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The Effect of Valproic Acid on Mesenchymal Pluripotent Cell Proliferation and Differentiation in Extracellular Matrices

Yuji Hatakeyama¹, Junko Hatakeyama¹, Atsushi Takahashi², Kyoko Oka¹, Eichi Tsuruga¹, Tetsuichiro Inai¹ and Yoshihiko Sawa¹

¹Department of Morphological Biology, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka 814-0193, Japan.

²Division of Disabled Dentistry, Tohoku University, Graduate School of Dentistry, 4-1, Seiryō-machi, Aoba-ku, Sendai 980-8575, Japan. Corresponding author email: yuji_hatakey@hotmail.com

Abstract: Valproic acid (2-*n*-propylpentanoic acid, VPA) is a widely used antiepileptic and anticonvulsant drug. Previous studies have reported that VPA effects osteogenesis *in vivo* and *in vitro*, yet it remains unclear whether VPA promotes cell differentiation of osteoblasts derived from mesenchymal cells. The purpose of this study was to clarify the effect of VPA on undifferentiated pluripotent mesenchymal cell proliferation and differentiation into osteoblasts while analyzing the impact of the absence or presence of extracellular matrices (ECMs). Mouse mesenchymal cells were cultured on non-coated plastic, type I collagen-coated, and fibronectin-coated plates in the absence or presence of VPA. A cell proliferation assay was performed in which modified formazan dye content was analyzed and proliferation nuclear antigen (PCNA)-positive cells were counted at various concentrations of VPA. A high concentration of VPA did not clearly alter cell morphology, but large numbers of stress fibers were observed in these cells and the cell proliferation ratio was decreased with positive PCNA counts. In the presence of matrices, the cell proliferation ratio decreased at low VPA concentrations compared with the ratio obtained in the absence of these ECMs. On the other hand, VPA promoted osteoblastic differentiation in the presence of type I collagen. These findings indicate that for undifferentiated mesenchymal cells, VPA promotes a decrease in the cell proliferation rate in the presence of ECMs and promotes osteoblastic differentiation, both of which could provide insight into additional mechanisms of osteoblastic cell differentiation caused by VPA.

Keywords: mesenchymal cells, valproic acid, cell proliferation

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Introduction

Valproic acid (2-*n*-propylpentanoic acid, VPA) is an effective and widely used antiepileptic and anticonvulsant drug, and recent studies have reported that VPA influences osteogenesis *in vivo* and *in vitro*.¹⁻⁴ It has been reported that VPA has no effect on osteogenesis identified with calcification nodule formation by a preosteoblast cell line derived from mouse calvaria, MC3T3-E1 cells.⁵ On the other hand, VPA promotes the cell proliferation of a pre-osteoblast cell line and the Runx2 gene, which plays a critical role in osteoblast differentiation, with transcriptional activity promoted in MC3T3-E1.⁴ These findings indicate that skeletal malformation caused by VPA may result in response to cell proliferation and/or cellular differentiation of preosteoblasts, and may not result in calcification by mature osteoblasts.

Bone is composed of not only osteoblasts and osteocytes but also chondrocytes and adipocytes, which are organized and integrated so as to maintain bone homeostasis. These various types of cells are all derived from mesenchymal stem cells. Mouse embryonic mesenchymal cells C3H10T1/2 are pluripotent cells which can be differentiated into osteoblasts, chondrocytes, adipocytes, and myoblasts *in vitro*.⁶⁻⁸ This cell line is widely used as a model of mesenchymal cell differentiation as it represents a homogeneous population of pluripotent cells that do not spontaneously differentiate under normal culture conditions.

Extracellular matrices (ECMs), a complex mixture of matrix molecules, including collagen and fibronectin, provide scaffolds for cellular support that are present in all tissues, organs, and cell-matrix adhesion signals, and regulate biological processes such as cell survival and proliferation.⁹ Both type I collagen and fibronectin play a critical role in osteogenic cell proliferation and cell differentiation both *in vivo* and *in vitro*;¹⁰⁻¹³ however, it remains unclear what the effect of VPA is on undifferentiated mesenchymal cell proliferation and cell differentiation in the presence of ECMs, especially to fibronectin and type I collagen.

The aim of this study is to investigate the effect of VPA on the cell differentiation and proliferation of mesenchymal cells which can differentiate into cells composed of mesenchymal tissues, including those found in bone. We observed a normal mesenchymal

cell shape but large number of stress fibers in cells in the presence of a high concentration of VPA, and these mesenchymal cells were also able to proliferate on a non-coated plastic plate. However, mesenchymal cell proliferation was inhibited when cells were cultured on ECM-coated plates, even in low concentrations of VPA, and VPA promoted the expression of osteoblastic cell marker genes. In an osteogenesis-inductive medium, VPA promoted osteogenesis on both non-coated plastic and collagen-coated plates. These observations may reflect the *in vivo* cell conditions and provide further insight into the osteoblastic cell differentiation mechanism caused by VPA.

