Valproic Acid Modulates the Multipotency in Periodontal Ligament Stem Cells via p53-Mediated Cell Cycle

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Abstract Human periodontal ligament stem cells (PDLSCs), a type of mesenchymal stem cell, are a promising source for dental regeneration and are identified in human periodontal ligaments from extracted third molars. Valproic acid (VPA) is a histone deacetylase inhibitor that has been used as a wide-spectrum antiepileptic drug and a medication for mood disorders. VPA has shown several effects on increasing the pluripotency of embryonic stem cells and controlling osteogenic differentiation, besides the prevention of seizures. However, its effect on proliferation and osteogenesis depends on the cell type and concentration. The aim of this study was to investigate the effects of cyclic and constant VPA treatment on PDLSCs. Proliferation and apoptosis of PDLSCs were determined with cyclic and constant VPA treatment. In cemento/osteogenic differentiation, osteogenic markers decreased significantly after cyclic treatment with 0.5 mM VPA. In contrast, VPA enhanced osteogenic differentiation after constant treatment. With cyclic VPA treatment, p53 levels related to apoptotic pathway decreased to induce proliferation. These findings indicated that VPA has different roles in proliferation and differentiation of PDLSCs *in vitro* and *in vivo* via p53-related pathway.

Keywords Periodontal ligament stem cell · Proliferation · Osteogenesis · Valproic acid · p53

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1 Introduction

Mesenchymal stem cells (MSCs), also called adult stem cells, have a limited differentiation potential and self-renewal capacity. MSCs divide symmetrically to increase their numbers or asymmetrically to self-renew. They can initiate regrowth to produce more differentiated descendants [1]. In dental research, periodontal ligament stem cells (PDLSCs) attached to human teeth have the same properties as other MSCs; express the genetic markers of stem cells; and can differentiate into osteoblasts, chondrocytes, and adipocytes [2–9]. PDLSCs have been suggested to be useful for therapeutic purposes, such as PDL regeneration [7, 9–11].

Valproic acid (N-dipropylpentanoic acid; VPA), a mood stabilizer and antiepileptic drug, is a histone deacetylase inhibitor (HDI) that includes a simple branched-chain fatty acid. HDIs are effective for neurodegenerative diseases, such

as Alzheimer's disease and Huntington's disease [12], and are used as potential therapies for inflammatory diseases such as rheumatoid arthritis and lupus erythematosus, as well as for cardiac diseases [13, 14]. Recently, HDIs, including VPA, have been clinically tested as anti-cancer drugs [15, 16]. VPA induces differentiation and apoptosis, inhibits proliferation, increases immunogenicity, and inhibits angiogenic proteins in cultured cancer cells [17, 18]. Other reports found that VPA enhances the proliferation of hematopoietic stem cells (HSCs) and neuronal cells [19–23]. The limited research on dental pulp stem cells (DPSCs) involves the study of proliferation and mineralization with HDI treatments. In the presence of HDI, DPSCs had reduced osteocalcin expression and decreased proliferation [24, 25].

Previous studies have indicated that HDIs are involved in DNA demethylation and accelerate reprogramming to induce pluripotent stem cells [26–29]. Fibroblasts transfected with the factors Oct4, Sox2, Klf4, and c-Myc, have more Oct4-GFP-positive cells with VPA treatment. However, induced pluripotent stem cells (iPSCs) are not an attractive source of stem cells for regenerative medicine because of their low efficiency and the risks involved with viral transduction. Several studies suggest that treating MSCs with VPA might improve the efficacy of stem cell therapy [30, 31], but the proliferative effect of VPA on dental MSCs, such as PDLSCs, is unknown. These controversial results on proliferation and osteogenesis with HDI treatment do not support the use of PDLSCs with HDI treatment. The purpose of this study was to understand how VPA treatment controls PDLSC proliferation and differentiation. In this study, we hypothesized that cyclic VPA treatment might affect proliferation through p53.

