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ARTICLE

Linalool Promotes Osteoblast Differentiation via SELENBP1-Dependent Signaling in MC3T3-E1 and C3H10T1/2 Cells

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ABSTRACT: Background: Linalool is a monoterpene alcohol with known anti-inflammatory and antioxidant properties, but its role in osteoblast differentiation remains unclear. This study aimed to investigate the osteogenic potential of linalool and to examine the role of selenium-binding protein 1 (Selenbp1) in mediating its effects. **Methods:** Murine MC3T3-E1 and C3H10T1/2 cells were treated with linalool under osteogenic conditions. Osteoblast differentiation was assessed by alkaline phosphatase (ALP) activity, Alizarin Red S staining, and expression of runt-related transcription factor 2 (Runx2) and distal-less homeobox 5 (Dlx5). The involvement of Selenbp1 was examined using siRNA knockdown and plasmid overexpression. A zebrafish caudal fin regeneration model was used to evaluate *in vivo* relevance. **Results:** Linalool significantly enhanced osteoblast differentiation, as evidenced by increased ALP activity (approximately 3–4-fold vs. control, $p < 0.01$) and matrix mineralization (approximately 2.5–4-fold increase, $p < 0.01$). The osteogenic transcription factors Runx2 and Dlx5 were significantly upregulated following linalool treatment ($p < 0.01$). Linalool also markedly induced Selenbp1 expression, showing an approximately 4–5-fold increase in MC3T3-E1 cells and an approximately 8–9-fold increase in C3H10T1/2 cells ($p < 0.01$). Silencing Selenbp1 attenuated the linalool-induced upregulation of osteogenic markers, whereas its overexpression restored marker expression and enhanced cellular responsiveness to linalool. *In vivo*, linalool significantly increased zebrafish caudal fin regeneration by approximately 30–45% compared with the vehicle control ($p < 0.001$). **Conclusion:** Linalool promotes SELENBP1-dependent osteoblast differentiation *in vitro* and enhances caudal fin regeneration *in vivo*, although the involvement of SELENBP1 in the latter was not examined.

KEYWORDS: Linalool; selenium-binding protein 1; preosteoblast; mesenchymal stem cell; osteoblast differentiation

1 Introduction

Osteoblast differentiation plays a central role in bone formation and skeletal maintenance [1,2]. This process is tightly regulated by transcription factors such as runt-related transcription factor 2 (Runx2) and distal-less homeobox 5 (Dlx5), which coordinate the activation of osteogenic genes and extracellular matrix maturation [3]. Notably, Dlx5 functions upstream of Runx2 and plays a critical role in early osteoblast lineage commitment, preceding the induction of late-stage differentiation markers such as Osterix and osteocalcin [4]. Impaired osteoblast activity is a major cause of osteoporosis [5] and contributes to delayed fracture healing [6].

Natural compounds have gained attention as modulators of bone metabolism [7]. Flavonoids, for instance, stimulate osteoblast differentiation via Wnt/β-catenin signaling [8], while terpenes exert osteogenic effects

through Mitogen-Activated Protein Kinases (MAP kinase; MAPKs) and Phosphatidylinositol 3-kinase (PI3K)/Akt pathways [9]. Recent reviews also highlight the antioxidant and anti-inflammatory actions of phytochemicals in bone remodeling [10]. However, the role of monoterpenes in osteoblast differentiation remains insufficiently defined [11]. Several phytochemicals illustrate the osteogenic potential of natural compounds [12]. Icariin from *Epimedium* species promotes osteoblast proliferation and differentiation by stimulating bone morphogenetic protein 2 (BMP2) signaling [13]. Resveratrol enhances osteogenesis in mesenchymal stem cells via estrogen receptor-dependent ERK1/2 activation [14]. Baicalin induces osteoblast differentiation by up-regulating Runx2 and osterix [15], while quercetin decreases osteoclast differentiation through inhibition of Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) signaling [16]. These findings indicate that phytochemicals regulate bone remodeling through diverse molecular mechanisms [10,17].

Linalool, a monoterpene alcohol present in essential oils of *Lavandula angustifolia* and *Coriandrum sativum* [11], is known for its anti-inflammatory, antioxidant, and neuroprotective effects [18,19]. It modulates redox balance and inflammatory pathways, both of which are closely linked to bone remodeling [20,21]. However, its role in osteoblast differentiation has not been clearly defined.

Selenium-binding protein 1 (Selenbp1) is a cytosolic protein implicated in redox regulation and cellular differentiation. Decreased Selenbp1 expression has been associated with tumor progression, whereas its upregulation supports lineage commitment in adipocytes and epithelial cells [22,23]. Selenium and selenoproteins have also been recognized as important regulators of bone homeostasis by modulating oxidative stress and cellular signaling [24,25]. However, the specific role of Selenbp1 in osteoblast biology has not yet been defined.

Therefore, this study aimed to investigate the effects of linalool on osteoblast differentiation and to elucidate the underlying molecular mechanism, focusing on the role of Selenbp1.

2 Materials and Methods

2.1 Cell Culture

MC3T3-E1 pre-osteoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in α-MEM (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. C3H10T1/2 (ATCC) cells were maintained in DMEM (Gibco) containing 10% FBS and 1% penicillin–streptomycin under the same incubation conditions. Cells were subcultured every 2–3 days and used at passages 5–15. Culture media were replaced at 2–3 day intervals. Mycoplasma contamination was routinely checked using a PCR-based detection kit (Takara, Shiga, Japan), and all cultures were confirmed to be negative.

