Effect of etched microgrooves on hydrophilicity of titanium and osteoblast responses: A pilot study

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PURPOSE. The aim of this pilot study was to investigate the effect of etched microgrooves on the hydrophilicity of Ti and osteoblast responses. **MATERIAL AND METHODS.** Microgrooves were applied on Ti to have 15 and 60 μm width, and 3.5 and 10 μm depth by photolithography, respectively. Further acid etching was applied to create Ti surfaces with etched microgrooves. Both smooth- and acid-etched Ti were used as the controls. The hydrophilicity of Ti was analyzed by determining contact angles. Cell proliferation and osteogenic activity of MC3T3 mouse preosteoblasts were analyzed by bromodeoxyuridine assay and alkaline phosphatase (ALP) activity test, respectively. One-way ANOVA, Pearson's correlation analysis and multiple regression analysis were used for statistics. **RESULTS.** Etched microgrooves significantly increased the hydrophilicity of Ti compared to the smooth Ti. 60 μm-wide etched microgrooves significantly enhanced cell proliferation, whereas the osteogenic activity showed statistically non-significant differences between groups. Result of the osteogenic activity significantly correlated with those of hydrophilicity and cell proliferation. Hydrophilicity was determined to be an influential factor on osteogenic activity. **CONCLUSION.** This study indicates that increase in hydrophilicity of Ti caused by etched microgrooves acts as an influential factor on osteogenic activity. However, statistically non-significant increase in the ALP activity suggests further investigation. **KEY WORDS.** Titanium, Etched microgrooves, Hydrophilicity, Osteoblast response **[J Adv Prosthodont 2010;2:18-24]**

INTRODUCTION

To promote the osteoblast responses to titanium (Ti) oral implants, the effects of surface microgrooves on *in vitro* cell behaviors have been reported extensively. Ti surface microgrooves were reported to induce changes in cell morphology,[1] cell-substratum adhesion,[2] and gene expression[3] *in vitro*. Microgrooves were also reported to have positive effects on the cell proliferation and osteogenic activity on defined surfaces of various biomaterials[4-7] as well as on Ti-coated silicone replica.[8-10] However, the effect of microgrooves applied by isotropic wet chemical photolithography for the native Ti on the early *in vitro* osteogenic activity has not yet been verified.

The hydrofluoric acid treatment with various treatment time periods has been verified to promote osteoblast responses, suggesting its efficacy as a submicron-scale topography secondary to the microtopography on Ti.[11] The hydrophilicity of Ti has been recently verified to affect profoundly the soft and hard tissue integration of implants.[12,13] Since the hydrophilicity of Ti was previously reported to be enhanced by various hydrofluoric-acid treatments[11] and the etched microgrooves of reasonable width on Ti triggered the cell proliferation of human gingival fibroblasts,[14] a strong expectation is made on the effect of Ti-surface etched microgrooves to increase hydrophilicity of Ti. Prior to investigate the effect of etched microgrooves on various Ti-surface characteristics and their influences on various cell behaviors of osteogenic cells, we designed a pilot study analyzing the effect of the corresponding surface on hydrophilicity and osteoblast responses.

The purpose of this pilot study was to investigate the effect of etched microgrooves on the hydrophilicity of Ti and osteoblast responses.

MATERIAL AND METHODS

Fabrication of titanium substrata

0.2-mm thick grade-2 commercially pure titanium (cp-Ti) sheets (TSM-TECH Co. Ltd., Ulsan, Korea) were mechanically polished to obtain a finish surface with Ra $\leq$ 0.1 μm. Microgrooves were applied on Ti to have 15 and 60 μm width, and 3.5 and 10 μm depth by photolithography (MEMSware Inc., Kwangju,

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Gyeonggi, Korea), respectively (NE15/3.5 and NE60/10). Details of the photolithography procedures were reported in our previous study.[15] Further acid etching was applied to create Ti surfaces with etched microgrooves (E15/3.5 and E60/10). Both smooth- and acid-etched Ti were used as the controls (NE0 and E0) (Fig. 1).

