Noninvasive Monitoring of Embryonic Stem Cells *In Vivo* with MRI Transgene Reporter

Jun Liu, M.S.,^{1,2,*} Eric C.H. Cheng, M.S.,^{2,*} Robert C. Long, Jr., Ph.D.,³ Shang-Hsun Yang, Ph.D.,^{2,4}
Liya Wang, M.D.,³ Pei-Hsun Cheng, M.S.,² Jinjing Yang,² Dong Wu, M.D.,⁵
Hui Mao, Ph.D.,³ and Anthony W.S. Chan, D.V.M., Ph.D.^{1,2,4}

Reporter gene-based magnetic resonance imaging (MRI) offers unique insights into behavior of cells after transplantation, which could significantly benefit stem cell research and translation. Several candidate MRI reporter genes, including one that encodes for iron storage protein ferritin, have been reported, and their potential applications in embryonic stem (ES) cell research have yet to be explored. We have established transgenic mouse ES (mES) cell lines carrying human ferritin heavy chain (FTH) as a reporter gene and succeeded in monitoring the cell grafts *in vivo* using T₂-weighted MRI sequences. FTH generated MRI contrast through compensatory upregulation of transferrin receptor (Tfrc) that led to increased cellular iron stored in ferritin-bound form. At a level sufficient for MRI contrast, expression of FTH posed no toxicity to mES cells and did not interfere with stem cell pluripotency as observed in neural differentiation and teratoma formation. The compatibility and functionality of ferritin as a reporter in mES cells opens up the possibility of using MRI for longitudinal noninvasive monitoring of ES cell-derived cell grafts at both molecular and cellular levels.

Introduction

IN *VIVO* IMAGING has been an integral part of stem cell research. As a widely available noninvasive imaging modality that offers great resolution, exquisite tissue contrast, and superb anatomical details, magnetic resonance imaging (MRI) has found extensive applications in stem cell imaging both in research and clinical settings.^{1–6} So far, MRI tracking of stem cells has largely relied upon *ex vivo* pre-labeling of stem cells with magnetic nanoparticles (mostly superparamagnetic iron oxide nanoparticles [SPIOs]), which can be internalized by the cells to generate strong MRI contrast on T₂- and T₂*-weighted images. An alternative approach to produce MRI contrast based on endogenous reporter gene expression has generated considerable interest in recent years,⁷ as it holds at least two unique potentials: (1) unlike SPIO labeling, transgene-based reporters are expected to be much less susceptible to signal loss through cell divisions and are uniquely suited for longitudinal monitoring of stem cell transplant, and (2) expression of reporter genes can be linked to that of therapeutic genes, effectively linking stem cell-based gene therapy to *in vivo* imaging of stem cells.

A number of candidate MRI reporter genes have been previously suggested, including ones that encode for β -galactosidase,⁸ tyrosinase,^{9,10} transferrin receptor,^{11,12} ferritin,^{13–15} MagA,¹⁶ and lysine-rich protein.¹⁷ Among the candidates, ferritin stands out as our choice for introduction into embryonic stem (ES) cells. Ferritin is a ubiquitous intracellular iron storage protein consisting of 24 subunits of heavy and light chains, and it is essential to life.¹⁸ Increased expression of ferritin shifts intracellular iron distribution toward ferritin-bound form and protects against damage from reactive oxygen species.¹⁹ This change in iron homeostasis in turn induces compensatory increase in iron uptake and cellular iron content that generates contrast in T₂- and T₂*-weighted MR images. One additional advantage of ferritin as an MRI reporter is that utilization of endogenous iron source can be sufficient for *in vivo* ferritin expression to generate MRI contrast, obviating external supplement of contrast agent.¹³ Ferritin heavy chain (FTH) is associated with the ferroxidase activity of the ferritin protein; it alone or in conjunction with the ferritin light chain has been previously reported to function as an MRI reporter.^{13–15}

¹Neuroscience Program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, Georgia.

²Yerkes National Primate Research Center; ³Department of Radiology, Center for MR Research; ⁴Genetics and Molecular Biology Program, Department of Human Genetics; Emory University School of Medicine, Atlanta, Georgia.

