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The role of integrin-linked kinase/β-catenin pathway in the enhanced MG63 differentiation by micro/nano-textured topography

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ABSTRACT

Micro/nano-texturing is a promising approach to produce biomaterials with better tissue integration properties, but the underlying mechanisms are only partially understood. We propose that the integrin-linked kinase (ILK)/β-catenin pathway may play a role in mediating the signals of topographical cues to cells. To confirm the hypothesis, human MG63 osteoblasts are cultured on the micro/nano-textured topographies (MNTs) to assess the cell differentiation in terms of collagen secretion, extracellular matrix mineralization, and osteogenesis-related gene expression. The expression of β-catenin, ILK and integrin β1 and β3 is assayed by real-time polymerase chain reaction and the protein levels of β-catenin, phosphorylated glycogen synthase kinase 3β (p-GSK3β) and ILK are determined by western blot. The ILK silenced MG63 induced by small interfering RNA is cultured on the samples and the cell functions and the levels of β-catenin, GSK3β and p-GSK3β are determined. The results show that the MNTs enhance MG63 differentiation and it is related to the higher expression of integrin β1 and β3 and ILK, which activate the β-catenin signaling by initiating β-catenin expression and inhibiting its degradation by phosphorylating GSK3β. ILK silencing attenuates the β-catenin signaling activation and the enhanced MG63 differentiation by the MNTs. Our results explicitly demonstrate the role of the ILK/β-catenin pathway in mediating the signals from topographical cues to osteoblasts to tailor differentiation and provide new target points for biomaterials modification and biofunctionalization to attain better clinical performance.

1. Introduction

Surface texturing of biomaterials is a good means to control cell functions for desirable biological responses, and various kinds of micro and nanoscale topographies have been developed. One impressive report from Dalby et al. [1] demonstrates that the slightly disordered nanopits with a diameter of 120 nm on polymethylmethacrylate are able to induce mesenchymal stem cell (MSC) osteogenic differentiation. Titania nanotubular topography constitutes another excellent example which has been widely documented to be a powerful modulator of cells shape, adhesion, proliferation and differentiation [2–6]. Specifically, the hierarchical micro/nano-textured topographies (MNTs) combined with nanotubes and micropitted topography can provide more abundant topographical cues on both the micro and nanoscale similar to natural bone extracellular matrix (ECM) and exhibit more pronounced effects on osteoblast maturation as well as MSC osteogenic differentiation [4,5]. However, the mechanisms mediating the response of cells to topographical cues are largely unknown thereby precluding precise understanding of the biological effect of topographical cues and making it difficult to optimize them systematically.

The β-catenin signaling pathway that plays a crucial role in osteoblast differentiation, maturation, and bone formation [7–9] is also involved in the biological effects of topographical cues on cells [10–13]. With regard to the titania nanotubular topography, Yu et al. have recently reported that β-catenin is the central gene to increase osteoblast proliferation and differentiation [12]. Our recent study also demonstrates that the Wnt/β-catenin pathway is

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involved in the effect of implant topography on osteoblast differentiation [13]. The Wnt/β-catenin pathway is initiated by the binding of a Wnt protein to its corresponding cell membrane receptor, leading to inhibition of a complex comprising Axin, glycogen synthase kinase 3β (GSK3β), and adenomatous polyposis coli (APC) that degrade the β-catenin in the cytoplasm. β-catenin then accumulates in the cytoplasm and translocates into the nucleus and in this way, the β-catenin signaling is activated [7,8]. It can be envisaged that the topographical cues may activate the β-catenin signaling though some special mechanisms different from the Wnt/receptor binding in the Wnt/β-catenin pathway, but the details of this process are relatively unknown.

It is recognized that the topographical cues modulate cell functions by altering direct and indirect mechanotransduction [14,15], and indirect mechanotransduction is the signal pathways that transduce the signals from focal adhesions (FAs). Integrin-linked kinase (ILK) is a multifunctional intracellular effector of cell–matrix interactions located in the FAs binding to the cytoplasmic domain of specific integrin β subunits [16–19]. In addition to being an adaptor protein, ILK functions as a serine/threonine kinase and GSK3β is characterized as its downstream target. ILK can inhibit the activity of GSK3β through phosphorylating and it in turn blocks the GSK3β/APC/Axin complex formation and consequently β-catenin cytoplasmic accumulation and nucleus translocation [16–19]. The present interest on ILK is mainly concerned with the studies on oncogenesis [19–21]. Meanwhile, ILK is also highly expressed in osteoblasts and mediates osteoblast differentiation as well as other functions [22–25]. Since the topographical cues modulate the cell function mainly *via* changing the shape, size and distribution of the FAs and ILK location in the FAs [15], the topographical cues may also influence the ILK expression, thus further modulating the β-catenin signaling through ILK. Actually, it has been reported that cysteine-rich protein 61, a ligand of integrin receptor, regulates osteoblast differentiation through a pathway involving ILK [22] and that ILK increases as cells adhere to type 1 collagen (ColI) dependent on integrin/collagen interaction [26]. To a certain degree, they support our hypothesis.

Herein, we propose that the biological effects of topographical cues may be mediated *via* an ILK/β-catenin pathway. For confirmation, we analyze the cell functions, ILK expression, and β-catenin signaling activation of the human MG63 osteoblasts on the MNTs with titania nanotubes fabricated in our laboratory [4,5]. Afterward, the ILK is silenced and then the supposed downstream events including β-catenin protein levels and cell functions are monitored. Our study serves to provide insights to the molecular mechanisms associated with topographical cues.