2 Materials and methods

2.1 Samples and cell culture conditions

Impacted third molars were collected from healthy volunteers (n=8) age 18–27 years at the Department of Oral and Maxillofacial Surgery of Seoul National University Dental Hospital, following a protocol approved by the Institutional Review Board at Seoul National University School of Dentistry (IRB No. S-D20080009). All isolation and cell culture procedures were detailed in a previous report [9, 32, 33]. The periodontal ligament was separated from the root surface and digested in 3 mg/mL collagenase type 1 (BioBasic Inc., Toronto, Canada) and 4 mg/mL dispase (GIBCO BRL, Grand Island, NY) for 1 h (37°C, 5% CO₂). Single-cell suspensions were obtained by passing cells through a 70-µm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA). Primary cells from PDL samples were seeded into 10-cm culture dishes (NUNC, Roskilde,

Denmark) with alpha-modification of Eagle's medium (GIBCO BRL) supplemented with 15% fetal bovine serum (Equitech-Bio Inc., Kerrville, TX, USA), 2 mM L-glutamine (GIBCO BRL), 100 µM L-ascorbic acid (BioBasic Inc.), and 100 U/mL antibiotic-antimycotics (GIBCO BRL). At 80–90% confluence, primary cells were seeded in subcultures. Cells were counted with a hemocytometer and 0.4% (w/v) trypan blue dye (GIBCO BRL). All cells used were from passages 2–6.

2.2 Valproic acid treatment

PDLSCs were seeded into dishes, plates, and chamber slides and incubated for 24 hours (37°C, 5% CO₂). For cyclic treatment, cells were treated with culture medium containing 0.5 or 1 mM VPA (Sigma-Aldrich, St Louis, MO, USA) for 24 hours, then washed and incubated for 48 hours in normal culture medium without VPA (1 cyclic). The cycle was repeated three times consecutively (2 cyclic and 3 cyclic). For constant treatment, the cells were incubated in VPA-containing medium continuously (1 constant, 2 constant, 3 constant). The experimental scheme is shown in Fig. 1A. Immunocytochemistry and flow cytometry for OCT4 and NANOG were performed on only the first cycle of VPA treatment.

2.3 Immunofluorescence staining

PDLSCs (10⁴ cells) were seeded into 2-chamber slides (NUNC) with VPA (0.5, 1 mM). After fixing with 4% paraformaldehyde (Sigma-Aldrich), blocking solution (10% goat serum and 0.1% Triton X-100 in PBS containing 1% bovine serum albumin) was added. For OCT4 and NANOG staining, primary anti-mouse/human OCT4 rabbit IgG antibody (1:200, Stemgent) or anti-mouse/human NANOG rabbit IgG antibody (1:200, Stemgent) were added and incubated at room temperature for 90 min. Secondary antibody (1:300, goat polyclonal secondary anti-rabbit IgG-H&L [FITC], Abcam) was added for 1 h at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), and mounting medium (Cytomation fluorescent mounting medium, DAKO, Glostrup, Denmark) was added for fluorescent imaging (confocal laser scanning microscope, FV300, Olympus America Inc., Center Valley, PA, USA).

2.4 *In vitro* proliferation

VPA-induced PDLSC proliferation was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution with 8 samples. The optical density of formazan crystals dissolved in dimethyl sulfoxide

Fig. 1 PDLSC proliferation with cyclic and constant VPA treatments. **A** Experimental scheme. Samples from both the cyclic and constant VPA treatment groups were collected at 3, 6, and 9d. **B** Direct cell counting and **C** MTT assays of 6 samples showed that 3 cyclic treatment with 0.5 mM VPA seemed to enhance cell proliferation compared 2 cycles. The data are shown as the mean $\pm$ SE. * $p < 0.05$

solution was read at 540 nm. Direct cell counting and MTT assays were performed after cyclic treatment.

