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| Original Article |

Specific Temporal Culturing and Microgroove Depth Influence Osteoblast Differentiation of Human Periodontal Ligament Cells Grown on Titanium Substrata

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Abstract : The purpose of this study is two-fold: to compare differences in the development of osteoblast differentiation processes between human bone marrow mesenchymal stem cells (MSCs) and human periodontal ligament cells (PLCs) cultured on microgrooved titanium (Ti) substrata and to investigate the effects of microgroove depth on PLCs' osteoblast differentiation. Using photolithography, 60 μm-wide and 10 or 20 μm-deep microgrooves were fabricated on the Ti substrata (NE60/10 or NE60/20). Subsequent acid etching was applied to the fabricated microgrooved Ti to yield the etched microgrooves (E60/10 and E60/20). Smooth and acid-etched Ti were used as the controls (NE0 and E0). Alkaline phosphatase activity and extracellular calcium deposition assays were performed after various timelines of culture on both MSCs and PLCs grown on NE0 and E60/10. For PLCs cultured on NE0, NE60/10, NE60/20, E0, E60/10 and E60/20, cell adhesion, cell proliferation, osteoblast differentiation were determined, followed by the analysis on various osteogenic gene expressions. By comparing the extracellular matrix maturation and mineralization processes on smooth and microgrooved Ti substrata, it was determined that PLCs require more time for osteoblast differentiation than MSCs. Also, E60/10 allowed for the highest levels of adhesion, proliferation, osteoblast differentiation, and osteogenic gene expression by PLCs. PLCs could be an excellent alternative to MSCs for use in future studies investigating cellular processes using microgrooved Ti substrata and other modified surfaces. Optimal Ti microgroove dimensions and osteogenic differentiation timelines are necessary to promote various cellular activities in human PLCs.

Key words: *titanium substrata, human periodontal ligament cells, osteoblast differentiation, microgroove depth, osteogenic gene expression*

1. Introduction

Standardized periodic surface microgrooves on titanium (Ti) substrata enhance cell adhesion and adhesion-dependent cell proliferation of human gingival fibroblasts.[1] Ti substrata with surface microgrooves 60 μm wide and 10 μm deep with subsequent acid etching have been determined as the most effective surface topography for enhancing the maturation of MG63 human osteoblast-like cells (MG63 cells).[2] However, osteosarcoma-derived cell lines such as MG63 cells are essentially proliferative and display poor sensitivity to differences in underlying substrates; therefore, the use of undifferentiated mesenchymal stem cells (MSCs) may be more appropriate for surface topography studies.[3] Like MSCs, cells derived from human periodontal ligament tissues (human periodontal ligament cells, PLCs) can differentiate into several types of specialized cells, including osteoblasts, chondrocytes, or adipocytes.[4] Furthermore, PLCs proliferate rapidly, show high expression levels of the osteocalcin gene when cultured on

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rough Ti surfaces,[5] and exhibit comparable utility to MSCs during alveolar bone regeneration in a canine peri-implant defect model,[6] suggesting the potential for using PLCs as an alternative to MSCs in oral implantology research.[4]

Since pluripotent bone marrow MSCs are the precursors of all osteoblastic cells that produce bone and bone marrow stroma, their potential for osteogenesis and ability to undergo osteoblast differentiation are crucial activities in investigating bone-implant surface interactions during the osseointegration process. Identifying the temporal peak expression levels of various osteogenic genes and proteins revealed that a particular sequence of osteoblast differentiation exists and is characterized by cell proliferation, extracellular matrix maturation, and mineralization procedures.[7] MSCs cultured on Ti surfaces with a nanoscale topography were reported to express significantly high levels of type I collagen and alkaline phosphatase (ALP) activity, osteocalcin synthesis, and extracellular matrix mineralization after 1, 2 and 3 weeks of culture, respectively.[8] Determining the most effective timeline and conditions of osteoblast differentiation of MSCs and PLCs cultured on microgrooved Ti substrata is the next step in optimizing future cellular surface experiments. Furthermore, while wider and deeper micromachined grooves have shown a superior ability in triggering fibroblast proliferation,[9] and 60 μm-wide and 10 μm-deep etched microgrooves have significantly enhanced osteoblast maturation,[2] subsequent investigations should examine whether microgrooves deeper than 10 μm can induce various *in vitro* cellular responses, such as osteoblast differentiation and related gene expression levels.