Materials and Methods

Cell culture

A mouse embryonic mesenchymal cell line, C3H10T1/2 Clone 8 cells, was obtained from Riken Bioresource Center Cell Bank (Tsukuba, Japan). Cells were cultured in basal medium Eagle (BME) containing 10% fetal bovine serum (FBS, Equitech-Bio Inc., Tokyo, Japan) and antibiotics (100 U/ml of penicillin-G and 100 mg/ml of streptomycin) at 37 °C in a humidified atmosphere of 5% CO₂ in air. The medium was changed twice a week. Before reaching confluence, the cells were passaged for use in experiments. VPA (valproic acid sodium, M.W. = 166.19) was purchased from Wako (Tokyo, Japan), diluted in sterilized phosphate-buffered saline at 100 mg/ml, and added to C3H10T1/2 at various final concentrations for indicated periods of time.

Cell counting

The proportional values of cells were counted using Cell-counting kit-8 (Dojindo Laboratories, Kumamoto, Japan), which measures a highly water-soluble formazan dye produced from tetrazolium salts. Cells were seeded at 1.0×10^3 cells per well on a 96-well plate and the medium was changed, along with various concentrations of VPA, after cell attachment to non-coated plastic, type I collagen-coated and fibronectin-coated 96-well plates (Becton-Dickinson, Tokyo, Japan). After 48- and 96-hour cell cultures, cells were incubated with the counting reagent for one hour, according to the manufacturer's instructions, and the relative cell number was determined by measuring light absorbance at a wavelength of 450 nm of the formazan dye product in the cultures. To determine the



number of viable cells cultured in VPA, a trypan blue dye exclusion test was used. Mesenchymal cells were cultured at various concentration of VPA for 48 hours and 96 hours on the non-coated plastic, fibronectin- and type I collagen-coated 24-well plate. After cell culture, 0.4% solution of trypan blue was added to equal volumes of the harvested cell suspensions. The ratio of stained to unstained cells was estimated using a hemocytometer.

Proliferating cell nuclear antigen (PCNA) staining and cytoskeleton staining

Before the experiment, C3H10T1/2 cells were trypsinized from a standard tissue culture plate and seeded in a Lab-Tek chamber slide (Nalge Nunc, Tokyo, Japan), to which they adhered during incubation overnight in a standard medium. After cell adherence, they were cultured for an additional 48 hours after changing the medium to include VPA concentrations of 10, 100 and 1000 $\mu\text{g/ml}$. After an additional 48-hour culture, cells were fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) and proliferating cell nuclear antigen (PCNA) and cytoskeleton staining was performed. PCNA was detected by a ZYMED PCNA staining kit (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Briefly, after fixation, cells were incubated with blocking solution and incubated with biotinylated mouse anti-PCNA for 60 minutes at room temperature. The color was developed using diaminobenzidine (DAB) as the substrate included in the kit. Negative cells were counterstained with hematoxylin. The PCNA-positive cell counts were expressed as a ratio of total cells, including both positive and negative PCNA within a defined area of 500 μm^2 taken from five different areas in each test group. For F-actin cytoskeleton observation, cells were incubated with Molecular Probes Alexa Fluor 488-FITC (Invitrogen) and DNA stained with 1, 4, 6-diamidino-2-phenylindole (DAPI).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Mouse mesenchymal cells were cultured on a non-coated plastic or type I collagen-coated plate in the absence or presence of 1 $\mu\text{g/ml}$ VPA in alpha-modified Eagle's minimum essential medium (α -MEM, Invitrogen) containing 10% FBS and

antibiotics, supplemented with 50 $\mu\text{g/ml}$ ascorbic acid 2-phosphate (Sigma, St. Louis, MO, USA) and 0.1 μM dexamethasone. After a one-week culture, total RNA was isolated from cultured cells using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. One-step RT-PCR was performed using a Superscript one-step RT-PCR with a Platinum Taq kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions, and a Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA, USA). To quantify the gene expression of collagen type I alpha 2 (collagen 1 α 2) and non-collagenous bone proteins (osteopontin, osteocalcin and bone sialoprotein (BSP)), semiquantitative RT-PCR, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed. Amplimers designed for these four molecules were previously reported.^{14,15} The PCR products were subjected to electrophoresis, and digital images were obtained and analyzed using IMAGE J (NIH image) software. Three populations of each cell type were studied.

Statistical analyses

All data are expressed as the mean \pm standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by post-hoc *t*-test. Differences were considered significant at a *P* value < 0.05 .

Results

Cell morphology

To examine the morphometric alteration of mesenchymal cells and their cytoskeleton in the presence of VPA, we observed the F-actin-stained cytoskeleton content at various concentrations of VPA after a 48-hour culture period. Both in the absence and presence of VPA, mesenchymal cells showed a flattened and extended shape (Fig. 1). This cell shape was not clearly altered even at a high concentration of VPA, but a high F-actin stained cytoskeleton content was observed in the cells (Fig. 1D). This data suggests that VPA was able to maintain normal cell shape but also synthesized a large number of stress fibers in cells at high concentrations.

