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STEM CELL IMAGING: FROM BENCH TO BEDSIDE

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Abstract

Although cellular therapies hold great promise for the treatment of human disease, results from several initial clinical trials have not shown a level of efficacy required for their use as a first line therapy. Here we discuss how *in vivo* molecular imaging has helped identify barriers to clinical translation and potential strategies that may contribute to successful transplantation and improved outcomes, with a focus on cardiovascular and neurological diseases. We conclude with a perspective on the future role of molecular imaging in defining safety and efficacy for stem cell clinical implementation.

Introduction: Basics of Molecular Imaging

Stem cell therapies provide unique opportunities for treating diseases of the heart and brain, which have limited regenerative capacity. Because somatic stem cells from the heart and brain are rare and difficult to isolate, therapeutic approaches using adult stem cells and differentiated cells derived from pluripotent stem cells provide a promising alternative source for regenerating cardiac and brain tissue (Garbern and Lee, 2013; Yu et al., 2013). Successful implementation of cell therapies will require a better understanding of cell fate after transplantation, which can be achieved by the application of molecular imaging. Molecular imaging enables the longitudinal, non-invasive assessment of cellular behavior *in vivo* following cell transplantation (Massoud and Gambhir, 2003). Cell tracking can be performed by labeling cells with molecular probes that enter the cell by active/passive transport and are trapped intracellularly (e.g., direct labeling). Alternatively, cells can be labeled by overexpression of specific reporter genes that integrate into the cellular genome via viral or non-viral vectors (e.g., reporter gene labeling) (Figure 1). Once integrated,

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reporter genes are transcribed into messenger RNA and translated into proteins that interact with a molecular probe for signal generation. Although reporter gene imaging requires genomic manipulation and poses potential safety issues, it is the preferred labeling strategy because signal generation is dependent on cell viability. Signal generated from cells labeled by either technique can then be visualized using imaging systems such as fluorescence imaging (FLI), bioluminescence imaging (BLI), single photon emission computed tomography (SPECT), positron emission tomography (PET), or magnetic resonance imaging (MRI). The advantages and disadvantages of each imaging system are summarized in Table 1 and can be found in other detailed reviews (Chen and Wu, 2011; Nguyen et al., 2011).

Akin to the use of pharmacokinetics for drug development, the overall goal of molecular imaging in regenerative medicine is to enhance therapeutic efficacy and decrease toxicity. Results from preclinical and clinical studies thus far suggest that cell imaging can and should be incorporated into more studies of cell transplantation in animals and humans. Continued application of molecular imaging for regenerative cell therapies will be critical for its successful implementation. In this review, we will discuss how stem cell imaging has helped identify the hurdles currently limiting the clinical translation of regenerative cell therapies for cardiovascular and neurological diseases, how it can be applied to define strategies to overcome these obstacles, and how it can be incorporated in the clinical implementation of regenerative stem cell therapies.

Defining Hurdles to Clinical Translation: Findings from Preclinical and Clinical Studies

Small and large animal studies have shown that stem cell therapies are effective in treating cardiovascular (van der Spoel et al., 2011) and neurodegenerative disease (Antonic et al., 2013; Lees et al., 2012). Based on these promising results, investigators have launched several Phase I and II studies to evaluate the safety and efficacy of stem cell therapies for the treatment of ischemic heart disease (Bolli et al., 2011; Hare et al., 2012; Heldman et al., 2014; Perin et al., 2012; Traverse et al., 2011; Traverse et al., 2012), peripheral vascular disease (Poole et al., 2013), spinal cord injury (Mothe and Tator, 2012), multiple sclerosis (Uccelli et al., 2011), and stroke (Bang et al., 2005; Kondziolka et al., 2005; Lee et al., 2010). While safety has been clearly demonstrated, efficacy remains more elusive (Clifford et al., 2012; Fadini et al., 2010). Based on these results, one may conclude that cell therapy itself may be inadequate or that better results could be achieved with different cell types. It is also possible that we have yet to apply these novel therapies effectively. Indeed, findings from current trials underscore the need to better understand the fate of transplanted cells and their correlation with structural (i.e., infarct size, left ventricular volume at end diastole) and functional outcome (i.e. left ventricular ejection fraction, neurocognitive and motor function) (Bang et al., 2005; Bolli et al., 2011; Kondziolka et al., 2005; Makkar et al., 2012; Perin et al., 2012; Traverse et al., 2011; Traverse et al., 2012).

The efficacy of any cell therapy depends on the interaction of many different factors such as disease etiology, cell type, delivery route, cell retention/engraftment, activation of resident cells, or functional integration. In order to optimize cell therapies, we need to improve our

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understanding of how these factors interact. The inclusion of molecular imaging into preclinical and clinical trials (Table 1) has helped answer some of these important questions, with further discoveries to come. Stem cell imaging studies, for example, have identified the following barriers, among others, to clinical translation in either adult or pluripotent stem cells: 1) limited cell engraftment, survival and proliferation; 2) poor cell differentiation and maturation; 3) immunogenicity with allogeneic transplantation; and 4) potential tumorigenicity with pluripotent stem cell derivatives (Figure 2).

Limited Cell Engraftment, Survival and Proliferation

To achieve their maximum clinical benefit, stem cells and their derivatives must engraft, survive, and integrate into the target transplantation tissue. Because the local microenvironment into which cells are delivered will have a substantial impact on retention, survival, and function of these cells, determining the optimal site and timing of delivery is critical. For example, delivering stem cell-derived cardiomyocytes into the normal myocardium next to infarcted tissue might improve their survival, but if these cells are expected to replace dead cardiomyocytes, they will have to migrate into the infarcted tissue. Given that neither situation is ideal, achieving high engraftment and survival rates after transplantation continues to be challenging.

A key factor in determining initial engraftment and retention is the mode of cell delivery. In animal studies of cardiovascular disease using molecular imaging (Huang et al., 2010; Kraitchman et al., 2005; Templin et al., 2012), less than 5% of transplanted stem cells and their derivatives engrafted when delivered intravenously, regardless of cell type (i.e., mesenchymal stem cells (MSCs), induced pluripotent stem derived-cardiomyocytes (iPSC-CMs), embryonic stem cell derived-endothelial cells (ESC-ECs)) and the number of cells implanted. In a direct head-to-head study comparing cell delivery methods, myocardial retention was highest when peripheral blood mononuclear cells (PBMNCs) were injected intramyocardially compared to intracoronary or intravenous delivery (11±3% vs. 2.6±0.1% vs. 3.2±1%) (Hou et al., 2005). Regardless of the delivery route, however, the majority of stem cells and their derivatives (i.e., MSCs and ESC-ECs) were found in the pulmonary vasculature, spleen, liver, and microvasculature (Gyöngösii et al., 2010; Huang et al., 2010; Toma et al., 2009), but not near the area of injury. Because cell size may potentially contribute to significant egress to extra-cardiac tissues, delivering cells as part of a tissue scaffold or graft may improve myocardial retention (Kraehenbuehl et al., 2011; Seif-Naraghi et al., 2013), but has not yet been tested in cell imaging studies.

