Original

Verification of osteoblast differentiation on airborne-particle abrasion, large-grit, acid-etched surface of titanium implants regulated by yes-associated protein and transcriptional coactivator with PDZ-binding motif

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Abstract: Although airborne-particle abrasion, large-grit, acid-etched (SLA) surface technology can promote implant osseointegration; its mechanism remains unclear. By preparing the SLA titanium (Ti) plate (SLA Ti) and Polished Ti plate (Polished Ti), this experiment investigates the expression and distribution of the Yes-associated protein (YAP) and transcriptional coactivator with the PDZ-binding motif (TAZ) in MC3T3-E1 cells. In addition, gene YAP and TAZ silencing on the SLA Ti was conducted to observe changes in the osteoblast differentiation markers, runt-related transcription factor-2 (Runx2) and bone sialoprotein (BSP). The results demonstrated that SLA Ti surface microtopography could induce YAP/TAZ's transfer from the cytoplasm to the nuclei of MC3T3-E1 cells. The expression of YAP/TAZ increased in terms of mRNA and protein. After silencing the YAP/TAZ genes, Runx2 and

BSP decreased, suggesting that YAP/TAZ plays an important regulatory role in this process. Meanwhile, the results also showed that SLA microtopography enhanced the expression of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$. After silencing the integrin $\alpha 1$, $\alpha 2$, and $\beta 1$ genes, YAP and TAZ decreased in terms of mRNA and protein. Therefore, this experiment was the first to confirm that SLA surface microtopography facilitates osteoblast differentiation by regulating YAP/TAZ and confirms that the process can be related to integrins $\alpha 1$, $\alpha 2$, and $\beta 1$.

Keywords: SLA Ti implant; YAP; TAZ; MC3T3-E1 cell; osseointegration.

Introduction

Titanium (Ti) and its alloys are widely used in dental implants. Osseointegration, referring to the direct interface between the bone and implant, either structurally or functionally, is regarded as a prerequisite for a successful dental implant (1). Conducting surface modifications for implants can improve implant osseointegration (2). Therefore, bone formation or angiogenesis can be promoted by changing the implants' surface microtopography or surface chemical properties or by designing a drug delivery system to mediate adhesion, migration,

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proliferation, and differentiation. This process can also provide antibacterial properties that may improve long-term success rates (3-7). Airborne-particle abrasion, large-grit, acid-etched (SLA) technology is one of the most commonly used surface modification methods in commercially-available implants.

SLA technology refers to applying large-grit sand to blast the implant surface. This leads to the formation of first-step pits. Then, specific acids are used to etch the surfaces of the first-step pits to form second-step pits in an overlapped manner to blunt the sharp sandblasting edges and generate multi-step microtopography on the implant surface, thereby increasing the implants' surface roughness. The surface topography formed by SLA technology increases the implant surface at the micron level, facilitating protein adsorption and cell adhesion and inducing osteoblast differentiation to promote bone formation and osseointegration (8). Clinical retrospective surveys showed that SLA implants effectively increased the bone mass in the immediate and early loading periods and that the implants' survival rate was above 97% (2). Ten years after implantation, the implants' survival rate was as high as 99.7%, whereas the incidence rate of peripheral inflammation was only 7% (9). Therefore, SLA technology provides beneficial clinical effects for promoting implant osseointegration and improving the implant success rate.

Yes-associated protein (YAP) and transcriptional coactivator with the PDZ-binding motif (TAZ) genes are ubiquitously expressed in multiple organs. The two proteins are transcriptional co-activators. They act in parallel to regulate cell proliferation and survival, organogenesis, and cancer development (10). YAP and TAZ in the cytoplasm are translocated into the cell nucleus under appropriate circumstances to exert effects on gene transcription. Research first identified YAP and TAZ as critical substrates of the Hippo signaling pathway, which negatively regulates cell proliferation and differentiation (10,11).

