Evaluation of anodized surfaces designed for improved soft tissue integration

Michael Nosswitz[1], Misha Teale[1], Stephanie Mathes[1], Andrea Venturato[2], and Angelines Gasser[2]

[1] Institute of Chemistry and Biotechnology, Zürich University of Applied Sciences, Wädenswil, Switzerland
[2] Nobel Biocare Services AG, Kloten, Switzerland

Correspondence to: stephanie.mathes@outlook.com

Published 12 March 2019

Abstract

A rapid and stable integration of dental implant systems into the surrounding tissues is achieved by enabling migration, adhesion, proliferation and differentiation of the cells surrounding the implant and abutment surfaces. Thus, a validation of abutment surface design requires a careful evaluation of their ability to provide a strong attachment for the connective tissue and the epithelium. To evaluate the effect of surface anodization on soft tissue integration, anodized (Xeal™) and machined surface titanium discs were used as culture substrates for human gingival epithelial cells (HGEPp) and primary human gingival fibroblasts (HFIB-G). The cellular response was evaluated by quantifying cell growth, viability, development of extracellular matrix (collagen-I protein levels) and by assessing cell morphology. HFIB-G cells did not show any significant preference between the two surfaces whereas HGEPp cells showed significantly faster growth on the Xeal™ surface. These results suggest that anodized surface has the potential to accelerate healing and promote soft tissue health by increasing peri implant healing and mucosa height.

Keywords: soft tissue, Xeal™, anodized surface, cell culture, keratinocytes, titanium

1. Introduction

Cellular behavior is strongly influenced by a material's surface chemical and physical properties (i.e. hydrophilicity, stiffness, porosity, roughness, topography).[1-3] In implant dentistry, titanium and its alloys are widely applied due to their ability to support and promote tissue integration while at the same time being biologically inert.[4-6]

The rapid formation of a soft tissue seal at the abutment level represents one of the key requirements to promote wound healing and to allow for a healthy integration of the implant.[6] The past years witnessed a remarkable effort aimed at the development of new or improved materials with enhanced properties and able to stimulate and support a better soft tissue adhesion.[6-9]

When an implant system is placed, its surface interacts with three main tissue types:

- The epithelia, formed by keratinocytes, which form a tight seal separating the body from the outside world. The cells are interconnected through cell-cell junctions that act as anchor points for actin filaments to provide support points for the cytoskeleton.[10]

- The connective tissue, formed by fibroblasts, which are responsible for the production of new matrix and glycoproteins.[11, 12] This matrix secreted by the fibroblasts contributes to tissue adhesion to the implant surface and its integration by mediating the interactions with structural proteins such as fibronectin, vitronectin and laminin.[1]
- The bone tissue, formed by osteoblasts, which are responsible for the osseointegration of the implant by the formation of new bone.[13]

Cell adhesion to a surface is partially achieved through focal adhesion points, which consist of a high density of protein aggregates involved in mediating cell-cell and cell-material adhesion, migration, mechano-transduction and signaling.[14, 15] An inappropriate interaction with the substrate could inhibit cell division and ultimately activate a pathway cascade leading to apoptosis.[16] Hence, focal adhesion points are key players in cell proliferation and affect cell behavior by triggering biochemical and biomechanical pathways. [1, 16] In this in-vitro study, anodized and nanostructured titanium surface[17] (Xeal™; Nobel Biocare AB, Göteborg, Sweden) was compared with a standard, machined titanium surface to evaluate their effect on cell adhesion and proliferation. To resemble the in-vivo conditions as closely as possible, primary human gingival epithelial cells (HGEPp) and primary human gingival fibroblasts (HFIB-G) were selected as test cell lines.

2. Materials and Methods

Two surfaces, machined without surface treatment as control (machined) and anodized (Xeal, Nobel Biocare AB), were produced from Ti alloy (Titanium-6 Aluminum-4 Vanadium ELI) discs (diameter: 6 mm). Anodized surface had regularly distributed nanostructures and an oxide layer approximately 150 nm thick.

2.1 Disc sterilization

Prior to cell seeding the discs were washed in EtOH 75% for 10min and three times in sterile ddH2O. Subsequently, they were air-dried, sterilized under UV light for 30min and distributed in 24 low adhesives well plates (Thermo Scientific, cat num 12567104).

