

Valproic Acid, a Histone Deacetylase Inhibitor, Decreases Proliferation of and Induces Specific Neurogenic Differentiation of Canine Adipose Tissue-Derived Stem Cells

Yasuhiro KURIHARA¹, Takehito SUZUKI¹, Motoharu SAKAUE¹, Ohoshi MURAYAMA², Yoko MIYAZAKI¹, Atsushi ONUKI¹, Takuma AOKI¹, Miyoko SAITO¹, Yoko FUJII¹, Masaharu HISASUE¹, Kazuaki TANAKA¹ and Tatsuya TAKIZAWA¹*

¹Graduate School of Veterinary Medicine, Azabu University, Fuchinobe, Chuo-ku, Sagamihara 252-5201, Japan

²School of Life and Environmental Science, Azabu University, Fuchinobe, Chuo-ku, Sagamihara 252-5201, Japan

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ABSTRACT. Adipose tissue-derived stem cells (ADSCs) isolated from adult tissue have pluripotent differentiation and self-renewal capability. The tissue source of ADSCs can be obtained in large quantities and with low risks, thus highlighting the advantages of ADSCs in clinical applications. Valproic acid (VPA) is a widely used antiepileptic drug, which has recently been reported to affect ADSC differentiation in mice and rats; however, few studies have been performed on dogs. We aimed to examine the *in vitro* effect of VPA on canine ADSCs. Three days of pretreatment with VPA decreased the proliferation of ADSCs in a dose-dependent manner; VPA concentrations of 4 mM and above inhibited the proliferation of ADSCs. In parallel, VPA increased *p16* and *p21* mRNA expression, suggesting that VPA attenuated the proliferative activity of ADSCs by activating p16 and p21. Furthermore, the effects of VPA on adipogenic, osteogenic or neurogenic differentiation were investigated morphologically. VPA pretreatment markedly promoted neurogenic differentiation, but suppressed the accumulation of lipid droplets and calcium depositions. These modifications of ADSCs by VPA were associated with a particular gene expression profile, viz., an increase in neuronal markers, that is, *NSE*, *TUBB3* and *MAP2*, a decrease in the adipogenic marker, *LPL*, but no changes in osteogenic markers, as estimated by reverse transcription-PCR analysis. These results suggested that VPA is a specific inducer of neurogenic differentiation of canine ADSCs and is a useful tool for studying the interaction between chromatin structure and cell fate determination.

KEY WORDS: adipose tissue-derived stem cell, cell proliferation, histone deacetylase inhibitor, pluripotency, valproic acid.

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Spinal cord injury (SCI) often occurs in dogs, due to motor vehicle accidents or intervertebral disease (IVDD). Most canine patients suffer from sustained incontinence and loss of walking ability, and the prognosis of severe SCI cases is poor. Unfortunately, no effective drug or surgical therapy has been established for severe SCI cases, and there is a need for new therapeutic approaches. One possibility is stem cell transplantation therapy, which is used as a radical cure treatment for refractory SCI [2, 14, 25, 26].

Adipose tissue-derived stem cells (ADSCs) are isolated from the stromal vascular fraction of adipose tissues [8, 24, 39]. ADSCs, similar to pluripotent adult mesenchymal stem cells, can differentiate into mesenchymal lineage cells, such as adipocytes, osteocytes, chondrocytes and myocytes [36, 38]. ADSCs have characteristics similar to those of bone marrow-derived mesenchymal stem cells (BMSCs), including gene expression and differentiation potential [1, 3, 5, 17, 19, 22, 33, 35, 37, 39]. Unlike BMSCs, however, the tissue

source of ADSCs can be obtained in large quantities and with low risks [11]. Therefore, it is reasonable that ADSCs will be the preferred adult stem cells for future clinical applications [24]. Moreover, the dog has been found to be a good animal model of human disease [32], and thus, the study of canine ADSCs is particularly useful. Stem cells derived from bone marrow and from olfactory ensheathing glia (OEG) have been studied for spinal cord regenerative therapy in dogs [6, 14, 16, 25, 26, 31]. However, only a few studies have been performed on the differentiation of canine ADSCs, except for a comparative study showing that BMSCs and ADSCs could be differentiated into neurospheres and neuron-like cells in dogs [2].

Valproic acid (VPA), a widely used antiepileptic and anticonvulsant drug, is an inhibitor of class 1 histone deacetylases (HDACs) [7, 27]. Histone acetylation correlates with gene activation [12, 13], and modification of histone N-terminal tails through acetylation or deacetylation can alter the interaction between histones and DNA, affecting the regulation of gene expression [9, 12, 13, 18, 34]. Therefore, HDAC inhibitors have been a useful tool for studying the association between chromatin modification and cell lineage specification. VPA has been found to promote differentiation of hippocampal neural progenitors into neurons, but inhibit their glial differentiation in adult rats [12].