Cell proliferation activity

To analyze mesenchymal cell proliferation activity when cells were cultured in VPA, we cultured cells

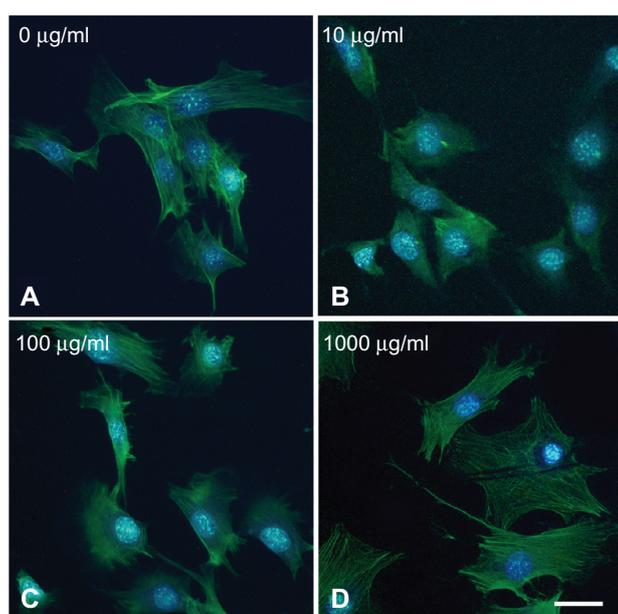


Figure 1. VPA does not alter mesenchymal cell shape even in high concentrations. Mesenchymal cells were cultured in the absence (0 $\mu\text{g/ml}$, **A**) or presence of VPA at various concentration (10 $\mu\text{g/ml}$, **B**., 100 $\mu\text{g/ml}$, **C**., and 1000 $\mu\text{g/ml}$ **D**) for 48 hours. Cells were stained with F-actin (green) and nuclear counterstained with DAPI (blue). Both in the absence or presence of high concentrations of VPA, mesenchymal cells maintained a flattened and extended shape.

Note: The bar in D represents 50 μm .

at various concentrations, from 10 to 1000 $\mu\text{g/ml}$, because therapeutic levels appear to range from 50 to 100 $\mu\text{g/ml}$ in human serum, and also because we had already reported that a VPA concentration of 1000 $\mu\text{g/ml}$ affects bone resorption in rat calvaria organ culture.¹⁶ The C3H10T1/2 mouse mesenchymal cell line was cultured in the absence (0 $\mu\text{g/ml}$) or presence of various concentrations of VPA (10, 100, 1000 $\mu\text{g/ml}$) for 48 hours on a non-coated plastic cell culture plate (Fig. 2A–D). After the cell culture period, cell proliferation activity was detected by PCNA staining. PCNA-negative cells were counterstained by hematoxylin. At all VPA concentrations from 10 to 1000 $\mu\text{g/ml}$, cells were attached to a non-coated plastic cell culture plate and PCNA-positive cells were observed at all VPA concentrations (Fig. 2A–D). When PCNA-positive cell counts were obtained from five randomly chosen areas following the 48-hour culture, the cell number decreased significantly only at a VPA concentration of 1000 $\mu\text{g/ml}$ and there was no difference noted at other concentrations (Fig. 2E). These data indicate that VPA had no effect on mesenchymal cell proliferation when cells were cultured on a non-coated plastic plate.

