

Ferritin heavy chain as a molecular imaging reporter gene in glioma xenografts

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Abstract

Purpose The development of glioma therapy in clinical practice (e.g., gene therapy) calls for efficiently visualizing and tracking glioma cells in vivo. Human ferritin heavy chain is a novel gene reporter in magnetic resonance imaging. This study proposes hFTH as a reporter gene for MR molecular imaging in glioma xenografts.

Methods Rat C6 glioma cells were infected by packaged lentivirus carrying hFTH and EGFP genes and obtained by fluorescence-activated cell sorting. The iron-loaded ability was analyzed by the total iron reagent kit. Glioma nude mouse models were established subcutaneously and intracranially. Then, in vivo tumor bioluminescence was performed via the IVIS spectrum imaging system. The MR imaging analysis was analyzed on a 7T animal MRI

scanner. Finally, the expression of hFTH was analyzed by western blotting and histological analysis.

Results Stable glioma cells carrying hFTH and EGFP reporter genes were successfully obtained. The intracellular iron concentration was increased without impairing the cell proliferation rate. Glioma cells overexpressing hFTH showed significantly decreased signal intensity on T₂-weighted MRI both in vitro and in vivo. EGFP fluorescent imaging could also be detected in the subcutaneous and intracranial glioma xenografts. Moreover, the expression of the transferritin receptor was significantly increased in glioma cells carrying the hFTH reporter gene.

Conclusion Our study illustrated that hFTH generated cellular MR imaging contrast efficiently in glioma via regulating the expression of transferritin receptor. This might be a useful reporter gene in cell tracking and MR molecular imaging for glioma diagnosis, gene therapy and tumor metastasis.

S. Cheng and R. Mi are co-authors.

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Introduction

Glioma, the most common primary brain tumor in adults, is characterized by high invasiveness and recurrence (Das and Marsden 2013; Swanson et al. 2002). Despite multiple therapeutic strategies such as neurosurgery, radiotherapy and chemotherapy, its prognosis remains unsatisfactory with a median survival of only 12–18 months (Combs et al. 2008; Grossman et al. 2010). Following the development of glioma therapy in clinical practice (e.g., gene therapy, oncolytic virus therapy and stem cell therapy), it is crucial to efficiently visualize and track glioma cells in vivo for

its diagnosis and therapy. This study aims to detect glioma cells *in vivo* using a human ferritin heavy chain by magnetic resonance imaging (MRI).

Compared to other graphic imaging techniques such as computed tomography (CT) and positron emission tomography (PET), MRI provides a more distinct view of brain tissue that ensures preoperative diagnosis and assessment of glioma without extra ionizing radiation exposure (Puttick et al. 2015). MRI of cells relies on the cellular contrast generated by two major principles: direct labeling and indirect labeling. In direct cell labeling, glioma cells were loaded with contrast agents prior to engraftment into the host. Superparamagnetic iron oxide particles (SPIO) are widely used MRI label agents for tracking various types of cells, including tumor cells, dendritic cells and microglia (Bulte and Kraitchman 2004; Fleige et al. 2001; Zhang et al. 2011). Tumors derived from SPIO-labeled cells show superiority in inchoate imaging, enabling one to create hyperintense images by T2WI and confirm the edematous brim of the tumor entity (Zhang et al. 2011). However, the injection of exogenous contrast agents is non-specific to tumor cells and can cause frustrating side effects including renal toxicity and allergic reactions (Kim et al. 2010b; Pierre et al. 2014). Moreover, the long-term tracking of engrafted cells is not possible using this direct labeling approach because the concentration can be diluted with cell division and thus results in the signal fading over time.

Modern molecular imaging has provided a new vision of tumor detection using a novel genetic reporter for imaging gene expression by MRI, which includes divalent metal transporters, β -galactosidase (Louie et al. 2000), tyrosinase (Weissleder et al. 1997), transferrin receptor (Weissleder et al. 2000), magA (Zurkiya et al. 2008) and ferritin (Genove et al. 2005). These molecular reporters could be categorized into three classes based on the encoded proteins: enzyme encoding reporter (e.g., tyrosinase), cell receptor encoding reporter (e.g., transferrin receptor) and endogenous reporter (e.g., ferritin). Ferritin is a wide-existing iron-bonding protein which can eliminate the cellular toxicity produced by excessive iron accumulation, and it has been proved to be an applicable reporter gene without generating toxicity or allergic reactions (Cohen et al. 2005; Genove et al. 2005; Zhao et al. 2006).

Ferritin consists of 24 heavy (H) and light (L) subunits (Harrison and Arosio 1996). Ferritin heavy chain (FTH) was one major candidate regulator of ferritin activity to promote iron oxidation and incorporation (Treffry et al. 1997). It was reported that FTH alone or in conjunction with ferritin light chain could work as an MR imaging reporter. Compared to SPIO, overexpression of ferritin heavy chain shows no cellular toxicity and can persistently produce a reduction of the signal intensity in MRI without fading over time (Cohen et al. 2005; Ono et al. 2009).

The implementation of ferritin's function depends on transferrin receptor (TfR), a transmembrane glycoprotein that transmits iron (binding with ferritin) from extracellular matrix into the cytoplasm (Muckenthaler et al. 2008). Moreover, the transferrin receptor was also previously reported to act as a gene reporter. In this study, we propose human ferritin heavy chain as an endogenous reporter for MRI in glioma, which promotes intracellular iron transferring and directly changes the MRI signal at its expression site.

Materials and methods

Cell culture

Rat C6 glioma cells and 293T cells were obtained from the Cell Bank of Peking Union Medical College (Beijing, China), cultured in DMEM (Gibco, Life Technologies, USA) and supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a 5% CO₂ incubator at 37 °C.

Lentivirus production and cell infection

The recombinant lentiviral vectors were constructed by Beijing Sky-Bio Technology Ltd. A third-generation lentivirus packaging system was used for lentivirus production. In brief, the lentivirus vector (pLV.ExBi.P/Puro-CMV-FTH1-IRES-EGFP) and the packaging plasmid (pMDL, pRev, and pVSVG) were co-infected into 293T packaging cells; lentiviruses carrying the hFTH gene were generated and infected the C6 glioma cells (MOI=1) as previously described (Jin et al. 2013).

Fluorescence-activated cell sorting analysis

To generate stable glioma cells, lentivirus-infected cells were selected with 1 μ g/ml puromycin and further sorted using a fluorescence-activated cell sorter (Beckman Coulter, Germany). Positive cells expressing hFTH and EGFP were maintained in 10% FBS with 1 μ g/ml puromycin.

Real-time quantitative reverse transcription polymerase chain reaction (real-time PCR)

Total cellular RNA was prepared from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription-PCR (RT-PCR) analyses were done with a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Quantitative real-time PCR was performed using an SYBR Premix Ex Taq™ II kit (Takara, Dalian, China) with the ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA, $n=3$). Reactions were run

at 95 °C for 2 min followed by 35 cycles of 95 °C for 10 s and 60 °C for 1 min. Gene expression was determined by the standard curve method and normalized to the level of GAPDH. PCR primers were described as follows: hFTH-F: 5'-CTTGGAACGTCAGAGGAGAAAC-3', hFTH-R: 5'-TGAACGGACAGGATGTAGGC-3', EGFP-F: 5'-CAG AAGAACGGCATCAAGGTG-3', EGFP-R: 5'-CTTCTC GTTGGGGTCTTTGCT-3', TfR-F: 5'-GCTATGAGGAAC CAGACCGCT-3'; TfR-R: 5'-AACAGAAGACCTGTT CCCACACT-3', GAPDH-F: 5'-TGGAGTCTACTGGCG TCTT-3' and GAPDH-R: 5'-TGTCATATTTCTCGTGGT TCA-3'. GAPDH was amplified as an internal control.

