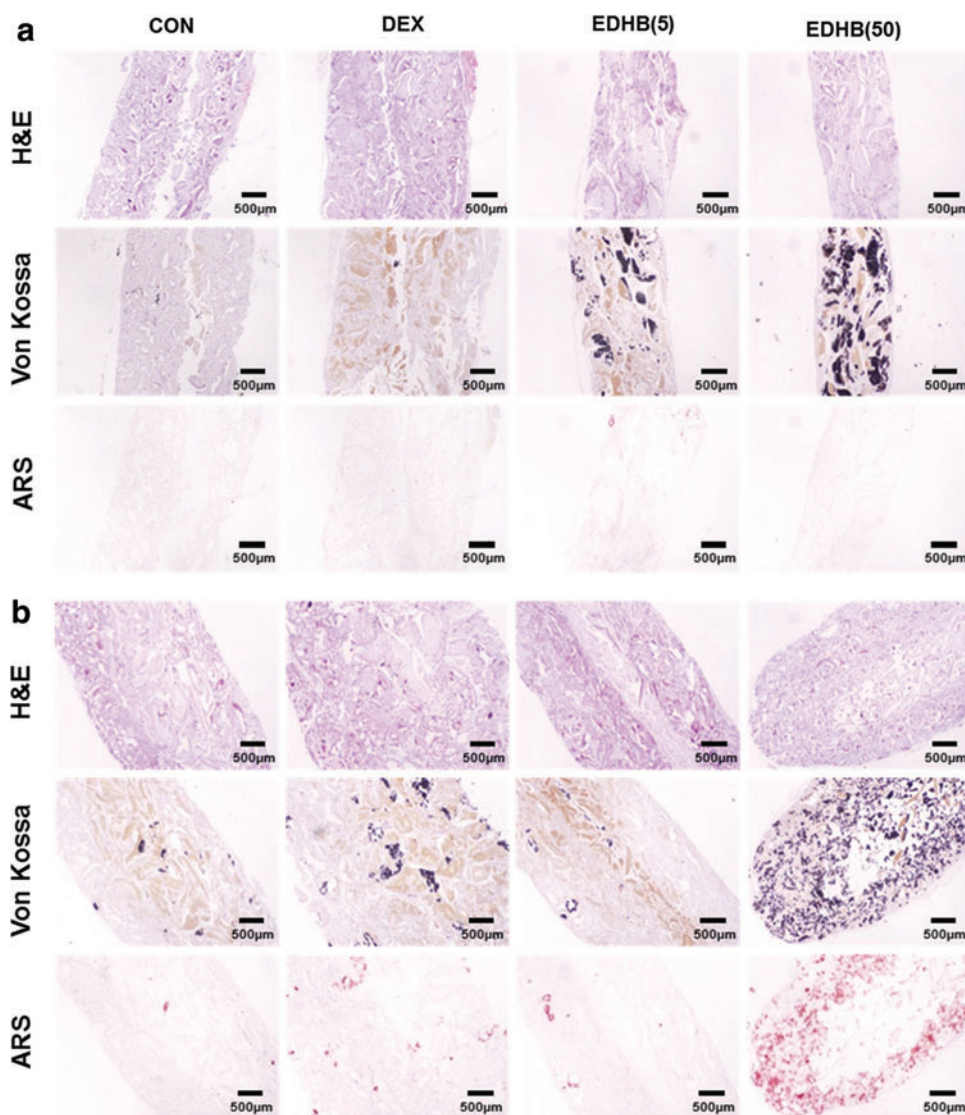
#### Histological analysis of hMSCs cultured in a bioreactor in alginate bead

To evaluate the osteoinductive activity in a three-dimensional (3D) dynamic culture system, hMSCs were cultured in a HARV bioreactor for 21 days. hMSCs cultured in a bioreactor with osteogenic media containing EDHB showed more red or black color using ARS and von Kossa staining methods, compared with the hMSCs cultured in osteogenic media (control). H&E staining confirmed well-formed 3D tissue formation within the alginate gel (Fig. 4).

#### In vitro EDHB release test

The release profiles obtained after the release of EDHB from alginate beads are shown in Figure 5. The amount of EDHB (25  $\mu\text{g}/\text{mL}$ ) and EDHB (250  $\mu\text{g}/\text{mL}$ ) released on the 5 h was  $\sim 48\%$  and 35%, respectively (Fig. 5a). Over a period of 168 h, 25 and 122  $\mu\text{g}/\text{mL}$  of EDHB was released from the 25  $\mu\text{g}/\text{mL}$ -EDHB in alginate beads and 250  $\mu\text{g}/\text{mL}$ -EDHB in alginate beads, respectively (Fig. 5b). EDHB was released from the alginate beads in a rapid burst over the first 5 h followed by a phase of slower release.

