

# Aucubin suppresses Titanium particles-mediated apoptosis of MC3T3-E1 cells and facilitates osteogenesis by affecting the BMP2/Smads/RunX2 signaling pathway

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Received October 25, 2017; Accepted March 28, 2018

DOI: 10.3892/mmr.2018.9286

**Abstract.** Aucubin represents an iridoid glucoside separated from multiple Chinese herbs, which has been demonstrated to possess numerous pharmacological activities. In the present study, the aim was to investigate the roles and mechanisms of aucubin in the suppression of mouse MC3T3-E1 osteoblast apoptosis induced by Titanium particles and the promotion of bone formation. MTT assay and flow cytometry were performed to analyze cell viability and apoptosis, respectively. ELISA and para-nitrophenyl phosphate colorimetry were carried out to evaluate the oxidative stress markers and alkaline phosphatase (ALP). Western blotting and reverse transcription-quantitative polymerase chain reaction assays were used to evaluate the associated mRNA and protein expression. The results revealed that aucubin enhanced the cell activity of MC3T3-E1 cells treated with Ti particles. Aucubin suppressed the apoptosis of Ti particles-induced MC3T3-E1 cells and facilitated osteogenesis by affecting the B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein, ALP and associated osteogenic factors expression. Aucubin reduced the oxidative stress in Ti particles-induced MC3T3-E1 cells. In addition, aucubin upregulated the bone morphogenetic protein 2 (BMP2)/Smads/runt related transcription factor 2 (RunX2) pathway in Ti particles-induced MC3T3-E1 cells. In conclusion, the present study confirmed that aucubin suppressed the Ti particles-mediated apoptosis of MC3T3-E1 cells and facilitated osteogenesis by affecting the BMP2/Smads/RunX2 signaling pathway.

## Introduction

The formation of bone tissues is a complex and orderly dynamic process regulated by bone growth factors of the organism (1). In this process, osteoblasts serve as the main functional cells of osteogenesis to responsible for the synthesis, secretion and mineralization of bone matrix (2,3). Various osteoblast specific genes such as collagen type I (COL I), alkaline phosphatase (ALP), and osteonectin (OPN) express in different periods of the osteoblast differentiation, which further produce the corresponding proteins and secrete to extracellular matrix thereby complete the osteogenesis (4). Moreover, osteocalcin (OCN) and osterix also serve as important factors in the development and process of osteogenesis (5). Previous studies have demonstrated that oxidative stress played a critical role in the progression of osteogenesis (6-8). As a consequence, the study of osteoblast differentiation and other regulatory mechanisms for the repair of bone tissues has important clinical significance.

Titanium (Ti) metal is characterized by better biocompatibility, hardness, inertia, and corrosion resistance, which has been widely used in the design and utilization of artificial arthroplasty (9-11). Ti particles have been proved to inhibit the mesenchymal stem cells (MSCs) osteoblast phenotype expression, bone sialoprotein (BSP) expression, cell proliferation, matrix mineralization and Type I collagen production (12). Furthermore, it also has been demonstrated that Ti particles could induce the apoptosis of MSCs (13). Different sizes of Ti particles and osteoblasts co-culturing could significantly reduce the expression levels of ALP, OPN, and OCN (14). And study has shown that Ti particles with a diameter of 1.5-4.0  $\mu\text{m}$  could markedly suppress the proliferation and functions of osteoblasts (15). Nevertheless, there are few reports in regard to the therapeutic agents and pharmacological mechanisms of Ti particles-mediated osteoblast apoptosis.

Bone morphogenetic proteins (BMPs) are a kind of growth factors that can induce undifferentiated mesenchymal cells to disintegrate into cartilage and bone (16). Smads/runt related transcription factor 2 (RunX2) represents one of the major transduction pathways of BMPs to transmit signals to cells (17). In addition, Smad1/5/8 act as key molecules in the signaling pathway to regulate the target

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**Key words:** aucubin, Titanium particles, MC3T3-E1, bone morphogenetic protein 2, Smads, runt related transcription factor 2

genes (18-20). In order to promote the cell differentiation towards osteogenesis, BMPs bind to the promoter regions of corresponding osteoblast-specific ALP and OPN through down-stream transcription factors such as RunX2 and Osterix (21,22). Studies have found that RunX2 was regulated by BMPs via Smads signaling pathway, and RunX2 could upregulate the expression of bone matrix proteins, including OPN and OCN (23-25). Nevertheless, we know little about the mechanisms of BMP2/Smads/RunX2 pathway in the Ti particles-induced osteoblasts apoptosis.

Aucubin represents an iridoid glucoside separated from multiple Chinese herbs involving leaves of *Aucuba japonica* and *Eucommia ulmoides* (26,27), which has been demonstrated to possess liver protective activities (28,29), anti-oxidative stress effects (30,31), and anti-inflammatory action (32). It has been well documented the extract of *Eucommiae Cortex* promoted the osteoblast proliferation and osteogenesis in postmenopausal osteoporosis (33). However, the therapeutic roles and accurate mechanisms of aucubin in the apoptosis of osteoblasts and osteogenesis have not been identified.

In our study, we explored whether aucubin could act as a novel therapeutic agent suppressing the apoptosis of MC3T3-E1 cells induced by Ti particles and facilitating osteogenesis. Furthermore, it was also fascinating to explore the related apoptosis proteins, osteogenic factors, and signal pathway expression in Ti particles-induced MC3T3-E1 cells treated with different concentration of aucubin.

## Materials and methods

**Reagents.** The products used in cell culture in our study were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibodies and aucubin were purchased from Abcam (Cambridge, UK) and Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), respectively. The Ti particles used in our study were obtained from XiLong Scientific Co., Ltd. (Shenzhen, China). The chemical composition of Ti was (wt%): Ti 99.3, Fe 0.039, O 0.35, N 0.035, C 0.025, Cl 0.034, H 0.024, and Si 0.0018. Sizing by means of Laser Particle Sizer (OMEC LS-POP III) revealed Ti particles had an average size of less than or equal to 5  $\mu\text{m}$  (95.98 wt% of Ti particles were in the range 3-4  $\mu\text{m}$ ).

**Cell culture.** Mouse MC3T3-E1 osteoblast cell line was obtained from the Cell Bank of Chinese Academy of Sciences. MC3T3-E1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) mixed with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO<sub>2</sub> atmosphere at 37°C. Afterwards, MC3T3-E1 cells were observed using an inverted microscope for growth status at 24, 48, and 72 h, respectively.

