

# Anthraquinone Glycoside Aloin Induces Osteogenic Initiation of MC3T3-E1 Cells: Involvement of MAPK Mediated Wnt and Bmp Signaling

Yutthana Pengjam<sup>1,2</sup>, Harishkumar Madhyastha<sup>1</sup>, Radha Madhyastha<sup>1</sup>, Yuya Yamaguchi<sup>1</sup>,  
Yuichi Nakajima<sup>1</sup> and Masugi Maruyama<sup>1,\*</sup>

<sup>1</sup>Department of Applied Physiology, Faculty of Medicine, University of Miyazaki, Miyazaki 889-1692, Japan

<sup>2</sup>Faculty of Medical Technology, Prince of Songkla University, HatYai, Songkhla 90110, Thailand

## Abstract

Osteoporosis is a bone pathology leading to increased fracture risk and challenging the quality of life. The aim of this study was to evaluate the effect of an anthraquinone glycoside, aloin, on osteogenic induction of MC3T3-E1 cells. Aloin increased alkaline phosphatase (ALP) activity, an early differentiation marker of osteoblasts. Aloin also increased the ALP activity in adult human adipose-derived stem cells (hADSC), indicating that the action of aloin was not cell-type specific. Alizarin red S staining revealed a significant amount of calcium deposition in cells treated with aloin. Aloin enhanced the expression of osteoblast differentiation genes, Bmp-2, Runx2 and collagen 1a, in a dose-dependent manner. Western blot analysis revealed that noggin and inhibitors of p38 MAPK and SAPK/JNK signals attenuated aloin-promoted expressions of Bmp-2 and Runx2 proteins. siRNA mediated blocking of Wnt-5a signaling pathway also annulled the influence of aloin, indicating Wnt-5a dependent activity. Inhibition of the different signal pathways abrogated the influence of aloin on ALP activity, confirming that aloin induced MC3T3-E1 cells into osteoblasts through MAPK mediated Wnt and Bmp signaling pathway.

**Key Words:** Aloin, MC3T3-E1 cell, MAPK, Wnt, Bmp signaling

## INTRODUCTION

Bone diseases such as osteoporosis and periodontitis result from an imbalance in bone remodeling that is characterized by excessive bone resorption by osteoclasts relative to the bone formation by osteoblasts. Abnormalities in bone remodeling can also produce a variety of bone-decreasing disorders like rheumatoid arthritis, besides effecting tumor metastasis into bone and Paget's disease (Deschaseaux *et al.*, 2009). Two different pharmacological approaches can be used to treat such diseases; anti-resorptive agents that inhibit osteoclastic bone resorption, and anabolic agents that stimulate osteoblastic bone formation. Anti-resorptive agents such as bisphosphonates, selective estrogen receptor (ER) modulators, and calcitonin are currently available for the treatment of osteoporosis (Romas, 2005). However, these anti-resorptive agents have disadvantages such as causing unusual bone fractures and flu-like symptoms (Feldbrin *et al.*, 2016).

Currently, parathyroid hormone (PTH) therapy replacing the bisphosphonates is available (Reid, 2015). However, limited availability of natural compounds agents to enhance bone mass is still a matter of concern.

Natural compounds have historically been used as agents for the prevention and treatment of lifestyle diseases such as cancer, heart disease, diabetes, high blood pressure and urokinase plasminogen activator mediated fibrinolysis (Madhyastha *et al.*, 2010). Natural compounds that stimulate osteoblast differentiation and bone formation could serve as useful anabolic agents. Phytochemicals, such as icariin (Chen *et al.*, 2005), genistein (Sugimoto and Yamaguchi, 2000), epigallocatechin-3-gallate (Vali *et al.*, 2007), resveratrol (Mizutani *et al.*, 1998) and harmine (Yonezawa *et al.*, 2011) can stimulate osteoblast differentiation and bone formation. Aloin, an anthraquinone glycoside, also known as barbaloin, is a bitter, yellow-brown colored compound with clinically proven pharmacological actions including anti-tumor, anti-inflammatory, anti-oxidant and

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**\*Corresponding Author**

E-mail: masugi@med.miyazaki-u.ac.jp  
Tel: +81-985 851785, Fax: +81-985 857932

anti-colitis properties (Park *et al.*, 2011; Cui *et al.*, 2014; Esmat *et al.*, 2015). Since this phytochemical has not been studied previously in the context of osteogenesis, this study aims to evaluate the efficacy of aloin to stimulate osteoblast differentiation with the detailed mechanism of action, using MC3T3-E1 cells. MC3T3-E1 possesses very high osteoblast differentiation potential, expressing osteoblast phenotypic marker genes and mineralizing after the addition of suitable inducible factors (Muhammad *et al.*, 2010; Wang *et al.*, 2011).

Bone morphogenetic proteins (Bmp) are potent inducers of osteoblastogenesis. Bmps activate the transcription factor, Runt-related transcription factor 2 (Runx2), which translocates into the nucleus and modulates the expression of many target genes (Chen *et al.*, 2004). Collagen 1a, osteopontin, osterix, and osteoprotegerin are essential proteins for osteoblast differentiation. Another important junction of osteoblast differentiation is MAPK family, which plays important roles in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis (Zhang and Liu, 2002).

We studied the effect of aloin on the expression of osteoblast marker genes Bmp-2, Runx2, collagen 1a, osteopontin, osterix, and osteoprotegerin. We also investigated the effect on MAPK and Wnt-dependent Bmp signaling cascades. Furthermore, we studied the effects of Bmp antagonist, noggin, p38 MAPK inhibitor, SAPK/JNK inhibitor and blockade of Wnt-5a signaling pathway using small interfering RNA (siRNA).

