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ABSTRACT
Osteoblast has been found to exert indispensable functions during fracture healing. This study aimed to investigate the effects of kaempferol, a natural flavonoid compound, on osteoblast proliferation, migration, and differentiation, as well as possible molecular mechanisms. qRT-PCR was performed to measure the expression level of microRNA-101 (miR-101). Cell viability and migration were respectively assessed using cell counting kit-8 (CCK-8) assay and two-chamber migration assay. Relative alkaline phosphatase (ALP) activity was evaluated using p-nitrophenyl phosphate (pNPP) as substrate. Western blotting was used to detect the protein expression levels of key molecules involved in cell proliferation, migration, differentiation, and Wnt/β-catenin pathway. Kaempferol treatment significantly promoted MC3T3-E1 cell proliferation, migration, and differentiation. Mechanistically, kaempferol notably enhanced the expression level of miR-101 in MC3T3-E1 cells. Knockdown of miR-101 obviously weakened the promoting effects of kaempferol on MC3T3-E1 cell proliferation, migration, and differentiation. In addition, kaempferol remarkably activated Wnt/β-catenin pathway in MC3T3-E1 cells via up-regulating miR-101. This research further confirmed the promoting effects of kaempferol on osteoblast proliferation, migration, and differentiation. The up-regulation of miR-101 and activation of Wnt/β-catenin pathway caused by kaempferol play critical roles in the promoting effects of kaempferol on osteoblast proliferation, migration, and differentiation.

Introduction
Bone fracture is a complete or partial breakage of the bone structure with high incidence, especially in children and elders [1]. Fracture healing is a very complex repair process that involves multi-stage integration of kinds of cells, growth factors, and the extracellular matrix [1,2]. Osteoblast is the main bone-forming cells that play critical roles during fracture healing [3]. The proliferation, migration and differentiation of osteoblast have been found to participate in the process of fracture healing [4]. It is worth believing that searching for effective medicines that can promote osteoblast proliferation, migration, and differentiation will be helpful for fracture healing.

Kaempferol galangal Linn. is a traditional herbal medicine in Asia for treating bruises, indigestion, enteritis, and toothache [5]. Kaempferol (3,5,7-trihydroxy-2- (4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a natural flavonoid compound firstly discovered in the rhizome of Kaempferol galangal Linn. and also existed in many edible plants, such as tea, cabbage, beans, and tomato [6,7]. Khedgikar et al. reported that kaempferol could facilitate cytoskeletal mineralization in osteoblasts by targeting keratin 14 (Krt-14) [8]. Chiou et al. indicated that kaempferol could promote osteoblast maturation through bone morphogenetic protein-2 (BMP-2)/p38 pathway to activate runt-related transcription factor 2 (Runx2) [9]. Guo et al. indicated that kaempferol could promote osteoblast differentiation via estrogen receptor signaling [10].

As a class of small and endogenous RNA molecules, microRNAs (miRNAs) have been found to be involved in the post-transcriptional regulation of multiple genes [11]. Increasing numbers of reports provide evidence that miRNAs play critical regulatory roles in the proliferation, migration, and differentiation of osteoblast [12,13]. A number of plant-derived medicines, including kaempferol, have been found to exert biological activities via regulating miRNAs [14,15]. miRNA-101 (miR-101) has been reported to promote goat skeletal muscle satellite cell proliferation and differentiation, as well as osteogenic differentiation of periodontal ligament cells [16,17]. However, there is no information available about the effects of miR-101 on osteoblast and effects of kaempferol on miR-101. Further experiments are still needed.

In the current research, we further explored the effects of kaempferol on osteoblast proliferation, migration, and differentiation. Then, we focused on our investigation on the
molecular mechanism of regulatory effects of kaempferol on osteoblast, which related to miR-101 and Wnt/β-catenin signaling pathway. The findings of this research will provide new evidence for further understanding the promoting effects of kaempferol on osteoblast proliferation, migration, and differentiation, as well as the treatment of bone fracture.

