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Natural Science

Effect of a novel compound from *Lycopodium obscurum* L. on osteogenic activity of osteoblasts *in vitro*

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ABSTRACT

We investigated the potential of an extract of *Lycopodium obscurum* L.; stigmastan-3-one-21-oic acid (SA), to enhance osteogensis of mouse osteoblastic MC3T3-E1 cells. SA at a concentration of 16 μM was found to have no significant effect upon the viability of the cells, thus concentrations of 8 μM and 16 μM of SA were used in all further experiments. Both concentrations of SA had an inhibitory affect upon alkaline phosphatase activity (ALP) after 8 days incubation, however, after 16 days activity was restored to control levels. However Alizarin red S staining showed increased levels of mineralization for both concentrations after 16 days culture. Real time PCR showed inhibition of genes Runx2 and Osterix genes responsible for the up-regulation of ALP. However early time point (8 days) up-regulation of bone matrix mineralization genes OPN and OCN, and late time point (16 days) up-regulation of both Jun-D and Fra-2 mRNA expression was significantly enhanced. These results suggest a potential mechanism of SA in enhancing bone fracture healing is through the up-regulating bone matrix mineralization.

Keywords: Osteogenesis; RT-PCR

1. INTRODUCTION

Lycopodium obscurum L., also known as Shen Jin Cao in China and ground pine in the USA, is a plant widely distributed in Hubei, Sichuan, Guizhou and Tibet in China. It has long been used in traditional Chinese medicine and traditional Tu medicine to treat arthritis pain, quadriplegia, dysmenorrheal and contusion [1]. It is also used in treatment of bone fractures [2,3]. However, little is known about the pharmacology of *Lycopodium obscurum* L. in bone fracture healing, which prevents its application in western medical practices.

The Lycopodium family is rich in alkaloids which have been reported to have strong bioactivity [4]. Currently more than 200 alkaloids and 140 serratane-type triterpenoids have been isolated from Lycopodium genus and theraputical effects (including anti-inflammation, analgetic and antibiosis, etc.) of some of these chemicals have been identified [5,6]. However, limited information is available on the chemical compounds isolated from *Lycopodium obscurum* L. and any associated theraputical value. Recently, a series of novel compounds have been isolated from *Lycopodium obscurum* L. These compounds include two new onoceranoid triterpenoids, (3*α*,8*β*,14*α*,21*β*)-26,27-dinoronocerane-3,8,14,21-tertrol and 26-nor-8*β*-hydroxy-*α*-onocerin [7] and stigmastan-3-one-21-oic acid (SA) (unpublished). Since *Lycopodium obscurum* L. has a long history of use in bone fracture healing in traditional Chinese Medicine, this study focused on identifying whether one of the isolated compounds, SA, could demonstrate osteogenic potential.

Developmental osteogenesis *in vivo* is a complex series of events involving a preosteoblast differentiation stage; the differentiation of MSC cells into osteoblast lineage. This stage is controlled in part, by two key genes, Runx2 and Osterix (OTX), expressed during osteoprogeniter cell differentiation towards osteoblasts. The bone matrix formation stage, which includes the expression of bone-related genes such as: alkaline phosphatase (ALP), type I collagen (COL1), osteopontin (OPN), osteonectin (SPARC), osteocalcin (OCN) and bone sialoprotein (IBSP), follows the preosteoblast differentiation stage.

This is followed by the bone matrix mineralization phase.

Bone-related genes play a key role in the formation of the osseous matrix and matrix calcification [8,9]. Runx2 binds to the promoter of osterix and up-regulate its expression [10]. OTX, a specific transcription factor found in osteoblasts, is only expressed in developing bone tissue, its expression is required during stem cell differenttiation toward the osteoblast lineage [11,12]. The genes c-jun, c-fos, Jun-D, Fra-1 and Fra-2 belong to the Ap-1 family, a transcription factor family expressed during the bone mineralization stage. ALP is a marker of early bone matrix formation [13,14], and is believed to be involved in the decomposition of organic phosphate-containing compounds to provide the phosphate needed during bone mineralization [15].