Some cells, however, do home to areas of injured myocardium, as shown in pre-clinical cell tracking studies using BLI and SPECT discussed below. Sheik et al. demonstrated greater homing of bone marrow mononuclear cells (BMMNCs) expressing firefly luciferase (FLuc) in murine hearts with ischemia/reperfusion compared to sham hearts within one week of delivery (Sheikh et al., 2007). Their findings were confirmed by histology and real-time PCR (for the male *SryY* gene from donor cells). Similarly, Kraitchman et al. showed a diffuse uptake of indium oxine (^{111}In-oxine) radiolabelled MSCs at 4 to 7 days post-transplantation in infarcted canine hearts (Kraitchman et al., 2005). In a murine model of hindlimb ischemia, Huang et al. also showed that ESC-ECs injected into the femoral vein

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initially lodged in the pulmonary circulation, but eventually homed in on the ischemic limb (Huang et al., 2010).

More promising results have been found in models of neurovascular disease. In a study directly comparing intra-arterial vs. intravenous injection of neural stem cells (NSCs) in a murine model of hypoxia using BLI, Pendharker et al. found 61% of NSCs were retained in the brain immediately after intra-arterial injection, which declined only slightly to 55% by 7 days (Pendharkar et al., 2010). The BLI signal in the brain was ~12 times higher after intra-arterial injection compared to intravenous injection, where the predominant signal was in the torso. Similar to findings from models of myocardial infarction as discussed previously, homing to the area of injury has been noted in models of cerebral injury. Investigators have tracked ESCs labeled with superparamagnetic iron oxide nanoparticles (SPIONs) and neuroprogenitor cells transfected with FLuc as they move from their injection site in the contralateral hemisphere, across the corpus callosum, and to the injured hemisphere using BLI and MRI, respectively (Hoehn et al., 2002; Kim et al., 2004). Importantly, cells injected into sham mice did not show any evidence of migration.

Reminiscent of results from preclinical studies discussed previously, cell-tracking studies in humans demonstrate low cell retention rates, regardless of delivery method. SPECT and PET are by far the most frequently applied techniques in clinical bio-distribution studies. The majority of studies have been performed in patients for the treatment of myocardial infarction (Hofmann et al., 2005; Kang et al., 2005; Karpov et al., 2005). In one of the initial cell tracking studies, Karpov et al. reported that only 6.8% of autologous BMMNCs labeled with ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) were retained in the heart at 2.5 hours after intracoronary cell infusion (Karpov et al., 2005). Hofmann et al. found higher retention rates with intracoronary delivery (2%) compared to coronary vein delivery (undetectable) at 1.5 hours after infusion of BMMCs (Hofmann et al., 2005). Interestingly, the subset of CD34$^+$ bone marrow cells had a significantly higher retention rate of almost 25%. A modest improvement in retention was also achieved by inflating a balloon to temporarily stop blood flow. Using this modified technique, retention of BMMNCs 24 hours after intracoronary artery or coronary vein delivery increased slightly to 10.3% and 3.1%, respectively (Silva et al., 2009). A more recent study demonstrated that intramyocardial injection of CD34$^+$ cells labeled with ^{99m}Tc-hexamethylpropylene amine oxime (^{99m}Tc-HMPAO) could significantly improve cell retention to 19% at 18 hours after cell delivery (Vrtovec et al., 2013). Unfortunately, the short half-life of radioactive tracers used in the aforementioned studies (i.e., 1.83 hours for ^{18}F-FDG, 6 hours for ^{99m}Tc-HMPAO) did not allow a longer duration of tracking. Using radioactive ^{111}In-oxine, which has a longer half-life of 2.8 days, Schächinger et al. found the number of retained cells dropped from 6.9% at 1 hour to 2% after 3-4 days (Schachinger et al., 2008).

In addition to initial cell engraftment, cell survival may be necessary to achieve maximal therapeutic benefit. Unfortunately, cell tracking studies using BLI in small animal models of myocardial infarction have demonstrated poor survival after transplantation of MSCs (van der Bogt et al., 2008), skeletal myoblasts (van der Bogt et al., 2008), BMMNCs (van der Bogt et al., 2008), adipose stromal cells (van der Bogt et al., 2008), cardiac resident stem cells (Li et al., 2009), ESC-ECs (Li et al., 2008; Li et al., 2007), and ESC-CMs (Cao et al.,

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2008). Similarly, in a large animal model of myocardial infarction, Gyöngösii et al. failed to detect the presence of porcine MSCs transfected with a PET reporter gene at 7 days after allotransplantation (Gyöngösii et al., 2008). Likewise, in murine models of peripheral vascular disease using BLI, the majority of MSCs (Hoffmann et al., 2010), ESC-ECs (Huang et al., 2010), and iPSC-ECs died shortly after transplantation (Rufaihah et al., 2011).

Interestingly, better long-term survival has been achieved in small animal models of brain injury using human NSCs (Guzman et al., 2007), human ESC-derived NSCs (Daadi et al., 2009), and bone marrow-derived MSCs (Sykova and Jendelova, 2007). In a MRI tracking study, Guzman et al. showed that an average of 51.3% (range: 38.9-74.6%) of human NSCs survived at 5 weeks after stereotactic injection in a rat model of distal middle cerebral artery occlusion, which was verified by histological staining for the human specific marker SC121 (Guzman et al., 2007). However, survival may be overestimated because of the engulfment of dead cells containing SPIONs by macrophages, leading to false-positive signal generation (Li et al., 2008; Terrovitis et al., 2008). To address this question, Daadi et al. labeled human NSCs with both SPIONs and the FLuc reporter gene and demonstrated stable SPIO and BLI signal up to 2 months post-transplantation (Daadi et al., 2009). Importantly, the *in vitro* cell dose-BLI relationship was maintained *in vivo* and correlated well with the SPIO dose relationship, suggesting that MRI of SPIONs might be a suitable method for cell tracking in stroke models. Taken together, these findings suggest that the environmental milieu of the central nervous system (CNS) may be less hostile to stem cell engraftment and survival than the cardiovascular system.

