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TITLE: Cardiomyocyte Chirality Defects in Congenital Heart Disease

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14. ABSTRACT A large proportion of congenital heart disease is caused by a defect in correct partitioning of the left and right compartments of the cardiac mesoderm. The result of this failure of laterality is a wide assortment of abnormal atrial, ventricular and arterial relationships. Aberration of early left right patterning is the underlying cause of heterotaxy. The focus of this grant is to use induced pluripotent stem cells to model and understand inherent cellular laterality. We have generated a genetic model of heterotaxy using a CRISPR interference system that targets the expression of a transcription factor, ZIC3, that has been implicated in inherited versions of heterotaxy. During this reporting period, we have also derived patient stem cell lines from patients with heterotaxy and no known genetic mutations. From these cell lines we characterized defects in cytoskeletal organization and gene expression differences that may underpin the development of heterotaxy.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	5
3. Accomplishments.....	6
4. Impact.....	10
5. Changes/Problems.....	11
6. Products.....	12
7. Participants & Other Collaborating Organizations.....	13
8. Special Reporting Requirements.....	15
9. Appendices.....	16 (None)

Introduction

Congenital heart disease is the most common birth defect and affects approximated 40,000 newborns per year in the United States. Because of surgical advances, mortality from congenital heart disease has declined significantly and the result has been an incredible increase in the number of surviving adults with significant congenital heart disease. A large proportion of congenital heart disease is caused by a defect in correct partitioning of the left and right compartments of the cardiac mesoderm. The result of this failure of laterality is a wide assortment of abnormal atrial, ventricular and arterial relationships. Aberration of early left right patterning is the underlying cause of heterotaxy. The focus of this grant is to use induced pluripotent stem cells to model and understand inherent cellular laterality. We have generated a genetic model of heterotaxy using a CRISPR interference system that targets the expression of a transcription factor, ZIC3, that has been implicated in inherited versions of heterotaxy. During this reporting period, we have also derived patient stem cell lines from patients with heterotaxy and no known genetic mutations. From these cell lines we characterized defects in cytoskeletal organization and gene expression differences that may underpin the development of heterotaxy.

Keywords

Heterotaxy

Laterality defect

Induced pluripotent stem cell

Cell chirality

ZIC3

Accomplishments

What were the major goals of the project?

The major goals of the project, along with the dates of completion, expected dates of completion and percentage completed. This table was taken from the approved SOW, amendment P00001, effective February 7th 2018.

Specific Aim 1(specified in proposal)	Initial Timeline	Completion/Expected/%
Major Task 1: Generate iPSC with inducible dCas9 targeting ZIC3	Months	
Subtask 1: Establish stable iPSC line with pHAGE TRE dCas9-KRAB and assess inducible expression of dCas9	1-2	Completed 6/2017
Subtask 2: Screen sgRNA ZIC3 in the cells from subtask 1 and establish stable line with second round of selection	2-4	Completed 7/2017
Milestone(s) Achieved: Inducible repression of ZIC3 expression in iPSC line.		<i>Milestone Achieved</i>
Major Task 2: Characterize ZIC3 interference during cardiac differentiation on gene expression and lineage fate		
Subtask 1: Differentiate cells and map ZIC3 expression	4	Completed 9/2017
Subtask 2: Induce suppression of ZIC3 expression at different time points of cardiac differentiation	5-6	Completed 10/2017
Subtask 3: Gene expression profiling and of ZIC3 suppression during differentiation and in cardiomyocytes versus WT	7-9	Completed 2/2018
Milestone(s) Achieved: Cardiomyocyte differentiation with and without ZIC3 and the resulting effect on state and lineage specific gene expression		<i>Milestone Achieved</i>
Major Task 3: Characterize electromechanical properties in cardiomyocytes that have lost ZIC3		
Subtask 1: Strain, area and contraction rate measurements	10-13	Exepcted 6/2018 (0%)
Subtask 2: Calcium handling	10-13	Expected 6/2018 (0%)
Subtask 3: microelectrode array	10-13	Expected 6/2018 (0%)
Milestone(s): electromechanical characterization of ZIC3 loss in iPSC derived cardiomyocyte		<i>Milestone Pending</i>
Major Task 4: Assess chirality		
Subtask 1: differentiate cell successfully on micro-patterned ring cultures	13-16	Expected 9/2018 (50%)
Subtask 2: Using phase contrast imaging, measure chirality of cells with and without ZIC3	13-16	Expected 9/2018 (50%)
Subtask 3: immunofluorescence confirmation and inhibition of actin-microtubules	13-16	Completed 4/2018
Milestone(s): measure and quantitate cell chirality in iPS cells differentiating into cardiomyocytes with and without ZIC3		<i>Milestone pending</i>
Specific Aim 2		
Major Task 1: Generate iPSC lines from patients with heterotaxy		
Subtask 1: IRB Approval (to be three months prior to start date)	0-1	Completed prior to start
Subtask 2: Recruit and collect from 4 pts with heterotaxy	1-12	Completed 4/2018
Subtask 3: Generate iPSC's from blood samples collected.	2-8 months	Completed 4/2018

