Assessment and Optimization of Cell Engraftment After Transplantation Into the Heart

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Abstract: Myocardial regeneration using stem and progenitor cell transplantation in the injured heart has recently become a major goal in the treatment of cardiac disease. Experimental studies and clinical applications have generally been encouraging, although the functional benefits that have been attained clinically are modest and inconsistent. Low cell retention and engraftment after myocardial delivery is a key factor limiting the successful application of cell therapy, irrespective of the type of cell or the delivery method. To improve engraftment, accurate methods for tracking cell fate and quantifying cell survival need to be applied. Several laboratory techniques (histological methods, real-time quantitative polymerase chain reaction, radiolabeling) have provided invaluable information about cell engraftment. In vivo imaging (nuclear medicine modalities, bioluminescence, and MRI) has the potential to provide quantitative information noninvasively, enabling longitudinal assessment of cell fate. In the present review, we present several available methods for assessing cell engraftment, and we critically discuss their strengths and limitations. In addition to providing insights about the mechanisms mediating cell loss after transplantation, these methods can evaluate techniques for augmenting engraftment, such as tissue engineering approaches, preconditioning, and genetic modification, allowing optimization of cell therapies. (Circ Res. 2010;106:479-494.)

Key Words: stem cells ▪ engraftment ▪ imaging ▪ reporter genes

Stem cell transplantation has emerged as a new therapeutic option for ischemic cardiomyopathy1–3 although not yet at the level of routine clinical utility. Numerous studies in recent years have shown that cell therapy administered after myocardial infarction can improve cardiac function and limit infarct size.4–5 Despite the encouraging initial results, the beneficial effects are modest in clinical applications to date, and much remains to be optimized. The best cell type and time and route of delivery are still under investigation. However, irrespective of the specific cell type used for...
transplantation, low retention and engraftment after cardiac cell delivery are persistent obstacles to successful myocardial regeneration.

Many different approaches have been used to augment cell survival. To accurately assess their efficacy, reliable methods for quantification of engraftment need to be developed. Ideally, these methods should be sensitive enough to detect even low numbers of cells, noninvasive, allow longitudinal tracking of cell fate, and be free from artifacts. At present, no technique satisfies all these requirements. Here, we review the various methods available for engraftment quantification, present their relative strengths and limitations, and briefly discuss methods that have been used effectively to improve the survival of the transplanted cells.

Assessment of Engraftment

Histological Methods

Applications

Techniques based on histology offer the possibility not only to track injected cells in the tissue but also to determine their fate.6–7 The majority of experimental studies that attempt to explore cell survival and function after intracardiac delivery apply a labeling method to allow detection by histological methods or immunohistochemistry. Cell labeling can be achieved by several, widely available techniques. Membrane (DiI [1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate]; PKH26) or nuclear (Hoechst 33342, DAPI [4',6-diamidino-2-phenylindole]) stains can be used, with simple and nontoxic protocols.8–10 Because direct labeling techniques are subject to dilution by cell proliferation, the use of genetic labeling and reporter genes is usually preferred. Plasmid transfection or viral transduction can be used to express reporter genes in the therapeutic cell before delivery in the heart. These genes encode proteins that allow subsequent detection of the cells by specific staining techniques (X-gal stain for β-galactosidase) or fluorescent microscopy.10–12 Green fluorescent protein (GFP) and enhanced (e)GFP are by far the most widely used fluorescent probes for this purpose. However, a wide array of proteins of different colors, excitation and emission wavelengths exist (red fluorescent protein, yellow fluorescent protein, mCherry, etc) and allow researchers to perform simultaneously detection of any desired combination of target molecules.13 Figure 1 shows a typical application, from our own work, of lentiviral transduction of cardiosphere-derived cells (CDCs) for eGFP overexpression and subsequent cell detection, by immunocytochemistry, after intramyocardial injection into infarcted mouse or rat hearts.

To overcome the limitations of cell labeling, specific antigens existing exclusively in the injected cells and not in the recipient can be targeted by immunofluorescence. This is possible in certain experimental settings, such as xenotransplantation (detection of species-specific antigens) or sex-mismatched (sex-specific antigens) transplantation.6 Differentiation of the cells can be addressed simultaneously, by using antibodies against lineage-specific markers. Figure 2 illustrates the use of this method to detect human CDCs (identified by human nuclear antigen expression) injected into immunodeficient (SCID) mice, demonstrating their differentiation into cardiomyocytes by expression of troponin I.

Finally, transgenic animals expressing the reporter gene, either constitutively or under the control of a tissue-specific promoter, can be used as cell donors, with wild type animals of the same strain or immunodeficient animals serving as recipients.7,14,15 Given that the animals carry the gene in the germline, no somatic gene transfer vectors are required. In one particularly trenchant transgenic application, genetic fate mapping can be used to determine the origin of potential cardiac progenitors or their fate after delivery in the myocardium.16,17 A bitransgenic MerCreMer-Z/EG mouse model was generated by crossing breeding cardiomyocyte-specific MerCreMer and Z/EG mice.18 The Z/EG reporter mouse has a cytomegalovirus enhancer/chicken β-actin promoter driving floxed β-galactosidase, followed by multiple stop codons and subsequently eGFP. Double heterozygous bitransgenic MerCreMer-Z/EG mice express eGFP exclusively in cardiomyocytes after induction of Cre recombination by 4-OH-tamoxifen treatment. Using this model, we demonstrated that c-kit+ cells in the one-week outgrowth of myocardial biopsies are not of cardiomyocyte origin.16

Quantum dots offer an alternative approach for cell labeling and detection by microscopy. These are nanoparticles that can be easily taken up by the cells, even without the need for transfection agents. They are very bright (require short exposure times), exert higher photostability than the more commonly used dyes, and have emission wavelengths in the red and near infrared range, where autofluorescence of the myocardium is less of a problem.19 These attributes of quantum dots lead to increased sensitivity for cell detection by fluorescence microscopy. Rosen et al developed an efficient protocol for labeling human mesenchymal stem cells (MSCs) with quantum dots.20 They demonstrated the lack of

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any detrimental effect of the labeling on cell properties (viability and differentiation), and were able to identify injected cells in the myocardium of rats or dogs, even at 8 weeks posttransplantation.