Recently, studies addressed YAP's and TAZ's roles in affecting mesenchymal stem cells (MSCs) and osteoblast differentiation by regulating runt-related transcription factor 2 (Runx2) transcriptional activity. Cytoplasmic YAP and TAZ translocate into the cell nucleus in response to a specific extracellular matrix (ECM), further exerting transcriptional coactivation of Runx2 and repressing peroxisome proliferator activated receptor gamma (PPAR γ), representing an osteogenesis differentiation-related gene (12,13). Improvement of bone mineral density and enhancement of bone mass were also observed. Both YAP and TAZ are involved in

regulating cell differentiation in primary osteoblasts and MC3T3-E1 cells (14,15).

Surface topography mediates YAP's and TAZ's contributions to differentiation of MSCs, which has been proven in the tissue engineering field. In response to micromorphology, YAP and TAZ induce cells to differentiate into osteoblasts. Microtopography and nanotopography, fabricated by different materials and treatments, exert distinct regulations on YAP and TAZ (15-17). However, the mechanism of SLA technology for commercial surface modification of dental implants remains elusive.

In terms of SLA microtopography, Olivares-Navarrete, Lai, Agarwal, and Gittens et al. (6,18-20) determined that integrins α 1, α 2, and β 1 can promote osteoblast differentiation. Tang found that integrin could regulate YAP/TAZ through the Rho GTP enzyme (21,22). Therefore, if SLA surface microtopography facilitates implant osseointegration by regulating YAP/TAZ, the process may be related to integrin.

Materials and Methods

Ti preparation and surface characterization

Ti-6Al-4V is sometimes called TC4, or Cr5 in the USA. As a classic implant material that is available on the market, its sound performance has been proven for a long time, with excellent biocompatibility and osseointegration properties, adequate yield strength and ultimate strength, and high dynamic strength (23,24). In this experiment, rods of Ti6Al4V alloy were fabricated (Aecc Beijing Institute of Aeronautical Materials, Beijing, P. R. China) with diameters of 19.5 mm and were cut into 2.5-mm thick disks. Each disk was polished to an average roughness of 0.2 mm and a thickness of 0.01 mm. The SLA disks were manufactured with large grit (250 μ m) jetted onto machined Ti surfaces after immersion in a mixed solution of 18% HCL and 48% H₂SO₄ at 60°C for 30 min. Ultrasonic cleaning occurred with concentrated cleaning solution (Micro-90, International Products Corporation, New York, NY, USA), absolute ethyl alcohol, and deionized water, in sequence. Polished Ti and SLA Ti disks were steeped in 75% alcohol for 12 h and exposed to ultraviolet (UV) radiation for 1 h. The surface topography of the Polished Ti and SLA Ti disks was examined with a field emission scanning electron microscope (FESEM) (SU-70, Hitachi, Tokyo, Japan). A Wyko NT9300 Optical Profiler (Veeco, New York, NY, USA) was used to examine the average surface roughness (Ra) of the Polished Ti and SLA Ti disks.

MC3T3-E1 cell culture

Osteoblast-like MC3T3-E1 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, P. R. China) were cultivated as previously described (25). The MC3T3-E1 cells were inoculated on the surface of the Polished Ti and SLA Ti disks, with a density of 4×10^4 cells, to assess the cellular localizations and expressions of YAP, TAZ, and integrins $\alpha 1$, $\alpha 2$, and $\beta 1$. The MC3T3-E1 cells were seeded on the surface of the SLA Ti disks at a density of 7×10^4 for the YAP, TAZ, and integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ knockdown. The MC3T3-E1 cells for gene transfection were inoculated with α -MEM containing fetal bovine serum (FBS). The MC3T3-E1 cells achieved 70-80% confluency and were pretreated with Opti-MEM (Invitrogen, Carlsbad, CA, USA). YAP, TAZ, and integrin $\alpha 1$, $\alpha 2$, and $\beta 1$ -siRNA were transfected into cells with Lipofectamine 3000 (Invitrogen). Then, the cells were incubated in a CO₂ incubator. After 6 h, the medium was exchanged with α -MEM containing FBS and the osteogenic supplement. The siRNA sequences are listed as follows:

Yap-1-siRNA: CGAGATGAGAGCACAGACA;
Wwtr1-siRNA: CAGAACTGACTTTAGAGAAT;
integrin $\alpha 1$ -siRNA: CCTTCTACATGTTGGATAA;
integrin $\alpha 2$ -siRNA: CCATCAATTTCCAAGCCTT; and
integrin $\beta 1$ -siRNA: GGAGAACCACAGAAGTTTA.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

After 3 days of incubation on the Polished Ti and SLA Ti, the mRNA expressions of YAP, TAZ, and integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ were examined using qRT-PCR. After 48 h of YAP and TAZ transfection, the mRNA expression of Runx2 and bone sialoprotein (BSP) were analyzed with qRT-PCR. After 48 h of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ transfection, the mRNA expression of YAP and TAZ, and the transfection efficiency of YAP, TAZ, and integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ were analyzed with qRT-PCR. QRT-PCR analysis was performed using a previously described protocol (25). The gene expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The qRT-PCR primers were as follows:

YAP: 5'-TGAGATCCCTGATGATGTACCAC-3' and
5'-TGTTGTTGTCTGATCGTTGTGAT-3';
TAZ: 5'-GACAAGGCAAGCAAGACGTG-3' and
5'-GCTGAAAGCAACAGCAGTCC-3';
ITGA1: 5'-CGTGTCATCTCCTTCCCTCG-3' and
5'-AATAGCCAGCTCTCGTTTCCG-3';
ITGA2: 5'-TGGTAGTTGTGACCGATGGC-3' and
5'-ACCCAAGAACTGCTATGCCG-3';
ITGB1: 5'-CGGACGCTGCGAAAAGATGAA-3' and

5'-CCGCAAGATTTGGCATTGCT-3';
Runx2: 5'-CCCAGCCACCTTTACCTACA-3' and
5'-TATGGAGTGCTGCTG GTCTG-3';
BSP: 5'-CAGGGAGGCAGTGACTCTTC-3' and
5'-AGTGTGGAAAGTGTGGCGTT-3'; and
GAPDH: 5'-AGGTCGGTGTGAACGGATTG-3' and
5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Immunofluorescence staining

To assess YAP's and TAZ's translocation from the cytoplasm into the nucleus, the MC3T3-E1 cells were incubated on the Polished Ti and SLA Ti disks for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 20 min. The nonspecific antibody interaction was blocked with goat serum for 30 min. The disks were incubated with rabbit monoclonal antibody to YAP (1:100 dilution; Cell Signaling Technology, Boston, MA, USA) and rabbit polyclonal antibody to TAZ (1:100 dilution; Abcam, Cambridge, UK) overnight at 4°C. Then they were incubated with a FITC-labeled goat anti-rabbit IgG (1:50 dilution; Jackson Immuno Research, Baltimore, MD, USA) to visualize the primary antibodies at 37°C for 50 min. The nuclei were counterstained with 4',6 diamidino-2-phenylindole (DAPI, Invitrogen) for 5 min. The disks were rinsed with PBS buffer in the interval of each of the two reagents. YAP's and TAZ's translocation was visualized and photographed (LSM 780 Spectral Confocal Laser Scanning microscope, Zeiss, Jena, Germany).