2.5 Flow cytometry for OCT3/4 and NANOG

PDLSCs (4×10^5 cells) were seeded onto 10-cm culture dishes and treated for 24 hours with VPA with 48 hours rest in normal proliferative culture medium. After fixation, cells were suspended in PBS containing 0.1% (w/v) saponin and 0.05% (w.v.) NaN_3 . PDLSCs were stained with anti-human/mouse Oct3/4-phycoerythrin (PE) monoclonal antibody (1:20) or rat IgG_{2B} isotype control-PE (1:20). Cells were also stained with anti-human NANOG-PE polyclonal

antibody (1:20) or goat IgG isotype control-PE (1:20). Antibodies for OCT3/4 and NANOG used in flow cytometry were from R&D Systems.

2.6 In vitro osteogenic differentiation

The composition of cemento/odontogenic/osteogenic induction medium was described previously [9]. Alizarin red S staining with 40 mM concentration was performed to detect the calcium accumulation in mineralized cells. After destaining with 10% cetylpyridinium chloride (Sigma-Aldrich) in 10 mM sodium phosphate (pH 7), the absorbance was measured at 562 nm. Alkaline phosphatase (ALPase) activity was determined by adding 0.3 N NaOH to release p-nitrophenol from 0.1 mg/ml para-nitrophenylphosphate (p-NPP; Sigma-Aldrich). The optical density was read at 405 nm.

2.7 Flow cytometry for apoptosis

PDLSCs were seeded at 4×10^5 cells on 10-cm culture dishes and treated with VPA as above, but without incubation after VPA cycles. This allowed for detection of apoptosis immediately after VPA treatment. The first VPA treatment was 24 hours, the second was 24 hours after the first cyclic treatment, and the third was 24 hours after the second. A FITC-annexin V apoptosis detection kit I (BD Biosciences, New Jersey) was used to detect apoptosis following the suggested protocol. Data were collected using FACSCAN (Becton Dickinson, Franklin Lakes, NJ, USA). Data analysis used FACSDiva software (Becton Dickinson).

2.8 Western blot

PDLSCs were cultured for 24 hours with VPA treatment and 48 hours in culture medium. Proteins were extracted in lysis buffer (Invitrogen) with protease inhibitor and phenylmethylsulfonyl fluoride. Proteins transferred to membranes were detected with phospho-p53, total p53, and p21^{CIP/Waf-1} primary antibodies (Cell Signaling Technologies, Boston, MA, USA) at 1:1000 dilution followed with an HRP-linked secondary antibody. For detection, an HRP chemiluminescent detection kit (SurModics, Eden Prairie, MN, USA) was used and measured with a Micro-Chemi analyzer (DNR Bio-image Analyzer).

2.9 RT-PCR

A PureLink RNA minikit (Invitrogen, Carlsbad, CA, USA) was used to collect RNA. CDNA synthesized by

SuperScript III First-Strand kit (Invitrogen) was examined with the following primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 57°C), forward, 5'-AGCCG CATCTTCTTTTGCCTC-3'; reverse, 5'-TCATATTTG GCAGGTTTTTCT-3', alkaline phosphatase [34], forward, 5'-TGGAGCTTCAGAAGCTCAACACCA-3'; reverse, 5'-ATCTCGTTGTCTGAGTACCAGTCC-3', osteocalcin (OCN), forward 5'-CATGAGAGCCCTCACA-3'; reverse 5'-AGAGCGACACCCTAGAC-3', collagen type 1 (COL1), forward, 5'-CAAAGAGTCTACATGTCTAG-3'; reverse, 5'-CATGGGGCCAGGCACGGAAA-3' [35, 36].

2.10 *In vivo* transplantation and immunohistochemistry

10⁵ cells were seeded and treated with three cycles of VPA at 0.1, 0.5, and 1 mM. About 5X10⁶ cells with 40 mg of hydroxyapatite/tricalcium phosphate particles (HA/TCP) (Zimmer, Warsaw, IN, USA) were implanted in the dorsal surface of immunocompromised beige nude mice (NIN-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN, USA). Eight weeks after transplantation, the mice were sacrificed and transplants were harvested. The transplants were fixed and decalcified with 10% EDTA (pH 8.0). Transplants were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Mouse anti-human mitochondria antibody (1:200, Chemicon, Temecula, CA), mouse anti-human osteocalcin antibody (1:100, Abcam, Cambridge, UK), and rabbit anti-human collagen type 1 (1:1000, Abcam) were used and detected with an anti-mouse/rabbit HRP/DAB detection kit (Abcam).