Western blotting analysis

Glioma cells and tumor tissues were collected and proteins were extracted using a protein extraction kit (Applygen Technologies Inc., China). The proteins were denatured at 98 °C for 10 min and the concentrations were measured by the bicinchoninic acid method (Beijing Tyd Biotech, Beijing China). The protein lysates were separated by SDS-PAGE and transferred onto PVDF membranes (Pierce Chemical, USA). The membranes were then incubated with the primary antibodies for hFTH (Abcam, Cambridge, MA, USA, ab75973, 1:1000), EGFP (Tianjin Real-ab Biotech, Tianjin, China, REK0061, 1:3000), TfR (Santa Cruz Biotechnology, Dallas, Texas, USA. sc-393719, 1:500) and GAPDH (Tianjin Real-ab Biotech, Tianjin, China, REK0005, 1:10000) at 4 °C for 12 h, followed by incubation with a secondary HRP-conjugated second antibody (Santa Cruz Biotechnology, Dallas, Texas, USA. sc-2005, 1:5,000) at room temperature for 2 h.

Cell proliferation assay

To confirm the proliferation rate of glioma cells, 1×10^3 cells per well were cultured in 96-well plates for continuous 7 days, and a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Shanghai, China, CK04) assay was performed each day according to the manufacturer's instruction. All data are presented as the mean \pm SD.

Iron concentration assay

To determine the iron-loaded ability of glioma cells, cells (1.5×10^3 cells per well) were plated in six-well plates with a supplement of ferric ammonium citrate (FAC, 25–500 μ M, Sigma-Aldrich Bio, Saint Louis, Missouri, USA) for 24 h. Cells were then washed with PBS and resuspended in HCl (6 N). The iron concentration was detected using total iron reagent kit (Nanjing Jiancheng Bio Institute, Nanjing, China, A039-2). All data are presented as the mean \pm SD.

Tumor xenografts and analysis

To appraise the efficiency of hFTH and EGFP as reporters in C6 glioma in vivo, hFTH-EGFP-C6 or EGFP-C6 cells ($1 \times 10^6/100 \mu$ l) were injected subcutaneously into the left hind limb of 4-week-old BALB/c nude mice ($n=5$) and imaged at 7 and 14 days after cell implantation.

To further confirm their efficiency in orthotopic tumors, cells were implanted into the brain of nude mice. Mice were fixed on a brain stereotaxic apparatus under gaseous anesthesia of isoflurane during the entire process. Cells ($2 \times 10^5/5 \mu$ l) suspended in PBS were pumped into a micro-injector and injected into the right caudate nucleus (coronal suture anteroposterior, +0.5 mm; sagittal suture mediolateral, 2.2 mm) of the 4-week-old BALB/c nude mice ($n=5$) at a depth of 2.5 mm. The injection was performed with a speed of 1 μ l/min for 5 min following a retention time of 10 min. Mice were imaged 7 and 14 days after cell implantation.

In vivo bioluminescent imaging

In vivo tumor imaging was performed 10 days after cell implantation via IVIS spectrum imaging system (PerkinElmer, Waltham, MA, USA). Mice were continuously anesthetized with gaseous isoflurane anesthesia, while bioluminescent imaging was performed in the system illuminated by fiber optic lighting at 465 nm.

MR imaging analysis

Analysis was performed on a 7T animal MRI scanner (Bruker BioSpin, Billerica, MA, USA). For MR imaging analysis in vitro, cells were cultured in 10% FBS with or without iron supplement of 100 μ M ferric ammonium citrate (FAC) for 24 h. Cells (1×10^7) were then suspended with 100 μ l of PBS in 200- μ l EP tubes for T_2 -weighted imaging. In vivo T_2 images were acquired 7 and 14 days after cell implantation by a 7 T MRI scanner. The T_2 measurement sequence parameters were as follows: repetition time (TR)=3000 ms, echo time (TE)=50 ms, flip angle (FA)=90.0°, field-of-view (FOV)=40.00 \times 40.00 mm², matrix size=256 \times 256, number of slices=16, slice thickness (TH)/gap=0.5 mm/0 mm, and number of examinations (NEX)=2.0.

T_2 relaxation time and signal intensity (SI) were acquired to evaluate the efficacy of hFTH in tumor imaging. T_2 relaxation times were calculated by fitting the SIs with increasing TEs. SI was analyzed by a standard MRI operating system software program. The final SI ratios were corrected using the signal score derived from the

muscle or counter lateral brain according to the following formula $SI = SI \text{ (tumor)}/SI \text{ (muscle)}$ or $SI = SI \text{ (tumor)}/SI \text{ (counter lateral brain)}$.

Histological analysis

Glioma xenograft samples were excised, fixed in 10% neutral-buffered formalin, and embedded in paraffin for the hematoxylin and eosin (HE) and immunofluorescence (IF) staining performed at the Pathology Centre of the Institute of Basic Medical Sciences (IBMS) as previously described (Zhou et al. 2014). Images were captured with optical and fluorescence microscope (Zeiss, Germany). All antibodies used were as follows: hFTH (Abcam, Cambridge, MA, USA, ab75973, 1:100), anti-EGFP (Abcam, Cambridge, MA, USA, ab111258, 1:100), Alexa Fluor 647-conjugated secondary antibody (Abcam, Cambridge, MA, USA, ab150115, 1:3000) and Alexa Fluor 488-conjugated secondary antibody (Abcam, Cambridge, MA, USA, ab150077, 1:3000) were used for hFTH and EGFP conjugation. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

All data are presented as the means \pm standard deviation (SD). An analysis was performed using Student's *t* test by SPSS 17.0. The differences were considered to be statistically significant at $P < 0.05$.

Results

Establishing stable glioma cells carrying both hFTH and EGFP reporter genes

A recombinant lentiviral vector carrying both hFTH and EGFP genes (or EGFP alone as control) was initially constructed (Fig. 1a). hFTH can be detected indirectly through EGFP because they were translated simultaneously via an internal ribosome entry site (IRES) in eukaryotic cells (Fig. 1a). We then generated subclonal C6 glioma cells carrying both hFTH and EGFP reporter genes (or EGFP alone) by lentivirus infection (Fig. 1b). Stable C6 glioma cells expressing both hFTH and EGFP (or EGFP alone) were obtained via fluorescence-activated cell sorting (Fig. 1b).

We then confirmed the expression of hFTH and EGFP in hFTH-EGFP-C6 cells by qPCR and western blot. The gene expression of EGFP showed no difference between hFTH-EGFP-C6 (hFTH) and EGFP-C6 (CON) cells ($P > 0.05$, Fig. 1c, d). However, the expression of hFTH is much higher in hFTH-EGFP-C6 cells compared with EGFP-C6 cells ($P < 0.05$, Fig. 1c, d).