**Ti particles treatment.** Cells were trypsinized by 0.25% Trypsin (Beyotime Institute of Biotechnology, Shanghai, China) and seeded into the 6-well plates containing type I collagen. After 24 h, cells were attached to the wall of culture bottles. Culture medium was removed and replaced by 2% FBS to starve the cells for 16 h. Before the Ti particles treatment, 2% FBS was replaced by 1% FBS. Ti particles were dissolved in the phosphate-buffered solution (PBS; XiLong Scientific

Co., Ltd.) and sterilized by autoclaved sterilization. After that, Ti particles ( $\leq 5 \mu\text{m}$ , 0.1 wt%; XiLong Scientific Co., Ltd.) were added into the cells.

**Grouping.** Here, five treatment groups were prepared for our study, including control group (cells treated with PBS), Ti group (cells coped with Ti particles), 0.1  $\mu\text{M}$  aucubin + Ti group (cells preprocessed with 0.1  $\mu\text{M}$  aucubin for 6 h, and then coped with Ti particles), 1  $\mu\text{M}$  aucubin + Ti group (cells preprocessed with 1  $\mu\text{M}$  aucubin for 6 h, and then coped with Ti particles), and 10  $\mu\text{M}$  aucubin + Ti group (cells preprocessed with 10  $\mu\text{M}$  aucubin for 6 h, and then coped with Ti particles).

**Cell viability analysis.** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to evaluate the cell vitality of MC3T3-E1 cells. MC3T3-E1 cells at a concentration of  $5 \times 10^3$  per well were seeded into the 96-well plates and incubated in a 5% CO<sub>2</sub> atmosphere at 37°C for 12 h. Afterwards, MC3T3-E1 cells were preprocessed with different concentration of aucubin (0.1, 1, and 10  $\mu\text{M}$ ) for 6 h. And then, the cells were coped with Ti particles prepared in advance for 12 h. After adding 10  $\mu\text{l}$  of MTT solution (5 mg/ml; Amerco, Reno, NV, USA), MC3T3-E1 cells were maintained at 37°C for 6 h. After that, MC3T3-E1 cells were centrifuged at 1,000 x g for 1 min, and the supernatant was removed. Cells were then treated with 100  $\mu\text{l}$  dimethyl sulfoxide (DMSO; XiLongScientific, Shenzhen, Guangdong, China) under low-speed oscillation for 10 min. The absorbance was detected at 490 nm wavelength using a microplate reader (BIO-RAD, California, USA). The cell viability and inhibition rate were calculated by the percentage of cell survival compared with control.

**Apoptosis assay.** Cell apoptosis was assessed by Flow cytometry (FCM). After washing by PBS, MC3T3-E1 cells were trypsinized by 0.25% Trypsin (Beyotime, Shanghai, China). MC3T3-E1 cells were centrifuged at 1,000 rpm/min for 1 min, and the supernatant was removed and the MC3T3-E1 cells for assessment were suspended in the incubation buffer at a density of  $1 \times 10^6$  cells/ml. And then cells were maintained with Annexin V-FITC and propidium iodide (PI; XiLong Scientific Co., Ltd.) at room temperature for 15 min in the dark. After that, cell apoptosis was assessed by FACSCalibur (BD Biosciences, San Diego, CA, USA).

**Para-nitrophenyl phosphate (pNPP) colorimetry.** Alkaline Phosphatase Colorimetric Assay kit (TE0003; Leagene, Beijing, China) was used in the assessment of ALP activity. The supernatant was collected after lysates of MC3T3-E1 cells centrifuging at 1,000 x g for 1 min. The collected supernatant and ALP assay buffer were added into the 96-well plates according to the specification. Afterwards, the plates were blended and incubated for 30 min at 37°C. Then, stop solution was added into the wells to stop reaction. Finally, the absorbance at 405 nm was detected by microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Enzyme linked immunosorbent assay (ELISA).** The kits used for the assessment of ROS (S0033), MDA (S0131), LDH (C0017), SOD (S0101), and GPx (S0056) in MC3T3-E1 cells

were obtained from Beyotime Institute of Biotechnology. MC3T3-E1 cells were added into the corresponding wells, and the wells were sealed up by adhesive tape and maintained at 37°C for 90 min. And then 100  $\mu$ l biotinylated antibody fluids were added into each well except for the blank wells. Afterwards, wells were sealed by adhesive tape and maintained at 37°C for 60 min. Plates were washed by PBS and 100  $\mu$ l enzyme solutions were added into each well. After that, wells were sealed up with adhesive tape and maintained at 37°C for 30 min. Chromogenic substrate was added into the wells except for the blank wells. Plates were maintained for 10-15 min in the dark at 37°C. Afterwards, stop solution was added into each well, and mixed in 10 min immediately. Finally, the OD450 value was detected by microplate reader (Bio-Rad Laboratories, Inc.).

**Western blot analysis.** Cell proteins lysates from MC3T3-E1 cells were partitioned by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). Blotting was carried out with specific antibodies [anti-B-cell lymphoma-2 (Bcl-2) associated X protein (Bax), 1:5,000 dilution, cat. no. ab32503; Abcam; anti-Bcl-2, 1:1,000 dilution, cat. no. ab692; Abcam; anti-OPN, 1:1,000 dilution, cat. no. ab8448; Abcam; anti-OCN, 1:1,000 dilution, cat. no. ab13418; Abcam; anti-Osterix, 1:1,000 dilution, cat. no. ab94744; Abcam; anti-BMP2, 1:500 dilution, cat. no. ab14933; Abcam; anti-Smad1, 1:1,000 dilution, cat. no. ab33902; Abcam; anti-Smad5, 1:1,000 dilution, cat. no. ab194661; Abcam; anti-Smad8, 1:5,000 dilution, cat. no. ab13723; Abcam; anti-RunX2, 1:500 dilution, cat. no. ab23981; Abcam; anti- $\beta$ -actin, 1:1,000 dilution, cat. no. ab8227; Abcam]. After that, horseradish peroxidase-conjugated secondary antibodies (bs-0293M; Bioss, Beijing, China) were added and maintained at room temperature for 1 h. The results were assessed by enhanced chemiluminescent reagents (EMD Millipore) using an ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from MC3T3-E1 cells by TRIzol reagent (Thermo Fisher Scientific, Inc.). Afterwards, two microliters of RNA was used for the cDNA synthesis with a first strand cDNA kit (Sigma-Aldrich; Merck KGaA) following the specification. RT-qPCR analysis was carried out using ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR cycles were under the following conditions: 10 min pretreatment at 94°C, 96°C for 15 sec, 62°C for 45 sec (45 cycles), a final extension at 75°C for 10 min and held at 4°C.  $\beta$ -actin and GAPDH were utilized as the control of the input RNA level. The primers used in RT-qPCR analysis were designed by Invitrogen (Shanghai, China) and were revealed in Table I.

