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GSK3 inhibitor AR-A014418 promotes osteogenic differentiation of human adipose-derived stem cells via ERK and mTORC2/Akt signaling pathway

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ABSTRACT

Small molecule-based bone tissue engineering is emerging as a promising strategy for bone defects restoration. In this study, we intended to identify the roles and mechanisms of AR-A014418, a highly selective inhibitor of GSK3, on the osteogenic differentiation. We found that AR-A014418 exhibited a dose-dependent effect on osteogenic differentiation of human adipose-derived stem cells (hASCs). hASCs treated with AR-A014418 showed higher activity of ERK and mTORC2/Akt signaling. Administration of ERK inhibitor U0126 or knockdown of RICTOR by siRNA attenuated AR-A014418 induced osteogenic differentiation of hASCs. Our results suggested that AR-A014418 significantly promoted osteogenic potential of hASCs partially by the activation of ERK and mTORC2/Akt signaling pathway, and might be used for bone tissue engineering as an osteo-inductive factor.

1. Introduction

Oral bone defects, resulting from systemic disease, congenital defects, tumor resection, inflammation or trauma, remains a great challenge for clinician. Autologous bone grafting is regarded as the “gold standard” for repairing such bone defects. Nevertheless, disadvantages such as donor site morbidity, protracted surgery and limited available volume, hinder its clinical application [1,2]. By combination of seed cells, bioactive carriers and growth factors, bone tissue engineering (BTE) is emerging as a promising strategy for regenerative medicine. Mesenchymal stem cells (MSCs) are widely applied for BTE due to their self-renewal and multiple differentiation potential [3,4]. Human adipose-derived stem cells (hASCs) is becoming a very promising method for BTE, taking advantage of their properties of abundant source, easy accessibility, and high yield efficiency [5]. Recently, several small molecules have been discovered with osteogenic activity through various signaling mechanisms [6–8]. Moreover, small molecules are non-immunogenic, more stable, easy to be synthetic and cost-effective [9]. Therefore, small molecule seems to be an attractive alternative for growth factor to amplify the stem cells population or direct their osteogenic differentiation.

Glycogen synthase kinase-3 (GSK3) belongs to serine-threonine protein kinase family. It participates in many pathophysiological courses such as cell proliferation, stem cell pluripotency and lineage commitment, embryogenesis and oncogenesis. GSK3 is an essential component of a variety of key signaling pathways such as Wnt/beta-catenin, BMP/Smad, mTOR and NF-κB [10–12]. It has been well documented that the lineage differentiation of MSCs is tightly regulated by the aforementioned signaling pathways. Therefore, small molecules targeting GSK3 may provide new therapeutic strategies for MSCs-based bone regeneration. However, conclusions of previous studies dealing with the effects of GSK3 on bone regeneration are controversial [13,14]. Moreover, mechanisms by which GSK3 regulated bone regeneration mostly related to BMP/Smad and Wnt/beta-catenin signaling in the previous studies [15]. Data is still scarce of the crosstalk between GSK3 and other signaling pathways in bone formation. Taken together, more researches are needed to discover new GSK3 inhibitors with powerful osteo-inductive potential and to unravel the intricate molecular

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mechanisms of GSK3 in MSCs lineage differentiation.

AR-A014418 is reported as a highly selective inhibitor of GSK3, which inhibits GSK3 activity in an ATP-competitive manner and does not inhibit CDK2, CDK5 or 26 other kinases [16]. In this study, we explored the roles of AR-A014418 on osteogenic differentiation of hASCs, and investigated the underlying molecular mechanisms.

2. Methods

2.1. Cell culture and osteogenic differentiation

Primary hASCs were obtained from ScienCell (Carlsbad, CA, USA). The experiments were repeated three times with third passage cells from three independent donors. Cells were cultured in proliferation media (PM) containing DMEM containing 10% fetal bovine serum, 100U/ml penicillin G and 100 mg/mL streptomycin. For osteogenic differentiation, cells were cultured in osteogenic media (OM) containing 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 0.2 mM ascorbic acid (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich).

2.2. Cell proliferation assay

AR-A014418 and U0126 were obtained from Sigma-Aldrich. The cell proliferative ability was detected with the Cell Counting Kit-8 assay kit (Dojindo, Kumamoto, Japan). At each time point, the supernatant of each group was discarded. Then cells were incubated with DMEM media added with CCK-8 reagent for 2 h at 37 °C, according to the manufacture's protocol. The absorbance value was measured at 450 nm using a microplate reader (ELX808, BioTek).