Materials and methods

Cell culture and treatment

The mouse pre-osteoblast cell line MC3T3-E1 cells were kindly provided by Cell Bank, Chinese Academy of Science (catalogue: GNM15, Shanghai, China). Cells were cultured in Minimum Essential Medium α (MEMα, catalogue: 11900024, Gibco, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, catalogue: 16140071, Gibco, Carlsbad, CA, USA) and 1% (v/v) penicillin-streptomycin solution (catalogue: 15140122, Gibco). 1.5 g/L NaHCO3 (catalogue: S6297, Sigma-Aldrich), 8.82 mg/L folic acid (F7876, Sigma-Aldrich) and 43.2 mg/L (catalogue: 15125, Sigma-Aldrich) were suspended into serum-free MEMα solution. Subsequently, non-migrated cells in the upper chamber were removed using cotton swab carefully. Migrated cells in the lower chamber were stained with 0.1 M M-PER mammalian protein extraction buffer (catalogue: L3000008, Invitrogen, Carlsbad, CA, USA) and added into the lower chamber. After incubation at 37 °C for 48 h, ME3T3-E1 cells were fixed with 4% paraformaldehyde (catalogue: 158127, Sigma-Aldrich) solution. Then, cells in each group were collected, washed with phosphate buffered saline (PBS, catalogue: P1020, Solarbio) twice and lysed with 0.1 M M-PER mammalian protein extraction reagent (catalogue: 78503, Thermo Fisher Scientific, Waltham, MA, USA). After centrifuging at 10,000 g for 10 min, the relative ALP activity of the supernatant was measured using p-nitrophenyl phosphate (pNPP) (catalogue: N7653, Thermo Fisher Scientific, Waltham, MA, USA). Cell viability (%) was calculated by the average absorbance of treatment (transfection) group/average absorbance of control group × 100%.

Relative alkaline phosphatase (ALP) activity assay

ME3T3-E1 cells were seeded into 96-well plate with 5 × 103 cells per well and treated by 1, 5, or 10 μg/ml kaempferol for 12 h. Then, 10 μl CCK-8 kit solution was added into the culture medium of each well and the 96-well plate was placed at 37 °C for 1 h. After that, the absorbance of each well at 450 nm was recorded using Micro-plate reader (Bio-Tek Instruments, Winóoski, VY, USA). Cell viability (%) was calculated by the average absorbance of treatment (transfection) group/average absorbance of control group × 100%.

Cell migration assay

Migration of ME3T3-E1 cells was assessed using QCM chemotaxis two-chamber migration assay (8 mm pore size, catalogue: ECM508, Millipore, Bedford, MA, USA). In brief, after different treatment or transfection, 1 × 103 ME3T3-E1 cells were suspended into serum-free MEMα (200 μl) and added into the upper chamber. Complete MEMα (600 μl) was added into the lower chamber. After incubation at 37 °C for 48 h, ME3T3-E1 cells were fixed with 4% paraformaldehyde (catalogue: 158127, Sigma-Aldrich) solution. Subsequently, non-migrated cells in the upper chamber were removed using cotton swab carefully. Migrated cells in the lower chamber were counted under a microscope (Nikon, Tokyo, Japan). Relative migration (%) was calculated by an average number of migrated cells in treatment (transfection) group/average number of migrated cells in control group × 100%.

Cell transfection

miR-101 inhibitor and its negative control (NC) were designed and synthesized by GenePharma Corporation (Shanghai, China). Lipofectamine® 3000 Transfection Reagent (catalogue: L3000008, Invitrogen, Carlsbad, CA, USA) was conducted to miR-101 inhibitor or NC transfection in line with the manufacturer's instruction. Quantitative reverse transcription PCR (qRT-PCR) was used to verify transfection efficiency.

qRT-PCR

qRT-PCR was performed to measure the expression level of miR-101 in MC3T3-E1 cells after different treatment or transfection. Briefly, total RNAs in MC3T3-E1 cells were isolated using MagMAX™ mirVana™ Total RNA Isolation Kit (catalogue: A27828, Applied Biosystems, Foster City, CA, USA). cDNA was synthesized by TaqMan™ MicroRNA Reverse Transcription Kit (catalogue: 4366596, Applied Biosystems). Subsequently, the expression level of miR-101 was measured using TaqMan™ MicroRNA Assay (catalogue: 4440886, Applied Biosystems). The expression level of U6 snRNA acted as an internal control. Data were analyzed using 2−ΔΔCt method [18].

