Effect of 3D microgroove surface topography on plasma and cellular fibronectin of human gingival fibroblasts

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ABSTRACT

Objectives: Fibronectin (FN), an extracellular matrix (ECM) glycoprotein, is a key factor in the compatibility of dental implant materials. Our objective was to determine the optimal dimensions of microgrooves in the transmucosal part of a dental implant, for optimal absorption of plasma FN and expression of cellular FN by human gingival fibroblasts (HGFs).

Methods: Microgroove titanium surfaces were fabricated by photolithography with parallel grooves: 15 μm, 30 μm, or 60 μm in width and 5 μm or 10 μm in depth. Smooth titanium surfaces were used as controls. Surface hydrophilicity, plasma FN adsorption and cellular FN expression by HGFs were measured for both microgroove and control samples.

Results: We found that narrower and deeper microgrooves amplified surface hydrophobicity. A 15-μm wide microgroove was the most hydrophobic surface and a 60-μm wide microgroove was the most hydrophilic. The latter had more expression of cellular FN than any other surface, but less absorption of plasma FN than 15-μm wide microgrooves. Variation in microgroove depth did not appear to affect FN absorption or expression unless the groove was narrow (~15 or 30 μm). In those instances, the shallower depths resulted in greater expression of cellular FN.

Conclusions: Our microgrooves improved expression of cellular FN, which functionally compensated for plasma FN. A microgroove width of 60 μm and depth of 5 or 10 μm appears to be optimal for the transmucosal part of the dental implant.

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1. Introduction

Soft tissue integration is a prerequisite for implant success and requires an effective seal between the soft tissue and implant to protect the underlying bone from microorganism invasion. Peri-implantitis begins at the soft tissue of implant is a risk factor in implant failure. The soft tissue interface consists of two zones: a thin epithelium and a thicker connective tissue. Connective tissues have poor mechanical resistance at the implant interface compared to natural teeth. Furthermore, as Jansen et al. state: “the quality of the connective tissue in the transitional area is apparently more important for the long-term prognosis of oral implants than the epithelial attachment”. Epithelial down-growth and attachment to the implant can be inhibited by a firm connection between the underlying connective tissue and the implant. Our study focused on reinforcing the attachment between connective tissue and titanium implants via well-defined surface topographies.

New advances in micro-electromechanical systems (MEMS) allow the fabrication of biological MEMS (BioMEMS) and have been used in the investigation of cell response to microgrooved surfaces. Microgrooves integrated into the structure of an implant have advantages compared with the traditionally smooth implant surface. For instance, epithelial down-growth could be inhibited by microgroove-induced contact guidance of connective tissue during growth. The microgroove surface has also affects cell interaction and behaviour, including modulation of cell adhesion, proliferation and gene expression. However, how the specific microgroove dimensions affect the extracellular matrix (ECM) has not yet been studied.

The organization and composition of the ECM mediate cell adhesion by controlling the degree to which cells can attach. Fibronectin (FN) is a high-molecular weight (~440 kDa) glycoprotein of the ECM that is a typical marker of the biocompatibility of new materials for connective tissue in dental implant studies. FN occurs in two forms: soluble plasma FN in blood, and insoluble cellular FN in ECMs or on cell surfaces. Plasma FN is a major protein component of blood plasma and is produced in the liver by hepatocytes. Cellular FN is secreted by various cells, primarily fibroblasts, as a soluble protein dimer and is then assembled into an insoluble matrix in a complex cell-mediated process. In both forms, FN is involved in tissue repair. The plasma form of FN is incorporated into fibrin clots, affecting platelet function and mediating the early stages of wound healing. Plasma FN also can be bound to the cell surface and assembled into extracellular fibrils. Cellular FN is synthesized and assembled by cells for attachment to the ECM as they migrate into a clot to repair damaged tissue. Both forms of FN are vital for establishing and maintaining tissue architecture and for regulating cellular processes and behaviours, such as adhesion, spreading, proliferation, migration and differentiation.

