Advanced Review

Genetically encoded iron-associated proteins as MRI reporters for molecular and cellular imaging

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All cell imaging applications rely on some form of specific cell labeling to achieve visualization of cells contributing to disease or cell therapy. The purpose of this review article is to summarize the published data on genetically encoded iron-based imaging reporters. The article overviews regulation of iron homeostasis as well as genetically encoded iron-associated molecular probes and their applications for noninvasive magnetic resonance imaging (MRI) of transplanted cells. Longitudinal repetitive MRI of therapeutic cells is extremely important for providing key functional endpoints and insight into mechanisms of action. Future directions in molecular imaging and techniques for improving sensitivity, specificity and safety of *in vivo* reporter gene imaging are discussed.

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INTRODUCTION

Regeneration of injured organs by transplantation of specific cell populations is an area of intense interest in translational medicine. Since the pioneering work of Dr. E. D. Thomas in bone-marrow transplantation in the late 1950s,[1] along with Dr. J. E. Murray who pioneered kidney transplantation,[2] a large number of clinical studies have been conducted using different cell types. Drs. Thomas and Murray received a Nobel Prize in physiology and medicine in 1990 for their achievements. Clinical studies have since demonstrated the safety and feasibility of transplanting adult stem cell derivatives (i.e., bone-marrow cells, mesenchymal stem cells, skeletal myoblasts, and cardiac/endothelial progenitor cells, adipose tissue-derived cells), but unfortunately, long-term benefits have been modest.[3,4] Considerable progress has been made in recent years in the development of new sources of transplantable cells [e.g., derivates of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)] and manipulation of endogenous stem cell pools[5] in applications to repair-specific organs, such as heart,[6,7] brain,[8–10] spinal cord,[11,12] retina,[13–16] liver,[17] and pancreas.[18] Despite considerable breakthrough in cell transplantation, many questions remain unanswered regarding survival and migration of the transplanted cells to an injury site, their engraftment and functional integration within host tissues, immunogenicity and tumorigenicity, etc. *Ex vivo* histological or immunohistochemical staining methods provide the most detailed picture of the transplant on cellular and tissue level with high spatial resolution, but they lack individual dynamic information and require the sacrifice of large numbers of animals in order to complete longitudinal studies. *In vivo* molecular imaging technology might serve as a valuable tool for guiding cell therapy by visualization of biological processes on the cellular

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and subcellular levels, enabling the longitudinal, noninvasive monitoring of the *in vivo* fate and function of transplanted cells.[19]

Various imaging modalities such as radionuclide, optical, magnetic resonance imaging (MRI), computed tomographic, and ultrasound can be used for anatomic and functional assessment of organs after cell therapy and for *in vivo* cell tracking. Specific research questions determine the choice of imaging modality. Several constructive reviews have been published in recent years outlining the uses of each imaging modality for cell tracking, achievable image resolution, and scan time.[20–25] Superior soft-tissue contrast, large availability of imaging techniques, variety of contrast mechanisms, multinuclear capability, absence of ionizing radiation (safety), as well as capability for monitoring functional, anatomical and metabolic information enable MRI to stand out. MRI contrast weighting techniques can detect areas of pathology (scar, inflammation),[26] volume loss,[27] complex tissue architecture,[28,29] chemical exchange within the macromolecular environment,[30,31] cellular death and inflammation,[32–34] metabolite concentration,[35–37] tissue perfusion,[38–40] and vascularity.[41–43] Recent advances in MRI reporter gene techniques have enabled *in vivo* imaging of specific cell populations of interest regarding cell survival, proliferation, migration, and differentiation,[24,44–50] which makes MRI a valuable technology among other molecular imaging modalities.

In molecular and cellular imaging, imaging probes are required to label cells or to target the biological processes of interest. The imaging probe usually consists of a carrier (such as nanoparticle, microbubble, liposome, etc.) and a signal element or contrast agent that is recognized by the corresponding imaging system. An imaging probe may or may not contain a targeting element so that the contrast agents either bind to specific cell surface proteins or are transported into the target cell by diffusion, endocytosis, or active transport (e.g., radiolabeled indium oxine and superparamagnetic iron oxide particles).[20,23,24] Another way to label cells is integration of reporter genes into cellular DNA. Transgene then will be transcribed into messenger RNA and translated into proteins that are detectable by an imaging modality or interact with a molecular probe for imaging signal generation, for example ferritin[51–54] and transferrin,[55–57] bivalent metal transporters DMT1[58] and Timd2,[59–61] CEST reporters,[32,33] expressible reporters based on gas vesicles,[62] etc.

Cell labeling by using a contrast agent carrier system (e.g., iron oxide nanoparticles) is relatively easy to accomplish; this labeling technique provides the strong signal on ^{1}H MRI that allows high-resolution visualization of the migration and homing of injected cells. However, this approach has several drawbacks for long-term imaging, because the imaging probe is diluted as cells divide, which results in a gradual disappearance of the contrast.[63–65] Moreover, nanoparticle-based imaging cannot distinguish live and dead cells[66–68] and high doses of iron-oxide nanoparticles used in MRI raise concerns of cellular toxicity.[69,70] Therefore, carrier imaging systems (such as iron nanoparticles) are best suited for noninvasive determination of transplanted cell localization and are not useful for longer-term monitoring of engraftment and survival. A transgene expression approach allows overcoming these disadvantages. With the right choice of gene-delivery method, reporter genes can incorporate stably into the DNA and will propagate to daughter cells. In this case, the resulting imaging signal reflects transplanted cell viability and quantity. Reporter gene expression is a better marker for monitoring cell viability, therefore this labeling concept is more suitable for assessing long-term grafted cell survival[68,71] (Figure 1).

The purpose of this review article is to summarize the published data on genetically encoded iron-associated reporters, to discuss their advantages and weaknesses. First, we provide overview of iron homeostasis regulation and genetically encoded iron-associated molecular probes used for noninvasive MRI of transplanted cells. Then we discuss the most important applications of iron-associated MR reporters in studies of cell transplantation in brain, heart, and tumors. In conclusion, we discuss the future directions in molecular imaging and techniques for improving sensitivity of *in vivo* MR reporter imaging and transition into the clinical trials.

IRON AND MRI CONTRAST

Effect of Iron on MRI Contrast

MRI contrast is dependent on tissue relaxation properties in a magnetic field following a radio frequency (RF) excitation pulse. The spin–lattice (T_1) relaxation time characterizes the return of nuclear spins to the equilibrium state along the direction of the static magnetic field (B_0). The spin–spin (T_2) relaxation time characterizes the loss of spin coherence and net magnetization in the transverse plane. A relaxation mechanism of coherent transverse magnetization, called T_2* decay, combines effects of B_0-inhomogeneities, susceptibility artifacts, and

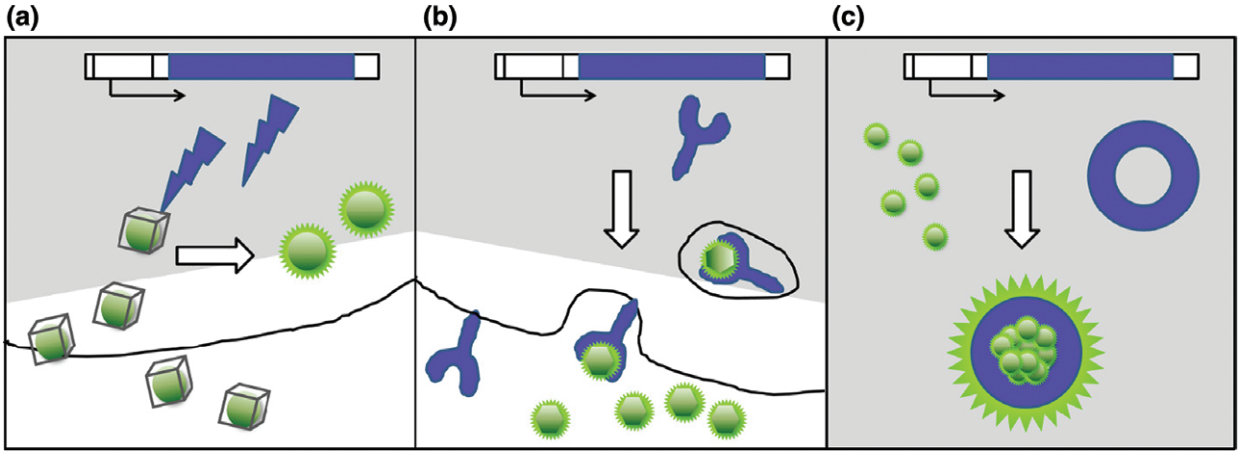

FIGURE 1 | Mechanisms for cell labeling through (semi-) genetic control of MR imaging contrast. *LacZ* is the gene that encodes β-galactosidase.[72] TfR: transferrin receptor. (a) A genetically expressed enzyme (blue) alters an exogenously administered imaging probe (green) that becomes active (green halo) upon enzyme processing (e.g., lacZ). (b) Gene expression leads to the synthesis of a cell surface protein (blue) that acts as a receptor for an exogenous contrast agent (green) and subsequently promotes agent internalization (e.g., TfR). (c) A gene directs endogenous production of protein (blue) that becomes a MRI contrast agent in complex with endogenous ions (green) (e.g., ferritin, magA).

chemical shifts that affect MRI contrast generation. Positive (bright) contrast is typically generated with contrast agents shortening T_1 relaxation, such as gadolinium (Gd^{3+}) or manganese (Mn^{2+}). Iron accumulation in cells and tissues affects the transverse (T_2) magnetic relaxation and T_2*-weighted MR images based on magnetic susceptibility effects. It creates dark areas of signal hypointensity, also referred to as negative contrast.

