The Effect of Fibronectin-Immobilized Microgrooved Titanium Substrata on Cell Proliferation and Expression of Genes and Proteins in Human Gingival Fibroblasts

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Abstract

BACKGROUND: We aimed to determine the effect of fibronectin (FN)-immobilized microgrooved titanium (Ti) on human gingival fibroblast proliferation, gene expression and protein expression.

METHODS: Photolithography was used to fabricate the microgrooved Ti, and amine functionalization (silanization) was used for FN immobilization on titanium surfaces. Cell proliferation, gene expression and protein expression were analyzed, followed by multiple regression analysis for determining the influential factors on cell proliferation.

RESULTS: FN-immobilized microgrooved Ti significantly enhanced the fibroblast proliferation in various timelines of culture, among which a burst of fivefold increase is induced at 96 h of culture compared to that on the control smooth Ti. We suggest a presence of the synergistic promotion effect of microgrooves and FN immobilization on fibroblast proliferation. Through a series of analyses on the expression of various genes and proteins involved in cell adhesion and proliferation, cyclin-dependent kinase 6, cyclin D1, integrin $\alpha 5$, oncogene c-Src, osteonectin, paxillin and talin-2 were determined as influential factors on promoting fibroblast proliferation induced by FN-immobilized microgrooved Ti.

CONCLUSION: FN-immobilized microgrooved Ti can act as an effective surface for enhancing fibroblast proliferation, and can be used for promoting soft tissue response on the connective tissue attachment zone of biomaterial surfaces.

Keyword Fibronectin · Microgrooved titanium · Cell proliferation · Gene and protein expression

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1 Introduction

Rapid advances in the understanding of osseointegration have recently led oral implantologists to focus on improving the peri-implant soft-tissue reaction [1]. Titanium (Ti)-surface microgrooves have been studied as a method for improving the peri-implant soft-tissue reaction by examining *in vitro* cell behavior. The cross section of the truncated V-shaped microgroove shows grooves and ridges of uniform width on Ti substrata. These microgrooves induce contact guidance and up-regulate the expression of various genes, including the fibronectin (FN), in human gingival fibroblasts (HGFs). However, cell adhesion and cell proliferation are not significantly induced [2]. Acid-etching on microgrooved Ti substrata significantly enhances cell proliferation and alters the expression of various genes and proteins, including FN and its protein product [3]. More specifically, 60- μm -wide and 10- μm -deep etched microgrooves and ridges trigger important *in vitro* HGF responses, such as cell adhesion and adhesion-dependent cell proliferation [4]. An earlier investigation examined the up-regulation of FN by microgrooves in fibroblasts. This study included multiple methods and concluded that microgrooved Ti modulates FN at the transcriptional and post-transcriptional levels. In addition, microgrooved Ti regulates the amount of FN that assembles into the extracellular matrix (ECM) [5]. Several studies suggest that Ti-surface microgrooves exclusively control endogenous FN expression. However, etched microgrooves and ridges also appear to increase the degree to which exogenous human plasma FN is adsorbed onto the Ti substrata [6]. These data suggest a need to determine if a secondary organic coating of FN on microgrooved Ti, such as immobilized FN, would further enhance the HGF response.

Because of the important roles of ECM proteins in cellular responses *in vivo*, various full-length ECM proteins have been applied on Ti surfaces to establish potential adhesive mimetic coatings for oral or orthopedic implants. Soluble plasma FN was first discovered to bind to collagen and gelatin and to play an important role in malignant growth [7]. Various extracellular proteins, including heparin and plasma FN, were later verified to bind to fibrous cellular FN. These data suggest the possibility that an immobilized plasma FN coating on silanized Ti [8] would

be an effective method for maximizing the numerous roles of FN in overall cell behavior and fate. There are potential disadvantages to using full-length FN coatings on Ti, such as low solubility, high cost, uncontrollable variation, and the presence of *in vivo* immunogenicity [9]. However, the numerous binding domains on FN for various ECM proteins [10] are important advantages for using an FN-immobilized surface as a functional cell-adhesive substratum. Fibroblasts need to form adhesions with ECM proteins, such as FN, for growth and proliferation. Integrin $\alpha 5 \beta 1$ is a representative FN receptor that connects the cell to FN and relays bi-directional signals [11]. FN is crucial for the completion of focal adhesion assembly and the generation of fibrillar cell adhesions [12, 13]. Moreover, despite the ability of collagen-I fibrils to self-assemble *in vitro*, they do not assemble *in vivo* without FN, FN/collagen-binding integrins, or collagen V [14]. These results suggest that FN is essential for collagen-I fibrillogenesis, which eventually comprises the soft connective tissue and bone around oral or orthopedic Ti implants. Thus, FN-immobilized coatings on Ti-surface-etched microgrooves and ridges would greatly benefit HGF adhesion, allowing a strong barrier to be established at the connective-tissue-attachment zone of oral implants or abutment surfaces [15].

The aim of this study was to determine the effects of FN-immobilized microgrooved Ti on cell proliferation and gene expression in HGFs. To fulfill our aim, we determined the optimal concentration of immobilized FN on Ti and compared the amount of immobilized FN between smooth and microgrooved Ti. We then examined HGF proliferation and analyzed the expression of various genes and proteins that are involved in cell adhesion and proliferation.