**MATERIALS AND METHODS**

**Reagents**

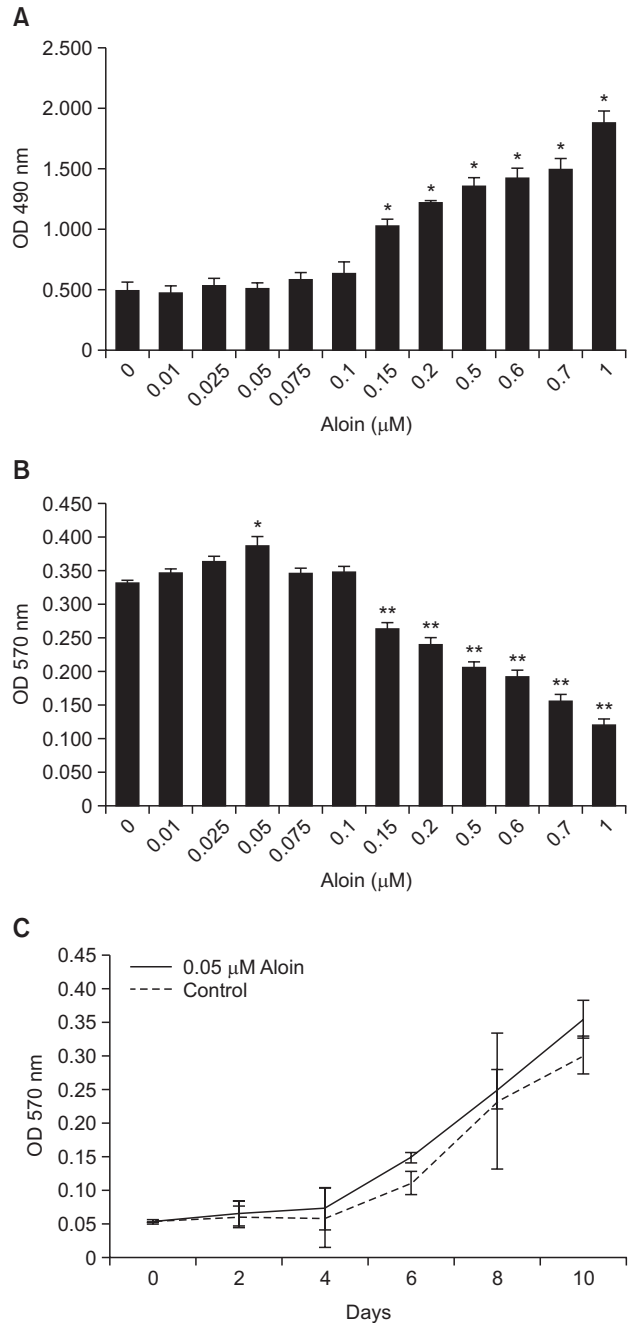
Aloin, recombinant human noggin dorsomorphin (SPR3227), p38 MAPK inhibitor (SB 203580), and SAPK/JNK inhibitor (SP 600125) were purchased from Sigma Chemical Co (St. Louis, USA). Cell culture medium ( $\alpha$ -MEM) was purchased from Gibco Co (Tokyo, Japan). All other reagents were obtained from Sigma Chemical Co. or Wako Pure Chemical Industries Ltd., Japan.

**Cell cultures**

MC3T3-E1 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in  $\alpha$ -MEM cell culture medium with 10% FBS and anti-bacterial cocktail (PNS) at humidified chamber (5% CO<sub>2</sub>, 37°C). At the semi-confluent stage, the cells were incubated with different concentrations of aloin for 16 h, 10 days or 2 weeks, as required.

**Cell viability and cytotoxicity assays**

MC3T3-E1 cells seeded at a density of 0.3×10<sup>3</sup> cells/ml onto 96 well plate were treated with different concentrations of aloin for 10 days, and subjected to MTT and LDH assays for cell viability and cell cytotoxicity, respectively. MTT assay followed the principle that yellow tetrazolium MTT is reduced by metabolically active cells into an insoluble formazan salt. The resulting intracellular purple formazan was solubilized and quantified with a spectrophotometer at an absorbance of 570 nm (Thermo scientific, Multiskan FC, Pittsburgh PK, United States). LDH assay was performed using a CytoTox 96® Non-Radioactive cytotoxicity Assay kit (Promega, Madison, USA). After incubating cells with lactate dehydrogenase enzyme buffer for 1 h, the amount of cytosolic enzyme released into the medium upon cell lysis was measured with a spectrophotom-

