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14. ABSTRACT Heart disease is the leading cause of death in both the United States and Hawaii. According to the Hawaii State Department of Health, over 1/3 of total deaths in the state are caused by cardiovascular disease, in which approximately 18% (hospital discharges) were associated with heart failure. The potential of stem cells for use in cell therapy to treat diseased or damaged organs is very promising due to the unique properties of these cells, namely the capacity for both long-term self-renewal and differentiation into various mature cell types. There are 2 main objectives for our research, 1) to examine the efficiency of repair and recovery of damaged heart tissue in stem cell based therapies by using enriched hematopoietic stem cells (HSC) and 2) study the efficiency of trans-differentiation of HSC into cardiomyocytes, following transplantation into damaged (ischemic) heart tissue to promote cardiogenesis.					
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Introduction

Heart disease is the leading cause of death in both the United States and Hawaii. According to the Hawaii State Department of Health, over 1/3 of total deaths in the state are caused by cardiovascular disease, in which approximately 18% (hospital discharges) were associated with heart failure (Balabis, Pobustsky, Kromer Baker, Tottori, & Salvail, 2007). Many patients with end-stage congestive heart failure (ESCHF) reach a state where medical therapy is not adequate to sustain acceptable cardiac function. After medical therapies have been optimized, further therapeutic options are limited to mechanical circulatory support (MSC) systems such as Ventricular Assist Devices (VADs), cardiac transplantation, or highly experimental stem cell procedures; none of these options are available in Hawaii.

The potential of stem cells for use in cell therapy to treat diseased or damaged organs is very promising due to the unique properties of these cells, namely the capacity for both long-term self-renewal and differentiation into various mature cell types. While it may be ideal to treat different organs or tissues with stem cells of the same type (i.e., treat brain with brain-derived stem cells), in many instances tissue- or organ-specific stem cells have either not yet been identified, or the isolation of these stem cells is impractical. Hematopoietic stem cells (HSC) are presently one of the most attractive types of stem cells for use in cell therapy because they are well characterized, are relatively easily purified, and can be isolated directly from either mobilized blood in adults or from cord blood. In particular, bone marrow HSC are of interest because they may be used in autologous transplants.

In this study, we proposed to further examine the potential and mechanism of HSC to treat damaged or diseased heart tissue, by (1) comparing the capacity of highly purified human HSC samples, bone marrow progenitor/stem samples, and whole bone marrow samples to promote the recovery of heart tissue in mice following transplantation to treat myocardial infarct, and (2) examine whether HSC can be re-programmed towards cardiogenesis, instead of hematopoiesis, by treating these cells with specific growth factors and/or expressing genetic regulators of cardiopoiesis in these cells.

If this proves possible, then the ability of these cells to promote the recovery of heart tissue in mice following transplantation to treat myocardial infarct will be compared to that of untreated HSC. These results should shed light on how important HSC are with respect to use in cell therapy to treat myocardial infarcts, and may also lead to an improved cell therapy based method, involving the re-programming of donor cells to a cardiogenic fate prior to transplantation, to treat damaged or diseased heart tissue.

Body

The following are a summary of activities during this period.

1. Complete all appropriate procedures with institutional review boards - *completed*

- a. Local University of Hawaii Animal Care & Use committee has approved this protocol on 15 January 2009.
 - i. Local University of Hawaii Animal Care & Use committee has approved Amendment #1 for this protocol on 16 July 2009.
 1. An alternative way to induce myocardial infarct in the mice has been identified and included as an alternative optional method to this protocol.
 2. Small change in protocol to utilize new end-point measures of angiogenesis, since the field is constantly changing and evolving, and we want use the best measure as we can.
- b. The USAMRMC Animal Care and Use Review Office has approved this protocol on 21 April 2009.
 - i. The USAMRMC Animal Care and Use Review Office has approved Amendment #1 of this protocol on 18 August 2009.
- c. Local University of Hawaii Committee on Human Studies has approved this protocol as an exempt study, under DHHS regulation, 45, CRF Part 46 on 21 January 2009.
- d. The USAMRMC Office of Research Protections, Human Research Protection Office has reviewed this protocol and, in accordance with 32 CFR 219.102(f), the HRPO determined that the proposal constitutes research not involving human subjects. Determination was received on 21 April 2009.