Plasma proteins are spontaneously adsorbed onto an implant surface within seconds and therefore help determine the bioactivity of implants. Plasma FN plays an important role in the interactions of implants with their surrounding ECMs by enhancing the attachment of cells to implant materials. Furthermore, the surface structure and wettability of implant materials determine the extent of protein adsorption. However, observations regarding the effects of surface wettability on protein have not been consistent. Though many previous studies have shown that FN adsorption is enhanced on hydrophobic surfaces, it has also been reported that FN adsorption is greater on hydrophilic surfaces. Anisotropic wettability is attributed to a liquid contact line encountering a physical discontinuity (e.g., a solid edge along a microgroove present on solid surfaces). The microgroove surface leads to anisotropic wetting and these micro-scale topographical surface structures may encourage macroscopic changes in droplet wetting behaviour. What is more, to the best of our knowledge there has been only a few studies on anisotropic wetting and the dimensions of microgrooves affecting plasma FN adsorption.

Cells continuously synthesize their own FN matrix, which they deposit and organize as a three-dimensional network. Material-mediated cellular FN reorganization is an important factor in determining the biocompatibility of a material. Cell attachment is required for cellular synthesis of FN. Cells react to micromaterial corrugation, possibly through membrane deformation and stretching. Thus, forces can be generated from just the process of cells recognizing topographical or other cues. This is relevant because mechanical forces are necessary for efficient (integrin-mediated) cellular FN assembly to fibrillar matrices. Furthermore, the forces placed upon cells, stimulated by material topography, can influence cellular FN assembly. The features alter surface wettability, adsorption of preferred proteins might result. We hypothesized that the dimensions of some microgroove surfaces might influence the synthesis and assembly of cellular FN more effectively than others.

The tissue repair function of FN is ubiquitous and useful for dental implant biocompatibility studies. The aim of this work was to investigate the effect of 3D microgroove surfaces on plasma and cellular FN. Through this effort, we hoped to identify an optimal set of dimensions for microgroove surfaces suitable for the transmucosal part of a dental implant. Plasma FN adsorption on different surface topographies was measured by immunofluorescence and ELISA. Human gingival fibroblasts (HGFs) were chosen for this study as they are most abundantly found in gingival connective tissues and have been used in numerous in vitro studies of implant integration. Cells were cultured on the topographically modified surfaces; real-time PCR and Western blotting were used to confirm enhanced cellular FN activity. The structure and properties of the microgrooves, including topography, groove dimensions, surface wettability in FN adsorption and synthesis are discussed in detail.

2. Materials and methods

2.1. Fabrication of micro-structured substrates

Microgroove surfaces were fabricated by photolithography with a micro-structured silicon substrate and an overlying 200-nm thick layer of titanium sputtering. Groove widths of
60 μm, 30 μm, and 15 μm, and groove depths of 5 μm and 10 μm were fabricated. The combinations of groove width and depth are hereafter denoted as T15/5, T15/10, T30/5, T30/10, T60/5, T60/10 (Fig. 1(A)). The cross-sectional shape of each groove was an inverted trapezoid, with an angle between the sidewall and groove-ridge of 54.74°. Group T0 (i.e. the controls) meanwhile was a sputter of titanium on a simple planar silicon substrate.

The key steps of the MEMS wet etching process (Fig. 1(B)) were as follows: a 4-in. silicon wafer was chemically cleaned (III: 4H2SO4 + H2O2; II: NH4OH + H2O2 + 5H2O; I: HNO3 + H2O2 + 5H2O), forming a 1.5-μm silicon dioxide layer on the wafer (by four micro-controlled diffusion system 4470) (1100 °C, 7 h). A photoresistant layer (AZ 5214-E Japan) was next coated to a thickness of approximately 1.8 μm, followed by pre-baking at 96 °C for 12 min. The wafer was exposed to UV light through a photo-mask containing patterns with 15-μm, 30-μm, or 60-μm wide lines for 15 s, and developed for 80 s; post-baking followed at 135 °C for 12 min. The pattern was transferred into the silicon by etching silicon dioxide. The photo-resistant layer was removed, silicon etching and silicon dioxide were removed, and the wafer was cleaned. Lastly, a 200-nm thick sputter of titanium was deposited on the silicon wafers. Etching time controlled the depth of the microgrooves, the patterned mask determined the width of the ridge and groove. The wafers were then cut to 10 mm × 10 mm and 20 mm × 20 mm chips. Small chips were placed in the bottom of 24-well plates for immunostaining, 20 mm × 20 mm chips were placed in the bottom of 6-well plates for real-time PCR and Western blotting. In all experiments, the substrates were cleaned by ultrasonication for 5 min in acetone, soaked in absolute ethanol for 2 h, washed another three times with distilled water and dried for 20 min in ultraviolet light before the cells were plated.