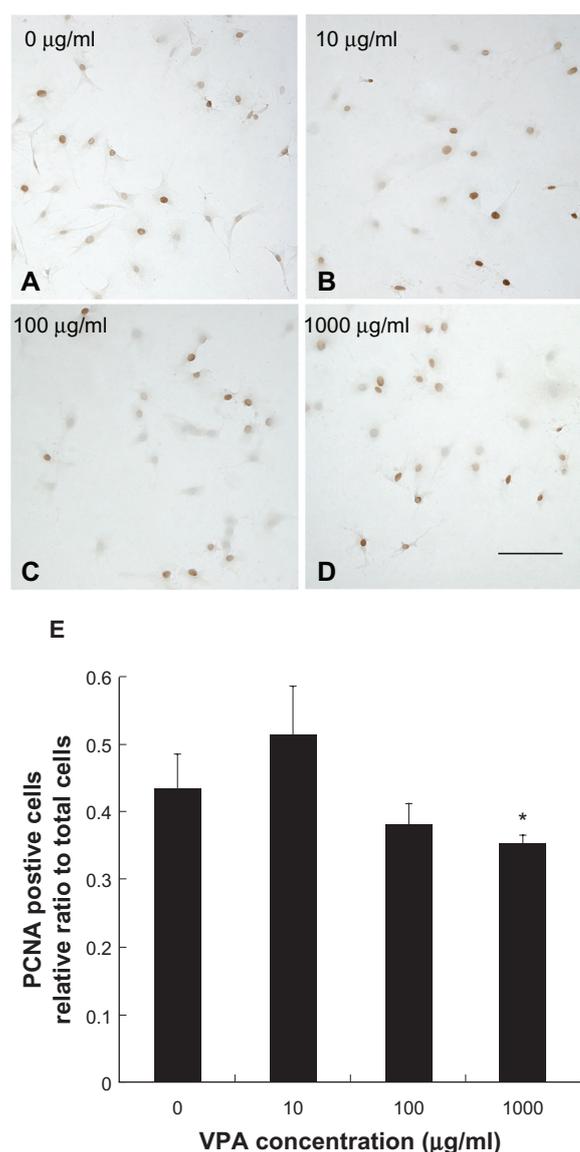


Figure 2. Proliferation of pluripotent mesenchymal cells is inhibited by VPA at a high concentration. C3H10T1/2, a mouse mesenchymal cell line, was cultured in the absence (0 $\mu\text{g/ml}$, **A**) or presence of VPA at various concentration (10 $\mu\text{g/ml}$, **B**., 100 $\mu\text{g/ml}$, **C**., and 1000 $\mu\text{g/ml}$, **D**) for 48 hours. Cell proliferation activity was detected by PCNA staining following cell culture. (**A–D**) PCNA-positive cells (brown) were present even at 1000 $\mu\text{g/ml}$. Negative cells were counterstained by hematoxylin (blue). **E**) Both PCNA-positive cell counts and total cell counts were obtained from five randomly chosen areas following the 48-hour culture period. The bar graph expresses the relative ratios of their average values. PCNA-positive cell numbers decreased significantly only at a VPA concentration of 1000 $\mu\text{g/ml}$ when compared with the numbers obtained from the absence of VPA.

Notes: * $P < 0.05$ when compared to 0 $\mu\text{g/ml}$. The bar in D represents 200 μm .

Effect of ECMs on cell number

For quantitative examination of the VPA effect on cell proliferation in the presence of ECMs, we performed a mesenchymal cell proliferation assay using fibronectin- and type I collagen-coated cell culture



plates and compared the results to an assay performed on the cells seeded on a non-coated plastic plate. Cell number on both the non-coated plastic plate and the ECM plates were estimated indirectly using highly water-soluble formazan dye as described in the Materials and Methods section. On the non-coated plastic plate (Fig. 3A), the cell counts at all doses of VPA, from 1 to 1000 $\mu\text{g/ml}$, were significantly higher following the 96-hour culture than those following the 48-hour culture ($n = 8$, $P < 0.01$). Following both the 48- and 96-hour culture periods, there were no significant cell number differences among the VPA doses, except for at the 1000 $\mu\text{g/ml}$ concentration on the non-coated plastic plate (Fig. 3A). These results indicated that only VPA at a concentration of 1000 $\mu\text{g/ml}$ reduced the mesenchymal cell proliferation level on the non-coated plastic plate; however in the fibronectin- and type I collagen-coated plate culture, the concentration of VPA at all concentrations, from 1 to 1000 $\mu\text{g/ml}$, lowered the mesenchymal cell proliferation level when compared to the 0 $\mu\text{g/ml}$ concentration on fibronectin- and

type I collagen -coated plates at each time period (Fig. 3B and C). In the absence of VPA, mesenchymal cell counts on the ECM plates were significantly higher than those on non-coated plastic plate following 48-hour culture periods (Fig. 3A, B and C). When we took trypan blue positive cell counts in a cell suspension following the 48-hour and 96-hour culture, ratios of viable cells in non-coated plastic plate were no significant differences found among the VPA concentrations (Fig. 3D, Normal). On the ECM plates, ratios of viable cells at the 10 $\mu\text{g/ml}$ were no significant differences comparing with 0 $\mu\text{g/ml}$ following the 48-hour and 96-hour culture (Fig. 3D). These results indicated that VPA could reduce mesenchymal cell proliferation level at a relatively high concentration in the absence of ECMs; however, this reducibility effect is enhanced at relatively low concentrations in the presence of ECMs.

