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CONTRACTING ORGANIZATION: University of California, Los Angeles

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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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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 (CMs), 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 CMs 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 Task 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 Task 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 Major Task 1 specified in this grant. The major activities in the **first** 12 months includes the following:

a) We have obtained the non-immunogenic hESC universal donor line (MHC I-/- Elf1 hESC cell line) from our collaborators.
b) 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.
c) We have used another control hESC line (H9) in parallel for comparison purposes.

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

During the **second 12 months** of this grant period, we have made significant progress in accomplishing Major Task 2, as follows:

a) As we had faced challenges in expanding our prior cell line, which resulted in significant delay in our progress to differentiate Elf1 hESCs into cardiomyocytes, we ordered a new Elf1(B2M-/Etrimer) cell line from WiCell.
b) The new Elf1 cells have been cultured on mouse embryonic fibroblasts (MEFs) and transitioned to a feeder-free system to avoid contamination with mouse fibroblast cells.
c) The new Elf1 cells have been expanded and differentiated into beating CMs in a monolayer fashion using our previously established first heart field (FHF) cardiac differentiation protocol.
d) The Elf1-derived CMs have been collected and characterized based on expression of cardiac-specific genes such as *TNNT2* and *ACTN2*.
e) The Elf1-derived CMs have been isolated and stained based on cardiac-specific protein (TNNT2).
f) Electrophysiological studies were completed on Elf1-derived CMs using voltage dye and cardiac-specific action potentials (APs) were recorded using MICAM Ultima.

We have made progress on Major Task 2 in expanding and characterizing the pluripotency of the new cell line. We purchased the Elf1(B2M-/Etrimer) from WiCell company. This cell line is the same as the one we received from Dr. Russell's lab. These cells had been maintained on a feeder system using MEFs. We have expanded these cells on MEF to obtain the adequate number of cryovials needed for this project. For successful cardiac differentiation of Elf1 cell line, two important criteria are worth mentioning. First, these cells need to be transitioned from MEF feeder system onto a feeder-free system such as Geltrex. To obtain the purified Elf1 population, the cells underwent several passages to gradually eliminate contaminating MEFs. After this process, we transitioned these cells from the normal Elf1 growth media into mTeSR media which is used for maintaining pluripotency. Figure 1 shows brightfield image of a new Elf1 cell cluster that we were able to expand and have assessed for pluripotency markers SOX2 and OCT4 via immunocytochemistry (ICC) (Figure 1).

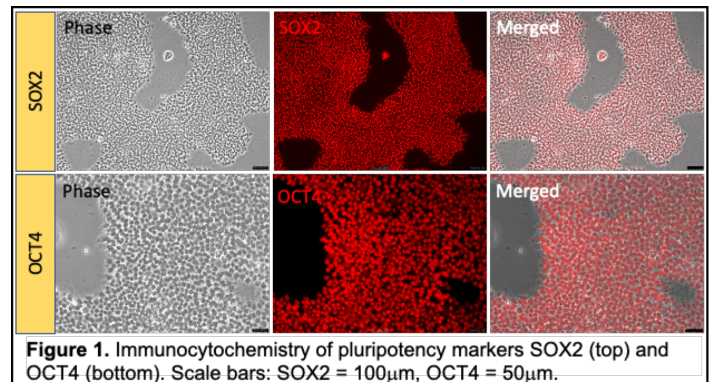

Figure 1. Immunocytochemistry of pluripotency markers SOX2 (top) and OCT4 (bottom). Scale bars: SOX2 = 100μm, OCT4 = 50μm.