⁵Department of Radiology, Fudan University School of Medicine and Zhongshan Hospital, Shanghai, China.

*These two authors contributed equally to this work.

Despite the strong appeal of a molecular MRI reporter system to stem cell research, no attempts to combine MRI reporter with ES cells have been reported, and the only study on introducing metalloprotein MRI reporters to an adult stem cell line failed to detect transgenic cells transplanted *in vivo*.²⁰ The lack of progress on this front may be attributable to additional challenges posed by stem cell applications. The major concerns over an MRI reporter–ES cell combination include the following:

- (1) Viability: Since most MRI reporter genes to date are involved in the critical cellular process of iron regulation and even ferritin, with the best safety profile among them, has been shown to significantly slow cell proliferation rate in certain cell models,^{21,22} whether introducing FTH into ES cells will impair cell viability remains an open question.
- (2) Pluripotency: One of the great appeals of ES cells to regenerative medicine is their potential to differentiate into any cell type of an organism, and it remains unexplored whether the introduction of metalloprotein transgene into ES cells will impair their pluripotency or differentiation potential.
- (3) Function: Transgene expression is usually less effective in ES cells and prone to silencing²³; this coupled with the possible incompatibility of high transgene expression level with stem cell viability and pluripotency, leaves much uncertainty whether molecular MRI reporters could function as effectively in ES cells as in other cell models.

To answer these questions, we have established clonal FTH transgenic mouse ES (mES) cell lines through lentiviral transduction to ensure stable transgene expression,²⁴ followed by *in vitro* and *in vivo* characterization of their viability, pluripotency, and reporter function. Here, we present the first report on introducing a metalloprotein-based MRI reporter gene into ES cells and successful noninvasive monitoring of the transgenic mES cell graft *in vivo*.

Materials and Methods

Construct design

A lentiviral vector FU-IRES-GW derived from FUGW²⁵ with internal ribosomal entry site (IRES) inserted before enhanced green fluorescent protein (EGFP) sequence is used in the current study. Full-length human ferritin, heavy polypeptide 1 (*FTH1*) cDNA (GenBank accession number: BC015156) was PCR modified to remove iron response element sequence and create hemagglutinin (HA)-tag at the N-terminus (primer sequences *FTH1* sense first, ATG TTC CAG ATT ACG CTA TGA CGA CCG CGT CCA CC; *FTH1* sense second, AGC TAG CAT GTA CCC ATA CGA TGT TCC AGA TTA CGC; *FTH1* antisense both times, CTT AGC TTT CAT TAT CAC TGT CTC CCA GGG) before subcloned into the multiple cloning site of FU-IRES-GW. The resultant pLVU-HA-FTH-IRES-EGFP (pLVU-HFG) vector constitutively expresses *FTH1* under the ubiquitin (U) promoter and coexpresses downstream EGFP (G) linked by IRES.

Maintenance of mES cells

mES cells (AB2.2 line) were cultured in mES maintenance medium composed of Dulbecco's modified Eagle's

medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), 1 mM glutamine (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO), and 1000 IU/mL of human recombinant leukemia inhibiting factor (Chemicon, Temecula, CA). Medium was changed daily, and mES cells passaged every 2 days at 1:10 to 1:15 ratio. On the day of passage, mES cells were treated with 0.05% trypsin-EDTA and dissociated into single cells before seeding onto mitomycin-C-inactivated mouse fetal fibroblast (MFF) feeder layer.