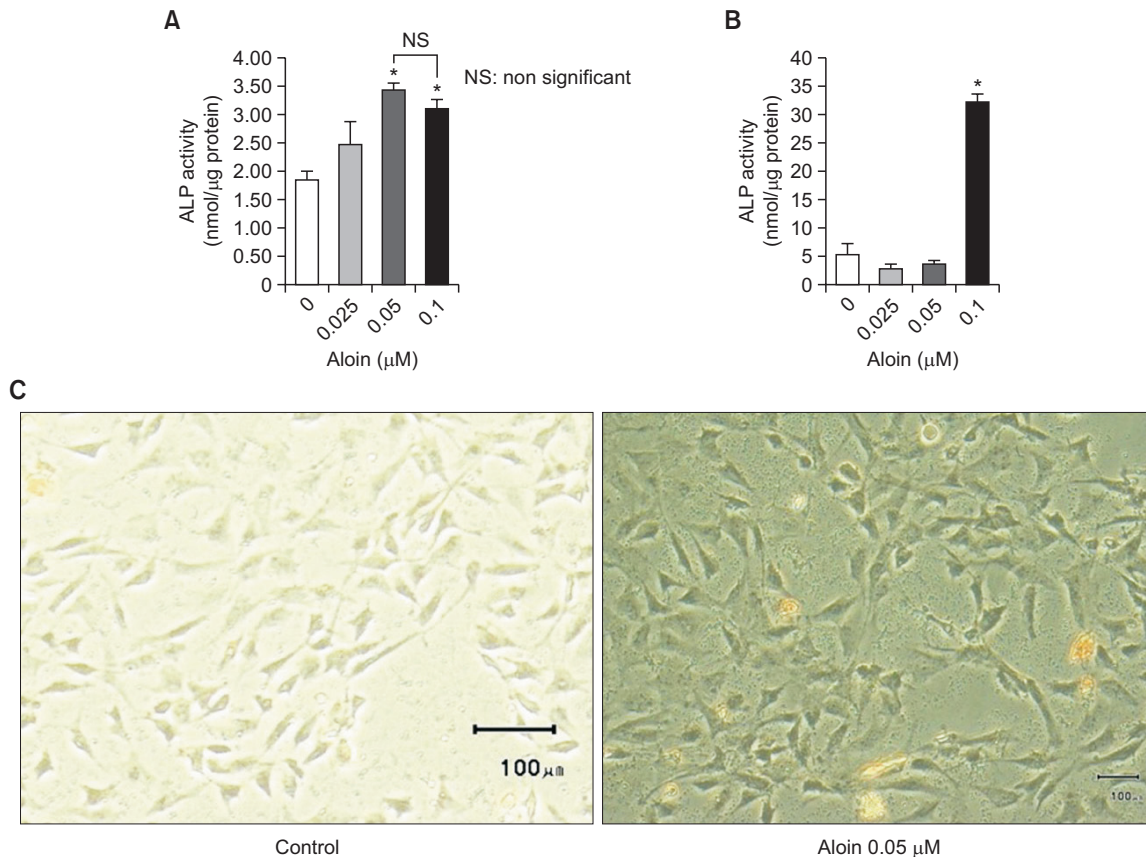


**Fig. 1.** Cytotoxicity and cell viability profile of aloin: MC3T3-E1 cells were treated with different doses of aloin for 10 days and assessed by LDH assay for cytotoxicity (A), and MTT assay for cell viability (B) and cell proliferation (C). The data represent mean ± SD of three experiments. \**p*<0.05 vs control cells, \*\**p*<0.05 vs 0.05 μM aloin group.

eter at an absorbance of 490 nm (Thermo scientific).

**Cell proliferation assay**

MC3T3-E1 cells were seeded at an initial density of 0.3×10<sup>3</sup> cells/ml in 96 well plates and treated with 0.05 μM of aloin. Cell proliferation assay was assessed by MTT assay, every alternate day, over a period of 10 days. The data represents



**Fig. 2.** Aloin increased the ALP activity in MC3T3-E1 cells (A) and human adult adipose derived stem cells (hADSC) (B). Cells treated with aloin were fixed and incubated in ALP substrate buffer for 30 min. The absorbance at 405 nm was measured as the p-nitrophenol released in nmol using a microplate reader. Values on y-axis represents the nmol ALP production (nmol/μg protein). The data represent mean  $\pm$  SD of three experiments. \* $p < 0.05$  vs control cells. (C) MC3T3-E1 cells treated with 0.05  $\mu$ M aloin for 10 days were stained for ALP content. ALP in the form of blue droplets were monitored in microscope and photographed using Olympus microscope (Model # 1X73).

the increase in cell number over the days.

#### ALP activity assay

MC3T3-E1 cells treated with aloin for 10 days were washed twice with cold PBS and lysed in cell lysis buffer. ALP activity was assayed using StemTAG™ Alkaline phosphatase activity assay kit (#CBA 301, Cell BioLabs Co. Ltd., San Diego, USA). Briefly, lysates were incubated in ALP substrate buffer (100 mM Tris-HCl pH 8.5, 2 mM MgCl<sub>2</sub>, 6.6 mM 4-nitrophenyl phosphate) for 30 min. Absorbance at 405 nm was measured as ALP activity using a microplate reader (Thermo scientific). In some studies, cells were either treated with noggin (1  $\mu$ g/ml), p38 inhibitor SB203580 (10  $\mu$ M) or JNK/SAPK inhibitor SP600125 (10  $\mu$ M) for 4 h or transfected with Wnt siRNA for 24 h, before treatment with aloin, followed by ALP activity assay.

#### ALP staining

MC3T3-E1 cells were treated with 0.05  $\mu$ M aloin for 10 days in a humidified chamber (5% CO<sub>2</sub>, 37°C). ALP staining was conducted using alkaline phosphatase staining kit (#AK20, Primary Cell Co Ltd., Tokyo, Japan) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS and fixed with 10% formalin neutral buffer solution for 20 min

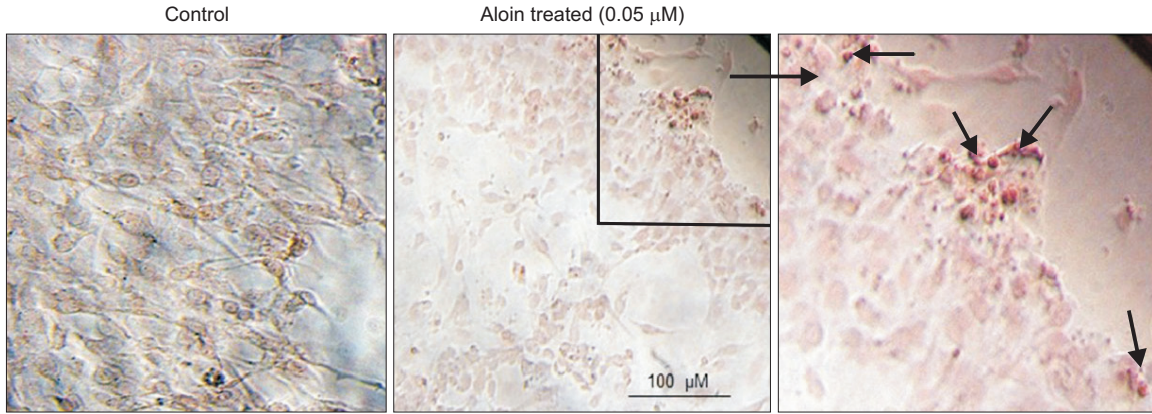
at room temperature. After washing with deionized water, cells were stained with the chromogenic substrate and incubated at 37°C for 20 min. The stained cells were dehydrated successively at room temperature for 1 h. Images were captured using a microscope coupled to a digital camera at  $\times 100$  magnification (Model # 1X73 Olympus, Tokyo, Japan).

