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.

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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...
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). Integrinlinked 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 GSCKβ/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 oncopogenesis [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 (Coll) 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 mm², 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 1M 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-S) 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% CO₂ 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 × 10⁴ 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, lipofectamine, 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 × 10⁴ 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 10⁻⁵ 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% w/v 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% cetylpyridinium 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 × 10⁴ cells/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 2 (ALP), Coll, 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 protease 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
expression. After culturing for 3 days, the MNTs up-regulate gene expression. After culturing for 3 days, the MNTs show more elongated with increasing nanotube diameter and spread and (Fig. 2A). On the smooth titanium, the cells exhibit a round, more morphologies are observed on the samples after 24 h incubation (Fig. 1). SEM pictures showing the morphology of the fabricated samples. The morphology of the samples is illustrated in Fig. 1. At a lower magnification, the polished surface appears relatively flat with parallel grooves, whereas R-5 and R-20 show similar micropitted morphology. The higher magnification pictures in the top right inset display the nanotopographies of the samples. The polished surface lacks obvious nanotopography, whereas R-5 and R-20 show an even distribution of nanotubes with diameters of 30 and 100 nm, respectively. The morphology of the samples is illustrated in Fig. 1. At a lower magnification, the polished surface appears relatively flat with parallel grooves, whereas R-5 and R-20 show similar micropitted morphology. The higher magnification pictures in the top right inset display the nanotopographies of the samples. The polished surface lacks obvious nanotopography, whereas R-5 and R-20 show an even distribution of nanotubes with diameters of 30 and 100 nm, respectively.

### 2.9. Statistical analysis

All data were expressed as mean ± standard deviation from at least three independent experiments. They were analyzed by one way ANOVA combined with Student-Newman–Keuls post hoc test, or Student’s t-test using SPSS 17.0 software (SPSS, USA). *p* < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Fabrication of the MNTs

The morphology of the samples is illustrated in Fig. 1. At a lower magnification, the polished surface appears relatively flat with parallel grooves, whereas R-5 and R-20 show similar micropitted morphology. The higher magnification pictures in the top right inset display the nanotopographies of the samples. The polished surface lacks obvious nanotopography, whereas R-5 and R-20 show an even distribution of nanotubes with diameters of 30 and 100 nm, respectively.

#### 3.2. MG63 differentiation on the MNTs

The cell shape, osteogenesis-related gene expression, collagen secretion, and ECM mineralization are monitored to investigate the response of MG63 to the MNTs (Fig. 2). Notably different cell morphologies are observed on the samples after 24 h incubation (Fig. 2A). On the smooth titanium, the cells exhibit a round, more spread and flattened shape, whereas on the MNTs, the cells appear smaller and more elongated with increasing nanotube diameter and show more filopodia extending from the leading edges. The gene expression quantification results in Fig. 2B demonstrate that in general, the MNTs up-regulate gene expression. After culturing for 3 and 7 d, the MNTs significantly increase the Runx2, ALP and BMP expression, and R-20 induces the highest. As shown in Fig. 2C and D, the MNTs, especially R-20, induce significantly denser collagen deposition and more ECM mineralization. Together, the MNTs effectively promote MG63 differentiation and R-20 does even better.

#### 3.3. β-catenin signaling activity

As shown in Fig. 3A, the MNTs significantly induce higher nuclear β-catenin levels and R-20 induces the highest. In addition, a similar trend is observed from the total β-catenin levels (Fig. 3B). With regard to the β-catenin transcription, as shown in Fig. 3D, the β-catenin mRNA expression is significantly up-regulated by MNTs, especially R-20. More importantly, the product of p-GSK3β, a protein leading to inhibition of β-catenin degradation, is also enhanced by the MNTs, especially R-20 (Fig. 3C). All in all, the MNTs activate the β-catenin signaling by simultaneously up-regulating the expression and inhibiting the degradation of β-catenin (Fig. 3E). ILK expression

The ILK mRNA levels on the samples during the first 168 h are monitored by real-time PCR (Fig. 4A). The ILK expression exhibits both time and surface-dependent patterns. The maximum ILK mRNA levels are observed at 12 h after plating. In general, the ILK expression shows a significant increase on R-20 followed by R-5. The ILK protein amount assay after 48 h culturing detected by western blot (Fig. 4B) shows that the R-20 induces the highest ILK protein level, followed by R-5. Hence, the MNTs increase the ILK expression and product and R-20 does even better.

