Ferric Ion Induction of Triggering Receptor Expressed in Myeloid Cells-2 Expression and PI3K/Akt Signaling Pathway in Preosteoclast Cells to Promote Osteoclast Differentiation

Lu-lin Liu, MM1,2†, Zi-hou Cao, MM1†, Chun-lei He, MM2, Yan-chun Zhong, MM2, Wu-yang Liu, MM2, Peng Zhang, MD1, Fan Yang, MD3, You-jia Xu, MD1,3

1Department of Orthopaedics, The Second Affiliated Hospital of Soochow University and 3Osteoporosis Institute of Soochow University, Suzhou and 2Department of Orthopaedics, The First Affiliated Hospital of Gannan Medical University, Ganzhou, China

**Objective:** Iron plays a significant role in multiple biological processes. The purpose of this study was to measure whether iron mediated osteoclast differentiation through regulation of triggering receptor expressed in myeloid cells-2 (Trem-2) expression and the PI3K/Akt signaling pathway.

**Methods:** The effects of six different concentrations of ferric ammonium citrate (FAC) (100, 80, 40, 20, 10 and 0 μmol/L) on RAW 264.7 cells proliferation were assessed by Cell Counting Kit-8 (CCK-8) gassay. Tartrate resistant acid phosphatase (TRAP) assay was performed to detect the effects of FAC on osteoclast formation. The expression of osteoclast differentiation-related (TRAP, NFATc-1, and c-Fos) and Trem-2 mRNA and proteins was analyzed by reverse transcription-polymerase chain reaction and western blot, respectively. Si-Trem-2 was constructed and transfected to RAW264.7 to measure the effects of Trem-2 on FAC-mediated osteoclast formation. TRAP assay and osteoclast differentiation-related gene analyses were further performed to identify the role of Trem-2 in osteoclastogenesis. The Search Tool for the Retrieval of Interacting Genes (STRING) was used to explore the target genes of Trem-2. Trem-2-related gene ontology and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were used for further in-depth analysis. PI3K/Akt pathway-related proteins were detected by immunofluorescence and western blot.

**Results:** In groups with FAC concentration of 10 (102.5 ± 3.1), 20 (100.5 ± 1.5), and 40 μmol/L (98.7 ± 3.1), compared with the control group (100.1 ± 2.2), cell viability was not significantly different from the control (P > 0.05). When the concentration of FAC exceeded 80 μmol/L, cell viability was significantly decreased (87.5 ± 2.8 vs 100.1 ± 2.2, P < 0.05). FAC promotes Trem-2 expression and osteoclast differentiation in a dose-response manner (P < 0.05). The number of osteoclast-like cells was found to be reduced following transfection with the siRNA of Trem-2 (42 ± 3 vs 30 ± 5, P < 0.05). We observed that most of Trem-2 target genes are primarily involved in response to organic substance, regulation of reactive oxygen species metabolic process, and regulation of protein phosphorylation. The STRING database revealed that Trem-2 directly target two gene nodes (Pik3ca and Pik3r1), which are key transcriptional cofactors of the PI3K/Akt signaling pathway. KEGG pathways include the “PI3K-Akt signaling pathway,” the “thyroid hormone signaling pathway”, “prostate cancer,” the “longevity regulating pathway,” and “insulin resistance.” Expression of p-PI3K and p-Akt protein, measured by immunofluorescence and western blotting, was markedly increased in the FAC groups. Trem-2 siRNA caused partial reduction of these two proteins (p-PI3K and p-Akt) compared to the FAC alone group.
Introduction

Osteoporosis is a common orthopaedic disorder characterized by low bone mass and volume and microstructural degradation of bone. Individuals with osteoporosis have an increased risk of fractures. In addition, osteoporosis is associated with a high rate of morbidity and mortality in the elderly. The number of osteoporosis patients in the USA is predicted to reach 61 million by 2020, while patients with osteoporosis that are >50 years of age in China had already reached 69.44 million in 2006. The incidence of osteoporosis in postmenopausal women is reported to be between 30% and 55%. With a worldwide aging population, the incidence of osteoporosis is rising year-on-year. Normal bone metabolism requires a balance of bone resorption (osteoclast activities) and bone formation (osteoblast activities). With the disruption of this balance when bone resorption exceeds the bone formation, the incidence of fractures due to osteoporosis may increase.