2.2 Reagents and Treatment

Linalool (≥98% purity, Sigma-Aldrich, Cat. No. L2602, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted with culture medium to final concentrations (1–100 μM). The final concentration of DMSO was kept below 0.1% (v/v). For osteogenic induction, cells (MC3T3-E1 and C3H10T1/2) were seeded and allowed to reach approximately 70–80% confluence before differentiation was initiated. Cells were then cultured in osteogenic medium consisting of α-MEM (Gibco, Cat. No. 12561-056) and DMEM (Gibco, Cat. No. 11885-084) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate. Dexamethasone was not included in the differentiation medium. The medium was refreshed every 2–3 days, and osteogenic induction was continued for up to 21 days unless otherwise indicated. Basal control cells were maintained in growth medium without

osteogenic supplements, while osteogenic control cells were cultured in osteogenic medium without linalool. Vehicle control cells were treated with the corresponding concentration of DMSO.

2.3 Cell Viability Assay

Cell viability was evaluated using an MTT-based colorimetric assay. MC3T3-E1 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and treated with linalool (0–200 μM) for 24 or 48 h. After treatment, 20 μL of MTT solution (5 mg/mL, Sigma-Aldrich, Cat. No. M2128) was added to each well and incubated for 4 h. The formazan crystals were dissolved in 150 μL DMSO, and absorbance was measured at 570 nm using a microplate reader (Tecan, infinite M200, Grödig, Austria).

2.4 Alkaline Phosphatase (ALP) Activity

MC3T3-E1 and C3H10T1/2 Cells were cultured in osteogenic medium presence or absence of 10 μM linalool for 7 days. Cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and fixed with 10% neutral-buffered formalin for 10 min at room temperature. After fixation, cells were rinsed twice with deionized water and incubated with BCIP/NBT substrate solution (Sigma-Aldrich, Cat. No. B1911) for 15 min at room temperature in the dark. The enzymatic reaction was terminated by gently washing the cells three times with deionized water. Stained cells were imaged using an Epson Perfection V37 scanner (Seiko Epson Corporation, Suwa, Nagano, Japan). For quantitative analysis, staining intensity was measured using ImageJ software (version 1.53; NIH, Bethesda, MD, USA) from at least three independent experiments.

2.5 Alizarin Red S (ARS) Staining

Matrix mineralization was evaluated by Alizarin Red S (ARS) staining. MC3T3-E1 and C3H10T1/2 Cells were cultured in osteogenic medium with or without 10 μM linalool for 21 days, with the medium refreshed every 2–3 days. At the end of the induction period, cells were washed twice with PBS and fixed with 10% neutral-buffered formalin for 10 min at room temperature. After fixation, cells were rinsed with deionized water and stained with 2% (w/v) Alizarin Red S solution (pH 4.2, Sigma, Cat. No. A5533) for 20 min at room temperature with gentle shaking. Excess dye was removed by washing the cells repeatedly with deionized water until the background was clear. Stained calcium deposits were scanned using an Epson Perfection V37 scanner (Seiko Epson Corporation). For quantitative analysis, staining intensity was measured using ImageJ software (NIH, USA) from at least three independent experiments.

2.6 Conventional Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from MC3T3E1 and C3H10T1/2 cells using TRIzol reagent (Bio Science Technology, Cat. No. TS200001, Gyeongsan, Korea) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically using an EzDrop1000 microvolume spectrophotometer (BLUERAY Biotech, Taipei, Taiwan), and RNA integrity was further confirmed by agarose gel electrophoresis, showing intact 28S and 18S rRNA bands. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the PrimeScript RT reagent kit (TOPscript™ RT DryMIX, Enzynomics, Daejeon, Korea) according to the manufacturer's protocol. PCR amplification was performed in a 20 μL reaction volume containing 10 μL of 2× PCR master mix, 0.5 μM of each forward and reverse primer, 1 μL of cDNA template, and nuclease-free water. The PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. Gene-specific primers were used for Dlx5, Runx2, Selenbp1, and β-actin as an internal control. The primers used are listed in Table 1.

PCR products were separated on a 1% agarose gel containing Midori Green (Nippon Genetics, Tokyo, Japan) prepared in 1× Tris–acetate–EDTA (TAE) buffer and electrophoresed at 120 V for 20 min. A 100 bp DNA ladder (Enzynomics, Daejeon, Korea). Gel images were acquired using a Vilber Bio-Print imaging system (Vilber Lourmat, Collégien, France). Band intensities were quantified using ImageJ software (NIH, USA).

Table 1: Specific primers for RT-PCR.

Target	F/R	Primer Sequence	Size	Annealing temp. Temperatures
Dlx5	F R	5′-CAG AAG AGT CCC AAG CAT CC-3′ 5′-GAG CGC TTT GCC ATA AGA AG-3′	193	63
Runx2	F R	5′-AGA TGA CAT CCC CAT CCA TC-3′ 5′-GTG AGG GAT GAA ATG CTG G-3′	238	60
Selenbp1	F R	5′-ACA GAT GGG CTG ATA CCC CT-3′ 5′-TCC CAG GCG CTG TAT AGT GA-3′	497	60
b-actin	F R	5′-GAC TAC CTC ATG AAG ATC-3′ 5′-GAT CCA CCT CTG GAA-3′	458	53

