

Molecular Imaging with Cell Tracking in the CNS

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Molecular and cellular MR imaging is a rapidly growing field aiming to visualization of macromolecules and cells in living organisms (1). These techniques allow *in vivo* monitoring of cell transplantation, transduction and migration, and until now they have been employed mainly by researchers for evaluation of cell trafficking, correction mechanisms and therapeutic efficacy in different animal models of human diseases. However, once novel therapeutic approaches, such as cellular and gene therapies, demonstrate to be useful and safe in pre-clinical models of Central Nervous System (CNS) disorders, molecular and cellular MR imaging might play an important role also in clinical practice, since accurate delivery and/or homing of cells to target organs is crucial for clinical success. Above all, the possibility to perform longitudinal studies on living subjects makes molecular and cellular MR imaging a helpful tool in both pre-clinical and clinical settings.

Neural Stem Cells (NSC) have recently raised interest because of their therapeutic potential in both cell-based and gene therapy applications. NSC are a heterogeneous population of immature progenitors, mitotically active, multipotent and self-renewing, present during development and in adult brain, both in primates and rodents (2-3). NSC can be cultured and grown *in vitro*, and, once transplanted *in vivo*, are able to generate a differentiated progeny capable of integrating and repairing the damaged neural tissue. This approach have been successfully employed in different CNS pathologies. In order to reconstitute the neural population in the *substantia nigra* that is progressively lost in Parkinson disease, NCS transplantation have been attempted in both animals and humans (4). NSC transplantation has been demonstrated to be useful also in animal models in traumatic lesions of the spinal cord (5), and in ischemic and hemorrhagic brain lesions (6,7). Further, intravenous injection of NSC induces recovery in a murine model of multiple sclerosis (8). Recent evidences suggest that endogenous neurogenesis can be exploited in adult brain *in vivo* for therapeutic purposes. Endogenous NSC can be modified *in vivo*, and thus recruited to damaged brain areas, where their progeny can differentiate and integrate in the tissue, replacing damaged or lost neurons (9-10). To this regard, the possibility to use viral vectors, such as lentiviruses (LV), to obtain sustained and long-term expression of therapeutic or trophic factors in NSC and their progeny is of particular interest (11).

Knowing the location of either delivered or genetically modified NSC in cell-based or gene therapy approaches is of utmost importance to assess therapeutic efficacy and analyze mechanisms of correction and cell distribution, and may represent a crucial step toward clinical application in humans. Diverse imaging approaches are available to track NSC, such as bioluminescence, fluorescence, and nuclear medicine approaches. Compared with these other techniques, MRI shows a greater resolution, allows direct anatomic correlation on the same image and can be performed and repeated several times on living

animals (1). Dynamic migration of transplanted or transduced stem cells and, alternatively, of gene expression can thus be studied during clinically useful time periods.

In order to make cells detectable at MR examinations, different strategies have been developed. The underlying rationale is to induce different signal intensities between labeled cells and not-targeted tissues. Gadolinium chelates and liposomes were the first to be employed for this purpose (12). However, nowadays gadolinium compounds are no more frequently used for cellular imaging applications since these agents exhibit low relaxivities that further decrease upon cellular internalization. Moreover, little is known about their potential toxicity following cellular dechelation over time (1). On the other hand, the development of Superparamagnetic Iron Oxides (SPIO) allowed a significant increase in the sensitivity of cellular and molecular MRI. These ferromagnetic agents are MRI negative contrast agents composed of a *core* of iron oxide nanoparticles, made of thousands of iron atoms, that create wide disturbances in the local magnetic field, leading to a rapid dephasing of protons and consequently to signal loss. Further, these contrast media are biodegradable along biochemical pathways for normal iron metabolism. The crystalline core is surrounded by a dextran or carboxi-dextran (13). Some SPIO agents, such as Endorem (Guebert) and Resovist (Shering) are already FDA-approved and widely used for liver MR imaging and for neuroimaging (14,15). Sinerem (Guebert) is on phase III-B, while other ultrasmall iron oxides particles such as CLIO (cross-linked iron oxide) and MION (monocrystalline iron oxide nanoparticles) are only for research purposes. All these SPIO agents differ for composition and diameter of the iron nanoparticle core. The demonstration that these SPIO can be internalized also by non-phagocytic cells made them the ideal MRI-sensitive tags for labeling mammalian cells (16). In order to be labeled, cells are to be grown *ex vivo* in a SPIO-containing culturing media. Cellular uptake can be further increased with different strategies, the easiest by mixing SPIO with transfection agents or protamine (17,18), that ameliorate the electrostatic interaction between iron particles and cellular membrane. Others and we tested labeling efficiency and toxicity of different commercially available SPIO contrast media (i.e. Endorem, Resovist and Sinerem) at different concentrations, on human and murine NSC, in the presence or absence of poly-L-lysine (PLL). After SPIO-labeling cell viability, differentiation and proliferation were completely preserved, while MRI relaxivity parameters (T2 and T2*) were significantly affected by the presence of these SPIO within cell bodies and showed linear decrease along with the cytoplasmatic iron content. In our and others experience, among different SPIO, Resovist turned out to be the most sensitive negative contrast media, even in the absence of transfection agents (19). Iron intra-cellular content may be semi-quantitatively evaluated with MR by calculating T2 and T2* maps. Precise quantification can be obtained using corrected T2* mapping technique, that balances the overestimation of the relaxation rate R_2^* due to large scale field inhomogeneities (20). These results indicate this method as suitable for tracking studies.