2 Materials and methods

2.1 Fabrication of titanium substrata and fibronectin immobilization

For the fabrication of titanium substrata and fibronectin immobilization, We used photolithography to fabricate 60- μm -wide and 10- μm -deep microgrooves on commercially-pure Ti sheets (0.14 mm-thick, grade-2; TSM-TECH Co. Ltd., Ulsan, Korea) (Fig. 1) as previously described [2]. Subsequent 1% hydrofluoric acid (HF) etching was performed for two seconds to generate a surface with etched microgrooves and ridges (E60/10) (Fig. 1). Titanium was ground with a cloth wheel (Yougar Enterprise Inc., Incheon, Korea) at 1800 rotations per minute on an angle grinder (GWS 20-230, Robert Bosch GmbH, Stuttgart, Germany) to obtain a polished surface. $R_a \leq 0.1 \mu\text{m}$ (NE0) and HF-etched (E0) Ti were both used as controls (Fig. 1). The floors of 96-, 24-, or 6-well tissue culture

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Fig. 1 Scanning electron microscopic images of the surfaces of NE0, E0, E60/10, NE0Si, and NE0FN ($\times 200$). Si: silanized Ti, FN: fibronectin-immobilized Ti. Scale bar: 100 μm

plates were removed. The remaining polystyrene cylinders were attached to the fabricated surfaces of 10-, 24- or 45-mm-diameter Ti discs with a silicone-bonding agent to prepare the 96-, 24-, or 6-well Ti substrata, respectively. Human serum FN was immobilized on 3-aminopropyltriethoxysilane (APTES)-functionalized (APTES-silanized) (Sigma-Aldrich, St. Louis, MO, USA) Ti surfaces. Amine functionalization (silanization) was performed in anhydrous toluene by adding 1 ml of APTES to 9 ml toluene solution per well of Ti substrata. The reaction was maintained at 120 °C in a nitrogen atmosphere with a reflux condenser for 24 h. After the reaction, Ti substrata were washed three times with methanol to remove residual silane. The amino-functionalized (silanized) Ti substrata were dried with a vacuum pump for 24 h. The surfaces of amino-functionalized (silanized) Ti substrata were washed three times for 20 min each time with distilled water in an

ultra-sonicator. Human plasma FN (human pFN, Sigma-Aldrich) was immobilized on the surface of amino-functionalized (silanized) Ti substrata at various concentrations ($\mu\text{g/ml}$) and maintained for 24 h at 4 °C. After the reaction, the pFN-immobilized Ti substrata were washed with distilled water to remove unbound pFN and dried at room temperature for 45 min. As a result, the FN-immobilized ground-Ti substrata (NE0FN), FN-immobilized acid-etched Ti substrata (E0FN), and FN-immobilized microgrooved-Ti substrata (E60/10FN) were fabricated and prepared (Table 1).

2.2 Surface characterization

Surfaces of NE0, E0, E60/10, NE0Si (silanized Ti) and NE0FN (FN-immobilized Ti) were imaged using field-emission scanning electron microscopy (FE-SEM, LEO

Table 1 Titanium substrata with various surface topographies and fibronectin (FN) immobilization used in this study

Surface treatment	NE0	E0	E60/10	NE0FN	E0FN	E60/10FN
Groove width (μm)	0	0	60	0	0	60
Groove depth (μm)	0	0	10	0	0	10
Bottom width (μm)	0	0	40	0	0	40
Subsequent acid-etching	Non-etched	Acid-etched	Acid-etched	Non-etched	Acid-etched	Acid-etched
FN immobilization	Non-immobilized	Non-immobilized	Non-immobilized	FN-immobilized	FN-immobilized	FN-immobilized

NE0, smooth titanium (Ti) substrata; E0, NE0 with subsequent hydrofluoric acid etching; E60/10, Ti substrata with surface microgrooves of 60 μm width and 10 μm depth; α FN, fabricated α Ti substrata with subsequent fibronectin immobilization. Note that the bottom width of microgrooves is calculated according to the isotropic principle of wet etching photolithography

SUPRA 55, Carl Zeiss, Oberkochen, Germany). EDS mapping was performed on non-silanized (NE0), silanized (NE0Si) and FN-immobilized (NE0FN) Ti substrata using GENESIS 2000 (EDAX, Carl Zeiss, Oberkochen, Germany). Also, the Fourier transform infrared (FTIR) spectra for NE0, NE0Si and NE0FN were analyzed using Spectrum One FT-IR Spectrometer (Perkin-Elmer, Waltham, MA, USA).

2.3 Cell culture and imaging

Healthy gingival tissues were acquired from patients receiving oral surgery for the removal of impacted wisdom teeth at the Department of Oral and Maxillofacial Surgery. Informed consent guidelines were followed as described in an approved Institutional Review Board protocol. HGFs were cultured from tissues as previously described [2]. Cells were used at passages three to five. At 16 h of adhesion, cells were then incubated with mouse monoclonal anti-vinculin-antibody (Ab; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After washing, cells were incubated with goat anti-mouse immunoglobulin G-fluorescein isothiocyanate (IgG-FITC; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, washed with PBS and mounted using mounting medium with diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Samples were imaged using confocal laser scanning microscopy (LSM510META®; Carl Zeiss, Oberkochen, Germany) (Fig. 2).

2.4 Optimization of the concentration of administered fibronectin on titanium substrata

To determine the optimal concentration of FN to induce the most favorable HGF response, cell proliferation was assessed at various FN concentrations. HGFs were plated on NE0FN, E0FN, or E60/10FN with 1 ml of FN solution

at the concentrations of 0, 1, 5, 10, 100, or 1000 µg/ml in 24-well Ti substrata. Proliferation was analyzed with the Cell Counting Kit (CCK-8, Dojindo, Kuramoto, Japan). The 0 µg/ml FN concentration represented a fabricated Ti-substrata surface lacking FN immobilization. HGFs were seeded at a density of 1×10^4 cells/well in 24-well Ti complexes comprising 18 different groups of substrata and cultured at 37 °C in 5% CO₂. After 24 h, 50 µl CCK reagent (Dojindo, Kuramoto, Japan) were added to each well of Ti complex and incubated for another 2 h. The reaction products were transferred to 96-well plates and monitored at 450 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

2.5 Quantification of the fibronectin immobilized on titanium substrata

The quantity of FN-immobilized on amino-functionalized (silanized) Ti substrata was determined by performing an enzyme-linked immunosorbent assay (ELISA). The supernatant obtained after human pFN was immobilized was tested with the human FN ELISA Kit (Abnova, Walnut, CA, USA). The FN-immobilized Ti substrata (NE0FN, E0FN, and E60/10FN) with 1 or 10 µg/ml FN were immersed in a microwell plate coated with rabbit polyclonal antibody against human FN. Fifty microliters of biotin-conjugate were immediately added and incubated at room temperature for 2 h. The microwell plate was then washed six times with wash buffer. Subsequently, 100 µl of streptavidin-horseradish peroxidase (HRP) was added and incubated at room temperature for 1 h. The microwell plate was washed another six times with wash buffer. Further, 100 µl of tetramethylbenzidine (TMB) substrate solution was added. The reaction was induced in the dark for 10 min (blue color) followed by 100 µl of Stop solution to stop the peroxidase-catalyzed reaction (yellow color). The absorbance was measured at 450 nm on a microplate reader (Bio-Rad, Hercules, CA, USA). A standard curve

