

Integration of genomics, proteomics, and imaging for cardiac stem cell therapy

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Abstract Cardiac stem cell therapy is beginning to mature as a valid treatment for heart disease. As more clinical trials utilizing stem cells emerge, it is imperative to establish the mechanisms by which stem cells confer benefit in cardiac diseases. In this paper, we review three methods—molecular cellular imaging, gene expression profiling, and proteomic analysis—that can be integrated to provide further insights into the role of this emerging therapy.

Keywords Stem cell · Coronary artery disease · Heart failure · Reporter gene imaging · Proteomics · Gene expression profiling

Introduction

Despite advances in medical therapy, heart disease remains the foremost killer of Americans. Mortality from acute and chronic ischemic heart disease is high, and the financial burden on the health care system alone exceeds \$140 billion per year [1]. In addition, the incidence of congestive heart failure as a consequence of ischemic heart disease continues to climb, as more patients are surviving the initial

ischemic insult but thereafter develop heart failure. Despite optimization of medical and interventional therapies, many patients experience deteriorating cardiac function, which is often caused by the death of heart cells via necrosis and apoptosis, myocardial fibrosis, and ventricular remodeling. Any novel therapy which impedes or prevents these processes would provide a significant clinical advancement.

Stem cell-based therapy for heart disease is emerging as an innovative treatment modality that has the potential to improve heart function. Although stem cells for oncologic and hematologic diseases have become a mainstay therapy over the years, their beneficial use in cardiac diseases has yet to be clearly demonstrated. An initial study reported in 2001 showed significant regeneration of new myocardium from injected bone marrow stem cells [2]. However, under closer scrutiny this finding has encountered considerable critiques, and subsequent studies were unable to validate their claims [3, 4]. The field of stem cell-based therapy for heart disease is therefore in a state of flux presently.

Nevertheless, many animal studies have shown that therapies using stem cells are able to decrease infarct size, improve ejection fraction, and maintain wall thickness via mechanisms such as angiogenesis, cell-to-cell fusion, and transdifferentiation, just to name a few [5–7]. Other studies, however, question whether these cells actually survive long enough in the myocardium to account for the improvements in these experiments [8, 9]. Despite the lack of a clear mechanistic understanding, the field has evolved rapidly, and multiple clinical trials have shown the potential of this emerging field. Recent larger randomized controlled clinical trials suggest that stem cell therapy can improve modestly cardiac function and short-term outcomes [10, 11], but a third study did not confirm these findings [12]. As stated in the accompanying editorial, investigators in the field should “guard against both premature declarations of

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victory and premature abandonment of a promising therapeutic strategy” [13]. Remarkably, no significant adverse effects for stem cell therapy have been found thus far, and additional larger-scale trials will likely follow to test the initial promise of this therapy.

The majority of cardiac stem cell transplantation experiments to date include variations of the following elements: (1) choice of stem cell lineage for transplantation, (2) method of cellular delivery, and (3) assessment of cell viability and functional improvement. Choice of stem cells is varied but limited, and has included bone marrow cells, skeletal myoblasts, circulating progenitor cells, and cardiac resident stem cells. Human embryonic stem (ES) cells have also seen limited use in animal studies. Methods of delivery include direct injection into the myocardial wall, intracoronary injection, and peripheral intravenous injection. Functional assessments have mostly relied on both noninvasive (echocardiography, magnetic resonance, nuclear perfusion scans) and invasive (left ventricular angiography) measurements. Multiple additional factors also come into play in application of this therapy to patients [14]. Further clinical studies are needed to identify the best patient population for this novel therapy, and to establish the most effective timing and method of cell delivery.

Given the limited understanding of this field, integration of currently available molecular tools can provide tremendous insights into the field. We will focus this review on three of these techniques—stem cell imaging, genomics, and proteomics—to discuss how combining these technologies can guide our future development of stem cell therapy.