Iron concentration was significantly increased in glioma cells expressing hFTH

To investigate the proliferation rate of glioma cells carrying the hFTH gene, the CCK-8 assay was performed for continuous 7 days. No statistically significant differences in cell proliferation were observed between the hFTH-EGFP-C6 (hFTH) and EGFP-C6 (CON) cells ($P > 0.05$), suggesting that overexpression of hFTH did not influence the cell proliferation rate (Fig. 2a). Then, the intracellular iron concentration was evaluated by an iron-loaded ability assay. Cells were cultured in supplemented medium including different concentration of ferric ammonium citrate (FAC) and the intracellular iron concentrations were detected. The results showed that the iron concentration varied in a dose-dependent manner, and hFTH-EGFP-C6 cells reached the highest intracellular iron concentration when treated with 100 μ M FAC (Fig. 2b).

Transferrin receptor expression was significantly higher in glioma cells carrying the hFTH gene

As we mentioned above, transferrin receptor (TfR) is a transmembrane glycoprotein with the function of transmitting iron (binding with ferritin) from the extracellular matrix into the cytoplasm. We wondered whether the overexpression of hFTH in glioma cells would influence the function of TfR. The results (both qPCR and western blot) showed that TfR was significantly higher in hFTH-EGFP-C6 cells compared to EGFP-C6 cells (Fig. 3a, $P < 0.05$), indicating that the overexpression of hFTH stimulated the expression of TfR.

MR imaging of glioma cells carrying both hFTH and EGFP reporter genes in vitro

Ferritin decreased signals on T_2 -weighted images (T2WI) and iron concentration directly proportional to R_2 ($R_2 = 1/T_2$). To investigate the contrasting effect of hFTH in vitro, we performed T_2 -weighted MR imaging of hFTH-EGFP-C6 (1×10^7) or EGFP-C6 (1×10^7) cells treated with or without ferric ammonium citrate (FAC) on a 7T MRI scanner. The dark signal was easily observed in hFTH-EGFP-C6 cells treated with 100 μ M FAC (Fig. 4a). The T_2 relaxation times of the hFTH-EGFP-C6 (hFTH) and EGFP-C6 (CON) cells without supplement with FAC were 254.4 ± 37.1 and 298.4 ± 22.9 ms, respectively (Fig. 4b). The T_2 relaxation times of the hFTH-EGFP-C6 (hFTH) and EGFP-C6 (CON) cells with an extra supplement of 100 μ M FAC were significantly lower, 239.5 ± 24.7 s and 280.8 ± 35.4 ms, respectively (Fig. 4b). Statistical analysis showed that C6 glioma cells expressing hFTH showed significantly lower signal

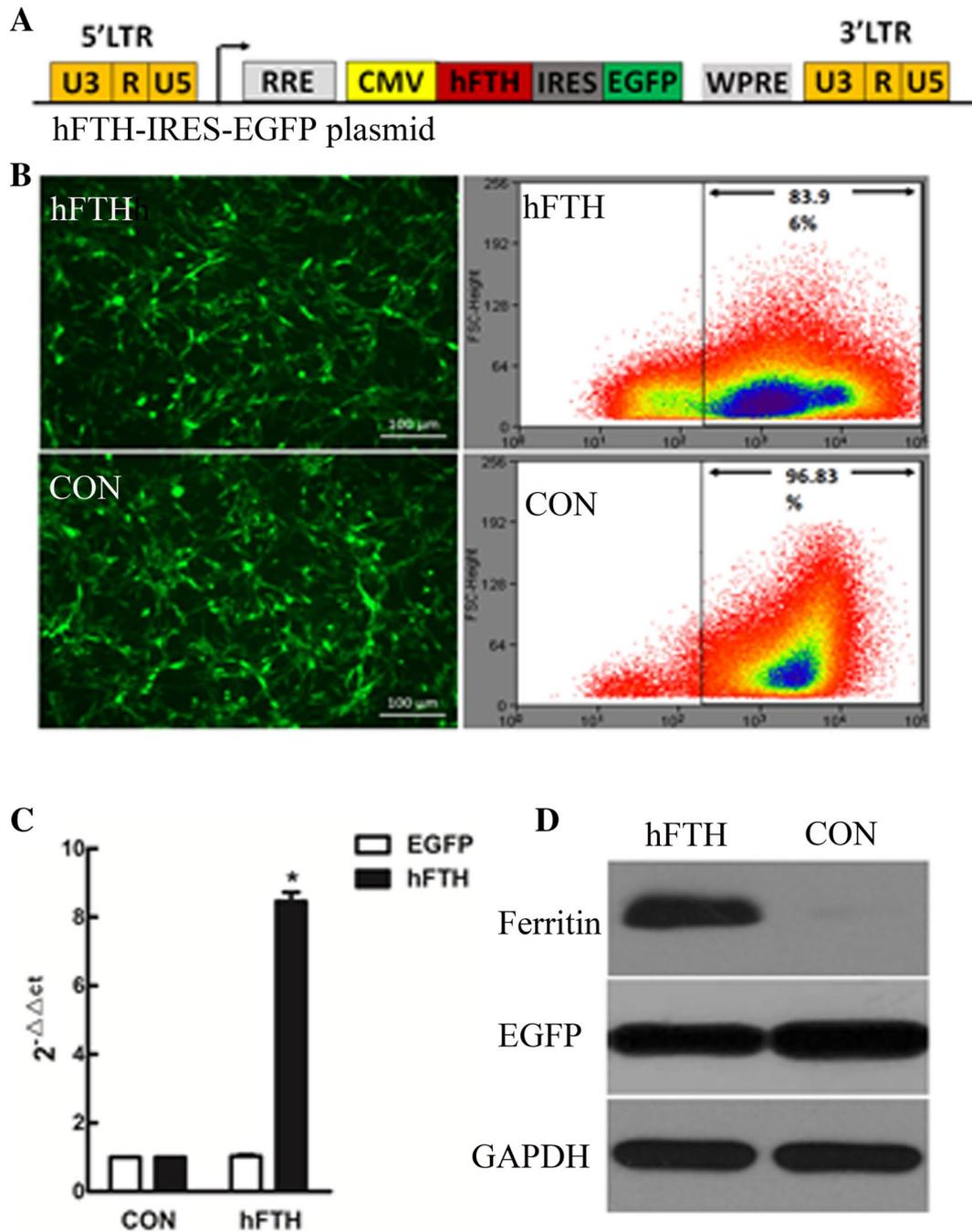


Fig. 1 Establishing the stable glioma cells carrying both hFTH and EGFP reporter genes. **a** Structures of the lentiviral vector carrying both hFTH and EGFP genes. **b** Fluorescent images of C6 cells carrying EGFP marker ($\times 100$), then the positive cells were analyzed and collected by the flow cytometer. **c** EGFP expression showed no difference between hFTH-EGFP-C6 (hFTH) and EGFP-C6 (CON) cells

(qPCR, $P > 0.05$), and the expression of the hFTH gene in hFTH-EGFP-C6 cells is significantly increased compared to EGFP-C6 cells (qPCR, $P < 0.05$). **d** Expression of hFTH and EGFP was confirmed in the collected cells by western blot. All data are presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

intensity compared to cells just expressing EGFP with or without FAC ($P < 0.05$, Fig. 4b), and glioma cells with FAC showed significantly lower signal intensity compared to the cells without FAC ($P < 0.05$, Fig. 4b).