We hypothesized that MSCs and PLCs would exhibit differences in the most effective timeline and culture conditions for osteoblast differentiation on microgrooved Ti substrata and that etched microgrooves deeper than 10 μm would enhance various *in vitro* PLC activities. The objectives of our present study were twofold: to compare differences in the development of osteoblast differentiation processes between MSCs and PLCs cultured on Ti substrata and to investigate the effects of microgroove depth on PLC osteoblast differentiation and gene expression.

2. Materials and Methods

2.1 Fabrication of Titanium Substrata

Commercially pure Ti sheets (0.14 mm-thick, grade-2; TSM-TECH Co. Ltd., Ulsan, Korea) were washed and dried in acetone, mechanically polished to obtain a finish surface with Ra 0.1 μm, and used as the control smooth Ti surface, NE0. Subsequent acid etching was applied to NE0 substrata using 1 % hydrofluoric acid (HF) for 10 s and used as another control group, E0. Photolithography was used to form truncated V-shaped surface microgrooves in cross-sections of 60 μm wide and 10 and 20 μm deep (NE60/10 and NE60/20) (Fig 1). Details of the photolithography procedure are described in our previous study.[10] Subsequent acid etching was applied to the entire surface of the fabricated microgrooved Ti substrata using 1% hydrofluoric acid (HF) for 2s (E60/10 and E60/20) (Table 1). In all experiments, fabricated Ti substrata were cleaned three times in an ultrasonic device with sterile distilled water for 30 min, washed another three times using distilled water, and dried at room temperature overnight before use.

2.2 Cell Culture

Human PLCs were acquired from the periodontal ligament tissues on freshly extracted bicuspid roots of patients undergoing orthodontic treatment at the Department of Orthodontics at Kyung Hee University Hospital in Gangdong. Tissues were harvested under the informed consent guidelines of an approved Institutional Review Board protocol of the same hospital. Immediately following extraction, teeth were placed in Dulbecco's Modified Eagle's Medium (DMEM: WelGene, Daegu, Korea) containing 10% fetal bovine serum (FBS;

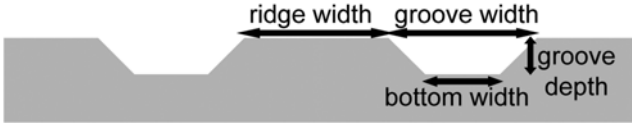

Figure 1. A schematic cross-sectional image and the structural nomenclature of the fabricated microgrooved titanium substrata using photolithography.

Table 1. Titanium substrata with various surface topographies used in this study.

	NE0	NE60/10	NE60/20	E0	E60/10	E60/20
Groove width (μm)	0	60	60	0	60	60
Groove depth (μm)	0	10	20	0	10	20
Bottom width (μm)	0	40	20	0	40	20
Subsequent acid-etching	non-etched	non-etched	non-etched	acid-etched	acid-etched	acid-etched

NE0, smooth titanium (Ti) substrata; NEα/β, Ti substrata with surface microgrooves of α μm width and β μm depth; E0, NE0 with subsequent hydrofluoric acid (HF) etching; Eα/β, NEα/β with subsequent HF etching.

Invitrogen, Carlsbad, CA, USA) and 1% of antibiotics/antimycotic (Invitrogen) solution containing penicillin G and streptomycin (Invitrogen). The periodontal ligament tissue was obtained from the middle third of the bicuspid root using a #15 surgical blade. Obtained tissues were treated with 1.1 units/ml dispase (Invitrogen) and 264 units/ml collagenase (Invitrogen) at 37°C under 5% CO_2 for 1 h. The tissue samples were then washed with DMEM and cultured in DMEM containing 10% FBS and 1% antibiotic/antimycotic solution.

Human bone marrow-derived MSCs were purchased from Lonza Inc. (Walkersville, MD, USA) and grown in the MSC-specific growth medium (MSCGM™; Lonza Inc.) at 37°C under 5% CO_2. Cells were maintained in DMEM containing 10% FBS and 1% antibiotic/antimycotic solution at 37°C under 5% CO_2. Cells at passages 3-5 were used in this study.

2.3 Alkaline Phosphatase Activity Assay

MSCs and PLCs were seeded on the 24-well Ti substrata of NE0 and E60/10 at 4×10^4 cells/well and cultured for 2 days to achieve confluence. Cells were then cultured in osteogenic media [DMEM supplemented with 10% FBS (Invitrogen), 50 μg/ml α-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich), 100 mM dexametasone (Sigma-Aldrich)] at 37°C under 5% CO_2 for 11, 14 and 17 days to investigate the ALP activity. The ALP activity assay was performed as previously described.[2] The reaction products were transferred to 96-well plates and monitored using a microplate reader (Bio-Rad, Hercules, CA, USA) at 405 nm, and measurements were compared using *p*-nitrophenol standards and normalized to total protein levels.

