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***In vivo* magnetic resonance imaging of ferritin-based reporter visualizes native neuroblast migration**

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Abstract

Adult neurogenesis research in mammals presents a challenge as most stem cells and progenitors are located deep in opaque brain tissues. Here, we describe an efficient ferritin-based magnetic resonance imaging (MRI) reporter and its use to label mouse subventricular zone progenitors, enabling *in vivo* visualization of endogenous neuroblast migration towards the olfactory bulb. We quantify the effect of the ferritin transgene expression on cellular iron transport proteins such as transferrin receptor, divalent metal transporter and steap reductase. Based on these data, we elucidate key aspects of the cellular pathways that the reporter utilizes to load iron and form its superparamagnetic core. This MRI reporter gene platform can facilitate the non-invasive study of native or transplanted stem cell migration and associated neurogenic or therapeutic molecular events in live animals.

Keywords

Magnetic resonance imaging; Reporter gene; Ferritin; Neuroblast migration; Adult neurogenesis

Introduction

The adult mammalian brain continuously generates new neurons in specific stem cell niches (Ming and Song, 2005). Subpopulations of astrocytes in these brain regions serve as the primary neural progenitors. During normal adult neurogenesis the progenitor cells transiently amplify and give rise to migrating neuroblasts (Doetsch et al., 1999). The subventricular zone (SVZ) is the largest stem cell niche in the brain, and neuroblasts born in the SVZ migrate long distances along the rostral migratory stream (RMS) over the course of several days to reach the olfactory bulb. Once in the bulb, they move radially into the cell layers and differentiate into inhibitory interneurons (Whitman and Greer, 2009). The functional significance of adult neurogenesis is still a topic of debate, however it is well recognized as playing an important role in neuronal circuit plasticity underlying learning and behavior (Lledo et al., 2006).

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New experimental approaches for non-invasive study of native stem cell migration are of interest to a wide community of neuroscientists. In addition, a number of neurodegenerative diseases are known to increase cell proliferation in the SVZ, and stem cells from this region migrate towards the affected brain sites to participate in different aspects of brain repair (Curtis et al., 2007). This naturally occurring process of neuroblast migration and local circuit integration is an important model for translational studies of stem cell therapy in the brain (Lindvall and Kokaia, 2006).

Most of our knowledge about the molecular and spatiotemporal characteristics of neuroblast migration originates from immunohistochemistry of fixed tissue slices (Ming and Song, 2005; Lledo et al., 2006). These traditional approaches are invasive and only look at a predefined two-dimensional plane. Chronological events are generally reconstructed from series of snapshots of different brains. Conversely, bioluminescence and fluorescence microscopy allow longitudinal *in vivo* studies, however the imaging depth and resolution are limited by the absorption and light scattering in tissues (Ntziachristos, 2010). These inherent constraints hamper the study of neurogenic events in deeper parts of the brain. The RMS in primates, for example, has very different spatial orientation than that of rodents, and this may be one of the major reasons for its late discovery (Gould, 2007).

A number of MRI studies have demonstrated the feasibility of *in vivo* stem cell tracking (Bulte et al., 2001; Zhang et al., 2003; Ruiz-Cabello et al., 2008; Panizzo et al., 2009). MRI is non-invasive, yields three-dimensional data, and has high spatial resolution, making it ideally suited for longitudinal studies of cell migration. The cell location in the brain can be co-registered with other MRI data such as tractography, functional MRI and spectroscopy of brain metabolites (Walter et al., 2010). Current approaches for imaging migrating stem cells by MRI rely on *ex vivo* labeling of the cells with superparamagnetic iron-oxide nanoparticles (Bulte et al., 2001; Zhang et al., 2003) or perfluorocarbon emulsions (Ruiz-Cabello et al., 2008) prior to brain engraftment. These approaches, while potentially having high relevance to emerging cell-based therapeutics, provide no information about native neurogenesis.

