# ORIGINAL RESEARCH Deferoxamine-induced high expression of TfRI and DMTI enhanced iron uptake in triple-negative breast cancer cells by activating IL-6/PI3K/AKT pathway

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Background: Deferoxamine (DFO) is a commonly used iron chelator, which can reduce the iron levels in cells. DFO is normally used to treat iron-overload disease, including some types of cancer. However, our previous studies revealed that DFO treatment significantly increased the iron concentrations in triple-negative breast cancer cells (TNBCs) resulting in enhanced cell migration. But the mechanism of DFO-induced increasing iron uptake in aggressive TNBCs still remained unclear.

Materials and methods: Iron metabolism-related proteins in aggressive breast cancer MDA-MB-231, HS578T and BT549 cells and nonaggressive breast cancer MCF-7 and T47D cells were examined by immunofluorescence and Western blotting. The possible regulatory mechanism was explored by Western blotting, co-incubation with neutralizing antibodies or inhibitors, and transwell assay.

Results: In this study, we found that DFO treatment significantly increased the levels of iron uptake proteins, DMT1 and TfR1, in aggressive TNBCs. Moreover, both TfR1 and DMT1 expressed on cell membrane were involved in high iron uptake in TNBCs under DFOinduced iron deficient condition. For the possible regulatory mechanism, we found that DFO treatment could promote a high expression level of IL-6 in aggressive MDA-MB-231 cells. The activated IL-6/PI3K/AKT pathway upregulated the expression of iron-uptake related proteins, TfR1 and DMT1, leading to increased iron uptakes.

**Conclusion:** We demonstrated that DFO could upregulate expression of TfR1 and DMT1, which enhanced iron uptake via activating IL-6/PI3K/AKT signaling pathway in aggressive TNBCs.

Keywords: deferoxamine, iron uptake, TfR1, DMT1

#### Introduction

A large body of data indicates that excessive accumulation of iron in the body may increase the risk factor of breast cancer.<sup>1-4</sup> Breast cancer undergoes broad changes in iron metabolism and exhibits increased ability to iron sequestration.<sup>5,6</sup> Numerous studies suggest that the iron level is much higher in the tissues and serum of the breast cancer patients than that in healthy people.<sup>3,7,8</sup> Iron accumulation in breast cancer cells promotes breast cancer initiation, growth and metastasis.<sup>9–12</sup>

Deregulation of cellular iron metabolism is reflected by the altered expression of iron-regulatory proteins in human breast cancer cells. The expression of iron import proteins, such as transferrin receptor (TfR1) and iron-storage protein, ferritin in aggressive breast cancer cells are frequently elevated, while the levels of the iron

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efflux protein, ferroportin is suppressed.<sup>5,13–16</sup> As a transmembrane receptor protein, TfR1 combines with iron transport protein transferrin (Tf) and assists iron uptake through endocytosis.<sup>17</sup> Divalent metal transporter 1 (DMT1) is identified as the non-transferrin-bound serum iron (NTBI) pathway, responsible for ferrous iron uptake.<sup>18,19</sup> DMT1 is also located on the surface of cell membrane and can transport extracellular ferrous ions.<sup>20,21</sup> Many studies have shown that irregular iron metabolism and the activated iron-uptake related pathways in breast cancer cells disrupt the iron regulatory network.<sup>22–24</sup>

Iron chelators could be used to prevent tumor progression in patients.<sup>25</sup> Deferoxamine (DFO) is the first iron chelator to be used as an anticancer drug. In our previous study, we used DFO to determine the iron metabolism in breast cancer cells.<sup>26</sup> Our results indicated that DFO could decrease iron concentration in non-aggressive estrogen receptor-alpha (ER $\alpha$ ) -positive breast cancer cell lines, while increase iron concentration in aggressive triplenegative breast cancer cell lines (TNBCs) leading to enhance migration of breast cancer cells.<sup>26</sup> But the mechanism of the increased iron uptake in TNBCs under the iron deficient condition induced by DFO was still remained unclear.

In order to investigate iron uptake in aggressive TNBCs under the iron-deficient condition induced by DFO, we hypothesized that DFO could up-regulate the expression of iron-related proteins to enhance iron uptake in aggressive TNBCs. In this study, three aggressive triplenegative breast cancer cells, Hs578T, BT549, MDA-MB -231, and two non-aggressive ERα-positive breast cancer cells, T47D, MCF-7, were used as cellular models treated with DFO to regulate intracellular iron metabolism to investigate the link between the altered iron concentration and the expression of iron-related proteins. The possible DFO-induced iron regulatory pathways in aggressive TNBCs were also investigated. Our study was shown that iron uptake-related proteins such as DMT1 and TfR1 expression were increased after 24 h following DFO treatment in aggressive TNBCs, which were involved in increasing intracellular iron uptake by activating IL-6/ PI3K/AKT pathways.