#### Mineralization assay

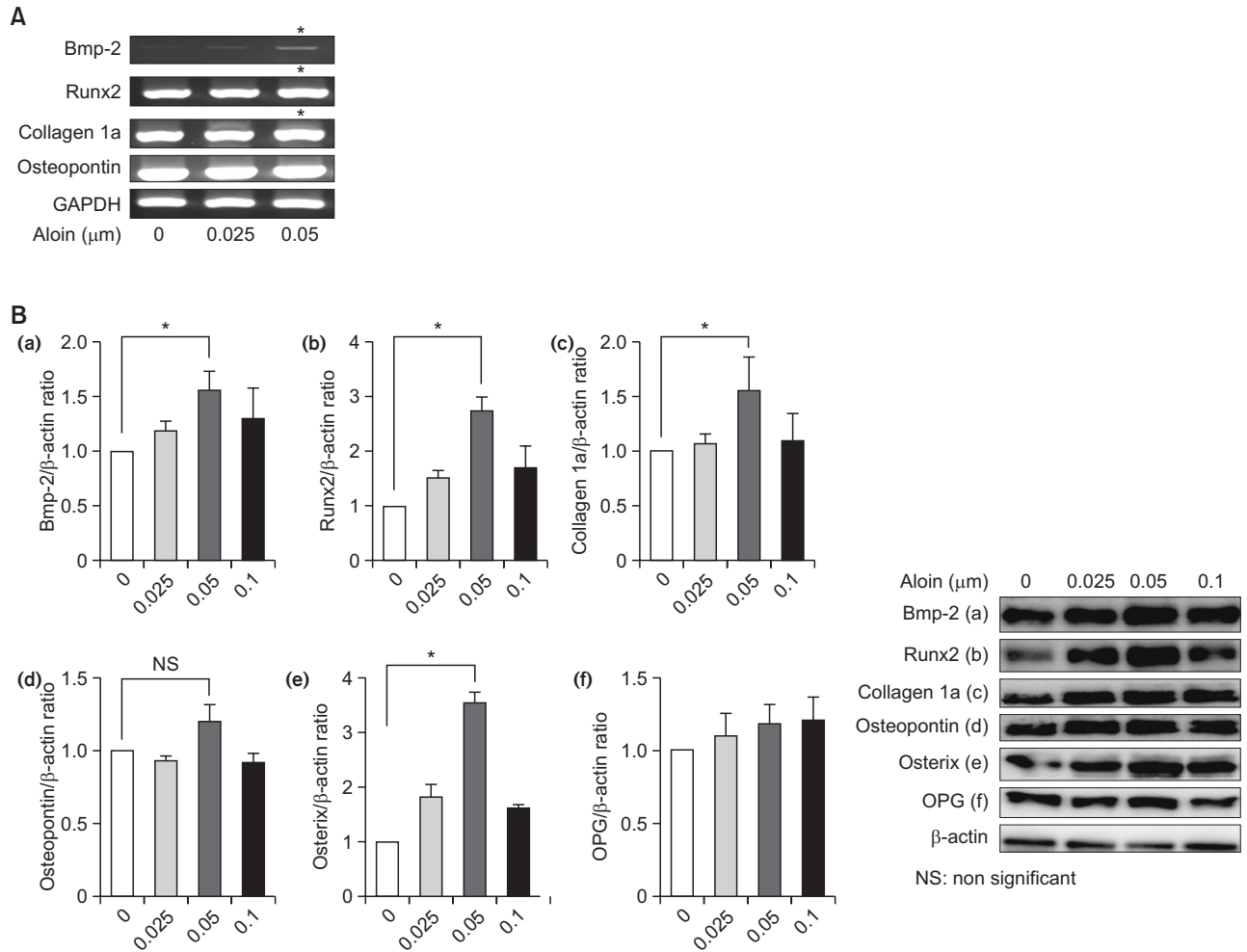
Mineralization of the extracellular matrix was determined by Alizarin red S staining, which detects calcium. Cells treated with aloin for 2 weeks were fixed in 0.4% paraformaldehyde and incubated in 1% Alizarin red S solution for 5 min at room temperature. Intracellular calcium deposition was monitored by light microscopy microscope (Nikon TMS 101, Tokyo, Japan).

#### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

MC3T3-E1 cells were cultured with or without aloin for 16 h. Total RNA was isolated using ISOGEN (Nippon Gene, Toyama, Japan), and cDNA was synthesized using ReverTra Ace qPCR (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Primers used were as follows;



**Fig. 3.** Alcin blue stimulated mineralization in the cells. Sub-confluent cells treated with/without 0.05  $\mu\text{M}$  alcin blue; Alcin blue were stained with Alizarin red S, to detect calcium nodules in the cell. Calcium deposits in the form of red droplets were monitored in microscope and photographed using Olympus microscope (Model # 1X73).



**Fig. 4.** Effect of alcin blue on osteogenic marker genes and proteins. (A) Total RNA isolated from MC3T3-E1 cells treated with different doses of alcin blue was subjected to reverse transcription polymerase chain reaction using specific primers. The band intensities were normalized to the housekeeping gene, GAPDH. The data represent mean  $\pm$  SD of triplicate determinations. \* $p < 0.05$  vs. control cells. (B) Relative protein expressions of (a) BMP-2, (b) RunX 2, (c) Collagen 1a, (d) Osteopontin, (e) Osterix and (f) Osteoprotegerin (OPG) were assessed by WB analysis.  $\beta$ -actin was used as housekeeping control. Data represents the results of three independent experiments. Level of significance was calculated at \* $p < 0.05$  vs. control cells.