**Statistical analysis.** Results in our study were showed as mean  $\pm$  SEM of at least three independent experiments. Statistical analysis was performed using IBM SPSS statistical software (version 19; IBM Corp., Armonk, NY, USA). The differences in characteristics between the 2 groups in cell viability analysis, apoptosis assay, pNPP colorimetry, ELISA, western blot analysis, and RT-qPCR analysis were examined

by Kruskal-Wallis and Tukey's test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**The identification of MC3T3-E1 mouse osteoblast cell line.** The morphologic characteristics of MC3T3-E1 mouse osteoblast cell line was observed by inverted microscope at 200 magnification times. As shown in Fig. 1A and B, we found that MC3T3-E1 cells attached to the wall of culture bottle and spread after culturing for 24 and 48 h. However, the confluence state of MC3T3-E1 cells was observed after culturing for 72 h (Fig. 1C). According to the previous literature, it has been proved that in logarithmic growth phase, MC3T3-E1 cells showed a fibroblastic morphology. The cell grew with a population doubling time. And on day 4 of culture, the cultures reached a confluent monolayer at a density of  $5-6 \times 10^4$  cells/cm<sup>2</sup>, showing a mosaic appearance (34). Hence, based on our observation of the cultured cells, we confirmed that the cell line was MC3T3-E1 mouse osteoblast.

**Aucubin enhanced the cell activity of MC3T3-E1 cells coped with Ti particles.** MTT assay data (Fig. 2) revealed that the cell viability of MC3T3-E1 cells treated with Ti particles for 12 h ( $55.86 \pm 5.9\%$ ) was distinctly lower than control ( $100 \pm 0\%$ ). However, compared with the MC3T3-E1 cells coped with Ti particles, we found that the cell viability of Ti particles-induced MC3T3-E1 cells in aucubin preprocessing groups was obviously enhanced, especially in the 10  $\mu$ M aucubin treatment group ( $86.96 \pm 4.5\%$ ). These consequences indicated that aucubin could enhance the cell viability of MC3T3-E1 cells suppressed by Ti particles.

**Aucubin suppressed the apoptosis of Ti particles-induced MC3T3-E1 cells.** As FCM data revealed in Fig. 3, the percentage of apoptosis MC3T3-E1 cell number in Ti group was  $20.13 \pm 2.06\%$ , which was markedly higher than control ( $6.44 \pm 0.42\%$ ). Nevertheless, after treating with different concentration of aucubin, the apoptosis rate of MC3T3-E1 cells was reduced to  $14.71 \pm 1.02$ ,  $11.15 \pm 0.89$ , and  $8.94 \pm 0.65\%$ , respectively. These data indicated that aucubin significantly weakened the apoptosis capacity of MC3T3-E1 cells which was enhanced by Ti particles. In addition, we also studied the apoptosis-associated proteins expression in MC3T3-E1 cells. According to the RT-qPCR and western blot data, we found that the Bax expression in Ti group ( $6.62 \pm 0.31$ ) was significantly higher than control ( $1 \pm 0.05$ ), while the Bax expression in Ti particles-induced MC3T3-E1 cells was obviously lessened by aucubin ( $3.73 \pm 0.15$ ,  $2.37 \pm 0.12$ ,  $1.52 \pm 0.25$ ; Fig. 4A and B). However, we also found that Ti particles markedly reduced the expression level of Bcl-2 in MC3T3-E1 cells ( $0.16 \pm 0.01$ ), while aucubin could evidently upregulate the Bcl-2 expression in Ti particles-induced MC3T3-E1 cells ( $0.72 \pm 0.02$ ,  $0.85 \pm 0.04$ , and  $0.96 \pm 0.04$ ; Fig. 4A and B). Based on these consequences, we confirmed that aucubin suppressed the apoptosis of Ti particles-induced MC3T3-E1 cells through regulating the Bax and Bcl-2 expression.

**Aucubin reduced the oxidative stress in Ti particles-induced MC3T3-E1 cells.** Malondialdehyde (MDA), lactate

Table I. Sequences of the primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Sequence (5'-3')	Product (bp)
Bax	Forward	TCTCCGGCGAATTGGAGATG	253
	Reverse	CTCACGGAGGAAGTCCAGTG	
Bcl-2	Forward	TGCGTGAAGGCTTGAGATGT	201
	Reverse	TCCCCCTTTCCTAGACCCAG	
OPN	Forward	GCCACAAGTTTCACAGCCAC	371
	Reverse	AAAATGCAGTGGCCGTTTGC	
OCN	Forward	GCACACCTAGCAGACACCAT	320
	Reverse	GGGCAGCACAGGTCCTAAAT	
Osterix	Forward	TGCTATACTCTGGGGGCTCT	296
	Reverse	ACAAAGCTCAGGGGGAATCG	
BMP2	Forward	TGAGCAAAGTGCTTGCACAC	360
	Reverse	AGCCCCCTGGAAGGGATTAT	
Smad1	Forward	AGTGGGCTTTCATCAGGCTC	318
	Reverse	TCTACATTTGCAGCCGTCGT	
Smad5	Forward	TCTGGGAATTTCTTTGCCTTACC	355
	Reverse	AATTGTTGGCCCAAAGCAGC	
Smad8	Forward	TAAGTCACGTCGTCAGCCAC	322
	Reverse	GTGTTTTCCATGTGGGGCAC	
RunX2	Forward	AGCGGCAGAATGGATGAGTC	280
	Reverse	ACCAGACAACACCTTTGACG	
$\beta$ -actin	Forward	GTTACAGGAAGTCCCTCACCC	194
	Reverse	CAGACCTGGGCCATTTCAGAAA	

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; OPN, osteonectin; OCN, osteocalcin; BMP2, bone morphogenetic protein 2; RunX2, runt related transcription factor 2; bp, base pairs.