In the present study, we have investigated the effects of VPA on the proliferation and differentiation of canine ADSCs isolated from subcutaneous adipose tissue in the

*CORRESPONDENCE TO: TAKIZAWA, T., Graduate School of Veterinary Medicine, Azabu University, Fuchinobe, Chuo-ku, Sagamihara 252-5201, Japan.

e-mail: takizawa@azabu-u.ac.jp

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Table 1. Primers used in RT-PCR and real-time PCR

Gene		Primer sequence (5'-3')	Product length (bp)
<i>p16</i>	Forward	CGATCCAGGTCATGATGATGG	145
	Reverse	ACCACCAGCGTGTCAGGAA	
<i>p21</i>	Forward	CATCCCTCATGGCAGCAAG	208
	Reverse	AGGCAGGGAGACCTTGGACA	
<i>PPARγ2</i>	Forward	ACACGATGCTGGCGTCTTGATG	119
	Reverse	TGGCTCCATGAAGTACCAAAAGG	
<i>FABP4</i>	Forward	ATCAGTGTAACGGGGATGTG	117
	Reverse	GACTTTTCTGTCATCCGCAGTA	
<i>LPL</i>	Forward	ACACATTCACAAGAGGGTCACC	134
	Reverse	CTCTGCAATCACACGGATGGC	
<i>BMP2</i>	Forward	CACTAACCACGCCATTGTTC	163
	Reverse	ACAACCCTCCACAACCATGTC	
<i>Dlx5</i>	Forward	TGCTCTCCTACCTCGGCTTC	224
	Reverse	TTGCCATTACCATCCTCAC	
<i>COL1A1</i>	Forward	GTAGACACCACCCTCAAGAGC	119
	Reverse	TTCCAGTCGGAGTGGCACATC	
<i>NSE</i>	Forward	GACCAACCCAAAGCGTATTGA	180
	Reverse	GCAATGAACGTGTCCTCAGTC	
<i>TUBB3</i>	Forward	AGCCAAGTTCTGGGAAGTCA	238
	Reverse	CCCCTCTGACCAAAGATGAA	
<i>MAP2</i>	Forward	AGAGGAGGTGTCTGCAAGGA	161
	Reverse	GTGATGGAGGTGGAGAAGGA	
<i>NEFH</i>	Forward	CTCAAAGGCACCAAGGACTC	244
	Reverse	CAAAGCCAATCCGACATTCT	
<i>GFAP</i>	Forward	AGATCCACGATGAGGAGGTG	104
	Reverse	TCTTAGGGCTGCTGTGAGGT	
<i>GAPDH</i>	Forward	GCTGAACGGGAAGCTCACTG	221
	Reverse	CGTCGAAGGTGGAAGAGTGG	

inguinal region.

MATERIALS AND METHODS

Animals: Subcutaneous adipose tissue was obtained from the inguinal region of 8 healthy laboratory beagles (age, 1–2 years) (Kitayama Labes, Ina, Japan). All animals were anesthetized with propofol, before tissue samples were obtained. After intubation, anesthesia was maintained with isoflurane (2.0%) in oxygen. At the end of each experiment, the animals were euthanized by additional doses of anesthesia (pentobarbital, 100 mg/kg). The protocol of this study was approved by the Committee for Animal Experimentation at Azabu University.

Isolation and culture of canine ADSCs: Adipose tissue samples were processed for ADSC isolation as described previously [23, 24] with a slight modification. Briefly, the adipose tissue removed was extensively washed with sterile phosphate-buffered saline (PBS) containing penicillin (100 U/ml) and streptomycin (0.25 μ g/ml) in order to remove

contaminating blood cells and local anesthetics. The tissue was minced into small pieces and then incubated in a solution containing 0.05% collagenase type IA (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37°C for 1 hr with vigorous shaking. The top lipid layer was removed, and the remaining liquid portion was centrifuged at 200 \times g for 10 min. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% newborn bovine serum (NBS, Invitrogen, Carlsbad, CA, U.S.A.) and spread in 100-mm collagen type I-coated dishes (Iwaki, Tokyo, Japan) at a density of 1 \times 10⁶ cells per dish. Cells were maintained in growth medium (DMEM supplemented with 10% NBS, penicillin [100 U/ml] and streptomycin [0.25 μ g/ml]) at 37°C and 5% CO₂. After 24 hr, the unattached cells were removed by washing with PBS. Canine ADSCs from passages 1–3 were used, and no difference was observed in ADSCs between these passages.