Superparamagnetic iron-oxide (SPIO) nanoparticles are the most popular agents for cell labeling. Cells may be easily directly labeled with SPIOs by adding these nanoparticles to cell media with or without transfection reagents. High intracellular concentrations of the SPIOs provide strong image contrast as signal void areas on T_2*-weighted images (decrease in signal intensity in areas of particle accumulation up to 80–90%[68]) or as areas of reduced T_2-relaxation on calculated T_2-maps.[68,73–75] SPIO-tagged cells can also be directly imaged by magnetic particle imaging.[76,77] This recently introduced technique directly detects the intense magnetization of synthetic iron-oxide tracers using low-frequency magnetic fields which enables monitoring of cellular grafts with high contrast, sensitivity, and provides highly quantitative 'hot spot' imaging.[78,79]

MRI contrast can also be enhanced by manipulation of the iron content in cells through the utilization of iron-associated proteins as contrast agents. Iron-associated gene reporters exhibit smaller changes in MRI contrast and transverse relaxation in T_2- and T_2*-weighted sequences in comparison with iron oxide nanoparticles: the observed decrease in signal intensity is in the range of 20–30 % in lower magnetic field strengths[68,80] and up to 50% at higher field strengths.[80–82] Weaker MRI contrast properties of iron-associated reporters are determined by the structure of the ferrihydrite mineral core, that contains fewer iron atoms[83] as well as alignment of the iron magnetic spin moments causing smaller net magnetization.[84,85] Not only the reporter transgene itself, but also the right choice of gene expression system is essential for optimal MRI contrast generation in the target cell population (Box 1).

Iron Homeostasis

Iron is one of the essential elements for life because of its ability to donate and accept electrons with relative ease. Many proteins and enzymes have iron as an integral cofactor critical for numerous biological functions, such as oxygen binding and transport (hemoglobins), oxygen metabolism (catalases, peroxidases), cellular respiration, electron transport (cytochromes), and fundamental cellular processes such as DNA synthesis, cell proliferation and differentiation (ribonucleotide reductase), gene regulation, drug metabolism, and steroid synthesis.[86] Iron deficiencies as well as iron overload lead to diverse pathological changes. Iron deficiency results from defects in acquisition or distribution of the metal and causes anemia. In opposite, high concentrations of iron are toxic for cells, because free iron catalyzes radical formation in oxygenated tissues (Fenton's reaction) leading to cell

BOX 1

REPORTER GENE EXPRESSION SYSTEMS

A plethora of options exist for transgene expression of a mammalian target cell population. Over the past few decades, different types of gene expression systems and delivery vectors have been engineered for genetic editing of mammalian cells, based on mixing and matching genes, promoters, and regulatory sequences found in nature. Not only for assembling the expression cassette and genome editing do there exist different elements and methods, also for delivery are there different viral and nonviral vehicles at hand, that are extensively reviewed elsewhere.[164–169] The characteristics of each technique for genetic labeling of cells will determine a great deal of the properties of contrast generation; whether this will be inducible, transient or stable, cell-type or tissue-specific, as well as transgene expression level. Therefore, not only the reporter transgene itself, but also the right choice of gene expression system is essential for optimal MRI contrast generation in the target cell population.

damage. Iron homeostasis is therefore tightly regulated in the organism for maintaining the balance between iron storage and utilization by controlling intestinal absorption of the metal from the diet along with the expression of iron transport and storage proteins such as transferrin, transferrin receptor (TfR), hepcidin, and ferritin.

The human body contains approximately 3–5 g of iron present as heme in hemoglobin of erythrocytes (>2 g), myoglobin of muscles (~0.3 g), in macrophages of spleen, liver, and bone marrow (~0.6 g). Excess of the metal is stored in the liver parenchyma within ferritin (~1 g).[87] All other cellular iron containing proteins and enzymes bind an estimated total of approximately 0.008 g of iron. Iron is delivered to erythroblasts and to most tissues via circulating *transferrin*, which carries approximately 0.003 g of the metal at steady-state. In humans, plasma transferrin is normally about 30% saturated with iron. A transferrin saturation of 45% is a sign of iron overload. When the saturation exceeds 60%, non-transferrin-bound iron begins to accumulate in the circulation and to damage parenchymal cells.[88]

Most mammalian cells acquire iron from circulating transferrin upon binding of the transferrin-iron complex to the TfR. The TfR is a homodimer transmembrane glycoprotein with an overall molecular weight of approximately 180 kDa, located at the cell surface. It is ubiquitously expressed in almost all cells,[89] with higher levels of expression in highly proliferating cells and those that have a functional need for iron.[86] TfRs are therefore naturally present in large numbers in erythrocyte precursors, placenta, and liver.[90–93] The main function of transferrin is mediation of iron internalization through receptor-mediated endocytosis. Most eukaryotic cells use receptor-mediated endocytosis almost exclusively for Fe(III)-TfR uptake.[94] Iron is released from the TfR complex by a pH-change in the endosomal compartment, caused by proton-pump ATPase-activity.[95] The TfR tightly controls cellular iron homeostasis by regulating cellular uptake of holo-transferrin.[96] Mutations in the transferrin gene cause severe anemia.[88] Targeted deletion of the mouse transferrin gene causes embryonic lethality at day E11.5 due to severe anemia, indicating that transferrin-mediated iron delivery is indeed critical for hematopoietic cells.[97]

Cytosolic iron is transported to intracellular sites either for local use or for storage in *ferritin*. Ferritin is found in most cell types of humans, animals, vertebrates, invertebrates, higher plants, fungi, and bacteria, but the concentration of the protein among different cell types can vary 1000-fold. The iron storage protein ferritin consists of a spherical polypeptide shell (apoferritin) surrounding a 6-nanometer inorganic core of the hydrated iron oxide ferrihydrite [$Fe_5O_3(OH)9$].[98] The mammalian apoferritin is a hollow sphere composed of 24 heavy (H; 21 kDA) and light (L; 19 kDA) folded chains. The apoferritin shell can accommodate up to 4500 iron atoms attached to the inner wall of the shell.[99–101] More recent data suggest as few as 1100 iron atoms in ferritin.[102] Iron load in ferritin is dependent of the H_2O_2 detoxification reaction and can be at low, intermediate, and high iron level.[103,104]

Ferritin stores iron in soluble form, available for use by enzymes and for DNA synthesis. Both ferritin subunits are ubiquitously expressed, but their expression ratios vary depending on the cell type and in response to stimuli such as inflammation or infection. Ferritin H-chain has the ferroxidase activity that is necessary for iron deposition into the nanocage, while ferritin L-chain facilitates iron nucleation and increases the turnover of the ferroxidase site. Ferritin provides cells with a means to lock up excess iron in a redox-inactive form to prevent iron-mediated cell and tissue damage.[105–107]

There are three variations in ferritin function in different cell types: (1) the storage of iron for other cells (e.g., recycling iron in macrophages, short- and long-term iron storage in red blood cells of embryos or in hepatocytes of adults); (2) the storage of iron for intracellular use (providing reserve of iron for cytochromes, nitrogenase, ribonucleotide reductase, hemoglobin, myoglobin); and (3) detoxification in iron excess.[108,109] Expression of ferritin is essential for life which is proved by the early embryonic lethality of ferritin H-chain knockout mice.[110]

