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In vivo MR Imaging and Quantification of Primary and Metastatic Cancers using Biomodal Lentiviral Vector Encoding Human Ferritin and Green Fluorescent Protein

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동시 발현 렌티바이러스 벡터를 이용한
원발성 및 전이암의 자기공명영상화 및
정량화 기법에 대한 연구

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In vivo MR Imaging and Quantification of Primary and Metastatic Cancers using Biomodal Lentiviral Vector Encoding Human Ferritin and Green Fluorescent Protein
Abstract

In vivo MR Imaging and Quantification of Primary and Metastatic Cancers using Biomodal Lentiviral Vector Encoding Human Ferritin and Green Fluorescent Protein

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A combination of reporter genes for magnetic resonance imaging (MRI) and optical imaging can provide an additional level of noninvasive and quantitative information about biological processes occurring in deep tissues. Cellular MRI with a reporter gene offers the opportunity to track small numbers of tumor cells and to study metastatic processes in their earliest developmental stages in the target organs of interest.

I developed a bimodal lentiviral vector to monitor deep tissue events using MRI to detect myc tagged human ferritin heavy chain (myc-hFTH) expression and fluorescence imaging to detect green fluorescent protein (GFP) expression. No cellular toxicity due to overexpression of myc-hFTH and GFP was observed in MTT and trypan blue exclusion assays. Iron accumulation was observed in myc-hFTH cells and tumors by Prussian blue staining and iron binding assays.
First of all, for primary tumor imaging, the transgene construct was stably transfected into MCF-7 and F-98 cells. After transplantation of the cells expressing myc-hFTH and GFP into mice or rats, serial MRI and fluorescence imaging were performed with a human wrist coil on a 1.5T MR scanner and optical imaging analyzer for 4 weeks. The myc-hFTH cells and tumors had significantly lower signal intensities in T2 weighted MRI than mock-transfected controls ($P \leq 0.05$). This is direct evidence that myc-hFTH expression can be visualized noninvasively with a 1.5T clinical MR scanner.

Second, for the noninvasive imaging and quantification of metastatic melanoma cells in the lymph nodes (LNs) of living mice, a B16F10 murine melanoma cell line expressing hFTH and GFP was constructed to allow the detection of cells by MRI and fluorescence imaging. Stable overexpression of hFTH and GFP in B16F10 murine melanoma cells was feasible and showed no cellular toxicity. In addition, hFTH cells were detectable by 9.4T MRI \textit{in vitro} and \textit{in vivo}, yielding significant changes in T2* relative to control cells. In BALB/c nude mice, the presence of hFTH and GFP expressing metastatic melanoma cells in deep seated axillary LNs was demonstrated as areas of low T2* on MRI, but the same LNs were not visible by fluorescence imaging because the light was unable to penetrate the tissue. Furthermore, the metastatic volume of each LN, which was assessed by cumulative histogram analysis of the T2* MRI data, correlated well with tumor burden, which was determined by histology ($r = -0.8773, p = 0.0001$).

This study shows that MRI and fluorescence imaging of transplanted cells or metastatic cancer cells at molecular and cellular levels can be performed simultaneously using my bimodal lentiviral vector system. In addition, this techniques can be used to monitor tumor growth, regression during cell and gene-based therapy in deep tissues.

**Keywords:** human ferritin heavy chain (hFTH), green fluorescent protein (GFP), MR reporter gene, Magnetic resonance (MR)

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Overall Introduction

Advances in cellular biochemistry and molecular biology provide innovative approaches for developing new molecular imaging probes for magnetic resonance imaging (MRI), which enables superior three-dimensional spatial resolution and multiple contrast mechanisms, yielding detailed anatomical and functional information. Such probes for classical reporter genes include β-galactosidase and the enzymes creatine and arginine kinase. In addition, other studies have suggested the use of genes associated with iron homeostasis, including the transferrin receptor, tyrosinase and ferritin. Among them, the contrast by transferrin receptor generated by inducing receptor mediated internalization of exogenously administered transferrin coupled to paramagnetic particles, and the contrast by tyrosinase-catalyzed melanin synthesis was generated by exogenously administered iron, which binds to the melanin. However, overexpression of either tyrosinase or transferrin receptor could elevate radical formation due to the increase in free iron concentration. Thus far, all the reporter genes for MRI developed rely on exogenous administration of contrast material. Therefore, delivery barriers and clearance must be considered.

Ferritin, a ubiquitous and highly conserved iron-binding protein, is assembled from 24 light and heavy polypeptide chains enveloping an iron oxide core of up to 4,500 iron atoms, which is depending on the tissue type and physiologic status of the cell. Cellular models in which ferritin expression was modulated revealed that the heavy (H) chain is the main regulator of ferritin activity. The H subunit has ferroxidase activity that promotes iron oxidation and incorporation. In contrast, the L-chain lacks detectable ferroxidase activity but facilitates the activity of the H-chain by offering sites for iron nucleation and mineralization, and by increasing the turnover at the ferroxidase center. Cells transfected with the H-chain ferritin responded by upregulation of transferrin receptor and increased iron uptake. The magnetic properties of ferritin and its effect on the transverse (R2 ¼1/T2) and longitudinal (R1 ¼1/T1) relaxation rates of water have been the focus of intensive investigation, and it has been shown that ferritin has peculiarly high relaxivity at very low iron loading and a unique linear dependence of R2 on the magnetic field. R2 induced by ferritin is substantially elevated in tissues relative to solution, an effect that was
attributed to spontaneous aggregation of ferritin particles. Thus, MRI was used for in vivo quantification of ferritin-bound iron in liver and brain nuclei in pathologies including b-thalassemia and Alzheimer’s disease.

Here, I performed two consecutive studies using a biomodal lentiviral vector to monitor deep tissue events using MRI to detect myc tagged human ferritin heavy chain expression and fluorescence imaging to detect green fluorescent protein expression. In the first study, the approach was performed for inducing MRI contrast in an *in vitro* and *in vivo* systems via expression of myc-hFTTH proteins in a transfected tumor cell line, which showed that the increased contrast effect by the iron accumulation within the transfected cells and tumors could be detected by MRI, and green fluorescent protein signals were also observed. The following second study revealed that the present system can be used for the in vivo imaging and quantification of the metastatic cancer cells in lymph nodes by using an animal model.
Chapter 1. *In vivo* imaging of Tumor Transduced with Bimodal Lentiviral Vector Encoding Human Ferritin and Green Fluorescent Protein on a 1.5T Clinical Magnetic Resonance Scanner

1.1 Introduction

Fluorescent or bioluminescent proteins have been used as reporter genes for noninvasive imaging of *in vivo* events, which can be used to assess the efficacy of gene therapy and metabolic activity (1), growth kinetics of transformed tumor cells (2, 3), and differentiation of stem cells (4, 5). However, fluorescence reporters are most useful for visualization of peripheral organs like skin, due to their limited ability to provide spatial resolution and anatomic information in deeper tissues, which is critical for visualization of events such as tumor metastases that occur throughout the body. However, combining fluorescent reporter genes with iron accumulating reporter genes for magnetic resonance imaging (MRI) provides noninvasive, high resolution, and quantitative information for biological processes occurring in deep tissues.

The main advantage of using iron accumulating reporter genes for MRI is that it overcomes the challenge of delivering the contrast agent to deep tissues (6). Recently, the ferritin gene has been used as an iron accumulating reporter gene for evaluating the efficacy of gene and cell therapy by MRI (7–12). Ferritin is an iron storage protein that can be ectopically expressed to augment endogenous iron uptake and produce signal changes in the surrounding environment that can be detected by MRI. Ferritin based imaging with high magnetic field magnetic resonance (MR) scanner, such as 4.7T or 7T scanner, has been performed for cells and tissues transfected with plasmid carrying the ferritin gene (9, 11, 12). The low MRI contrast and signal intensities in these studies are likely to be due to the inherent limits of gene delivery by plasmid. Therefore, the development of a suitable vector for MR reporter gene delivery is a major goal.
that must be reached to produce detectable signal and contrast for clinical MR applications.

Reportedly, lentiviral vectors derived from HIV type 1 have efficient delivery, integration, and long term and stable expression of transgenes for mitotic and nonmitotic cells both in vivo (13, 14). Furthermore, in contrast to adenoviral vectors, lentiviral vector delivery systems do not produce humoral responses to injected viral antigens (15). These critical features of lentiviral vector delivery have made them the logical choice for gene therapy treatment of cancer or diseases with a genetic basis (14, 16, 17). I believe that the generation of safe and efficacious lentiviral vectors will significantly advance the prospects of multimodal imaging technique using 1.5T MR scanners.