Establishment of clonal transgenic mES cell lines

Bicistronic lentiviral vector pLVU-HFG that constitutively expresses iron response element-free human FTH and EGFP was used to establish clonal transgenic mES cell lines. Human and rodent ferritin share high sequence homology, and human ferritin is known to function in murine cells.²⁶ Lentivirus was generated by cotransfecting pLVU-HFG with packaging plasmid p Δ 8.9 and envelope vector pVSV-G into 293FT packaging cells (Invitrogen). Culture medium was collected 48 h posttransfection. mES cells were passaged as single cells and seeded at low densities onto inactivated MFF. Once the cells attach, the medium was changed to freshly collected VSVG-LVU-HFG-containing medium supplemented with polybrene (8 μ g/mL) and cultured overnight. The following day, viral medium was replaced with mES medium; 48 h after transduction, positive colonies were selected manually based on EGFP expression. Each positive colony was passaged as single cells onto individual 35 mm culture plates with MFF, and the process was repeated if necessary until clonal transgenic lines were established. One transgenic mES cell line that has maintained stable and the highest level of FTH expression among the group was chosen for the current study, and named HFG-mES. It is worth pointing out that genetic engineering of human ES cells is expected to be significantly more challenging than that of mES cells, and lentiviral vectors are considered one of the best options in achieving stable transgene expression both in primate and murine ES cells. We expect pLVU-HFG used in the current study to be applicable to other ES cell models as well.

Cell growth

Cell growth rate was estimated as doubling time based on cell count. Specifically, 1×10^5 of wild-type (WT) mES and HFG-mES cells were seeded as single cell onto gelatin-coated plates with inactivated MFF feeder layer. mES cells were cultured following the maintenance protocol with daily medium change. After cell attachment, mES cells were harvested and counted at three different time points during their exponential growth phase (21, 49, and 74 h). For each time point, data were averaged from three replicates.

Differentiation of mES cells

Neural differentiation of mES cells followed the five-stage differentiation protocol previously described with minor modifications.^{27,28} Briefly, mES cells were cultured on MFF feeder in ES medium to maintain them in an undifferentiated state. To initiate differentiation, mES cells were suspended as single cells in mES medium without leukemia inhibiting factor at a concentration of 1.5×10^5 cells/mL and transferred

to nonadherent plates for embryoid body formation in suspension. After 4 days, embryoid bodies were transferred to adherent cell culture dishes, allowed to attach overnight and medium switched to serum-free ITSFn (insulin/transferrin/selenium/fibronectin) medium to select for neural progenitor cells (NPCs). After 8 days in ITSFn medium, expansion of nestin-positive NPCs was initiated by switching ITSFn medium to N2 medium supplemented with basic fibroblast growth factor. The expansion phase lasted for 4 days, at the end of which basic fibroblast growth factor was removed from N2 medium to initiate neuronal differentiation. The differentiation stage lasted for 6–10 days.

Western blot

Samples were lysed in prechilled radio immunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail and homogenized with a sonicator. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA), and equal amounts (30 µg) of proteins from each sample were loaded into 15% sodium dodecyl sulfate (SDS) polyacrylamide gel and separated by electrophoresis. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) using Bio-Rad's transblot, the membranes were blocked for 3 h in 5% milk in TBST, and incubated overnight at 4°C with monoclonal HA antibody (1:1000; Chemicon) and monoclonal Tfrc antibody (1:1000; Zymed, San Francisco, CA), followed by 45 min incubation with horseradish peroxidase (1:10,000; Jackson ImmunoResearch, West Grove, PA) at room temperature. Proteins were viewed with Amersham ECL kit (Amersham, Piscataway, NJ).

Teratoma formation

All animal procedures applied in this study were approved by the Institutional Animal Care and Use Committee (IACUC) and Biosafety Committees of Emory University. Teratoma formation of HFG-mES cells was confirmed by two subcutaneous (s.c.) inoculations of 1×10^6 HFG-mES cells into severe combined immunodeficiency (SCID) mice. At day 30 postinoculation, tumors were removed, paraffin embedded, sectioned at 8 µm, and stained with hematoxylin–eosin (H&E) to view tissue morphology characteristic of different germ layers.

Magnetic resonance imaging

CD-1 nude mice ($n = 4$) were chosen for *in vivo* MRI study. Following a within subject design, 3×10^6 WT mES and HFG-mES cells were inoculated s.c. onto opposite flanks of each mouse to facilitate direct comparison. Tumor growth was monitored daily, and first MRI scans were performed on day 14 posttransplant when tumor diameters reached the 7–10 mm range, with repeat scans on three remaining mice from the same group performed on day 21.