Measurement of proliferation potential: The effects of VPA or valpromide (VPM; Wako Pure Chemical Ind., Osaka, Japan), an analogue of VPA with no HDAC inhibi-

tory activity, on ADSC proliferation were measured using a 3-(4, 5-dimethyl-thiazol-2-yl)-2, 3-diphenyltetrazolium bromide (MTT) assay kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Briefly, canine ADSCs were plated in 96-well plates at a density of 1×10^4 per well and cultured in growth medium for 48 hr. VPA or VPM (0–8 mM) was then added to the medium, and cultures were incubated for 3 days. Subsequently, 10 μ l of MTT stock solution was added, and the plates were further incubated for 4 hr at 37°C. Diluted HCl (100 μ l) was then added to solubilize the formazan crystals, and the absorbance of each well at 570 nm was measured with a microplate reader LS-PLATE manager 2004 (Wako Pure Chemical Ind.); the average of measurements of 6 wells per sample has been presented.

In vitro differentiation assay: *In vitro* assay of cell differentiation into adipogenic, osteogenic and neurogenic lineages was performed as described previously [4, 29, 39] with a slight modification. Briefly, ADSCs were seeded into 35-mm dishes at a density of 1×10^5 cells per dish. The cells were incubated on glass coverslips in growth medium containing 4 mM VPA for 3 days and then transferred to adipogenic induction medium (DMEM supplemented with 10% FBS, 1 μ M dexamethasone, 10 μ M insulin and 0.5 mM isobutylmethylxanthine) or to osteogenic induction medium (DMEM supplemented with 10% FBS, 0.1 μ M dexamethasone, 50 μ M L-ascorbate-2-phosphate and 10 mM glycerophosphate) for 14 days [39] and then to neurogenic induction medium (DMEM supplemented with 100 μ M dibutylryl cyclic adenosine monophosphate and 125 μ M isobutylmethylxanthine) for 2 hr [23, 24]. Intracellular lipid accumulation, as an indicator of adipogenic differentiation, was visualized by oil red O staining. Osteogenic differentiation was confirmed by positive staining with alizarin red S, a specific marker for calcium deposition. Neurogenic differentiation was assessed by immunofluorescence staining for β III-tubulin or neuron-specific enolase (NSE). Reagents for this induction medium were purchased from Wako Pure Chemical Ind.

Immunofluorescence staining: Immunocytochemical analyses of HDAC1, acetylation of histone H3 (ace H3), β III-tubulin and NSE were performed. ADSCs were incubated in growth medium for 3 days as described above. Some cultures were processed for neurogenic induction. At the end of incubation, the cells were fixed in PBS containing 3.7% formaldehyde for 15 min at 4°C. After PBS washes, the cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature. The cells were then incubated with an anti-HDAC1 antibody (sc-7872, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), an anti-histone H3 (acetyl K9) antibody (Novus Biologicals, Littleton, CO, U.S.A.), an anti- β III-tubulin antibody (ab78078, Abcam, Cambridge, U.K.) or an anti-NSE antibody (PA1-46203, Thermo Scientific, Billerica, MA, U.S.A.) for 1 hr at room temperature. After further PBS washes, cells were incubated with secondary antibody (Cy3-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG, Jackson ImmunoResearch, West Grove, PA, U.S.A.) for 30 min at room temperature. The cells were then washed with PBS and counterstained

with 4', 6-diamidino-2-phenyl-indole (DAPI) for nuclear staining before fluorescence microscopic observation.

Reverse transcription-PCR and real-time PCR: Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan) and reverse-transcribed to single-strand cDNA using oligo-dT primer and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed using *Taq* DNA polymerase (KAPA Biosystems, Woburn, MA, U.S.A.) and using specific primers, and each cycle consisted of the following steps: denaturation for 10 sec at 98°C, annealing for 30 sec at 53–65°C and a 30-sec elongation at 72°C (Table 1). Reaction products were electrophoresed on a 2.0% agarose gel and visualized with ethidium bromide. Real-time PCR of the mRNAs for *p16*, *p21* and *GAPDH* was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's instructions. Analysis of the results was carried out using ABI PRISM 7500 Dissociation Curve Software v 1.0 (Applied Biosystems Japan). The relative amount of mRNA was normalized to that of *GAPDH*.

Statistical analysis: Results are expressed as the mean \pm standard error. Multiple comparisons were done with the Turkey–Kramer test after one-way analysis of variance. A *p*-value < 0.05 was considered statistically significant.