Milestone(s) Achieved: Generate 4 iPSC lines from patients with heterotaxy		<i>Milestone achieved</i>
Major Task 2: Characterize cardiac differentiation of these lines		
Subtask 1: Successfully differentiate cells into cardiomyocytes	8-9	Completed 4/2018
Subtask 2: Characterize lineage and gene expression of differentiation and cardiomyocytes	10-12	Expected 8/2018 (75%)
Milestone(s): successful differentiation and characterization of cardiomyocytes from patient specific iPS lines		<i>Milestone Pending</i>
Major Task 3: Characterize electromechanical properties in heterotaxy cardiomyocytes		
Subtask 1: Strain, area and contraction rate measurements	13-15	Expected 9/2018 (0%)
Subtask 2: Calcium handling	13-15	Expected 9/2018 (0%)
Subtask 3: microelectrode array	13-15	Expected 9/2018 (0%)
Milestone(s): electromechanical characterization of heterotaxy iPSC derived cardiomyocytes		<i>Milestone pending</i>
Major Task 4: Assess chirality		
Subtask 1: differentiate cell successfully on micro-patterned ring cultures	16-18	Expected 10/2018 (0%)
Subtask 2: Using phase contrast imaging, measure chirality of heterotaxy iPSC and iPSC-CM compared to wt	16-18	Expected 10/2018 (0%)
Subtask 3: immunofluorescence confirmation and inhibition of actin-microtubules	16-18	Expected 10/2018 (0%)
Milestone(s): measure and quantitate cell chirality in heterotaxy iPSC's		<i>Milestone pending</i>

What was accomplished under these goals

In the first aim of the project, we established a stable iPS transgenic line that expresses both a dCAS9 interference system as well as a gRNA that directly targets ZIC3 expression. iPS wild type line WTC11, obtained as a gift from Bruce Conklin (Gladstone Institute) was transduced with two lentiviruses: pHAGE-TRE dCAS9-KRAB which encodes an endonuclease dead version of CAS9 (dCAS9) fused with a transcriptional repressor KRAB all under a tetracycline inducible promoter, and a gRNA under a U6 promoter designed targeting the first exon of ZIC3 (Dharmacon). We tested this CRISPR interference system expression in response to doxycycline by both assessing RNA and protein of CAS9. CAS9 protein expression was robust over three weeks by immunofluorescence (Figure 1a) though RNA levels did drop from initial induction (Figure 1b). We also mapped ZIC3 levels in response to induction in iPS cells and showed an 85% reduction in levels three days after exposure to doxycycline (Figure 1c).

