

## Original Article

# Effects of IRAK-4 gene silencing by siRNA on the mitogen-activated protein kinase signaling pathway in osteoblast-like cells

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**Abstract:** Background: The molecular mechanism for osteolysis around prostheses remains unclear. Artificial joint loosening is typified by osteolytic resorption. Interleukin-1 affects bone resorption via the MAPK signaling pathway. The aim of this study was to observe the effects of siRNA-silenced expression of interleukin-1 receptor-associated kinase-4 (IRAK-4) gene on the mitogen-activated protein kinase (MAPK) signaling transduction pathway in human osteoblast-like cells MG63, and to provide experimental evidence for the prevention and treatment of osteolysis around prostheses after artificial joint replacement. Methods: IRAK-4-siRNA was transfected into MG63 cells by using Lipofectamine 2000 as the vector. The cells were divided into a blank group, a control group and a silencing group, which were not transfected, transfected with scrambled siRNA and with specific IRAK-4-siRNA respectively. The protein expressions of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (c-JNK) and p38MAPK in target cells were detected by Western blot. Results: Compared with the control group, the IRAK-4 mRNA and protein expression levels of the silencing group were significantly lower ( $P < 0.05$ ). After IRAK-4 expression was down-regulated, the expressions of c-JNK1/2 p46, ERK1/2 and p-p38 MAPK were also down-regulated by 62%, 64% and 68% respectively ( $P < 0.05$ ). Conclusion: Silencing IRAK-4 gene expression with siRNA inhibited the expressions of ERK, c-JNK and p38 MAPK in human osteoblast-like cells MG63.

**Keywords:** Osteoblast-like cell, interleukin 1, gene silencing, mitogen-activated protein kinase

## Introduction

Aseptic loosening, which is mainly responsible for the long-term failure of artificial joint replacement [1], is typified by abnormally active osteolysis around prostheses. Prevention and treatment of pathological osteolysis through specific siRNA-inhibited target gene expressions have attracted global attention [2, 3].

Interleukin-1 (IL-1), as one of the important osteolysis-related inflammatory cytokines [4], is highly expressed around prosthetic implants due to the inducing effects of artificial joint-related materials (including poly (methyl methacrylate) and titanium particles) [5]. Transduction of extracellular signals on the cell surface into the cell nucleus shares the mitogen-activated protein kinase (MAPK) pathway [6].

Interleukin-1 receptor-associated kinase-4 (IRAK-4) is a key protein in the intracellular signaling pathway for IL-1 receptor, but whether it affects osteoblasts by changing the MAPK signaling pathway to participate in osteolysis in the inflammatory environment around prostheses remains elusive. MG63 cells have been widely used to study metabolic bone diseases due to multiple characteristics of osteoblasts [7, 8].

Based on a previous study concerning the effects of IL-1 on bone resorption via the MAPK signaling pathway [9, 10], we herein designed and synthesized siRNA to specifically silence IRAK-4 gene expression, aiming to evaluate the influence on this pathway in osteoblast-like cells, to clarify the molecular mechanism for osteolysis around prostheses, and to provide

## IRAK-4 gene silencing

**Table 1.** Primer sequences

Primer	Sequence (5'-3')
IRAK-4 Upstream	TGCAGCTAATGGCATCAATTT
IRAK-4 downstream	GTCATGACTGTCTGGGCAAA
GAPDH Upstream	GTGGACCTGACCTGCCGTCT
GAPDH Downstream	GGAGGAGTGGGTGTCGCTGT

experimental evidence for the prevention and treatment of it after artificial joint replacement.

### Materials and methods

#### Materials

Human osteoblast-like cells MG63 were purchased from China Center for Type Culture Collection. Fetal bovine serum and DMEM were bought from Hyclone (USA). BCA protein quantitative kit was obtained from Sigma (USA). Trizol kit and Lipofectamine 2000 were purchased from Life Technologies (USA). Reverse transcription kit was obtained from TaKaRa (Japan). SYBR-qPCR kit was bought from Toyobo (Japan). Primary antibodies against IRAK-4, p-p38 MAPK, p-c-Jun N-terminal kinase (c-JNK) 1/2 and GAPDH were purchased from Santa-Cruz (USA). Goat anti-mouse secondary antibody was obtained from Abcam (USA).