#### 3.5. Role of ILK in MG63 differentiation

We down-regulate the ILK expression by ILKsi in order to check whether ILK is essential regarding the promoting effects of MNTs on MG63 differentiation. The transfection efficiency detected by real-time PCR 48 h after transfection (Fig. 5) shows that ILKsi successfully decreases the ILK mRNA expression by 49%. ILKsi depresses the expression of ALP, BMP, OPN and Coll on the MNTs but not on the smooth control surface (Fig. 6A). For the gene Runx2, down-regulation by ILKsi is only observed on R-20 but not R-5. The OCN expression is not affected by ILKsi. On the smooth control surface, the expression of nearly all the genes except OPN is not apparently changed by ILKsi (Fig. 6A). ILKsi results in obvious decrease in both the collagen secretion and ECM mineralization on the MNTs but it doesn’t affect those on the smooth control surface (Fig. 6B and C). According to the quantitative analysis, ILKsi depresses collagen deposition by about 20% and 25% and decreases ECM mineralization by 45% and 40% on R-5 and R-20, respectively. To summarize, ILK mediates the enhanced MG63 differentiation by the MNTs.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence (5′–3′)</th>
<th>Reverse primer sequence (5′–3′)</th>
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<tr>
<td>Runx2</td>
<td>CACCGGCTGCAAGACAGA</td>
<td>GATCCGGACGTCAAGAAAT</td>
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<td>CCTGACGCGCAGGACCTTCTAG</td>
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<td>OCN</td>
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<td>BMP</td>
<td>CACCTGACGCTGTCTGTAC</td>
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</tr>
<tr>
<td>Coll</td>
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<td>GCCCGGGTTTATAGACAACTTC</td>
</tr>
<tr>
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<td>AAGCTGATGCTGCTCCCCGTAT</td>
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<tr>
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<td>CGTACGGTCGACGTCTGCTAG</td>
</tr>
<tr>
<td>ITGβ3</td>
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<td>CGGAATACGGTCGACCAGTATTT</td>
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<tr>
<td>β-cat</td>
<td>AATAGTGCACGTTGTTTAG</td>
<td>TTTGAAGCAGCTGCTGTA</td>
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<tr>
<td>β-actin</td>
<td>TGGCAACCGAGCAATGAA</td>
<td>CTAAGCTCATAGTCGCGCTAGA</td>
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**Table 1:** Primers used in real-time PCR.
3.6. Relation of ILK with the β-catenin signaling

To determine whether ILK is the upstream of the β-catenin signaling and modulates it in MG63 on the samples, we observe the changes of the β-catenin signaling after ILK silencing (Fig. 7). ILKsi attenuates the enhanced nuclear and total as well as cytoplasmic β-catenin amounts by the MNTs (Fig. 7A, B and C). The up-regulating effect of the MNTs on the β-catenin mRNA expression is also significantly abated by ILKsi (Fig. 7F). Though ILKsi doesn’t affect the GSK3β amounts (Fig. 7E), it significantly attenuates the
enhanced p-GSK3β amounts on the MNTs (Fig. 7D). Therefore, the enhanced β-catenin expression and the inhibited β-catenin degradation by the MNTs are mediated by ILK.

3.7. ITGβ1 and ITGβ3 expression

The mRNA levels of ITGβ1 and ITGβ3 which are the upstream of ILK are monitored during the first 72 h (Fig. 8). On the MNTs, especially R-20, the ITGβ1 mRNA level is greatly increased 12 h after plating. Similarly, a higher level of ITGβ3 is also observed on R-20 at 12 h. Hence, the MNTs enhance the ITGβ1 and ITGβ3 expression.

4. Discussion

The surface micro/nano-texture on biomaterials has been documented to significantly modulate cell and tissue functions and provides an excellent strategy to achieve better tissue integration [2–6]. However, the detailed underlying mechanism is not well understood. In this study, the MNTs are used to study the signal pathway related to the topography. In terms of surface chemistry, the MNTs and the smooth control are all composed of titania, and the main difference is that there are residual fluoride and phosphorus on the MNTs from the electrolyte. Such residues at low concentration level cannot account for the observed biological effects [27], so the observed mechanism in this study can be attributed to the topographical cues. It’s found that the MNTs enhance MG63 differentiation and this is related to the higher ITGβ1 and ITGβ3 expression, enhanced ILK expression, and activation of β-catenin signaling. ILKsi attenuates the activation of β-catenin signaling and the consequent enhancement of MG63 differentiation by the MNTs. Our results demonstrate that the biological effects of topographical cues to cells are mediated by the
expression is found among the samples (data not shown) and it may be related to the short culturing period of this study since OCN is a late marker of osteogenic differentiation only expressed by mature osteoblasts [28]. These results to some extent are consistent with our previous results that MNTs promote primary cultured osteoblast cell differentiation as indicated by the higher ALP activity, collagen secretion, and ECM mineralization [5]. In addition, the MNTs induce MSC osteogenic differentiation in the absence of exogenous osteogenic supplements [4]. The presently available evidence unequivocally demonstrates the osteoblast differentiation promoting ability of the MNTs.

