AWARD NUMBER: W81XWH-17-1-0112

TITLE: Cardiomyocyte Chirality Defects in Congenital Heart Disease

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CONTRACTING ORGANIZATION: The Trustees of Columbia University in the City of New York, New York

REPORT DATE: Nov 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Nov 2019	Final	05/01/2017 - 07/31/2019

4. TITLE AND SUBTITLE

Cardiomyocyte Chirality Defects in Congenital Heart Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0112

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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New York, NY 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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 final 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. We report here successful generation of left sided iPSC derivatives in response to ZIC3 loss. To our knowledge, this is the first description of left-right asymmetry in iPSC and genetic manipulation affecting that axis.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	20	19b. TELEPHONE NUMBER *(include area code)*

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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 final 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. We report here successful generation of left sided iPSC derivatives in response to ZIC3 loss. To our knowledge, this is the first description of left-right asymmetry in iPSC and genetic manipulation affecting that axis.

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	Completed 6/2019
Subtask 2: Calcium handling	10-13	Completed 4/2019
Subtask 3: microelectrode array	10-13	Not completed
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	Not completed 7/2019 (0%)
Subtask 2: Calcium handling	13-15	Completed 4/2019
Subtask 3: microelectrode array	13-15	Not completed (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 4/2019 (100%)
Subtask 2: Using phase contrast imaging, measure chirality of heterotaxy iPSC and iPSC-CM compared to wt	16-18	Completed 7/2019 (100%)
Subtask 3: immunofluorescence confirmation and inhibition of actin-microtubules	16-18	Completed 7/2019(100%)
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

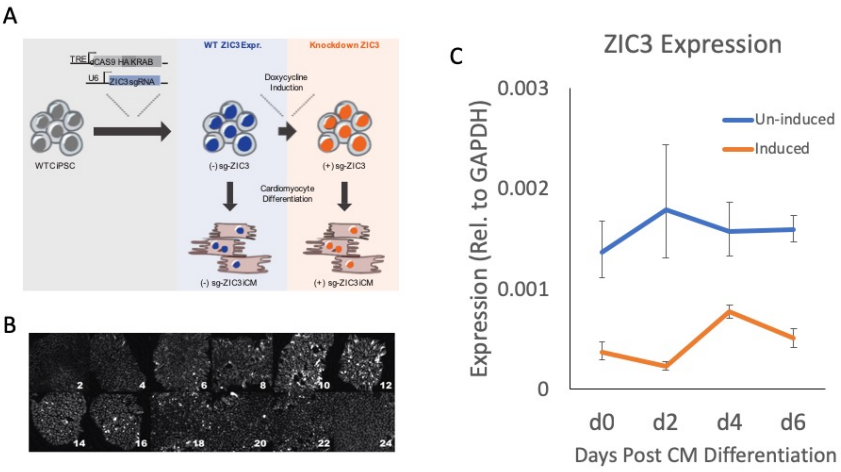


Figure 1: Inducible ZIC3 knockdown in iPS cells. A) Schematic of doxycycline induction of dCAS9-KRAB B) Immunofluorescence of CAS9 in transduced iPS cells up to 24 hour after exposure to doxycycline. C) Durable knockdown of ZIC3 (in orange) throughout differentiation of iPS cells into cardiomyocytes.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

