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TITLE: Cardiomyocyte Chirality Defects in Congenital Heart Disease

PRINCIPAL INVESTIGATOR: Barry Fine

CONTRACTING ORGANIZATION: The Trustees of Columbia University in the City of New York
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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 utilized our genetic and patient based stem cells lines to understand the cellular differences that occur in heterotaxy. Specifically we characterize fundamental differences in cell architecture and adhesion and the signaling cascades that underpin them.

15. SUBJECT TERMS

Heterotaxy, laterality defect, induced pluripotent stem cell, cell chirality, ZIC3

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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 utilized both genetically altered and patient derived lines to characterize the cellular phenotype of heterotaxy, specifically addressing defects in cell migration and movement.

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.		*Milestone Achieved*
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		*Milestone Achieved*
Major Task 3: Characterize electromechanical properties in cardiomyocytes that have lost ZIC3		
Subtask 1: Strain, area and contraction rate measurements	10-13	Exepcted 6/2019 (0%)
Subtask 2: Calcium handling	10-13	Completed 4/2019
Subtask 3: microelectrode array	10-13	Expected 6/2019(0%)
Milestone(s): electromechanical characterization of ZIC3 loss in iPSC derived cardiomyocyte		*Milestone Pending*
Major Task 4: Assess chirality		
Subtask 1: differentiate cell successfully on micro-patterned ring cultures	13-16	Completed 4/2019
Subtask 2: Using phase contrast imaging, measure chirality of cells with and without ZIC3	13-16	Completed 4/2019
Subtask 3: immunofluorescence confirmation and inhibition of actin-microtubules	13-16	Completed 5/2019
Milestone(s): measure and quantitate cell chirality in iPS cells differentiating into cardiomyocytes with and without ZIC3		*Milestone achieved*
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		*Milestone achieved*
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	Completed 1/2019
Milestone(s): successful differentiation and characterization of cardiomyocytes from patient specific iPS lines		*Milestone achieved*
Major Task 3: Characterize electromechanical properties in heterotaxy cardiomyocytes		
Subtask 1: Strain, area and contraction rate measurements	13-15	Expected 7/2019 (0%)
Subtask 2: Calcium handling	13-15	Completed 4/2019
Subtask 3: microelectrode array	13-15	Expected 7/2019 (0%)
Milestone(s): electromechanical characterization of heterotaxy iPSC derived cardiomyocytes		*Milestone pending*
Major Task 4: Assess chirality		
Subtask 1: differentiate cell successfully on micro-patterned ring cultures	16-18	Completed (100%)
Subtask 2: Using phase contrast imaging, measure chirality of heterotaxy iPSC and iPSC-CM compared to wt	16-18	Expected 7/2019 (0%)
Subtask 3: immunofluorescence confirmation and inhibition of actin-microtubules	16-18	Expected 7/2019 (0%)
Milestone(s): measure and quantitate cell chirality in heterotaxy iPSC's		*Milestone pending*

Description of Accomplishments

Generation of *ZIC3*^{iKRAB} IPSCs

Using lentiviruses encoding a tetracycline responsive element controlled dCAS9-KRAB cassette and guide RNAs targeting the first exon of ZIC3, we successfully generated double transgenic lines in WTC11 iPSC's. Doxycycline was able to induce dCAS9-KRAB expression within several hours of exposure and we were successful in knocking down *ZIC3* expression to compared to uninduced cells during differentiation (**Figure 1**). Because dCAS9-KRAB irreversibly binds DNA, maintenance of knockdown was not dependent on persistent exposure to doxycycline. We observed that knockdown of *ZIC3* over a period of more than 7 days resulted in spontaneous differentiation of iPS cells (data not shown). *ZIC3* is not expressed in differentiated cardiomyocytes which is in line with other published literature demonstrating that *ZIC3* expression is limited to embryonic tissue. Because of this, we narrowed our experimental objective to knockdown of *ZIC3* in iPS cells only. All studies on *ZIC3* knockdown in cardiomyocytes

