Stem cell therapy for cardiovascular disease : answering basic questions regarding cell behavior

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CHAPTER 1

General introduction

STEM CELLS

Bovine non-identical twins that share the same placenta and circulation before birth, can keep producing a pool of blood cells that are a genetic mix of themselves as well as their brother's.[1, 2] Although not immediately recognized as such, it was as early as 1945 that this observation by Owen already suggested that the bone marrow hosted cells from early developmental origin that could produce more specialized progeny (blood cells) for a prolonged period throughout life. How else would it be possible that one of the twins produced cells that genetically "belonged" to his twin brother while the two had lost physical connection long ago? Despite these early suggestions, it took nearly 20 years before Mc Culloch and Till discovered a portion of bone marrow cells that could renew themselves extensively without losing their ability to produce a variety of other organ-specific cells.[3, 4] These two characteristics (self-renewal and differentiation capacity into more specialized cell types) have now been widely accepted as the requirements for calling a cell a "stem cell". As one can imagine, these characteristics automatically pose such cells as ideal candidates for both cellular and organ replacement therapies. However, before going into the therapeutic possibilities, it is important to gain some insight into different classes of stem cells. The most widely used classification of stem cells is based upon their origin and separates two groups of cells: "Embryonic stem cells (ESC)" and "adult stem cells".

Four to 5 days after fertilization, the early stage embryo consists of around 150 cells called the blastocyst. ESC from the inner mass of this blastocyst can be isolated and expanded indefinitely under strict culture circumstances and supported by a feeder layer of mouse embryonic fibroblasts.[5] The unique property of these cells lies within the capacity to develop into all three germ layers: Endo-, ecto-, and mesoderm, a phenomenon best described as pluripotency. After making the first transition to these germ layers, the cells become more restricted in their developmental potential and differentiate within germ layer boundaries to more specialized cell types, resembling the natural process of organogenesis.

On the other hand, adult or somatic stem cells are cells that have already differentiated further down the path of development. Although still capable of differentiating into multiple specialized cell types, these cells are restricted by germ layer boundaries and as such have already been programmed to become and replace cells specific to their biological environment or function. Because of this limitation, adult stem cells are referred to as being multi- but not pluripotent. Adult stem cells reside in the adult body in various places where they play a role in tissue homeostasis and repair, but are usually low in number and difficult to isolate and expand. One least imaginative example of tissue-specific stem cells is epidermal stem cells that reside in the skin where they are responsible for the fast turnover and accelerated production of progeny in case of injury.[6]

REGENERATIVE MEDICINE

Because of the above described properties of pluri- and multipotency, stem cells have contributed significantly to the field of "regenerative medicine". As the name already suggests, this discipline aims to heal disease by regeneration of damaged tissue. Adult stem cells carry this property by nature, as these cells are biologically destined to repair, for example, skin[6], gut[7], and liver[8], or to replace blood cells[9] throughout life. However, there is a range of diseases where endogenous repair fails including diabetes, Parkinson's, or coronary and peripheral artery disease. In this respect, it may prove beneficial to isolate, expand, and transplant adult stem cells to induce increased healing capacity. This approach carries great advantage because the cells are isolated from the patient and are thus not rejected through immunogenicity. It has even been reported that mesenchymal stem cells, which can be isolated from the bone marrow, may alleviate immune reaction.[10] However, most adult stem cells can prove difficult to isolate and expand, and may not be able to restore a complete spectrum of different cells needed for functional recovery. Moreover, these transplanted cells may not be able to survive in the diseased, often hostile environment after transplantation. Lastly, the cells may not integrate into the host tissue and as such cannot contribute to functional improvement.