Bmp-2; Forward: AGTTCTGTCCCCAGTGACGAGTTT  
Reverse: GTACAACATGGAGATTGCGCTGAGRT  
Osteopontin; Forward: TCACCATTGGATGAGTCTG  
Reverse: ACTTGTGGCTCTGATGTTCC  
Runx2; Forward: CCGCACGACAACCGCACCAT  
Reverse: CGCTCCGGCCCAAAATCTC  
Collagen 1a; Forward: TTCCTGGTGCTGATGGTGTGCT  
Reverse: GCCTTCCAGTTCTCCAGCGG  
GAPDH; Forward: AAATGGTGAAGGTCGGTGTG  
Reverse: GAATTTGCCGTGAGTGGAGT

PCR was performed with FastStart SYBR Green Master (Roche Diagnostic, Mannheim, Germany) in Gene Atlas thermocycler (Astec, Tokyo, Japan). The relative expression levels of target genes against the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were calculated using Image Quant TL (GE Healthcare Life Sciences) with a digital imaging system (LAS4000, Fujifilm, Tokyo, Japan).

### Western blotting

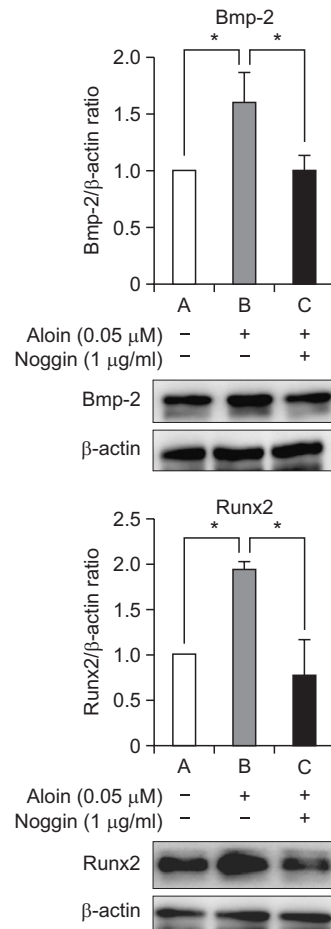
Total cell lysates were prepared using RIPA buffer (Nacal Chemicals, Tokyo, Japan). Cytoplasmic and nuclear proteins were obtained using NE-PER reagent (ThermoFisher Scientific Inc, Waltham, USA) following manufacturer's instruction. Protein fractions were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer containing 2-mercaptoethanol, and boiled at 95°C for 5 min. Protein samples were subjected to SDS-PAGE in 10% polyacrylamide gel and subsequently electroblotted onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, NJ, USA). After blocking non-specific binding sites for 1 h in 3% nonfat milk in TBST (TBS and 0.1% Tween 20), membranes were incubated overnight at 4°C with specific primary antibodies. Antibodies for Bmp-2, Runx2, collagen 1a, p38, p-p38, JNK/SAPK, p-JNK/SAPK, ERK1/2, p-ERK1/2 and  $\beta$  actin were purchased from Cell Signaling (Massachusetts, USA). Antibodies for osteoprotegerin, Sp7/osterix and osteopontin were purchased from Abcam Inc (San Francisco, USA). The membranes were washed in TBST and incubated further with horseradish peroxidase-conjugated secondary antibodies at room temperature. Protein bands were detected using an enhanced ECL kit (GE Healthcare, Tokyo, Japan) with the digital imaging system (LAS4000).

### Treatment with Bmp antagonist noggin

Subconfluent cells were treated with noggin (1  $\mu$ g/ml) for 4 h, followed by 16 h incubation with 0.05  $\mu$ M aloin. Cell lysates were prepared using RIPA buffer. Cell lysates were used for western blot detection of Bmp-2 and Runx2.  $\beta$  actin was used as a housekeeping control.

### Wnt-5a siRNA transfection

Cells were plated into a 6-well plate with  $\alpha$ -MEM cell culture medium with 10% FBS and anti-bacterial cocktail (PNS) at humidified chamber (5% CO<sub>2</sub>, 37°C) as described previously. Wnt-5a siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, Biotechnology, Inc., Texas, USA). siRNA transfection was performed according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were treated with aloin. Cell lysates were analyzed for expression of Bmp-2 and Runx2 proteins by western blotting, as described earlier.