2.2 Disc coating

The two surfaces were divided into two groups and coated respectively with FBS and laminin. FBS coated surfaces were prepared by incubation in 20% FBS overnight (37°C, 5% CO2). After incubation, the discs were washed 1x with PBS and air-dried prior to cell seeding. Laminin (Sigma-Aldrich, cat num L4544-100UL) coating was performed according to manufacturer's guidelines.

2.3 Cell seeding

Cells were seeded on 6mm Ø discs at a concentration of 10'000 cells/cm2. Per disc, 40µL of cell suspension was pipetted leading to a drop sticking onto the surface. Cells were allowed to settle for 4 hours in an incubator (37°C / 5% CO_2). On laminin coated discs, cells seeding was performed dispending 30µL of cell suspension. Next, the discs were flushed with cell culture medium (2mL/well) and incubated until analysis was performed (37°C / 5% CO_2).

2.4 Proliferation assay

Cell proliferation was assessed after 1, 3 and 6 days by PrestoBlue® (Invitrogen, cat num A13262) assay according to the manufacturer's protocol. Defined cell concentrations (1500, 3000, 6000, 12000, 25000, 50000, 100000 cells) were seeded into wells to enable correlation to cell number. Using the linear regression fit of the standard curve the cell numbers for each disc was calculated (n = 4).

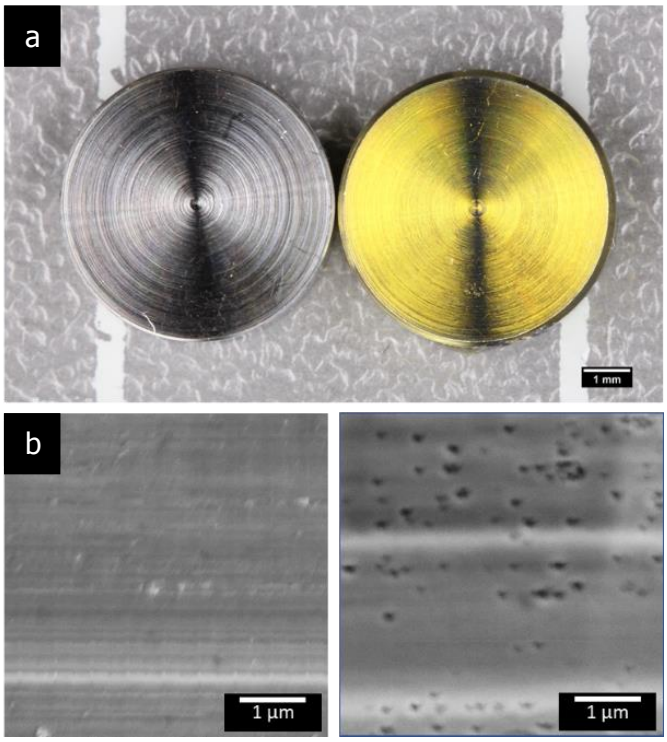

Fig 1 a) Representative image of machined (left) and Xeal (right) titanium discs. Scale bar: 1 mm. b) SEM image of the machined (left) and Xeal (right) surface. Scale bars: 1 µm.

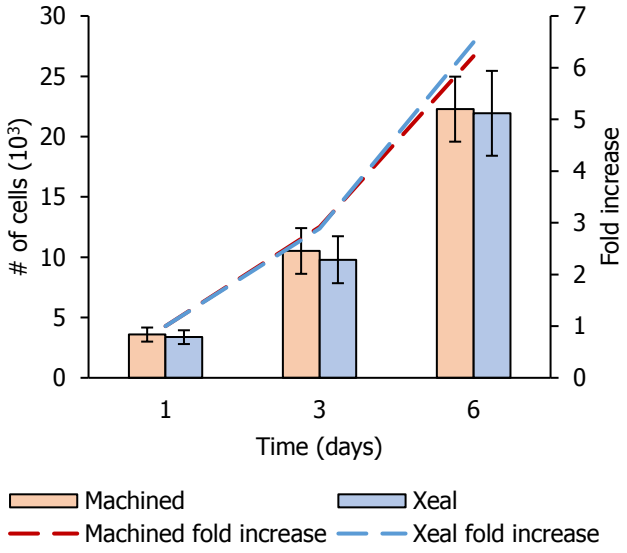

Fig 2 Mean HFIB-G cell number at 1, 3 and 6 days on machined and Xeal surfaces. Right axis, fold increase of HFIB-G cells over time normalized to the cell number at 1 day. Error bars represent standard deviation.