Here, I developed a lentiviral vector construct that expresses both recombinant human ferritin [myc-tagged human ferritin heavy chain (myc-hFTH)] and green fluorescent protein (GFP) for simultaneous MRI and fluorescence imaging in vitro and in vivo. In this system, myc-hFTH cells and tumors had more iron accumulation than mock transfected controls, which produced a pronounced increase in T2* contrast effects on a 1.5T clinical MR scanner. This lentiviral myc-hFTH vector system overcomes the present limitations of reporter gene delivery for MRI by providing direct, strong, and noninvasive imaging of deep tissues in vivo using a 1.5T clinical MR scanner.
1.2 Material and Methods

1.2.1 Construction of dual promoter lentiviral vectors encoding hFTH and GFP

The recombinant lentiviral vector (LentiM1.41) was constructed in the laboratory of Yeon-Soo Kim (Inje University, Gimhae, South Korea). The phosphoglycerate kinase promoter was inserted immediately upstream of GFP in the lentiviral vector into a BamHI-EcoRI site available for insertion of a gene of interest under the control of the cytomegalovirus (CMV) immediate early promoter and puromycin resistant gene by the IRES system. Briefly, the hFTH gene was amplified from pOBT7-human FTH (BC073750) by PCR with the primers forward, BamHI 5'-CGGGATCCGCCACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGACGACCCGCTCCACC-3' and reverse, EcoRI 5'-CGGAATTCTTAGCTTTCATTATCATTACTGAT-3', followed by digestion with BamHI and EcoRI and insertion in the restriction sites downstream of the CMV promoter. The NH2 terminus of hFTH was tagged at the NH2 terminus with the mouse c-myc gene to produce recombinant myc-hFTH. The sequence of the cloned cDNA was confirmed by DNA sequencing. The constructed lentiviral vector expressing myc-hFTH was named Lenti myc-hFTH (Figure 1.1A). Lentiviral vector without GFP and hFTH was used for control (mock) transfections.

1.2.2 Lentivirus production

The recombinant lentivirus was produced by Macrogen LentiVector Institute. Briefly, three plasmids, a transfer vector, a VSV-G expression vector, and a gag-pol expression vector, were cotransfected into 293T cells at a 1:1:1 molar ratio. The culture supernatant containing viral vector particles was harvested 48 hours after transfection, clarified with a 0.45 μm membrane filter (Nalgene), and immediately stored at −70°C. Titers were determined by p24 ELISA or infection of HeLa cells. The GFP expression of transduced cells was observed and imaged with a fluorescence microscope and camera system. In routine preparations, the unconcentrated titer was \( \sim 10^6 \) to \( 10^7 \) transduction units/mL.

1.2.3 Generation of MCF-7 and F-98 cells stably expressing myc-hFTH and GFP

Human breast cancer cells (MCF-7) and rat glioma cells (F-98) were obtained from American Type Culture Collection, cultured in DMEM supplemented with 10% fetal bovine serum and...
1% penicillin/streptomycin, and passaged in my laboratory for <6 months after resuscitation. MCF-7 cells expressed the estrogen receptors. F-98 cells provided a reproducible tumor model when inoculated into the brains or under the skin of Fisher 344 rats. For stable cell generation, MCF-7 and F-98 cells were transduced with lentivirus supernatants for 6 to 10 hours in the presence of 4 to 8 μg/mL polybrene. Transduced cells were selected using 1 μg/mL puromycin in the media for 3 to 4 weeks and sorted using fluorescence activated cell sorter. myc-FTH and GFP expressions in all clones were analyzed by reverse transcription PCR (RT-PCR), Western blot, immunostaining, and fluorescence microscopy (Nikon). Clones that express high levels of myc-hFTH and GFP were passaged and maintained in a growth medium containing 1 μg/mL puromycin. To further confirm the stable expression of myc-hFTH and GFP of selected clones, Western blot and immunostaining were performed before in vivo and in vitro experiments.

**1.2.4 Fluorescence-activated cell sorting analysis**

Fluorescence of GFP in transduced cells was analyzed using a FACSCalibur flow cytometer (BD Biosciences) equipped with a 530 nm filter (bandwidth, ±15 nm), a 585 nm filter (bandwidth, ±21 nm), and a CellQuest software (BD Biosciences). Sorted GFP expressing cells were used for in vivo and in vitro studies.

**1.2.5 RT-PCR**

Transduced MCF-7 and F-98 cells were selected by puromycin (2.5 μg/mL) resistance. Human FTH mRNA expression was detected by RT-PCR analysis. RNA was isolated from confluent 10 cm culture plates using the TRIzol reagent (Invitrogen), and RT-PCR was performed using the Superscript III One-Step RT-PCR kit (Invitrogen) according to the manufacturer's protocol.

**1.2.6 Western blot**

myc-hFTH protein levels were evaluated by Western blot analysis. The cells were washed with cold PBS and lysed with NP40 lysis buffer including protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL pepstatin A, and 1 μg/mL chymostatin). The lysate protein concentration was evaluated with the bicinchoninic acid method (Pierce Biotechnology), and ~30 μg of protein were loaded per lane on a
polyacrylamide denaturing gel for electrophoresis. Protein was transferred to nitrocellulose membranes for blotting. The antibodies used were rabbit polyclonal antibody to FTH (Santa Cruz Biotechnology), mouse monoclonal antibody to myc (Santa Cruz Biotechnology), rabbit polyclonal antibody to GFP (Santa Cruz Biotechnology), and mouse monoclonal antibody to β-actin for loading controls (Sigma). Primary antibodies were detected by horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology).

1.2.7 Assessment of iron accumulation

For iron loading, the cells were grown in supplemented medium that included ferric ammonium citrate (FAC; 20–500 μmol/L) for 72 to 98 hours. Briefly, cells were cultured in a medium supplemented with increasing concentrations of FAC, washed three times with PBS, resuspended in 6 N HCl, and incubated at 70°C for 30 minutes. The iron amount was determined using a total iron reagent kit (Pointe Scientific). Average iron loadings were calculated by dividing the total mean values by total protein amounts. Prussian blue iron staining was used to assay for iron accumulation. Cells were grown on eight-well chamber slides in a medium with or without FAC for 72 to 98 hours. The cells were washed thoroughly in PBS and fixed in 4% paraformaldehyde for 10 minutes before staining. The eight-well chamber slides were placed in a staining solution (1% potassium ferrocyanide and 5% HCl) for 30 minutes, washed twice in PBS, counterstained with fast red, and mounted on slides with a water soluble mounting medium (Gel/Mount; Biomeda Corp.). Prussian blue staining for iron in tumor sections was performed as described above.

1.2.8 Immunofluorescence

Cells were cultured on eight-well chamber slides and rinsed in PBS before fixation in 2% paraformaldehyde in PBS for 30 minutes. Fixed cells were incubated with primary antibodies directed against myc and GFP in cells, and staining was visualized using Alexa 594–conjugated and Alexa 488–conjugated secondary antibodies (Invitrogen). After s.c. transplantation of MCF-7 or F-98 cells, tumors were collected at 2 to 4 weeks and fixed in 10% formalin. Prepared paraffin sections (4 μm thickness) were dewaxed, hydrated, and treated with 0.01% protease XXIV (Sigma) in PBS for 20 minutes at 37°C. Sections were then incubated with primary
antibodies to myc and GFP according to the manufacturer's instructions. myc-hFTH and GFP were visualized with Alexa 549–conjugated and Alexa 488–conjugated secondary antibodies.

1.2.9 Cell toxicity assay

To determine cell viabilities and proliferative abilities, cells were initially seeded in 96-well plates at $10^4$ per well. Cell viability was assessed using a standard MTT assay. Cell proliferation was evaluated using trypan blue exclusion assays. The proliferative activities were expressed as the relative percentage of cell numbers at day 0.

1.2.10 Determination of the mitochondrial membrane potential

Changes in the mitochondrial membrane potential of cells cultivated in an iron rich medium treated with FAC were determined with the vital mitochondrial dye JC-1 (Molecular Probes). Cells were grown in an eight-well chamber tissue culture dish. The cells were either treated with 100 μmol/L FAC or untreated for 72 hours. After treatment, the medium was replaced with a serum free medium containing 10 μg/mL JC-1, a potential-dependent J-1 aggregate-forming lipophilic cation. Cells were incubated at 37°C for 10 minutes, followed by washing with PBS. Immediately, the cells were visualized by a confocal laser scanning microscope (Leica SP2). The monomer and JC-1 aggregate forms were simultaneously excited by 488 nm argon ion laser sources (18). Mitochondrial depolarization was marked by punctate orange-red fluorescence.