Contact angle determination

A drop shape analysis system goniometer, EasyDrop® contact angle measuring instrument (KRÜSS GmbH, Hamburg, Germany), was used for contact angle measurement. Distilled water (6 μl per drop) was used as a probe for contact angle calculation. Measurements were taken for each drop after 15 s deposition from 3 independent samples of each control and experiment Ti substrata. The drop images were captured serially 5 times by a video camera in the directions parallel with as well as perpendicular to the surface microgrooves. The contact angles used for data were the averages of the angles of 5 serial captures of each water drop calculated by an image analysis system.

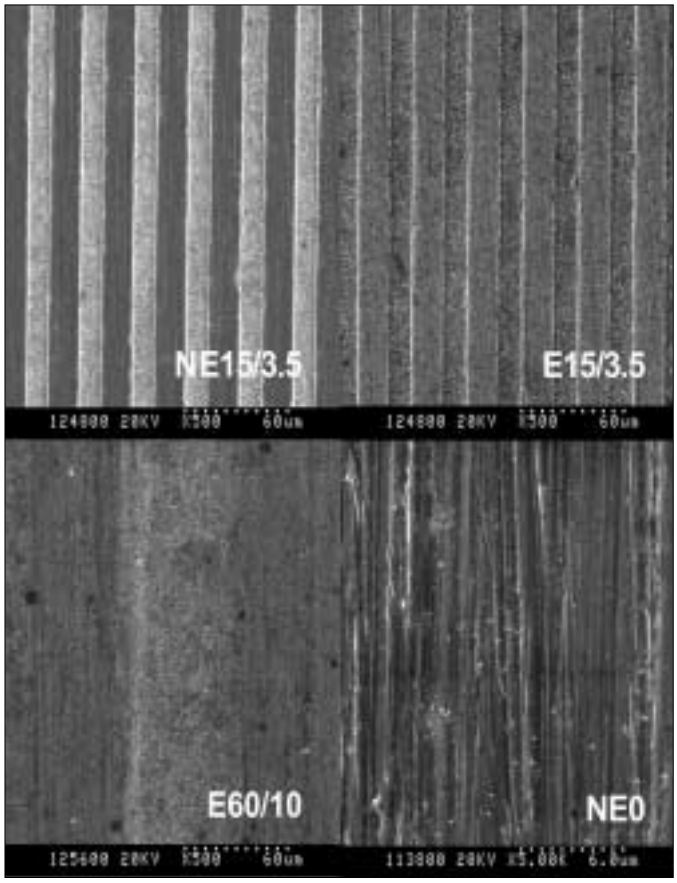

Fig. 1. Scanning electron microscopic (SEM) images of NE15/3.5 ($\times$ 500), E15/3.5 ($\times$ 500), E60/10 ($\times$ 500) and NE0 ($\times$ 5000).

Cell culture

MC3T3-E1 mouse preosteoblasts (MC3T3 cells) were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in α-modified Eagle's medium (α-MEM; WelGene, Daegu, Korea) containing 10% fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) and incubated at 37℃ in a humidified atmosphere of 5% CO_2 for 12 h. Non-adherent cells were aspirated, adherent cells were cultured and expanded and the medium was refreshed every 2 days for confluence till the 3rd-5th passages of culture were obtained. The cells were washed in 10 ml phosphate-buffered salines (PBS; Gibco BRL, Grand Island, NY, USA) and removed by trypsin-EDTA solution (0.25% trypsin and 0.1% glucose dissolved in 1 mM of EDTA-saline; Sigma-Aldrich Co., St. Louis, MO, USA). To induce osteogenic activity, MC3T3 cells were cultured in an osteogenic media [DMEM (Dulbecco's modified Eagle's medium, WelGene, Daegu, Korea) supplemented with 10% FBS (Sigma-Aldrich Co., St. Louis, MO, USA), 50 ug/ml of α-ascorbic acid, 10 mM of β-glycerophosphate, 100 nM of dexametasone and antibiotics (Invitrogen, Carlsbad, CA, USA)].

