



Surface Free Energy of Titanium Disks Enhances Osteoblast Activity by Affecting the Conformation of Adsorbed Fibronectin

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This study evaluated the influence of surface free energy (SFE) of titanium disks on the adsorption and conformation of fibronectin (FN) and the biological behavior of osteoblasts cultured on the FN-treated modified surfaces. High [H]-SFE titanium disks were irradiated by a 30 W UV light, while low (L)-SFE titanium disks received no treatment. The surface characteristics of the titanium disks were examined using scanning electron microscope, optical surface profilometer, X-ray photoelectron spectroscopy, and contact angle measurements. Adsorbed FN on different groups was investigated using attenuated total reflection-Fourier transform infrared spectroscopy. MG-63 cells were cultured on FN-treated titanium disks to evaluate the *in vitro* bioactivity. The experiment showed H-SFE titanium disks adsorbed more FN and acquired more β -turn content than L-SFE group. MG-63 cells cultured on FN-treated H-SFE titanium disks showed better osteogenic responses, including adhesion, proliferation, alkaline phosphatase activity and mineralization than that on FN-treated L-SFE titanium disks. Compared to L-SFE titanium disks, integrin- β 1, integrin- α 5 and Rac-1 mRNA levels were significantly higher in MG-63 cells on FN-treated H-SFE after 3 h of culture. These findings suggest that the higher SFE of H-SFE compared to L-SFE titanium disks induced changes in the conformation of adsorbed FN that enhanced the osteogenic activity of MG-63 cells.

Keywords: conformation, fibronectin, osteoblast, titanium, surface free energy

INTRODUCTION

Titanium and titanium-based alloys are widely used as orthopedic implant materials. Osteointegration is a major factor influencing the success rate of orthopedic implants. Osteointegration is related to implant macroscopic surface structure, surface topography, surface wettability, surface charge, surface free energy (SFE), surface hydrophilicity, pore structure, release of bioactive molecule (Chen et al., 2018), and coating (Smeets et al., 2016). Osteoblasts are not in direct contact with the implant surface immediately after implantation (Barberi and Spriano, 2021). Rather, implants adsorb a thin layer of proteins, including immunoglobulins, vitronectin, fibrinogen and fibronectin (FN), which modulate a pro-inflammatory response and clotting to create the correct microenvironment for osteointegration (Raphel et al., 2016; Barberi and Spriano, 2021). Recently, extensive research efforts have focused on the analysis of protein adsorption to synthetic surfaces (Keselowsky et al., 2003). Findings have showed that altering the surface of titanium-implant

materials affects protein adsorption, cell–substrate interactions, and tissue integration (Rapuano et al., 2012; Isoshima et al., 2019; Yao et al., 2019; Zeng et al., 2019; Bayrak et al., 2020; Lin et al., 2020).

FN is a glycoprotein in the extracellular matrix that promotes osteoblast adhesion to a substrate. FN is a dimer of subunits joined by two disulfide bonds at their C-terminal ends. Each subunit has six domains, which perform different functions (Maurer et al., 2016). The arginine-glycine-aspartate (Arg-Gly-Asp, RGD) domain is a binding receptor for integrins, and directly associated with cell adhesion (Asghari Sana et al., 2017). The RGD domain is located in a β -turn structure in FN, and increasing β -turn content is associated with improved cell adhesion properties (Hasan et al., 2018). FN has a dynamic structure and must transition from a compact to an extended conformation to expose the RGD domain and mediate cell adhesion (Maurer et al., 2016).

Studies show that implant material and surface properties, including pH, temperature, surface polarity and surface charge, affect the adsorption and conformation of FN (Osterlund, 1988; Kowalczyńska et al., 2009; Lv et al., 2017; Gossart et al., 2018; Hasan et al., 2018); however, the influence of the SFE of an implant on the adsorption and conformation of FN, and the biological behavior of osteoblasts cultured on the FN-treated surface, remains to be elucidated. SFE is of fundamental importance when characterizing interactions between liquids and solids as it relates to adhesion, binding affinity, adsorption and interfacial attractive forces. To date, the most widely used method of determining SFE is by measuring the contact angle between the liquid and solid (Zhang et al., 2019). In this study, we exposed titanium disks to ultraviolet-C (UVC) light to evaluate the effects of changes in the SFE of titanium implants on the adsorption and conformation of FN and the biological behavior of cultured osteoblasts.

MATERIALS AND METHODS

Specimens

Titanium disks were 15 mm in diameter and 1 mm thick. Titanium disks were abraded with a sequence of successively finer silicon carbide papers; washed with acetone, anhydrous ethanol and dH₂O for 15 min each; dried for 1 h at room temperature; etched in mixed acid solution (water: H₂SO₄:HCl 2:1:1 v/v); and washed 3 times with water. Disks were divided into two groups. The SFE of one group of titanium disks (high [H]-SFE) was increased by exposing the disks to a 30 W light tube that emitted UV light at wavelengths of 200–275 nm (Philips, Holland) for 24 h. The disks in the low (L)-SFE group received no treatment.

The surface morphology of the titanium disks was observed with a scanning electron microscope (SEM, Regulus8239, Hitachi, Japan).

Surface roughness of the titanium disks was measured with an optical surface profilometer (BMT EXPERT, BMT, Germany). The arithmetic mean roughness (Sa), and average height over the measurement field (Sz) were calculated as amplitude parameters to characterize surface topography.

Contact angles were measured with a contact angle meter (OCA40 Micro, dataphysics, Germany).