Stem cell imaging

Assessment of stem cell viability, differentiation, and function post transplant is imperative for establishing the potential therapeutic role of cell-based therapy for cardiovascular disease. In the absence of a rigorous analysis of cell fate and differentiation, it is difficult to draw useful conclusions on the mechanism of benefit from such therapy. Conventional assessment in animal studies has relied on postmortem histology using donor cells that may harbor markers such as green fluorescent protein (GFP). Human studies are even more limited in that marker proteins such as GFP are difficult to use, and biopsy samples or postmortem histology offer only limited assessment of stem cell survival and efficacy. Other functional assessments such as echocardiograms and nuclear perfusion studies are limited because they provide only *indirect* assessment of potential stem cell benefits. These limitations have created a need for direct, accurate, and reliable imaging of cell-based therapy. For such imaging techniques to prove useful, they will need the capacity to localize the cellular location over a protracted period of time

to establish viability and engraftment. To date, three modes of tracking cells have been utilized: (1) radionuclide imaging, (2) magnetic resonance imaging, and (3) reporter gene imaging. These techniques will each be described in further detail below.

Radionuclide imaging

Radionuclide imaging using single-photon emission computed tomography (SPECT) and positron emission tomography (PET) has been validated through clinical use for decades. SPECT and PET imaging are routinely used for detection of malignancy and evaluation of myocardial ischemia. Application of this technique for stem cell transplantation is recent, with first use in animal studies in 2003 and subsequent human studies being reported in 2005 [8, 15]. Using bone marrow cells labeled with radioactive ^{18}F -2-fluoro-2-deoxy-D-glucose (^{18}F]FDG), Hofmann and colleagues were able to follow stem cells after injection into humans. They showed that there was minimal cardiac retention of unselected bone marrow cells (1.3–2.6%), but when CD34+ purified cells were used, this fraction was much higher (14–39%) (Fig. 1). Even though bone marrow-derived cells were used for these studies, there is no limitation for application of this imaging modality to other types of cell. Although this method is useful for visualizing cells following delivery, the main

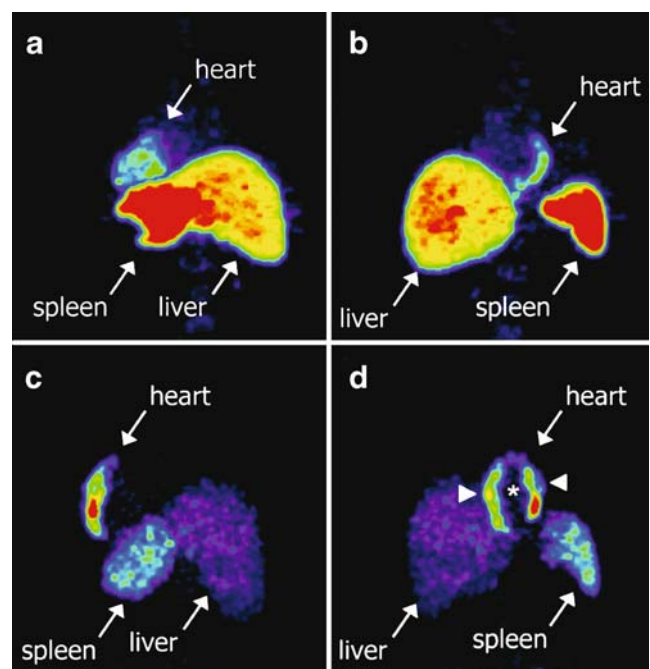


Fig. 1 Myocardial homing and biodistribution of ^{18}F]FDG-labeled bone marrow cells. Unpurified bone marrow cells (**a**, **b**) show much lower homing to the infarcted myocardium than do CD34-enriched bone marrow cells (**c**, **d**). Non-myocardial homing is also present in the liver and the spleen. (Reprinted from *Circulation* 2005;111:2198–202)

limitations lie in the fact that these cells can only be labeled prior to injection, and the duration of imaging is limited by the half-life of the radioactive tracer. In addition, this method works for localization of signal but does not assess viability of the cells. Moreover, the effects of the radioactive tracer on the stem cell function are presently unknown, and there may be alteration of signaling pathways that are important for the proper differentiation and function of these cells.