At present, therapeutic agents of osteoporosis include osteoporosis-specific medication (bisphosphonate and teriparatide), life factor change (smoking cessation, diet and exercise), as well as increased calcium (Ca) and vitamin D intake. However, these anti-osteoporosis therapies do not achieve satisfactory results because the precise mechanism of osteoporosis remains unclear. Moreover, potential complications, including a few serious complications, have continued to be reported. One of the most severe complications of the bisphosphonate treatment is osteonecrosis of the jaw. Osteonecrosis of the jaw is a serious complication, currently with no specific therapy. Thus, revealing the pathogenesis of osteoporosis is an urgent problem to prevent it and treat it.

Various risk factors are associated with osteoporosis, including age, obesity, and excessive alcohol consumption. The etiology and pathogenesis of osteoporosis have not yet been fully clarified. Iron is present in many proteins and is indispensable for life. Recent research studies revealed that bone tissues were new targets of iron. Iron plays a significant role in multiple biological processes, including oxygen transport, enzymatic reactions, protein synthesis, and DNA repair. Weinberg et al. found that osteoporosis was closely associated with iron overload and that lactoferrin can promote bone growth. In particular, iron overload may lead to osteopenia or even osteoporosis. In addition, in vitro experiments demonstrated that iron overload decreases osteoblast activity and bone deposition by inducing osteoblast apoptosis. Further in vivo research found that iron overload significantly promotes the degradation of type I collagen. These results suggest that iron overload might induce bone loss; however, the specific molecular mechanisms require further investigation.

Osteoclasts play an essential role in regulating bone homeostasis. Osteoclasts are derived from osteoclast precursor cells, which originate from the monocyte/macrophage lineage. Osteoclasts are the main cells responsible for bone resorption and, subsequently, bone loss. The excessive activity of osteoclasts leads to osteoporosis, osteoarthritis, and rheumatoid arthritis. Our previous study found that iron overload facilitates osteoclast differentiation and bone resorption by regulating the reactive oxygen species (ROS). However, in-depth exploration of iron overload for osteoclastogenesis is lacking. Therefore, it is imperative to explore the special mechanism of iron overload in cell differentiation to osteoclast from osteoclast precursor cells.

Triggering receptor expressed in myeloid cells-2 (Trem-2) is an immunoglobulin-like cell surface receptor and functions as a co-receptor common to RANKL receptor. It has been shown that osteoclast differentiation is dramatically arrested in Trem-2 deficient patients. These results suggest that Trem-2 is a key mediator of the osteoclast differentiation to osteoclastic bone resorption. However, whether Trem-2 is involved in the iron overload-mediated osteoclast differentiation is not yet known.

Triggering receptor expressed in myeloid cells-2 also regulates multiple signaling pathways, including the TLR, p38, and PI3K pathways. The key molecules involved in the PI3K/Akt pathway include PTEN, PI3K, and Akt. Upon PI3K activation, Akt is recruited to the membrane where AKT is phosphorylated (pAKT). The PI3K/Akt pathway is a key signaling pathway that regulates cell proliferation and differentiation. Some studies have shown that the PI3K/Akt pathway is involved in endothelial progenitor cell differentiation, osteosarcoma migration, and osteoblast differentiation. Recently, other studies have characterized the PI3K/Akt signaling pathway also involved in the osteoclast differentiation. Therefore, Trem-2 mediated the PI3K/Akt signaling pathway, which demonstrated crucial roles in the control of cell differentiation, and certainly played a key role in the osteoclastogenesis. However, whether iron overload affecting Trem-2 mediated the PI3K/Akt signaling pathway was unknown.

We performed microarray analysis first and validated differentially expressed genes between iron overload and control groups in male fractured mice. Among these differentially expressed mRNA, we revealed that Trem-2 is more dramatically increased in the iron overload than control group (unpublished data). Considering the possible role and function.

Conclusion: The FAC promoted osteoclast differentiation through the Trem-2-mediated PI3K/Akt signaling pathway. However, its regulation osteoclastogenesis should be verified through further in vivo studies.

Key words: Ferric ammonium citrate; Osteoclast differentiation; PI3K/Akt; Trem-2
of Trem-2 in osteoclast differentiation, we speculated that iron overload regulates Trem-2 and subsequently activates the PI3K/Akt signaling pathway to stimulate osteoclast differentiation.

Therefore, the purpose of this study was to examine: (i) the optimal dose of iron for RAW 264.7 cell proliferation; (ii) whether iron overload exhibited a positive role in osteoclast differentiation; and (iii) whether the promotion effects of iron overload for osteoclast differentiation was mediated by the Trem-2/PI3K/Akt axis.

