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

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CHAPTER 10

Summary and Discussion

INTRODUCTION

Over the past years, stem cell therapy has raised tremendous enthusiasm as a potential treatment for cardiovascular diseases. However, questions remain about the *in vivo* behavior of the cells after transplantation and the mechanism of action with which the cells could potentially alleviate disease symptoms. The objective of the research described in this thesis was to visualize the survival, proliferation, and migration of both embryonic and adult stem cells using non-invasive molecular imaging techniques in small animal models of cardiovascular diseases. The major findings can be described as follows: (1) Non-invasive bioluminescence imaging is a validated tool to monitor donor cell survival, proliferation, migration, and misbehavior; (2) Embryonic stem cells (ESC) are a potential source for a true regenerative therapy; (3) ESC form teratomas; (4) Adult stem cell survival is short-lived, but of all adult stem cells currently used in the clinic, mononuclear cells show the most prolonged survival; (5) Transplantation of mononuclear cells can preserve cardiac function in the short term after myocardial infarction in mice; (6) Compared to other measurements of cardiac function in mice, Micro-CT is a superior, three-dimensional, non-invasive method to assess cardiac geometry and function; and (7) Transplantation of mononuclear cells in peripheral artery disease is hampered by dismal cell survival and homing.

MONITORING EMBRYONIC STEM CELL THERAPY

In this thesis we have provided, largely for the first time, insight in the *in vivo* cellular behavior of adult and ESC. In **Chapter 2**, we have shown that ESC transplantation results in superior preservation of cardiac function in the short term compared to fibroblasts, as measured by small animal MRI that was confirmed by invasive hemodynamic measurements. However, novel bioluminescence imaging of the GFP+/Fluc+ ESC revealed a robust increase in BLI signal as early as two days post-transplant. After having shown that increasing BLI signal is in fact representative of an increase in cell number *in vitro* by BLI and that *in vivo* BLI signals correlated well with *ex vivo* TaqMan PCR, the increasing signal *in vivo* was clearly a consequence of cell proliferation. This early, *in vivo* suggestion of teratoma formation was supported by gross histology showing intense accumulation of donor cells.

As a follow-up study, **Chapter 3** nicely outlined the importance of using BLI to monitor cell fate after ESC transplantation. Indicative of the sensitivity of this technique, we observed increasing signals, implying teratoma formation, as early as one week after subcutaneous transplantation of as little as 1000 ESC. Correlating with the BLI results, teratoma formation was indeed confirmed by post-mortem histology showing differentiation of ESC to progeny from all three germ layers. The chapter also showed the importance of being able to perform whole-body imaging as ESC migrated through the body and formed teratomas at distant locations that

were easily identified by BLI. Finally, the stable integration of the GFP/Fluc construct in the donor ESC and the persistence of cell retention in the heart was illustrated by experiments showing follow-up of intramyocardially transplanted ESC *in vivo* as long as 10 months. These above mentioned advantages of using molecular imaging techniques to monitor ESC transplantation were summarized and related to the great potential of ESC to treat a wide variety of diseases in **Chapter 4**. **Chapter 5**, on the other hand, focused on the potential of ESC to differentiate into cardiomyocytes while indicating some of the hurdles that need to be overcome before ESC-derived-cardiomyocyte therapy will face the clinic. Next to the possibility of teratoma formation following inefficient pre-differentiation and subsequent transplantation of a heterogeneous population, this chapter also discussed the problems of immunogenicity and stable integration within the host myocardium.