2.4 Extracellular Calcium Deposition Assay

Extracellular calcium deposition on the Ti substrata was quantified to investigate the steps of osteoblast differentiation. MSCs and PLCs were seeded on 24-well Ti substrata of NE0 and E60/10 at 4×10^4 cells/well and cultured for 2 days to achieve confluence. Cells were then cultured in osteogenic media at 37°C under 5% CO_2 for 18, 21 and 24 days on NE0 and E60/10. In a subsequent experiment, only PLCs were cultured in osteogenic media for 24 days to compare the osteoblast differentiation process when grown on NE0, NE60/10, NE60/20, E0, E60/10 and E60/20 substrata. Cells were rinsed in phosphate buffered solution (PBS; Gibco BRL, Grand Island, NY, USA) and the substrates with remaining calcium deposits were incubated with 0.5 N HCl at 4°C overnight. After centrifugation, the amount of calcium present in the acidic supernatant was quantified using Calcium Liquicolor (Stanbio Laboratory, Boerne, TX, USA). The reaction products were transferred to 96-well plates and monitored using a microplate reader (Bio-Rad) at 650 nm. Total calcium (μg/well) was calculated from standard curves of absorbance versus concentrations of calcium in controls measured in parallel with the experimental samples.

2.5 Scanning Electron Microscopy

The surfaces of NE60/10, NE60/20, E60/10 and E60/20 were observed using scanning electron microscopy (S-800 FE-SEM®, HITACHI, Tokyo, Japan).

2.6 Bromodeoxyuridine Cell Adhesion Assay

PLCs were seeded on the 96-well Ti substrata of NE0, NE60/10, NE60/20, E0, E60/10 and E60/20 at 3×10^3 cell/well and incubated for 16 h at 37°C under 5% CO_2. One milliliter of bromodeoxyuridine (BrdU) reagent (Roche Diagnostics GmbH, Mannheim, Germany) was labeled to each well, the cells were incubated for 2 h at 37°C and the BrdU assay [Cell Proliferation ELISA, BrdU (colorimetric); Roche Diagnostics GmbH] was performed as previously described.[11] The reaction products were transferred to 96-well plates and monitored using a microplate reader at 370 nm.

2.7 Cell Count Kit (CCK) Cell Proliferation Assay

PLCs were seeded on the 24-well Ti substrata of NE0, NE60/10, NE60/20, E0, E60/10 and E60/20 at 1×10^4 cell/well and cultured for 48 and 72 h at 37°C under 5% CO_2. Twenty microliters of Cell Count Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) were added to each well and incubated for 2 h. The reaction products were transferred to 96-well plates and monitored using a microplate reader at 450 nm.

2.8 RT-PCR and Quantitative Real-Time PCR

PLCs were seeded on 6-well Ti substrata of NE0 and E60/10 for RT-PCR, and NE0, NE60/10, NE60/20, E0, E60/10 and E60/20 for quantitative real-time PCR at 2×10^5 cells/well and cultured for 2 days to achieve confluence. PLCs were then cultured in osteogenic media for 14 days and total RNA was extracted using Trizol (Invitrogen) and the RNA concentration was determined using the NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). One microgram of total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). The mRNA expression levels of ALP, runt-related transcription factor 2 (RUNX2), osteocalcin (OC) and GAPDH mRNA (internal control) was determined using the TaqMan Gene Expression Assays Kit (Applied Biosystems, Foster City, CA, USA) with a predesigned probe and primer set (Primer3 Input, version 0.4.0;

Table 2. Gene-specific primers used in RT-PCR.

Gene (human)	GeneBank	Sense(5'-3')	Anti-sense(5'-3')	Bp
ALP	NM_000478.3	5'-GCGAACGTATTTCTCCAGACCCAG-3'	5'-TTCCAAACAGGAGAGTCGCTTCAA-3'	367
RUNX2	NM_004348.3	5'-TTGCAGCCATAAGAGGGTAG-3'	5'-GTCACTTTCTTGGAGCAGGA-3'	470
OC	NM_199173.2	5'-CATGAGAGCCCTCACA-3'	5'-AGAGCGACACCCTAGAC-3'	315
GAPDH	NM_002046.3	5'-GAGTCAACGGATTTGGTCGT-3'	5'-TGTGGTCATGAGTCCTTCCA-3'	512

ALP: alkaline phosphatase transcript variant 1, RUNX2: runt-related transcription factor 2, OC: osteocalcin

gene ID: ALP, Hs01029144_m1; RUNX2, Hs01047978_m1; Osteocalcin, Hs00609452_g1; GAPDH, Hs99999905_m1) (Table 2). Following Chromo4 Reverse Transcription-Polymerase Chain Reactions (Bio-Rad Laboratories, Hemel Hempstead, UK) with IQ Supermix (Bio-Rad), the MJ Opticon Monitor Analysis Software (Bio-Rad) was used to quantify the gene expression levels. The relative expression levels were analyzed by normalizing the experimental values with those of GAPDH and were presented as fold changes relative to the control Ti substrata, NE0.