# Materials and methods

## Cell culture

Triple-negative breast cancer cell lines (Hs578T, BT549, MDA-MB-231), and ER $\alpha$ -positive breast cancer cell lines

(T47D, MCF-7) were purchased from ATCC, USA. MCF-7, T47D, MDA-MB-231, and Hs578T cells were cultured at 37 °C in an atmosphere of 5 % CO<sub>2</sub> and 95% air in DMEM with 10% FBS. BT549 cells were cultured at 37 ° C in an atmosphere of 5% CO<sub>2</sub> and 95% air in Roswell Park Memorial Institute-1640 medium (RPMI-1640) with 10% FBS.

# Inductively coupled plasma mass spectroscopy (ICP-MS) analysis

Collected cells and samples were digested in nitric acid (Sigma) and diluted with  $DDH_2O$ . The ICP-MS (Thermo Fisher Scientific) was used to determine the iron content of samples. Iron concentration was normalized to the weight or cells number of each sample. Each experiment was repeated 3 times.

## Migration assay

A 6.5 mm (0.8 µm pore size) 24-well transwell chamber system (Corning) was used in Migration assay.  $3 \times 10^5$  Cells were suspended in 100 µl serum-free DMEM, and added into the upper chamber. 600 µl DMEM with 10% FBS was added into the bottom of 24-wells. After culture at 37 °C for 24 h, cells were co-incubated with DMT1 and TfR1 neutralizing antibody and DFO for 24 h. The upper chamber was washed with PBS for 10 s 3 times, and the cells on the on the upper layer of the filter were scrubbed. Then the upper chamber was fixed in 4% paraformaldehyde for 10 min, and treated with crystal violet for another 15 min. Images were captured in 5 random fields by an invert Microscope with a 5× objective. 33% acetic acid was used to dissolve the migrated cells which were stained by crystal violet. Migrated cells were measured at OD 595 nm of the eluted crystal violet. All experiments were performed in triplicate.

# Quantitative RT-PCR

Collected  $1 \times 10^6$  cells, and isolation RNA was performed using a Trizol Reagent (Invitrogen). Then cDNAs derived from RNA were made by using PrimeScript RT reagent kit (TaKaRa). Primers were synthesized by Invitrogen. The forward primer sequences for IL-6 are 5'-TCTCCACAAGCGCCTTCG-3' and reverse primer sequences for IL-6 are 5'- CTCAGGGCTGAGATGCCG-3'. RT-PCR was completed on the ABI 7900HT sequence detection system, and SDS software (Applied Biosystems) using SYBR Premix Ex Taq (TaKaRa). All data were normalizations to GAPDH levels, the fold changes were analyzed by the double  $\Delta$ Ct method. All experiments were performed in triplicate.

#### Western blot

Cells were lysed by RIPA. After being shocked and icebath for 30 min, cells were centrifuged for 20 min at 22,000 rpm at 4°C. The supernatants were collected. Cell membrane protein and cytoplasmic protein were separated using Mem-PER<sup>TM</sup> Plus Membrane Protein Extraction Kit (Thermo). The concentration of protein was accessed using BCA. 20-40 µg protein was separated by 8-12.5% SDS-PAGE, then transferred to polyvinylidene fluoride membrane (Immobilon-P) using Trans-Blot Turbo Transfer System (Bio-Rad). DMT1, IRP1, IRP2, TfR1, TfR2, ferritin, T-PI3K, phospho-PI3K antibodies were obtained from Abcam. T-STAT3 (0.1 mg/ml), phospho-STAT3 tyr705 (1 mg/ml) were acquired from R&D. T-ERK, phospho-ERK, pan-AKT, phospho-AKT (1:1000) were obtained from Cell signaling technology. GAPDH and  $\beta$ -actin antibodies were purchased from Biotime. After being cultured with primary antibodies overnight at 4 °C, proteins were cultured with the HRP-labeled secondary antibody (KPL). Proteins were visualized using enhanced chemiluminescence (Pierce). All experiments were performed in triplicate.

#### Immunofluorescence assay

Cells were grown on coverslips inside 24-wells plates overnight. After being crawled on slides, cells were washed by PBS for 3 times, treated with 4% paraformaldehyde for 10 min, and treated in 0.3% Triton-X 100 for 5 min. After being blocked with 5% bovine serum albumin for 1 h, cells were incubated with PBS diluted primary antibodies overnight at 4 °C. Slides were washed by PBS for 3 times, and incubated with fluorescent secondary antibody protect from light for 1 h. Nuclear were stained by DAPI. Confocal laser scanning microscope (Leica TCS SP5) was used to capture images.