2.7 Overexpression and Small Interfering RNA (siRNA) Transfection

MC3T3-E1 cells were plated in 24-well culture plates or 35-mm culture dishes and allowed to attach for 24 h in α-MEM supplemented with 10% fetal bovine serum (FBS). For SELENBP1 overexpression, cells were transiently transfected with 2 μg of a pCMV-SELENBP1 expression construct using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). The SELENBP1 overexpression vector (pCMV-Selenbp1) was obtained from the Korea Human Gene Bank (KRIBB, Daejeon, Korea). To suppress endogenous SELENBP1 expression, two independent small interfering RNAs targeting SELENBP1 (siSELENBP1-1 and siSELENBP1-2) were chemically synthesized (Bioneer, Daejeon, Korea), purified, and annealed according to the manufacturer's instructions. Cells were transfected with siRNAs at a final concentration of 100 nM using Lipofectamine® 2000. The nucleotide sequences of the siRNAs are provided in Table 2.

Table 2: Specific sequence for siRNA.

Target	Primer Sequence		Cat. No.
Negative control	Sense Antisense	UUC UCC GAA CGU GUC ACG U ACG UGA CAC GUU CGG AGA A	SN-1003
Selenbp1-1	Sense Antisense	AAU UUG AAG CAU CAU GGA G CUC CAU GAU GCU UCA AAU U	20341-1
Selenbp1-2	Sense Antisense	GCA ACG UGA GCA GUU UGC A UGC AAA CUG CUC ACG UUG C	20341-2

2.8 Western Blot Analysis

MC3T3-E1 and C3H10T1/2 Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (WSE-7420, ATTO Corporation, Tokyo, Japan). Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a standard. Briefly, cell lysates were mixed with Bradford reagent (B6916, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 10 min at room temperature. Absorbance was measured at 595 nm using a microplate reader (Tecan Group Ltd., Männedorf, Switzerland), and protein concentrations were calculated from a standard curve generated with serial

dilutions of BSA (0332, GENERAY Biotech Co., Ltd., Shanghai, China). Equal amounts of protein (30 μg) were separated by SDS-PAGE, and the animal ethics approval statement has been added to the revised manuscript, as requested. PVDF membranes (Cat. No. 03010040001, Roche Diagnostics, Basel, Switzerland). Membranes were blocked with 5% skim milk and incubated with primary antibodies against Dlx5 (Abcam, Cambridge, UK), Runx2, Selenbp1, and β-actin (Santa Cruz, Dallas, TX, USA) overnight at 4°C. After washing, membranes were incubated with HRP conjugated secondary antibodies, and signals were detected using enhanced chemiluminescence (ECL) detection reagent (Cat. No. RPN2232, Cytiva, Marlborough, MA, USA). Detailed information on primary and secondary antibodies used in this study, including host species, clonality, supplier, and catalog numbers, is provided in Table 3.

Table 3: Specific antibodies for Western blot.

Target	Antibody Type	Host Species	Supplier	Cat. No.	Dilution
Dlx5	Polyclonal	Rabbit	abcam	Ab64827	1:2000
Runx2	Monoclonal	Mouse	Santa Cruz	sc-390715	1:1000
Selenbp1	Monoclonal	Rabbit	Santa Cruz	sc-373726	1:1000
b-actin	Monoclonal	Mouse	Santa Cruz	sc-47778	1:1000
Secondary (anti-rabbit IgG)	Polyclonal	Goat	Jackson	111-035-003	1:5000
Secondary (anti-mouse IgG)	Polyclonal	Goat	Jackson	115-035-003	1:5000

2.9 Quantitative Analysis of Zebrafish Caudal Fin Ray Regeneration

Wild-type zebrafish (Danio rerio; mixed sex; body length 3.4–4.4 cm) were maintained under standard laboratory conditions in recirculating tanks (30 ± 1°C; 300 mL per tank, three fish per tank) with a 14 h light/10 h dark photoperiod. Fish were fed daily with commercial flake food (Tetra Bits Complete; Tetra, Melle, Germany) and Artemia salina nauplii. For fin amputation, zebrafish were anesthetized by brief immersion in cold water, and approximately 50% of the caudal fin rays (lepidotrichia) were surgically removed using a sterile scalpel. The fish were randomly assigned to three groups: control (vehicle-treated), 10 μM linalool, and 20 μM linalool (n = 6 per group) using a random number table. Linalool was dissolved in the tank water at the indicated concentrations, and fish were incubated for 10 days. The treatment solution was refreshed every 2 days. At the end of the experimental period, zebrafish were euthanized and fixed in 10% neutral-buffered formalin for 24 h. Fixed specimens were incubated in 25% saturated sodium tetraborate for 2 h, washed thoroughly with distilled water, and stained with 1% KOH containing 1 mg/mL Alizarin Red S (ARS). To visualize mineralized structures, tissues were cleared in 1% KOH supplemented with 3% H_2O_2 for 12 h. Regenerated caudal fin rays were imaged using a flatbed scanner and an optical microscope. The extent of bone regeneration was quantified by measuring the regenerated fin ray length using ImageJ software (NIH, Bethesda, MD, USA). Measurements were performed by an investigator blinded to the treatment groups. Statistical comparisons among groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The sample size (n = 6 per group) was selected based on previous zebrafish fin regeneration studies and pilot observations indicating sufficient sensitivity to detect treatment-related differences in regenerative growth. All animal experiments were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of Daegu University (approval number: DUIACC-12020/4-0313-006).