2.9 Statistical Analyses

ALP activity test and extracellular calcium deposition assay of MSCs and PLCs on NE0 and E60/10 were performed simultaneously and independently for five times, and the mean values were compared using independent samples *t* test. The BrdU cell adhesion, CCK cell proliferation and extracellular calcium deposition assays, and quantitative real-time PCR of PLCs on NE0, NE60/10, NE60/20, E0, E60/10 and E60/20 were performed simultaneously and independently for four times, and the mean values from these experiments were compared using a one-way analysis of variance (ANOVA). SPSS 17.0 software was used for all statistical analyses.

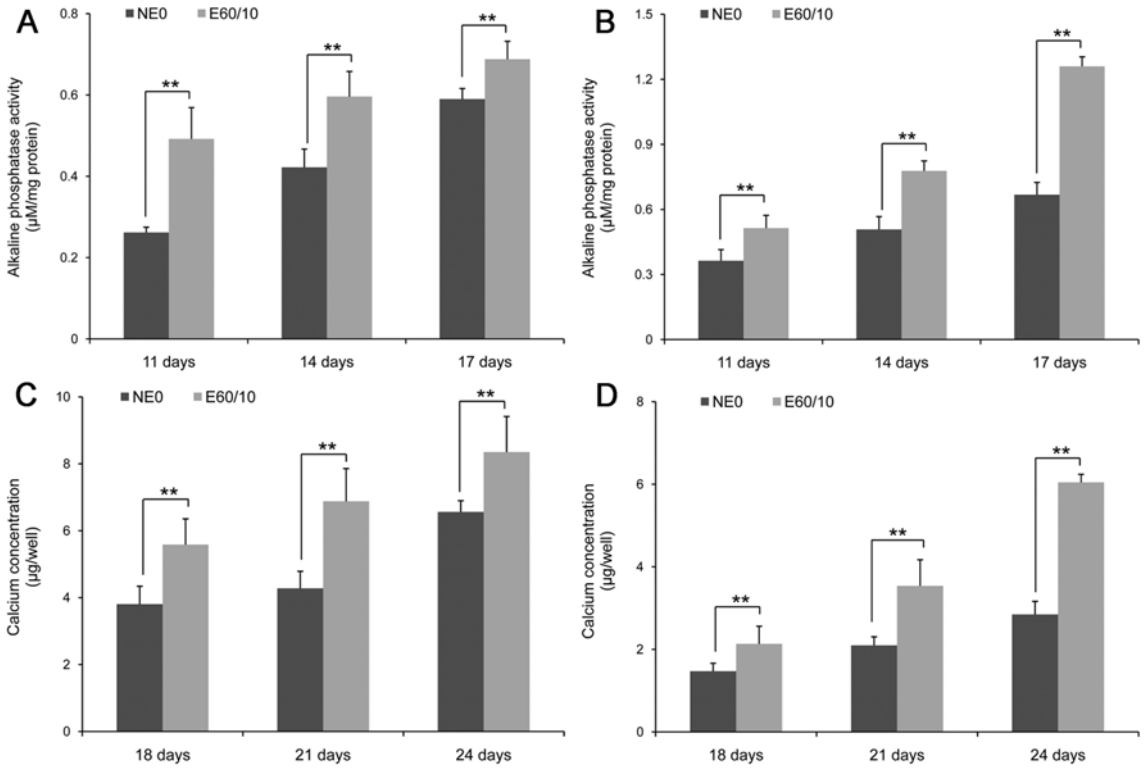

Figure 2. Comparison results of the alkaline phosphatase activity of MSCs (A) and PLCs (B) on NE0- and E60/10 titanium substrata after 11, 14 and 17 days of osteogenic culture, respectively. Comparison result of the osteoblast differentiation of MSCs (C) and PLCs (D) on NE0- and E60/10 titanium substrata after 18, 21 and 24 days of osteogenic culture using extracellular calcium deposition assay, respectively. Student's *t* test with equal variances assumed. N = 5. **: significant difference ($p < 0.01$).