Cell differentiation

Since a previous report indicated that VPA promoted osteoblastic cell differentiation from a pre-osteoblastic

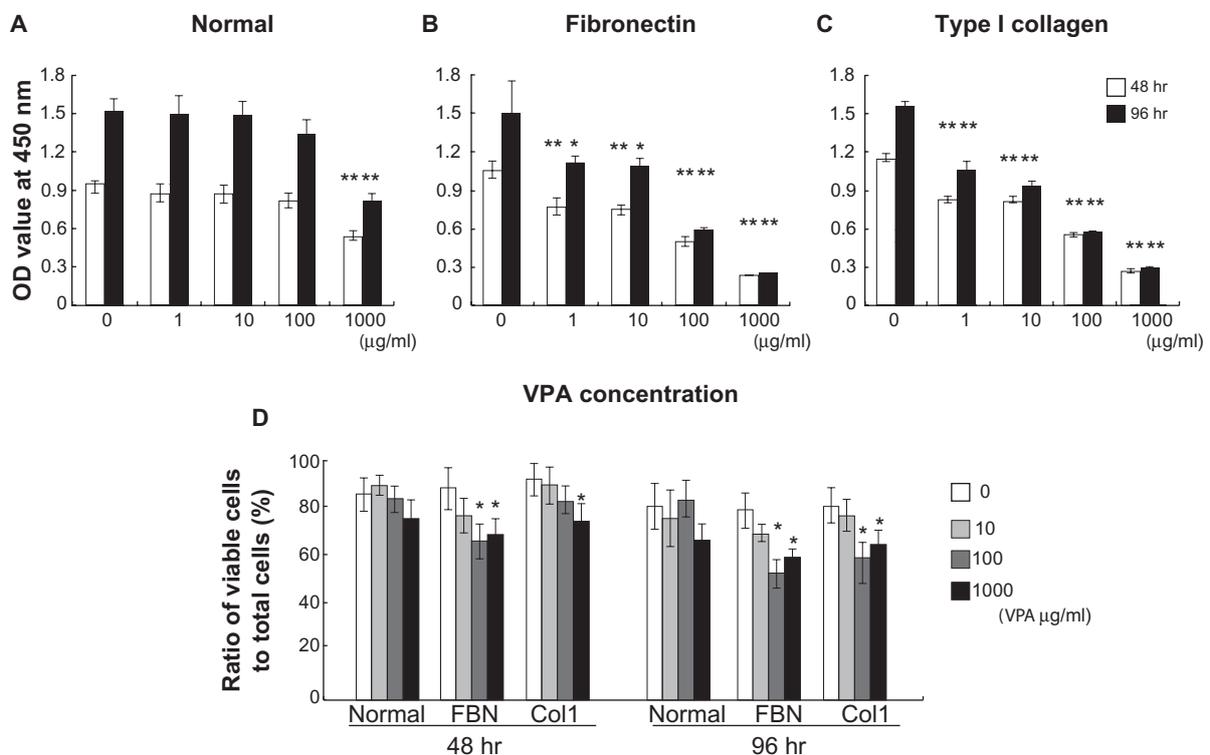


Figure 3. VPA reduced mesenchymal cell proliferation level in the presence of ECMs. Mesenchymal cells were cultured in the absence (Normal, **A**) or presence (Fibronectin (**B**) and Type I Collagen (**C**)) of extracellular matrices for 48 and 96 hours. Relative cell number was indicated by light absorbance at a wavelength of 450 nm of a formazan dye product in the culture. VPA, at relatively low concentrations, had no obvious inhibitory effect on mesenchymal cell proliferation when cultured in the absence of ECM (**A**); however, in the presence of ECM, VPA reduced mesenchymal cell proliferation level in a dose-dependent manner (**B**, **C**). **D** The ratios of viable cells on the ECM plates (Fibronectin (FBN), Type I Collagen (Col1)) were not decreased significantly at 10 $\mu\text{g/ml}$ following 48 hr- and 96 hr-culture period. $n = 8$ in each experiment group.

Notes: * $P < 0.05$; ** $P < 0.01$ compared to 0 $\mu\text{g/ml}$ for each time period.



cell line,⁴ we tested whether VPA promotes the expression of osteoblastic marker genes during sequential cell differentiation from mesenchymal cells to osteoblasts both in the absence or presence of an extracellular matrix, when cultured on non-coated plastic or type I collagen-coated plates. In the absence of VPA, the gene expressions of collagenous and of non-collagenous bone matrices (ie, collagen 1 α 2, osteopontin and osteocalcin, which are osteoblastic marker genes) were detected in the mesenchymal cells cultured on a non-coated plastic plate with an inductive medium of osteoblastic cell differentiation after a one-week culture (Fig. 4A, B, C, D; Normal). The expression levels of these marker genes were significantly promoted in the presence of 1 μ g/ml VPA, except in the case of BSP. Similar to the results in the cell culture on the non-coated plastic plate, VPA significantly promoted the expression level of all genes, except for that of BSP, when compared with the absence of VPA in the cell culture on the collagen-coated plate (Fig. 4A, B, C, D; Collagen). Furthermore the increase in these gene expression levels was higher than that of the non-coated plastic plate both in the absence or presence of VPA. These results indicate that VPA promoted osteoblastic cell differentiation from mesenchymal cells cultured on the non-coated plastic plate and the co-presence of an extracellular matrix promoted osteoblastic differentiation.