MONITORING ADULT STEM CELL THERAPY

Chapter 6 was designed to answer the basic question: Which adult stem cell already clinically used in heart failure trials in humans can best preserve cardiac function after myocardial infarction in mice? To monitor cell fate after transplantation, all cells were isolated from donor mice that transgenically expressed both GFP and Fluc. After multimodality *in vitro* validation of reporter gene expression in all cell types, *in vivo* BLI results showed extensive donor cell death from skeletal myoblasts (SkMb), mesenchymal stem cells (MSC), and fibroblasts (Fibro, a cellular control) within three to four weeks after transplantation. Mononuclear cells (MN), on the other hand, were still present after 6 weeks, although in low numbers. Interestingly, these cells proliferated during the inflammatory phase (first ten days) after infarction, while some cells that had leaked into the circulation homed to the spleen, liver, and bone marrow later on. Our echocardiography studies, validated by invasive hemodynamic measurements, showed significantly improved cardiac function in mice that had received mononuclear cells as compared to negative (saline injection) and cellular (fibroblast injection) controls, while skeletal myoblasts were only significantly better than the negative controls. On the contrary, mesenchymal stem cells had no significant functional effect. Interestingly, however, deterioration of cardiac function was observed between 4 and 6 weeks in all cellular groups without such an effect in the saline control group, suggesting that the preservation lasts only for a short time. However, this can only be confirmed by future long-term studies.

In addition to the findings described in the previous chapter, **Chapter 7** provided for the first time *in vivo* information on the cellular kinetics of adipose tissue-derived stromal cells when transplanted into the infarcted mouse heart. This recently discovered cell population is believed to be similar to mesenchymal stem cells from the bone marrow. Indeed, these cell types shared similar morphology, cell surface expression patterns, and *in vitro* behavior. Unfortu-

nately, however, neither cell type was capable of surviving the ischemic environment of the infarcted heart and cell death ensued within 4 weeks of transplantation. Moreover, we did not observe any functional effect by echocardiography and pressure-volume loops.
In the studies described above, echocardiography proved to be an easy-to-use, quick modality to measure cardiac function in mice, and acquired diameters were generally correlative to ventricular volumes measured by conductance catheters. However, echocardiography is limited by means of its two-dimensionality while performing pressure-volume loops with a conductance catheter is a terminal procedure. In **Chapter 8**, we therefore introduced a novel, three-dimensional, *in vivo* modality to our inventory. The model of murine myocardial ischemia was used to show good correlations between Micro-CT and the more conventional imaging modalities. However, Micro-CT proved to gain the most detailed, precise measurements of systolic and diastolic cardiac geometry and subsequent functional parameters. Moreover, *in vivo* images acquired with Micro-CT resembled *ex vivo* post-mortem histological pictures of ventricular morphology.

Finally, **Chapter 9** provided insight into the kinetics of mononuclear cells (MN) after intramuscular and intravenous transplantation into a mouse model of peripheral artery occlusive disease. Intramuscular injection, either by single or repeated dosages, resulted in dismal cell survival without any effects on restoration of perfusion as measured by Laser-Doppler Perfusion Imaging. Following intravenous injection, regular *in vivo* BLI revealed homing to the injured area, although not exclusively. Signals were also observed from liver, spleen, and bone marrow. Moreover, *ex vivo* BLI showed that signals from the injured area were predominantly the result of homing to the scarred skin and the manipulated subcutaneous fat pad rather than the ischemic muscle. These findings translated into a lack of functional effect on paw perfusion.

NON-INVASIVE MOLECULAR IMAGING: KEEPING AN EYE ON TRANSPLANTED CELL SURVIVAL, PROLIFERATION, MIGRATION, AND MISBEHAVIOR.

The common technique used in the studies described in this thesis was non-invasive bioluminescence imaging. The double-fusion reporter construct carrying GFP and Fluc, on which this imaging technique was based, proved to be stably integrated into the donor cell's DNA as confirmed by *in vitro* BLI and luminometry. Moreover, *in vivo* BLI signals were validated by *ex vivo* quantitative PCR techniques as well as post-mortem histology and flow-cytometry with staining for GFP. Thus, BLI is a validated tool to image cell quantity as its signal is representative of cell number due to the equal transmission of the reporter genes to daughter cells. Subsequently, the adult stem cell studies have shown this technique to be suitable to image cell survival and migration. The fact that dead cells lack the transcriptional and translational process of reporter protein production underlies the capability to monitor cell death by loss of BLI signal.