Materials and Methods

Cell Culture
Mouse macrophage cell line RAW 264.7 was acquired from Procell (Wuhan, China) and cultured in the low-glucose Dulbecco’s modified Eagle’s medium with 10% FBS and 1% antibiotics. All cells were cultured at 37°C at 5% CO₂ in a humidified atmosphere. RAW 264.7 at passage 3–5 were used for the following experiments.

Cell Counting Kit-8
The CCK-8 (Dojindo, Kumamoto, Japan) assay was performed to determine the viability of cells in a control group and cells with different concentrations of ferric ammonium citrate (FAC). In brief, RAW264.7 were seeded in 96-well culture plates and received varying concentrations of FAC citrate (FAC). In brief, RAW264.7 were seeded in 96-well plates. The cells were cultured at 37°C for 24 h. Then, CCK-8 solution (10 μL) was added to each well and then incubated at 37°C for 1.5 h. The absorbance was read at 450 nm using a microplate reader. Absorbance was read at 450 nm and was recorded and calculated as the mean absorbance normalized to the control.

Tartrate Resistant Acid Phosphatase Staining
The activities of FAC on osteoclasts were visualized using TRAP staining kit (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) according to the instruction manual. Osteoclast-like cells were characterized as TRAP-positive osteoclasts with >3 nuclei.

Polymerase Chain Reaction
Total RNA of RAW264.7 cells were extracted with TRIzol (Takara Bio, Otsu, Shiga, Japan) solution. Afterwards, mRNA levels were determined using the PrimeScript RT Reagent Kit (Takara, Japan) on the polymerase chain reaction (PCR) system (7300 Real-Time PCR System, Applied Biosystems). The primers used were designed and synthesized by GeneChem (Shanghai, China), represented as follows:

- Trem-2 forward 5'-CGTGCCGTTATTGGTATA AAC-3', reverse 5'-GAGCAGGGTCCGAGGT-3'; GAPDH forward 5'-GAGTCCACTGGCGTCTTCAC-3', reverse 5'-ATCTTGAAGCTGTTGCTACATTCTTC-3'.

The 2^ΔΔCt method was used to calculate the cycle threshold value of each sample with GAPDH as the internal reference.

Western Blot Analysis
The protein expression level of osteoclast-related was determined by western blot analysis. RAW264.7 was lysed in RIPA lysis buffer including protease inhibitor phenylmethylsulfonyl fluoride for protein extraction. To evaluate the concentration of protein, the BCA method was applied. Isolated protein was denatured for 5 min at 95°C. Next, 20 μg protein for each well was separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride (PVDF) membrane. Next, 5% fat-free milk was used to block the PVDF membrane for 1 h at 37°C. Primary antibodies were prepared to incubate the PVDF membrane at 4°C overnight. After draining with TBST (three times for 5 min), the membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h at 37°C. The enhanced chemiluminescence reagent was used to visualize protein bands and the analysis of western blot was performed using image J software.

The antibodies (Abcam, Cambridge, MA, USA) included primary antibodies PI3K (Abcam, Cambridge, UK, 1:30000), p-PI3K antibody (Abcam, Cambridge, UK, 1:100), Akt (Abcam, Cambridge, UK, 1:3000), p-Akt (Abcam, Cambridge, UK, 1:3000), GAPDH (Abcam, Cambridge, UK, 1:2500) and a corresponding HRP-labeled secondary antibody (Abcam, Cambridge, UK, 1:50000).

Fig. 1 Effects of ferric ammonium citrate (FAC) in different concentrations (100, 80, 40, 20, 10, and 0 μmol/L) on RAW264.7 cell proliferation via CCK-8 assay. *P < 0.05 versus FAC at concentration of 0 μmol/L.
Immunofluorescence Assay

Immunofluorescence assay was performed as described previously. The RAW264.7 was seeded in 6-well plates at a density of 2 × 10⁵ cells/well. Twenty-four hours after siRNA transfection, the cells were fixed with 4% paraformaldehyde for 10 minutes and then washed three times with phosphate buffered saline (PBS). The cells were then permeabilized with 0.1% Triton in PBS for 5 min. The cells were incubated with primary antibody against (Abcam, Cambridge, UK, 1:100) and p-Akt (Abcam, Cambridge, UK, 1:3000) overnight. Then, fluorescein isothiocyanate-conjugated antirabbit antibody and Alexa Fluor-conjugated antirabbit antibody were incubated for 2 h. Fluorescence was observed under a confocal microscope (Olympus, Tokyo, Japan).

