Hesperetin affects osteoclast differentiation via MAPK signaling pathway

Jingxian Fan[1,2,3,A–D,F], Chengfeng Xu[1,3,A–C,F], Hui Shi[1,3,A–D], Xun Wang[3,4,A–C], Tiantian Zheng[4,A–C], Minyu Zhou[3,A–D], Zhiqiang Zhang[3,A–C], *Yingxiao Fu[1,3,A–F], *Baoding Tang[3,A,D–F]

[1] Department of Biotechnology, Anhui Province Key Laboratory of Translational Cancer Research, Bengbu Medical College, China
[2] Department of Public Fundamentals, Bengbu Medical College, China
[3] Department of Life Sciences, Bengbu Medical College, China
[4] Key Laboratory of Neural Regeneration, Nantong University, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Baoding Tang
E-mail: baodtang_16@163.com

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Conflict of interest
None declared

*Yingxiao Fu and Baoding Tang contributed equally to this work.

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Abstract

Background. The number and activity of osteoblasts and osteoclasts play an important role in skeletal biology, especially in bone reconstruction. Scientific and rational regulation of osteoclast formation and activity has become a critical strategy aimed at inhibiting the loss of bone mass in the body and alleviating the occurrence of bone diseases. Currently, there are only a few reports related to hesperetin-regulated osteoclast differentiation.

Objectives. To investigate the influence of hesperetin on osteoclast-like cell differentiation and formation, and determine whether the MAPK signaling pathway is involved in the differentiation process.

Materials and methods. The RAW264.7 cells were induced and cultured in vitro to promote their differentiation into osteoclast-like cells. Tetrazolium bromide was utilized to determine the effects of different concentrations (100, 200, 400, and 600 μM) of hesperetin on the proliferation of osteoclast-like cell precursors. Osteoclast-like cell differentiation was conducted using tartrate-resistant acid phosphatase (TRAP) staining assay. The status of nuclei and actin filaments of differentiated osteoclast-like cells was observed with the use of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and actin-tracker green staining experiments. Changes in key proteins of the MAPK signaling pathway were detected using western blot.

Results. The results of TRAP staining experiments showed that the number of osteoclast-like cells decreased with the increase in hesperetin concentration. The DAPI and actin-tracker green staining demonstrated that the nuclei of differentiated osteoclast-like cells reduced in size with the increase in hesperetin concentration, and the osteoclast-like cells became smaller. Western blot for key MAPK signaling pathway proteins revealed that phospho-ERK and phospho-p38 protein levels were not significantly inhibited, but phospho-JNK protein levels were reduced.

Conclusions. Hesperetin inhibits the differentiation of osteoclast-like cells. Further studies revealed that hesperetin also affects the activation level of phospho-JNK, a key signaling protein of the MAPK signaling pathway, in the induced differentiation of osteoclast-like cells.

Key words: MAPK signaling pathway, hesperetin, osteoclast-like cell

Background

The maintenance of the total skeletal mass of the body depends on the dynamic balance between new bone formation mediated by osteoblasts and the resorption of old bone mass mediated by osteoclasts.[1–5] Osteoblasts are specialized mesenchymal cells responsible for bone matrix production and mineralization, with both the receptors and catabolic enzymes required to internalize and utilize circulating lipids. Disruption of these receptors or enzymes could impair osteoblast function and lead to bone defects.[6] Osteoclasts are multinucleated macrophage lineage cells found uniquely in the bone.[7] They interact with the extracellular matrix of fibronectin, collagen, bone salivary protein, and bone bridging proteins to adhere to the bone surface and migrate, which degrades the bone matrix components. Osteoclasts may be the only cells in the body with bone resorption activity, and they are essential for the establishment and maintenance of bone homeostasis and repair after bone injury.[8] A growing number of studies have shown that abnormal osteoclast activity is an important factor in various bone diseases, such as osteoporosis, osteosclerosis, osteoarthritis, and Paget's disease. Scientific and rational regulation of osteoclast formation and activity has become a key strategy to inhibit the loss of bone mass in the body and alleviate the occurrence of bone diseases.[9] The currently available clinical drugs for the treatment of skeletal disorders, such as bisphosphonates and vitamin D, have not achieved satisfactory results.[10] Natural herbs are expected to be potential therapeutic agents for many diseases, such as those related to the skeletal system, due to their high activity and low side effects.[11] Some flavonoids, such as quercetin, epimedoside, naringin, ephedrine, and gerberoside, have been reported to be used, or attempted to be used, in the prevention and treatment of abnormal bone metabolism diseases.[12] Hesperetin also belongs to the flavonoid family of compounds and is one of the main active ingredients of traditional Chinese medicine tangerine peel and it is found in high levels in the pulp and peel of citrus plants of the *Rutaceae* family. Hesperetin has been shown to significantly inhibit the fibrotic process in the lung, liver, kidney, and heart muscle,[13–16] while another study found that hesperetin could promote osteogenesis.[17] However, few reports on the regulation of osteoclast differentiation by hesperetin are available, and no cases of its application in the prevention and treatment of clinical skeletal diseases have been reported. The tertiary members of the mitogen-activated protein kinase (MAPK) cascade pathway are MAPK, MAPKK, and MAPKKK. These 3 kinases are activated sequentially and simultaneously regulate various important physiological/pathological effects, such as cell growth, differentiation, stress, and inflammatory responses. Previous reports have suggested that the MAPK signaling pathway is involved in osteoclast differentiation and activation.[18] Reports also indicated that hesperidin inhibits RANKL-induced osteoclast formation.[19,20] However, only low concentrations (0–150 μM) of hesperetin were used in the above study to explore the effects of hesperetin on osteoclast differentiation and its potential mechanisms. Therefore, the effects of high concentrations of hesperetin on osteoclast differentiation and its mechanisms need to be further investigated. Herein, we investigated whether high concentrations of hesperetin affect osteoclast differentiation and whether the MAPK signaling pathway plays a role in this process. The results may provide a theoretical reference for the prevention and treatment of skeletal diseases caused by abnormal osteoclast activity in clinical settings.