#### Design and synthesis of siRNA sequences, cell grouping and transfection

The most effective interfering target was screened from human chondrocytes after BLAST analysis that excluded other homologous coding sequences. siRNA sequences were synthesized and purified by Shanghai Biotend Biotechnologies Co., Ltd. The content of full-length double-stranded siRNA was higher than 97%. Sense strand: 5'-GCUCAUGACAUGCAAGA-UUTT-3', anti-sense strand: 5'-AAUCUUGCAUGUCAUGAGCTT-3'. The cells were divided into a blank group, a control group and a silencing group, which were not transfected, transfected with scrambled siRNA and with specific IRAK-4-siRNA (final concentration: 75 nmol/L) respectively. Then the cells were used for further experiment on the same day.

MG63 cells were digested and counted 24 h before transfection, cultured in high-sugar DMEM containing 10% fetal bovine serum overnight, and then in serum-free, antibiotics-free DMEM/F12. Afterwards, the blank group was incubated in a mixture of Lipofectamine 2000

(6  $\mu$ L) and Opti-MEM (300  $\mu$ L) at room temperature for 5 min. The control group was incubated in a mixture of IRAK-4-siRNA, scrambled siRNA (final concentration: 75 nmol/L) and Opti-MEM (300  $\mu$ L) at room temperature for 5 min. Lipofectamine 2000 was mixed with siRNA at room temperature for 20 min, forming a Lipofectamine-siRNA complex. The silencing group was incubated with the Lipofectamine-siRNA mixture (600  $\mu$ L) in 5% CO<sub>2</sub> at 37°C for 6 h. After the mixture was discarded, 3 mL of high-sugar DMEM containing 10% fetal bovine serum was added. Complete culture medium was discarded 48 h after interference. RNA was extracted for RT-PCR, and total protein was collected for Western blot.

#### RNA extraction and real-time PCR

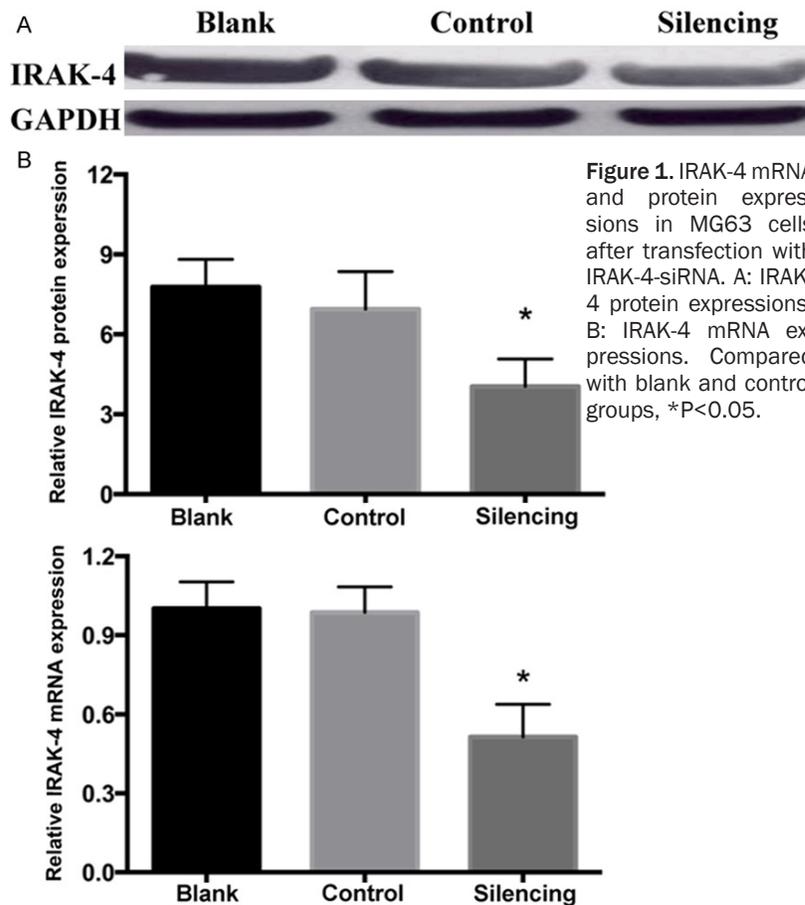
Total RNA was extracted 48 h after transfection to detect the silencing of target gene by real-time PCR. Primers were designed with Primer Premier 5.0 (PREMIER Biosoft international, Palo Alto, CA, USA) (Table 1), synthesized and sequenced by Beijing Genomics Institute.