Bromodeoxyuridine cell proliferation assay

MC3T3 cells were plated on the control and experiment Ti substrata that were previously attached to the bottom of the 96-well tissue culture plates (96-well Ti substrata) at a population density of 3×10^3 cell/ml and incubated in a humidified incubator at 37℃ with 5% CO_2 in 95% air for 16 and 24 h. 1 ml of bomodeoxyuridine (BrdU) labeling reagent (Roche Diagnostics GmbH, Mannheim, Germany) was added to each well and the cells were reincubated for 2 h at 37℃. Details of the BrdU assay procedure human gingival fibroblasts were reported in our previous study.[16] In all groups, the reaction products were transferred to 96-well plates and the absorbance was measured by monitoring the light absorbance of the solution at 370 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Alkaline phosphatase activity test

To estimate the osteogenic activity, the level of alkaline phosphatase (ALP) activity was measured. MC3T3 cells were plated on the 12-well Ti substrata of the control and experiment groups at a density of 5×10^4 cells/ml and cultured for 2 days for confluence. The cells were then incubated in an osteogenic media [DMEM (WelGene, Daegu, Korea) supplemented with 10% FBS (Sigma-Aldrich Co., St. Louis, MO, USA), 50 ug/ml of α-ascorbic acid, 10 mM of β-glycerophosphate, 100 nM of dexametasone and antibiotics (Invitrogen, Carlsbad, CA, USA)] at 37℃, 5% CO_2 for 1, 7 and 14 days. The cultured cells were washed with PBS (Gibco BRL, Grand Island, NY, USA), removed by trypsin-EDTA solution, lysated with 0.1% Triton X-100 buffer and sonicated in ice. Aliquots of 50 μl were

incubated with 100 μl of 1 M Tris-HCl (pH 9.0), 5 mM $MgCl_2$, 20 μl of 5 mM p-nitrophenyl phosphate solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37℃. The reaction was quenched by adding 250 μl of 1 N NaOH and placing the mixture in ice. The level of p-nitrophenol production in the presence of ALP was measured by monitoring the light absorbance of the solution at 405 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Measurements were compared using p-nitrophenol standards and normalized using the total protein amounts to account for differences in the number of cells on different titanium substrata at individual time points.

Statistical analysis

Experiments involving the contact angle determination, BrdU assay and ALP activity test were repeated simultaneously and independently in triplicate and the mean values and standard deviations were calculated. One-way analysis of variance (one-way ANOVA) was used to compare the mean values between the groups of NE0, NE15/3.5, NE60/10, E0, E15/3.5 and E60/10. Pearson's correlation analysis was used to analyze the correlations between the results from the contact angle determination, BrdU assay and ALP activity test. Multiple regression analysis was used to determine the influential factors, among the hydrophilicity of Ti and cell proliferation, on the osteogenic activity. The SPSS 17.0 software program was used for all statistical analyses in this study.

RESULTS

Contact angle determination

In ANOVA, multiple comparisons of the data from the contact angle determination in parallel direction with the microgrooves showed the mean contact angle value of NE60/10 to be significantly smaller compared to that of NE0 ($P < .01$) and the mean values of the E-series to be significantly smaller compared to those of the NE-series ($P < .01$) (Fig. 2). In the perpendicular direction, the mean value of the NE0 was significantly greater compared to those of NE15/3.5, NE60/10, E0, E15/3.5, or E60/10 ($P < .01$) and the mean value of E15/3.5 was significantly smaller compared to that of NE60/10 ($P < .01$) (Fig. 2). Independent t-test (Student t-test), comparing the results between parallel and perpendicular contact angles, showed no differences in the mean values in all groups, except for NE15/3.5, where the contact angles measured in the direction perpendicular to the microgrooves were significantly smaller compared to those measured in the parallel direction ($P < .01$) (Table I). All other comparisons between groups were not statistically significant.