Not only does the host environment change depending on the organ transplanted, but it may also vary based on the time after injury. Shortly after myocardial infarction, for example, the host environment may be hostile to cell viability because of the presence of inflammatory cells that are recruited to repair the injured myocardium. As scar tissue forms in the myocardium, the injured area may be devoid of vasculature, which may also impair cell survival. Interestingly, a preliminary study by Swijnenburg et al. showed that timing of delivery actually has little impact on the overall survival of BMMNCs and subsequent change in left ventricular ejection fraction (LVEF) in both acute (<2 hours) and subacute (7 days) models of myocardial infarction (Swijnenburg et al., 2010). Additional imaging studies are needed to determine whether delivery of cells at later times (e.g., 2 to 4 weeks post myocardial infarction when the inflammatory response has subsided) may indeed improve cell engraftment.

Interestingly, timing of delivery has also been shown to affect cell differentiation in stroke models. Rosenblum et al. used the reporter genes FLuc and green fluorescent protein (GFP) to assess *in vivo* survival and *ex vivo* phenotypic differentiation after intra-arterial delivery of NSCs, respectively. Improved survival was noted three days after transplantation when levels of vascular adhesion molecules that promote cell homing were highest (Rosenblum et al., 2012). Moreover, early transplantation (i.e., 6 and 24 hours) led to greater differentiation into astrocytes than neurons, which were more predominant with later (7 and 14 days) transplantation times. Transplantation at 3 days post injury resulted in a more even distribution of astrocytes and immature neurons.

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Long-term cell tracking in humans presents a greater challenge. The use of radioactive tracers with short half-lives that are more commonly used in clinical stem cell imaging studies is not suitable to assess cell migration due to a short tracking period and limited resolution. The persistence of SPIONs in labeled cells together with the high resolution and superior soft tissue contrast makes long-term clinical cell tracking with MRI feasible. De Vries et al. showed that SPIO-labeled dendritic cells injected into lymph nodes could be readily detected, in addition to their migration to draining lymph nodes two days after injection (de Vries et al., 2005). By co-labeling these cells with ^{111}In-oxine for visualization by SPECT, the investigators also demonstrated that high spatial resolution for MRI is critical for successful transplantation. Because of its lower spatial resolution, SPECT did not accurately locate the lymph nodes in three patients, resulting in injection of dendritic cells into the surrounding fat. Moreover, SPECT provided limited visualization of migration to several draining lymph nodes, which were resolved by MRI. In a second study, Callera et al. used SPIO-labeled CD34$^+$ cells injected into the spine of patients with spinal cord injury to assess their migratory capacity (Callera and de Melo, 2007). MRI at 20 and 35 days after cell delivery was able to detect injected cells and showed their migration towards the side of injury. MRI-based detection of cell migration has also been performed for NSCs injected into patients with brain trauma for up to 3 weeks after cell injection (Zhu et al., 2006). These and other examples demonstrate the ability of MRI for cell tracking (see Table 2).

The advantages of MRI are high resolution, good contrast, and label persistence. However, it is difficult to estimate biodistribution, initial cell retention, or cell numbers with MRI. In addition, the persistence of SPIONs can also be a disadvantage because resident macrophages can take up the particles after delivered cells have died, leading to false positive signals for cell retention or survival (Li et al., 2008; Terrovitis et al., 2008). These potential confounding factors need to be considered when interpreting cell migration of magnetically labeled cells with MRI, particularly for long-term cell tracking. Interestingly, the ability of macrophages to take up free SPIONs can be used to label them in the human body and track their migration to sites of inflammation (Yilmaz et al., 2013). Combining radioactive labeling or reporter gene imaging with MRI tracking might be the best solution if initial cell retention and spatial localization have to be determined.

Although reporter gene imaging has become very widespread in preclinical studies as discussed previously, only one study has shown a proof-of-principle application for cytolytic T-cell therapy in humans (Yaghoubi et al., 2009). Viability of genetically modified T-cells expressing herpes simplex virus type 1 thymidine kinase (HSV1-tk) could be assessed a few days after cell delivery. This approach could track transplanted cells in the human body as long as they remain alive and would also be suitable to assess proliferative capacity of transplanted cells. Reporter gene imaging, however, requires genetic modification of transplanted cells, which increases the regulatory complexity for their approval and poses additional risks for mutagenesis. Furthermore, the repeated use of radioactive tracers would expose the patient to higher radiation doses. Nevertheless, the potential value of the information that could be gained and improved methods for genetic transformation may justify the use of this technique in the future.

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Whether cellular proliferation after implantation can compensate for low levels of retention and survival remains unclear. Signal increase is frequently inferred as proliferation and often occurs early after transplantation of adult progenitor or differentiated cells prior to signal decline as cells die. Progressive signal increase has been reported after transplantation of undifferentiated ESCs and iPSCs (Cao et al., 2009; Cao et al., 2007b; Gutierrez-Aranda et al., 2010; Lee et al., 2009), corresponding to the development of a teratoma. Overreliance on this measure, however, may lead to the underestimation of true proliferation rates because replacement of dying cells is not accounted for. Direct imaging of cell proliferation requires the utilization of radiotracers involved in the thymidine salvage pathway of DNA synthesis (e.g., thymidine analogues), because thymidine contains the only pyridine or purine base that is unique to DNA. Direct imaging of *in vivo* stem cell proliferation has not yet been performed, but it has been reported in studies tracking tumor progression (Bading and Shields, 2008).

Poor Differentiation, Maturation, and Integration

It is hoped that once cells are delivered and engraftment takes place, they will differentiate, mature, and integrate into the target organ, resulting in tissue regeneration. However, *in vivo* differentiation of ESCs and iPSCs is hampered by the difficulty of directing differentiation towards the target cell type because the host tissue milieu may lack the complex array of signaling sequences required for directed differentiation and maturation (Cao et al., 2008), and hence resulting in the development of teratomas (Cao et al., 2009; Cao et al., 2007b; Gutierrez-Aranda et al., 2010; Lee et al., 2009). Due to the challenges of *in vivo* differentiation and the relative ease of *in vitro* differentiation where reagents can be added under controlled conditions, numerous protocols have emerged for *in vitro* cardiac (Burridge et al., 2012) and neural differentiation (Kim et al., 2009). Although robust differentiation to cardiac and neural cells is now feasible, generating specific cell subtypes can be more difficult (e.g., atrial vs. nodal vs. ventricular), highlighting the need for better understanding of the signaling pathways associated with cellular differentiation.