2.5 Fixation

The samples were washed 1x with PBS and then cells were fixed by incubation with a 10% neutral buffered formalin solution for 20min at RT. After incubation, formalin excess was removed by washing 3x with PBS. Samples were stored at 4°C until further use.

2.6 DAPI quantification

Quantification of cell nuclei was performed by DAPI staining. DAPI signal was read at 358 nm using 40x microscope magnification. Cell nuclei were counted automatically with the "count and measure" tool in CellSens dimension 1.6 software.

2.7 Collagen-I protein levels

The determination of collagen production was performed with a MicroVue™ CICP ELISA kit (Quidel, cat num 8003) according to the manufacturer's protocol. Collagen-I amount was calculated per 1000 cells to normalize the value to the cell population (n = 8).

2.8 Focal adhesion staining

Fixed cells were permeabilized with 0.1% Triton X-100, blocked for 30min in 1% BSA in PBS, incubated with primary mouse α vinculin antibody (1:250) for 1h, incubated with secondary Alexa Fluor 488 α mouse antibody (1:400) and TRITC-conjugated phalloidin for 1h and subsequently stained for DAPI (300ng/mL).

2.9 Picture acquisition

The discs were analyzed on an inverted Olympus IX81 microscope. CellSens Dimension software version 1.6 was used to acquire fluorescent pictures and to take overview images for DAPI cell count.

2.10 Statistical analysis

Statistical analysis was carried out with SigmaStat 3.5 software. One Way ANOVA was used to test for differences between the surface types. Significant results were further analyzed by Tukey's post-hoc test in order to compare each surface type against every other.

3. Results and Discussion

The anodization process of titanium and its alloys has been reported to induce physical and chemical changes on the surface of the material.[18] Visual inspection of the Xeal surface showed a change of the surface color from silver to yellow (Fig 1a). Nanostructures could be readily observed on the Xeal disc surface, suggesting an increase of the total surface area (Fig 1b and 1c).

3.1 Effect of anodization on fibroblast behavior

The ability of fibroblasts to adhere to and grow on the two surfaces was assessed at 1, 3, and 6 days of cell culture. At all evaluated timepoints, the total cell number and the growth rate of HFIB-G cells were comparable between the two surfaces (Fig 2). Similarly, the protein levels of collagen-I, an indicator of fibroblast activity and tissue regeneration,[19-21] were similar at all three time -

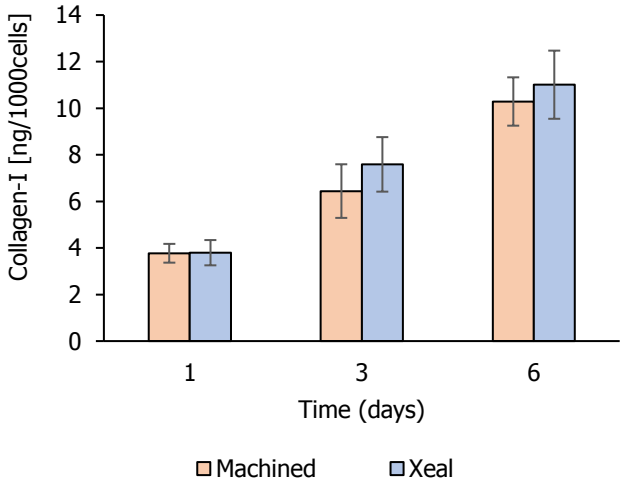

Fig 3 Mean amount of collagen-I per 1000 HFIB-G cells at 1, 3 and 6 days on machined and Xeal surfaces. Error bars rep-resent standard deviation.

