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Induction of differentiation and mineralization in rat tooth germ cells on PVA through inhibition of ERK1/2

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ABSTRACT

Poly(vinyl alcohol) (PVA) has been widely used in the field of biomedical applications because of its hydrophilic properties for desired functions. Nonetheless, the role of PVA in tooth germ (TG) cell differentiation and mineralization has seldom been explored. To test the capacity of PVA in regulating TG cell differentiation and mineralization, TG cells obtained from 4-day-old Wistar rats were cultured on the PVA substrate. It was found that PVA was able to promote TG cell exhibiting high levels of alkaline phosphatase (ALP) activity, mineralization, and mRNA expression of osteocalcin (OCN), osteopontin (OPN), dentin matrix protein 1 (DMP1) and enamelin. Even when the additives routinely administrated in the differentiation medium such as dexamethasone, β-glycerophosphate and ascorbic acid were removed from the culture system, PVA itself still stimulated TG cells with the differentiation and mineralization ability. By showing the direct suppression of extracellular signaling-regulated kinase1/2 (ERK1/2) of TG cells treated with U0126, known to suppress the activation of ERK1/2, and significant synergistic effects between PVA and U0126, we demonstrated the suppression of ERK1/2 pathway is one of the effects of PVA-promoted TG cell differentiation and mineralization. Taken together, this study demonstrated a novel role of PVA in promoting the differentiation and mineralization of TG cells through ERK1/2 acting as a negative regulator.

1. Introduction

The tooth germ (TG), sometimes called the tooth bud, contains a heterogeneous population of cells including ameloblasts, odontoblasts and dental pulp cells. During tooth development, TG cell differentiation and mineralization proceed synchronously eventually forming a tooth [1]. Thus, similar to other osteoblast-like cells [2–4], TG cells also can serve as a model to elucidate the cell differentiation and mineralization in vitro and to determine the effects of growth factors, cytokines, and mechanical stimulus implicated in cell differentiation and mineralization.

We recently reported that the adhesion and proliferation of TG cells were sensitive to changes in surface hydrophilic properties of biomaterial [5]. Especially, the very hydrophilic biomaterial poly(vinyl alcohol) (PVA) could maintain TG cells with a three-dimensional spherical structure, resembling in vivo physiological condition. It is well known that cell behavior on biomaterial is crucial to many biomedical applications, yet the molecular pathways responsible for converting PVA signals into TG cell responses are still being elucidated. Therefore, the purpose of the study was to investigate the effect of PVA on TG cell differentiation and mineralization, and the possible signaling pathway involved in regulating TG cell change in response to PVA biomaterial.

The mitogen-activated protein kinase (MAPK) signaling pathway is tightly related to the regulation of cell proliferation, differentiation, motility and death [6,7]. Three central elements of the MAPK family have been identified in mammalian cells, referred to as extracellular signal-regulated kinase1/2 (ERK1/2), p38 kinase, and c-Jun-N terminal kinase (JNK) [8–10]. This study made use of Western blot analysis to examine the role of individual MAPK pathway in enhancing alkaline phosphatase (ALP) activity and mineralization of TG cells by blocking the pathway using specific inhibitor. To our knowledge, TG cells were not employed to examine how MAPK signaling pathways can be triggered by PVA. Our findings show PVA is an effective substrate that promotes TG cell differentiation and mineralization and the ERK1/2 dependent

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pathway plays an important role in negatively mediating function of TG cells.

2. Materials and methods

2.1. Preparation of PVA substrate

A 5 wt% solution of PVA (Chemika Fluka, MW = 72,000 g/mol, Switzerland) was prepared by dissolving PVA in distilled water at 95 °C. For preparing PVA-coated wells, 140 μl of PVA solution was added into 24-welled tissue culture polystyrene (TCPS) plates (Costar, USA). The solution was then allowed to dry at 60 °C for 24 h to form a thin membrane. Before cell culture, the PVA-coated wells were sterilized in 70% alcohol overnight and rinse extensively with phosphate buffered saline (PBS). As controls, uncoated TCPS wells were treated by the same way as PVA-coated wells.

2.2. Cell culture

The animal study was performed according to a protocol approved by the Review Committee of College of Medicine, National Taiwan University. The method for isolating TG cells from rat mandibular molar TG was described previously [5]. In brief, rat mandibular molar TG was removed from 4-day-old Wistar rats using the explant outgrowth technique without collagenase treatment. Ten first molar TGs in total were isolated from five rats from both sides of lower jaws of each rat. The TGs were placed in PBS and then were cut into small fragments about 1 mm^3 in size, in which the TG cells were released. Subsequently, the excised fragments of TG and released cells were placed into a 15 ml centrifuge tube and centrifuged at 900 rpm for 5 min. After removal of the upper layer solution, cells with tissue fragments were mixed with 10 ml Dulbecco' s modified Eagle medium (DMEM, Chemicon, USA) supplemented with 10% fetal calf serum (Gibco-RBL Life Technologies, UK), antibiotic/antimycotic (penicillin G sodium 100 U/ml, streptomycin 100 g/ml, amphotericin B 0.25 g/ml, Gibco-BRL Life Technologies, UK) placed in a 100-mm cell culture dish (Costar, USA) and then cultured at 37 °C with 5% CO_2 atmosphere in a humidified incubator. TG cells released from the tissue fragments were grown to confluence in approximately 6–8 days. At approximately 90% confluence, tissue fragments were removed and used for another culture to release more TG cells, and subcultured in 100-mm cell culture dishes (Costar, USA) in fresh culture medium for another two weeks. The total number of cells obtained from each primary culture increased to approximately 1×10^8 cells after 30 d in culture. In this work, TG cells used for the subsequent analysis were in the third passage and the medium was changed every 3 or 4 days.

