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TiO_2 nanorod arrays modified Ti substrates promote the adhesion, proliferation and osteogenic differentiation of human periodontal ligament stem cells

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ABSTRACT

Nanostructure coating on titanium (Ti) implants is well known as a cue for directing osteoblast behavior and function. However, effects of nanostructure coatings on dental stem cells have been rarely explored. In this work, assembled TiO_2 nanorod arrays (TNRs) were fabricated on the polished Ti substrates using hydrothermal and sintering methods. The adhesion, morphology, proliferation and osteogenic differentiation of human periodontal ligament stem cells (PDLSCs) seeded onto TNRs substrates were evaluated. Ti substrates were used as control. Rougher TNRs showed better hydrophilicity and protein adsorption capacity compared with Ti control. When seeded on TNRs substrates, PDLSCs exhibited more stretched morphology and higher proliferation rate. Cytoskeletal F-actin expression was markedly promoted for PDLSCs cultured on TNRs substrates under osteogenic induction. Alkaline phosphatase (ALP) activity and mineral deposition were also enhanced by TNRs. Moreover, osteogenesis-related markers of ALP, runt related transcription factor 2 (Runx2) and osteopontin (OPN) of PDLSCs cultured on TNRs substrates were significantly up-regulated at both gene and protein levels when compared to Ti substrates. In conclusion, the unique structure of TNRs provided a biocompatible platform for modulating morphology and function of PDLSCs. The promotion of osteogenic differentiation indicated that the surface modification of implants with TNRs may improve the osteogenic activity of implants and the bone-implant integration in future clinical applications.

1. Introduction

Titanium (Ti) and its alloys have been widely used as implants in orthopedic and oral implantology, due to their high strength and superior biocompatibility [1]. However, failure and loss of implants because of inefficient osteoinduction of Ti metal are still critical issues bothering researchers and clinicians. After artificial biomaterial implanted into body, cell responses and cell-biomaterial interactions were considered as crucial factors for the clinical success of implants [2]. Surface properties of implant, such as topography, charge distribution and chemical composition, were reported to influence osteoblast responses and the osseointegration between bone and implant [3,4]. Normally, responses of mesenchymal stem cells (MSCs) to implant surface were vital indicators for evaluating the osteogenic activity of implant [4,5]. Since nature bone is composed of nanoscaled components, surface modifications of Ti implants at nanometer level have been applied to simulate the architecture of human bone surface and improve the bioactivity of implants [6–8]. Recently, surface modification with TiO_2 nanostructures has been extensively investigated to improve the osteoinduction and osseointegration of implants. TiO_2 nanotubes or nanorods constructed on different substrates have good performance in promoting the osteogenic differentiation tendency of MSCs [8–10]. Our previous study demonstrated that a vertically grown TiO_2 nanorod arrays (TNRs) on FTO ($F:SnO_2$) conductive glass significantly promoted the osteogenic differentiation of bone marrow mesenchymal stem cells (BMMSCs) *in vitro* [10].

As a promising dental stem cell population, human periodontal ligament stem cells (PDLSCs) possess osteogenic, adipogenic and chondrogenic characteristics under inductive culture conditions *in vitro* [11]. Transplantation of PDLSCs has the potential to facilitate the formation of bone, cementum and functional periodontal ligament

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(PDL) in damaged periodontium in animal models [12,13]. Considering the inconvenience of obtaining BMMSCs, PDLSCs might be an alternative to human BMMSCs for assessing the osteogenic activity of Ti surface and evaluating the osseointegration of dental implants [14,15]. PDLSCs cultured on Ti surface were demonstrated to have the tendency of osteogenic differentiation and the potential of bone formation [14]. Moreover, the osteogenic differentiation of PDLSCs could be influenced by roughness and hydrophilicity of Ti surface, along with modulations of differential Wnt pathways and signaling molecules [16]. Recently, TiO_2 nanotubes normally fabricated by anodic oxidation method have been widely employed to biologically modify Ti implants. Gao firstly reported that TiO_2 nanotubes layered on Ti substrates provided a biocompatible scaffold for PDLSCs and obviously enhanced the adhesion, spreading and osteogenic functions of PDLSCs [17]. However, fabricating TiO_2 nanostructures with hydrothermal method is simpler and more environmentally friendly than the anodic oxidation method [18]. Our group previously fabricated TNRs using hydrothermal method and found that TNRs promoted the proliferation, osteogenic differentiation of BMMSCs [10].

In this present study, rutile TNRs were fabricated on Ti substrates as a cell culture platform for PDLSCs. The adhesion, proliferation and osteogenic differentiation potentials of PDLSCs were examined to evaluate the effects of TNRs structure on PDLSCs *in vitro*.