SFE was calculated using contact angle data and the Owens-Wendt model:

$$[\gamma_{SL} = \gamma_S + \gamma_L - 2(\gamma_S^D \cdot \gamma_L^D)^{1/2} - 2(\gamma_S^P \cdot \gamma_L^P)^{1/2}]$$

The Owens-Wendt model (Zhang et al., 2019) considers dispersion and polar contributions (e.g., $\gamma_S = \gamma_S^D + \gamma_S^P$) to describe the solid (S)-liquid (L) interfacial tension (γ_{SL}). Photo-induced changes in the SFE of titanium disks are temporary (Rupp et al., 2010); therefore, our analyses were performed immediately after irradiation.

The chemical composition of the titanium disks was confirmed with X-ray photoelectron spectroscopy (XPS, ESCALAB 250, Thermo-VG, America), using C_{1s} at 284.8 eV as the charge reference and by comparing the amount of surface hydroxyl (OH) groups.

Protein Adsorption and Secondary Structure Analysis

Human plasma FN (Sigma-Aldrich, MO, United States) was adsorbed onto titanium disks from a 1 mg/ml FN solution at 37°C for 3, 6, and 24 h. FN was desorbed from the titanium disks by shaking with 5% SDS in PBS at 37°C for 1 h. Protein was estimated using a BCA Protein Assay kit (MA0082, United States).

The secondary structure of adsorbed FN was investigated using attenuated total reflection-Fourier transform infrared (ATR-FTIR) (Nicolet 6700-Contiūm, Thermo, England) spectroscopy, particularly in the amide I band IR absorption range, 1,600–1700 cm⁻¹ (Buchanan and El-Ghannam, 2009). After FN adsorption for 24 h, titanium disks were dried at 37°C and ATR-FTIR spectra were acquired. The secondary structure of FN was determined by evaluating the amide I band through second derivative/Gaussian curve fitting analysis.

Effect of SFE of Titanium Disks on Osteoblast Behavior

Human plasma FN (Sigma) was adsorbed onto titanium disks from a 1 mg/ml FN solution at 37°C for 24 h prior to investigating osteoblast behavior.

Cell Culture

Human osteosarcoma cells (MG-63) were provided by Procell Life Science and Technology Co., Ltd. Cells were cultured in α -Minimum Essential Medium (α -MEM, Gibco, United States) supplemented with 10% fetal bovine serum (FBS, Gibco, United States) and 1% P/S (Gibco, United States) at 37°C and 5% CO₂. Culture media was replaced every 2 days, and cells were passaged by trypsinization at 80% confluence.

Cell Adhesion and Proliferation

MG-63 cells were seeded at 1×10^4 cells/titanium disk, incubated for 3, 6 and 24 h, and rinsed 3 times with PBS to remove

TABLE 1 | Lists of primer sequences used for RT-PCR analysis in this study.

Gene	Forward primers (5'→3')	Reverse primers (5'→3')
Integrin-β1	CCTACTTCTGCACGATGTGATG	CCTTTGCTACGGTTGGTTACATT
Integrin-α5	CATGATGAGTTTGCCGATTTG	CCCCAGGAAATACAAACACTA
FAK	TGGGCGGAAAGAAATCCTGC	GGCTTGACACCCTCGTTGTA
Src	TGAGGCATGAGAAGCTGGTG	AGTCCAGCAAACCTCCCCTTG
Rac-1	TCCGCAAACAGATGTGTTCTTA	CGCACCTCAGGATACCCTTT
β-actin	GTCACCAACTGGGACGACAT	TAGCAACGTACATGGCTGGG
RhoA	AGCCAAGATGAAGCAGGAGC	TACCCAAAAGCGCCAATCCT

nonadhered cells. Cells were permeabilized with DAPI (P0131, Beyotime) for 30 min and visualized with an inverted fluorescent microscope (X50; DMi8, Leica, Germany). 10 fields of view per disk were captured to determine the mean number of adhered cells. Images were analyzed with ImageJ software.

1×10^4 MG-63 cells were seeded at 1×10^4 cells/titanium disk, incubated for 1, 3, 5, and 7 days, and culture media was removed. MG-63 proliferation was evaluated with a CCK-8 assay (CA1210, Solarbio) using absorbance at 450 nm (SpectraMax Plus384, MD, United States), according to the manufacturer's instructions.

Alkaline Phosphatase Assay

Differentiation of osteoblasts was investigated by evaluating alkaline phosphatase (ALP) activity. ALP is a mineralization-related protein and an important marker of early osteoblast differentiation (Xu et al., 2015). MG-63 cells were seeded at 1×10^4 cells/titanium disk and cultured for 7 and 14 days. Culture media was removed and disks were rinsed 3 times with PBS. Cells were lysed with 200 μl cell lysis buffer (P0013J, Beyotime, China), and centrifuged at 14,000 g at 4°C for 5 min. ALP activity in the supernatant was determined with an ALP assay kit (P0321S, Beyotime, China) using absorbance at 405 nm, according to the manufacturer's instructions.

Mineralization

Mineralization of osteoblasts was investigated with alizarin red staining. MG-63 cells were seeded at 1×10^4 cells/titanium disk and incubated for 14 days. Disks were rinsed 3 times in PBS, incubated with 1 ml alizarin red solution (G1450, Solarbio) for 30 min, rinsed with ddH₂O, and observed under a light microscope (S9i, Leica, Germany). Images were analysed with ImageJ software.

Real-Time Reverse Transcription Polymerase Chain Reaction

To evaluate MG-63 cell adhesion, integrin-β1, integrin-α5, FAK, Src, Rac-1, and RhoA mRNA levels were analyzed by RT-PCR. MG-63 cells were cultured at 2×10^5 cells/disk for 3 and 6 h on FN-treated H-SFE and L-SFE titanium disks. Total RNA was isolated with a SteadyPure Universal RNA Extraction Kit (Accurate Biotechnology Co., Ltd., AG21017, China). cDNA was synthesized using an Evo M-MLV RT Mix Kit (Accurate Biotechnology Co., Ltd., AG11728, China), according to the manufacturer's instructions. Gene expression analysis was

performed using a SYBR Green Premix Pro Tap HS qPCR Kit (Accurate Biotechnology Co., Ltd., AG11701, China) on an Real-Time PCR system (Loghtcyclor 96, Roche, Switzerland). Sequence specific primers (Table 1) were purchased from Telenbiotech DNA Technologies (China). Differential gene expression was calculated using mRNA levels from MG-63 cells cultured on L-SFE titanium disks as the reference.