Total RNA was extracted according to the instruction of Trizol reagent. cDNA was synthesized according to the instruction of reverse transcription kit, which was used the template to amplify the target gene, with GAPDH as the internal standard template. Reaction conditions: Pre-denaturation at 95°C for 60 s, denaturation at 95°C for 15 s, annealing and extension at 60°C for 60 s, 40 cycles in total. Absorbance was recorded during each extension. After PCR, DNA double strands were allowed to DNA duplex to fully integrate by denaturation at 95°C for 15 s and cooling down to 60°C. Then absorbance was recorded 15 s at 95°C and 15 s at 60°C. The melting curve was plotted, and relative gene expression was expressed as  $2^{-\Delta\Delta Ct}$ .

#### Detection of expressions of IRAK-4 and proteins in the MAPK pathway by Western blot

The culture medium was discarded 48 h after transfection, and the cells were treated with RIPA lysate for 10-15 min according to the kit's instructions. After lysis, the cell debris and lysate were transferred into 1.5 ml EP tubes, and the cells were treated with ultrasonic cell disrupter (200 W, four times, 5 s each time, 2 s of interval), and centrifuged at 4°C and 12000 r/min for 10 min. The supernatant was transferred into 0.5 ml EP tubes. Standard curve for

## IRAK-4 gene silencing



protein concentration was plotted by using BCA protein quantitative kit on a UV-vis spectrophotometer to calibrate the concentration of sample protein. Protein (30  $\mu$ g) was denatured at 100°C, separated by 10% SDS-PAGE, electronically transferred at 4°C and 110 V for 150 min to a PVDF membrane, blocked in TBST+5% skimmed milk at room temperature for 1 h. Primary antibodies against IRAK-4, p-JNK, p-extracellular signal-regulated kinase (ERK), p-p38 MAPK and GAPDH were diluted with the blocking solution, with which the membrane was incubated by shaking at 4°C overnight. Subsequently, the membrane was washed three times with TBST, 10 min each time. Then it was incubated with corresponding diluted horseradish peroxidase-labeled goat anti-rabbit secondary antibody at room temperature for 2 h, and washed three times with TBST, 10 min each time. Chemiluminescence reaction was conducted with ECL solution to expose and develop the membrane that was scanned into image files. Gray value was directly recorded with GlycoBand Scan 5.0 (ProZyme, Hayward,

CA, USA), with GAPDH as the internal reference.

### Statistical analysis

All data were expressed as  $\bar{x} \pm s$ , analyzed with SPSS 18.0 (SPSS, Chicago, IL, USA), and plotted with GraphPad Prism 5.01. Inter-group comparisons were performed by using Tukey's test based on one-way analysis of variance. Dunnett's T3 test was used in the case of variance heterogeneity. P<0.05 was considered statistically significant.

### Results

#### *IRAK-4 mRNA and protein expressions in MG63 cells after transfection with IRAK-4-siRNA*

After transfection, the IRAK-4 mRNA and protein expressions in the silencing group were significantly lower than those of blank and control groups (P<0.05). IRAK-4-siRNA effectively down-regulated IRAK-4 mRNA and protein expressions in MG63 cells (Figure 1).

