To ensure the long-term success of dental implants, a functional attachment of the soft tissue to the surface of the implant abutment is decisively important in order to prevent the penetration of bacteria into the implant–bone interface, which can trigger peri-implant disease. Here a surface modification approach is described that includes the covalent immobilization of the extracellular matrix (ECM) proteins fibronectin and laminin via a crosslinker to silanized Ti6Al4V and Y-TZP surfaces. The surface properties are evaluated using static contact angle, X-ray photoelectron spectroscopy (XPS), and atomic force microscopy (AFM). The interaction of human gingival fibroblasts (HGFs) with the immobilized ECM proteins is verified by analyzing the localization of focal contacts, cell area, cell morphology, proliferation rate, and integrin expression. It is observed in the presence of fibronectin and laminin an increased cellular attachment, proliferation, and integrin expression of HGFs accompanied by a significantly higher number of focal adhesions. The presented approach holds great potential to enable a stronger bond between soft tissue and implant abutment surface. This could potentially help to prevent the penetration of bacteria in an in vivo application and thus reduce the risk of periimplant disease.

1. Introduction

Oral rehabilitation with the help of dental implants is a well-established treatment option for completely and partially edentulous patients and has become widespread in recent decades.[1] Dental implants create an interface with both the bone and mucosal tissue. In addition to the osseointegration of the implant material's surface, the adhesion of the abutment to the gingival tissue is crucial in order to ensure a biological seal against deleterious stimuli in the oral cavity.[2] However, the mucosa around the implant abutment displays numerous differences from that surrounding natural teeth: The collagen fibers run parallel to the implant surface,[3] blood supply is reduced due to the lack of a periodontal ligament,[1] and fewer fibroblasts are present.[4,5] Taken together, these factors result in a reduced sealing capacity of the gingiva against bacterial invasion and compromise the longevity of the implant.

The soft tissue barrier, the so-called biological width around natural teeth, is usually ≈2 mm whereas the width around implants is generally 3–4 mm.[1] This enables bacteria such as Streptococcus oralis to more easily enter this gap and compete with host cells to cover the implant, which is described in literature as a “race for the surface.”[6–8] As soon as pathogens cover the implant surface, a plaque biofilm will form that protects bacteria from displacement through host cells.[9] Consequently, the penetration of bacteria into the implant/tissue interface can cause an accelerated destruction of the epithelial and connective tissue due to biofilm formation[10] and further lead to periimplant bone destruction.

Titanium and its alloys are widely used as an implant abutment material due to their excellent cytocompatibility,[10] mechanical properties,[11] and corrosion resistance.[12] In addition to titanium, high-performance oxide ceramics, such as zirconia, are preferred as an abutment material primarily because of their aesthetics and biocompatibility.[13] However, the non-bioactive character of these materials represents a limitation for establishing a direct contact with the surrounding mucosa.

In order to achieve a successful integration of an implant in the biological environment, the choice of suitable material properties is crucial because numerous events such as protein adsorption...
take place at the interface of the implant. The non-specific adsorption of proteins can trigger undesirable biological reactions, which ultimately lead to the failure of the implant. To circumvent such non-specific reactions, interfaces must be modified in such a way that biocompatibility is guaranteed.\textsuperscript{[14]}

Surface modification of materials that are relevant for implant therapies is a well-established approach to ensure enhanced compatibility.\textsuperscript{[15]} Novel strategies for surface modification include acid-etching,\textsuperscript{[16]} plasma spraying,\textsuperscript{[17]} grinding,\textsuperscript{[18]} sand blasting,\textsuperscript{[19]} and bioinspired coatings\textsuperscript{[13]} through the application of agents such as collagen,\textsuperscript{[20]} chitosan,\textsuperscript{[21]} and hydroxypatite.\textsuperscript{[22]} In addition, the modification of surfaces with extracellular matrix (ECM) proteins is a promising option to actively control cell responses by providing sites for cellular attachment.\textsuperscript{[9,20,21]}

Fibronectin is a ubiquitous ECM glycoprotein dimer that consists of two almost identical subunits linked by two disulfide bridges.\textsuperscript{[24]} It plays an important role in many biological processes such as cell adhesion, migration, and differentiation.\textsuperscript{[25]} Various fibronectin isoforms are formed by alternative splicing.\textsuperscript{[26]} Moreover, fibronectin contains various amino acid motifs such as arginine-glycine-aspartic acid (RGD) that are recognized and bound by integrins. Besides fibronectin, laminin, a major component of the basal laminae, is an essential ECM regulator of cell adhesion and matrix-mediated signaling.\textsuperscript{[24,25]}

Laminin is a heterodimer, consisting of three different chains ($\alpha$, $\beta$ and $\gamma$), encoded by different but homologous genes. Not only RGD sequences are found as binding sequences in laminins, but also other recognition motifs that can interact with cell surface receptors such as proline-aspartic acid-serine-glycine-arginine (PDSGR), tyrosine-isoleucine-glycine-serine-arginine (YIGSR) and isoleucine-lysine-valine-alanine-valine (IKVAV).\textsuperscript{[27]} Both laminin\textsuperscript{[28–30]} and fibronectin\textsuperscript{[31]} are known to enhance fibroblast activity and upregulate focal contacts in vitro.