**FIG. 6.** *In vivo* Balb/c nude mice model for bone tissue engineering. Histological images of human mesenchymal stem cells (hMSCs) inserted in alginate beads with different types calcium deposition. H&E staining, von Kossa staining, and ARS staining (**a**) at 5 weeks after implantation and (**b**) 8 weeks after implantation. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)



### Histological/immunohistochemical analysis of implanted alginate bead to study *in vivo* bone formation

To examine osteogenic differentiation, hMSCs inserted into alginate beads containing dexamethasone or EDHB were transplanted into Balb/c nude mice. H&E staining was used to determine cell distribution and cell density (Fig. 6). At 5 and 8 weeks following transplantation of hMSCs into nude mice, alginate beads containing dexamethasone or EDHB showed much denser cell distributions. However, alginate bead containing only hMSCs showed smaller amounts of cells.

ARS and von Kossa staining of hMSCs embedded onto alginate beads containing dexamethasone or EDHB were analyzed to evaluate the calcium levels produced *in vivo*. Osteoblast cells converted from hMSCs that were embedded into alginate beads containing dexamethasone or EDHB had increased calcium that was secreted and accumulated by hMSCs. EDHB induced a significant increase in the mineral deposition area in a dose-dependent manner. However, alginate beads with only hMSCs did not affect calcium accumulation. Calcium accumulation by hMSCs showed that EDHB enhanced calcium deposition through differentiation of hMSCs into osteoblast cells. This result indicated that hMSCs differentiated into osteoblasts that could produce calcium and suggested that EDHB could help bone formation through increased osteoblast differentiation. EDHB-enhanced bone formation may have dual functions in regulating both osteoblasts and osteoclasts *in vivo*.

Collagen type I and osteocalcin were analyzed to determine whether hMSCs inserted into alginate gel differentiated into bone cells in response to stimulation with EDHB (Fig. 7). The expression of collagen type I was similar upon treatment with either dexamethasone or EDHB (Fig. 7a). As a middle marker, collagen type I is typically expressed

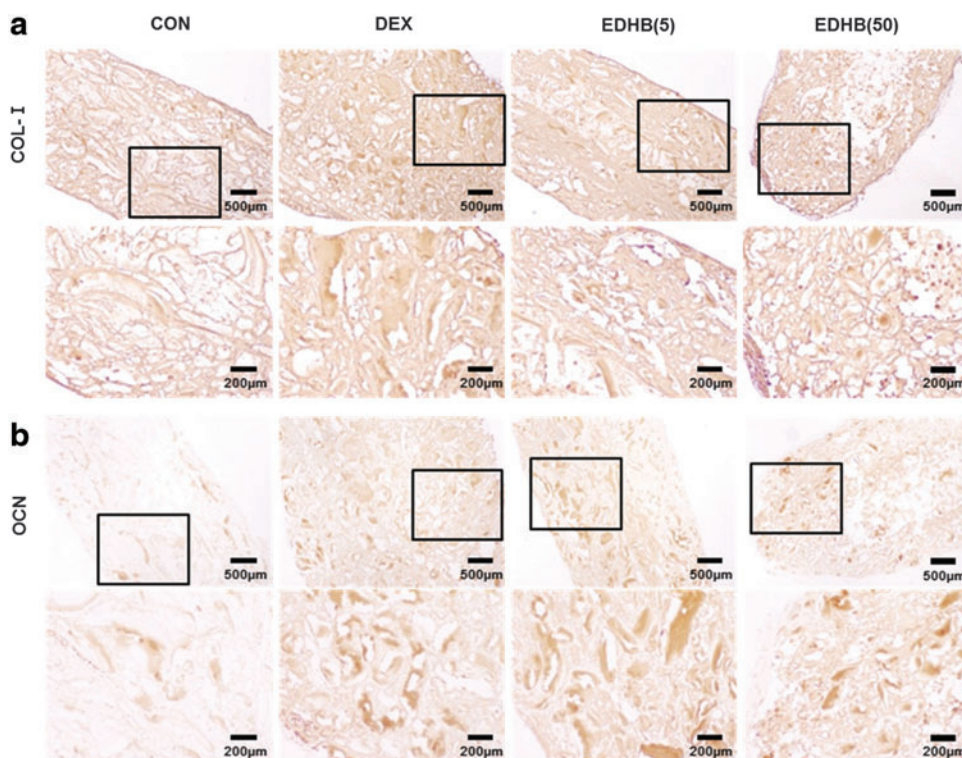
within 8 weeks, and its level of expression was not significantly different from either dexamethasone or EDHB. In the controls, osteocalcin staining was weak. On the other hand, the increased level of osteocalcin we found indicates that the implanted hMSCs differentiated into osteoblasts when treated with dexamethasone or EDHB (Fig. 7b).

### Discussion

In this study, we investigated whether EDHB inhibits osteoclast differentiation and stimulates osteoblastic differentiation *in vitro* and *in vivo*. We confirmed the osteoinductive effect of EDHB and analyzed the cell response. Also, we demonstrated that EDHB caused an increase in the calcification of the ECM in MC3T3-E1 cells without any significant cytotoxic effects on the cells (Supplementary Fig. S1; Supplementary Data are available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)). The ability of EDHB to induce osteogenic differentiation was confirmed by increases in ALP expression, collagen deposition, bone nodule formation, Runx2 expression, and bone regeneration.