2. Materials and methods

2.1. Specimen fabrication

Pure titanium foil (99.9%, 10 × 10 × 1 mm^3, Northwest Institute for Nonferrous Metal Research, China) was used in this study. After polishing using SiC sandpaper from 400 to 1500 grits and ultrasonic cleaning, the samples were treated by 0.5 wt% hydrofluoric acid for 30 min, immediately rinsed with distilled water, and then dried. The samples were anodized for 1 h in an electrolyte containing 0.5 wt% hydrofluoric acid and 1 M phosphoric acid using a DC power supply with a platinum electrode as the cathode at 5 and 20 V, respectively. After ultrasonic cleaning, all the samples, including the two MNTs, namely acid-etched/anodized at 5 V (R-5) and acid-etched/anodized at 20 V (R-20), and the polished smooth control surface (S) were sterilized by cobalt 60 irradiation before cell plating.

2.2. Cell culture

The human MG63 osteoblasts were obtained from ATCC company. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Only cells of early passage were used in the experiments.

2.3. Scanning electron microscopy observation and cell morphology

The morphology of the titanium samples was inspected by field-emission scanning electron microscopy (FE-SEM, S-4800, Hitachi). The cells were seeded at a density of 2×10^4 cells/well on the samples placed in 24 well plates. After culturing for 24 h, the cells on the substrates were washed with phosphate buffered saline (PBS) and fixed with 2.5% w/v glutaraldehyde (Sigma) in PBS at 4 °C overnight. After fixation, they were washed thrice with PBS for 15 min each. The cells were then dehydrated in a graded series of ethanol (50, 70, 90 and 2 × 100 vol%) for 30 min each and dried in a critical point dryer (E-1045, Hitachi). The dried samples were sputter-coated with gold (ES-2030, Hitachi) before the morphology of the adhered cells was observed by FE-SEM.

2.4. RNA interference

Small interfering RNA (siRNA) specific to ILK and control siRNA was synthesized by RiboBio (China). The siRNA were transiently transfected into MG63 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, the plasmids were incubated with Lipofectamine 2000 in OPTI-MEM™ reduced serum medium (Gibco) for 20 min at room temperature before transfection. The plasmid-Lipofectamine 2000 was added to individual wells containing the cells and growth medium with no antibiotic and incubated for 4 h at 37 °C. The RNA was isolated after 48 h and subjected to real-time polymerase chain reaction (PCR) to determine the efficiency of target gene silencing. The regular medium, lipofactamine, control plasmid were used as controls.

2.5. Collagen secretion

The wild type (WT) and ILKsi MG63 cells were seeded on the substrates at a density of 2×10^4 cells/well and cultured in the osteogenic medium. The osteogenic medium was supplemented with 10 mM β-glycerophosphate (Sigma), 50 μg/mL ascorbic acid (Sigma), and 10^{-7} M dexamethasone (Sigma). To avoid gradual loss of the siRNA effect due to cell proliferation, at day 7, the ILKsi plasmid-Lipofectamine 2000 complex was added to the individual wells once again. After 4 h transfection, the culture medium was changed to fresh medium for continuous incubation. After culturing for 14 d, the cells were fixed in 4% paraformaldehyde and stained for collagen secretion in a 0.1 wt% solution of sirius red (Sigma) in saturated picric acid for 18 h. Afterward, the cells were washed with 0.1 M acetic acid and images were taken. In the quantitative analysis, the stain on the specimens was eluted in 500 μl of destain solution (0.2 M NaOH/methanol 1:1) and the optical density at 540 nm was then measured on the spectrophotometer.

2.6. ECM mineralization

The cell culture was the same as the collagen secretion assay. After culturing for 14 d, the cells were washed with PBS and then fixed in 60% isopropanol for 1 min at room temperature. After rehydration in distilled water for 3 min, the cells were stained with 1 wt% alizarin red (Sigma) for 3 min at room temperature. After washing in distilled water thrice, images were taken. To quantify the red-stained nodules, the stain was solubilized within 10% cetylpyridinum chloride in 10 mM sodium phosphate and the absorbance values were measured at 620 nm.

2.7. Quantitative real-time PCR

The WT and ILKsi MG63 cells were seeded on the substrates at a density of 2×10^4/well and cultured for 7 d. The total RNA was extracted with Trizol (Invitrogen). Total RNA (1 μg) was converted to cDNA using the PrimeScript™ RT reagent kit (TaKaRa). The real-time PCR reactions were performed using SYBR Premix Ex™ Taq II (TaKaRa) on the CFX96™ PCR System (Bio-rad), in order to evaluate the gene expression of the runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), bone morphogenetic protein 2 (BMP), ColI, ILK, integrin β1 (ITGβ1), integrin β3 (ITGβ3) and β-catenin. β-actin was used as a housekeeping gene. The primers are listed in Table 1.

2.8. Protein isolation and western blot analysis

After 48 h of culturing on samples, the total proteins were extracted from the cells by lysed in RIPA buffer (10 mM Tris-HCL, 1 mM EDTA, 1% sodium dodecyl sulfate, 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mM β-glycerophosphate, and 50 mM sodium fluoride) to determine the product of ILK. After WT and ILKsi MG63 plating on samples for 7 d, the cytoplasmic and nuclear proteins were extracted with the Nuclear Extraction Kit (Millipore) to assay the protein amounts of cytoplasmic β-catenin and nuclear β-catenin, and the total proteins were also extracted the same as above to measure the amounts of total β-catenin, GSK3β and p-GSK3β. The protein concentration in the extracted lysates was measured using a BCA protein assay kit (Beyotime). Aliquots of 20–30 μg of the cell lysates per sample were separated by 10% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Bio-Rad). After blocking with 5% bovine serum albumin (BSA, Gibco) for 1 h, the membranes were incubated with primary antibodies overnight. The