Objectives

This study aimed to investigate the effects of hesperetin on osteoblast differentiation and whether the MAPK signaling pathway is involved in this process.

Materials and methods

Materials

RAW264.7 cells were purchased from the Shanghai Institute of Life Sciences of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium alpha (MEM-α) were obtained from Hyclone Laboratories, Inc. (Logan USA). RANKL (PeproTech, Thermo Fisher Scientific, Waltham, USA), tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich, St. Louis, USA), fetal bovine serum (FBS; GE Healthcare, Chicago, USA), hesperetin (MedChemExpress, Monmouth Junction, USA), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), actin-tracker green, FR180204, SP600125, and SB203580 were bought from Beyotime Biotechnology (Shanghai, China). p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2), SAPK/JNK antibody, phospho-SAPK/JNK antibody, p38 MAPK antibody, and phospho-p38 MAPK were obtained from Cell Signaling Technology (Danvers, USA).

Determination of the effects of hesperetin on precursors of osteoclast-like cell proliferation using MTT method

RAW264.7 cells were inoculated into 96-well plates (3,000/well) and incubated at 37°C and 5% CO_2. RANKL cytokines were added following cell wall attachment. At the end of the induction culture, 100, 200, 400, and 600 μM of hesperetin were added to each experimental group, and each group contained 5 replicate wells. Dimethyl sulfoxide (DMSO) without hesperetin was used as the control group. After 24 h of hesperetin treatment, MTT (100 μL/well) was added, and the MTT solution was aspirated and discarded after 4 h. The DMSO solution was added and incubated overnight at 37°C in a 5% CO_2 incubator and the optical density (OD) values were measured.

TRAP staining to observe the effects of hesperetin on osteoclast-like cell formation

RAW264.7 cells were inoculated in 96-well cell culture plates (500/well), and RANKL cytokine was added after cell wall attachment. At the end of the induction culture, 100, 200, 400, and 600 μM of hesperetin were added to each group and incubated overnight, followed by TRAP staining. Next, 5 mL of ultrapure water was added to a 15 mL centrifuge tube heated in a 37°C water bath. The medium was aspirated, and 4% paraformaldehyde solution was added for 10 min to fix the cells. Then, 50 μL of freshly prepared fast garnet GBC base solution and 50 μL sodium nitrite solution were added in a 1.5 mL Eppendorf (EP) tube and incubated at room temperature for 2 min. The cells were fixed and washed twice with ultrapure water. All the prepared reagents were transferred to the pre-warmed ultrapure water. Thereafter, 50 μL NA-B solution, 200 μL AS solution, and 100 μL tartrate solution reagent were added and mixed well with 150 μL staining solution per well. The plate was incubated at 37°C for 60 min. The staining solution was then aspirated and discarded, and 100 μL ultrapure water was added per well to prevent drying. Then, the samples were observed with a light microscope and images were acquired (model IX71; Olympus Corp., Tokyo, Japan).

Effects of DAPI and actin-tracker green staining on osteoclast-like cell formation by hesperetin

RAW264.7 cells were inoculated in 96-well cell culture plates (500/well), and RANKL cytokine was added after cell wall attachment. A total of 100, 200, 400, and 600 μM of hesperetin were added to each cell well at the end of the induction culture. The cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min, and washed thrice with PBS containing 0.1% Triton X-100 (Beyotime Biotechnology). After actin-tracker green staining solution was added, the sample was incubated for 60 min at room temperature in the dark, washed 3 times with PBS containing 0.1% Triton X-100 for 5 min each time, and finally, DAPI was added (working concentration 6 μg/mL) and incubated for 15 min in the dark. The staining solution was discarded, washed twice in PBS for 3–5 min each time, observed, and images were acquired under a fluorescent microscope (model IX71; Olympus Corp.).

