# Manipulation of Iron Transporter Genes Results in the Suppression of Human and Mouse Mammary Adenocarcinomas

XIAN P. JIANG, ROBERT L. ELLIOTT and JONATHAN F. HEAD

*Elliott-Elliott-Head Breast Cancer Research and Treatment Center, Baton Rouge, LA 70816, U.S.A.*

**Abstract.** *Since malignant cells often have a high demand for iron, we hypothesize that breast cancer cells may alter the expression of iron transporter genes including iron importers [transferrin receptor (TFRC) and solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (SLC11A2)] and the iron exporter SLC40A1 (ferroportin), and additionally that the growth of breast cancer can be inhibited by manipulating iron transporter gene expression. To test our hypothesis, reverse transcription polymerase chain reaction (RT-PCR) was used to determine mRNA expression of iron transporter genes in normal human mammary epithelial MCF-12A cells and human breast cancer MCF-7 cells. Antisense oligonucleotides were employed to suppress the expression of TFRC gene in the 4T1 mammary adenocarcinoma in both cell culture and a mouse tumor model. We found the following: i) the MCF-7 cells have higher expression of TFRC and SLC11A2 compared with MCF-12A epithelia; ii) SLC40A1 was only expressed in MCF-12A epithelia but not in MCF-7 cells; iii) iron increased mRNA levels of the SLC11A2 gene in both MCF-12A and MCF-7 cells; iv) TFRC antisense oligonucleotides reduced TFRC mRNA levels and intracellular total iron, and inhibited the proliferation of the 4T1 cells in cell culture; v) TFRC antisense oligonucleotide inhibited tumor growth and lung metastases in the 4T1 mammary adenocarcinoma mouse model. In conclusion, breast cancer cells up-regulate the expression of iron importer genes and down-regulate the expression of iron exporter SLC40A1 to satisfy their increased demand for iron. Suppression of transferrin receptor by antisense results in inhibition of tumor growth and lung metastasis in the 4T1 mammary adenocarcinoma mouse model.*

*Correspondence to:* Dr. Xian P. Jiang, Elliott-Elliott-Head Breast Cancer Research and Treatment Center, 17050 Medical Center Drive, 4th Floor, Baton Rouge, LA 70816, U.S.A. Tel: +1 2257553070, Fax: +1 2259062620, e-mail: xjiang@eehbreastca.com

*Key Words:* Breast cancer, antisense, transferrin receptor, iron transport, cell culture.

Iron is an essential micronutrient necessary for nearly all living cells. It is required by a large number of heme and non-heme enzymes, which have essential functions in oxygen transport and oxidative phosphorylation (1). Iron is a cofactor of ribonucleotide reductase, which is a salvage enzyme that converts ribonucleotides to deoxyribonucleotides and therefore is a key enzyme in DNA synthesis. Ribonucleotide reductase turns over rapidly and needs a continuous supply of iron to maintain its activity (2, 3). Thus, iron is directly associated with cell proliferation.

Cellular iron homeostasis assures adequate iron supply for the various metabolic needs of individual cells, as long as extracellular iron concentrations remain in the normal range. Iron circulates in plasma and extracellular fluid bound to transferrin. Cells take up iron-transferrin by transferrin receptor (TFRC)-mediated endocytosis and store iron predominantly as cytoplasmic ferritin. It has been shown that *TFRC* expression correlates with cellular proliferation and is higher in rapidly dividing cells (including malignant cells). The density of the TFRC has also been correlated with the rate of DNA synthesis and the metastatic potential of tumor cells (4, 5). Two iron regulatory proteins (IRP1 and IRP2) function as cytoplasmic iron sensors. When cellular iron is low, IRPs stabilize *TFRC* mRNA, thus increasing the number of TFRCs for iron uptake (6, 7). Besides TFRCs, other iron importers, including SLC11A2 (also known as DMT1, NRAMP2, and DCT1), have been identified in the tissues where major iron uptake occurs, including duodenal enterocytes, placental trophoblast, macrophages and hepatocytes. SLC11A2 is also found in endosomes of erythroid precursors. SLC11A2 transports iron into cells, which makes it a likely candidate for transferrin-independent uptake of iron (8, 9). Besides iron importers, some cells also express iron exporter genes. An iron exporter, SLC40A1, also called MTP1 and IREG1, is also essential for iron homeostasis, as it transports iron out of cells. An iron regulatory hormone, hepcidin, can bind to SLC40A1 and induce its endocytosis and degradation. As a result, hepcidin controls iron flow out of the cells and into the plasma compartment. SLC40A1 is not expressed in erythroid cells

but is present in the placenta, intestine, reticuloendothelial macrophages, and hepatocytes (10-12).