The aim of this study was to investigate the potential of the isolated compound stigmastan-3-one-21-oic acid (SA) from *Lycopodium obscurum* L. to induce bone formation. Firstly this study aimed to determine any harmful effect SA potentially had upon cells in culture by a viability assay, and established working concentrations. These concentrations will be tested for both ALP activity and mineralization. Finally real time PCR will be used to assess expression of key genes responsible for bone formation, early osteogenetic differentiation and mineralization.

2. MATERIALS AND METHODS

2.1. Material Preparation and Isolation

Whole plants of *Lycopodium obscurum* L. were collected from Jianshi County, Hubei Province, China. Plants were first air-dried and then ground to fine powder. Samples then underwent a MeOH extraction three times at room temperature. The MeOH extract was resuspended in 3% tartaric acid/H_2O (pH = 3) and then partitioned with ethyl acetate (EtOAc). The EtOAc extract was resuspended in 90% H_2O/MeOH and then successively partitioned with petroleum ether (PE), EtOAc and n-BuOH. The EtOAc extract was subjected to column chromatography (CC) (silica gel, PE/Acetone 9:1, 8:2, 7:3, 1:1, 3:7, 0:1, v/v) to give 9 fractions (Fr.1-Fr.9). Fr.2 was subjected to CC (silica gel, cyclohexane/acetone 1:0 → 1:1) to give compound A.

2.2. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectra analysis was performed to identify the structure of compound A. Spectra runs were performed on a Bruker AM-400 spectrometer with TMS as internal standard. Electron ionized mass spectrometry (EIMS) and high resolution EIMS were measured via a Finnigan MAT 95 instrument.

Compound A: white amorphous powder, C29H48O3 [α] D = +53.0 (c = 0.965, C5H5N) ^{1}H NMR (400 MHz, C5D5N) and ^{13}C-NMR (100 MHz, C5D5N) as shown in **Table 1**; HR EIMS m/z 444.3593 (calcd for C29H48O3, 444.3604), EI MS: m/z 444 (M+, 33)，426 (30)，411 (5), 398 (5), 332 (4), 271 (8), 246 (23), 231 (100), 163 (13), 121 (12), 107 (12), 95 (15), 81 (14), 55 (10). Therefore, compound A was identified as stigmastan-3-one-21-oic acid (SA) (**Figure 1**).

A stock solution of 16 mM SA was made by dissolveing SA in DMSO with vortexing and heated to 60˚C for 30 minutes. The stock solution was kept at −20˚C until use. Pure DMSO was used as control (0 μM).

2.3. Cell Culture Medium

For cell expansion, Mouse osteoblastic MC3T3-E1 subclone 14 were cultured in growth medium (α-MEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL streptomycin).

For the ALP activity assay, Alizarin red mineralization assay, and mRNA expression of osteogenic related genes, a differentiation medium (growth medium supplemented with 50 μg/mL of ascorbic acid and 10 mM glycerophosphate and 10^{-8} M of dexamethasone) was used for the duration of the experiment.

2.4. Cell Viability Assay

Any potential toxic effect of SA upon cell viability was assessed via an Alamar Blue assay. MC3T3-E1 cells were seeded at a density of 1 × 103 cells/well in 150 μL growth media in opaque 96-well plates (Nunc). After 1 day culture, media was removed from the 96 well plate and cells were treated with a dose of SA (1, 2, 4, 8, 16 μM in differentation medium) for 3 days (n = 3 wells per dose). Then 18 μL of Alamar Blue reagent (Invitrogen) was added to the medium to create a final concentration of 10% v/v and the plates were incubated @37˚C for a period of 4 - 5 hours. After incubation, fluorescence (excitation @544 nm, emission @590 nm) was measured using a fluorescence plate reader (BMG PolarStar Optima), all samples were run in triplicates. The fold-change of cell numbers based on metabolic activity was calculated for each time point. Differentiation medium with 1% DMSO was used as an osteogenic control, (SA concentration 0 μm). Statistical difference of test groups vs control was measured via ANOVA were $p < 0.05$ was considered a significant difference.