Conduct stem cell research for the treatment of congestive heart failure. Studies will examine the potential and mechanism of hematopoietic stem cells to treat damaged or diseased heart tissue – *completed*

During this reporting period, two no-cost extensions were requested. As stated in the monthly reports, in the initial months of the experiments, we experienced somewhat high post-surgical mortality rate (~60-50%) in the first few transplant experiments performed. However, over the proceeding few months, the post-surgical mortality rate markedly improved and we were able to reach a 20-25% mortality range, which is good for this type of invasive surgery on immune compromised mice. The additional time extensions allowed us to add additional mice to the stated numbers per group, such that statistical significance could be properly evaluated. We had requested an additional 50 SCID mice for additional experiments to amend for the high post-surgical mortality rate in the initial experiments performed.

We would also like to note that we were unable to acquire the Gaq*44 mice transgenic mouse strain despite repeated requests for this strain from the laboratory that developed it, as it is no longer available. Therefore, we have been unable to perform the proposed experiments with this strain. It is important to emphasize that this component only plays a relatively minor part in the

testing for this hypothesis (Hypothesis 1), and we were still able to evaluate this hypothesis from our current experiments. A reason why the Gaq*44 mice transgenic mouse strain is not available is likely because this mouse strain has been reported to have some non-cardiac related side effects associated with the transgene expression. We became aware of this after the start of our study. The experiments we proposed using the Gaq*44 strain, while having the potential to further verify the utility of HSC to treat heart disease, would have to be performed using murine HSC (since this is not an immune compromised mouse strain), and therefore would not involve transplantation of the more relevant human HSC. However, the unavailability of these mice affords us to purchase additional SCID mice to increase our sample size.

a. ***Hypothesis: Are hematopoietic stem cells (HSC) required to promote recovery in cell-based therapies that utilize bone marrow derived samples to treat myocardial infarct?***

We successfully trained lab members in both the initial protocol (the LAD suture procedure) and alternative protocol (cryo-ablation), to induce myocardial infarct. 40 C57BL6 mice were used to develop the model was complete in 2009.

We have successfully received high quality bone marrow samples from Hawaii Transplant Center, in sufficient quantity to serve as a source of HSC for the duration of this study (**completed Jan 2010**). Optimization of our stem cell sort protocol was performed with these samples was completed shortly after receiving these samples (**completed Feb 2010**).

We have completed all transplant experiments as described in the initial proposal (90 SCID mice) (**completed Dec 2010**), and completed all transplant experiments with the additional 50 SCID mice, and analyzed and interpreted all results (**completed March 2011**).

b. ***Hypothesis: Will exposure of HSC to specific re-programming factors promote the ability of HSC to trans-differentiate into cardiomyocytes following transplantation into damaged heart tissue?***

We have completed the cloning of the human Nkx2.5 cDNA, and assembled the lentiviral expression vector to use in experiments to re-program HSC into cardiac progenitor cells in vitro (**completed in 2009**). Frozen aliquots of the packaged lentivirus have been prepared and stored for use (**completed in 2009**). We have optimized the protocol for efficient transduction and re-programming of HSC with the lentiviral expression construct (**completed summer 2010**), and completed all transplant experiments with the 40 SCID mice, and analyzed and interpreted all results (**completed March 2011**).

2. **Analyze data, interpret results, and draft manuscript for publication – completed**

a. ***Hypothesis: Are hematopoietic stem cells (HSC) required to promote recovery in cell-based therapies that utilize bone marrow derived samples to treat myocardial infarct?***

To specifically assess the capacity of HSC (the CD34+Lin^{Neg} sub-population of whole bone marrow)) to facilitate the recovery of mice from induced infarcts, we systematically altered the number of HSC in bone marrow derived samples, and subsequently used these samples to treat mice in our human → mouse transplantation model (described in original proposal). For all recipient mice, a total of 250-300 thousand cells were transplanted. Four weeks post-transplant, the recipient mice were

