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Focal adhesion formation of primary human gingival fibroblast on hydrothermally and in-sol-made TiO_2-coated titanium

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Abstract

Optimal cell adhesion of the gingival fibroblasts to dental implants is important for maintaining good implant integration. The aim of this study was to discover, if the nanoporous TiO_2-coating on titanium alloy substrates is able to increase the cell adhesion of the human gingival fibroblasts (HGF). The study consisted of three differently produced titanium groups: hydrothermally produced TiO_2-coating (HT), novel TiO_2-coating made in sol (SOL), and noncoated control group. Primary HGF cells were initiated from gingival biopsies from patients having a third molar extraction. HGF were cultivated on titanium discs for 2 and 24 h to determine the initial attachment with confocal microscope. The cell spreading and adhesion protein signals were measured. In addition, expression of adhesion proteins vinculin, paxillin, and focal adhesion kinase (FAK) were measured after 3 days of cultivation by using Western Blotting. Higher protein levels of paxillin, vinculin, and FAK were induced on both coated discs compared to noncoated discs. The difference was statistically significant ($p < 0.05$) concerning expression of paxillin. The cell spreading was significantly larger on SOL discs after 2 and 24 h when comparing to noncoated controls. The confocal microscope analyses revealed significantly higher adhesion protein signals on both HT- and SOL-coated titanium compared to control group. This study showed, that both methods to produce TiO_2-coatings are able to increase HGF adhesion protein expression and cell spreading on titanium surface. Accordingly, the coatings can potentially improve the gingival attachment to titanium implant surfaces.

KEYWORDS
dental implant, fibroblast, focal adhesions, nanoparticles, titanium

Summary Box

What is known

Gingival attachment forms a barrier against oral microbes. Therefore, an optimal cell attachment plays important role in maintaining desirable peri-implant health. Novel method to produce sol–gel-derived TiO_2-coating in sol has been shown to improve keratinocyte attachment to titanium.

What this study adds

Aim of this research was to study, if the in-sol-made TiO_2-coating or hydrothermally treated titanium are able to enhance the quality of fibroblast attachment to titanium surface. The results

evidenced higher focal adhesion formation, adhesion molecule expression, and cell spreading on both coated surfaces. The results are promising and indicate potential of these coatings to improve mucosal attachment.

1 | INTRODUCTION

Dental implants are in contact with three different tissues, bone, connective tissue, and epithelium.[1] To maintain a proper bone attachment and prevent peri-implant inflammation, it is important to prevent bacterial invasion of peri-implant area. The connective tissue and epithelial attachment play an important role in preventing microbes from invading deeper into tissues.

Even though zirconia abutments are increasing their popularity, titanium is still the most commonly used implant material due to its favorable properties such as high mechanical resistance, good biocompatibility, and corrosion resistance.[2] The adverse property of titanium abutments is their dark color, which can lead to unpleasant aesthetic results if the abutment is revealed after gingival recession. Ensuring a proper soft tissue attachment to implant surface minimizes the risk of gingival recession and prevents microbial invasion. Therefore, supporting a decent mucosal adhesion formation is crucial.[3]

Gingival epithelium is able to attach to implant surface in a similar manner as it does to natural tooth, via hemidesmosomes.[4] However, the connective tissue does not attach directly to implant or abutment surface, but the gingival fibers are organized in parallel to the implant surface forming a capsule like structure around oral implants.[5,6] The capsule makes an easier access to peri-implant area and can expose to peri-implant infection. The gingival connective tissue consists mainly of fibroblasts, collagen fibers, and blood vessels. Around oral implants the connective tissue consist of two zones, where the inner zone close to implant surface is rich with fibroblasts meanwhile the lateral zone contains more collagen fibers and blood vessels.[7] Focal adhesions bind the cell cytoskeleton to matrix with anchorage mechanism consisting of focal adhesion molecules. Important molecules in fibroblast focal adhesions are different integrins, which penetrate through the cell membrane to extracellular matrix in addition to vinculin, paxillin, and focal adhesion kinase (FAK), which locate in the cell cytoplasm.[8] Regarding oral fibroblast, the main integrin subunits expressed are α2, α4, α5, β1, and β3.[9,10] In turn, vinculin connects integrins to actin cytoskeleton. It forms from head and tail domains, where the head attaches to actin-binding proteins such as talin whereas the tail binds to paxillin and F-actin.[11] Vinculin is also able to increase cell spreading by stabilizing focal adhesions.[12] Concerning paxillin, it locates at the end of actin fibers and as it is able to bind to vinculin molecules, it is an important part in focal adhesion organization.[13] FAK and Src (Steroid receptor coactivator) are significant kinases in focal adhesion, as they are able to affect cell behavior and focal adhesion dynamics through binding. All these molecules play a role in focal adhesion signaling, tyrosine phosphorylation named as one.[14] The expression of these important focal adhesion molecules can be used to study the quality of connective tissue attachment to implant surface. In this study, we are focusing on the expression of vinculin, paxillin, and FAK.