The β-catenin signaling is critical to osteogenesis [7–9]. In this respect, several groups are investigating the role of β-catenin signaling in mediating cell response to biomaterial topography. Concerning the titania nanotubular topography, increasing evidences indicate that the β-catenin signaling is involved in the response of cells to it [12,13]. β-catenin nucleus translation and accumulation constitute the marker for β-catenin signaling activation [29], and so we detect the nuclear β-catenin protein level. Higher nuclear β-catenin protein levels are found on the MNTs, confirming the activation of β-catenin signaling by the MNTs. Increased total β-catenin amounts by the MNTs are also found. Thus, the higher nuclear β-catenin protein levels on the MNTs may be reasonably attributed to the higher total β-catenin amounts. Under silent conditions, the cytoplasmic β-catenin is continuously degraded by the GSK3β/APC/Axin complex, and hence, the cytoplasmic β-catenin concentration is restricted to a base line to keep β-catenin signaling inactivated [7,8]. Theoretically, the increased total β-catenin amounts can be ascribed to two possible reasons: increased β-catenin product and decreased β-catenin degradation. It’s noticed that the mRNA expression of β-catenin is increased by the MNTs, indicating that the elevated total β-catenin on MNTs shall contribute, at least partially, to increased β-catenin products. This finding to a great extent is consistent with Yu et al.’s study which reveals enhanced MG63 β-catenin transcription on the 70 nm titania nanotubular topography [12]. As described above, phosphorylation of GSK3β prevents the GSK3β/APC/Axin complex formation thus preventing β-catenin degradation. The amount of p-GSK3β is higher on the MNTs, indicating that the MNTs also prevent β-catenin degradation. Galli et al. find that the rough titanium surface reduces the expression of Axin 2, a member of GSK3β/APC/Axin complex that phosphorylates and degrades β-catenin, thus possibly inhibiting β-catenin degradation [11]. Galli et al.’s results support ours. The evidence demonstrates that the topographical cues may modulate β-catenin degradation via different routes and the MNTs can activate the β-catenin signaling via the dual effects of enhancing β-catenin transcription and inhibiting β-catenin degradation.

Regarding the upstream events of the β-catenin signaling activation on the MNTs, we pay attention to ILK. There is abundant evidence supporting the correlation between ILK and the β-catenin signaling [17,19,21,30]. As a functional serine/threonine protein kinase, ILK can inhibit the activity of GSK3β through directly phosphorylating it on Ser9 and consequently blocks β-catenin degradation resulting in β-catenin cytoplasmic accumulation and nuclear translocation [18,31]. We propose that ILK may be the upstream of β-catenin signaling in osteoblasts triggered by the topographical cue. It’s found that the ILK transcription exhibits a time and surface-dependent pattern on the titanium samples. The peak activity of ILK is reached within 12 h after cell plating and R-20 induces the highest ILK expression. It has been previously demonstrated that the peak levels of ILK induced by tensile strain emerge after 8 and 12 h in the 3 and 2 dimensional cultures, respectively [32]. This report strongly supports our observation, since the force and the topography influence cell function via the
Fig. 6. Effects of ILK silencing on MG63 functions: (A) Real-time PCR analysis of Runx2, ALP, BMP, OPN, Coll and OCN expression in WT and ILKsi cells cultured on samples after 7 d of incubation. The data is shown as fold change in relation to the WT cells cultured on the smooth sample for each gene; (B) Optical images and colorimetric quantitation of collagen secretion by WT and ILKsi cells cultured in osteogenic medium for 14 d; (C) Optical images and colorimetric quantitation of ECM mineralization by WT and ILKsi cells cultured in osteogenic medium for 14 d. *p < 0.05 compared to each WT counterpart.
Fig. 7. ILKsi depresses the β-catenin mRNA expression and the protein levels of nuclear, total cellular and cytoplasmic β-catenin as well as p-GSK3β on the MNTs. Western blot and semi-quantitative analysis of (A) nuclear β-catenin, (B) total cellular β-catenin, (C) cytoplasmic β-catenin, (D) p-GSK3β and (E) GSK3β in WT and ILKsi cells cultured on samples for 7 d. The expression levels are normalized to either α-tubulin or β-actin. (F) β-catenin mRNA expression in WT and ILKsi cells on samples after 7 d of culturing, expressed as fold change in relation to those of WT cells on the smooth surface. *p < 0.05 compared to each WT counterpart.
same mechanism — mechanotransduction [14,15,33]. Afterward, β-catenin signaling and cell functions are observed after silencing ILK. ILKsi attenuates the enhanced gene expression, collagen secretion, and ECM mineralization by the MNTs, demonstrating that ILK indeed mediates the enhancing effect of the MNTs on MG63 differentiation. ILKsi also attenuates the enhanced nuclear, cytoplasmic, and total cellular β-catenin amounts by the MNTs, thereby confirms our hypothesis that ILK is the upstream of β-catenin signaling in conveying the topographical signals to cells. In addition, ILKsi attenuates the enhanced β-catenin transcript and p-GSK3β amount on the MNTs, suggesting that ILK modulates the β-catenin signaling by enhancing the β-catenin product and preventing its degradation by phosphorylating GSK3β. All in all, our experimental data demonstrate that the topographical cues activate cell β-catenin signaling through ILK.