Several groups used this or similar efficient labeling strategies for *in vivo* magnetic tracking of different cell types in several animal models of CNS disorders. By magnetic labeling, it was possible to monitor non invasively the migration of intra-cisternally transplanted NSC into ischemic lesion in rodents with experimental stroke (21). Similarly, after intraventricular injection, labeled NSC migration into demyelinating lesions was monitored in a mouse model of multiple sclerosis. In this context, the rate of labeled NSC migration was assessed and correlated to the degree of inflammation (22-23). A critical issue is whether this labeling strategy may affect the functional properties of NSC. To this regard, it has been shown that olfactory ensheathing cells and oligodendrocyte precursors, once labeled, retain their therapeutic potential and are able to myelinate normally after transplantation (24-25).

It has been recently shown by our co-investigators that NSC, when administered intravenously, have an unexpected capability of crossing the blood brain barrier, and selectively reach the multiple sites of myelin damage within the injured CNS of Experimental Autoimmune Encephalomyelitis (EAE) mice. This finding was associated to an overt clinical benefit (8). In order to better dissect these events, we monitored CNS homing of intravenously transplanted, labeled NSC with MRI. Adult NSC were labeled with Resovist (0.2mg/ml) and then injected into the bloodstream of symptomatic Experimental EAE mice. Starting from day 1 after transplantation, labeled cells were detected in the brain, within previously MR documented demyelinating lesions. Cells were still detectable up to 15 days after transplantation. These findings were confirmed by histopathology. To directly assess the feasibility of this strategy in its future human applications, these experiments were performed using human MR scanners (1.5 and 3 Tesla), since magnetic susceptibility increases in high field animal dedicated bores. Detection of labeled cells in animal models by standard 1.5T scanners has also been reported in literature by other groups (26). Clinical application of cellular tracking with SPIO has been recently achieved. De Vries and co-workers monitored migration to lymphonodes of SPIO labeled dendritic cells in melanoma patients, thus providing the first evidence of safety and feasibility of this technique in humans (27). Overall, these results constitute proof of principle that MRI might allow tracking of labeled NSC *in vivo* in the human brain.

This labeling strategy for MR cellular imaging, which demonstrated to have great potential for clinical use, has however some major limitations that deserve discussion. As all other negative MR contrast agents, SPIO determine a local area of signal loss. They create a “black hole” that prevents direct anatomical MR evaluation, and that can be easily mistaken for other susceptibility artifacts (1). Most importantly, progressive dilution of SPIO upon cell division occurs. The *ex vivo* endocytosed iron nanoparticles are progressively lost during cell division after transplantation, and labeled cells become no more detectable after three or four mitosis. Thus, despite the great efficacy demonstrated for short term monitoring, SPIO cannot be used for studying the long-term fate of highly proliferating cells such as stem cells. MR markers for stable, robust and long-lasting cell labeling, capable to persist along with cell division, are still to be developed.

We are addressing this crucial issue with an alternative approach, based on LV and MR reporter genes, allowing accumulation of magnetic material, such as melanin or iron, within cells. With this approach we might also non-invasively monitor gene expression *in vivo*. LV are powerful tools for gene transfer and therapy. These vectors are capable of transducing dividing and non-dividing cells with high efficiency. They integrate in the genomic DNA of target cells and allow long-term expression of a reporter or therapeutic gene. Most importantly, the cDNA that have been inserted in the host genome is duplicated at the time of mitosis together with cellular chromosomes and transmitted to the progeny of transduced cells, thus enabling long-term persistence of gene expression along with cell division. Candidate MR reporter genes, encoding proteins detectable with MRI, comprise tyrosinase and intracellular ferritins. The former is an enzyme that catalyzes two fundamental reactions during melanin synthesis. Tyrosinase over-expression might induce melanin accumulation in non melanotic cells. Melanin itself has a high iron-binding capacity that is responsible for intensified MR signals (28). Intracellular ferritins, which have an extraordinary high superparamagnetism within the crystalline ferrihydrite core, can be used as MR reporter since they enable intracellular iron accumulation. The first direct proof of MR *in vivo* visualization of gene expression came recently (29). Genove and co-workers expressed H and L ferritin by adenoviral vectors in striatal neurons upon direct intraparenchymal injection of the vector, and made these cells detectable by MR. However,

since the vector used does not integrate in the cellular genome, and can be lost as SPIO along with cell division, it cannot be used for long-term tracking, particularly of highly proliferating stem cells. To overcome this limitation, we constructed LV carrying these candidate MR reporter genes and tested them *in vitro* on neural and hematopoietic stem cells. Moreover, a bi-directional LV (30), allowing simultaneous expression of two transgenes within the same cell, was constructed, encoding tyrosinase and green fluorescent protein (GFP), the latter as conventional reporter gene. After transduction and several cell divisions, cells remained detectable in T2 and T2* weighted images. No toxicity was documented, and stem cells proliferating and differentiating capacities were preserved. Further, the mono and bi-cistronic, tyrosinase-encoding LV were tested *in vivo* in the CNS. After direct vector injection into the striatum of wild type mice, a distinct MR signal was detected at the injection site, thus validating tyrosinase as MR reporter *in vivo*. These data suggest that tyrosinase encoding LV might represent potential new tools for tracking either transplanted or *in vivo* transduced NSC in normal or pathologic conditions. Moreover, if associated to a therapeutic gene in the bi-directional LV, our MR reporter could allow monitoring of therapeutic gene expression in disease models.

In conclusion, as soon as cellular and gene therapies will become clinically available for CNS disorders, SPIO and, in the long run, MR reporter genes will constitute fundamental tools for *in vivo* monitoring of cell fate and gene expression.

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