Furthermore, the ESC studies have emphasized the excellent value of BLI to monitor cell location, proliferation, and misbehavior. Taken together, BLI is an indispensible tool for imaging the effects and safety of cell therapy. However, BLI uses low-energy 2-3 eV photons, which leads to photon attenuation and scattering within deep tissues.[1] Moreover, at present the imaging system containing the ultrasensitive CCD camera is unavailable for large animals or humans. These factors make this technique currently unsuitable for large animal or clinical safety studies. Instead, clinical molecular imaging techniques are currently based upon the utilization of Positron Emission Tomography (PET) with its associated reporter construct herpes simplex virus thymidine kinase (HSV-tk). Following injection of a radiolabeled thymidine analog (e.g. [18F]fluoro-3-hydroxymethylbutylguanine or [18F]-FHBG), the donor cells carrying HSV-tk will phosphorilate and subsequently trap the probe inside the cell, producing a signal consisting of high-energy photons strong enough for deep tissue imaging. This HSV-tk reporter gene construct has been used in small[2] and large animal[3] studies as well as in human trials.[4] Due to the fact that every imaging modality has its advantages and drawbacks, it is important to develop reporter gene constructs that combine different techniques. In this respect, we have shown the promise of the double fusion construct carrying Fluc and GFP for BLI and immunohistochemistry (IHC) in the studies described, respectively. Moreover, our group has developed a triple fusion construct containing Fluc, red fluorescent protein (RFP), and tk, thereby enabling BLI, IHC, and PET imaging.[5] Additionally, reporter gene imaging can be combined with magnetic labeling to enable superior imaging of acute localization of transplanted cells by MRI.[6]

CLINICAL UPDATE

To date, over 250 and almost 30 clinical trials are registered for heart disease and peripheral artery occlusive disease, respectively (http://clinicaltrials.gov/), illustrating the huge enthusiasm for cell therapy among doctors, patients, and media. As to cardiac cell therapy, studies greatly differ in patient population (acute vs. chronic ischemia), cell type and the administered quantity, method and timing of delivery, and duration of follow-up. Moreover, the majority of results comes from non-blinded trials.

So far, two meta-analyses have been published, both of which analyzed the efficacy of cell therapy for acute ischemic heart disease. Abdel-Latif and colleagues analyzed 18 controlled studies and generally, cell injection showed no increase in adverse events. Improvements in cardiac function with cell transplantation included a significant 3.66% increase in left ventricular ejection fraction and a significant 5.49% reduction in infarct size.[7] A second meta-analysis was performed by Lipinski and colleagues. After analysis of 10 studies, intracoronary cell therapy showed a significant decrease in recurrent myocardial infarction, but no difference in mortality risk and rehospitalization. Cell therapy resulted in a 2.97% increase in ejection fraction,

decreased end-systolic volume, and a 5.28% reduction in perfusion defect size.[8] Despite the statistically significant numbers from both meta-analyses, these studies do not clearly provide information whether these numbers translated into clinically relevant improvements in quality of life.

A recent Cochrane systematic review by Martin-Rendon and colleagues described 13 randomized controlled trials with a cumulative of 880 patients that received percutaneous intracoronary infusion of cells following acute ischemia. This review modestly concluded that cell therapy for acute myocardial infarction may be safe and moderately beneficial. However, the trials included were too small to demonstrate whether this therapy may have an effect on the incidence of mortality and morbidity.[9]

Regarding cell therapy for peripheral artery occlusive disease, the data are still rather preliminary, as most studies do not provide adequate patient numbers for definitive conclusions. Although small studies such as the initial TACT investigation show promising results including a 4-week increased ankle-brachial index, decreased rest pain and increased pain-free walking time,[10, 11] these observations require large, multicenter randomized trials to confirm the ability of cell therapy for relieving symptoms and improving quality of life in peripheral artery occlusive disease.

FUTURE DIRECTIONS

This thesis has shown that there are certain advantages and drawbacks of stem cell therapy for cardiovascular diseases. On adult stem cells, a functional benefit, if present, may be the result of paracrine signaling protecting host cells from dying, attracting native stem cells, attenuating remodeling, and inducing arterio-/ angiogenesis. However, this activity may be limited by poor cell survival which may explain short-term effects in our studies and some large clinical trials.[12] Therefore, one major goal should be to develop strategies that improve cell survival or increase the downstream effects as described above. In this regard, it may be of significant benefit to stimulate the cells using specific growth factors. Overexpression of certain factors might both increase survival as well as augment the biological function of adult stem cells. Supporting this hypothesis, researchers have shown that transfection of mesenchymal stem cells with the pro-survival gene *Akt* not only increased cell survival, but also augmented the functional effect on the infarcted heart.[13] More research will nevertheless be needed to discover the optimal combination of transcriptional factors needed to establish a significant, clinically relevant benefit of cell therapy. However, one must keep in mind the mechanism that might lead to this objective. If the stem cells do not have the capacity to become cells of the target tissue (cardiomyocytes in case of intramyocardial transplantation or endothelial cells when used in peripheral