Mammalian cells can obtain iron via multiple routes based on the specific biochemical requirements. Additionally to transferrin-mediated endocytosis, cells can acquire iron complexed with proteins or small molecules. For example, internalization of ferritin via ferritin-specific receptors such as T-cell immunoglobulin and mucin-domain containing protein-2 (TIM-2)[59] or scavenger receptor family class A, member 5 (Scara5) facilitates iron import.[111] Cells can internalize iron indirectly via a heme carrier protein-1, HCP-1.[112] Iron can also be transported from lysosomes to the cytosol by the heme response gene-1, HRG-1.[113]

Cells also express mitochondrial ferritin (Ft_{mt})[114] that, similarly to H-ferritin, possesses ferroxidase activity. In contrast to cytosolic ferritin, the expression of Ft_{mt} is restricted to few tissues and is not iron-regulated.[115] Ft_{mt} serves as a molecular sink to prevent accumulation of unshielded iron in mitochondria, which protects the organelle against iron's toxicity. The iron storage capacity of Ft_{mt} is similar to the ubiquitous ferritin; however, Ft_{mt} has greater avidity for iron[116] and higher iron load when Ft_{mt} is expressed as a transgene in a cell line.[117]

The presence of ferritin is not limited to the intracellular compartment. It is also found in body fluids, such as serum, cerebral-spinal fluid, and synovial fluids. The origin of the extracellular ferritin may originate from cell damage or from active and regulated ferritin secretion. Assessing the concentration of serum ferritin is a clinically useful index of body iron status. Low serum ferritin levels indicate depleted stores and iron deficient anaemias, whereas increased levels may indicate iron overload. Serum ferritin concentration also increases in inflammation, infection, liver diseases, cancer, and in response to oxidative stress.[88,118]

At high iron loading, ferritin aggregates in the lysosomes to form hemosiderin.[119–121] Hemosiderin is a mixture of ferritin, lipids, and iron[122,123] that is often formed during pathological processes and neurodegenerative diseases associated with altered brain iron metabolism such as Alzheimer's and Parkinson's diseases, and multiple sclerosis.[124–127] Iron deposition in the brain also increases as a result of the normal aging process, and these changes are detectable by MRI.[128]

ENDOGENOUS IRON-ASSOCIATED PROTEINS TURNED INTO MRI REPORTERS

The properties of endogenous proteins involved to regulation of the cellular iron content allowed to study those as potential imaging reporters capable in MRI contrast modulation. Examples of genetically encoded iron-associated proteins employed to image of distinct cell populations are shown below.

Transferrin Receptor

The TfR was the first MRI reporter gene proposed by Koretsky et al. in 1996,[55] based on its function of facilitating metal transport across membranes by internalizing the transferrin-iron complex through receptor-mediated endocytosis.[129] It raises the level of labile iron in the cells, therefore can be used as one potential way to generate MRI contrast. The TfR has a fast rate of turnover (2×10^4 transferrin molecules internalized per minute), which results in the quick uptake of large amounts of iron.[130] The synthesis of the endogenous receptor is tightly controlled through an iron-dependent negative feedback in cells; this provides an adequate supply of the essential metal in function of the iron need, while guarding against toxic excess of iron.[96,131–133] Genetic modification of cells to express a TfR lacking mRNA destabilization motifs leads to constitutively overexpressed high levels of TfR with subsequently increased accumulation of iron in cells, resulting in higher MR contrast.[134]

The TfR is a receptor that can be (over-) expressed in very high numbers (several million copies per cell) on the cell membrane. Fibroblasts overexpressing TfR exhibited an approximately threefold increase in iron content yielding significant MRI contrast in T_2-weighted sequences.[55] In the absence of iron, the binding sites of transferrin can accommodate a number of other metals including gallium, copper, chromium, cobalt, manganese, vanadium, aluminum, terbium, plutonium, europium, indium, and platinum.[130] The feasibility of manganese labeling of murine hepatocytes via the transferrin-receptor-dependent and/or -independent metal-transport pathways has been documented.[135] This method of cell labeling is based on the chemical similarities between manganese and iron

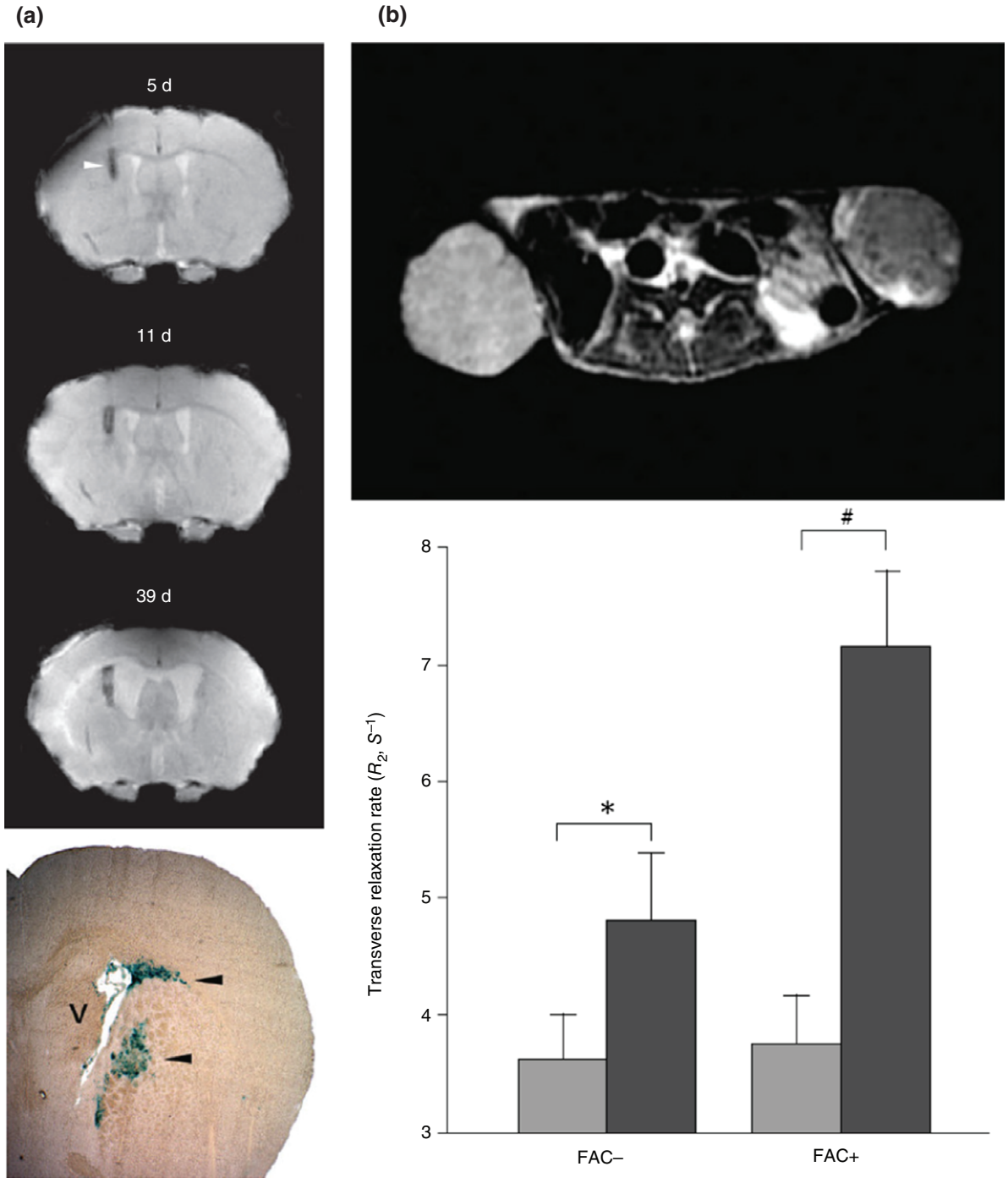

FIGURE 2 | *In vivo* MRI detection of ferritin expression. (a) T_2*-weighted images of the mouse brain at days 5, 11, and 39 after inoculation of an adenovirus containing the MRI reporter ferritin into the striatum. Images were acquired at 11.7 T with a 0.75-mm slice thickness and an in-plane resolution of 102 μm. Bottom image is the Prussian blue staining for iron representing similar pattern to the MRI. v, ventricle (Reprinted with permission from Ref 52. Copyright 2005 Nature). (b) Representative T_2-weighted images of mice inoculated with tumors over-expressing ferritin heavy chain *in vivo* (right tumor) and parent cells (left). External iron supplementation enhances MRI contrast based on ferritin overexpression. (Reprinted with permission from Ref 136. Copyright 2012 Hindawi)

(e.g., similar ionic radii; similar valence states—(II) and (III)—under physiological conditions; and similar binding affinities for transferrin). The results of these studies demonstrate that Mn(III)-transferrin is an effective MRI contrast agent for labeling murine hepatocytes.