dehydrogenase (LDH), reactive oxygen species (ROS), glutathione peroxidase (GPx), and superoxide dismutase (SOD) represent the most important markers of oxidative stress in cellula. In our investigation, ELISA was carried out to evaluate the levels of oxidative stress markers in MC3T3-E1 cells treated with Ti particles and different concentration of aucubin. According to the results, we found that the ROS ( $18.23 \pm 1.20$ ), MDA ( $1.56 \pm 0.09$ ), and LDH ( $129.89 \pm 3.00$ ) content in MC3T3-E1 cells treated with Ti particles were markedly higher than control, while aucubin significantly lessened the ROS ( $15.63 \pm 1.00$ ,  $12.03 \pm 0.89$ , and  $8.99 \pm 0.56$ ), MDA ( $1.25 \pm 0.08$ ,  $1.03 \pm 0.01$ , and  $0.59 \pm 0.03$ ), and LDH ( $112.36 \pm 2.90$ ,  $108.56 \pm 2.50$ , and  $85.36 \pm 3.10$ ) content in Ti particles-induced MC3T3-E1 cells (Fig. 5A-C). Nevertheless, Ti particles were observed to reduce the SOD ( $55.23 \pm 15.37$ ) and GPx ( $7.96 \pm 0.23$ ) activities in MC3T3-E1 cells. After treating with different concentration of aucubin, the SOD ( $69.36 \pm 18.45$ ,  $98.02 \pm 20.30$ , and  $128.69 \pm 18.36$ ) and GPx ( $9.89 \pm 0.23$ ,  $12.23 \pm 0.33$ , and  $14.52 \pm 0.41$ ) activity in Ti particles-induced MC3T3-E1 cells was distinctly enhanced (Fig. 5D and E). To sum up, we confirmed that aucubin reduced the ROS, MDA, and LDH content, while enhanced the SOD and GPx activity in Ti particles-induced MC3T3-E1 cells. Due to the modulation of oxidative markers in Ti particles-induced MC3T3-E1 cells, it was demonstrated that aucubin reduced the oxidative stress in Ti particles-induced MC3T3-E1 cells.

*Aucubin facilitated osteogenesis through regulating the related osteogenic factors expression.* In order to explore the effects of aucubin in bone formation, we further studied the related osteogenic factors expression in MC3T3-E1 cells coped with Ti particles and aucubin. The pNPP colorimetry data indicated that activity of ALP in MC3T3-E1 cells was markedly reduced by Ti particles ( $10.02 \pm 0.23$ ), while increases were observed in Ti particles-induced MC3T3-E1 cells treated with aucubin ( $28.50 \pm 0.98$ ,  $35.20 \pm 1.85$ , and  $45.90 \pm 2.89$ ; Fig. 5F). Moreover, the mRNA expression levels of OPN ( $0.24 \pm 0.05$ ), OCN ( $0.38 \pm 0.01$ ), and Osterix ( $0.13 \pm 0.01$ ) in MC3T3-E1 cells were significantly decreased by Ti particles (Fig. 6A). After treating with different concentration of aucubin, the OPN ( $0.33 \pm 0.01$ ,  $0.53 \pm 0.03$ , and  $0.65 \pm 0.02$ ), OCN ( $0.75 \pm 0.03$ ,  $0.73 \pm 0.03$ , and  $0.85 \pm 0.04$ ), and Osterix ( $0.33 \pm 0.01$ ,  $0.46 \pm 0.02$ , and  $0.64 \pm 0.03$ ) expression in Ti particles-induced MC3T3-E1 cells was markedly enhanced (Fig. 6A). Additionally, the protein expression levels of OPN ( $0.14 \pm 0.01$ ;  $0.21 \pm 0.01$ ,  $0.33 \pm 0.03$ , and  $0.51 \pm 0.03$ ), OCN ( $0.11 \pm 0.01$ ;  $0.22 \pm 0.01$ ,  $0.27 \pm 0.02$ , and  $0.38 \pm 0.02$ ), and Osterix ( $0.16 \pm 0.01$ ;  $0.25 \pm 0.01$ ,  $0.35 \pm 0.02$ , and  $0.55 \pm 0.02$ ) in MC3T3-E1 cells treated with Ti particles and aucubin verified the RT-qPCR results (Fig. 6B). Hence, it was determined that Ti particles suppressed the OPN, OCN, and Osterix expression in MC3T3-E1 cells, while aucubin strengthened the OPN, OCN, and Osterix expression in Ti particles-induced MC3T3-E1 cells. According to

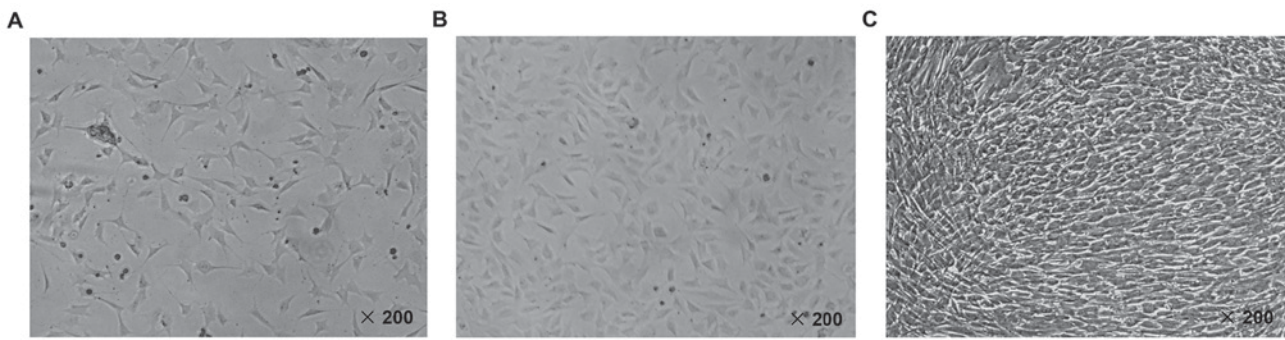


Figure 1. Identification of MC3T3-E1 mouse osteoblast cell line. The MC3T3-E1 cells were observed using an inverted fluorescence microscope at x200 magnification following culture for (A) 24 h, (B) 48 h and (C) 72 h, respectively.

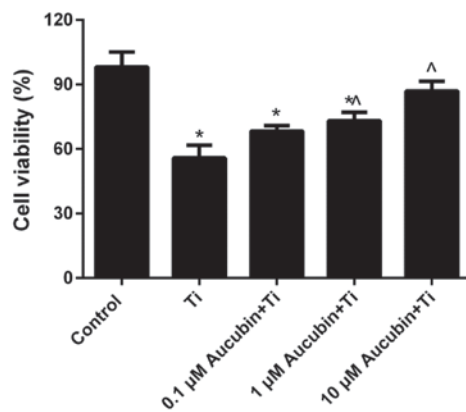


Figure 2. Aucubin enhances the cell viability of Ti particles-induced MC3T3-E1 cells. MC3T3-E1 cells were preprocessed with different concentration of aucubin (0.1, 1 and 10  $\mu$ M) for 6 h in advance, and then treated with Ti particles ( $\leq 5 \mu$ m, 0.1 wt%) for 12 h. MTT assay was performed to assess the cell viability of MC3T3-E1 cells. Data are presented as the mean  $\pm$  standard error mean (n=3). \*P<0.05 vs. control; ^P<0.05 vs. Ti particles. Ti, titanium.

these conclusions, we conjectured that aucubin facilitated osteogenesis through enhancing the activity of ALP and upregulating the osteogenesis-related genes expression.