Western blot for detection of protein changes

RAW264.7 cells were inoculated in 6-well cell culture plates (800,000/well), and RANKL cytokine was added after cell apposition. A total of 100, 200, 400, and 600 μM of hesperetin were added to each group at the end of the induction culture, and the total protein was extracted at the end of the cell culture. After the protein was quantified, it was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to a nitrocellulose (NC) filter membrane. The NC membrane was then blocked using Tris-buffered saline with Tween (TBST) containing 5% skim powdered milk for 90 min by shaking at room temperature. Next, the primary antibody was added, followed by incubation on ice overnight. The sample underwent TBST washing 3 times, for 5 min each time, followed by the secondary antibody incubation for 90 min at room temperature. The sample was then washed in TBST 3 times for 10 min each time. Finally, enhanced chemiluminescence (ECL) detection was performed.

Effects of key protein inhibitors of MAPK signaling pathway on osteoclast-like cell differentiation

RAW264.7 cells were inoculated in 96-well cell culture plates (500/well), and FR180204 (ERK inhibitor, 20 mM), SP600125 (JNK inhibitor, 20 mM), and SB203580 (p38 inhibitor, 20 mM) were added to each group after cell wall attachment. RANKL cytokines were added to each experimental group at the end of drug treatment. The TRAP staining was performed at the end of the culture.

Statistical analyses

All data were analyzed using GraphPad Prism v. 8.0 (GraphPad Software, San Diego, USA), and results are displayed as mean ± standard deviation (M ±SD). The Kruskal–Wallis one-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test was used for 3 or more groups of nonparametric data. The experiments were repeated independently 3 times. A value of $p < 0.05$ indicated statistical significance.

Results

Effects of hesperetin on the proliferation of differentiated osteoclast-like cell precursors

The MTT results showed that hesperetin significantly inhibited the proliferation of differentiated osteoclast-like cell precursors. The hesperetin groups of 100, 200, 400, and 600 μM all demonstrated significantly reduced cell proliferation rates compared to the DMSO group in a dose-dependent manner (Fig. 1).

Effects of hesperetin on osteoclast-like cell formation

The TRAP staining showed that the differentiated osteoclast-like cells were large and had many nuclei and a burgundy cytoplasm and colorless nuclei; by contrast,

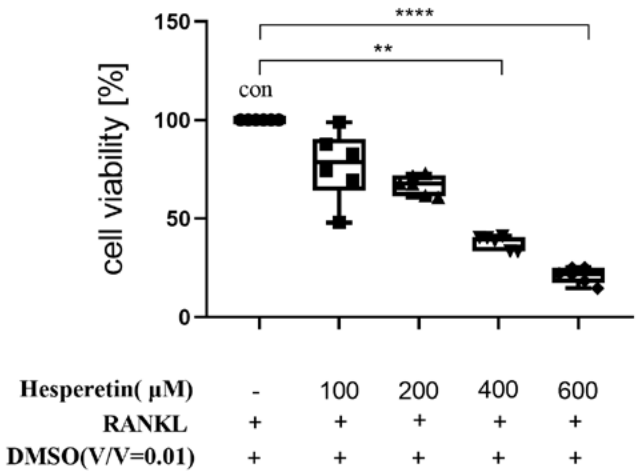

Fig. 1. Effects of hesperetin on the viability of osteoclast-like cell precursors. The results showed that the cell proliferation rates of hesperetin groups of 100, 200, 400, and 600 μM were significantly lower than those of the control group in a dose-dependent manner. The Kruskal–Wallis test (K–W) with Dunn's post hoc test was applied in the statistical analysis

**p = 0.002, 400 μM compared with the control group; ****p < 0.001, 600 μM compared with the control group; K–W, Dunn's post hoc test.

the undifferentiated cells showed mononuclear aggregates (Fig. 2A). The number of TRAP-positive cells indicated that hesperetin affected the differentiation of osteoclast-like cells, with a reduction in TRAP-positive cells with the increase in hesperetin concentration. The number of TRAP-positive cells was significantly reduced in the high-concentration hesperetin groups of 400 (6 ±0.67) and 600 μM (2.67 ±0.19) compared with the control group (Fig. 2B).

Effects of hesperetin on the cytoskeleton and nucleus of osteoclast-like cells

The DAPI staining highlighted that the nuclei of differentiated osteoclast-like cells in the control group were tightly clustered together, forming a relatively independent region. The cytoskeletal microfilament proteins were more widely distributed and exhibited a more complete cell outline. The differentiated cells in the low-concentration hesperetin groups of 100 μM and 200 μM had a higher number of nuclei than those in the control group, and the nuclei in the high-concentration group were also aggregated. However, the results of actin-tracker green staining showed that the aggregated cells in the hesperetin-treated group were not differentiated multinucleated osteoclast-like cells but only single RAW264.7 cells aggregated together, and the number of differentiated osteoclasts decreased with increasing hesperetin concentration. Moreover, the differentiated osteoclasts were larger, with extensive distribution of intracellular actin filaments, whereas the undifferentiated cells were smaller, with clear intercellular boundaries. Hesperetin reduced the size of differentiated osteoclast-like cells and decreased the number of nuclei in a dose-dependent manner compared with controls (Fig. 3).