worked up for analysis of cardiac function and repair. Cardiac function was assessed in live animals prior to termination by measurement of the left ventricular ejection fraction (LVEF) using echocardiography. The results for mice receiving samples varying in the number of HSC only are shown in figure 1, and indicate enhanced recovery of cardiac function in mice receiving higher doses of HSC. Control recipient mice receiving samples depleted in either T cells, B cells, myeloid cells or hematopoietic progenitors were not significantly compromised in their ability to facilitate recovery of cardiac function, as assessed by both analysis of LVEF or amount of scar tissue, relative to the cohort receiving whole bone marrow (to be reported as supplemental data in a future manuscript). To assess the extent of repair in the region of induced infarct, we measured the thickness of cardiac wall and the extent of scarring (measured by Masson's Trichrome stain analysis of collagen deposition). The thickness of the cardiac wall, which is atrophied as a result of induced infarct, showed significantly greater recovery for mice receiving samples enriched in HSC (Figure 2). The extent of scarring within the region of induced infarct, as measured by the ratio of epicardial wall/endocardial wall length within the infarct region (as defined by Masson's Trichrome staining) was also significantly improved for recipients of HSC-enriched samples. Importantly, the results from Figures 1 to 3 show a general trend of improved cardiac function and repair dependent on the dose of HSC (HSC-enriched samples > whole bone marrow > HSC depleted samples). Together, these results strongly support a positive role for HSC in promoting recovery from induced cardiac infarcts in our human → mouse model system.

To assess the mechanism by which HSC facilitate repair in the infarct zone, we measured the density of new capillaries in the infarct zone by staining for Iso-lectin B4, a marker of endothelial cell proliferation. Quantitative analysis of the amount of lectin staining in the infarct zone indicated enhanced re-vascularization of the infarct region as a function of the number of HSC transplanted (Figure 4), indicating that HSC may promote repair of the damaged cardiac tissue by inducing angiogenesis.

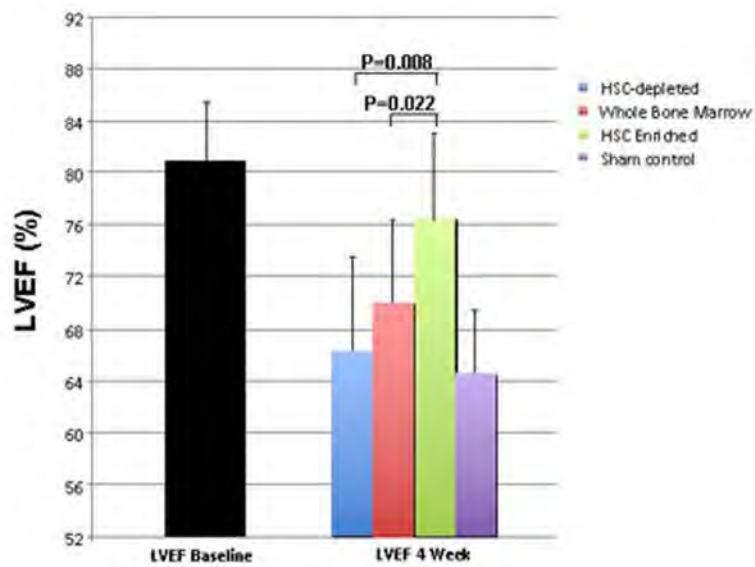


Figure 1. Analysis of left ventricular ejection fraction for mice used to evaluate the capacity of bone marrow-derived stem cells to facilitate recovery from induced infarcts.

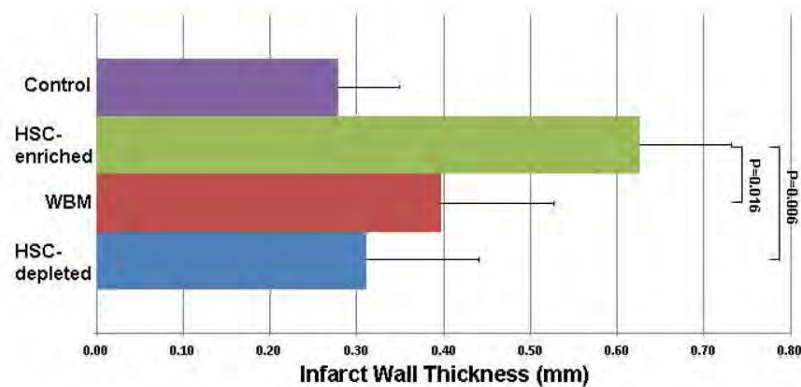


Figure 2. Analysis of cardiac wall thickness within the infarct zone in mice used to evaluate the capacity of bone marrow-derived stem cells to facilitate recovery from induced infarcts.