### Cultured with antibody or inhibitors

Inhibitor LY294002 (Cell Signaling Technology) was dissolved in DMSO. Cells were pretreated for 1 h with or without LY294002 and then co-cultured with or without 200  $\mu$ M DFO for 24 h. Cells were treated with IL-6, DMT1 or TfR1 neutralizing antibody combined with DFO for 24 h.

#### Statistical analysis

All results were presented as mean  $\pm$  SD. Statistical significance between means was analyzed by one-way analysis of variance. Multiple comparisons were determined with a least significant difference test. Statistically significant was set at *P*-value <0.05.

## Results

# Effects of DFO on the expression of iron metabolism-related proteins in nonaggressive $ER\alpha$ -positive and aggressive tnbcs

In our previous study, we identified that DFO treatment enhanced intracellular iron concentration in aggressive TNBCs leading to enhance cell migration.<sup>26</sup> To address the question why the intracellular iron concentration was increased in aggressive TNBCs after DFO treatment, the expression of iron-uptake and iron-storage proteins, such as DMT1, TfR1, TfR2 and ferritin in non-aggressive ERα-positive breast cancer MCF-7 cells and aggressive triple-negative MDA-MB-231 breast cancer cells were determined by western blotting (Figure 1). In the cell lysate, the expression of TfR2 was not changed after 24 h of DFO treatment in both MDA-MB-231 and MCF-7 cells, as compared with the control group (Figure 1A and C), and on cell membrane, the expression of TfR2 was decreased in both MCF-7 and MDA-MB-231 cells (Figure 1B and D), which indicated that TfR2 would not have the key role of iron uptake in DFO-treated MDA-MB-231 cells. The expression of ferritin was decreased significantly in both MDA-MB -231 and MCF-7 cells after DFO treatment as compared with the control group, respectively (Figure 1A and C). Interestingly, after 24 h following DFO treatment, the expression of DMT1 in the cell lysate and on cell membrane was markedly increased in aggressive MDA-MB-231 cells, while they were decreased significantly in non-aggressive MCF-7 cells (Figure 1A-D), which suggested that DMT1 would participate in iron uptake in MDA-MB-231 cells after 24 h following DFO treatment. Moreover, the protein level of TfR1 was increased in both MDA-MB-231 and MCF-7 cells after DFO treatment as compared with the control group, respectively (Figure 1A-D). The expression of TfR1, DMT1 and ferritin was further identified by using immunofluorescence staining (Figure 2). All the above results were



Figure I Effects of DFO treatment on the expression of iron-uptake and iron-storage proteins in MCF-7 and MDA-MB-231 cells. (**A**) MDA-MB-231 and MCF-7 cells were treated with or without 200  $\mu$ M DFO. Proteins from cell lysate were analyzed using Western blotting. (**B**) TfR1, TfR2 and DMT1 on cell membrane were detected by Western blotting. (**C**, **D**) Western blotting quantification with anti- $\beta$  actin antibody: values were the means of three independent experiments ±SD. \*p<0.05, \*p<0.01, \*\*p<0.01.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; TFR2, transferrin receptor 2; DMT1, divalent metal transporter 1; ns, no statistical difference.

verified using other breast cancer cell lines such as aggressive triple-negative HS578T cells, BT549 cells and no-aggressive ER $\alpha$ -positive breast cancer T47D cells (Figures S1–S3). These results indicated that DFO treatment led to different effects on the expression of iron-related proteins between non-aggressive ER $\alpha$ -positive and aggressive TNBCs. Moreover, we suggested that DFO-increased iron concentration in MDA-MB-231 cells observed in our previous study would be mainly due to the up-regulated expression of TfR1 and DMT1 resulted in enhanced iron uptake under the iron deficient condition induced by DFO.

# TfRI and DMTI enhanced iron uptake in aggressive TNBCs after DFO treatment

To ask the question why DFO treatment increased intracellular iron concentration in MDA-MB-231 cells observed in our previous study, we postulated that TfR1 and DMT1 would be related with increasing iron uptake in MDA-MB





Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1; DAPI, 4',6-diamidino-2-phenylindole; MFI, mean fluorescence intensity.

-231 cells after DFO treatment. To investigate the potential roles of TfR1 and DMT1 in the iron uptake of DFO-treated MDA-MB-231 cells, MDA-MB-231 cells were added with 5 µg/ml DMT1 or TfR1 neutralizing antibody alone, and two neutralizing antibodies together, iron content in DFO-treated or untreated MDA-MB-231 cells were measured by ICP-MS (Figure 3). As shown in Figure 3, iron levels in DFO-treated MDA-MB-231 cells were significantly increased, as compared with that in untreated MDA-MB-231 cells. Interestingly, there was no difference about iron concentration in MDA-MB-231 cells treated with DMT1, TfR1 neutralizing antibody alone and two neutralizing antibodies. However, the iron concentration was obviously decreased in DFO-treated MDA-MB-231 cells with DMT1 or TfR1 neutralizing antibody alone, as compared with DFO-treated MDA-MB-231 cells respectively. Particularly, the iron concentration was markedly reduced in DFO-treated MDA-MB -231 cells with DMT1 and TfR1 neutralizing antibody together. These results were verified using aggressive triplenegative HS578T and BT549 cells (Figure S4). These data indicated that, under normal cell culture condition, DMT1 and TfR1 did not involve in iron uptake in aggressive TNBCs, however, under the iron-deficient condition induced by DFO, DMT1 and TfR1 jointly participated in enhancing