2.10 Statistical Analysis

Data are expressed as mean ± standard deviation (SD) from at least three independent biological experiments, each performed using separately prepared cell cultures on different days. Data normality and

homogeneity of variances were assessed using standard tests implemented in GraphPad Prism prior to ANOVA. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (GraphPad Prism 5.0, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Cytotoxicity of Linalool in Osteoblastic Cells

The chemical structure of linalool is shown in Fig. 1A. To determine the cytotoxicity of linalool, MC3T3-E1 and C3H10T1/2 cells were treated with increasing concentrations (1–200 μM) for 1 or 2 days, and cell viability was assessed by the MTT assay. As shown in Fig. 1B, cell viability was not affected at concentrations up to 10 μM. However, significant reductions were observed at 50 μM and above, in a dose- and time-dependent manner (**$p < 0.01$ vs. control). These results indicate that linalool is cytotoxic at higher concentrations, and doses ≤5 μM were used in subsequent experiments.

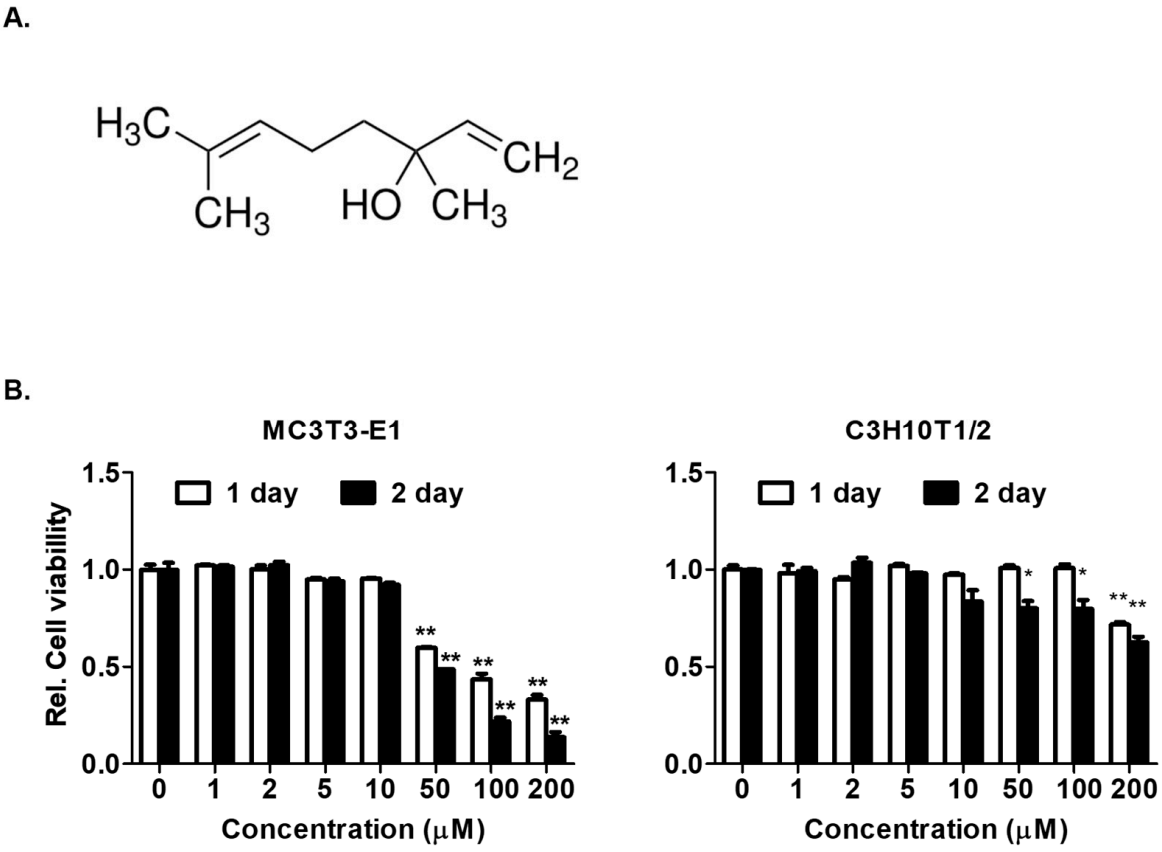

Figure 1: Chemical structure of linalool and its effects on cell viability. (**A**) The chemical structure of linalool (3,7-dimethyl-1,6-octadien-3-ol), a naturally occurring monoterpene alcohol. (**B**) The cytotoxicity of linalool was evaluated in MC3T3-E1 and C3H10T1/2 cells using the MTT assay after 1 and 2 days at various concentrations (0–200 μM). Linalool showed no significant cytotoxicity up to 10 μM, whereas higher concentrations (≥50 μM) markedly reduced cell viability (*$p < 0.05$, **$p < 0.01$). Data are expressed as mean ± SD of three independent experiments.