A few pioneering studies succeeded in labeling SVZ progenitors *in vivo* by injecting large concentrations of micron-sized iron-oxide particles directly into the ventricles (Shapiro et al., 2006; Panizzo et al., 2009; Sumner et al., 2009; Nieman et al., 2010). The cellular mechanism of this *in vivo* uptake is not clear and has a low efficiency. Moreover, the approach results in considerable image artifacts from the ventricles and labels cells non-specifically (Shapiro et al., 2006; Sumner et al., 2009). Importantly, the label dilutes with every cell division, and clearance dynamics for dead cells containing the contrast agent is difficult to characterize. Microglia readily engulf cell debris and extracellular contrast agent, which results in non-specific labeling (Shapiro et al., 2006). Another source of a false positive is the possible diffusion of contrast agent and dead cells along the flow of cerebrospinal fluid that parallels the neuroblast migration path (Sawamoto et al., 2006).

A partial solution to the above challenges is the use of a reporter gene such as green fluorescent protein (GFP) and luciferase. Reporter genes are only active in live cells, and they can be inducible or continuously expressed under promoters that are cell specific; moreover, they can be integrated into the genome or remain episomal (Serganova and Blasberg, 2005). Most neurogenic events however, take place deep into the opaque brain tissue, and *in vivo* information is difficult to obtain using optical reporter molecules. MRI reporter genes offer an efficient combination of the high resolution imaging power of MRI with the elegant tools of molecular biology.

In this paper we demonstrate that an engineered, ferritin-type, iron storage protein can be utilized as a 'probeless' MRI reporter gene (Cohen et al., 2005; Genove et al., 2005; Deans

et al., 2006; Cohen et al., 2007) for endogenous stem cell tracking *in vivo*. In mammalian cells, two genes, heavy (H) and light (L) ferritin, code for separate polypeptide chains, and 24 of these subunits assemble into ferritin shell. Ferritin loads iron *in situ* and stores it in a paramagnetic ferrihydrite core (Genove et al., 2005). The exact molecular pathway used by cells to sequester bioavailable iron when overexpressing ferritin has not been characterized.

We recently designed a chimeric ferritin molecule (L*H) that fuses the L and H subunit with a flexible polypeptide linker. The L*H has higher iron loading and significantly larger MRI contrast enhancement compared to wild-type ferritin (Iordanova et al., 2010).

The present work describes a powerful imaging approach applicable to a multitude of neuroscience applications entailing *in vivo* tracking of native and therapeutic stem cells. We use the L*H chimera to label native primary neuronal progenitors in the mouse brain and visualize their migration *in vivo* from the SVZ to the mouse olfactory bulb. In addition, we elucidate the molecular mechanisms employed by the L*H ferritin expressing cells to load iron when used as a gene reporter in mammalian cells, by quantifying the change in expression of several key iron-import proteins.

Methods

Cell line and viral vectors

U251 human astrocytoma cell line was a gift from Victor Levin, University of Texas. To express transgenes, we used replication-deficient, type 5 adenovirus (AdV5, deleted for E1/E3) based on the Adeno-XTM Expression System (BD Biosciences Clontech, Carlsbad, CA). Molecular clones of human H and L were kindly provided by Paolo Arosio, University of Brescia, Italy. The cDNAs were inserted into appropriate restriction sites in pShuttle-2 and subcloned into pAd-X plasmid. The viruses were titered on HEK293 cells. The titers were as follows: LacZ AdV 3.7×10^{10} pfu/mL, L*H AdV 2×10^{10} pfu/mL, H AdV 1.4×10^{10} pfu/mL, L AdV 4.5×10^{10} pfu/mL. The EGFP AdV titer was 1×10^{11} pfu/mL and purchased from the University of Pittsburgh Vector Core Facility.