MR and fluorescent imaging of subcutaneous glioma xenografts carrying hFTH and EGFP reporter genes

Fluorescent imaging of glioma xenografts expressing EGFP was performed 7 days after the subcutaneous

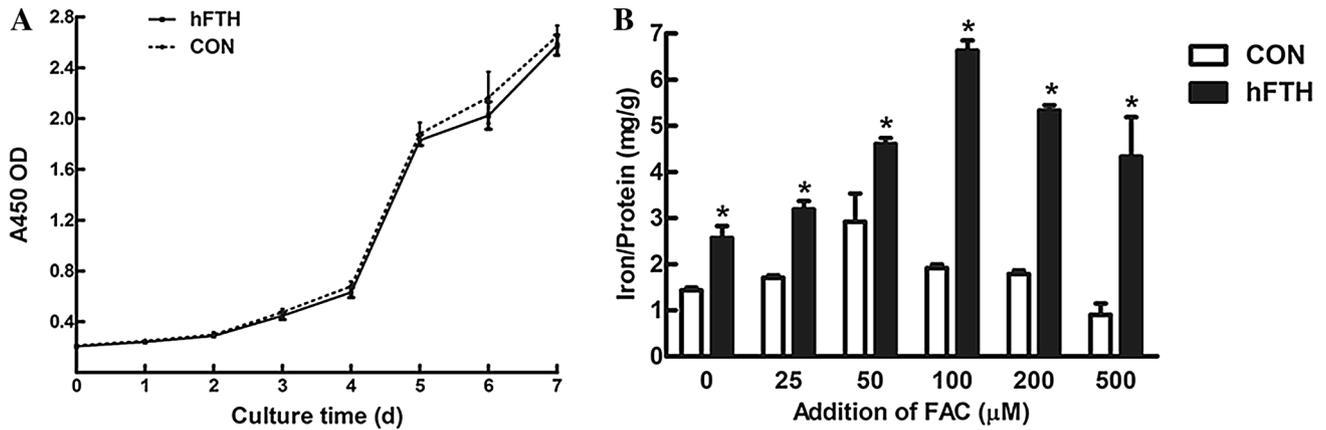


Fig. 2 Cell proliferation and iron concentration assay. **a** Proliferation rate curve showed no significant difference between hFTH-EGFP-C6 (hTFH) and EGFP-C6 (CON) cells (CCK-8 assay, $P > 0.05$). **b** Iron

concentration in the infected cells with addition of certain concentrations of FAC for 24 h. All data are presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

Fig. 3 qPCR and western blot analysis of transferrin receptor. **a** TfR expression was significantly higher in hFTH-EGFP-C6 cells (hTFH) than in EGFP-C6 cells (CON) (qPCR, $P < 0.05$). **b** TfR expression was significantly higher in hFTH-EGFP-C6 cells (hTFH) than in EGFP-C6 cells (CON) (western blot, $P < 0.05$). The data were presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

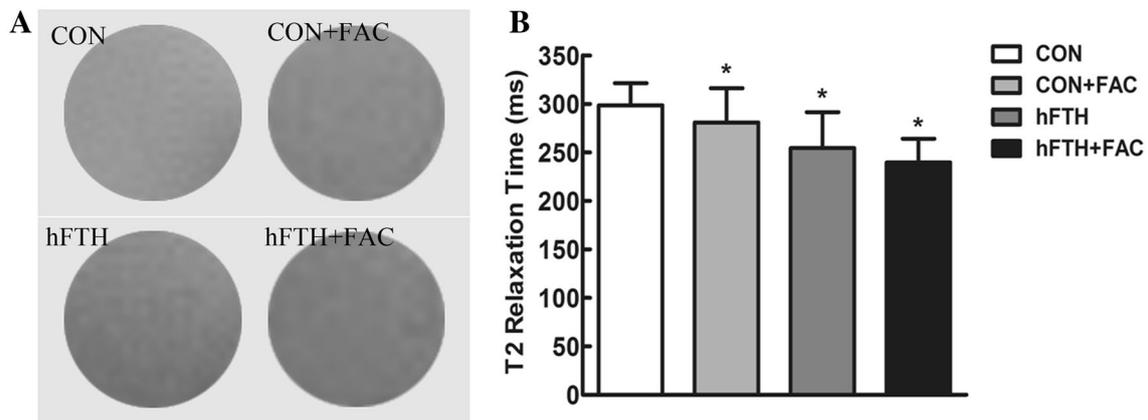
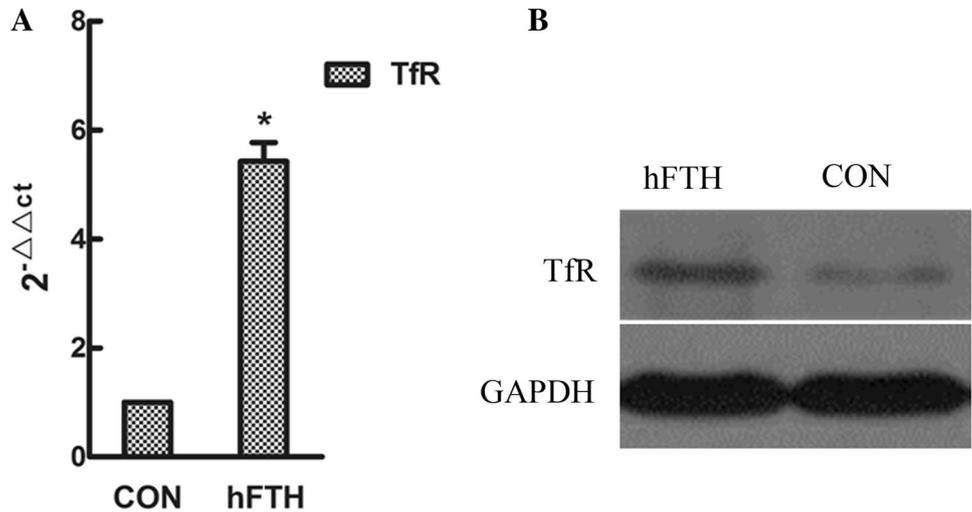


Fig. 4 In vitro MR imaging of glioma cells carrying hFTH and EGFP reporter genes. **a** T_2 -weighted images of hFTH-EGFP-C6 cells (hTFH) or hFTH-EGFP (CON) cells treated with or without FAC respectively. **b** T_2 relaxation times of hFTH-EGFP-C6 cells (hTFH)

or hFTH-EGFP (CON) cells treated with or without FAC for 24 h. The data were presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

implantation of C6 glioma cells on the left hind limb of nude mice ($n=5$, Fig. 5a). MR imaging was performed to detect the signal intensity 7 and 14 days after the subcutaneous implantation of glioma cells. The results indicated that the signal intensity of hFTH subcutaneous xenografts was lower compared with the CON xenografts (Fig. 5a).

For further evaluation, we measured the T_2 relaxation time and signal intensity of subcutaneous glioma xenografts. The T_2 relaxation times of hFTH and CON glioma xenografts on the seventh day were 47.8 ± 3.8 and 89.6 ± 9.3 ms, respectively (Fig. 5b). The T_2 values of hFTH glioma xenografts were significantly lower than that of the CON xenografts ($P < 0.01$, Fig. 5b). Additionally, the signal intensity in the hFTH glioma xenografts was significantly lower than the CON xenografts both 7 and 14 days after cell implantation ($P < 0.05$, Fig. 5c).