*Aucubin affected the BMP2/Smads/RunX2 pathway.* Furthermore, we assessed the BMP2, Smad1/5/8, and RunX2 expression in MC3T3-E1 cells from each group. The RT-qPCR and western blot results indicated that the BMP2 (0.08 $\pm$ 0.001), Smad1 (0.24 $\pm$ 0.01)/5 (0.02 $\pm$ 0.001)/8 (0.35 $\pm$ 0.001), and RunX2 (0.21 $\pm$ 0.01) expression in MC3T3-E1 cells coped with Ti particles were significantly lower than control. However, distinct increases of BMP2 (0.33 $\pm$ 0.01, 0.43 $\pm$ 0.02, and 0.71 $\pm$ 0.02), Smad1 (0.44 $\pm$ 0.02, 0.67 $\pm$ 0.02, and 0.81 $\pm$ 0.04)/5 (0.54 $\pm$ 0.02, 0.80 $\pm$ 0.03, and 0.85 $\pm$ 0.02)/8 (0.56 $\pm$ 0.02, 0.67 $\pm$ 0.03, and 0.86 $\pm$ 0.04), and RunX2 (0.52 $\pm$ 0.02, 0.71 $\pm$ 0.03, and 0.89 $\pm$ 0.02) expression were observed in the Ti particles-induced MC3T3-E1 cells treated with aucubin (Fig. 7A-C). Therefore, it was affirmed that BMP2/Smads/RunX2 pathway could be upregulated by aucubin in MC3T3-E1 cells induced by Ti particles.

## Discussion

In the traditional sense, osteoblasts are mainly derived from the primary cell culture of living tissues, which is closer to

the physiological condition of organism (35). However, *in vitro* culture of primary cells susceptible to extraction conditions, culture environment, and other factors, which might impact the cell proliferation and differentiation of osteoblasts. In addition, different batches of primary cells often unable to maintain the genetic stability (36). Thus, we chose MC3T3-E1 cells as the study object in the current research. MC3T3-E1 cell line was first separated from the newborn C57BL/6 mouse skull bone and established as osteoblasts cell line by a Japanese scholar Kodama in 1981 (34). MC3T3-E1 cell line possesses stable proliferation, infinite cell passage function, and multiple biological characteristics of osteoblasts, involving ALP activity, COL1 synthesis, and matrix mineralization. Hence, MC3T3-E1 cells were often used as the cell model in the bone metabolism research (37,38).

Aucubin represents an iridoid glucoside separated from multiple Chinese herbs involving leaves of *Aucuba japonica* and *Eucommia ulmoides*, which has been demonstrated to possess numerous pharmacological activities (26,27). It has been reported that the components of *Eucommiae Cortex* activated the osteoblast and further facilitated osteogenesis (33). Recent study also has proved that the extract of *Eucommia ulmoides* leaves antagonized H<sub>2</sub>O<sub>2</sub>-induced mouse MC3T3-E1 apoptosis via suppressing the expression of Caspases 3/6/7/9 (39). Up to now, although many studies were in regard to aucubin and osteoblasts, the apoptosis and related mechanisms of Ti particles-induced osteoblasts treated with aucubin is not clear. In our study, it was confirmed that aucubin evidently enhanced the cell activity of Ti particles-induced MC3T3-E1 cells. Hence, we conjectured whether aucubin possesses the functions in the suppression of MC3T3-E1 cell apoptosis. We further evaluated the effect of Ti particles and aucubin on the apoptosis of MC3T3-E1 cells. Experimental data indicated that Ti particles led to high percentage of apoptosis cell number, while aucubin significantly inhibited the apoptosis of Ti particles-induced MC3T3-E1 cells. Furthermore, the apoptosis-associated mechanisms in MC3T3-E1 cells coped with Ti particles and aucubin were investigated. It was revealed that aucubin obviously reduced the Bax expression, while upregulated the Bcl-2 expression in Ti particles-induced MC3T3-E1 cells. Therefore, we could draw the conclusion that aucubin inhibited the Ti particles-mediated apoptosis of MC3T3-E1 cells through regulating the expression levels of Bax and Bcl-2.

Mitochondria play a crucial part in the cell growth and death and possess the function of ROS generation and