For imaging of animals, mice were scanned using a 4.7 tesla (T) horizontal bore (33 cm) MRI scanner (Oxford Magnet Technology, Oxford, UK), interfaced to a Unity INOVA console (Varian, Palo Alto, CA). Animal was placed in a custom-built volume coil (5 cm ID and 8 cm long) and anesthetized using 2% isoflurane delivered via a mask throughout the MRI experiments. Animal was kept warm in the scanner using

circulating water blanket. A set of survey images was obtained using T₂-weighted fast spin echo (FSE) imaging sequence with TR of 5000 ms and TE of 20 ms. This is followed by high-resolution images of selected field of view covering the full extent of tumors with T₂-weighted FSE sequence with TR of 5000 ms and multiple effective TE of 20, 40, 60, and 80 ms, matrix 256 × 256. Typically, field of view of 40 × 70 mm, slice thickness of 0.5 mm, and a gap of 0.15 mm were used. Care was taken to maintain the same animal positions and same imaging parameters across different scan sessions. Multiecho images of slices cutting through the center of tumors across samples were used for calculating T₂ maps using MRI analysis calculator plugin (Dr. Karl Schmidt of Harvard University) of ImageJ (National Institutes of Health).

Histology and immunohistochemistry

For *in vitro* stem cell characterization, cell cultures at both undifferentiated stage and multiple stages during neural differentiation were fixed in 4% paraformaldehyde for 15 min followed by thorough washes. Samples were then incubated overnight with the following primary antibodies: monoclonal Oct-4 (1:250; Santa Cruz, Santa Cruz, CA), monoclonal stage specific embryonic antigen-1 (SSEA-1) (1:200; Chemicon), monoclonal nestin (1:500; Chemicon), monoclonal beta III-tubulin (1:250; Chemicon), rabbit polyclonal tyrosine hydroxylase (1:500; Chemicon), monoclonal human FHC (rH02, 1:200; Ramco Lab, Stafford, TX), and monoclonal HA antibody (1:1000; Chemicon), followed by incubation with secondary antibodies conjugated with appropriate fluorochromes.

To confirm transgene expression in cell transplants, animals were euthanized upon completion of MRI scan. Tumor tissues were removed, and cryostat sectioned at 20 µm followed by fluorescence microscopy of EGFP expression and confirmation of HA-tagged FTH expression with monoclonal HA antibody (1:1000; Chemicon).

Determination of iron content

Snap-frozen WT and FTH transgenic tumor samples were sent to CAIS/Chemical Analysis Laboratory at University of Georgia for inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis (20 elements) to determine Fe content normalized to tissue dry weight.

In vitro MRI with superparamagnetic iron oxide labeling

Cell labeling was performed on AB2.2 mES cells with 13 nm magnetic iron oxide nanoparticles (Ocean Nanotech, Fayetteville, AZ). Each condition has 3 million WT mES cells coincubated with SPIO in culture medium for 2 h before thoroughly washed and evenly suspended in agarose gel (500 µL). Concentrations of SPIOs were 0.25 mg/mL for standard (undiluted) condition, followed by serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/256, and 1/1024) down to 0.24 µg/mL for 1/1024 dilution. HFG-mES cell samples were similarly prepared without SPIO or any other iron supplement in culture medium.

T₂-weighted MRI was performed on a 3T unit (Siemens, New York, NY) with FSE sequence (multi-TE points using TR of 2 s, 15 TE values: 10–150 ms with a 10 ms increment). Images were processed, and R₂ values of each sample were calculated from multi-TE data using ImageJ.

Statistical analysis

Data were presented as mean $\pm$ SD. Two-tailed *t*-test was used in data analysis with $p < 0.05$ considered statistically significant. All experiments have at least three replicates.

Results

Generation of transgenic MRI reporter mES cell line

Stable homogenous FTH transgene expression in HFG-mES cells was confirmed with ICC using antibody against HA (Fig. 1B) as well as antibody specific for human FHC (data not shown). Western blot using antibody against HA revealed FTH protein expression at expected molecular weight (22 kDa with HA tag) in HFG-mES cell samples but not WT mES controls (Fig. 1C). Under standard ES cell culture conditions, we observed that HFG-mES cells had normal morphology and a growth rate comparable to that of the WT mES cell controls. The doubling times were estimated at 12.25 h for transgenic line and 12.15 h for its WT control, and the difference was not statistically significant ($p = 0.236$; $n = 3$; Fig. 1D).