Bioinformatic Analysis of Trem-2

A protein–protein interaction (PPI) network of Trem-2 and neighborhood genes was constructed from the seed genes using the STRING Protein–Protein Interaction Network Database (https://string-db.org/). Filter criteria for protein identification was set at the minimum required interaction score of 0.4 and P-value less than 0.05. Furthermore, Trem-2-related gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were used for further in-depth analysis. There were three independent GO categorization hierarchies, including biological process (BP), cellular component (CC), and molecular function (MF).

Statistical Analysis

Data were shown as mean ± standard deviation and comparisons in more than three groups were analyzed by one-way ANOVA followed by Tukey’s post-hoc test. Comparisons of two groups were assessed with Student’s t-test. Statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, USA) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). A value of P < 0.05 was considered statistically significant.

Fig. 2 Effect of different concentrations of ferric ammonium citrate (FAC, 40, 20, 10, and 0 μmol/L) on RANKL-induced osteoclast differentiation of RAW264.7 (A). Osteoclast differentiation-related genes and triggering receptor expressed in myeloid cells-2 (Trem-2) gene expression analysis in different concentrations of ferric ammonium citrate (FAC) were examined by quantitative reverse transcriptase-PCR (B). Osteoclast differentiation-related proteins and Trem-2 protein expression analysis in different concentrations of ferric ammonium citrate (FAC) were examined by quantitative western blotting (C). *P < 0.05 versus FAC at concentration of 0 μmol/L.
Results

Viability of Cells

To study the function of FAC in osteoclast differentiation of RAW264.7, the dose-dependent effects and optimal concentrations need to be determined. CCK-8 with FAC concentration of 10, 20, 40, 80, and 100 μmol/L were determined, respectively. As illustrated in Fig. 1, in groups with FAC concentration of 10, 20, and 40 μmol/L, compared with the control group, cell viability was not significantly different from the control ($P > 0.05$, Fig. 1). When the concentration of FAC exceeded 80 μmol/L, cell viability was significantly

![Image of Fig. 3](image-url)
decreased \((P < 0.05, \text{Fig. 1})\). The concentration \((10, 20, \text{and} 40 \mu\text{mol/L})\) of FAC was selected in the following experiment based on the above results.

**Activities of Ferric Ammonium Citrate**

To further explore whether FAC could promote osteoclast differentiation, TRAP staining was performed in different concentrations \((10, 20, \text{and} 40 \mu\text{mol/L})\) of FAC. The number of osteoclast-like cells was significantly decreased in a dose-dependent manner in the FAC treatment groups compared with the control group \((P < 0.05, \text{Fig. 2A})\). The effects of FAC for RAW264.7 on the expression of genes related to osteoclast differentiation induced by RANKL were investigated. FAC increased the osteoclast marker genes such as NFATc1, TRAP, and c-Fos in a dose-dependent manner; Trem2 also increased in a dose-dependent manner \((P < 0.05, \text{Fig. 2A})\). The results of western blotting were consistent with those of qRT-PCR \((\text{Fig. 2C})\). These results indicate that FAC promotes Trem-2 expression and osteoclast differentiation in a dose-response manner.

**Role of Trem-2 in Ferric Ammonium Citrate-Induced Osteoclast Differentiation**

To demonstrate the role of Trem-2 in FAC-induced osteoclast differentiation, RAW264.7 was treated with siRNA to Trem-2, and the TRAP staining and osteoclast differentiation markers were further measured by qRT-PCR. As illustrated in Fig. 3A, FAC significantly increased the number of osteoclasts; however, this promotion effect of FAC was partially attenuated when cells were simultaneously pretreated with siRNA of Trem-2. We further explored the role of Trem-2 siRNA on osteoclast markers \((\text{TRAP, NFATc1, and c-Fos})\). In line with the TRAP staining finding, the expression of osteoclast markers \((\text{TRAP, Cathepsin K, and NFATc1})\) was significantly higher in the FAC treatment group, whereas the Trem-2 siRNA group partially mobilized the promotion effects of FAC \((\text{Fig. 3B})\). The results of western blotting were consistent with those of qRT-PCR \((\text{Fig. 3C})\). Altogether, our data suggest that Trem-2 is indispensable for FAC-mediated osteoclast differentiation.