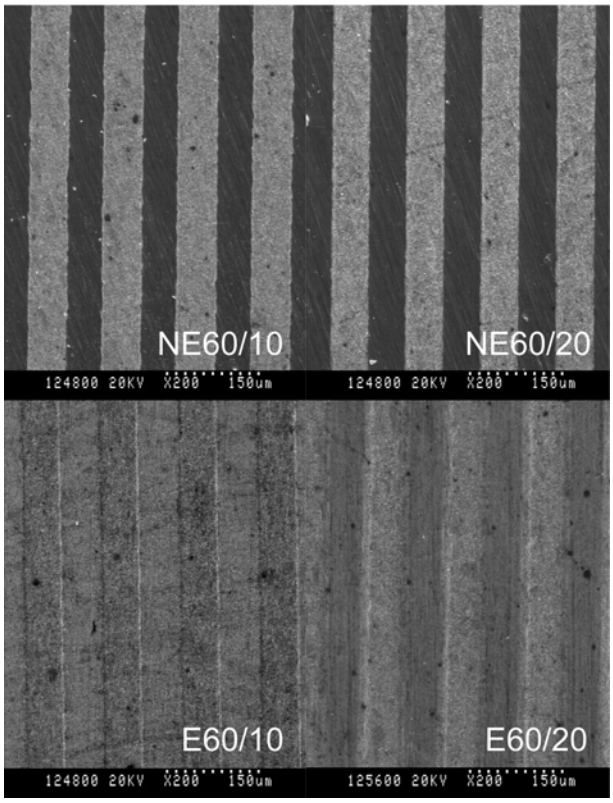

Figure 3. Scanning electron microscopic images of NE60/10, NE60/20, E60/10 and E60/20 (X200). Note that polished surface remains on the ridge-top surfaces of NE60/10 and NE60/20, whereas acid-etched ridge-top surfaces were observed in E60/10 and E60/20. Microgroove walls with obtuse angles towards the bottom surface were observed. In general, periodic microgrooves and ridges with identical widths were observed. See table 1 for nomenclature of the titanium substrata and their microstructural dimensions.

3. Results

3.1 Comparison of Osteoblast Differentiation Timelines in Human Primary Cells

The ALP activities of both MSCs and PLCs were tested after 11, 14, and 17 days of osteogenic culturing. The 14-day time point was selected based on previous studies reporting a peak in ALP activity after 14 days in both human bone marrow-derived MSCs cultured on tissue culture plates[12] and in rat calvarias primary osteoblasts cultured on Ti substrata with V-shaped machined surface grooves.[13] The extracellular calcium deposition assay was performed after 18, 21 and 24 days of osteogenic culturing. Since human primary mesenchymal populations are exceptionally sensitive to the environmental cues such as defined nanoscale topographies on titanium surfaces,[14] we compared the optimal timelines of osteogenic culturing of both MSCs and PLCs with regard to ALP activity and extracellular calcium deposition. We found that cultured MSCs showed the highest increase of ALP activity on E60/10 relative to NE0 on day 11 (Fig 2A), whereas PLCs showed the highest increase of ALP activity on E60/10 relative to NE0 on day 17 (Fig 2B). Similarly, MSCs showed a peak in the difference of osteoblast differentiation (signaled by extracellular calcium deposition) on E60/10 substrata on day 21 (Fig 2C), compared to the peak in calcium deposition of PLCs on day 24 (Fig 2D).

3.2 Effect of Microgroove Depth on *In Vitro* Responses of Periodontal Ligament Cells

The scanning electron microscopic images of NE60/10, NE60/20, E60/10 and E60/20 are presented in Fig 3. “Surface