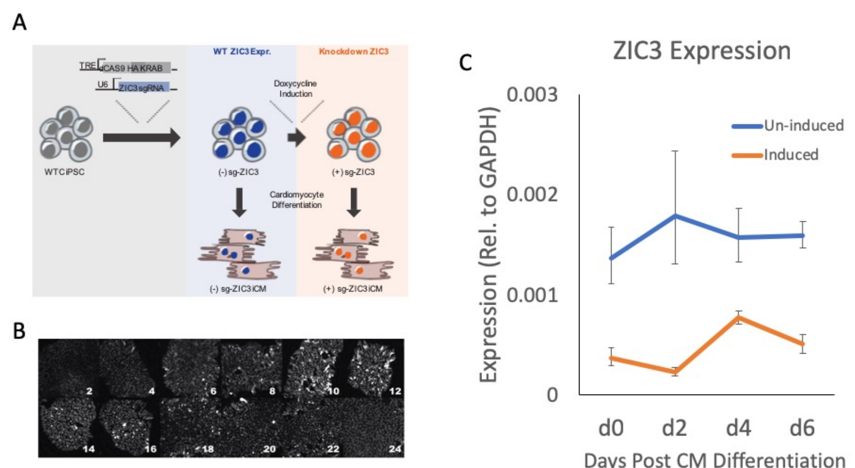
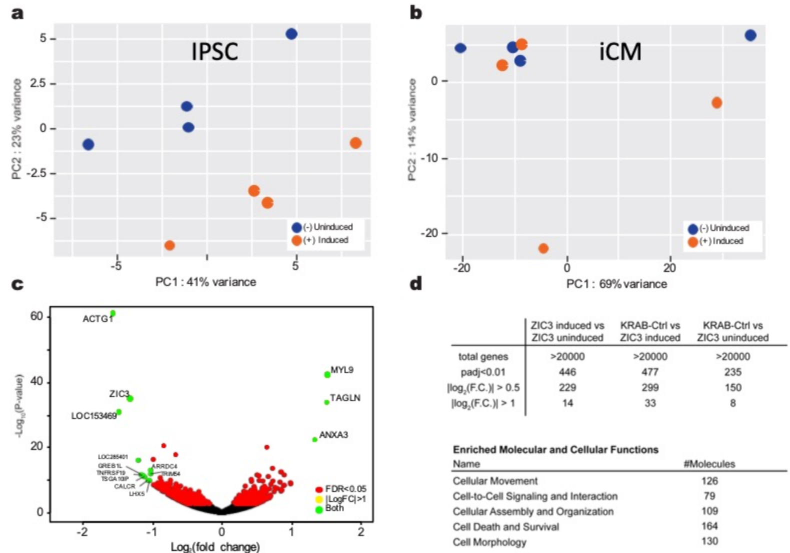


Figure 1: Inducible ZIC3 knockdown in iPS cells. A) Schematic of doxycycline induction of dCAS9-KRAB B) Immunofluorescence of CAS9 in transduced iPS cells up to 24 hour after exposure to doxycycline. C) Durable knockdown of ZIC3 (in orange) throughout differentiation of iPS cells into cardiomyocytes. N=4 sample replicates per data point

were performed in differentiated iCM's from iPSC's with or without CRISPRi induction. The pluripotency of parent cell line was confirmed with staining for *NANOG*, *SOX* and *OCT4* and its ability to generate cardiomyoytes using derivation of a published protocol was also demonstrated(6) (data not shown).



	ZIC3 induced vs ZIC3 uninduced	KRAB-Ctrl vs ZIC3 induced	KRAB-Ctrl vs ZIC3 uninduced
total genes	>20000	>20000	>20000
padj<0.01	446	477	235
$\lvert log_2(F.C.) \rvert > 0.5$	229	299	150
$\lvert log_2(F.C.) \rvert > 1$	14	33	8

Enriched Molecular and Cellular Functions

Name	#Molecules
Cellular Movement	126
Cell-to-Cell Signaling and Interaction	79
Cellular Assembly and Organization	109
Cell Death and Survival	164
Cell Morphology	130

Figure 2: PCA analysis of RNA-Seq of ZIC3 knockdown in (A) iPS cells and (B) differentiated cardiomyocytes. (C) Plot of gene expression changes by fold change (X axis) and significance (Y axis). (D) Gene enrichment analysis by IPA (Qiagen)