Bromodeoxyuridine cell proliferation assay

In ANOVA, multiple comparisons of the MC3T3 cell proliferation data from the BrdU assay at 24 h incubation showed the mean OD value of E60/10 to be significantly greater compared to that of NE15/3.5, E0 or E15/3.5 ($P < .05$) (Fig. 3). All other comparisons between groups, including those at 16 h incubation, were not statistically significant.

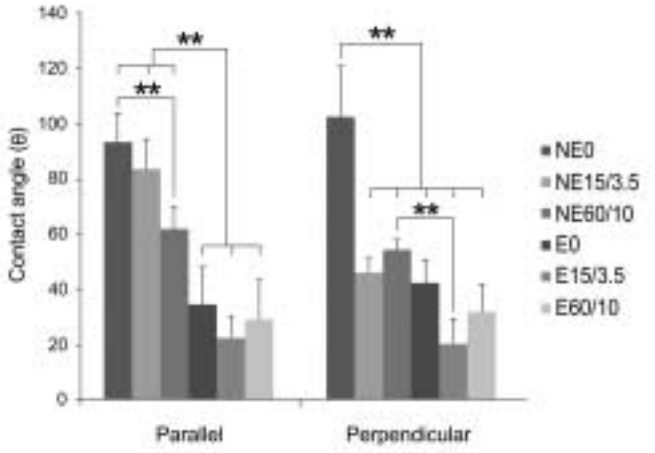

Fig. 2. Multiple-comparison result of the contact angle determination on titanium substrata with various surface topographies measured in directions parallel with and perpendicular to the microgrooves. Statistical significances were tested among NE0, NE15/3.5, NE60/10, E0, E15/3.5, and E60/10 using one-way ANOVA (n = 3). **: significant difference ($P < .01$).

Table I. Comparison of the contact angles on Ti substrata between those measured in the directions parallel with and perpendicular to the microgrooves (θ).

	Parallel (n = 3)	Perpendicular (n = 3)	Sig.[1)]
NE0	93.5 ± 10.2	102.4 ± 18.0	ns 0.497
NE15/3.5	83.3 ± 11.1	46.1 ± 4.9	< 0.01
NE60/0	62.0 ± 7.8	54.2 ± 3.6	ns 0.195
E0	34.8 ± 13.4	42.0 ± 8.5	ns 0.479
E15/3.5	22.7 ± 7.7	20.4 ± 8.8	ns 0.753
E60/0	29.5 ± 14.6	31.8 ± 9.7	ns 0.826

1) Statistical significances were tested by Student t-test with equal variances assumed. ns: non-significant.
See table I for nomenclature.

Alkaline phosphatase activity test

In ANOVA, multiple comparisons of the MC3T3 cells' osteogenic activity data from the ALP activity test after 1, 7 and 14 days of osteogenic culture showed no significant differences in the mean ALP activity values (Fig. 4).

Correlation and regression analyses

The results from seven experiments such as the contact angle determination in the directions parallel with and perpendicular to the microgrooves (Parallel and Perpendicular);

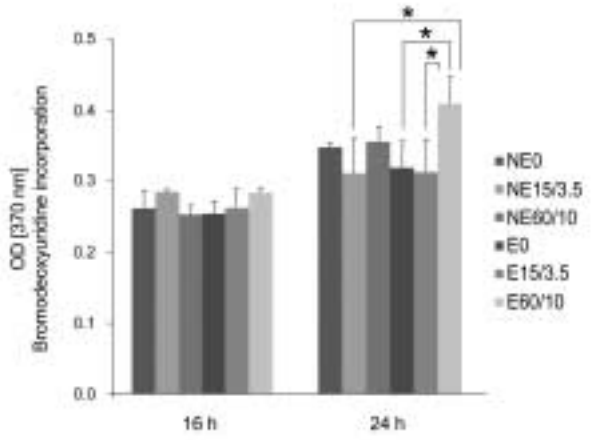

Fig. 3. Multiple-comparison result of the cell proliferation of MC3T3 mouse preosteoblasts on titanium substrata with various surface topographies after 16 and 24 h of culture using bromodeoxyuridine assay. Statistical significances were tested among NE0, NE15/3.5, NE60/10, E0, E15/3.5, and E60/10 using one-way ANOVA ($n = 3$). *: significant difference ($P < .05$).