Discussion

VPA is an effective and widely used antiepileptic and anticonvulsant drug which influences osteogenesis *in vivo* and *in vitro*.¹⁻³ In terms of osteogenesis, osteoblasts play a critical role in cooperating with various types of cells, which are all derived from mesenchymal stem cells. The murine embryonic mesenchymal cell line, C3H10T1/2, is a pluripotent cell line which can be differentiated into osteoblasts, chondrocytes, adipocytes, and myoblasts *in vitro* and is used in numerous studies as a model for mesenchymal cell differentiation.⁶⁻⁸ ECMs secreted by mesenchymal cells, including osteoblasts, also play a critical role in osteogenesis, and two ECM components, type I collagen and fibronectin, are known to effect osteogenic cell proliferation and differentiation both *in vivo* and *in vitro*.¹⁰⁻¹³ In this study, we attempted to examine the effect of VPA in mesenchymal cell proliferation and cell differentiation *in vitro* in both the presence and absence of ECMs.

First we observed that normal cell shape was maintained, but a large number of stress fibers were formed in high concentrations of VPA after a 48-hour culture. Previous studies have reported that high concentrations of VPA induced morphological changes in mouse osteoblast cell line MC3T3-E1 cells⁴ and mouse fibroblast cell line L929.¹⁷ Microarray analysis of somatic tissue of mouse embryos with applied VPA revealed that gene expression in the microtubule cytoskeleton and actin filament may be associated with axis skeletal malformations² and increasing levels of F-actin were also observed in mouse fibroblasts.¹⁷ Our observations on the morphology of undifferentiated mesenchymal cells cultured in a VPA-containing medium is consistent with these reports and suggests that VPA could rearrange the cytoskeleton of various types of cells.

In the cell proliferation on a non-coated plastic plate, our results showed that mesenchymal cells were positive for PCNA at all VPA concentrations, from 1 to 1000 μ g/ml, after 48 hours and we detected no significant differences between the control (0 μ g/ml) and 100 μ g/ml populations cultured for 48 hours when PCNA-positive cell counts were obtained from a randomly chosen area and also when the estimated cell number was determined from an assay utilizing water-soluble formazan dye. Previous studies were performed at 0.1 μ M to 3 mM VPA concentrations (corresponding to 0.08 to 480 μ g/ml values) for cell viability and proliferation assays in osteoblasts and neural crest cells.^{4,18} In a mouse osteoblast cell line, MC3T3-E1, VPA promoted cell proliferation at low concentrations of 0.1 to 50 μ M (corresponding to 0.08 to 4 μ g/ml values) and decreased the cell proliferation rate at a high concentration of 1 mM.⁴ On the other hand, neural crest cells could proliferate at 1.5 mM (240 μ g/ml) concentrations of VPA and this was not significantly different to the control results.¹⁸ Taken together, our data suggested that the effect of VPA on cell proliferation is dependent on the maturation level of the cells, namely that immature cells have resistance to high concentrations of VPA.

In the present study, VPA had inhibitory effect on the mesenchymal cell proliferation with culture on an ECM coat of fibronectin and type I collagen. Fibronectin and type I collagen are major components of ECMs and well known to promote mesenchymal cell proliferation.¹⁰⁻¹⁹ Consistent with these reports, our data showed that fibronectin or type I alone had the effect of increasing cell proliferation in the absence