3.2 Linalool Upregulates Osteogenic Transcription Factors and Promotes Osteoblast Differentiation

To examine the effect of linalool on osteogenic marker expression, RT-PCR was performed in MC3T3-E1 and C3H10T1/2 cells. Linalool treatment increased the mRNA levels of *Runx2* and *Dlx5* in a time-dependent

manner, with induction observed as early as day 1 and sustained up to day 4 (Fig. 2A). Consistently, Western blot analysis demonstrated that the protein levels of Runx2 and Dlx5 were markedly elevated following linalool treatment in both cell lines (Fig. 2B). The functional effects of linalool on osteoblast differentiation were further evaluated by ALP staining after 7 days of culture in osteogenic medium. Linalool markedly enhanced ALP activity compared with control cells (Fig. 2C). Long-term differentiation assays showed that linalool also promoted matrix mineralization, as demonstrated by increased calcium deposition visualized by Alizarin red S staining after 21 days (Fig. 2D). Together, these results indicate that linalool enhances osteoblast differentiation by upregulating osteogenic transcription factors and promoting matrix maturation. Although both MC3T3-E1 and C3H10T1/2 cells showed overall increases in osteogenic marker expression, ALP activity, and matrix mineralization following linalool treatment, slight differences in the magnitude and temporal patterns of these responses were observed between the two cell lines.

