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CONTRACTING ORGANIZATION: Johns Hopkins University

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6. AUTHOR(S)

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cardiomyopathy (heart muscle disease) is a leading cause of heart failure, and congenital heart disease often leads to heart failure in adults. Heart transplantation remains the gold standard for treating patients with advanced heart failure, and many people desperately need a donor heart. For this reason, there are ongoing trials to create artificial hearts with tissue engineering approaches. However, a number of challenges remain to be overcome due to the complexity of the real heart. This proposal aims to overcome the complexity by utilizing the machinery present in a developing organism with cells that serve as building blocks to make the heart during fetal development. The successful demonstration of generating such hearts will provide unprecedented insights into generating transplantable hearts from patients' own cells.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction

Heart transplantation remains the gold standard for treating patients with advanced heart failure. However, hearts are in short supply, and many people desperately need a donor heart. Moreover, transplant rejection, which occurs even in recipients with closely matched organs, continues to be the most fearful obstacle to the success of transplantation. The main goal of this proposal is to establish a proof of concept to make donor-specific hearts by in-vivo engineering. We have developed novel techniques to generate heartless embryos by suppressing the formation of embryonic cardiac progenitor cells—the building blocks to generate the heart during fetal development—and to reconstruct their hearts with foreign embryonic stem cells-derived cardiac progenitor cells by somatic mosaicism. Based these findings, we formulated the overall hypothesis that pluripotent stem cell-derived cardiac progenitor cells can be used as donor cells to generate make donor-specific hearts. To test this, we will determine if induced pluripotent stem cells can be used as donor cells to generate a donor-specific heart in a recipient of different species and if cardiac progenitor cells can be directly used as donor cells in generating a donor-specific heart.

2. Keywords

Pluripotent stem cells, Cardiac progenitor cells, Cardiac differentiation, Blastocyst complementation, Somatic mosaicism, Cardiogenesis

3. Accomplishments

a. **What were the major goals of the project?**

Aim 1	**Timeline**	**Completion**
Major Task: To determine if iPSCs can be used as donor cells to generate a donor-specific heart in a recipient of different species.	Months	
Subtask 1: To generate and label iPSCs	1–3	100%
Subtask 2: To optimize cardiac differentiation	4–6	100%
Subtask 3: To generate mutant blastocysts	7–12	100%
Subtask 4: To analyze contributions of donor cells	13–18	0%
Milestone(s): We expect to generate iPSC lines and confirm their cardiac differentiation in the first 6 months. We will focus on generating mutant cells for the next 6 months. Blastocyst complementation experiments will be conducted afterward, and we will analyze the donor-derived cells by 18 months.		
Aim 2	**Timeline**	**Completion**
Major Task: To determine if CPCs can be directly used as donor cells in generating a donor-specific heart.	Months	
Subtask 1: To generate and label CPCs from PSCs	13–18	100%
Subtask 2: To analyze contributions of donor CPCs	18–24	100%
Milestone(s): We expect to isolate CPCs at different stages from 13–18 months. Blastocyst complementation experiments will be conducted afterward, and we will analyze the donor-derived CPC development until the end of the funding period.		

b. **What was accomplished under these goals?**

- Major Activities: During the final (no cost extension) year of support, we injected fluorescent protein-labeled mouse cardiac progenitor cells (CPCs) and embryonic stem cells (ESCs) into blastocysts and analyzed whether they contribute to the developing embryos.

- Specific Objectives

(1) To generate mutant embryos with defective heart formation

(2) To generate cardiac progenitor cells (CPCs) derived from pluripotent stem cells (PSCs)