Most differentiated stem cell populations exhibit immature features, which may hamper their ability to treat disease. ESC-CMs and iPSC-CMs, for example, exhibit a neonatal form of gap junction distributions, which have slower conduction velocities (Chen et al., 2009). These gap junction connections are randomly distributed at the contact interfaces and do not align properly, making the action potentials across these interfaces less predictable and less homogeneous, which may lead to the development of arrhythmias after cell transplantation.

Because of the slow maturation of certain neuronal subtypes, generating mature functional neurons has also been challenging. Most interneuron precursors derived from human ESCs retain undifferentiated features and even those in more differentiated state still express neural precursor markers within 1 to 2 months after transplantation (Maroof et al., 2013). Development into functional GABAergic interneurons can take up to 7 months *in vitro* and after transplantation (Nicholas et al., 2013).

In addition to maturation, structural and functional (i.e., electrical and mechanical) integration within the target organ is needed for effective tissue regeneration. Preliminary studies based on *ex vivo* histopathological data indicate that transplanted stem cells do, at

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least partially, integrate into the heart and brain (Kehat et al., 2004; Shiba et al., 2012; Wernig et al., 2004). Kehat et al. demonstrated that transplanted human ESC-CMs successfully paced the hearts of swine with complete heart block (Kehat et al., 2004). Although electroanatomical mapping and subsequent histopathological analysis demonstrated that the focus of ventricular activation was the site of cell transplantation, it is possible that pacing resulted from the effects of the ESC-CMs on the host myocardium rather than the cells themselves. Further support for electrical-mechanical coupling was provided by Shiba et al, who showed that guinea pigs grafted with human ESC-CMs had improved mechanical function and lower incidence of arrhythmias (Shiba et al., 2012). By creating human ESC-CMs grafts that stably transfected a fluorescent calcium sensor, the authors showed that calcium fluorescent signal recorded in transplanted grafts synchronized 1:1 with systole (i.e., the phase of left ventricular contraction) on the electrocardiogram (Shiba et al., 2012).

Likewise, Wernig et al. showed that synapses form between the embryonic rat host and donor ESC-derived neuroprecursors in the brain, as supported by the expression of post-synaptic density 95 (PSD-95) of donor cell dendrites on *ex vivo* histopathological analysis and confirmed by ultra-structural and electrophysiological data (Wernig et al., 2004). However, not all of the transplanted neuronal cells expressed regionally appropriate transcription factors, which are important regulators of the brain's regional specific activity.

Currently, evaluation of stem cell differentiation, maturation, and integration relies primarily on *ex vivo* histological examination and, thus, has been limited to animal studies as discussed previously. *In vivo* monitoring will enable investigators to potentially direct these processes to improve efficacy. Investigators reported an approach to image differentiation using a promoter of a differentiation-specific gene to drive the expression of an established reporter gene, the sodium/iodide symporter (NIS). Kang et al. created a transgenic mouse carrying a reporter gene construct driven by an alpha myosin heavy chain (MHC) promoter in transgenic mice. When exposed to radioactive iodine, the myocardium of transgenic mice showed rapid, increased uptake that was abolished by oral administration of potassium perchlorate, a NIS inhibitor (Kang et al., 2005). Using an alternative strategy, Xie et al. monitored changes in the expression of signal transducers and activators of transcription 3 (STAT3) using a 7-repeat STAT3 reporter construct driving Renilla Luciferase (RLuc) during the *in vitro* differentiation of mouse ESCs (Xie et al., 2009).

Immunogenicity Associated with Allogeneic Transplantation

As in solid organ transplantations, the immune system remains a formidable barrier to the clinical implementation of stem cell therapy. Poor cell survival after stem cell implantation is partly due to cellular rejection by the immune system (de Almeida et al., 2013; Pearl et al., 2012). Even pluripotent stem cells, which were once considered potentially immunoprivileged due to their lack of major histocompatibility complex (MHC) Class I, MHC Class II, and costimulatory molecules as well as their expression of immune-modulating molecules (Abdullah et al., 2007; Koch et al., 2008; Magliocca et al., 2006), have been found to elicit a donor-specific immune response when transplanted into immune competent mice (Drukker et al., 2006; Swijnenburg et al., 2008a). Although the discovery of

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iPSCs offered a potential solution to immune rejection (Takahashi et al., 2007; Yu et al., 2007), Zhao et al. reported that murine iPSCs were also rejected after transplantation into syngeneic recipients (Zhao et al., 2011). However, two recent studies by Araki et al. and Guha et al. have disputed that claim; the authors showed that transplantation of tissue grafts derived from iPSC-derived mice (e.g., skin and bone marrow) and terminally differentiated iPSC-derivatives (e.g., endothelial cells, hepatocytes, and neuronal cells), respectively, may be less immunogenic, reigniting hope that iPSCs can still be used to circumvent the immune system (Araki et al., 2013; Guha et al., 2013). Both of the aforementioned studies relied on *ex vivo* histopathological analysis.

Improvement in survival can be achieved by transplantation into a more immune-tolerant tissue environment. Because the blood-brain barrier separates the CNS from the systemic immune system, the brain is considered more immunoprivileged than other sites of the body, enabling the long-term survival of stem cells as discussed previously (Daadi et al., 2009; Guzman et al., 2007). An alternative strategy is to transplant cells into a more immune tolerant recipient host, which has been shown to be successful in the following small animal cell tracking studies. Using BLI, Swijnenburg et al. found poor survival of cells transplanted into immunocompetent mice compared to immuodeficient mice, with marginal improvement in survival achieved by administration of traditional immunosuppressive regimens (i.e., tacrolimus, sirolimus, and myocophenolate mofetil) (Swijnenburg et al., 2008b). In a follow-up study using BLI, improved engraftment and survival of ESCs, iPSCs, and their differentiated derivatives was achieved by the administration of co-stimulatory receptor blocking agents such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4)-Ig, anti-CD40 ligand (anti-CD40L), and anti-lymphocyte function-associated antigen 1 (anti-LFA-1) (Huber et al., 2013; Pearl et al., 2011). More recently, a study by Rong et al. based on *ex vivo* histopathological data found that constitutive expression of CTL4-Ig and programmed death ligand-1 (PD-L1) in human ESCs were not rejected in humanized mice and showed reduced infiltration of human T-cells (Rong et al., 2014). Furthermore, infiltrating T-cells expressed high levels of immunosuppressive cytokines.