2.2. Scanning electron microscopy

The surfaces and profiles of the samples were imaged using a scanning electron microscope (1000×) (Philips-XL30; Netherlands).

2.3. Contact angle determination

Surface hydrophilicity was determined by measuring liquid contact angles. A 1-μl drop of distilled water (about 2 mm in diameter) was placed on the disc perpendicular to the surface microgrooves and then photographed. The contact angle between the surface and the drop tangent was measured on the photograph (Dataphysics OCA20, Germany). The mean value was calculated from five separate measurements.

2.4. Culture of HGFs

Healthy gingival tissues were obtained from orthodontic patients who had their premolar teeth removed. The process was approved by the Ethics Committee of Affiliated Stomatological Hospital of Fujian Medical University. Tissues from different donors were minced to about 3 mm³, placed in 6-well plates and covered by a cover slip to prevent tissues from floating. Tissues were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, USA) supplemented with 10% foetal bovine serum (FBS, Hyclone, USA) and antibiotics with 1% (v/v) penicillin–streptomycin solution (Hyclone, USA). Cultures were maintained in a humidified atmosphere of 5% (v/v) CO₂ at 37 °C. After reaching 80% confluence the HGFs were digested with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA, and subcultured at a 1:3 ratio. The cells from passages 3–5 were used in this study.

2.5. Immunofluorescence staining of adsorbed plasma FN

Samples were cut to 10 mm × 10 mm and placed in separate wells in 24-well plate prepared for immunofluorescence staining of adsorbed proteins. Human plasma FN (Sigma, F 2006) was reconstituted to a concentration of 20 μg/ml in PBS. The low concentration value was taken with the fundamental interest of understanding the adsorption behaviour and morphology of plasma FN as in previous studies.35 The surfaces of experimental samples were immersed in the prepared plasma FN solution (1 ml) for
2 h at room temperature with gentle rotation. Additional samples were also immersed in PBS solution, without protein, to be used as control surfaces. After incubation, the solutions were removed and the samples were carefully washed three times with PBS to eliminate any unbound protein. The patterned and control surfaces were removed from PBS and incubated in 2% bovine serum albumin (BSA, Sigma, A2058) solution for 30 min at room temperature, in order to block non-specific antibody binding. The immunofluorescence procedure was performed with mouse anti-human FN (1:50, Sigma, F 0791) for 1 h. Following a PBS wash, samples were incubated for 30 min in anti-mouse FITC (1:32, Sigma, F 0257). The sample was then washed with PBS. Finally, each sample surface was mounted with fluorescence mounting medium (DAKO, S3023). The protein adsorbed on each surface was observed with a fluorescence microscope (OLYMPUS BX43). Plasma FN was visualized as green dots. Colour fluorescence micrographs were converted to black-and-white bitmaps with ‘Image J’ (from NIH and available at http://rsb.info.nih.gov/ij/); its operation and use were described previously. The white spots represented plasma FN. Image J’s “Analyze particles” was used to count the FN spots in each bitmap. Seven digital images of each sample group were captured and analysed for adhesion in the smooth and microgroove areas.