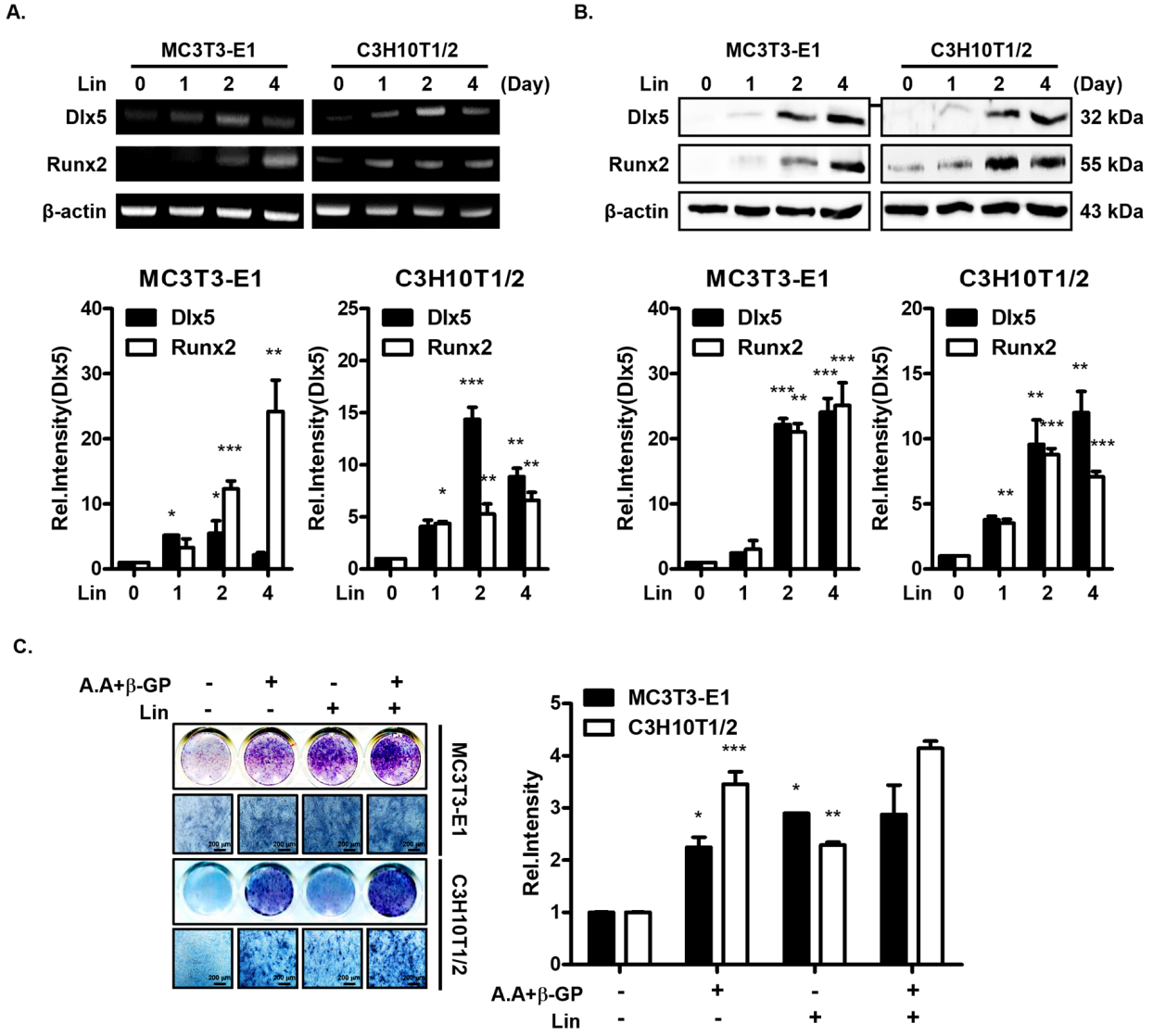

Figure 2: *Cont.*

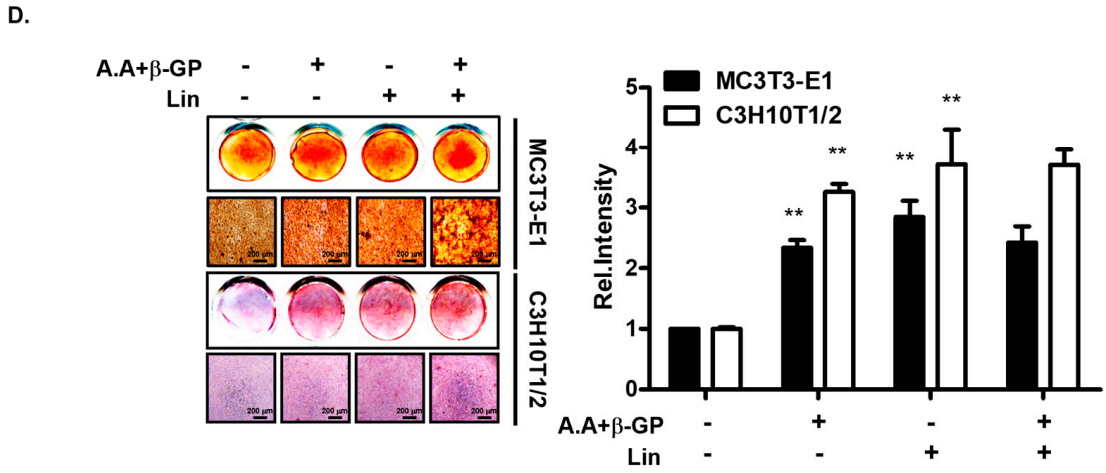

Figure 2: Linalool promotes osteogenic differentiation of MC3T3-E1 and C3H10T1/2 cells. (**A**) RT-PCR analysis showing time-dependent increases in Dlx5 and Runx2 mRNA expression in MC3T3-E1 and C3H10T1/2 cells treated with linalool (10 μM) for 0–4 days. β-actin was used as an internal control. (**B**) Western blot analysis confirming the upregulation of Dlx5 and Runx2 protein levels by linalool under the same conditions. (**C**) Alkaline phosphatase (ALP) staining showing enhanced ALP activity in MC3T3-E1 and C3H10T1/2 cells during osteogenic induction. Cells were cultured in the absence (−) or presence (+) of osteogenic supplements (ascorbic acid and β-glycerophosphate; AA + β-GP), and in the absence (−) or presence (+) of linalool, as indicated above each panel. (**D**) Alizarin Red S (ARS) staining demonstrates increased mineralized nodule formation in linalool-treated cells compared with untreated controls under osteogenic conditions. Symbols (−) and (+) indicate the absence or presence of osteogenic supplements (AA + β-GP) and linalool treatment, respectively. Data are presented as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. untreated control. Scale bars = 200 μm.

3.3 Selenbp1 Is Induced by Linalool and Regulates Osteogenic Differentiation

Linalool treatment induced a gradual increase in *Selenbp1* mRNA and protein expression in both MC3T3-E1 and C3H10T1/2 cells, with upregulation evident from day 1 and sustained through day 4 (Fig. 3A,B). To further assess the functional role of Selenbp1, we performed gain-of-function experiments using pCMV-Selenbp1. Overexpression of Selenbp1 markedly increased the expression of the osteogenic transcription factors *Runx2* and *Dlx5*, supporting its positive regulatory role in osteoblast differentiation (Fig. 3C,D). Conversely, silencing of Selenbp1 with specific siRNA significantly reduced basal expression of these markers and completely abolished the linalool-induced upregulation of Runx2 and Dlx5 (Fig. 3E,F). These findings demonstrate that SELENBP1 is not only upregulated by linalool but also acts as an important regulatory factor associated with its pro-osteogenic activity, linking redox-associated regulation with transcriptional control of osteoblast differentiation.

3.4 Linalool Promotes Caudal Fin Regeneration in Zebrafish

At day 6 after caudal fin amputation, vehicle-treated zebrafish showed limited regeneration, whereas linalool treatment significantly enhanced regenerative outgrowth. Both 10 μM and 20 μM linalool increased fin regrowth compared with the vehicle control, as evidenced by longer regenerated fin rays and improved tissue extension (Fig. 4). These findings indicate that linalool promotes caudal fin regeneration *in vivo* and supports its potential role in enhancing bone-related regenerative processes.