Fig. 2 Confocal laser scanning microscopic images of human gingival fibroblast morphology and adhesion at 16-h incubation (×200). Fluorescent green: vinculin. Blue: nucleus by diamidino-2-phenylindole staining. Scale bar: 50 µm. (Color figure online)

was prepared from seven standard dilutions of FN, and the concentration of FN was determined in each sample. Measurements were subtracted from the initial amount of FN (1 or 10 µg/ml) that was added during the immobilization procedure to determine the concentration of FN that remained on the Ti substratum.

2.6 Cell proliferation assay

HGFs were seeded in 24-well plates at a density of 1×10^4 cells/well in Ti substrata of NE0, E0, E60/10, NE0FN, E0FN, or E60/10FN and cultured for 48, 72, and 96 h at 37 °C in 5% CO₂. The concentration of immobilized FN on Ti was determined to be 10 µg/ml with the optimization procedure described above. A cell density of 1×10^4 cells/well was applied for the optimization procedure described above. However, 1×10^4 cells/well resulted in an excessive level of cell proliferation by 72 or 96 h, yielding data that were beyond the limit of measurement for the proliferation assay method used in this study. Thus, a density of 5×10^3 cells/well was used for the cell proliferation assay. Fifty microliters of CCK reagent were added to the 24-well Ti substrata and incubated for 2 h. The reaction products were transferred to 96-well plates and monitored at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.7 Gene expression

The expression levels of 76 genes involved in cell adhesion and proliferation were analyzed by RT-PCR. HGFs were seeded in 24-well Ti substrata of NE0 and E60/10FN at a density of 1×10^4 cells/well and cultured for 48 h at 37 °C in 5% CO₂. PCR primers were designed with PRIMER3 software (Primer3 Input, version 0.4.0). The primer sequences are shown in Supplementary Table 1. Extraction of total RNA and RT-PCR were performed as previously described [3]. Based on the RT-PCR results, 44 of 76 genes were selected for mRNA expression analysis by quantitative real-time PCR. HGFs were seeded in 24-well Ti substrata of NE0, E0, E60/10, NE0FN, E0FN, or E60/10FN at a density of 1×10^4 cells/well and cultured for 48 and 72 h at 37 °C in 5% CO₂. Extraction of total RNA and quantitative real-time PCR were performed as previously described [16]. Relative expression levels were analyzed by normalizing the experimental values to those of the internal control, GAPDH. The relative expression levels obtained on E0, E60/10, NE0FN, E0FN, or E60/10FN are presented as fold-change relative to the levels obtained on the control Ti substrata, NE0, at 48 h.

2.8 Western blotting and protein quantification

Based on the gene expression results, 10 of 44 genes were selected, and the corresponding protein expression was

confirmed by western blotting and protein quantification. HGFs were seeded at a density of 2×10^5 cells/well and cultured for 48 h in six-well Ti substrata of NE0, E0, E60/10, NE0FN, E0FN, or E60/10FN. Western blotting and protein quantification were performed in all of the substrata groups to detect and quantify the expression levels of cyclin-dependent kinase 6 (CDK6), v-myc avian myelocytomatosis viral oncogene homolog (c-Myc), v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (c-Src), cyclin D1, extracellular signal-regulated kinase 1 (ERK1), integrin $\alpha 5$, nuclear factor κ B (NF κ B), osteonectin (ON), paxillin, and talin-2 as previously described [16]. Blots were re-probed with an anti- β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as an internal control. Protein expression levels on E0, E60/10, NE0FN, E0FN, and E60/10FN are presented as fold-change relative to the results obtained on the control Ti substrata, NE0.

2.9 Statistical analyses

ELISAs for FN quantification, cell proliferation assays, quantitative real-time PCR analyses, and western blotting/protein quantification were repeated simultaneously and independently five times. Mean values and standard deviations were calculated for each result. One-way analysis of variance (ANOVA) was calculated to compare the mean values of the results from NE0, E0, E60/10, NE0FN, E0FN, and E60/10FN. Two-way ANOVA was performed to identify the effects of interactions between Ti topography and FN immobilization on HGF proliferation. Pearson's correlation analysis was performed to determine correlations among the experimental results. Multiple regression analysis with the stepwise method was performed to identify proteins that have statistically significant influences on HGF proliferation. All statistical analyses were performed with the SPSS 18.0 software program.

3 Results

3.1 Surface characteristics

From the EDS mapping results, NE0 showed a 100% atomic percent of Ti, whereas NE0Si showed 12.25% C, 3% Si, and 84.75% Ti. Also, NE0FN showed 8.62% C, 6% N, 7.76% O, 1.54% Si, and 76.08% Ti (Supplementary data). The FTIR analyses showed that NE0Si exhibited the characteristic band at 950–1130 cm⁻¹, which was ascribed to the asymmetric stretching vibrations of the Si–O bonds [17]. For NE0FN, we found additional expression of amide I (mainly C=O stretching of amide coupled to NH₂ in-plane bending) and II bands (N–H bending coupled to a C–N stretching) at around

1680 cm^{-1} and 1540 cm^{-1} , respectively, originating from the peptide bonds of the relevant proteins [18]. From these results, we could identify the silanized surface of NE0Si and the FN-immobilized surface of NE0FN. (Supplementary Data).