Gene expression profiling of ZIC3 knockdown

In order to evaluate the consequence of *ZIC3* loss in iPSC's, RNA-seq was performed on both induced and uninduced ZIC3^{iKRAB} iPSC's as well as iCM's differentiated from those cultures. *ZIC3* loss was highly associated with alterations in pathways involving cellular movement, cell-cell interaction, cell assembly and cell morphology (**Figure 2**). Analysis of significant (FDR <0.05) gene expression differences revealed enrichment for genes involved in congenital heart disease including *NODAL, ACTC1, ADRA2A, COL1A2, CITED2* and *GJA1*. Additionally, multiple components of small GTPase signaling pathways, critical for the cellular cytoskeleton, movement and motility, were also altered. The most highly downregulated gene other than *ZIC3* was gamma actin. These results were confirmed by RTPCR (not shown). There were however no FDR corrected significant gene expression differences in iCM's. This experiment was limited to heterogenous differentiated culture system and is now being repeated in isolated cardiomyocytes.

ZIC3 loss impairs iPS cell movement

In light of the impact of *ZIC3* loss on gene expression that controls cell movement and motility, we assessed iPS cell mobility in response to induction of *ZIC3* knockdown. Movement was assessed using live cell imaging with both nuclear and actin staining over several hours. *ZIC3* loss was associated with significant reduction total distance, nuclear displacement as well as speed of iPS cell migration (**Figure 3)**. `

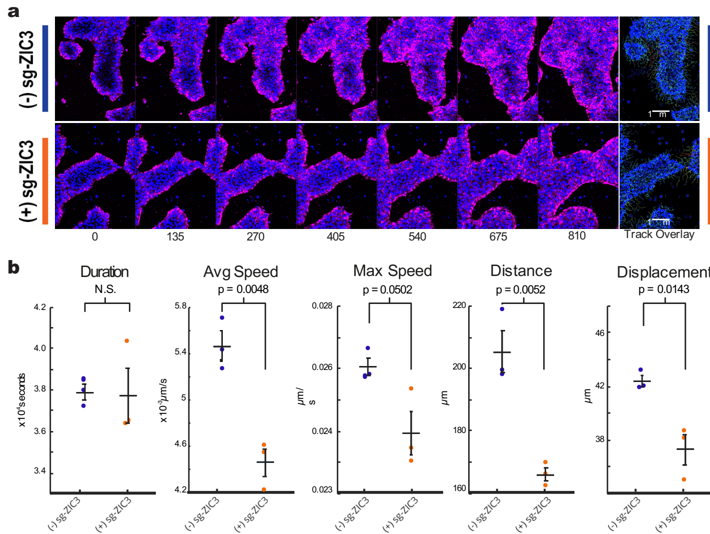


Figure 3: A) live cell nuclear (DAPI) and actin (red) tracking of IPSc with (bottom) and without (top) knockdown of ZIC3. B) Measurements of movement over 14 hours. Each dot represents the mean of all cells in one field of observation Images and measurements processed by Matlab.

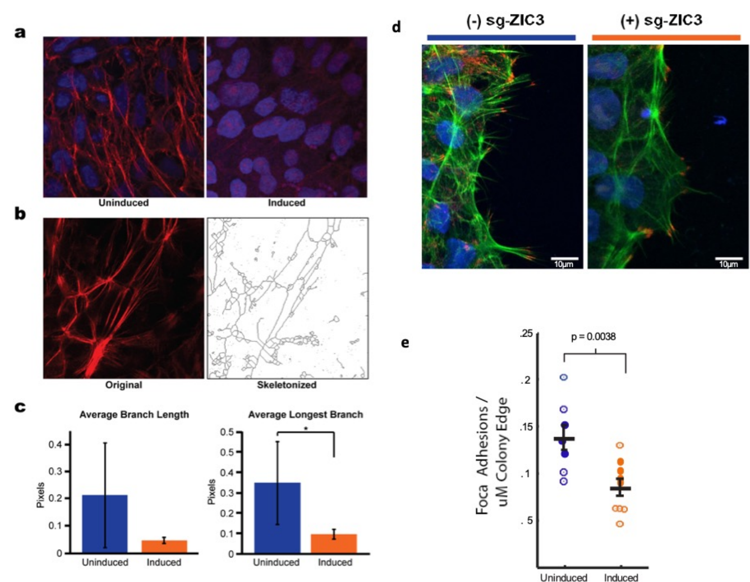


*Figure 4: A) Filamentous actin reduced in ZIC3 knockdown iPSC's B) Graphical representation of image processing C) Reduction in average branch length and longest branch of actin network in iPSc (*p<0.05) D) Staining for focal adhesion formation with paxillin (red) E) Reduction in number of focal adhesions at the migratory edge of an iPSC colony*