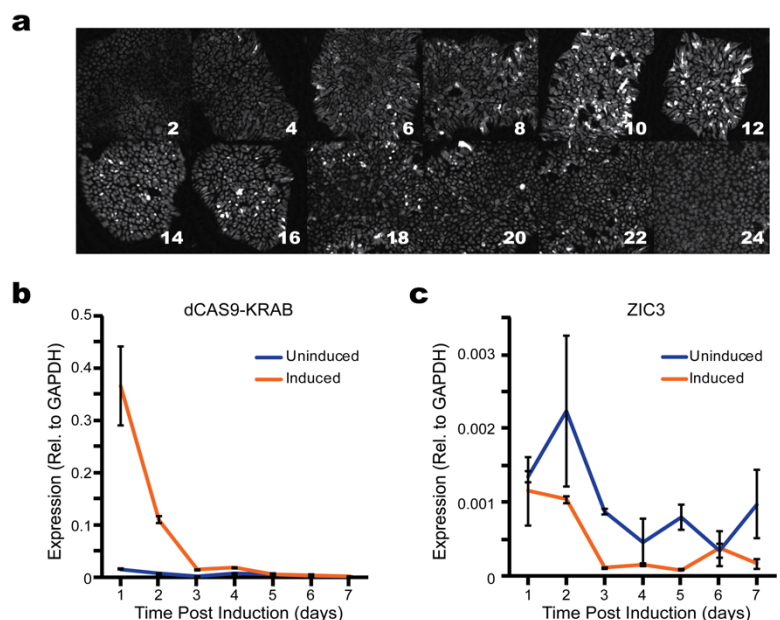


Figure 1: Induction of CRISPRi with tetracycline a) immunofluorescence of CAS9 in iPS cells exposed to doxycycline at Day 0. B) CAS9 RNA expression in response to doxycycline during differentiation C) ZIC3 RNA in induced versus uninduced iPS cells during 7 days of exposure to doxycycline

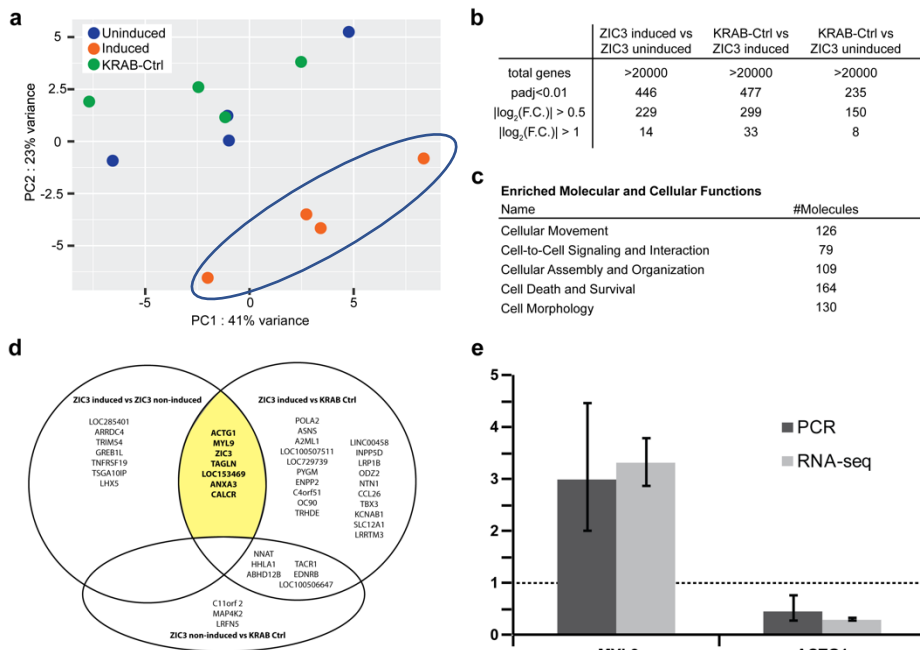


Figure 2: Gene expression changes in response to loss of ZIC3. RNA-seq was performed three days after doxycycline induction in iPS cells. A) PCA analysis of induced and two control lines (uninduced and KRAB control) B) number of genes with significant differences and fold changes between different comparisons C) Gene with high fold changes common to both control comparisons D) parallel results of RTPCR and RNA-seq for top two differentially expressed genes

we were able to detect its loss in the experiment by RNA-seq. There were several proteins involved in the cytoskeleton whose expression was significantly different. We confirmed the top two candidates, ACTG1 and MYL9 by RTPCR and showed excellent correlation with our RNA-seq results (Figure 2e)