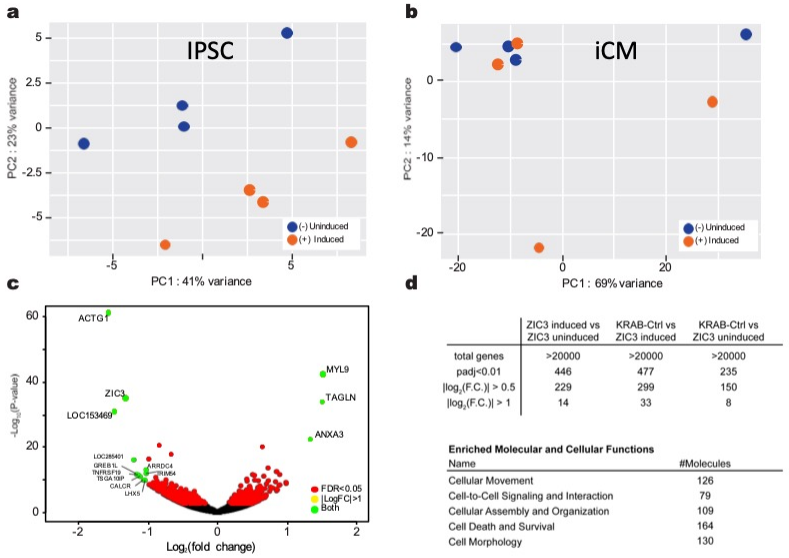


Figure 2: PCA analysis of RNA-Seq of ZIC3 knockdown in (A) iPS cells and (B) differentiated cardiomyocytes. (C) Plot of gene expression changes by fold change (X axis) and significance (Y axis). (D) Gene enrichment analysis by IPA (Qiagen)

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)**. `

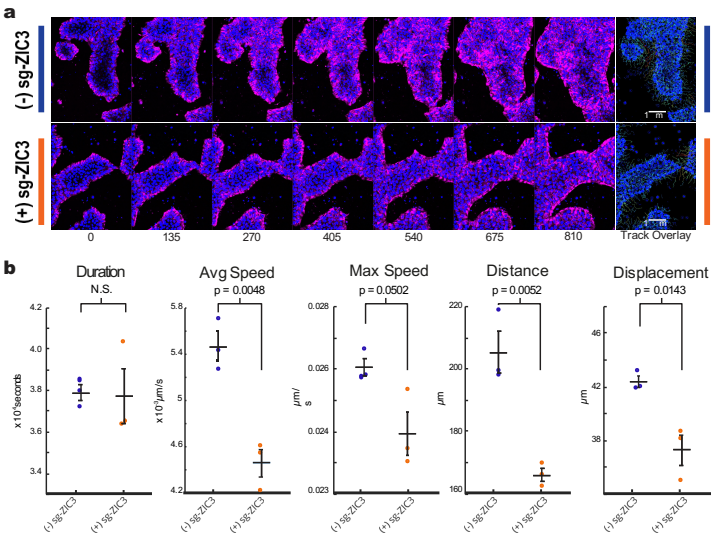


Figure 3: A) live cell nuclear (DAPI) and actin (red) tracking of IPSc with (bottom) and without (top) knockdown of ZIC3. B) Measurements of movement over 14 hours. Each dot represents the mean of all cells in one field of observation Images and measurements processed by Matlab.

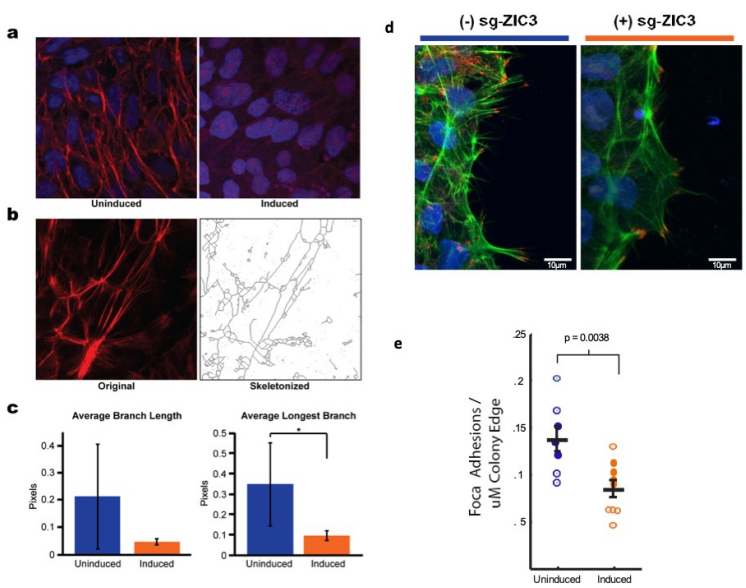


*Figure 4: A) Filamentous actin reduced in ZIC3 knockdown iPSC's B) Graphical representation of image processing C) Reduction in average branch length and longest branch of actin network in iPSc (*p<0.05) D) Staining for focal adhesion formation with paxillin (red) E) Reduction in number of focal adhesions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