Figure 3 TfR1 and DMT1 enhanced iron uptake in MDA-MB-231 cells after DFO treatment. The iron concentration in DFO-treated or untreated MDA-MB-231 cells with 5  $\mu$ g/ml DMT1, TfR1 neutralizing antibody and both was measured using ICP-MS, respectively. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1; Ab, antibody; ICP-MS, inductively coupled plasma mass spectrometry; ns, no statistical difference.

iron uptake to increase intracellular iron concentration in aggressive TNBCs.

# TfRI and DMTI enhanced iron uptake in aggressive TNBCs leading to promote cancer cell migration under DFO-induced iron-deficient condition

In our previous study, the viability of MDA-MB-231 cells and MCF-7 cells was not significantly changed after 200 µM DFO treatment, but the iron levels in MDA-MB -231 cells significantly increased after 200 µM DFO treatment leading to increase the expression of mesenchymal markers and promote MDA-MB-231 cell migration. The above results verified that the increased intracellular iron concentration in MDA-MB-231 cells after being treated with DFO was attributed to the increased expression of TfR1 and DMT1. We supposed that inhibiting expression of TfR1 and DMT1 would disturb intracellular iron metabolism, which could affect MDA-MB-231 cells migration. To prove the hypothesis, transwell cell migration assay was performed (Figure 4). The results suggested that the migration of MDA-MB-231 cells was promoted after being cultured with DFO for 24 h, as compared with the untreated MDA-MB-231 cells. However, with the addition of DMT1 and TfR1 neutralizing antibody in untreated MDA-MB-231 cells, cell migration was not obviously affected. Especially, in DFO-treated MDA-MB-231 cells with DMT1 and TfR1 neutralizing antibody, cell migration was significantly diminished (Figure 4A and B). The results suggested that DFO treatment enhanced MDA-MB-231 cells migration would be attributed to the up-regulated expression of TfR1 and DMT1 increasing intracellular iron uptake after DFO treatment. These results were verified using aggressive triple-negative HS578T and BT549 cells (Figure S5). Taken together, all these results suggested that TfR1 and DMT1 in aggressive TNBCs could play the vital role of increasing intracellular iron uptake under iron-deficient condition induced by DFO.

# The expression level of IL-6 was increased in DFO-treated triple-negative breast cancer MDA-MB-231 cells

The mechanism for up-regulated expression of TfR1 and DMT1 in triple-negative MDA-MB-231 cells under the iron-deficient condition induced by DFO was still unknown. Previous reports have indicated that TfR is regulated by the PI3K/AKT pathway.<sup>27–29</sup> PI3K/AKT



Figure 4 TfR1 and DMT1 affected MDA-MB-231 cells migration. (A) Cells were allowed to migrate in transwell assays. MDA-MB-231 cells, MDA-MB-231 cells treated with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, DFO-treated MDA-MB-231 cells, DFO-treated MDA-MB-231 cells with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. The images were acquired using an upright microscope with the 10× objective. (B) Quantitative analysis of the cell migration was performed by Image Pro Plus software. \*p<0.05, \*\*p<0.01, \*\*\*p<0.01.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1; Ab, antibody; ns, no statistical difference.

pathway can be regulated by pro-inflammatory cytokine interleukin 6 (IL-6).<sup>30-32</sup> The secreted IL-6 - IL-6R complex activated MAPK/ERK, PI3K/AKT, and JAK/STAT3 pathways.<sup>33</sup> IL-6 is primarily autocrine secreted by highly aggressive TNBCs such as MDA-MB-231 and Hs578T, whereas IL-6 is not expressed in non-aggressive ERapositive breast cancer cell lines such as MDA-MB-361, MCF-7, and T47D.<sup>34,35</sup> Thus we supposed that IL-6/PI3K/ AKT signaling in aggressive MDA-MB-231 cells could regulate iron-uptake under DFO-induced iron-deficient condition. The expression level of IL-6 was detected in triple-negative MDA-MB-231 and ERa-positive MCF-7 cells using RT-qPCR (Figure 5). IL-6 mRNA expression level was significantly increased in DFO-treated MDA-MB-231 cells, as compared with control MDA-MB-231 cells. In contrast, IL-6 mRNA level in DFO-treated MCF-7 cells was obviously decreased (Figure 5). The results indicated that DFO treatment could promote expression of IL-6 in aggressive MDA-MB-231 cells under DFOinduced iron-deficient condition.

