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Enhanced osteogenic differentiation of rat bone marrow mesenchymal stem cells on titanium substrates by inhibiting Notch3

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ABSTRACT

Objective: The role of the Notch pathway has already been identified as a crucial regulator of bone development. However, the Notch signaling pathway has gone largely unexplored during osseointegration. This study aims to investigate the role of Notch signaling on osteogenic differentiation of rat derived bone marrow mesenchymal stem cells (BMSCs) on sandblasted, large-grit, acid-etched (SLA) treated Ti disks.

Methods: The involved target genes in Notch pathways were identified by *in vitro* microarray and bioinformatics analyses with or without osteogenic induction. Adhesion, proliferation, and osteogenic related assay were subsequently conducted with target gene shRNA treatment.

Results: We found that 11 genes in the Notch signaling pathway were differentially expressed after osteogenic induction on SLA-treated Ti disks, which included up-regulated genes (*Notch2, Dll1, Dll3, Ncstn, Ncor2,* and *Hes5)* and down-regulated genes *(Notch3, Lfng, Mfng, Jag2* and *Maml2)*. With Notch3 shRNA treatment, the adhesion and proliferation of BMSCs on SLA-treated Ti disks were inhibited. Moreover, the expression levels of alkaline phosphatase (ALP), osteocalcin (OCN), calcium deposition, BMP2 and Runx2 increased significantly compared with that observed in control groups, suggesting that the function of Notch3 was inhibitory in the osteogenic differentiation of BMSCs on SLA-treated titanium.

Conclusions: Inhibition Notch3 can enhance osteogenic differentiation of BMSCs on SLA-treated Ti disks, which potentially provides a gene target for improving osseointegration.

1. Introduction

Titanium dental implants are widely developed in clinics, mainly due to their superior mechanical property and biocompatibility. A host of methods have been developed for improving surface properties of titanium implants, such as titanium plasma spraying (Koshuro, Nechaev, & Lyasnikova, 2016), grit-blasting (Gil, Eduardo Espinar, Jose Maria Llamas, & Sevilla, 2014), acid etching (Yoo et al., 2015), microarc oxidation (YeonWook, 2008), and electrodeposit (Shifang, Jue, Fuming, Liu, & Guoli, 2014). Generally, the sandblasted, large-grit, acid-etched (SLA) implants are particularly representative combined method. A multitude of studies (Buser et al., 2004; Calderón et al., 2011) have reported that SLA implants can significantly accelerate osseointegration.

Osseointegration after implant placement is a complex process involving several biological mechanisms including an early immuno-inflammatory response, angiogenesis, and osteogenesis (Ralf et al., 2016). Initially, mechanisms of cellular and plasmatic hemostasis lead to fibrin polymerization and the formation of a blood clot. Then, bone marrow mesenchymal stem cells (BMSCs) migrate to the surface of the implant cavity, differentiate, and lead to the formation of new bone. Research efforts have been focused on designing novel implant surfaces such as SLA-treated titanium to promote osteoblast differentiation and increase bone to implant contact (Wennerberg & Albrektsson, 2009). However, the signals responsible for modification-induced implant osseointegration have remained elusive.

Several signaling pathways including WNT/β-catenin (Lumetti et al., 2014; Wang et al., 2012; Yang et al., 2016), TGFβ/BMP (Vlacic-Zischke, Hamlet, Friis, Tonetti, & Ivanovski, 2011), and MAPK (Huang, Tan, Cai, & Yang, 2012) have been studied in their regulation of bone-implant osseointegration, thus providing clues into molecular management. It has been reported that on

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roughened titanium surfaces, osteogenic genes (BMP2, SP1, and so on) were significantly up-regulated through the TGFβ/BMP pathway (Vlacic-Zischke et al., 2011). However, the role of the Notch pathway, which has recently been identified as a crucial regulator of bone development (Dong et al., 2010; Engin et al., 2008; Hilton et al., 2008; Tao et al., 2010; Zanotti et al., 2008), has gone largely unexplored during osseointegration.

Notch signaling plays a crucial role in regulating cell fate determination, proliferation, differentiation, and stem cell self-renewal in both embryonic and adult organs (Artavanis-Tsakonas, Rand, & Lake, 1999; Chiba, 2006; Chillakuri, Sheppard, Lea, & Handford, 2012; Konishi et al., 2007; Lai, 2004). Previous studies have shown that inhibitory Notch signaling regulated the osteogenesis in ST-2 marrow stromal cells (Deregowski, Gazzerro, Priest, Rydziel, & Canalis, 2006), mesenchymal progenitor cells Kusa (Shindo et al., 2003), and osteoblastic cells MC3T3-E1 (Sciaudone, Gazzerro, Priest, Delany, & Canalis, 2004). Moreover, recent evidence has demonstrated that Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation (Dong et al., 2010; Hilton et al., 2008), whereas inactivation of Notch signaling in BMSCs enhances bone formation (Hilton et al., 2008). These results suggest that Notch signaling could be responsible for enhanced osteogenesis. However, there is no clear genetic evidence demonstrating a direct role for Notch signaling in BMSCs during the formation of osseointegration.

The purpose of this study was to investigate the effects of Notch signaling on the adhesion, proliferation, and differentiation of BMSCs both at cellular and molecular levels on SLA-treated titanium substrates.

