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Original Article

TWIST1 promotes cell viability and migration, but inhibits apoptosis in MC3T3 cells via regulating PI3K and p16 pathways

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Abstract: Background: Osteoporosis, a disease of the skeleton, results in an increased risk of fracture. Its characters are the loss of bone mass and degeneration of bone microstructure. This study was aimed to demonstrate an essential function of Twist-related protein 1 (TWIST1)-on osteoporosis. Methods: The expression of TWIST1 was overexpressed or silenced by specific transfection in MC3T3 cells and then were confirmed by real-time polymerase chain reaction (RT-PCR) and Western blot. The cell viability and cell migration were determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) and Transwell cell migration assay, respectively. Furthermore, cell apoptosis was analyzed by flow cytometry, and the protein expression of phosphatidyl inositide 3-kinases (PI3K) or p16 pathways related proteins was measured. Results: Here, this study showed that MC3T3 cells viability and migration were significantly increased by TWIST1 overexpression compared to the control group ($P<0.05$), but the cell apoptosis was statistically decreased ($P<0.05$). However, while TWIST1 silencing, the cells viability and migration were significantly appeared to be decreased, accompanied by apoptosis increasing ($P<0.05$). Furthermore, the protein expression of PI3K and protein kinase B (p-Akt) were significantly increased ($P<0.05$), but p16 and phosphorylate retinoblastoma protein (p-RB) were significantly decreased ($P<0.05$). Conclusions: These results highlight the importance of TWIST1 to the target of treating osteoporosis. TWIST1 could promote cell viability and migration, but inhibit apoptosis in MC3T3 cells via regulating PI3K and p16 pathways.

Keywords: Osteoporosis, twist-related protein1, cell viability and migration, apoptosis, phosphatidyl inositide 3-kinases, P16

Introduction

New bone formation may be desirable in clinical settings, such as in osteoblast, osteoporosis, and skeletal deformities [1]. Drugs that promote bone formation are thus in demand [2]. The further study of mechanism of osteoblast proliferation and other biological behaviors can help understanding the pathogenesis of osteoporosis, which is essential to provide theoretical basis for research and development of treatment for osteoporosis [3].

Twist-related protein1 (TWIST1) is one member of the Basic helix-loop-helix- (bHLH) transcription factor family, which is associated with the development of the skeleton [4]. TWIST1 plays an important role in cellular determination, and is related to the proliferation and differentiation of several lineages containing myogenesis [5], osteogenesis [6], neurogenesis [7] and cardiogenesis [8]. Mouse TWIST1 has been demonstrated to be a negative regulator of mouse embryonic calvarium gene activation [9, 10]. Based on spatial and temporal genetic deletions of TWIST1 in the developing of mouse head, TWIST1 was related to a variety of functions during craniofacial development. Related to the migration of MC3T3 cells derived from mouse embryonic calvarium, TWIST1 is directed into the first branchial arch and proliferation of first arch derivatives [11-13]. In addition, it has been reported that TWIST1 expression is increased in the early stages of mouse osteoblast cell lines, and decreased in the later stages of osteoblast [14]. Moreover, TWIST1 negatively regulates osteoblast differentiation [8, 15, 16].

However, the functional role of TWIST1 in osteoblast MC3T3 cells viability, migration, and apoptosis is not clear. Therefore, we report that TWIST1 gene can promote MC3T3 cells viability and migration, and inhibit apoptosis, which is via the phosphatidyl inositide 3-kinases (PI3K) and p16 pathways.

Material and methods

Cell culture

The MC3T3 cell line was purchased from the American Type Culture Collection (ATCC, California, USA) and was maintained in α-modified Eagle's Minimum Essential Medium (EMEM, Gibco, Life technologies, USA) complete medium supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA) [17]. The MC3T3 cells were cultured in 96 well-plates (Corning, USA) at initial seeding cell densities of 1×10^6 cells/ml under controlled condition of temperature (37°C) a humidified atmosphere of 5% CO_2. Medium was replaced with fresh medium every 2-3 days.

Lentivirus vectors construction

Lentivirus-encoded small hairpin RNAs (shRNAs), retroviral vector (pBABE-puro), and pBABE-puro-TWIST1 were purchased from Invitrogen (California, USA). 5'-GCCAGGTACATCGACTTCCTCT-3' and 5'-TCCATCCTCCAGACCGAGAAGG-3' are the two sense strand sequences for the TWIST1 shRNAs. The transfection was performed by using Lipofectamine 2000 (Invitrogen, USA). In a small volume of serum-free Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life technology, USA), cells were cultured with lentivirus for 4 h at 37°C. Then, adding 10% DMEM, the cells were continued to place in an incubator for 24 h for the following experiments.