For expansion, the new ELF1 cell line was grown on Geltrex to 90% confluency then harvested as single cell suspension and resuspended in mTeSR1 containing 10 μM ROCK inhibitor. Cells were replated onto Geltrex coated plates at 1.3 x 10^5 cells/cm^2 density. Cells were kept for 3 days prior to the addition of small molecules CHIR and IWP2 for induction of cardiac differentiation, as outlined in the diagram (Figure 2A). This differentiating strategy yielded clusters of Elf1-derived CMs, which started beating about 12-15 days after cardiac

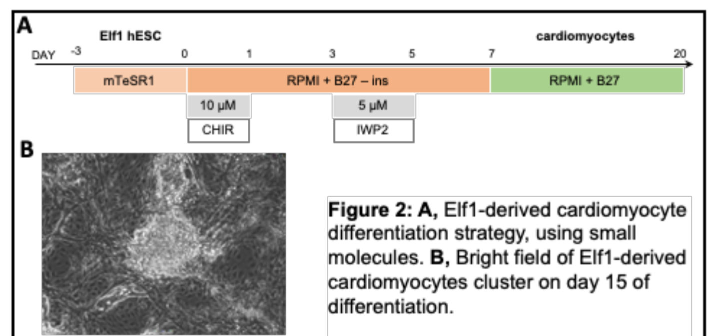

Figure 2: A, Elf1-derived cardiomyocyte differentiation strategy, using small molecules. **B,** Bright field of Elf1-derived cardiomyocytes cluster on day 15 of differentiation.

induction (Figure 2B and video 1). As expected, qPCR analysis confirmed significantly higher expression of cardiac structural genes *TNNT2* and *ACTN2* in Elf1-derived CMs compared to undifferentiated cells (Figure 3). ICC analysis of isolated Elf1-derived CMs support the gene expression results and confirm their CM phenotype with positive staining of TNNT2 (Figure 4). Interestingly, these Elf1-derived CMs exhibited sarcomeric organization, as seen by striations of TNNT2 on high power magnification (Figure 4 inset). Finally, electrophysiological properties of Elf1-derived CMs were analyzed using optical mapping. Differentiated cells were stained with FluVolt dye (voltage dye) and optically mapped and typical ventricular cardiac APs (with plateau) were recorded.

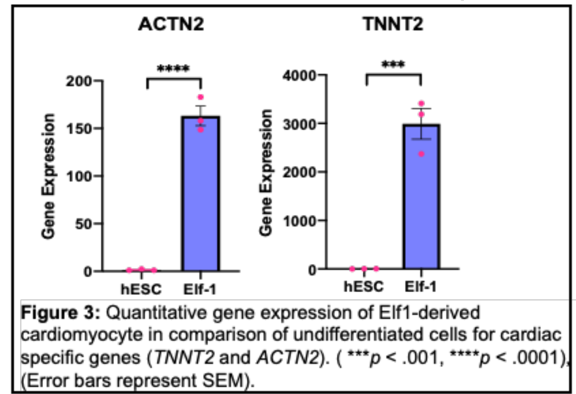

Figure 3: Quantitative gene expression of Elf1-derived cardiomyocyte in comparison of undifferentiated cells for cardiac specific genes (*TNNT2* and *ACTN2*). (***$p < .001$, ****$p < .0001$), (Error bars represent SEM).

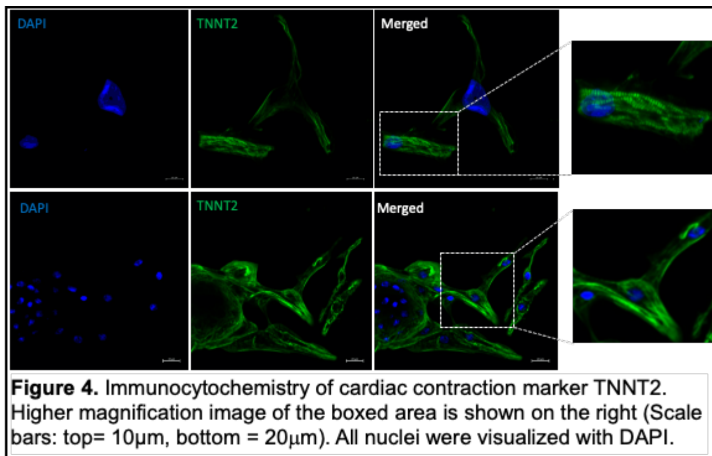

Figure 4. Immunocytochemistry of cardiac contraction marker TNNT2. Higher magnification image of the boxed area is shown on the right (Scale bars: top= 10µm, bottom = 20µm). All nuclei were visualized with DAPI.