2.3. ALP activity and mineralization assays

TG cells were cultured on PVA and TCPS at a density of 1×10^5 cells/well for 1, 4 and 7 days in the above medium (regarded as the regular medium) and differentiation medium. The differentiation medium was supplemented with 100 nM dexamethasone (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA) and 50 μg/ml ascorbic acid (Sigma, USA). ALP activity was assayed using *p*-nitrophenylphosphate as a substrate following the method described previously [11]. The amount of *p*-nitrophenol produced was measured spectrophotometrically at 410 nm. The degree of mineralization was measured by staining with Alizarin Red S (ARS, Sigma, USA) as described by Ratisoontorn et al. [12]. Data were expressed as units of ARS released (1 unit = 1 unit of optical density at 562 nm).

2.4. MAPK inhibition studies

The role of individual MAPK pathway in ALP activity and mineralization of TG cells was assessed by blocking the pathways using specific inhibitor. The ERK1/2 pathway was blocked with U0126 (Cell signaling, USA), which inhibits MEK1/2, an upstream molecule of the ERK phosphorylation cascade [13]. The p38 MAPK was specifically inhibited with SB203580 (Sigma, USA) [14] and JNK was inhibited with SP600125 (Sigma, USA) [15]. For assessing ALP activity, mineralization and gene expression of mineralization- and differentiation-related markers, TG cells were treated with or without inhibitor for 7 days. For Western blot analysis, TG cells were cultured in the differentiation medium for 4 h followed by treatment with inhibitor for 30 min.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from TG cells by using Trizol (Invitrogen Life Technologies, CA). The RNA (1 μg) was reverse transcribed into first-strand cDNA using the iScript cDNA Synthesis kit (BIO-RAD, CA) for RT-PCR. The oligonucleotide RT-PCR primers for glyceraldehydes-3-phosphate (GAPDH), osteocalcin (OCN) [16], osteopontin (OPN) [17], dentin matrix protein 1 (DMP1) and enamelin are listed in Table 1. The PCR amplification was performed as follows: 32 cycles of denaturation at 94 °C for 45 s, annealing for 45 s, and extension at 72 °C for 30 s. The amplified products were analyzed by electrophoresis through 1.5% agarose gel containing 10 μg/ml ethidium bromide, electrophoresed at 100 mV, and visualized on a UV transilluminator (Alpha Innotech, CA). All bands were scanned and analyzed using AlphaEase FC 4.0 software.

Table 1
Oligonucleotide primer sequences utilized in the RT-PCR.

Target cDNA		Primer sequence (5′–3′)	T_{hyb} (°C)	Product size (bp)	NCBI no. or Ref.
GAPDH	F	ATGGGAAGCTGGTCATCAAC	51.8	375	NM017008
	R	CCACAGTCTTCTGAGTGGCA			
OCN	F	ATGAGGACCCTCTCTCTFCTC	56.3	293	[16]
	R	GTGGTGCCATAGATGCGCTTG			
OPN	F	TCCAAGGAGTATAAGCAGCGGGCCA	58	200	[17]
	R	CTCTTAGGGTCTAGGACTAGCTTCT			
DMP 1	F	CTGGTATCAGGTCGGAAGAATC	55	499	NM206493
	R	CTCTCATTAGACTCGCTGTCAC			
Enamelin	F	CACCGTACCTTAGAGGCAATAC	54.8	463	NM000106
	R	GAGGTCCATGAAGGAAAGAGAG			

2.6. Western blot analysis

Cells were collected by gentle shaking of the wells and washed twice with PBS. Cell lysates were prepared with ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail II; Calbiochem, Germany) for 30 min and then were sonicated at 4 °C for 15 s. Lysates were clarified by centrifugation at 14,000 rpm for 30 min at 4 °C and the resulting supernatant was saved for protein analysis and Western blot analysis.

Protein concentration was measured by using the commercial protein assay reagent (Bio-Rad, CA). For Western blotting, the supernatant was added to an equal volume of Laemmli sample buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% *b*-mercaptoethanol) and heated to 95 °C for 10 min. Proteins (35 μg total protein per lane) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST buffer (Bio-Rad, CA), probed with primary rabbit antibodies against ERK1/2 and phospho-ERK1/2 (Cell Signaling, USA) at a dilution of 1:1000, and were incubated at 4 °C overnight. After washing, the blots were incubated with anti-rabbit IgG-HRP conjugated secondary antibodies (Cell Signaling, USA) at a dilution of 1:5000 for 2–3 h. Finally, the proteins on the membranes were detected using the ECL Plus chemiluminescence system. Densitometric quantification of Western blots was done using AlphaEase FC 4.0 software.

2.7. Statistical analysis

Results are presented as the mean ± standard deviation (SD) of 3–5 independent cultures. Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by post hoc procedure (Bonferoni analysis) ($p < 0.005$ was considered significant).