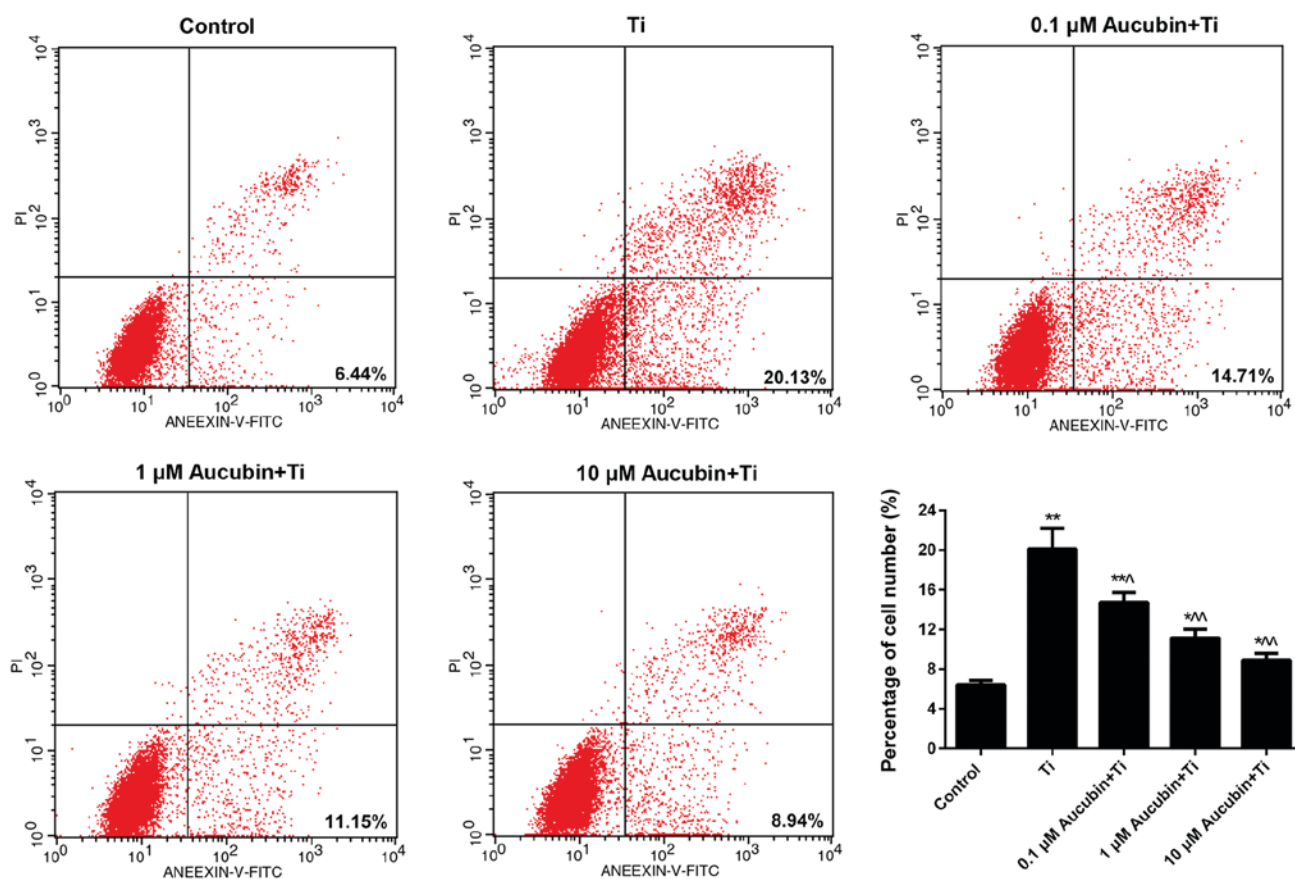


Figure 3. Aucubin reduces the apoptosis of Ti particles-induced MC3T3-E1 cells. MC3T3-E1 cells were preprocessed with different concentration of aucubin (0.1, 1 and 10  $\mu$ M) for 6 h in advance, and then treated with Ti particles ( $\leq 5 \mu$ m, 0.1 wt%) for 12 h. Cell apoptosis was evaluated by flow cytometry. Data are presented as the mean  $\pm$  standard error mean (n=3). \*P<0.05 and \*\*P<0.01 vs. control; ^P<0.05 and ^^P<0.01 vs. Ti particles. Ti, titanium; FITC, fluorescein isothiocyanate; PI, propidium iodide.

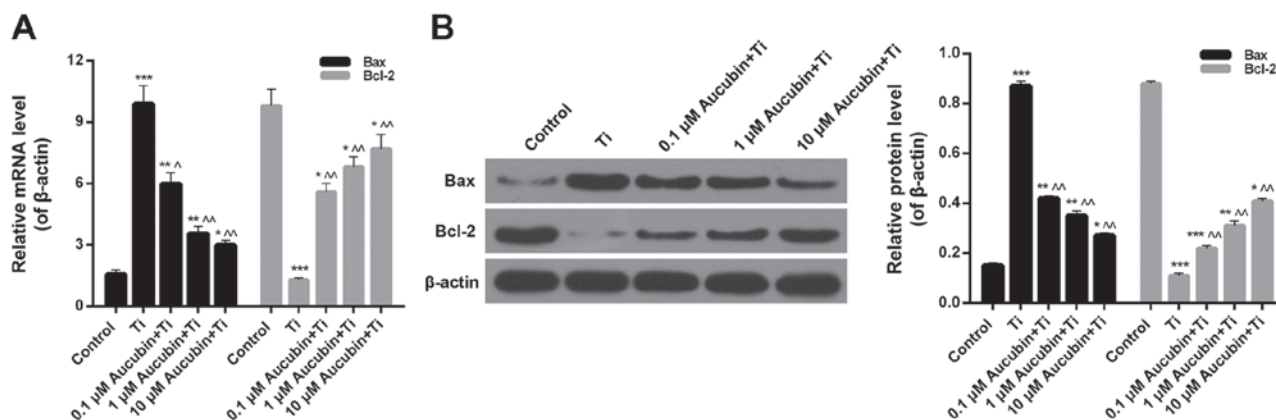


Figure 4. Aucubin regulates the apoptosis-associated genes and proteins expression in Ti particles-induced MC3T3-E1 cells. MC3T3-E1 cells were preprocessed with different concentration of aucubin (0.1, 1 and 10  $\mu$ M) for 6 h in advance, and then treated with Ti particles ( $\leq 5 \mu$ m, 0.1 wt%) for 12 h. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot assays were performed to determine Bax and Bcl-2 expression in MC3T3-E1 cells. Data are presented as the mean  $\pm$  standard error mean (n=3). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. control; ^P<0.05 and ^^P<0.01 vs. Ti particles. Ti, titanium; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

detoxification (40,41). It has been demonstrated that at high concentration, ROS might lead to severe injury to cells, which referred to the 'oxidative stress' (42-44). Aucubin has been reported that possessed the anti-oxidation activity (45,46). Due to the ability of aucubin in the suppression of MC3T3-E1 cell apoptosis, it was arrestive that whether aucubin could affect the oxidative stress in MC3T3-E1 cells. Hence, we assessed

the oxidative stress markers in MC3T3-E1 cells treated with aucubin, including ROS, MDA, LDH, SOD, and GPx. Obvious reductions of ROS, MDA, and LDH content were observed in the Ti particles-induced MC3T3-E1 cells treated with aucubin. Additionally, we also found that aucubin enhanced the activities of SOD and GPx in Ti particles-induced MC3T3-E1 cells. Thus, according to these results, it was confirmed that aucubin

