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14. ABSTRACT

Many veterans suffer from ischemic cardiomyopathy partly resulting from exposure to Agent Orange. Due to the limited supply of donor hearts and potential complications from chronic immunosuppressive therapy, investigators have turned to therapeutic approaches aimed at improving myocardial function, namely, cell transplantation. The purpose of this proposal is to generate non-immunogenic human embryonic stem cell-derived left ventricular cardiomyocytes as a potential off-the- shelf candidate for cardiac cell transplantation. Success of this proposal would address the increasing health burden of veterans suffering from ischemic cardiomyopathy. A non-immunogenic universal donor cell line (Elf1) was previously developed to bypass host immune recognition and response. We have made progress in expanding and characterizing the pluripotency of this cell line. We confirmed that these cells express high levels of pluripotency markers indicative of their stem cell state. We are currently transitioning them from a feeder to feeder-free system as well as switching from
Naïve to Primed state that is required for our cardiac differentiation.

15. SUBJECT TERMS
Discovery - Tissue regeneration

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TABLE OF CONTENTS

1. Introduction

Human embryonic stem cells (hESCs) can be used as an unlimited source for generation of tissue specific cell types for the purpose of organ transplantation. This approach offers hope for millions of people who suffer from end stage heart failure, in whom damage has led to irreversible loss of cardiomyocytes, or the essential contractile muscle cells of the heart. Experimental studies, including preclinical studies in large animal models, have generally been encouraging, although the functional benefits that have been attained are modest and inconsistent. The efficacy of current strategies is limited by many factors, but perhaps a significant hurdle to the progress in this field is the immunogenicity of the hESC-derived cardiomyocytes upon transplantation. It is widely accepted that expression of human leukocyte antigens (HLAs), encoded by genes in the major histocompatibility complex (MHC), creates an immunologic barrier that hinders survival of transplanted cells. In particular, HLA class I molecules play a central role in allogeneic rejection through their presentation of peptide antigens to CD8+ T cells. To circumvent the immunogenicity of hESC-derived cells, a non-immunogenic hESC universal donor line (MHC I-/- Elf1 hESC cell line) was developed through concomitant overexpression of HLAE protein, a natural inhibitor of NK cell-dependent lysis2. In this proposal we have utilized this genetically engineered cell line to generate non-immunogenic hESC-derived left ventricular CMs as a potential off-the-shelf candidate for cardiac cell transplantation.

2. Keywords

Human embryonic stem cell, left ventricular cardiomyocyte, cardiac cell transplantation, non-immunogenic cell line, regenerative therapy, myocardial infarction, oxygen-generating hydrogel

3. Accomplishments

What were the major goals of the project?

Major Goal 1: To optimize differentiation protocol for the enrichment of left ventricular CMs

i. Optimization of non-immunogenic hESC universal donor line cardiac differentiation for First Heart Field
ii. Optimization of non-immunogenic hESC universal donor line cardiac differentiation for Ventricular like CMs

Major Goal 2: To characterize structural and functional properties of HLAE-LV-CMs using biochemical and electrophysiology studies

i. Characterization of CMs-derived from universal donor hESC line based of specific genes expression and performing Immunocytochemistry
ii. Electrophysiological studies to characterize CMs-derived from universal donor hESC line.

What was accomplished under these goals?

During the first 12 months of this grant period, we have made significant progress in accomplishing the aims specified in this grant. The major activities in the first 12 months includes the following:

1. We have obtained the non-immunogenic hESC universal donor line (MHC I-/- Elf1 hESC cell line) from our collaborators.

2. These cells have cultured on mouse embryonic fibroblasts (MEFs) and we have successfully transitioned them to a feeder-free system to avoid contamination with any other cell types.

3. We have used another control hESC line (H9) in parallel for comparison purposes.

4. We have established a differentiation protocol for generation of first heart filed (FHF)-specific cardiomyocytes from differentiating hESCs. This has been a major accomplishment since we now are capable to get a highly pure population of FHF cells that preferentially differentiate to left ventricular cardiomyocytes.

5. We have successfully generated FHF cardiomyocytes and have characterized their identity using immunostaining, gene expression, and electrophysiology studies.