Magnetic resonance imaging

Another well-established imaging modality which has gained interest in stem cell imaging is magnetic resonance imaging (MRI). Cells are enriched with contrast agent to allow for increased signal intensity. Agents range from gadolinium-based particles to superparamagnetic iron oxide (SPIO) particles. Gadolinium-based agents are limited by their intrinsically low sensitivity for detection, making iron oxide particles the preferred agent for this modality of imaging. Initial proof-of-principle experiments in pig ischemic models have detected signals in the heart even as long as 3 weeks after transplant using mesenchymal stem cells [16, 17]. A major drawback of MRI, however, is that a positive signal does not necessarily distinguish dead cells from live cells. In addition, once these cells undergo proliferation, the signal within each daughter cell will be decreased because the physical quantity of iron particles cannot increase in amount [18].

Reporter gene imaging

Reporter gene imaging is providing a molecular and biologic approach to assess cell-based therapies. Reporter genes have been used to track patients with cancer [19, 20] as well as to image viral gene transfer to the heart in rats and pigs [21, 22]. The technique of reporter gene imaging involves introduction of the vector encoding the reporter protein into the cells of interest. The expression of such proteins can be under either constitutively expressed promoter or tissue-specific promoter. These reporter proteins produce a quantifiable signal when exposed to an imaging probe.

Reporter gene technology provides a number of advantages over direct labeling of cells with imaging agents. First, the reporter signal requires *transcription* of the reporter gene, *translation* of the reporter mRNA, and enzymatic *activity* of the reporter protein. All three steps require cellular viability. Direct labeling of cells does not discriminate cellular viability, as signals may still be present after cell death owing to local accumulation. Second, reporter imaging can be performed repeatedly at later time points without being limited by the half-life of the labeling agents. In fact, the reporter probe can be injected on a daily or weekly basis to interrogate the physiologic state of stem cells expressing reporter genes. Third, unlike radionuclide and MRI modalities, reporter genes can track daughter cells with equal efficacy as the progenitor cells. This is because the reporter genes are often integrated into the chromosomal structure and passed to subsequent generations of cells.