1.2.11 Tumor model

To evaluate the efficacy of the myc-hFTH gene imaging approach in vivo, MCF-7 and F-98 cells transduced with a Lenti-myc-hFTH vector or a control vector were transplanted s.c. into mice or rats. MCF-7 cells ($2 \times 10^7$) were suspended with Matrigel and injected s.c. into the shoulders of 6-week old BALB/c nude mice ($n = 2$). Mice were imaged at 2, 7, and 21 days after transplantation. To further evaluate rat tumor models, F-98 cells ($1 \times 10^6$) were suspended in PBS and injected s.c. into the shoulders of 6-week-old Fisher 344 rats ($n = 9$), and the rats were imaged at 1, 2, 3, and 4 weeks after transplantation.

1.2.12 Fluorescence imaging

All fluorescence imaging was performed using the fluorescence imaging system from Maestro Cambridge Research and Instrumentation. For in vitro fluorescence imaging, the
collected cells were prepared in 1.5 mL centrifuge microtubes and imaged using excitation at 470 nm and emission at 535 nm. Whole body in vivo and ex vivo tumor imaging was performed in a fluorescent light box illuminated by fiber optic lighting at 465 nm.

1.2.13 MRI

To evaluate the detection sensitivity by FTH overexpression compared with other methodologies involving uptake of Feridex (Advanced Magnetics), the T2* relaxation times on T2* weighted MRI of phantoms consisting of $2 \times 10^2$, $2 \times 10^3$, $2 \times 10^4$, $2 \times 10^5$, $2 \times 10^6$, $2 \times 10^7$, and $2 \times 10^8$ cells were measured in mock, myc-hFTH, and Feridex labeled cells. Feridex is a clinically approved and commercially available superparamagnetic iron oxide (SPIO) contrast agent. Feridex labeled cells were collected after 24 hours of incubation with 12.5 μg Fe/mL Feridex. To prepare an MR phantom, mock, myc-hFTH, myc-hFTH + FAC, and Feridex labeled cells were suspended in 1% agarose gel and transferred into 1.5 mL centrifuge microtubes. In vitro and in vivo T2* weighted images of phantoms and tumors at different times after the transplantation were acquired on the 1.5T MR scanner with a wrist coil or a head coil (GE Signa Excite). A conventional CPMG sequence with 12 multiple TEs (TR/TE = 3300 ms/13, 26, 39, 52, 70, 140, 210, 280, 400, 800, 1200, and 1600 ms) for T2* measurements was performed. The measurement parameters were as follows: TR = 3400 ms, TE = 100 ms, FOV = 60 × 60 mm², ETL = 16, matrix = 256 × 256, slice thickness/gap = 2.0 mm/0 mm, and NEX = 2.0.

For analysis of MRI data, T2* relaxation times were calculated by fitting the signal intensities (SI) with increasing TEs. The mean SI within the region of interest was also computed using a standard MRI operating system software program. The MRI SI data were normalized using the mean signal score derived from the muscle. The ratios of SI changes for tumor versus muscle on T2 weighted images were calculated according to the formula

$$\text{SI} = \left(\frac{\text{SI}_{\text{tumor}}}{\text{SI}_{\text{muscle}}}\right)$$

1.2.14 Statistical analysis

Data were presented as means ± SDs of more than three independent experiments. Comparisons were performed using the Student’s t test. Differences were considered significant
at $P$ values of $\leq 0.05$. 
1.3 Results

1.3.1 Expression of myc-hFTH and GFP in cancer cells

The dual promoter lentiviral vector was successfully constructed to express myc-hFTH and GFP proteins (Figure 1.1A). The transduction efficiency of MCF-7 and F-98 cells expressing GFP abundantly was >90% using the Lenti-myc-hFTH vector (Figure 1.1B). GFP positive cells were isolated by FACS and used for all experiments. The presence of myc-hFTH and GFP expression in transfected cells and their absence in the no-vector and mock controls were confirmed using immunofluorescence and Western blot, respectively (Figure 1.1C and D).

1.3.2 Increased iron accumulation in cells expressing myc-hFTH

I investigated if myc-hFTH overexpression with lentivirus influenced cell viability and growth. No differences in cell viability and growth were observed between the myc-hFTH, mock, and no-vector cells (Figure 1.2A and B). The viability and mitochondrial membrane potential of myc-hFTH cells after iron overload further investigated by treatment with FAC. Iron is a cofactor for enzymes involved in many metabolic processes, but free iron can also be harmful, because its excess is known to enhance the production of reactive oxygen species and induce cell injury/apoptosis via a mitochondrial permeability transition (19). The most widely implemented application of JC-1 is for the detection of mitochondrial depolarization occurring in the early stages of apoptosis. The dye undergoes a reversible change in fluorescence emission from green to orange-red as mitochondrial membrane potential increases. Therefore, the orange-red/green ratio can help to identify the viability status of a cell. As shown in Figure 1.2C, no-vector, mock, and myc-hFTH normal cells untreated with FAC or H$_2$O$_2$ (Figure 1.2C) fluoresced green. However, all cells treated with 100 μmol/L H$_2$O$_2$ for 3 hours fluoresced orange-red, which indicated mitochondrial potential collapse. The treatment with 100 μmol/L FAC caused higher mitochondrial polarization in no-vector and mock cells than in myc-hFTH cells (Figure 1.2C). The treatment with FAC (20–500 μmol/L) for 72 hours did not cause cellular toxicity in myc-hFTH cells, whereas the viability of no-vector and mock cells decreased after treatment with FAC (Figure 1.2D). The results imply that myc-hFTH gene expression was protective against FAC toxicity. Iron accumulation of all cells was increased in a dose
dependent manner by treatment with FAC (20 and 500 μmol/L). myc-hFTH cells had more accumulation of intracellular iron than mock and no-vector cells (Figure 1.2E). As shown in Figure 1.2F, the accumulated iron of myc-hFTH cells cultivated in a medium supplemented with FAC (500 μmol/L) was detected by Prussian blue stain.

1.3.3 *In vitro* MRI and fluorescence imaging of myc-hFTH cells

T2* weighted MRI and fluorescence imaging of the agarose phantom containing $1 \times 10^7$ mock and myc-hFTH cells treated with or without FAC were performed on a fluorescence imaging system and a 1.5T MR scanner. GFP fluorescence imaging was obtained from the agarose phantom containing myc-hFTH cells (Figure 1.3A). The dark signal was easily discernable in myc-hFTH cells treated with FAC (300 μmol/L; Figure 1.3B). T2* relaxation times of mock and myc-hFTH cells treated without FAC were 30.3 ± 0.65 and 26.01 ± 1.45 ms, respectively (Figure 1.3C). T2* relaxation times of mock and myc-hFTH cells in the presence of FAC were significantly lower, 22.03 ± 1.14 ms and 18.3 ± 0.31 ms, respectively (Figure 1.3C). In detection sensitivity studies, as the number of cells increased, the T2* relaxation times on T2* weighted MRI of phantoms and all groups (mock, myc-hFTH, and Feridex labeled cells) gradually decreased (Figure 1.3D). The signal drops on T2* weighted images were not observed for the $2 \times 10^2$ myc-hFTH cells and Feridex labeled cells compared with mock cells. However, I could detect significant signal drops for $2 \times 10^3$ myc-hFTH cells and Feridex labeled cells compared with the same number of mock cells. T2* relaxations of $2 \times 10^3$ mock, myc-hFTH, myc-hFTH + FAC, and Feridex labeled cells were 444.4 ± 96.2 ms, 305.6 ± 48.1 ms, 277.8 ± 48.1 ms, and 169.8 ± 28.7 ms, respectively. T2* relaxations of $2 \times 10^4$ mock, myc-hFTH, myc-hFTH + FAC, and Feridex labeled cells were 444.4 ± 96.3 ms, 241.3 ± 103.8 ms, 188.9.8 ± 19.24.1 ms, and 144.8 ± 20.9 ms, respectively. The detection sensitivity of *in vitro* MRI of Feridex labeled cells was greater than that of myc-hFTH cells ($P < 0.05$).
Figure 1.1 Analysis of myc-hFTH and GFP expression in F-98 cells transfected with Lentiviral vector. A, schematic of lentiviral vector with dual promoter, myc-hFTH, and GFP. B, over 90% purity of GFP-positive cells from transduced F-98 cells sorted by fluorescence activated cell sorter. C, immunofluorescence staining in fixed myc-hFTH and GFP-transduced cells with anti-myc and anti-GFP antibodies. Immunofluorescence images for myc-hFTH and GFP were acquired in myc-hFTH–transduced cells. D, Western blots for myc-hFTH and GFP using anti-myc, anti-FTH, and anti-GFP antibodies.
Figure 1.2 In vitro cytotoxicity test and iron accumulation in F-98 cells transfected with Lentimyc-hFTH vector. A, viability (left) and proliferation (right) of transduced cells measured by MTT and trypan blue exclusion assay. B, mitochondrial membrane potential of cells treated with FAC (100 μmol/L) or H$_2$O$_2$ (100 μmol/L) evaluated by JC-1 fluorescence. C, viability of cells treated with increasing concentrations of FAC (50–500 μmol/L) for 72 hours measured by MTT assay. D, average iron amount of cells treated with increasing concentrations of FAC (20–500 μmol/L) for 72 hours calculated by using a total iron reagent kit (left). Prussian blue staining for iron (arrows) in myc-hFTH cells treated with FAC (500 μmol/L) for 72 hours (right). All values are the means ± SDs of four independent experiments. *, $P$ value was statistically significant ($P \leq 0.05$).
Figure 1.3 Analysis of *in vitro* MRI of agarose phantom suspended F-98 cells transfected with Lenti-myc-hFTTH vector. A, fluorescence images of agarose phantoms of $1 \times 10^7$ myc-hFTTH cells treated with or without FAC (300 μmol/L) for 72 hours. B, T2* weighted images of agarose phantom of myc-hFTTH cells treated with FAC showed discernible dark signal. C, T2* relaxation times of mock and myc-hFTTH cells treated with or without FAC were measured from T2* weighted images. D, the graphs show T2* relaxation times calculated from T2* weighted images of different numbers ($2 \times 10^2$, $2 \times 10^3$, and $2 \times 10^4$) of mock, myc-hFTTH, myc-hFTTH + FAC, and Feridex labeled cells. Values are the means ± SDs of five independent experiments. *, \(P\) value was statistically significant (\(P \leq 0.05\)).
1.3.4 In vivo MRI and fluorescence imaging of myc-hFTH tumors