ILK locates in the FAs by binding to the cytoplasmic domain of the ITGβ1 and ITGβ3 and linking to the actin structures and acts as an important mediator of the integrin related signal transduction [16–19]. The topographical cues can influence the expression and distribution of integrins [34]. We have checked whether the expression of ITGβ1 and ITGβ3, the upstream molecules of ILK, is changed by the MNTs. The expression of ITGβ1 and ITGβ3 is up-regulated by the MNTs and especially, these changes occur within the time frame of the peak expression of ILK. It has been previously reported that the engagement of integrins to the ECM stimulates the kinase activity of ILK rapidly and transiently [19]. The concurrent expression of ITGβ1, ITGβ3 and ILK is in line with and further verifies the close link between integrin β subunits and ILK in the FAs. The quick up-regulation of their expression at 12 h is related to the formation and maturation of the FAs in the early period of cell spreading on the biomaterials.

With the objective of providing a clear understanding of the mechanisms underlying the biological effects of topographical cues, we confirm that the ILK/β-catenin pathway is involved in the effects of the MNTs. More evidences from other topographies are needed to draw a conclusion on the versatility of the ILK/β-catenin pathway.

**Fig. 8.** The mRNA expression of (A) ITGβ1 and (B) ITGβ3 in MG63 cells cultured on the samples during the first 72 h, displayed as fold change in relation to that of the smooth sample at 0.5 h *p < 0.05 compared to the smooth surface.*

**Fig. 9.** Illustration of the detailed ILK/β-catenin pathway in osteoblasts on the topography. The topographical cue up-regulates the ILK expression, which on one side initiates β-catenin expression resulting in more β-catenin product and on the other side promotes the p-GSK3β amount thereby inhibiting the β-catenin degradation. The dual effects of ILK together lead to β-catenin cytoplasmic accumulation and nuclear translocation. By this way, the β-catenin signaling is activated to initiate the osteogenesis-related gene transcription.
pathway in different topographies. It is relatively clear that the topographical cues influence cell functions mainly via FAs [14,15], while there must be many downstream signal pathways mediating the signals from FAs since FAs control many aspects of cell functions. Another one relatively widely recognized downstream pathway of FAs is the focal adhesion kinase (FAK) dependent signal cascade [15]. It is noticed that ILK and FAK can activate some same signal pathways, such as the β-catenin signaling [17,19,21,29,30] and MAPK pathway [15,22], so it can be reasonably deduced that the signals from ILK and FAK may have downstream crosstalk on the points of β-catenin and MAPK but studies are needed to confirm this. Furthermore, ILK can be modulated not only by the cell/ECM interaction, but also by many other factors such as force [32], hypoxia [23], and dexamethasone [25]. Thus, it can be envisaged that ILK may integrate and transduce signals from the topography, force, hypoxia, dexamethasone and possibly growth factors and also experiments are needed to verify this. Whatever, our results greatly advance our understanding on the FA related signal transduction by revealing the ILK/β-catenin pathway and may provide new target points for the surface modification and bio-functionalization of biomaterials to accomplish better clinical performance.

5. Conclusions

The MNTs enhance MG63 differentiation by the ILK/β-catenin pathway and the ILK/β-catenin pathway is described to provide insights to the mechanism underlying the biological effect of topography. It may lead to biomaterials boasting better tissue integration and attract clinical interest concerning modification or bio-functionalization targeting at the ILK/β-catenin pathway. Further studies are needed to confirm whether the ILK/β-catenin pathway is generally involved on all the topographies and has crosstalk with other FA dependent pathways as well as signals from other stimuli such as force and growth factors.

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