To avoid the potential side effects of immunosuppressants that can increase morbidity and mortality, studies using *ex vivo* histopathological analysis are currently evaluating encapsulation of MSCs into an artificial semi-permeable membrane as a means for evading the immune system for applications that do not require direct interaction of transplanted cells with the host tissue (Levit et al., 2013). In animal models of myocardial infarction, transplantation of encapsulated human MSCs has been shown to improve cell survival and efficacy, compared to implantation of non-encapsulated counterparts (Levit et al., 2013)

Direct monitoring of transplant cell rejection has not been evaluated, and is currently inferred indirectly by the progressive decline of labeled cells post transplantation. Christen et al. proposed a promising technique to directly image immune cells involved in transplant rejection, namely, macrophages, which release proteases and phagocytose dead or dying cells (Christen et al., 2009). Using a fluorescent protease sensor and a magnetofluorescent phagocytosis marker, the investigators created a 3D functional map showing higher phagocytosis activity during rejection on MRI and higher protease activity on tomographic FLI in mice receiving cardiac allografts than those receiving isografts. Strategies such as this

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will enhance the discovery of novel tolerance-inducing agents to improve survival of transplanted cells.

Potential Tumorigenicity Associated with Pluripotent Stem Cell Transplantation

The risk for tumor formation is low for adult somatic stem cells and progenitors, whose differentiation is largely limited to the different cell types of their tissue of origin. Given their better safety profile, somatic cells as well as progenitor cells are currently being evaluated in clinical trials. Nevertheless, *in vivo* cell tracking is still needed to monitor long-term safety after delivery of these cells, as demonstrated by a recent case report of a boy with ataxia telangiectasia who developed a glioneuronal brain tumor 4 years after intracerebellar and intrathecal injection of fetal neural stem cells (Amariglio et al., 2009).

Because they arise from pluripotent stem cells, ESC- and iPSC-derivatives may pose a greater risk for tumor development. These cells can malignantly transform, resulting in the formation of a teratoma, a tumor consisting of cells from all three germ layers. Alternatively, it is possible that a preparation of ESC or iPSC-derivatives could be contaminated with undifferentiated or incompletely differentiated cells that can proliferate and form a teratoma (Cao et al., 2007b; Gutierrez-Aranda et al., 2010; Lee et al., 2009).

In order to prevent tumor development, a pure cell product must be obtained prior to transplantation. Product purity can be achieved by depletion of undifferentiated cells (Ben-David et al., 2013a; Ben-David et al., 2013b; Tang et al., 2011) or enrichment of differentiated cells based on their expression of specific surface markers (Dubois et al., 2011), using either fluorescent activated cell sorting (FACS) or magnetic activated cell sorting (MACS), the latter being more amenable for high-throughput processing. Because 100% purity may be difficult to attain, it will be important to define a minimum threshold of undifferentiated cells that can be tolerated. Based on a previous study using BLI to track the growth of teratomas originating from ESCs transduced with a construct expressing FLuc and GFP in immunodeficient mice, it appears that a “pure” cell product cannot contain more than 10^4 undifferentiated cells, the threshold required to induce teratoma formation in the heart (Lee et al., 2009). Even lower thresholds may be required based on other cell tracking reports (Cao et al., 2007b).

The removal of potential tumorigenic cells, however, will not preclude the need for tumor surveillance by more traditional imaging techniques to identify abnormal growths (e.g., ultrasound, computed tomography, or MRI) or cellular activity (e.g., PET). Earlier detection may require the use of molecular imaging probes, such as the PET reporter probe ^{64}Cu-radiolabeled cyclic Arg-Gly-ASP (RGD) peptides to image expression of $\alpha_V\beta_3$ integrins, which play an important role in angiogenesis and metastasis. Cao et al. demonstrated the superiority of this technique compared to more standard ^{18}F-FDG and ^{18}F-Fluorothymidine (^{18}F-FLT) PET imaging for detecting teratoma formation following subcutaneous injection in mice (Cao et al., 2009). Alternatively, the PET reporter gene herpes simplex virus truncated thymidine kinase (HSV-ttk) can serve as both a monitor for tumor development and a suicide gene for ablative therapy (Cao et al., 2007a). Specifically, Cao et al. transplanted mice with ESCs labeled with either a double-fusion (GFP and FLuc) or a triple-fusion construct (GFP, RLuc, and HSV-ttk). BLI detected teratoma formation in both groups

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after 5 weeks, but only those teratomas expressing HSV-ttk were ablated by the administration of ganciclovir, an inhibitor of herpes viral DNA polymerase.

The Future of Stem Cell Imaging

Although significant insight has been gained from incorporating *in vivo* imaging in preclinical studies, its application in clinical trials remains underutilized and perhaps undervalued. Molecular imaging provides much needed “pharmacokinetic” information for the safe and effective administration of cellular therapies. For example, when examining a patient’s response to stem cell therapies in clinical trials, individual variation is significant (Alper, 2009; Bang et al., 2005; Bolli et al., 2011; Hare et al., 2012; Heldman et al., 2014; Kondziolka et al., 2005; Perin et al., 2012), with some patients in the therapy group achieving little or no improvement while some have significant functional recovery. Unfortunately, these pivotal trials did not incorporate cell tracking, leaving us with many unanswered questions. It is possible if not likely that individual patients might have different reactions to the therapy administered, and the transplanted cells did not engraft or survive in those that did not respond to therapy. As ESC- and iPSC-derivatives enter clinical trials (Garber, 2013), it will also be important to determine whether inadequate differentiation, maturation, and integration could also lead to poor functional outcome. Although strategies to monitor the latter processes are still in their infant stages and will need to be further explored in animal models, short-term tracking of engraftment and survival of cells directly labeled with imaging probes is currently feasible in humans and could be utilized in future stem cell trials (see Table 2).

At a minimum, molecular imaging should be incorporated into clinical phase II trials to generate a dose response curve to identify the optimal dose and dosing frequency. Depending on the efficacy readout, estimating dose response could be performed purely on the number of cells engrafted. In line with that, both preclinical and clinical studies using molecular imaging have found a considerable variability in the degree of cell retention, which was not correlated with the number of administered cells (Barbosa da Fonseca et al., 2011; Correa et al., 2007; Dedobbeleer et al., 2009; Schachinger et al., 2008). This makes dose response estimates more difficult because of the need for substantial group sizes. Despite the small number of patients enrolled, two recent clinical studies were able to find a positive correlation between early cell retention by cell imaging and late improvement in cardiac function six months after cell delivery (Silva et al., 2009; Vrtovec et al., 2013).