Protein extraction and Western blot analysis

The protein expression of YAP, TAZ, and integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ on the Polished Ti and SLA Ti after 3 days, the protein expression of Runx2 and BSP after 48 h of YAP and TAZ transfection, and YAP's and TAZ's protein expression after 48 h of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ transfection were examined with Western blot analyses using a previously described protocol (25). The membrane was incubated overnight at 4°C with rabbit monoclonal antibody to YAP; rabbit polyclonal antibody to TAZ; rabbit monoclonal antibody to Runx2 (1:1,000 dilution; Cell Signaling Technology, Boston, MA, USA); rabbit polyclonal antibody to BSP (1:500 dilution; Cell Signaling Technology); rabbit polyclonal antibody to ITGA1 (1:1,000 dilution; Affinity, Cincinnati, OH, USA); rabbit monoclonal antibody to ITGA2 (1:1,000 dilution; Abcam, Cambridge, Britain); rabbit polyclonal antibody to ITGB1 (1:500 dilution; Wanleibio, Shenyang, Liaoning, P. R. China); or rabbit polyclonal antibody to GAPDH (1:10,000 dilution; ProteinTech, Chicago, IL, USA). The

Fig. 1 Images of Polished Ti and sandblasted, large-grit, acid-etched (SLA) Ti under different power lenses: (a) General image of Polished Ti and SLA Ti; (b) surface topography images of Polished Ti and SLA Ti under scanning electron microscope (SEM) with low-power ($\times 50$) and high-power ($\times 500$) lenses; (c) SLA Ti surface topography under SEM with high-power lens ($\times 2.00k$); (d) average surface roughness (Ra) of Polished Ti and SLA Ti under an optic profiler; *** $P < 0.0001$ when SLA Ti and Polished Ti are compared, and the difference is statistically significant.

polyvinylidene difluoride membrane was rinsed with 1% TBST buffer and incubated with goat anti-rabbit IgG peroxidase conjugate at room temperature (RT) for 1 h. The membrane, incubated with HRP-conjugated rabbit polyclonal antibody to β -actin (1:5,000 dilution; Protein-Tech), was directly placed at RT for 1 h. Immunodetection was performed by SageCapture software (Beijing SaiZhi Venture Technology Co., Ltd., Beijing, P. R. China) with an enhanced chemiluminescence kit.

Statistical analysis

Statistical software (SPSS 24.0, IBM Corp., Armonk, NY, USA) was used to analyze the experimental results,

and Shapiro-Wilk test was used to analyze data normality. The test level was $\alpha = 0.05$, and the data were in normal distribution ($P > 0.05$). The representative data of YAP, TAZ, Runx2, and BSP were presented as mean $\pm$ standard deviation using statistical software (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA). The t -test was used to detect differences between two groups, and one-way ANOVA (Dunnett's multiple comparisons test) was used to compare the mean of each group with a control group. Statistical significance was defined as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

Results

Topography features of SLA

After grinding and polishing, the smooth Ti6Al4V specimen appeared as a silver-white lustrous metal with scratches visible to the naked eye. The SLATi6Al4V specimen produced with SLA technology based on the previously produced smooth Ti specimen appeared as a gray, rough, and lackluster metal (Fig. 1a). After cleaning, the Polished Ti and SLA Ti underwent vacuum freeze-drying followed by metal spraying. When observing the surface microtopography of the Ti plate with FESEM, the surface of the Polished Ti ($\times 50$, $\times 500$) was relatively smooth with several scratches visible in different directions. After sandblasting and acid-etched treatment, the SLA Ti specimen ($\times 50$, $\times 500$) acquired a large number of surface pits, which were continuously overlapped (Fig. 1b). Under a high magnification lens, the surface topography of the SLA Ti ($\times 2.0k$) showed first-step pits with a pit diameter of approximately 30 μm . There were several second-step pits with a pit diameter of approximately 4–8 μm , and discontinuous, short scratches were still visible (Fig. 1c). To further observe the roughness of the two specimens, an optical profilometer was used to measure Ra. The results were $0.2358 \pm 0.005199 \mu m$ for Ra of the Polished Ti and $2.587 \pm 0.09597 \mu m$ for Ra of the SLA Ti; thus, the Ra of the SLA Ti was significantly higher than that of the Polished Ti ($P < 0.0001$) (Fig. 1d).