ZIC3 loss impairs actin cytoskeleton and focal adhesions

Because of the predominance of cytoskeletal proteins enriched in our gene expression analysis and a defect in movements seen in *ZIC3* loss, we investigated the actin cytoskeleton in response to *ZIC3* knockdown. Analysis of the actin network showed a dramatic decrease in filamentous actin when staining with phalloidin in response to *ZIC3* loss (**Figure 4a)**. Image analysis using processed images revealed significant decreases in both branch length and longest branch points (**Figure 4b,c)**. Staining for ACTG1, the top most downregulated gene in *ZIC3* knockdown, demonstrated redistribution of ACTG1 to the nucleus (not shown). As focal adhesions are an integral element in cell movement, we further assessed changes in focal adhesion formation at the leading edge of migrating cells. Confocal microscopy of iPS cells stained for both paxillin and actin revealed a significant decrease in the formation of focal adhesions at the leading edge in response to *ZIC3* knockdown (**Figure 4d,e)**.

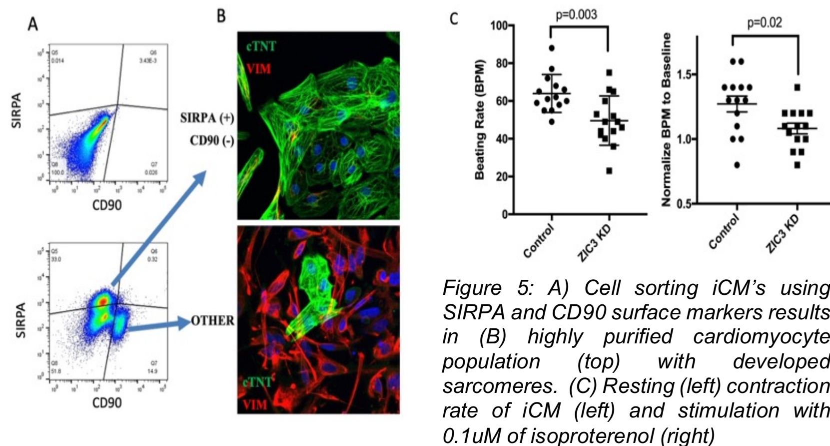


Figure 5: A) Cell sorting iCM's using SIRPA and CD90 surface markers results in (B) highly purified cardiomyocyte population (top) with developed sarcomeres. (C) Resting (left) contraction rate of iCM (left) and stimulation with 0.1uM of isoproterenol (right)