**Functional Interaction of Trem-2**

To reveal functional interaction of the Trem-2, we performed PPI network analysis and identified two gene nodes \((\text{Pik3ca and Pik3r1})\) in the interaction network of Trem-2 \((\text{Fig. 4A})\). The GO enrichment analysis resulted in the identification of 85 enriched GO terms \((24 \text{ BP, 6 CC, and 52 MF})\). Biological processes of the top 10 GO-enriched terms are shown in Fig. 4B. We observed that most of these genes are primarily involved in response to organic substance, regulation of the ROS metabolic process, and regulation of protein phosphorylation. For CC, neighboring genes of Trem-2 mainly function as a protein-containing complex and plasma membrane bounded cell projection \((\text{Fig. 4C})\).
Notably, 52MF terms were found to be enriched, including transcription factor binding, protein phosphatase binding, protein kinase binding, and protein kinase activity (Fig. 4D). According to the results, we noticed that KEGG pathways included "PI3K-Akt signaling pathway," "thyroid hormone signaling pathway," "prostate cancer," "longevity regulating pathway," and "insulin resistance" (Fig. 4E). We therefore examined the effect of Trem-2 knockdown on the PI3K/Akt signal transduction pathway.

**Association Between Triggering Receptor Expressed in Myeloid Cells-2 and PI3K/AKT Signaling Pathway**

The PI3K/AKT signaling pathway is a crucial regulator of cell differentiation. In the current study, we first detected the expression of p-PI3K and p-Akt in RAW264.7 cells after infection with Trem-2-siRNA. Expression of p-PI3K (Fig. 5A) and p-Akt (Fig. 5B) protein, as measured by immunofluorescence, was markedly increased in FAC groups. There was partial reduction of these two proteins (p-PI3K and p-Akt) by adding the Trem-2 siRNA. In RAW264.7 cells, we further assayed the expression of total-PI3K, total-AKT, p-PI3K, and p-AKT by western blotting. The difference in protein expression of total-PI3K and total-AKT between these three groups was the same for each protein loading. However, phosphorylation of PI3K and AKT was notably decreased in RAW264.7 cells in the si-Trem-2 group compared to the FAC group (Fig. 6). Taken together, these data demonstrated that the PI3K/Akt pathway mediates FAC-induced Trem-2 expression for osteoclast differentiation.

**Discussion**

**Main Findings**

The key findings of this study are as follows: (i) we found that FAC significantly enhanced osteoclast differentiation;
and (ii) we identified that Trem-2 deficiency inhibited osteoclast differentiation through regulation of the PI3K/Akt signaling pathway.

**Potential Function of Ferric Ammonium Citrate in Osteoclastogenesis**

In previous research, our team has found that FAC plays a vital role in promoting osteoclast differentiation through regulation of reactive oxygen in an animal model. To explore the role of FAC on RANKL-induced osteoclast differentiation, we first explored the cytotoxicity of FAC in RAW264.7 using CCK-8 assays. When FAC concentration was 80 μmol/L, the cytotoxicity was 25%, and when 100 μmol/L of FAC was added, the cytotoxicity was reduced to 34%. Thus, concentration of FAC between 10 to 40 μmol/L was selected for further study. FAC dose-dependently promoted osteoclast differentiation, and the osteoclasts were three times higher at 40 μmol/L FAC.

Many extracellular receptors recognize FAC, including transferrin receptor (CD71). FAC Trem-2 was upregulated during osteoclast differentiation, which suggests that Trem-2 is involved in the control of osteoclast differentiation or function. Another most significant finding in this study was that silencing Trem-2 attenuates FAC-induced osteoclast differentiation through downregulation of PI3K and Akt, which is a key modulator for osteoclast differentiation. It is known that Trem-2 promotes osteoclast differentiation and Trem-2-deficient individuals would have impaired bone resorption in vitro. Moreover, the induction of ROS also requires the activity of Trem-2. Trem-2 promotes macrophage killing by enhancing ROS production but not nitric oxygen production.

**Trem-2 Affecting PI3K/Akt Signaling Pathway**

Bioinformatic analysis results revealed that Trem-2 directly targets Pik3ca and Pik3r1, which are two key proteins of the PI3K/Akt signaling pathway. Trem-2 and neighboring genes are mainly enriched in the PI3K/Akt signaling pathway. Further in-depth analysis of these data revealed that siRNA of Trem-2 could significantly decrease the phosphorylation of PI3K and Akt. It is well known that the PI3K/Akt signaling pathway is involved in the osteoclast differentiation.

**Conclusion**

The data presented here support the hypothesis that FAC plays important roles in osteoclast differentiation by regulating the Trem-2-mediated PI3K/Akt signaling pathway. Further study using Trem-2 knockout mice to determine the role of Trem-2 in the iron overload-induced osteoporosis may provide a new therapeutic strategy for bone diseases.

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