# The activated PI3K/AKT pathway enhanced the expression of TfRI and DMTI leading to the increased iron uptake in triple-negative MDA-MB-23I cells after DFO treatment

Since DFO promoted high expression of IL-6 in triplenegative MDA-MB-231 cells, whether IL-6 could activate PI3K/AKT pathway to regulate iron metabolism in MDA-MB-231 cells following DFO treatment was investigated (Figure 6). The expression of PI3K, and transcription factors, such as STAT3, ERK and AKT were assessed by western blotting. As shown in Figure 6A and B, after DFO treatment for 24 h, phosphorylated PI3K, AKT in MDA-MB-231 cells was significantly increased, but the level of phosphorylated STAT3 was not obviously changed and the level of phosphorylated ERK was decreased, as compared with untreated MDA-MB-231 cells, respectively. At the same time, phosphorylated PI3K was markedly decreased in MCF-7 cells after DFO treatment, but



Figure 5 IL-6 mRNA expression levels in MDA-MB-231 and MCF-7 cells were measured using RealTime-qPCR. \*\*\*p<0.001. Abbreviations: DFO, deferoxamine; IL-6, interleukin 6.

phosphorylated STAT3, ERK and AKT level were no difference between DFO-treated MCF-7 cells and untreated MCF-7 cells. And the two iron regulatory protein1 and 2 (IRP1 and IRP2) control iron metabolism proteins expression at the post-transcriptional level. The expression of IRP1 and IRP2 in MDA-MB-231 cells was obviously increased after DFO treatment, while they were decreased in DFO-treated MCF-7 cells. The results suggested that PI3K/AKT/IRPs pathway in triple-negative MDA-MB-231 cells was activated after DFO treatment.

To explore the relationship between PI3K/AKT activation and the expression of iron metabolism-related proteins in MDA-MB-231 cells after exposure to DFO, the inhibitor of PI3K, LY294002 were used to treat MDA-MB-231 cells. After the addition of LY294002 for 1 h, MDA-MB-231 cells were treated with DFO for 24 h, PI3K/AKT pathway in MDA-MB-231 cells was further verified. As shown in Figure 6C and D, the expression of iron-related proteins, such as IRP1, IRP2, TfR1 and DMT1 were up-regulated in DFO-treated MDA-MB-231 cells as compared with the untreated MDA-MB-231 cells. While when being pretreated with LY294002 for 1 h and treated with DFO for 24 h, IRP1, IRP2, TfR1 and DMT1 in MDA-MB-231 cells were down-regulated (Figure 6C and D). The same effect was observed in the expression of iron uptake-related cell membrane proteins such as TfR1 and DMT1 (Figure 6E and F). Next, the iron concentration was detected in DFOtreated MDA-MB-231 cells with LY294002 pretreatment (Figure 6G). There was no difference about iron concentrations between MDA-MB-231 cells and LY294002 pretreated MDA-MB-231 cells. After treated with DFO for 24 h, the iron concentration was significantly increased in MDA-MB-231 cells, as compared with untreated MDA-MB-231 cells. However, the iron concentration was markedly decreased in DFO-treated MDA-MB-231 cells with LY294002 pretreatment. Taken together, all of the data demonstrated that DFO treatment could trigger PI3K/AKT/IRPs activation to up-regulate the expression of TfR1 and DMT1 leading to enhance iron uptake in aggressive MDA-MB-231 breast cancer cells.

# IL-6/PI3K/AKT pathway involved in upregulating the expression of TfR1 and DMT1 leading to enhance iron uptake and promote cell migration in MDA-MB-231 cells after DFO treatment

The other question was addressed whether IL-6 directly activated PI3K/AKT to regulate iron metabolism in DFOtreated MDA-MB-231 cells. MDA-MB-231 breast cancer cells were cultured with 200 µM DFO and 5 µg/ml IL-6 neutralizing antibody for 24 h. As shown in Figure 7A and B, there was no obvious difference about the expression of phosphorylated PI3K and AKT, IRP1, IRP2, TfR1 and DMT1 between MDA-MB-231 cells and MDA-MB-231 cells treated with IL-6 neutralizing antibody, whereas the phosphorylated PI3K and AKT were significantly overexpressed in MDA-MB-231 cells response to DFO treatment. With the addition of IL-6 neutralizing antibody, phosphorylated PI3K, AKT and iron-related proteins, such as IRP1, IRP2, TfR1 and DMT1 in DFO-treated MDA-MB-231 cells were obviously decreased as compared with DFO-treated MDA-MB-231 cells respectively. With the addition of IL-6 neutralizing antibody in DFOtreated MDA-MB-231 cells, the expression of TfR1 and DMT1 on the cell membrane (Figure 7C and D) was obviously decreased as compared with the DFO-treated MDA-MB-231 cells respectively. Next, the iron