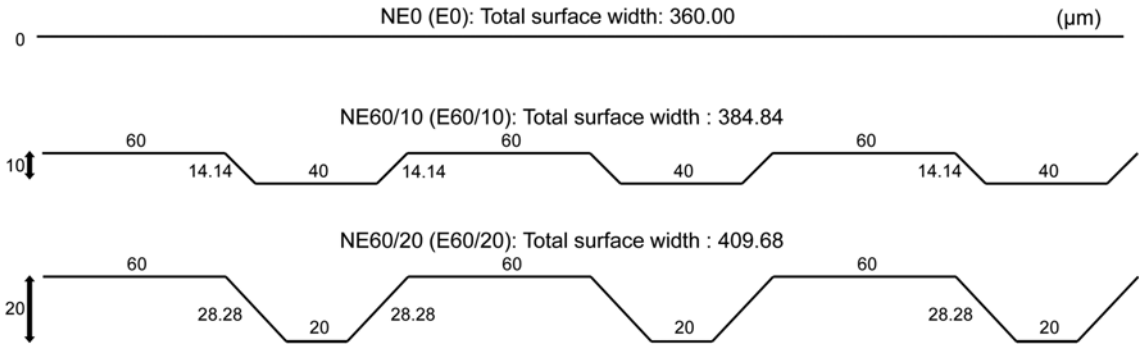

Figure 4. Schematic cross-sectional illustrations of NE0, NE60/10, NE60/20, microstructural dimension and the calculated surface widths. In this study, surface width is defined as the total sum of the groove, ridge, bottom and inclination widths in 360 μm-cross sectional width of NE0. Note that the ridge width and groove width were designed to be uniform in dimension. According to the isotropic principle, the bottom width inside the microgrooves with truncated V-shape can be calculated as [(groove width) - 2(groove depth)] and the inclination width as the square root of 2(groove depth)^2. See Fig 1 and table 1 for nomenclature.

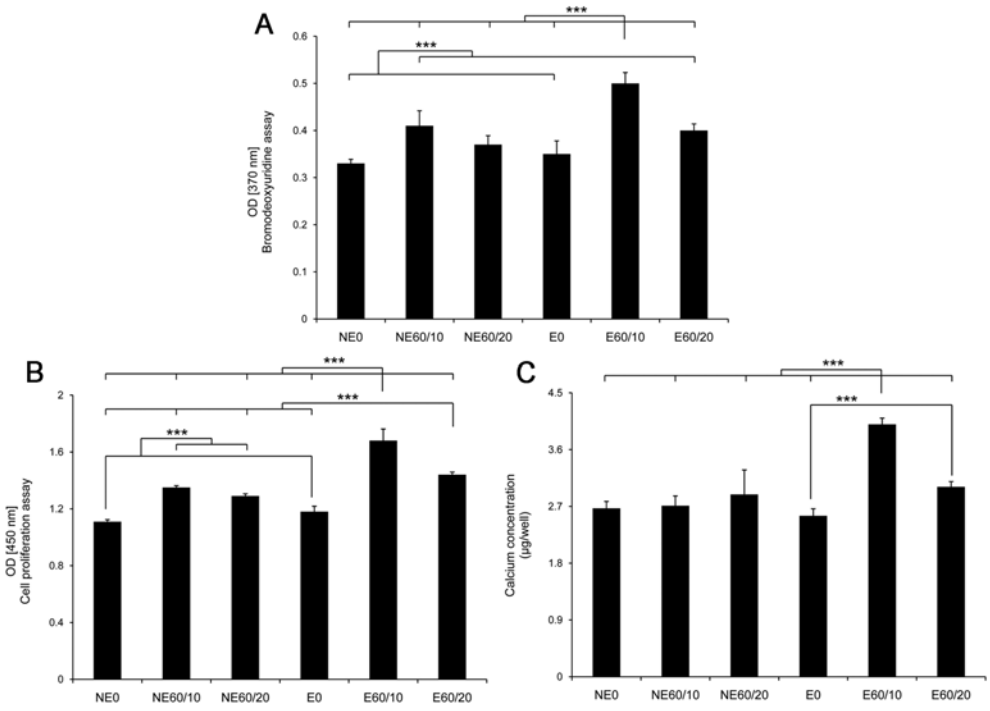

Figure 5. Multiple-comparison results of the BrdU cell adhesion assay at 16 h of incubation (A), the cell proliferation assay using Cell Count Kit after 72 h of culture (B) and the osteoblast differentiation using extracellular calcium deposition assay after 24 days of culture (C) using one-way ANOVA (n = 4). ***: significant difference ($p < 0.001$).

width" is defined as the total sum of the groove, ridge, bottom and inclination widths in 360 μm-cross sectional width of NE0. It is presented in the schematic cross-sectional illustration that NE60/20 and E60/20 would yield greater surface areas than NE60/10 and E60/10 in schematic cross-sectional illustrations (Fig 4). However, the highest cell adhesion and proliferation was found in PLCs cultures on E60/10 compared with NE60/20 or E60/20 (Fig 5A and 5B). Twenty-four days of optimal osteogenic culturing of PLCs and subsequent results from the extracellular calcium deposition assay showed that E60/10 was also the strongest inducer of PLC osteoblast differentiation when compared to all other Ti substrata topographies used in this study (Fig 5C).