2.5. Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was used as method for measuring osteogenesis. MC3T3-E1 cell were seeded into wells in 6-well plates at a density of 3 × 10^4

cells/well (n = 3 wells per dose). Differentiation medium (2 mL) was added to each well and the medium was changed once a week. After cell attachment, cells were treated with SA of different concentration (8 μM and 16 μM). Differentiation medium with 1% DMSO (SA concentration 0 μm) was used as an osteogenic control. At 8 days and 16 days of treatment, medium was aspirated off and the cells were washed 2 times with PBS. The samples were then incubated with 1 ml of 0.1% Triton X100 in 0.2 M Tris-HCl at −20˚C for 10 min. Samples were harvested with a cell scraper and were transferred to 1.5 ml Eppendorf tubes. Then, they were centrifuged at 10,000 rpm for 10 min at 4˚C. Each supernatant (100 μL) was transferred into wells in 96-well microplate. *para*-Nitrophenylphosphate (pNPP) substrate solution (200 μL) was added to each well and the plate was incubated in dark for 30 min at room temperature with gentle shaking. After the incubation, the plate was read @405 nm in a multi-well plate reader (n = 3). Statistical difference of test groups vs control was measured via ANOVA were $p < 0.05$ was considered a significant difference.

2.6. Alizarin Red S Staining

Alizarin red S staining was used as a method for assessing cell mineralization. MC3T3-E1 cells were seeded into wells of 48-well plates at a density of 7.5 × 103 cells/well and cultured with differentiation medium supplemented with 2 different concentrations of SA (8 μM and 16 μM). To determine matrix mineralization, at 8 days and 16 days treatment, triplicate samples were washed twice with PBS and fixed with ice cold methanol (−20˚C) for 10 minutes at room temperature. They were then washed twice with dH_2O and incubate with 1% Alizarin red S (Sigma-Aldrich). After aspiration of the unincorporated dye, samples are washed several times with dH_2O and air-dried. Stained monolayers were photographed with inverted phase microscopy. To quantify the amount of mineralization, acetic acid (800 μL 10% v/v) was added to each well, and the plate incubated at room temperature for 30 minutes with shaking. The monolayers were scraped from the plate with a cell scraper and transferred in 10% acetic acid to a 1.5 mL microcentrifuge tube. After vortexing for 30 seconds, the slurry was overlaid with 200 - 300 μL mineral oil (Sigma-Aldrich), heated to 85˚C for 10 minutes, and transferred to ice for 5 minutes. Then, the slurry was centrifuged at 20,000 g for 15 minutes and 300 - 400 μL of each supernatant was removed to a new 1.5 mL microcentrifuge tube. Ammonium hydroxide (200 μL, 10% v/v) was added to neutralize the acid. Aborption of 150 μL of the supernatant at 405 nm in 96-well normal plates (Nunc) (n = 3). Statistical difference of test groups vs control was measured via ANOVA were $p < 0.05$ was considered a significant difference.

2.7. Evaluation of mRNA Expression

Cells were seeded into 48-well plates at a density of 7.5×10^3 cells/well and cultured with differentiation medium containing SA at either 8 μM or 16 μM final concentrations, for 8 or 16 days (n = 3). At respective time points the cells were harvested in TRIzol (Life Techologies, Australia) and total RNA isolated following the manufacturer's instructions. The first strand of cDNA was synthesized using a SuperScript™ III First-Strand Synthesis SuperMix kit (Life Technologies). Real-time PCR was performed using Brilliant SYBR Green Master Mix (Life Technologies). Real-time PCR primers (GeneWorks, The Barton, SA, Australia) were the same as those described earlier in **Table 1**. [16]. The RT-qPCR assays were performed in a 384 well format, using an Eppendorf epMotion 5075 robot for liquid transfers, and a 7900HT Fast Real-time system (Applied Biosystems) was used for the thermal cycling. The PCR cycling parameters were as follows: enzyme activation step: 95˚C for 10 min; denaturing: 95˚C for 15 s; annealing and extension: 60˚C for 60 s; number of cycles: 45. PCR reactions for all samples were run in triplicate and the data was collected with software of Applied Biosystems SDS 2.2.2 and analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). GAPDH was used as a control gene. Statistical difference of fold change was measured via ANOVA with a post hoc test were $p < 0.05$ was considered a significant difference.

3. RESULTS AND DISCUSSION

3.1. The Effect of SA on Osteoblast Proliferation

As detected by the Alamar blue, assay, SA had no detectable effect upon the metabolic activity of MC3T3 cells at any of the five tested concentration, up to 3 days, as shown in **Figure 2**. Following these results, the two highest concentrations of SA (8 μm and 16 μm) were used for all subsequent experiments.