HFG-mES cells maintained pluripotency

One major concern about introducing a reporter gene into ES cells is whether it will have a negative impact on the pluripotency of the stem cell. Three methods were adopted to evaluate the effect of constitutive FTH expression on HFG-mES pluripotency: expression of stem cell markers, neuronal differentiation, and teratoma formation.

Under standard ES culture conditions, expression of early stem cell markers in HFG-mES cells was confirmed by immunostaining using antibody specific for Oct 4 and SSEA-1, and HFG-mES cells were also positive for alkaline phosphatase activity (Fig. 2A). Following differentiation protocol,^{27,28} HFG-mES cells were successfully induced to undergo transitions from embryoid bodies to NPCs to mature neurons (Fig. 2B), without morphologic or temporal differences in differentiation to those of WT controls. Stable and homogenous transgene expression has been maintained throughout the differentiation process (Fig. 2B). For teratoma formation, which is considered the gold-standard assay for pluripotency, HFG-mES cells were inoculated s.c. into SCID mice and allowed to proliferate and differentiate. Tumors were extracted 30 days after transplant and histologically confirmed to be teratoma, with H&E staining revealing tissues representative of all three germ layers (Fig. 2C).

MRI detection of FTH expression

To validate the function of FTH as an MRI reporter in ES cells *in vivo*, WT and HFG-mES cells were grafted into CD-1 nude mice ($n = 4$) following a within subject design with

each mouse receiving WT mES and HFG-mES transplants s.c. onto opposite flanks. Given that ferritin shortens transverse relaxation time (T_2) to greater extent than it shortens longitudinal relaxation time (T_1), and the linear dependency of ferritin transverse relaxation rate R_2 (i.e., $1/T_2$) on magnetic field strength,²⁹ T_2 -weighted sequences were chosen to assess transgene-induced change in T_2 relaxation time and MRI contrast.

On day 14 posttransplant when average tumor diameter reached 8 mm, the first *in vivo* MRI on a 4.7T scanner with FSE sequences (TR = 5000 ms, effective TE = 20, 40, 60, and 80 ms) was performed on all four mice. Care has been taken in selection of image slices to cover the full extent of transplants on both sides. Measurements of MRI signal decay at multiple TE points showed significantly ($p = 0.038$) decreased T_2 relaxation time in transgenic mES cell transplants overexpressing FTH (Fig. 3A–C, day 14), with a corresponding increase in transverse relaxation rate ($R_2 = 1/T_2$) of 15% between WT ($R_2 = 14.5/s$) and FTH transgenic transplants ($R_2 = 16.6/s$) (Fig. 3G, day 14). One week later, repeat scans were performed with the same parameters on three remaining mice ($n = 3$) from the original group, again with significant ($p = 0.019$) decrease in T_2 relaxation time in transplants of HFG-mES origin (Fig. 3D–F, day 21). An average 28% increase in R_2 was observed in FTH transgenic transplants ($R_2 = 18.1/s$) as compared to WT controls ($R_2 = 14.1/s$) (Fig. 3G, day 21).

Postmortem analysis

Throughout the experimental period, transplants of both WT and HFG-mES cells maintained comparable growth rate *in vivo* as measured by tumor size based on MR image. On day 14 average tumor diameters measured at 8.3 ± 1.06 mm for WT and 9.1 ± 1.53 mm for FTH transgenic transplants, on day 21 the measurements were 15.0 ± 2.71 mm (WT) and 16.9 ± 4.09 mm (HFG), respectively, with no sign of negative impact on cell survival from ferritin expression ($p = 0.266$ on day 14 and $p = 0.215$ on day 21; $n = 3$; Fig. 4A).