2. Materials and methods

2.1. Sample preparation

TA2 commercially pure Ti sheets ($10 \times 10 \times 1$ mm^3) were bought from Xin Dongying Metal Product Co. Ltd. (Baoji, China). After polished with *SiC* sandpaper and ultrasonic cleaning, TNRs were synthesized on well-polished Ti sheets as previously reported with slight modification [19]. Briefly, 10 ml deionized water was mixed with 9 ml concentrated hydrochloric acid (36.5%–38% by weight). Then, 850 μl titanium butoxide was added to the mixture under stirring. After stirring for another 5 min, the mixture was transferred to a 25 ml poly (tetrafluoroethylene)-lined autoclave with 4 pieces of 10×10 mm Ti sheets and heated at 160 °C for 1.5 h to fabricate TNRs onto Ti sheets. After cooling to room temperature, TNRs sheets were taken out, and then were rinsed extensively with deionized water and subsequently with ethanol. Then, TNRs sheets were annealed at 500 °C for 2 h at a heating rate of 5 °C/min. Well-polished Ti sheets were used as control substrates. Prior to use, Ti and TNRs substrates were first sterilized with 75% ethanol for 12 h and then were washed thrice with sterile phosphate-buffered solution (PBS).

2.2. Surface characterization

Crystallinity of the as-synthesized TNRs substrate was identified by an X-ray diffractometer (XRD; D8, Bruker, Germany) with Cu-Kα radiation ($\lambda = 0.15406$ nm). These two types of substrates (Ti/TNRs) were observed under a scanning electron microscope (SEM; S-8020, Hitachi, Tokyo, Japan). The individual TiO_2 nanorod (TNR) was further characterized using a transmission electron microscope (TEM; JEM 2100, JEOL, Tokyo, Japan) with an operating voltage of 200 kV.

Topographic features and roughness of Ti and TNRs surfaces were studied under an atomic force microscope (AFM; Dimension Icon, Bruker). The root mean square average (Ra) roughness of these two substrates was calculated from 1×1 μm^2 image areas. Values of four different areas were obtained to ensure the validity of data.

2.3. Contact angle measurement and protein adsorption assay

Water contact angles on the substrates were examined to show the wettability of surface using the Contact Angle Instrument (DSA10, Kruss, Germany). Briefly, a volume of 2 μl deionized water was dropped onto each sample. Images of contact areas were captured by the camera. Subsequently, the contact angle was calculated using the SCA20 video software (DataPhysics, Germany). All the measurements were conducted at room temperature (26 ± 1 °C).

Ti and TNRs substrates were put in 24-well plates (Coring, NY, USA) and three parallel replicates for each group were used. 1 ml Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, BI, Kibbutz Beit Haemek, Israel) were added to each well. After incubated at 37 °C for 24 h, the substrates were transferred to a new 24-well plate and were rinsed with PBS. Then 0.5 ml 1% sodium dodecyl sulfate (SDS; Amresco, Solon, OH, USA) solution was added to each well. The proteins adsorbed onto the substrates were rinsed into the SDS solution by oscillation at room temperature for 1 h. The protein concentration was assayed using a BCA protein assay kit (KeyGEN, Nanjing, China) by measuring the absorbance of the reaction solution at 562 nm.

2.4. Isolation and cultivation of human PDLSCs

All the procedures were conducted in accordance with the experiment protocols approved by the Medical Ethics Committee of School of Stomatology, Shandong University, Jinan, China (Protocol Number: GR201603). Healthy premolars extracted for orthodontic reason were used for the collection of human PDL tissue. Written informed consent was obtained from each donor. PDL tissues, scraped from the middle third of the root surface, were cultured by the enzymatic dissociation method as previously described [20,21]. Briefly, small PDL tissues were digested with enzymatic mixture containing 3 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/ml dispase (Sigma-Aldrich) at 37 °C for 1 h and then single-cell suspension was obtained by filtering through a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ,USA). After cell counting, single cell suspension was plated at a concentration of 60 cells/cm^2 on non-treated 10 cm Petri dishes for single cell-derived colony selection, and was cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin (Solarbio, Beijing, China). Single cell-derived colonies were identified as PDLSCs and then were pooled for further amplification. PDLSCs at passages 3–5 were utilized for further experiments.

2.5. Cell adhesion and proliferation

PDLSCs were seeded on Ti and TNRs substrates at a density of 2.0×10^4 cells/cm^2 in 24-well plates. After 24 h, the substrates were rinsed with PBS to remove the non-adherent cells. The adherent cells remaining on the plates were fixed with 4% paraformaldehyde for 30 min at 4 °C and then were incubated with 4′,6-diamidino-2-phenylindole (DAPI, Solarbio) for 5 min to stain the cell nuclei. Finally, the substrates were observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan). Six high-power microscopic fields (×100) were randomly selected for counting the numbers of adherent cells.

A cell counting kit-8 (CCK8; Dojindo, Tokyo, Japan) was used to evaluate the proliferation of PDLSCs. Briefly, after incubated for 3, 5 and 7 days, the substrates were transferred to a new 24-well plate. Then 500 μl DMEM containing 50 μl CCK8 was added to each well and incubated at 37 °C for 3 h. Finally, the absorbance was measured at 450 nm using a microplate reader (SPECTROstarNano, BMG, Germany).

2.6. Morphology of human PDLSCs on Ti and TNRs substrates

To observe the morphology of human PDLSCs on these two substrates, cells were seeded at a density of 2.0×10^4 cells/cm^2 onto the substrates in 24-well plates. After incubated for 72 h, the morphology of human PDLSCs attached on the substrates was examined under an SEM. The substrates with attached PDLSCs were washed with PBS, fixed with 2.5% glutaraldehyde solution for 1 h and and dehydrated in