Statistical Analysis

Statistical analyses were performed with SPSS 19.0 (SPSS Inc., United States). All experiments were repeated in triplicate. Data are reported as means and standard deviations. Comparisons between H-SFE and L-SFE disks were performed with the independent samples *t*-test and nonparametric Mann-Whitney *U* test. $p \leq 0.05$ was considered statistically significant.

RESULTS

Surface Characteristics of H-SFE and L-SFE Titanium Disks

The surface characteristics of the H-SFE and L-SFE titanium disks are shown in Figure 1, Tables 2, 3. L-SFE (Figures 1A,B) and H-SFE (Figures 1C,D) titanium disks had pore sizes of 2–3 μm. There were no significant differences in amplitude parameters (Figures 1E,F) *S*_a or *S*_z (Table 2) between H-SFE and L-SFE titanium disks. The water contact angle (Figures 1G,I; Table 3) at room temperature was significantly higher for L-SFE compared to H-SFE titanium disks; there was no significant difference in the diiodomethane contact angle (Figures 1H,J; Table 3) at room temperature.

SFE was significantly higher for H-SFE compared to L-SFE titanium disks ($p < 0.001$; Table 3).

The surfaces of the L-SFE and H-SFE titanium disks had high percentages of C, O, N, Ti. The C_{1s} and O_{1s} XPS spectra for L-SFE and H-SFE titanium disks are shown in Figure 2.

Adsorbed FN

The FN adsorption capabilities of H-SFE and L-SFE titanium disks after immersion in a 1 mg/ml FN solution at 37°C for 3, 6, and 24 h are shown in Figure 3. FN adsorption levels were significantly increased on H-SFE compared to L-SFE titanium disks after immersion for 3 and 6 h, but there was no significant difference in FN adsorption level after immersion for 24 h

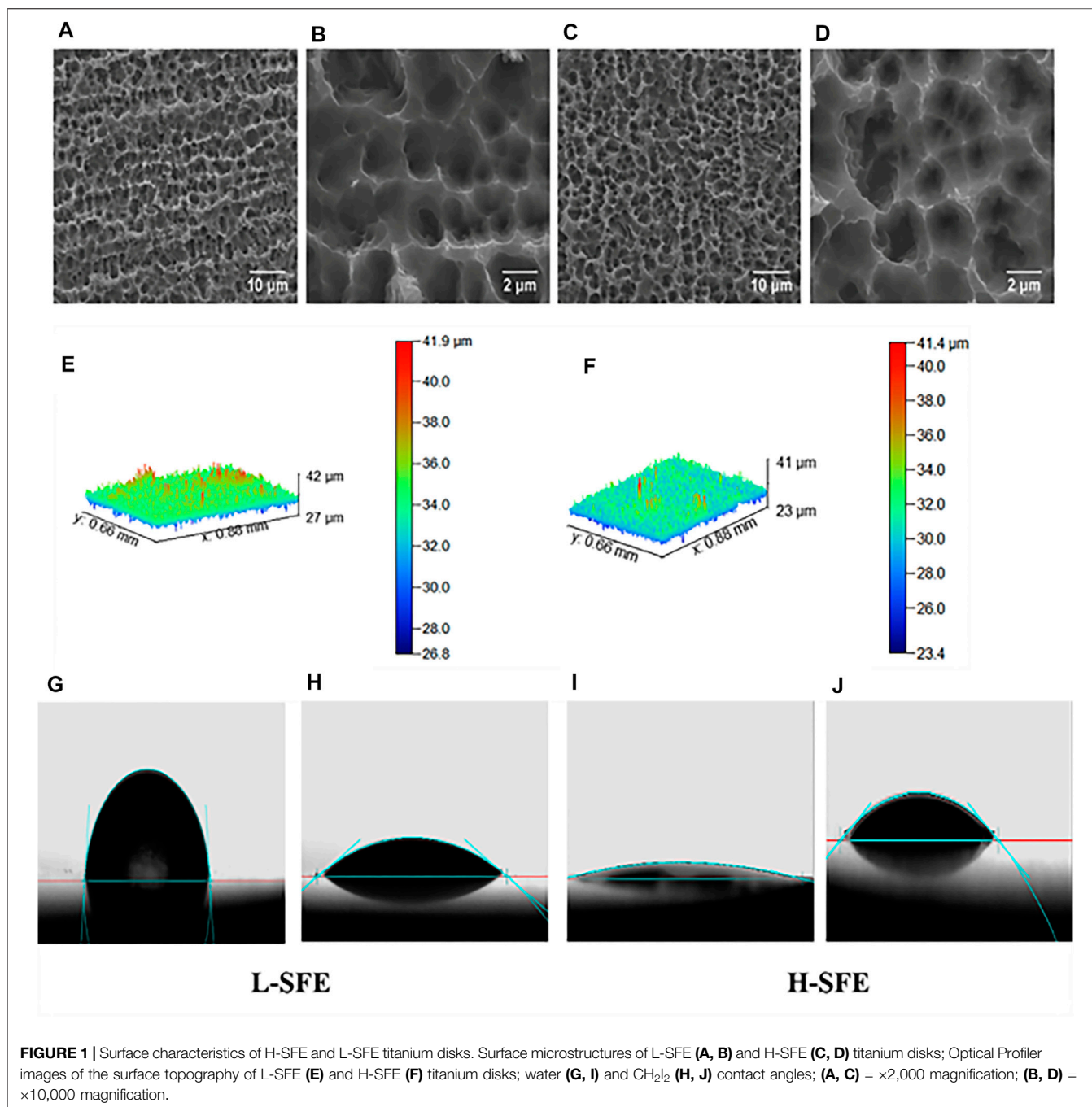


TABLE 2 | Surface topography of H-SFE and L-SFE titanium disks.