To confirm FTH expression *in vivo*, animals were euthanized upon completion of MRI and the transplants extracted for analyses. Transgenic mES tumor could be easily distinguished under fluorescence microscope with EGFP expression, and immunostaining of tissue sections confirmed homogenous expression of HA-tagged FTH (Fig. 4B).

To evaluate the impact of FTH expression on iron regulation, the expression levels of both Tfrc- and HA-tagged FTH were determined by Western blot. Significant upregulation of Tfrc, with average increase of 35.5% ($135.5\% \pm 18.1\%$) over WT controls as estimated by densitometry, was observed in transplants of HFG-mES origin (Fig. 4C). Iron content of both

FIG. 2. Characterization of pluripotency of HFG-mES cells. (A) HFG-mES cell culture was EGFP positive and expressed early stem cell markers of SSEA-1, Oct 4, and alkaline phosphatase. (B) Neuronal differentiation of HFG-mES cells: immunostaining of nestin at neural progenitor stage and β -III tubulin and tyrosine hydroxylase at neuronal stage. HFG-mES-derived neurons maintained homogenous transgene expression shown here with immunostaining for HA. This confirmed that HFG-mES cells were capable of neuronal differentiation while maintaining FTH transgene expression. (C) HFG-mES-derived teratoma contains differentiated tissues representing all three germ layers: ectoderm (top to bottom: neural epithelium and squamous epithelium with keratin deposition), mesoderm (cartilage and striated muscle), and endoderm (ciliated epithelium and gut epithelium). Scale bar: 50 μ m.

FIG. 1. Generation of transgenic MRI reporter mES cell line. **(A)** Schematic of lentiviral construct: FUGW vector was modified to express HA-tagged human FTH under the control of ubiquitin promoter and EGFP linked by IRES. **(B)** The homogenous expression of transgene was confirmed by expression of EGFP and immunostaining using antibody that specifically recognized HA tag. All images were from the same HFG-mES colony. Scale bar: 50 μ m. **(C)** Western blot of HA-tagged FTH in WT (left) and FTH transgenic (right) mES cell lines (α -tubulin was used as an internal control). Expression of tagged human FTH (predicted molecular weight, 22 kDa) was confirmed in HFG-mES cells. **(D)** When seeded at 1×10^4 per cm^2 and quantified at different time points (21, 49, and 74 h) during their exponential growth phase, HFG-mES cells retained similar growth rate as WT controls ($p = 0.236$, two-tailed unpaired t -test, $n = 3$). Data are represented as mean $\pm$ SD. MRI, magnetic resonance imaging; HA, hemagglutinin; FTH; ferritin heavy chain; EGFP, enhanced green fluorescent protein; IRES, internal ribosomal entry site; WT, wild-type.

control and FTH transgenic tissue samples was quantified using ICP-OES. An average 80% ($p = 0.029$; $n = 3$) increase in iron content normalized to tissue dry weight was found in transgenic mES transplants (Fe 226.9 ppm) when compared to that of control samples (Fe 126.1 ppm) (Fig. 4D).

Discussion

To become a useful tool for stem cell research and therapy, a transgenic MRI reporter is expected to meet the criteria of compatibility and functionality in host cells. Our characterizations of mES cells constitutively expressing FTH suggest that a proper balance can be found in a ferritin-ES cell combination to achieve both.

Host compatibility encompasses the concerns of both viability and interference with pluripotency, and our findings support ferritin as a safe MRI reporter in both aspects. The growth rate of HFG-mES cells closely matched that of their WT controls both *in vitro* (under standard ES cell culture conditions) and *in vivo* (as cell grafts in nude mice). Previous study of FTH overexpression in HeLa cells found that it induced an iron-deficient phenotype with significantly reduced cell growth, which was reverted by incubation in iron-supplemented medium.²¹ In the current study, overexpression of FTH transgene did not reduce cell growth even without iron supplement. This by no means contradicted previous findings as the different outcomes likely reflected variations in transgene expression level between cell models. Instead of suggesting FTH safety under any conditions, our findings should be interpreted as feasibility of maintaining sufficient level of FTH expression *in vitro* and *in vivo* without impairments to cell growth. Based on our own unpublished observations, significantly higher FTH transgene expression (>500% of the current level in mES cells) could be achieved in C6 glioma lines, and significant reduction in growth rate was indeed observed under the high FTH transgene expression conditions. When transplanted and monitored *in vivo* following the same protocol, transgenic C6 line generated similar level of MRI contrast as HFG-mES transplants despite much higher FTH expression level. This implies that a moderate level of FTH expression is sufficient for its function as MRI reporter, beyond which endogenous iron supply, rather than transgene expression level, may become the limiting factor for MRI contrast.