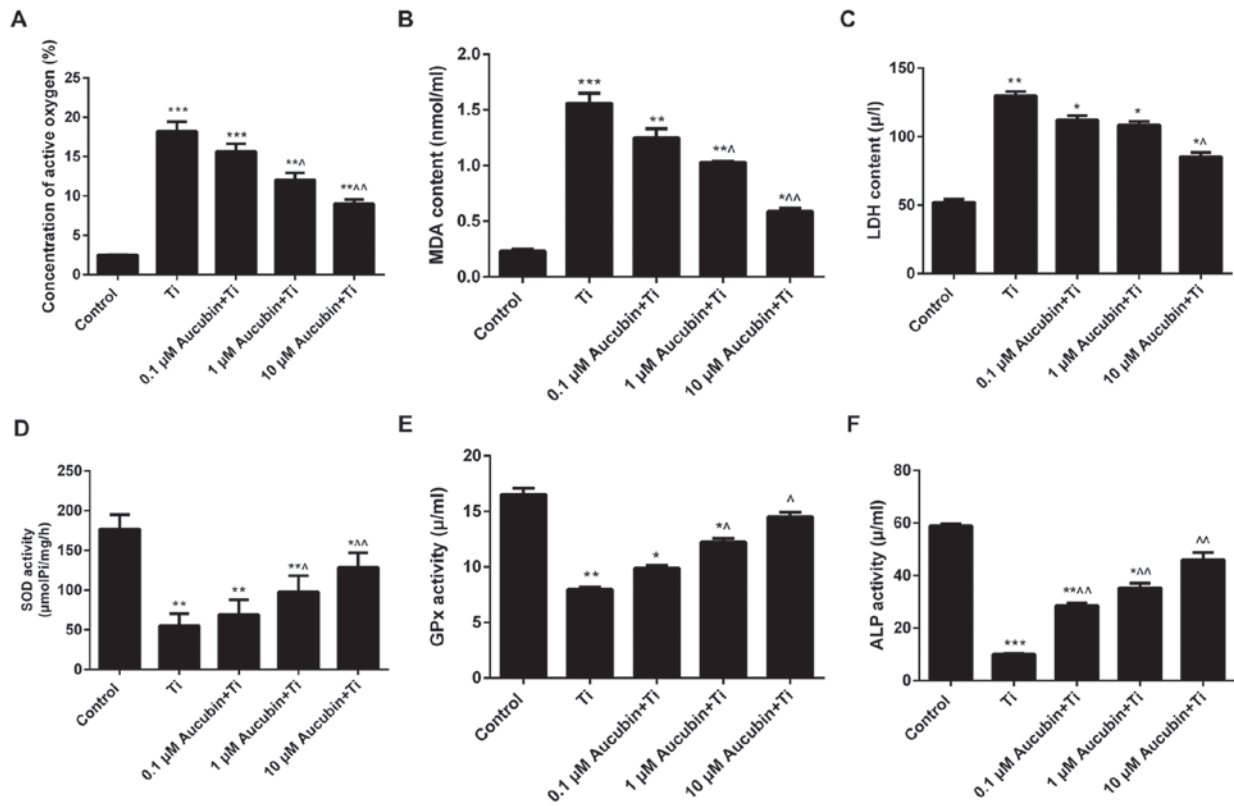


Figure 5. Aucubin reduces the stress. MC3T3-E1 cells were preprocessed with different concentration of aucubin (0.1, 1 and 10  $\mu\text{M}$ ) for 6 h in advance, and then treated with Ti particles ( $\leq 5 \mu\text{m}$ , 0.1 wt%) for 12 h. ELISA was performed to assess the levels of (A) ROS, (B) MDA, (C) LDH, (D) SOD and (E) GPx in MC3T3-E1 cells (n=3). (F) pNPP colorimetry was carried out to assess the ALP activity in MC3T3-E1 cells. Data are presented as the mean  $\pm$  standard error mean (n=3). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control; ^ $P < 0.05$  and ^^ $P < 0.01$  vs Ti particles. Ti, titanium; ROS, reactive oxygen species; MDA, malondialdehyde; LDH, lactate dehydrogenase; SOD, superoxide dismutase; GPx, glutathione peroxidase; pNPP, para-nitrophenyl phosphate; ALP, alkaline phosphatase.

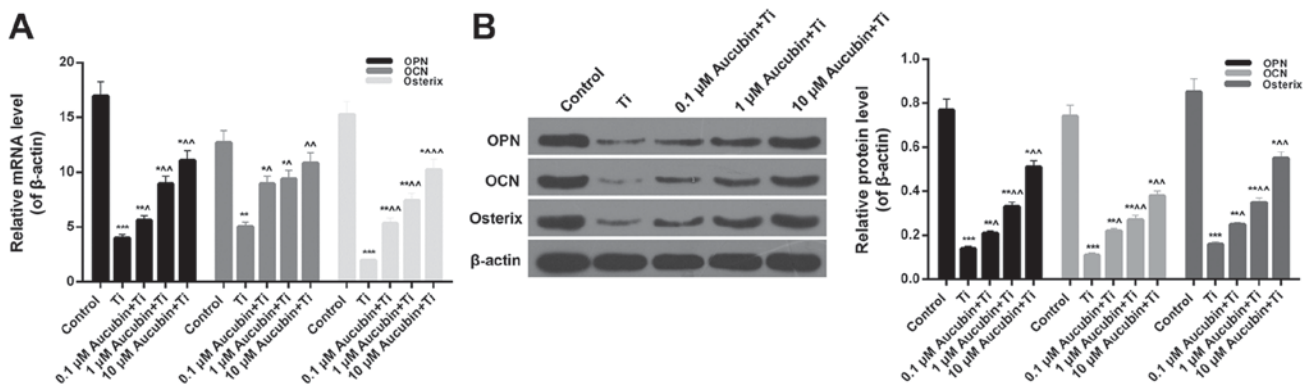


Figure 6. Aucubin facilitates osteogenesis. MC3T3-E1 cells were preprocessed with different concentrations of aucubin (0.1, 1 M and 10  $\mu\text{M}$ ) for 6 h in advance, and then treated with Ti particles ( $\leq 5 \mu\text{m}$ , 0.1 wt%) for 12 h. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot assays were performed to measure the expression levels of OPN, OCN and Osterix in MC3T3-E1 cells. Data are presented as the mean  $\pm$  standard error mean (n=3). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control; ^ $P < 0.05$ , ^^ $P < 0.01$  and ^^ $P < 0.001$  vs. Ti particles. Ti, titanium; OPN, osteonectin; OCN, osteocalcin.

distinctly reduced the oxidative stress activated by Ti particles. At present, we proved that aucubin possessed the functions of suppressing the apoptosis and reducing the oxidative stress of Ti particles-induced MC3T3-E1 cells. Thus, the protective effects of aucubin on the MC3T3-E1 cells induced by Ti particles were demonstrated. Moreover, on account of MC3T3-E1 cells play an important role in the progression of osteogenesis. We thereby speculated that aucubin might impact the osteogenesis.

Based on the previous study (47), ALP, OPN, OCN, and Osterix were selected as osteoblast specific factors to

evaluate the effect of aucubin in osteogenesis. In the current study, MC3T3-E1 cells acted as precursor osteoblasts, which could be gradually differentiated into osteoblasts in the specific medium. We found that the ALP activity, OPN, OCN, and Osterix expression in Ti particles-induced MC3T3-E1 cells was enhanced by aucubin. In consequence, it was proved that aucubin might facilitate osteogenesis through enhancing ALP activity and upregulating the expression levels of OPN, OCN, and Osterix in Ti particles-induced MC3T3-E1 cells.