Conversely, ESCs can be expanded in culture rapidly thus providing a possible "off-the-shelf" therapeutic. However, these cells must be directed into the desired cell type before transplantation to prevent uncontrolled differentiation and subsequent malignant potential if residual undifferentiated cells are present. In this respect, success has been achieved by driving ESCs towards brain and skin derivates as well as pancreatic cells or muscle, bone and cardiac lineages.[11] However, one major challenge remains eliminating undesired cell types as well as undifferentiated cells. A second hurdle is that, similar to organ transplants, the cells are from a different genetic background and may provoke immunorejection. Despite these problems and considerable ethical debate about the derivation of these cells, a clinical trial using ESC-derived oligodendrocyte precursor cells to treat spinal cord injury has just been initiated.[12]

STEM CELL TREATMENT FOR CARDIOVASCULAR DISEASE

Annually, more people die from cardiovascular diseases (CVDs) than from any other cause, representing 30% of all global deaths (http://www.who.int, factsheet 317). Thus, despite a wide variety of treatments ranging from medication to heart transplantation, there clearly remains a great need for new therapeutic approaches. In 2001, Orlic and colleagues reported that transplantation of bone marrow stem cells in the damaged mouse heart not only yielded an improvement in function, but also new cardiomyocytes (cardiac muscle cells) that originated directly from the transplanted cells.[13] For reasons described above, the observation that bone marrow cells were capable of differentiation into cardiomyocytes was heavily discussed and refuted

by, among others[14], our laboratory.[15] Despite these conflicting reports, it was Orlic's study that raised tremendous enthusiasm for stem cell therapy for heart disease and subsequent ultra-rapid initiation of clinical trials using bone marrow cells. Similarly, clinical trials with bone marrow cells for treatment of peripheral artery occlusive disease were initiated.[16] In the meantime, experimental studies showed promise for other adult stem cell types as well, including skeletal myoblasts[17], mesenchymal stem cells[18], and adipose-derived stromal cells.[19] While outcomes from these studies were generally promising, questions remained about the mechanism of action and the cellular behavior following cell transplantation. Unfortunately, there was few available data on the *in vivo* cellular kinetics thus leaving an unknown gap of what happened to the cells once they were transplanted into the animal.

IN VIVO MOLECULAR IMAGING OF STEM CELL KINETICS

To study the mechanism by which stem cells might or might not preserve function after transplantation, it is of great importance to gain insight into cellular behavior. Usually, this is approached by labeling cells with conventional reporter genes such as Green Fluorescent Protein (GFP, which is isolated from luminescent jelly fish).[20] However, to image GFP, extrinsic excitation light is needed, which produces significant background signal and has poor tissue penetration. This makes GFP unsuitable for reliable *in vivo* imaging of stem cells, and therefore GFP-labeled cells are typically identified histologically. Unfortunately, this requires the isolation of the target tissue and thus provides only a single time point rather than following a series of events in real time. In order to reliably investigate the behavior of transplanted stem cells, however, one must be able to track the cells longitudinally over time, whilst keeping the animal alive. To establish this goal, our group has developed novel molecular imaging techniques.[21]

Molecular imaging is defined as the *in vivo* characterization of cellular and molecular processes.[22] The backbone of reporter gene-based molecular imaging technique is the design of a suitable reporter construct. This construct carries a reporter gene linked to a promoter that can be inducible, constitutive, or tissue specific. The construct can be introduced into the target tissue by molecular biology techniques using either viral or nonviral approaches. Transcription of DNA and translation of mRNA lead to the production of reporter protein. After administration of a reporter probe, this probe reacts with the reporter protein, giving rise to signals that are detectable by a charged-coupled device (CCD) camera, positron emission tomography (PET), single photon emission computed tomography (SPECT), or magnetic resonance imaging (MRI).[23]