Antibodies

Primary antibodies used for staining of molecular markers were mouse anti-FLAG (F3165, Sigma-Aldrich, St. Louis, MO), rabbit anti-mouse glial fibrillary associated protein (GFAP) (G9269, Sigma), rabbit anti-mouse neurofilament (N4142, Sigma), rabbit polyclonal to Ki67 (ab15580, Abcam, Cambridge, MA), goat polyclonal to doublecortin (C-18 epitope, sc-8066, Santa Cruz Biotechnology, CA), and rabbit polyclonal to polysialylated neural cell adhesion molecule (PSA-NCAM, AB5032, Millipore, Billerica, MA). Secondary antibodies were donkey anti-rabbit Alexa Fluor 488 (A-21206, Molecular Probes, Carlsbad, CA), rabbit anti-goat Alexa Fluor 488 FAB (A-21222, Molecular Probes), goat anti-mouse Alexa Fluor 594 FAB (A-11020, Molecular Probes), and donkey anti-mouse Alexa Fluor 594 (A-21203, Molecular Probes). Primary antibodies used for the western blot analysis were mouse monoclonal for β -actin (sc-47778, Santa Cruz), mouse monoclonal for iron regulatory protein-2 (IRP2, sc-33682, Santa Cruz), mouse monoclonal to transferrin receptor-1 (TfR1, T8199-41, US Biological, Swampscott, MA), rabbit polyclonal to divalent metal transporter-1 (DMT1, NRAMP24-A, Alpha Diagnostic, San Antonio, TX), rabbit polyclonal to DMT1+IRE (NRAMP22-A, Alpha), where IRE is iron regulatory element, and goat polyclonal to the metalloredutase STEAP3 (sc-20531, Santa Cruz). Secondary antibodies were goat anti-rabbit horseradish peroxidase (HRP) conjugate (1858415, Pierce, Rockford, IL) and goat anti-mouse HRP conjugate (1858413, Pierce).

Immunocytochemistry

U251 cells were grown on glass slides and transduced with reporter transgenes. At 48 h post-transduction, cells were fixed using 4% paraformaldehyde (PFA). The cells were washed with phosphate buffer saline (PBS) and 0.2% Tween 20 (Bio-Rad, Hercules, CA) and then probed using antigen-specific antibodies followed by the secondary reagents as described above.

Western blot and enzyme-linked immunosorbent assay (ELISA)

U251 cells expressing the different ferritins and control reporter (LacZ) were incubated for 48 h post-transduction in 95% Dulbecco's Modified Eagle Medium, 5% fetal bovine serum and 1 mg/mL holotransferrin (#T0665, Sigma). Total protein content was measured with a bicinchoninic acid (BCA) assay kit (Pierce). Equivalent amounts of clarified samples were resolved on 4–20% polyacrylamide gradient gels (Pierce). We used chemiluminescence to expose the immunoreactive bands on film (1651454, Eastman Kodak, Rochester, NY). The films were scanned at 600 dpi and 16 bit grayscale using a flat bed scanner (Hewlett-Packard 8200), and the lanes were quantified using ImageJ software (<http://rsbweb.nih.gov>). All protein levels were expressed as a percent of LacZ expression. For enzyme-linked immunosorbent assay (ELISA) analysis, cells were assayed with the Quantikine TfR kit (R&D Systems, Minneapolis, MN).

Animal studies

All animal experiments were approved by the Carnegie Mellon Institutional Animal Care and Use Committee (IACUC). Adult female C57BL mice (Harlan, Indianapolis, IN), 5–7 weeks old, were anesthetized using an intraperitoneal cocktail of ketamine and xylazine and placed in a head stereotactic device. Animals were injected with 5 μ L of L*H AdV in the right striatum (anterior, lateral and ventral coordinates from Bregma 1.0, 2.2, 3.0 mm, n=7) and SVZ area (−1.0, −1.0, 2.2 mm, n=20). The contralateral control side was injected with the same viral load using LacZ AdV or GFP AdV. Animals were monitored until recovered and housed with food and water *ad libitum*. Mice injected in the striatum were imaged at day 5, and mice injected in the SVZ were imaged at 10 days post-injection. After imaging, animals were perfused transcardially with PBS and then with 4% PFA, and the brains flash frozen in optimal cutting temperature (OCT) compound (EMS, Hatfield, PA) and stored at −80 °C.