2.6. Plasma FN adsorption by enzyme-linked immunosorbent assay (ELISA)

Plasma FN was suspended in PBS to a concentration of 200 μg/ml (close to the physiological concentration of FN in plasma) to determine the relative adsorption of plasma FN on each group using ELISA absorbance. The procedure for preparing samples pre-coated with plasma FN was as previously described (Section 2.5), the only difference being the specific concentration of FN in solution between the two tests. Negative controls without FN were also assayed. After blocking with 2% BSA for 30 min, samples were then incubated with mouse anti-human FN (1:50, Sigma F 0791) as the primary antibody for 1 h and anti-mouse-alkaline phosphatase antibody (1:10,000, Sigma A-1293) as the secondary antibody for 30 min. Unbound antibodies were rinsed away with two PBS washes, using p-nitrophenyl-phosphate (pNPP, SigmaN2765) as substrate for 30 min. The reaction was stopped with 3 M NaOH. Finally, the reaction solution was transferred to a 96-well plate and the absorbances of each well were recorded with a microplate reader at 405 nm. Corrected absorbances were obtained by subtracting the negative control absorbances from the experimental values. All experiments were performed in triplicate.

2.7. Morphology of cellular FN by immunofluorescence

Samples were introduced to separate wells in a 24-well plate. HGFs were seeded onto the samples at a density of 5 × 10⁴ cells/well for one day. Cells were then fixed with 4% formaldehyde for 30 min and permeabilized for 5 min with 0.1% Triton X-100 in PBS. A solution of 1% BSA in PBS was added for 30 min before samples were subjected to the primary antibody, mouse anti-human FN (1:50, Sigma, F 0791), for another 60 min at 37 °C. Following a PBS wash, cells were stained with the secondary antibody, anti-mouse-FITC (1:32, Sigma, F 0257), for 30 min, then rhodamine-labelled with Phalloidin (1:400, Cytoskeleton, Cat. # PHDR1) for 30 min to view the cytoskeletal actin. Finally, 6-diamidino-2-phenylindole (DAPI) (1:1000, sigma, D9542) was added for nuclear fluorescence at 1:1000 for 3–5 min. Samples were washed three times in PBS and mounted with fluorescence mounting medium (DAKO, S3023) before analysis with by confocal laser scanning microscopy (CLSM, LSM710, Carl Zeiss, Germany). We used a laser combiner featuring a diode laser (405 nm; blue fluorescence) to visualize the cell nucleus, an argon-ion laser (488 nm; green fluorescence) for cellular FN, and a helium-neon laser (543 nm; red fluorescence) for cytoskeletal actin. For smooth controls (T0) we collected 2D images. For microgroove groups, optical sections were gathered in 1-μm steps perpendicular to the microscope optical axis. Assembling a stack of these 3D images from successive focal planes created the full image.

2.8. Quantification of mRNA levels of cellular FN by real-time PCR

Samples were cut down to 20 mm × 20 mm and were prepared for real-time PCR. Six of each sample type were introduced onto a 6-well plate. HGFs were seeded onto the sample surfaces simultaneously at a density of 3 × 10⁵ cells/well and incubated for three days until confluent. Total RNA was extracted using Trizol (Invitrogen). The RNA concentration was determined using a NanoDrop 1000 (Thermo, USA). Total RNA (1 μg) was then reverse transcribed with a cDNA Reverse Transcription Kit (TaKaRa PrimeScript® RT reagent Kit DRR037A). TaKaRa real-time PCR primers for human FN (GenBank Accession NM_212482) included a forward primer: (AGGAAAGCCAGGTTT-TAACTG) and a reverse primer: (AGGACCGCTATAAGTGT-CACC), primers for human β-actin (GenBank Accession NM_001101) included a forward primer (CATGTACGGTCT ATCCAGGC) and a reverse primer (CTCCCTATGTCACCCG- GAT). For each experimental condition, reverse-transcribed cDNA was amplified using SYBR® Premix Ex TaqTM II (Tli RNaseH Plus; TaKaRa Code:DRR820A) for each gene on an ABI Prism 7500 real-time PCR cycler (Applied Biosystems). Data were analysed by the Ct method; values for FN were normalized to β-actin for each sample. Data are the means ± SD of three independent experiments.