Application of self-assembled monolayers (SAMs) is another way to chemically activate surfaces.\textsuperscript{[32]} Specifically, SAMs are nm thick monolayers made of molecules that covalently bind to the substrate material and, due to the intermolecular interactions, form a closed layer with a locally highly ordered structure. The molecules are characterized by three functional sections: i) a head group, which forms a covalent bond with the substrate material, ii) an organic backbone, and iii) a terminal group that determines the surface properties and thus its interaction with the surface environment.\textsuperscript{[33]} Different functional groups such as $\text{–OH}$, $\text{–CH}_3$, $\text{–COOH}$, $\text{–NH}_2$ can be used to control hydrophobicity or to induce reactivity.\textsuperscript{[12–14]}

In our study, we tested the hypothesis that the presence of fibronectin and laminin on Ti6Al4V and Y-TZP surfaces facilitates enhanced integrin expression, and adhesion and proliferation of human gingival fibroblasts (HGFs). Here, fibronectin and laminin were covalently immobilized on silanized Ti6Al4V and Y-TZP surfaces through crosslinking using bis(sulfosuccinimidyl)suberate (BS\textsuperscript{3}). The different modified specimens were examined via XPS, AFM, and static contact angle measurements. HGFs were used for in vitro studies because this cell type is the major cellular constituent of the fibrous connective tissue and essential for the maintenance of the soft tissue barrier at the abutment interface.\textsuperscript{[35]} Cell responses were assessed in vitro by investigating phosphorylation of focal adhesion kinase (pFAK-Y397), integrin expression, cell spreading, and cell proliferation.

2. Results and Discussion

2.1. Identification of HGFs via Immunofluorescence and Flow Cytometry

Human gingival fibroblasts were obtained after seven days through the explant technique. The isolated cells can be recognized by their typical spindle-shaped morphology (Figure 1A). Characterization of fibroblasts was achieved by immunostaining the marker’s vimentin and CD90 (Thy-1) (Figure 1B,C) since these markers are typically expressed in fibroblasts. Moreover, analysis by flow cytometry revealed high expression of CD10 and CD63 (Figure 1F), whereas the expression of epithelial cell markers CD24 and CD326 (EpCAM), which were used as negative markers, were almost undetectable (Figure 1E). These data confirmed that the isolated cells are gingival fibroblasts.

2.2. Characterization of the Individual Surfaces

The topography of the applied ADPS monolayer on sapphires was monitored by AFM. The AFM images clearly show the untreated sapphire (Figure 2A) and the presence of homogeneously distributed APDS on functionalized sapphires (Figure 2B). The application of APDS resulted in a grained structure and revealed an increased Sa value of 0.52 nm compared to those from untreated sapphires (Sa: 0.08 nm). The height profiles of untreated and APDS treated sapphires are visible in the bottom panel (Figure 2C,D).

The atomic chemical composition of the different modified surfaces was characterized by XPS. The XPS survey of silanized Y-TZP and Ti6Al4V is illustrated in Figure 3 and the atomic percentage of both specimens is listed in Table 1.

Piranha-treated Y-TZP discs showed almost no differences compared to native Y-TZP. In contrast, treatment of Ti6Al4V with piranha solution reflected an increased content of titanium from 8.7 ± 0.4% to 15.4 ± 2.0%, as well as an increased oxygen content up to 50.2 ± 1.9% (Table 1). Furthermore, the associated increase of carbon content to 31.5 ± 3.5% indicated the reduction of organic residues and the formation of a hydrated titanium oxide layer. It is worth noting that the alumina content within Ti6Al4V was almost undetectable in all specimens. Based on the EDX spectrum, however, it was confirmed that the alumina content within Ti6Al4V was almost undetectable in all specimens. Based on the EDX spectrum, however, it was confirmed that the titanium alloy Ti6Al4V was present (Figure S1, Supporting Information). After APDS application, the element silicon was detectable with 2.7 ± 1.3% on Y-TZP and on Ti6Al4V with a content of 1.6 ± 1.7%. In the presence of APDS the amount of carbon increased due to the alkyl chain within the organosilane, indicating the successful application of the silane.

The immobilization of biomolecules at the implant interface can be realized using a variety of methods and is a crucial step for its interaction with the surrounding biological environment.\textsuperscript{[36]} Here, immunostaining was performed to demonstrate that the protein fibronectin was successfully coupled to the surface of the Y-TZP and Ti6Al4V discs. To accomplish this, the specimens
Figure 1. Characterization of fibroblasts isolated from human gingival tissue. A) Bright field image of human gingival fibroblasts (HGFs), which started to grow out of tissue explants after 7 d. B) CD90 and C) vimentin expression of HGFs cultured on glass slides for 3 d was visualized by indirect immunofluorescence. Apart from immunofluorescence of HGFs, flow cytometry analysis was performed: D) Forward scatter (FSC-A) plot against side scatter (SSC-A) plot was used to gate fibroblasts and to exclude cell debris. E) CD24 and CD326 served as negative markers, while F) CD63 and CD10 represented the positive markers with 98.58% of gated cells. Scale bars: 100 µm.