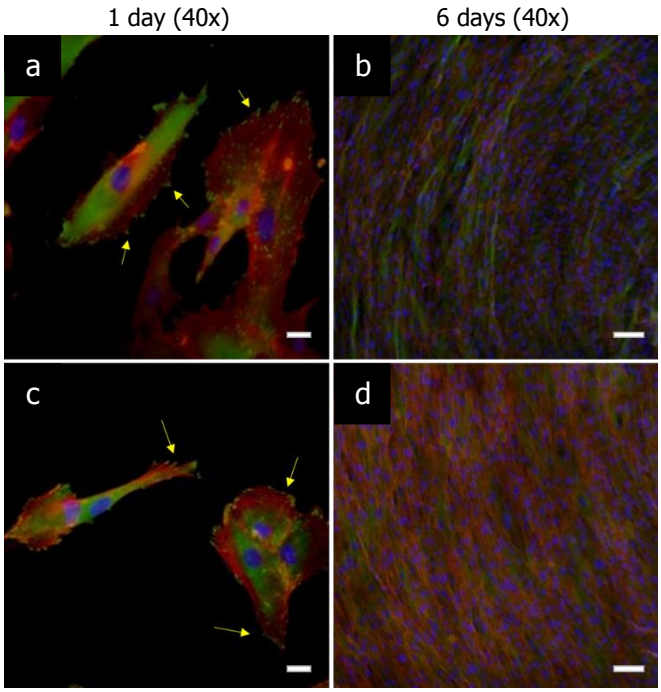

Fig 4 Triple fluorescent straining of HFIB-G cells for actin (red), DAPI (blue) and vinculin (green). a) and b) HFIB-G cells on machined surfaces after 1 day and 6 days, respectively. After 1 day focal contacts can be observed as small green dots outlining the cells. c) and d) HFIB-G cells on Xeal surfaces after 1 and 6 days, respectively. Scale bars: 20 µm for a) and c), 100 µm for b) and d).

points (all $p>0.05$); Therefore, the collagen-I levels were not affected by the anodization process.

Next, the fibroblast morphology was analyzed through triple fluorescent staining of actin filaments, as component of the cytoskeleton, vinculin, as mediator of focal adhesion, and DAPI nuclear staining. Focal adhesion sites were visible as small green dots outlining the cells on both machined and Xeal surfaces (Fig 4a and c). After 6 days of cell culture, fibroblasts reached confluency and aligned radially along the grooves present on the surfaces (Fig 4b and d). No major differences in fibroblast morphology were observed on machined versus Xeal surfaces.

3.2 Effect of anodization on keratinocyte behavior

Following the fibroblast evaluation, keratinocyte growth was assessed in the same cell culture model. After the first day, no significant differences could be observed between machined and Xeal surfaces (Fig 5a). After 3 days, the cell number on Xeal surface was significantly higher than the cell number on machined surface (Fig 5a), and after 6 days the difference between the epithelial cells number on the two surfaces was even bigger, suggesting Xeal surface could stronger promote keratinocyte proliferation. Calculation of the fold increase confirmed the faster cell growth on Xeal surface. The small difference in the cell number at day 1 was likely related to the cells adapting to the new surface and therefore having a low proliferation rate.

In addition to the assessment of cell viability, quantification of cell numbers was performed through DAPI staining at 1, 3 and 6 days. The results confirmed the previous finding, showing higher total cell numbers on the Xeal surface (Fig 5b). To evaluate to which extent the observed differences between Xeal and machined surfaces were induced by the sole properties of the Xeal surface, a coating of the discs with either laminin or FBS