SLA induced the translocation of YAP and TAZ

To confirm that the surface topography of SLA technology was conducive to YAP/TAZ's nuclear migration, the MC3T3-E1 cells were cultured on the Polished Ti and SLA Ti for 24 h. Then, the distributions of the YAP/TAZ proteins in the cells were observed, by immunofluorescence staining, in the nucleus and cytoplasm of Polished Ti, whereas the distribution in the cytoplasm was more widespread. On SLA Ti, the YAP proteins mainly clustered in the nucleus, and few were observed in the cytoplasm surrounding the nucleus. Nearly all the

Fig. 2 Intracellular localization of Yes-associated protein (YAP) (a) and transcriptional coactivator with PDZ-binding motif (TAZ) (b) on Polished Ti and SLA Ti under immunofluorescent staining detection for 24 h. “Merge” represents the blended image of the target protein (YAP/TAZ) and nucleus.

TAZ was gathered in the nucleus (Fig. 2). These results were influenced by the surface microtopography of SLA technology, and YAP/TAZ was transferred from the cytoplasm to the nucleus.

SLA enhanced YAP and TAZ expression

To further study SLA topography’s influence on YAP/TAZ expression in osteoblasts, qRT-PCR was used to detect changes in the mRNA levels of YAP/TAZ on Polished Ti and SLA Ti. For the MC3T3-E1 cells cultured for 3 days on the two materials, the results showed that the mRNA level of YAP on SLA Ti (1.895 ± 0.1548 , $P < 0.01$) increased remarkably compared to that on Polished Ti (1.000 ± 0.08451). TAZ’s mRNA level (2.645 ± 0.05891 , $P < 0.0001$) also increased compared to that on a Polished Ti (1.000 ± 0.07173), with both having statistical significance (Fig. 3a). Meanwhile, Western blot demonstrated that the SLA’s surface microtopography also increased YAP/TAZ’s protein level when compared

Fig. 3 mRNA expression and protein expression of YAP and TAZ on Polished Ti and SLA Ti: (a) mRNA expression after GAPDH standardization of YAP and TAZ on Polished Ti and SLA Ti, detected with qRT-PCR on the third day; (b) protein expression of YAP and TAZ on Polished Ti and SLA Ti, detected with Western blot on the third day; $**P < 0.01$ when SLA Ti and Polished Ti are compared, and the difference is statistically significant; $***P < 0.0001$ when SLA Ti and Polished Ti are compared, and the difference is statistically significant.

to Polished Ti (Fig. 3b). The above results indicated that SLA microtopography could increase YAP/TAZ expression in terms of the transcription.

YAP and TAZ knockdown attenuated SLA-induced MC3T3-E1 osteogenic differentiation

To explore the regulation of SLA microtopography over osteogenic differentiation by YAP and TAZ, the MC3T3-E1 cells were cultured on SLA Ti, and the genes *yap1* and *wtr1* of YAP and TAZ were silenced through siRNA to observe changes in the osteogenic differentiation markers Runx2 and BSP. On the second day of YAP gene silencing, the mRNA level of Runx2 (0.5808 ± 0.04188 , $P < 0.01$) was significantly lower than that of the transfected nonspecific sequence group (1.000 ± 0.06271), while the mRNA level of BSP (0.6345 ± 0.05408 , $P < 0.05$) was also lower than that of the control group (1.000 ± 0.06521) (Fig. 4a). Similarly, after TAZ gene silencing, the mRNA level of Runx2 (0.5640 ± 0.01611 , $P < 0.0001$) was significantly lower than that of the control group (0.9997 ± 0.01729), and the mRNA level of BSP (0.2363 ± 0.02777 , $P < 0.01$) was also significantly lower than that of the control group (1.000 ± 0.06521) (Fig. 4b). The protein levels of Runx2 and BSP were further detected using Western blot. It was