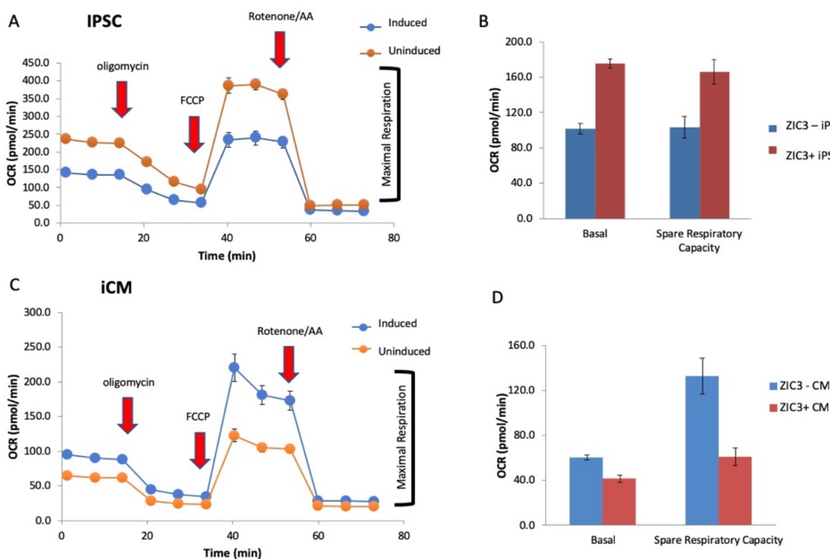


Figure 6: Mitochondrial Seahorse assay of iPSC's and iCM. (A) Maximal mitochondrial respiratory capacity (measured after FCCP addition) of ZIC3 knockdown (induced, blue) and control (uninduced, orange) iPSC's along with (B) basal measurements of oxygen consumption rate and spare respiratory capacity. (C) Maximal mitochondrial respiratory capacity of iCM's derived from ZIC3 knockdown iPS cells (orange) and uninduced control iPSC's (blue) along with basal and spare respiratory capacity.

Metabolic potential and performance of cardiomyocytes is impacted by *ZIC3* loss

Despite the lack of significant gene expression changes in cardiomyocytes in response knockdown of ZIC3 in their iPS progenitors, this did not exclude potential phenotypic differences in individual cardiomyocytes. Differentiated iCM cultures are often heterogeneous with multiple different cell types within the culture. Thus we optimized a new protocol to isolate a purified cardiomyocytes for *in vitro* culture. Cell sorting a population of cells that is SIRPA+/CD90- yielded a culture that is >95% cardiomyocytes (**Figure 5a,b**). The basal beating rate of *ZIC3* knockdown cardiomyocytes was diminished both alone and in response to isoproterenol (**Figure 5c)**. We assessed the metabolic potential of these cells using the Seahorse Mitochondrial Assay (Agilent). This assay revealed that *ZIC3* loss caused a significant decrement in oxygen consumption rate indicating lower levels of both basal and total mitochonrdrial respiratory function (**Figure 6)**.

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 further explore chirality in both iPS lines and cardiomyocytes using new techniques that we have developed that have generated promising though preliminary data. We will also explore more fully the mechanism by which ZIC3 impacts cell movement by exploring WNT signaling and specifically how ZIC3 inhibits WNT signaling in early cardiac mesoderm differentiation. Gene set enrichment analysis shows a significant upregulation of WNT with ZIC3 loss.

Specific Aim 2: We will analyze the RNAseq data generated from heterotaxy patient lines and explore both functional assays with respect to cell movement at the iPS stage and cardiomyocyte contractility in differentiated cells. Similar to our ZIC3 knockdown cells, we will also asses whether WNT signaling is altered in these cell lines compared to controls.

IMPACT

What was the impact on the development of the principal discipline(s) of the project
In this project, we have been able to understand how a particular mutation in humans leads to a disease called heterotaxy. In this disease, the left and right sides of the body develop incorrectly and there are often left right asymmetry errors. Mutations in a gene called ZIC3 account for an inherited version of this disease and we have used human stem cells to show that ZIC3 is important for cell movement and cell attachment. These changes are largely based on changes of the actual cellular skeleton. This is the first cell model of heterotaxy and it will lead to further understanding of the mechanism behind why congenital heart disease occurs.

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
In order to complete many of the sub-aims associated with characeterizing cardiomyocytes, we had to generate a new protocol to isolate an enriched and nearly homogenous single cell culture of cardiomyocytes using cell sorting of SIRPA and CD90 surface markers. This allowed us to do single cell contraction analysis, metabolism studies. A final ongoing hurdle has been reliable patterning of cells using cell matrix printing on culture slides. We have recently been able to culture spheroids of iPS cells that then appear to show polarization of cell chirality on 2 dimensional imaging. We are still analyzing this data using both cell membrane and golgi apparatus staining.

Actual or anticipated problems or delays and actions or plans to resolve them
Due to an initial delay in recruitment of a post-doctoral fellow during the previous reporting period as well as the need to develop and optimize new techniques for cardiomyocyte isolation, we applied for and were granted a no cost extension until July of 2019.

Changes that had a significant impact on expenditures
There were no changes that had a significant impact on expenditures

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
There were no changes in use or care of human subjects.

