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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Heart disease is the leading cause of death in both the United States and Hawaii, including men and women of the Armed Forces. Furthermore, chest trauma in military men and women can lead to cardiac injury and cardiomyopathy. There is presently no good therapy for injured myocardium. Stem cells hold great promise in the field of regenerative medicine, and may be the key to developing new therapies to treat cardiac damage. Our prior study showed that blood stem cells can, to some extent, facilitate repair of cardiac damage in a mouse model of cardiac infarct. Adipose stem cells however, are more accessible and abundant than blood stem cells, and are multipotent (can produce several different tissues types other than adipose). The latter property may make directed re-programming of these cells into the cardiac lineage more efficient. The objectives for the present study are: (#1) Test the hypothesis that the multipotent adipose-derived stem cells will perform better than bone marrow-derived stem cells in facilitating recovery from heart disease. (#2) Test the hypotheses that cardiac-like progenitors, derived via re-programming of different types of stem cells, will further enhance the therapeutic potential of stem cell-based methodologies in the treatment of heart disease and (#3), the delivery of re-programming factors into stem cells using a transposase-mediated gene delivery system will enhance the re-programming efficiency of stem cells towards the cardiac lineage. The results to date have confirmed that adipose stem cells may be re-programmed into the cardiac lineage with an efficiency of ~25%, substantially higher than ~1% efficiency we had previously observed for blood stem cells. We are presently working an analysis of the relative potential of adipose stem cells and re-programmed adipose stem cells to facilitate recovery of induced infarcts in our murine model					
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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 et al, 2007). Many patients with endstage congestive heart failure reach a state where present medical therapy is not adequate to sustain acceptable cardiac function. Though this option is presently not available in Hawaii, an exciting new therapy that is emerging is the use of stem cell procedures (Assmus et al, 2006). 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. Adipose stem cells (ASC) have recently emerged as one of the most attractive types of stem cells for use in cell therapy (Gimble et al, 2011) because they are well characterized, are relatively easily purified, and are much more abundant than many other types of stem cells, including hematopoietic stem cells (HSC).

In our previous research, we specifically tested the relative importance of the HSC component of human bone marrow samples to facilitate repair from induced myocardial infarcts using a human to mouse transplantation model. We found that the degree of recovered cardiac function, as indicated by measurements of left ventricular ejection fraction (LVEF), and repair of damaged cardiac tissue post-induction of infarct, as measured by the reduction in size of the infarct area and the degree of re-vascularization of the infarct zone, positively correlated with the number of HSC in the transplanted sample

In this study, we proposed to examine the potential and mechanism of ASC to treat damaged or diseased heart tissue, by 1) testing whether highly purified human ASC samples perform better at facilitating recovery from induced infarcts than bone marrow derived HSC samples, (2) assess whether cardiac-like progenitors, derived via re-programming of different types of stem cells, will further enhance the therapeutic potential of stem cell-based methodologies in the treatment of heart disease, and 3), assess whether the delivery of re-programming factors into stem cells using a transposase-mediated gene delivery system will enhance the re-programming efficiency of stem cells into towards the cardiac lineage. These results should shed light on how important ASC 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 reprogramming of donor cells to a cardiogenic fate prior to transplantation, to treat damaged or diseased heart tissue. The technical objectives are to elucidate the following questions- 1) Are ASC a more therapeutically valuable source of stem cells to facilitate recovery from ischemic damage and heart disease in cardiac patients than bone-marrow derived stem cells; 2) Are induced cardiac-like cells, obtained by re-programming stem cells using specific transcriptional regulators, a better source of cells to use in transplantation therapies to treat ischemic damage and heart disease in cardiac patients than untreated stem cells ?; 3) Do

transposase-mediated methods of gene delivery allow for more efficient re-programming of cells into induced cardiac-like cells ?

BODY:

***TASK A** Test the hypothesis that the multipotent adipose-derived stem cells will perform better than bone marrow- derived stem cells in facilitating recovery from heart disease.*

*1. Add Principal Investigator (PI) to existing Institutional Review Board (IRB) protocol for collection of adipose specimens and get ACURO approval for the proposed animal work.
100% Complete*

Local University of Hawaii Animal Care & Use committee approved this protocol on February 17, 2012. Animal Care and Use Review Office (ACURO) approval was obtained June 22, 2011. The project was exempt from IRB approval as both ASC and HSC samples are obtained from anonymous donors. Project received HRPO approval on April 14, 2012.