Fig. 4 Comparison of runt-related transcription factor 2 (Runx2) and bone sialoprotein (BSP) in different groups by qRT-PCR and Western blot: (a) mRNA expression after GAPDH standardization of Runx2 and BSP, detected with qRT-PCR on the 2nd day when YAP genes are silenced; (b) mRNA expression after GAPDH standardization of Runx2 and BSP, detected with qRT-PCR on the 2nd day, when TAZ genes are silenced; (c) mRNA expression after GAPDH standardization of Runx2 and BSP, detected with Western blot on the 2nd day when YAP and TAZ genes are silenced; (d) gene silencing efficiency of YAP and TAZ, detected with qRT-PCR on the 2nd day. * $P < 0.05$ is considered statistically significant; ** $P < 0.01$ is considered statistically significant; *** $P < 0.0001$ is considered statistically significant.

discovered that, upon YAP silencing, Runx2's and BSP's protein levels were decreased; the same thing occurred upon TAZ silencing (Fig. 4c). Figure 4d shows that YAP's silencing efficiency on the second day of gene silencing was approximately 70% ($P < 0.01$), while TAZ's was approximately 75% ($P < 0.05$). The above results showed that when YAP and TAZ were silenced, both of the osteogenic differentiation markers Runx2 and BSP in the cells were downregulated, which had statistical significance.

SLA enhanced the expression of integrin $\alpha 1$, integrin $\alpha 2$, and integrin $\beta 1$

QRT-PCR was used to detect changes in the mRNA

levels of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ on Polished Ti and SLA Ti. After the MC3T3-E1 cells were cultured for 3 days on the two materials, results showed that the mRNA levels of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ on SLA Ti (0.008952 ± 0.0004110 ; $7.327e^{-006} \pm 2.241e^{-006}$; 0.02978 ± 0.003258 ; $P < 0.05$) increased remarkably compared to those on Polished Ti (0.002926 ± 0.0001434 ; $3.547e^{-005} \pm 8.474e^{-006}$; 0.01247 ± 0.003680 , $P < 0.05$) (Fig. 5a). Meanwhile, Western blot demonstrated that SLA's surface microtopography also increased the protein levels of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ compared with (Fig. 5b) above results indicated that SLA microtopography could increase the expression of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ in terms of the transcription.

Fig. 5 mRNA and protein expression of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ on Polished Ti and SLA Ti: (a) mRNA expression after GAPDH standardization of integrin $\alpha 1$, integrin $\alpha 2$, and integrin $\beta 1$ on Polished Ti and SLA Ti, detected with qRT-PCR on the 3rd day; (b) protein expression of integrin $\alpha 1$, integrin $\alpha 2$, and integrin $\beta 1$ on Polished Ti and SLA Ti, detected with Western blot on the 3rd day; * $P < 0.05$ when SLA Ti and Polished Ti are compared; ** $P < 0.01$ is considered statistically significant when SLA Ti and Polished Ti are compared; *** $P < 0.0001$ is considered statistically significant when SLA Ti and Polished Ti are compared.

Fig. 6 Comparison of YAP and TAZ in different groups by qRT-PCR and Western blot: (a) mRNA expression after GAPDH standardization of YAP and TAZ, detected with qRT-PCR on the 2nd day when integrin $\alpha 1$, integrin $\alpha 2$, and integrin $\beta 1$ genes were silenced; (b) protein expression after GAPDH standardization of YAP and TAZ, detected with Western blot on the 2nd day when integrin $\alpha 1$, integrin $\alpha 2$, and integrin $\beta 1$ genes were silenced; (c) gene silencing efficiency of integrin $\alpha 1$, integrin $\alpha 2$, and integrin $\beta 1$, detected with qRT-PCR on the 2nd day. * $P < 0.05$ is considered statistically significant; ** $P < 0.01$ is considered statistically significant; *** $P < 0.0001$ is considered statistically significant.