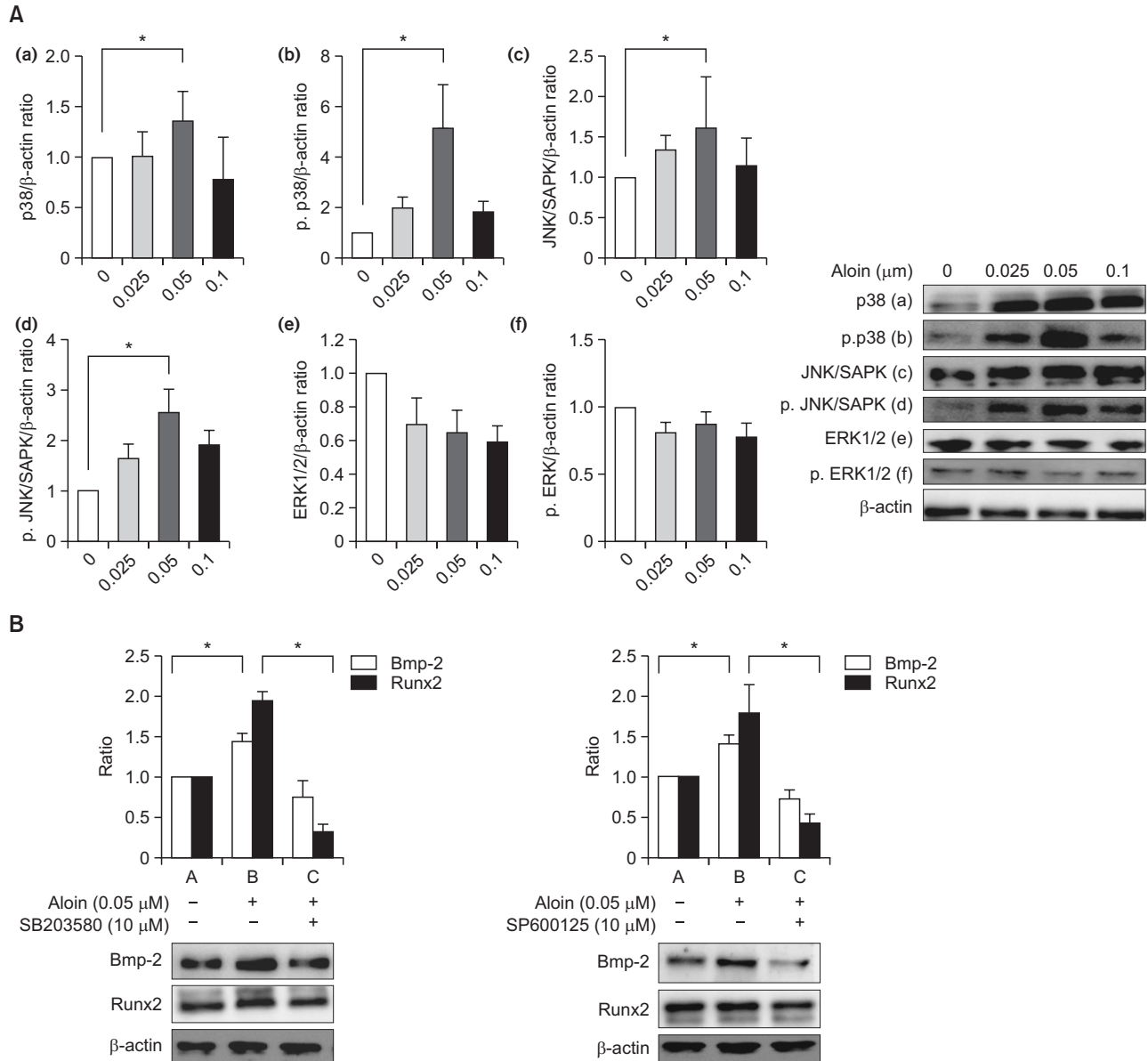
**Fig. 5.** The influence of aloin was annulled in the presence of noggin. Cells were treated with aloin (0.05  $\mu$ M) in the presence of Bmp antagonist noggin (1  $\mu$ g/ml). Protein expressions of Bmp-2 and Runx 2 were assessed by WB analysis. The data represent mean  $\pm$  SD of triplicate determinations. \* $p$ <0.05 vs. control cells.

### Culture of adult human adipose-derived stem cells (hADSC)

Adult human adipose-derived stem cells were purchased from a commercial supplier (Zen-Bio, Inc., NC 27709, USA). Cells were cultured in pre-osteogenic DMEM/Ham's F-12 medium. The initiation medium contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.4, fetal bovine serum (10%), penicillin, streptomycin, and amphotericin B. Cells were incubated under standard condition (5% CO<sub>2</sub> and 37°C) until they reached semi-confluence. Cells (8 $\times$ 10<sup>4</sup> cells/well) were treated with various concentrations of aloin and ALP activity was determined as described earlier.

### Statistical analysis

All data are expressed as mean  $\pm$  SD. Statistical analyses of the significance of differences among values were carried out by one-way ANOVA with post hoc Dunnett's test or Students T-test with  $n=4$  independent experiments. Values of  $p$ <0.05 were considered to indicate statistical significance.



**Fig. 6.** (A) Effects of aloin on MAPK signaling pathway. MC3T3-E1 cells were treated with or without aloin and analyzed for (a) p38, (b) phospho-p38, (c) JNK/SAPK, (d) phospho- JNK/SAPK, (e) ERK1/2 and (f) phospho-ERK1/2 by western blotting.  $\beta$ -actin was used as house-keeping protein. The data represent mean  $\pm$  SD of triplicate determinations. \* $p$ <0.05 vs. control cells. (B) Effect of inhibitors of p38 (SB203580) and SAPK/JNK (SP600125) on the activity of aloin: Cells were treated with aloin (0.05  $\mu$ M) in the presence of SB203580 or SP600125. Protein expressions of Bmp-2 and RunX 2 were assessed by WB analysis.  $\beta$ -actin was used as housekeeping protein. Data represents the results of three independent experiments and significant level (\*) was calculated at  $p$ <0.05 vs. control cells.