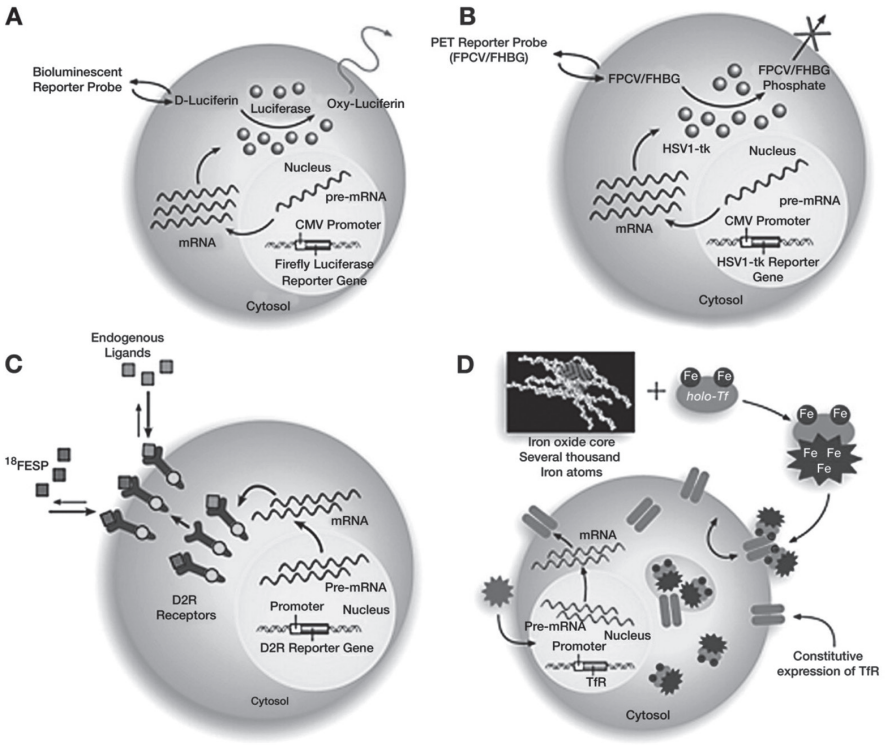

Figure 1. Examples of reporter gene and probe imaging. (a) Enzyme-based bioluminescence imaging. Expression of the firefly luciferase (Fluc) reporter gene leads to the firefly luciferase reporter enzyme, which catalyzes the reporter probe (D-luciferin) that results in a photochemical reaction. This yields low levels of photons that can be detected, collected, and quantified by a CCD camera. (b) Enzyme-based PET imaging. Expression of the herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene leads to the thymidine kinase reporter enzyme (HSV1-TK), which phosphorylates and traps the reporter probe (F-18 FHBG) intracellularly. Radioactive decay of F-18 isotopes can be detected via PET. (c) Receptor-based PET imaging. F-18 FESP is a reporter probe that interacts with D2R to result in probe trapping on or in cells expressing the D2R gene. (d) Receptor-based MRI imaging. Overexpression of engineered transferrin receptors (TfR) results in increased cell uptake of the transferrin–monocrystalline iron oxide nanoparticles (Tf-MION). These changes result in a detectable contrast change on MRI. Reprinted with permission from Wu et al.[24]

Recent studies from our group have shown that it is possible to monitor experimental stem cell transplantation by bioluminescence imaging (BLI).[25, 26] By introducing the Firefly Luciferase gene into the isolated cells, these donor cells can be imaged after transplantation in a naïve host. The Luciferase gene produces Luciferase protein, which will react with D-Luciferin (the

probe that is injected into the recipient animal before every imaging session) to produce a donor cell-specific signal that will be detected by the CCD camera. This approach potentially carries four major advantages: (1) It is *in vivo*, thus keeping the animal alive and permitting repeated imaging over time; (2) Luciferase protein will only be produced by donor cells that are alive, thus providing insight into cell viability; (3) D-Luciferin is systemically distributed while the CCD camera can image the whole animal, thereby monitoring signal from donor cells independent of cell location; and (4) There is no need of extrinsic excitation light, keeping background signals within acceptable limits. Following these advantages, this technique would be specifically suited to answer basic questions regarding cellular behavior after (embryonic and adult) stem cell transplantation in small animal models of cardiovascular disease.

STEM CELL THERAPY FOR CARDIOVASCULAR DISEASES: ANSWERING BASIC QUESTIONS REGARDING CELL BEHAVIOR

The scope of this thesis is to utilize molecular imaging techniques to address for the first time some basic but critical issues of both embryonic and adult stem cell therapy. Specifically, these issues involve *in vivo* cell survival, proliferation, migration, and misbehavior.