Cell viability

Cell viability was determined using MTT colorimetric methods. Logarithmic growth phase cells were collected, and suspended with culture medium. Cells were seeded in a 96 well plate at a density of 2.5×10^5 cells/ml, and 100 μl cell suspension was added to each well in 96 well plates, then cultured in condition of temperature (37°C) a humidified atmosphere of 5% CO_2 for various days. For colorimetric assay, 10 μl MTT was added to each well at different time points (1 d, 2 d, 3 d, and 4 d), incubated in 37°C 5% CO_2 for 4 h, and detected at 492 nm by microplate reader (Thermo, USA). Three independent experiments were repeated.

Cell migration

Using a modified two-chamber migration assay with a pore size of 8 mm, cell migration was performed [18]. For migration assay, MC3T3 cells were seeded on the upper compartment of 24-well Transwell culture chamber suspending in 200 ml serum-free medium, and 600 ml complete medium was added to the lower compartment. After incubating for 12 h at 37°C, cells were fixed with methanol. Under the filter with a cotton swab, non-traversed cells were carefully intercepted on the upper surface of the filter, while traversed cells were on the lower side of the filter which were stained with crystal violet, and then were counted.

Cell apoptosis

To identify and quantify the apoptotic cells, flow cytometry analysis was performed by using Annexin V-FITC/prodium iodide (PI) apoptosis detection kit (Shanghai Kaifang Biotechnology, Shanghai, China). The MC3T3 cells (100,000 cells/well) were seeded in 6 well-plates. Treated cells were washed twice with precool phosphate buffered saline (PBS) and resuspended in buffer. According to the manufacturer's instruction, the adherent and floating cells were combined and treated, and then measured by flow cytometer (Beckman Coulter, USA).

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from transfected cells by using TRIzol reagent (Invitrogen, USA) and treated with DNaseI (Promega, USA). Using the Multiscribe RT kit (Applied Biosystems) and random hexamers or oligo (dT), reverse transcription was performed. The reverse transcription was performed in condition of 10 min at 25°C, followed by 30 min at 48°C, and 5 min at 95°C. The sequences of the primers were as follows, TWIST1 forward primer: 5'TCCGCAGTCTTACGAGGAGC'3, reverse primer: 5'GCTTGAGGGTCTGAATCTTGCT'3. GAPDH forward primer: 5'GCACCGTCAAGGCTGAGAAC'3, reverse primer: 5'TGGTGAAGACGCCAGTGGA'3.

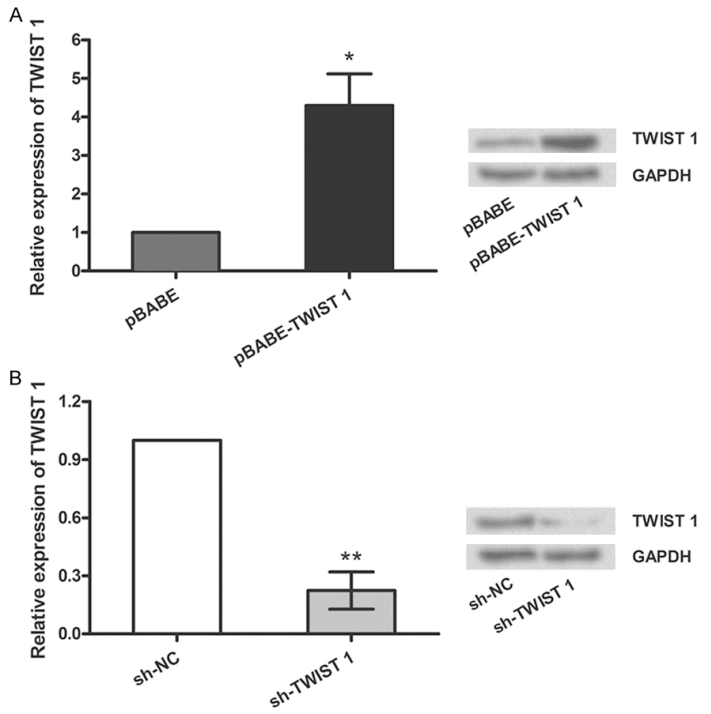

Figure 1. The expression level of TWIST1 overexpression or silencing. The MC3T3 cells were cultured with lentivirus or the corresponding negative control, and then the expression level of TWIST1 was analyzed by RT-PCR and Western blot. A: TWIST1 was overexpressed by pBABE-TWIST1 ($P<0.05$). B: The expression level of TWIST1 was silenced by shRNA ($P<0.01$). MC3T3, mouse calvaria-derived cells; TWIST1, Twist-related protein 1; shRNA, small hairpin RNA; RT-PCR, real-time polymerase chain reaction. *$P<0.05$ or **$P<0.01$ compared to the control.