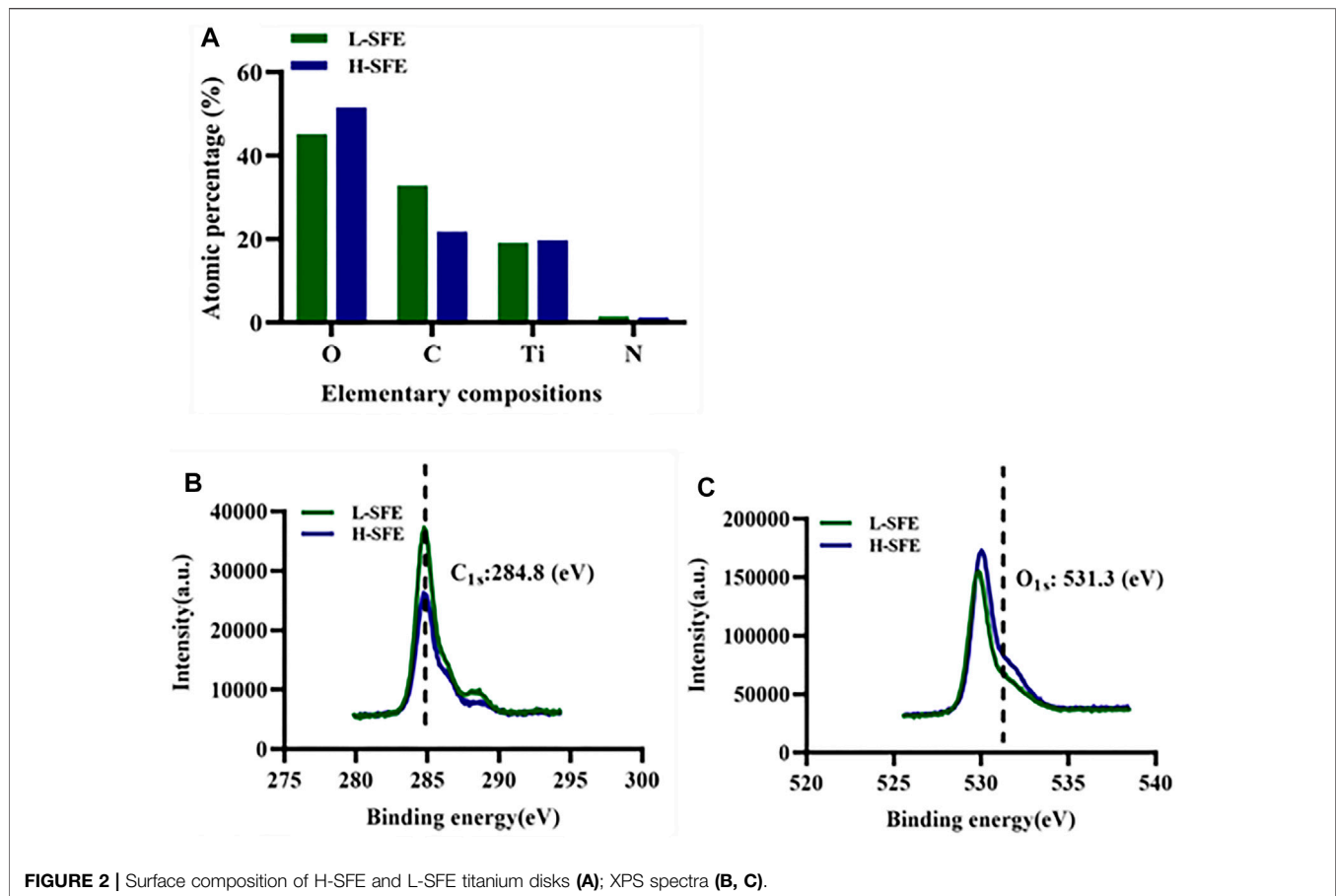
Group	Sa	Sz
H-SFE	0.83 ± 0.08	16.29 ± 1.80
L-SFE	0.85 ± 0.08	14.86 ± 1.47
P	0.29	0.19

Data are mean ± standard deviation. p ≤ 0.05 was statistically significant.

TABLE 3 | Contact angles and surface free energy (SFE) of H-SFE and L-SFE titanium disks.

Group	dH ₂ O(°)	CH ₂ I ₂ (°)	SFE(mN/m)
H-SFE	8.03 ± 1.33	22.37 ± 5.95	73.78 ± 0.68
L-SFE	67.27 ± 14.59	21.90 ± 3.58	50.66 ± 4.44
P	0.019	0.913	0.001

Data are mean ± standard deviation. p ≤ 0.05 was statistically significant.



(Figure 3A). ATR-FTIR spectra showed the β -turn content in adsorbed FN was significantly increased on FN-treated H-SFE compared to L-SFE titanium disks (Figures 3B,C).

Osteoblast Behavior

MG-63 cell adhesion on FN-treated H-SFE and L-SFE titanium disks after 3, 6, and 24 h of culture is shown in Figure 4A. The number of MG-63 cells adhered to FN-treated H-SFE and L-SFE titanium disks increased with time, with maximum MG-63 cell adhesion on FN-treated H-SFE titanium disks after 24 h of culture (Figure 4B).