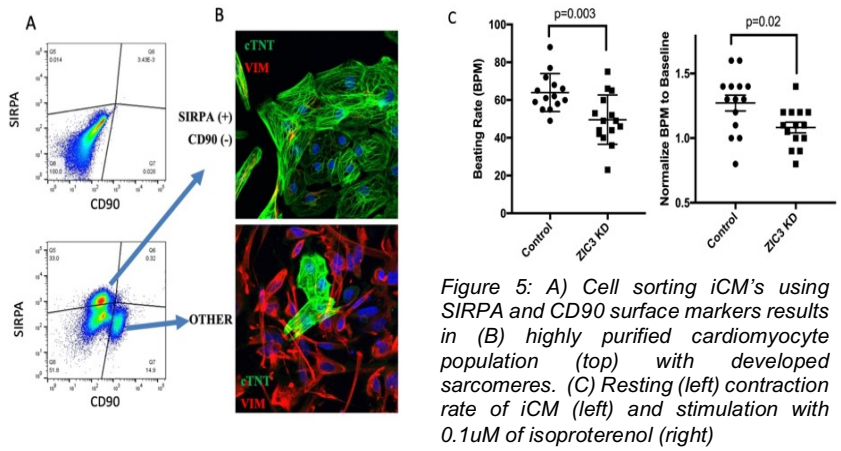


Figure 5: A) Cell sorting iCM's using SIRPA and CD90 surface markers results in (B) highly purified cardiomyocyte population (top) with developed sarcomeres. (C) Resting (left) contraction rate of iCM (left) and stimulation with 0.1uM of isoproterenol (right)

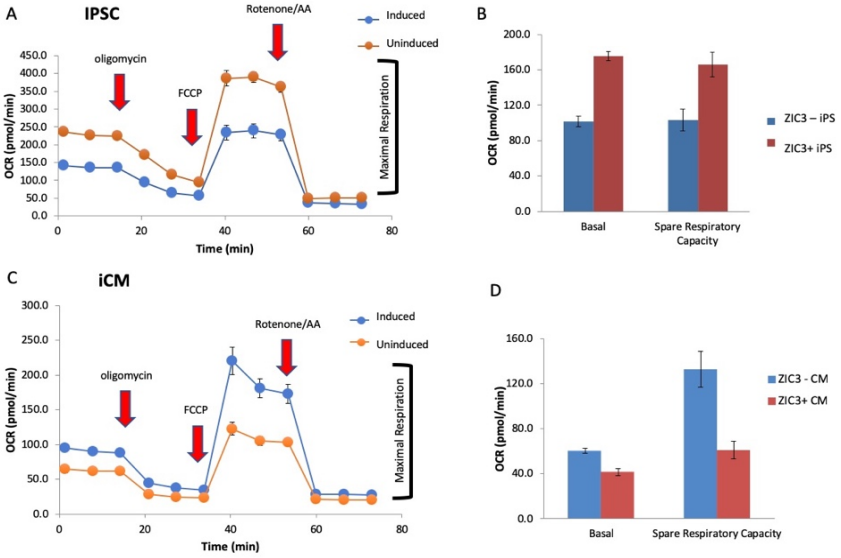


Figure 6: Mitochondrial Seahorse assay of iPSC's and iCM. (A) Maximal mitochondrial respiratory capacity (measured after FCCP addition) of ZIC3 knockdown (induced, blue) and control (uninduced, orange) iPSC's along with (B) basal measurements of oxygen consumption rate and spare respiratory capacity.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

ZIC3 inhibits WNT signaling The TCF family of transcription factors are the downstream effectors of canonical WNT signaling(39). Using transfected 293 cells, ZIC3 was able to inhibit a gradient of WNT activation of a TCF luciferase reporter (Fig. 7A). Human mutations found in ZIC3 in published heterotaxy patients, K408X and C253S (40), were attenuated in their ability to suppress WNT activation of TCF. ZIC3 was able to suppress β-catenin mediated TCF activation, demonstrating that it is likely downstream of β-catenin (Fig 7B).