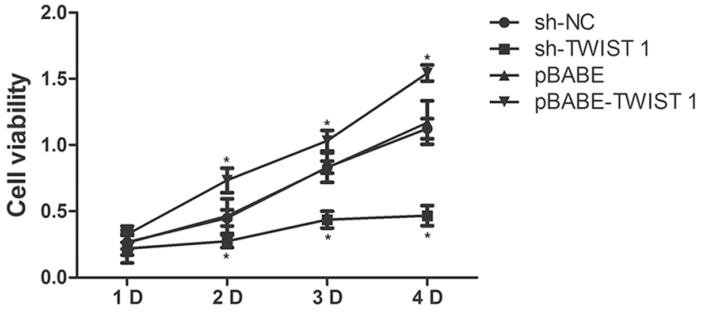

Figure 2. The effects of TWIST1 on cell viability. MC3T3 cells viability was assessed by MTT after transfection. The TWIST1-retroviral vector (pBABE-TWIST1) overexpression significantly increased cells viability, while shTWIST1 silencing decreased cells proliferation. MC3T3, mouse calvaria-derived cells; MTT, 3-(4,5-dimethly-2-thiazolyl)-2,5-di phenyl-2-H-tetrazolium bromide; shTWST1, small hairpin TWIST1. *$P<0.05$ compared to the control.

Western blot

Total protein was isolated from MC3T3 cells after transfection by using RIA lysis buffer (Sangon Biotech, Shanghai, China) supplemented with protease inhibitors (Sangon Biotech, Shanghai, China). The protein concentration was determined by using the BCA™ Protein Assay Kit (Sigma, USA). According to the manufacturer's instructions, a 10-12% sodium dodecyl sulfate (SDS)-polyacryla-mide gel electrophoresis (PAGE) was established to use as the western blot system. Rabbit-anti-mouse TWIST1 antibody was purchased from Univ-bio (Shanghai, China); GAPDH, p-Akt, PI3K, and Retinoblastoma protein (p-RB) antibody was all purchased from Sigma (Sigma, USA). Preparing the primary antibodies was used with 5% blocking buffer at a dilution of 1:1,000. The first perform was incubated with the primary antibody at 4°C overnight, followed by wash buffer, and then bonding with secondary antibody which was marked by horseradish peroxidase (HRP, Takara, Dalian, China) for 1 h at room temperature. After wash, PVDF were transferred into the Bio-Rad ChemiDoc™ XRS system. After capturing the signals, the quantified intensity of the bands was analyzed by using the Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

Three independent experiments were repeated. The values are presented as the mean of ± standard deviation (SD). SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses. By using a one-way analysis of variance (ANOVA), the *P*-values were calculated. A *P*-value of <0.05 was considered statistically significant.

Results

TWIST1 promotes MC3T3 cells viability

As shown in **Figure 1A**, the results suggested that the expression level of TWIST1 in MC3T3

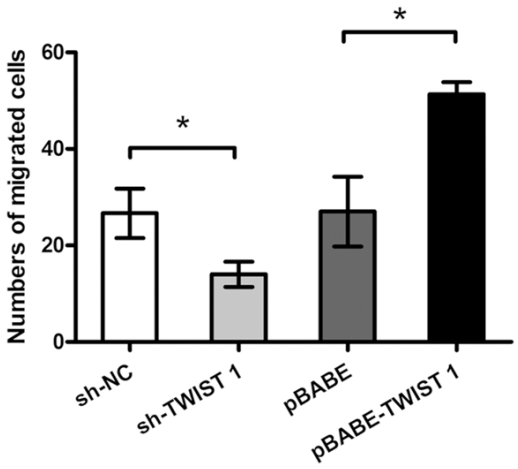

Figure 3. MC3T3 cells migration assay under TWIST1 overexpression or silencing. pBABE-TWIST1 overexpression significantly increased numbers of migrated cells (P<0.05). Compared to sh-TWIST1 silencing, numbers of migrated cells were reduced (P<0.05). MC3T3, the mouse calvaria-derived cells; TWIST1, Twist-related protein1; shTWIST1, small hairpin-TWIST1. *P<0.05 compared to the control.