**Strengths and Limitations**

Histological microscopic approaches for cell tracking have several strengths. Technologies, reagents, and expertise are widely available. Information about cell viability, location, and fate as well as quantitative results can be obtained. However, there are equally important limitations that hamper the applicability of these approaches for cell tracking:

- Animals have to be biopsied or euthanized for the collection of specimens; therefore, longitudinal tracking of cell fate is difficult or impossible in the same animal.
- A large number of experiments is required to obtain information about long-term cell fate, because several animals need to be euthanized at each time point.
- Quantitation is subject to variability and sampling errors. Only a few sections (and usually only a limited number of optical fields per section), sampled from the whole heart, are typically examined.
- Importantly, results are susceptible to artifacts, particularly when immunofluorescence is used to track labeled cells. The myocardium is notorious for high levels of autofluorescence; therefore, researchers must be cautious with the interpretation of their results and always examine appropriate controls. Confocal microscopy should also be used for these types of experiments, particularly when more than one target is examined and information about cell fate is being sought; otherwise, superimposed cells can lead to erroneous interpretations. Even more subtle errors might confound histological assessments. Laflamme and Murry elegantly demonstrated that macrophages can phagocyte-labeled cells including the organelles labeled by fluorescent dyes (membranes or nuclei). Immunocytochemistry can subsequently detect the label in a viable, healthy cell and lead to the assumption that this is the injected stem or progenitor cell. Fusion events can confound histological interpretation and more complicated labeling techniques (dual reporter systems or recombination systems) need to be applied to rule out this possibility. Limitations of labeling techniques should also be taken into account. Intercalating dyes such as DAPI can be toxic for the cells. Nuclear stains, such as Hoechst 33342, released by dead cells, can label neighboring host cells and yield false-positive results. The same theoretical concern also exists for quantum dots, because nanoparticles released by dead, labeled cells can be readily taken up by macrophages and generate spurious results.
- Genetic labeling is superior from the particle persistence/reuptake point of view; however, limitations also exist with this approach. Simple transfection and adenoviral vectors do not confer stable transgene expression, therefore the absence of reporter gene detection does not necessarily mean cell loss. Adeno-associated viruses and lentiviruses are more appropriate vectors for engraftment studies; however, the possibility of transgene silencing, particularly when nonmammalian promoters are used, should always be taken into account. Even cells from transgenic animals are not immune to transgene silencing. Therefore, careful validation experiments should accompany these types of studies, to confirm stable reporter gene expression.

**Fluorescent In Situ Hybridization**

Fluorescent in situ hybridization (FISH) is an invaluable technique for cell detection by microscopy. FISH allows,
with the use of an appropriate probe, the detection of a target cell-specific sequence. In the same section, several other targets can be explored by plain immunofluorescence simultaneously, to determine cell identity and fate. The usual applications of these techniques are in xenotransplantation or sex-mismatched cell transplantation models. FISH can detect male-specific sequences in the Y chromosome or human-specific sequences, when human cells are injected in immunocompromised animals.6,14,21,29,30 Figure 3 shows the application of this technique to detect human CDCs in the myocardium of SCID mice, using a human-specific pan-centromeric sequence as the target. The advantage of this approach lies in the fact that it does not require cell labeling for detection of the injected cell. The targets are stably present in the cells (species- or sex-specific genomic sequences), and in that way, they are not subject to silencing. However, the technique is labor-intensive, susceptible to artifacts (false-positives or negatives), requires careful optimization of the staining protocol for optimal sensitivity and specificity and requires appropriate controls. It also has the same generic limitations as other histology–microscopy techniques, namely the requirement for animal euthanasia for tissue harvest and limited quantitation potential.

**Ex Vivo Quantitation of Particles**

In vitro labeling with particles has also been used to quantify cell survival after cell delivery. In a swine model, Freyman et al injected iridium-labeled mesenchymal stem cells and evaluated cell survival at 14 days, by ex vivo quantitation of the amount of iridium in the heart, using a standard curve and atomic absorption spectrometry.31 The technique requires animal euthanasia and does not allow longitudinal tracking of cell fate but, more importantly, like all particle approaches, is susceptible to overestimation of engraftment. Signal from particles that persist in the heart despite cell death (in macrophages or in the interstitium) will be misinterpreted as indicating cell survival.

**Ex Vivo Quantitation of Reporter Gene Products**

Enzymatic activity of reporter gene products, such as firefly luciferase or β-galactosidase, can also be quantified ex vivo and provide information about cell engraftment. To correlate enzyme activity to cell numbers, standard curves are constructed from serial dilutions of known number of cells mixed with myocardial tissue, in vitro. Suzuki et al used an ex vivo assay for β-galactosidase to quantify engraftment of skeletal myoblasts (derived from transgenic animals stably expressing β-galactosidase) in rat hearts, after retrograde coronary vein infusion, up to 14 days after delivery.32 Robey et al injected myoblasts retrovirally transduced with β-galactosidase into infarcted mice and used a chemiluminescent assay for ex vivo β-galactosidase activity measurement.33 They even applied correction for the activity derived from enzyme released by dead cells, based on the half-life of free β-galactosidase in the myocardium.

Luciferase is a very popular reporter gene, because it can be used for both in vivo imaging and ex vivo luciferase activity assessment. Luciferase assays have the advantage of
very high sensitivity, and background is essentially zero. We have successfully used ex vivo luciferase assay to quantify engraftment in rats and pigs,34 24 hours after intramyocardial or intracoronary injection of lentivirally transduced CDCs expressing firefly luciferase. A standard curve is created with known numbers of cells mixed with rat or pig tissue and used for absolute quantification. The standard is used to convert luciferase activity (relative light units) measured in tissue lysates into cell numbers. It is essential to use a standard curve derived from the same cell preparation that was injected, because transduction efficiency and transgene copy number per cell may vary by experiment, even if the same multiplicity of infection is used every time. An example of quantification of cell engraftment by this method, in 3 rats 24 hours after direct intramyocardial CDC delivery is shown in Figure 4. Figure 4A shows the standard curve used in the experiment; Figure 4B, the absolute number of cells per whole rat heart. Because 1 million cells were injected in each rat, an average of 3.8% was retained in the heart 24 hours postinjection.