2.11 Statistical analysis

One-way ANOVA tests were used to evaluate statistical significance among groups, followed by Tukey's HSD tests with SPSS 21 (SPSS Inc., Chicago, IL, USA). Data are presented as mean with standard errors. A statistically significant difference between groups was assigned if $p < 0.05$.

3 Results

3.1 Proliferation of PDLSCs with VPA

Samples from cyclic and constant treatment at days 3, 6, and 9 were collected to study PDLSC proliferation. Direct cell counting showed increased cell proliferation after cyclic treatment with 0.5 mM VPA (Fig. 1B). Similar results were obtained with MTT assays (Fig. 1C). Absorbance rates for cells treated with 0.5 mM VPA were higher than those for control cells after the third treatment cycle. In contrast, proliferation rates through the second treatment cycle did not differ significantly between control and

experimental groups (Fig. 1B, C). Cell numbers decreased significantly with constant VPA treatment (Fig. 1B, C). Constant VPA treatment inhibited PDLSC proliferation.

3.2 OCT3/4 and NANOG expression in PDLSCs with cyclic treatment

Expression of OCT3/4 and NANOG, markers of undifferentiated cells, in PDLSCs was examined by immunofluorescence after 1 cyclic treatment of VPA to induce proliferation. OCT4 was significantly higher after treatment with 0.5 mM VPA for 24 hours with 48 hours rest, compared to 1 mM VPA treatment (Fig. 2A). However, NANOG was highly expressed after cyclic treatment at both 0.5 and 1 mM (Fig. 2B). These results were confirmed by flow cytometry (Fig. 2C, D). Flow cytometry showed that 0.5 mM VPA treatment slightly increased OCT3/4 and NANOG expression compared with the control and 1 mM groups. Cyclic VPA treatment increased in OCT3/4, but constant VPA treatment did not.

3.3 Decreased apoptosis during cyclic treatment

To further analyze increased proliferation during cyclic treatment, flow cytometry was performed to detect apoptosis in cells receiving cyclic VPA treatment (Fig. 3). After 24 hours with 0.5 mM VPA (first VPA treatment), cells showed a slight decrease in annexin V-positive cells, indicating a lower rate of cell death compared to the control. However, cells treated with 1 mM VPA had higher apoptosis after 24 hours of treatment. The results were similar after the second VPA treatment, when cells treated with 0.5 mM VPA had decreased apoptosis. However, the third VPA treatment produced no difference between the control and cells treated with 0.5 mM VPA. The similarity of the results demonstrated that cyclic treatment of PDLSCs with 0.5 mM VPA promoted proliferation and inhibited apoptosis.

3.4 Apoptosis related to p53-mediated cell cycle

We analyzed p53 and p21 proteins, which are tumor suppressor genes that are related to apoptosis by regulating the cell cycle (Fig. 4). PDLSCs after 24 hours with VPA and 48 hours rest (cyclic) or 72 hours with VPA (constant) were cultured for western blotting. Immunoblotting showed that cyclic VPA treatment suppressed phospho-p53 in PDLSCs. On the other hand, constant treatment did not change phospho-p53 expression levels (Fig. 4B). Cyclic VPA treatment did not affect p21^{CIP-1/Waf-1} expression levels while constant treatment increased expression (Fig. 4C, D). VPA regulated the PDLSC cell cycle differently depending on the treatment.