3.2. The Effect of SA on Alkaline Phosphatase Activity

As shown in **Figure 3(a)**, after 8 days of treatment, both tested concentrations of SA had a significant effect of inhibiting alkaline phosphatase activity when compared to the control. However, by 16 days incubation, the alkaline phosphatase activity for both SA concentrations had been restored to control levels.

3.3. The Effect of SA on Bone Matrix Mineralization

Alizarin red S was used as a semi-quantification of bone matrix mineralization. As shown in **Figure 3(b)**,

Table 1. Nuclear Magnetic Resonance spectra of compound A, identifying it as stigmastan-3-one-21-oic acid.

No.	^{1}H-NMR	-NMR	No.	^{1}H-NMR	-NMR
1	1.60 (1H, m) 2.28 (1H, m)	38.7	15	1.59 (2H, m)	24.4
2	1.73 (1H, m) 2.11 (1H, m)	38.5	16	1.51 (2H, m)	28.4
3	----	210.8	17	1.01 (1H, m)	56.4
4	2.10 (1H, m) 2.24 (1H, m)	45.2	18	0.99 (3H, s)	12.3
5	1.34 (1H, m)	47.1	19	0.88 (3H, s)	11.7
6	1.66 (2H, m)	29.4	20	1.18 (1H, m)	46.0
7	0.81 (1H, m) 1.58 (1H, m)	32.3	21	----	178.1
8	1.32 (1H, m)	35.9	22	1.60 (1H, m) 1.86 (1H, m)	31.0
9	1.88 (1H, m)	53.6	23	1.35 (1H, m) 1.95 (1H, m)	28.0
10	----	36.1	24	2.55 (1H, m)	49.1
11	1.37 (2H, m)	21.9	25	1.21 (1H, m)	29.8
12	1.07 (1H, m) 1.70 (1H, m)	39.0	26	0.84 (3H, d, J = 6.6 Hz)	20.4
13	----	43.1	27	0.84 (3H, d, J = 6.6 Hz)	19.5
14	0.59 (1H, m)	54.3	28	1.33 (2H, m)	23.6
			29	0.92 (3H, m)	12.8

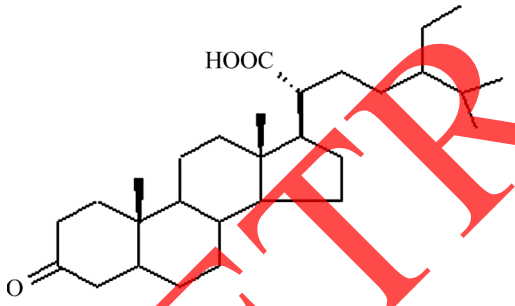

Figure 1. Molecular structure of SA.

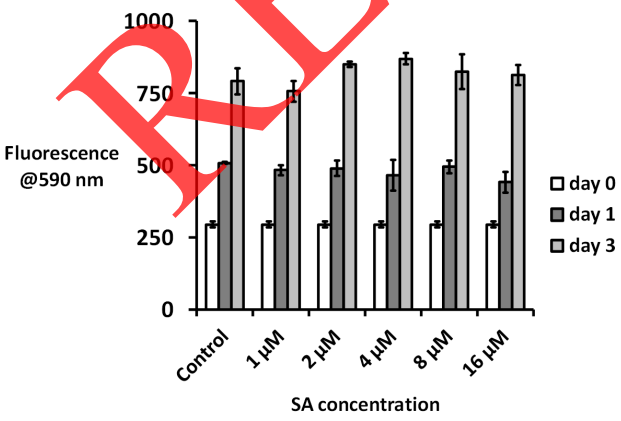

Figure 2. Alamar blue cell metabolic activity assay for assessing any toxic effect that SA has on MC3T3s. Error bars displayed are 1 SD (n = 3).

mineralization of the matrix was significantly higher than control at 16 days time point.

Quantitative results shown in **Figure 4**, indicated that after 16 days treatment cells treated with SA at both tested concentrations had a significantly enhanced level of mineralization when compared to the untreated control.