Figure 3. FISH image of human cardiac-derived stem cells injected into infarcted SCID mice, at 4 days after injection. Human-specific sequences (A: red dots; B: white dots), as well mouse-specific sequences (A: green dots; C: white dots), were used as targets in the nuclei (A: blue). Red arrows in A point to the nuclei containing human sequences (human cardiac derived cells). In B, red arrows point to the area corresponding to the human nuclei in A. Green arrows in A point to the nuclei containing mouse sequences (mouse cardiomyocytes). In C, green arrows point to the area corresponding to the mouse nuclei.

Figure 4. A, Standard curve correlating ex vivo luciferase activity in rat heart homogenates with known cell numbers of luciferase-expressing cardiac-derived stem cells, indicating the excellent sensitivity and linearity of the assay. B, Absolute quantification of CDC number at 24 hours after direct intramyocardial injection, in 3 rats, using ex vivo luciferase assay. C, Quantitative real-time PCR standard curve used for ex vivo quantification of male cell numbers injected in female hearts. A genomic sequence of the SRY gene is used as target. The curve shows excellent sensitivity of the assay, high efficiency of the reaction and linearity of the relationship between SRY gene copies and cell numbers. D, Amplification plots of the reactions corresponding to the points of the standard curve. Data were analyzed with the SDS 2.1 software (Applied Biosystems).
Ex vivo assays can provide reliable quantitative results; however, they require animal euthanasia and destruction of the tissue samples. They provide information about surviving cell number but not about cell fate or differentiation. In addition, gene silencing or unstable transfection may confound the results of these assays, particularly with long-term applications (weeks to months).

**Direct Radiolabeling for Ex Vivo Quantitation of Radioactivity**

Direct radiolabeling of stem cells with radiopharmaceuticals is also used for quantitation of engraftment. The method is not difficult to use in laboratories that are experienced with handling radioactive material. Tracers with long half-lives have been attached to molecules that can then accumulate in a cell. Fluoro-deoxyglucose, a glucose analog that is taken up by the cell through glucose transporters, can be labeled with $^3$H. Thymidine, labeled with $^3$H or $^{14}$C, can be incorporated into the DNA as thymine. After cell labeling, baseline radioactivity contained within the cells is measured. At several time points after cell transplantation, animals are euthanized, tissue is lysed, and radioactivity is measured in appropriate scintillation counters. Suzuki et al described a protocol for labeling myoblasts with $[^{14}$C]thymidine before intramyocardial injection in infarcted mice. At several time points after cell transplantation, animals were euthanized, whole hearts were lysed, radioactivity was measured in the lysates, and the percentage of engrafted cells was reported.

The method has the important advantage of accurate quantitation. However, it also has significant disadvantages:

- Handling of radioactivity requires significant caution, because these assays frequently involve the use of tracers with long half-lives.
- Animal euthanasia precludes longitudinal testing.
- Importantly, interpretation of results is not straightforward. There can be “contamination” of the signal from radioactivity released by dead cells that have not been washed out rapidly enough.
- Cell proliferation cannot be assessed, because there is only a fixed initial amount of radioactivity that can be distributed in daughter cells. Results at any time point can indicate the loss of the injected cell population but, if proliferation has occurred, there is underestimation of the actual size of the engrafted cell population.

**Real-Time Polymerase Chain Reaction**

Another approach that bypasses the drawbacks of gene transfer and genetic modification is quantitative, real-time polymerase chain reaction (PCR), with cell-specific sequences as targets for the reaction. It is used in xenotransplantation models (human-specific sequences such as the Alu can serve as targets) or in sex-mismatched transplantation, where male cells are injected into female animals and the Y chromosome specific SRY gene is the target. A standard curve derived from dilutions of genomic DNA (human or male, depending on the experimental setting) is used for quantification (Figure 4C and 4D). Numerous experimental studies have been published where engraftment was quantified by real-time PCR.

Real-time PCR can provide absolute quantification and, because the whole heart of a rodent can be homogenized and assessed, the results are not susceptible to sampling errors. No genetic modification of cells is needed, and limitations of cell labeling techniques are avoided. More importantly, cell proliferation can be assessed, because the genetic marker is stably present in the genome of the cell. Indeed, Suzuki et al elegantly demonstrated the superiority of real-time quantitative (q)PCR over direct radiolabeling in measuring the true size of the engrafted cell population, in experiments involving the delivery of muscle precursor cells in mice. The radioactivity-based assay showed a decrease of the cell population at 3 days after cell injection, whereas qPCR demonstrated an increase attributable to cell proliferation. On the other hand, real-time PCR has the disadvantages of all the ex vivo assays. Tissue is destroyed, results can be obtained only at single time points, and no information about cell differentiation and localization is available.

**Noninvasive Imaging Methods**

It is obvious from the above-mentioned assays that no laboratory method is ideal for assessing cell engraftment. Even those that allow accurate quantification require animal euthanasia and preclude longitudinal cell tracking. There is a clear need for noninvasive quantitative methods; imaging modalities have the potential to play a major role in this setting.

**Bioluminescence Imaging**

Optical bioluminescence imaging (BLI) is used frequently for cell tracking in cell transplantation studies. Genetically engineered cells overexpressing firefly luciferase are transplanted into the recipient animals. Subsequently, the animals are injected with the substrate of luciferase D-luciferin. In the presence of ATP, O$_2$, and Mg$^{2+}$, luciferin is oxidized and photons of visible light are emitted. Animals undergo imaging in a dark chamber and light is detected using highly sensitive cameras. Numerous studies involving BLI for cell tracking in the heart have been published. We have used BLI to study CDC engraftment in a syngeneic rat model of direct intramyocardial injections in the infarcted heart. A third-generation lentivirus was used to stably transduce the cells with firefly luciferase, driven by the constitutively active CAG promoter. Imaging results were validated by qPCR, and both techniques provided similar results (Figures 5 and 6). BLI has many significant advantages. It is an easy technique, provides quantitative results, is sensitive ($\approx$1000 cells can be visualized in a rat, even smaller numbers in a mouse), is nontoxic, and allows serial imaging at consecutive time points. Importantly, only viable cells can generate signal, because the reaction requires transcription, translation, and ATP. However, there are also several limitations:

- Light in vivo is significantly scattered, the spatial resolution is low, and cells cannot be located accurately.
- Only surface images are obtained; therefore, the results are sensitive to the depth of signal origin. In the case of cardiac imaging, the same number of cells will yield a...
much smaller signal if the cells are injected in the posterior wall of the left ventricle, instead of the anterior.