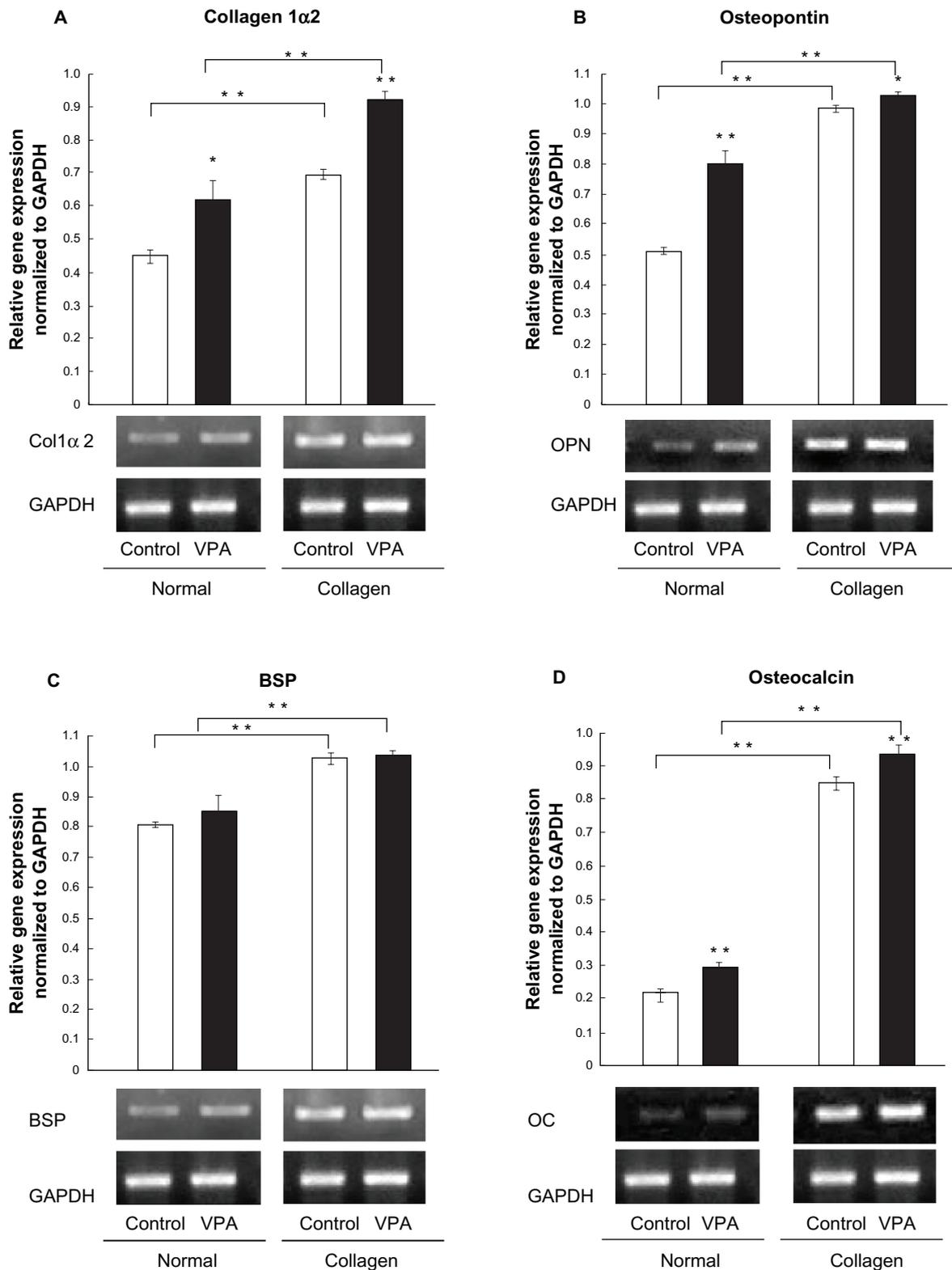


Figure 4. VPA promoted osteoblastic cell differentiation both in the absence or presence of ECM. Mesenchymal cells were cultured in the absence (Control) or presence of VPA (VPA) on a non-coated plastic plate (Normal) or type I collagen-coated plate (Collagen) for each of the conditions for one week. The expression level of osteoblastic marker genes, Collagen type 1 α 2 (Col 1 α 2), osteopontin (OPN), bone sialoprotein (BSP) and osteocalcin (OC) were assayed by semi-quantitative RT-PCR and each marker gene was normalized against GAPDH. On both non-coated plastic (Normal) and collagen-coated (Collagen) plates, VPA promoted the expression level of each marker gene significantly compared to the Control results (A, B, D), except in the case of BSP (C). $n = 3$ in each experiment group.

Notes: * $P < 0.05$; ** $P < 0.01$ comparing Control or Normal with Control or VPA.



of VPA as compared with normal culture. Therefore inhibitory effect of VPA on cell proliferation in the presence of fibronectin and collagen type I cultured with VPA may depend on that interactions between mesenchymal cells and ECM increased sensitization of the cells to proliferation inhibition induced VPA.

Based on the current results of the effect of VPA on mesenchymal cell proliferation inhibition, we assayed the effect of cell differentiation, especially of osteoblastic cell differentiation, in the absence or presence of ECMs. Our results showed that VPA promoted osteoblastic cell differentiation in the presence of an extracellular matrix, the collagen-coated plate. This result indicates that the inhibitory effect of VPA on cell proliferation on the collagen-coated plate at lower concentrations compared with that on the non-coated plastic plate would be dependent on the effect of VPA on osteoblastic cell differentiation from mesenchymal cells to mature osteoblasts in cooperation with collagen, which is well known as a promoter of osteoblastic cell differentiation.²⁰ Because it has been reported that VPA promoted cell differentiation from preosteoblast to osteoblast, which indicated the effect of VPA on cell differentiation in committed cells such as from osteoblast to mature osteoblast,⁵ our results suggested that VPA could promote sequential cell differentiation from uncommitted mesenchymal cells to mature osteoblasts in the presence of ECMs. Interestingly, VPA increased the gene expression level of osteoblastic marker genes even on the non-coated plastic plate, in which VPA had no inhibitory effect on cell proliferation in low concentrations. Since we added minimum supplements for osteoblastic cell differentiation including ascorbic acid and dexamethasone, which are widely used for basal induction of osteoblastic cell differentiation of various mesenchymal cells including C3H10T1/2,^{21–23} VPA may enhance osteoblast cell fate determination and promote cell differentiation in the presence of ECMs.