*2. Train new technician, obtain additional cryo-preserved bone marrow (BM) samples, optimize FACS analysis and sorting of adipose-derived stem cell (ASC) samples.
90% complete*

New post doctoral fellow, Phil Davy, is now fully trained and proficient in the processes of

- i) purifying ASC using fluorescent activated cell sorting (FACS)
- ii) re-programming ASC towards a cardiogenic fate, and
- iii) performing the surgical procedure in mice to induce cardiac infarct using left anterior descending artery (LAD) ligation, and the intra-cardiac transplantation of cells.

A limited amount of good quality cryo-preserved BM samples has been obtained from the Hawaii Transplant Centre, however we are still awaiting the collection of additional samples. We have been waiting for additional BM samples for ~ 5months, and anticipate receiving an additional donor in the next 1-2 months.

*3. Perform comparative analysis of the effect of BM stem cell and ASC samples on recovery from induced infarcts in the murine transplantation model system
50% complete*

The transplantation of equal numbers (200,000) of bone marrow derived stem cells and ASC into severe combined immune deficient (SCID) mice has now been performed (initiated June 2012), and end point analysis of the results has begun. Three cohorts were included in the analysis, 2 receiving either HSC or ASC and 1 sham control group (no stem cells transplanted)(n=12 per group). The procedure, briefly, was to induce infarcts in the SCID mice using the LAD ligation technique, followed immediately by inter-cardiac injection of the stem cell samples directly into the site of infarct. End point analyses will be performed 1 month post-transplantation. Specifically, measurements of left ventricular ejection fraction (as assessed using echocardiography), infarct area, and number of new capillaries (capillaries will be detected by Isolectin B staining and counted) at the site of infarct will be compared between the 2 cohorts using Student's t Test.

4. Complete all data analysis and draft manuscripts to publish results

0% Complete

***TASK B** Test the hypotheses that (1) Cardiac-like progenitors, derived via reprogramming of different types of stem cells, will further enhance the therapeutic potential of stem cell-based methodologies in the treatment of heart disease and (2), the delivery of re-programming factors into stem cells using a transposase mediated gene delivery system will enhance the re-programming efficiency of stem cells into towards the cardiac lineage.*

1. Obtain and/or assemble expression vectors for all transcription factors to test in the stem cell-to-cardiogenic lineage re-programming protocol

100% Complete

The re-programming factor expression vector has now been assembled and tested. Specifically, we sub-cloned cDNAs encoding the cardiogenic re-programming factors Nkx2.5, Gata4, Mef2C and Tbx5 into the Piggy Bac transposase-based delivery expression vector, with the help of Dr. Moisyadi. Correct assembly of the vector was confirmed by DNA sequence analysis. The vector was then transfected into the ASC cells to verify, 48 hours post-transfection, that the re-programming factors were being expressed as predicted (based on RT-PCR analysis).

2. Optimize transduction and re-programming of BM stem cell (HSC) and ASC samples with the lentiviral and transposase-based system for stable gene expression in mammalian cells

90% complete

Optimization of re-programming of ASC cells is complete. For optimization of delivery of the re-programming vector, we tried both the Extreme Gene lipofection method (a technique to introduce DNA into cells using lipid vesicles which can easily merge with the cell membrane), electroporation (a technique where cells are briefly exposed to an electrical field which increases the permeability of the cell membrane to allow DNA to get into the cell), and Amaxa transfection method (which combines features of both lipofection and electroporation) to deliver the re-programming vector to ASC, and found that the Amaxa method was substantially superior in efficiency (>50% cells were transfected with Amaxa, as compared to 10% and <1% with lipofection and electroporation respectively). Using Amaxa, our preliminary data indicate are able to re-program ASC towards the cardiac lineage, based on troponin staining (troponin is an established marker of cardiomyocytes) at an efficiency of ~25%. Will plan to use the Amaxa technology to also deliver the re-programming vector into HSC. Optimization of HSC will commence Aug 2012. We consider this specific task to be ~90% complete as we put significantly more weight on the importance of the experiments using ASC than with HSC, as we ideally prefer to ultimately use ASC in any future therapies since they are more practical to work with.