3. Results

In the present study, the very hydrophilic PVA substrate with the air–water contact angle of 54.0 ± 2.0° was used to maintain TG cells with a three-dimensional spherical structure [5]. In addition, monolayered TG cells on commercial TCPS with the air–water contact angle of 62.7 ± 2.4° [5] was compared.

3.1. ALP activity and mineralization of TG cells on PVA and TCPS

To determine the effect of PVA on TG cell differentiation, ALP activity, an early marker of odontoblasts differentiation [18,19], and ARS assay, a traditional approach for evaluating the calcium deposition, were measured. In the culture system, cells were confluent on TCPS due to the high seeding density and cells formed aggregates suspending above PVA as reported previously [5] (data not shown). Fig. 1 shows both TCPS and PVA expressed increasing levels of ALP activity and ARS assay through 7 days of culture in the differentiation medium. Nonetheless, TG cells grown on PVA showed greater expression and significantly higher than those on TCPS at every time point, regardless of ALP activity and ARS assay ($p < 0.005$).

Generally, culture medium was routinely changed and added with dexamethasone, β-glycerophosphate and ascorbic acid, which had been reported to be beneficial to differentiation of bone-like cells [20]. Therefore, to further explore whether PVA per se promoted the differentiation and mineralization of TG cells, regular medium without dexamethasone, β-glycerophosphate and ascorbic acid was

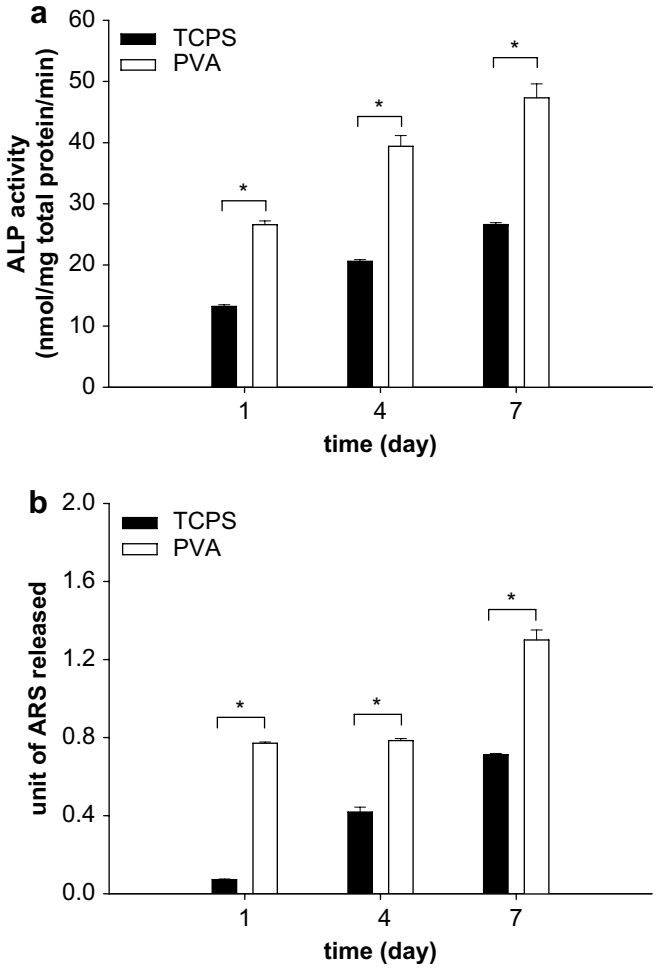

Fig. 1. (a) ALP activity and (b) mineralization (ARS assay) in TG cells cultured on TCPS (closed) and PVA (open) in the differentiation medium after 1, 4 and 7 days of culture. Results represent the mean ± SD from 2–5 independent cultures and determinations. Asterisk denotes significant difference ($p < 0.005$) compared with TCPS, determined by post hoc procedure (Bonferoni analysis).