Integrins $\alpha 1$, $\alpha 2$, or $\beta 1$ knockdown attenuated SLA-induced expression of YAP and TAZ

To verify that SLA microtopography enhanced YAP and TAZ expression by integrins $\alpha 1$, $\alpha 2$, or $\beta 1$, MC3T3-E1 cells were cultured on SLA Ti, and the integrins $\alpha 1$, $\alpha 2$, or $\beta 1$ genes were silenced through siRNA to observe changes in YAP and TAZ expression. On the third day of gene silencing of integrins $\alpha 1$, $\alpha 2$, or $\beta 1$, mRNA levels of YAP and TAZ were significantly lower than those of the transfected nonspecific sequence groups ($P < 0.05$) (Fig.

6a). This study further detected the protein levels of YAP and TAZ using Western blot and discovered that upon integrins $\alpha 1$, $\alpha 2$, or $\beta 1$ being silenced, YAP's and TAZ's protein levels decreased (Fig. 6b). Figure 6c shows the silencing efficiency of integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ on the second day of gene silencing. The above results showed that when integrins $\alpha 1$, $\alpha 2$, and $\beta 1$ were silenced, both YAP and TAZ in the cells were downregulated, which had statistical significance.

Discussion

SLA technology is a commonly used surface modification method for dental implants. The hierarchical microtopography formed by SLA technology not only allows implant-osseous tissue to form proper mechanical chimerization, but also increases the superficial area of the implant-osseous tissue interface and further uplifts the material's surface roughness. This experiment investigated differences in SLA Ti and Polished Ti in surface microtopography with a scanning electron microscope and discovered that the SLA plate surface showed a hierarchical micropore morphology, with the pit diameter of the first-step pits being approximately 30 μm and that of the second-step pits being approximately 3-8 μm . Surface roughness is usually expressed with Ra. In this experiment, an optical profilometer detected that SLA Ti's RA was approximately 2.6 μm and that of Polished Ti was approximately 0.24 μm . This is consistent with the Ra of the optimum Ti alloy surface, 2-3 μm , obtained by Olivares-Navarrete et al. (19).

Osteoblasts are sensitive to implants' surface microtopography. The microtopography of SLA implants can promote osteoblasts differentiation, thus facilitating bone formation around implants (26). Multiple signaling pathways are involved in regulating SLA microtopography over osteoblasts. $\alpha 2\beta 1$ integrins may induce the release of TGF- $\beta 1$ by activating PKC, ultimately promoting osteoprotegerin expression (27). Yang et al. (28) found that the β -catenin/LRP5 pathway also participated in the process of SLA surface microtopography, promoting bone marrow-derived mesenchymal stem cell osteogenic differentiation. Rho-associated coiled-coil forming protein kinase (ROCK) downstream of RhoA also mediated the SLA microtopography process, promoting osteoblast differentiation by activating the Wnt/ β -catenin pathway (29).

Both YAP and TAZ are transcriptional co-activators in mammalian cells. The hardness of the extracellular matrix and surface microtopography can promote MSC and osteoblast differentiation through regulating YAP/TAZ inner cellular localization (30). The relatively hard extracellular matrix can promote YAP/TAZ nuclear migration and facilitate osteoblast and MSC differentiation. Previous research showed that YAP/TAZ migrated into the nucleus under the effect of specific surface microtopographies and combined with Runx2, promoting MSCs' osteoblast differentiation.

Pan et al. (31) adopted sandblasting to prepare the pure Ti surface micron structure and used alkali heat treatment to prepare the pure Ti surface micron-nano structure. They observed that, compared to the surface

of Polished Ti, the micron-structured pure Ti surface could promote YAP migration into the nucleus, and YAP migration on a micron-nano multilevel structure surface was more obvious. Furthermore, the YAP content was also increased. In this experiment, immunofluorescent staining results showed that SLA microtopography could promote YAP and TAZ migration from the cytoplasm into the nucleus. Meanwhile, qRT-PCR and Western blot experimental results showed that the mRNA and protein expression of YAP/TAZ in cells on SLA Ti significantly increased compared to cells on Polished Ti. Therefore, SLA microtopography could further change intranuclear signaling pathways by increasing YAP/TAZ's intracellular content of and promoting their nuclear migration, influencing the osteoblasts' biological changes.