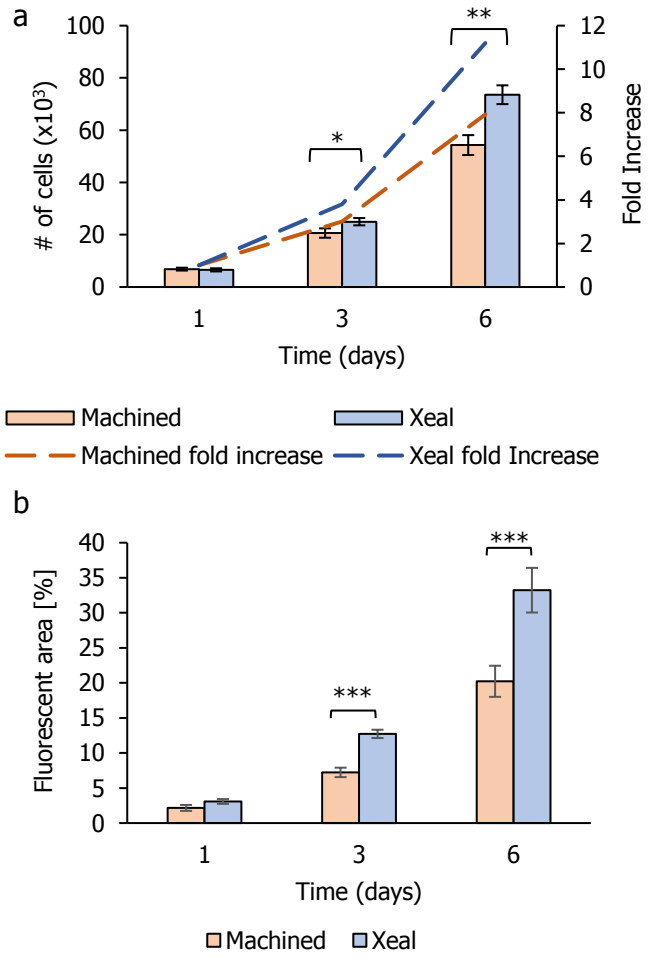

Fig 5 a) Mean HGEPp cell number at 1, 3 and 6 days on machined and Xeal surfaces. Right axis, fold increase of HGEPp cells over time normalized to the cell number at 1 day. b) Quantification of the total DAPI fluorescent area of cells on machined and Xeal surfaces at 1, 3 and 6 days. Error bars represent standard deviation. Significance levels: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

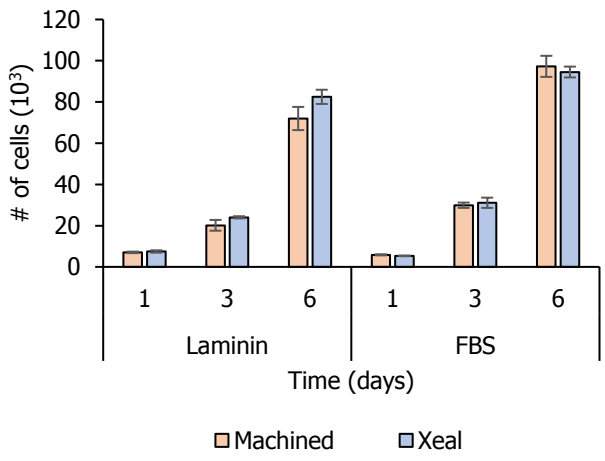

Fig 6 (Left) Mean HGEPp cell number at 1, 3 and 6 days on machined and Xeal surfaces coated with laminin. (Right) Mean HGEPp cell number at 1, 3 and 6 days on machined and Xeal surfaces coated with FBS. Error bars represent standard deviation.

was performed. Laminin is a key component of the basal membrane and is well known to provide excellent support,[22] while FBS is a standard coating used to support keratinocyte proliferation. HGEPp cells adhered to and spread on both machined and Xeal surfaces coated with laminin or FBS; however, no significant differences in the cell number were observed at any of the three timepoints (Fig 6) confirming that the previously described differences were indeed due to the changes in surface properties of the two kinds of the titanium discs.

A qualitative evaluation of cell morphology on the different surfaces was performed through triple fluorescent staining and showed cell attachment and formation of focal adhesion points on all surface types (Xeal and machined surfaces, uncoated and coated with laminin or FBS) (Fig 7). Cell morphology looked very similar: in all cases actin filaments were observed to support cell-substrate and cell-cell adhesion. Interestingly, actin filaments were evenly distributed throughout the cell body (Fig 7, yellow arrows), indicating active rearrangement of the cytoskeleton suggesting that the cells were proliferating and migrating on the substrate surface. Vinculin proteins were detected in focal adhesion points in all cases, suggesting that the Xeal surface is a substrate able to support focal adhesion attachment similarly to the control surface.

4. Conclusions

The quantification of cell viability and cell number confirmed that the Xeal surface has the ability to enhance proliferation of HGEPp cells. These results corroborate previously reported studies where anodization was shown to improve the interaction with laminin, activate integrins and subsequently focal-adhesion kinase (FAK) in epithelial cells, thus inducing a proliferative cellular response.[23] As expected, when machined and Xeal surfaces were coated with laminin or FBS to mask chemical and physical differences between the two surfaces, no major differences in cell proliferation were detected. Interestingly, HFIB-G cell proliferation, collagen-I protein levels and cell morphology were comparable between the two surfaces, confirming that fibroblasts and keratinocytes have different responses to certain surfaces. These data confirm that the chemical and physical changes induced by the Xeal layer are the principal mediators of the observed differences in cell growth. This finding is supported by scientific literature demonstrating how surface chemistry, and nano-topographies can strongly influence cell behavior.[18, 23, 24] In conclusion, this study