RESULTS

VPA induces acetylation of histone H3 and decreased cell proliferation: To confirm the effect of VPA on HDAC1 and acetylation of histone H3, we examined the expression of HDAC1 and acetylation of histone H3 by immunofluorescence staining. Minimal HDAC1 and acetylation of histone H3 were observed in the control ADSCs. VPA flattened the morphology of ADSCs and increased the expression of HDAC1 and the acetylation of histone H3 in ADSCs after 3 days of treatment (Fig. 1A and 1B). In contrast, VPM did not cause any morphological changes or significant changes in HDAC1 and histone H3 staining in ADSCs.

Moreover, VPA treatment significantly decreased the proliferation of ADSCs in a dose-dependent manner: about 20% (2 mM VPA), 40% (4 mM VPA) and 80% (8 mM VPA) of the control group (Fig. 1C). However, VPM treatment did not substantially affect ADSC proliferation. No dead cells were observed in any VPA-treated groups by phase-contrast microscopy.

VPA induces upregulation of cyclin-dependent kinase inhibitors: To assess the effect of VPA on the expression of cyclin-dependent kinase (CDK) inhibitors, we examined the expression of these genes by real time-PCR. The expression levels of *p16* mRNA significantly increased in the ADSCs treated with 4 mM VPA (4.6 fold vs. control); however, *p16* mRNA expression levels did not change in the cells treated with 8 mM VPA (2.1 fold vs. control; Fig. 2A). *p21* mRNA expression levels significantly increased in the cells treated with 8 mM VPA (Fig. 2B). The expression levels of *p21* mRNA were approximately 2.6 fold (4 mM VPA) and 3.0 fold (8 mM VPA) of that of the control group.

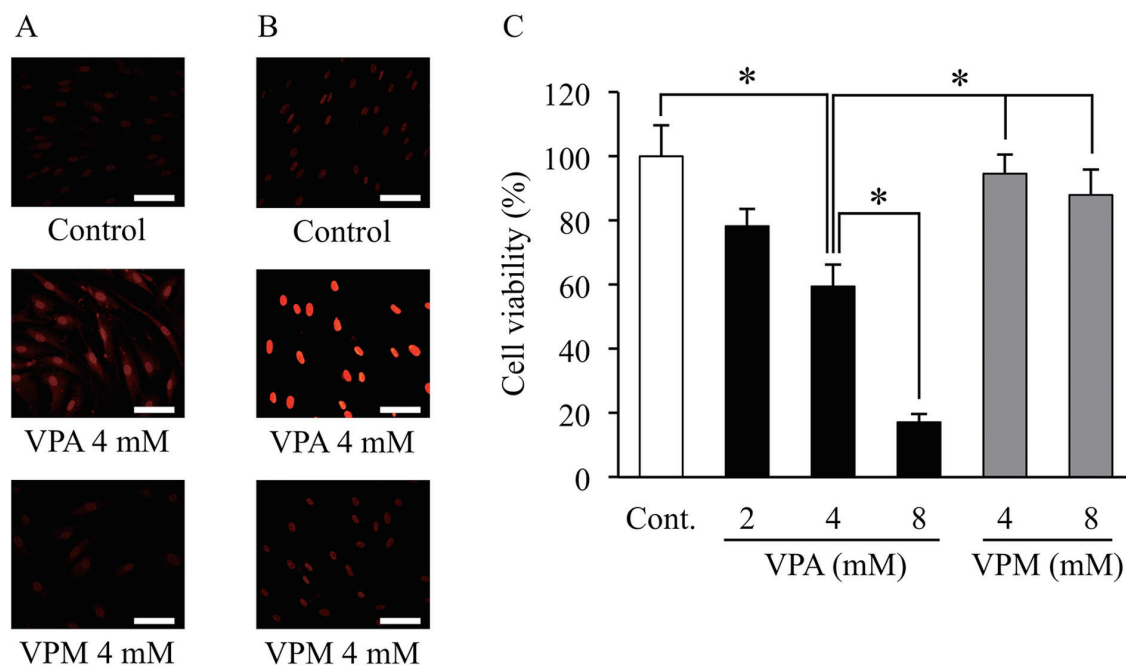


Fig. 1. Effects of valproic acid on histone deacetylase 1 expression, histone H3 acetylation and cell proliferation. Canine adipose tissue-derived stem cells (ADSCs) were treated with valproic acid (VPA) or valpromide (VPM). (A) ADSCs were then processed for immunofluorescence staining with an anti-histone deacetylase 1 (HDAC1) or an anti-histone H3 (acetyl K9) antibody and a secondary antibody (Cy3-conjugated goat anti-rabbit IgG). Red fluorescence indicates positive staining for HDAC1 (A) and acetylation of histone H3 (B). Scale bar, 50 μ m. (C) Cell proliferation was measured by MTT assay and expressed as percentage of the negative control (DMSO). Data represent the means \pm S.E. (% of control) of 4 independent experiments; each measurement was the average for 6 wells. * P <0.05, significant difference among the indicated groups.