3.4. The Effect of SA on Gene Expression

Real time PCR was used to analysis key osteogenic gene expression, and results summarized in **Figure 5**. and **Table 2**. Real-time PCR data revealed that short-term treatment with SA had an inhibitory effect on ALP mRNA expression although the down regulation observed from the 16 µM SA dose was not considered to be statistically significant (**Figure 5(A)**). After 16 days the treatment of 8 µM SA, had a significant up regulatory effect of ALP expression. In contrast the 16 µM SA treatment had a significant down regulation effect of ALP expression.

Short-term treatment (8 days) of SA had an inhibitory effect of COL1 expression at both test concentrations (**Figure 5(B)**), whereas long-term treatment (16 days) did not have a significant effect on expression.

OPN expression was significantly enhanced by both concentrations of SA after 8 days treatment, whereas SA only at 16 µM stimulated OPN expression at the 16 days

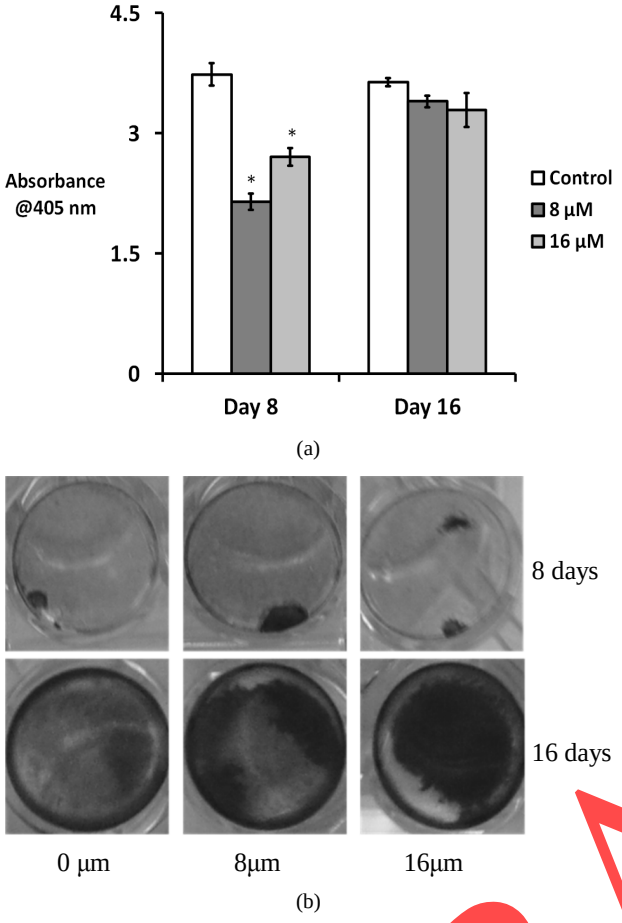

Figure 3. (a) The effect of SA on Alkaline Phosphatase activity of M3T3C cells. Error bars displayed are 1 SD (n =3). Significance from the control is highlighted by $^{*}(p < 0.05)$. (b) Photograph of the effect of SA on M3T3C mineralization as detected by Alizarin red.

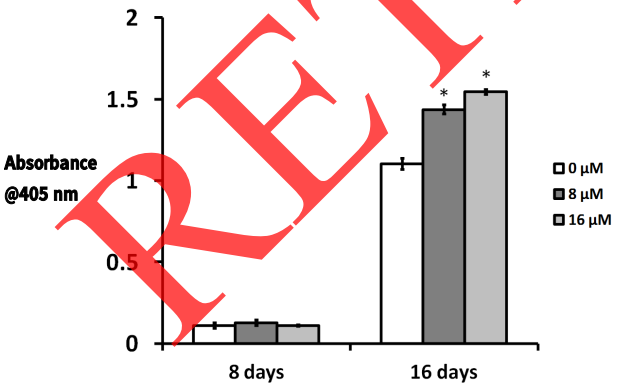

Figure 4. Quantification of mineralization of osteoblasts treated with SA of different concentrations. Error bars displayed are 1 SD (n = 3). Significance from the control (0 µm) is highlighted by $^{*}(p < 0.05)$.

treatment time point (**Figure 5(C)**). The expression of IBSP mRNA was inhibited by the 16 µm SA treatment at 8 days, while there was as inhibitory trend with the 8 µm concentration this was not statistically significant. An inhibitory effect was observed at 16 days by both concentrations (**Figure 5(D)**). For OCN, SA treatment at of both concentrations at 8 days stimulated mRNA expression (**Figure 5(E)**); Expression was unaffected at 16 days for either concentration. The mRNA expression of BMP2 (shown in **Figure 5(F)**) was unaffected.