Fig. 2 PDLSCs expressed OCT4 and NANOG after cyclic VPA treatment as measured by immunofluorescence and flow cytometry ($n=3$). **A, B** OCT4 and NANOG expression was higher when treated with VPA for 24 hours and 48 hours rest. Bar 20 μm ($\times 400$). **C, D**

Flow cytometry for OCT3/4- and NANOG-expressing PDLSCs. **C** OCT4-positive PDLSC showed the highest percentage after 0.5 mM VPA treatment. **D** NANOG-positive PDLSCs showed the highest percentage after 0.5 mM VPA treatment

3.5 Opposite effects on *in vitro* osteogenic differentiation by different VPA treatment conditions

Cyclic and constant VPA treatment had opposite results on osteogenic differentiation. Cyclic treatment with a higher VPA concentration inhibited calcium accumulation for osteogenic induction. Alizarin red S staining showed fewer mineralized nodules after cyclic treatment. On the other hand, constant treatment boosted nodule formation (Fig. 5A). After 2 weeks of osteogenic induction, ALPase activity was measured (Fig. 5B). With cyclic treatment, ALPase activity decreased. However, ALP activity increased significantly with constant VPA treatment. These results were similar to alizarin red S staining patterns. The expression of osteogenic markers was unlikely to differ from the patterns of alizarin red S staining and ALPase activity. In cyclic treatment, 0.5 mM VPA suppressed expression of ALP and OCN, osteogenic differentiation markers. However, constant treatment increased ALP expression in a dose-dependent manner. Interestingly, ALP OCN were suppressed by cyclic treatment with 0.5 mM VPA. At 0.5 mM on both cyclic and constant treatments, VPA affected ALP and OCN expression (Fig. 5C). Cyclic VPA treatment inhibits osteogenic differentiation; however, constant VPA treatment enhances osteogenesis in PDLSCs.

3.6 Enhancement of bone formation on *in vivo* osteogenic differentiation by VPA

After cyclic VPA treatment of PDLSCs, cells were transplanted in the back of nude mice with HA-TCP carriers for 8 weeks. Although cells were treated cyclically, the VPA-treated group had enhanced hard tissue formation. Cells treated with 1 mM VPA were more differentiated and formed more hard tissue structures than did controls (Fig. 6A). To confirm transplanted cells, human mitochondria were stained (Fig. 6B). Human mitochondria were detected on the surface of hard tissue. A higher degree of hard tissue formation in the VPA-treated group indicated that the role of VPA in osteogenic differentiation *in vivo* differed from that *in vitro*. Hard tissue formed by PDLSCs was positively stained with osteocalcin and collagen type I antibodies.

4 Discussion

VPA, as a successful treatment for seizures and bipolar disorder, has been studied in many areas. VPA has the potential to affect to stem cells by regulating histone acetylation in regions of active pluripotency genes. On the contrary, VPA also affected the osteogenic differentiation

Fig. 3 Decreased apoptosis during cyclic treatment. Cyclic treatment with 0.5 mM VPA decreased apoptosis compared to other concentrations. **A** On the first and second cycles, 0.5 mM VPA inhibited apoptosis. **C** However after the third cycle, both controls and 0.5-mM treated cells had the same level of apoptosis. First VPA treatment indicated 24 hours treatment of VPA. Cells collected for flow cytometry were stained with FITC-annexin V and PI for the first VPA cycle treatment (24 hours VPA treatment), second VPA treatment (1 cycle followed by 24 hours VPA treatment), and third VPA treatment (2 cycles followed by 24 hours VPA treatment)

of MSCs. However, the mechanism by which VPA controls stem cell pluripotency or osteogenic differentiation was not obvious.

Antiepileptic therapeutic concentrations of VPA vary from 0.41 to 1.04 mM [21]. In one report, 0, 0.5, 1, 2, and 4 mM VPA had little cytotoxicity when used for neural differentiation of rat muscle-derived stem cells [37]. In other studies, VPA has been suggested to decrease MSC proliferation. VPA treatment at 0.4–10 mM of MSCs derived from human or mouse adipose tissue, umbilical cord blood, or bone marrow for 1–6 days inhibited proliferation and self-renewal [38, 39]. However, 0.065 mM VPA significantly increased *ex vivo* expansion of human hematopoietic stem cells [40]. Studies have found different cellular proliferation and apoptosis responses depending on the VPA concentration. The concentrations used in this study were within the therapeutic ranges currently used to control various diseases [12, 41]. Treatment with a low VPA concentration (0.1–50 μ M) for 3 days stimulated proliferation of a mouse osteoblast cell line and primary osteoblasts, whereas a higher VPA concentration (1 mM)