MRI

All MRI was performed at 11.7 Tesla using a Bruker microimaging system (Bruker, Billerica, MA). T₂*-weighted images were acquired using a 3D gradient-echo (GRE) sequence. The mice were anesthetized, intubated, placed on a mechanical ventilator, and maintained on 0.75% isoflurane in 70% O₂ and 30% N₂O inhalation gas during the imaging sessions. For the *in vivo* imaging we used a 2.5 cm diameter, laboratory-built surface coil, 3D GRE with TE/TR=7/50 ms, 20 flip angle, 4 averages, 200×200×200 image points, field of view=1.2×1.2×1.2 cm and 60 μ m isotropic resolution. The total *in vivo* scan time was 4.4 hours. For the *ex vivo* imaging we used a 20 mm diameter volume coil, 3D GRE with TE/TR=8/100 ms, 20° flip angle, 9 averages, 256×256×256 image points, field of view=1.28×1.28×1.28 cm and 50 μ m isotropic resolution. The total *ex vivo* scan time was 16.5 hours.

Immunohistochemistry and histology

Frozen brain tissue was cryosectioned in 20 μ m thick slices and incubated for one hour at room temperature with primary antibodies in a humidified chamber, washed and incubated for 45 min with secondary antibodies. Nuclei were counterstained with Hoechst 3342

(Calbiochem, La Jolla, CA). The control was an incubation of slices with secondary antibodies without the presence of primary. For the Perls' iron stain the slices were immersed in water with 2% potassium ferrocyanide and 2% concentrated hydrochloric acid for 30 min. Cell nuclei were counterstained with 0.5% nuclear fast red (H-3403, Vector Labs, Burlingame, CA). The X-gal stain for detection of β -galactosidase activity was performed using 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (B4252, Sigma).

Confocal microscopy

Glass cover slips were mounted after the final wash, and the slides with tissue slices were imaged using a Carl Zeiss LSM 510 Meta UV DuoScan inverted confocal laser-scanning microscope. RGB channels were collected sequentially with a matrix resolution of 1024×1024 and 4 averages.

Statistical analysis

The significance of the western blot quantification was calculated using Origin 7.5 software (Northampton, MA). We used one-way analysis of variance (ANOVA) to look at differences of the group means, followed by Tukey's method for pair-wise differences with a confidence interval of 0.95. We also used a paired student t-test with a confidence interval of 0.95.

Results

Iron transport proteins utilized by the ferritin reporter

We first evaluated the effects of the L*H reporter on the cellular iron metabolism and key iron uptake proteins. In order to demonstrate that the iron in the contrast-bearing cells is stored inside the ferritin reporter and not part of the labile iron pool, we quantified the expression of IRP2. At stable oxygenation levels the IRP2 presence in the cytoplasm is inversely correlated with the size of the labile iron pool (Rouault, 2006). We transduced U251 astrocytoma cell line with adenovirus coding for L*H ferritin and compared the IRP2 expression to that in cells expressing H ferritin, or H and L expressed concurrently. We used the same multiplicity of infection for all ferritin groups and have shown previously that it results in similar transgene expression levels and no cellular toxicity (Iordanova et al., 2010). We verified that the astrocytoma cells express the transgenes and maintain normal morphology (Fig. 1). We found that IRP2 expression increased in all ferritins comparing to LacZ control and L*H ferritin had the highest IRP2 levels (Fig. 2A). These results are consistent with previous findings that ferritin expression controls the labile iron pool in cells (Cozzi et al., 2000).