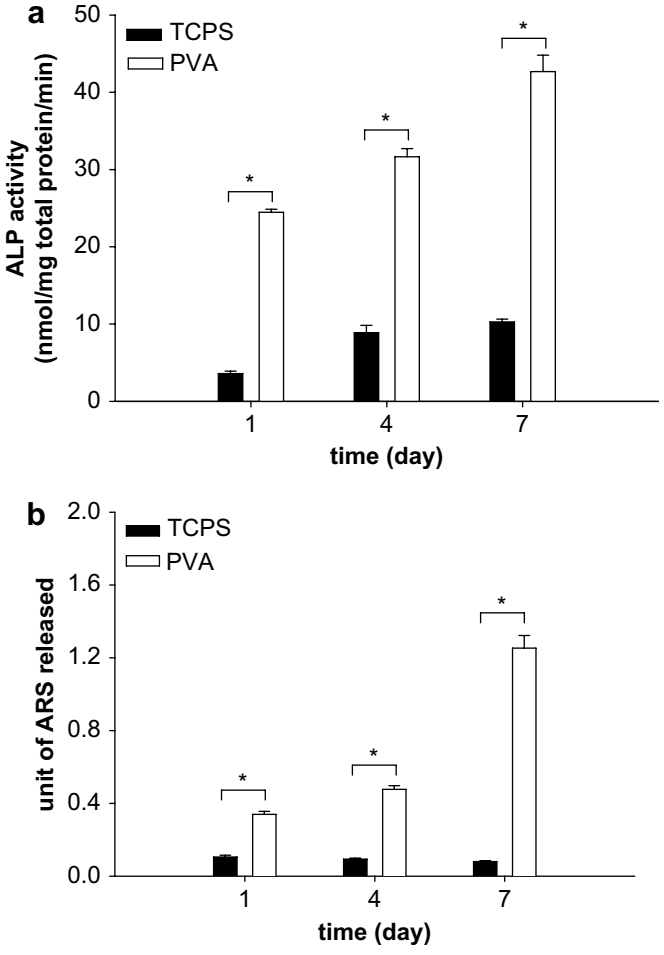

Fig. 2. (a) ALP activity and (b) mineralization (ARS assay) in TG cells cultured on TCPS (closed) and PVA (open) in the regular medium without the addition of dexamethasone, β-glycerophosphate and ascorbic acid after 1, 4 and 7 days of culture. Results represent the mean ± SD from 4–5 independent cultures and determinations. Asterisk denotes significant difference ($p < 0.005$) compared with TCPS, determined by post hoc procedure (Bonferoni analysis).

prepared. Fig. 2 shows TG cells grown on TCPS could not exhibit significant effects on production of ALP and calcium accumulation in the absence of differentiation medium. In contrast, PVA stimulation still resulted in increased levels of ALP activity and ARS assay with culture time. Similar to Fig. 1 the expression of ALP activity and ARS assay on PVA was always significantly higher than those on TCPS during the culture period ($p < 0.005$). Therefore, TG cells could exhibit high levels of ALP activity and mineralization on PVA, regardless of the presence or absence of differentiation medium.

3.2. Effect of U0126 on TG cells differentiation and mineralization

It has been well documented that growth factor, cytokine, and mechanical stimulation often induce changes in cell differentiation and mineralization through the activation of MAPK, particularly through ERK1/2 pathway [21–24]. Therefore, it is interesting to investigate the effects of MAPK inhibitor on promoting or inhibiting ALP activity and mineralization in TG cells. U0126, a widely used inhibitor of MEK1/2, the upstream kinases of ERK1/2 [21] was used in this study. Fig. 3 shows ALP activity and ARS assay of TG cells grown on TCPS with differentiation medium in the presence of U0126 ranging from 0 to 50 μM after culture for 7 days. Clearly, ERK1/2 inhibitor promoted TG cell differentiation in a dose-dependent manner and its effect was significant for all concentration used ($p < 0.005$). In addition, to address a possible involvement of MAPKs other than ERK1/2 pathway, specific inhibitors for p38 and JNK were also examined. It was found that inhibition of p38 by SB203580 (50 μM) and of JNK by SP600125 (50 μM) after culture for 7 days did not exhibit any effect on promoting ALP activity and mineralization of TG cells (data not shown).

3.3. Synergism of PVA and U0126 on TG cell differentiation and mineralization

Based on the above results, the possibility that U0126 inhibitor was able to interact synergistically with PVA substrate to enhance the differentiation and mineralization of TG cells was tested. Cells grown on PVA with the addition of 50 μM of U0126 were harvested after 7 days of incubation for analysis of ALP activity and ARS assay. The results successfully showed that elevation of ALP activity and mineralization of TG cells on PVA by U0126 treatment, regardless of using regular or differentiation medium (Fig. 4). In addition, the increasing degree of TG cell differentiation and mineralization exceeded the effect of PVA and U0126 alone, indicating there was a significant synergism between PVA and U0126.

3.4. Gene expression of TG cells on PVA and TCPS

In order to examine the synergistic effects between PVA and U0126 on known mineralization- and differentiation-related

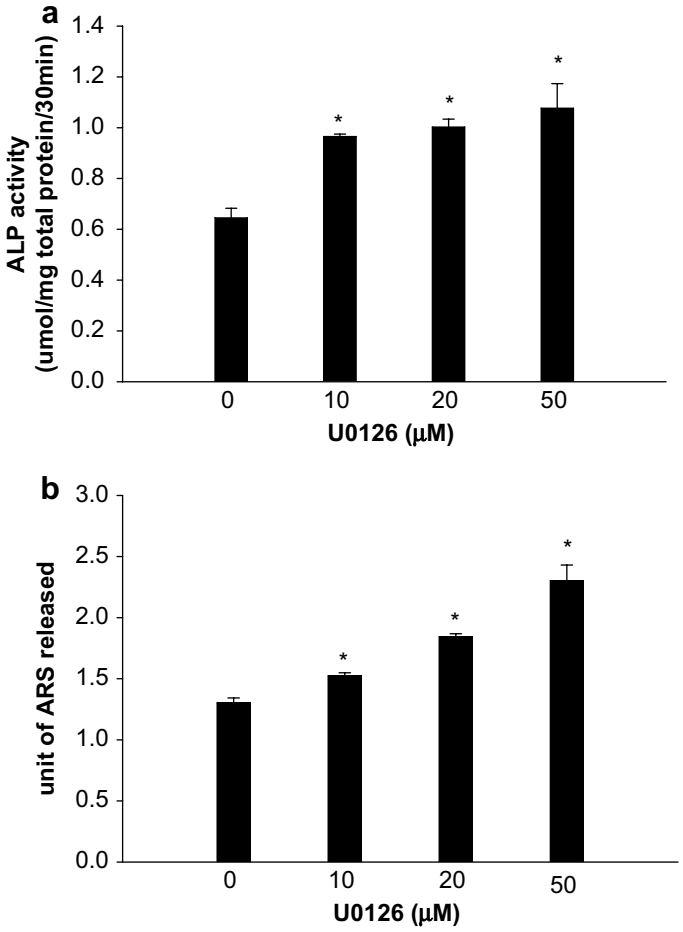

Fig. 3. Effects of ERK1/2 inhibitor U0126 on (a) ALP activity and (b) mineralization (ARS assay) of TG cells cultured on TCPS in the differentiation medium in a dose-dependent manner on the 7th day of culture. Results represent the mean ± SD from 4–5 independent cultures and determinations. Asterisk denotes significant difference ($p < 0.005$) compared with no inhibition, determined by post hoc procedure (Bonferoni analysis).