However, many questions remain unanswered in breast cancer. Do human breast cancer cells express SLC11A2 and SLC40A1? How do breast cancer cells regulate the expression of these molecules? Does suppression of iron importer gene expression inhibit the growth of tumors? In order to answer these questions, this study measured mRNA levels of *TFRC, SLC11A2* and *SLC40A1* with RT-PCR. We also treated mammary adenocarcinoma bearing mice with antisense oligonucleotides targeted to *TFRC*, and examined tumor growth and metastasis.

## Materials and Methods

*Cell lines and mouse model*. The human breast cancer cell line MCF-7, human normal mammary epithelial cell line MCF-12A, and the mouse mammary adenocarcinoma cell line 4T1 were obtained from the Americian Type Culture Collection (Rockville, MD, USA). The MCF-7 and 4T1 cells were grown in alpha-minimun essential medium (MEM) supplemented with 10% fetal calf serum, 1 mM glutamine and 0.05 mg/ml gentamicin (GIBCO Invitrogen, Carlsbad, CA, USA). Normal MCF-12A cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium containing 0.1 μg/ml cholera enterotoxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 μg/ml epidermal growth factor and 5% horse serum (Sigma Chemical Co., St. Louis, MO, USA). All cell lines were incubated in 5% $CO_2$ at 37˚C. Cells were detached from tissue culture flasks by digestion with 0.05% trypsin and 0.53 mM EDTA.

*Determination of TFRC, SLC11A2 and SLC40A1 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)*. The MCF-12A and MCF-7 cells were incubated in complete media containing $FeCl_3$ or the iron chelator deferoxamine mesylate (DFOM) for 72 hours. To test the effects of hypoxia on the expression of the iron transporter genes, the cell lines were also incubated in 1% $O_2$ environment. Cells were detached from culture flasks. Total RNA was purified by RNA microisolation kit (GIBCO Invitrogen). One μg of RNA was used as the template for reverse transcription (RT). The RT reaction mixture contained 0.5 mM dNTP, 0.1 nM Oligo$(dT)_{12-18}$, and 0.25 units AMV reverse transcriptase (Promega, Madison, WI, USA), and was incubated at 42˚C for 60 minutes. The PCR was performed in a total volume of 25 μl with 2 μM forward and reverse primers, 5 mM MgCl2, 20 μM each of dGTP, dATP, dCTP and dTTP, and 0.125 unit Taq DNA polymerase. The primers used for amplification of *SLC11A2* were 5'-TGCTGGGTCCTGAACAGAAG-3' (forward) and 5'-GCAACTTAAATCCAGCCACTG-3' (reverse) and were designed according to the published *SLC11A2* cDNA sequence (13). The primers for SLC40A1 were 5'-TCCTTGGCCGACTACCTGAC-3'and 5'-TCCCTTTGGATTGTGA TTGC-3' (14). The primers for human *TFRC* were 5'-TATCTGCTATGGGACTATTGC-3' and 5'-CGCCA CATAACCCC CAGGATT-3' (15). The primers for mouse *TFRC* were 5'-ATGCCCTCTCTGGTGACATTTGGA-3' and 5'-ACCCTCCACA AGCACACTCTTTCT-3' (16). β-Actin gene (*ACTB*) was used as an internal control for gene expression. The primers for amplification of *ACTB* gene were 5'-CTTCTACAATGAGCTG CGTG-3' and 5'-GAGGATCTTCATGAGGTAGTC-3'. The amplification procedure involved denaturing at 94˚C for 30 seconds, annealing at 56˚C for 45 seconds, and extension at 72˚C for 90 seconds in an Omn-E DNA Thermal Cycler (National Labnet Co., Woodbridge, NJ, USA). After 35 PCR cycles, there was a final elongation step at 72˚C for 10 minutes. The PCR products were run on a 2% agarose gel and visualized by ethidium bromide fluorescence. The sizes of PCR products were determined by comparison to standard PCR DNA markers. The band densities on the gel were measured. Signal intensities of *SLC11A2, SLC40A1* and *TFRC* were normalized to the signal intensities of the *ACTB* amplification. The experiments were repeated three times independently.