Twenty one days after s.c. transplantation of MCF-7 cells, fluorescence imaging of GFP in myc-hFTH tumors in nude mice was detected, but not in mock tumors (Figure 1.4A, left). I detected lower signals on T2 weighted images of myc-hFTH tumors than of the mock tumors on a 1.5T MR scanner (Figure 1.4A, right).

Further evaluation was performed using MRI and fluorescence imaging of rat tumor models. A hypointense signal in T2 and T2* weighted images of myc-hFTH tumors was detected (Figure 1.4B, left). T2* relaxation times of mock and myc-hFTH at 3 weeks after transplantation were $\sim 63.3 \pm 7.5$ ms and 35.9 $\pm$ 6.1 ms, respectively (Figure 1.4B, right). T2* values of myc-hFTH tumors were significantly lower than the mock tumor ($P < 0.05$). The signal intensity of myc-hFTH tumors decreased at 2 to 4 weeks (Figure 1.4C). A significant decrease in signal intensity of myc-hFTH tumors was detected at 3 to 4 weeks ($P < 0.05$). Two weeks after s.c. transplantation of F-98 cells, ex vivo fluorescence images of GFP protein in myc-hFTH tumors were acquired (Figure 1.4D).

1.3.5 Histologic analysis of myc-hFTH tumors

Western blotting showed that myc-hFTH and GFP proteins were only expressed in myc-hFTH tumors (Figure 1.5A). Immunostaining of mock and myc-hFTH tumors with anti-myc antibody and anti-GFP antibody was performed. The expression of GFP and myc-hFTH proteins in myc-hFTH tumors was detected, and the sites expressing myc-hFTH also expressed GFP (Figure 1.5B). To verify the ability of myc-hFTH protein to augment iron storage, I performed Prussian blue staining to detect the accumulated iron in myc-hFTH tumors 3 weeks after transplantation. Large deposits of accumulated iron were detected in the myc-hFTH tumors (Figure 1.5C).
Figure 1.4 Analysis of in vivo MRI and fluorescence imaging of subcutaneous tumors expressing myc-hFTH. A, in vivo fluorescence images (left) and T2 weighted images (right) of MCF-7 tumors expressing myc-hFTH cells 21 days after s.c. transplantation of myc-hFTH cells into nude mice. GFP fluorescence images and dark signals for myc-hFTH tumor. B, in vivo MRI in T2 and T2* weighted images of F-98 tumor 2 weeks after s.c. transplantation of myc-hFTH and mock cells (left) and T2* relaxation times of mock and myc-hFTH tumors were measured from T2* weighted images (right). C, the changes in signal intensity of subcutaneous F-98 tumor up to 4 weeks after s.c. transplantation of myc-hFTH and mock cells by in vivo MRI. All values are the means ± SDs of five independent experiments. *, P value was statistically significant (P ≤ 0.05). D, ex vivo fluorescence images of F-98 tumors collected 2 weeks after transplantation of mock and myc-hFTH cells. GFP fluorescence images of a myc-hFTH tumor.
Figure 1.5 Histologic analysis of myc-hFTH tumors. A, Western blots for myc-hFTH and GFP using anti-myc, anti-FTH, and anti-GFP antibodies of lysates from F-98 tumors collected 3 weeks after transplantation of mock and myc-hFTH cells. B, immunofluorescence staining of myc-hFTH and GFP in mock and myc-hFTH tumors with anti-myc and anti-GFP antibodies. C, Prussian blue staining of iron deposits in mock and myc-hFTH tumors collected at 3 weeks.
1.4 Discussion

Of all the imaging modalities, MRI provides the highest spatial resolution and the most anatomic and physiologic information in deep tissues, which makes it readily translatable to clinical applications (20, 21). Multimodality imaging is widely considered to involve the incorporation of two or more imaging modalities, usually within the setting of a single examination, using optical studies, nuclear medicine, computed tomography, or MRI (22–24). Noninvasive multimodality imaging techniques have emerged as an essential tool for imaging of the exact localization, extent, and metabolic activity of the target tissue.

Other groups have shown that in vitro and in vivo MRI of SPIO labeled single cells can be acquired using an optimized MR hardware and a 1.5T clinical MR scanner (25–27). However, MRI contrast agents can cause unpleasant side effects including toxic, allergic, and hypersensitivity reactions (28, 29). Furthermore, long-term retention of SPIO in dividing cells is a limitation of this approach (29). The loss of intracellular SPIO from dividing cells is attributed to the dilution due to cellular division or exocytosis. An advantage of using MRI reporter genes to introduce contrast agents is that long term imaging is possible and the negative side effects of exogenous MR contrast agents are avoided. Ferritin, the iron storage protein of mammals, can store up to 4,500 iron and has been accepted as a universal MRI reporter gene (7–12, 30). The presence of ferritin in organs influences the contrast of T2 weighted MRI at very low iron concentrations. However, recent studies for a visualization of cancer cells and tumor tissues using plasmid vector encoding ferritin has been performed on high magnetic field MR scanners (Table 1.1) and reported that native ferritin is a weak T2 contrast enhancing agent in practice. Despite this, Liu and colleagues recently showed that, when ferritin is introduced with lentiviral vector, it is useful for noninvasive MRI monitoring embryonic stem cells in vivo (12).

Lentiviral vectors have become attractive vehicles for gene transfer and expression because lentivirus can maintain target gene expression for up to 6 months. The ability of lentiviral vectors to integrate into both non dividing cells and terminally differentiated cells and to provide stable and long term gene expression in vivo is a desirable attribute for many scientific approaches (17, 31). Lentiviral vector is the preferred method for stable integration of reporter
genes. Therefore, I developed a lentiviral vehicle to transfer ferritin effectively into target cancer cells and tissues and to increase the ferritin expression level and duration for more effective contrast during MRI. The viral vector system in this study was designed to allow MRI and fluorescence imaging simultaneously. It had very high transduction efficiency and produced stable expression in cultured cells without decreasing viability or proliferation. I obtained dark signals and a significant decrease in T2* relaxation time from T2* weighted MRI of cells and tumors expressing myc-hFTH on a 1.5T clinical MR scanner. Previous studies have not been able to show that previously available ferritin reporters are sensitive enough for detection of cells and tissues using a 1.5T clinical MR scanner. Thus, this lentiviral vector system is the first ferritin reporter to provide sufficient sensitivity for the detection of $2 \times 10^3$ cells with a clinical MR scanner. Although I did not detect ferritin transduced single cells in vitro or in vivo, this lentiviral vector was more effective for ferritin gene transfer and expression and for the detection of cancer cells and tumor tissues than previously described expression systems. This improvement in the sensitivity of ferritin transduced cell detection by MRI is critical for clinical applications. MRI detection sensitivity can also be increased by combining ferritin heavy and light polypeptide chimera expression with iron supplementation (32, 33). My future goals are to improve the MRI sensitivity for detecting single cells and small populations of cells by combining this lentiviral expression system with ferritin light polypeptide expression and iron supplementation. The development of improved MRI sensitivity on the single cell level will facilitate progress in understanding the mechanisms of metastasis and the improvement of cancer therapeutics.