Figure 2. AFM images and height profiles. AFM surface measurements of A) polished untreated sapphires and B) sapphires after silanization procedure with a resolution of 5 µm × 5 µm. C) The height profile of native sapphire is represented. D) Height profile of APDS treated sapphire.
Surface wettability is a key factor for efficient cell attachment and proliferation. Several studies indicate that surface wettability affects cell behavior, but there is disagreement about which kind of surface wettability stimulates cell attachment. However, we know that cells tend to attach more on hydrophilic than hydrophobic surfaces. Wei et al. showed that fibroblasts exhibited greater cell spreading on hydrophilic surfaces, and other studies have demonstrated that surface modifications that induce hydrophilicity result in increased cell proliferation and enhanced fibronectin expression. In addition, the expression of pro-inflammatory molecules like tumor necrosis factor alpha (TNF-α) is decreased on hydrophilic surfaces compared to uncoated and hydrophobic surfaces. Hydrophilicity thus plays a key role in surface modification and promotes soft tissue attachment.

The homogenous distribution of fibronectin was demonstrated by immunofluorescence on both specimens (Figure 4A). The homogenous distribution of fibronectin on Y-TZP and Ti6Al4V surfaces is illustrated in Figure 3 by immunofluorescence on both specimens (Figure 4B,C), but the detachment of the biomolecules.

Table 1. Atomic percentages of native (Z; Ti), piranha solution (Z-A; Ti-A), and APDS (Z-AS; Ti-AS) treated Y-TZP/Ti6Al4V discs examined by XPS.

|          | O 1s  | C 1s  | Zr 3d | Y 3d  | Si 2p | Ti 2p | Al 2p |
|----------|-------|-------|-------|-------|-------|-------|-------|
| Z        | 58.0 ± 2.2 | 15.5 ± 3.9 | 24.9 ± 2.8 | 1.5 ± 0.1 | –     | –     | –     |
| Z-A      | 51.6 ± 2.3 | 25.2 ± 4.3 | 21.9 ± 2.8 | 1.3 ± 0.1 | –     | –     | –     |
| Z-AS     | 44.8 ± 2.9 | 32.8 ± 4.5 | 18.9 ± 3.5 | 0.8 ± 0.1 | 2.7 ± 1.3 | –     | –     |
| Ti        | 31.8 ± 2.0 | 58.2 ± 4.0 | –     | –     | –     | 8.7 ± 0.4 | –     |
| Ti-A      | 50.2 ± 1.9 | 31.5 ± 3.5 | –     | –     | –     | 15.4 ± 2.0 | –     |
| Ti-AS     | 46.0 ± 1.7 | 34.5 ± 3.8 | –     | –     | 1.6 ± 1.7 | 14.6 ± 1.6 | 1.9 ± 1.0 |

Figure 3. XPS survey obtained from the silanized Y-TZP (in black) and Ti6Al4V (in gray) surfaces.

SEM images of the individual modified surfaces are visible in Figure S3 (Supporting Information).

2.3. Fibroblast Behavior on Modified Surfaces

FAK plays a key role in cell adhesion. Since Tyr 397 autophosphorylation (pFAK-Y397) is important for FAK activation and therefore a prominent marker for downstream signal transmission, the pFAK-Y397 antibody was used to analyze integrin signaling.

To assess the influence of native, APDS, BSA, fibronectin, and laminin modified samples on cellular adhesions, HGFs were seeded under serum-free conditions on the individual specimens. Cell adhesion was investigated at 30 min (Figure 5A,B) and 24 h (Figure 6A–F) after cell seeding by confocal microscopy. pFAK-Y397 immunostaining of HGFs seeded on fibronectin and laminin modified surfaces for 30 min revealed an increased activation of pFAK in comparison to all other surfaces of Ti6Al4V and Y-TZP (Figure 5A,B). It should be noted that cells on APDS-modified surfaces also showed enhanced pFAK activation. In contrast, cells cultivated on native specimens showed significantly reduced pFAK activation compared to cells seeded on ECM protein-modified surfaces. BSA coated samples served as a negative control, where the lowest pFAK activation was found. Looking more closely at the actin cytoskeleton at 30 min, the presence of the ECM proteins seems to favor cell spreading. Here, the cell area was much more extended in comparison to cells cultivated on native Y-TZP and Ti6Al4V discs, which exhibited a typical narrow shape due to their surfaces being less favorable to cells. In addition to microscopic illustration, this phenomenon was also quantified as shown in the diagrams in the bottom panel (Figure 5C,D). Cell area on BSA coated samples is not depicted due to an insufficient number of attached cells.
After 24 h, the actin network of fibroblasts was clearly visible in stress fibers and filopodia on laminin and fibronectin-coated surfaces (Figure 6A–D). Furthermore, extended cells started to form connections with adjacent cells. Specifically, the pFAK-Y397 expression at the ends of actin filaments was greatly increased on ECM protein-modified surfaces (highlighted in Figure 6 with white arrows), whereas poor activation was detectable on native surfaces. On uncoated samples, the cells exhibited a rounded, non-elongated morphology and fewer cell protrusions. In particular, filopodia were rarely visible (Figure 6E,F).