2.9. Quantification of protein levels of cellular FN by Western blotting

Six of each sample type (20 mm × 20 mm) were introduced onto a 6-well plate. HGFs were seeded at a density of 3 × 10⁵ cells/well and incubated for three days until confluent. Samples with attached cells were removed to a new plate, washed with ice cold PBS three times and subsequently scraped in RIPA buffer (RIPA Lysis Buffer, Strong, P0013B, Beyotime, China) containing a 1% Halt Protease Inhibitor Cocktail (Thermo, USA). Protein concentration was determined by BCA Protein Assay Kit (P0012, Beyotime, China). Equal amounts of proteins were applied to 10% polyacrylamide SDS gels (SDS-PAGE), separated electrophoretically and blotted using PVDF membranes. For detection of FN
expression, the membrane was incubated with mouse anti-human FN (1:300, F7387, Sigma) overnight, washed three times in TBS-T, incubated subsequently with anti-mouse-peroxidase (1:80,000, A2304, Sigma) for an hour, then washed again and developed with ECL reagent. GAPDH detected with Anti-GAPDH (1:20,000, G8795, Sigma) was used as the loading control. Blotting was simultaneously and independently repeated three times in HGFs.

2.10. Statistical analysis

The statistical analysis took into consideration statistical pitfalls common in dental research. One-way analysis of variance (ANOVA) in SPSS 17.0 (SPSS Inc., Chicago, USA) was used to compare the mean values of the data between the groups of T0, T15/5, T15/10, T30/5, T30/10, and T60/5, T60/10. Followed by post hoc statistical tests using an SNK-q test for

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Fig. 2 – (A) High magnification SEM images of the right side show microgroove and ridge width (1000×). (B) High magnification SEM images of the profile show microgroove depth (1000×). (C) Photographs of water droplets on the smooth and patterned surfaces (100×).
each pair of samples compared (p-values below 0.05 considered significant).

3. Results

3.1. Topographical characterization and static contact angles

Microgrooves were arranged in parallel (albeit with different dimensions) on the experimental surfaces (Fig. 2(A)). The depth of each microgroove group is shown in Fig. 2(B). Drop images were captured by a video camera in the direction perpendicular to the surface microgroove (Fig. 2(C)). Multiple comparisons made by one-way ANOVA (Fig. 3) revealed that sample T60 showed the smallest water contact angle (WCA) compared with that of other surface samples of smaller widths (p < 0.001). Furthermore, T60/5 had the greatest surface hydrophilicity, as demonstrated by its small contact angle. However, T15/10 had the greatest surface hydrophobicity, even more than T0 (unmodified control). From these results, it seems that with narrowing groove width and deepening of the groove, the greatest surface hydrophobicity was achieved.

3.2. Effects of microgroove titanium surfaces on adsorption of plasma FN by immunofluorescence staining and ELISA

Fluorescence staining was used to identify the extent of plasma FN adhesion to the surfaces of samples (Fig. 4). FN was detected as green fluorescence (shown in monochrome to better display contrast). The T15/5 and T15/10 sample sets showed the greatest degrees of FN adhesion, especially compared to the control (T0), and the most hydrophilic microgroove surface (T60). The highest level of fluorescence was on the T15 sample (p < 0.001), followed in descending degrees of FN aggregation by the T30, T60 and T0 samples (Fig. 5(A)). Different microgroove depths, of the same width, did not affect plasma FN absorption. These observations were confirmed using ELISA (Fig. 5(B)).

3.3. Influence of microgroove titanium surfaces on cellular FN and fibroblast morphology

Fibroblasts on the microgroove experimental surfaces (T15/5, T15/10, T30/5, T30/10, T60/5, and T60/10) had contact guidance parallel to the microgrooves, whereas the cells on T0 were oriented randomly (Fig. 6). Most of the cells on T15/5 and T15/10 were found on the ridge surfaces. As width of the grooves increased, cells were located not only on the ridge surfaces, but also into the microgrooves themselves. Newly synthesized cellular FN was found exclusively in cell aggregates and existed both on cell surfaces and within the ECM (Fig. 6). Cellular FN coating on the cells was arranged just like the cytoskeletal actin, with the extracellular FN scattered along the groove. Based on 3D images (from microgroove top to bottom), T15/5, T60/5, and T60/10 samples expressed more cellular FN than other groups.