Ferritin is an antiapoptotic gene that protects cells and tissues against oxidative stress (34). Free iron is toxic because it facilitates the generation of highly reactive oxygen radical species that can damage cellular constituents. Balancing the deleterious and beneficial effects of iron is an essential aspect of cell survival (35). Overexpression of myc-hFTH protein had no deleterious effects on cell viability or the mitochondrial membrane potential of cells, although it increased iron storage. My results imply that myc-hFTH can be safely used for the long term tracking of transplanted cells and tissues by MRI, as well as for protection of transplanted cells.
and tissues against oxidative stress. In summary, I have successfully simultaneously imaged myc-hFTH and GFP transduced cells and tumors, both *in vitro* and *in vivo*, with a 1.5T clinical MR scanner and fluorescence imaging analyzer. This technology can be applied to monitor tumor growth, metastasis, and regression and multidrug cell and gene based therapy in deep tissues.
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1.5 References


Chapter 2. Imaging and Quantification of Metastatic Melanoma Cells in Lymph Nodes with a Ferritin MR Reporter in Living Mice

2.1 Introduction

Many primary malignancies spread through the lymphatic system. Tumor cells passing through, or residing in, lymph nodes (LNs) can be a reservoir of cells leading to distant, lethal metastases. The detection of metastases in sentinel LNs and other LNs within the regional bed provides clinically important information for tumor staging, choice of treatment and prediction of patient outcome (1,2). Sensitive and specific noninvasive imaging techniques to visualize and quantify cancer cells in vivo are critical to obtain information about tumor progression and LN metastasis (3,4).

Of the various in vivo imaging modalities, MRI is one of the most common methods to evaluate tumor size, location and metastatic burden because of its high resolution, exquisite soft tissue contrast and ability to produce images of entire organs/organisms without the use of ionizing radiation (4). MRI is especially useful for the study of cancer dynamics in deep tissues, which makes it readily translatable to clinical applications. Several previous reports have traced cell fate using MRI and iron oxide particles (5–8). In addition, MRI has been used to monitor the changes in T2* values of metastasized cancer cells labeled with iron oxide particles over time, which provides information on the location of the cells (7–9). A potential limitation of the in vitro labeling and tracking schemes, however, is the loss of cell detection that may be expected to occur with cell division and dilution of the iron label (3). Another limitation is that iron oxide particles from dead cells can remain in the tissue or undergo uptake by phagocytic cells and give a signal unrelated to the originally labeled cells (10). MRI contrast agents can also cause unpleasant side effects, such as toxic, allergic and hypersensitivity reactions (11).

The availability of gene products capable of altering local MRI signals will be useful for the
monitoring of tumor growth or gene expression in deep tissues. Recently, the ferritin gene has been used as an iron accumulating reporter gene for the evaluation of tumor growth and the efficacy of gene therapy by MRI (12–18). Ferritin, an endogenous iron storage metalloprotein in mammals, consists of 24 light and heavy polypeptide chains encapsulating an iron oxide core with up to 4500 iron atoms (13). Because the local magnetic field is altered when the heavy chain of ferritin binds to iron oxide, cells that overexpress ferritin can be detected by MRI (14,15,19).

In this study, a B16F10 murine melanoma cell line expressing human ferritin heavy chain (hFTH) and green fluorescent protein (GFP) allowing detection of the cells by both in vivo MRI and fluorescence imaging was prepared. The present data demonstrated that MRI can be used to visualize and quantify LN metastasis of melanoma cells expressing hFTH in living mice. By employing a novel cumulative histogram analysis of T2* changes, quantitative assessment of tumor burden in LNs was achieved in vivo with MRI, which correlated strongly with histological findings.
2.2 Material and Methods

2.2.1 myc-hFTH and GFP expression in B16F10 melanoma cell line

Murine B16F10 melanoma cells were chosen because of their high metastatic potential to regional LNs (20). B16F10 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37°C. To establish a B16F10 cell line expressing myc-tagged hFTH (myc-hFTH) and GFP, I used dual promoter (cytomegalovirus and phosphoglycerate kinase promoter for myc-hFTH and GFP, respectively) lentiviral vectors (LentiM1.41) encoding myc-hFTH and GFP, which have been described previously (18). B16F10 cells were transduced with lentivirus for 6–10 hours in the presence of 4–8 mg/mL polybrene. Transduced cells were selected using puromycin and analyzed by fluorescence microscopy using green filters (Nikon, NY, USA). Microscopic images were photographed using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Uninfected B16F10 cells were used as control cells.

2.2.2 Fluorescence activated cell sorting analysis

Fluorescence of GFP in transduced cells was analyzed using a FACSCalibur cell sorter (BD Bioscience, San Jose, CA, USA) equipped with a 530 nm filter (bandwidth, ±15nm), a 585 nm filter (bandwidth, ±21 nm) and Cell Quest software (BD Bioscience). Sorted cells were used for further in vivo and in vitro studies.

2.2.3 Western blot

The protein levels of myc-hFTH were evaluated by western blot analysis. Cells were lysed in ice cold RIPA buffer [0°C; 20 mM tris(hydroxymethyl)aminomethane (pH 7.4), 137 mM NaCl, 10% glycerol, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid, 200 mL phenylmethysulfonyl fluoride (1 mM) and protease inhibitor cocktail (Sigma, St Quentin, France)], and the lysate protein concentration was evaluated with the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). Approximately 30 mg of protein were loaded in each lane of a polyacrylamide denaturing gel for electrophoresis. After electrophoresis, the protein was
transferred to nitrocellulose membranes for blotting. I used a rabbit polyclonal antibody to FTH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rat monoclonal antibody to myc for myc tagged hFTH (Santa Cruz Biotechnology), a rabbit polyclonal antibody to GFP (Santa Cruz Biotechnology) and a rabbit monoclonal antibody to actin (I-COR Biosciences, USA). Primary antibodies were detected by horseradish peroxidase-conjugated goat anti-rat or goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology).

2.2.4 Immunocytochemistry

Control or myc-hFTH cells were cultured on eight-well chamber slides. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 30 minutes at 37°C and blocked with 3% bovine serum albumin containing 0.1% Triton X-100 in phosphate buffered saline. The cells were stained with primary antibodies (Santa Cruz Biotechnology) directed against myc-hFTH and GFP, and visualized on a confocal laser scanning microscope (Leica SP2, Bannockburn, IL, USA) using Alexa 594 and Alexa 488 conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA).

2.2.5 Cell viability assay

To determine cell viability, cells were initially seeded in 96-well plates at 10^3 cells per well. The viability of control and myc-hFTH cells was assessed using a standard 3,5-diphenyltetrazolium bromide assay. Optical densities were read at 540 nm.

2.2.6 Determination of in vitro iron concentration

For iron loading, cells were grown in supplemented medium that included 0, 100 or 200 mM ferric ammonium citrate (FAC; Sigma Chemical Co.) for 5 days. To determine the iron levels, control or myc-hFTH cells were cultured in medium supplemented with FAC. The cells were washed three times with phosphate buffered saline, and 2 × 10^6 cells were harvested and resuspended in a 6 M solution of HCl and incubated at 70°C for 30 minutes. The iron concentration was determined using a total iron reagent kit (Pointe Scientific, Canton, MI, USA). The average iron loading was calculated by dividing the total mean value by the cell number.
2.2.7 Preparation of cell pellets for in vitro imaging

Control or myc-hFTH cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100 or 200 mM FAC. Medium containing FAC was changed every 24 hours. After 5 days of incubation with the iron supplement, $5 \times 10^7$ cells were washed, harvested, transferred to 0.2 mL thin wall strip tubes (Axygen, USA) and centrifuged. Cells without exposure to FAC were prepared in an identical manner. For in vitro MRI, the strip tubes of cell pellets were set on 1% agar plates for stable and homogeneous MRI measurements.

2.2.8 Metastatic LN model

This experiment was approved by the animal care committee at Seoul National University Hospital. First, the in vivo expression of the myc-hFTH gene of myc-hFTH tumors was evaluated using the BALB/c nude mice model. Second, the following procedure was used to produce metastatic brachial and axillary LNs: (i) a 1 cm incision was made in the bilateral upper brachial area of anesthetized BALB/c nude mice (n = 9); (ii) the skin was inverted to expose the brachial LNs; (iii) 5 mL of $2 \times 10^6$ B16F10 cells were injected slowly into the fatty area adjacent to the bilateral brachial area (right, myc-hFTH cells; left, control cells) using a Hamilton syringe and an investigational microneedle (Hamilton Syringe); (iv) the dermal incision was closed with a 5–0 Prolene purse string (Harrell Medical, USA). One week after cell implantation, I performed in vivo MRI and in vivo and ex vivo fluorescence imaging to examine the metastatic LNs.