Runx2 and osterix are two key genes expressed by osteoprogeniter cell during differentiation to osteoblasts. In our study, only the 8 µM concentration at the 16-day time point had a significant up-regulatroy effect upon Runx2 expression, although at the 8 day time the effect was inhibtaory (**Figure 5(G)**). There was slight inhibitory effect on Runx2 mRNA expression at 16 µM SA concentration at day 16, and a trend at day 8, although this was not significant. OTX expression, on the other hand, was inhibited by both SA concentrations at both time points (**Figure 5(H)**). As for c-jun, there was a near three-fold increased mRNA at day 8 with 16 µM SA, but a reduction in expression at 16 days (shown in **Figure 5(I)**); the 8µm concentration had no noticeable effect upon regulation.

SA treatment did not affect c-fos mRNA expression at either concentrations or time points (**Figure 5(J)**). The expression of Jun-D and Fra-2 showed very similar patterns: the mRNA expression was reduced by the 8 µm dosage at day 8, but was significantly enhanced after 16 days (**Figures 5(K)** and **(M)**) although Jun-D displayed significant up regulation at 8 days with the 16 µm, it was not as pronounced as the 16 day time point. SA treatment at 16 µM resulted in significantly enhanced Fra-1 mRNA expression at 8 and 16 days; but at the 16 day time point 8 µM SA concentration the expression of Fra-1 was indistinguishable from the control, while the at 8 days up regulation was observed (**Figure 5(L)**).

4. DISCUSSION

With the development of molecular biology and pharmacology and the opening of China to the rest of the world, traditional Chinese herbal medicine has attracted much research interest and could provide potential solutions to some serious diseases currently affecting the world population. *Lycopodium obscurum* L. has been used in traditional Chinese herbal medicine and traditional Tu Nationality herbal medicine for many centuries; primarily for healing contusion and bone fractures in some ethnic groups in China. However, little detail is known about the biological mechanism of the therapies. In order to understand more about this traditional Chinese herbal medicine, we investigated the effect an extract of one of the compounds, stigmastan-3-one-21-oic acid (SA) from plant *Lycopodium obscurum* L. had upon osteogenic activity of M3T3C cells.

Table 2. mRNA expression of bone-related genes, early osteogenic differentiation gene and mineralization stage after culture with SA (8 μM and 16 μM) after 8 or 16 days. Statistical significant change of expression from the control (0 μm SA) shown by ↑ for up regulation or ↓ for down regulation ($p < 0.05$).

	SA concentration	Gene	Expression change		SA concentration	Gene	Expression change
Day 8	8 μm	ALP	↓	Day 8	8 μm	Osterix	↓
	16 μm		-		16 μm		↓
Day 16	8 μm		↑	Day 16	8 μm		↓
	16 μm		↓		16 μm		↓
Day 8	8 μm	COL1	↓	Day 8	8 μm	C-Jun	-
	16 μm		↓		16 μm		↑
Day 16	8 μm		-	Day 16	8 μm		-
	16 μm		-		16 μm		↓
Day 8	8 μm	OPN	↑	Day 8	8 μm	C-Fos	-
	16 μm		↑		16 μm		-
Day 16	8 μm		-	Day 16	8 μm		-
	16 μm		↑		16 μm		-
Day 8	8 μm	ISBP	-	Day 8	8 μm	Jun-D	↓
	16 μm		↓		16 μm		-
Day 16	8 μm		↓	Day 16	8 μm		↑
	16 μm		↓		16 μm		↑
Day 8	8 μm	OCN	↑	Day 8	8 μm	Fra-1	↑
	16 μm		↑		16 μm		↑
Day 16	8 μm		-	Day 16	8 μm		-
	16 μm		-		16 μm		↑
Day 8	8 μm	BMP-2	-	Day 8	8 μm	Fra-2	↑
	16 μm		-		16 μm		↓
Day 16	8 μm		-	Day 16	8 μm		↑
	16 μm		-		16 μm		↑
Day 8	8 μm	Runx-2	-				
	16 μm		↓				
Day 16	8 μm		↑				
	16 μm		↓				

Our initial investigation showed that SA has no noticeable toxic effect upon the M3T3Cs up to the maximum tested concentration of 16 μm, as measured by the MTT assay. When looking at mineralization by staining with Alizarin red increased mineralization was observed, however the effect of the test dosages of SA on ALP activity actually caused a decrease in observed activity on M3T3C cells.