*Measurement of intracellular total iron*. Cells were digested with trypsin-EDTA, counted, centrifuged for 5 minutes, washed 2 times with Hank's balanced salt solution (HBSS). One ml of water was added to the cell pellets. Cells pellets were homogenized for 5 minutes with a Polytron (Brinkmann Intruments, Westbury, NY, USA). The cell solution was centrifuged and the supernatant was stored at –20˚C for total iron measurement. For the quantitative determination of intracellular total iron, Total Iron Reagent Set was purchased from Pointe Scientific, Inc. (Lincoln Park, MI, USA). The procedure is described as follows: 1.25 ml Iron Buffer Reagent was added to all tubes; 0.25 ml of above supernatants, standards, and iron-free water were added to respective tubes; the spectrophotometer was zeroed at 560 nm with the reagent blank; absorbance of all tubes was read and recorded ($A_1$ reading); 25 μl of Iron Color Reagent was added to all tubes and mixed; all tubes were placed in a heated bath at 37˚C for 10 minutes; the spectrophotometer was zeroed at 560 nm with the reagent blank; absorbances of all tubes was read and recorded ($A_2$ reading).Total iron (μg/dl) was calculated as: $[(A_{2test}–A_{1test}) \div (A_{2standard}–A_{1standard})] \times$ concentration of standard. Finally, the intracellular total iron per million cells was calculated. The experiments were repeated three times independently.

*Preparation of transferrin receptor (TFRC) sense and antisense oligonucleotides*. The sense (m*TFRC*S) and antisense (m*TFRC*AS) phosphorothioate oligonucleotides were designed according to the published mouse *TFRC* cDNA sequence. The oligonucleotides were synthesized on an automated DNA synthesizer and purified by high-pressure liquid chromatography (Invitrogen). The m*TFRC*AS was a 24-mer nucleotides complementary to the sequence covering the translation initiation AUG code of mouse *TFRC* gene. A 24-mer DNA sequence (m*TFRC*S) complementary to the sequence of m*TFRC*AS was used as a control. The sequences were as follows: 5'-TGGCTTGATCCATCATTCTCAGCT-3' (m*TFRC*AS) and 5'-AGCTGAGAATGATGGATCAAGCCA-3' (m*TFRC*S). In addition phosphorothioated random sequence oligonucleotides (Oligo-R) were synthesized.

*[$^3$H]-Thymidine incorporation assay*. 4T1 mouse mammary adenocarcinoma cells (2,000 cells/well) were plated in 96-well tissue culture plates. After 24 hours of incubation, the culture medium was replaced with medium containing m*TFRC*S or m*TFRC*AS or Oligo-R and cells were further incubated for 72 hours. [$^3$H]-Thymidine (0.1 μCi/well; MP Biomedical, Santa Ana, CA, USA) was added to the wells for the last 16 hours of incubation. The cells were removed from the plates by trpsin-EDTA digestion and harvested onto a glass-fiber filter (Skatron Basic 96 Harvester; Skatron Inc., Sterling, VA, USA). The filters were placed into scintillation fluid, and the radioactivity was counted by liquid

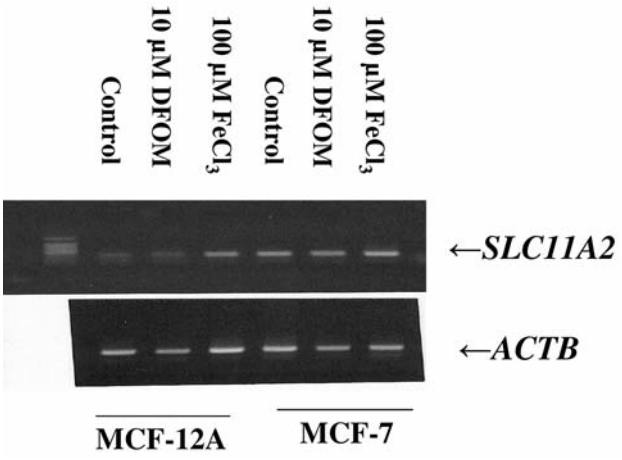


Figure 1. *Electrophoresis of RT-PCR products for analysis of SLC11A2 mRNA expression in the MCF-12A human normal mammary epithelia and MCF-7 human breast cancer cells.*

scintillation (LS 1800; Beckman Co., Fullerton, CA, USA). Cell proliferation was quantified by [$^3$H]-thymidine incorporation and expressed as a percentage of the untreated control. All the [$^3$H]-thymidine incorporation experiments were carried out in triplicate and were repeated three times independently.

*Treatment of 4T1 mammary adenocarcinoma with TFRC antisense oligonucleotides in a mouse model*. The protocol for animal care and use was approved by Louisiana State University School of Veterinary Medicine. Under isoflurane gas anesthesia, 24 eight-week-old female BALB/c mice (Lousiana State University School of Veterinary Medicine, Baton Rouge, LA, USA) were injected subcutaneously in the dorsal cervical area above the mammary gland chain with $2\times10^5$/0.5 ml/mouse 4T1 mammary adenocarcinoma cells. When tumors were palpable, tumor sizes were measured. The 24 mice were divided into two groups (12 mice per group) for which tumor size was not significantly different. The control group was injected with 100 μl HBSS. The remaining m*TFRC*AS group was injected with 100 μl phosphorothioated m*TFRC*AS oligonucleotides dissolved in HBSS and administered *via* subcutaneous implantation of Alzet osmotic mini-pumps (LxD: 1.5 cm × 0.6 cm) (DURECT Corporation, Cupertino, CA, USA) on the back under isoflurane gas anesthesia. The pumps can continuously deliver the 100 μl of oligonucleotides at a rate of 0.25 μl/h over a period of 2 weeks. Each mouse in the m*TFRC*AS group received a total of 5 mg phosphorothioated oligonucleotides in 100 μl HBSS.