MG-63 cell proliferation on FN-treated H-SFE and L-SFE titanium disks increased with time, with maximum MG-63 cell proliferation on FN-treated H-SFE titanium disks after 7 days of culture (Figure 4C).

MG-63 cell adhesion and proliferation were significantly greater in FN-treated H-SFE compared to L-SFE titanium disks at each time point.

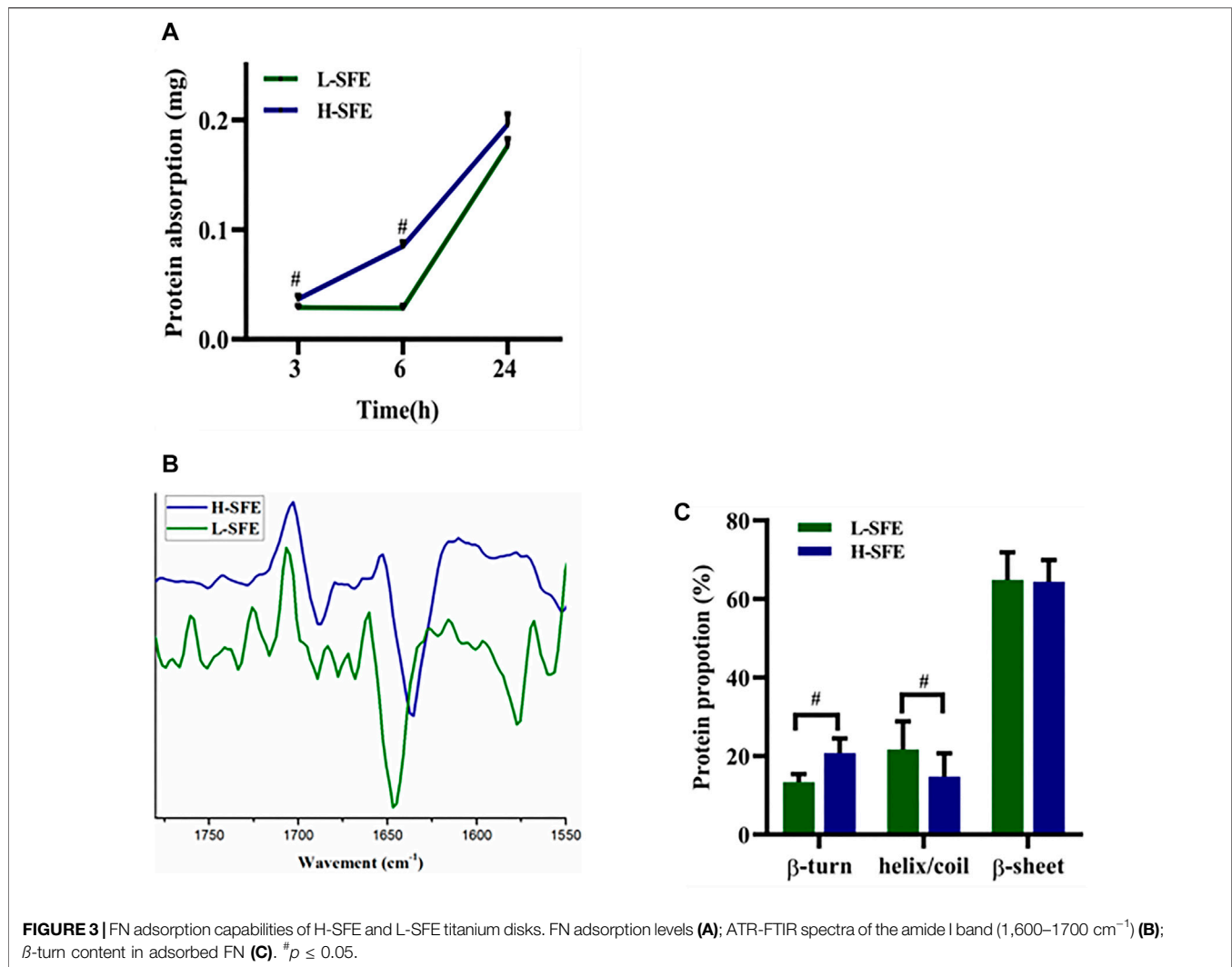
ALP activity of MG-63 cells was significantly increased in FN-treated H-SFE compared to L-SFE titanium disks after 7 and 14 days of culture (Figure 5A). MG-63 cell mineralization was significantly increased in FN-treated H-SFE compared to L-SFE titanium disks after 14 days of culture (Figures 5B,C).

mRNA Levels of Cell Adhesion Molecules in Osteoblasts

Integrin- β 1, integrin- α 5, FAK, Src, Rac-1, and RhoA mRNA levels in MG-63 cells cultured on FN-treated H-SFE and L-SFE titanium disks are shown in Figure 6. Integrin- β 1, integrin- α 5, and Rac-1 mRNA levels were significantly higher in MG-63 cells on FN-treated H-SFE compared to L-SFE titanium disks after 3 h of culture, while there were no significant differences in FAK, Src, and RhoA mRNA levels (Figure 6A). Integrin- β 1, integrin- α 5, FAK, Src, and RhoA mRNA levels were significantly higher in MG-63 cells on FN-treated L-SFE compared to H-SFE titanium disks after 6 h of culture, while there was no significant difference in Rac-1 mRNA level (Figure 6B).