- A major drawback is the difficulty in obtaining reliable baseline signal intensity. It has been reported that bioluminescence signal in vivo reaches its peak value approximately 24 hours after cell injection. This was also the case in our experience with the method. The reason is not clear, but this limitation essentially does not allow any information about cell viability during the first 24 hours to be retrieved, although a very significant amount of cell loss occurs during this period. Because the results are sensitive to the timing of acquisition, significant errors can be made if normalization is performed according to day 1 postinjection signal intensities that do not really represent the peak values. These inherent limitations result in significant variability in the quantitative results obtained by BLI. Figure 6 shows the large standard deviations of engraftment results obtained by BLI, in comparison to qPCR.

- As with all transcriptionally dependent reporters, gene silencing may lead to a falsely low estimate of engraftment.

**Direct Radiolabeling for Single Photon Emission Tomography or Positron Emission Tomography**

Direct radiolabeling of stem cells with radiotracers for in vivo imaging is a widely available and frequently used method for quantitation of cell engraftment. Because there is significant experience in nuclear medicine with white blood cell labeling for detection of clinically occult sites of inflammation, it is not surprising that radiolabeling of cells has already been used for cell tracking in many experimental and several clinical applications. [111In]oxine and [99mTc]hexamethyl-propyleneamine oxime have been used in conjunction with single photon emission computed tomography (SPECT) imaging, and 18F-fluoro-deoxy-glucose (18FDG) with positron emission tomography (PET) for tracking bone marrow–derived or endothelial progenitor cells delivered to the myocardium intravenously or via the coronary arteries. Many interesting observations have been made using these imaging approaches. They have made clear that only a small percentage of the injected cells is retained in the heart, even at relatively short periods of time after infusion. In one clinical study, CD34+ selected cells showed significantly higher retention rates in comparison to the unselected mononuclear bone marrow population. In an experimental study, the intracoronary delivery of a single, large intracoronary bolus of cells resulted in higher retention rates than the standard technique of intermittent cell infusions during balloon occlusion of antegrade flow, calling into question the efficacy of the method applied in most clinical studies until now. However, a major safety concern not addressed in this experimental study was the possibility that infusing a concentrated cell population in the myocardial microcirculation may lead to cell plugging in the capillaries and creation of microinfarcts. Indeed, the increased cell retention could be secondary to such microvascular sludging, a phenomenon previously reported with intracoronary administration of mesenchymal stem cells (but which, for CDCs at least, can be avoided by systematic optimization of dosage and delivery). Imaging studies, both experimental and clinical, have also demonstrated that, with certain stem and progenitor cell populations such as MSCs and circulating progenitor cells...
post–cell injection. $^{13}$NH$_3$ (green) was used as perfusion tracer to delineate normally perfused myocardium. In vivo PET images are coregistered with CT images, for better anatomic detail and attenuation correction. (A: transverse image orientation; B: coronal; C: sagittal.)

Figure 7. PET images of directly radiolabeled with $^{18}$FDG cardiac-derived stem cells (red) injected in the infarct area of rats, at 1 hour post–cell injection. $^{13}$NH$_3$ (green) was used as perfusion tracer to delineate normally perfused myocardium. In vivo PET images are coregistered with CT images, for better anatomic detail and attenuation correction. (A: transverse image orientation; B: coronal; C: sagittal.)

(cPCs), homing to the infarcted myocardium is possible even after intravenous cell administration, although the percentage of cells accumulating in the heart is again very low (<3%). We have used radiolabeling of cardiac stem cells with $^{18}$FDG and assessed cardiac retention of cPCs by in vivo PET imaging. 1 hour after direct intramyocardial delivery, in a rat myocardial infarction model (Figure 7). Even with this direct approach, retention was on average <20%. Importantly, we validated the results obtained using real-time qPCR, as described above, and found almost identical retention rates, proving the accuracy of this imaging approach.

Both SPECT and PET can be used for tracking radiolabeled stem cells. SPECT is widely available, simple and requires tracers that are applied in everyday clinical practice. However, there are problems with acquiring accurate quantitative data, mainly attributable to errors derived from photon scattering. PET is more sensitive, has higher spatial resolution, and quantitation is more straightforward and clinically applicable. Unfortunately, PET scanners are more expensive, not widely available, and usually require an on-site or nearby cyclotron (for production of the necessary tracers).

Nuclear imaging techniques have several important advantages for studying cell engraftment. They provide information about cell localization, homing, and migration. Newer hybrid scanners, combining computed tomography (CT) with PET or SPECT, are even more appropriate for these studies. Anatomic CT images have higher resolution and quantitative data are more accurate, because the coregistered CT facilitates attenuation correction. In addition, in vivo imaging can allow quantitation of cell persistence at several time points in the same animal. This of course is only feasible when tracers with sufficiently long half-lives are used, such as $^{111}$In for SPECT (4.2 days). Most PET tracers, such as $^{18}$FDG (half-life of 110 minutes), have very short half-lives and can only be used to assess acute retention. Importantly, the successful use of the positron emitter $^{64}$Cu-PTSM has also been reported for cell tracking. This compound has a half-life of 12 hours and therefore can allow the use of PET imaging for cell tracking for up to 3 days.