3.2 Optimization and quantification of immobilized fibronectin

CCK cell proliferation assays showed significantly lower levels of cell proliferation at 24 h on NE0FN, E0FN, and E60/10FN with 0 $\mu\text{g}/\text{ml}$ FN (NE0, E0, and E60/10, respectively) compared to those of other FN-immobilized Ti substrata (Fig. 3). In addition, E60/10FN with either 1 or 5 $\mu\text{g}/\text{ml}$ FN produced significantly lower levels of cell proliferation compared to those of E60/10FN with 10, 100, or 1000 $\mu\text{g}/\text{ml}$ FN (Fig. 3). Our goal was to determine the optimal concentration of FN for microgrooved-Ti substrata. Among 0, 1, 5, 10, 100, and 1000 $\mu\text{g}/\text{ml}$ FN, 10 $\mu\text{g}/\text{ml}$ FN was determined to be the optimal concentration for E60/10FN to enhance HGF proliferation. The optimal concentration was determined by the absence of significant differences in cell proliferation on E60/10FN with 10, 100, or 1000 $\mu\text{g}/\text{ml}$ FN (Fig. 3), leading to the selection of 10 $\mu\text{g}/\text{ml}$ as the strong candidate for both minimal and optimal concentration of immobilized FN. Similarly, a previous study showed that the response to FN-immobilized Ti remains the same after a specific quantity and concentration of FN is reached [8]. The amount of FN that remained after immobilizing 10 $\mu\text{g}/\text{ml}$ FN was indirectly quantified by measuring the FN concentration in the reaction solution created by immersing the Ti substrata. The remaining FN concentration in the 1 $\mu\text{g}/\text{ml}$ FN group was also analyzed for comparison. Indeed, E60/10FN showed

significantly higher levels of residual FN in the 1 and 10 $\mu\text{g}/\text{ml}$ FN groups compared to those of NE0FN or E0FN (Fig. 4). These results suggest that the increased surface area at the microscale level produced by the microgrooves affected the amount of immobilized FN. However, E0FN also induced higher levels of FN immobilization in the 10 $\mu\text{g}/\text{ml}$ group compared to that of NE0FN (Fig. 4). Thus, the increased surface area at the sub-microscale level caused by the acid-etched roughness inside the microgrooves or at the top of the ridges also influenced the amount of immobilized FN.

3.3 Cell proliferation and imaging

Contract guidance of HGFs on E60/10FN was observed in CLSM (Fig. 2). The addition of microgrooves and immobilization of FN on Ti significantly enhanced the proliferation of HGFs compared to that achieved with the control substratum after 48, 72, and 96 h of culture. A significant increase in proliferation of up to fivefold was induced by E60/10FN compared to that with NE0 or E0 at 96 h (Fig. 5). An approximately twofold increase in HGF proliferation was achieved on E60/10 compared to that on NE0 according to a previous study [4]. Thus, we re-analyzed the fivefold increase induced by FN-immobilized Ti with two-way ANOVA. A consistently higher level of HGF proliferation was observed on NE0FN and E0FN compared to that on E60/10. These results suggested that FN immobilization triggered higher levels of HGF proliferation than that achieved by microgrooves alone. Indeed, two-way ANOVA revealed that an interaction effect existed between factors, such as FN immobilization and Ti topography (Tables 2 and 3). These data suggested strong synergism between Ti-surface microgrooves and FN

Fig. 3 Multiple comparison result of human gingival fibroblast proliferation on smooth, acid-etched, and microgrooved titanium substrata with various concentrations of immobilized human plasma fibronectin after 24 h of culture using Cell Count Kit cell proliferation assay (optical density value). One-way ANOVA ($n = 5$). ***Significant difference ($p < 0.001$)

Fig. 4 Multiple comparison result of the amount of fibronectin (FN)-immobilized on titanium substrata using the human FN ELISA Kit at 24 h incubation after the administration of 1 and 10 µg/ml FN (µg/ml). One-way ANOVA ($n = 5$). ***Significant difference ($p < 0.001$)

immobilization for enhancing HGF proliferation. In addition to altering the biology and chemistry of Ti substrata, FN produces nanoscale topography secondary to the sub-microscale acid-etched roughness and microscale grooves. The fivefold increase in HGF proliferation was probably due to the unique combination of nanoscale, sub-microscale, and microscale topographies and the favorable ECM environment. However, this possibility requires further investigation and characterization of the Ti surface,

including a deeper understanding of the chemistry, physical properties, nanoscale morphology, and the degree to which hydrophilicity is altered.

3.4 Expression of genes and proteins

We analyzed 76 genes involved in cell adhesion and proliferation. These genes were selected due to previous evidence that they were significantly up- or down-regulated in HGFs cultured on microgrooved-Ti substrata compared to that in HGFs cultured on smooth substrata [2, 3, 16]. RT-PCR analysis indicated that 43 genes were up-regulated, and six genes were down-regulated in HGFs cultured on E60/10FN compared to that of the control, NE0. The genes that were expressed by more than 1.1-fold (up-regulation) or less than 0.9-fold (down-regulation) on E60/10FN compared to that on NE0 were selected. Focal adhesion kinase (*FAK*) was removed from further experimental analysis due to a technical problem. The phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunits alpha, beta, and delta (*PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively) and regulatory cAMP-dependent protein kinase type I beta, type II alpha and type II beta (*PRKAR1B*, *PRKAR2A*, and *PRKAR2B*, respectively) were also removed. Instead, only the isoforms *PIK3CG* and *PRKARIA* were analyzed to avoid redundant analyses of isomers or analogues. In contrast, genes encoding integrin αv (0.99-fold relative expression), integrin $\beta 1$ (1.01-fold relative expression), and cyclin-dependent kinase inhibitor 1A (*p21CIP1*, 1.02-fold relative expression) were added for effective comparisons with the expression of other genes in the same family or those with associated functions. As a result, 44 of 76 genes were selected, and relative mRNA expression levels were analyzed. Based on the gene

Fig. 5 Multiple comparison result of human gingival fibroblast proliferation on smooth (NE0), acid-etched (E0), microgrooved (E60/10), and fibronectin-immobilized (NE0FN, E0FN, and E60/10FN) titanium substrata after 48, 72 and 96 h of culture using Cell Counting Kit cell proliferation assay (optical density value). One-way ANOVA ($n = 5$). ***Significant difference ($p < 0.001$)