3.4. HGF-related cellular expression of FN gene

The relative changes in mRNA expression of FN in HGFs after 3-day culture are expressed as the ratio of the mRNA levels of a reference gene, β-actin, followed by a standardization of the threshold cycle (Ct) expressions on the control surface (T0) as 1. In this study, our results demonstrate how the presence and dimension of microgrooves and depths can affect the up-regulation and down-regulation of gene expression of FN in HGFs. T60/5 and T60/10 induced significantly higher mRNA expression levels of FN than any other surface (Fig. 7(A)). T15/5 and T30/5 exhibited intermediate expression levels that were somewhat higher than for the controls and T15/10. Taken together, these results suggest that wider microgrooves (i.e. T60), even with different depths (5 μm and 10 μm), resulted in higher cellular FN expression than any other width. Depth seemed important for the more narrow microgrooves (i.e. T15) – the shallower depth (5 μm) resulted in higher cellular FN RNA expression than the deeper grooves (10 μm). FN RNA expression levels for the T30/5 and T30/10 surfaces did not change significantly. All microgroove surfaces yielded greater FN expression than the smooth surface.

3.5. HGF-related cellular FN protein expression

The quantity of FN for the T60/5 and T60/10 surfaces was greater than that of any other surface (Fig. 7(B)). FN expression on the T15/5 and T30/5 surfaces was significantly greater than on T15/10, T30/10 and T0. Results of protein expression had a trend similar to results of real-time PCR.

4. Discussion

4.1. Design and characterization of microgroove titanium surfaces

As our microgroove materials were fabricated with photolithography in one direction, they can be defined as anisotropic...
Fig. 4 - (A) Fluorescence micrographs of plasma FN adsorption on the smooth and patterned surfaces (200×). Green fluorescence spots represent plasma FN. (B) Fluorescence micrographs converted to black-and-white images; white spots indicate plasma FN. Control micrograph also shown.
surfaces. SEM images also supported this classification (Fig. 2(A) and (B)). The anisotropic surface texture seemed to influence the growth and proliferation of cells, leading to contact guidance, which depended upon the specific micro-pattern and size of geometrical elements. Previously, it was shown that cells experience contact guidance on grooves, but the extent to which they react varies by cell type. Gingival fibroblasts are among the major cell types found in peri-implant connective tissues, so the microgroove dimension used here were of suitable size. When the grooves or ridges are larger than the cell size, cell orientation is only marginally affected. With the reduction of the groove/ridge width to less than the width of the cells, effects on orientation become more pronounced. The mean size of the HGF was about 110 μm (measured from 30 cells). We decided that the reasonable dimensions of grooves would be 15 μm, 30 μm, or 60 μm in width. However, previous studies showed that the depth of the groove also plays a role in the cell reaction. Furthermore, as groove depth increased (1 μm, 5 μm and 10 μm) contact guidance increased. Thus, in this study, a reasonable depth of 5 μm and 10 μm was selected to ensure that contact guidance was seen.

4.2. Anisotropic wetting characteristics on microgroove surfaces

Unlike isotropic wetting, where wetting properties are identical in all directions, water contact angles on the anisotropic microgrooved titanium substrate were measured in the direction perpendicular to the microgrooves. The apparent contact angles are different when observed perpendicular or parallel to the direction of the grooves. The contact angles measured from the direction parallel to the grooves were larger than those measured from the perpendicular direction, in a study by Zhao et al. Thus far, experimental studies on anisotropic wetting have been conducted using microliter-volume drops. In our study, we decided that it was reasonable to assume that the 1-μl droplet size was millimetre scaled and much larger than the dimension of surface asperities. Our results using titanium surfaces are consistent with previous studies, decreasing groove widths and spacing, or increasing groove depth amplified the anisotropy for the contact angle (equilibrium contact angle). These trends are likely due to an increasing energy barrier. It should be pointed out that many studies demonstrate that surface topographic structure and chemical composition can result in anisotropic wettability. This wettability can be further attributed to the difference in the energy barrier between different directions due to geometrical and chemical heterogeneity. In our study, geometrical parameters such as groove width, depth and spacing played an important role in unique anisotropic wetting behaviour of the surface. For microgrooved titanium surfaces, decreasing groove width and increasing depth amplified the surface hydrophobicity.