MR and fluorescent imaging of intracranial glioma xenografts carrying hFTH and EGFP reporter genes

Fluorescent imaging of glioma xenografts expressing EGFP was performed 7 days after the intracranial implantation of C6 glioma cells on the right caudate nucleus of

nude mice ($n=5$, Fig. 6a). MR imaging was performed to detect the signal intensity 7 and 14 days after intracranial cell implantation on nude mice. Similar to the subcutaneous results in vivo, lower signals were detected in hFTH intracranial xenografts compared to CON xenografts on the 7T MRI scanner (Fig. 6a).

The T_2 relaxation times of the hFTH and CON glioma intracranial xenografts were 52.3 ± 1.3 and 76.5 ± 3.5 ms, respectively (Fig. 6b). The T_2 value of the hFTH xenografts was significantly lower than that of the CON xenografts ($P < 0.01$, Fig. 6b). Moreover, the signal intensity of the hFTH xenografts was significantly lower than that of the CON xenografts 7 and 14 days after cell implantation ($P < 0.05$, Fig. 6c).

Protein expression analysis in glioma xenografts

Western blot analysis and immunofluorescence (IF) were performed to confirm the expression of hFTH and EGFP in glioma xenografts. The results showed that the expression of EGFP could be detected both in hFTH and CON xenografts, while hFTH was expressed only in the hFTH glioma xenografts (Fig. 7a, c, S1 Fig A, C). In addition, the

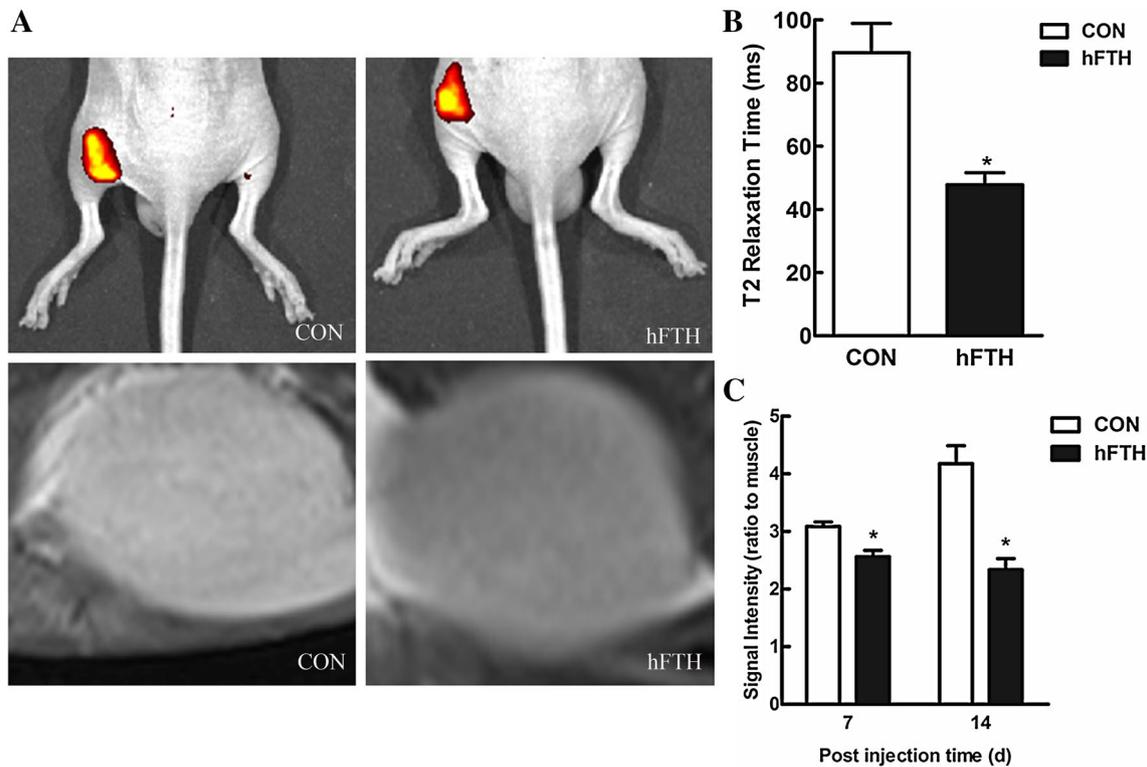


Fig. 5 MR and fluorescent imaging of subcutaneous glioma xenografts. **a** In vivo fluorescent and MR (T_2 -weighted) imaging of glioma subcutaneous xenografts (on the *left* hind limb of nude mice) 7 days after transplantation of hFTH-EGFP (hTFH, $n=5$) or EGFP-C6 (CON, $n=5$) cells. **b** T_2 relaxation time of hFTH and CON xenografts 7 days after cell implantation ($n=5$). **c** MR imaging signal intensity of subcutaneous xenografts 7 and 14 days after cell implantation ($n=5$). All values were presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

ografts 7 days after cell implantation ($n=5$). **c** MR imaging signal intensity of subcutaneous xenografts 7 and 14 days after cell implantation ($n=5$). All values were presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

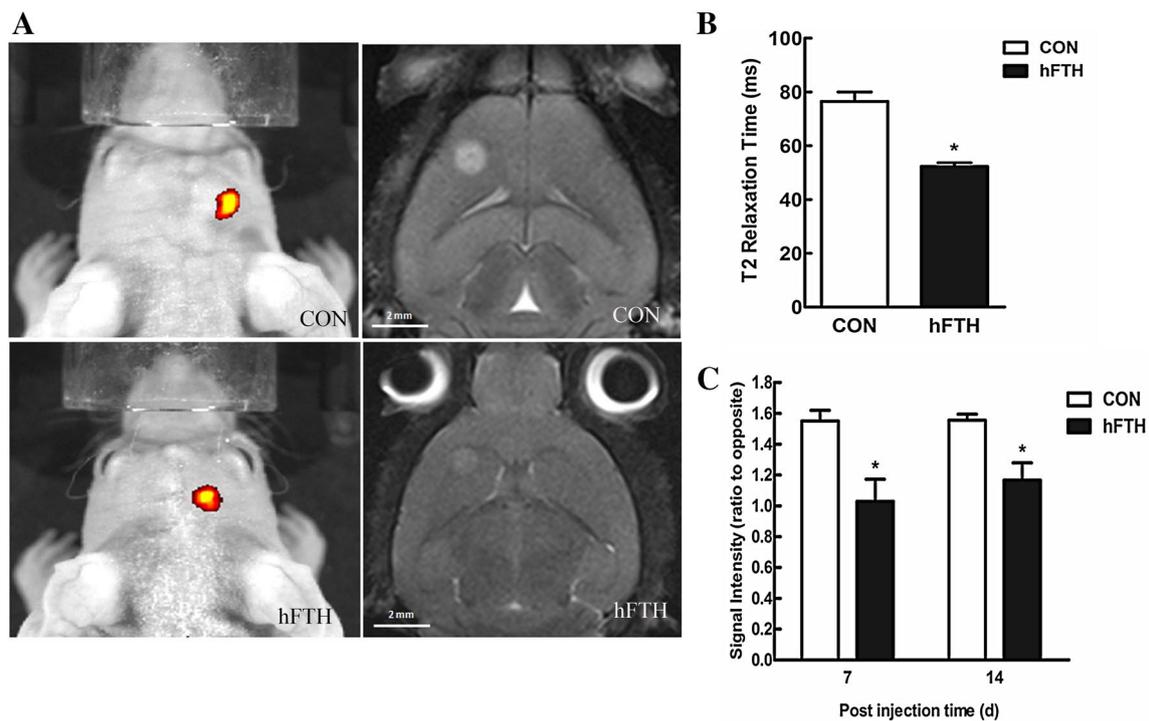


Fig. 6 MR and fluorescent imaging of intracranial glioma xenografts. **a** In vivo fluorescent and MR (T_2 -weighted) imaging of intracranial hFTH-EGFP-C6 (hFTH, $n=5$) and EGFP-C6 (CON, $n=5$) xenografts 7 days after cell implantation. **b** T_2 relaxation time

of hFTH and CON xenografts 7 days after cell implantation ($n=5$). **c** MR imaging signal intensity of intracranial xenografts 7 and 14 days after cell implantation ($n=5$). All data are presented as the mean \pm SD. * P value was statistically significant ($P < 0.05$)