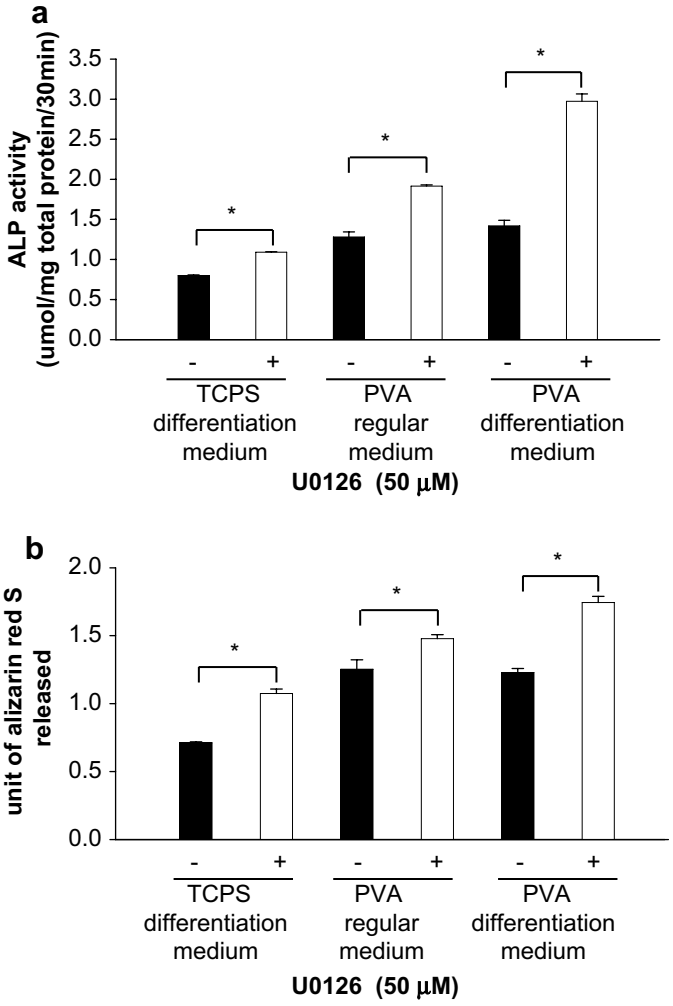

Fig. 4. Effects of ERK1/2 inhibitor U0126 (50 μM) on (a) ALP activity and (b) mineralization (ARS assay) of TG cells cultured in the regular or differentiation medium on the 7th day of culture. Results represent the mean ± SD from 4–5 independent cultures and determinations. Asterisk denotes significant difference ($p < 0.005$) compared with no inhibition, determined by post hoc procedure (Bonferoni analysis).

markers, the gene expression of OCN, OPN, DMP 1, and enamelin of TG cells cultured on TCPS and PVA with or without U0126 treatment for 7 days was examined by semi-quantitative RT-PCR (Fig. 5). Compared to TG cells cultured on TCPS without addition of U0126, exposure of cells to U0126 on TCPS or cells cultured on PVA without U0126 resulted in an increase in OCN mRNA expression [25], regardless of the presence or absence of differentiation medium. When cells cultured on PVA and exposed to U0126, the combination exhibited a more dramatic effect on increasing transcripts on OCN. Furthermore, compared to TG cells cultured on TCPS, cells grown on PVA also exhibited high level of expression of OPN mRNA, no matter in the regular or the differentiation medium. Interestingly, similar to the effect of PVA, exposure of TG cells to U0126 exhibited an increase expression of OPN mRNA, even cells cultured on TCPS. This is consistent with the previous report by Higuchi et al. that cells treated with ERK1/2 inhibitor showed increased levels of mineralization-related markers [26]. In contrast, TG cells cultured on TCPS, regardless of the presence or absence of U0126, could not exhibit the expression of DMP 1 and enamelin mRNA, but PVA itself still induced the mRNA levels for them. DMP 1, a dentin-specific protein, plays an important role in controlling dentin formation [27,28]. Enamelin, a specific protein of enamel matrix and secreted by ameloblasts, takes part in amelogenesis [29,30]. Thus, the induction of DMP1 and enamelin mRNA further confirmed the effects of PVA on TG cell differentiation to produce dentin and enamel mineralization. In addition, the synergistic effects between PVA and U0126 on expression of DMP1 and enamelin mRNA of TG cells were still could be detected. Thus, PVA alone or with U0126 inhibitor might be competent to induce the production of desired factors by activating TG cells.