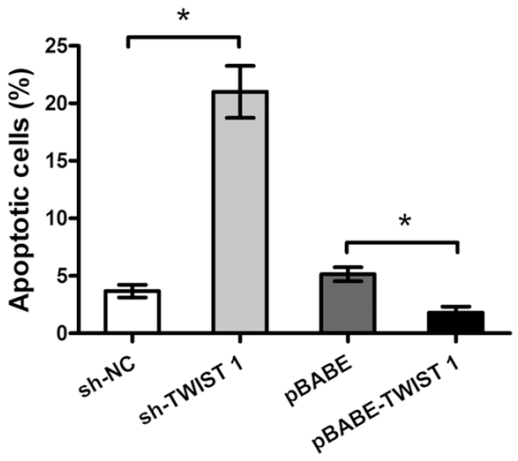

Figure 4. Apoptotic cells assay via TWIST1 overexpression or silencing. The TWIST1-retrovial vector (pBABE-TWIST1) overexpression decreased apoptotic cells. However, for the sh-TWIST1 silencing, the apoptotic cells was above the average (P<0.05). TWIST1, Twist-related protein1; shTWIST1, small hairpin-TWIST1. *P<0.05 compared to the control.

cells was significantly increased by TWIST1 overexpression compared to the control group (P<0.05), but was statistically decreased by TWIST1 silencing (P<0.01, **Figure 1B**). To examine the effects of MC3T3 on cell viability under TWIST1 overexpression or silence, MTT was performed at different time points (1 d, 2 d, 3 d and 4 d) after transfection in MC3T3 cells. Results showed that TWIST1 overexpression could significantly promote the viability of MC3T3 cells, but TWIST1 silence could suppress the MC3T3 cells viability (P<0.05, **Figure 2**).

TWIST1 promotes MC3T3 cells migration

To investigate the effects of TWIST1 expression on MC3T3 cell migration, Transwell experiments were performed. **Figure 3** showed that the MC3T3 cells migration was effectively increased by TWIST1 overexpression compared to the control group (P<0.05). While making TWIST1 silencing, the capacity of MC3T3 cells migration was significantly decreased (P<0.05).

TWIST1 inhibits MC3T3 cells apoptosis

To explore the effects of TWIST1 expression on MC3T3 cell apoptosis, flow cytometry were performed. As shown in **Figure 4**, the results showed that the overexpression of TWIST1 statistically decreased the cell apoptosis (P<0.05). Without TWIST1 expression, the proportion of cell apoptosis was significantly increased (P<0.05).

TWIST1 regulates the protein expressions in PI3K and p16 pathways

PI3K and p16 are important activators for cell apoptosis. To investigate the influence of TWIST1 expression on the protein expression PI3K and p16 pathways associated proteins, we carried out Western blot. **Figure 5** showed that the protein expression level of PI3K and p-Akt was significantly increased (P<0.05), followed by p16 and p-RB expression level statistically decreased (P<0.05). However, while the TWIST1 silencing, PI3K and p-Akt expression level was significantly decreased (P<0.05), with p16 and p-RB significantly increased (P<0.05). The results demonstrated that TWIST1 could activate PI3K pathway, but suppress p16 signal pathway.

Discussion

In this study, under TWIST1 overexpression, the MC3T3 cell viability and migration was increased, but apoptotic cells was statistically decreased. While the TWIST1 silencing, the MC3T3 cell viability and migration were signifi-