Mucosal attachment can be improved with different surface modifications, which improve surface properties for example by increasing surface activity or nanotopography.[15] Oxidized titanium surface has been shown to form a longer connective tissue seal compared to machined titanium.[16] TiO_2-coating has ability to make the surface more hydrophilic, nanostructured and consequently increase the bioactivity of titanium surface.[17] In addition, non-resorbable, thin coating layer is rather easy to produce. Earlier, the sol–gel derived TiO_2-coating was produced by dip-coating method, which had limitations when it comes to coating more complicated structures.[18–21] In this study, two easier methods to produce TiO_2-coating have been used instead of dip coating. The first novel coating is made directly in sol with polycondensation method (SOL), whereas the second coating is produced with hydrothermal treatment under high pressure (HT). These coating protocols make it easier to coat more complicated structures like implant abutments when compared to dip coating method. Especially, coating made in sol needs no advanced technology, rather it can be produced with basic laboratory equipment.

We have shown recently that in-sol-made TiO_2-coating is able to improve epithelial cell spreading as well as adhesion protein expression on titanium and zirconia surface.[22] Also, earlier studies have indicated enhanced fibroblast adhesion and proliferation rates on sol–gel derived and hydrothermally treated titanium.[23] The aim of this study is to evaluate whether the in-sol-made and HT-derived TiO_2-coatings are able to influence the initial attachment of gingival fibroblast on molecular level by enhancing the focal adhesion formation or cell spreading, and whether those reflect as improved cell adhesion.

2 | MATERIALS AND METHODS

2.1 | Coating procedures

In this study, round titanium discs stamped from a plate (Grade 5, titanium 90%, vanadium 6%, aluminum 4%) with a diameter of 10 mm were used. Altogether, 90 titanium discs were polished with 1200-grit sandpaper (LaboPol 21, Struers A/S, Rodovre, Denmark). Thereafter, the samples were washed with acetone and ethanol (5′ + 5′) before coating.

The study consisted of TiO_2-coating produced with a coating-made-in-sol polycondensation technique, described earlier by Riivari and colleagues.[22] and HT-modified TiO_2-coating, which were compared against noncoated control group.

Firstly, 98% titanium isopropoxide (Acros Organics) was mixed with 95% ethanol. Secondly, 99% 2-ethoxyethanol (Acros Organics), 1 M Hydrogen chloride, and ethanol were mixed together. After, the second solution was pipetted to the first solution during affective mixing. This is followed by sol aging at room temperature for 24 h. After

1 day of sol aging at room temperature, titanium discs were covered with TiO_2–sol on a petri dish and set in a freezer (−18°C, 2 h). Lastly, the discs were washed three times with ethanol and heated in oven at 500°C for 10 min.

The production of hydrothermal coating also began with solution production. Titanium dioxide powder (3.2 g, Acros Organics) was mixed with purified water (160 g) and tetramethylammonium hydroxide in water (10%, 8.96 mL, Sigma Aldrich). The titanium discs were set in a Teflon cylinder with the produced sol. The cylinder was slowly rolled in an oven at 150°C for 48 h. Before beginning the cell cultures, all the samples were washed again in acetone + ethanol (5′ + 5′) in ultrasonic bath and sterilized in an autoclave.

Scanning electron microscopy (SEM) imaging (2 kV) was accomplished with Apreo S field-emission SEM (Thermo Scientific, Netherlands) to compare the surface topography between HT-coated, in-sol-coated, and noncoated samples. Two different magnifications (8000×, 50 000×) were used.

2.2 | Collecting the primary fibroblasts

Primary gingival fibroblasts were used in this study. Small gingival biopsy samples were collected from healthy gingiva of healthy adult patients undergoing a surgical extraction of third molar (Oral Health Care, City of Turku, Finland). The biopsy samples were transported to cell laboratory in cell medium [Gibco Dulbecco's Modified Eagle Medium (DMEM), Thermo Fisher Scientific] mixed with antibiotics (Pen Strep, Penicillin 10 000 units/mL, Streptomycin 10 000 µg/mL, Gibco). The gingival samples were cut into smaller pieces, set on a petri dish, and covered with DMEM. The pieces were left to grow for 3 days, before changing the media for the first time to prevent disturbing the cell attachment. The cells were cultured for 2 weeks, changing the media three times a week. The collection of biopsy samples was approved by the Ethics Committee, Hospital District of Southwest Finland (63/1801/2020).