With direct radiolabeling of cells for in vivo imaging, there is no need for systemic radiotracer administration. This is an important advantage, because there is no blood pool activity and image background is minimal. Cells can be visualized even when they are labeled with very low amounts of radioactivity, therefore systemic radiotoxicity can be avoided. This is particularly true for PET imaging that has higher sensitivity than SPECT. Using $^{18}$FDG for cell labeling and a dedicated small animal PET scanner, we were able to visualize cells and reliably quantify intramyocardial retention, even when injected activities were consistently less than 1 μCi.

A word of caution is also necessary in relation to potential radiotoxicity to the cells themselves, because of labeling. Several reports have found measurable toxicity related to in vivo labeling of blood cells, hematopoietic stem cells, or mesenchymal stem cells. In our experience, cardiac-derived stem cells were also very sensitive to exposure to $^{18}$FDG; very small doses had to be used to avoid adverse effects of the labeling procedure on cell viability and proliferation. We believe that simply checking acute viability after radiolabeling with Trypan blue (standard method in most published studies) is not adequate to rule out radiotoxicity to the cells. Radioactivity could damage more subtle functions of the cells. As a minimal precaution for dividing cells, the absence of any effect on cell proliferation for a few days after labeling should be confirmed before a radiolabeling method is considered safe. This is even more important if clinical applications are being considered.

Finally, the limitations of radiolabeling for ex vivo radioactivity measurements also apply to the imaging studies. Tracer released from dead cells can contaminate the signal for a certain period of time until it is washed out from the myocardium. For tracers with sufficient half-lives to allow longitudinal imaging, it should always be kept in mind that cell proliferation cannot be assessed and engraftment may be underestimated. In addition, efflux of the radiolabel from viable cells can also confound the quantitative results, because it is indistinguishable from cell loss.

**Reporter Genes for SPECT or PET**

Reporter genes offer the possibility to circumvent the aforementioned limitations of direct radiolabeling. Reporter genes for in vivo imaging encode a membrane receptor, transporter, or enzyme that is not normally expressed in the target cell. This protein allows the uptake and accumulation of a systemically injected radiotracer exclusively in the
genetically modified cells. In that way, the signal is specific, and because tracer uptake requires protein synthesis and metabolic activity of the cells, only viable cells can generate signal. When the transgene is stably integrated in the host genome (which is the case when lentiviruses and retroviruses are used as vectors), daughter cells also express the reporter gene. In that way, proliferating cells can also be visualized, allowing longitudinal and reliable cell tracking.

Three reporter genes have been used successfully for cardiac nuclear imaging. (1) The herpes virus thymidine kinase, either as the wild type (HSV1-tk), which phosphorylates and traps intracellularly the pyrimidine derivative 2-deoxy-2-fluoro-5-iodo-1-b-D-arabinofuranosyluracil (FIAU), labeled with $^{124}$I or $^{18}$F for PET imaging, or its truncated mutant form (HSV1-sr39tk), which uses as substrate the acycloguanosine analog 9-[4-$^{18}$F-fluoro-3-(hydroxymethyl)butyl]guanine (18F-FHBG).30,41,45,64 (2) The sodium-iodide symporter (NIS) can be ectopically expressed in cells and used for PET imaging with $^{125}$I tracer or for SPECT imaging with $^{99m}$Tc (pertechnetate). (3) The D2 dopaminergic receptor, which can be used for PET imaging with the $^{18}$F-labeled tracers 2-$^{18}$F-(fluoroethyl)spiperone, $^{18}$F-fluoroclobopride, and $^{18}$F-4-fluorobenzyltrozaminic.64 Only thymidine kinase and NIS have been used for stem cell tracking studies until now. Wu et al injected rat cardiomyoblasts expressing the mutant thymidine kinase after adenoviral transduction into the myocardium of nude athymic rats. They were able to detect the cells by in vivo PET on day 2 after injection.41 Imaging results were confirmed by autoradiography. Our group injected lentivirally transduced CDCs overexpressing the reporter gene.25 In addition, there are safety concerns about mutagenesis and oncogenicity of this type of viral vector, because they integrate preferentially adjacent to oncogenes, that may be activated by this process.68 Reporter gene approaches also have important limitations. Genetic modification may alter cell properties and compromise the functional benefit from cell transplantation.63 Transgene expression in the cell progeny is not guaranteed, even when viral vectors are used.66 Adenoviruses are cleared by the immune system. The recombinant adenovirus-associated viruses currently used for gene transfer rarely integrate in the host genome but remain as episomes in the host nucleus.66 Because they are nonreplicating, the episomes become diluted with cell proliferation.67 Even when viruses that integrate in the host genome are used, transgene expression can be silenced by DNA methylation, particularly when strong viral promoters such as cytomegalovirus drive the expression of the reporter gene.24 In addition, there are safety concerns about mutagenesis and oncogenicity of this type of viral vector, because they integrate preferentially adjacent to oncogenes, that may be activated by this process.68 Reporter gene approaches have certain drawbacks from the imaging point of view as well:

- Quantification is not as straightforward, because uptake (percentage of injected dose) in the areas of interest depends not only on the number of labeled cells but also on the dose injected, arterial concentration of the tracer, regional blood flow, and tracer uptake kinetics.69 Therefore, complex kinetic models need to be constructed and validated, if absolute quantification is required.
- In general, reporter gene approaches are less sensitive than direct radiolabeling, which means that more viable cells are needed for detection.
- Tracers have to be injected systemically to reach the genetically modified cells. Therefore, there is a substantial amount of radioactivity circulating in the blood pool that increases the background and decreases the contrast. This is particularly important for cardiac studies, where blood in the atrial and ventricular cavities can be a significant source of signal. In this type of study, an optimal time window should be defined where accumulation of radiotracer in the cells is adequate while blood pool activity has sufficiently decreased, to image with the highest possible contrast. This also explains why reporter genes that encode for transporters or intracellular enzymes (NIS or thymidine kinase) are potentially superior to the ones encoding membrane receptors (dopamine receptors), because they allow accumula-
tion of tracer in the labeled cell, in contrast to reversible extracellular binding. Indeed, in a study where thymidine kinase and D2 receptor were compared as reporter genes for PET, after adenoviral transduction of the rat myocardium, signal to background was higher with thymidine kinase. In addition, even with thymidine kinase and NIS, a degree of tracer leak from the cell (resulting in signal loss) is inevitable, stressing the importance of defining the optical timing for image acquisition.