Figure 6 The activated PI3K/AKT pathway enhanced the expression of TfR1 and DMT1 leading to increased iron uptake in MDA-MB-231 cells after DFO treatment. (A, B) The expression levels of phosphorylated PI3K, STAT3, ERK, AKT, IRP1 and IRP2 were assessed in MDA-MB-231 and MCF-7 cells by western blotting. (C, D) MDA-MB-231 cells were pretreated with or without LY294002 for 1 h, and treated with or without DFO treatment for 24 h. Cellular lysates proteins were analyzed by Western blotting. (E, F) With or without LY294002 pretreatment for 1 h, and with or without DFO treatment for 24 h, TfR1 and DMT1 from cell membrane were detected by Western blotting. (G) With or without LY294002 pretreatment for 1 h, and with or without DFO treatment for 24 h, iron concentration in MDA-MB-231 cells was tested by ICP-MS, respectively. \*\*p<0.01, \*\*\*p<0.001.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1; IRP1, iron regulatory protein1; IRP2, iron regulatory protein 2; ICP-MS, inductively coupled plasma mass spectrometry; ns, no statistical difference.

concentration was detected in DFO-treated MDA-MB-231 cells with an IL-6 neutralizing antibody (Figure 7E). There was no difference about iron concentration between MDA-MB-231 cells and MDA-MB-231 cells treated with IL-6 neutralizing antibody. However, the iron concentration was markedly decreased in DFO-treated MDA-MB-231 cells with IL-6 neutralizing antibody as compared with DFO-treated MDA-MB-231 cells. The results identified that DFO treatment could induce IL-6/PI3K/AKT signaling activation to up-regulate the expression of iron-related proteins enhancing iron uptake in aggressive MDA-MB-231 breast cancer cells.

To explore that DFO activated IL-6/PI3K/Akt signaling to lead migration in TNBC cells, transwell cell migration assay was performed (Figure S6). The results suggested that the migration of MDA-MB-231 cells was promoted after being cultured with DFO for 24 h, as compared with the untreated MDA-MB-231 cells. With the addition of IL-6 neutralizing antibody in untreated MDA-MB-231 cells, cell migration was not obviously affected. However, in DFO-treated MDA-MB-231 cells with IL-6 neutralizing antibody, cell migration was significantly diminished (Figure S6A and B). Collectively, all these results suggested that DFO activated IL-6/PI3K/AKT signaling pathway in MDA-MB-231 cells up-regulated the expression of TfR1 and DMT1 leading to increasing intracellular iron uptake and enhancing cell migration.

#### Discussion

In this study, we observed that: 1) DFO treatment significantly increased the expression of the iron uptake proteins, TfR1 and DMT1 in aggressive TNBCs. 2) DFO treatment



**Figure 7** IL-6/PI3K/AKT pathway involved in upregulating the expression of TfR1 and DMT1 leading to enhance iron uptake in MDA-MB-231 cells after DFO treatment. (**A**, **B**) MDA-MB-231 cells were treated with or without 5  $\mu$ g/ml IL-6 neutralizing antibody, and with or without 200  $\mu$ M DFO for 24 h. The expression of phosphorylated PI3K and AKT, IRP1, IRP2, TfR1, and DMT1 in MDA-MB-231 cells were assessed by Western blotting. (**C**, **D**) After being treated with or without 5  $\mu$ g/ml IL-6 neutralizing antibody, and with or without 200  $\mu$ M DFO for 24 h, TfR1 and DMT1 on cell membrane were detected by Western blotting. (**E**) After being treated with or without 5  $\mu$ g/ml IL-6 neutralizing antibody, and with or without 200  $\mu$ M DFO for 24 h, TfR1 and DMT1 on cell membrane were detected by Western blotting. (**E**) After being treated with or without 5  $\mu$ g/ml IL-6 neutralizing antibody, and with or without 200  $\mu$ M DFO for 24 h, iron concentration in MDA-MB-231 cells was tested by ICP-MS, respectively. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1; IRP1, iron regulatory protein1; IRP2, iron regulatory protein 2; ICP-MS, inductively coupled plasma mass spectrometry; ns, no statistical difference.

could promote high expression of IL-6 in aggressive triplenegative MDA-MB-231 cells. 3) The aggressive TNBCs exhibited the activated IL-6/PI3K/AKT signaling to upregulate the expression of TfR1 and DMT1, leading to increased iron uptake.

In our previous study, we originally applied DFO to decrease the iron concentrations in breast cancer cells.<sup>26</sup> However, exposure to DFO significantly enhanced the iron concentrations in aggressive TNBCs. More importantly, the increased intracellular iron concentrations in aggressive TNBCs led to enhance cell migration.<sup>26</sup> But the mechanism of the increased iron uptake in aggressive TNBCs after DFO treatment still remained unclear.