Because of the predominance of cytoskeletal proteins enriches in our gene expression analysis, we investigated the actin cytoskeleton in response CRISPRi of ZIC3. ZIC3 loss resulted in a dramatic decrease in actin staining with phalloidin (Figure 3a). Analysis of the actin network using image processing (Figure 3b) showed significant decreases in branch length and longest branch points (Figure 3c). Staining for ACTG1, a top target from the RNA-seq analysis demonstrated a shift in the localization towards the nucleus (Figure 3d-f).

Over the next 6 months we will be assaying several parameters with this CRISPRi system. First, we will be assessing cell chirality at both the iPS and cardiomyocyte stage using patterned cell culture on rings

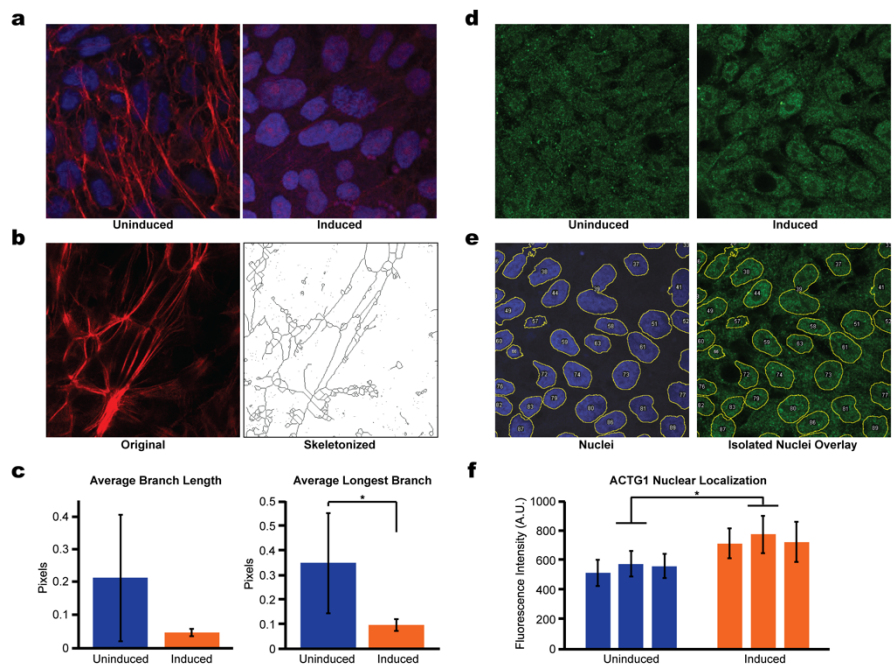


Figure 3: Actin cytoskeleton of iPS cells with ZIC3 knockdown. a) phalloidin stain of iPS cells with ZIC3 knockdown (right). b) representative skeletonized image used for processing c) average branch length and longest branch of actin cytoskeleton d) ACTG1 staining e) nuclear localization image processing f) ACTG1 nuclear fluorescence intensity

described in the original grant. For this assay, we will use live cell imaging using live centromere, nuclear and actin staining. We will assess cell chirality with respect to the nuclear-centromere angle with the nearest curved border using automated image processing at both the iPS and cardiomyocyte stage. Cardiomyocyte contractility, conduction and calcium handling will also be measured in response to loss of ZIC3 during the differentiation. We also plan to delineate ZIC3 transcriptional targets using CHIP-seq in order to better elucidate potential transcriptional targets of ZIC3 that may underlie failure of proper left right patterning in response to ZIC3's loss.