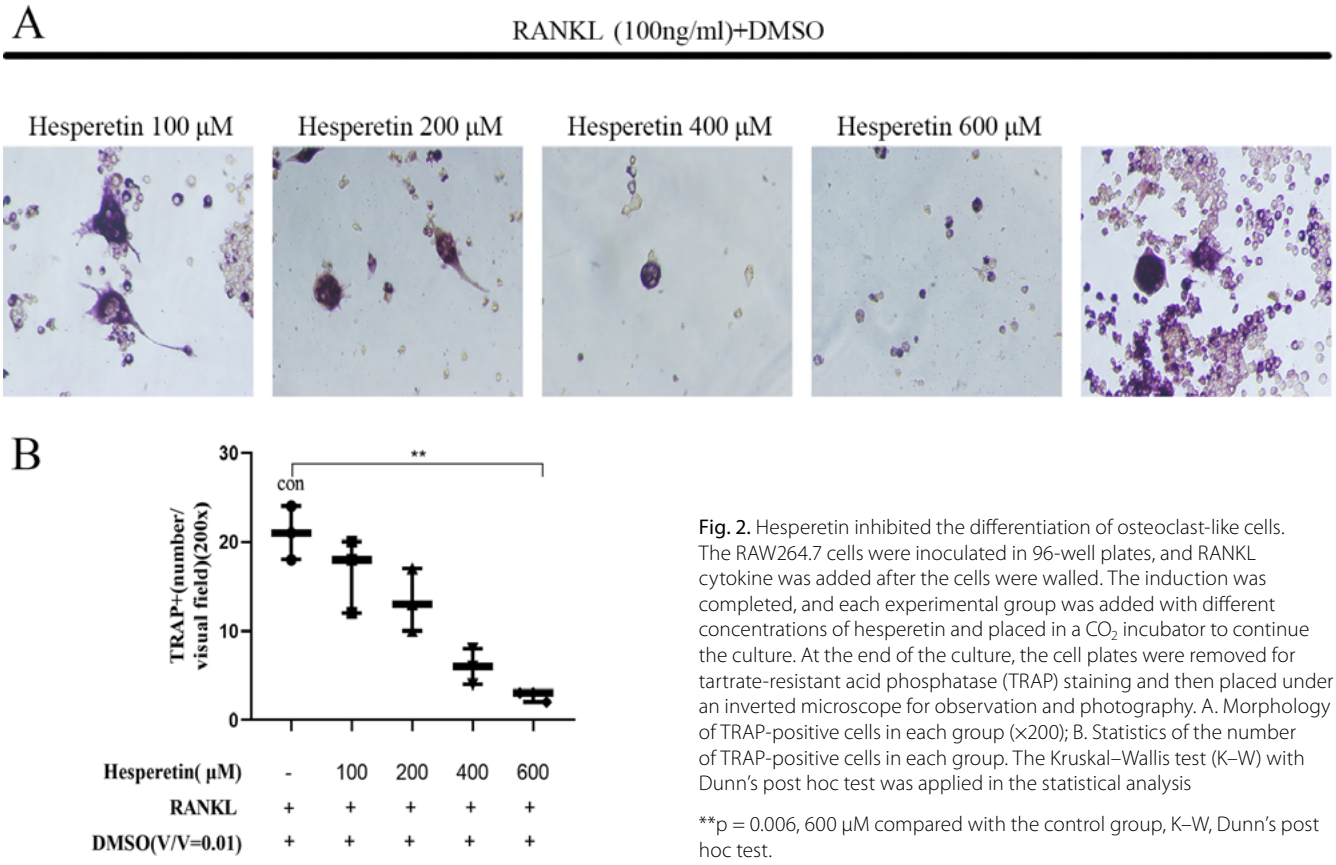

Fig. 2. Hesperetin inhibited the differentiation of osteoclast-like cells. The RAW264.7 cells were inoculated in 96-well plates, and RANKL cytokine was added after the cells were walled. The induction was completed, and each experimental group was added with different concentrations of hesperetin and placed in a CO_2 incubator to continue the culture. At the end of the culture, the cell plates were removed for tartrate-resistant acid phosphatase (TRAP) staining and then placed under an inverted microscope for observation and photography. A. Morphology of TRAP-positive cells in each group (×200); B. Statistics of the number of TRAP-positive cells in each group. The Kruskal–Wallis test (K–W) with Dunn's post hoc test was applied in the statistical analysis