3. Characterize the phenotype of the re-programmed BM and ASC samples. Perform comparative analysis of the effect of the re-programmed cell samples, derived from either BM stem cell or ASC samples, on recovery from induced infarcts in the murine transplantation model system

10% Complete

20 SCID mice have been ordered for this task, and have recently arrived (arrived July

2012).

4. Complete all data analysis and draft manuscripts to publish results

- 15% Complete

The construction of the re-programming vector and preliminary promising data on its potential use to re-program ASC towards the cardiogenic lineage has been reported at the annual ISSCR meeting in Yokohama Japan this summer (Jun 2012).

KEY RESEARCH ACCOMPLISHMENTS:

- Established local IACUC and ACURO approval for planned animal work
- Hired and trained a postdoctoral fellow who will perform the majority of the planned work, including cell re-programming and the surgical procedures of induced cardiac infarcts in mice and intra-cardiac transplantation of cells.
- Optimized re-programming of ASC towards the cardiogenic lineage, and showed that this process is ~10X more efficient for ASC than HSC.
- Completed transplantation of equal numbers of HSC and ASC into mice following induction of infarct, to assess the relative capacity of these cells to facilitate recovery (Task A).

REPORTABLE OUTCOMES:

We have developed a non-viral transposase-based delivery system vector to efficiently deliver re-programming factors, encoded in the vector, into cells, in particular, stem cells. We have also shown that, compared to HSC, ASC may be re-programmed towards the cardiogenic lineage at ~10X the efficiency.

CONCLUSION:

In summary, we believe stem cells, including ASC, hold promise for the development of a new method to treat cardiac infarcts that could potentially enhance the efficiency and degree of recovery of cardiac function. The utility of treatment methods using ASC and derived re-programmed cells also has the capacity to complement other present methods to treat cardiac infarcts (eg. balloon angiograms). Overall, we are on track for accomplishing both tasks for this study by the end of year 2. All proposed work has received the required ACURO and HRPO approval, personnel has been hired and trained, and ASC re-programming towards the cardiogenic lineage has been shown to be successful. In addition, transplantation experiments to test the ability of the ASC and re-programmed cells to facilitate recovery from induced infarcts have begun.

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Appendices

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REPLY TO
ATTENTION OF

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

June 22, 2011

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number 10214010, Award Number W81XWH-11-1-0457 entitled, "Analysis of the Therapeutic Potential of Stem Cells to Facilitate Recovery from Cardiac Disease and Damage"

Principal Investigator Richard Allsopp
University of Hawaii, Honolulu
Honolulu, HI

Dear Dr. Allsopp:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, protocol 10214010 entitled, "Therapeutic Evaluation of Hematopoietic Stem Cells for Cardiac Therapy," IACUC protocol number 09-673-3 is approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the University of Hawaii, Manoa IACUC.

When updates or changes occur, documentation of the following actions or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)
- USDA annual program/facility inspection reports
- Reports to OLAW involving this protocol regarding
 - a. any serious or continuing noncompliance with the PHS Policy;
 - b. any serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
 - c. any suspension of this activity by the IACUC
- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program

- AAALAC, International status change (gain or loss of accreditation only)

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: acuro@amedd.army.mil.

Sincerely,

A rectangular box containing a handwritten signature in black ink. The signature appears to be "Alec Hail". In the top right corner of the box, there is a small yellow square icon with a black question mark.