artery disease) the question remains whether prolonged survival is necessary. If the effect of adult stem cells is merely a consequence of paracrine signaling, an approach whereby a slow-release cocktail of cytokines is infused might be just as effective. Alternatively, a gene therapy approach whereby the host tissue is modified to express pro-angiogenic or pro-survival genes could still be very promising. Taken together, in the case of cardiovascular disease, adult stem cell therapy seems to be more of a preservative therapy rather than a true regenerative therapy and has yet to be optimalized on the basis of more mechanistic studies.

ESC, on the other hand, have shown to be capable of really rebuilding the heart muscle, as we have observed that they form cardiomyocytes *in vivo*. Even if the frequency of this rare event can be increased or if ESC-derived cardiomyocytes can be purely grown in culture and subsequently transplanted, it remains questionable if these cells can truly integrate with native tissue and, importantly, will contract synchronously and respond effectively to the natural pacing of the heart. This problem has an extra dimension because ESC-derived cardiomyocytes appear to consist of both atrial and ventricular types that may react differently upon pacing. This illustrates the great caution warranted when using these cells for transplantation. Two other major problems we have visualized or addressed in this thesis concern tumorigenicity and immunogenicity. The development of more efficient pre-differentiation systems may make it possible to obtain 100% pure populations of a desired cell type from undifferentiated ESC cultures, thus limiting the possibility of present undifferentiated, potentially tumorigenic cells. Accordingly, it should be stressed that imaging cell therapy is indispensible as malignant events should be detected at an early stage. In this respect, one major advantage of using reporter gene imaging with the HSV-tk construct is the possibility to use this construct as a suicide gene as it is responsive to gancyclovir treatment. As such, the donor cells can be targeted when imaging reveals misbehavior, possibly preventing teratoma formation.[5]

One other ESC-related problem involves immunorejection. Great progress has recently been made by our group to characterize the immunogenic pattern of ESC. Similar to organ transplants, the rejection of embryonic stem cell grafts is CD4-mediated which can be largely overcome by treatment with immunosuppressive drugs.[14] Although these findings illustrate the increased understanding of ESC biology and development, consequently embryonic stem cell therapy will pose the patient to a life-long treatment to immunosuppressive drugs including the associated complications. Lastly, and very important, are the ethical issues that are associated to the derivation of cells from embryonic tissue. However, a great breakthrough has been established that has changed the field of stem cell research dramatically.

Recently, a Japanese group has published a report showing the possibility of using transcrip-

tion factors to reprogram adult stem cells (fibroblasts) to less differentiated states, where after these cells regain capability to differentiate into all germ layers.[15] These observations may redefine the differentiation patterns as described in the introduction of this thesis, now showing that, by *in vitro* manipulation, germ layer- or tissue lineage boundary limitations can be overcome. These so-called induced pluripotent stem cells (iPSc) seem to resemble ESC, but circumvent ethical and immunogenicity problems, offering the possibility to develop patient- and disease specific stem cells. Not only could this lead to new cell replacement therapies for tissue otherwise incapable of endogenous regeneration, but iPSc can also serve as *in vitro* surrogates for testing drug efficacy and toxicity specific to a disease or patient. While the opportunities seem innumerable, iPSc remain to be characterized more thoroughly regarding, among others, differentiation capacity and energy metabolism.