4.3. Adsorption of plasma FN on microgroove surface

Plasma FN circulates in the blood and functions during early wound-healing responses. Various techniques have been developed for their study in protein–material interactions or to quantify protein adsorption. The most straightforward method to quantify protein is by labelling with fluorescent probes. Another method to follow adsorption is verified by measuring the ELISA by light absorbance. In our study, we used fluorescent probes and ELISA measurements to determine the extent of FN adsorption on different micro-groove surfaces. T15/5 and T15/10 had the greatest surface hydrophobicity, but absorbed the highest plasma FN. These data showed that hydrophobic microgroove surfaces with narrower width might be considered more protein-adsorbent than hydrophilic surfaces with larger groove widths. Surface chemistry, topography and wettability influence the changes in protein absorption. Strong hydrophobic forces found near these surfaces are in direct contrast to the repulsive solvation forces arising from strongly bound water at the hydrophilic surface. Interactions between proteins and surfaces include van der Waal’s and electrostatic interactions, hydrogen bonding, and hydrophobic interactions. Hydrophobic interactions are likely to be the principal binding forces between proteins and surfaces. Water molecules play a significant role in protein adsorption, as proteins are generally hydrophilic in character. When the surface is hydrophobic, the water dipoles near it have to be ordered. Upon protein adsorption, these ordered molecules are released to the bulk solution increasing the entropy of the system, thereby
decreasing free energy, and enhancing adhesion on hydrophobic surfaces.\textsuperscript{18,54,55}

4.4. Cellular FN assembly on microgroove surface

All microgroove surfaces showed successful contact guidance for HGFs to migrate through the groove. Cellular FN was then synthesized and assembled by the cells as they migrated. Fibroblasts depend on classical focal adhesions (FAs) to the substrate, which are mediated by integrin $\alpha_5\beta_1$. On the intracellular side this interacts with the actin cytoskeleton; on the extracellular side, ligands that anchor to integrin include FN. FAs function as both adhesion and signal transducers.\textsuperscript{56} Cell-generated contractile forces generated from the stiffness or topography of the cell attachment to a substrate is of significance in our study.\textsuperscript{27-29,57} Within the microgroove environment, cells are affected by signals from the ECM and neighbouring cells.\textsuperscript{58} Microgrooves lead to cell elongation; the mechanical stresses stemming from the microgrooves result in mechano-sensitivity signals through

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Fig. 6 - Immunofluorescence staining of HGFs, CLSM overlay of triple stain with DAPI (blue), cytoskeleton-actin stress fibres (red) and FN (green)(400×). (A) 2D image of the smooth surface (T0). Cellular FN was found as a coating on cell surfaces and in the ECM. FN coating on the cell surface assembled into a complex fibrillar network that resembled a web throughout the cell (red arrows); extracellular FN assembled near the cell (white arrows). (B–G) 3D microgroove samples are shown as overlay pictures (from top to bottom) for each microgroove type. (b–g) 3-D microgroove samples are shown as a series of pictures in 1 μm axial steps (from top to bottom) for each microgroove type.
Fig. 7 – (A) Relative gene expression of FN in HGFs on different surfaces after day 3 of incubation. The results are expressed as a ratio of the quantified gene expression levels to the reference protein, β-actin, followed by a standardization of the expressions on the control surface; T0 as reference (one-way ANOVA, ***p < 0.001, **p < 0.01, *p < 0.05, mean ± SD, N = 3). (B) Result from Western blotting for the expression of cellular FN after 3 days of culture on each surface; comparison of protein quantity on each surface. The results are expressed as a ratio of the quantified protein expression levels to the reference protein, GAPDH, followed by a standardization of the expressions on the control surface (T0), as reference (one-way ANOVA, ***p < 0.001, **p < 0.01, *p < 0.05, mean ± SD, N = 3).