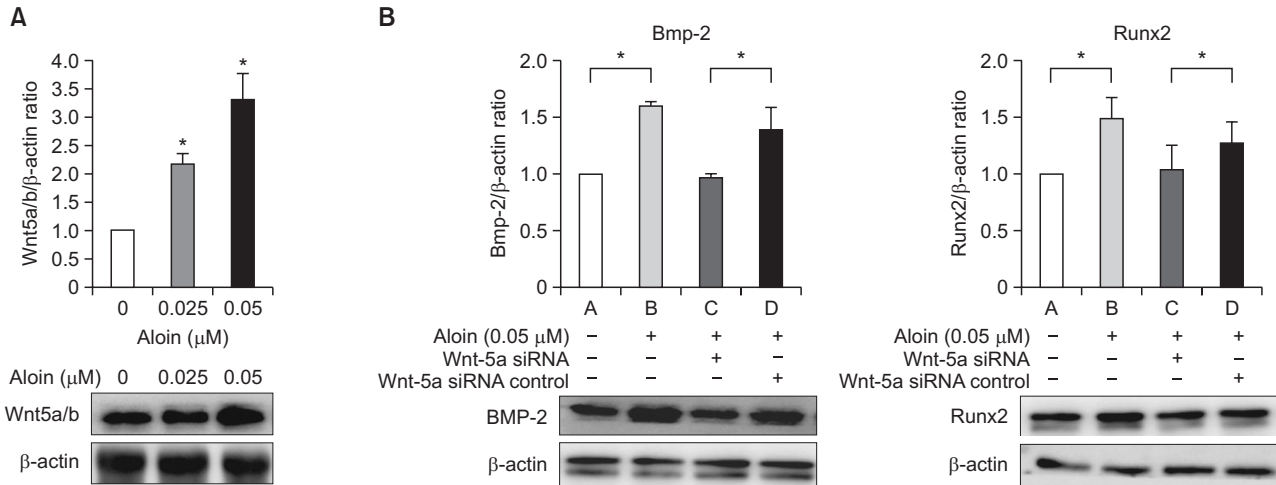
**RESULTS**

Preliminary studies were conducted to assess the effect of aloin on cell viability, proliferation, and toxicity. LDH assay showed that aloin at concentrations up to 0.1  $\mu$ M was not cytotoxic to the cells (Fig. 1A). MTT assay revealed that aloin had no adverse effect on viability (Fig. 1B) or rate of proliferation of the cells (Fig. 1C), at concentrations up to 0.1  $\mu$ M. Higher concentrations of aloin proved cytotoxic. Further experiments were performed using 0.05  $\mu$ M aloin.

Alkaline phosphatase (ALP) activity (an early phase marker

of osteoblast differentiation) and mineralization (late phase marker) were evaluated to assess the effect of aloin on osteogenic induction. Aloin significantly increased ALP activity of MC3T3-E1 cells (Fig. 2A) and hADSC (Fig. 2B) at 0.05  $\mu$ M and 0.1  $\mu$ M concentrations respectively. Aloin's effect on two different distinct cell types clearly implies that effect is not cell type specific. Aloin also increased the intracellular ALP content in MC3T3-E1 cells, as evident from ALP staining (Fig. 2C).

Cells treated with aloin for 2 weeks showed significant deposits of calcium, indicating stimulation of mineralization (Fig. 3). Next, effect of aloin on the expression of osteoblast marker



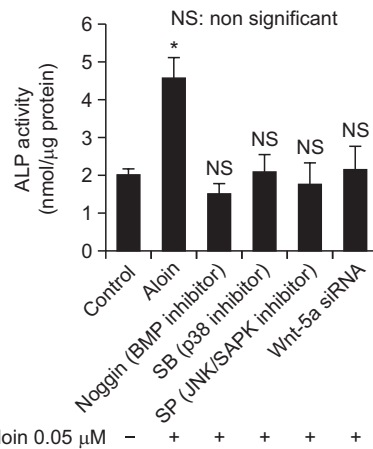
**Fig. 7.** (A) Effect of aloin on Wnt signaling pathway. MC3T3-E1 cells were treated with or without aloin and analysed for Wnt 5a/b expression by western blotting. The data represent mean ± SD of triplicated determinations. \**p*<0.05 vs. control cells. (B) Effect of Wnt-5a silencing on activity of aloin. Cells were transfected with Wnt-5a siRNA or control siRNA before treatment with aloin (0.05 μM). Total protein was analysed by western blotting for Bmp-2 and RunX 2 proteins. The data represent mean ± SD of triplicated determinations. \**p*<0.05 vs. control cells.

genes, namely Bmp-2, Runx2, collagen 1a and osteopontin was evaluated. Results revealed a dose-dependent induction of Bmp-2, Runx2, and collagen 1a at the RNA level (Fig. 4A) as well as protein level (Fig. 4B). The most significant effect was observed in Bmp-2 mRNA, where 0.05 μM aloin caused a 6.2 fold increase and Runx2 protein (>2 fold increase). Aloin did not show any stimulatory effect on osteopontin, either at gene or protein level. Collectively the results indicate that aloin targets the upstream molecules like Bmp-2 and Runx2 in osteoblastogenesis pathway. The influence of aloin on expressions of Bmp-2 and Runx2 proteins was annulled in the presence of Bmp antagonist, noggin (Fig. 5), implying that Bmp activation is necessary for their expression. Results so far indicate that aloin could stimulate osteogenic differentiation of MC3T3-E1 cells through the induction of Runx2 via Bmp signaling cascade.

**Involvement of signaling pathways**

Members of the MAPK signaling pathways (p38, JNK/SAPK and ERK1/2) were studied to elucidate the possible participation of MAPK signaling pathways in the effects of aloin. Treatment with aloin caused a significant increase in the activation of p38 and JNK/SAPK proteins; on the other hand, ERK1/2 was downregulated (Fig. 6A). To test the role of MAPK activation in the regulation of Bmp-2 and Runx2, cells were treated with aloin in the presence of p38 inhibitor (SB203580) and SAPK/JNK inhibitor (SP600125). Co-treatment with the inhibitors attenuated the effect of aloin on Bmp-2 and Runx2 proteins (Fig. 6B), confirming the involvement of MAPK members, p38, and JNK/SAPK.

Besides MAPK, Wnt family also plays important roles in many aspects of osteogenesis. In the present investigation, we selected Wnt 5a/b to evaluate the Wnt signaling pathway. Aloin (0.05 μM) increased Wnt 5a/b protein activity significantly (Fig. 7A). To study the involvement of Wnt on the activity of aloin, we employed the siRNA-mediated silencing technique to knock down Wnt5a. Silencing of Wnt-5a inhibited both



**Fig. 8.** The rate of ALP production in the presence of signal pathway inhibitors. Cells were treated with aloin (0.05 μM) in the presence of Bmp-2 antagonist noggin (1 μg/mL), p38 inhibitor SB203580 (10 μM), JNK/SAPK inhibitor SP600125 (10 μM) or Wnt-5a siRNA, and cultured for a period of 10 days. ALP activity was assessed and expressed as nmol ALP produced/mg protein. The data represent mean ± SD of triplicate determinations. \**p*<0.05 vs. control cells.