Tumor measurements were made on the 4th, 7th, 10th and 14th days post-treatment. Tumor volumes were calculated as follows: volume ($mm^3$) = [length (mm)×width (mm)×depth (mm)] ÷ 2. Mice were sacrificed with $CO_2$ on the 14th day after implantation of the pump (study endpoint). Subcutaneous tumor nodules were excised and weighed. Tumor metastasis to internal organs was checked during necropsy. Local tumors, lungs, liver, spleen and kidneys were collected and fixed in 10% buffered formalin. The tissues were embedded and cut into sections. The sections were stained with H&E and examined by microscopy. Lung tumor metastasis were scored as follows (all lung lobes): 0=no metastasis, 1=1-2 foci/slide, 2=3-10 foci/slide, 3=11-20 foci/slide and 4>20 foci/slide.

Table I. *The mRNA expression levels of SLC11A2 gene were examined by RT-PCR in the MCF-12A human normal mammary epithelia and MCF-7 human breast cancer cells.*

| Cell lines | *SLC11A2* mRNA level (relative to *ACTB*) | | |
|---|---|---|---|
| | Control | 10 μM DFOM | 100 μM $FeCl_3$ |
| MCF-12A | 0.33±0.12 (n=3) | 0.37±0.19 (n=3) NS | 0.64±0.17 (n=3) $p<0.05$[2] |
| MCF-7 | 0.74±0.21 (n=3) $p<0.05$[1] | 0.86±0.27 (n=3) NS | 1.1±0.31 (n=3) $p<0.05$[2] |

DFOM: Deferoxamine mesylate. NS, not significant compared to cell line control. [1]Compared to MCF-12A control; [2]compared to cell line control.

*Statistical analysis*. Student's *t*-test was employed to test statistical significance, and *p*-values less than 0.05 were judged to be of statistical significance.

## Results

*The expression of TFRC mRNA is regulated by, iron, an iron chelator and hypoxia*. RT-PCR was used to determine *TFRC* mRNA expression in MCF-12A human normal mammary epithelia and the MCF-7 human breast cancer cells. MCF-7 cells had higher levels of *TFRC* mRNA expression than the MCF-12A normal mammary epithelia [*TFRC* mRNA relative to ACTB mRNA: 1.20±0.31 *versus* 0.41±0.12, respectively, $p<0.01$]. The addition of iron (100 μM) to culture media significantly reduced the expression of *TFRC* mRNA of MCF-7 cells [*TFRC* mRNA relative to *ACTB* mRNA: 0.25±0.11 (100 μM $FeCl_3$) *versus* 1.20±0.31 (control), $p<0.01$]. However, hypoxia or addition of the iron chelator DFOM significantly increased the expression of *TFRC* of MCF-7 cells [*TFRC* mRNA relative to *ACTB* mRNA: 1.94±0.35 (1% $O_2$) *versus* 1.20±0.31 (control), $p<0.05$; 2.12±0.24 (10 μM DFOM) *versus* 1.20±0.31 (control), $p<0.05$].

*Iron increases the expression of SLC11A2 mRNA in MCF-12A normal epithelia and MCF-7 cancer cells*. The MCF-12A and MCF-7 cells were incubated in media with or without the addition of 100 μm $FeCl_3$ or 10 μm DFOM for 72 hours. The total RNA was purified and mRNA levels of the iron importer gene *SLC11A2* were measured by RT-PCR. The MCF-7 cancer cells had significantly higher mRNA levels of *SLC11A2* than the MCF-12A normal mammary epithelia, $p<0.05$. However, in contrast to *TFRC* mRNA, RT-PCR showed that iron increased the mRNA expression of *SLC11A2* gene in both MCF-12A and MCF-7 cells (both at $p<0.05$). In addition, the iron chelator DFOM did not change the level of *SLC11A2* mRNA in MCF-12A or MCF-7 cells (see Figure 1 and Table I).