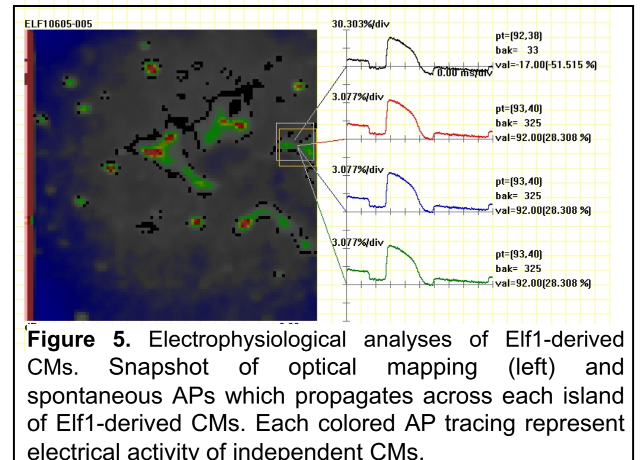

Figure 5. Electrophysiological analyses of Elf1-derived CMs. Snapshot of optical mapping (left) and spontaneous APs which propagates across each island of Elf1-derived CMs. Each colored AP tracing represent electrical activity of independent CMs.

In summary, within the prior funding period, we have made tremendous progress in Major Task 2 of this grant. We were able to obtain a new Elf1 cell line that was able to expand more effectively and be differentiated into functional CMs based on molecular and functional studies. In the remaining funding period, we plan to further optimize the differentiation efficacy of this new cell line for *in vivo* transplantation and immune infiltration studies, as described in Major Tasks 3 and 4 of this grant.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
This research has allowed us to generate potential universal donor cardiomyocytes from pluripotent stem cells, which is an important step for a safe and successful cell therapy for regenerative medicine.

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

As described in the Accomplishments section above, we faced challenges in expanding and differentiating the original Elf1 cell line received from Dr. Russell's group. Fortunately, by purchasing the new Elf1 cell line from WiCell we were able to expand and differentiate the ELF1 cells into CMs. However, an additional requirement for cell transplantation is achieving high differentiation efficacy that would enable isolation of adequate number of cells needed for cell transplantation. To overcome this obstacle, we will optimize the efficiency of our protocol by changing the initial cell seeding density before starting the cardiac induction and modifying the CHIR concentration during the first day of cardiac induction. The CMs from the optimized cardiac protocol will be characterized for cardiac-related genes and proteins.

During this past funding period, we continued to have research restrictions due to COVID-19, with limited access to Core Facilities. The primary investigator was also infected with COVID-19 and was away from work for 1.5 months.

June 2020 – Jan 2021: 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

Publications, conference papers, and presentations

1. **Arash Pezhouman**, James L. Engel, Ngoc B. Nguyen, Rhys J.P. Skelton, W. Blake Gilmore, Debashis Sahoo, David A. Elliott, Peng Zhao, and Reza Ardehali; Isolation and characterization of hESC-derived heart field-specific cardiomyocytes unravels new insights into their transcriptional and electrophysiological profiles; Cardiovasc Res. 2021 Mar 21:cvab102. doi: 10.1093/cvr/cvab102. PMID: 33744937; Published; Acknowledgment of federal support (yes).
2. **Arash Pezhouman**, Ngoc B. Nguyen, Allison Shevtsov, Rong Qiao, Reza Ardehali; *In vitro* characterization of heart field specific cardiomyocytes. *Method in Molecular Biology*; accepted; acknowledgment of federal support(yes).

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Arash Pezhouoman
Project Role:	Associate Project Scientist I

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)

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, surgery for MI
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