2.3 | Western blotting

HGF were cultivated in media (DMEM, Gibco) at 37°C at a density of 30 000 cells/cm^2. After 3 days, when cell density reached 80%, the samples were washed with PBS and lysed with 95°C warm TXLB-buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-X, 0.5% glycerol, 1% SDS, Complete protease inhibitor (Sigma-Aldrich), and phos-stop tablet (Sigma-Aldrich)]. The lysate was collected in eppendorf tubes and heated again at 95°C (10 min) and stored in the freezer (−20°C). The amount of proteins in each sample was measured with Protein Assay Reagent (Bio-Rad). From each sample, equal amounts of protein were mixed with 8× sample buffer, pipetted on Mini Protean TGX Precast SDS-PAGE Gels (Bio-Rad), and transferred to membrane (Trans-Blot Turbo Transfer System, Bio-Rad). The membranes were washed twice with ultrapure water (mQ) and once with Tris-buffered saline with tween (TBST). Thereafter, the membranes were set in 5% milk in TBST for 1 h. The membranes were stained with primary antibodies [vinculin (1:1000, v9131, Sigma-Aldrich), paxillin (1:5000, 612405, BD Biosciences), Focal adhesion kinase (FAK, 1:5000, 610088), GAPDH (1:20 000, 5G4MaB6C5, Hytest)] diluted in 5% milk overnight (+4°C). The following day, the membranes were washed three times with TBST and treated with secondary antibody [IRDye 680 RD Donkey Anti-Mouse (1:5000, LI-COR Biosciences)] for 1 h. Again, the membranes were washed three times with TBST and imaged (Li-Cor, Infrared Imager, Odyssey).

2.4 | Cell cultures for immunofluorescence staining and confocal microscopy

The cells were cultivated in media at 37°C for 2 and 24 h to define the focal adhesion formation and cell spreading on each surface. After 2 and 24 h, the samples were fixed with paraformaldehyde (4%) for 15 min, washed with PBS (phosphate buffered saline), and stored (+4°C). Afterward, the samples were treated with TRITON-X-100 in PBS (0,5%, 300 µL) for 15 min. The discs were incubated overnight in

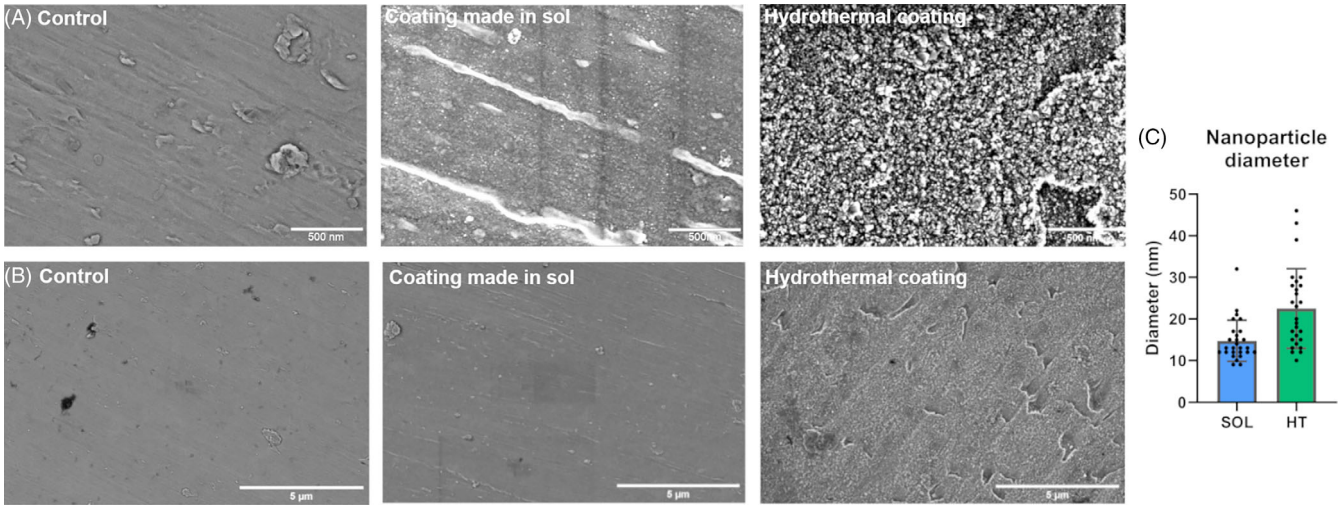

FIGURE 1 Surface topography of noncoated, coating-made-in-sol, and hydrothermally coated titanium imaged with SEM. Representative images of surface topography imaged with (A) 50 000× and (B) 8000× magnification. (C) Nanoparticle diameters on SOL- and HT-coated titanium. Shown are means ± SD + individual values.