Ferritin, Iron Storage Protein

The intracellular level of ferritin can be genetically altered. The higher number of ferritin complexes per cell causes higher iron accumulation, thus directly affecting MRI contrast. The possibility of using ferritin in biomedical imaging and cell labeling was shown in 1992 by Meldrum et al.[98] In 2005 Cohen et al. used ferritin expression and eGFP under tetracycline control to visualize C6 glioma cells transplanted to the mouse.[51] Genove et al. demonstrated the use of ferritin as an MRI reporter gene by injecting an adenoviral vector for transient overexpression of human ferritin (H-chain) into the brain parenchyma of mice, resulting in significant loss of signal at the site of vector injection[52] (Figure 2(a)).

During the last decade, ferritin overexpression has been extensively explored for a variety of applications, such as imaging of transgenic tumors.[51,137,138] Ferritin overexpression has been detected *in vivo* in the mouse brain,[48,52] *in utero* in transgenic mice[139] and in liver hepatocytes.[140] A ferritin shell was used in material science as a precursor for making nano-composite particles as an effective MRI contrast agent for macrophage tracking[141] and for noninvasive imaging of atherosclerosis.[142] Replacing the native iron core in ferritin with a synthetic paramagnetic core[143] has proven to result in effective T_2 relaxation enhancement both *in vitro*[144] and *in vivo*.[145]

Additionally to cell labeling, it was shown that a ferritin expression vector can also be used to monitor *in vivo* gene expression. GL261 mouse glioma cells expressing ferritin–DsRed fusion protein under b-actin promoter showed reduced T_2-weighted signal intensity for *in vitro* and *in vivo* MRI studies as well as DsRed fluorescence in optical imaging.[138]

First application of mitochondrial ferritin (Ft_{mt}) as an MRI reporter has been proposed by Iordanova et al.[146] In that study, mitochondrial ferritin was modified to localize within the cell cytoplasm for efficient iron load and MRI tracking on olfactory sensory neurons.

It is important to emphasize that no exogenous iron supplementation is required for MRI contrast generation by overexpressed ferritin. The fact that ferritin-tagged grafts cause strong signal intensity can be explained only by redistribution of endogenous iron and its accumulation from the host tissue in the overexpressed ferritin complexes.[51,52,80] However, external iron supplementation can enhance MRI contrast based on ferritin overexpression[136] (Figure 2(b)).

To date, several molecular biology approaches and different vectors have been used to overexpress ferritin: adenoviral,[52] retroviral,[51] lentiviral,[54,81,137,147] and adeno-associated virus gene delivery[54]; different plasmid vectors have also been used.[53,57,138,148] The technique chosen for reporter gene expression is crucial as its properties will determine whether contrast generation will be transient or stable, constitutive, inducible, or tissue-specific. Continuous expression is best suited for tracking the survival and the migration of labeled cells. Therefore, several studies investigating MRI reporter genes for long-term tracking of transplanted cells have focused on methods to introduce reporter genes stably into the cells of interest, such as lentiviral (LV) and adeno-associated viral (AAV) vectors for reporter gene expression. AAV-mediated ferritin overexpression through direct vector injection into the brain was shown to be the most promising way for *in vivo* reporter gene imaging in the brain that resulted in significantly enhanced contrast to background on T_2*-weighted MRI in comparison with other delivery systems[54] (Figure 3). By introducing a genetic switch, ferritin expression can be turned on or off and changes in the imaging signal can be detected within several days thereafter.[51,140] Being able to control overexpression is important in avoiding potential toxicity issues associated with constitutive overexpression of ferritin, even though overexpression of ferritin results in suppression of the damaging Fenton reaction and thus can protect cells from oxidative damage enhancing the survival of the administered cells.[53] These results suggest that ferritin holds significant promise as translational MRI reporter gene for *in vivo* imaging of live transplanted cells.

Bacterial Magnetosome-Associated MRI Reporters (*magA* and *mms6*)

Iron-associated biomolecules were found not only in mammalian cells, but also in bacteria, and explored as potentially interesting MRI reporter genes. Formation of iron biominerals is a naturally occurring phenomenon among magnetotactic bacteria which produce magnetite (Fe_3O_4) in a subcellular compartment termed the magnetosome. There are two main components to the magnetosome: the biomineral and the lipid bilayer that surrounds it. Magnetotactic bacteria naturally synthesize these intracellular magnetic structures which help to direct motility of the bacteria based on the earth's magnetic field.[149,150] The magnetosome-associated proteins are important for vesicle formation, cytoskeletal attachment, iron transport, and crystallization.[151] Expression of magnetosome genes in nonbacterial cells

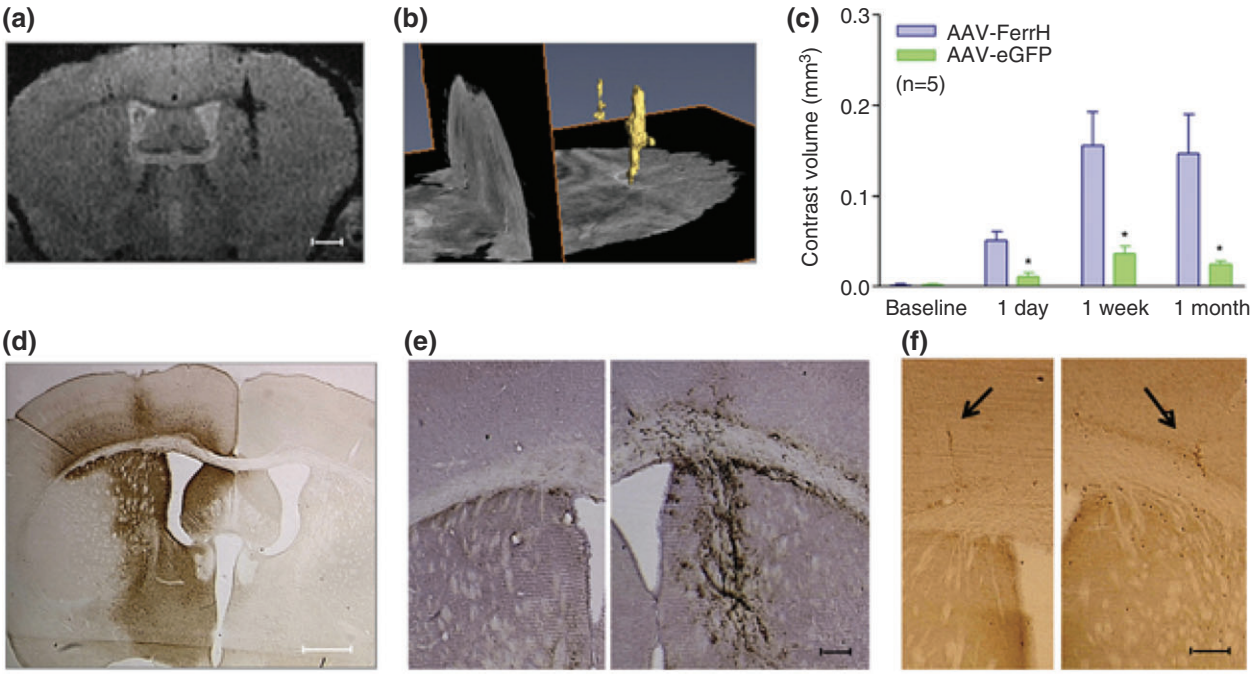

FIGURE 3 | AAV-FerrH-mediated contrast evaluation by MRI. (a) 3D T_2*-weighted *in vivo* MR image acquired 1 month after injection of AAV-FerrH-T2A-fLuc and AAV-eGFP-T2A-fLuc vectors in the right and left striata, respectively. (b) 3D graphic representations of the contrast volume from the same mouse. (c) Quantification of the contrast volume using the normalized 3D T_2*-weighted *in vivo* MR images at different time points post-injection. (d) Immunohistochemical staining for eGFP shows a large area of transgene expression after AAV transduction in the brain. (e) DAB-enhanced Prussian blue staining, showing the presence of iron at the site of AAV-FerrH injection in contrast to the contralateral control injection site where very little iron is detectable. (f) immunohistochemical staining on an adjacent section using CD11b antibody, showing very few CD11b-positive cells at the left and right injection tracts (arrows). White scale bars represent 500 mm, black scale bars 100 mm. (Reprinted with permission from Ref 54. Copyright 2011 Nature)

provides new opportunities for development of reporter gene-based contrast for MRI.