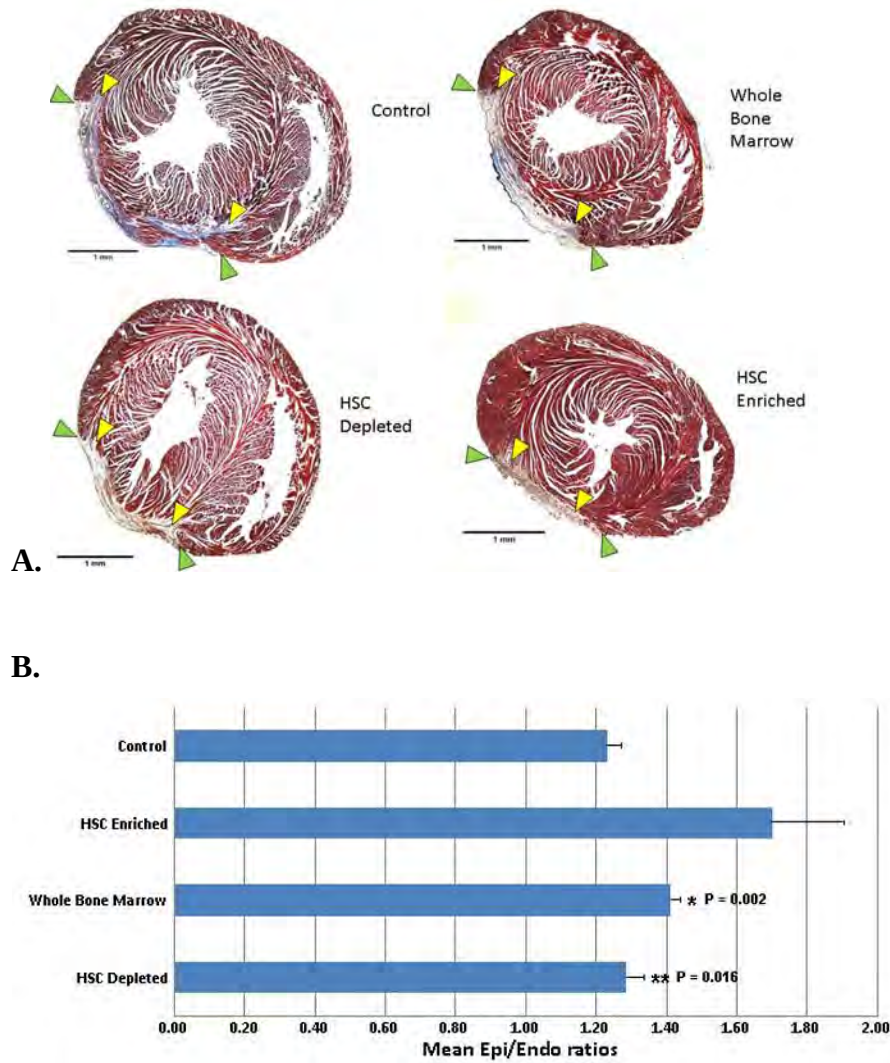


Figure 3. Analysis of infarct zone area and scarring in mice used to evaluate the capacity of bone marrow-derived stem cells to facilitate recovery from induced infarcts.

A. Sample images of Trichome stained heart sections for cohorts varying in number of HSC received. Green and yellow arrowheads delimit the length of the epicardial wall and endocardial wall respectively within the infarct zone. Trichome stain for collagen is revealed as the bluish-clear stained region.