(3) To isolate and inject PSC-derived CPCs into mouse blastocysts

- Significant Results/Key Outcomes: To test whether lineage committed cells can serve as donor cells for blastocyst injection and contribute to organ formation, we conducted experiments injecting either mouse ESCs or CPCs into the E3.5 blastocysts and analyzed the trace of the injected cells at E9.5. In the first two experiments with CPC injection only, we were not able to detect any RFP^+ donor cells. This could be due to the low cell quality caused by cell sorting and handling, thus we conducted a third injection including ESC injections with the same cell preparation process as a control. We observed RFP^+ chimeric embryos in ESC-injected group, but we were not able to detect any RFP^+ donor cells in all CPC-injected embryos. Based on the three injections, we concluded that cardiac progenitor cells don't have the same potential as the embryonic stem cells in contributing to embryo development after injected into blastocysts. This could be due to the developmental stage mismatch of the donor cells and the host embryo. The donor cardiac progenitor cells were not able to find th e right place and get the right signals to survive and differentiate in blastocyst stage host embryos. It is also possible that they have the potential, but the engraftment rate is extremely low. It may be necessary to increase the number of injected embryos or to optimize the injection condition to get one successful engraftment. Alternatively, instead of using wildtype hosts, we could use heart-mutant blastocysts for complementation experiments to further study the potential of using lineage committed cells for blastocyst injection.

(1) Blastocyst injections with CPCs: We differentiated the TGFP-RFP c5 mESCs into cardiac progenitor cells with 3μM CHIR and 0.3 ng/ml of BMP4. We sorted GFP^+ cardiac progenitor cells on differentiation day 3 using the Sony cell sorter (SH800). We sent the FACS sorted GFP^+ cardiac progenitor cells to the transgenic core at Johns Hopkins University for blastocyst injection. Two injections were performed. In the first injection, 12-15 cells were injected into E3.5 wildtype mouse blastocysts and 10 injected blastocysts were transferred to 1 wildtype surrogate female mouse. In the second injection, 44 injected blastocysts were transferred to 4 wildtype surrogate female mice. We sacrificed the females and dissected the embryos on E9.5 for analysis. We were not able to detect any RFP signals under the fluorescent microscope (Fig. 1).

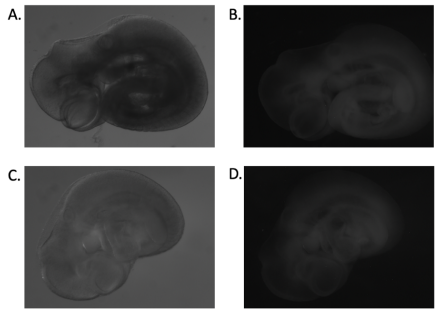

Fig. 1: Embryos after CPC injection. Two E9.5 embryos under the brightfield (A and C) and RFP (B and D) channel.

(2) Blastocyst injection with ESCs and CPCs: In order to rule out the possibility that the failure of donor cell engraftment was due to cell sorting induced cell stress or mis-operation during the cell handling process, we performed a third injection and included a control group with mouse embryonic stem cell(ESC) injections. ESC injection is widely used to generate chimeric mice, and thus can be served as an indicator of whether the cell preparing process affected their ability of survival after injection. The undifferentiated mouse TGFP-RFP ESCs were used for control group injection. We dissociated mouse TGFP-RFP ESCs from the cell culture flask and FACS sorted the RFP^+ ESCs for injection. The cell

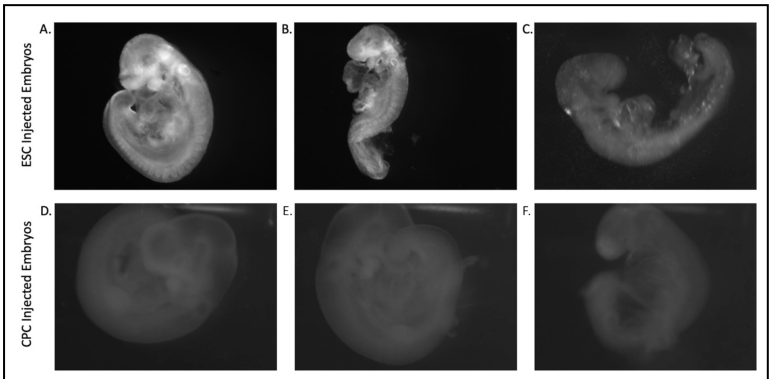

Fig. 2. RFP^+ donor cells were detected in ESC-injected embryos but not in CPC-injected embryos in the third injection. (A&B) Images of two E9.5 ESC-injected embryos under the RFP channel showing positive signal. (C) Image of the one E9.5 ESC-injected embryo showing negative RFP signal under the RFP channel. (D,E,&F) Images of three E9.5 CPC-injected embryos showing no RFP signal under the RFP channel.