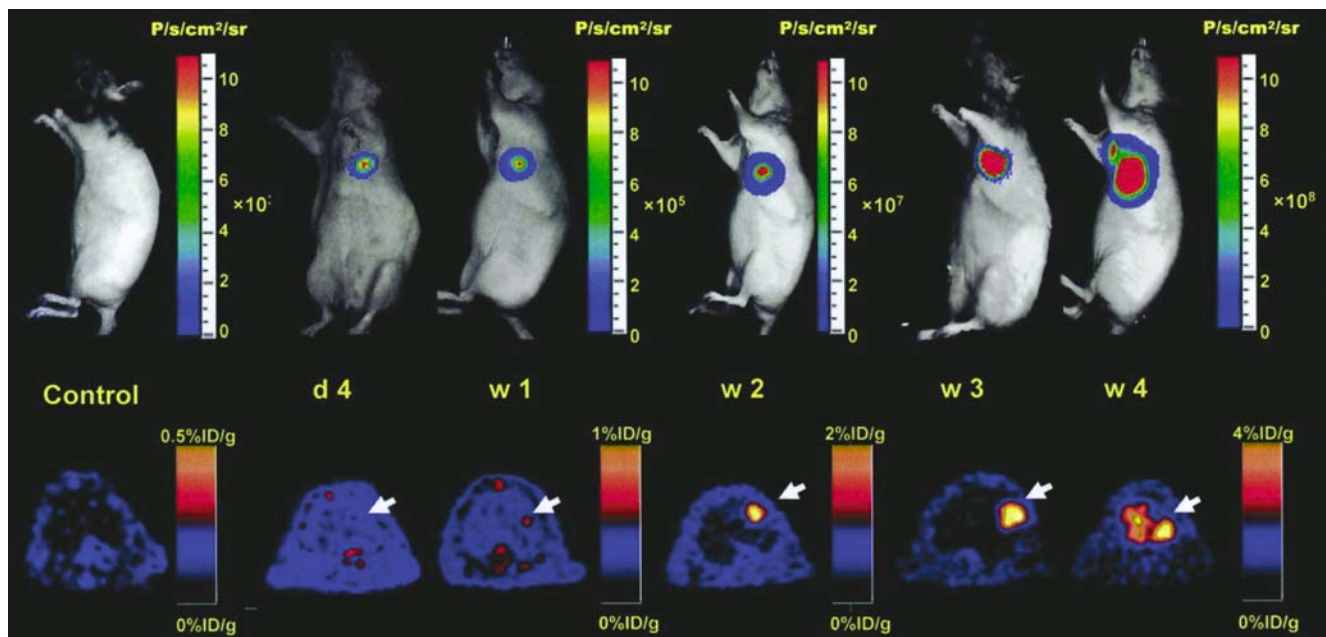


Fig. 2 Imaging of transplanted mouse embryonic stem cells with bioluminescence and PET imaging. A representative study animal injected with embryonic stem cells expressing reporter genes showed

significant bioluminescence (*top*) and PET (*bottom*) signals at day 4, week 1, week 2, week 3, and week 4. (Reprinted from *Circulation* 2006;113:1005–14)

Fourth, this technology also offers the unique advantage of combining various promoters and reporter genes to create a multimeric construct. Such a construct could be engineered not only to assess for cellular viability, but also to determine cellular differentiation via the use of cell type-specific promoter.

Two well-established examples of reporter gene assays are herpes simplex virus thymidine kinase (HSV-tk)-based PET imaging and firefly luciferase (Fluc)-based optical bioluminescence imaging. PET imaging with HSV-tk reporter protein has been widely used with a number of radiolabeled thymidine analog probes [20, 23]. Bioluminescence imaging with the Fluc reporter protein is based on the oxidation of D-luciferin reporter probe to generate low-energy photons. This signal is emitted from the cell through the tissue and detected using ultrasensitive CCD cameras (Fig. 2).

Despite its many advantages, reporter gene imaging does have some inherent limitations. First, a vector delivery method that allows for chromosomal integration and stable expression of the reporter gene is required. Although this can be achieved by the use of lentiviral or retroviral vectors, there is a low risk of insertional mutagenesis. In addition, potential adverse effects on cell viability and function are not yet clearly defined. A recent study from our group addressed this particular concern by performing gene expression analysis comparing murine ES cell lines with or without lentiviral integration of a reporter construct encoding fluorescence, bioluminescence, and PET reporter genes [24]. Despite slight variability in expression profiles, the overall function of the cells (including cell viability, proliferation, and differentiation) remained the same between the two cell lines. Further use of such modified cell lines is supported by published clinical studies using adenoviral gene transfer HSV-tk into patients with glioblastoma and hepatocellular carcinoma for PET imaging [19, 20]. Future experiments with stem cells will build upon the results in these earlier studies to demonstrate the efficacy and safety of reporter gene imaging.

Gene expression profiling of stem cells

A detailed understanding of the complex regulatory mechanisms governing stem cell self-renewal and differentiation has been hindered by our inability to efficiently study the global gene transcriptional processes that underlie their physiology. An ideal technology would quickly and accurately analyze whole genome expression patterns that drive stem cell survival, renewal, and ultimately differentiation. Specific to cardiac stem cell transplantation, this technology must also temporally analyze stem cells' genomic transcriptional changes after transplantation to

native or non-native tissue. In recent years, DNA microarray has given investigators an important tool for efficient whole genome analysis [25, 26]. Microarray technology has enabled rapid and accurate analysis of genome-wide expression. It is now possible to not only measure the temporal gene expression patterns of both individual genes and genomes taken from any number of cellular environmental conditions, but also to predict the function of poorly understood genes based on their expression profile.