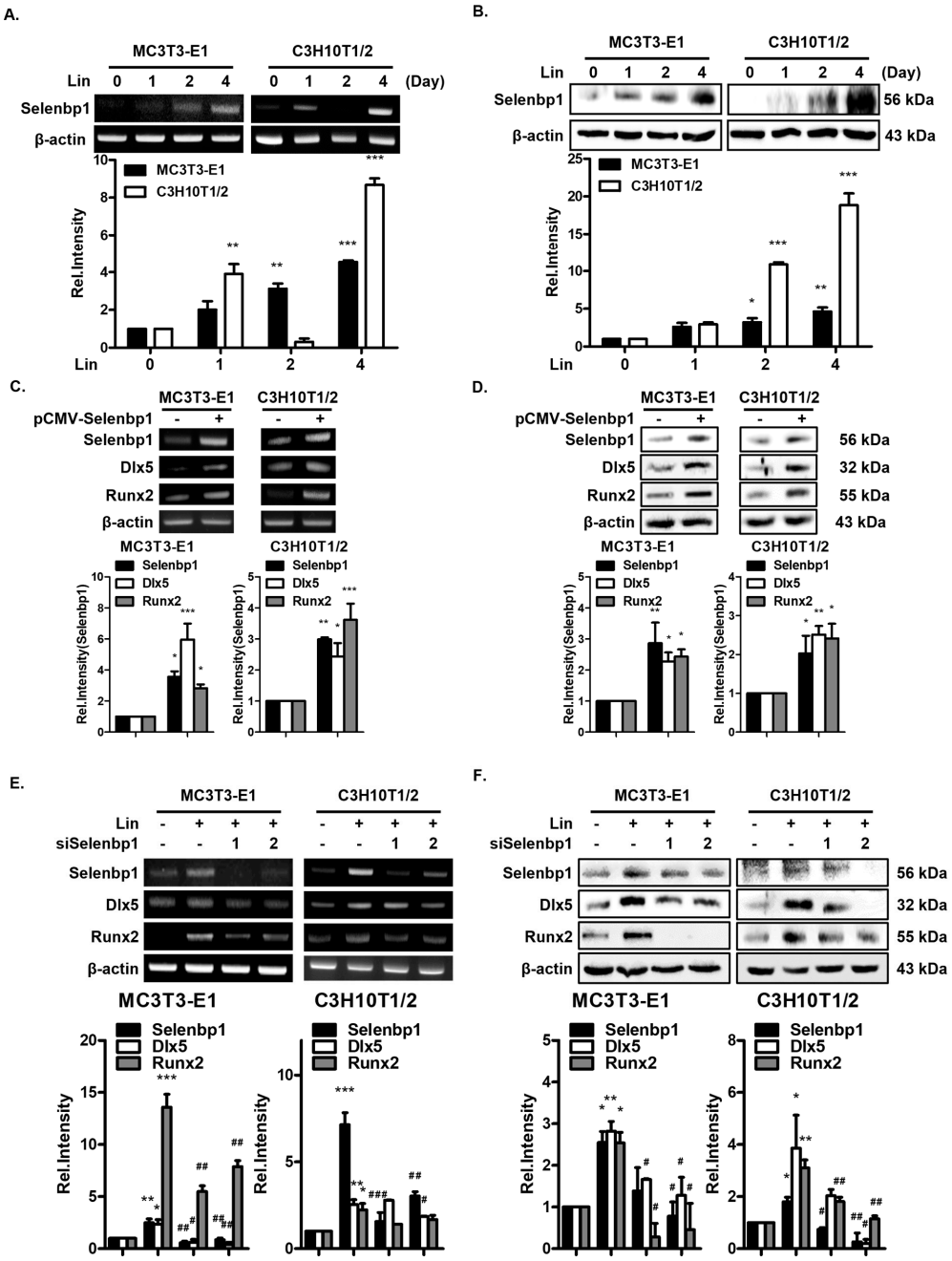

Figure 3: Selenbp1 regulates osteogenic differentiation in MC3T3-E1 and C3H10T1/2 cells. (**A**) RT-PCR analysis showing the time-dependent induction of Selenbp1 mRNA in MC3T3-E1 and C3H10T1/2 cells treated with linalool (10 μM) for 0–4 days. β-actin served as an internal control. (**B**) Western blot analysis confirms the increase in Selenbp1 protein levels under the same conditions. (**C**) RT-PCR analysis of cells transfected with pCMV-Selenbp1, showing enhanced Selenbp1 expression accompanied by increased Dlx5 and Runx2 mRNA levels. (**D**) Western blot analysis demonstrates that Selenbp1 overexpression elevates Dlx5 and Runx2 protein expression in both cell lines. (**E**) RT-PCR analysis of Selenbp1 knockdown using siRNA (siSelenbp1), showing reduced Selenbp1 levels together with decreased Dlx5 and Runx2 mRNA expression in linalool-treated cells. (**F**) Western blot analysis confirms that Selenbp1 silencing diminishes linalool-induced Dlx5 and Runx2 protein expression. β-actin was used as a loading control in all experiments. Data are presented as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. untreated control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Linalool-treated group.