primary antibodies (Vinculin V9131, 1:100, Anti-Paxillin ab32084, 1:500) in horse serum mixed with PBS (30%). The following day the discs were washed three times with PBS and handled with secondary antibodies [Anti-Mouse A21202, 1:400, Anti-Rabbit A110111:400 (ThermoFisher Scientific) and DAPI (nucleus staining, 1:200) and Phalloidin Atto (1:400, Sigma-Aldrich)] diluted in 20% horse serum for 1 h. After this, the discs were washed again with PBS and glued to microscope glass (Mowiol, Sigma-Aldrich). The stained discs were imaged with a spinning disc confocal microscope (63× Zeiss Plan-Apochromat, Hamamatsu sCMOS Orca Flash4.0, 3i CSU-W1 Spinning Disk). The signal of adhesion molecules paxillin and vinculin was measured from the bottom layer of cells with ImageJ, Fiji-program. Area of the cells and amount of focal adhesions per area from each group were calculated with ImageJ. Also, the size of focal adhesions stained with vinculin were measured from confocal images as earlier described by Horzum and colleagues.[24]

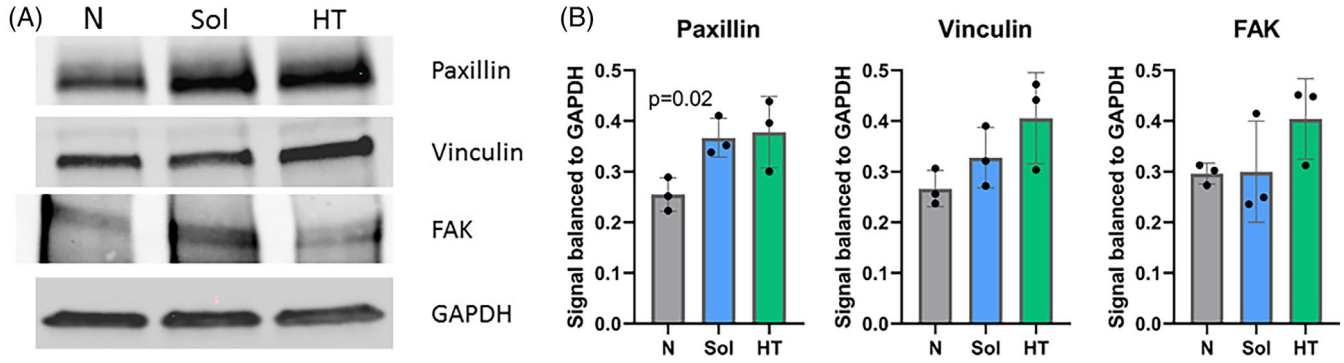

FIGURE 2 Expression of focal adhesion proteins paxillin, vinculin, and focal adhesion kinase (FAK) on noncoated, coating-made-in-sol, and hydrothermally coated titanium. (A) Western blotting. (B) Signal levels of paxillin, vinculin, and FAK balanced to GAPDH on coated and noncoated titanium surfaces after 3 days of cell culture. HT, hydrothermal coating; N, noncoated; Sol, coating-made-in-sol. $n = 3$ biological replicates, mean ± SD + individual values. Significant p values ($p < 0.05$) are marked in the figures.