3.5. Effect of PVA on inhibiting ERK1/2 phosphorylation

Previous studies have demonstrated that the cascade of ERK1/2 pathway can be inhibited by inhibitor, U0126 [7,21]. Therefore, if the ERK1/2 pathway is also involved in PVA-promoted TG cell differentiation and mineralization, it is reasonable to assume that the ERK1/2 phosphorylation will be reduced or abolished for TG cells cultured on PVA. Here, we examined the ability of U0126 and PVA to inhibit ERK1/2 activation of TG cells by immunocytochemistry with a widely used phospho-specific ERK1/2 antibody which recognizes the phosphorylation of the two sites known to be responsible for the ERK1/2 pathway. Fig. 6 shows TG cells cultured on TCPS in the absence of U0126 for 4 h were capable of activating the ERK1/2 phosphorylation to a high level in Western blot analysis. Consistent with the effect of ERK1/2 inhibitor, when TG cells cultured on TCPS for 4 h and then treated with U0126 for 30 min, the ERK1/2 activity of TG cells was considerably decreased.

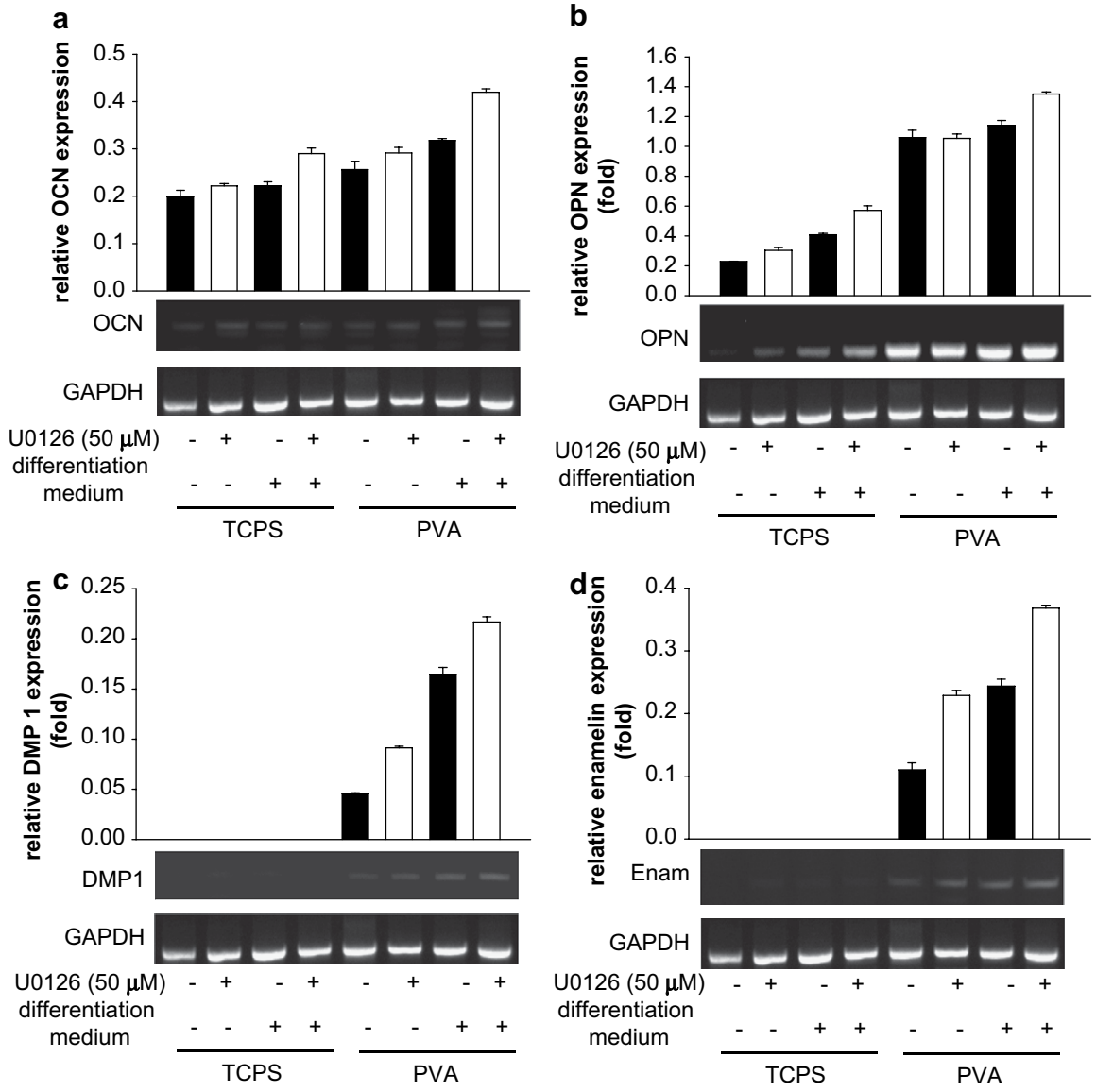

Fig. 5. Effects of ERK1/2 inhibitor U0126 (50 μM) on mRNA expression of typical mineralization-related markers in TG cells on the 7th day of culture (a) OCN, (b) OPN, (c) DMP 1 and (d) enamelin. The bar charts show the average density ± SD of the PCR product from three independent experiments. GAPDH is the normalization control.