**p = 0.006, 600 μM compared with the control group, K–W, Dunn's post hoc test.

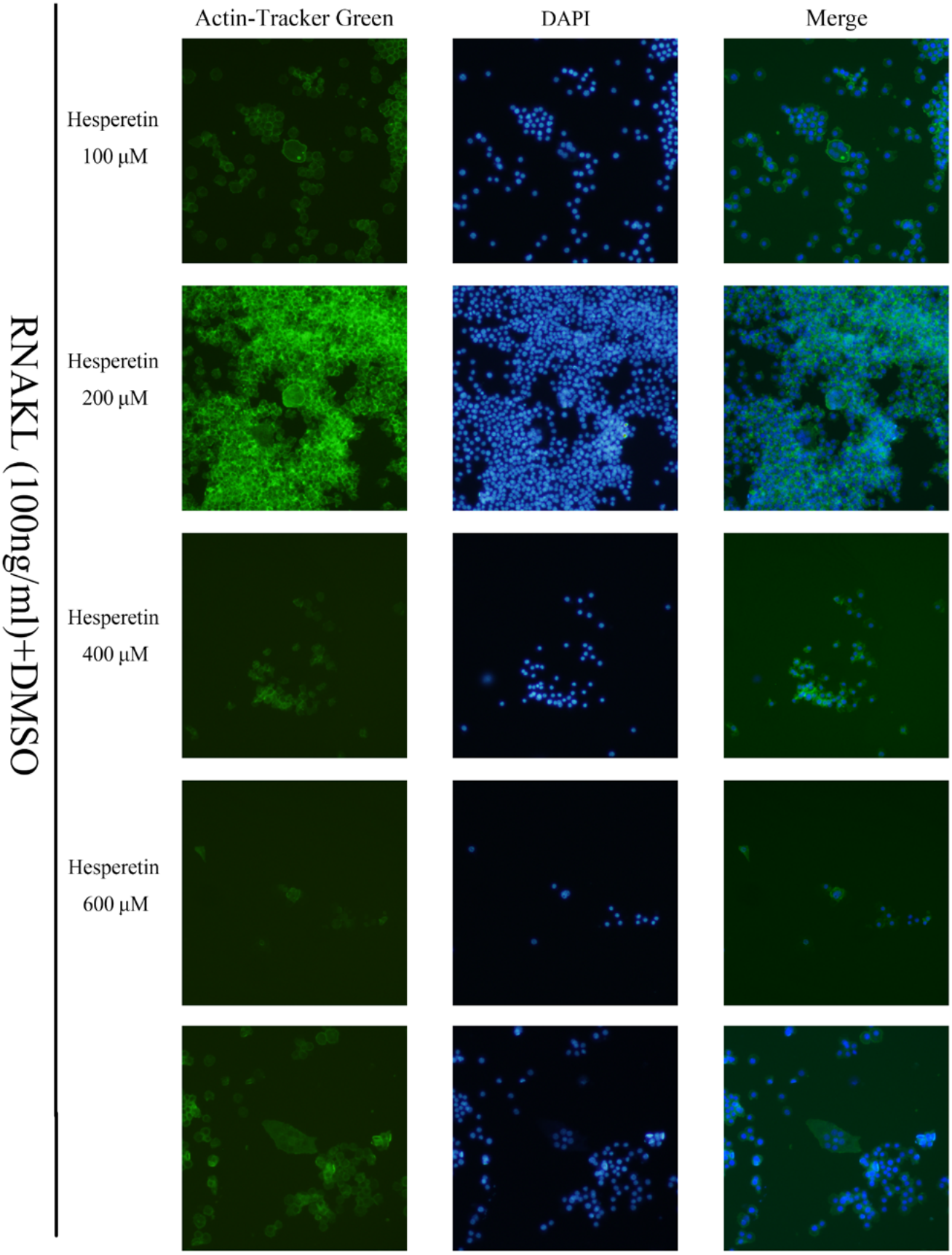

Fig. 3. Hesperetin reduced the number of osteoclast-like nuclei and decreased the cell volume. The RAW264.7 cells were inoculated in 96-well cell plates, cultured overnight, and induced by adding RANKL cytokine. The procedure was followed by the addition of different concentrations of hesperetin for each experimental group. Incubation was continued in a CO_2 incubator. At the end of the culture, the medium was aspirated and discarded to add paraformaldehyde for fixation, followed by actin-tracker green staining and DAPI (4',6-diamidino-2-phenylindole) staining. When the staining was completed, the sample was immediately observed under an inverted fluorescent microscope and photographed (×100)

Effects of hesperetin on the expression of key proteins of MAPK signaling family in osteoclast-like cells

The western blot experiments showed that the expression of phospho-ERK, phospho-JNK, and phospho-p38, which are key proteins of the MAPK signaling family, changed with the increase in hesperetin concentration relative to the DMSO group. The phospho-p38 and phospho-ERK protein levels were not significantly inhibited by the increase in hesperetin concentration, but phospho-JNK protein levels decreased (Fig. 4).

Effects of key protein inhibitors of MAPK signaling pathway on osteoclast-like cell differentiation

The TRAP staining showed that the formation of osteoclast-like cells was significantly inhibited in the groups treated with the MAPK signaling pathway protein inhibitors FR180204 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) (Fig. 5A). The number of TRAP-positive cells was significantly reduced in the groups treated with FR180204 (ERK inhibitor, 11 ±0.33), SP600125 (JNK inhibitor, 10 ±1), and SB203580 (p38 inhibitor, 9 ±0.33) compared with that in the control group (23.33 ±0.51, Fig. 5B).