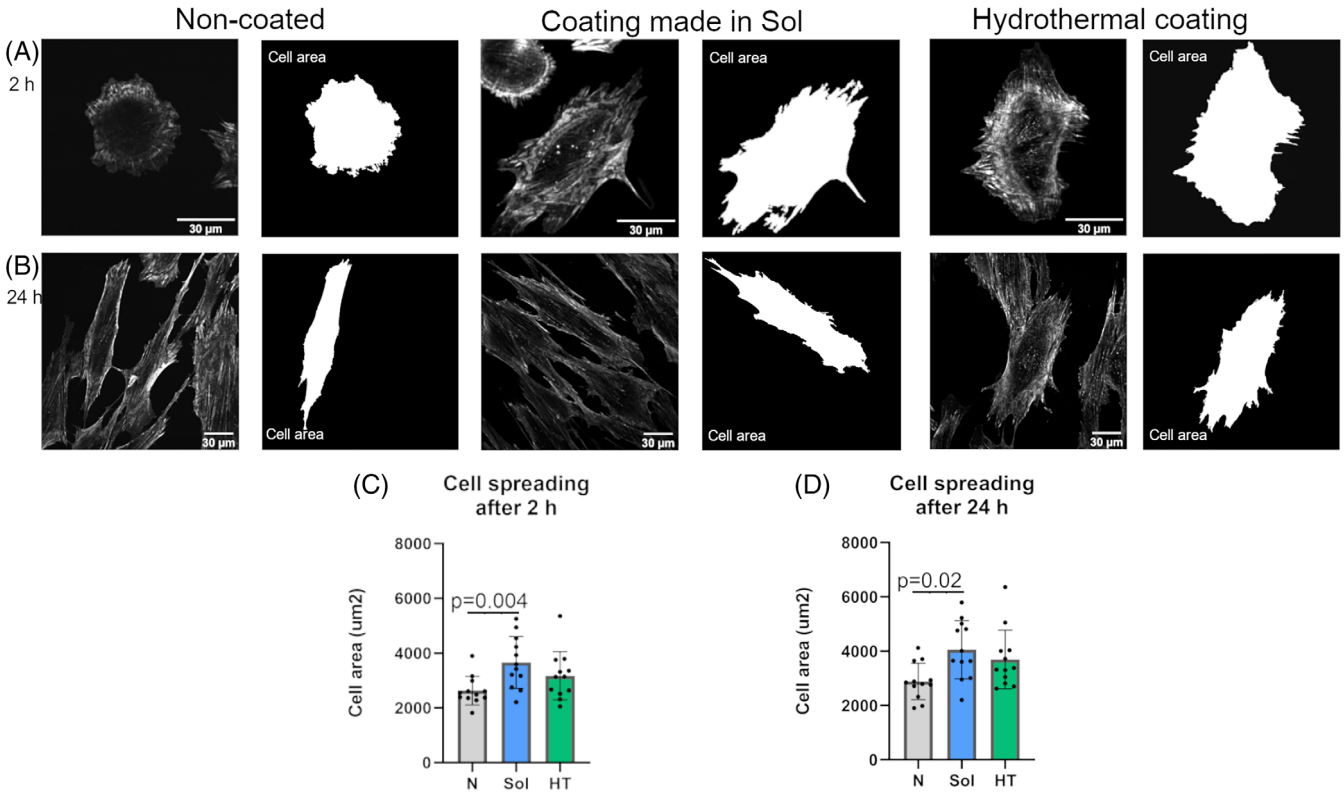

FIGURE 3 Effect of directly in sol-made and hydrothermally produced TiO_2-coatings on cell spreading after 2 and 24 h. (A) Representative images of Actin cytoskeleton and cell area after 2 and (B) 24 h. (C) Quantifications of cell area after 2 and (D) 24 h. HT, hydrothermal coating; N, noncoated; Sol, coating made in sol. $n = 12$ technical replicates, mean ± SD + individual values. Significant p values ($p < 0.05$) are marked in the figures.