Theoretically thymidine kinase should have an advantage as a reporter gene over NIS, because phosphorylating the substrate leads to its intracellular entrapment, whereas iodide or pertechnetate are free to diffuse out of the cell. However, in a study where the efficiency as PET reporter genes of both wild-type and mutant thymidine kinase was compared to that of NIS, after adenoviral transduction of the rat myocardium, signal to background was higher with NIS. In addition, thymidine kinase is a viral enzyme which can elicit immunogenic reactions that may not only limit its long-term efficiency but even harm the labeled cells. For this purpose, a human mitochondrial thymidine kinase was developed and used recently as a reporter gene for tracking genetically engineered cytolytic T cells in the brain, in a small clinical study. This novel reporter gene has not yet been used to track cells in the myocardium. NIS, as a human protein, on the other hand, has great potential for clinical translation. It can be used for SPECT imaging with widely available tracers, the other hand, has great potential for clinical translation. It can be used for PET imaging, although 124I has a long half-life eliminating the need for the complex radiosynthesis required for production of the thymidine kinase substrates. It can also be used for PET imaging, although 124I has a long half-life and potential toxicity to the thyroid, therefore its clinical applicability is problematic. However, the positron emitter 99mTc pertechnetate can be an alternative PET tracer to track NIS-labeled cells in future clinical applications.

**Magnetic Resonance Imaging**

Cell labeling with iron particles for visualization by MRI is one of the most frequently applied methods for cell tracking. Several iron formulations have been used for this purpose, including dextran-coated iron oxide nanoparticles (ferumoxides), cross-linked iron particles and micron-sized iron particles. This approach is attractive, because MRI provides anatomic information of the highest quality for cell localization and does not require ionizing radiation. In addition, most of the protocols that have been used for cell labeling have repeatedly been found to be nontoxic for cells. However, there are important pitfalls that limit the usefulness of these techniques. We and others have demonstrated that iron nanoparticles released by dead cells are taken up by macrophages and persist in the myocardium for up to 5 weeks, generating signals that could readily be misinterpreted as representing robust cell survival. We used a model of xenogeneic transplantation, where human CDCs or MSCs, labeled with ferumoxides and genetically engineered to overexpress β-galactosidase, were injected in the myocardium of immunocompetent rats. Three weeks after cell injection, MRI could clearly identify black spots in myocardium (areas of signal loss) corresponding to the presence of iron. The iron-specific Prussian blue stain showed many iron-containing cells in myocardial tissue sections. However, X-gal stain could not identify any surviving β-galactosidase-positive cells, and immunostaining revealed that all the iron-containing cells were macrophages. In addition to iron labeling and 1H-MRI, cells have been labeled with perfluorocarbon particles and detected by 19F-MRI. The positive signals (cells) appear as “hot” spots and can be localized anatomically after superimposition of the conventional proton MRI picture to that of 19F. This approach has the advantage of low background and high sensitivity, however, requires specialized technical expertise and is susceptible to the same kind of artifacts with any other particle approach, namely persistence of the label after cell death resulting in false-positive signals. Such methods may prove to be particularly useful for quantification of acute cell retention and biodistribution, rather than long-term engraftment.

**Optimization of Engraftment**

**Factors Influencing Acute Retention and Long-Term Engraftment**

Irrespective of the method used to assess engraftment, the cell type and transplantation model, it is widely recognized that only a small fraction of the delivered cells are acutely retained (within the first hour); cell loss is compounded by further attrition over the first 1 to 2 days and again in weeks. During intracoronary or direct intramyocardial injection, cells can be washed away by blood flow. In the case of direct intramyocardial injection, a significant portion of the cells are also lost because of leak from the injection site, pushed by the contracting myocardium, and washed by local bleeding. We have shown that both myocardial contraction, by accentuating leakage, and coronary blood flow, by washing away the cells, have a profound effect on acute cell retention. When cells are injected in the normally contracting and perfused myocardium, less than 20% are retained 1 hour postinjection (measured by in vivo PET). When cells are injected after induction of cardiac arrest, with the heart still and no coronary flow, retention was increased to more than 70%. A number of fundamental questions need to be addressed and resolved, to improve acute retention. The optimal delivery route (intracoronary, retrograde transvenous, intramyocardial) has not been determined yet. This of course depends on the clinical setting (acute myocardial infarction, chronic ischemic cardiomyopathy, transcatheter, or open-chest approaches). In an experimental swine model where the intramyocardial, intracoronary, and retrograde transvenous routes for cell delivery were compared using 111In-labeled peripheral mononuclear cells, direct intramyocardial cell delivery resulted in the higher engraftment, whereas the retrograde transvenous approach was proven feasible and superior to the intracoronary route.

The optimal cell dose for any given delivery route remains an open question. It may appear reasonable that a higher dose would result in better long-term engraftment; however, delivering a large number of cells is not always feasible and safe. In a dose-finding study conducted in a swine model, Johnston et al have shown that increasing the number of intracoronary-administered CDCs to more than 2.5×10^7 in-
deed augments engraftment but, at the same time, results in myocardial damage (as evidenced by increases in the serum ischemic biomarker troponin I). Bone marrow mononuclear cells can be safely administered in higher doses via the intracoronary route, because of their smaller size. For direct intramyocardial cell injections in rodents, delivery of larger number of cells has resulted in better engraftment. For clinical applications, the optimal cell concentration and volume and number of injections remain to be determined. Interestingly, in one study conducted in sheep, the functional benefit of directly administered MSCs was higher in the subgroups that received the lower cell numbers. Unfortunately, cell retention was not directly measured in this study.

Myocardial substrate is another factor affecting cell retention and engraftment. In the acute myocardial infarction setting, retention after direct intramyocardial cell delivery was lower when reperfusion was established. In a rat infarct model, MSCs were directly injected at 1 hour, 1 week, and 2 weeks after myocardial infarction. Functional benefit and engraftment were better at 1 week, suggesting that, at later time points, lack of homing signals in the injured myocardium may have adversely affected cell retention, whereas cell administration too early after an acute myocardial infarction may lead to suboptimal engraftment, because of the intensity of the inflammatory response in the acute stage.