Cell viability assay

Cell counting kit-8 (CCK-8) assay (catalogue: CA1210, Solarbio, Beijing, China) was used to determine the viability of MC3T3-E1 cells. In brief, transfected or non-transfected MC3T3-E1 cells were seeded into 96-well plate with 5 × 103 cells per well and treated by 1, 5, or 10 μg/ml kaempferol for 12 h. Then, 10 μl CCK-8 kit solution was added into the culture medium of each well and the 96-well plate was placed at 37 °C for 1 h. After that, the absorbance of each well at 450 nm was recorded using Micro-plate reader (Bio-Tek Instruments, Winóoski, VY, USA). Cell viability (%) was calculated by the average absorbance of treatment (transfection) group/average absorbance of control group × 100%.

Cell transfection

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Sigma-Aldrich) as the substrate. Briefly, 50 µl sample was mixed with 50 µl pNPP (1 mg/ml) in 1 M diethanolamine buffer containing 0.5 mM MgCl₂ (pH 9.8) at 37°C for 15 min on a bench shaker. The reaction was stopped by adding 200 µl of 2 M NaOH to the reaction mixture. Total protein content was determined by Micro BCA™ Protein Assay Kit (catalogue: 23235, Thermo Fisher Scientific). One unit of the standard will hydrolyze 1 µmol of pNPP per min at 37°C.

Western blotting
After different treatment or transfection, total proteins in ME3T3-E1 cells were isolated using RIPA buffer (catalogue: R0020, Solarbio) containing Halt™ Protease Inhibitor Cocktail (catalogue: 78430, Thermo Fisher Scientific). Then, equal amounts of total proteins in each group were electrophoresed using Bis-Tris polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% bovine serum albumin (BSA, catalogue: A1933, Sigma-Aldrich) solution at room temperature for 1 h. Subsequently, the PVDF membranes were incubated with anti-proliferating cell nuclear antigen (PCNA) antibody (ab18197), anti-Cyclin D1 antibody (ab16663), anti-matrix metalloproteinase 9 (MMP-9) antibody (ab38898), anti-tissue inhibitor of metalloproteinase 1 (TIMP-1) antibody (ab179580), anti-TIMP-2 antibody (ab180630), anti-Runx2 antibody (ab76956), anti-osterix (Osx) antibody (ab209484), anti-Wnt3a antibody (ab28472), anti-β-catenin antibody (ab6302), and anti-β-actin antibody (ab8226, Abcam Biotechnology, Cambridge, MA, USA) at 4°C for 12 h, respectively. After that, the PVDF membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Hercules, CA, USA). Followed by adding 200 µl Immobilon Western Chemiluminescent HRP Substrate (catalogue: P36599A, Millipore, Bedford, MA, USA) to the surface of the PVDF membranes, the signals of the proteins and the intensities of bands were analyzed using Image Lab™ software (Bio-Rad Laboratories).

Statistical analysis
All experiments in this study were repeated three times in triplicate. The results of multiple experiments were expressed as the mean ± standard deviation (SD). Graphpad 6.0 statistical software (Graphpad, San Diego, CA, USA) was conducted for statistical analyses. The p values between the two groups were calculated using Student’s t-test. The p values with more than three groups were calculated using one-way analysis of variance (ANOVA). p < .05 was considered to be of significant difference.

Results

**Kaempferol promoted MC3T3-E1 cell proliferation and migration**

Firstly, the effects of kaempferol on MC3T3-E1 cell viability, proliferation, and migration were assessed. The results of Figure 2(A) showed that 1 µg/ml kaempferol treatment had no appreciable effect on MC3T3-E1 cell viability, while 5 or 10 µg/ml kaempferol treatment enhanced the viability of MC3T3-E1 cells (p < .05). Figure 2(B) displayed that 5 µg/ml kaempferol treatment up-regulated the protein expression
levels of PCNA and Cyclin D1 in MC3T3-E1 cells (p < .05 or p < .01), which suggested that kaempferol could promote MC3T3-E1 cell proliferation. Figure 2(C) presented that the migration of MC3T3-E1 cells was also increased after 5 μg/ml kaempferol treatment (p < .05). The protein expression level of MMP-9 was elevated, as well as the protein expression levels of TIMP-1 and TIMP-2 were reduced in MC3T3-E1 cells after 5 μg/ml kaempferol treatment (Figure 2D, p < .05 or p < .01). Altogether, these above results suggested that kaempferol could promote MC3T3-E1 cell viability, proliferation, and migration.