In summary, this study reported on the relationship of VPA and ECMs in mesenchymal cell proliferation and cell differentiation. Additionally, this study could reflect the effects of VPA in *in vivo* conditions, but further research would be necessary to clarify the mechanism of cell proliferation in the co-presence of VPA and other ECMs to provide further insight into the effect of VPA in mesenchymal tissue development.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

References

- Sheth RD, Wesolowski CA, Jacob JC, et al. Effect of carbamazepine and valproate on bone mineral density. *J Pediatr*. 1995;127(2):256–62.
- Massa V, Cabrera RM, Megegola E, Giavini E, Finnell RH. Valproic acid-induced skeletal malformations: associated gene expression cascades. *Pharmacogenet Genomics*. 2005;15(11):787–800.
- Ardinger HH, Atkin JF, Blackston RD, et al. Verification of the fetal valproate syndrome phenotype. *Am J Med Genet*. 1988;29(1):171–85.
- Schroeder TM, Westendorf JJ. Histone deacetylase inhibitors promote osteoblast maturation. *J Bone Miner Res*. 2005;20(12):2254–63.
- Okada A, Shiomi T, Aoki Y, Fujiwara M. Phenytoin stimulates chondrogenic differentiation in mouse clonal chondrogenic cells, ATDC5. *J Toxicol Sci*. 2005;30(3):145–56.
- Zehentner BK, Leser U, Burtscher H. BMP-2 and Sonic hedgehog have contrary effects on adipocyte-like differentiation of C3H10T1/2 cells. *DNA Cell Biol*. 2000;19(5):275–81.
- Ahrens M, Ankenbauer T, Schroder D, Hollnagel A, Mayer H, Gross G. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell Biol*. 1993;12(10):871–80.
- Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, et al. The non-osteogenic mouse pluripotent cell line, C3H10T1/2 is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. *Biochem Biophys Res Commun*. 1990;172(1):295–9.
- García AJ, Reyes CD. Bio-adhesive surfaces to promote osteoblast differentiation and bone formation. *J Dent Res*. 2005;84(5):407–13.
- Andrianarivo AG, Robinson JA, Mann KG, Tracy RP. Growth on type I collagen promotes expression of the osteoblastic phenotype in human osteosarcoma MG-63 cells. *J Cell Physiol*. 1992;153(2):256–65.
- Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: Modification of expression genes supporting cell growth, adhesion, and extracellular matrix mineralization. *Exp Cell Res*. 1995;216(1):35–45.
- Weiss RE, Reddi AH. Synthesis and localization of fibronectin during collagenous matrix-mesenchymal cell interaction and differentiation of cartilage and bone in vivo. *Proc Natl Acad Sci U S A*. 1980;77(4):2074–8.
- Yamada KM, Olden K. Fibronectins, adhesive glycoproteins of cell surface and blood. *Nature*. 1978;275(5677):179–84.
- Hatakeyama J, Hatakeyama Y, Takahashi I, Suzuki O, Sasano Y. Proliferation and adhesion of periodontal ligament cells on synthetic biomaterials. *Oral Dis*. 2007;13(5):500–6.



15. Huojia M, Muraoka N, Yoshizaki K, Fukumoto S, et al. TGF-beta3 induces ectopic mineralization in fetal mouse dental pulp during tooth germ development. *Dev Growth Differ.* 2005;47(3):141–52.
16. Takahashi A, Onodera K. Valproic acid induced osteopenia and its prevention with alfacalcidol and alendronate. *J Hard Tissue Biology.* 2005; 14(2):275–6.
17. Walmod PS, Skladchikova G, Kawa A, Berezin V, Bock E. Antiepileptic teratogen valproic acid (VPA) modulates organization and dynamics of the actin cytoskeleton. *Cell Motil Cytoskeleton.* 1999;42(3):241–55.
18. Fuller LC, Cornelius SK, Murphy CW, Wiens DJ. Neural crest cell motility in valproic acid. *Reprod Toxicol.* 2002;16(6):825–39.
19. Terranova VP, Aumailley M, Sultan LH, Martin GR, Kleinman HK. Regulation of cell attachment and cell number by fibronectin and laminin. *J Cell Physiol.* 1986;127(3):473–9.
20. George J, Kuboki Y, Miyata T. Differentiation of mesenchymal stem cells into osteoblasts on honeycomb collagen scaffolds. *Biotechnol Bioeng.* 2006; 20;95(3):404–11.
21. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology.* 1994;134(1):277–86.
22. Shea CM, Edgar CM, Einhorn TA, Gerstenfeld LC. BMP treatment of C3H10T1/2 mesenchymal stem cells induces both chondrogenesis and osteogenesis. *J Cell Biochem.* 2003;15;90(6):1112–27.
23. Makhijani NS, Bischoff DS, Yamaguchi DT. Regulation of proliferation and migration in retinoic acid treated C3H10T1/2 cells by TGF-beta isoforms. *J Cell Physiol.* 2005;202(1):304–13.

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