Bone is continuously destroyed and reformed in vertebrates to maintain bone volume and calcium homeostasis. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Osteoblasts produce bone matrix proteins, including type I collagen, the most abundant extracellular protein of bone, and also cause tissue mineralization.<sup>37</sup> Osteoblasts, chondrocytes, myocytes, and adipocytes are all derived from a common progenitor called undifferentiated mesenchymal cells. During cell differentiation, progenitor cells acquire specific phenotypes under the control of their respective regulatory factors.<sup>38</sup>

In our *in vitro* study using RAW264.7 cells, we found that EDHB at concentrations of 2 and 4  $\mu\text{g}/\text{mL}$  dose-dependently inhibited RANKL-induced osteoclast differentiation. The



**FIG. 7.** Immunohistochemical analysis of alginate beads 8 weeks following implantation. (a) Images showing collagen type I (COL-I) in alginate beads with hMSCs in the presence of dexamethasone (10 nM) or EDHB (5  $\mu\text{g}/\text{bead}$  and 50  $\mu\text{g}/\text{bead}$ ) 8 weeks following implantation. (b) Images showing osteocalcin (OCN) in alginate beads with hMSCs in the presence of dexamethasone (10 nM) or EDHB (5  $\mu\text{g}/\text{bead}$  and 50  $\mu\text{g}/\text{bead}$ ) 8 weeks following implantation. Black rectangle boxes indicate the enlarged photograph of the images below. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

total TRAP activity was most dramatically reduced with EDHB treatment. This might be attributable to the presence of TRAP-positive mononuclear cells in EDHB-treated cultures. Therefore, we found that EDHB dose-dependently suppressed the osteoclast differentiation and bone resorption activity at an early stage of osteoclastogenesis in cultured RAW264.7 cells. Also, we found that EDHB induced a biphasic effect on osteoclast differentiation, inducing differentiation at lower concentrations and inhibiting differentiation at higher concentrations. Karst *et al.*<sup>39</sup> reported that low-dose TGF- $\beta$  stimulation of spleen and marrow precursors cultured with stromal support cells may be accounted for by regulation of the RANKL/osteoprotegerin (OPG) expression ratio in stromal cells. Accordingly, EDHB may be capable of stimulating RANKL expression at any concentration.

Tissue engineering for bone repair has received considerable attention in reconstructive surgery during the past decade. The most common strategy to engineer bone is performed using three components: a scaffold, growth factors, and MSCs. There are a number of reports of successful bone formation achieved with a combination of these three elements in animal experiments.<sup>40</sup> However, there are only a few reports of successful bone formation using an injectable gel as a scaffold for the cells.<sup>41</sup> In this study, the alginate gel was completely resorbed without forming new bone when the alginate gel contained only MSCs. We used calcium alginate as carrier material for several reasons. First, alginate is a biocompatible polysaccharide that is structurally similar to the glycosaminoglycans present in cartilage.<sup>42–44</sup> Also, alginate gelation properties allow gel formation directly on bone defects by the simple addition of a calcium chloride solution. Thus, it is a fluid material that can fill the shape of any defect and incorporate various bioactive agents (e.g., proteins and growth factors).<sup>45–48</sup> Alginate has been extensively studied in cartilage tissue regeneration.

Osteoclast differentiation is regulated by many systemic hormones and local cytokines (e.g., tumor necrosis factor- $\alpha$ , interleukin-1, interleukin-6, parathyroid hormone, and macrophage colony-stimulating factor).<sup>49</sup> Recently, a more direct anabolic role of osteoclasts in stimulating bone formation has been suggested, one in which osteoclasts secrete anabolic growth factors that mediate osteoblast chemotaxis, proliferation, differentiation, and mineralization.<sup>50–53</sup> However, the mechanism of osteoclast-mediated bone formation is not well understood. Cytokines and growth factors released from osteoclasts may assist in maintaining a healthy balance between adipogenic and osteogenic differentiation.<sup>54</sup> RANKL, a stimulator of osteoclast differentiation and activity, and OPG, the inhibitor of osteoclastogenesis, are differentially expressed during osteoblast development.<sup>55</sup>

Bone regeneration may be influenced by stimulating osteoblast differentiation and preventing osteoclast differentiation. We suggest that EDHB could help bone remodeling by stimulating osteoblast differentiation, while, at the same time, preventing bone loss by inhibiting osteoclast differentiation.

## Conclusions

In conclusion, this study examined the utility of EDHB to increase osteoinductive activity and decrease bone resorption. We demonstrated that EDHB can be an effective bioactive compound material and that it plays a role in inducing

osteoblast differentiation and inhibiting osteoclast differentiation. We verified the osteoinductive activity through animal testing. Therefore, EDHB is a strong candidate for dual regulation of osteoblasts and osteoclasts in bone tissue engineering. Our future objective is to further investigate the mechanism of this dual regulation.

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## Disclosure Statement

No competing financial interests exist.

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