caused cell death [42]. Our results showed that cell viability increased with cyclic 0.5-mM VPA, a relatively low concentration, by MTT assay, although the difference was not significant. We concluded that a higher VPA concentration, 1 mM, did not improve PDLSC proliferation with constant or cyclic treatment. Constant VPA treatment also did not induce stem cell proliferation. This result indicates that the 0.5 mM VPA concentration is a critical to increase proliferation with cyclic treatment.

Oct4 and Nanog are key proteins for maintaining MSC properties [43] as shown by studies of peripheral blood stem cells, umbilical cord blood stem cells, and bone marrow stem cells [44]. Recent research shows that the PI3K/Akt/mTOR pathway activates the Oct4 promoter after VPA treatment of mouse myoblast cells [45, 46]. We confirmed that PDLSCs expressed OCT4 and NANOG by immunocytochemistry after cyclic VPA treatment. PDLSCs treated with VPA for 24 hours with 48 hours rest had increased self-renewal capacity. By flow cytometry, the percentage of OCT3/4-positive and NANOG-positive cells increased slightly with cyclic 0.5 mM VPA treatment.

Fig. 4 Apoptosis related to the p53-mediated cell cycle. **A** Immunoblot data for phospho-p53, total-p53, and p21^{CIP1/WAF1} tumor suppressor genes related to apoptosis after cyclic (24 hours VPA with 48 hours rest) and constant treatment (72 hours VPA) of PDLSCs. **B–D** Fold changes of each gene were normalized to β -actin. The data are shown as the mean $\pm$ SD of triplicate determinations. * $p < 0.05$

These results demonstrated that 0.5 mM VPA treatment for 24 hours with 48 hours of rest increased the self-renewal capacity of the stem cells.

VPA has a biphasic effect on apoptosis of activated lymphocytes by dose-dependently modulating multiple signaling pathways. At low doses, less than 1.1 mM, VPA promotes early lymphocyte activation and reduces apoptosis, but, at high doses, it attenuates activation and enhances apoptosis [47]. In our study, apoptosis in the 0.5-mM group was lower than in the control and 1-mM group after both the first and second cycles. However, the third VPA cycle had different results, which may reflect over-

Fig. 5 Effects of VPA on cemento/odontoblastic differentiation of PDLSCs. **A** Alizarin red S staining shows that mineral nodules in PDLSCs were more abundant after constant VPA treatment. On the contrary, cyclic VPA treatment caused little accumulation of mineralized nodules in PDLSCs. **B** ALPase activity in PDLSCs after cyclic VPA treatment decreased in a dose-dependent manner. In contrast, constant treatment at higher concentrations increased ALPase activity. **C** Constant VPA treatment enhanced the expression of osteogenic differentiation markers, OCN and ALP. However, cyclic treatment at 0.5 mM VPA inhibited OCN expression. At specific concentration, 0.5 mM VPA had different effects on the expression of osteogenic differentiation markers. The data are shown as the mean $\pm$ SD of triplicate determinations. * $p < 0.05$

confluency in the control and 0.5-mM groups. As the culture progressed, apoptosis increased in the control and 0.5-mM VPA groups according to culture confluency. In contrast, the 1-mM VPA group had decreased apoptosis in the third cycle, with much fewer viable cells than in the other groups. Therefore, we conclude that cyclic treatment with relatively high VPA concentrations or constant treatment (data not shown) negatively affected cell survival, whereas cyclic treatment with 0.5 mM VPA enhanced PDLSC proliferation.