Currently, our studies provided the evidence that DFO treatment produced different effects on the expression of iron uptake proteins between non-aggressive ERα-positive and aggressive TNBCs. TfR1, TfR2 and DMT1 are wellknown proteins related to iron uptake in cells. In this study, DMT1 and TfR1 were found to be up-regulated to increase iron uptake in aggressive TNBCs under the irondeficient condition induced by DFO. Though TfR1 was also up-regulated in ERa-positive MCF-7 breast cancer cells after DFO treatment, the intracellular iron concentration in DFO-treated MCF-7 cells was declined sharply, whether up-regulated expression of TfR1 in DFO-treated MCF-7 breast cancer cells was related with iron uptake should be further studied. After DFO treatment, the expression of TfR2 in the cell lysate of MCF-7 and MDA-MB-231 cells was not changed, and the expression of TfR2 on cell membrane was both decreased, it seems that the major role of TfR2 is sensing intracellular iron levels rather than iron uptake.<sup>36,37</sup>

The iron regulatory proteins IRPs (IRP1 and IRP2) could sense intracellular iron status and regulate the iron homeostasis by binding to iron-responsive elements (*IREs*) of DMT1 and TfR1.<sup>38</sup> Much more abundant IRP1 than IRP2 in variety cells is reported.<sup>20,39</sup> Similarly, in our study, the expression of IRP1 was up-regulated much higher than IRP2 in DFO-treated MDA-MB-231 cells, and the expression of IRP1 and IRP2 in MDA-MB-231 cells was increased via activation *of* IL-6/PI3K/AKT pathway after DFO treatment, thus we suggested that both IRP1 and IRP2 responded to DFO-induced iron deficiency in mediating the regulation of DMT1 and TfR1.<sup>20,39</sup>

It is noteworthy that iron metabolism pathways are closely related to inflammatory stressors.<sup>23</sup> Proinflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or IL-6 influence the posttranscriptional control of iron homeostasis by modulating the binding affinity of IRP1 and IRP2 to IREs in human monocytic cells and neuron cells.<sup>40-42</sup> However, the role of IL-6 in mediating iron uptake in tumor cells remained to be elucidated. Under the iron deficient condition induced by DFO, triple-negative MDA-MB-231 cells were triggered to up-regulate the expression level of IL-6, but the situation in ER $\alpha$ -positive MCF-7 cells was just on the contrary. As an inflammatory cytokine, IL-6 is higher expressed in highly aggressive TNBCs, whereas is almost not expressed in non-aggressive ERa-positive breast cancer cells.<sup>34,35</sup> Meanwhile, IL-6 were associated with iron homeostasis.43,44 After DFO treatment, the activation of IL-6/PI3K/AKT pathway led to increase expression of IRP1 and IRP2 in MDA-MB-231 cells. IRPs regulates TfR1 and DMT1 mRNA stability, ultimately increasing protein levels of TfR1 and DMT1 to promote iron uptake in TNBC cells.<sup>38</sup>

The present results were suggested that IL-6 involved in iron uptake through the activated PI3K/AKT pathway under the iron-deficient condition induced by DFO. In this study, we suggested that both TfR1 and DMT1 were involved in increasing iron uptake in triple-negative MDA-MB-231 cells under DFO-induced iron-deficient condition, but the intracellular iron transport and iron storage remained unsolved. The further studies were in process to elucidate the route of the intracellular iron transport, and intracellular iron storage in aggressive TNBCs under the iron-deficient condition induced by DFO.

Collectively, our study suggested that aggressive TNBCs exhibited the activated IL-6/PI3K/AKT signaling to up-regulate the expression of TfR1 and DMT1 leading to increased iron uptake under the iron-deficient condition induced by DFO. Our study also suggested that when DFO was applied to treat breast cancer cells, it should be considered that DFO has different effects on iron metabolism in breast cancer cells with different phenotype leading to distinct biological outcomes.

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### Disclosure

The authors report no conflicts of interest in this work.