Interestingly, TG cells cultured on PVA for 4 h also displayed low extent of ERK1/2 phosphorylation, while the effect of PVA was not as stronger as that of U0126. However, synergistic effects between PVA and U0126 were noted. No levels of ERK1/2 phosphorylation were detected for TG cells cultured on PVA under the stimulation of U0126. These results suggested that both U0126 inhibitor and PVA substrate suppressed the ERK1/2 phosphorylation and the inactivation of ERK1/2 activity helped to induce TG cell differentiation and mineralization.

To further confirm the role of ERK1/2 activity in TG cell differentiation and mineralization, ERK1/2 activity of TG cells cultured on TCPS and PVA with differentiation or regular medium was measured at different time points. Fig. 7(a) indicated the level of ERK1/2 phosphorylation was declined on both TCPS and PVA in the differentiation medium. Combined with Fig. 1, PVA could induce TG cell differentiation and mineralization, and could inhibit ERK1/2 activation more efficiently than TCPS. Fig. 7(b) shows that the ERK1/2 phosphorylation could still be negatively regulated when TG cells cultured on PVA with regular medium. However, cells cultured on TCPS without differentiation medium were unable to inhibit ERK1/2 activation, even enhanced ERK1/2 activation. Based on Figs. 1, 2 and 7, ERK1/2 phosphorylation negatively regulated ALP activity and mineralization in TG cells.

4. Discussion

The effects of growth factors [2], cytokines [3], and mechanical stimulus [4] on cell differentiation and mineralization have been the subject of intense investigation recently. On the other hand, it is well documented that biomaterials govern cellular responses and ultimately affect the success of application and, therefore, might be useful for controlling cell differentiation and mineralization. For example, neural stem cells respond to different substrates, and their fate determination depends on the chemical properties of the substrates [31]. In addition, it has been reported that PVA had a positive effect on HpG2 cell functionality in terms of albumin synthesis [32].

Traditionally, if cells are able to differentiate toward an osteoblast phenotype, culture medium will be routinely added with dexamethasone, β-glycerophosphate and ascorbic acid [33], which

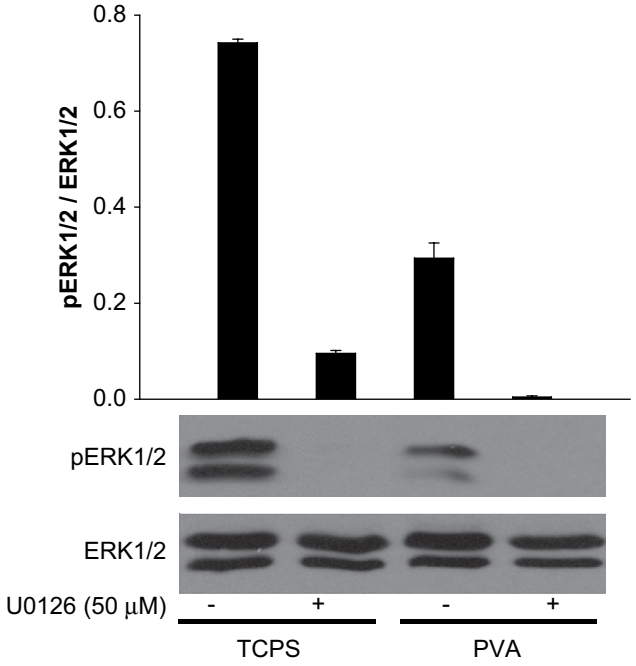

Fig. 6. Western blots were performed with anti-phosphorylated ERK1/2 and anti-ERK1/2 antibodies for TG cells cultured on TCPS and PVA in the differentiation medium for 4 h followed by treatment with or without U0126 for 30 min. The upper panel shows the ratio of phosphorylated to total ERK1/2, determined by band densitometry analysis. Results are mean ± SD from three independent experiments.

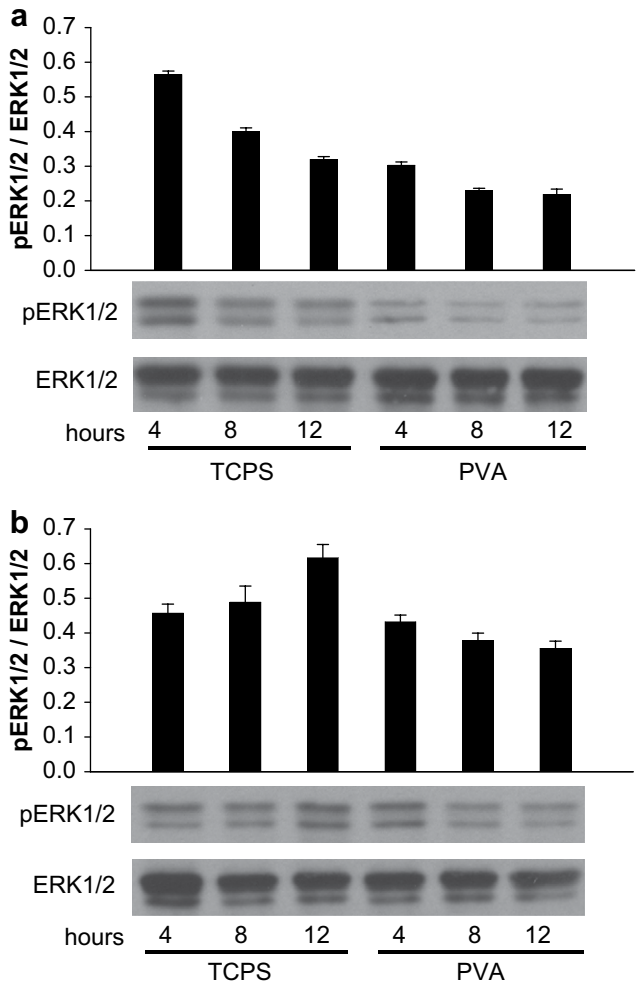

Fig. 7. Western blots were performed with anti-phosphorylated ERK1/2 and anti-ERK1/2 antibodies for TG cells on TCPS and PVA in the (a) differentiation and (b) regular medium for 4, 8, and 12 h. The upper panel shows the ratio of phosphorylated to total ERK1/2, determined by band densitometry analysis. Results are mean ± SD from three independent experiments.