P53 is involved in activating apoptosis through the extrinsic and intrinsic pathways, depending on the cell type

Fig. 6 **A** Hard tissue formation by VPA-treated PDLSCs *in vivo*. Eight weeks after transplantation, PDLSCs and VPA-treated PDLSCs (0.1, 0.5 and 1 mM) differentiated into cement-/osteoblast-like cells that formed hard tissue on the surface of hydroxyapatite tricalcium phosphate carriers. **B** Immunohistochemical staining with anti-human

mitochondria antibodies showed that PDLSCs were positive and generated a hard structure. **C** Osteocalcin and **D** type 1 collagen antibodies positively stained the hard tissue and extracellular matrix. Scale bar 50 μ m. N=3. HA HA/TCP scaffold, H hard tissue

[48]. p53, a transcription factor, trans-activates apoptotic genes by binding at specific DNA regions. p53 regulates target genes, such as Oct4 or Nanog, by repressing mouse embryonic stem cells [49–52]. Also, p53 activated p21 in HSCs to regulate HSC proliferation [53]. We demonstrated that the first cyclic treatment of 0.5 mM VPA downregulated p53 phosphorylation, reducing apoptosis. This result supports that cyclic VPA treatment might control proliferation/apoptosis through p53 in PDLSCs. Activating p53 suppressed proliferation via the beta-catenin-Ras-ERK-p21^{CIP1/WAF1} pathway [19, 54]. In previous studies on cancer, HDI promoted the expression of p21^{CIP1/WAF1}, a cell-cycle inhibitor, which inhibited proliferation to arrest cancer cell growth [55]. Constant VPA treatment also enhanced p21^{CIP1/WAF1} expression, inhibiting PDLSCs proliferation and enhancing apoptosis. In contrast, cyclic VPA treatment, especially at 0.5 mM, did not change p21^{CIP1/WAF1} expression. These results show that p53 regulated the PDLSC proliferation after cyclic treatment with 0.5 mM VPA. Understanding that VPA acts through p53 enables a broader use of VPA to control stem cells.

In MSCs, VPA decreased the multilineage differentiation capability. VPA triggered selective-directional differentiation toward osteogenesis in MSCs [38, 39] while decreasing adipogenic, chondrogenic, and neurogenic differentiation [32, 39]. Several experiments showed similar results that reduced oxygen tension promoted expression of

extracellular matrix components, such as type I collagen or fibronectin, with HDI treatment [56, 57]. On the contrary, one report showed that VPA downregulated osteocalcin in dental pulp stem cells by controlling histone deacetylases [25]. These controversial results showed that HDI control of transcription factors during osteogenic differentiation depended on cell type and HDI concentration. In this study, osteogenic differentiation was inhibited after cyclic VPA treatment. However, constant treatment increased mineral accumulation and alkaline phosphatase activity in a dose-dependent manner. We confirmed that VPA also affects osteogenesis *in vivo*. With high VPA cytotoxicity, too few PDLSCs survived constant treatment for *in vivo* transplantation. Transplanted PDLSCs cyclically treated with VPA produced more hard tissue in a dose-dependent manner. The hard tissue was detected with osteocalcin and collagen type I antibodies. These conflicting results indicate that osteogenic differentiation varies according to the VPA treatment modality, concentration, and experimental scheme (*in vitro*, *in vivo*). Moreover, *in vivo* results reflect the complex body environment in which multiple factors might influence osteogenic differentiation.

Previous studies suggested that the effects of VPA might depend on cell type, concentration, and application mechanism. However, the specific mechanisms of VPA have not been explained, except for the control of epigenetic gene expression and regulation [58, 59]. Our study suggested

that VPA acts as an important modulator controlling dental MSCs proliferation and differentiation. Moreover, the p53-mediated cell cycle with cyclic VPA treatment is also important for controlling dental stem cell proliferation. Our experiments showed that VPA has dual effects on proliferation and differentiation, depending on the mode of treatment and concentration. With the limited understanding of the specific condition to control proliferation, further studies are needed to confirm our results in animal models and different cell types. Our approach to two modes of VPA treatment can be used to promote proliferation and induce differentiation of dental stem cells.

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Compliance with ethical standards

Conflict of interest The authors have no potential conflicts of interest to declare.

Ethical statement This study was approved by the Institutional Review Board at Seoul National University School of Dentistry (IRB No. S-D20080009).

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