Based on the involvement of *magA* in the synthesis of magnetosomes in *Magnetospirillum* bacterial species,[152–154] the use of this gene has been explored as an inducer of MRI-detectable contrast. It has been shown that *magA* can be expressed in mammalian cells and its expression leads to the formation of magnetic nanoparticles that strongly affect the MRI signal[155] (Figure 4). However, it is not yet known whether *magA* expression leads to the formation of the same iron oxide crystals seen in magnetotactic bacteria. MRI detection of *magA* expression has been shown in mouse neuroblastoma (N2A) cells[156] and in the human 293FT cell line.[155] The iron-based MRI reporter *magA* resulted in larger increase in R_2 relaxation: cell pellets expressing *magA* consistently showed an increase in R_2 of approximately three- to fourfold,[155] comparing to an increase in R_2 of approximately 2.5-fold for ferritin expression.[52]

Escherichia coli expressing *magA* showed increased iron uptake in membrane vesicles when cells were supplemented with iron and ATP. However, this response was limited when ATP was excluded, suggesting that *magA* function is coupled to ATP hydrolysis.[157] *MagA* might be expressed in a controllable fashion using a doxycycline-inducible promoter.[155] This has the advantage that reporter gene expression can be switched on or off, which is important for controlling *magA* expression levels in order to avoid cytotoxic effects at high levels of overexpression.[158] *In vivo*, *magA* overexpression did not lead to pathological alterations in transgenic mice and could even attenuate oxidative damage due to iron overload.[159] An additional iron supplementation of *magA*-expressing cells increased transverse relaxation rates compared to nonsupplemented cells,[160] also *in vivo*.[159] It was shown that expression of both magnetotactic bacterial gene *magA* and mammalian iron storage protein ferritin in MDA-MB-435 tumor cells sequestered iron within a membrane-enclosed vesicle and permitted iron biomineralization with simultaneous regulation of mammalian iron homeostasis.[160] These results highlight the potential of magnetotactic bacterial gene expression for improving

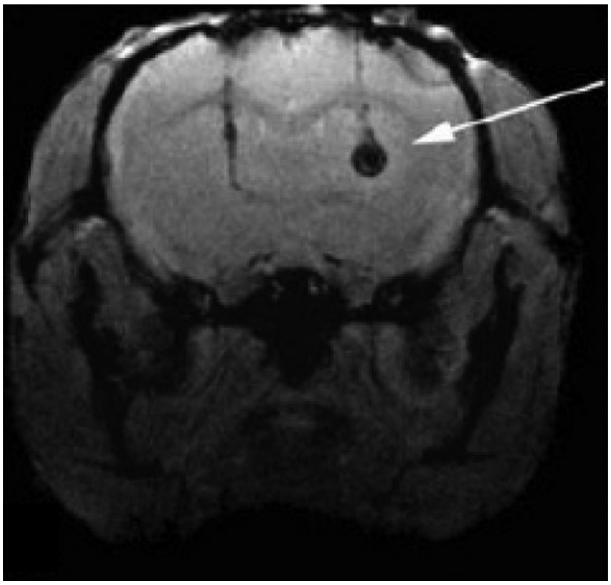

FIGURE 4 | T_2*-weighted image of mouse brain with transplanted *magA* cells (right) and GFP control cells (left) after 5 days of induction. These cells were neither induced nor incubated with iron supplement prior to transplantation. The *magA* cells (white arrow) exhibit significantly lower MRI signal, reflecting an increase in R_2, suggesting that *magA* cells are able to use endogenous iron sources. The control cells on the left do not show such an effect. (Reprinted with permission from Ref 155. Copyright 2008 Harvard Catalyst)

MR contrast. However, *magA*'s status as a putative MR reporter gene is controversial as its implication in magnetosome formation has been contested.[154]

Another bacterial gene, magnetosome-associated *mms6*, has been proposed as a reporter gene for MRI of mammalian cells.[161] *In vitro* experiments show that *mms6*-expressing cells form clusters of nanoparticles within and outside membrane-enclosed structures and produce changes in MR contrast, most likely by increasing iron uptake of intracellular iron. *In vivo* MRI experiments demonstrate that *mms6*-expressing tumors can be distinguished from parental tumors not expressing *mms6*, even in the absence of exogenous iron supplementation. Whether *mms6* is an efficient genetic MR reporter remains to be established, and potential toxicity issues should be clarified. Mms6 protein fused to the C-terminal of murine h-ferritin has been proposed as a novel chimeric magneto-ferritin reporter gene—ferritin-M6A, enabling magnetite biomineralization of ferritin and enhancing R_2 relaxivity of ferritin-M6A-expressing C6-glioma cells.[162]

Timd2, Mediator of Ferritin Endocytosis

T-cell immunoglobulin and mucin domain containing protein receptor (Timd2 or Tim-2) are expressed primarily on immune cells, where it plays a role in signaling and mediates ferritin endocytosis.[59,60,163] Timd2 expression elsewhere is limited, but is also found in both liver and kidney, where it functions primarily as a ferritin receptor to remove ferritin from the blood,[59] as well as on oligodendrocytes, where it is upregulated during myelination to meet increased iron demands.[60] It has been shown that cells expressing Timd2 and incubated with ferritin or manganese-loaded apoferritin showed large increases in R_2 and R_1, respectively. Cells expressing Timd2 implanted as xenografts in mice also showed changes in R_2 following intravenous injection of ferritin, although these changes in contrast were more modest than those observed *in vitro*.[61] Therefore, Timd2 represents another potential MRI-based gene reporter.

CELL-TRACKING APPLICATIONS

Neural Stem Cell Migration

It was a long-standing paradigm that brain damage occurring during adulthood is irreparable, until this paradigm started shifting with the discovery of neural stem-like cell pools residing in discrete brain regions.[170] When active populations of neural stem cells (NSC) were also found to generate new neurons in the adult human brain,[171] hope was raised that possible stimulation of this endogenous stem cell pool could have the potential for neuro-regenerative therapy after brain injury, without having to transplant exogenous cells. Because the often invasive standard methods used to monitor the proliferation, migration, differentiation, and functional integration of endogenous NSCs and their progeny are limited in studying dynamic processes, novel techniques and contrast mechanisms for *in vivo* imaging of neurogenesis have been developed and successfully applied.[172] *In vivo* labeling of endogenous neuronal progenitor cells *in situ* with magnetic nanoparticles has proven useful for tracking neuroblast migration with MRI,[173–178] but only labeling with genetic reporters can overcome the issues with dilution of contrast agents and subsequent signal loss upon cell division and has potential for long-term monitoring of neurogenesis. Using LVs expressing ferritin to label the endogenous NSCs, the stem cell progeny migrated and integrated in the olfactory bulbs could be detected and quantified with *ex vivo* MRI, but for *in vivo* tracking of the migrating stem cell progeny, the system lacked sensitivity.[48]

In search of improving the contrast generating properties of ferritin, linking the light and heavy

chains of ferritin resulted in a slightly larger protein cage and improved MR contrast *in vitro*.[147] Using an adenoviral vector encoding this chimeric ferritin reporter to label endogenous neural progenitor cells, migrating cells could be visualized along the rostral migratory stream with *in vivo* MRI at 11.7T.[179] However, no contrast was seen in the olfactory bulbs where these cells arrive and integrate. Although possibly due to the vector system used for gene transfer, improving the sensitivity of ferritin nevertheless remains a challenge. The strategy to combine constitutive overexpression of both ferritin heavy chain and the TfR in mouse NSCs implanted into the mouse brain did not lead to spectacularly improved results[57] (Figure 5). This work provides an example of how gene reporter-based contrast improves at 7T in comparison with the standard clinical field strength of 1.5 T.