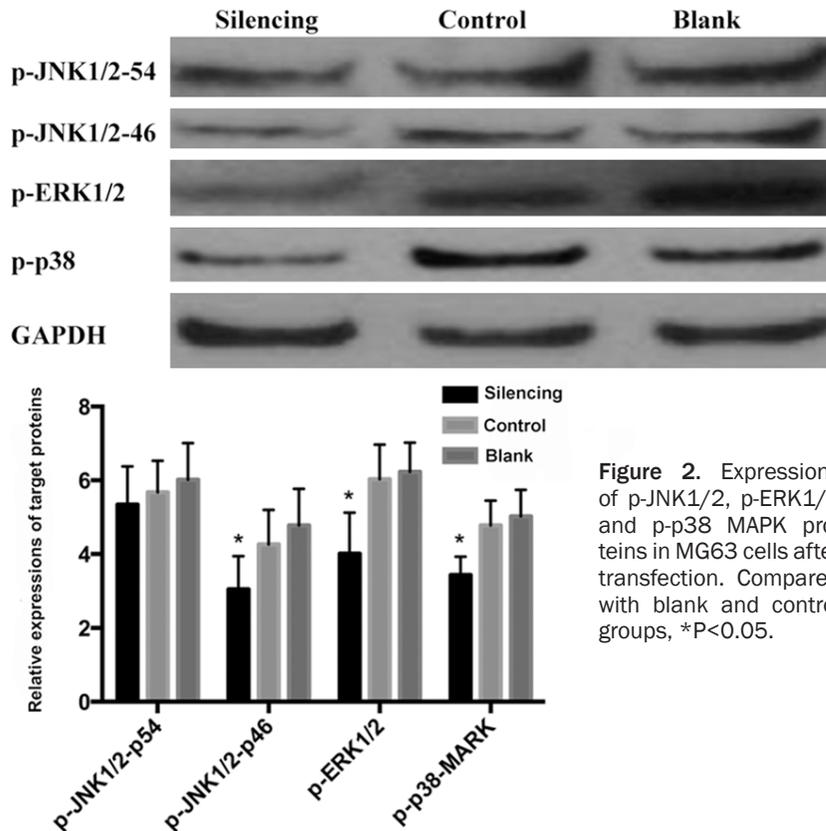
#### *Effects of transfection with IRAK-4-siRNA on the MAPK signaling pathway*

After transfection, the expressions of p-JNK1/2, p-ERK1/2 and p-p38 MAPK in the silencing group were down-regulated compared with those of blank and control groups. Particularly, the expressions of p-JNK1/2 p46 (including JNK1 $\alpha$ 1, JNK1 $\beta$ 1, JNK2 $\alpha$ 1 and JNK2 $\beta$ 1), p-ERK1/2 and p-p38 MAPK were down-regulated by 62%, 64% and 68% respectively (P<0.05). The expression levels of p-JNK1/2 p54 (including JNK1 $\alpha$ 2, JNK1 $\beta$ 2, JNK2 $\alpha$ 2 and JNK2 $\beta$ 2) were similar in the three groups (Figure 2).

### Discussion

Currently, target genes have been widely silenced by siRNAs to exert therapeutic effects

## IRAK-4 gene silencing



**Figure 2.** Expressions of p-JNK1/2, p-ERK1/2 and p-p38 MAPK proteins in MG63 cells after transfection. Compared with blank and control groups, \*P<0.05.

functions are dominantly controlled by downstream cascades of polypeptide factors and receptors. It has previously been reported that IL-1 is highly expressed around prosthetic material particles [5]. Proteins in the IL-1 receptor pathway all play crucial roles in the resistance to infections and autoimmune diseases [14]. Thus, studying the signaling pathway of IL-1 in osteoblasts is beneficial to clarifying the mechanism for osteolysis around prostheses.

### *Relationship between IL-1 and MAPK intracellular signaling pathway*

[11]. In this study, we constructed a siRNA to effectively silence the expression of IRAK-4 gene and to transfect human osteoblast-like cells, aiming to verify the down-regulatory effects of such inhibited expression on the expressions of proteins in the MAPK pathway.