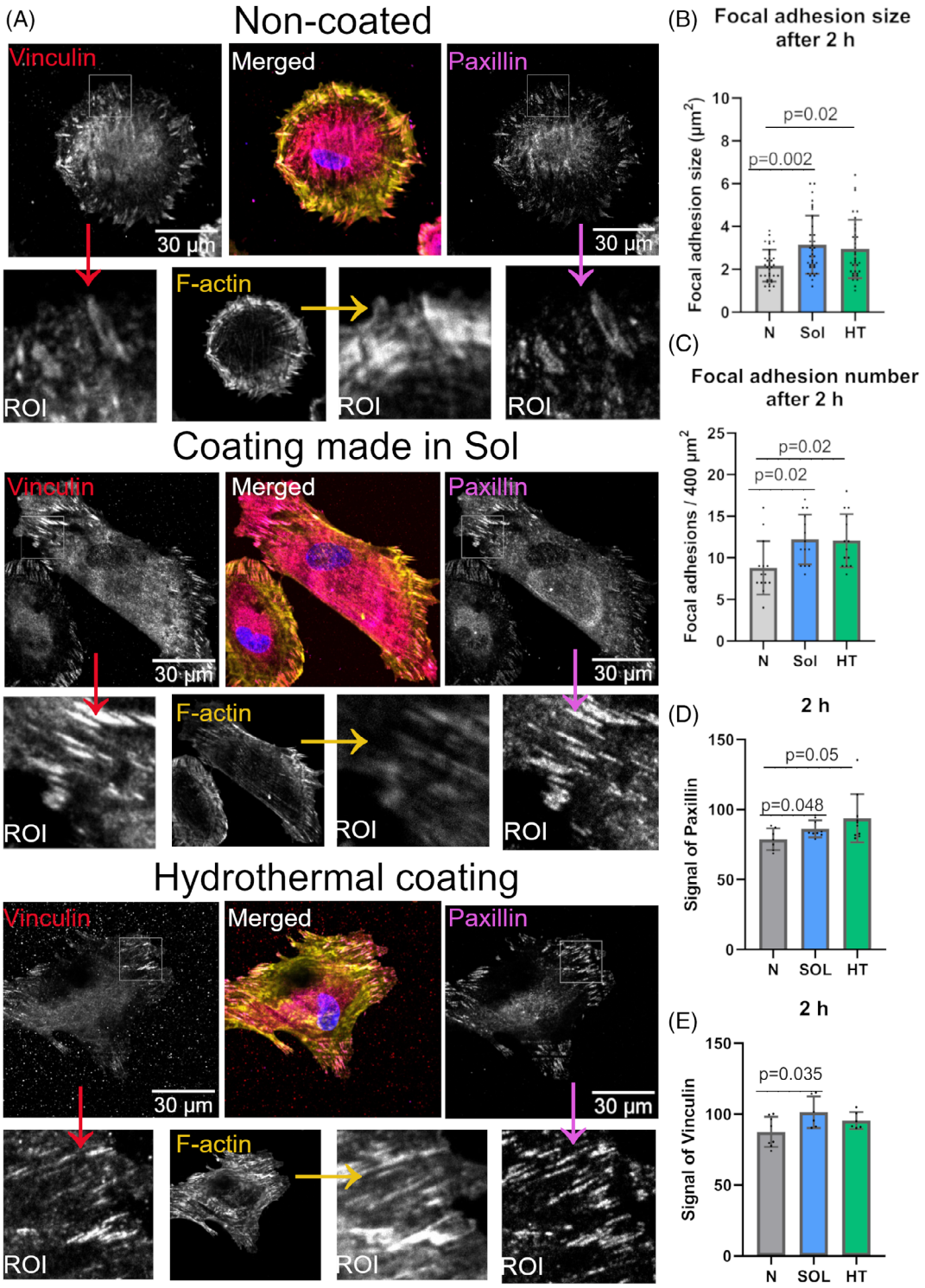

FIGURE 4 More rapid focal adhesion formation on TiO_2-coated titanium compared to noncoated titanium. (A) Shown are representative confocal microscopy images from the bottom layer of cells after 2 h of cell culture. The expression of stained paxillin, vinculin, F-Actin, and DAPI (blue nucleus in merged images). ROI, region of interest (imaged with 3i CSU-W1 Spinning Disk with 63× Zeiss Plan-Apochromat objective and Hamamatsu sCMOS Orca Flash4.0 camera). Confocal microscope images were done with three biological replicates. (B) Size of focal adhesions on TiO_2-coated and noncoated titanium after 2 h. (C) The amount of focal adhesions per area (400 μm^2) on coated and noncoated surfaces after 2 h. Signal intensities of (D) paxillin and (E) vinculin on the bottom layer of HGF on coated and noncoated titanium surfaces after 2 h. Data represent mean ± SD + individual values. Significant p values ($p < 0.05$) are marked in the figures.