VPA suppresses adipogenic and osteogenic differentiation: To assess the effect of VPA on the pluripotency of ADSCs, we investigated whether VPA treatment alters the differentiation of ADSCs into adipogenic and osteogenic lineages using an *in vitro* differentiation assay. Oil red O staining revealed that ADSCs that differentiated into the adipogenic lineage accumulated lipid droplets in the cytosol, as compared to undifferentiated cells, which did not accumulate lipid droplets (Fig. 3A). VPA pretreatment followed by adipogenic induction significantly suppressed the accumulation of lipid droplets. RT-PCR analysis showed that the mRNA expression levels of adipogenic markers, peroxisome proliferator-activated receptor γ 2 (*PPAR* γ 2), fatty acid binding protein 4 (*FABP4*) and lipoprotein lipase (*LPL*) were elevated by adipogenic induction (Fig. 3B). On the other hand, VPA pretreatment followed by adipogenic induction significantly reduced the *LPL* mRNA expression level, in parallel with the decreased accumulation of lipid droplets. Alizarin red S staining revealed that ADSCs differentiated into osteogenic lineage cells with accumulated calcium deposition, as compared with the undifferentiated cells, which demonstrated no calcium deposition (Fig. 4). VPA pretreatment followed by osteogenic induction significantly reduced calcium deposition (Fig. 4A). mRNA expression levels of osteogenic markers, viz., bone morphogenetic protein 2 (*BMP2*) and distal-less homeobox 5 (*DLX5*), were also elevated by osteogenic induction, but were not significantly

affected by VPA pretreatment (Fig. 4B).

VPA promotes neurogenic differentiation: We further examined the effect of VPA on the neurogenic lineage induction of ADSCs. β III-tubulin immunofluorescence staining revealed that ADSCs that differentiated into neurogenic cells had typical neuron-like cell protrusions and higher β III-tubulin levels than undifferentiated cells. β III-tubulin-positive cells also stained for NSE. VPA pretreatment followed by neurogenic induction significantly enhanced the level of β III-tubulin-positive cells with approximately 90% of ADSCs showing β III-tubulin expression (Fig. 5A).

mRNA levels of neurogenic markers, viz., *NSE*, *TUBB3* and microtubule-associated protein 2 (*MAP2*), were also elevated by neurogenic induction, but mRNA expression of the glial cell marker, *GFAP*, was not observed in any groups (Fig. 5B). Pretreatment with VPA followed by neurogenic induction increased the expression of *NSE*, *TUBB3* and *MAP2* and of neurofilament heavy polypeptide (*NEFH*), as compared to that in the neurogenic induction group. VPA pretreatment increased the number of β III-tubulin-positive cells, even without neurogenic induction. Furthermore, VPA elevated the mRNA expression levels of neurogenic markers in ADSCs with and without neurogenic induction.