2.3. Alkaline phosphatase (ALP) activity analysis

Cells were cultured in osteogenic media for 7 days in 12-well plates. ALP staining was performed using a BCIP/NBT staining kit (CWBIO, Beijing, China) according to the manufacture's protocol. For ALP activity quantification, cells were washed thrice with PBS and lysed with 1% TritonX-100 (Sigma-Aldrich) for 10 min on ice. The cells were harvested and centrifuged at 12,000 rpm for 30 min at 4 °C. Protein concentration of the collected supernatants was measured using a BCA protein assay kit (Pierce Thermo Scientific, MA, USA). The ALP quantification assay was detected using an ALP activity kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Relative ALP activity was compared with that in the controls and normalized by the total protein concentration.

2.4. Alizarin Red S staining and quantification

After cultured with OM for 14 days, cells were fixed with 4% paraformaldehyde, and rinsed three times with distilled water. Then cells were incubated with 2% Alizarin Red S staining solution (pH 4.2, Sigma-Aldrich). For quantification of mineralization, the staining was incubated in 100 mM cetylpyridinium chloride (Sigma-Aldrich) for 1 h. The optical density value was measured at 562 nm and normalized by the total protein concentration detected in a duplicate plate.

2.5. RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted with a PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). Quantitative real-time PCR assays were conducted using SYBR Green PCR Master Mix as described on an ABI 7500 real-time PCR system (Applied Biosystems, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal control for normalization. The primers used are listed in Table 1.

2.6. Western blotting

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Roche). Lysates were centrifuged at 12000 rpm at 4 °C for 30 min. Protein concentrations of the supernatants were measured using a BCA protein assay kit (Thermo Scientific). Aliquots (30 μg) of each sample were subjected to 10% SDS PAGE and then transferred to polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 5% skim milk for 1 h to avoid non-specific reactivity. Then the blots were incubated with indicated primary antibodies overnight at 4 °C. Then the blots were incubated with peroxidase-linked secondary antibodies at room temperature for 1 h. The membranes were detected using an ECL kit (CWBIO) to visualize the bound antibodies. The antibodies used were anti-β-Catenin, anti-GAPDH; anti-phospho-GSK3α/β (Ser21/9), anti-GSK3α/β (Ser21/9), anti-phospho-ERK1/2 (Thr202), anti-ERK1/2, anti-RICTOR, anti-p-Akt (Ser473), anti-Akt (Cell Signaling, Danvers, MA, USA). GAPDH was used as internal control.

2.7. Bone formation in vivo

Cells were cultured to 100% confluence *in vitro*, resuspended and incubated with β-TCP (Bicon, Boston, MA, USA). The nude mice was transplanted with two groups of cells on the dorsal subcutaneous space: hASC/Dimethyl Sulphoxide (DMSO), hASC/AR-A014418 (10 μM) in two sites. Transplants were collected at 4 weeks, fixed in 4% paraformaldehyde and then decalcified in 10% EDTA (PH 7.4) for 10 days. Following dehydration, the samples were embedded in paraffin. Sections (5 μm) were used for the histological staining, including haematoxylin and eosin (H&E), Masson's trichrome stain, and immunohistochemical (IHC) staining for Osteocalcin (OCN) (Abcam). All animal experiments in this study were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch.

2.8. Small interfering RNA

siRNAs targeting *RICTOR* and negative control siRNA (NC-FAM) were obtained from Genepharma. siRNA sequences were: si*RICTOR*-1: AAGCAGCCTTGAACTGTTTA-A; si*RICTOR*-2: AAACTTGTGAA-GAATCGTATC. Cells at 70%–80% confluence were transfected with 5 nM of siRNA using Lipofectamine RNAi-MAX (Invitrogen) and cultured for the indicated days.

2.9. Statistical analysis

Statistical calculation were conducted using the GraphPad Prism software (San Diego, CA, USA). All values were shown as the

Table 1
Sequences primers of selected genes designed for real-time PCR.

Genes	Forward primer	Reverse primer
GAPDH	TGTTCGACAGTCAGCCGCAT	CGCCCAATACGACCAAATCCGT
RUNX2	ACCACAAGTGCGGTGCAAAC	ACTGCTTGCAGCCTTAAATGACTCT
ALP	TAAGGACATCGCCTACCAGCTC	TCTTCCAGGTGTCAACGAGGT
OCN	CACCATGAGAGCCCTCACACTC	CCTGCTTGGACACAAAGGCTGC
BSP	TGCCAGAGGAAGCAATCACCA	GTGGCCTGTACTTAAAGACCCCA
CCND1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
MYC	ATTTGGGGACACTTCCCCGC	GGGAGGCTGCTGGTTTTCCA

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