We have made progress on Major Goal 1 in expanding and characterizing the pluripotency of this cell line. We received one vial each of Elf1 and Elf1 MHCI$^{-/-}$ HLAE$^{+/+}$ from Dr. David Russell. These cells had been maintained on a feeder system using mouse embryonic fibroblast (MEF). We have expanded these cells on MEF to obtain the adequate number of cryovials needed for this project. This task took us longer than anticipated and will be discussed under the "problem" section below. Figure 1 shows brightfield image of an Elf1 cell cluster (Fig. 1A). From the cells that we were able to expand, we assessed their pluripotency via immunocytochemistry (ICC) using Nanog, Sox2, and Oct3 antibodies (Fig. 1B).

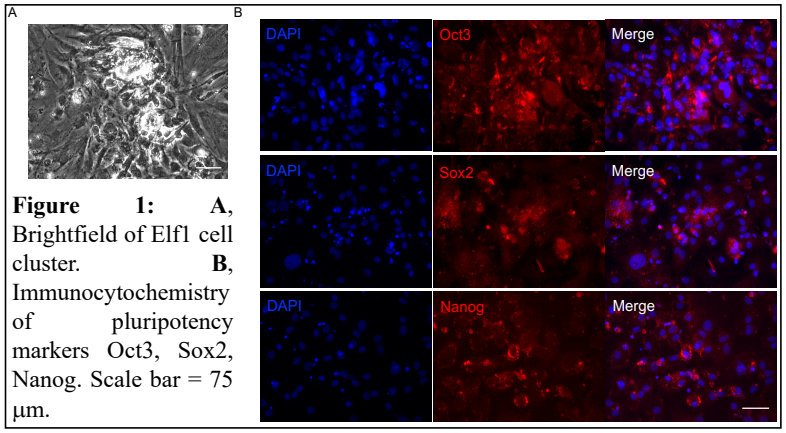

Figure 1: A, Brightfield of Elf1 cell cluster. **B**, Immunocytochemistry of pluripotency markers Oct3, Sox2, Nanog. Scale bar = 75 µm.

For successful cardiac differentiation of these cell lines, two important criteria are worth mentioning. First, these cells need to be transitioned from MEF feeder system onto feeder-free system such as Geltrex or Matrigel. To obtain the purified Elf1 population we needed to undergo several passages of the cells to gradually eliminate the MEFs from the cell culture. After this process we also need to transition these cells from the normal Elf1 growth media into mTeSR media which is used for maintaining pluripotency. Second, we were informed that these Elf1 cell lines are in the Naïve state. Studies have shown that differentiation of Naïve cells is challenging and inefficient. As this project requires a high number of cardiomyocytes for transplantation, we decided it was necessary to induce them to a primed state for more efficient cardiac differentiation. To promote efficient differentiation of hESCs towards LV-CMs required in Major Goal 1, it is necessary to elucidate and control signaling pathways that regulate their formation during embryonic development. Recent studies have

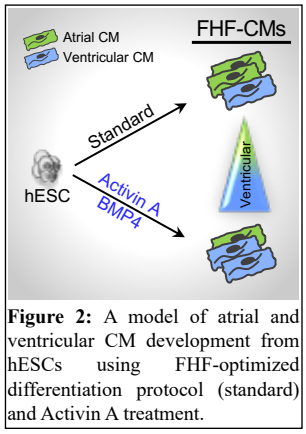

Figure 2: A model of atrial and ventricular CM development from hESCs using FHF-optimized differentiation protocol (standard) and Activin A treatment.

demonstrated that a tight regulation in the Activin/Nodal pathway is required for ventricular specification during embryonic development (Fig. 2). We are interested to determine whether addition of Activin A to our optimized FHF differentiation protocol will shift CMs toward a ventricular phenotype. If this is the case, we can then apply Activin A to the cardiac differentiation protocol that we will use for the Elf1 cell line. We used our previously generated Hes3 TBX5$^{TdTomato/W}$/NKX2-5$^{eGFP/W}$ reporter line to test varying concentrations of Activin A. The reporter line was grown on Geltrex to 90% confluency then harvested as single cell suspension and resuspended in mTeSR1 containing 10μM ROCK inhibitor. Cells were replated onto Geltrex coated plates at 1.3 x 10^5 cells/cm^2 and undergo differentiation as outlined in the diagram (Fig. 3A). These cells were treated with varying concentrations of Activin A to achieve the highest efficiency of cardiac differentiation into FHF CMs, as determined by co-expression of TBX5 and NKX2-5. Fig. 3B). The highest cardiac differentiation was achieved using 10 ng/ml Activin A. We then sorted these cells for gene expression analysis for ventricular markers (Fig. 3C). These results recapitulated observations developmental biology, where adjustment in Activin supplementation during early stages of differentiation resulted in generation of ventricular CMs.