Next, we examined the L*H reporter's effect on TfR1, an importer of transferrin bound iron, which is known to upregulate in cells overexpressing ferritin (Cozzi et al., 2000). TfR1 has been also shown to increase in somatic tissues of H ferritin transgenic models (Wilkinson et al., 2006). Quantification of western blots showed significant increase of TfR1 in U251 cells expressing ferritin (Fig. 2B), and a subsequent enzyme-linked immunosorbent assay confirmed these results (Fig. 2C). Iron taken up by TfR1-mediated endocytosis is transported into the cytosol across the endosomal membrane by DMT1. The DMT1 on the cell surface can also participate in the import of non-transferrin bound iron, and two of four possible DMT1 isoforms contain the IRE (Garrick and Garrick, 2009). We found that in U251 cells expressing ferritin, the level of DMT1 is slightly elevated, with L*H being the only ferritin with a statistically significant increase (Fig. 2D). We then looked specifically for the expression of the DMT1 isoform that contains IRE and found that all ferritins had a significant increase of DMT1 (+IRE) levels compare to control (Fig. 2E). The transport of iron ions via DMT1 requires reduction of Fe^{+3} to Fe^{+2} . The STEAP3 reductase is often

found on the cell membranes in proximity to DMT1 and acts synergistically (Moos et al., 2007; Garrick and Garrick, 2009). The expression of STEAP3 was elevated in all astrocytoma cells expressing ferritins, however only the L*H expressing cells had a significant increase compared to controls (Fig. 2F).

Expression of L*H ferritin reporter in the mouse striatum

To initially evaluate *in vivo* detection of the L*H reporter, we stereotactically injected adenovirus expressing L*H into the striatum of C57 female mice (n=6). We imaged the live mice at 5 days post-injection. The L*H expressing region showed robust contrast in T₂*-weighted images; where no contrast was observed on the contralateral side injected with the same amount of adenovirus coding for LacZ (Fig. 3A). The *in vivo* contrast-to-noise ratio in the region of L*H expression against background tissue was 26.4 ± 1.8 (standard error for n=6). We also imaged the same brains *ex vivo* with high spatial resolution (50 μ m isotropic voxels). We confirmed strong contrast on the side of L*H ferritin reporter, and additionally, we were able to detect the needle track from the LacZ control illustrating the amount of signal change solely due to the injection procedure (Fig. 3B). Using histology, we observed colocalization of the FLAG tag on the L*H reporter with the astrocytic marker GFAP in striatal cell populations transduced by adenovirus (Fig. 3C). There was minimal colocalization between FLAG and the neuronal marker neurofilament (NF) (Fig. 3D). We also demonstrated a lack of reporter toxicity at the injection site, showed cellular iron accumulation at the region of MRI contrast and confirmed the LacZ expression on the contralateral side (Fig. 4).

In vivo MRI visualization of native neuroblast migration

Using the same L*H reporter adenovirus, we stereotactically targeted native progenitor cells in the SVZ region of the mouse brain (n=20). We also injected the same region contralaterally with control adenovirus coding for GFP. At 10 days post-inoculation, we imaged the mice *in vivo* and observed a hypointense stream from the site of reporter transduction along the RMS, towards the olfactory bulb (Figs. 5A,B). There was no observable change in image contrast on the GFP control side. We also imaged the brains *ex vivo* and confirmed the presence of a hypointense migratory stream towards the bulb (Figs. 5C,D). In order to validate these findings and establish the phenotype of the migrating cells, we performed a series of immunohistological stainings using different molecular markers. Figure 6A shows the SVZ region double-stained for FLAG tag on the L*H reporter and Ki67, a mitotic marker. Numerous cells around the lateral ventricle double-stained indicating that cells expressing the MRI reporter undergo proliferation and give rise to neuroblasts. PSA-NCAM is present on the cell surface of migrating cells such as neuroblasts (Ming and Song, 2005). We observed a considerable number of cells along the RMS region that double-stained for FLAG and PSA-NCAM (Fig. 6B). The location of the migrating neuroblasts spatially corresponds to the hypointense trail observed by MRI (Fig. 5). We also looked at the expression of the neuroblast and early neuronal marker doublecortin (DX). We found cells in the RMS (data not shown) and olfactory bulb (Fig. 6C) double-stained for FLAG and DX indicating that cells labeled with the reporter have reached the bulb and migrated radially to integrate into the local circuits. Most double-stained cells on Fig. 6C were in the center of the olfactory bulb at the end of the tangential path. Fig. 6C also shows double-stained cells that have moved radially into the layers and assumed granule cell morphology, as well as a few superficial cells displaying glomerular cell phenotype. We were unable to observe this radial migration by MRI since the cells were no longer clustered together as in the RMS, and the concentration of L*H expressing cells were below the level necessary to generate T₂*-weighted contrast.