had been reported to be beneficial to cell differentiation and mineralization in the course of culture [20,33]. Therefore, ALP activity and ARS assay of TG cells on TCPS were greatly reduced with regular medium, when compared with medium containing differentiation-induced additives (Figs. 1 and 2). Interestingly, compared to TCPS, PVA provided a more preferential environment for TG cells to express higher levels of the ALP activity and mineralization, no matter in the regular or differentiation medium (Figs. 1 and 2). These findings suggested that PVA act as an important role to induce the differentiation and mineralization of TG cells as the supplement of dexamethasone, β-glycerophosphate and ascorbic acid [20].

Several studies indicated that the MAPK signaling pathway, particularly through ERK1/2 pathway, plays a critical role in the regulation of cell growth and differentiation [21–23]. However, there were contradictory results about the relationship between ERK1/2 phosphorylation and osteoblast differentiation. Kono et al. [34], Higuchi et al. [26] and Nakashima et al. [35] reported that ERK1/2 activation negatively regulates the differentiation in different cells. In contrast, Lai et al. [36] demonstrated that ERK1/2 inhibitor suppressed osteoblast differentiation and mineralization. In order to investigate the direct role of ERK1/2 in the process of TG cell differentiation and mineralization, cells were treated with U0126, known to suppress the activation of ERK1/2. Interestingly, direct suppression of ERK1/2 by U0126 treatment enhanced ALP activity and mineralization of TG cells grown on TCPS (Fig. 3), indicating a critical involvement of ERK1/2 and acting as a negative regulator of the differentiation and mineralization of TG cells. Compared to TCPS, treatment of U0126 further enhanced the ALP activity and mineralization of TG cells grown on PVA, regardless of the absence or presence of differentiation medium (Fig. 4). In each case, the increasing expression of ALP activity and ARS assay exceeded that of PVA or U0126 added alone, indicating that PVA substrate and U0126 inhibitor synergistically promoted TG cell differentiation and mineralization. In addition to enhanced ALP activity and ARS assay, the synergistic effect was further confirmed by increased expression of OCN, OPN, DMP 1, and enamelin (Fig. 5). These results suggested that TG cells, a heterogeneous population of cells [37], still had the differentiation and mineralization ability when they cultured on PVA and treated with U0126.

Based on these results, we focused on defining the specific signaling pathway involved in PVA-promoted TG cell differentiation and mineralization. Direct inhibition of ERK1/2 phosphorylation by the inhibitor of ERK1/2 U0126 has been related to the osteoblast differentiation in C2C12 and MC3T3-E1 cells [32,33]. In this study with TG cells, either cells on TCPS treated with U0126 or on PVA without U0126 treatment inhibited ERK1/2 phosphorylation (Fig. 6). Although the effect of PVA was not as stronger as that of U0126, PVA itself still had the inhibition effect on the activation of ERK1/2. Furthermore, cells exposed to PVA and U0126 in combination seemed to exhibit a complete inhibition in ERK1/2 activity when compared with cells exposed to PVA or U0126 alone (Fig. 6). The greater decrease in ERK1/2 activity coincides with the synergistic effects between PVA and U0126 that TG cells exhibiting more enhanced ALP activity and ARS assay and more increased expression of OCN, OPN, DMP 1, and enamelin (Figs. 4 and 5). Analysis of the signaling mechanism responsible for the ability of PVA to promote differentiation and mineralization of TG cells identified the ERK1/2 pathway play a direct and critical role, while neither the p38 nor JNK pathway appeared to be involved (data not

shown). In addition, in comparison with cells cultured on TCPS in the regular medium, ERK1/2 phosphorylation was reduced in a time-dependent manner for TG cells cultured on TCPS in the differentiation medium and on PVA in the regular or differentiation medium (Fig. 7). More importantly, decrease of ERK1/2 activation was accompanied by an increase in ALP activity and mineralization (Figs. 1 and 2). These results suggest that the ERK1/2 activation negatively regulates differentiation and mineralization of TG cells not only cultured on PVA but also treated by U0126. This study demonstrated a novel role of PVA in promoting the differentiation and mineralization of TG cells through ERK1/2 acting as a negative regulator.

5. Conclusion

In summary, the exact regulatory mechanism of differentiation and mineralization of TG cells by ERK1/2 pathways still remains unclear. However, we demonstrated that the suppression of ERK1/2 pathway is one of the effects of PVA-promoted TG cell differentiation and mineralization. PVA substrate can suppress ERK1/2 activation and exposure of cells to U0126 further improves the inhibitory effect. The results provide support for the potential applications of PVA in TG cell investigation and the future development of tooth regeneration.

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