Table 2 Analysis on the association between and interaction effect of the fibronectin (FN) immobilization and titanium (Ti) surface topography on the cell proliferation of human gingival fibroblasts after 72 h of culture using two-way ANOVA

Source	Sum of squares	Degrees of freedom	Mean square	F-statistics	P value ^a
Corrected model	2.582	5	0.516	8195.342	< 0.001
FN immobilization	2.067	1	2.067	40,552.795	< 0.001
Ti topography	0.502	2	0.251	87.522	< 0.001
FN immobilization × Ti topography	0.013	2	0.006	124.436	< 0.001
Error	0.001	12	5.10E–005 (0.000051)		

^aStatistical significances were tested by two-way analysis of variance among groups based on Tukey's multiple comparison tests

Table 3 Analysis on the association between and interaction effect of the fibronectin (FN) immobilization and titanium (Ti) surface topography on the cell proliferation of human gingival fibroblasts after 96 h of culture using two-way ANOVA

Source	Sum of squares	Degrees of freedom	Mean square	F-statistics	P value ^a
Corrected model	19.482	5	0.516	3261.366	< 0.001
FN immobilization	16.060	1	16.060	15,748.010	< 0.001
Ti topography	3.175	2	1.588	158.104	< 0.001
FN immobilization × Ti topography	0.247	2	0.124	121.260	< 0.001
Error	0.012	12	0.001		

^aStatistical significances were tested by two-way analysis of variance among groups based on Tukey's multiple comparison tests

expression results, we selected genes that were expressed by more than 1.3-fold (up-regulation) or less than 0.7-fold (down-regulation) on E60/10FN at 72 h compared to those on NE0 at 48 h. As a result, 10 of 44 genes were selected. The expression levels of the proteins encoded by these 10 genes were compared. CDK6, c-Myc, c-Src, cyclin D1, ERK1, integrin α 5, NF κ B, ON, paxillin, and talin-2 showed significant differences in expression among and within groups, with E60/10FN showing the highest expression level of each protein (Fig. 6).

3.5 Determination of influential protein expression on cell proliferation

The Pearson's correlation analysis indicated that there were significant correlations present between the expression level of each of the ten proteins and cell proliferation at 48, 72, and 96 h (Table 4). To determine which proteins influenced cell proliferation, protein expression was used as an independent variable, and cell proliferation at different time points served as a dependent variable. Multiple regression analyses showed that expression of CDK6, c-Src, cyclin D1, integrin α 5, ON, paxillin, and talin-2 had the most significant influence on HGF proliferation at 48 h on Ti substrata with various topographies and FN immobilization (Table 5). The expression patterns of cyclin D1,

integrin α 5, ON, and talin-2 influenced HGF proliferation at 72 h, whereas cyclin D1, integrin α 5, and talin-2 were influential factors at 96 h (Table 5). Thus, CDK6, c-Src, cyclin D1, integrin α 5, ON, paxillin, and talin-2 were significant factors that promoted HGF proliferation in response to FN-immobilized microgrooved-Ti substrata (Table 5). Among the 76 genes that were initially analyzed and among the seven proteins that were finally selected as influential factors, a statistical stepwise deletion of dependent variables from the multiple regression analyses showed that integrin α 5 was the most influential factor promoting fibroblast proliferation (Table 5).

4 Discussion

Effective adhesion of fibroblasts to the surface is required for the formation of a strong connective-tissue barrier around the connective-tissue-attachment zone of oral or orthopedic implants. The mechanism by which fibroblasts adhere to the underlying substratum can be represented *in vitro* by the formation of focal adhesions, leading to eventual cell–matrix adhesion [19]. Fibroblasts adhere to the substratum through the ECM and form an adhesion complex by generating a continuous cascade of trans-membrane proteins, such as integrins, cytoplasmic focal

Fig. 6 Multiple comparison result of the expression of CDK6, c-Myc, c-Src, Cyclin D1, ERK1, Integrin $\alpha 5$, NF κ B, ON, Paxillin and Talin-2 in human gingival fibroblasts on smooth (NE0), acid-etched (E0), microgrooved (E60/10), and fibronectin-immobilized (NE0FN, E0FN, and E60/10FN) titanium substrata after 48 h of culture using

western blotting and protein quantification. The relative expression levels were analyzed by normalizing with β -actin, and are presented as fold changes relative to the control, NE0. One-way ANOVA ($n = 5$). ***Significant difference ($p < 0.001$)

Table 4 Pearson correlation coefficients between the results of western blotting/protein quantification and cell proliferation assay

Protein marker	CDK6	c-Myc	c-Src	Cyclin D1	ERK1	Integrin $\alpha 5$	NF κ B	ON	Paxillin	Talin-2	CCK 48 h	CCK 72 h
c-Myc	0.922**											
c-Src	0.837**	0.912**										
Cyclin D1	0.940**	0.952**	0.846**									
ERK1	0.756**	0.848**	0.893**	0.844**								
Integrin $\alpha 5$	0.762**	0.878**	0.900**	0.874**	0.977**							
NF κ B	0.749**	0.856**	0.905**	0.794**	0.927**	0.917**						
ON	0.941**	0.966**	0.852**	0.973**	0.789**	0.837**	0.784**					
Paxillin	0.929**	0.945**	0.813**	0.984**	0.815**	0.859**	0.781**	0.982**				
Talin-2	0.912**	0.943**	0.881**	0.965**	0.888**	0.919**	0.872**	0.944**	0.959**			
CCK 48 h	0.724**	0.831**	0.907**	0.757**	0.877**	0.913**	0.904**	0.777**	0.750**	0.859**		
CCK 72 h	0.602**	0.751**	0.847**	0.686**	0.904**	0.929**	0.888**	0.679**	0.680**	0.792**	0.964**	
CCK 96 h	0.581**	0.725**	0.827**	0.674**	0.904**	0.927**	0.876**	0.654**	0.661**	0.784**	0.954**	0.995**

**Correlation is significant at the 0.01 level

N = 30

Table 5 The influential proteins of human gingival fibroblast proliferation after respective 48, 72 and 96 h of culture, as determined by multiple stepwise regression analysis