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 and a biomedical engineering symposium at Duke University in May of 2019

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:	*Barry Fine*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	*Principal Investigator in charge of all aspects of the project*
Funding Support:	*NIH (NHLBI), Institutional Support*

Name:	*Bohao Liu*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Derivation of the CRISPRi interference line targeting ZIC3 and characterization of iPS cells from that line*
Funding Support:	*MSTP NIH*

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

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

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 Lisa Dean Moseley Award titled "iPS cells in Congenital Heart Disease." The award covers sequencing and CRISPR correction of iPS lines generated with the PRMRP award. There is no technical overlap with this award but it does further our ability to create control cell lines for characterization of our heterotaxy models and their phenotype-genotype relationship.

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

Nothing to report

QUAD CHARTS

Nothing to report

APPENDICES

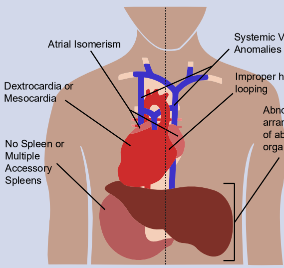
Columbia University
Medical Center

Modulation of *ZIC3* Expression in Pluripotent Stem Cells using CRISPR-interference to Model Heterotaxy

Roberta Lock*,[1] Bohao Liu*,[2,3] Lynn Bi,[1] Jordan Metz,[2] Gordana Vunjak-Novakovic,[1,3] Barry Fine [3]

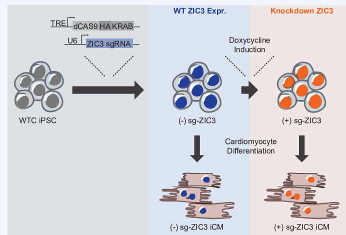
[1] Department of Biomedical Engineering; [2] College of Physicians and Surgeons; [3] Department of Medicine, Columbia University

Introduction



Failure to establish proper left-right patterning during embryogenesis can result in a range of congenital malformations. Aberration from this early asymmetric patterning causes abnormal arrangements in thoracic and abdominal organs relative to the left-right axis as well as complex cardiovascular malformations typical of improper heart looping. Collectively, these laterality defects are referred to as heterotaxy. While much of the underlying molecular and genetic mechanisms remain unknown, linkage analysis in familial cases of heterotaxy has identified the zinc finger transcription factor *ZIC3* as one of the few genes that plays a causal role in the development of heterotaxy. Despite animal models demonstrating that *ZIC3* deletion can recapitulate the human heterotaxy phenotype, little is known about the mechanistic basis of *ZIC3* function in cardiac development and left-right patterning. With recent advances in CRISPR-interference (CRISPRi) enabling temporal control of gene expression, human-induced-pluripotent-stem cells (iPSCs) and iPSC-derived cardiomyocytes (iCMs) can now be used to develop a human based model for the study of heterotaxy. To study the mechanism of *ZIC3* action at a molecular, cellular, and functional level, we used CRISPRi to create an inducible system allowing for robust knockdown of ZIC3 in iPSCs. Subsequent differentiation of ZIC3-knockdown iPSCs into cardiomyocytes allows for analysis of the effect of *ZIC3* on cardiac development. Gene expression analysis revealed significant differences in ZIC3-knockdown iPSCs, but not in ZIC3-knockdown iCMs. Pathway analysis of differentially expressed genes suggests *ZIC3* plays a notable role in modulating cell movement. Fluorescent staining of actin cytoskeleton demonstrated that ZIC3 knockdown results in a reduction of filopodia-like structures in iPSCs. Finally, live cell fluorescent imaging revealed that ZIC3 knockdown reduced iPSCs motility. Together, these findings demonstrate that *ZIC3* plays an important role in cellular motility which may contribute to the mechanisms underlying heterotaxy.

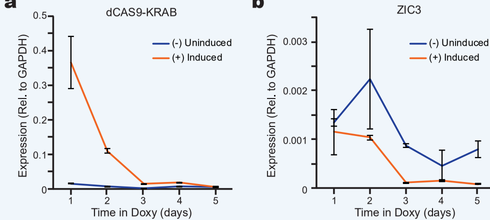
Research Design



We transduced the commercially available pHAGE-TRE-dCas9-KRAB construct into WTC10 iPSCs. This construct expresses the CRISPRi recombinant protein dCas9-KRAB under the control of a doxycycline inducible tetracycline responsive promoter. dCas9-KRAB is a human codon-optimized, catalytically inactive version of Cas9 (dCas9) fused to a Krüppel-associated box (KRAB) repressor domain. When combined with a sgRNA targeted to exon one of *ZIC3*, dCas9-KRAB allows for the inducible repression of *ZIC3* in iPS cells while maintaining parent cell line pluripotency. ZIC3-knockdown iPSCs can then be differentiated into cardiomyocytes.