Molecular imaging can play a more extensive role by evaluating strategies to improve the viable cell concentration at the target site. As demonstrated in a growing number of preclinical studies but only a handful of clinical studies, cell imaging can help define the optimal cell type, delivery method (Hofmann et al., 2005; Vrtovec et al., 2013), timing of delivery (Rosenblum et al., 2012; Swijnenburg et al., 2010), and host microenvironment (Pearl et al., 2011; Swijnenburg et al., 2008b). Large-scale clinical studies incorporating molecular imaging are needed to determine which strategies will prove to be the most efficacious.

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The other important role that cell imaging provides is to decrease toxicity to patients. The optimal cell dose must not only lead to maximal benefit, but it will also result in minimal risk of tumor formation, which is likely specific to the cell subtype and the injection site (Lee et al., 2009), and perhaps even specific to the patient. Molecular imaging can identify early transformation of cell grafts into tumors by imaging the proliferation and/or expression of tumor specific markers (Cao et al., 2009), which cannot be detected by traditional imaging techniques.

Further optimization of cellular therapy will require implementation of reporter gene imaging in humans, which will enable long-term assessment of survival and proliferation as well as differentiation, maturation, and integration. Adequate tumor surveillance will also require long-term imaging. Although the use of reporter gene imaging for long-term cell tracking in humans is currently restricted by regulatory hurdles, reservations about safety may be addressed by the use of human endogenous versions of reporter genes (e.g., human ferritin reporter for MRI (Campan et al., 2011) and human mitochondrial thymidine-kinase-2 for PET (Ponomarev et al., 2007). To avoid the problem of insertional mutagenesis, safety can be further enhanced by applying newer methods for genomic manipulation such as site-specific integration using phage integrases (Karow et al., 2011; Lan et al., 2012) and safe harbor mediated integration by zinc-finger nuclease (ZFN) (Ochiai et al., 2012; Wang et al., 2012), transcription activator-like effector nuclease (TALEN) (Sommer et al., 2014), or clustered regularly interspaced short palindromic repeats (CRISPR) (Yang et al., 2013).

In the future, we predict that molecular imaging will be performed regularly throughout the course of treatment. From the time of delivery, patients will be monitored for safety and efficacy to identify patients at risk for tumor formation or those that may benefit from repeat transplantation, respectively (Figure 3). PET/MRI will likely emerge as the imaging modality of choice, given that it provides exquisite functional and anatomical detail with minimal radiation exposure. Although molecular imaging in humans is restricted to short-term monitoring of engraftment and survival, investigators are encouraged to use the tools at hand to guide the clinical translation of stem cell therapy. With rapid advancements in imaging technology, visualization of such intricate processes as stem cell differentiation and integration will become feasible as well as longer-term tracking, which will enable tracking cell fate beyond engraftment and survival. Finally, it is vital that we conduct more studies to evaluate the relationship between cell fate and functional improvement to confirm the efficacy of cell transplantation.

Summary

Although significant progress has been made in the field of regenerative medicine, stem cell therapies are still in their infancy. Preclinical studies have helped identifying barriers to clinical translation, but additional studies must be performed to define strategies to overcome these hurdles. Disappointing results from clinical trials thus far suggest that we should perhaps reconsider fundamental strategies while continuing to broaden the application of stem cell imaging for future clinical trials. This could be achieved by refining and expanding the available technologies to enable long-term tracking of cell fate.

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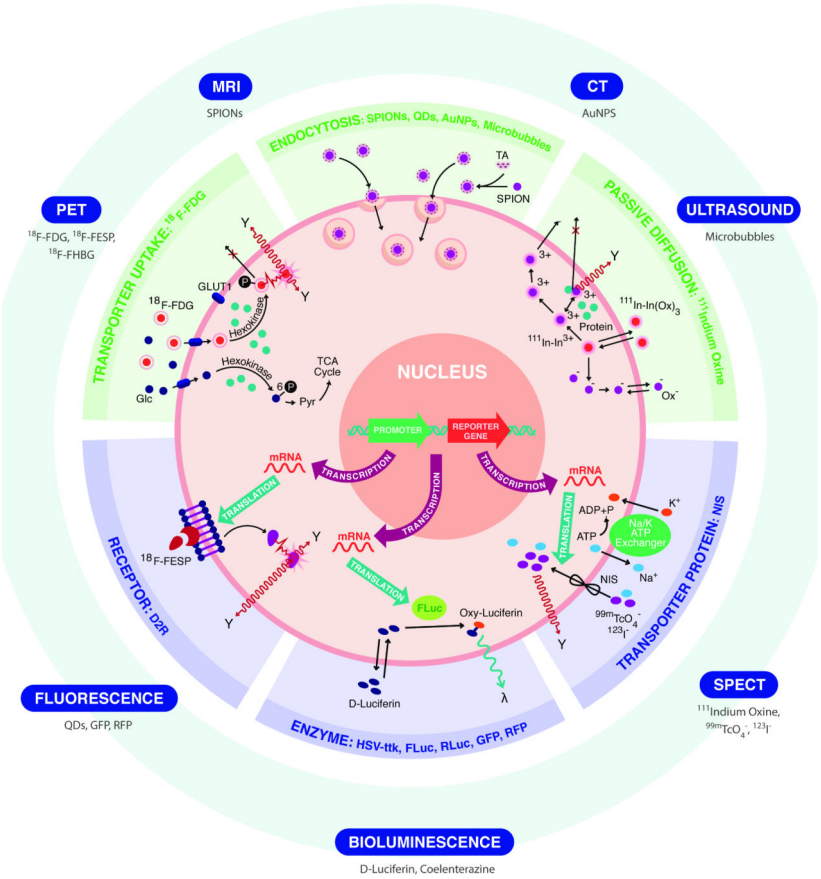

Figure 1.