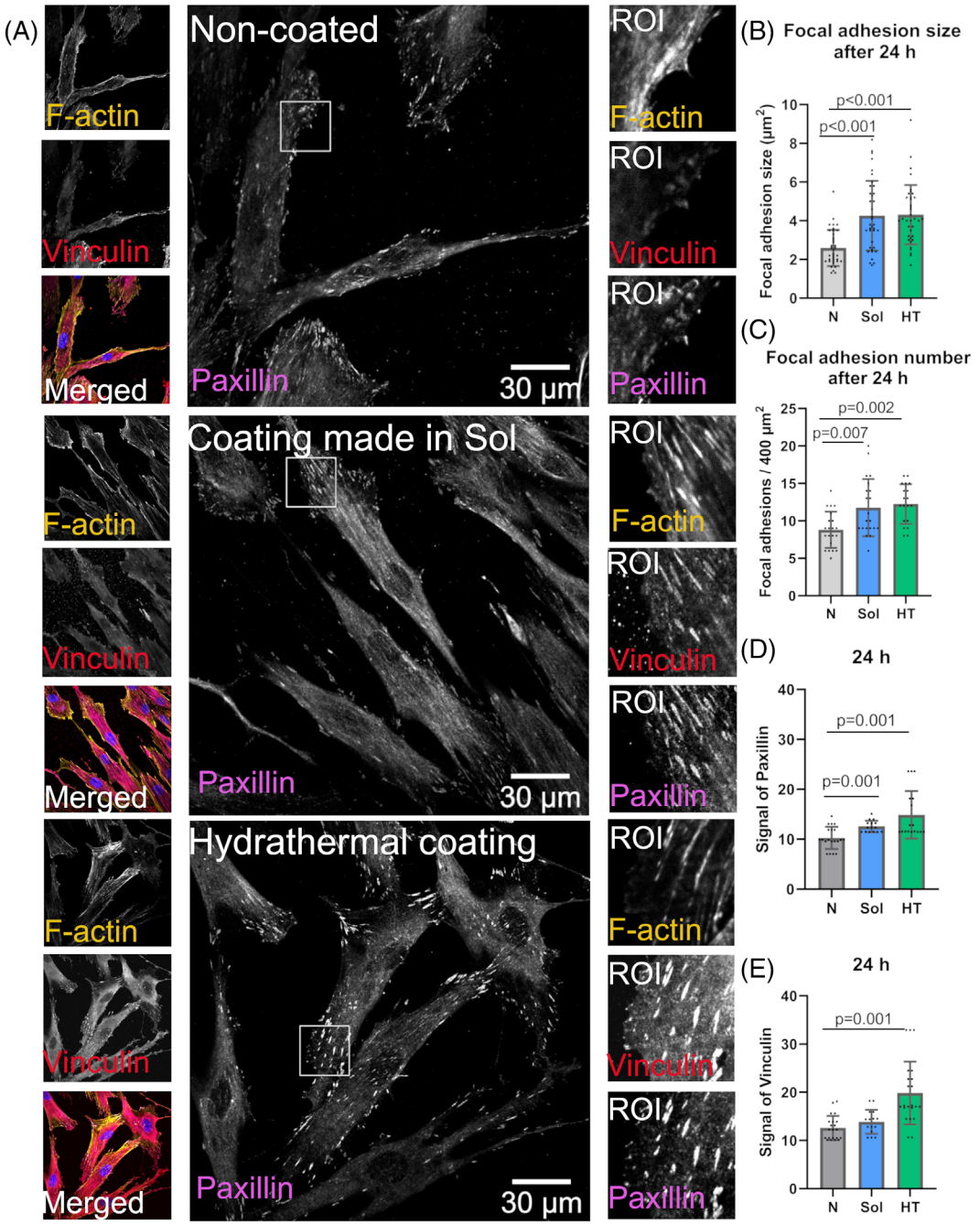

FIGURE 5 Larger focal adhesions with higher volume on TiO_2-coated titanium. (A) Presentable confocal microscope images of oral fibroblasts bottom layer after 24 h of cultivation. (B) Focal adhesion size on noncoated, SOL, and HT surfaces after 24 h. (C) The number of focal adhesions per area (400 µm^2) after 24 h of cell culture. Expression signal of (D) paxillin and (E) vinculin on the bottom layer of the cells after 24 h. HT, hydrothermal coating; N, noncoated; Sol, coating made in sol. Data represent mean ± SD + individual values. Significant p values ($p < 0.05$) are marked in the figures.

2.5 | Data analyses

Western Blot and confocal microscope images were analyzed with ImageJ, Fiji-program. GraphPad Prism program was used to analyze the data and design the graphs. One-way ANOVA with Tukey's multiple comparisons test was used to determine statistical significance between the mean values.

3 | RESULTS

3.1 | HT- and SOL-coated surfaces have different topographies

Surface topography was analyzed from both types of coated discs and noncoated control discs. With 8000× magnification, the difference in

surface topography is rather unclear (Figure 1B). With a higher magnification (50 000×), at nano level, small, round particles appear on both coated titanium surfaces. On HT surface, the nanostructure seems to have greater peak-to-valley differences as well as surface porosity compared to SOL coatings that appear smoother overall. However, the peak-to-valley height differences achieved by particle stacking (in sol) or crystal growth (hydrothermal) in both coated surfaces are within nanometers. Such features are absent in the control surfaces (Figure 1A). The nanoparticle size varies from 10 to 30 nm on SOL surface, meanwhile, the range is a bit wider on HT surface varying from 10 to 50 nm (Figure 1C).

3.2 | HT and SOL coatings increase Paxillin expression

In order to study if HT or SOL coatings are able to induce HGF adhesion, focal adhesion protein expressions were determined with Western Blotting. The Western Blot analysis indicated, that the expression of paxillin was significantly higher on SOL surfaces compared to noncoated titanium. No significant difference was found between HT and SOL coating (Figure 2). These results imply that titanium coating increases the expression of Paxillin.

3.3 | SOL coating increases cell spreading

To determine whether the TiO_2-coatings can affect cell spreading, the approximate areas of attached HGF were calculated from confocal microscope images. The fibroblasts were significantly more spread on SOL surfaces compared to noncoated surfaces after 2 and 24 h (Figure 3A–D). Also, on HT-coated titanium the cell areas were larger compared to control group, but the difference was not significant.

3.4 | TiO_2-coating increases focal adhesion size and number

The representative confocal microscope images (Figures 4A and 5A) express the signals of focal adhesion proteins paxillin and vinculin on the bottom layer of HGF after 2 and 24 h. There proteins are located peripheral at focal adhesion spots. To determine, whether the higher adhesion protein expression correlates with actual focal adhesion formation, the amounts of focal adhesions per area and the size of focal adhesions on each surface were measured. Focal adhesion size was significantly larger on TiO_2-coated surfaces after 2 and 24 h when compared to noncoated titanium (Figures 4B and 5B). Also, the amounts of focal adhesions per area were significantly higher on both coated samples compared to noncoated controls after 2 and 24 h (Figures 4C and 5C).

Also, the signal of paxillin on the bottom layer was significantly higher after 2 and 24 h on both coated discs compared to noncoated discs (Figures 4D and 5D), whereas signal level of vinculin was significantly higher on SOL discs after 2 h and on HT discs after 24 h compared to noncoated control discs (Figures 4E and 5E).