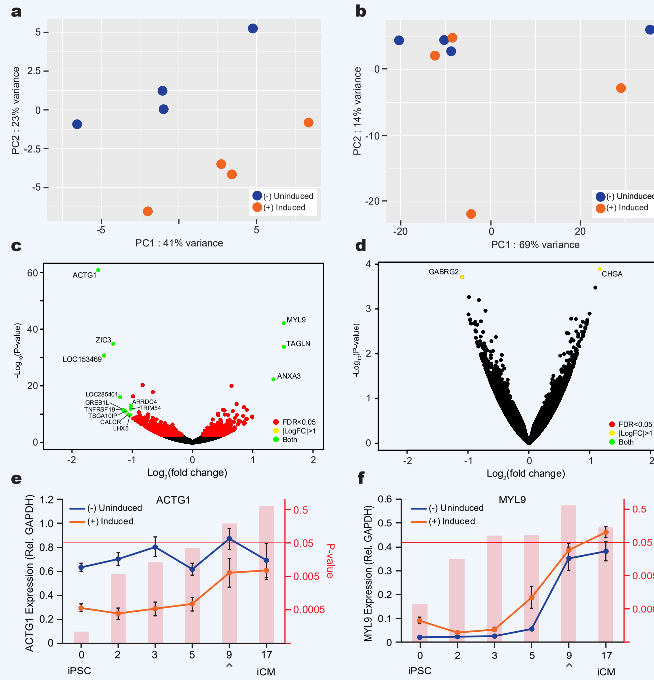
Results

Induction of dCAS9-KRAB expression with doxycycline results in knockdown of *ZIC3* expression in sg-ZIC3 iPSCs



qPCR time course analysis was conducted to compare *dCAS9-KRAB* **(a)** and *ZIC3* **(b)** expression between induced and uninduced populations, confirming that doxycycline induces a robust knockdown of *ZIC3* expression by 72 hours, with a fold change of 0.12 compared to uninduced.

RNA sequencing reveals significant differences in ZIC3-knockdown iPSCs but not ZIC3-knockdown iCMs



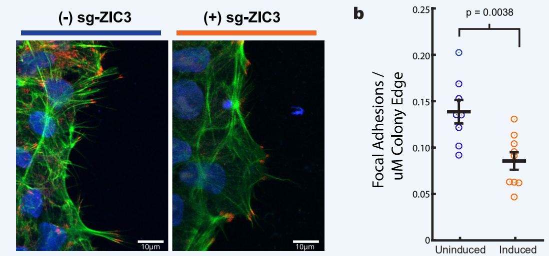
RNA sequencing was used to generate a gene expression profile of *ZIC3* loss in iPSCs and iCMs. We applied DESeq2 to identify differentially expressed genes between induced and uninduced cells. Principal component analysis (PCA) was then used to explore the broad gene expression differences in iPSCs **(a)** and iCMs **(b)**, identifying two distinct populations in iPSCs, but not in iCMs. In iPSCs, 935 genes were significantly differentially expressed (adjusted $p < 0.05$), and of those, 14 genes had an absolute fold change of greater than 2 **(c)**. In iCMs, only 2 genes had an absolute fold change of greater than 2 **(d)**. To validate the RNA-seq results, the expression of two of the most significantly differentially expressed genes in iPSCs -gamma actin 1 (*ACTG1*) and myosin light chain 9 (*MYL9*)- were analyzed in induced versus uninduced populations by qPCR time course **(e,f)**. sg-ZIC3 iPSCs were initially induced with doxycycline for 72 hours before the initiation of cardiac differentiation. By day 9 (grey arrow), when spontaneously contractioning cardiomyocytes become apparent, expression differences between induced and uninduced cells for both *ZIC3*-regulated genes had become insignificant (P-value > 0.05) suggesting that ZIC3 knockdown has little impact on differentiated cardiomyocytes.