2.2.9 MRI

The in vitro MRI was performed for cell pellets. For in vivo MRI, mice were anesthetized with isoflurane (1% in 100% oxygen) using a nose cone with a vacuum for scavenging. To stabilize the body temperature of the mice during the MRI experiments, an animal warming system (Bruker Biospin, Ettlingen, Germany) was used, consisting of a warm water (39°C) reservoir with a pump and hoses placed underneath the animal bed.

MRI studies were conducted on a 9.4-T Bruker animal scanner (Bruker Biospec 94/20 USR; Bruker Biospin). A transmit only volume coil (Bruker Biospin) was used for excitation and a four channel surface coil (Bruker Biospin) for signal reception. For the estimation of $T2^*$, a
gradient echo pulse sequence was used with the following imaging parameters: TR/TE = 5000/3.1, 7.6, 12.1, 16.5, 21.0, 25.5, 29.9, 34.4 ms; flip angle, 90°; field of view, 45 × 45 mm; matrix size, 256 × 256; slice thickness, 1 mm; pixel resolution, 0.18 × 0.18 mm; no intersection gap; one signal average. The total number of image slices was 20 per mouse.

2.2.10 Fluorescence imaging

Fluorescence images for cell pellets were obtained using a 12 bit charge coupled device camera (Kodak Image Station 4000MM) and a filter set selected for GFP (excitation, 470 nm; emission, 535 nm).

Mice were transferred to a fluorescence imaging system immediately after MRI, and in vivo fluorescence images were obtained using the Maestro In Vivo Imaging System (CRi Inc., Woburn, MA, USA) for data acquisition and analysis. Before imaging, the mice were anesthetized by an intraperitoneal injection of a solution containing zolazepam (5 mg/kg, Zoletil®W, Virbac, France) and xylazine (10 mg/kg, Rompun®W, Bayer-Schering Pharma AG, Berlin, Germany). After in vivo fluorescence measurements had been taken for tumors from bilateral brachial and axillary LNs (n = 9) from metastatic LN models, the mice were sacrificed, and the tumors or LNs were removed for ex vivo fluorescence imaging. In all cases, a bandpass filter from 445 to 490 nm and a long pass filter above 515 nm were used for excitation and emission light. The tunable filter was automatically stepped in 10 nm increments from 500 to 800 nm, and the camera captured images at each wavelength interval with constant exposure. Fluorescence imaging for each in vivo and ex vivo experiment was performed three times.

2.2.11 Analysis of MRI data

Regions of interest (for cell pellets, LNs) were defined in the individual slices acquired at the shortest TE, and these data were used to generate T2* maps of the regions of interest using pixel by pixel analyses across the eight-point MR images in MATLAB™ (MathWorks Inc., Natick, MA, USA) and assuming single exponential decay (Figure 2.1) (i.e. SI = SI₀ × exp (–TE/T2*), where SI is the signal intensity and SI₀ is the proton density).

A T2* histogram was obtained for each animal by including all pixels in the regions of interest
Figure 2.1 Figures show the generation of the T2* histograms for each LN; MR images of the mouse axillary LN (the highest column) and subsequent generation of T2* maps (the middle column) and histograms (the lowest column). Finally, a T2* histogram was obtained for each animal by including all pixels in the ROIs (e.g., each LN) across all slices.
(e.g. each LN) across all slices. In the histogram, the number of pixels on the y-axis was expressed as a percentage by dividing each bin by the total number of pixels of the histogram. For further quantitative analysis of metastatic burden, cumulative T2* histograms were produced from the T2* histograms, and the cumulative number of pixels was expressed as a percentage and plotted on the y-axis. In this step, the median value of the histogram was defined as R50 (21) (i.e. R50 in the cumulative T2* histogram is the T2* value at which the cumulative number of pixels in the percentage becomes 50%). Such R50 values were estimated for all cumulative T2* histograms and used as an ultimate MRI measure for the assessment of the tumor burden in the metastatic LNs. Finally, the efficacy of using R50 in the noninvasive tumor assessment was evaluated by examining the correlation between the R50 values and the histopathology.

2.2.12 Histological analysis

Brachial and axillary LNs removed from mice were fixed in 10% buffered formalin. Paraffin-embedded LNs were cut into 4 mm thick sections. Staining methods included hematoxylin and eosin, which was used to visualize tumor and node morphology, and immunohistochemistry, which was used to assess the expression of myc-hFTH and GFP. For immunohistochemistry, prepared paraffin sections were dewaxed, hydrated and treated with 0.01% protease XXIV (Sigma Chemical Co.) in phosphate buffered saline for 20 minutes at 37°C. Sections were then stained with primary antibodies to myc-hFTH and GFP according to the manufacturer’s instructions. The expression of myc-hFTH and GFP was visualized with Alexa 594- and Alexa 488-conjugated secondary antibodies (Invitrogen), respectively.

For the volume measurement, all microscopic images of hematoxylin and eosin staining were captured and saved on a computer using a DP-70 camera with a color charge coupled device sensor mounted on a microscope (Olympus, Tokyo, Japan). To determine the volumes of LNs and metastases, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Volumes based on diameter measurements were calculated by the modified ellipsoidal formula (22,23):

\[
\text{Volume} = \frac{1}{2} (\text{length} \times \text{width}^2)
\]
For each metastatic LN, the following formula was used to calculate the tumor burden, which was defined as the volume percentage between metastatic tissue and overall LN tissue:

\[
\text{Tumor burden (\%)} = \left( \frac{\text{volume of metastatic tissue}}{\text{volume of LN}} \right) \times 100
\]

2.2.13 Statistical analysis

For all statistical analyses, a two-tailed \( p \) value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using commercially available software (MedCalc, version 11.1.1.0; MedCalc Software, Mariakerke, Belgium). Single-group data were assessed using Student’s t-test or the Mann–Whitney test. Repeated measurements analysis of variance (ANOVA) with Tukey–Kramer post hoc comparisons was performed for multiple comparisons. The histopathological volume (\%) of the metastatic burden of the LNs was compared with both the T2* and R50 values derived from cumulative histogram analysis of LNs, using Pearson’s correlation analysis, from which partial correlation coefficients were derived.
2.3 Results

2.3.1 In vitro analysis of control and myc-hFTH cells

Over 90% purity of GFP-positive cells was achieved from myc-hFTH cells by sorting with a fluorescence activated cell sorter. These cells were used for all experiments (Figure 2.2A). Abundant expression of myc-hFTH and GFP was confirmed in myc-hFTH cells using immunocytochemistry and western blotting. Importantly, the expression of myc-hFTH and GFP was not detected in control cells (Figure 2.2B, C). myc-hFTH cells showed an increase in proliferation relative to control cells from culture day 2 ($p<0.001$, Student’s t-test) (Figure 2.2D).

All pellets of myc-hFTH cells showed fluorescence, whereas no signals were observed in pellets of control cells (Figure 2.3A). The T2* values of control and myc-hFTH cell pellets without the addition of FAC were $33.1 \pm 2.4$ (mean standard deviation) and $29.2 \pm 2.2$ ms ($p<0.01$, Student’s t-test), respectively. When exposed to 100 mM FAC, both control and myc-hFTH cell pellets showed decreased T2* values ($20.3 \pm 2.8$ and $13.0 \pm 2.8$ ms, respectively; $p<0.01$, Student’s t-test), which were significantly lower than without exposure to FAC ($p<0.01$, Student’s t-test). Further decreases in T2* ($14.9 \pm 4.0$ and $11.4 \pm 2.9$ ms, respectively; $p<0.01$, Student’s t-test) were observed in control and myc-hFTH cell pellets exposed to 200 mM FAC (Figure 2.3B, C), which showed statistical significance compared with exposure to 100 mM FAC ($p<0.01$, Student’s t-test). These MRI findings were further confirmed by the iron concentration assay; the results are summarized in Figure 2.3D, which clearly demonstrates a greater accumulation of intracellular iron in myc-hFTH cells relative to control cells ($p<0.05$, Student’s t-test).