The L*H ferritin reports neuroblast migration via the iron load into its protein cage. Perls' iron staining of the tissue showed positive areas at the SVZ injection site, and we also observed numerous iron loaded (blue) cells along the white matter tracks and RMS region (Fig. 7A). The contralateral side injected with GFP did not display any iron staining, however, we observed GFP expression optically in the SVZ, striatum and along the RMS (Fig. 7B).

Discussion

In the present work, we characterize and apply *in vivo* a versatile ferritin-based MRI reporter. Reporter genes generate contrast only in live cells, and they can be expressed in specific cell populations (Serganova and Blasberg, 2005). This imaging technology platform can be used not only for non-invasive visualization of neuroblast migration, but also for tracking cellular therapeutics, such as stem cells, and monitoring event-related promoter activity *in vivo*.

Despite prior use of ferritin as an MRI reporter, very little is known about the molecular routes it activates upon expression in order to sequester iron. This knowledge is important not only for our basic understanding of brain iron metabolism and avoiding toxicity, but also for exploring the potential of multicistronic reporters to achieve better MRI sensitivity.

A critical factor for the potency of a ferritin-based reporter is the iron-loading efficacy *in situ*. We used human astrocytoma cell line for the *in vitro* characterization of the molecular pathway employed by the reporter to approximate the primary cell types observed to be transduced *in vivo*. Astrocytomas have been shown to originate from astrocytic progenitors and stem cells, and they display similar phenotype and molecular markers (Alcantara Llaguno et al., 2009). These gliomas have comparable proliferating activity, employ analogous metabolic pathways, and preferentially express stem cell related genes (Sanai et al., 2005). We showed that the L*H ferritin expressing cells had the highest levels of IRP2 demonstrating that L*H loads iron more efficiently than H expressed alone, or H and L concurrently (Iordanova et al., 2010) (Fig. 2A). All cells expressing ferritin also increased the expression of TfR1 (Figs. 2B,C). The change of the labile iron pool, IRP2, and TfR1 was previously demonstrated in H ferritin expressing cells and tissues (Cozzi et al., 2000; Wilkinson et al., 2006). We were surprised to find that the level of TfR1 expression in cells expressing of L*H was not higher than the cells expressing other ferritins, consistent with the observed increased iron loading and IRP2 levels. There was a small change in TfR1 levels when the media was not supplemented with transferrin, however the cells expressing L*H ferritin still loaded more iron (data not shown). We speculate that when expressed as an MRI reporter, ferritin reporters may also use nontransferrin bound iron sources. We note that brain interstitial fluid has high levels of iron carried by citrate. Astrocytes, normally with low TfR1 levels, are thought to secrete citrate and use it as alternative route for iron import (Moos et al., 2007). In an effort to address this, we looked at DMT1 levels, as this transporter is also known to be expressed on the cell surface and import iron directly, in addition to pumping iron out of TfR1 endosomes (Garrick and Garrick, 2009). We found that the DMT1+IRE was significantly increased (Fig. 2E), suggesting that this alternate (non TfR1) pathway may play a role.