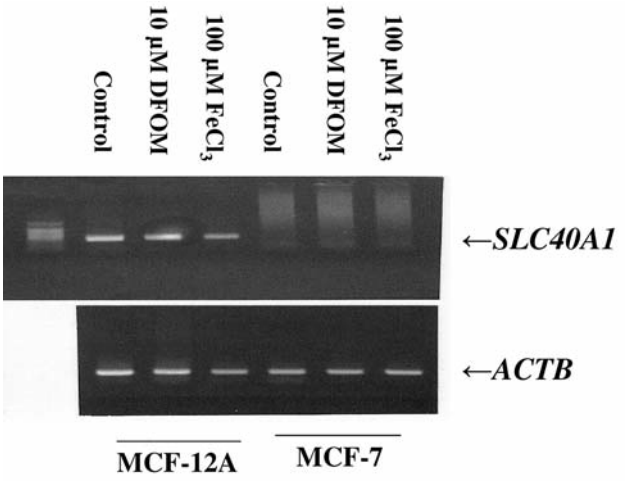


Figure 2. *Electrophoresis of RT-PCR products for analysis of SLC40A1 mRNA expression in the MCF-12A human normal mammary epithelia and MCF-7 human breast cancer cells. MCF-7 cells do not express SLC40A1.*

*The MCF-7 breast cancer cell line has lost the expression of the iron exporter gene SLC40A1*. The mRNA levels of the iron exporter gene SLC40A1 in the MCF-12A and MCF-7 cells were examined by RT-PCR. The MCF-12A normal mammary epithelia expressed the *SLC40A1* mRNA, but the MCF-7 human breast cancer cells did not. In contrast to iron importer genes, such as *TFRC* and *SLC11A2*, the expression of *SLC40A1* was not changed by the addition of iron or an iron chelator to the MCF-12A cells [*SLC40A1* mRNA relative to *ACTB* mRNA: 1.12±0.12 (control) *versus* 1.23±0.21 (10 μM DFOM), $p>0.05$; 1.12±0.12 (control) *versus* 0.98±0.08 (100 μM $FeCl_3$), $p>0.05$] (Figure 2).

*TFRC antisense oligonucleotides suppress cell proliferation and reduce total intracellular iron in the 4T1 mammary adenocarcinoma cell line*. The phosphorothioated antisense oligonucleotide targeted to the mouse transferrin receptor (*mTFRCAS*) suppressed the proliferation of the 4T1 cells in a dose-dependent manner, but sense oligonucleotides (*mTFRCS*) and random sequence oligonucleotides (Oligo-R) did not significantly inhibit the proliferation of the 4T1 cells (Figure 3). The 50% and 90% inhibitory concentrations ($IC_{50}$ and $IC_{90}$) of *mTFRCAS* were 8 μM and 100 μM, respectively (for 3 days of incubation). To determine the mechanism of suppression of cell proliferation, the 4T1 cells were treated with 8 μM and 100 μM of m*TFRC*AS for 3 days. The 4T1 cells were also treated with these same concentrations of Oligo-R or m*TFRC*S. *TFRC* mRNA levels and total intracellular iron were measured. The *mTFRCAS* significantly reduece the *TFRC* levels and those of total intracellular iron, but Oligo-R and m*TFRC*S had no effect (Table II).

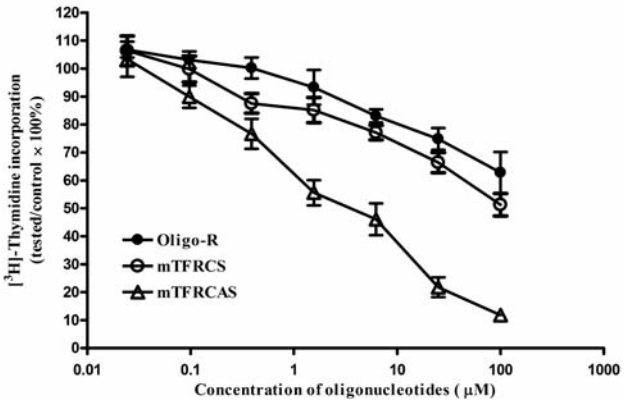


Figure 3. *Dose effects of phosphorothioated random-sequence oligonucleotides (Oligo-R), mTFRCS and mTFRCAS on the proliferation of 4T1 mouse mammary adenocarcinoma cells. mTFRCAS inhibits the proliferation of 4T1 cells in a dose-dependent manner.*

Table II. *TFRC antisense oligonucleotides (mTFRCAS) inhibit mRNA expression of transferrin receptor TFRC and reduce total intracellular iron of 4T1 mouse mammary adenocarcinoma cells.*

| Oligonucleotides (μM) | *TFRC* mRNA levels (relative to *ACTB*) | Total intracellular iron (ng/$10^6$ cells) |
|---|---|---|
| Control (0) | 1.13±0.31 (n=3) | 55.6±7.5 (3) |
| Oligo-R (8) | 1.08±0.22 (n=3) | 47.6±5.5 (3) |
| | NS | NS |
| Oligo-R (100) | 0.89±0.42 (n=3) | 42.2±8.8 (3) |
| | NS | NS |
| m*TFRC*S (8) | 0.98±0.35 (n=3) | 50.4±4.5 (3) |
| | NS | NS |
| m*TFRC*S (100) | 0.88±0.38 (n=3) | 45.6±6.4 (3) |
| | NS | NS |
| m*TFRC*AS (8) | 0.52±0.18 (n=3) | 31.2±4.5 (3) |
| | $p<0.05$[1] | $p<0.01$[1] |
| m*TFRC*AS (100) | 0.15±0.06 (n=3) | 8.8±2.5 (3) |
| | $p<0.01$[1] | $p<0.01$[1] |

NS, Not significant compared to control. [1]Compared to control.