ZIC3 knockdown in iPSCs specifically affects pathways related to organ development and cellular movement

Molecular and Cellular Functions		Physiological System Development and Function	
Name	p-value	Name	p-value
1. Cellular Movement	1.55E-10	Organismal Survival	8.52E-11
2. Cell-to-Cell Signaling and Interaction	7.10E-10	Cardiovascular System Development	4.17E-10
3. Cellular Assembly and Organization	7.10E-10	Organismal Development	4.79E-09
4. Cell Death and Survival	9.38E-10	Tissue Morphology	1.76E-08
5. Cell Morphology	1.43E-09	Skeletal and Muscular System Development	3.74E-08

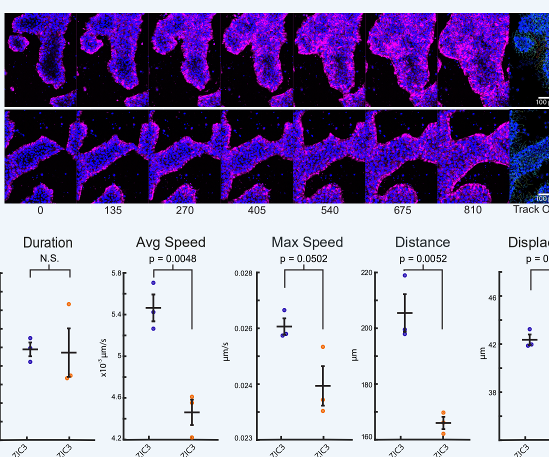
To predict cellular processes affected by ZIC3 knockdown, we applied Qiagen's Ingenuity Pathway Analysis to differentially expressed genes. Gene ontology analysis revealed significant enrichment in cellular processes related to cellular movement, assembly, and morphology as well as in physiological processes related to organism survival, cardiovascular system development and tissue morphology. Together, these results suggest that ZIC3 knockdown in iPSCs can recapitulate the effects of ZIC3 on heterotaxy as well as implicates the role of *ZIC3* in cellular movement pathways.

ZIC3 knockdown in iPSCs reduces presence of filopodia-like structures



The effect of *ZIC3* knockdown on the actin cytoskeleton was investigated using scanning confocal microscopy. Induced and uninduced sg-ZIC3 iPSCs were fluorescently stained for focal adhesions (Paxillin-red), filamentous actin (Phalloidin-green), and nuclei (DAPI-blue) **(a)**. Quantification of images showed ZIC3 knockdown in iPSCs reduced the presence of filopodia-like projections as well as focal adhesions at the edges of the cell colonies **(b)**. Filopodia are actin based cytoplasmic protrusions found at the leading edge of migrating cells and are often linked to migration and adhesion abilities. Therefore, the reduction of filopodia-like projections in induced iPSCs suggests that ZIC3 knockdown may impede cellular movement.

ZIC3 knockdown in iPSCs reduces cell movement



To further explore functional differences caused by ZIC3 knockdown we conducted live fluorescent imaging of iPSCs stained with SiR-Actin (Magenta) and Hoescht nuclear (Blue) stains. The resulting timelapses demonstrated visible reduction in colony movement in induced iPSCs compared to uninduced over 16 hours. Subsequent image analysis using ImageJ Trackmate tracked the movement of individual cells through nuclei position over the entire time course **(a)**. Quantification of migration parameters demonstrated a significant reduction in average speed, maximum speed, distance, and displacement in induced iPSCs when compared to uninduced **(b)**. In concordance with differential gene expression analysis, these results suggest that ZIC3 knockdown impacts iPSC cytoskeleton and cell mobility.

Conclusion

In this study, we demonstrate that human iPSCs, in combination with CRISPRi and cardiomyocyte differentiation technologies, can be used to mechanistically study the complex genetic underpinnings of heterotaxy. Our model allowed us to determine that ZIC3 knockdown resulted in deficiencies in iPSC mobility and differences in cellular cytoskeletal structure, suggesting a novel mechanism of *ZIC3*-mediated heterotaxy. Importantly, the ability to temporally control gene expression in human iPSCs and subsequent cardiomyocyte differentiation delineates a new paradigm for exploring forms of congenital heart disease at a molecular, cellular, and functional level.

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