B. Quantitative analysis of the size of the scarred region. ‘Epi’- epicardial wall, ‘Endo’- endocardial wall.

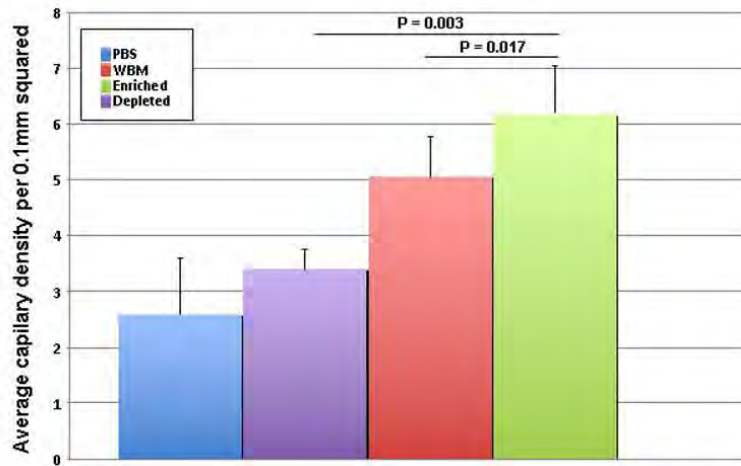


Figure 4. Analysis of re-vascularization within the infarct zone in mice used to evaluate the capacity of bone marrow-derived stem cells to facilitate recovery from induced infarcts. Capillaries were detected by Iso-lectin B4 staining, and the number of capillaries within the infarct zone were counted visually.

- b. ***Hypothesis: Will exposure of HSC to specific re-programming factors promote the ability of HSC to trans-differentiate into cardiomyocytes following transplantation into damaged heart tissue?***

To assess and compare the ability of induced cardiogenic cells, prepared by re-programming HSC to a cardiogenic fate, to facilitate recovery from induced infarcts relative to resting HSC, we developed an Nkx2.5 lentiviral expression vector. This vector is designed to introduce the expression construct into quiescent cells, including HSC, that are normally resistant to transduction by conventional viral expression systems, and to allow constitutive expression of the transcription factor Nkx2.5, known to be essential for cardiac development during mammalian embryogenesis. Using this vector, we were able to successfully transduce HSC, and optimize re-programming of HSC to the cardiogenic lineage, as assessed by staining for the cardiac specific marker Troponin, to achieve a re-programming efficiency of 1.5-2%, or 3000-6000 cells which we evaluated, based on previous studies, as having a potential therapeutic effect. In these experiments, all mice received samples of 250-300 thousand cells.

The re-programmed HSC samples, along with whole bone marrow, whole bone marrow enriched in HSC, or sham (no cells) control samples, were then used to treat mice following induced infarct. The relative ability of these samples to facilitate recovery of cardiac function and repair was then assessed by measurement of LVEF, cardiac wall thickness, amount of scar tissue and Iso-lectin staining, as described for hypothesis 1 and in the original grant proposal. The data is shown in figures 5-8, and indicate that, in our human → mouse model system, the re-programmed samples are capable of facilitating recovery from induced infarcts approximately as efficiently as the HSC-enriched samples. While still somewhat preliminary, we believe this observation is encouraging because we were able to achieve similar levels of recovery from induced cardiac infarct using 10 times the number of re-programmed HSC

(~3000-6000) per sample than the total HSC in the HSC-enriched samples (~100,000).

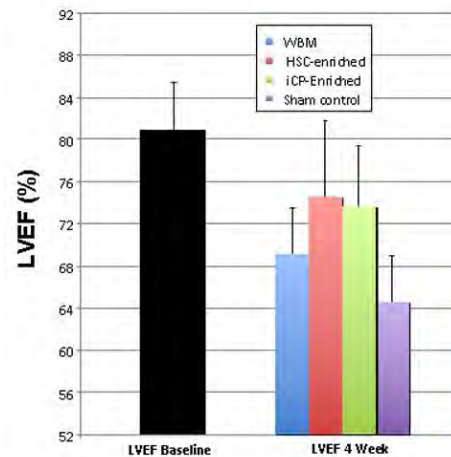


Figure 5. Analysis of left ventricular ejection fraction for mice used to evaluate the capacity of re-programmed stem cells to facilitate recovery from induced infarcts. The results for samples enriched in re-programmed cells are indicated as ‘iCP-enriched’ (iCP- induced cardiogenic progenitor).