As an iron ion crosses a cell membrane via a transport protein such as DMT1, it requires an enzyme to change its oxidative state. The knowledge about cellular reductases in the brain is incomplete (Garrick and Garrick, 2009). We looked at the most common cellular reductase, STEAP3 and found a slightly significant increase in L*H expressing cells (Fig. 2F). There are several other STEAP isoforms that could be facilitating the DMT1 iron import (Garrick and Garrick, 2009). A recent study looking at iron import in astrocytes found that in addition

to DMT1, these cells have an independent route for ferric iron (Lane et al., 2010). It is not known how the astrocytic progenitors and stem cells import the iron needed for DNA replication and ATP production. In addition, there is no full understanding of how the cells ultimately incorporate and recycle the iron from the exogenous paramagnetic particles widely used for MRI cell tracking. We believe that our findings may be a useful starting point for future studies of iron based contrast agents and their molecular effects in the brain.

Using adenovirus expressing the L*H reporter we were able to target and visualize neuroblast migration from SVZ to the olfactory bulb. Adenovirus DNA remains episomal and does not replicate with cell divisions. Lentiviruses, which incorporate into the cell genome are generally preferred for labeling dividing cells. Adenoviruses can carry larger transgenes, do not diffuse in the brain, and target both mitotic and post-mitotic cells (Yoon et al., 1996; Serganova and Blasberg, 2005). In order to reduce the number of variables in this proof-of-principle experiment, we chose an adenoviral vector system that is well established for targeting adult progenitors and neuronal precursors in the mouse brain (Yoon et al., 1996). We had previously shown that high titer adenoviral transduction of the L*H transgene results in robust and continuous expression, such that all daughter cells remain labeled over several divisions (Iordanova et al., 2010).

Every day about 30,000 cells leave the SVZ and travel few centimeters towards the olfactory bulb (Lois and Alvarez-Buylla, 1994). With average speed of 70–100 m/h it takes about 5 to 12 days to reach the destination (Whitman and Greer, 2009; Nieman et al., 2010). At any given time the number of migrating cells along the RMS is between 50,000 and 100,000 cells (Alvarez-Buylla et al., 2001). We were able to visualize the migrating cells during their tangential path along the RMS as they cluster together and the contrast is amplified. When the neuroblasts reach the bulb, they disconnect from the chains and move radially into the cell layers (Whitman and Greer, 2009). We were unable to see this radial movement since the signal becomes diluted as the labeled cells separate. In the histological sections, we observed numerous FLAG positive cells in the core of the bulb and in the cellular layers with granule and periglomerular morphology (Fig. 6C) indicating that the cells reached the bulb layers and integrated into the circuits. The detection sensitivity of the ferritin-labeled cells is not at the single cell level; we estimate that cell cluster containing on the order of 10^4 cells can be detected, which is reasonable for neurogenesis and cell therapy imaging studies.

To our knowledge, this is the first report of non-invasive visualization of native neuroblast migration using an MRI reporter gene. This work may serve as a platform for future *in vivo* studies of stem cells both in health and disease.

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Fig. 1.

U251 astroglioma cells are efficiently transduced with adenovirus expressing the ferritin reporter genes. Immunocytochemistry shows similar expression levels of MRI reporter transgenes 48 hours post-transduction. (A) Confocal image of the cells expressing L*H ferritin reporter stained for FLAG tag (red). Cell nuclei are blue. Scale bar = 20 μ m. (B) Confocal image of the cells expressing H ferritin reporter (red) at the same multiplicity of infection. (C) Confocal image of non-transduced control cells. (D) Western blot for the FLAG tag on the L*H reporter shows a band in the L*H expressing cells. NV = no virus used (control).

Fig. 2.