### *Relationship between the IL-1 intracellular signaling pathway and osteolysis around prostheses*

Aseptic loosening, which is characterized by chronic inflammatory response induced by prosthesis wear particles, leads to bone resorption (osteolysis) around the prostheses and limits them from being fixed [12]. Prosthesis failure has now mainly been attributed to aseptic loosening [1]. Since osteoblasts tend to express osteolysis-related cytokines and hormone surface receptor [13], they provide a signaling pathway for the activation of osteoclasts. In other words, osteoblasts not only participate in bone formation, but also regulate bone resorption during the metabolic process of normal bone tissues. The activity of osteoblasts is regulated by signals originating from direct intercellular contact, and their differentiation and

Transduction of extracellular signals on the cell surface into the cell nucleus shares the MAPK pathway [6] among which ERK, c-JNK and p38 MAPK pathways have been most frequently accessed [15]. Besides nuclear transcription factor  $\kappa$ B, IL-1 is also activated by p38 and JNK pathways, further promoting the production of pro-inflammatory cytokines and chemical substances [16].

### *Silencing IRAK-4 expression inhibited the proliferation of osteoblasts*

Although being a key protein in the IL-1 receptor signaling pathway [17], IRAK-4 has seldom been related with the proliferation and activity of osteoblasts upon expression changes. Magdalena et al. [18] found KRNXNOD (K/BxN) serum-induced inflammatory osteolysis was mitigated in the joints of mice with IRAK-4 gene knocked down. After IRAK-4 gene was silenced, the proliferation of osteoblasts is inhibited [19]. Moreover, IL-1 can facilitate the proliferation of osteoblasts [20]. IRAK-4 is a key adaptor protein in the IL-1 receptor signaling pathway, so its gene expression blocks IL-1 intracellular signal transduction, which may be one of the rea-

sons responsible for inhibited secondary proliferation of osteoblasts and then participate in osteolysis. Based on these, we herein explored the relationship between inhibited proliferation of osteoblasts owing to silencing of IRAK-4 expression and intracellular signaling pathway.

### *siRNA interfered with IRAK-4 and inhibited activation of proteins in the MAPK signaling pathway*

After specific IRAK-4-siRNA transfection, IRAK-4 mRNA and protein expressions were significantly down-regulated in the silencing group compared with those of the control group, suggesting that specific siRNA-mediated RNA interference effectively silenced the expression of target gene. Meanwhile, the expressions of p-JNK1/2, p-ERK1/2 and p-p38 MAPK were also down-regulated, indicating that IRAK-4, as a key molecule in the IL-1 intracellular signaling pathway, affects common downstream MAPK pathways.

As mentioned above, the IL-1 intracellular signaling pathway is activated by p38 and JNK pathways, further facilitating the production of pro-inflammatory cytokines and chemical substances [10]. However, silencing IRAK-4 expression in this study affected all MAPK signaling pathways including the ERK pathway, suggesting that changes in intracellular IRAK-4 expression may affect downstream cytokines via other unknown pathways. Regardless, whether activation of IRAK-4 activated ERK, JNK and p38 MAPK in osteoblast-like cells directly or via other intermediate pathways remains unclear. ERK, p38 MAPK and JNK pathways all participate in the signaling transductions for differentiation and proliferation of osteoblasts, and play critical roles in stress, apoptosis, bone metabolism and inflammation [21, 22]. These findings suggested that inhibited proliferation of osteoblast-like cells after silencing of IRAK-4 gene may be associated with down-regulation of ERK, JNK and p38 MAPK pathways. Probably, the inflammatory environment around prostheses affected the proliferation of osteoblast-like cells and altered local bone reconstruction through IRAK-4 via the MAPK pathway. Nevertheless, detailed molecular mechanisms for the induction of each MAPK pathway as well as the interactions between these pathways still need in-depth studies.

In summary, the expressions of p-JNK1/2, p-ERK1/2 and p-p38 MAPK were down-regulated in MG63 cells after siRNA transfection that silenced IRAK-4 expression. Changes in IRAK-4 gene expression may suppress the proliferation of osteoblasts via the MAPK pathway and further participate in the inflammatory osteolysis around prostheses. Given that the role of IRAK-4 in inflammatory osteolysis remains unknown, this study provided evidence for unraveling the relationship between IRAK-4 and osteoblast-like cells and exploring the inflammatory environment around prostheses. Hence, it is necessary to study such environment before clarifying the influence of IRAK-4 on inflammatory osteolysis.

### **Disclosure of conflict of interest**

None.

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