These results suggest that HGFs recognize the RGD sequence or any other adhesive motifs within fibronectin and laminin with integrin receptors, resulting in improved cell adhesion. The RGD motif was identified in many ECM proteins, including fibronectin, laminin, and vitronectin, and is recognized by integrins. As multifunctional adhesion receptors, integrins are involved in many cellular processes such as cell adhesion, spreading, migration, proliferation, differentiation, survival, and cell cycle progression. Furthermore, the formation of focal adhesions is mainly caused by integrins. The integrin-stimulated FAK-dependent signaling pathway is initiated by oligomerization of the integrins, which leads to the recruitment of FAK. This in turn leads to autophosphorylation on Tyr 397, thus forming a binding site for the family of Src kinases, which phosphorylate FAK on further tyrosine residues. With the activation of these signaling pathways, a range of cellular reactions such as invasion, adhesion, motility, and survival can occur.

In findings similar to ours here, Yang et al. showed that the application of RGD sequences on zirconia significantly enhances the biological activities of HGFs. Jian et al. demonstrated that titanium surfaces with a microgroove structure modified with fibronectin significantly enhanced cell adhesion, proliferation of HGFs, and cell orientation along these structures. However, fibronectin was connected to silanized titanium surfaces without...
Figure 5. Detection of pFAK-Y397 and Actin in HGFs after 30 min of cultivation on the individual specimens by confocal microscopy. HGFs were seeded onto native, APDS and ECM protein modified Y-TZP and Ti6Al4V surfaces. A,B) Actin cytoskeleton of HGFs was stained with Alexa Fluor 488-Phalloidin (green) and pFAK-Y397 with Alexa Fluor 555 (red). C,D) Cell area was determined by ImageJ from immunofluorescence images. Statistical significance values are indicated as $p < 0.05^*$, $p < 0.001^{***}$, error bars show ± standard error of the mean. Scale bars: 5 µm.
using a crosslinker. Within our study, we used a crosslinker that binds a significantly higher amount of protein compared to non-specific protein adsorption on silanized surfaces. Böke et al. verified that the covalent immobilization of the desired protein via BS1 crosslinker on silanized ceramic surfaces prevents protein displacement in culture medium. In addition, crosslinking and coupling of the proteins takes only a few hours in our approach and is therefore a highly time-efficient method. The present study demonstrates clearly, that through this novel surface modification a much higher amount of stable coupled ECM proteins can be achieved, which resulted in enhanced cell adhesion, cell proliferation, and integrin expression.

To evaluate the cell–substrate interaction a centrifugation assay was performed whereby cells were cultivated for 30 min on the individual surfaces and exposed to centrifugal forces up to 50 × g (Figure 7A). Significantly more HGFs remained on ECM protein-modified surfaces after this procedure compared to non-functionalized surfaces, indicating an increased strength of cell adhesion on the fibronectin and laminin modified surfaces.

The proliferation rate of HGFs on different samples was monitored over seven days by CCK-8 assay (Figure 7B). At day 1, HGFs showed almost the same proliferation level on all surface conditions. From day 3 on, an increase in the proliferation of HGFs on ECM protein-modified surfaces compared to native surfaces was evident. The difference was even more pronounced on day five and day seven of cultivation.

As mentioned above, colonization of the implant surface with bacteria in place of host cells will result in biofilm formation and reduced longevity of the implant. Our results demonstrate that the presence of the ECM proteins fibronectin and laminin greatly support cellular attachment and cell adhesion compared to non-functionalized surfaces. This suggests that host cells at the modified implant interface, in particular HGFs, establish a more robust tissue cuff. Whether fewer bacteria accumulate at the ECM functionalized surfaces will be addressed in further studies.

In addition, the expression of integrin subunit β1 was evaluated, since β1 is a common mediator of fibroblast attachment to different ECM proteins and crucial for cutaneous tissue repair. The expression of integrin subunits on pure Ti6Al4V/Y-TZP

Figure 6. Confocal microscopy images of HGFs 24 h after cultivation on pure and ECM protein modified surfaces. Immunostaining indicates pFAK-Y397 (red) and phalloidin-staining (green) of HGFs seeded on specimens coated with A,B) laminin, C,D) fibronectin, and E,F) native Ti6Al4V/Y-TZP discs. White arrows label pFAK-Y397 expression at the end of actin filaments. Scale bars: 20 µm.
Figure 7. Analysis of cell adhesion and proliferation rate of HGFs. A) Cells were cultivated on bare and ECM–protein coated Ti6Al4V/Y-TZP for 30 min and subsequently exposed to centrifugal forces (50 × g). B) The proliferation rate of HGFs on the individual samples was monitored over seven days via CCK-8 assay. The absorbance was measured at 450 nm using a plate reader. Statistical significance values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, error bars show ± standard error of the mean. Experiments were performed in triplicates (n = 3) for each condition. CCK-8 assay was analyzed with two-way ANOVA.