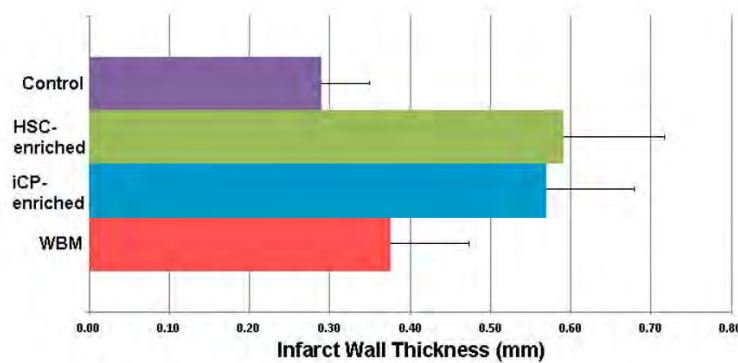


Figure 6. Analysis of cardiac wall thickness within the infarct zone in mice used to evaluate the capacity of re-programmed stem cells to facilitate recovery from induced infarcts. The results for samples enriched in re-programmed cells are indicated as ‘iCP-enriched’ (iCP- induced cardiogenic progenitor).

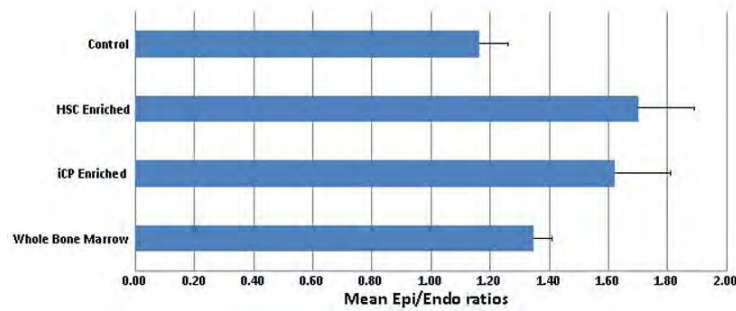


Figure 7. Analysis of infarct zone area and scarring in mice used to evaluate the capacity of re-programmed stem cells to facilitate recovery from induced infarcts.

Quantitative analysis of the size of the scarred region. Epi-epicardial wall, endo-endocardial wall. The results for samples enriched in re-programmed cells are indicated as 'iCP-enriched' (iCP- induced cardiogenic progenitor).

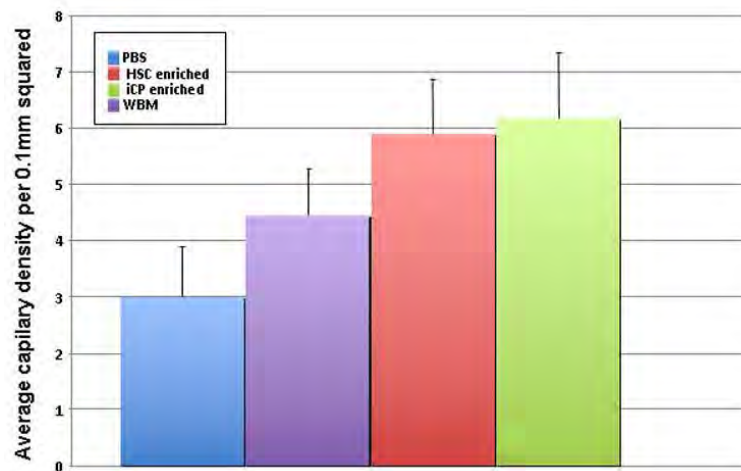


Figure 8. Analysis of re-vascularization within the infarct zone in mice used to evaluate the capacity of re-programmed stem cells to facilitate recovery from induced infarcts. Capillaries were detected by Iso-lectin B4 staining, and the number of capillaries within the infarct zone were counted visually. The results for samples enriched in re-programmed cells are indicated as 'iCP-enriched' (iCP- induced cardiogenic progenitor).

Key Research Accomplishments

1) Task 1. Complete all appropriate procedures with institutional review boards

- a) Protocols for this research has been submitted and approved by the following organizations.
 - i) University of Hawaii Animal Care & Use
 - ii) University of Hawaii Committee on Human Studies
 - iii) USAMRMC Animal Care and Use Review Office
 - iv) USAMRMC Office of Research Protections, Human Research Protection Office

2) Task 2. Conduct stem cell research for the treatment of congestive heart failure. Studies will examine the potential and mechanism of hematopoietic stem cells to treat damaged or diseased heart tissue

Hypothesis 1: Are hematopoietic stem cells (HSC) required to promote recovery in cell-based therapies that utilize bone marrow derived samples to treat myocardial infarct?