Dependent variable	Model	Regression results	R	R ²	Sig. ^a
CCK 48 h	1	CCK 48 h = $-0.023 + 0.382 \cdot [\text{Integrin } \alpha 5]$	0.913	0.834	< 0.001
	2	CCK 48 h = $-0.057 + 0.213 \cdot [\text{Integrin } \alpha 5] + 0.186 \cdot [\text{c-Src}]$	0.934	0.872	< 0.001
	3	CCK 48 h = $-0.030 + 0.290 \cdot [\text{Integrin } \alpha 5] + 0.227 \cdot [\text{c-Src}] - 0.153 \cdot [\text{Cyclin D1}]$	0.945	0.893	< 0.001
	4	CCK 48 h = $0.020 + 0.184 \cdot [\text{Integrin } \alpha 5] + 0.200 \cdot [\text{c-Src}] - 0.454 \cdot [\text{Cyclin D1}] + 0.382 \cdot [\text{Talin-2}]$	0.964	0.930	< 0.001
	5	CCK 48 h = $0.004 + 0.247 \cdot [\text{Integrin } \alpha 5] + 0.146 \cdot [\text{c-Src}] - 0.727 \cdot [\text{Cyclin D1}] + 0.344 \cdot [\text{Talin-2}] + 0.314 \cdot [\text{ON}]$	0.973	0.947	< 0.001
	6	CCK 48 h = $-0.020 + 0.315 \cdot [\text{Integrin } \alpha 5] + 0.035 \cdot [\text{c-Src}] - 0.572 \cdot [\text{Cyclin D1}] + 0.416 \cdot [\text{Talin-2}] + 0.639 \cdot [\text{ON}] - 0.483 \cdot [\text{Paxillin}]$	0.979	0.959	< 0.001
	7	CCK 48 h = $-0.022 + 0.338 \cdot [\text{Integrin } \alpha 5] - 0.562 \cdot [\text{Cyclin D1}] + 0.429 \cdot [\text{Talin-2}] + 0.701 \cdot [\text{ON}] - 0.554 \cdot [\text{Paxillin}]$	0.979	0.958	< 0.001
	8	CCK 48 h = $-0.199 + 0.386 \cdot [\text{Integrin } \alpha 5] - 0.659 \cdot [\text{Cyclin D1}] + 0.363 \cdot [\text{Talin-2}] + 0.622 \cdot [\text{ON}] - 0.494 \cdot [\text{Paxillin}] + 0.299 \cdot [\text{CDK6}]$	0.982	0.965	< 0.001
CCK 72 h	1	CCK 72 h = $-0.482 + 0.958 \cdot [\text{Integrin } \alpha 5]$	0.929	0.863	< 0.001
	2	CCK 72 h = $-0.334 + 1.434 \cdot [\text{Integrin } \alpha 5] - 0.644 \cdot [\text{Cyclin D1}]$	0.964	0.929	< 0.001
	3	CCK 72 h = $-0.371 + 1.466 \cdot [\text{Integrin } \alpha 5] - 1.349 \cdot [\text{Cyclin D1}] + 0.691 \cdot [\text{ON}]$	0.973	0.946	< 0.001
	4	CCK 72 h = $-0.313 + 1.319 \cdot [\text{Integrin } \alpha 5] - 1.614 \cdot [\text{Cyclin D1}] + 0.602 \cdot [\text{ON}] + 0.440 \cdot [\text{Talin-2}]$	0.977	0.954	< 0.001
CCK 96 h	1	CCK 96 h = $-1.826 + 2.615 \cdot [\text{Integrin } \alpha 5]$	0.927	0.859	< 0.001
	2	CCK 96 h = $-1.390 + 4.021 \cdot [\text{Integrin } \alpha 5] - 1.900 \cdot [\text{Cyclin D1}]$	0.967	0.936	< 0.001
	3	CCK 96 h = $-1.201 + 3.515 \cdot [\text{Integrin } \alpha 5] - 3.160 \cdot [\text{Cyclin D1}] + 1.558 \cdot [\text{Talin-2}]$	0.974	0.949	< 0.001

CCK α h, human gingival fibroblast proliferation assay result after α h of culture using the Cell Counting Kit (CCK-8; Dojindo, Kumamoto, Japan) assayR, coefficient of multiple correlations; R², coefficient of determination^aSignificances of each regression model were tested by analysis of variance (n = 30)

adhesion proteins, and cytoskeletal structures, including actin filaments [20]. We initially focused on the focal adhesion proteins c-Src, FAK, integrin-linked kinase (ILK), p21cip-activated kinase (PAK), paxillin, protein kinase C (PKC), talin, and vinculin and performed a thorough analysis of gene expression. Downstream signaling molecules, including *c-Crk*, *c-Fos*, *c-Fyn*, *c-Jun*, *c-Myc*, *Elk1*, *ERK*, *JNK*, *MEK*, *MKP*, *MLC*, *MLCK*, *NFκB*, *p130CAS*, *PAK*, *PIK3*, *PLC*, *PKA*, *regulatory PKA*, *PKB* (*AKT*), *RAC1*, *RAF1*, *RHOA*, and *ROCK* were also included in the gene expression analyses. Among the 76 genes that were initially selected, all of the above genes, except *regulatory PKA*, were significantly up-regulated in HGFs cultured on E60/10FN compared to those on NE0. Expression analyses of genes and proteins finally determined that *c-Src*, *paxillin*, and *talin-2* were influential factors promoting cell proliferation (Table 5). A series of analyses, including optimization and quantification of immobilized FN, cell proliferation, RT-PCR, quantitative real-time PCR, Pearson's correlation, and multiple regression, suggested that FN-immobilized microgrooved-Ti substrata promote HGF proliferation by activating focal-adhesion and cell–matrix-adhesion signaling. These results substantiated the validity of our experimental design.