preparation process was the same as the experimental group with cardiac progenitor cell injection. We also increased the number of cells injected into each embryo from 12-15 cells to 20 cells to increase the chance of engraftment. In this injection, 53 E3.5 blastocysts injected with cardiac progenitor cells were transferred to 4 wildtype surrogate female mice, and 14 embryos injected with ESCs were transferred to 1 wildtype surrogate female mouse. We collected the embryos at E9.5 and imaged them. We observed RFP signals in 7 out of 8 embryos from the ESC injected control group but none in the rest embryos injected with cardiac progenitor cells (Fig. 2). We then sectioned the CPC injected embryos, looking for evidence of RFP^+ cells. In sections stained with anti-RFP antibody and imaged under the confocal microscope, we observed some small, round cells with dim RFP signal, which could be the auto-fluorescence of the red blood cells, not the donor cells (Fig. 3).

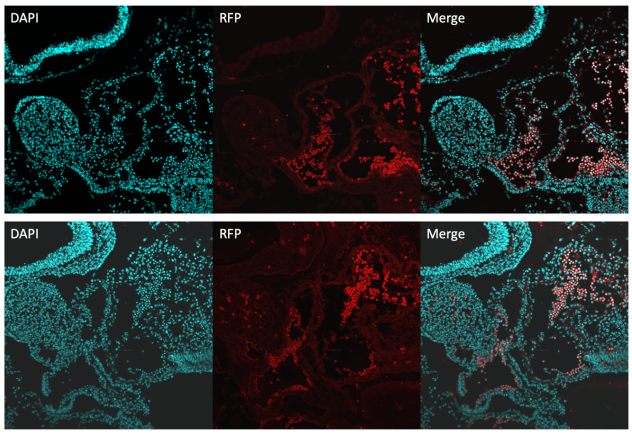

Fig. 3. Confocal images of sagittal sections of E9.5 CPC-injected embryos stained with anti-RFP antibody, focusing on the heart region. The RFP^+ cells located in the cavity region and throughout the tissue are red blood cells, not the injected donor cells.

c. **What opportunities for training and professional development has the project provided?**

Nothing to Report

d. **How were the results disseminated to communities of interest?**

Nothing to Report

e. **What do you plan to do during the next reporting period to accomplish the goals?**

N/A

4. Impact

a. **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to Report

b. **What was the impact on other disciplines?**

Nothing to Report

c. **What was the impact on technology transfer?**

Nothing to Report

d. **What was the impact on society beyond science and technology?**

Nothing to Report

5. Changes/Problems

Nothing to Report

6. Products

Publications

- Miyamoto M, Kannan S, Anderson M, Liu X, Suh D, Htet M, Li Biyi, Kakani T, Murphy S, Tampakakis E, Lewandoski M, Andersen P, Uosaki H, Kwon C. (2023) Cardiac Progenitors Instruct Second Heart Field Fate through Wnts. *PNAS*. 120(4)e2217687120

7. Participants & Other Collaborating Organizations

a. What individuals have worked on the project?

Name:	*Chulan Kwon*
Project Role:	*PI*
Researcher Identifier (ORCID ID)	0000-0002-4298-2778
Nearest person month worked:	1.8
Contribution to Project:	*Dr. Kwon coordinated all research activities, including formulating experimental strategies, analyzing data, troubleshooting, and supervising trainees.*

Name:	*Biyi Li*
Project Role:	*Graduate student*
Researcher Identifier (ORCID ID)	0000-0002-1084-4050
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Li has performed cell injection and embryo analysis.*

b. 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

c. What other organizations were involved as partners?

Nothing to Report

8. Special Reporting Requirements

Not Applicable

9. Appendices

None