**Kaempferol promoted MC3T3-E1 cell differentiation**

Then, we also investigated the effects of kaempferol on MC3T3-E1 cell differentiation. The results in Figure 3(A) displayed that the protein expression levels of Runx2 and Osx in MC3T3-E1 cells were both increased after 5 μg/ml kaempferol treatment (p < .05 or p < .01). Figure 3(B) showed that the relative ALP activity was also enhanced in MC3T3-E1 cells after 5 μg/ml kaempferol treatment (p < .05). These findings indicated that kaempferol also could promote the differentiation of MC3T3-E1 cells.

**Kaempferol enhanced the expression level of miR-101 in MC3T3-E1 cells**

Next, the expression levels of miR-101 in MC3T3-E1 cells after 5 or 10 μg/ml kaempferol treatment were measured. As presented in Figure 4, kaempferol treatment up-regulated the expression level of miR-101 in MC3T3-E1 cells in a concentration-dependent manner (p < .05 or p < .01), which suggested that miR-101 might play key roles in the kaempferol-caused enhancements of MC3T3-E1 cell proliferation, migration and differentiation.

**miR-101 played critical roles in the kaempferol-caused enhancements of MC3T3-E1 cell proliferation, migration and differentiation**

To analyze the roles of miR-101 in kaempferol-caused enhancements of MC3T3-E1 cell proliferation, migration and differentiation, miR-101 inhibitor was transfected into MC3T3-E1 cells. Figure 5(A) displayed that miR-101 inhibitor transfection drastically reduced the expression level of miR-101 in MC3T3-E1 cells. Figure 5(B) presented that the protein expression levels of Runx2 and Osx in MC3T3-E1 cells were both increased after 5 μg/ml kaempferol treatment (Figure 5C, p < .05 or p < .01). The protein expression levels of PCNA and Cyclin D1 were both decreased in kaempferol + NC group (Figure 5C, p < .05). Similar result was found in Figure 5(D), which presented that miR-101 inhibitor transfection also mitigated the kaempferol-caused enhancement of MC3T3-E1 cell migration (p < .05). Figure 5(E) illustrated that compared to kaempferol + NC group, the protein expression level of MMP-9 was reduced, as well as the protein expression levels of TIMP-1 and TIMP-2 were enhanced in MC3T3-E1 cells in kaempferol + miR-101 inhibitor group (p < .05). In addition, the results of Figure 5(F) showed that miR-101 inhibitor transfection also mitigated the enhancements of Runx2 and Osx protein expression levels caused by kaempferol treatment in MC3T3-E1 cells (p < .05). Compared to kaempferol + NC group, the relative ALP activity in MC3T3-E1 cells was also decreased in kaempferol + miR-101 inhibitor group (Figure 5G, p < .01). Altogether, these above findings suggested that miR-101 played critical roles in the kaempferol-caused enhancements of MC3T3-E1 cell proliferation, migration and differentiation.

**Kaempferol activated wnt/β-catenin pathway in MC3T3-E1 cells via up-regulating miR-101**

Finally, the effects of kaempferol and miR-101 inhibitor on Wnt/β-catenin pathway in MC3T3-E1 cells were explored.
Figure 6 displayed that 5 μg/ml kaempferol treatment remarkably activated Wnt/β-catenin pathway in MC3T3-E1 cells via up-regulating the protein expression levels of Wnt3α and β-catenin (p < .05 or p < .01). Moreover, miR-101 inhibitor transfection significantly alleviated the kaempferol-caused activation of Wnt/β-catenin pathway in MC3T3-E1 cells via down-regulating the protein expression levels of Wnt3α and β-catenin (p < .05). These findings indicated that kaempferol could activate Wnt/β-catenin pathway in MC3T3-E1 cells via up-regulating miR-101.