Prussian blue staining showed much more iron accumulation in hFTH glioma xenografts compared to the CON xenografts (Fig. 7b, S1 Fig B).

Discussion

In the past decades, the limited survival time of patients with glioma especially glioblastoma multiforma (GBM) reminds us to pay more attention to efficient glioma diagnosis and therapy. Using a lentivirus, we successfully established a rat C6 cell line carrying both hFTH and EGFP reporter genes. It was demonstrated that the overexpression of hFTH promoted the MR imaging contrasting effect in both glioma cells and xenografts. Interestingly, the expression of TfR significantly increased during this process. Our data illustrated hFTH as a useful gene reporter to tracking glioma cells in glioma diagnosis and therapy.

Whether the overexpression of hFTH and iron accumulation has potentially adverse impacts on cellular homeostasis and tumor growth deserves concern. In our study, the overexpression of hFTH did not influence the proliferation rate of C6 glioma cells, which was consistent with previous results (Cohen et al. 2005; Kim et al. 2015; Zhang et al. 2015). However, there is an opposite

result declaring that the cell proliferation rate was significantly decreased due to the overexpression of FTH (Feng et al. 2012). The effects of FTH on cell proliferation and growth may depend on the cell type and the expression level of FTH. Moreover, the potentially adverse impacts could be reduced by a Tet-On/Tet-off switch controlled system to avoid the continuous gene expression of ferritin and the subsequent iron accumulation in cells (Cohen et al. 2005; He et al. 2015).

Another deficiency of the MR imaging reporter gene is that it provides much weaker imaging contrast compared with the direct cell-labeling methods such as superparamagnetic iron oxide particles (SPIOs). However, the signal would be rapidly diluted following cell proliferation, division and differentiation when using SPIOs to track cells for a long time (Cromer Berman et al. 2013; Hsiao et al. 2008; Kedziorek et al. 2010). Moreover, SPIOs may have side effects on cell differentiation. Thus, a genetic MRI reporter is much more suitable for the longitudinal and long-term MRI monitoring of cells, while the SPIO cell-labeling method is fit for the horizontal and short-term MRI monitoring of cells. For the above reasons, FTH had a clear advantage in labeling glioma cells (especially glioma stem cells) with a high proliferation rate.

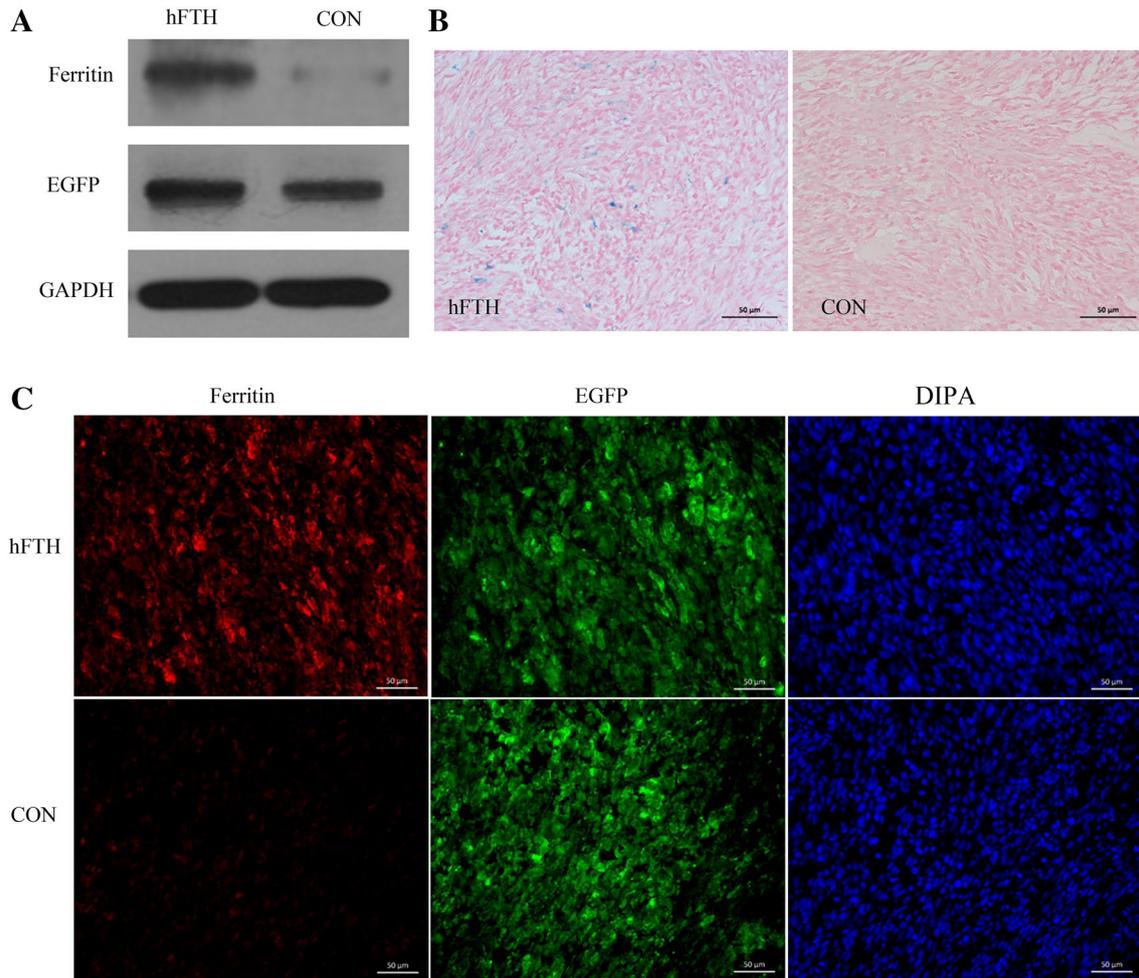


Fig. 7 Western blot and histological analysis of subcutaneous glioma xenografts. **a** Expression of hFTH and EGFP in subcutaneously implanted xenografts was confirmed by western blot. **b** Prussian blue staining showed iron accumulated much more in hFTH glioma xeno-

grafts compared to CON xenografts ($\times 400$). **c** Immunofluorescent analysis of implanted xenografts expressing hFTH (red) or EGFP (green) alone ($\times 400$)