2.3.2 In vivo MRI, in vivo and ex vivo fluorescence imaging and histological analysis

Thirty-six LNs isolated from nine mice of the metastatic LN model were evaluated by MRI, fluorescence imaging and histological analysis. myc-hFTH and control cells metastasized to the right ($n = 9$) and left ($n = 7$) brachial LNs, respectively, and to the right ($n = 4$) and left ($n = 5$) axillary LNs, respectively. The volume percentages of metastatic LNs of myc-hFTH and control...
Figure 2.2 myc-tagged human ferritin heavy chain (myc-hFTH) and green fluorescent protein (GFP) expression in the B16F10 melanoma cell line. (A) Greater than 95% purity of GFP-positive cells from transduced myc-hFTH cells sorted by fluorescence activated cell sorting. (B) Immunocytochemistry (400× magnification) in fixed control or myc-hFTH cells with anti-GFP and anti-myc antibodies. DAPI, 4’,6-diamidino-2-phenylindole. (C) Western blots of myc-hFTH and GFP. (D) myc-hFTH cells showed an increase in proliferation relative to control cells from culture day 2 (p<0.001, Student’s t-test).
**Figure 2.3** *In vitro* imaging of myc-hFTH cell pellets and intracellular iron concentration. (A) Fluorescence images of phantoms of $5 \times 10^7$ myc-hFTH cells with or without ferric ammonium citrate (FAC) treatment for 5 days. (B, C) T2* color map of control and myc-hFTH cell pellets with or without FAC, and comparison of the T2* values. (D) A total iron reagent kit was used to calculate the average iron concentration in cells treated with increasing concentrations of FAC (100 and 200 mM) for 5 days. All values are the means ± standard deviations of four independent experiments. *p < 0.05, Student’s t-test.
cells were in the ranges 14.9–94.7% (mean, 50.3%) and 13–100% (mean, 62.0%), respectively. T2* maps from *in vivo* MRI showed that the T2* values of metastases in the right axillary and brachial LNs of myc-hFTH cells were lower than the T2* values of metastases in the left axillary and brachial LNs of control cells. Histograms showed that the pixel numbers of low T2* were higher in mychFTH cell metastases than in control cell metastases (Figure 2.4A). *In vivo* fluorescence imaging revealed GFP signals from metastatic myc-hFTH cells at the brachial LNs, but no signals were observed at metastatic LNs of control cells. In addition, GFP signals were not detected from metastatic myc-hFTH cells at the axillary LNs. However, *ex vivo* fluorescence imaging showed a GFP signal from metastatic myc-hFTH cells at both brachial axillary LNs (Figure 2.4B). The metastatic cells in the LNs were evaluated by hematoxylin and eosin staining, and immunohistochemistry confirmed mychFTH and GFP expression in the metastatic LNs of myc-hFTH cells, but expression was not detected in metastatic LNs of control cells (Figure 2.4C).

The mean T2* values of normal (n = 11) and metastatic LNs of myc-hFTH (n = 13) and control (n = 12) cells were 13.4 ± 0.7, 11.8 ± 1.3 and 17.4 ± 1.8 ms, respectively (p<0.05, ANOVA test). Importantly, T2* values of metastatic LNs of myc-hFTH cells were negatively correlated with the histopathological volume (%) (p = 0.0001, Pearson’s correlation analysis), indicating that the expression of hFTH in LNs leads to a decrease in T2* (Figure 2.5A). In contrast, there was no significant correlation between T2* of metastatic LNs of control cells and histopathological volume (%) (p = 0.8323, Pearson’s correlation analysis) (Figure 2.5B). In three of four LNs with a small metastatic burden of myc-hFTH cells, T2* values were greater than those of normal LNs (red box in Figure 2.5A).

Cumulative histograms of metastatic LNs with myc-hFTH cells started to increase at lower T2* values than did those of normal LNs (Figure 2.6A). This suggests that the cumulative histograms of metastatic LNs with myc-hFTH cells contain more pixels at low T2* values, indicating hFTH overexpression. The cumulative histograms of metastatic LNs with myc-hFTH cells were shifted to the left in the manner of metastatic burden dependence (volume percentage). In contrast, the cumulative histograms of metastatic LNs with control cells were
shifted to the right, and the degree of shift was not related to the metastatic burden (Figure 2.6B).

R50 values were calculated from the cumulative histograms. The mean R50 value of normal LNs was 12.7 ms, whereas the R50 values of metastatic LNs with myc-hFTH and control cells were in the ranges 9.2–12.5 and 14.5–19.9 ms, respectively. All R50 values of metastatic LNs with myc-hFTH cells were lower than the mean value of normal LNs, whereas all metastatic LNs with control cells showed higher R50 values than normal LNs. In addition, a significant correlation was only observed between R50 values of metastatic LNs with myc-hFTH cells and histopathological volume (%), whereas R50 values of metastatic LNs with control cells did not show a significant correlation with histopathological volume (%) (Figure 2.6C, D).
Figure 2.4 *In vivo* imaging and histological analysis of metastatic cells expressing myc-hFTH in lymph nodes (LNs). (A) T2* map and histograms of metastasis from myc-hFTH and control cells in the right and left axillary (Â) and brachial (B) LNs in nude mice. (B) *In vivo* and *ex vivo* fluorescent imaging of metastatic myc-hFTH and control cells in the brachial (arrows) and axillary (curved arrows) LNs. (C) The metastatic axillary LNs evaluated by hematoxylin and eosin (H&E, 100x magnification) and immunofluorescence (200x magnification) staining. DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein.
Figure 2.5 Correlation of mean T2* values and nodal tumor burden. (A) T2* values of metastatic lymph nodes (LNs) of myc-tagged human ferritin heavy chain (myc-hFTH) cells were inversely correlated with histological volume (%) ($r^2 = 0.803$, $p = 0.0001$, Pearson’s correlation analysis). Three of four LNs with a small metastatic burden of myc-hFTH cells had a T2* value greater than that of normal LNs (red box). (B) There was no significant correlation between the T2* values of metastatic LNs with control cells and histological volume (%) ($r^2 = 0.005$, $p = 0.8323$, Pearson’s correlation analysis).
Figure 2.6 (A, B) Cumulative T2* distribution of normal and metastatic lymph nodes (LNs) of myc-hFTH and control cells. The numbers on the right of the figures indicate the histological volume (%) of metastatic burden in each LN. Correlation of R50 values and LN tumor burden (%) of LN metastasis. (C, D) Although a significant correlation was observed between the R50 values of metastatic LNs with myc-hFTH cells and histological volume (%) ($r^2 = 0.770$, $p = 0.0001$, Pearson’s correlation analysis), there was no correlation between the R50 values of metastatic LNs with control cells and histological volume (%) ($r^2 = 0.017$, $p = 0.6884$, Pearson’s correlation analysis).
2.4 Discussion

This study demonstrates the feasibility of using the MR reporter ferritin for noninvasive imaging and quantification of melanoma cells in mouse LNs. Although previous investigations have quantified cells labeled with iron oxide particles in murine brains or LNs (9,24), this study is the first to use MRI for the quantification of metastatic burden in LNs with the ferritin gene as an MR reporter. Stable overexpression of ferritin in B16F10 murine melanoma cells was detectable by MRI in vivo and yielded significant changes in T2* compared with control cells. Although the median values (R50) calculated from cumulative T2* histograms correlated well with the histologically measured metastatic burden in LNs, the overall T2* value of each metastatic LN with myc-hFTH cells was limited by the detection of small (less than 25%) metastatic burden. For the quantitative assessment of metastatic cells in LNs, this results suggest that the analysis of T2* values themselves is not sufficient. Indeed, an additional analytical scheme, such as R50, which was employed in this study, may be required.

Much of my current understanding of the metastatic process has come from the use of various in vivo imaging modalities (3). One of the most powerful modalities in terms of optical imaging for tumor growth and metastasis is fluorescent protein imaging. Fluorescent protein imaging is known for its sensitivity in detecting a small number of cells, its stability of signal and its use of multicolor imaging (25–27). However, fluorescent protein imaging is seriously limited by spatial resolution and the penetration of light through optically opaque tissues, even in mice (28). In this study, fluorescence imaging was unable to detect B16F10 cell metastases expressing GFP at deeply located LNs (e.g. axillary LNs). Indeed, in live mice, the metastatic melanoma cells expressing GFP in the axillary LNs were only seen with MRI.

The primary advantage of MRI as a molecular imaging system is its ability to provide excellent soft tissue contrast and functional information by exploiting proton density, perfusion, diffusion and biochemical contrasts (29). This feature allows the co-registration of molecular information and anatomical information within a single imaging mode. MRI also provides high resolution (<1 mm) and offers good depth penetration (30). Ultrasmall superparamagnetic iron oxide enhanced MRI allows the identification of metastatic areas within LNs, which results
from the absence of macrophage activity replaced by malignant cells, and therefore there is a lack of ultrasmall superparamagnetic iron oxide uptake in LNs (31). Foster et al. (3) reported that cellular MRI allows the detection of as few as 100 melanoma cells labeled with micrometer sized iron oxide particles within the LN after direct injection of the cells, which showed obvious regions of signal void. The primary disadvantage of MRI is its inherently lower sensitivity for the detection of targeted agents compared with other molecular imaging modalities, which can be partially overcome by signal amplification strategies that generate higher contrast between the target and the background (32). In this regard, I prepared a B16F10 murine melanoma cell line that stably expressed ferritin. The lentiviral vector system in this study was the first ferritin reporter to provide sufficient sensitivity for the detection of $2 \times 10^3$ cancer cells with a 1.5T clinical MR scanner (18).