The initial part of this thesis describes the advantages and drawbacks of embryonic stem cell (ESC) transplantation. In **Chapter 2**, the effects of undifferentiated mouse ESC transplantation into the infarcted heart are described. Different *in vivo* modalities were used to assess short-term functional effects while histology tested the true regenerative capacity of these cells by means of staining for GFP and cardiomyocyte-specific markers. Moreover, non-invasive BLI and gross histology were performed to follow the fate of donor cells, and to image possible migration or misbehavior. Following this study, **Chapter 3** was designed to test some important characteristics of ESC-derived teratoma formation. Specifically, it tested the possibility that these cells can migrate through the body and form teratomas in distant locations after intramyocardial transplantation. Moreover, the potent teratogenic potential of undifferentiated mESC was visualized by testing how many undifferentiated cells are sufficient to provoke teratoma formation. The importance of these kinds of studies was acknowledged in a comment by Rao.[27]

Chapter 4 provides an overview of the potential of guided *in vitro* ESC differentiation and the wide variety of therapeutic possibilities. While giving insight into the basics of molecular imaging, it provides an introduction to the drawbacks of ESC. Getting more into detail, **Chapter 5** focuses on ESC-derived cardiomyocytes. It describes the clinical hurdles concerning differentiation efficiency, purification, integration, and immune rejection of embryonic stem cell-derived cardiomyocytes. Moreover, it outlines the role that molecular imaging can and should play in identifying these problems in both experimental and clinical settings.

After discussing the hurdles for clinical translation of ESC, the second part of this thesis focuses on the applicability of adult stem cells in cardiovascular diseases. **Chapter 6** focuses on non-invasive molecular imaging of different clinically utilized adult stem cell types. This was the first study to directly compare mononuclear cells from the bone marrow, skeletal myoblasts, mesenchymal stem cells, and fibroblasts in a mouse model of heart failure and revealed the survival of these cells in the ischemic environment of the infarcted heart. Moreover, it clarified which cell type resulted in superior functional preservation and if any of these cell types formed cardiomyocytes.

An alternative kind of mesenchymal stem cells can be isolated from the fat (adipose-derived stromal cells). These were compared to the "traditional" mesenchymal stem cell population from the bone marrow as described in **Chapter 7**. The *in vitro* morphological and growth characteristics of both cell types were analyzed. Thereafter, the cells were transplanted into the infarcted mouse myocardium and cell survival was monitored by *in vivo* BLI, while cardiac function was monitored by echocardiography. The echocardiography data was validated by pressure-volume loop measurements followed by histological analysis. Additional experiments using *in vivo* BLI examined the possibility that immunogenicity of GFP or the sex mismatch model used were of significant influence on donor cell survival in this study. This study was featured on the cover of *Transplantation*.

In **Chapter 8**, a novel small animal imaging modality to assess cardiac function is introduced. By comparison to traditional modalities such as echocardiography and catheter-based hemodynamic measurements, novel Micro-CT was tested for its ability to reliably and precisely assessing cardiac geometry and ventricular function of the infarcted mouse heart in an *in vivo*, three-dimensional fashion.

Next, **Chapter 9** makes the transition from cardiac studies to the field of stem cell therapy for peripheral artery occlusive disease. Using a mouse model of hind limb ischemia, different mononuclear cell transplantation techniques were tested while the patterns of cell survival and migration were visualized in live animals using molecular imaging. The results from this study reveal the patterns of cell survival and homing to the affected area after intramuscular and intravenous transplantation, respectively. Moreover, the effect on paw perfusion was monitored by Laser Doppler Perfusion Imaging (LDPI).

Finalizing this thesis, **Chapter 10** summarizes and discusses the most important findings and implications from the research conducted, and brings forward my opinion on the future directions of stem cell therapy for cardiovascular diseases. A synopsis in Dutch is provided in **Chapter 11**.

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