Lentivirus is an attractive carrier for gene delivery because it can efficiently infect cells (both dividing and non-dividing cells) and form stable cells carrying inserted genes for a long period of time. Kim HS et al. reported the ability to detect 2×10^3 cells at a clinical MR scanner with a ferritin reporter (Kim et al. 2010a). In addition, single SPIO-labeled cells have been detected using an optimized MR hardware and a clinical MR scanner (Heyn et al. 2006). It might, therefore, be predicted to track a single glioma cell in the presence of an endogenous gene reporter such as FTH. To some extent, the realization of this may rely on a more effective transfer vehicle and a high expression level of ferritin for more effective imaging contrast. Oncolytic viruses (HSV-1) had been investigated for cancer treatment due to their ability to selectively infect and lyse neoplastic cells while sparing normal cells (Zhang et al. 2014; Zhu et al. 2011). HSV-1 was shown to be more efficient than the

other virus and has the capacity to incorporate large or multiple transgenes into the viral genome to facilitate genetic engineering. HSV-1 could be a more efficient and safe way for gene delivery (e.g., p53 and FTH genes) to track cells following gene therapy and oncolytic therapy. Improving the sensitivity of MR imaging could also be achieved by other methods, such as combining hFTH with other gene reporters (e.g., TfR) and supplementing with iron.

Because of their effectiveness and safety in MR imaging, molecular reporters could be used more widely to monitor tumor growth, tumor invasion, tumor metastasis, etc. Furthermore, ferritin was reported to generate MR imaging contrast in C3H10T1/2 stem cells under the control of a Tet-On switch (He et al. 2015). Human ferritin heavy chain (hFTH) had also labeled human mesenchymal stem cells (hMSCs) in the mouse brain and led to a significant increase in R_2^* values (Pereira et al.

2015). Whether glioma stem cells could be detected by the endogenous reporter of hFTH needs to be further characterized. In addition, hFTH reporter genes could be used in glioma gene therapy via cloning just behind the therapeutic gene to track the entirety of the cellular therapeutics.

Transferrin receptor (TfR) is a homodimer transmembrane glycoprotein that widely exists in almost all mammalian cells with a molecular weight of 170–200 kDa. The iron-loaded transferrin binds to TfR and forms the TfR–Tf–Fe complex, which will be rapidly internalized through endocytosis. As we mentioned above, TfR is another reporter gene in MR imaging. The overexpression of TfR will increase the iron concentration in cells and decrease the following T_2 relaxation time. In our study, it was surprising to find that the expression of TfR was significantly increased in hFTH-EGFP-C6 glioma cells, indicating that the overexpression of hFTH could promote the expression of TfR to facilitate intracellular iron transferring and molecular imaging.

Optical imaging techniques (e.g., bioluminescent imaging and fluorescent imaging) have also been investigated for tracking cells. However, they are commonly considered to be unfeasible for monitoring grafted cells in vivo due to the rapid deterioration of sensitivity and spatial resolution in deeper tissues via light scattering. In our study, EGFP was used to monitor hFTH expression in glioma cells. The results showed that EGFP fluorescent imaging could be detected in subcutaneous and intracranial tumors. Our data supported fluorescent protein to be used as reporter gene for noninvasive imaging in glioma, especially when combined with other endogenous reporter genes such as hFTH.

In summary, we would like to propose hFTH as a reporter gene in MR imaging for glioma diagnosis, gene therapy and metastasis research. We found for the first time that the overexpression of ferritin could promote the expression of transferrin receptor to facilitate intracellular iron transferring and MR imaging.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animal research was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Beijing Tiantan Hospital, Capital Medical University. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Beijing Tiantan Hospital, Capital Medical University. All efforts were made to minimize the suffering of animal subjects.

References

- Bulte JW, Kraitchman DL (2004) Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed* 17:484–499 doi:[10.1002/nbm.924](https://doi.org/10.1002/nbm.924)
- Cohen B, Dafni H, Meir G, Harmelin A, Neeman M (2005) Ferritin as an endogenous MRI reporter for noninvasive imaging of gene expression in C6 glioma tumors. *Neoplasia* 7:109–117. doi:[10.1593/neo.04436](https://doi.org/10.1593/neo.04436)
- Combs SE, Wagner J, Bischof M, Welzel T, Wagner F, Debus J, Schulz-Ertner D (2008) Postoperative treatment of primary glioblastoma multiforme with radiation and concomitant temozolomide in elderly patients. *Int J Radiat Oncol Biol Phys* 70:987–992 doi:[10.1016/j.ijrobp.2007.07.2368](https://doi.org/10.1016/j.ijrobp.2007.07.2368)
- Cromer Berman SM, Kshitiz, Wang CJ, Orukari I, Levchenko A, Bulte JW, Walczak P (2013) Cell motility of neural stem cells is reduced after SPIO-labeling, which is mitigated after exocytosis. *Magn Reson Med* 69:255–262 doi:[10.1002/mrm.24216](https://doi.org/10.1002/mrm.24216)
- Das S, Marsden PA (2013) Angiogenesis in glioblastoma. *N Engl J Med* 369:1561–1563. doi:[10.1056/NEJMcibr1309402](https://doi.org/10.1056/NEJMcibr1309402)
- Feng Y, Liu Q, Zhu J, Xie F, Li L (2012) Efficiency of ferritin as an MRI reporter gene in NPC cells is enhanced by iron supplementation. *J Biomed Biotechnol* 2012:434878 doi:[10.1155/2012/434878](https://doi.org/10.1155/2012/434878)
- Fleige G, Nolte C, Synowitz M, Seeberger F, Kettenmann H, Zimmer C (2001) Magnetic labeling of activated microglia in experimental gliomas. *Neoplasia* 3:489–499. doi:[10.1038/sj/neo/7900176](https://doi.org/10.1038/sj/neo/7900176)
- Genove G, DeMarco U, Xu H, Goins WF, Ahrens ET (2005) A new transgene reporter for in vivo magnetic resonance imaging. *Nat Med* 11:450–454 doi:[10.1038/nm1208](https://doi.org/10.1038/nm1208)
- Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M, Fisher J, NABTT CNS Consortium (2010) Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the US. *Clin Cancer Res* 16:2443–2449. doi:[10.1158/1078-0432.CCR-09-3106](https://doi.org/10.1158/1078-0432.CCR-09-3106)
- Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* 1275:161–203
- He X, Cai J, Liu B, Zhong Y, Qin Y (2015) Cellular magnetic resonance imaging contrast generated by the ferritin heavy chain genetic reporter under the control of a Tet-On switch. *Stem Cell Res Ther* 6:207 doi:[10.1186/s13287-015-0205-z](https://doi.org/10.1186/s13287-015-0205-z)
- Heyn C, Ronald JA, Mackenzie LT, MacDonald IC, Chambers AF, Rutt BK, Foster PJ (2006) In vivo magnetic resonance imaging of single cells in mouse brain with optical validation. *Magn Reson Med* 55:23–29 doi:[10.1002/mrm.20747](https://doi.org/10.1002/mrm.20747)
- Hsiao JK, Chu HH, Wang YH, Lai CW, Chou PT, Hsieh ST, Wang JL, Liu HM (2008) Macrophage physiological function after superparamagnetic iron oxide labeling. *NMR Biomed* 21:820–829. doi:[10.1002/nbm.1260](https://doi.org/10.1002/nbm.1260)
- Jin G, Zhou Y, Chai Q, Zhu G, Xu F, Liu F (2013) VP22 and cytosine deaminase fusion gene modified tissue-engineered neural stem