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#### Α В Control DFO Cell membrane 3 Control DFO TfR1/actin 5 TfR1 TfR1 DMT1 DMT1 Ferritin 0 Control OFO Actin Actin 1.0 1.0 1.5 1.5 0.8 DMT1/actin 0.1 DMT1/actin 0.5 0.1 TfR1/actin 0.5 0.6 0.4 0.2 0.2 0.0 0.0 0.0 0.0 Control OFO Control OFO Control Control 0HO 040 С D Control DFO **Cell membrane** TfR1 Control DFO 1.5 \*\*\* TfR1/actin 0.1 TfR1 DMT1 DMT1 Ferritin 0.0 Control Actin OFO Actin 0.15 0.5 1.5 1.5 Ferritin/actin 0.1 2.0 \*\* 0.4 DMT1/actin DMT1/actin 20.02 TfR1/actin 5.0 0.3 0.2 0.1 0.0 0.00 0.0 0.0 control Control Control off<sup>O</sup> Control SFO 0HO 0FO

# Supplementary materials

Figure S1 Effects of DFO treatment on the expression of iron-uptake and iron-storage proteins in Hs578T and BT549 cell lines. (**A**) Hs578T cells were treated with or without 200  $\mu$ M DFO for 24 h. Proteins from cell lysates were analyzed using Western blotting. (**B**) TfR1 and DMT1 on cell membrane were detected by Western blotting. (**C**) BT549 cells were treated with or without 200  $\mu$ M DFO for 24 h. Proteins from cell lysates were analyzed using Western blotting. (**B**) TfR1 and DMT1 on cell membrane were detected by Western blotting. (**C**) BT549 cells were treated with or without 200  $\mu$ M DFO for 24 h. Proteins from cell lysates were analyzed using Western blotting. (**D**) TfR1 and DMT1 on cell membrane in BT549 cells were detected by western blotting. Western blotting quantification with anti- $\beta$  actin antibody: values were the means of three independent experiments ±SD. \*\*p<0.01, \*\*\*p<0.001.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1.



Figure S2 The expression of iron-uptake and iron-storage proteins in (left) Hs578T and (right) BT549 cell lines after 200  $\mu$ M DFO treatment was observed using immunofluorescence staining. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

Abbreviations: dFO, Deferoxamine; TfRI, transferrin receptor I; DMTI, divalent metal transporter I.



Figure S3 Effects of DFO treatment on the expression of iron-uptake and iron-storage proteins in T47D cells. (A) Cells were treated with or without 200  $\mu$ M DFO for 24 h. Proteins from cell lysates were analyzed using Western blotting. (B)Western blotting quantification with anti- $\beta$  actin antibody: values were the means of three independent experiments ±SD. (C) TfR1 and DMT1 on cell membrane were detected by western blotting. (D) Western blotting quantification with anti- $\beta$  actin antibody: values were the means of three independent experiments ±SD. \*p<0.05, \*\*p<0.01, \*\*\*p<0.01.

Abbreviations: DFO, deferoxamine; TfRI, transferrin receptor I; DMTI, divalent metal transporter I.



**Figure S4** TfR1 and DMT1 enhanced iron uptake in aggressive human breast cancer cells after DFO treatment. (**A**) The iron concentration in DFO-treated or untreated Hs578T cells with 5  $\mu$ g/ml DMT1, TfR1 neutralizing antibody and both was measured by using ICP-MS. (**B**) The iron concentration in DFO-treated or untreated BT549 cells with 5  $\mu$ g/ml DMT1, TfR1 neutralizing antibody and both was measured by using ICP-MS. (**B**) The iron concentration in DFO-treated or untreated BT549 cells with 5  $\mu$ g/ml DMT1, TfR1 neutralizing antibody and both was measured by using ICP-MS. \*p<0.01, \*\*p<0.001. **Abbreviations:** DFO, Deferoxamine; TfR1, transferrin receptor 1; DMT1, Divalent metal transporter 1; Ab, antibody; ns, no statistical difference.



Figure S5 TfR1 and DMT1 affected Hs578T and BT549 cell migration. Cells were allowed to migrate in trans-well assays. (A) Hs578T cells treated with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, DFO-treated Hs578T cells, DFO-treated Hs578T cells with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. (B) BT549 cells treated with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. (B) BT549 cells treated with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. (B) BT549 cells treated with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. (B) BT549 cells treated with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. (B) BT549 cells treated BT549 cells with 5  $\mu$ g/ml DMT1 and TfR1 neutralizing antibody, respectively. The images were acquired using an upright microscope with the 10× objective. (C, D) Quantitative analysis of the cell migration was performed by Image Pro Plus software. \*p<0.05, \*\*p<0.01.

Abbreviations: DFO, deferoxamine; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1; Ab, antibody; ns, no statistical difference.



Figure S6 IL-6 affected MDA-MB-231 cells migration. (A) Cells were allowed to migrate in transwell assays. MDA-MB-231 cells, MDA-MB-231 cells treated with 5 µg/ml IL-6 neutralizing antibody, DFO-treated MDA-MB-231 cells, DFO-treated MDA-MB-231 cells with 5 µg/ml IL-6 neutralizing antibody, respectively. The images were acquired using an upright microscope with the 10× objective. (B) Quantitative analysis of the cell migration was performed by Image Pro Plus software. \*\*\*p<0.001. Abbreviations: DFO, Deferoxamine; IL-6, interleukin 6; Ab, antibody; ns, no statistical difference.

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