Of the cells that are retained in the heart 24 hours after delivery, a small percentage (from 1% to 20% depending on the cell type) will stably engraft as indexed at 3 weeks. A combination of factors have been implicated in this process, with lack of connection to the host extracellular matrix (anoikis), ischemic death, and apoptosis apparently playing the most important roles. It is therefore reasonable to expect that a combination of approaches will be required to achieve meaningful increases in cell retention and engraftment.

### Tissue-Engineered Matrices

Tissue-engineered 3D matrices have been used as vehicles for cell delivery, to reduce washout from the injection site. Matrices can also provide a scaffold for the cells to attach and prevent apoptosis triggering attributable to anoikis. In addition, 3D matrices can serve as carriers for growth factors that can increase cell survival and proliferation after transplantation. Kutschka et al seeded rat cardiomyoblasts into a collagen matrix, mixed with Matrigel, with or without vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), and implanted them into infarcted hearts, in an heterotopic transplantation rat model. Engraftment was assessed by optical BLI. They were able to show that engraftment was increased, both at early (day 5 after cell injection) and late (day 14) time points, when cells were transplanted embedded in the 3D collagen matrices. The addition of FGF or VEGF did not further increase engraftment.

In another tissue engineering approach, Davis et al used biotinylated oligopeptides, able to self-assemble into nanofibers, as a delivery vehicle of insulin-like growth factor (IGF)-1 to the myocardium. In a rat model of acute myocardial infarction, neonatal cardiomyocytes delivered together with these nanofibers exerted higher functional benefit in comparison to cells or nanofibers alone. Engraftment was not quantified in this study; therefore, it is not possible to attribute the favorable effect to increased cell survival, although such a mechanism is certainly plausible.

Memon et al compared the efficacy of delivering myoblasts either by direct injection or by implantation of monolayer cell sheets epicardially, in a rat acute myocardial infarction model. They demonstrated more significant attenuation of remodeling in the animals of the cell sheet group, in terms of ejection fraction, left ventricular dimensions, fibrosis, and capillary density. Recently, Matsuura et al used the same technique to administer clonally expanded cardiac-derived stem cell sheets in mice and reported similar favorable effects on left ventricular function after myocardial infarction.

Christman et al injected skeletal myoblasts embedded in fibrin glue in the hearts of rats, 7 days after the induction of myocardial infarction. They demonstrated increased myoblast survival at 5 weeks by histology and less infarct expansion, in the cells plus fibrin glue group. Although fibrin glue is an injectable material, there are concerns that intra-vascular injections of the activated thrombin may promote thrombosis and lead to increases in infarct size. Several clinical cases of thrombosis have been reported after inadvertent intravascular application of fibrin glue. Therefore, we developed a modified protocol, where, after intramyocardial injection of cardiac-derived stem cells, the glue was applied only epicardially, as a plug to the injection site, to prevent leak of the injected cells after removal of the needle.

We were able to demonstrate an almost doubling of the acute retention of the cells, by in vivo PET and real-time PCR. Increased retention rate resulted in a significant increase in engraftment at 3 weeks and a larger functional benefit by CDC therapy, in comparison to intramyocardial injections of cells suspended in plain vehicle (PBS).

### Preconditioning of Cells

Several pretreatments with cytokines, growth factors, prosurvival factors, and physical stimuli have been used to increase cell survival; after transplantation. Suzuki et al subjected skeletal myoblasts to heat shock before ischemic/reoxygenation injury. They observed less ischemic and apoptotic death of heat-shocked cells in comparison to nontreated cells. Subsequently, in an in vivo experiment, they compared survival of heat-shocked myoblasts to untreated cells, after intracoronary delivery in rat hearts, using a heterotopic cardiac transplantation model. They found a significant increase in cell survival at 8 weeks, assessed by a β-galactosidase assay. Similarly, Laflamme et al demonstrated that heat shock was effective in promoting human embryonic stem cell engraftment in the hearts of athymic rats. One week after cell injection, they observed that the grafts formed by heat shock–treated cells were nearly 3 times larger than the respective ones formed by untreated cells.

Ischemic preconditioning is a powerful way to significantly reduce ischemic cell death in the setting of myocardial infarction. Another possibility to enhance cell survival takes advantage of drugs that mimic the effects of ischemic preconditioning, by opening mitochondrial potassium channels. Niagara et al showed that pretreatment of skeletal
myoblasts with diazoxide, a mitochondrial potassium channel opener, increased their survival almost 2-fold after direct intramyocardial injection in the infarcted rat heart.\textsuperscript{92} Importantly, the functional benefit in the animals that received diazoxide-treated cells was also greater, indicating that increased cell survival is closely related to the effectiveness of cell transplantation.

A variety of growth and angiogenic factors have also been used to enhance cell engraftment. Kofidis et al injected eGFP-labeled mouse embryonic stem cells with or without IGF-1 and observed better functional improvement and enhanced cardiomyocyte differentiation of embryonic stem cells in the animals treated with cells and IGF-1.\textsuperscript{93} Engraftment was not quantified in this study. Pasha et al pretreated mesenchymal stem cells with stromal cell-derived factor (SDF)-1 before intramyocardial injection in the infarcted rat heart.\textsuperscript{94} They were able to demonstrate a significant increase in cell survival (by histology) compared to transplantation of nonpretreated cells. In addition, fibrosis in the infarct area was less, and improvement in cardiac function was greater, when pretreated cells were used. These favorable effects of SDF-1 were abolished by a selective antagonist of CXCR4, the receptor of SDF-1. VEGF has also been used effectively in several studies to enhance survival and function of various cell types. Pons et al injected MSCs and VEGF in infarcted mouse hearts and demonstrated increased cell engraftment and better cardiac function in comparison to animals injected with MSCs or VEGF alone.\textsuperscript{95} Recently, Higuchi et al used PET to track endothelial progenitor cells genetically engineered to overexpress the human NIS, in infarcted rat hearts.\textsuperscript{96} Using iodine-124 as tracer, they showed increased cell engraftment after pretreatment of cells with VEGF and atorvastatin.