Genomics studies typically require the isolation of mRNA from a test cell group at temporal or environmental points of interest for comparison with mRNA of a reference cell group. Most DNA microarray systems utilize two-color hybridization on a single microarray plate to visualize and measure the relative gene expression levels of test and reference samples. The total mRNA isolated from test samples and that isolated from reference samples are each fluorescently labeled with a distinct dye (for example Cy3 or Cy5) during reverse transcription. The samples are then competitively hybridized to a DNA microarray and the ratio of fluorescent wavelengths of both dyes is measured by scanning the microarray slide using wavelengths specific to each dye. The relative abundance of test versus reference mRNA is the measured fluorescent intensity ratio of the two dyes. Further review of this technology is provided elsewhere [27].

In the investigation of the pathophysiology of cardiovascular diseases, DNA microarray studies have been used to evaluate tens of thousands of gene transcripts simultaneously [28]. However, only a few studies have focused on stem cell biology using transcriptional profiling. One exception is Beqqali et al. who compared temporal gene expression patterns of human ES cells with fetal cardiomyocytes [29]. They identified genes that were rapidly downregulated upon human ES cell differentiation, including OCT4 as well as previously unknown genes that may play roles in stem cell self-renewal and differentiation. Because DNA microarrays assess only mRNA levels and not downstream protein levels, the resulting gene expression profiles cannot serve as surrogates for protein expression. Important posttranscriptional modification processes (e.g., glycosylation, phosphorylation, ubiquitination, and methylation) are neglected when protein expression is assumed to be identical to gene transcription. For example, Tian et al. evaluated the correlation of mRNA and protein expression in mammals and found that the differential expression of mRNA accounted for at most 40% of the variation in protein expression [30]. They concluded that a better understanding of stem cell regulatory mechanisms requires an integrated analysis of both proteins and mRNAs. In addition, microarray experiments are subject to random fluctuations resulting from either the experimental procedures or inherent biological variations [25]. Our group found that gene transcription profiles between biological replicate

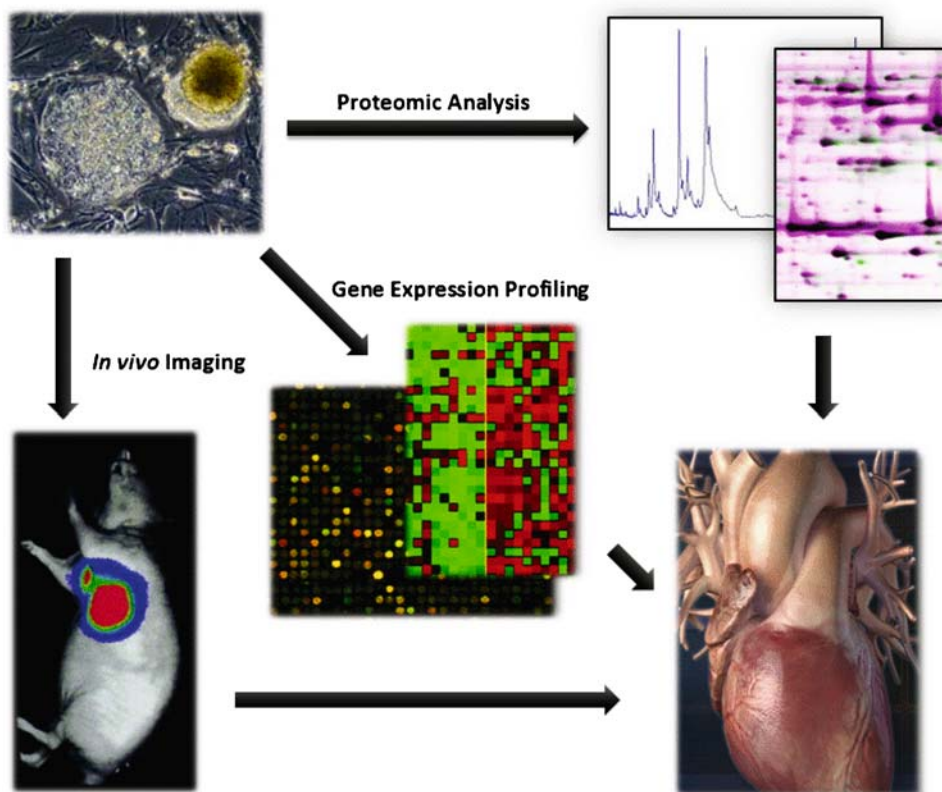
microarrays of mouse ES cells was at best 80% [24]. It is therefore advantageous to perform at least replicate experiments under standardized experimental conditions.