Cyclin D1 couples with CDK4 and CDK6 to activate the retinoblastoma protein and stimulate activity of the E2F transcription factor [21–23]. These events are important for G1/S transition and cell-cycle progression. The prominent activity of CDK6 in mid-G1 is tightly regulated by Cyclin D1 and the CDK inhibitors p21cip1 and p27kip1 [24]. Cyclin D1 and CDK6 were determined as significant influential factors in this study (Table 5). Thus, we suggest that FN-immobilized Ti substrata stimulate cell-cycle progression from G1 to S phase, promoting fibroblast proliferation. This hypothesis corresponds with our previous results, which showed up-regulation of *cyclin D1*, *cyclin E*, and *CDK2/4/6* gene expression on E60/10 compared to that on NE0 [3]. However, *cyclin D1* expression is reduced on Ti surfaces that contain microgrooves and polished ridge tops, but not acid-etching, compared to that on smooth Ti surfaces [12]. Our previous and current expression analyses of genes involved in cell-cycle progression suggested that microgrooves alone did not up-regulate genes on Ti surfaces. Instead, a secondary treatment of surfaces, such as acid etching or FN immobilization, was required for up-regulation of genes. In this study, the *p27kip1* gene was up-regulated on E60/10FN compared to that on NE0. These data corresponded with those of our previous study, which suggested a possible role for p27kip1 in mediating and maintaining the cyclin D1-CDK4 complex. This function is in addition to the classically known function of p27kip1, which is to inhibit cyclin-CDK complex activation and negatively regulate cell-cycle

progression. Interestingly, *p21cip1* was up-regulated on E60/10 but not on E60/10FN compared to expression levels on other Ti substrata. These results are also consistent with our previous results and may be due to three-dimensional cell sensitivity [25]. The results of the cell-cycle gene expression analyses in the current study are mostly consistent with the results of our previous studies. However, there were some inconsistent results. In particular, the finding that E60/10 induced a significantly higher level of *p21cip1* expression than that on E60/10FN needs to be further confirmed. Our results indicated that FN-immobilized Ti substrata promoted fibroblast proliferation by effectively regulating the expression of specific genes involved in cell-cycle progression.

Ti substrata with microgrooves that are less than 10 μm in width induce changes in cell shape [26] and alter the expression of specific genes [5]. However, an earlier quantitative analysis revealed that these relatively small microgrooves can induce contact guidance and cell orientation without promoting fibroblast proliferation [27]. In contrast, the proliferation of fibroblasts is significantly enhanced on truncated, V-shaped, etched microgrooves that are of sufficient width and depth to induce fibroblast crawling or migration [3]. An FN coating on Ti enhances the long-term success of percutaneous implants by promoting soft-tissue attachment and possibly preventing infection or epithelial downgrowth [28, 29]. Thus, we designed a microgrooved-Ti surface silanized with FN that would synergistically induce fibroblast proliferation to a far greater extent than that on smooth Ti or microgrooved Ti. This unique microgrooved-Ti surface silanized with FN can be applied to various oral or orthopedic biomaterials to create a tight soft-tissue seal. Indeed, a fivefold increase in fibroblast proliferation was observed on E60/10FN compared to that on NE0 or E0 at 96 h (Table 3). These results were consistent with the results of several studies that showed increased dermal fibroblast attachment and attachment strength on FN-coated Ti alloys for up to 96 h in culture [8, 30]. We attempted to establish a mechanism for this synergy by thoroughly analyzing the expression levels of genes and proteins that are involved in cell adhesion and proliferation. There were seven highly influential proteins that were associated with fibroblast proliferation on FN-immobilized Ti surfaces (Table 5). All seven proteins are essential for cell-cycle progression, cell adhesion, cell proliferation, and cell survival. Integrin α5 was the most influential factor in this study (Table 5). Integrin α5 is critical for FN-mediated focal-adhesion formation and FN fibrillogenesis during matrix assembly [31, 32]. In association with integrin β1, integrin α5 functions as a receptor for FN [33]. Thus, plasma FN appears to be an excellent choice as a coating protein to induce maximum cell proliferation on biomaterial surfaces.

Although we initially intended to use the numerous protein-binding sites and domains of FN [34], it was challenging to use plasma FN as the coating protein on Ti due to first-phase, non-continuous recruitment and binding of proteins, in contrast to cellular fibrous FN. However, we observed a significant increase in fibroblast proliferation and potentially strong role for integrin $\alpha 5$. Therefore, immobilization of plasma FN on microgrooved Ti appeared to stimulate FN-mediated focal-adhesion formation and FN fibrillogenesis (i.e., matrix assembly), inducing fibroblast proliferation.

In conclusion, we found that the combination of FN immobilization and etched microgrooves and ridges on Ti substrata significantly enhanced fibroblast proliferation and altered the expression of genes and proteins that control cell adhesion and proliferation. Among the numerous genes and proteins that we analyzed, CDK6, c-Src, cyclin D1, integrin $\alpha 5$, ON, paxillin, and talin-2 were the most influential factors that promoted fibroblast proliferation on FN-immobilized microgrooved-Ti substrata. Future studies should include a larger number of gene expression profiling experiments in HGFs. In addition, further characterization of the unique Ti surfaces developed in this study is required to fully interpret the specific cellular responses observed in this study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This study was approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong (KHNC MD IRB 2010-011).