Heart

Molecular and cellular imaging is essential in the evaluation of graft survival and cardiac repair after cell transplantation. Myocardial infarction leads to significant loss of cardiomyocytes, scar formation, and impaired contractile function. Since the heart has very limited intrinsic regenerative capabilities, transplantation of new cardiomyocytes offers a promising treatment approach. MRI is the perfect imaging modality for noninvasive visualization of soft tissues. It is valuable for assessment of heart contractile function, chamber morphology, vascularity, inflammation, infarct size, tissue viability, metabolism, as well as for evaluation of the transplanted cell localization and graft size. Most of the cell tracking studies have been done using SPIOs nanoparticles that provide robust MR signal detection, useful for short-term monitoring of cell localization and migration. It

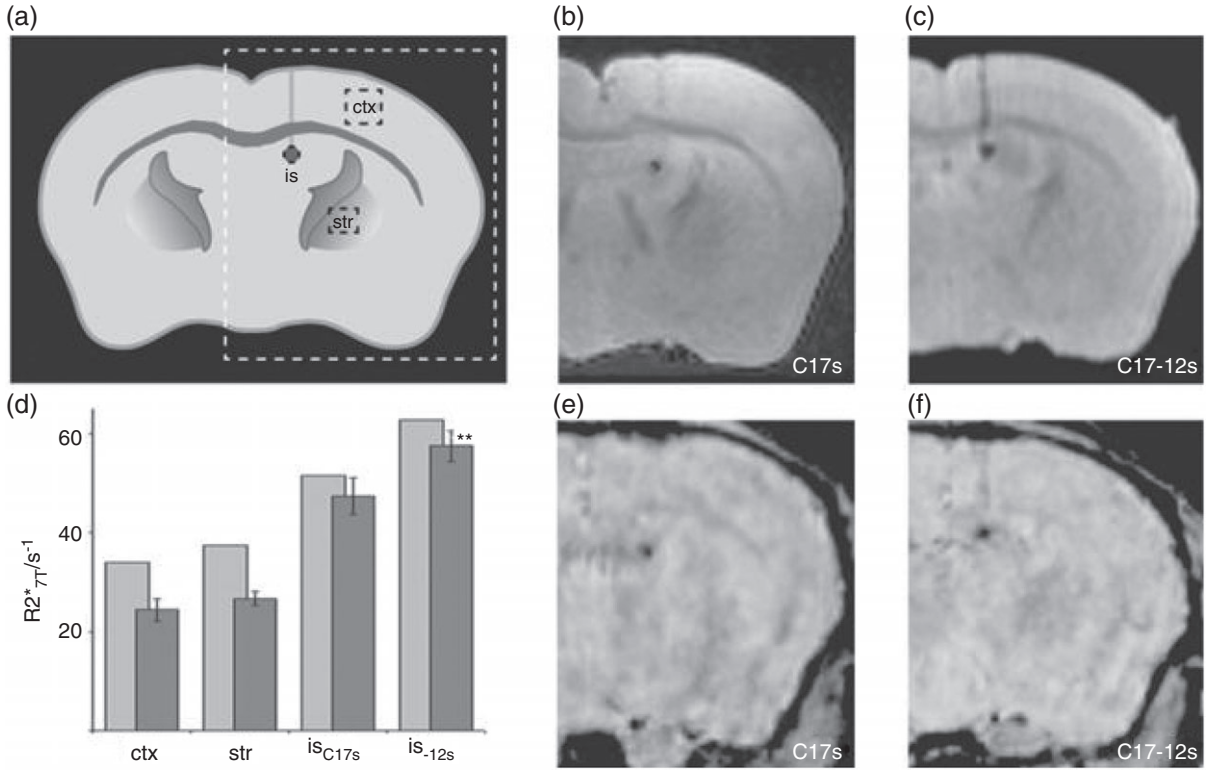

FIGURE 5 | T_2*-weighted MR images of mouse brain at 7T and changes in R_2* relaxivity in transgenic cell samples. (Reprinted with permission from Ref 57. Copyright 2006 Wiley) (a) Schematic of a coronal section of an adult mouse brain shows the location of the MRI images (b, c, e, and f) shown, including the injection site (is), and control regions in cortex (ctx), and striatum (str). T_2*-weighted images from *ex vivo* mouse brains injected with C17s control cells (b) or C17-12s transgenic cells (c) clear contrast between the transplanted cells and surrounding brain tissue. (d) Mean R_2* measurements (dark bars) were made on *ex vivo* brains for ROIs within the injection site of either the C17s injected brains or the C17-12s injected brains, as well as for region of interest (ROIs) within ctx and str. Error bars denote the SD in each case. R_2* was significantly increased in is-12s versus isC17s (**$P < 0.04$). Light bars indicate the R_2* values measured in the same regions from an *in vivo* multi-GE acquisition of a C17s injected brain and a C17-12s injected brain. *In vivo* T_2*-weighted images from C17s (e) and C17-12s transplanted mice (f) were acquired from mice later used in *ex vivo* imaging (data included in b and c, respectively).

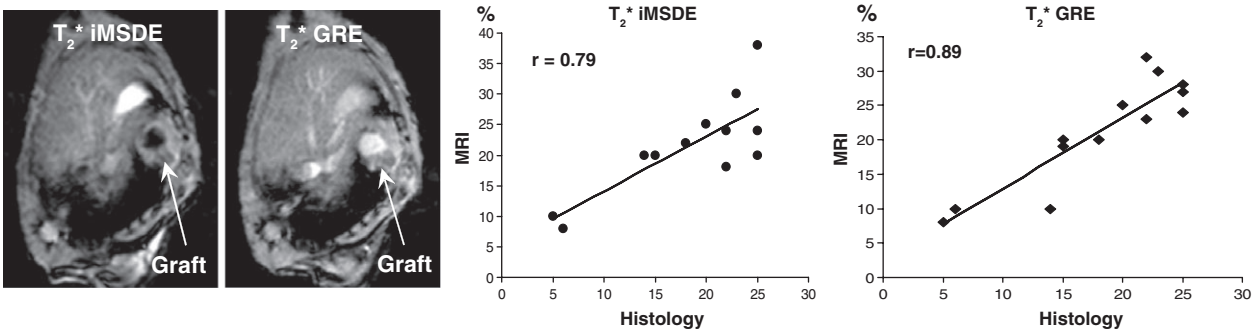

FIGURE 6 | MRI detection of ferritin-tagged graft in the infarcted mouse heart using T_2* iMSDE and T_2* GRE pulse sequences and correlation of the graft size measurements between MRI and histology (Reprinted with permission from Ref 80. Copyright 2012 Wiley).

has been shown that direct injection of cells into the myocardium is the most effective delivery for heart repair.[180] Gene reporter expression is better suitable for longitudinal follow-up of changes in graft size and viability. *In vivo* MRI detection of cardiac grafts overexpressing ferritin in infarcted mouse hearts was shown to be feasible.[53,68,80] MRI thereby provided morphological measurements of graft size with reasonable accuracy and precision[53,80] (Figure 6).

In the context of longitudinal graft imaging, the MRI contrast provided by ferritin over-expression does not diminish with cell proliferation and represents live grafted cells, while imaging signal from iron-oxide particles is not related to graft viability and size, but may be a better marker for high-resolution detection of cell location by MRI.[68,181]

Campan et al. employed ferritin expressing swine cardiosphere-derived cells for serial imaging on a 1.5 T MRI scanner after transplantation to a rat model of myocardial infarction.[182] The hypointense signal on MRI was detectable at 1 week after infarction and its size did not change significantly after 4 weeks,[182] which is a surprising finding since immune rejection of the pig cells is expected in Wistar rats. Prussian blue staining confirmed the presence of differentiated, iron-accumulating cells containing mitochondria of porcine origin.

An example of plasma membrane bound contrast complexes was described for cardiac applications of ESCs engineered to express the reporter proteins myelocytomatosis (myc) and hemagglutinin (HA) and transplanted into infarcted myocardium in mice. Regions of surviving/proliferating ESC were detected on T_2*-weighted images following intravenous injection of iron oxide nanoparticles conjugated to either anti-myc, or anti-HA monoclonal antibodies. Further, by spacing follow-up scans appropriately to allow for washout of labeled SPIO particles, a clear time-course of proliferation and teratoma formation was determined.[71] The combination of a gene-expression- and particle-based cell labeling approaches may help to overcome the limitations of each individual labeling technique.