Figure 8. Integrin subunit expression analyses via flow cytometry and confocal microscopy on native and laminin treated Ti6Al4V/Y-TZP surfaces. A–D) HGFs were cultivated on native Ti6Al4V/Y-TZP and E–H) on laminin modified surfaces for 48 h, after which the expression of CD29 and CD49a was analyzed via flow cytometry. Expression of CD29 (red) and actin staining with Alexa Fluor 488-Phalloidin (green) of HGFs seeded onto I,J) native and K,L) laminin coated Ti6Al4V and Y-TZP surfaces was verified by confocal microscopy. Scale bars: 50 µm.

(Figure 8A–D) and on laminin-coated specimens (Figure 8E–H) was analyzed by flow cytometry. In particular, the expression of CD29 (integrin β1) and CD49a (integrin α1) were verified due to the fact that the combination of β1 and α1 form a cell surface receptor for laminin and collagen.\(^{[52]}\) The histograms revealed an enhanced expression of CD29 and CD49a on laminin-modified Ti6Al4V surfaces compared to non-functionalized surfaces (Figure 8A,B,E,F). In addition to flow cytometry, confocal images were taken in order to analyze integrin expression (Figure 8I–L). These clearly show that more integrin β1 (CD29) was expressed on laminin-coated Ti6Al4V surfaces compared to non-modified surfaces (Figure 8K). In results similar to the Ti6Al4V laminin modified surfaces, increased expression of CD29 was also detectable on the laminin-coated Y-TZP specimens (Figure 8L).
These results are in contrast to the flow cytometry analyses of Y-TZP discs, which showed almost no differences in integrin expression between modified and non-modified surfaces (Figure 8C,D,G,H). Finally and of great importance, a higher number of cells and a larger cell-covered area were visible on the laminin-coated specimens (Figure 8K,L).

In contrast to our findings, Palaiologou et al. found that gingival fibroblasts but not periodontal ligament fibroblasts adhere poorly to laminin coated surfaces. However, they showed that both cell types express the laminin receptors (e.g., CD24, and CD326) and assumed that more than one receptor is involved in cell-binding to ECM or that the specific receptor is inactive. In our study, we demonstrated that gingival fibroblasts exhibited enhanced cell adhesion on laminin-coated surfaces and revealed a higher expression of the integrin compared to non-functionalized surfaces. The receptor thus seems to be active and to recognize adhesivemotifs within laminin. The laminin molecules may also be more accessible after immobilization through the coupling procedure presented in this study.

3. Conclusion

The results of this study indicate that the covalent conjugation of fibronectin and laminin on Ti6Al4V and Y-TZP supports cell adhesion, proliferation and spreading, and integrin expression. It can be concluded that the covalent immobilization of the ECM proteins fibronectin and laminin to silanized surfaces via a crosslinker holds promise for creating an effective “seal” between the abutment surface and the attaching gingiva in clinical applications, reducing the risk of bacteria penetration and thus potentially preventing or minimizing diseases like peri-implantitis.

4. Experimental Section

Isolation of Human Gingival Fibroblasts (HGFs): Human gingival tissue was obtained with written informed consent (EK 266/19) as required by the ethics committee of the Faculty of Medicine of RWTH Aachen University from clinically healthy donors undergoing routine surgical interventions. For collection of HGFs, the gingival tissue was mechanically separated into epithelial and connective tissue during surgical procedures. The obtained tissue was disinfected in 70% ethanol and washed in phosphate-buffered saline solution (PBS, Gibco, USA) containing 1% (v/v) penicillin–streptomycin. The connective tissue was cut into small pieces and the explants were placed into 25 cm² culture flasks. After the explants had 30 min to adhere to the bottom of the culture flask, they were carefully covered with 3 mL of culture medium (Dulbecco’s modified Eagle’s medium, DMEM, high glucose, Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS, PAN Biotech, Germany) and 2% (v/v) l-glutamine (Sigma-Aldrich). The connective tissue was disinfected in 70% ethanol and washed in phosphate-buffered saline solution (PBS, Gibco, USA) containing 1% (v/v) penicillin–streptomycin. The connective tissue was cut into small pieces and the explants were placed into 25 cm² culture flasks. After the explants had 30 min to adhere to the bottom of the culture flask, they were carefully covered with 3 mL of culture medium (Dulbecco’s modified Eagle’s medium, DMEM, high glucose, Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS, PAN Biotech, Germany) and 2% (v/v) l-glutamine (Sigma-Aldrich). The connective tissue was disinfected in 70% ethanol and washed in phosphate-buffered saline solution (PBS, Gibco, USA) containing 1% (v/v) penicillin–streptomycin. The connective tissue was cut into small pieces and the explants were placed into 25 cm² culture flasks. After the explants had 30 min to adhere to the bottom of the culture flask, they were carefully covered with 3 mL of culture medium (Dulbecco’s modified Eagle’s medium, DMEM, high glucose, Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS, PAN Biotech, Germany) and 2% (v/v) l-glutamine (Sigma-Aldrich).