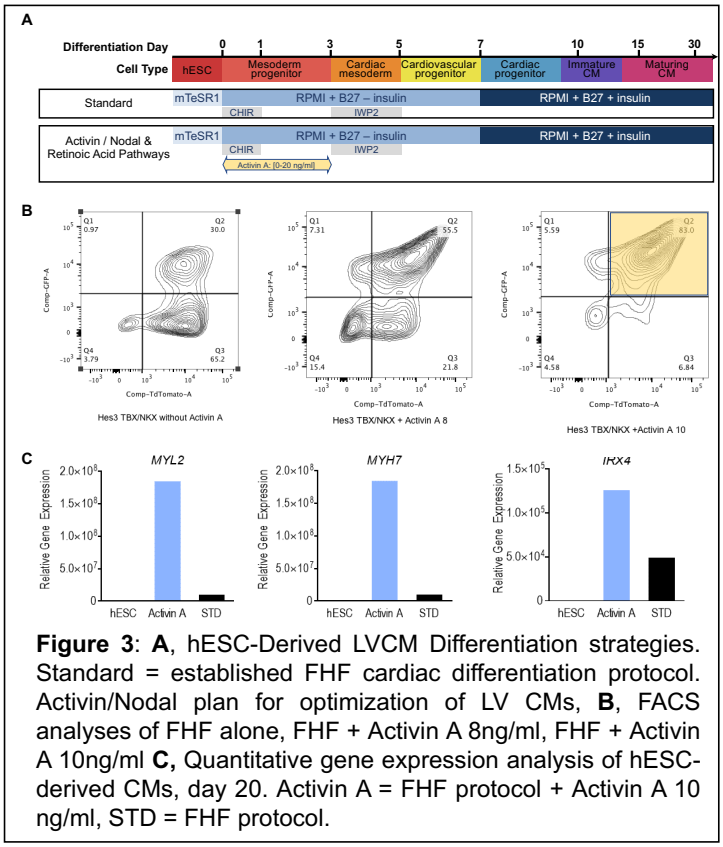

Figure 3: **A**, hESC-Derived LVCM Differentiation strategies. Standard = established FHF cardiac differentiation protocol. Activin/Nodal plan for optimization of LV CMs, **B**, FACS analyses of FHF alone, FHF + Activin A 8ng/ml, FHF + Activin A 10ng/ml **C,** Quantitative gene expression analysis of hESC-derived CMs, day 20. Activin A = FHF protocol + Activin A 10 ng/ml, STD = FHF protocol.

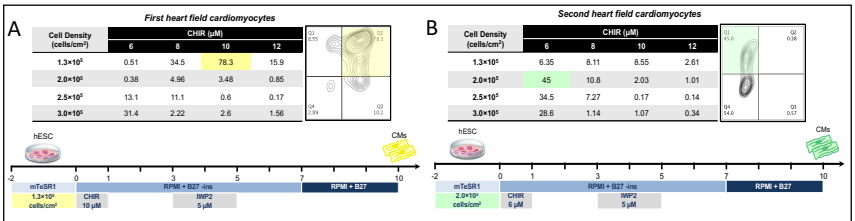

Figure 4: **A**, Optimization of FHF and **B**, SHF CM populations via small-molecule modulation of Wnt signaling and cell seeding densities. Flow cytometric analysis at Day 10 of cardiac differentiation showing the expression of TBX5 and NKX2-5. Column represents varying seeding densities and row shows varying concentrations of CHIR99021 (CHIR)

Other Achievement

Our differentiation protocol for generation of FHF-cardiomyocytes also shed light as to how hESCs can be used as an in vitro model for cardiac development. By altering the seeding density of the starting hESCs and the combination of cytokines, we were able to generate second heart field (SHF) cardiomyocytes in addition to FHF-cardiomyocytes. We optimized the differentiation conditions via altering seeding density and concentration of GSK-3β inhibitor (CHIR99021) to efficiently generate FHF- or SHF-cardiomyocytes. Using a plating density of 1.3x10^5 cells/cm^2 with 10μM CHIR99021 or 2.0x10^5 cells/cm^2 with 6μM CHIR99021 in the differentiation protocol (Figure 4 A-B) we were able to efficiently generate TBX5$^+$NKX2-5$^+$ (76.7 ± 1.3%) and TBX5$^-$NKX2-5$^+$ cells (41.5 ± 1.8%) respectively, as indicated by fluorescence microscopy, spontaneous beating, and flow cytometry analysis at day 10 of differentiation (Figure 5).