Less was known about the relationship between ferritin overexpression and ES cell pluripotency as the current study constitutes the first attempt on ferritin reporter gene-ES cell combination. HFG-mES cells matched their WT controls in stem cell marker expressions and have passed the critical test of teratoma formation. Success with neuronal differentiation

provided additional support for pluripotency, and suggested that the transgenic mES cells could be effectively differentiated *in vitro* with standard protocols. It should be emphasized that the current differentiation study only looked at one cell type in the neural lineage out of the possibility of hundreds of tissue types; whether FTH expression may impact differentiation in other cell types awaits further confirmation. Based on our *in vitro* and *in vivo* observations, especially of teratoma formation, the evidence is strongly in favor (though not absolute confirmation) with noninterference to pluripotency from FHC overexpression. This is promising for the applications of genetically engineered MRI reporter across the wide range of ES cell research. Currently, we cannot speculate on how pluripotency might respond to extremely high level of FTH expression; however, our experience does suggest that further increase in FTH expression is probably not necessary for its future function as an MRI reporter. Considering that under the current design FTH has been constitutively and stably expressed throughout the experimental period with mES cell growth and pluripotency intact, there is strong support for FTH compatibility with ES cell biology.

In their original proposition of ferritin as an MRI reporter, Cohen *et al.*¹³ postulated that overexpression of ferritin would shift the labile iron pool into more ferritin-bound storage form, which in turn induces compensatory upregulation of Tfrc and increases iron uptake from the environment to restore iron homeostasis. Our observations of *in vivo* Tfrc upregulation and iron content increase in FTH transgenic grafts provide further support to this model. Considering no noticeable change in cell growth or pluripotency was observed, the extent of the iron regulatory changes was significant with 35.5% upregulation in Tfrc expression and 80% increase in iron content. These changes in iron regulation could be effectively detected with high field strength MRI units. On a 4.7T scanner with T_2 -weighted sequences, we observed up to 28% increase in R_2 relaxation rate from transgenic transplants. The changes in R_2 relaxation rate were statistically significant and, more importantly, comparable with or greater than the R_2 changes reported by previous studies of murine ferritin transgene expression under the same field strength.^{13,14} This suggested that, despite potential limitations, FTH as an MRI reporter could function as effectively in mES cells as in other cell models.

There are a number of issues worth consideration in the potential applications of FTH as an MRI reporter. It is worth pointing out that when compared to SPIOs, ferritin makes a much weaker MRI contrast agent, with particle magnetic moment several orders of magnitude smaller than chemically synthesized nanoparticles.⁷ In our *in vitro* MRI characterization comparing HFG-mES cell pellets (no iron

FIG. 4. Postmortem analyses of tumor tissues. (A) Diameter of WT and HFG-mES tumors measured in millimeter based on MRI image on days 14 and 21 posttransplant. No statistically significant difference in growth rate was observed between WT and transgenic transplants ($p = 0.266$ on day 14 and $p = 0.215$ on day 21, two-tailed paired t -test, $n = 3$). (B) Homogenous transgene expression in HFG-mES tumor sections was confirmed with fluorescence microscopy of EGFP and immunostaining of HA-tagged FTH. Scale bar: 100 μ m. (C) Western blot using antibodies against HA tag and Tfrc further confirmed FTH transgene expression in HFG-mES tumors and showed *in vivo* upregulation of Tfrc in FTH transgenic tumor transplants compared to WT controls, with an average increase of 35.5% estimated by densitometry. (D) Inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis of Fe content measured in parts per million (ppm) tissue dry weights of WT and FTH transgenic tumors. Iron content in HFG-mES tumors was 80% higher than that of WT controls ($p = 0.029$, two-tailed t -test, $n = 3$). Data are represented as mean $\pm$ standard deviation (SD). mES, mouse embryonic stem.