Identification of HGFs via Flow Cytometry: Flow cytometry was used to confirm the expression of the surface antigen markers CD10, CD63, CD24, and CD326. To accomplish that, 1 x 10⁶ of HGFs at passage two were used. The cells were incubated with the fluorescently labeled antibodies APC/Cyanine7 CD10, PE CD63, Brilliant Violet 421 CD24 and FITC CD126 (EpCAM) (1:20 dilution, anti-human, #312212, #353003, #311121 and #324209 all from BioLegend, USA) for 15 min at 4 °C in the dark. After this incubation step, cells were washed with 2 mL of PBS containing 2% FCS and centrifuged at 4 °C, 1300 rpm for 5 min. Subsequently, the supernatant was discarded and the cell pellet was dissolved in 150 µL PBS with 2% FCS. The labeled cells were filtered into flow cytometry tubes using a 100 µm cell strainer and were analyzed using a BD FACS Canto II (BD Biosciences, USA). Flow cytometry data were analyzed via FCS Express 7 (De Novo Software, USA).

Indirect Immunostaining of HGFs: In addition to flow cytometry analysis, immunofluorescence staining of vimentin and CD90 (Thy-1) was performed, as these markers are mostly expressed in fibroblasts. 1 x 10⁵ cells/mL were seeded on coverslips and after 3 days of incubation fixed with 4% (v/v) paraformaldehyde (PFA, Thermo Fisher Scientific, USA) for 10 min. Thereafter, HGFs were permeabilized with 0.1% v/v Triton X-100 (Fluka, Germany) for 10 min and blocked with 1% (v/v) bovine serum albumin (BSA; Sigma Aldrich, Germany) for 1 h at room temperature. Fibroblasts were labeled with vimentin (1:400 dilution, #MA110459, mouse monoclonal, Invitrogen, USA) and CD90 (1:200 dilution, #DIA 100 M, mouse monoclonal, Dianova, Germany) diluted in 0.1% (v/v) BSA and incubated overnight at 4 °C. In order to visualize labeled cells under the fluorescence microscope (Axio Imager 2, Carl Zeiss, Germany), HGFs were incubated with secondary antibody Alexa Fluor 488 (for vimentin, 1:2000 dilution, #A21121, goat anti-mouse, Invitrogen, USA) and Alexa Fluor 555 (for CD90, 1:2000 dilution, #A31570, donkey anti-mouse, Invitrogen, USA) for 45 min at room temperature under continuous agitation. Finally, samples were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, USA).

Preparation of Titanium and Ceramic Discs: Cylindrical Ti6Al4V bars with a diameter of 9 mm were obtained from EWGE Wagener (Germany) and Ti6Al4V discs (thickness 1.5 mm) were prepared by uniaxial dry pressing using a stainless steel cylindrical mold. Subsequently, Y-TZP discs were sintered at 1500 °C for 2 h (Therm-Aix, Germany). Afterwards, the discs were ground (No. 40 and 74) and polished using diamond pastes (ATM, Germany). On both specimens a roughness of 0.2 µm was adjusted. In order to remove potential organic residues, ceramic discs were heated up to 450 °C for 15 min. Finally, the discs were cleaned by sonication in 70% ethanol (Schmittmann GmbH, Germany) and Milli-Q water (Sartorius, Germany).

Pretreatment and Silanization of the Specimens: For the silanization procedure, the specimens were cleaned and hydroxylated by immersion in piranha solution (3:1 ratio of concentrated sulfuric acid (Sigma Aldrich, USA) and 30% hydrogen peroxide (Merck, Germany)) for 1 min and rinsed thoroughly in Milli-Q water (Sartorius, Germany). After drying at room temperature, the Ti6Al4V and Y-TZP discs in a round bottom flask equipped with a magnetic stirrer were soaked in a 5 vol% 3-aminopropyltrimethoxysilane (APDS, Gelest, USA) solution mixed with anhydrous toluene (Sigma-Aldrich, USA). The reaction was performed under reflux conditions at 120 °C for 3 h in an oil bath. Immediately afterwards, samples were thoroughly cleaned by 3-minute wash steps with toluene (VWR, USA) and Milli-Q water (Sartorius, Germany). The specimens were dried overnight at room temperature.

Biofunctionalization—Immobilization of Fibronectin and Laminin on Silanized Surfaces: To immobilize the proteins fibronectin (Merck, Germany) or laminin (Merck, Germany) on silanized samples, BS3 crosslinker (Thermo Fisher Scientific, USA) was used. Specifically, 0.25 × 10⁻³ M of this water-soluble bi-functional crosslinker reagent was used to react with the primary amines of the previously silanized specimens at 4 °C for 1 h in borate buffered saline (BBS, pH 8.5) under agitation. Afterwards, the specimens covered with BS3 solution were rinsed with precooled BBS. Subsequently, the discs were immersed in protein solution (1 µg mL⁻¹ in BBS) for 2 h at 4 °C with frequent shaking in order to enable covalently bonded proteins on the individual surfaces. In order to remove unbound proteins, the samples were washed three times with precooled BBS. Table 2 lists the nomenclature for identifying the various surfaces examined.