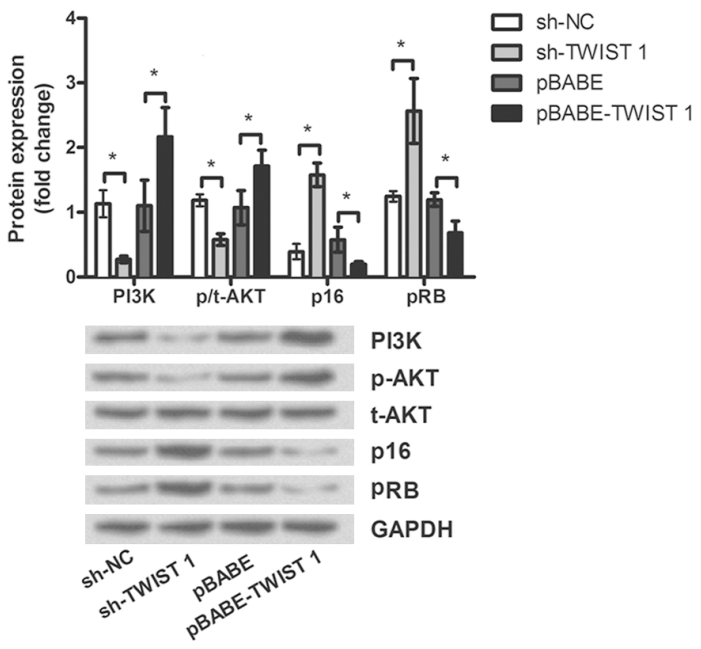

Figure 5. The protein expression of PI3K and p16 pathways. The pBABE-TWIST1 overexpression increased PI3K protein expression accompanying with decreased p16 protein expression ($P<0.05$). In the sh-TWIST1 silencing, PI3K protein expression decreased combining with p16 protein expression rising ($P<0.05$), GAPDH was the internal reference protein. PI3K, phosphatidyl inositide 3-kinases; shTWIST1, small hairpin-TWIST1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; t-Akt, the total protein kinase B. *$P<0.05$ compared to the internal reference.

cantly decreased, but cell apoptosis was markedly increased. The protein expression level of PI3K and p-Akt was significantly increased, but p16 and p-RB expression level was obviously decreased by TWIST1 overexpression. On condition of TWIST1 silencing, these results were reversed. These results indicated TWIST1 overexpression could inhibit apoptosis, but promote the viability and migration of MC3T3 cells, and the mechanism might be by regulating PI3K and p16 pathways.

TWIST1 has been reported to regulate cellular processes including proliferation [19], migration [20], apoptosis [21, 22] and protein transport [23, 24]. For example, Dijkers *et al.* found that cell was chiefly regulated by activation of their pro-apoptotic target gene [25]. Ho, K., S. *et al.* suggested that the cell proliferation was activated by a number of mechanisms, including inhibition of the TWIST1 expression [26, 27]. Consequently, it is necessary to explore the mechanisms of regulation to the cell functional role in the osteogenesis by TWIST1. Here, we investigated the effect on cell viability, migration and apoptosis by TWIST1 overexpression or silencing. We found TWIST1 overexpression could promote cell viability, migration, and inhibit cell apoptosis. We also found the progress of TWIST1 expression was via PI3K or P16 pathways.

Previous study have shown that PI3K plays an important role in osteogenesis and protein expression [28]. Endochondral bone growth is suppressed by inhibition of PI3K/Akt signaling [29, 30]. There is also substantial evidence that PI3K/Akt signaling is required for osteogenesis *in vitro* cell culture studies. Osteogenesis requires PI3K/Akt signaling in cultures of MC3T3 osteoblast precursor cells [31]. This is also confirmed by osteogenesis of murine bone marrow-derived MSCs and in MC3T3 cells [32]. PI3K/Akt is required early in the transcriptional activation of osteogenesis in mouse cells. For example, the expression of PI3K was upregulated subunits p16 and p-BR by the overexpression of TWIST1, in an immature osteoblast cell line (MC3T3-E1) derived from mouse calvaria [33]. It shows that PI3K/Akt signaling has a positive feedback loop to induce osteogenesis, and expression and activity of TWIST1 need to induce by PI3K/Akt signaling. However, this mechanism is unclear, because the phosphorylation of TWIST1 was not activated directly by Akt. In this paper, we have shown that PI3K/Akt signaling is required for the TWIST1 expression in MC3T3 cells *in vitro*.

Furthermore, we have shown that this signaling is influenced by P16 pathways. There was a tremendous increase in the expression of the osteocyte specific TWIST1, accompanied by a decrease in expression of the p16, indicating that TWIST1 down-regulates the expression of p16. As our results pointed out that we have measured the expression level of p16, and it remains possible that p16 protein was suppressed and complete elimination of all p16 protein expression might completely

suppress biomineral deposition. Although, the preliminary data presented here, convincingly that p16 inhibits MC3T3 osteogenesis and PI3K/Akt signaling is pro-osteogenic [34]. Our results suggested PI3K/Akt signaling is required for MC3T3 proliferation and migration, during the progression of which TWIST1 expression increases, but p16 pathways contributes to the suppression of osteogenesis. This study indicated a therapeutic approach of the target for treating osteogenesis.

In conclusion, this study highlights the essential role of TWIST1 in the target of treating osteoporosis. TWIST1 could regulate PI3K and p16 pathways to promote cell viability and migration, but inhibit apoptosis in MC3T3 cells.

Disclosure of conflict of interest

None.

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