The feed back of ‘inside-out’ signalling regulates FN-matrix secretion and assembly.59-61 Mechanical stresses produced by cytoskeletal elements provide a mechanism for regulating matrix dynamics and are required for FN assembly.30–32 A mechanism of bio-mechanotransduction especially,57,60 We can infer that the quantity of cellular FN expression in response to different mechanical stresses of the cell may come from the different dimensions of a microgroove. From the CLSM we found that T60, still smaller than HGF, successfully guided the cells to attach along the microgroove ridge. The 60-μm width is enough to guide cell attachment and proliferation in the groove. The cells on this substrate may have received much higher mechanical stresses than on other grooves including protruding and bending stress from the groove ridge during cell attachment. These stresses cause groove sidewall and cell–cell stresses as the cells migrate into the groove; similar mechanical stresses have been described before.62,63 However, the T15 groove width was too narrow for cells to migrate into the groove to set up a full 3-D environment. In these circumstances, most mechanical stresses came only from the ridge protrusion and a lack of crushing stress from the profile of the groove and the intercellular extrusion. Although the width of 15 μm was much smaller than the cell, this surface size received much more protrusion than the surface with a microgroove width of 30 μm. However T30 was between T15 and T60 in terms of how much a cell could migrate into the microgroove (Fig. 6). So the mechanical stresses for this group may have been less than that for the T60 attaching cells.

Cells on the T60 surface received the highest mechanical stresses, and cellular FN expression was greater than on any other surface (Fig. 7). For cells attaching to the T60 surface, the depth of the groove did not make much difference in cellular expression of FN. However, the narrower microgrooves (T15 and T30) with a shallower depth (5 μm) had greater expression of cellular FN than the 10 μm depths (Fig. 7(B)). We can infer that a smaller ridge platform with deeper grooves might result in poor adhesion for the cell and poor formation of FAs. The corresponding reduction of signal transmission of mechanical stresses would lead to lower expression of cellular FN. It would seem reasonable to suggest that the effects on cellular expression of FN in response to the topography of the substrate surface, is a response to the forces induced upon them and is scalable depending on width and depth of the groove.

4.5. Plasma FN and cellular FN have different microgroove reactions

We found that the most hydrophobic samples (T15/5 and T15/10) absorbed the greatest amount of plasma FN but relatively low cellular FN. T60/5 and T60/10 had the opposite tendency – a greater tendency to express cellular FN but a lower absorbency of plasma FN. Although the two FN forms are not identical, they appear to be interchangeable in some respects. Plasma FN assemblies into preexisting or newly assembled cellular FN matrices.10,26 Plasma and cellular FN could potentially perform similar, but not necessarily the same, functions. Plasma FN is known to enhance the attachment of cells15,16; cellular FN is a basic requirements for cell attachment. Cellular FN-deficient fibroblasts have defects in adhesions and mechanotransduction.64 There are also studies showing that conditional plasma FN knockout mice have normal wound healing and hemostasis, suggesting possible compensation by cellular isoforms of FN.55,86
Moreover, the observation that wider grooves are effective in stimulating cell proliferation in vitro.6
Our data also indicate that the 60-µm grooves were best at inducing cells to secrete cellular FN. This greater secretion may compensate for the decreasing absorption of plasma FN, since it has been shown that cellular FN function can compensate for plasma FN.

5. Conclusion

Groove widths of 60 µm, 30 µm, and 15 µm, and groove depths of 5 µm and 10 µm were fabricated. Narrower and deeper microgrooves amplified surface hydrophobicity. These results have implications for the design and application of anisotropic wetting surfaces.

The effects of microgroove width on plasma FN and cellular FN were quite different: wide microgrooves were favourable for adhesion of cellular FN while narrower grooves were favourable for plasma FN. Microgroove depth did not affect plasma FN absorption. While groove depth did not affect cellular FN expression to wide microgrooves (T60), narrow microgrooves of 5-µm depth (T15/5 and T30/5) resulted in higher cellular FN adhesion than deeper grooves of the same width. In general, forces generated from the stiffness or topography of the cells during attachment to a substrate is of some considerable biological significance. These parameters need to be considered during the design of 3D biomaterials as they provide a means to influence the "mechanical stress effect" on cells and therefore their physiological and biochemical behaviour.

Due to the adaptability and functionality of FN, our results pertain to the design of implant surfaces. It seems that the microgroove width of 60 µm with a depth of either 5 µm or 10 µm would be the best choice for the transmucosal part of the dental implant.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdent.2013.08.004.

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