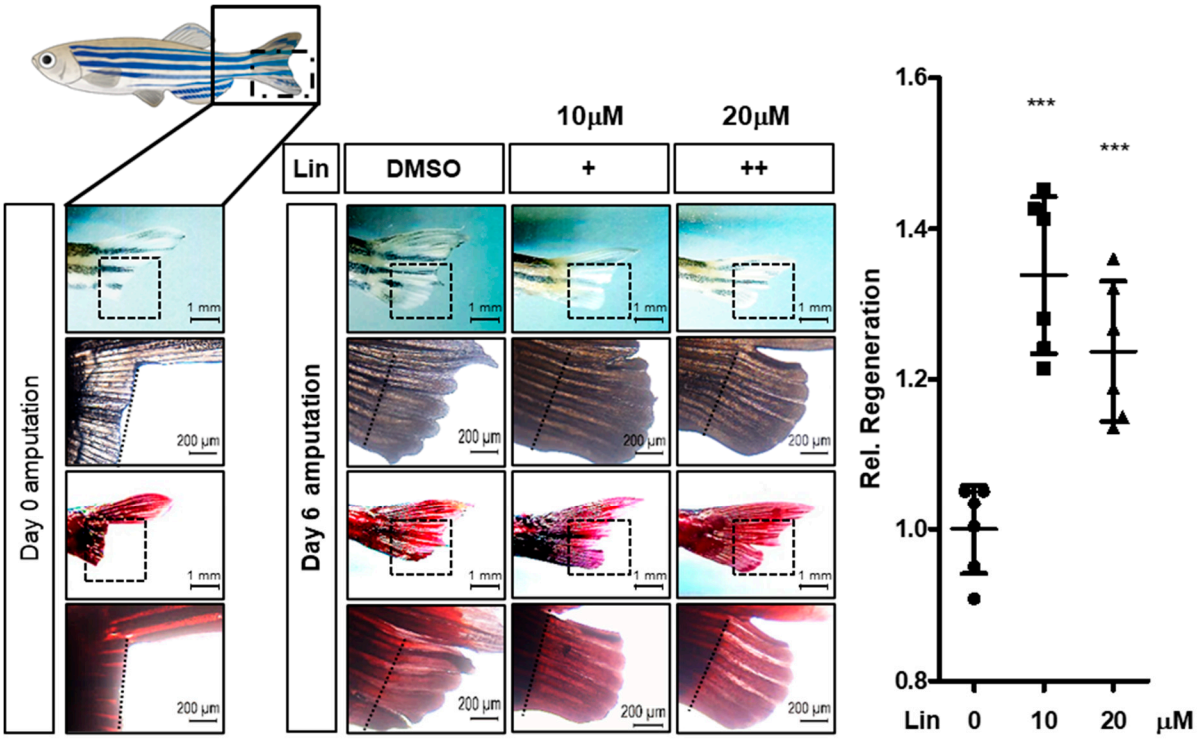

Figure 4: Linalool enhances caudal fin regeneration in zebrafish. Representative images of zebrafish caudal fins at Day 0 and Day 6 post-amputation following treatment with DMSO, 10 μM linalool, or 20 μM linalool. Linalool promoted fin regeneration, with the strongest effect observed at 10 μM. In the figure, "+" indicates treatment with 10 μM linalool and "++" indicates treatment with 20 μM linalool. Magnified views of the boxed regions are shown. Scale bars = 1 mm for the original images and 200 μm for the magnified images. Quantification of regenerated fin length is shown on the right. Data are presented as mean ± SD (n = 6). ***$p < 0.001$ vs. DMSO.

4 Discussion

In this study, we demonstrated that linalool enhances osteoblast differentiation and promotes vertebrate tissue regeneration under both *in vitro* and *in vivo* conditions. Linalool was well tolerated at low concentrations and increased the expression of key osteogenic transcription factors, including Dlx5 and Runx2, which play central roles in osteoblast lineage commitment and bone matrix maturation [1,3]. These molecular changes were accompanied by elevated alkaline phosphatase activity and enhanced mineral deposition, indicating progression toward mature osteoblast phenotypes. Similar osteogenic actions have been described for other phytochemicals such as icariin, resveratrol, and baicalin, which modulate bone formation through diverse signaling pathways [13–15]. Our findings extend this body of evidence by identifying linalool as another natural compound capable of stimulating osteogenesis.

A notable aspect of this work is the identification of Selenbp1 as a mediator of linalool-induced osteogenic activity. Selenbp1 has been previously associated with cellular differentiation and redox-related functions in non-skeletal tissues [22,23]. Selenium-related biology has also been increasingly recognized as an important modulator of bone remodeling and osteoclast–osteoblast balance [24,25]. However, Selenbp1 itself had not been implicated in osteoblast regulation. The present data demonstrate that Selenbp1 is upregulated during linalool treatment and is required for the induction of Dlx5 and Runx2, as silencing of Selenbp1 attenuated the expression of these transcription factors. Conversely, Selenbp1 overexpression restored osteogenic marker expression, supporting the functional relevance of Selenbp1 in osteoblast

differentiation. These findings suggest that Selenbp1 may serve as an upstream regulatory component linking redox-associated mechanisms to osteogenic transcriptional control.

The *in vivo* zebrafish regeneration model further supported the biological relevance of linalool. Regeneration of the caudal fin is a well-established model for studying bone and tissue repair processes [26], and several natural compounds—including curcumin, ginsenoside Rg1, and quercetin—have been reported to enhance fin regeneration through diverse mechanisms [17]. Consistent with our *in vitro* observations, linalool significantly accelerated fin outgrowth at the tested concentrations, suggesting coordinated effects on osteogenic differentiation and overall regenerative capacity.

A limitation of this study is that the functional effects of SELENBP1 knockdown on ALP activity and matrix mineralization were not directly examined. Therefore, although SELENBP1 regulates osteogenic marker expression, its requirement for the full osteogenic phenotype remains to be determined. Another limitation of this study is that additional osteoblast-specific markers such as osterix (SP7), bone sialoprotein (BSP), and osteocalcin (OCN) were not evaluated. Although ALP activity and the transcription factors Runx2 and Dlx5 are widely used indicators of osteogenic differentiation, these markers are not exclusively restricted to osteoblasts and may also be expressed in other skeletal cell types, including hypertrophic chondrocytes [1,5]. Therefore, future studies examining more specific osteoblast markers will be important to further confirm the osteoblast-specific effects of linalool.

Collectively, the present findings suggest that linalool promotes osteoblast differentiation *in vitro* and that Selenbp1 may contribute to this process. In addition, linalool enhances vertebrate tissue regeneration *in vivo*. These results expand the biological context of SELENBP1 and identify linalool as a potential natural agent for bone anabolic applications. Further studies in mammalian models are warranted to evaluate its therapeutic relevance and to elucidate the downstream pathways connecting SELENBP1 to osteogenic transcriptional networks.

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Author Contributions: Kyeong-Min Kim and A-Rang Kim; methodology, validation, formal analysis, investigation, resources, Kyeong-Min Kim and Won-Gu Jang; data curation, Kyeong-Min Kim; writing—original draft preparation, Kyeong-Min Kim and Won-Gu Jang; writing—review and editing, Won-Gu Jang; supervision, project administration, funding acquisition. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the Corresponding Author, [Won-Gu Jang], upon reasonable request.

Ethics Approval: All animal procedures were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of Daegu University (approval number: DUIACC-12020/4-0313-006).

Conflicts of Interest: The authors declare no conflicts of interest.

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