One last modality that will likely regain significant interest is gene therapy. As the effect of cell therapy may largely depend on the paracrine action of the cells rather than their structural, long lasting support, it would perhaps make more sense to 'train' the host cells to exhibit the paracrine character needed to stimulate, for example, angiogenesis. In order to provide a significant beneficial effect, it is yet to be investigated which factor is a key modulator of the process of angiogenesis, and create a vector that is non-immunogenic and grants an efficient, long lasting transfection. Ultimately, reporter gene imaging can be combined with gene therapy, allowing for *in vivo* monitoring of gene expression and dosing. Combining knowledge from imaging, cell therapy, and angiogenesis studies, we are currently moving forward in achieving these characteristics and hope to provide a novel gene therapy agent for cardiovascular diseases in the near future.

FINAL REMARKS

The field of adult stem cell therapy for cardiovascular disease has provoked an enormous amount of enthusiasm, leading to a clinical translation of experimental findings with unprecedented rapidity. Such transitions cannot solely be based on findings in experimental rodent models, as the results from the studies described in this thesis are not always concordant to findings from clinical studies. This emphasizes the current gap that exists between animal models and human disease and justifies extensive investigations in large animals or even primates before proceeding to any kind of clinical trial with cardiomyocytes. Regarding ESC or iPSc, it is of main importance to characterize these cells, explore the way they differentiate, and portray their genomic and proteomic patterns. Luckily, research in this field has been given a boost by recent developments since president Obama issued Executive Order 13505 entitled "Removing Barriers to Responsible Scientific Research Involving Human Stem Cells" (http://stemcells.nih.gov). As a result, federal funding of embryonic stem cell research from the National Insti-

tutes of Health was restored after being strongly limited for eight years under the previous administration. Hopefully, this will lead to a better understanding of developmental biology, disease, and therapeutic targets for the large range of diseases that can benefit from new treatments, not at the least for cardiovascular diseases (**figure 1**). In all these settings, molecular imaging should and will indisputably form an important tool in assessing the efficacy in both experimental and clinical settings of cell therapy.

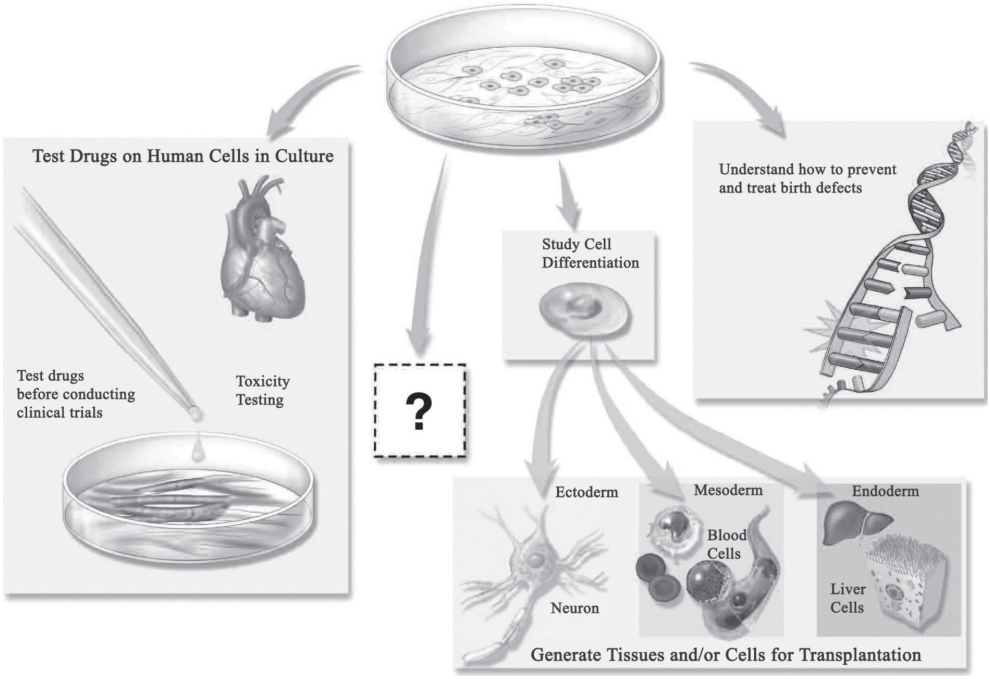

Figure 1. Promises and directions of stem cell research (www.nih.gov)

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