Discussion

In this paper, the differentiation of osteoclast-like cells was inhibited with increasing concentrations of hesperetin, as evidenced by a decrease in the number of multinucleated cells. In addition, the number of nuclei in the differentiated osteoclast-like cells was reduced, and the cell size decreased in the high-hesperetin-concentration groups. Further studies revealed that hesperetin also affected the level

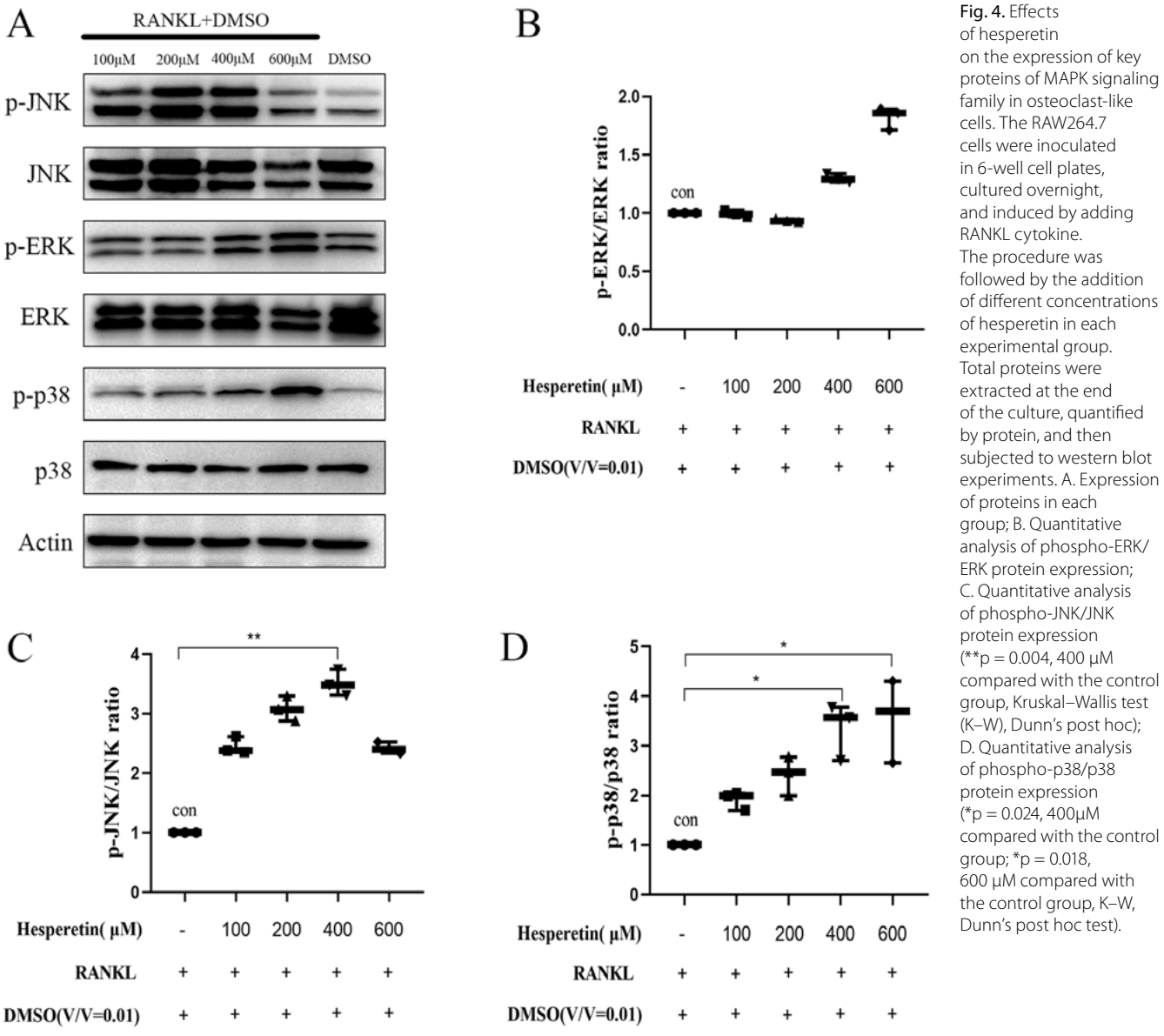

Fig. 4. Effects of hesperetin on the expression of key proteins of MAPK signaling family in osteoclast-like cells. The RAW264.7 cells were inoculated in 6-well cell plates, cultured overnight, and induced by adding RANKL cytokine. The procedure was followed by the addition of different concentrations of hesperetin in each experimental group. Total proteins were extracted at the end of the culture, quantified by protein, and then subjected to western blot experiments. A. Expression of proteins in each group; B. Quantitative analysis of phospho-ERK/ERK protein expression; C. Quantitative analysis of phospho-JNK/JNK protein expression (**p = 0.004, 400 μM compared with the control group, Kruskal–Wallis test (K–W), Dunn's post hoc); D. Quantitative analysis of phospho-p38/p38 protein expression (*p = 0.024, 400μM compared with the control group; *p = 0.018, 600 μM compared with the control group, K–W, Dunn's post hoc test).