References

- Rompen E, Domken O, Degidi M, Pontes AE, Piattelli A. The effect of material characteristics, of surface topography and of implant components and connections on soft tissue integration: a literature review. *Clin Oral Implants Res.* 2006;17 Suppl 2:55–67.
- Lee SW, Kim SY, Rhyu IC, Chung WY, Leesungbok R, Lee KW. Influence of microgroove dimension on cell behavior of human gingival fibroblasts cultured on titanium substrata. *Clin Oral Implants Res.* 2009;20:56–66.
- Kim SY, Oh N, Lee MH, Kim SE, Leesungbok R, Lee SW. Surface microgrooves and acid-etching on titanium substrata alter various cell behaviors of cultured human gingival fibroblasts. *Clin Oral Implants Res.* 2009;20:262–72.
- Lee SW, Kim SY, Lee MH, Lee KW, Leesungbok R, Oh N. Influence of etched microgrooves of uniform dimension on in vitro responses of human gingival fibroblasts. *Clin Oral Implants Res.* 2009;20:458–66.
- Chou L, Firth JD, Uitto VJ, Brunette DM. Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J Cell Sci.* 1995;108:1563–73.
- Lee MH, Oh N, Lee SW, Leesungbok R, Kim SE, Yun YP, et al. Factors influencing osteoblast maturation on microgrooved titanium substrata. *Biomaterials.* 2010;31:3804–15.
- Engvall E, Ruoslahti E. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int J Cancer.* 1977;20:1–5.
- Middleton CA, Pendegrass CJ, Gordon D, Jacob J, Blunn GW. Fibronectin silanized titanium alloy: a bioinductive and durable coating to enhance fibroblast attachment in vitro. *J Biomed Mater Res A.* 2007;83:1032–8.
- Shekaran A, Garcia AJ. Nanoscale engineering of extracellular matrix-mimetic bioadhesive surfaces and implants for tissue engineering. *Biochim Biophys Acta.* 2011;1810:350–60.
- Leiss M, Beckmann K, Girós A, Costell M, Fässler R. The role of integrin binding sites in fibronectin matrix assembly in vivo. *Curr Opin Cell Biol.* 2008;20:502–7.
- Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. *Annu Rev Cell Dev Biol.* 2003;19:173–206.
- Zamir E, Katz BZ, Aota S, Yamada KM, Geiger B, Kam Z. Molecular diversity of cell-matrix adhesions. *J Cell Sci.* 1999;112:1655–69.
- Cohen M, Joester D, Geiger B, Addadi L. Spatial and temporal sequence of events in cell adhesion: from molecular recognition to focal adhesion assembly. *ChemBioChem.* 2004;5:1393–9.
- Kadler KE, Hill A, Canty-Laird EG. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. *Curr Opin Cell Biol.* 2008;20:495–501.
- Moon IS, Berglundh T, Abrahamsson I, Linder E, Lindhe J. The barrier between the keratinized mucosa and the dental implant. An experimental study in the dog. *J Clin Periodontol.* 1999;26:658–63.
- Lee MH, Kang JH, Lee SW. The significance differential expression of genes and proteins in human primary cells caused by microgrooved biomaterial substrata. *Biomaterials.* 2012;33:3216–34.
- Karakassides MA, Gournis D, Petridis D. An infrared reflectance study of Si–O vibrations in thermally treated alka-lisaturated montmorillonites. *Clay Miner.* 1999;34:429–38.
- Meade AD, Lyng FM, Knief P, Byrne HJ. Growth substrate induced functional changes elucidated by FTIR and Raman spectroscopy in in vitro cultured human keratinocytes. *Anal Bioanal Chem.* 2007;387:1717–28.
- Jockush BM, Bubeck P, Giehl K, Kroemker M, Moschner J, Rothkegel M, et al. The molecular architecture of focal adhesions. *Ann Rev Cell Dev Biol.* 1995;11:379–416.
- Jansen JA, den Braber ET, Walboomers XF, de Ruijter JE. Soft tissue and epithelial models. *Adv Dent Res.* 1999;13:57–66.
- Tam SW, Theodoras AM, Shay JW, Draetta GF, Pagano M. Differential expression and regulation of Cyclin D1 protein in normal and tumor human cells: association with Cdk4 is required for Cyclin D1 function in G1 progression. *Oncogene.* 1994;9:2663–74.
- Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol.* 1994;14:2077–86.
- Johnson DG. Regulation of E2F-1 gene expression by p130 (Rb2) and D-type cyclin kinase activity. *Oncogene.* 1995;11:1685–92.
- Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 1999;13:1501–12.
- Li S, Lao J, Chen BP, Li YS, Zhao Y, Chu J, et al. Genomic analysis of smooth muscle cells in 3-dimensional collagen matrix. *FASEB J.* 2003;17:97–9.

26. den Braber ET, de Ruijter JE, Ginsel LA, von Recum AF, Jansen JA. Quantitative analysis of fibroblast morphology on micro-grooved surfaces with various groove and ridge dimensions. *Biomaterials*. 1996;17:2037–44.
27. den Braber ET, de Ruijter JE, Smits HT, Ginsel LA, von Recum AF, Jansen JA. Quantitative analysis of cell proliferation and orientation on substrata with uniform parallel surface micro-grooves. *Biomaterials*. 1996;17:1093–9.
28. Holgers KM, Bjursten LM, Thomsen P, Ericson LE, Tjellström A. Experience with percutaneous titanium implants in the head and neck: a clinical and histological study. *J Invest Surg*. 1989;2:7–16.
29. Brånemark PI, Albrektsson T. Titanium implants permanently penetrating human skin. *Scand J Plast Reconstr Surg*. 1982;16:17–21.
30. Pendegrass CJ, Middleton CA, Gordon D, Jacob J, Blunn GW. Measuring the strength of dermal fibroblast attachment to functionalized titanium alloys in vitro. *J Biomed Mater Res A*. 2010;92:1028–37.
31. Pankov R, Cukierman E, Katz BZ, Matsumoto K, Lin DC, Lin S, et al. Integrin dynamics and matrix assembly: tensin-dependent translocation of $\alpha_5\beta_1$ integrins promotes early fibronectin fibrillogenesis. *J Cell Biol*. 2000;148:1075–90.
32. Clark K, Pankov R, Travis MA, Askari JA, Mould AP, Craig SE, et al. A specific $\alpha_5\beta_1$ integrin conformation promotes directional integrin translocation and fibronectin matrix formation. *J Cell Sci*. 2005;118:291–300.
33. Mould AP, Askari JA, Aota Si, Yamada KM, Irie A, Takada Y, et al. Defining the topology of integrin $\alpha_5\beta_1$ -fibronectin interactions using inhibitory anti- α_5 and anti- β_1 monoclonal antibodies. *J Biol Chem*. 1997;272:17283–92.
34. Olivares-Navarrete R, Raz P, Zhao G, Chen J, Wieland M, Cochran DL, et al. Integrin $\alpha_2\beta_1$ plays a critical role in osteoblast response to micron-scale surface structure and surface energy of titanium substrates. *Proc Natl Acad Sci U S A*. 2008;105:15767–72.