In this study, the expression of Osterix was generally down-regulated by the supplementation of SA to the media. Runx2 was down regulated at day 8, but up regulated at day 16; this suggests that SA is not promoting the early stages of differentiation of mesenchymal stem cells toward osteoblasts lineage.

As previously stated, SA treatment of both test concentrations had an inhibitory effect upon ALP activity at the 8 day time point, and this result matches the rtPCR trend of ALP expression. In addition, our data revealed that COL1 mRNA expression was significantly inhibited at the 8 day time point of SA treatment, although normal expression levels were returned at the 16-day time point. These results suggest that SA treatment may cause a delay in short term bone matrix formation but not the late stage. Since ALP and COL1 are two bone-related genes highly expressed in the early stages of bone matrix formation, our data on ALP and COL1 mRNA expression suggests that SA does not promote this early stage bone matrix formation, and may even have an inhibitory function. Therefore, from the above result, we may hypothesis that, if SA exerts a positive effect on osteogenesis, it may do this by up-regulating bone matrix mineralization, as opposed to preosteoblast differentiation and bone matrix formation.

This hypothesis is supported by the effect SA has upon OCN expression; where it promotes upregulation at the 8 day time point, and the mineralization assay showing

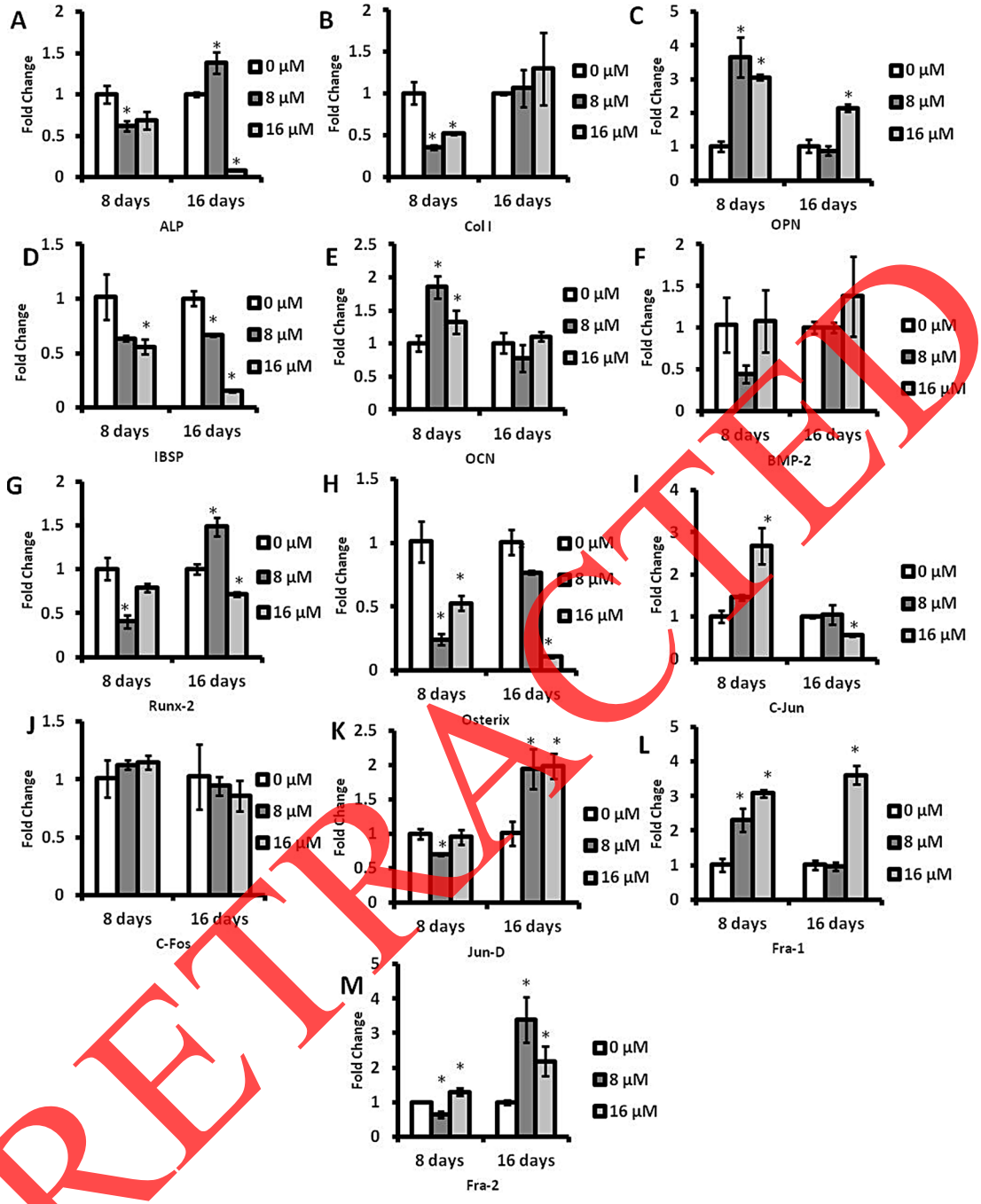

Figure 5. mRNA expression of bone-related genes (A-F), early osteogenic differentiation genes (G-H) and mineralization stage (I-M) after culture with SA (8 μM and 16 μM) after 8 or 16 days. Error bars displayed are 1 SD (n = 3). Significance from the control (0 μm) is highlighted by $^{*}(p < 0.05)$.

increased levels of matrix mineralization. OCN, which was at some stage thought to be the only bone-specific gene, is strongly related to calcium ion binding to the bone matrix and is believed to be the marker of bone mineralization. It is expressed highly in the stages of bone mineralization [17]. In our study, OCN mRNA expression was enhanced after short-term treatment (8 days) of osteoblasts with both concentrations, which suggested that SA could enhance early stage bone mineralization. Meanwhile, our Alizarin red S staining assay result further confirmed that *in vitro* mineralization level in SA treated samples was significantly higher than control after 16 days.