- cells for glioma therapy. *J Cancer Res Clin Oncol* 139:475–483 doi:[10.1007/s00432-012-1347-3](https://doi.org/10.1007/s00432-012-1347-3)
- Kedziorek DA, Muja N, Walczak P, Ruiz-Cabello J, Gilad AA, Jie CC, Bulte JW (2010) Gene expression profiling reveals early cellular responses to intracellular magnetic labeling with superparamagnetic iron oxide nanoparticles. *Magn Reson Med* 63:1031–1043 doi:[10.1002/mrm.22290](https://doi.org/10.1002/mrm.22290)
- Kim HS, Cho HR, Choi SH, Woo JS, Moon WK (2010a) In vivo imaging of tumor transduced with bimodal lentiviral vector encoding human ferritin and green fluorescent protein on a 1.5 T clinical magnetic resonance scanner. *Cancer Res* 70:7315–7324. doi:[10.1158/0008-5472.CAN-10-0241](https://doi.org/10.1158/0008-5472.CAN-10-0241)
- Kim HS, Oh SY, Joo HJ, Son KR, Song IC, Moon WK (2010b) The effects of clinically used MRI contrast agents on the biological properties of human mesenchymal stem cells. *NMR Biomed* 23:514–522. doi:[10.1002/nbm.1487](https://doi.org/10.1002/nbm.1487)
- Kim HS, Woo J, Lee JH, Joo HJ, Choi Y, Kim H, Moon WK, Kim SJ (2015) In vivo Tracking of dendritic cell using MRI reporter gene, ferritin. *PLoS One* 10:e0125291. doi:[10.1371/journal.pone.0125291](https://doi.org/10.1371/journal.pone.0125291)
- Louie AY, Hüber MM, Ahrens ET, Rothbächer U, Moats R, Jacobs RE, Fraser SE, Meade TJ (2000) In vivo visualization of gene expression using magnetic resonance imaging. *Nat Biotechnol* 18:321–325. doi:[10.1038/73780](https://doi.org/10.1038/73780)
- Muckenthaler MU, Galy B, Hentze MW (2008) Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annu Rev Nutr* 28:197–213. doi:[10.1146/annurev.nutr.28.061807.155521](https://doi.org/10.1146/annurev.nutr.28.061807.155521)
- Ono K, Fuma K, Tabata K, Sawada M (2009) Ferritin reporter used for gene expression imaging by magnetic resonance. *Biochem Biophys Res Commun* 388:589–594. doi:[10.1016/j.bbrc.2009.08.055](https://doi.org/10.1016/j.bbrc.2009.08.055)
- Pereira SM, Moss D, Williams SR, Murray P, Taylor A (2015) Overexpression of the MRI reporter genes ferritin and transferrin receptor affect iron homeostasis and produce limited contrast in mesenchymal stem cells. *Int J Mol Sci* 16:15481–15496 doi:[10.3390/ijms160715481](https://doi.org/10.3390/ijms160715481)
- Pierre VC, Allen MJ, Caravan P (2014) Contrast agents for MRI: 30+ years and where are we going? *J Biol Inorg Chem* 19:127–131. doi:[10.1007/s00775-013-1074-5](https://doi.org/10.1007/s00775-013-1074-5)
- Puttick S, Bell C, Dowson N, Rose S, Fay M (2015) PET, MRI, and simultaneous PET/MRI in the development of diagnostic and therapeutic strategies for glioma. *Drug Discov Today* 20:306–317 doi:[10.1016/j.drudis.2014.10.016](https://doi.org/10.1016/j.drudis.2014.10.016)
- Song C, Wang J, Mo C, Mu S, Jiang X, Li X, Zhong S, Zhao Z, Zhou G (2015) Use of ferritin expression, regulated by neural cell-specific promoters in human adipose tissue-derived mesenchymal stem cells, to monitor differentiation with magnetic resonance imaging in vitro. *PLoS One* 10:e0132480. doi:[10.1371/journal.pone.0132480](https://doi.org/10.1371/journal.pone.0132480)
- Swanson KR, Alvord EC Jr, Murray JD (2002) Virtual brain tumours (gliomas) enhance the reality of medical imaging and high-light inadequacies of current therapy. *Br J Cancer* 86:14–18. doi:[10.1038/sj.bjc.6600021](https://doi.org/10.1038/sj.bjc.6600021)
- Treffry A, Zhao Z, Quail MA, Guest JR, Harrison PM (1997) Dinuclear center of ferritin: studies of iron binding and oxidation show differences in the two iron sites. *Biochemistry* 36:432–441. doi:[10.1021/bi961830l](https://doi.org/10.1021/bi961830l)
- Weissleder R, Simonova M, Bogdanova A, Bredow S, Enochs WS, Bogdanov A Jr (1997) MR imaging and scintigraphy of gene expression through melanin induction. *Radiology* 204:425–429. doi:[10.1148/radiology.204.2.9240530](https://doi.org/10.1148/radiology.204.2.9240530)
- Weissleder R, Moore A, Mahmood U, Bhorade R, Benveniste H, Chiocca EA, Basilion JP (2000) In vivo magnetic resonance imaging of transgene expression. *Nat Med* 6:351–355 doi:[10.1038/73219](https://doi.org/10.1038/73219)
- Zhang F, Xie J, Liu G, He Y, Lu G, Chen X (2011) In vivo MRI tracking of cell invasion and migration in a rat glioma model. *Mol Imaging Biol* 13:695–701. doi:[10.1007/s11307-010-0401-2](https://doi.org/10.1007/s11307-010-0401-2)
- Zhang G, Jin G, Nie X, Mi R, Zhu G, Jia W, Liu F (2014) Enhanced antitumor efficacy of an oncolytic herpes simplex virus expressing an endostatin-angiostatin fusion gene in human glioblastoma stem cell xenografts. *PLoS One* 9:e95872. doi:[10.1371/journal.pone.0095872](https://doi.org/10.1371/journal.pone.0095872)
- Zhao G, Arosio P, Chasteen ND (2006) Iron(II) and hydrogen peroxide detoxification by human H-chain ferritin. An EPR spin-trapping study. *Biochemistry* 45:3429–3436. doi:[10.1021/bi052443r](https://doi.org/10.1021/bi052443r)
- Zhou Y, Jin G, Mi R, Dong C, Zhang J, Liu F (2014) The methylation status of the platelet-derived growth factor-B gene promoter and its regulation of cellular proliferation following folate treatment in human glioma cells. *Brain Res* 1556:57–66. doi:[10.1016/j.brainres.2014.01.045](https://doi.org/10.1016/j.brainres.2014.01.045)
- Zhu G, Su W, Jin G, Xu F, Hao S, Guan F, Jia W, Liu F (2011) Glioma stem cells targeted by oncolytic virus carrying endostatin-angiostatin fusion gene and the expression of its exogenous gene in vitro. *Brain Res* 1390:59–69. doi:[10.1016/j.brainres.2011.03.050](https://doi.org/10.1016/j.brainres.2011.03.050)
- Zurkiya O, Chan AW, Hu X (2008) MagA is sufficient for producing magnetic nanoparticles in mammalian cells, making it an MRI reporter. *Magn Reson Med* 59:1225–1231 doi:[10.1002/mrm.21606](https://doi.org/10.1002/mrm.21606)