Despite these encouraging results, it should be remembered that multiple mechanisms mediate cell loss after transplantation; thus, pretreatment with single factors may not suffice to achieve a clinically meaningful increase in engraftment. With this rationale, Laflamme et al used a combination of growth factors and drugs to enhance engraftment of human embryonic stem cell derived cardiomyocytes in the infarcted hearts of immunodeficient rats.\textsuperscript{97} Their cocktail consisted of Matrigel (a cell attachment material to prevent anoikis, 2 components for suppression of mitochondrial death pathways (a cell permeant Bcl-XL-derived peptide and cyclosporin A), pinacidil as a preconditioning agent (mitochondrial ATP-dependent potassium channel opener), IGF-1 as a prosurvival factor (activation of Akt pathways) and a broad-spectrum caspase inhibitor (ZVAD-fmk), for apoptosis inhibition. This "kitchen sink" approach yielded a significant increase in histologically assessed graft formation, in comparison to animals that received nonpretreated cells (control), cells subjected to heat shock (very poor engraftment), or heat shock–treated cells in Matrigel (better engraftment than control but less than the cocktail-treated cells). Robey et al, from the same group, demonstrated the efficacy of a combination approach using carbamylated-erythropoietin (as a prosurvival factor) and heat shock for pretreatment of human embryonic stem cell–derived cardiomyocytes to enhance engraftment (assessed by real-time PCR) in the infarcted mouse heart.\textsuperscript{95}

**Genetic Engineering of Cells**

Despite the success in experimental models of cell preconditioning, the effect of the pretreatment is only transient and therefore the magnitude of the increase in cell engraftment may (or may not) be adequate for a clinically meaningful result. Another approach in this direction is the genetic modification of the cells, to stably express prosurvival or angiogenic factors. Mangi et al transduced mesenchymal stem cells with a retrovirus to overexpress Akt, an important prosurvival signal, and injected them intramyocardially in infarcted rats.\textsuperscript{98} They showed a significantly larger functional benefit and smaller infarct size in the animals treated with Akt-overexpressing cells in comparison to the ones that received lacZ-expressing cells. In this proof-of-concept study, cell engraftment was not quantified directly. However, in a similar experiment, Shuja et al transduced MSCs with adenovirus to overexpress angiopoietin-1 and Akt and injected them in the myocardium of infarcted rats.\textsuperscript{99} Three months after transplantation, they were able to demonstrate, by real-time PCR, increased cell survival in the animals that received genetically engineered cells in comparison to the ones transplanted with nontransduced cells. In addition, functional improvement and vascular density in the infarct area were increased in the animals treated with Akt and angiopoietin-1 overexpressing cells. Kutschka et al injected embryonic stem cell–derived cardiomyoblasts, adenovirally transduced to overexpress the antiapoptotic gene Bcl-2, in infarcted rat hearts, using a heterotopic cardiac transplantation model.\textsuperscript{100} Bcl-2–overexpressing cells survived better than control nontransduced cells at 4 weeks (assessed by bioluminescence), although cardiac function was similarly preserved in both groups. A similar increase in cell survival was observed with Bcl-2–overexpressing MSCs, in a rat myocardial infarction model.\textsuperscript{101} Tang et al transiently transduced mouse MSCs with a hypoxia inducible vector overexpressing human heme oxygenase (HO)-1. HO-1 has been shown to confer protection in the myocardium during ischemia, through antioxidant and antiapoptotic activity. HO-1- and lacZ-overexpressing MSCs were injected intramyocardially into the myocardium of infarcted mice, 1 hour after MI induction.\textsuperscript{102} At 7 days, MSC engraftment was 5 times higher in the HO-1 group (assessed by real-time PCR), indicating a prosurvival effect of the hypoxia-induced expression of this enzyme. Left ventricular function was also more effectively preserved by HO-1–overexpressing MSCs.

Genetic modifications have several limitations that need to be considered, particularly with regard to clinical translatability:

- Any intervention in the gene expression profile of the cell should not have detrimental effects on the desired function of the cell.
- Prosurvival gene overexpression carries the risk of oncogenesis, at least theoretically. This concern has been raised specifically for Akt overexpression strategies.\textsuperscript{103}
- Vector safety is also an issue, particularly with lentiviruses and retroviruses that stably integrate in the cell genome. If only transient transgene expression is required, plain transfection may be preferable.104
- The regulatory hurdles to clinical application of genetically modified cells will be substantial, because safety criteria for both cell therapy and gene therapy will have to be satisfied.

Conclusions
Suboptimal cell engraftment appears as one of the major hurdles for successful myocardial regeneration with stem cell therapy. Many experimental studies have connected improved engraftment with superior functional benefits of cell therapy. The development of reliable, accurate quantitative tools for tracking cell fate and assessing cell viability after transplantation is crucial, not only to understand the mechanisms of cell attrition but also to enhance cell survival. Within this setting, in vivo imaging will undoubtedly play major role in the future, because reporter gene technologies, combined with recent improvements in multimodality imaging, will be able to provide the required information noninvasively. Improvement of engraftment demands interventions in many different technical and biological aspects of cell transplantation. Delivery methods should be optimized to increase acute cell retention. Resistance of cells to ischemia by preconditioning, inhibition of apoptotic death, amelioration of acute inflammatory reactions, stimulation of cell proliferation by growth factors, and gene therapy are all potential targets for augmenting engraftment. In this regard, preclinical studies in clinically relevant large-animal models (acute myocardial infarction or chronic ischemic cardiomyopathy) should be carefully designed and conducted, before these interventions can be translated into clinical trials. The risks of these approaches to cell function and to the health of the recipient should be carefully evaluated, particularly if they involve genetic modifications or the use of viral vectors. The encouraging, albeit modest, functional benefits observed in stem cell clinical trials have occurred despite the universally acknowledged low rates of cell survival. These support the notion that improvement of long-term cell engraftment is a goal that should be pursued aggressively, to take full advantage of the potential of myocardial regeneration therapy.

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