Cell labeling strategies and detectors for stem cell imaging. For direct labeling (in green), cells are incubated with imaging probes that enter the cell via transporter uptake (i.e., ^{18}F FDG, ^{18}F-FESP, and ^{18}F-FHBG), endocytosis (i.e., SPIONs, QDs, Au NPS, and microbubbles), or passive diffusion (i.e., ^{111}In-ox). In reporter gene imaging (in blue), cells are transfected or transduced with the reporter gene construct. Transcription of the reporter gene under the control of a promoter followed by translation of its mRNA, leads to accumulation of different reporter proteins such as receptors (i.e., D2R), enzymes (HSVtk, FLuc, RLuc, GFP, and RFP), and transporter proteins (NIS). Introduction of a reporter gene probe (i.e., ^{18}F-FESP, D-luciferin, coelenterazine) results in signal generation. Labeled cells are detected by imaging systems such as PET, MRI, CT, and ultrasound. Abbreviations: ^{18}F-FDG, ^{18}F-fluorodeoxyglucose; ^{18}F-FESP, 3-(2′-[18F]-fluoroethyl)-spiperone; ^{18}F-FHBG, 9-(4-18Ffluoro-3-[hydroxymethyl]butyl) guanine; SPIONs, superparamagnetic iron oxide nanoparticles; QDs, quantum dots; Au NSPs, gold nanoparticles; ^{111}In-ox, indium oxine; Asp, aspartic acid; Ser, serine; NIS, sodium iodide symporter; I, iodine; ^{99m}TcO $^{-}_{4}$, technetium pertechnetate; D2R, dopamine 2 receptor; HSV-ttk, herpes simplex virus truncated thymidine kinase; FLuc, firefly luciferase; RLuc, renilla luciferase; GFP, green fluorescent protein; RFP, red fluorescent protein); PET, positron emission tomography; MRI, magnetic resonance imaging; CT, computed tomography; SPECT, single photon emission computed tomography; GLUT1, glucose transport type I.

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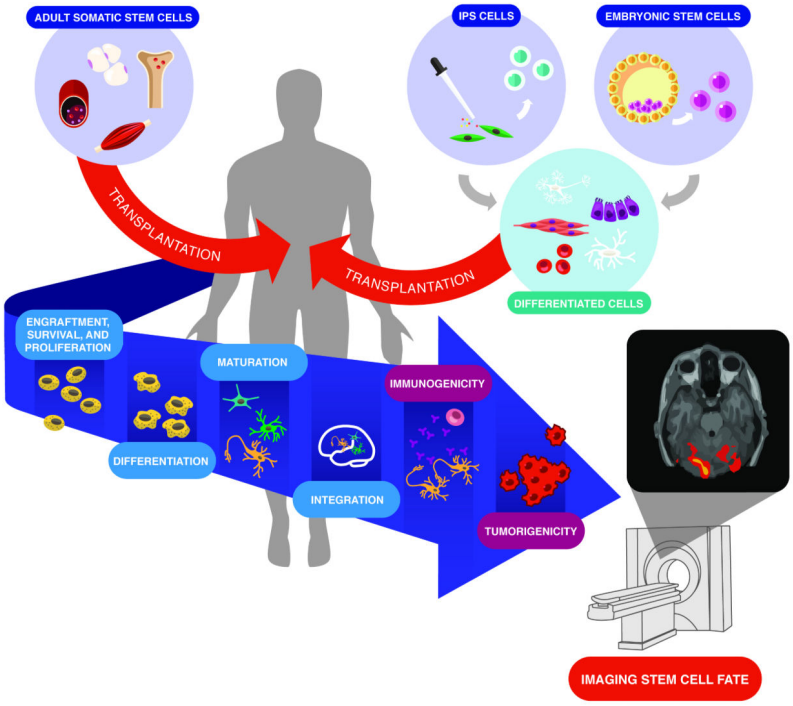

Figure 2.

Barriers to clinical translation. A variety of stem cell types (e.g., adult somatic cells, induced pluripotent stem cells, and embryonic stem cells) are available for transplantation, each with their advantages and disadvantages. Adult somatic stem cells are most commonly transplanted directly into the recipient, whereas embryonic and induced pluripotent stem cells undergo *in vitro* differentiation prior to transplantation. Regardless of the stem cell type, their successful application in regenerative therapy faces similar clinical hurdles, including: 1) limited engraftment, survival, and proliferation; 2) poor differentiation, maturation and integration; 3) immunogenicity with allogeneic transplantation; and 4) potential tumorigenicity with pluripotent stem cell derivatives. Cell imaging plays a pivotal role in overcoming these hurdles and will help guide the translation of this promising therapy.

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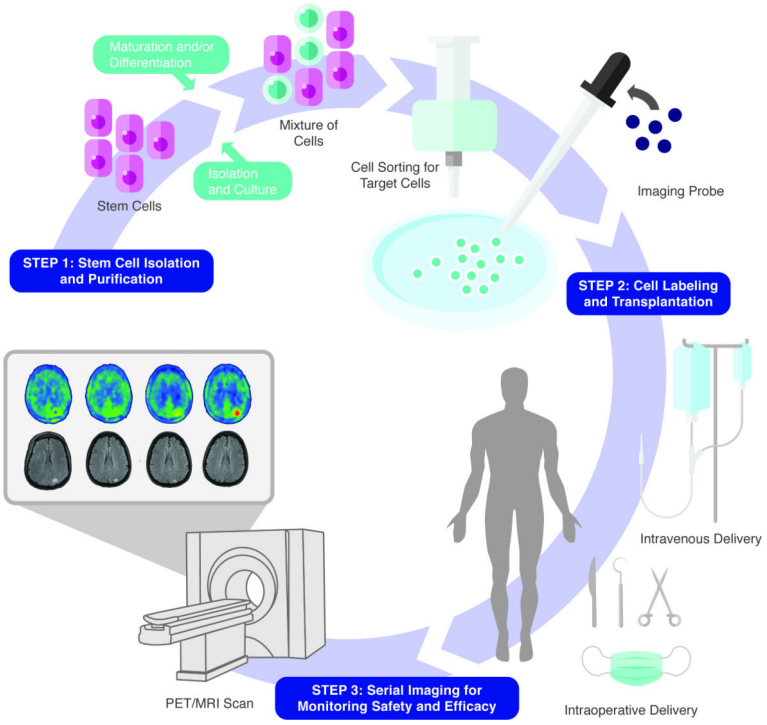

Figure 3.

A step-by-step approach to successful routine application of stem cell therapy. Step 1: Stem cell isolation and purification. Stem cells must be initially isolated and purified from a tissue source. Because of poor *in vivo* differentiation, cells are often differentiated *in vitro* prior to implantation. These techniques, however, are imperfect and can result in a mixture of undifferentiated and differentiated cells. Cell sorting is then required to select for the cell population that can be safely transplanted with maximal therapeutic benefit. Step 2: Cell labeling and transplantation. Prior to transplantation, cells are labeled to track cell fate. Selected cells are labeled *in vitro* using direct labeling or reporter gene techniques and then implanted at the bedside via intravenous delivery or in the operating room/angiography suite, via intra-lesional or intra-arterial delivery, respectively. Step 3: Serial imaging for safety and efficacy monitoring. After delivery, labeled cells are tracked using PET/MRI, which provides high sensitivity for cell detection as well as excellent spatial localization. Cells can be tracked serially to determine whether they engraft, survive, and proliferate in the target organ. Cells can also be monitored for any potential unwanted effects including tumor formation and migration to other sites. In addition, PET/MRI can provide serial data on the functional and structural recovery of damaged tissues.