**Discussion**

Bone fracture is a very common body injury and fracture healing which is a very complex biologic process [1,19]. In this study, we further revealed the promoting effects of kaempferol on osteoblast cell proliferation, migration and differentiation. Mechanistically, we found that kaempferol could up-regulate the expression level of miR-101 in osteoblast. miR-101 played pivotal roles in the promoting effects of kaempferol on osteoblast proliferation, migration and differentiation. Furthermore, kaempferol could activate Wnt/β-catenin pathway in osteoblast by up-regulating miR-101.

As one of the most important cell types in bone tissue, osteoblast has been found to exert indispensable functions in bone formation and remodeling [4,20]. The proliferation, migration and differentiation of osteoblast are helpful for fracture healing [21]. In the current study, kaempferol treatment significantly enhanced MC3T3-E1 cells viability, elevated the protein levels of PCNA and Cyclin D1, which are the key
cell proliferation marker molecules [22]. MMP-9 has a positive effect on cell migration, while TIMP-1 and TIMP-2 have negative effects on cell migration [23]. We found that kaempferol could promote osteoblast migration by enhancing MMP-9 expression and reducing TIMP-1 and TIMP-2 expressions. In addition, the protein levels of Runx2 and Osx in osteoblast, which both play essential roles in cell differentiation [24], were enhanced after kaempferol treatment. The activity of ALP in osteoblast, an indicator of cell differentiation [25], was also increased after kaempferol treatment. These results collectively indicated that kaempferol might be helpful for fracture healing via promoting osteoblast proliferation, migration and differentiation.

The breakthrough progress of RNA research is one of the most significant achievements in the biomedical field in the past 30 years [26]. Until now, more than 2000 miRNAs have been found in human cells and it is believed that they are closely related to the regulation of multiple biological processes in cells [11]. Previous studies reported that miR-101 could facilitate goat skeletal muscle satellite cell proliferation and differentiation, as well as osteogenic differentiation of periodontal ligament cells [16,17]. A critical finding in this research was that kaempferol elevated the expression level of miR-101 in osteoblast. Moreover, we found that knockdown of miR-101 could weaken the promoting effects of kaempferol on osteoblast proliferation, migration and differentiation, which suggested that kaempferol promoted osteoblast proliferation, migration and differentiation at least in part by up-regulating miR-101.

It is well known that Wnt/β-catenin signaling pathway participates in the regulation of cell proliferation, migration and differentiation [27]. The activation of Wnt/β-catenin pathway has been found to promote osteoblast differentiation and bone formation [28,29]. Many traditional Chinese medicine formulas, including formulas containing kaempferol, have been reported to accelerate osteoblast proliferation and differentiation by activating Wnt/β-catenin pathway [30]. In the present experiment, we revealed that kaempferol could activate Wnt/β-catenin pathway in osteoblast by elevating the expression levels of Wnt3α and β-catenin. Besides, knockdown of miR-101 could mitigate kaempferol-caused activation of Wnt/β-catenin pathway in osteoblast by reducing the expression levels of Wnt3α and β-catenin. These findings suggested that kaempferol might promote osteoblast proliferation, migration and differentiation through up-regulating miR-101 and then activating Wnt/β-catenin pathway.

In total, our research further confirmed the promoting effects of kaempferol on osteoblast proliferation, migration and differentiation. Moreover, we found that the up-regulation of miR-101 and activation of Wnt/β-catenin pathway caused by kaempferol play critical roles in the promoting effects of kaempferol on osteoblast proliferation, migration and differentiation. This finding expands the understanding about the molecular mechanism of kaempferol on osteoblast and provides a theoretical basis for deeply exploring the healing of fracture by using kaempferol.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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Figure 6. Kaempferol activated Wnt/β-catenin pathway in MC3T3-E1 cells via up-regulating miR-101. After 5 μg/ml kaempferol treatment and/or miR-101 inhibitor transfection, the protein expression levels of Wnt3α and β-catenin in MC3T3-E1 cells were assessed using Western blotting. miR-101: MicroRNA-101. *p < .05; **p < .01.
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