Ferritin-based transgene in U251 astrocytoma cells activates native iron loading pathways. Cells were transduced with the transgenes LacZ, H, H and L concurrently (H+L), and L*H ferritin. All error bars represent standard deviation from the mean. Representative blots are shown on the bottom of each graph. All lanes are in the same order as the bars on the graphs. (A) IRP2 levels were increased in all ferritins compared to LacZ control (ANOVA, $n=4$). The L*H ferritin expressing cells had significantly higher IRP2 levels than all other groups (Turkey test, $n=4$ and t-test). (B) All ferritin expressing cells had increased transferrin receptor levels (TfR1) (ANOVA, $n=4$). (C) Enzyme-linked immunosorbent assay confirms significant increase of TfR1. (D) DMT1 levels were increased in cells expressing the L*H reporter ($P=0.036$, $n=3$). The difference between control and all other ferritins was not significant (NS). (E) DMT1 with iron responsive element (DMT1+IRE) had significantly higher levels in all ferritin expressing cells (ANOVA, $n=3$). (F) Iron reductase STEAP3 had significantly elevated levels in L*H ferritin expressing cells (t-test, $n=3$).

Fig. 3.

Expression of the L*H ferritin reporter in striatal astrocytes generates robust MRI contrast. (A) *In vivo* T₂*-weighted image 5 days after injection shows contrast at the site of L*H expression (arrow). (B) Same mouse *ex vivo* with arrow at the L*H contrast region. Asterisk indicates the control LacZ side. (C) Confocal image of the injection site. The FLAG tag on the L*H ferritin (red) colocalizes with (green), an astrocytic marker. (D) Confocal image shows a lack of colocalization between FLAG (red) and neurofilament (NF, green), a neuronal marker. Cell nuclei (blue) are stained with Hoechst dye. Scale bar = 20 μ m.

Fig. 4. Histochemistry of mouse brains expressing the L*H reporter. (A) Hematoxylin and eosin stain at the injection site of the L*H reporter. The site of the needle track and transgene expression shows no apparent cytotoxicity. (B) Perls' iron stain demonstrating iron accumulation at the site of the MRI contrast. (C) X-gal stain verifying the expression of LacZ control on the contralateral side. Inserts show high magnification views of the regions of interest.

Fig. 5.

MRI of L*H reporter visualizes native neuroblast migration. (A) Axial projection from several contiguous slices from the T_2^* -weighted *in vivo* dataset acquired at 60 μm isotropic resolution 10 days after adenoviral injection in the SVZ. Asterisks indicate the injection sites. The arrow points to a hypointense trail of migrating neuroblasts along the RMS. There is no similar feature on the contralateral GFP control side. (B) Sagittal projection from another mouse imaged *in vivo* with the same parameters shows the injection site (asterisk). An arrow shows a hypointense trail of cells in the RMS region. (C) Axial projection over the RMS region of an *ex vivo* 3D T_2^* -weighted image acquired with 50 μm isotropic voxels. (D) Sagittal 3D projection of another mouse imaged *ex vivo* with the same parameters. The asterisk is the injection site, where the arrow shows a neuroblast stream entering the olfactory bulb.

Fig. 6.

Progenitor cells and migrating neuroblasts express the L*H reporter. (A) Confocal images of the SVZ of mouse injected with the L*H reporter. Several cells stained for FLAG (red) are also positive for the mitotic marker Ki67 (green). Scale bar = 20 μ m. (B) Cells in the RMS double-stain for FLAG (red) and PSA-NCAM (green) indicating migration. (C) Doublecortin (DX, green) positive cells in the olfactory bulb also stain for FLAG (red). Cell nuclei (blue) are stained with Hoechst dye.

Fig. 7.

Cells in brain regions of L*H transduction showing MRI contrast also display iron accumulation. (A) Perls' iron stain of brain sections shows iron rich (blue) cells. High magnification views are shown on the panels to the right. Blue cells are also apparent along the white matter tracks (top), proximal to the olfactory bulb (middle), and in the RMS region (bottom). (B) The contralateral side of the ferritin reporter expresses the control reporter GFP. The large panel shows brain section abundant with green cells around the lateral ventricle (LV) and in the striatum. Scale bar = 100 μ m. The panel on the top right shows a confocal image of the SVZ area. The panel on the bottom right shows a confocal image of the RMS distant from the injection site containing green cells in migratory chains. Scale bar = 50 μ m. Cell nuclei are stained with Hoechst blue.