3.3 Effect of Microgroove Depth on the Osteogenic Gene Expressions of Periodontal Ligament Cells

By investigating the effect of microgroove depth on the expression levels of core genes related to osteoblast differentiation in PLCs, microgrooved surfaces were found to upregulate the expression of ALP, RUNX2, and OC genes when compared to cells grown on smooth or acid-etched substrata controls (Fig 6). ALP removes phosphate groups from nucleotides, proteins, and alkaloids. Since accumulation of ALP occurs from increased osteoblast activity, elevated ALP level indicates a presence of active bone formation process. Also, OC is an important osteoblast differentiation hormone whose level is tightly controlled by the mater transcription factor of osteoblast differentiation, RUNX2. We confirmed our earlier result that E60/10 allowed for the highest level of osteoblast differentiation among all other groups of Ti substrata (Fig 5C).

4. Discussion

PLCs used in this study were acquired from the periodontal ligament tissue on root surface of freshly extracted teeth. This procedure is much simpler and less invasive than that required for MSC acquisition. Moreover, the PLC responses were examined in this study for pre-evaluating a possible osseointegration of oral Ti implants placed into fresh extraction sockets, so called immediate implantation, where some of the periodontal ligament tissues could remain inside after tooth extraction. Consistent with other studies using various shapes and dimensions of microgrooves on various biomaterials,[13,15,16] we verified that the etched microgrooves on the Ti substrata used in this study triggered osteoblast differentiation in both

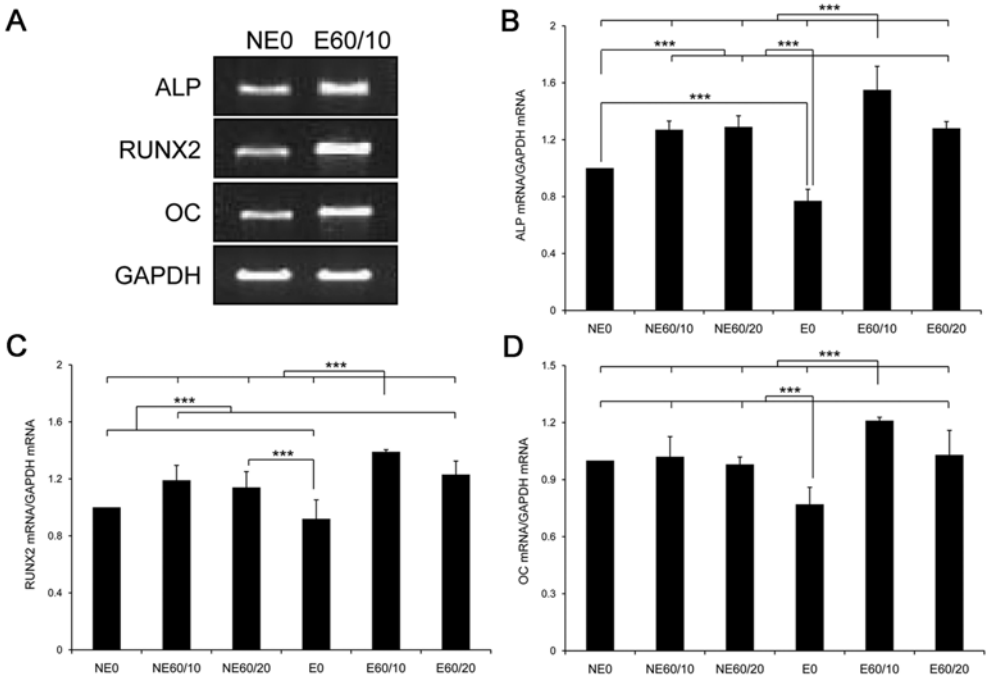

Figure 6. Analysis on the expressions the ALP-, RUNX2- and OC genes after 14 days of osteogenic culture of human periodontal ligament cells using RT-PCR (A), and relative mRNA expressions of ALP (B), RUNX2 (C) and OC (D) on NE0, E0, E15/3.5, E30/10 and E60/10 titanium substrata after 14 days of PLCs culture. The relative expression levels were analyzed by normalizing with GAPDH, and are presented as fold changes relative to the control, NE0. One-way ANOVA (n = 4). ***: significant difference ($p < 0.001$).

MSCs and PLCs (Fig 2). By comparing the temporal sequences of osteoblast differentiation of human primary cells on smooth and microgrooved substrata, we demonstrated that PLCs require more time to undergo osteoblast differentiation and show a slower rate of extracellular matrix maturation and mineralization compared to MSCs. This difference contributes to our understanding that optimal culture conditions are necessary for osteoblast differentiation in PLCs. Moreover, this difference should be taken into account when designing experiments that investigate the effects of microgrooved Ti substrata and other surface modifications on cellular responses, which could make PLCs an excellent alternative to MSCs in future studies.