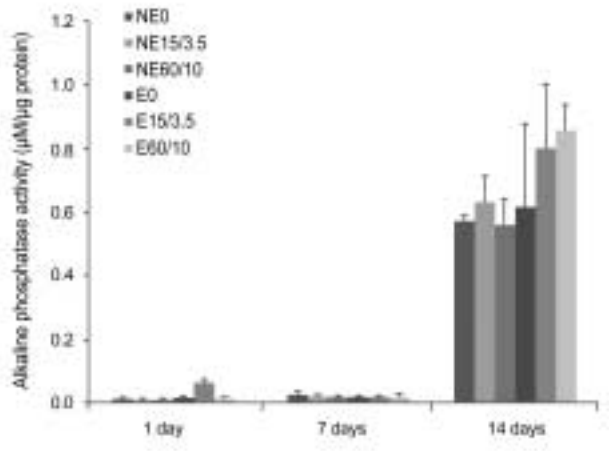

Fig. 4. Multiple-comparison result of the alkaline phosphatase activity test of MC3T3 mouse preosteoblasts on titanium substrata with various surface topographies after 1, 7 and 14 days of osteogenic culture. Statistical significances were tested among NE0, NE15/3.5, NE60/10, E0, E15/3.5, and E60/10 using one-way ANOVA ($n = 3$). The result showed no significant differences in the osteoblastic differentiation between and within all groups ($P < .05$).

Table II. Pearson correlation coefficients between the results from the contact angle determination, bromodeoxyuridine assay and alkaline phosphatase activity test

	Parallel	Perpendicular	BrdU 16 h	BrdU 24 h	ALP 1 day	ALP 7 days
Perpendicular	0.831**					
BrdU 16 h	-0.103	-0.337				
BrdU 24 h	-0.207	-0.053	0.364			
ALP 1 day	-0.378	-0.362	0.043	-0.017		
ALP 7 days	0.240	0.261	0.466	0.256	0.052	
ALP 14 days	-0.654**	-0.598**	0.624**	0.387	0.302	0.230

**: Correlation is significant at the 0.01 level (2-tailed). N = 18. Parallel and Perpendicular: the result from the contact angle analysis in the directions parallel with and perpendicular to the microgrooves. BrdU α h: the result from the bromodeoxyuridine assay of MC3T3 mouse preosteoblasts at α h incubation. ALP β day(s): the result from the alkaline phosphatase activity test of MC3T3 mouse preosteoblasts after β day(s) of osteogenic culture.

the BrdU assay of MC3T3 cells at 16 and 24 h incubation (BrdU 16 h and BrdU 24 h); and the ALP activity test after 1, 7 and 14 days of osteogenic culture (ALP 1 day, ALP 7 days and ALP 14 days) were used as the variables in the Pearson's correlation analysis. Significant correlations were present between Parallel and Perpendicular, Parallel and ALP 14 days, Perpendicular and ALP 14 days and BrdU 16 h and ALP 14 days ($P < .01$) (Table II and Fig. 5). Using ALP 14 days as the dependent variable and using Parallel, Perpendicular, BrdU 16 h and BrdU 24 h as the independent variables, multiple regression analysis in the enter method showed that all independent variables were determined to influence ALP 14 days (Table III). In multiple stepwise regression analysis using the dependent and independent variables identical to the enter method, both Parallel and BrdU 16 h were determined as the influential factors on ALP 14 days in the regression model 2, whereas Parallel was determined as the greatest influential factor on ALP 14 days in the regression model 1 (Table III).