DISCUSSION

In this study, we exposed titanium disks to UVC light to evaluate the effects of changes in the SFE of titanium implants on the adsorption and conformation of FN and the biological behavior of osteoblasts cultured on the FN-treated modified surfaces. Pure titanium quickly forms a nanometer thick layer of titanium oxide when exposed to air. Titanium oxide is a semiconductor and will



undergo a photocatalytic reaction when irradiated with ultraviolet light (Sharmin and Ray, 2012; Khanna and Shetty, 2013; Assadi et al., 2014). This reaction will decompose hydrocarbons and water on the surface of H-SFE titanium disks to reduce C content and form a large number of hydroxyl groups (Diebold, 2003; Bikondoa et al., 2006; Sun et al., 2012). Therefore, our H-SFE titanium disks had a high SFE and hydrophilicity, consistent with the results of studies. Exposure to UV irradiation does not change the surface morphology and roughness of pure titanium, eliminating the influence of these surface characteristics on our findings.

As a component of the extracellular matrix, FN is involved in cell-implant interaction events. It can bind to integrins on the cell membrane, resulting in a conformational change in these proteins (Marconi et al., 2021). In our study, the FN adsorption capabilities of H-SFE and L-SFE titanium disks increased with time. FN adsorption levels were significantly increased on H-SFE compared to L-SFE titanium disks after titanium disks were immersed in a 1 mg/ml FN solution at 37°C for 3 and 6 h. This suggests high SFE can promote the early adsorption of

FN on a pure titanium surface. Although there was no significant difference in FN adsorption level on H-SFE compared to L-SFE titanium disks after immersion for 24 h, the β -turn content in FN was significantly increased on FN-treated H-SFE compared to L-SFE titanium disks. OH groups on titanium surfaces may react with the amino groups of an adsorbed protein through electrostatic interactions and cause a change in protein conformation (Hong et al., 2014). This may expose β -turns. In the present study, XPS spectra showed the binding energy at 531.5 eV, the position expected for surface OH groups, was higher for H-SFE compared to L-SFE titanium disks, while ATR-FTIR spectra showed the β -turn content in FN was significantly increased on FN-treated H-SFE compared to L-SFE titanium disks. These data suggest H-SFE titanium disks may adsorb FN faster than L-SFE titanium disks by optimizing β -turn exposure.

FN interacts with integrins on the cell surface, arbitrates mechanical anchoring, and establishes focal adhesions between intracellular actin bundles and the extracellular matrix. Extracellular signals are translated into cellular responses at focal adhesions (Marconi et al., 2021). The biological behavior

