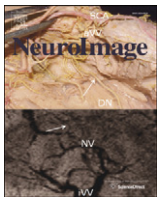
NeuroImage 59 (2012) 1004–1012

Contents lists available at SciVerse ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/ynimg



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

Bistra Iordanova, Eric T. Ahrens *

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA
Pittsburgh NMR Center for Biomedical Research, Pittsburgh, PA, USA

ARTICLE INFO

Article history:
Received 23 June 2011
Revised 17 August 2011
Accepted 21 August 2011
Available online 13 September 2011

Keywords:
Magnetic resonance imaging
Reporter gene
Ferritin
Neuroblast migration
Adult neurogenesis

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 toward 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.

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).

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 toward 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 (Lledo et al., 2006; Ming and Song, 2005). 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; Panizzo et al., 2009; Ruiz-Cabello et al., 2008; Zhang et al., 2003). 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 (Nieman et al., 2010; Panizzo et al.,

* Corresponding author at: Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA. Fax: +1 412 268 7083.
E-mail address: eta@cmu.edu (E.T. Ahrens).

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doi:10.1016/j.neuroimage.2011.08.068

2009; Shapiro et al., 2006; Sumner et al., 2009). 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; Cohen et al., 2007; Deans et al., 2006; Genove et al., 2005) 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 subunits 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 double-cortin (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 metalloreductase 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 μL 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.