X-Ray Photoelectron Microscopy (XPS): The elemental surface composition of the individual specimens was analyzed by X-ray photoelectron microscopy (XPS). For XPS measurements, an Ultra Axis spectrometer...
Table 2. Nomenclature of the individual specimens.

| Specimens | Sample description |
|-----------|--------------------|
| Ti        | Native Ti6Al4V, untreated (just ground and polished) |
| Ti-A      | Ti6Al4V activated with piranha solution (3:1) |
| Ti-AS     | Ti-A followed by silanization with APDS |
| Ti-AS-FN  | Ti-AS followed by covalent immobilization of fibronectin (via BS\(^5\) crosslinker) |
| Ti-AS-LN  | Ti-AS followed by covalent immobilization of laminin (via BS\(^5\) crosslinker) |
| Z         | Native Y-TZP, untreated (just ground and polished) |
| Z-A       | Y-TZP activated with piranha solution (3:1) |
| Z-AS      | Z-AS followed by silanization with APDS |
| Z-AS-FN   | Z-AS followed by covalent immobilization of fibronectin (via BS\(^5\) crosslinker) |
| Z-AS-LN   | Z-AS followed by covalent immobilization of laminin (via BS\(^5\) crosslinker) |

(Kratos, UK) equipped with monoenergetic AlK\(_{\alpha,2}\) (1486.7 eV, 144 W) radiation was used. Survey scans were recorded using a pass energy of 160 eV.

Atomic Force Microscopy (AFM): The NanoStation II (SiS, Germany) was used in the tapping mode to assess the silane adsorption profile on sapphire (Y:ZrO\(_2\)–YSZ, CrysTeC, Germany). The PPP-NCL cantilever (NANOSENSORS, Switzerland) was placed randomly on the individual samples (native sapphire and sapphire treated with APDS) and three images with a resolution of 5 \(\mu\)m \(\times\) 5 \(\mu\)m were recorded. All atomic force microscopy (AFM) images were analyzed using NanoScope 9.1 software (Bruker, USA) to observe the surface topography.

Static Contact Angle: The surface wettability of various activated samples was assessed by the sessile drop method. For this, 10 \(\mu\)L of Milli-Q water (Sartorius, Germany) was deposited at random onto the respective surfaces. Contact angles were measured directly after drop deposition. Three images of each specimen were taken via digital camera (Canon EOS 7D, Japan) and analyzed in Fiji with the DropSnake plugin.[54]

Fluorescence Labeling of the Immobilized ECM Proteins: In order to confirm the presence of applied human fibronectin and laminin on the sapphire substrate, immunofluorescence microscopy was performed. The immobilized proteins were fixed with 4% (w/v) PFA for 10 min and blocked with horse serum (Sigma-Aldrich, USA) for 1 h at room temperature. Next, the specimens were incubated overnight at 4 °C with the primary antibodies anti-human fibronectin/anti-laminin (1:200 dilution, #A1918, mouse monoclonal, R&D Systems, USA and 1:100 dilution, #SA4200719, mouse monoclonal, Sigma Aldrich, USA). The remaining antibody solution was removed by 5 min wash steps with PBS and after washing samples were incubated with Alexa Fluor 488 (1:200 dilution, #A21121, goat anti-mouse, Invitrogen, USA) or Alexa Fluor 555 (1:2000 dilution, #A31570, donkey anti-mouse, Invitrogen, USA) for 1 h at room temperature. Finally, bound antibodies were detected by fluorescence microscopy (Axio Imager Z1, Carl Zeiss, Germany).

Confocal Microscopy: For visualization of the focal adhesion kinase (FAK) phosphorylation via antibody staining, HCFs were cultured on various surfaces (nativ discs, coated with APDS, coated with fibronectin, coated with laminin). Furthermore, expression of integrin \(\beta1\) on pure and laminin-coated specimens was assessed by immunostaining. HCFs were seeded onto the discs at a density of 1 \(\times\) 10\(^4\) cells \(\text{cm}^2\) in serum-free medium. After 30 min/24 h incubation at 37 °C and 5% CO\(_2\), cells were fixed with 4% (w/v) PFA in PBS and permeabilized with buffered Triton X-100 solution for 5 min at room temperature. To prevent unspecific antibody binding, samples were blocked with 5 wt% horse serum in PBS containing 0.3 vol% Triton X-100 for 1 h. After washing with 3% (w/v) BSA, specimens were incubated overnight with anti-pFAK antibody (1:1000 dilution, #8555, rabbit monoclonal, Cell Signaling Technology, USA). In order to visualize integrin \(\beta1\) the primary antibody (1:100 dilution, #14-0299-82, mouse monoclonal, Invitrogen, USA) was incubated overnight at 4 °C. The samples were washed as described above and incubated with the secondary antibody (Alexa Fluor 555, 1:200 dilution for pFAK, #A13152, donkey anti-rabbit, 1:2000 dilution for integrin \(\beta1\), #A131570, donkey antimouse, Invitrogen, USA) for 1 h at room temperature. After this incubation step, actin was stained with Alexa Fluor 488-Phalloidin (1:250 dilution, #A12379, Invitrogen, USA) for 45 min under constant agitation. Finally, the stained samples were mounted with Mowiol 4-88 (Carl Roth, Germany) and placed in eight-well slides (ibidi, Germany). Imaging was conducted using a Zeiss LSM 710 confocal microscope.