2. Materials and methods

2.1. Preparation of SLA titanium disks

To prepare the SLA substrates, Ti disks were prepared from 2-cm diameter and 1-mm thick sheets of grade 2 unalloyed Ti (Ningbo, Zhejiang, China). Briefly, the degreased disks were grit-blasted with 0.25–0.50 mm corundum grit and then submerged for 10 min in a 0.11 mol/L ammonium fluoric acid and 0.09 mol/L nitric acid solution (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. Subsequently, Ti disks were processed for 30 min in a 5.8 mol/L hydrochloric acid and 8.96 mol/L sulfuric acid solution (Sigma-Aldrich, St. Louis, MO, USA) at 80 °C. Scanning electron microscopy assay (SEM) (SU-70, Hitachi, Japan) was performed to characterize the SLA-treated Ti surfaces.

2.2. Isolation and culture of rat BMSCs

Three-week-old male Sprague-Dawley rats were used. Isolation and culture of BMSCs were performed as described elsewhere (Lennon & Caplan, 2006). Briefly, after euthanasia, BMSCs were aspirated from the bone marrow of femurs and tibias and then cultured in basal medium, which was alpha-modified minimum essential medium (aMEM; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), 0.272 g/L-glutamine (Sigma, USA), and 1% antibiotic solution (penicillin and streptomycin (Gibco, USA)). In this study, a total of 9 rats were used for culturing of BMSCs. These studies were approved by the Institutional Animal Care and Use Committee of Zhejiang University, Hangzhou, China.

2.3. Microarray analysis

In a 6-well plate, BMSCs were seeded on 3-cm diameter SLA-treated Ti disks at a density of 1×10^5 cells/well. Cells were cultured in osteogenic medium (aMEM containing 10% FBS, 0.1 mmol/L dexamethasone (Sigma-Aldrich), 10 mmol/L β-sodium glycerophosphate (Sigma-Aldrich), and 50 mg/mL ascorbic acid (Sigma-Aldrich)). The basal medium was used as a negative control. After maintaining the cells in the medium for 7 days, they were subjected to microarray analysis (Yang et al., 2016). The analysis was conducted by Bohao Biotech Co., Ltd. (Shanghai, China). No standard was set up on how much the ratios of gene expression should differ between 2 specimens for that difference to be considered real (Hanagata, Takemura, & Minowa, 2016). For the osteogenic condition groups, we selected genes that were differentially expressed by at least 2-fold higher or 30 percent lower relative to that of the control groups based on the log2-transformed expression value (MATLAB/Bioinformatics Toolbox, Math Works, Natick, MA, USA). In addition, we used the KEGG pathway database for pathway analysis to investigate Notch signaling.

2.4. Construction of Notch3 lentivirus and transfection

We designed 4 shRNA sequences of rat Notch3. Our preliminary experiment showed that the most efficiency shRNA sequences was lentiviral vector-Notch3 (LV-Notch3): CGC CTA GTC CAG CAA CTG C, lentiviral vector negative control (LV-NC): TTC TCC GAA CGT GTC ACG T (GenePharma Co., Ltd., Shanghai, China). Lentiviral vectors carried the enhanced green fluorescent protein (EGFP) gene and puromycin-resistant gene. The optimal multiplicity of infection (MOI, transduction units (TU)/number of cells) was explored. Cells were transfected with increasing MOI (100, 200, and 400) with LV-Notch3. After transfection for 72 h, BMSCs were fixed in 4% paraformaldehyde for 30 min. As a counterstain for nuclei, 4′-6 diamidino-2-phenolindole (DAPI, Sigma, USA) was used. The final transfection effcacy (GFP-positive cells/the number of nuclei) was calculated by image analysis software Image-Pro Plus (version 6.0; Media Cybernetics; Rockville, MD; USA). After transfection for 72 h, 2 μg/mL of puromycin dihydrochloride (Sigma, SI-P8833) was added to screen transfected cells. Cells seeded on SLA-treated Ti disks were divided into 3 groups: Blank (BMSCs without transfection), NC (BMSCs transfected with LV-NC), and Notch3 (BMSCs transfected with LV-Notch3). The procedures for lentivirus transfection, cell screening, and osteogenic differentiation assay on SLA-treated Ti disks are shown in Fig. 2A.

2.5. Cell adhesion assay

In a 24-well plate, 3 groups of BMSCs were seeded on $1 \times 1\text{-cm}^2$ SLA-treated Ti disks at a density of 1×10^4 cells/well in the basal medium. After incubating the cells for 2 h and 4 h, BMSCs were fixed in 4% paraformaldehyde for 30 min. Rhodamine Phalloidin (1:1500, Life Technologies, Grand Island, NY) was used to label actin filament (F-actin). As a counterstain for nuclei, DAPI was used. Cells were incubated with DAPI for 5 min and washed 3 times with 1× PBS. The cell morphology and cytoskeletal arrangement were analyzed using a fluorescence inversion microscope system (AX10, Zeiss, Germany). Three random images of each sample were taken, and more than 30 cells were chosen to measure cell morphology. All images were analyzed with Image J software. Cell morphology was described using a shape factor φ, which was expressed as $\varphi = 4\pi A/p^2$ (A was the footprint area and p was the perimeter of the cell) (Schuler et al., 2006). The better cells adhered on the substrates, the smaller value of φ was.

2.6. Cell proliferation assay

In a 24-well plate, 3 groups of BMSCs were seeded on $1 \times 1\text{-cm}^2$ SLA-treated Ti disks at a density of 3×10^4 cells/cm^2. The medium was replaced every 2 days. On days 1, 3, and 7, cells were incubated in medium supplemented with 10% Cell Counting Kit-8 (CCK-8,