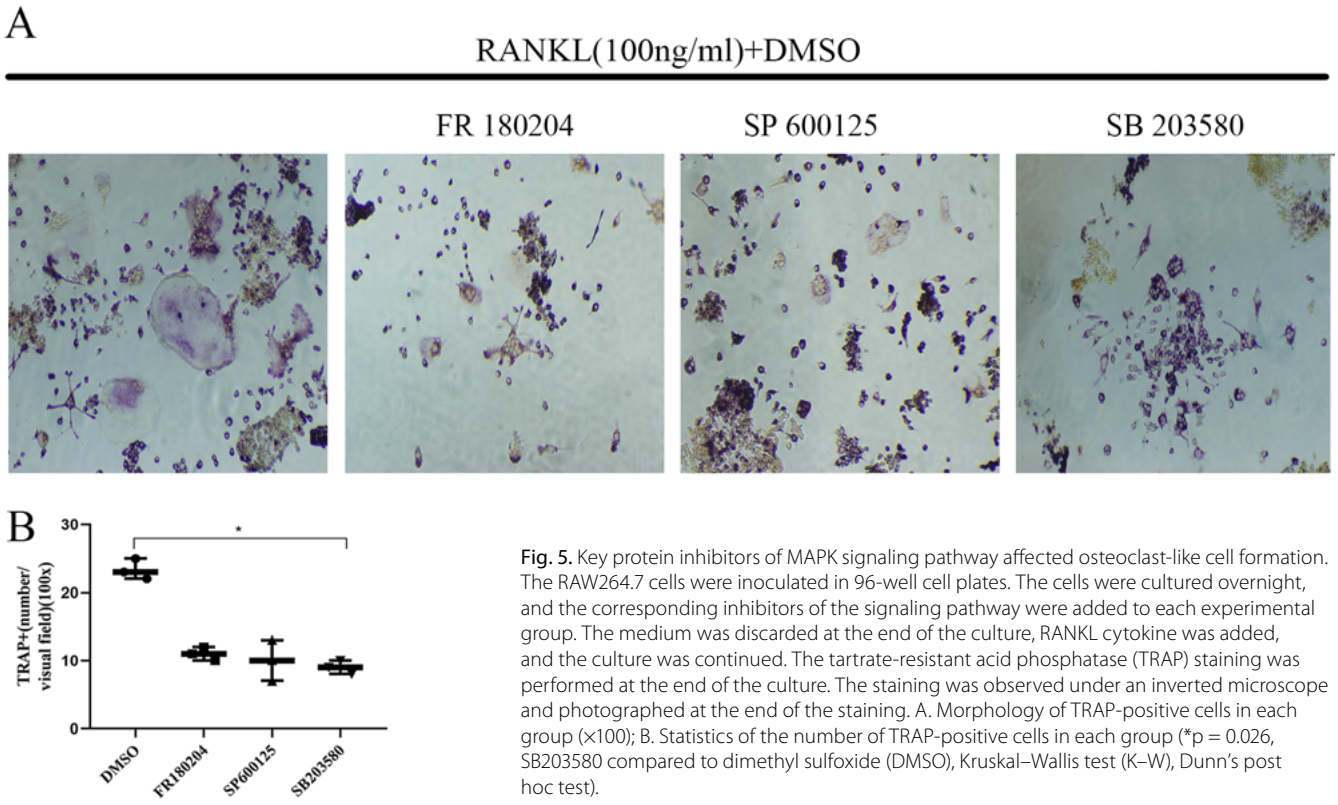

Fig. 5. Key protein inhibitors of MAPK signaling pathway affected osteoclast-like cell formation. The RAW264.7 cells were inoculated in 96-well cell plates. The cells were cultured overnight, and the corresponding inhibitors of the signaling pathway were added to each experimental group. The medium was discarded at the end of the culture, RANKL cytokine was added, and the culture was continued. The tartrate-resistant acid phosphatase (TRAP) staining was performed at the end of the culture. The staining was observed under an inverted microscope and photographed at the end of the staining. A. Morphology of TRAP-positive cells in each group (×100); B. Statistics of the number of TRAP-positive cells in each group (*p = 0.026, SB203580 compared to dimethyl sulfoxide (DMSO), Kruskal–Wallis test (K–W), Dunn's post hoc test).