DISCUSSION

Here, we demonstrated that VPA flattened the morphol-

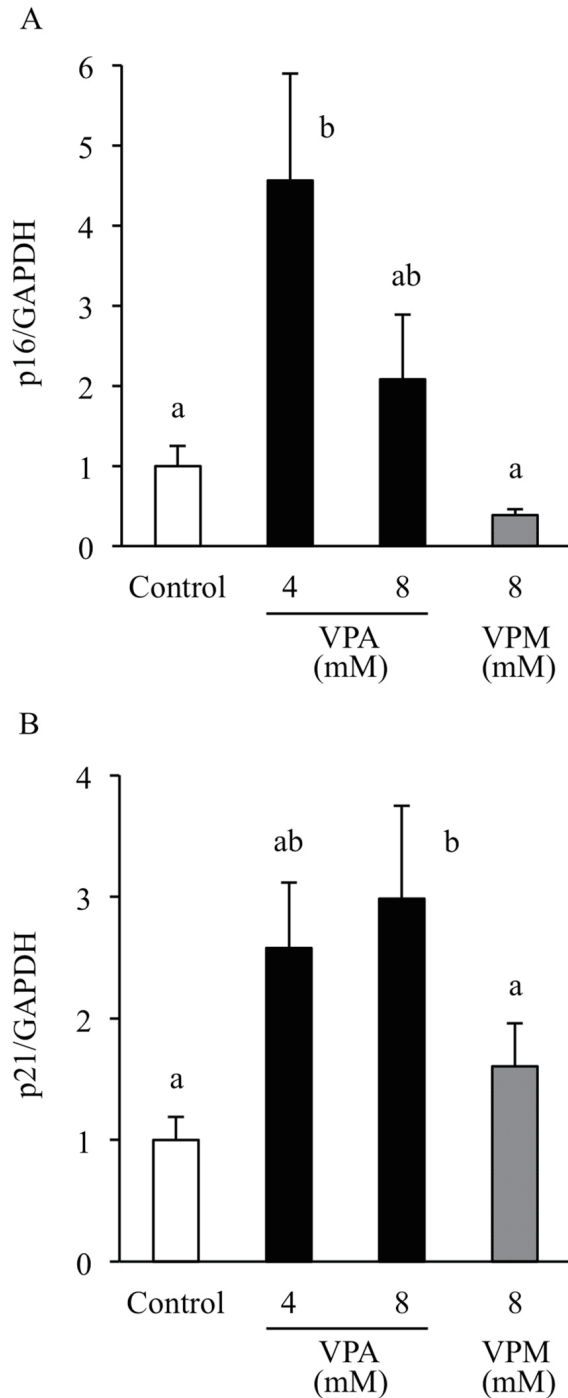


Fig. 2. Effects of valproic acid on cyclin-dependent kinase inhibitor expression. Adipose tissue-derived stem cells (ADSCs) were treated with valproic acid (VPA) or valpromide (VPM). Total RNA was extracted from ADSCs after 3 days of treatment with VPA (4 or 8 mM) or VPM (8 mM). The relative expression of the cyclin-dependent kinase (CDK) inhibitors *p16*(A) and *p21*(B) was quantified by real time-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard. Data are the means \pm S.E. of 4–7 independent experiments. a, b: bars with different letters at the top differ significantly; a vs. b, $P < 0.05$.

ogy of canine ADSCs and markedly induced their expression of HDAC1 and acetylation of histone H3. In contrast, VPM, an analogue of VPA with no HDAC inhibitory activity, did not cause any morphological changes and had no significant effects on HDAC1 and histone H3, indicating that the H3 acetylation was increased by VPA. These observations support the findings of Lee *et al.* [20]. Thus, our results clearly indicated that VPA induced H3 acetylation by reducing HDAC1 activity in canine ADSCs.

VPA, but not VPM, induced a significant and dose-dependent decrease in the proliferation of ADSCs, suggesting that VPA suppresses ADSC proliferation through acetylation of histone H3. *p21* and *p16*, well-known CDK inhibitors, regulate cell cycle arrest. VPA induces expression of these CDK inhibitors in human ADSCs and mesenchymal stem cells [20, 30]. We found that VPA significantly induced mRNA expression levels of *p16* at 4 mM and of *p21* at 8 mM without inducing cell death. *p21* is also a well-known HDAC-inhibitor responsive gene that is upregulated by hyperacetylation of histones H3 and H4 [10, 20, 28]. In addition, using immunofluorescence, we showed that H3 acetylation was markedly increased by VPA and that *p21* mRNA was significantly increased by 8 mM VPA; thus, cell viability was further reduced by 8 mM VPA treatment, again supporting the findings of Lee *et al.* [20] who reported that VPA causes cell cycle arrest through increased *p21* expression in the absence of *p16* mRNA expression in human ADSCs. Therefore, the inhibitory effect of VPA on proliferation of canine ADSCs was due to cell cycle arrest, although the underlying mechanism needs to be further examined.

Furthermore, VPA promoted differentiation of approximately 90% of ADSCs into a neuronal cell lineage after 3 days of treatment. The differentiated cells have neuron-like morphology and significantly expressed β III-tubulin protein. Pretreatment with VPA followed by neurogenic induction also promoted mRNA expression of the neuronal markers *NSE*, *TUBB3*, *MAP2* and *NEFH* as compared to the pretreatment without VPA, suggesting that promotional effects of VPA on neuronal differentiation of ADSCs were induced by upregulation of these genes. Previous reports have shown that VPA promotes differentiation of neural stem cells into neurons in adult rats [12, 15]. To our knowledge, the present study is the first report that VPA promotes neuronal differentiation of ADSCs.