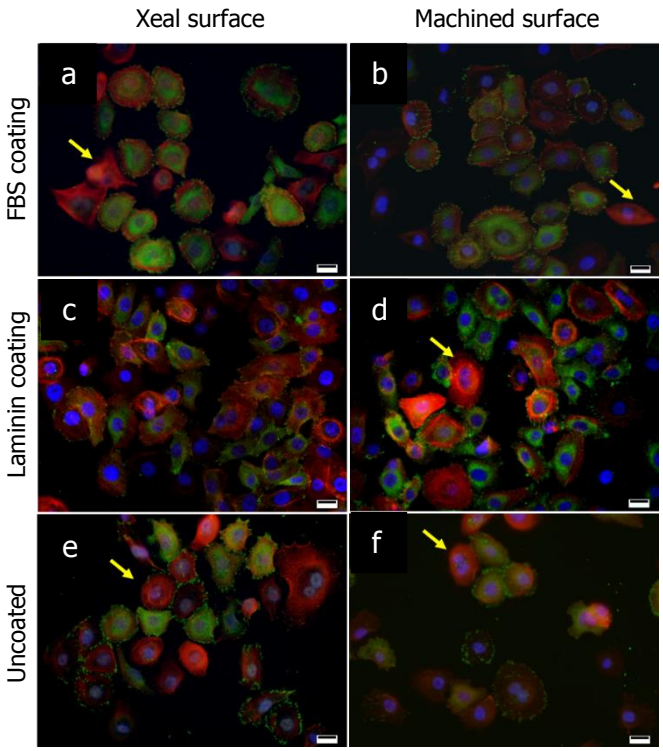

Fig 7 Triple immune-fluorescent straining of HGEPp cells for actin (red), DAPI (blue) and vinculin (green). a) Xeal surface and b) machined surface coated with FBS. c) Xeal surface and d) machined surface coated with laminin. e) Xeal surface and f) machined surface not coated. Scale bars: 20 μm.

suggests that anodization of titanium alloys has the potential to improve proliferation of HGEPp cells while maintaining the chemical and physical properties to support fibroblast proliferation and collagen-I production. Further studies have been conducted to confirm the performances of the newly developed surface and to gather a better understanding of the contribution of surface chemistry and topography on the improved tissue health.[25, 26] Moreover, a recently reported outcome of a randomized controlled study on Xeal abutments showed consistently healthier mucosa in comparison to machined abutments.[27]

Conflict of interest

The authors declare no conflict of interest related to this study. Andrea Venturato and Angelines Gasser are currently employees of Nobel Biocare Services AG and participated in the study as contributing scientists.

Funding

Testing materials and reagents for the study were provided and funded by Nobel Biocare Services AG.