I propose that ferritin, a natural iron storage protein, will be useful for the noninvasive monitoring of metastatic melanoma cells in LNs. Studies have shown that the overexpression of the ferritin heavy chain induces the expression of the transferring receptor and increases iron uptake (33). Therefore, there are natural mechanisms that shift the iron pool into a ferritin bound storage form, which restores iron homeostasis and prevents iron cytotoxicity. Recent work by Liu et al. (17) has confirmed that transgenic cells overexpressing ferritin are characterized by increased iron content and upregulation of transferrin receptors. The present study showed that ferritin positively interfered with the viability and LN metastasis of B16F10 cells. High ferritin expression has been shown to enhance cell growth and increase resistance to oxidative stress in metastatic melanoma cells by interfering with their cellular antioxidant system (34). In addition, ferritin has been reported to stimulate the growth of various cancer cells, such as neuroblastoma, leukemia, melanoma and lung cancer cells (35–37). Thus, I believe that B16F10 cells expressing ferritin are a suitable model for the study of tumor growth and LN metastasis in vivo.

Ferritin overexpression provided sufficient MRI contrast to make transduced cells detectable in vitro and in vivo in LNs. In addition, the myc-hFTH cell burden in LNs was strongly correlated with the results of T2* cumulative histograms. Indeed, the myc-hFTH cells showed a greater decrease in T2* than did both normal LNs and control cells. Even though ferritin
overexpression decreased T2* of myc-hFTH cells, T2* histograms (Figure 2.6A, B) showed that the T2* distribution of the cells was sufficiently broad to overlap those of normal LNs and control cells. As a result, it was difficult to determine the threshold T2* value to discriminate myc-hFTH cells from normal LNs or control cells. In addition, the correlation between the T2* values of metastatic LNs of myc-hFTH cells and histopathology revealed that a small metastatic burden (less than 25%) did not decrease significantly the overall T2* value of an LN. I believe that this was mainly because the low T2* pixel number was too small to decrease the overall T2* value of an LN. Ziv et al. (38) also reported that chronic overexpression of ferritin could cause a shift in the R2 (1/T2) histogram of the liver to the left, which did not show a significant difference in average R2 of the liver from control groups on 4.7T MRI. Therefore, I propose that T2* cumulative histogram analysis, using the R50 value, is more suitable for the quantification of tumor burden in vivo. For instance, in the present study, the cumulative histograms of metastatic LNs of myc-hFTH cells were shifted to the left with respect to those of normal LNs. In addition, the extent of the shift was strongly correlated with histology. In the present study, R50 values represent the degree of shift of the cumulative histograms. I believe that the R50 value from a cumulative histogram is a useful parameter for the evaluation of small changes in T2*. The quantification method presented here, which was based on a ferritin MR reporter, advances the study of metastatic cells in LNs because it is currently the only noninvasive method of which I am aware that is capable of detecting, localizing and quantifying the metastatic cell population in LNs without suffering from signal decay over time.

In this study, I prepared mice models with metastatic LNs by injection of melanoma cells near brachial LNs, which induced metastasis in brachial and axillary LNs. This method seems to be limited by the difficulty in simulating a situation of metastatic spread because of the proximity of the injection site to the LNs. However, I believe that this method has strength with regard to the study of sentinel LNs and the high production yield of metastatic LNs. This is the first MRI study to both visualize and quantify ferritin gene expression in LN metastasis. Although R50 (i.e. median) values calculated from cumulative T2* histograms correlated well with histologically measured metastatic burden in LNs, overall T2* values for each metastatic
LN with myc-hFTH cells were limited by the detection of a small (less than 25%) metastatic burden. Potential future applications of this technique include the tracking of tumor progression and metastasis and the monitoring of transgene expression in deep tissues.
2.5 References


Overall Conclusions

A number of MRI reporters of gene expression were studied so far, all of which were rely on exogenous contrast agents for the detection of contrast changes. In the study reported here, the heavy chain of ferritin was applied as the endogenous intracellular reporter of gene expression that is detectable by MRI, both in vitro (in cell) and in vivo (in primary and metastatic tumors). In addition, the exogenous contrast agents were not required in both cases. Independent validation of MRI reporters is important due to the indirect detection of contrast changes in MRI. The expression of both GFP and myc-tagged ferritin was placed using a biomodal lentiviral vector to gain such a validation. Thus, independent detection and cross-validation of changes in the expression of the reporter gene were provided by measurements of fluorescence and MR relaxation rates in genetically identical cells or tumors. Sensitivity of the contrast by the ferritin was high and activation of ferritin expression resulted in significant changes in relaxation rates. In addition, this approach suggested many additional results which are tracking the metastatic cancer cells.

In summary, the results in this study demonstrate that the ferritin as a reporter for in vivo mapping of gene expression by MRI is feasible. The first demonstration of this approach, as reported here for tumors, may aid the tracking of tumor cell migration. The use of endogenous reporter gene as developed here would be particularly beneficial in those cases where administration of contrast material is compromised by barriers, including embryonic development and the central nervous system.
초 록

자기공명영상(MRI)과 광학영상(Optical imaging)을 위한 이중영상 리포터 유전자는 심부 조직에서 발생하는 생물학적 변화에 대한 비침습적이며 정량적인 정보 획득을 가능하게 한다. 또한, 리포터 유전자를 이용한 세포 자기공명영상 기법은 적은 수의 종양세포를 추적하고 전이암의 초기 단계 연구에 이용 될 수 있다.

본 연구는 자기공명영상을 이용하여 심부 조직에 있는 세포 및 종양을 영상화 할 수 있는, Myc이 표지 된 사람 페리틴 heavy chain(Myc-hFTH) 과 형광영상을 위한 초록 형광단백질(GFP) 을 동시 발현하는 렌티바이러스 벡터를 구축하였다. MTT 와 트립반블루(Trypan blue) 실험을 통하여 Myc-hFTH와 GFP의 과발현에 의한 세포 독성이 없음을 확인하였다. 프루시안 블루(Prussian blue)와 철결합 분석법(Iron binding assays)을 이용하여 Myc-hFTH가 발현하는 세포와 종양내에 철이 축적됨을 확인하였다.

첫번째 연구에서는 종양영상을 위하여, MCF-7과 F-98세포에 렌티바이러스 벡터를 이용하여 Myc-hFTH와 GFP 유전자를 과발현 시켰다. Myc-hFTH와 GFP가 동시 발현한 세포를 마우스나 랫드에 이식 후 1.5T 자기공명영상기기와 광학영상 분석기를 이용하여 자기공명영상과 광학영상을 4주 동안 시행하였다. Myc-hFTH와 GFP가 동시 발현한 세포와 종양의 T2 자기공명영상 신호가 Mock를 발현시킨 대조군보다 통계적으로 유의하게 감소함을 알 수 있었다(p ≤ 0.05).

두번째 연구에서는 살아있는 마우스의 림프절에 전이된 피부암 (melanoma) 세포를 비침습적으로 영상화 하고 정량화 하기 위하여, Myc-hFTH와 GFP의 동시발현 유전자를 B16F10세포에 과발현 시켰다. 마찬가지로 Myc-hFTH와 GFP를 동시 과발현한 B16F10세포에서 과발현에 의한 독성은 관찰 되지 않았다.
또한 Myc-hFTH 발현 세포는 9.4T 자기공명영상에서 신호 검출 가능 하였고 대조군 세포와 비교하였을 때 통계학적으로 유의하게 T2* 값이 감소함을 알 수 있었다. 그러나 생체 형광영상에서는 빛이 심부 조직에 침투하지 못하는 관계로 형광 신호를 관찰 할 수 없었다. 또한, T2* MRI data의 축적히스토그램 분석 기법(Cumulative histogram analysis)을 이용하여 림프절에 전이된 암의 부피가 T2* 신호강도 변화와 일치함을 확인하였다(r = −0.8773, p = 0.0001).

본 연구에서는 세포수준에서 이식 세포와 전이 암세포를 이중영상 리포터 유전자시스템을 이용하여 자기공명영상과 형광 영상에서 생체 영상화를 구현하였다. 향후 본 연구진의 기술은 심부 조직암 치료에 있어 세포 및 유전자 치료에 대한 치료 반응 평가에 이용 될 수 있을 것으로 기대된다.

주요어 : human ferritin heavy chain (hFTH), green fluorescent protein (GFP), MR reporter gene, Magnetic resonance (MR)

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