DISCUSSION

Our result from the contact angle determination shows two unique characteristics. First, the contact angles measured in the directions parallel with (Parallel) and perpendicular to (Perpendicular) the microgrooves were not significantly different except NE15/3.5, on which Perpendicular was significantly smaller compared with Parallel (Table I). While the result has scarcely been reported related to the microgrooved Ti surfaces, one study reported the wetting characteristics on the polymer surfaces with nano-scale grooves.[17] In the study, the contact angles measured parallel with various dimensions of nanogrooves were larger than those measured in the per-

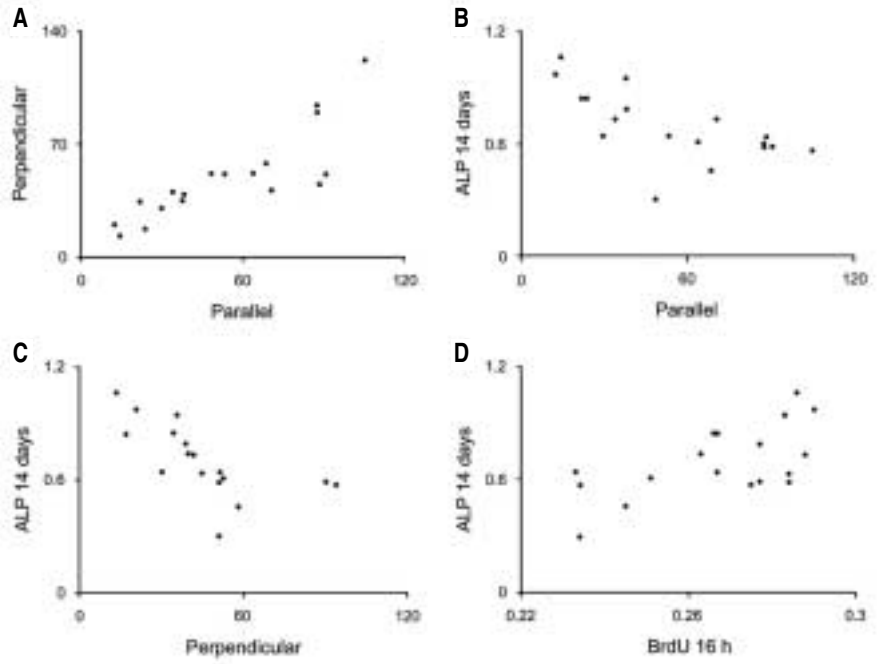

Fig. 5. Scatter-plot results from the Pearson's correlation analysis. Correlations of the data and results between A Parellel and Perpendicular, B Parellel and ALP 14 days, C Perpendicular and ALP 14 days and D BrdU 16 h and ALP 14 days are presented. Significant correlations were present A, B, C and D ($P < .01$). See table II for nomenclature and the overall results.

Table III. Factors influencing osteoblastic differentiation of MC3T3 mouse preosteoblasts cultured on microgrooved titanium substrata as determined by multiple regression analysis

Method	Dependent variable	Independent variables	Regression results	R	R^2	Sig.[1)]
Enter	ALP 14 days	Parallel	ALP 14 days = -0.794 - 0.006 · [Parallel]	0.878	0.771	< 0.001
		Perpendicular	-0.002 · [Perpendicular]			
		BrdU 16 h	+6.403 · [BrdU 16 h]			
		BrdU 24 h	+0.088 · [BrdU 24 h]			
Stepwise	ALP 14 days Model 1	Parallel	ALP 14 days = 0.923 - 0.004 · [Parallel]	0.654	0.428	< 0.01
		Perpendicular (excluded)				
		BrdU 16 h (excluded)				
		BrdU 24 h (excluded)				
	ALP 14 days Model 2	Parallel	ALP 14 days = -0.546 - 0.004 · [Parallel]	0.861	0.741	< 0.001
		Perpendicular (excluded)	+ 5.428 · [BrdU 16 h]			
		BrdU 16 h				
		BrdU 24 h (excluded)				