Interestingly, VPA pretreatment in the absence of neurogenic induction caused moderate differentiation into neuron-like cells and also increased mRNA expression levels of neurogenic markers in ADSCs. Thus, our data indicated that VPA could induce neurogenic differentiation in the absence of neurogenic induction. We also demonstrated that VPA increased the acetylation of histone H3 that has been correlated with gene activation [12, 13]; thus, VPA caused gene expression in part through H3 acetylation. In addition, we showed that the neuron-like differentiated cells all stained positively for β III-tubulin. Using NG108-15 cells, Liu *et al.* [21] recently reported that neuronal differentiation could modulate gene transcription, translation and post-translational modulation of Ca^{2+} channels to change the Ca^{2+} ion

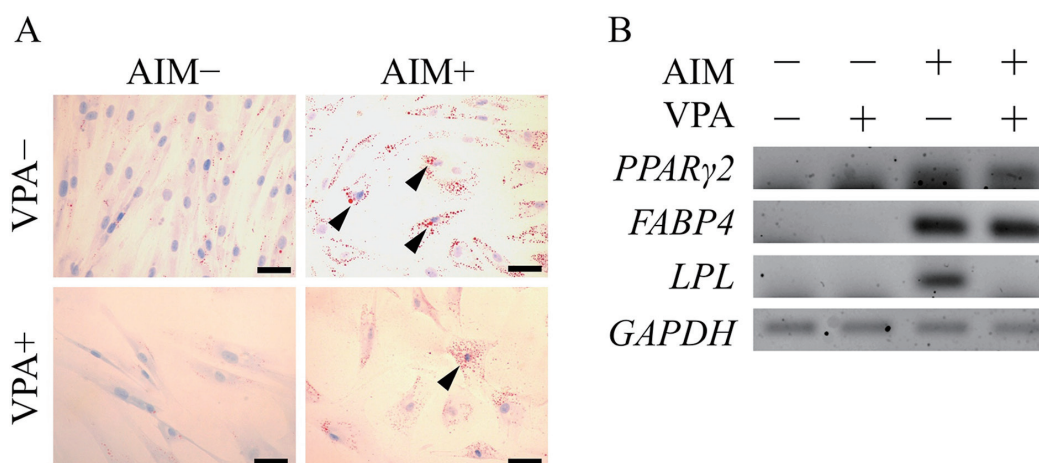


Fig. 3. Valproic acid suppresses accumulation of lipid droplets. Adipose tissue-derived stem cells (ADSCs) were pretreated with valproic acid (VPA) for 3 days followed by adipogenic induction for 14 days. (A) Adipogenic differentiation was visualized by oil red O staining after 14 days of induction with adipogenic medium. Arrowheads show cells that accumulated lipid droplets. Scale bar, 50 μ m. (B) RT-PCR analysis of adipogenic markers, *PPARγ2*, *FABP4* and *LPL*, was performed using total RNA extracted from ADSCs after 14 days of adipogenic induction. AIM, adipogenic induction medium.

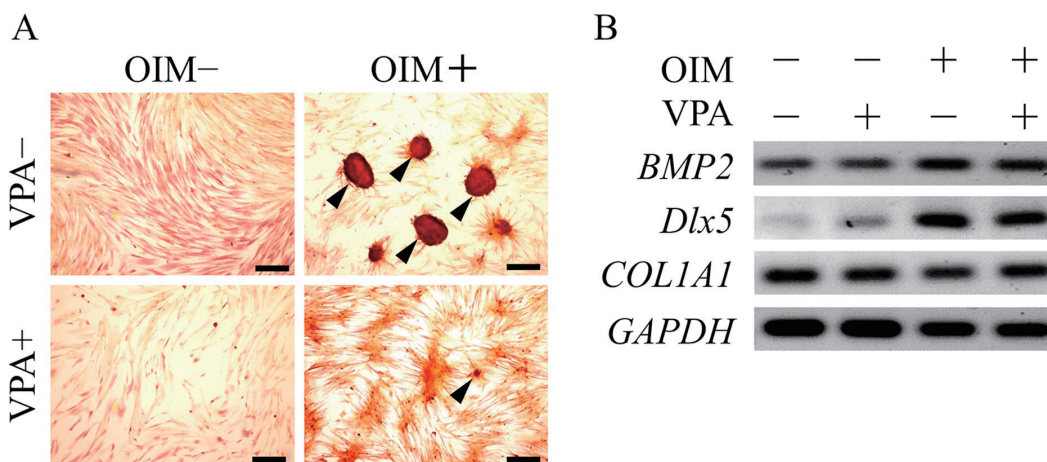


Fig. 4. Valproic acid suppresses calcium deposition. Adipose tissue-derived stem cells (ADSCs) were pretreated with valproic acid (VPA) for 3 days followed by osteogenic induction for 14 days. (A) Osteogenic differentiation was evaluated by alizarin red S staining after 14 days of induction with osteogenic medium. Arrowheads show cells that accumulated calcium in the cytosol. Scale bar, 200 μ m. (B) RT-PCR analysis of osteogenic markers, *BMP2*, *Dlx5* and *COL1A1*, was performed using total RNA extracted from ADSCs after 14 days of osteogenic induction. OIM, osteogenic induction medium.

currents; our results suggested that neuronal differentiation could modulate *TUBB3* transcription and translation. In the present study, VPA promoted ADSCs differentiation into neuronal cells in the absence of neurogenic induction factors and induced acetylation of histone H3, indicating that VPA is a useful tool for studying the interaction between chromatin structure and cell fate determination. Further studies are needed to examine the molecular mechanism underlying the neurogenic differentiation induced by VPA using canine

ADSCs.