Bmp-2 and Runx2 protein expressions (Fig. 7B), highlighting that the effect of aloin on Bmp-2 and Runx2 was indeed, Wnt-dependent.

ALP production is the hallmark of differentiation of MC3T3-E1 cells into osteoblasts, through a complex process of signaling circuits (Chen et al., 2004). Inhibition of Bmp-2, p38, JNK/SAPK and Wnt pathways annulled the effect of aloin on ALP activity (Fig. 8).

## DISCUSSION

Many research groups are currently attempting to identify molecules that stimulate osteoblast differentiation, for the development of drugs to treat osteoporosis. Plant-derived natural compounds including flavonoids, polyphenols, lignans, coumarins, terpenoids, carotenoids, and alkaloids can stimulate *in vitro* osteoblast cellular differentiation and *in vivo* bone mass formation (Woo *et al.*, 2010). Phytochemicals can differentiate mesenchymal stem cells and MC3T3-E1 cells, to osteoblasts, through several crucial molecular and cellular processes in bone formation, and can be used as agents to treat various bone diseases (Woo *et al.*, 2010).

In this study, we attempted to clarify the effect of aloin, an anthrocylic glycoside, on *in vitro* osteogenic induction and the associated mechanisms, employing MC3T3-E1 cells. Undifferentiated cells such as MC3T3-E1 and C3H10T1/2 are model cell lines utilized for *in vitro* studies on osteoblast differentiation. 3T3 fibroblasts, which are already committed to a specific differentiation phenomenon, can be induced to express osteoblast markers, but these cells have to be re-programmed by adding epigenetic modifiers (Muhammad *et al.*, 2010). MC3T3-E1 cells can also differentiate into chondrocytes, adipocytes and myoblasts by physiological inducers through Bmp, Wnt signaling circuits (Kobayashi *et al.*, 2008).

Aloin stimulated the process of osteoblast induction through an increase in ALP production at the initial stage, and mineralization at the later stage. It is reported that the methoxyl substituent in anthraquinone derivatives is important to elicit osteogenic activity (Lee *et al.*, 2008). Several natural compounds are reported to enhance the ALP activity and calcium deposition during initial osteogenesis process (Chen *et al.*, 2005; Lee *et al.*, 2008). Since aloin has methoxyl group, we believe that structure-activity relationship of aloin could be important for inducing initial osteogenic activity.

In this study, aloin induced Bmp-2 gene at the initial stage (Fig. 4A), stimulated ALP accumulation (Fig. 2A) at an early stage, and intracellular calcium deposition at a later stage (Fig. 3). Taken together, these findings collectively indicate that aloin induced molecular initiation of osteoblastogenesis in MC3T3-E1 cells.

MAPK family regulates multiple cellular activities related to osteoblast initiation process, and can be activated in response to a wide range of external stimuli including natural compounds (Trzeciakiewicz *et al.*, 2009). Various reports highlight that the MAPK pathway can phosphorylate Runx2 and osterix, implying that MAPK is an obligatory transducer for bone healing (Xiao *et al.*, 2000; Celil and Campbell, 2005). In addition, MAPK family proteins, p38 and JNK, are reported to regulate osteoblast differentiation via activation of transcriptional factors such as activator protein 1 (AP-1) (Lee *et al.*, 2008). MAPK activation can induce Runx2 dependent osteocalcin and osteopontin genes (Zhang and Liu, 2002). Stimulation of cells with aloin resulted in the activation of p38 and JNK/ SAPK MAPK pathways and also in an increased expression of Runx2 and osterix proteins. Inhibition of MAPK using specific inhibitors annulled the effect of aloin on Runx2 and Bmp-2 proteins, indicating that osteogenesis parameters are initiated through MAPK members. Runx2 is a key transcription factor associated with differentiation of bone forming cells (Holleville *et al.*, 2007). It can differentiate mesenchymal stem cells to osteochondroblast progenitor through Bmp signaling

pathways, and also differentiate pre-osteoblast to mature osteoblast through MAPK signaling pathways (Nakashima *et al.*, 2002; Ge *et al.*, 2007). Bmp pathway is crucial for progression and maturation of osteogenesis (Nohe *et al.*, 2002; Chen *et al.*, 2004; Seib *et al.*, 2009). Bmp-2 is also crucial for proliferation and differentiation of osteogenesis through pre-osteoblast cells, which could depend on the transcription factor osterix acting downstream of Runx2 (Lum and Beachy, 2004). Inactivation of Bmp-2 using specific inhibitor, noggin, attenuated the increase in Runx2 protein caused by aloin.

In addition to MAPK and Bmp pathways, aloin also induced Wnt signaling. Wnt signaling is required for commitment of mesenchymal stem cells to the osteoblast lineage (You *et al.*, 2004; Baron and Kneissel, 2013; Kumawat *et al.*, 2014). Wnt 5a/b has a significant role in bone formation (Liu *et al.*, 2008; Bennett *et al.*, 2005; Bodine *et al.*, 2005). Silencing of Wnt signaling via siRNA technique nullified the effect of aloin on Bmp-2 and Runx2 proteins.

In conclusion, our study reveals that aloin can stimulate osteogenic initiation of MC3T3-E1 cells via the induction of transcription factors (Runx2, osterix) and osteogenic factors (Bmp-2). These inductive effects are in turn mediated by the regulation of MAPK (p38, JNK/SAPK) and Wnt pathways.

The present investigation revealed that aloin differentiates MC3T3-E1 cells and adipose-derived stem cells to osteoblasts. First, the cells become osteochondroblast progenitor cells as evidenced by overexpression of transcription factor Runx2, through Bmp signaling pathway. Later, transcription factors Runx2 and osterix induce the osteochondroblast progenitors to mature osteoblasts. These processes are mediated by Wnt and MAPK signaling pathways. Taken together, these results indicate that aloin differentiates MC3T3-E1 cells into osteoblasts through MAPK- and Wnt-dependent Bmp signaling pathways.

## ACKNOWLEDGMENTS

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