1) Significances were tested by analysis of variance. R: coefficient of multiple correlations. R^2: coefficient of determination. N = 18. See table II for nomenclature.

pendicular direction, which do not correspond with our contact angle result in general. Second, the etched microgrooves and ridges showed higher hydrophilicity compared to the microgrooves-only in this study, suggesting that increase in wettability of Ti requires combined surfaces of micron- and submicron topography. We suggest that our contact angle result upgraded the pre-existing knowledge that tissue integration is mainly influenced by surface hydrophilicity, rather than by microtopography.[12]

We have reported in our previous studies that E60/10 promotes human gingival fibroblast proliferation and that E15/3.5 could also be a strong candidate.[14,16] However, the experiment surfaces used in the studies included only smooth Ti and the E-series as the culture substrata. Since the differences in the proliferation data were not statistically significant at 24 h among 24, 48, 72, and 96 h in our previous study,[14] we attempted to seek for another possibility by selecting 24 h in this study. Indeed, the BrdU-assay result showed that E60/10 Ti substrata were potent promoters of MC3T3-cell proliferation also at the early time point of culture, whereas the presence or absence of acid-etched roughness on Ti had no effect. However, lack of correspondence in the result from the BrdU assay

with that from the contact angle determination suggests that microgroove-dimension and submicron-geometry rather than hydrophilicity affect the MC3T3-cell proliferation.

Unfortunately, we failed to directly correlate the result of contact angle determination with that of the MC3T3 cell proliferation, in which none of the results from the BrdU assay at 16 and 24 h incubation (BrdU 16 h and BrdU 24 h) correlated with Parallel or Perpendicular (Table II). Consistently, MG63 cells have previously been reported to be highly proliferative on the grooved Ti surfaces that showed inferior hydrophilicity.[18] However, the osteogenic activity of MC3T3 cells after 14 days of osteogenic culture (ALP 14 days) showed a significant correlation with both Parallel and Perpendicular in this study. Since the MC3T3 cells in this study showed statistically non-significant increase in the osteogenic activity on E15/3.5 and E60/10 compared to the controls, further investigations into the osteogenic activity and its correlation with the hydrophilicity analyzing more groups of microgroove dimensions are strongly required. Among various factors, the non-significant result is considered mainly due to the increased standard deviations in E0, E15/3.5 and E60/10.

Taken together, two major differences were found from the comparison of the cell proliferation and osteogenic activity in this study. First, E60/10 promoted cell proliferation but did not statistically enhance osteogenic activity. Second, the hydrophilicity of Ti correlated with the osteogenic activity but not with the cell proliferation. Interesting is that BrdU 16 h and ALP 14 days showed strong correlation to each other (Table II and Fig. 5) affecting the result of the regression result in this study. In the multiple regression analysis, both hydrophilicity and cell proliferation were determined as the influential factors on osteogenic activity (Table III), suggesting that the Ti surfaces, such as the combined micron- and submicron-scale topographies in this study, that are capable of enhancing hydrophilicity and cell proliferation could also lead to an alteration in osteogenic activity. Determination of Parallel as the greatest influential factor on osteogenic activity is considered partly due to the greater values of Parallel compared to Perpendicular on NE15/3.5.

CONCLUSION

We have demonstrated that etched microgrooves of 60-μm width and 15-μm depth on Ti substrata significantly enhance the cell proliferation but have no statistically significant effect on the osteogenic activity of MC3T3 mouse preosteoblasts. We have also demonstrated a finding that the hydrophilicity of Ti enhanced by etched microgrooves is one of the major influential factors on osteoblastic differentiation. A further investigation using various Ti topographies with a wide range of combined micron- and submicron-scale dimensions is strongly required.

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