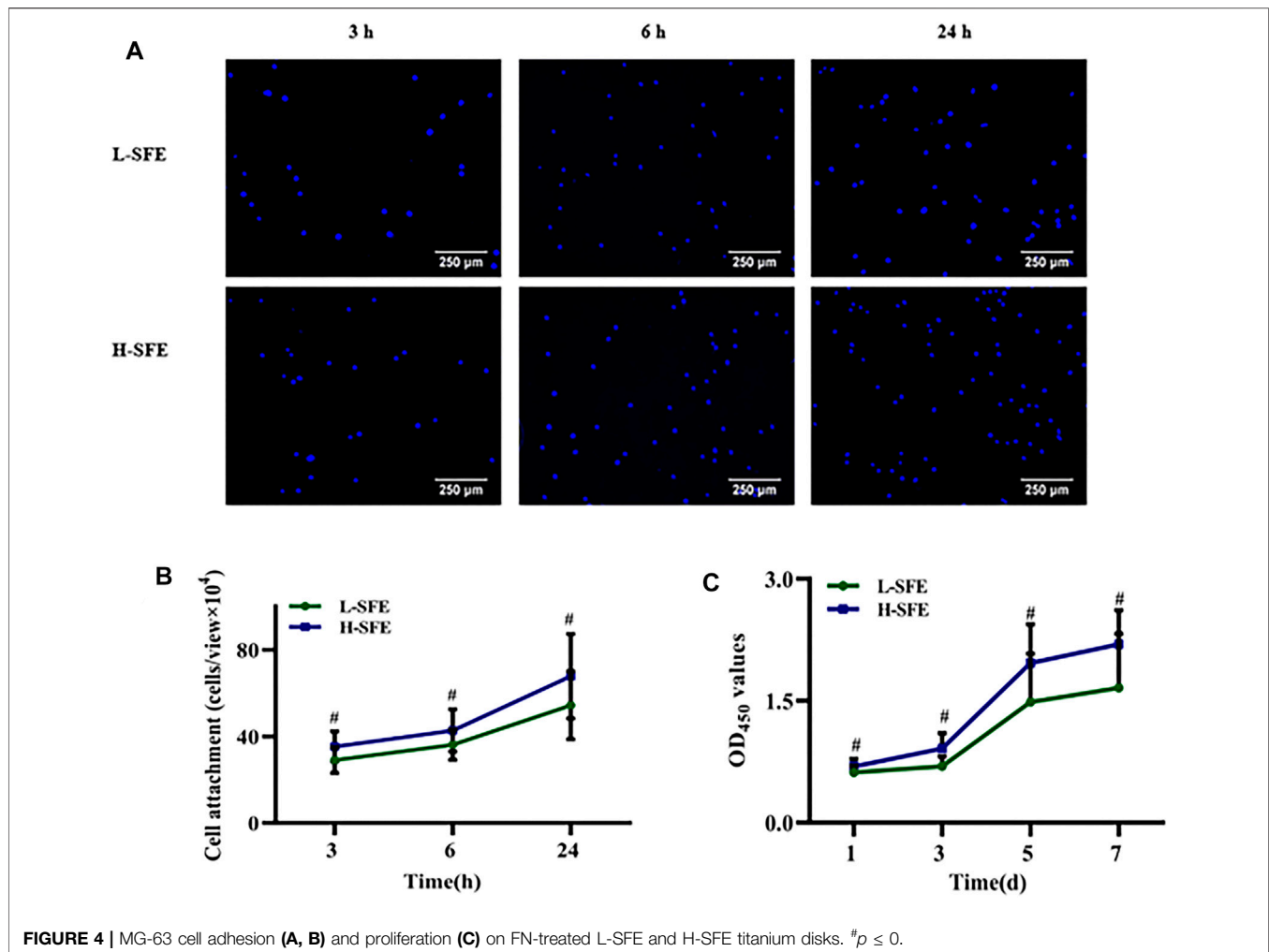
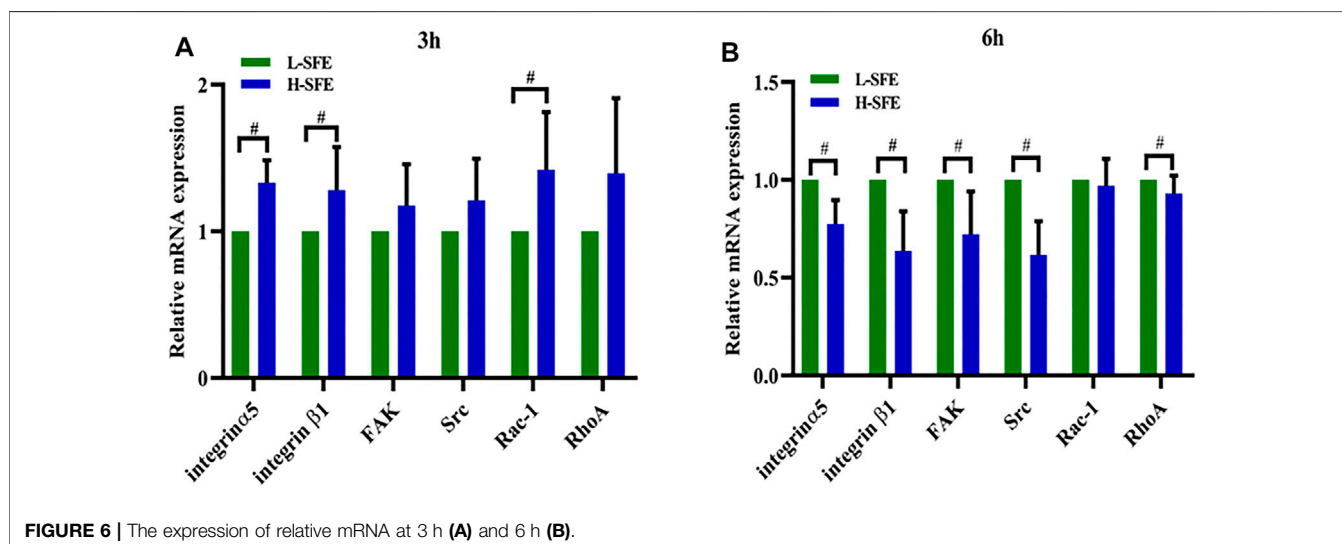
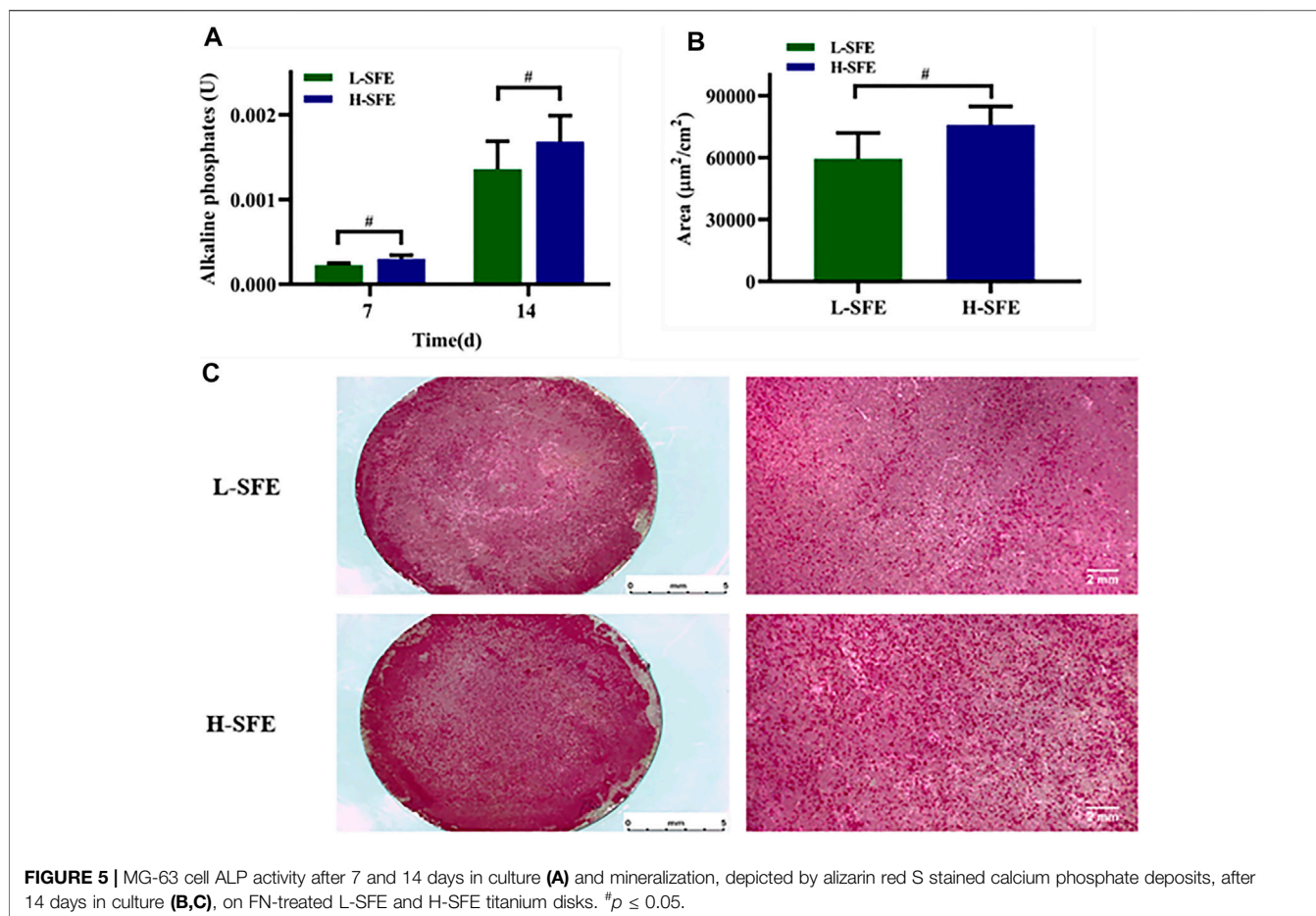