supplement) with WT mES cell pellets prelabeled with serial dilutions of SPIOs (with 2 h incubation with 0.25 mg/mL SPIOs as the undiluted condition), it was found that SPIO-labeled cells continued to induce stronger MRI contrast until we reached a dilution factor of 1024 (Supplemental Fig. S1, available online at www.liebertonline.com). This suggested that transplant labeled with SPIOs may need to undergo on average ≥ 10 cell divisions or through other mechanisms lose more than 99% of nanoparticles before its signal reduced to the level typical of FTH reporter. We thus expect SPIOs to have a clear advantage over MRI reporters for tasks that focus on cellular tracking over a relatively short period of time. Rather than competing with SPIOs under such conditions, FTH is more likely to find applications that focus on MRI monitoring of molecular events (such as reporter gene expression linked to cell differentiation, gene therapy, etc.) or those that require longitudinal monitoring beyond the reach of the prelabeling approach. In any of these applications, improving the detection sensitivity for ferritin reporter would be desirable, which could conceivably come from both MRI hardware (increase in field strength) and sequence designs (using T_2^* -weighted sequence). The impact of long-term transgene expression is another concern. Despite strong evidence in support of ferritin's safety and its protection against reactive oxygen species,¹⁹ caution should be exercised when generalizing these claims over longer time frames. Kaur *et al.*³⁰ have observed in a ferritin transgenic mice model that ferritin expression over the short term protected against MPTP toxicity; however, prolonged expression beyond 8 months saturated the storage capacity of ferritin and increased susceptibilities to neurotoxins in aging mice.³¹ Currently, there is no telling whether careful calibration of ferritin expression level in ES cells could avoid this long-term susceptibility. Alternatively, it is conceivable that an inducible expression system would be adopted in experimental designs that incorporate longitudinal monitoring.

The current study provided the first demonstration of metalloprotein reporter gene-based noninvasive monitoring of ES cells *in vivo*. An MRI reporter-ES cell combination is not merely an appealing theoretical possibility, but the evidence of its safety and effectiveness warrants further exploration of its practical applications. To put the future applications of MRI reporter systems in stem cells into proper perspective, we wish to stress that, given the clear superiority of SPIOs over native ferritin in MRI contrast, ferritin and other MRI molecular reporter systems should not seek to replace SPIOs when it comes to short-term stem cell tracking, but to complement the traditional magnetic nanoparticle prelabeling approach by opening up possibilities previously unavailable. These include, at the cellular level, MRI-based longitudinal tracking of stem cell-derived cell grafts, without the need for external administration of contrast agents or concerns over signal loss over cell division, and at the molecular level, where genetic modifications of stem cells for therapeutic or research purposes can be linked to the expression of reporter gene and noninvasively monitored *in vivo*. For researchers who have access to the high field strength MRI units and are interested in greater flexibility in experimental design, longitudinal monitoring, or imaging at the molecular level in stem cell research, a ferritin-based MRI reporter system is worthy of considerations.

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Author Contribution

J.L., E.C., and P.C. generated the construct and established FTH transgenic mES line; J.L., E.C., and J.Y. conducted cell culture and *in vitro* characterization; R.L., H.M., J.L., L.W., and D.W. performed MRI experiment and data analysis; J.L., S.Y., and H.M. performed animal studies and histological and iron content analysis; J.L., A.C., and H.M. prepared the manuscript; A.C. and H.M. designed and supervised the study.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Anthony W.S. Chan, D.V.M., Ph.D.
Yerkes National Primate Research Center
Emory University School of Medicine
Room 2212, Neuroscience Building
954 Gatewood Road N.E.
Atlanta, GA 30329

Hui Mao, Ph.D.

Department of Radiology, Center for MR Research
and Emory Center for Systems Imaging
Emory University School of Medicine
1364 Clifton Road N.E.
Atlanta, GA 30322

E-mail: achan@genetics.emory.edu

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