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Table 1

Comparison of imaging techniques for cell therapies

Imaging modality	Spatial resolution	Acquisition time	Detection limit: cells	Labeling strategy	Advantages	Disadvantages
Bioluminescence imaging (BLI) (preclinical only)	5-20 mm depending on depth of signal	seconds	~ 10^3	Reporter gene	Cheap, simple, high throughput	Small animals only, low resolution, only 2D images
Fluorescence tomography (FMT) (preclinical only)	2-3 mm	seconds to minutes	~ 10^6	Reporter gene, fluorescent dye	Cheap, simple	Low resolution, cells need to be close to surface
Ultrasound (US) (preclinical and clinical)	150 μm − 2 mm depending on depth	seconds to minutes	not well characterized	Reporter gene, antibody with microbubble	Cheap, relatively simple	Limited 3D capabilities, low signal to noise ratio
Single photon emission computed tomography (SPECT) (preclinical and **clinical)**	1-2 mm *8-12 mm*	minutes	~ 10^5	Reporter gene, incubation with radiotracer	3D imaging	Anatomic reference required, radioactive tracer required
Positron emission tomography (PET) (preclinical and **clinical)**	~1 mm **4-6 mm**	seconds to minutes	~ 10^4	Reporter gene, incubation with radiotracer	3D imaging, high sensitivity	Anatomic reference required, radioactive tracer required
Magnetic resonance tomography (MRI) (preclinical and **clinical**)	25-500 μm 0.5-5 mm	minutes to hours	~ 10^4	Internalization or surface labeling with nanoparticles or specific ions	3D imaging, good soft tissue contrast, no radiation, high resolution	Very expensive, complicated
Computed tomography (CT) (preclinical and **clinical)**	< 50 μm <**1 mm**	seconds to minutes	not well characterized	Internalization or surface labeling with nanoparticles	3D imaging, relatively cheap, high resolutio	Uses ionizing radiation

Fields in bold indicate parameters specific to clinical systems if they are different from preclinical systems.

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Table 2

Selected clinical studies for human cell transplantation

First Author / Year	# of patients	Disease	# of cells/ type	Delivery method	Imaging modality	Labeling technique	Cell retention / survival [%]	Study observations
Studies based on nuclear medicine techniques (SPECT, PET, GC)								
Hofmann et al (2005)	9	STEMI	2-4.5×10^9 BMC	ICA ICV+ICA	PET	^{18}F-FDG BMC or ^{18}F-FDG CD34+ fraction of BMC	2 (ICA 1.5h) 3.8 (ICV+ICA 1.5h) 25 (ICA, CD34+ 1.5h	Homing only to perfused area of delivery artery, no retention after ICV, higher retention for CD34$^+$ cells
Karpov et al (2005)	44	Transmural MI	9×10^7 BMMNC	ICA	SPECT	^{99m}Tc-HMPAO	6.8 (2.5h) 3.2 (24h)	No differences in cardiac function between control and treatment groups
Kang et al (2006)	20	STEMI	PBMNC	ICA ICV	PET	^{18}F-FDG	1.5 (ICA 2h) 0 (ICV 2h)	Cell retention in old and new infarcts are not different
Correa et al (2007)	1	Ischemic stroke	3×10^7 BMMNC	OTW LCMA	SPECT	^{99m}Tc-HMPAO	n/a	Substantial amount of delivered cells in brain
Schächinger et al[7] (2008)	19	Acute-chronic MI	1.5×10^7 PBMNC	OTW ICA	GC	^{111}In-oxine	6.9 (1h) 2 (3-4 days)	Reduced retention in chronic compared to acute MI
Silva et al (2009)	30	STEMI	1×10^8 BMMNC	OTW: ICA or ICV	SPECT	^{99m}Tc-HMPAO	10.3 (ICA, 24h) 3.1 (ICV, 24h	6 month LVEF improvement is correlated with cell retention and higher in ICA group
Barbosa da Fonseca et al (2010)	6	Ischemic stroke	1-5×10^8 BMMNC	MCA	SPECT WB GC	^{99m}Tc	1.7 (2h)	Uptake primarily in the hemisphere with the stroke lesion
Barbosa da Fonseca et al (2011)	6	Chagasic Cardio-myopathy	1-10×10^8 BMMNC	ICA	SPECT WB GC	^{99m}Tc	5.4 (1h), 4.3 (3h), 2.3 (24h)	Homing not correlated with number of cells administered Poor uptake in areas with perfusion deficit
Vrtovec et al (2013)	40	DCM	1×10^8 CD34$^+$ from PBMNC	ICA, IM	GC	^{99m}Tc-HMPAO	4.4 (IC, 18h) 19.2 (IM, 18h)	6 month LVEF improvement is correlated with cell retention and higher in IM group
Studies based on magnetic resonance imaging (MRI)								
Zhu et al (2006)	2	Brain trauma	NPC	Stereo-tactical injection	3T MRI	SPIO	n/a	Migration of cells from injection site to boarder of lesion, signal persistent for 7 weeks
Callera et al (2007)	16	Spinal cord injury	0.7×10^6 CD34$^+$ from PBMNC	Lumbar puncture	1T MRI	Antibody - SPIO	n/a	Cell migration 35 days after injection, cells were not detected in 50% of patients

STEMI: ST elevation myocardial infarction, BMC: bone marrow cells, ICA: intra-coronary artery infusion, ICV: intra-coronary vein infusion, PET: positron emission tomography, ^{18}F-FDG: ^{18}F-fluorodeoxyglucose, MI: myocardial infarction, BMMNC: bone marrow mononuclear cells, SPECT: single photon emission computed tomography, ^{99m}Tc-HMPAO: ^{99m}Tc-hexamethylpropyleneamine

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oxime, PBMNC: peripheral blood mononuclear cells, OTW: over the wire balloon infusion, LCMA: left cerebral middle artery, GC: gamma scintillation camera, LV-EF: left ventricular ejection fraction, MCA: middle cerebral artery, WB GC: whole body gamma scintillation camera, DCM: dilated cardiomyopathy, SPIO: superparamagnetic iron oxide nanoparticle, NPC: neuronal progenitor cells.