Further application of this technology to cardiac stem cell research will help answer some of the fundamental questions surrounding the field. Although the mechanism of functional improvement from stem cell therapy remains unresolved, gene expression analysis provides a valuable tool to compare expression profiles of these cells before and after their transplantation. Identification of signaling pathways that are either up-regulated or down-regulated in these stem cells after transplantation would shed useful insights. The major difficulty in comparison of the cells at these two stages is that transplanted cells would need to be isolated somehow from the endogenous myocardial tissue. Preliminary experiments in our laboratory have utilized stem cells that are stably expressing reporter genes such as GFP and injecting these cells into mouse myocardium. Using fluorescence activated cell sorting (FACS) technology, we have been able to isolate a sufficient number of GFP-positive cells for subsequent use for mRNA purification and gene expression analyses. These ongoing studies will help us to better understand the genetic changes in these cells and provide mechanistic clues to their function.

Proteomic analysis of stem cells

Although gene expression profiling is a powerful tool, ultimately the analysis of protein expression patterns will provide the most biologically relevant clues behind the mechanism of these cells. Proteomic analysis is an effective method to study proteins and their roles in complex biological systems. Using this method, the delicate balance of protein interactions associated with changes in protein modifications and abundance can be evaluated. Although protein expression is regulated at the level of transcription and splicing of the mRNA, the assumption of a direct correlation between the mRNA expression and protein level does not consistently hold, as described earlier. Proteomic studies of stem cells have shown promise in promoting our understanding of stem cell biology. For example, recent data of Baharvand et al. showed proteomic comparison of mouse ES cell and neonatal-derived cardiomyocytes [31]. In their study, they performed differentiation of embryonic stem cells into beating cardiomyocytes. Using 2-DE gel electrophoresis, they demonstrated an expression similarity of more than 95% between ES cell-derived and neonatal-derived cardiomyocytes, whereas undifferentiated ES cells and ES cell-derived cardiomyocytes had only a 20% similarity. They concluded that ES cell-derived cardiomyo-

Fig. 3 Schematic incorporating molecular assays to further elucidate the mechanisms behind cardiac stem cell therapy



cytes share a close relation with neonatal-derived cardiomyocytes. This finding provides additional support for future use of these cells in cell-based therapy. With maturation of mass spectrometry methods, the field of proteomics has been applied to establish the abundance of thousands of proteins concurrently [32]. Our group recently tested this method to compare protein expression levels in native mouse ES cells and mouse ES cells that were transduced with lentiviral vector that contains reporter genes used for stem cell imaging [33]. Our results showed that there were no significant differences between the native ES cells and the ES cells expressing the reporter genes. Application of the proteomic field to cardiac stem cell therapy is at best in its infancy, and further application of this technology to stem cell biology will provide additional important clues to the mechanism behind this therapy.

Conclusions

Current gaps in management of coronary artery disease and heart failure have necessitated the development of an exciting, novel treatment such as stem cell therapy. Recent clinical trials have begun to establish the potential efficacy of this therapy, and larger trials will further assess the potential benefits. However, the lack of a clear mechanism by which these stem cells function has hindered this field from developing more quickly. The three techniques described here—stem cell imaging, genomics, and proteomics—are generating high interest in their potential to define this field (Fig. 3). Although each technique by itself has limitations, innovative integration of the three is showing considerable promise in unfolding the mystery of stem cells. Continued advancement of the modalities described here will help bring about advances in cardiac stem cell therapy as we continue to apply this field to a wider range of clinically important problems.

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