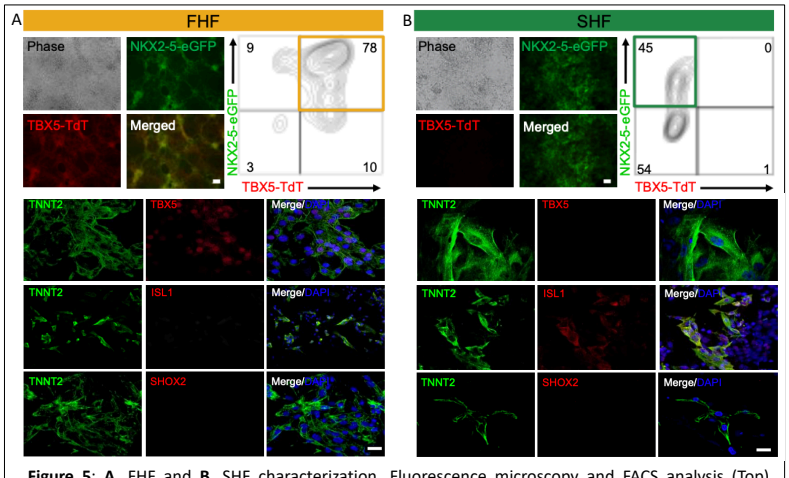

Figure 5: **A**, FHF and **B**, SHF characterization. Fluorescence microscopy and FACS analysis (Top). Immunocytochemistry analyses (Bottom).

Using our double reporter line to optimize the FHF and LV cardiomyocyte helped us to establish the protocol that can be used in differentiating of our Elf1 MHCI$^{-/-}$HLAE$^{+/+}$ line.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
This research has allowed us to generate heart-field specific cardiomyocytes from pluripotent stem cells, which is a necessary step for future successful cell therapy to treat heart disease.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. Changes/Problems

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

We have made progress in expanding and characterizing the pluripotency of this cell line, however, this took us longer than anticipated given the following reasons: 1) These non-immunogenic cell lines are very slow growing, 2) Transition of these cells from feeder to non-feeder system that is needed for our cardiac differentiation requires a systematic and gradual approach to obtain a pure population of Elf1 cells which is also time consuming. A potential reason why these cells have been growing slowly is they may have been cryopreserved for an extended period of time and were slow to recover. We expected to passage these cells every three to four days, however, it has been taking about twice as long for the cells to reach confluency. For the same reason, the process of transitioning of these cells off MEFs onto Geltrex have been taking longer than anticipated. If the slow growth of these cells hinders the progress of this project, we will purchase these cell lines from WiCell company (lot numbers: WB17042, WB67154). For the moment, we will continue to work on these cells to minimize the unnecessary expenditures.
Research Ramp-Down/Suspension Due to COVID-19: During this funding period, we have had to ramp down research due to the COVID pandemic. For this reason, our lab was shut down completely from March 23 – June 8. As of June 8, our lab has received approval for research ramp-up (phase 2) during which, we have not still had access to our Core Facilities (Flowcytometry, Confocal Microscopy, and surgery rooms). I am now focused on getting the in vitro assays. Due to the limited access to our FACS core facility during the Covid ramp-down we used our double reporter cell line to establish the LV-CM protocol by which we will be able to differentiate our Elf1 cell line into left ventricular cardiomyocyte.

March 23 – June 8, 2020: Essential personnel only, remote learning, no experiments could be done.
June 8 – present: Phase II (10-25% operation, limited core facility/services)

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report for all categories

6. Products

Nothing to report for all categories

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Arash Pezhouoman
Project Role:	Assistant Project Scientist III
Researcher Identifier (e.g ORCID ID):	0000-0001-9106-7136
Nearest person month worked:	3
Contribution to Project:	Cell expansion and transition, FHF and Left Ventricular cardiomyocyte optimization.
Funding Support:	DOD (PR182456), R01(HL148714-01), BSCRC

Name:	Peng Zhao	
Project Role:	Adjunct Assistant Professor	
Researcher Identifier (e.g ORCID ID):	0000-0002-6610-6217	
Nearest person month worked:	2.28	
Contribution to Project:	Cell expansion, Pluripotency assessment IHC staining	
Funding Support:	DOD (PR182456), R01(HL148714-01)	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

8. SPECIALREPORTINGREQUIREMENTS

N/A

9. APPENDICES

N/A