In contrast, VPA suppressed the late stage of differentiation into adipogenic and osteogenic lineage cells. Two of the 3 adipogenic marker genes examined showed no reduction after VPA pretreatment; however, lipid accumulation appeared to be suppressed, suggesting that the VPA inhibits accumulation of lipid droplets rather than an inhibiting the whole adipogenic differentiation process.

Similarly, osteogenic marker genes showed no changes

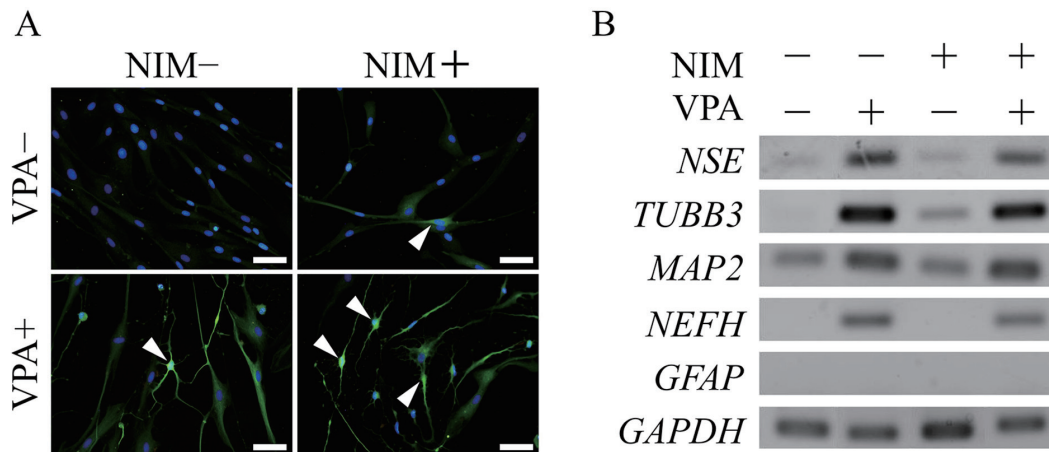


Fig. 5. Valproic acid promotes neurogenic differentiation. Adipose tissue-derived stem cells (ADSCs) were pretreated with valproic acid (VPA) for 3 days followed by neurogenic induction. (A) Neurogenic differentiation was assessed by immunofluorescence staining using an anti- β III-tubulin antibody and a secondary antibody (FITC-conjugated goat anti-mouse IgG) after 2 hr of induction with neurogenic medium. Arrowheads show cells that expressed β III-tubulin. Scale bar, 50 μ m. (B) RT-PCR analysis of the neurogenic markers, *NSE*, *TUBB3*, *MAP2* and *NEFH* and the glial marker, *GFAP*, was performed using total RNA extracted from ADSCs after 2 hr of neurogenic induction. NIM, neurogenic induction medium.

after VPA treatment, but accumulation of calcium deposition by osteogenic induction was significantly reduced, suggesting that VPA inhibits calcium deposition rather than inhibiting the osteogenic differentiation process as a whole. The mechanism underlying the differential effects of VPA on the pluripotent capacity of ADSCs remains unclear. A previous report has shown that VPA decreases adipogenic and neurogenic differentiation, but increases osteogenic differentiation in human ADSCs [20]. The reason for VPA acting as a stimulator for differentiation of canine ADSCs and as a suppressor for that of human ADSCs is not immediately clear. Therefore, chromatin structure and cell fate determination need to be further examined in relation to the pluripotency of ADSCs, including this difference between humans and dogs.

In conclusion, pretreatment with VPA dose-dependently decreased proliferation of canine ADSCs. In parallel with its inhibitory effects, VPA increased *p16* and *p21* mRNA expression, implying induction of cell cycle arrest through activation of p16 and p21. In addition, pretreatment with VPA followed by adipogenic, osteogenic or neurogenic induction markedly promoted *in vitro* neurogenic differentiation, but suppressed accumulation of lipid droplets and calcium deposition. These *in vitro* modifications of ADSCs by pretreatment with VPA were associated with changes in expression of relevant markers. These results suggested that VPA is a specific inducer of neurogenic differentiation of canine ADSCs and is a useful tool for studying the interaction between chromatin structure and cell fate determination.

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