of phospho-JNK, a key signaling protein of the MAPK signaling pathway, in the differentiated osteoclast-like cells. Hesperetin is a dihydroflavonol compound with molecular formula C16H14O6 and a molecular weight of 302.28 Da. Its structure contains ketocarbonyl, ether, methoxy, and several phenolic hydroxyl groups, which give it a wide range of pharmacological effects. Hesperetin has been shown to have antibacterial, anti-inflammatory, antioxidant, antitumor, and immune-modulating effects.[21] In the present study, the effects of hesperetin on the formation of osteoclast-like differentiation were investigated, and we found that hesperetin affects osteoclast differentiation through the MAPK signaling pathway. The activity of osteoclasts in the organism is mainly reflected by the ability to differentiate bone marrow mesenchymal stem cells into osteoclasts and the strength of their osteolytic function.[22] Osteoclasts are multinucleated giant cells that originate from clones of mononuclear macrophages differentiated from hematopoietic stem cells. They have an abundant intracytoplasmic enzyme system and a series of signature proteins that can be used as markers to identify osteoclasts and determine their differentiation stage. Among them, TRAP is a specific marker for osteoclasts, and TRAP staining that shows TRAP activity in the cytoplasm of osteoclasts is a specific staining method for identifying these cells.[23] Our TRAP staining results highlighted that the formation of osteoclast-like cells was inhibited with the increase in hesperetin concentration. The cytoskeleton is a three-dimensional (3D) network structure composed of microtubules, microfilaments, and intermediate fibers in eukaryotic cells. Their main functions are to maintain the structure and morphology of cells, influence cell motility,[24] and play an important role in proliferation, differentiation, and apoptotic activities. Moreover, microfilaments play a key role in the attachment of osteoclasts to the bone matrix, and the microenvironment formed by the adhesion of osteoclasts to the bone matrix is a prerequisite for osteoclasts to undergo bone resorption. Therefore, the dynamic changes in microfilaments could be used as an indicator of osteoclast activity. This activity could be determined by observing the microfilament-mediated attachment, migration, and the "osteolytic" function of osteoclasts on the bone matrix.[25] In the present study, actin-tracker green staining revealed that hesperetin disrupted the cytoskeleton (the cell outline maintained by microfilaments) in osteoclast-like cells, which then reduced the cell size and inhibited their differentiation into osteoclast-like cells. Reports have shown that the actin ring is a cytoskeletal protein unique to osteoclasts that undergo bone resorption.[26] Future investigations will likely focus on whether hesperetin could disrupt the structure of the actin ring and pseudopods in osteoclasts, thereby affecting bone resorption activity.

We found that RANK within osteoclasts and their precursors could activate 3 signaling pathways of the MAPK family, namely, ERK, JNK, and p38, which promote osteoclast differentiation and activation.[18] TAK1/TAB2, which is a downstream protein of TARF6 in the RANK signaling pathway, activates JNK with p38 protein.[27] Furthermore, downstream signaling of the ERK and JNK pathways include AP-1 transcription factors, Fos family

dimers (c-Fos, FosB, Fra-1, and Fra-2) and Jun family (c-Jun, JunB, and JunD).[28] ERK could induce and activate c-Fos, while JNK could enhance the transcriptional activity of AP-1 through the phosphorylation of c-Jun. AP-1 could also initiate the encoding of genes, such as matrix metalloproteinases (MMPs), which promote the differentiation, survival, and fusion of osteoclast precursors and the activation of mature osteoclasts. Activated ERK enters the nucleus and stimulates the transcription factor Elk, which binds to a sequential regulatory element in the c-Fos gene promoter and controls the transcription of the c-Fos gene. This results in mature macrophages being converted into osteoclast precursors.[29] In the present study, the phospho-p38 and phospho-ERK protein levels were not significantly inhibited by the increase in hesperetin concentration, but the phospho-JNK protein levels were reduced. Further studies revealed that the inhibitors of the key proteins of the MAPK signaling pathway could inhibit the differentiation and formation of osteoclasts. These results suggest that the MAPK signaling pathway is involved in regulating osteoclast-like cell formation, and that hesperetin could affect this process by downregulating the phosphorylation level of JNK.

Limitations

This study only focused on the in vitro cellular model, making the results and findings relatively limited. In vivo animal experiments are needed to further validate the effects of hesperetin on osteoclast differentiation.

Conclusions

Hesperetin inhibits the differentiation process of osteoclast-like cells, and further studies revealed that hesperetin could also affect the activation level of phospho-JNK, a key signaling protein of the MAPK signaling pathway, in differentiated osteoclast-like cells.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10020626. The package contains the following files:

Supplementary Fig. 1. Detailed results of MTT experimental data analyzed with Kruskal-Wallis method.

Supplementary Fig. 2. Detailed results of TRAP experimental data analyzed with Kruskal-Wallis method.

Supplementary Fig. 3. Detailed results of Western blot experimental data analyzed with Kruskal-Wallis method.

Supplementary Fig. 4. Detailed results of Effects of key protein inhibitors of MAPK signaling pathway data analyzed with Kruskal-Wallis method.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

ORCID iDs

Jingxian Fan https://orcid.org/0009-0005-3029-0453
Chengfeng Xu https://orcid.org/0009-0003-6776-4400
Hui Shi https://orcid.org/0009-0002-3202-4851
Xun Wang https://orcid.org/0009-0000-1595-2344
Tiantian Zheng https://orcid.org/0009-0004-4301-0230
Minyu Zhou https://orcid.org/0009-0003-1792-3768
Zhiqiang Zhang https://orcid.org/0009-0007-4155-2249
Yingxiao Fu https://orcid.org/0009-0002-1963-8138
Baoding Tang https://orcid.org/0009-0009-3153-5175

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