Alec Hail, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:

Ms. Catherine Henry, US Army Medical Research Acquisition Activity
Dr. Stanley M. Saiki, Jr., Tripler Army Medical Center
Ms. Brigit Ciccarello/MCMR-ZB-PH
Dr. Sylvia Kondo, University of Hawaii, Manoa
Mr. Mark Yabui, University of Hawaii, Honolulu

Classification: **UNCLASSIFIED**

Caveats: NONE

SUBJECT: Determination for the Proposal, "Analysis of the Therapeutic Potential of Stem Cells to Facilitate Recovery From Cardiac Disease and Damage," Submitted by Richard Allsopp, PhD, University of Hawaii, Honolulu, Proposal Log Number 10214010, Award Number W81XWH-11-1-0457, HRPO Log Number A-16859

1. The subject proposal and supporting documents received on 12 January 2012 with supporting documentation received in February in the U.S. Army Medical Research and Materiel Command, Office of Research Protections, Human Research Protection Office (HRPO) have been reviewed for applicability of human subjects' protection regulations.
 2. The research involves a comparison of the ability of human-derived hematopoietic stem cells (HSC) to adipose-derived stem cells (ADSC) to facilitate recovery of ischemic damage in a murine model of induced infarct. Human-derived cells will be obtained, engineered, and introduced into the model for *in vivo* testing. The HSC will be obtained from a human cadaver donated through the Life Legacy of Hawaii body donation program. The ADSC will be obtained from Tissue Genesis, Incorporated. No information that could be used to identify the donors of either the HSC or the ADSC will be provided with the cells.
 3. In accordance with 32 CFR 219.102(f), the HRPO determined that the project is research not involving human subjects, as it does not involve living individuals about whom an investigator conducting research obtains data through intervention or interaction with the individual or identifiable private information. The project may proceed with no further requirement for review by the HRPO. The HRPO protocol file will be closed.
 4. In the event that there is a change to the subject research or statement of work (SOW), the Principal Investigator must notify the Contracting Officer's Representative (COR)/Grant Officer's Representative (GOR) and send a description of the change to the HRPO at hrpo@amedd.army.mil referencing both the proposal log number and the HRPO log number listed in the "Subject" line above. The HRPO will re-open the protocol file if necessary.
- Any changes to the SOW that the COR/GOR determines could involve research with human subjects (as defined above) must be reviewed by the HRPO prior to approval by the Contracting Officer/Grants Officer.
5. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting officer regarding the expenditure of funds for your project.
 6. Further information regarding the award can be obtained by contacting the assigned Contract Specialist, Catherine Henry, at 301-619-1249.
 7. Further information regarding technical oversight can be obtained by contacting the assigned COR, Dr. Stanley Saiki.
 8. Further information regarding this review may be obtained by contacting Sarah L. Donahue, PhD, MPH, CIP at 301-619-1118 or Sarah.L.Donahue@us.army.mil.

LAURA R. BROSCH, PhD
Director, Office of Research Protections
Director, Human Research Protection Office
U.S. Army Medical Research and Materiel Command

Therapeutic Application of Human Adipose Stem Cells to Repair Cardiac Damage and Disease

P. Davy, R. Allsopp

Institute for Biogenesis Research, University of Hawai'i, John A. Burns School of Medicine, Honolulu, HI, USA

ABSTRACT

Stem cell therapy for myocardial infarction offers the potential for enhanced restoration of the loss of heart function associated with cardiac disease and infarcts that affects millions of people each year. Adipose stem cells (ASC) in particular represent a bountiful and readily accessible source of autologous adult stem cells with demonstrated capacity for cardiac lineage differentiation¹.

We have previously demonstrated that the CD34⁺ hematopoietic stem cells (HSC) in human bone marrow are responsible for the regenerative revascularization of bone marrow aspirate transplanted into infarcted hearts. We directly compared the efficacy of HSC with ASC to test the hypothesis that the latter offer greater therapeutic potential. Human adult liposarcoma obtained through Tissue Genesis, Inc. was shown to contain a population of CD90⁺, CD105⁺, CD45^{neg}, CD31^{neg} plastic-adherent cells capable of proliferating for multiple passages. These cells effect improvement in cardiac output and reduce scarring following transplantation into SCID mice directly after induced myocardial infarction. Driving the differentiation of ASC towards cardiac lineages prior to transplantation may increase the efficacy of ASC myocardial treatments. A transposon vector for the constitutive expression by the cmv promoter of the three early cardiac lineage genes *GATA4*, *MEF2C*, and *TBX5* (GMT) and a GFP transfection indicator was constructed and transfected into freshly processed human liposarcoma and stable ASC lines derived from the same. GFP expression analysis by FACS showed transfection efficiency of around 50%. Following 14 days of culture in cardiomyocyte media, the transfected cells showed expression of cardiomyocyte-specific proteins cardiac-troponin T and alpha-actinin by immunocytochemical staining.