4 | DISCUSSION

This study focused on the evaluations of initial cell adhesion after 2 h followed by cell spreading that was studied after 24 h of cell culture. Our results show that HGF were more widely spread and focal adhesion molecule expressions were higher on TiO_2-coated titanium surfaces in both time points indicating higher initial cell adhesion and spreading. In addition, signals of vinculin and paxillin on focal adhesion areas were higher on coated surfaces. What is more, the coated surface seems to induce the size and amounts of focal adhesions. The size of focal adhesions has shown to correlate with cell spreading.[25] Both methods of producing TiO_2-coating seemed to have favorable properties on cell adhesion on molecular level when compared to noncoated titanium. Furthermore, this study demonstrated, that both methods of producing TiO_2-coatings enable to produce a nanostructure on titanium surface, which has been shown to be convenient for cell adhesion.[26] The nanotopography and more hydrophilic surface on coated titanium seem to result in better cell spreading and focal adhesion formation on titanium surface. TiO_2 surface does not seem to be only favorable for soft tissue cells, but it has also shown enhancement on osteoblast behavior.[27] Improved cell attachment may reflect enhanced gingival tissue attachment to implant surface, form a stronger barrier against oral microbes and in that way decrease the risk of peri-implantitis. Even though the TiO_2 coating seems to enhance cell attachment, it has not been shown to increase bacterial colonization.[28]

Previous studies have indicated that bioactive TiO_2-coating is favorable for soft tissue integration and fibroblast attachment.[29–31] Furthermore, sol–gel coating produced with dip-coating method has improved HGF proliferation in vitro.[20] As this study evidenced higher focal adhesion molecule expression and larger cell spreading on coated titanium surface, it indicates better cell attachment qualitatively, and supports the results of earlier studies. However, there are also studies where no significant difference has been found concerning fibroblast adhesion on nano-structured oxidized titanium surface.32

The earlier study done with oral keratinocytes, proved that TiO_2-coating is able to increase epithelial cell adhesion and proliferation on implant surfaces.[18] Furthermore, the keratinocytes were more spread and the hemidesmosomal molecules more induced on TiO_2-coated surfaces.[22] This study with HGF is in the same line with earlier studies performed with epithelial cells. The gingival fibroblasts were more spread and had higher expression levels when it comes to vinculin and paxillin on coated surfaces compared to noncoated titanium. This is notable, because vinculin and paxillin are some of the main proteins in focal adhesions.[13] While in the previous study with keratinocytes, paxillin, and vinculin were mostly located diffusely in cytoplasm, in the present study the paxillin and vinculin were located peripheral at focal adhesion spots. This difference can be explained by the differences in

cell adhesion mechanisms. The epithelial cells attach to implant surface mostly via hemidesmosomes whereas fibroblast attach via focal adhesions, where paxillin and vinculin play more important role.

The TiO_2-coating produced directly in sol or with HT process had both positive effects on cell attachment properties. However, no significant difference was found in cell adhesion between the differently coated surfaces, even though HT coating had an appearance of higher peak to valley height differences of its nanostructures on the coated surface. Besides the increased hydrophilicity shown as lower water contact angles,[19,23] the TiO_2-coating has been shown to induce calcium phosphate growth on the surface, which can also induce the formation of mucosal attachment.[17] The biggest difference between the coatings is their production method. Earlier used dip-coating is rather time-consuming method and it has some limitations when it comes to coating of objects with different 3D shapes. In sol-made coating is relatively simple method and does not require specific devices or laboratory conditions whereas HT coating needs specific hydrothermal autoclave. When TiO_2-coating is made directly in sol, after treatment the samples are washed three times with ethanol and set to oven on a metal net. The bottom side of the samples have spots, where the metal has been in contact during the heating process, which may affect on attachment of coating on these spots. Furthermore, holding a coated device on a metal net can be challenging in the case of coating more complicated 3D objects. When it comes to HT-produced coating, the samples are set inside of rotating Teflon vessel in an oven for 48 h, which enables the solution to attach every part of the sample. In addition, the coating thickness is more uniform on HT method, as coating process does not depend on the operator effect. Nevertheless, this study showed that both methods result in the formation of nanoporous TiO_2 coating that support HGF cell adhesion even on molecular level.

5 | CONCLUSION

To conclude, TiO_2-coating is able to increase gingival fibroblast adhesion protein expression and cell spreading on titanium surface. Consequently, the coating has good potential to offer biological adhesion of gingival tissues to implant surface.

AUTHOR CONTRIBUTIONS

Sini Riivari: Study design, sample preparation, cell culture, data analysis, drafting the manuscript. **Elisa Närvä:** Study design, collaboration in western blot and immunofluorescence analyses, review of the manuscript. **Ilkka Kangasniemi:** Collaboration in coating preparations, review of the manuscript. **Jaana Willberg:** Study design, expertise in cell cultures, review of the manuscript. **Timo Närhi:** Study design, review of the manuscript, supervisor, and research group leader. The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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