Highlight of major accomplishments

- c. Training of Dr. Allsopp's lab members in both the initial protocol and alternative protocol to induce myocardial infarct is complete. 40 C57BL6 mice were used to develop the model. (**completed in 2009**)
- d. Received high quality bone marrow samples from Hawaii Transplant Center, in sufficient quantity to serve as a source of HSC for the duration of this study (**completed Jan 2010**).
- e. Optimization of our stem cell sort protocol was performed with these samples was completed shortly after receiving these samples (**completed Feb 2010**).
- f. Completed all transplant experiments as described in the initial proposal (90 SCID mice) (**completed Dec 2010**). Requested an additional 50 SCID mice for additional experiments to amend for the high post-surgical mortality rate in the initial experiments performed.
- g. Completed all transplant experiments with the additional 50 SCID mice, and analyzed and interpreted all results (**completed March 2011**).

Hypothesis 2: Will exposure of HSC to specific re-programming factors promote the ability of HSC to trans-differentiate into cardiomyocytes following transplantation into damaged heart tissue?

- h. Completed the cloning of the human Nkx2.5 cDNA, and assembled the lentiviral expression vector to use in experiments to re-program HSC into cardiac progenitor cells in vitro (**completed in 2009**).
- i. Frozen aliquots of the packaged lentivirus have been prepared and stored for use (**completed in 2009**).
- j. Optimized protocol for efficient transduction and re-programming of HSC with the lentiviral expression construct (**completed summer 2010**).
- k. Completed all transplant experiments with the 40 SCID mice, and analyzed and interpreted all results (**completed March 2011**).

Conclusions

In accordance with the newly established Armed Forces Institute of Regenerative Medicine (AFIRM), our research aims to find innovative ways to treat heart disease. Regenerative medicine offers hope for better treatment and cures for chronic diseases, which will improve the lives of veterans and citizens. As proof, bone marrow transplantation is a well-established therapy for a variety of blood diseases; however, other applications using adult stem cells are still largely in the research phases. In order to demonstrate the safety and efficacy, extensive research is needed. Results of this research will benefit all citizens, including military retirees, recuperating from congestive heart failure.

Heart failure and heart disease are one of the most common afflictions of the elderly and are also one of the most common causes of both morbidity and mortality (Cosentino & Ostro, 2007). Therefore, the development of new methodologies to treat and improve prognosis of cardiac patients is highly desired. One of the key points of significance of this research is that the results could ultimately lead to the development of a novel and improved method to treat heart disease and ischemic damage. Relative to other stem cell-based therapies that have been proposed, for example procedures which rely on embryonic stem cells or cardiac stem cells, our procedure has the advantage of using stem cells that are readily available from the patients own bone marrow (Strauer, Brehm, & Schannwell, 2008). In addition, autologous transplantation of the patients' own HSC would eliminate the risk of rejection of the transplanted cells by the host immune system.

The results from this research suggest that the answer to the question posed in hypothesis 1 is yes, namely, HSC do play a very important role in facilitating recovery from cardiac damage in recipients receiving bone marrow-derived samples as therapy. Furthermore, the research results also suggest that the answer to the question posed in hypothesis 2 is also yes, namely, that cells re-programmed towards a cardiogenic fate are also capable of facilitating recovery from cardiac damage. Methods to both further improve the re-programming efficiency of stem cells toward the cardiogenic lineage as well as characterize the re-programmed cells in detail are desired in future efforts. Overall, the results are encouraging with respect to the ultimate goal of translating stem cell therapy and/or re-programmed cell therapy, to treat human cardiac patients.

References

- Balabis, J., Pobustsky, A., Kromer Baker, K., Tottori, C., & Salvail, F. (2007). *The Burden of Cardiovascular Disease in Hawaii 2007* Hawaii State Department of Health.
- Cosentino, F. & Osto, E. (2007). Aging and endothelial dysfunction. *Clinical Hemorheology & Microcirculation*, 37, 143-147.
- Strauer, B. E., Brehm, M., & Schannwell, C. M. (2008). The therapeutic potential of stem cells in heart disease. *Cell Proliferation*, 41, 126-145.