*TFRC antisense oligonucleotides inhibit tumor growth and lung metastasis of the 4T1 mammary adenocarcinoma mouse model*. Tumor volumes are shown in Table III. Tumor volumes were not significantly different between the control and m*TFRC*AS groups pre-treatment (0 day). At the 4th, 7th, 10th and 14th days post-treatment, tumor volumes of the m*TFRC*AS group (total dosage of 5 mg per mouse) were significantly smaller than those of the control group (with HBSS injection) (Table III). Mice were sacrificed on the 14th day post-treatment and local tumors were weighed. Tumor weight of the m*TFRC*AS-treated group was significantly less than those of the control. Tumor weights were 4.3±1.3 g (12 mice) for the control group and 3.3±1.0 g (12 mice) for

Table III. *TFRC antisense oligonucleotides suppress the growth of the 4T1 mammary adenocinoma mouse model. Tumor sizes were measured pre-treatment (0 days) and post-treatment.*

| Group (number of mice) | Tumor volume ($mm^3$) Day | | | | |
|---|---|---|---|---|---|
| | 0 | 4 | 7 | 10 | 14 |
| Control with HBSS (12) | 50.1±36.5 | 218.1±64.2 | 433.0±168.4 | 738.0±240.6 | 1405.7±605.9 |
| *mTFRCAS* (12) | 54.3±30.9<br>NS | 155.2±77.6<br>$p<0.05$[1] | 301.8±138.0<br>$p<0.05$[1] | 533.4±216.1<br>$p<0.05$[1] | 980.3±370.1<br>$p<0.05$[1] |

NS, not significant compared to control. [1]Compared to control.

the m*TFRC*AS group ($p<0.05$). During necropsy, 91.7% (11 out of 12) of the mice in the control group had visible tumor nodules on the lung surfaces, but only 16.7% (2 out of 12) of the mice in the m*TFRC*AS group had visible tumor on the lung surface. This difference was significant ($p<0.001$). Microscopy showed that all the mice in both the control and treated groups had lung metastases. However, the lung tumor metastasis score was significantly lower in the m*TFRC*AS group (1.75±1.49) than in the control group (3.25±1.06) ($p<0.01$).

## Discussion

Iron is an essential micronutrient for all living cells, including tumor cells. Low iron or inhibition of normal iron metabolism with iron chelators results in inhibition of DNA synthesis, arrest of cell division, suppression of tumor growth, and apoptosis of tumor cells (17-19). Cellular iron homeostasis is maintained by the iron importer genes of transferrin receptors and *SLC11A2*, and the iron exporter gene *SLC40A1* (8-12). Human cancer, including breast cancer, usually has higher levels of transferrin receptor expression than normal cells (5, 7). The reason may be that tumor cells with increased proliferation demand more iron than normal cells. This is supported by the fact that down-regulation of transferrin receptor expression by transferrin receptor antibodies and antisense oligonucleotides suppresses the growth of tumor cells (7, 20) and that the expression of the transferrin receptor is regulated by iron levels and oxygen levels (21, 22). In agreement with previous research, we found that MCF-7 human breast cancer cells had significantly increased expression of *TFRC* mRNA compared to the MCF-12A human normal mammary epithelia. We also found that the addition of iron reduced *TFRC* mRNA expression, and hypoxia and the iron chelator DFOM (which decreases intracellular iron levels) increased the *TFRC* expression in MCF-7 cells. These results showed that transferrin receptors play an important role in intracellular iron homeostasis.

Another iron importer gene, *SLC11A2*, is often identified in tissues where major iron uptake is found, including duodenal enterocytes, placental trophoblast, macrophages and hepatocytes (8, 9). SLC11A2 is also found in human bronchial epithelial cells and its mRNA expression is up-regulated after exposure to iron. The increase in SLC11A2 expression results in an elevated transport of iron into cells and its probable detoxification by these cells (12). Thus, SLC11A2 is a likely candidate for transferrin-independent uptake of iron in peripheral tissues. However, the role of *SLC11A2* in human carcinoma is not clear. Boult *et al*. reported that progression to adenocarcinoma is associated with increased expression of iron import protein SLC11A2 (23).