In the second aim of the project, an IRB was approved to collect blood samples from heterotaxy patients. In total, blood was collected from 4 patients with heterotaxy. At least three iPS clones were derived from each blood sample by the Columbia Stem Cell Core Facility. iPS cells were tested for pluripotency and stem cell markers and karyotyped. One clone (with a confirmed normal karyotype) from each patient was selected for experiments and successfully differentiated into cardiomyocytes. We are currently performing gene expression analysis on these cell lines to compare them with the ZIC3 knockdown. Over the next 6 months we plan to phenotype both iPS and cardiomyocyte stages from these cell lines with respect to cell chirality, motility and electromechanical performance.

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

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

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

Specific Aim 1: We will assess cell chirality at the iPS and cardiomyocyte stages as well as outputs of cardiomyocyte performance in response to loss of ZIC3. Cell chirality will be assessed with live imaging of cells stained with nuclear, actin and centromere markers. Mobility of iPS cells will be measured as will changes in actin cytoskeleton in response to inhibition or activation of small GTPases involved in cytoskeletal signaling. Cardiomyocyte contractility will be measured by strain imaging. Calcium handling will be assayed with fluorescent calcium ionophores. Cardiomyocyte conduction velocity will be measured with a multielectrode array.

Specific Aim 2: The four heterotaxy lines will undergo RNA-seq at both the iPS and cardiomyocyte level in order to compare gene expression differences with ZIC3 loss from Aim 1. Similar assays characterizing the cytoskeletal morphology, cell chirality and cardiomyocyte performance will be measured for each cell line.

IMPACT

What was the impact on the development of the principal discipline(s) of the project

Nothing to report

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.

CHANGES/PROBLEMS

Changes in approach and reasons for change

During this reporting period, the IRB to collect blood samples for Specific Aim 2 was altered to incorporate the addition of minors to recruitment. This alteration was made because of slower than expected recruitment of adult patients with heterotaxy. Because heterotaxy is a more common pediatric congenital heart disease, we decided to add minors to speed up recruitment to four patients. This change was made in conjunction with the USAMRMC ORP HRPO and was an approved revision of the SOW.

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

We had a delay in enrollment. Please see above and how we rectified the problem

Changes that had a significant impact on expenditures

We were able to recruit a very highly trained post-doctoral fellow in cardiovascular biology for the project. However, due to extenuating circumstances and prior commitments, she was unable to start the project until 4/01/2018. During this reporting period, a graduate student who was funded by the MSTP program at Columbia University and a part time technician funded by division sources worked on the project and made significant progress. However, given the remaining funds still available for salary support, we will be able to fund both the post-doctoral fellow as well as a technician during this time which should accelerate the project significantly. This change has been communicated with the Grants Management Specialist at the USAMRAA and did not require prior approval because the change does not involve key personnel.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

As stated above, we expanded our IRB to include minors and this was approved by the DOD within a Revised Statement of Work. We have fulfilled all enrollment at this time.

PRODUCTS

Publications, conference papers, and presentations

An abstract detailing these findings over the prior project year have been accepted for poster presentation at the BCVS meeting in San Antonio Texas July 2018.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>Barry Fine</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Principal Investigator in charge of all aspects of the project</i>
Funding Support:	<i>NIH (NHLBI), Institutional Support</i>

Name:	<i>Bohao Liu</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Derivation of the CRISPRi interference line targeting ZIC3 and characterization of iPS cells from that line</i>
Funding Support:	<i>MSTP NIH</i>

Name:	<i>Roberta Locke</i>
Project Role:	<i>Masters Student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>5</i>
Contribution to Project:	<i>Ms. Locke has been focused on differentiation of iPS cells into cardiomyocytes and their characterization</i>
Funding Support:	<i>Columbia University</i>

Name:	<i>Xiaokan Zhang</i>
Project Role:	<i>Post Doctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Zhang is optimizing differentiation of cardiomyocytes from iPS cells and exploring their signaling changes</i>
Funding Support:	<i>NIH, AHA</i>

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

The PI was awarded a K08 from the NIH:

1 K08 HL140201-01, 12/07/2017-11/30/2022

The STK25 Signaling Pathway in Human Cardiac Cells

Overlap: NONE

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

Nothing to report

QUAD CHARTS

Nothing to report

APPENDICES

Nothing to report