Another gene, OPN mRNAs expression was generally enhanced in the early time point by SA treatment. OPN is strongly associated with calcium or hydroxyapatite bind-

ing in bone mineralization [18,19] and bone remodeling [20], therefore, this result further suggests that SA treatment is havening a significant enhancing effect upon bone mineralization and remodeling. Since remodeling is a necessary step in bone fracture healing, the enhancive effect of SA in fracture healing may involve the stimulation of remodeling on bone fracture interface.

Jun (c-Jun, JunB, JunD) and fos (c-fos, Fra-1, Fra-2 etc.) proteins are the components of transcription factor AP-1. AP-1 is a heterodynamic protein which plays a key role in modulating several gene expressions including the process of osteogenesis and remodeling [21]. It has been demonstrated that some of these genes' expression is highly differentiation stage-regulated. In the early stage of differentiation, fos and Jun are highly expressed. After extracellular matrix formation and mineralization, their expression declines and Fra-2 and Jun-D become the major components of the AP-1 complex [22]. In our study, both Jun-D and Fra-2 mRNA expression was significantly enhanced at the 16 day time point of treatment with SA of both concentration when compared with control while c-fos expression was not affected by SA treatment. These findings further suggest that osteoblast mineralization could be stimulated by SA treatment. The early stage enhanced expression of Fra-1 is also helpful to osteoblast maturation and mineralization in this study [23].

Therefore, we may conclude that SA treatment may be favorable for the bone development and bone fracture healing, with a potential mechanism of stimulating bone matrix mineralization and bone fracture interface remodeling.

However, our result showed that SA treatment was not favorable for bone sialoprotein expression, although other results suggest that SA stimulates bone mineralization. As a major extracellular bone matrix protein, bone sialoprotein is responsible for the nucleation of HA crystal [24] and mineralization in bone and cementum [25,26]. Further studies are needed to explore the mechanism.

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