To investigate the role of *SLC11A2* in human breast cancer, we used RT-PCR to examine the mRNA expression of *SLC11A2* in the human breast cancer cell line MCF-7 and human normal mammary epithelia MCF-12A. RT-PCR showed both cancer cells and normal epithelia expressed *SLC11A2* mRNA. Moreover, the level of *SLC11A2* mRNA in the MCF-7 cells was higher than in the MCF-12A cells. The addition of iron to the media increases mRNA levels of *SLC11A2* in both MCF-7 and MCF-12A cells. These results are similar to those previously reported for *SLC11A2* expression in human bronchial epithelia (12). In addition, reducing intracellular iron with the iron chelator DFOM did not significantly change the mRNA levels of *SLC11A2*. The mechanism responsible for these results remains to be elucidated.

The iron exporter gene *SLC40A1* plays a significant role in transporting intracellular iron out of cells. It is not expressed in erythroid cells but is present in the placenta, intestine, reticuloendothelial macrophages and hepatocytes (10-11). It has been rarely reported that tumors express the *SLC40A1* gene. We found that MCF-12A human normal mammary epithelia expressed *SLC40A1* mRNA, but that MCF-7 human breast cancer cells did not. The levels of mRNA were not changed by addition of iron or the iron chelator DFOM. The increase in the expression of the iron importer genes for *TFRC* and *SLC11A2*, and loss of

expression of the iron exporter gene *SLC40A1* in the MCF-7 cells reflect an increased demand for bioavailable iron and a high iron turnover in breast cancer cells.

We have previously reported that the down-regulation of the expression of the *TFRC* gene suppresses the proliferation of human breast cancer cell lines (7). m*TFRC*AS significantly suppressed the proliferation of 4T1 cells, and reduced mRNA expression of *TFRC* and total intracellular iron of the cultured 4T1 cells. In the present study, we investigated the effects of *TFRC* antisense oligonucleotides on the 4T1 mouse mammary adenocarcinoma in mice. After 14 days of treatment, m*TFRC* antisense effectively suppressed the growth of local tumors and reduced gross and microscopic lung metastasis (Table III). To exclude nonspecific inhibition by oligonucleotides of the tumor, we also used mouse interleukin 6 (IL-6) receptor antisense oligonucleotides (mIL-6RAS) as an additional control. The mIL-6RAS has similar percentages of (G+C) and (A+T) to m*TFRC*AS. We also used the same total injection dose (5 mg/mouse) and administration method (Alzet osmotic pump) as for m*TFRC*AS. We found that mIL-6RAS did not inhibit tumor growth and lung metastasis to the lungs (data not shown). These results in cell culture and in mice suggest that the down-regulation of *TFRC* by its antisense oligonucelotide resulted in intracellular iron depletion and thus suppressed the tumor growth and lung metastasis in mice.

The results of this study demonstrate that human breast cancer cells increase their iron requirement through the up-regulation of the expression of the iron importer genes *TFRC* and *SLC11A2*, and the down-regulation of expression of the iron exporter gene *SLC40A1*. We found that *TFRC* antisense oligonucleotides reduce the total intracellular iron and suppress mouse mammary adenocarcinoma. We conclude that complete blocking of all iron-importer gene pathways will produce maximum iron depletion and tumor inhibition. In addition, increased expression of the iron exporter gene *SLC40A1* by transfection of cancer cells with a plasmid containing the *SLC40A1* gene may enhance the effect of iron deprivation on cancer cells, thus inhibiting their proliferation. Therefore depleting iron through a combination of reduced uptake and increased export might become a viable method of cancer therapy.

## Acknowledgements

Financial support from The Sallie Astor Burdine Breast Foundation, Baton Rouge, LA, U.S.A.

## References

1 Cammack R, Wrigglesworth JM and Baum H: Iron-dependent enzymes in mammalian systems. *In*: Iron Transport and Storage. Ponka P, Schulman HM, Woodworth RC (eds.). Boca Baton, Florida: CRC Press, 1990.

2 Thelander L, Graslund A and Thelander M: Continual presence of oxygen and iron required for mammalian ribonucleotide reductase: possible regulation mechanism. Biochem Biophys Res Commun *110*: 859-865, 1983.

3 Thelander M, Grasland A and Thelander L: Subunit M12 of mammalian ribonucleotide reductase. J Bio Chem *250*: 2737-2741, 1985.

4 Neckers LM: Regulation of transferrin receptor expression and control of cell growth. Pathobiology *59*: 11-18, 1991.

5 Inoue T, Cavanaugh PG, Steck PA, Brunner N and Nicolson GL: Differences in transferrin response and numbers of transferrin receptors in rat and human mammary carcinoma lines of different metastatic potentials. J Cell Physiol *156*: 212-217, 1993.