Energy Dispersive X-Ray Spectroscopy (EDX): Energy-dispersive X-ray spectroscopy (EDX, Falcon Genesis EDAX, USA) was used for the identification of the elemental composition of the specimens Ti6Al4V and Y-TZP. Imaging was conducted at 10 kV.

Centrifugation Assay: In this study, the adherence of fibroblasts on the individual samples was assessed by centrifugation assay, adopted from Reyes and Garcia.[55] Cells were seeded at a density 1 \(\times\) 10\(^5\) cells \(\text{mL}^{-1}\) onto each specimen in a 24-well plate and incubated for 30 min at 37 °C and 5% CO\(_2\). After incubation, the serum-free medium was discarded and samples were glued into the cavities of a 24-well plate. The samples were then centrifuged speeded up to 50 \(\times\) g for 5 min at room temperature. The persisting adherent cells on the different surfaces were visualized by crystal violet staining. For staining, cells were fixed with 4% (w/v) PFA for 10 min at room temperature. Subsequently, 500 \(\mu\)L of the 0.1 wt% crystal violet staining solution was pipetted into the wells, and the plate within the samples was incubated at room temperature for 20 min on a plate shaker. Immediately thereafter, the dye was removed and the samples were washed until the water showed no residue of the staining solution. Then the specimens were air-dried overnight. As a control, non-modified Ti6Al4V and Y-TZP discs were used. Five images of each sample were recorded in order to count the remaining cells by Image.[56]

Cell Proliferation Assay CCK-8: The CCK-8 assay (Dojindo Laboratories Inc., Japan) was performed to measure the proliferation rate of HGFs. Fibroblasts (1 \(\times\) 10\(^5\) cells \(\text{mL}^{-1}\)) were seeded on the individual samples in a 48-well plate. After an incubation period of 1, 3, 5, and 7 days, the different specimens were washed with PBS and the extent of cell proliferation was quantified using 30 \(\mu\)L of CCK-8 reagent. The CCK-8 solution was added to each well and the well-plate was subsequently incubated at 37 °C for 3 h. In order to measure the absorbance at 450 nm using a multilabel reader (SpectraMax M2/M2e, Molecular Devices, USA), the 100 \(\mu\)L of supernatant was transferred from a 48-well plate to a 96-well plate.

Integrin Expression Analysis via Flow Cytometry: The integrin expression of the cells on the different treated surfaces was analyzed by flow cytometry. For this purpose, 5 \(\times\) 10\(^4\) cells \(\text{mL}^{-1}\) were seeded onto the discs and incubated for 48 h at 37 °C and 5% CO\(_2\) in a humidified atmosphere. After that, cells were detached from the surfaces using 0.05% trypsin/0.02% EDTA and collected in 2 \(\mu\)L tubes. The tubes within the cells were centrifuged at 13 000 rpm and immediately afterwards, the supernatant was discarded and the cell pellet was dissolved in fresh medium. Next, the cells were labeled with anti-integrin antibodies, especially \(\beta1\) subchain (1:2 dilution, PerCP/Cyanine5.5 anti-human CD29, #303023, BioLegend, USA) and \(\alpha1\) (1:200 dilution, APC anti-human CD49a, #328313, BioLegend, USA) and incubated for 15 min at 4 °C protected from light. Unlabeled cells served as a control. After washing with 2 \(\mu\)L PBS supplemented with 2% FCS, cells were centrifuged at 13 000 rpm for 5 min. Shortly after, the supernatant was removed and the cell pellet was dissolved in 150 \(\mu\)L PBS with 2% FCS. Cells were analyzed using BD FACSCanto II (BD Biosciences, USA) and for data analysis the software FCS Express 7 (De Novo Software, USA) was used.

Statistical and Image Analysis: Unless otherwise stated, all experiments were performed in triplicates. The cell culture experiments were repeated in three independent experiments with at least three different HCF donors. Results are presented as mean ± standard deviation. In this study, one-way analysis of variance (ANOVA) with Tukey’s post-hoc test was applied with a significance level of \(p < 0.05\). Cells were visualized with fluorescence microscope (Axio Imager 2; Carl Zeiss, Germany) and confocal microscope (Zeiss LSM 710) using 10 \(\times\) and 20 \(\times\) objectives.
respectively. Images were recorded at room temperature and randomly selected areas of the samples were captured. In order to avoid bias, the samples for confocal microscopy were blinded. Finally, images were analyzed using ImageJ.[18] All of the statistical analyses were determined with GraphPad Prism Version 8.0 (GraphPad Software, USA).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

Research data are not shared.

**Keywords**
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