Tumors

Molecular imaging has already became an indispensable tool in basic oncology research to identify not only tumor location and size, but also critical biological pathways involved in oncogenesis and cancer progression.[183] Most cancer imaging has been done with optical and nuclear imaging technology, whereas MRI and MR spectroscopy have been used for assessment of nanomedicine-based strategies for cancer treatment and in image-guided enzyme/prodrug cancer therapy.[184,185] MRI gene reporters, such as ferritin and transferrin, have mostly been assessed in tumor models as proof-of-principle approach. Tetracycline-controlled ferritin overexpression for MRI visualization was demonstrated in a tumor formed by C6 glioma cells. Inoculation of these tumor cells in nude mice revealed a significant decrease in T_2 on tetracycline withdrawal[51] (Figure 7, study 1). In studies of Moore et al.[56,134] and Weissleder[186] 9L gliosarcoma cells were genetically engineered to express human TfR (hTfR). It was demonstrated that a conjugate of transferrin and monocrystalline iron oxide nanoparticles can be used for amplification of receptor expression and MRI visualization in this model (Figure 7, study 2). MCF-7 and F-98 cells could be tagged by a bimodal lentiviral vector encoding myc-tagged human ferritin and green fluorescent protein. These transgenic tumors had significantly decreased T_2 and T_2* relaxation times on 1.5T MRI, 1–4 weeks after transplantation in mice and rats[137] (Figure 7, study 3). Subcutaneous teratoma formation in nude mice was imaged at the 4.7 T magnet at 14 and 21 days post inoculation of mouse stem (mES) cells expressing ferritin[81] (Figure 7, study 4). The added value of increased MRI contrast for the detection of solid tumors in these studies remains unclear, but the low level of contrast generated by the reporter genes

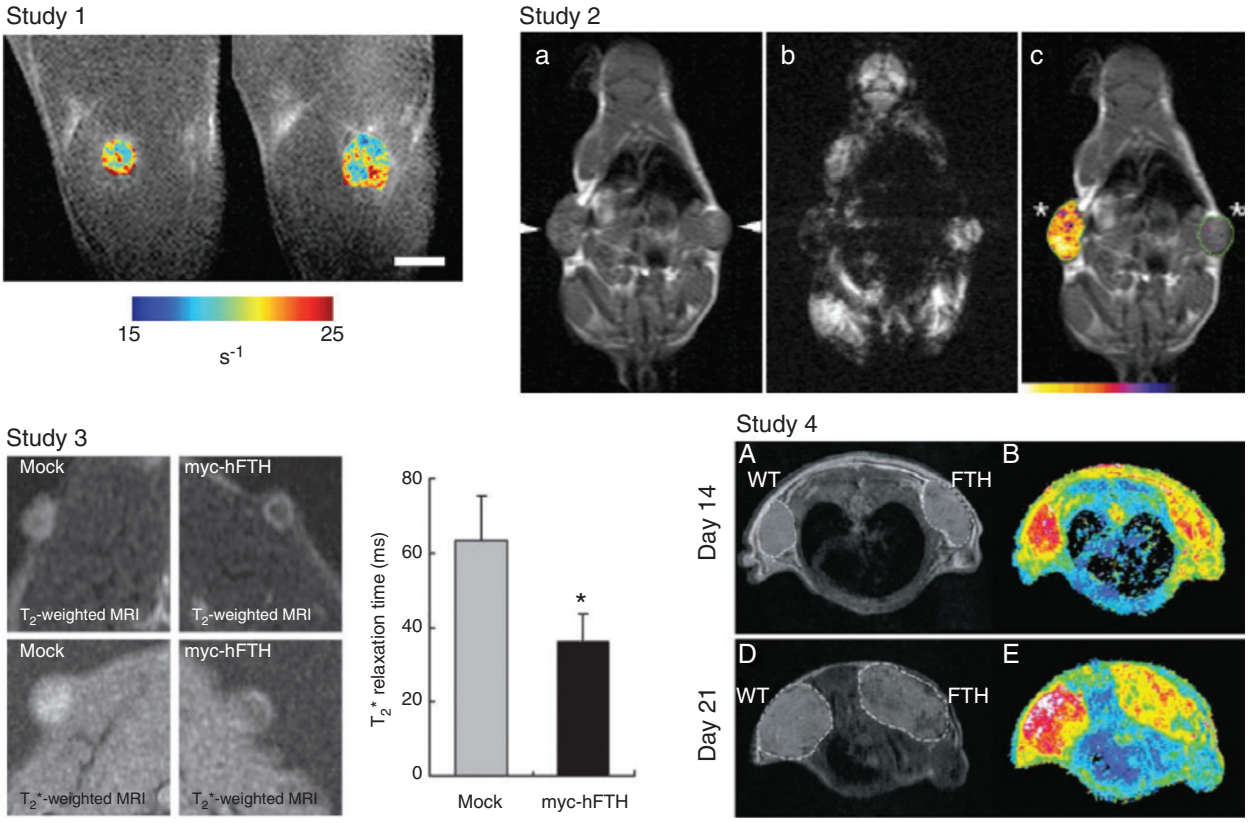

FIGURE 7 | MR imaging of iron-oxide reporters in cancer applications. Study 1: *In vivo* R_2 maps of C6 tumors expressing ferritin in hind limb of CD1-nude mice. Scalebar 2.5 mm (Reprinted with permission from Ref 51. Copyright 2005 Elsevier). Study 2: *In vivo* MRI of a mouse with transplanted glyosarcoma cells expressing human transferrin receptor (left arrowheads) and naïve (right arrowheads) flank tumors. (a) T_1-weighted coronal spin-echo image. (b) T_2-weighted gradient-echo image corresponding to the image in (a). Transferrin-mediated cellular accumulation of the superparamagnetic probe decreases signal intensity. (c) Composite image of a T_1-weighted spin-echo image obtained for anatomic detail with superimposed R_2 changes after Tf-MION administration, as a color map (Reprinted with permission from Ref 186. Copyright 2000 Nature). Study 3: *In vivo* T_2- and T_2*-weighted MRI of F-98 tumor 2 weeks after subcutaneous transplantation of myc-hFTH and mock cells (left) and T_2* relaxation times of mock and myc-hFTH tumors were measured from T_2*-weighted images (right) (Reprinted with permission from Ref 137. Copyright 2010 AACR). Study 4: *In vivo* MRI detection of FTH transgene-induced MRI contrast in mES cell grafts. (A, D) Representative T_2-weighted fast spin echo (FSE) images showing the same pair of tumors grown from mES grafts (left, WT; right, FTH transgenic), at days 14 and 21 postinoculation. (B, E) Corresponding color-coded T_2 maps from multiecho measurements of T_2 relaxation time showed significant reduction of T_2 relaxation time in the tumor overexpressing FTH transgene at both time points (Reprinted with permission from Ref 81. Copyright 2009 Mary Ann Liebert Inc).

makes the use of MRI for tracking of metastatic cancer cells highly unlikely.

Gene reporter imaging has its advantages for longitudinal monitoring of tumor growth without the need for external administration of contrast agents or concerns over signal loss over cell division. However, biomedical applications might be limited to preclinical research on animals, since transgenic manipulations are required for creation of stable cell lines.

CHALLENGES, LIMITATIONS, AND IMPROVEMENT STRATEGIES

Despite the significant value that reporter gene imaging brings to the field of molecular imaging and cell tracking with MRI, there are several limitations and challenges that remain to be resolved and that warrant further research attention before the reporter gene-based contrast paradigm can be exploited to its full potential for MRI applications.

Low Sensitivity

The low sensitivity of MRI compared to other modalities in visualization of reporter genes remains a major challenge. The majority of iron-associated MRI gene reporters are yielding imaging signal attenuation rather than signal gain. Detection of those signals requires development of complicated time-consuming acquisition and processing protocols.[187–193] Many technical and methodological

improvements, such as higher field strength, dedicated RF coils, superconducting RF coils, and new pulse sequences could be implemented for substantial improvements of signal detection generated by iron-associated MRI reporters. In contrast to genetic cell labeling, synthetic iron oxide nanoparticles, possess much higher R_2* relaxivity, enabling *in vivo* MRI tracking of a single cell,[194] which is a level of sensitivity not possible with native and engineered reporter gene cell labeling. Further improvements in MRI sensitivity for reporter gene detection are possible, by intensifying the MRI signal by external iron supplementation,[136] by transferrin labeled contrast media[186] or by manipulating mineralization and engineering of mutant ferritin proteins.[102] A chimeric ferritin with a fixed subunit stoichiometry by fusion of the L and the H subunits (L*H and H*L) was created and improved the sensitivity of MRI for ferritin detection to some extent.[147] Cellular MRI contrast could also be slightly enhanced via co-expression of the TfR and ferritin.[57] The modulation of ferritin aggregation by binding to cytoskeletal elements might be another useful strategy to increase transverse relaxivity.[195] Another way to enhance MRI sensitivity of gene reporter imaging is to combine expression of specific genes with targeted delivery of iron oxide nanoparticles and increased iron storage within selected cells. For example, intravenous administration of holo-transferrin covalently bound to iron oxide nanoparticles generated significant contrast on T_2*-weighted images in cells overexpressing transferrin,[186,196,197] or the administration of antibody-tagged SPIOs targeted to tag sequences expressed on transplanted stem cells into infarcted heart,[71] as we mentioned above. Taken together, the sensitivity of reporter gene induced contrast for MRI remains limited for cell tracking applications, in particular for those involving small population sizes. More sensitive MRI reporters, and particularly those that would help to gain the signal rather than attenuating it, would be a significant advance in this field. Examples pursuing such an approach are all based on uptake of manganese or gadolinium-based agents that enhance T_1 contrast, rather than iron-oxide-based contrast that modulates the T_2.[58,198,199]