INTRODUCTION

Adipose tissue is an accessible source of stem cells for cell therapy that may be abundant enough to circumvent the need for in vitro expansion for autologous application². Pertinent to their use in cell therapy, ASC have angiogenesis-promoting properties³ and can differentiate into beating cells that express cardiac-specific proteins when co-cultured with cardiomyocytes⁴. Both freshly isolated and cultured ASC have been demonstrated to enhance cardiac function following infarction⁵. Evidence suggests that driving the differentiation of stem cells towards cardiac lineages prior to transplantation improves the benefits of stem cell therapy⁶. Expression of GMT has previously been shown to reprogram fibroblasts to cardiomyocytes⁷. We are attempting to direct the differentiation of ASC towards the cardiac lineage by the CMV-driven expression of GMT from a transposon insertion cassette. The final goal of this project will be the determination of the therapeutic value of partially or terminally differentiated cells derived from ASC.

METHODS

For this project human adipose stromal cells containing stem cells (ASC) were obtained from liposarcoma processed by the Tissue Genesis iCellator Cell Isolation System. The efficacy of these cells were compared to bone marrow lineage-negative, HSC-enriched cells in the mouse LAD infarct model with 2.5×10^5 cells/heart. Heart function was assessed prior to infarction (baseline) and 30 days after treatment by echocardiography. Mason's trichrome and isolectin B4 staining were utilized to determine the area of infarction and capillary density respectively. Immunocytochemistry with troponin-T and alpha-actinin specific antibodies was performed on ASC cells 14 days after Fugene6-mediated transfection with the transposon vector GENIE3 containing the GMT genes.

Figure 1. Enrichment of the Lineage negative – hematopoietic stem cell (HSC) enriched – cell population in human bone marrow (BM) treatment of myocardial infarction increases left ventricular ejection fraction compared to unadjusted BM and controls (* $P < 0.05$) [A]. ASC-enriched stromal cells show similar properties (* $P > 0.05$) to HSC-enriched BM treatment of myocardial infarction in recovery of ejection fraction [B]. Left ventricular remodeling as assessed by percentage of the left ventricle scarred showed no difference ($P > 0.05$) between ASC- and HSC- enriched treatments [C]. The capillary density of the peri-infarct areas suggests ASC-enriched and HSC-enriched cell treatments have similar revascularization capacities ($P > 0.05$) [D].

Figure 2. To create stable transfection of ASC with the GMT genes a transposon vector was created from the piggyBac plasmid GENIE3. Constitutive expression of *GATA4*, *MEF2C*, and *TBX5* is driven by the CMV promoter, as is the reporter gene eGFP [A]. Standard liposomal transfection of cultured ASC was performed with Fugene6. 48 hours after transfection fluorescence assisted cell sorting analysis was used to determine the percentage of GFP positive cells. Transfection efficiency was approximately 50% [B].

Figure 3. Co-staining with antibodies to cardiac Troponin T (cTnT) and cardiac alpha-actinin (ACTN1) showed differentiation of ASC towards cardiomyocytes 14 days after transfection with pmGENIE3-GMT [A]. No positive staining was observed in non-transfected ASC cultured under the same conditions [B].

CONCLUSIONS AND FUTURE DIRECTIONS

- Freshly isolated adipose stromal cells containing hASC have similar impact on heart function and remodeling as HSC-enriched BM (Fig 1.)
 - hASC can be transfected at high efficiency by standard liposomal methods (Fig 2B.)
 - hASC expressing GMT express cardiac-specific proteins with sarcomeric structure after 14 days of culture (Fig 3A.)
- Determine differentiation endpoint for successfully transfected cells.
 - Compare therapeutic efficacy of transfected hASC to nontransfected hASC and freshly isolated adipose stromal cells.

ACKNOWLEDGEMENTS

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S. Moisyadi, PhD for the pmGENIE3 plasmid
A. Chow and L. Wong for data analysis. K. Lye, MD for review
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