References

1. Bacakova, L., Filova, E., Parizek, M., Ruml, T. & Svorcik, V. Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. Biotechnology Advances 29, 739-767 (2011).
2. Teng, F.-Y. et al. A comparison of epithelial cells, fibroblasts, and osteoblasts in dental implant titanium topographies. Bioinorganic chemistry and applications 2012, 687291-687291 (2012).
3. Anselme, K. & Bigerelle, M. Topography effects of pure titanium substrates on human osteoblast long-term adhesion. Acta Biomaterialia 1, 211-222 (2005).
4. Feng, B., Weng, J., Yang, B.C., Qu, S.X. & Zhang, X.D. Characterization of surface oxide films on titanium and adhesion of osteoblast. Biomaterials 24, 4663-4670 (2003).
5. Annunziata, M. & Guida, L. The Effect of Titanium Surface Modifications on Dental Implant Osseointegration. Front Oral Biology 17, 62-77 (2015).
6. Rompen, E., Domken, O., Degidi, M., Farias Pontes, A.E. & Piattelli, A. The effect of material characteristics, of surface topography and of implant components and connections on soft tissue integration: a literature review. Clinical Oral Implants Research 17, 55-67 (2006).
7. Kononen, M., Hormia, M., Kivilahti, J., Hautaniemi, J. & Thesleff, I. Effect of surface processing on the attachment, orientation, and proliferation of human gingival fibroblasts on titanium. J Biomed Mater Res 26, 1325-41 (1992).
8. Mustafa, K., Lopez, B.S., Hultenby, K., Wennerberg, A. & Arvidson, K. Attachment and proliferation of human oral fibroblasts to titanium surfaces blasted with TiO2 particles. A scanning electron microscopic and histomorphometric analysis. Clinical Oral Implants Research 9, 195-207 (1998).
9. Hormia, M., Könönen, M., Kivilahti, J. & Virtanen, I. Immunolocalization of proteins specific for adhaerens junctions in human gingival epithelial cells grown on differently processed titanium surfaces. Journal of Periodontal Research 26, 491-497 (1991).
10. Bruce, A.A., Johnson; Julian, Lewis; David, Morgan; Martin, Raff; Keith, Roberts; Peter, Walter. Molecular Biology of the Cell (2015).
11. Velnar, T., Bailey, T. & Smrkolj, V. The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res 37, 1528-42 (2009).
12. Shaw, T.J. & Martin, P. Wound repair at a glance. J Cell Sci 122, 3209-13 (2009).
13. Anselme, K. Osteoblast adhesion on biomaterials. Biomaterials 21, 667-681 (2000).
14. Humphries, J.D. et al. Vinculin controls focal adhesion formation by direct interactions with talin and actin. J Cell Biol 179, 1043-57 (2007).
15. Case, L.B. et al. Molecular mechanism of vinculin activation and nanoscale spatial organization in focal adhesions. Nature Cell Biology 17, 880 (2015).
16. Frisch, S.M. & Screaton, R.A. Anoikis mechanisms. Current Opinion in Cell Biology 13, 555-562 (2001).
17. Milleret, V. et al. Rational design and in vitro characterization of novel dental implant and abutment surfaces for balancing clinical and biological needs. Clinical Implant Dentistry and Related Research, epub ahead of print (2019).
18. Kim, K., Lee, B.-A., Piao, X.-H., Chung, H.-J. & Kim, Y.-J. Surface characteristics and bioactivity of an anodized titanium surface. Journal of periodontal & implant science 43, 198-205 (2013).
19. Marsell, R. & Einhorn, T.A. The biology of fracture healing. Injury 42, 551-555 (2011).
20. Wang, Y., Zhang, Y. & Miron, R.J. Health, Maintenance, and Recovery of Soft Tissues around Implants. Clinical Implant Dentistry and Related Research 18, 618-634 (2016).
21. Sculean, A., Gruber, R. & Bosshardt, D.D. Soft tissue wound healing around teeth and dental implants. Journal of Clinical Periodontology 41, S6-S22 (2014).
22. Tjin, M.S. et al. Biologically relevant laminin as chemically defined and fully human platform for human epidermal keratinocyte culture. Nature Communications 9, 4432 (2018).
23. Takebe, J., Miyata, K., Miura, S. & Ito, S. Effects of the nanotopographic surface structure of commercially pure titanium following anodization–hydrothermal treatment on gene expression and adhesion in gingival epithelial cells. Materials Science and Engineering: C 42, 273-279 (2014).
24. Huang, H.-H., Wu, C.-P., Sun, Y.-S., Yang, W.-E. & Lee, T.-H. Surface nanotopography of an anodized Ti–6Al–7Nb alloy enhances cell growth. Journal of Alloys and Compounds 615, S648-S654 (2014).

25. Susin, C., Fiorini, T., Demoner Ramos, U. & Schüpbach, P. Safety and Efficacy of a Novel Anodized Abutment on Soft Tissue Healing in Yucatan Minipigs. Clinical Implant Dentistry and Related Research, epub ahead of print (2019).
26. Roffel, S. et al. Evaluation of a novel oral mucosa in vitro implantation model for analysis of molecular interactions with dental abutment surfaces. Clinical Implant Dentistry and Related Research, epub ahead of print (2019).
27. Hall, J., Neilands, J., Davies, J.R., Ekestubbe, A. & Friberg, B. A randomized, controlled, clinical study on a new titanium oxide abutment surface for improved healing and soft tissue health. Clinical Implant Dentistry and Related Research, epub ahead of print (2019).

How to cite this article: Michael Nosswitz, Misha Teale, Stephanie Mathes, Andrea Venturato, and Angelines Gasser. Evaluation of anodized surfaces designed for improved soft tissue integration. Foundation for Oral Rehabilitation (FOR) 2019, pp. 1-7. www.for.org/scientific-article/001