In our previous studies, E60/10 allowed for superior cell adhesion, cell proliferation and ostoeblast maturation to the Ti substrata with etched microgrooves of identical width but 5 μm depth,[1,2] suggesting that the microgroove depth could be an influential factor for enhancing *in vitro* cell responses if the microgroove widths are consistent. In another earlier study, we had focused only on creating substrata with periodic parallel microtopography whose ridges and grooves showed identical widths; the rationale for examining this specific variable resulted from previous study which suggested that groove and ridge width, rather than depth, is important in controlling osteo-specific cellular functions, such as osteoblast differentiation.[17] Cells respond more strongly to deeper microgrooves than to shallow ones with regard to the alignment and elongation of the microgrooves and ridges.[18] Specifically, one study observed a high proliferation rate of fibroblasts grown on substrata with relatively deep microgrooves (up to 20 μm) coupled with a 30 μm width.[9] Moreover, the number of bone-like nodules found on native Ti- and hydroxyapatite-coated substrata increased as the groove depth increased from 3 to 30 μm in a previous study using rat calvarias primary osteoblasts.[13]

Based on these previous reports, we examined an additional parameter referred to as "surface width", which describes the surface areas of various dimensions of grooves on Ti substrata at the microscale level. The isotropic principle of wet etching used in phophtolithography states that an increase of microgroove depth leads to the creation of increased inclination width and decreased bottom width. We were unable to apply a microgroove depth of 30 μm or more because at that depth, the microgroove bottoms would be eliminated and would hinder the cells from forming natural focal adhesions inside the grooves. Instead, we applied a depth of 10 and 20 μm for comparison and expected that approximately 40 and 20 μm of

the bottom width of the microgroove could be theoretically maintained, respectively. Contrary to our prediction that the greater surface areas of microgrooves in NE60/20 or E60/20 would induce higher levels of cell adhesion and proliferation, we found that E60/10 promoted the highest levels of PLC adhesion, proliferation and osteoblast differentiation (Fig 5). These results indicate that in addition to a greater surface area, microgroove width and bottom width, cross-sectional shape of the microgrooves, and secondary submicroscale topographies are also important factors in enhancing various PLC cellular activities.

The 14-day osteogenic culture time point (Fig 6) was selected for gene expression analysis based on previous studies which showed that the nanoscale topography of growth substrata upregulated the expression of various genes responsible for the sequential progress of osteoblast differentiation in MSCs grown on Ti.[19-21] We showed that microgroove depth positively affects *in vitro* cellular activities and osteogenic gene expressions of PLCs. These results suggest that truncated V-shaped, relatively shallow microgrooves with bottoms wide enough to allow cell settling are superior to deeper microgrooves with decreased bottom widths in their ability to induce various PLC functions. From these data, we can report the optimal dimensions, shape, and secondary submicroscale topography of Ti substrata microgrooves that will exert the strongest positive impact on the cellular behaviors, such as osteoblast differentiation. Further investigations of the relationship between microgroove dimensions and the temporal expression of additional osteogenic genes will greatly contribute to our understanding the effects of substrata topography and PLCs' osteoblast differentiation.

Enhanced cytoskeletal organization and osteoblast differentiation, as indicated by high expression levels of osteocalcin and osteopontin genes in this study, were described in a previous study to be concurrent with increased cell spreading of MSCs on nanoscale pits; conversely, MSCs were restricted from spreading but showed increased osteoblast differentiation on microgrooved substrata.[14] Another study reported that cells elongated and spread along microgrooves rather than spreading radially, which decreased their overall surface area contact with the underlying substrate, but the cells still maintained or at times elevated their activities.[22] However, these studies used narrow and shallow microgrooves in which the cells were unable to sense an increase in overall surface area at the microscale level. The wider and deeper microgrooves used in this study not only allowed cells to utilize an increased surface area and enhance their proliferation, but they also induced contact guidance to improve overall cellular functions.[1] Therefore, the PLCs examined in this study were able to concurrently increase their levels of cell adhesion, cell proliferation, osteoblast differentiation, and osteogenic gene expression in a manner unique from what has been previously described.[14,22]

Since the E60/10 substrata used in this study provides optimal conditions for a variety of enhanced *in vitro* cellular activities, further investigation is recommended to explore the application of nano- and other submicroscale topographies in various cellular assays. Using E60/10 as a standard microtopography, the potential for different substrata, such as osteogenic matrix-protein immobilization or inorganic coatings, to enhance cell proliferation and osteoblast differentiation in human primary cells can be effectively investigated.

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