6 Hentze MW, Muckenthaler MU and Andrews NC: Balancing acts: molecular control of mammalian iron metabolism. Cell *117*: 285-297, 2004.

7 Yang DC, Jiang XP, Elliott RL and Head JF: Inhibition of growth of human breast carcinoma cells by an antisense oligonucleotide targeted to the transferrin receptor gene. Anticancer Res *21*: 1777-1788, 2001.

8 Abboud S and Haile DJ: A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Bio Chem *275*: 19906-19912, 2000.

9 McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW and Simpson RJ: A novel duodenal iron-regulated transporter, IRRG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell *5*: 299-309, 2000.

10 Ganz T: Cellular iron: ferroportin is only way out. Cell Metabolism *1*: 155-157, 2005.

11 Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S and Andrews NC: The iron exporter ferroportin/SLC40A1 is essential for iron homeostasis. Cell Metabolism *1*: 191-200, 2005.

12 Wang X, Ghio AJ, Yang F, Dolan KG, Garrick MD and Piantadosi CA: Iron uptake and NRAMP2/DMT1/DCT1 in human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol *282*: L987-995, 2002.

13 Knutson M, Menzies S, Connor J and Wessling-Resnick M: Developmental, regional, and cellular expression of *SFT/UbcH5A* and *DMT1* mRNA in brain. J Neurosci Res *76*: 633-641, 2004.

14 Zaahl MG, Merryweather-Clarke AT, Kotze MJ, van der Merwe S, Warnich L and Robson KJ: Analysis of gene implicated in iron regulation in individual presenting with primary iron overload. Hum Genet *115*: 409-417, 2004.

15 Schneider C, Owen MJ, Banville D and Williams JG: Primary structure of human transferrin receptor deduced from the mRNA sequence. Nature *311*: 675-678, 1984.

16 Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, Altschul SF, Zeeberg B, Buetow KH, Schaefer CF, Bhat NK, Hopkins RF, Jordan H, Moore T, Max SI, Wang J, Hsieh F, Diatchenko L, Marusina K, Farmer AA, Rubin GM, Hong L, Stapleton M, Soares MB, Bonaldo MF, Casavant TL, Scheetz TE, Brownstein MJ, Usdin TB, Toshiyuki S, Carninci P, Prange C, Raha SS, Loquellano NA, Peters GJ, Abramson RD, Mullahy SJ, Bosak SA, McEwan PJ, McKernan KJ, Malek JA, Gunaratne PH, Richards S, Worley KC, Hale S, Garcia AM, Gay LJ, Hulyk SW, Villalon DK, Muzny DM, Sodergren EJ, Lu X, Gibbs RA, Fahey J, Helton E, Ketteman M, Madan A, Rodrigues S, Sanchez A,

Whiting M, Madan A, Young AC, Shevchenko Y, Bouffard GG, Blakesley RW, Touchman JW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Krzywinski MI, Skalska U, Smailus DE, Schnerch A, Schein JE, Jones SJ and Marra MA: Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci USA *99*: 16899-16903, 2002.

17 Hoyes KP, Hider RC and Porter, JB: Cell cycle synchronization and growth inhibition by 3-hydroxypyridin-4-one iron chelators in a leukemia cell line. Cancer Res *52*: 4591-4599, 1992.

18 Wang F, Elliott RL and Head JF: Inhibitory effect of deferoxamine mesylate and low iron diet on the 13762 NF rat mammary adenocarcinoma. Anticancer Res *19*: 445-450, 1999.

19 Jiang XP, Wang F, Yang DC, Elliott RL and Head JF: Induction of apoptosis by iron depletion in the human breast cancer MCF-7 cell line and 13762NF rat mammary adenocarcinoma *in vivo*. Anticancer Res *22*: 2685-2692, 2002.

20 Taetle R, Castognola J and Mendelsohn J: Mechanism of growth inhibition by anti-transferrin receptor monoclonal antibodies. Cancer Res *46*: 1759-1763, 1986.

21 Head JF, Wang F and Elliott RL: Antineoplastic drugs that interfere with iron metabolism in cancer cells. Adv Enzyme Regul *37*: 147-169, 1997.

22 Tacchini L, Gammella E, De Ponti C, Recalcati S and Cairo, G: Role of HIF-1 and NF-kappaB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals. J Biol Chem *283*: 20674-20686, 2008.

23 Boult J, Roberts K, Brookes MJ, Hughes S, Bury JP, Cross SS, Anderson GJ, Spychal R, Iqbal T and Tselepis C: Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma. Clin Cancer Res *14*: 379-387, 2008.

*Received September 25, 2009*
*Accepted January 12, 2010*