Runx2, also called core-binding factor a1 (Cbfa1), serves as the key transcription factor in osteoblast differentiation. Moreover, Runx2 is also the switch for osteoblast differentiation (32-35). BSP belongs to the family of small integrin-binding ligand N-linked glycoproteins and is one of the marker genes for osteoblast differentiation. BSP expression visibly increases in maturely differentiated osteoblasts, and BSP is highly expressed in the early stage of bone deposition (36). BSP can also regulate the formation of osteoclasts. The lack of BSP influences the function of osteoblasts and osteoclasts (37). BSP knockdown mouse models have shown a relatively low level of bone remodeling for lowered osteoblast and osteoclast activity (38).

This experiment adopted siRNA to inhibit YAP/TAZ in MC3T3-E1 cells on SLA microtopography surfaces. Then, qRT-PCR and Western blot were used to detect the expression of the osteoblast marker genes Runx2 and BSP. The results showed that the mRNA and protein levels of Runx2 and BSP obviously declined after YAP or TAZ silencing. This indicated that YAP/TAZ could promote the expression of Runx2 and BSP on the SLA microtopography surface, which was consistent with the above conclusion of regulating YAP/TAZ to promote MSC osteoblast differentiation in the microtopography prepared by histological engineering (16).

In terms of SLA microtopography, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin may promote osteoblast differentiation (3,18-20). Integrin can form physical connections with ECM and cytoplasm fibers on cell membranes through mutual effects with various signaling proteins, namely, focal adhesion. As the mechanical stimulation receptor on the bone tissue cell surface, focal adhesion-cytoskeleton plays an important role in cell adhesion, survival, proliferation, migration, and invasion (39,40). Pan et al. (31) found that the multilevel structure microtopography

surface generated effects on myosin via the integrin pathway, thus changing the cytoskeleton's tension. The mechanical conduction caused by the tension promoted YAP/TAZ's nuclear migration and combined them with the transcription factors Runx2 and PPAR γ inside the nucleus, determining the BMSCs osteoblast or fat differentiation. This study found that SLA microtopography enhanced the expression of integrin α 1, integrin α 2, and integrin β 1. After the integrin α 1, α 2, and β 1 genes were silenced, YAP and TAZ decreased in terms of mRNA and protein, suggesting that integrins α 1, α 2, and β 1 participate in the process of YAP/TAZ regulating the differentiation of osteoblasts on SLA microtopography surface.

The Rho GTP enzyme, downstream of integrin, takes part in the composition of focal adhesion. It belongs to the small G protein superfamily. It was recently proven that integrin can regulate YAP/TAZ through the Rho GTP enzyme. Tang et al. discovered that MT1-MMP (membrane-type 1 matrix metalloproteinase) could activate the Rho GTP enzyme through β 1 integrin, enabling YAP/TAZ's nuclear migration and promoting skeletal stem cell osteoblast differentiation (22). On the surface of TNTs, focal adhesion kinase, downstream of integrin in the MC3T3-E1 cells, could regulate YAP through RhoA (17). The Rho GTP enzyme also regulated YAP by mechanical stress or cytoskeleton tension (21). Therefore, this study determined that the microtopography, formed by SLA technology on the Ti surface, changed cell spreading and form, activated integrins on the cell surface, conducted focal adhesion assembly, and activated the Rho GTP enzyme through a series of kinases. Meanwhile, the change of the cell form also formed micro-mechanical acting forces due to the change in cytoskeleton tension, which similarly worked on the Rho GTP enzyme. The activated Rho GTP enzyme may regulate Runx2 transcriptional activity through regulating YAP/TAZ's nuclear migration, thus promoting osteoblast differentiation.

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Conflict of interest

The authors declare that they have no competing interest.

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