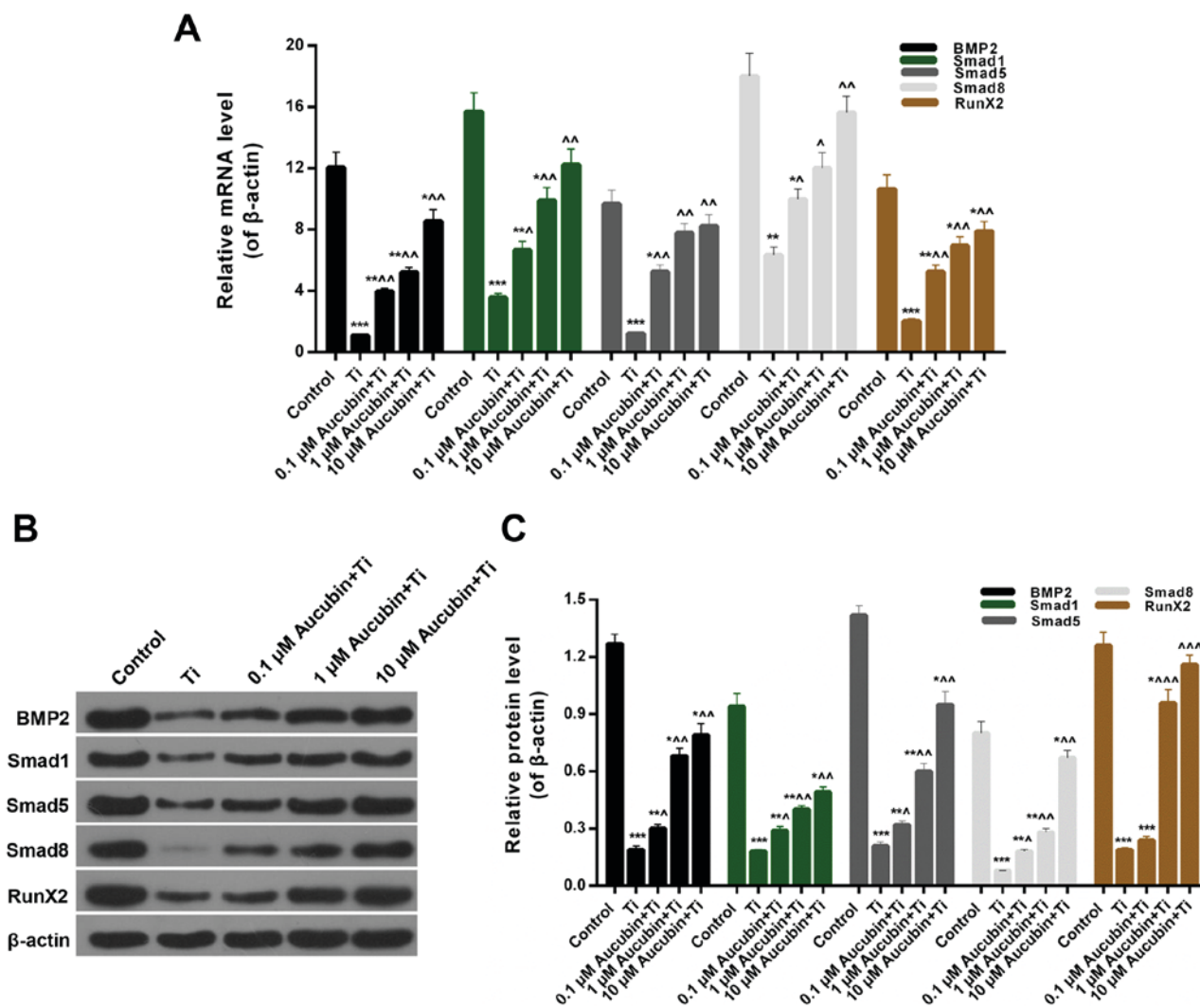


Figure 7. Aucubin affects the BMP2/Smads/RunX2 signaling pathway. MC3T3-E1 cells were preprocessed with different concentrations of aucubin (0.1, 1 and 10  $\mu$ M) for 6 h in advance, and then treated with Ti particles ( $\leq 5 \mu$ m, 0.1 wt%) for 12 h. (A) Reverse transcription-quantitative polymerase chain reaction and (B and C) western blot assays were performed to evaluate the expression levels of BMP2, Smad1/5/8, and RunX2 in MC3T3-E1 cells. Data are presented as the mean  $\pm$  standard error mean (n=3). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control;  $\wedge P < 0.05$ ,  $\wedge\wedge P < 0.01$  and  $\wedge\wedge\wedge P < 0.001$  vs. Ti particles. Ti, titanium; BMP2, bone morphogenetic protein 2; RunX2, runt related transcription factor 2.

Additionally, previous studies also have indicated that BMP2/Smads/RunX2 pathway might participate in the apoptosis and the process of osteogenesis (48,49). Nevertheless, the accurate role and mechanism of aucubin in the regulation of BMP2/Smads/RunX2 pathway in osteoblasts apoptosis and osteogenesis is unclear. Thus, we further explored the probable mechanism of BMP2/Smads/RunX2 pathway in the suppression of osteoblasts apoptosis and promotion of osteogenesis. According to the western blot data, it was confirmed that the BMP2, Smad1/5/8, and RunX2 expression in Ti particles-induced MC3T3-E1 cells was strengthened by aucubin. Thus, we confirmed that aucubin could impact the BMP2/Smads/RunX2 pathway in Ti particles-induced MC3T3-E1 cells. All together, to the best of our knowledge, it was first proved that aucubin inhibited Ti particles-induced MC3T3-E1 cell apoptosis and facilitated osteogenesis by upregulating BMP2/Smads/RunX2 pathway.

In summary, our present work highlights that aucubin suppressed Ti particles-mediated apoptosis of MC3T3-E1 cells and facilitated osteogenesis through affecting

BMP2/Smads/RunX2 pathway. The findings of our research have crucial influence on the mechanisms of aucubin and osteoblasts. The potential effects of aucubin on the promotion of osteogenesis suggest that aucubin might be an effective target for osteogenesis promotion.

#### Acknowledgements

Not applicable.

#### Funding

This work was supported by Zhejiang Provincial Medical Science and Technology on General Item (grant nos. 2015KYA014 and 2017KY011).

#### Availability of data and materials

All data generated or analysed during this study are included in this published article.



### Authors' contributions

YC designed the experiments and wrote the article. ZZ conducted the cell culture and treatment sections, and analysed the function of Aucubin on the BMP2/Smads/RunX2 pathway. QX performed the MTT and flow cytometry assays to detect cell viability and apoptosis. SZ carried out ELISA and para-nitrophenyl phosphate colorimetry to evaluate the oxidative stress markers and alkaline phosphatase levels. YH was involved in the detection of the expression levels of apoptosis-associated factors.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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