FIGURE 4 | MG-63 cell adhesion (A, B) and proliferation (C) on FN-treated L-SFE and H-SFE titanium disks. # $p \leq 0$.

of osteoblasts *in vitro* can be used as a reference that reflects the biological activity of artificial materials. There was no significant difference in FN adsorption level on H-SFE compared to L-SFE titanium disks after immersion for 24 h, but osteogenic responses were significantly improved in MG-63 cells cultured on H-SFE compared to L-SFE titanium disks after 24 h FN adsorption. This implies that the osteogenic responses of MG-63 cells are related to protein adsorption level and the secondary structure of the adsorbed proteins. Protein adsorption is the first step in cell-biomaterial interactions (Hubbell et al., 2009; Petros and DeSimone, 2010; Scharnagl et al., 2010; Grafahrend et al., 2011). *In vivo*, proteins that are adsorbed onto an implant surface play a key role in cell/implant interactions (García et al., 1999; Hynes, 2002; Mao and Schwarzbauer, 2005; Roach et al., 2005; Vogel and Sheetz, 2006; Tsapikouni and Missirlis, 2008). We have shown that the physicochemical properties of the surface of a biomaterial impact the secondary structure of adsorbed proteins, and then impact cell adhesion and the behavior of attached cells (Toworfe et al., 2009; Kushiro et al., 2016). FN is a dimeric glycoprotein that promotes osteoblast adhesion, migration, and differentiation (Pankov and Yamada, 2002; Cantini et al., 2012). Unfolding of FN may expose binding

sites (Felgueiras et al., 2014), such as the RGD sequence, which can be recognized by cell integrins (Shen et al., 2008). In FN, the RGD domain is located in a β -turn structure, and increasing β -turn content is associated with improved cell adhesion properties (Hasan et al., 2018). These data suggest that the higher SFE of H-SFE compared to L-SFE titanium disks may have induced changes in the conformation of adsorbed FN that enhanced the osteogenic activity of MG-63.

Integrins play key structural roles in cells as they are transmembrane proteins that engage with the extracellular matrix and regulate the organization of the actin cytoskeleton. In addition, α and β integrins are important initiating and regulating factors in signal transduction (Juliano et al., 2004) and integrin- $\alpha 5$ and integrin- $\beta 1$ recognize and connect to the RGD domain in FN, resulting in focal adhesions FAK and Src are key components of the signaling pathways controlling focal adhesions. Cytoplasmic tails ($\beta 1$, $\beta 3$, and $\beta 5$) of β integrin promote activation of FAK through an undefined mechanism (Toutant et al., 2002). Src can be activated by binding directly to the cytoplasmic domain of β integrin (Arias-Salgado et al., 2003), and Src can also activate FAK through phosphorylation. Interactions



between Src and FAK and integrin-mediated signaling pathways can activate the Rho family of small GTPases, which mediate the biological activities of cells (Turner, 2000; Wozniak et al., 2004; Nayal et al., 2006; Morgan et al., 2009; Tang et al., 2018; Rajah

et al., 2019). Rac-1 regulates the formation of plate-shaped pseudopodia and promotes cell migration (Tang et al., 2018; Liu et al., 2020), while RhoA is involved in maintaining cell adhesions (Warner et al., 2019).

In the present study, the expression of some genes related to focal adhesions, and Rac-1, the gene related to cell migration, was significantly higher in MG-63 cells on FN-treated H-SFE compared to L-SFE titanium disks after 3 h of culture. After 6 h of culture, the expression of genes related to focal adhesions was significantly higher in MG-63 cells on FN-treated L-SFE compared to H-SFE titanium disks, while there was no significant difference in the expression of Rac-1. We speculate that MG-63 cells on FN-treated H-SFE titanium disks were migrating and developing focal adhesions after 3 h in culture, and proliferating and differentiating after 6 h in culture. In contrast, MG-63 cells on FN-treated L-SFE titanium disks were migrating and developing focal adhesions after 6 h in culture. These data suggest that increasing the SFE of titanium disks promotes conformational changes in FN and biological behavior in cultured osteoblasts.

CONCLUSION

In this study, titanium disks with high SFE were generated by UVC irradiation. There were no significant differences in surface characteristics such as pore size, Sa, or Sz between H-SFE and L-SFE titanium disks. MG-63 cells cultured on FN-treated H-SFE titanium disks showed better osteogenic responses, including adhesion, proliferation, ALP activity and mineralization, than MG-63 cells cultured on FN-treated L-SFE titanium disks. Although the underlying mechanisms remain to be elucidated, UVC irradiation may have increased the number of OH groups on the surface of FN-treated H-SFE titanium disks, which induced changes in the conformation of adsorbed FN, exposed RGD binding sites, and enhanced the biological activity of MG-63 cells.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JL and SL proposed and designed the experiments. JL and HD carried out the experiments with the help of YW and XZ. JL and HD drafted the article and interpreted the data. JL and SL revised the article. All the authors approved the final version of this article.

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