MRI Signal Specificity Issues

MRI contrast based on iron-associated reporter gene overexpression may also suffer from specificity issues when the endogenous ferritin or iron content is elevated.[200,201] Signal specificity issue is generally pertains to any cell labeling strategies based on negative contrast. The signal void caused by iron accumulation is almost indistinguishable in MRI from that caused by hemosiderin deposition in the damaged tissues[80,202,203] or hemorrhage caused by needle insertion to the brain.[201] Under pathological conditions, the total amount of tissue iron and the proportion stored as hemosiderin can increase, while the capacity of ferritin to store iron is overwhelmed.[99,107,204,205] Therefore, hemosiderin deposition areas may obscure contrast from iron-associated reporter gene expression.[80,202,203,205] Care should therefore be taken with interpretation of areas of signal void is, especially for small grafts that cannot be precisely identified by a known anatomical location. These limitations could potentially be overcome by the application of technical approaches, such as analysis of non-monoexponential signal decay,[187–189] advanced acquisition modes[190,191] or postprocessing methods[192,193] that enable generation of positive contrast in the presence of paramagnetic materials, such as iron oxides. Although DNA and protein degradation occur rapidly after cell death,[206] there are no data as to how rapidly ferritin complexes undergo degradation after the death of transplanted cells and for how long the MRI signal from iron persists. Cessation of *magA* induction resulted in a return of iron content in 293FT cells to control values within 6 days, suggesting activation of iron degradation pathways.[155] It will be important to determine how quickly other iron-associated proteins can be produced by daughter cells and how quickly ferritin complexes can bind a sufficient amount of iron from the extracellular environment to be detected by MRI.

Administration of Cells

One of the challenges in imaging of transplanted cells is their administration route. Cell therapies ideally involve systemic administration of cells with subsequent homing of cells to the site of injury. Nevertheless, systemically administered cells distribute to multiple organs with primary localization to the lungs, liver, spleen, or marrow spaces and relatively few cells homing to diseased sites.[207–209] For these applications where low cell numbers are to be found, MR reporter genes are unlikely to be adequate labels given their low sensitivity. All published MR reporter gene studies to date involved a bolus injection of transgenic cells or viral agent into a target organ with subsequent accumulation of labeled cells. Direct injection of a labeling agent allows local distribution and higher concentration of the label, needed for robust MRI detection. While preclinical cardiovascular and cancer models utilized concentrated injections of large numbers (millions) of cells, the typical

injections in neurological studies utilize orders of magnitude less cells (thousands), presenting a potential sensitivity barrier to the use of MRI reporter genes for neurological studies. In this case, as well as for imaging of cells administered systemically, synthetic nanoparticles remain the more suitable option for short-term MRI tracking due their higher iron payload.

Expression Level

The site of transgene insertion in the genome and the number of transgene copies can significantly affect the expression level. Use of novel cloning techniques, such as zinc finger nuclease (ZFN),[210–212] clustered regulatory interspaced short palindromic repeats (CRISPR)[213,214] or transcription activator-like effector nucleases (TALENs)[215] would make transgene insertion targeted to a specific locus in the chromosomes. Targeted genome addition can provide persistent expression of the transgene while avoiding gene silencing and insertional mutagenesis caused by viral vector-mediated random integration. 'Safe harbours' can be chosen for insertion of a transgene, such as ROSA26[216] or AAVS1.[217,218] To direct transgene integration, ZFN and TALENs methods use DNA-binding modules engineered to match a target DNA sequence. The CRISPR system uses RNA-guided Cas9 DNAse activity to generate sequence-specific target cleavage.[214]

Safety

The toxicity and interference associated with any cell label or expressed reporter gene should be addressed carefully. Reported changes in the cell's phenotype either accompanied with or without alterations in proliferation or viability, do raise concerns about possible toxic effects of iron-associated gene overexpression.[57,200,219,220] For example, chronic overexpression of H-ferritin (FTH1) in a transgenic mouse resulted in progressive age-related neurodegeneration.[221] The constitutive expression of *magA* from *Magnetospirillum magnetotacticum* was tolerated by human embryonic kidney (HEK) cells but induced a strong toxic effect in murine mesenchymal/stromal cells and kidney-derived stem cells, restricting its use as a reporter gene.[200] As an alternative solution to constitutive expression, an inducible system might be advantageous: controllable expression of gene reporter can minimize the adverse effects of constitutive expression (e.g., toxicity, reduced proliferation rate, possible impact on differentiation potential). Another solution is addition of suicide genes, such as herpes simplex virus type 1 thymidine kinase (HSV1-tk), for targeted deletion of the transplanted cells in case of adverse effects.[222,223]

For longitudinal studies of proliferation, migration, differentiation, and functional integration of therapeutic cells, long-term safety and stability are required. Applications of gene reporters in clinical studies are still problematic because of the concerns regarding potential toxicity and tumorigenicity of transgenic manipulations with cells. In this context, the development of novel imaging methods that could provide alternative contrast properties capable of identifying specific cell types is of particular interest. Recently, several MRI methods exploiting endogenous contrast-generation mechanisms were proposed. For example, chemical exchange saturation transfer (CEST) contrast agents can identify the presence of certain enzymes, and their application holds great promise in potential 'multi-color' MRI.[31–33] Another method, based on macromolecular proton fraction (MPF) mapping is determining the relative amount of immobile macromolecular protons involved in magnetization exchange with mobile water protons and can provide quantitative information about tissue composition and collagen content.[224]

Imaging Multimodality

Multimodal imaging approaches seem the most interesting option to overcome limitations of individual imaging techniques regarding specificity, sensitivity, tissue penetration, etc. There is an enormous yet so far under-exploited potential for synergy by combining the strengths of different approaches available. In this view, future perspectives behold the combined visualization of processes with different reporter genes with different functions integrated in one expression cassette.

FUTURE DIRECTIONS

Molecular imaging technology provides invaluable tools for researchers to study transplanted cells non-invasively: their survival, engraftment, proliferation, and possible mechanisms of benefit. Despite the extensive use of reporter genes in preclinical studies, application of molecular imaging in clinical trials is limited. The main obstacle for implementation of the reporter gene imaging for cell tracking in patients is the lack of regulatory confidence in the safety and specificity of genomic manipulations. Additional studies are needed to proof the safety and understanding of the long-term behavior of transplanted

cells with integrated transgenes. Noninvasive imaging can provide quantitative and qualitative assessment of transplanted cell dynamics that would lead to personalized patient care. Functional outcome after cell transplantation is dependent on several aspects, including engraftment success, graft survival, structural, and functional integration within the host tissue. Noninvasive imaging can help to define the optimal cell type, delivery method and time, changes in host microenvironment as well as provide information about off-target behavior and oncogenic events. A particle-based cell labeling is most useful for short-term cell visualization with MRI, for confirmation of the targeted delivery and tracking of cell migration, while a reporter gene approach offers longitudinal monitoring of transplanted cell viability, proliferation, and migration. To minimize safety concerns, human natural iron-associated proteins, such as ferritin, might be used, or advanced MRI techniques based on endogenous proton exchange contrast mechanisms, such as CEST or MPF, might be implemented for patient trails. Selection of the optimal labeling technique and imaging modality will depend on the studied cellular processes. Since each imaging modality has its own strengths and limitations, a multimodal imaging approach with multimodal gene reporters integrated in one expression cassette might be a potential solution for tracking the fate of transplanted cells and functional benefits. Future studies will likely employ advanced imaging technology to patient's benefits ensuring cell therapy efficacy and safety.

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