ZIC3 knockdown leads to an increase in WNT signaling. RNA-seq was performed on both induced and uninduced ZIC3^{iKRAB} iPSC's. Analysis of significant (FDR <0.05) gene expression differences revealed an enrichment for genes involved in congenital heart disease including *NODAL, ACTC1, ADRA2A, COL1A2,*

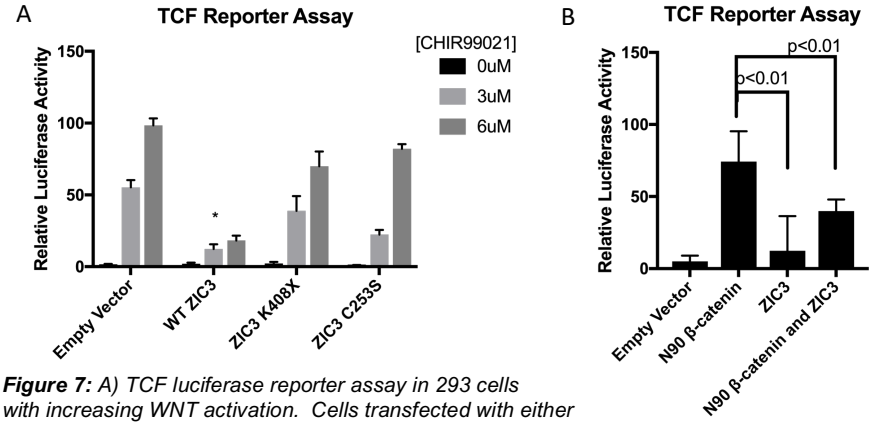


Figure 7: *A) TCF luciferase reporter assay in 293 cells with increasing WNT activation. Cells transfected with either WT ZIC3 or ZIC3 mutants found in heterotaxy patients (K408X and C253S). Values normalized to empty vector without CHIR99021. N=6 for each condition *p<0.05 by ANOVA compared to empty. C) TCF luciferase reporter assay in 293 cells. N=6 for each condition. Cells transfected with indicated constructs. N90 β-cateninin is a constitutively active mutant.*

CITED2 and *GJA1*. These results were confirmed by RTPCR (not shown). *ZIC3* loss was highly associated with alterations in pathways involving cellular movement, cell-cell interaction, cell assembly and cell morphology. Gene set enrichment analysis demonstrate WNT pathway activation in response to *ZIC3* knockdown (FDR q=0.13) (Fig. 3A). AXIN2, a feedback β-catenin inhibitor, was blunted in response to mesoderm induction in CHIR99021 treated ZIC3 knockdown iPSCs which may provide the basis for upstream WNT pathway modulation as well.

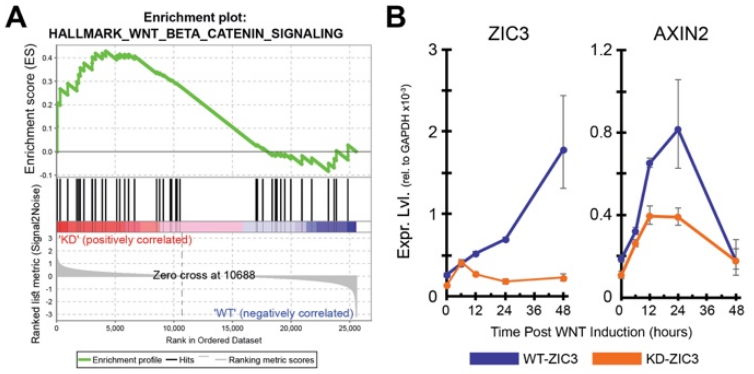


Figure 8: *A) GSEA analysis of RNA-seq data showing enrichment of WNT activation in ZIC3 knockdown B) Levels of ZIC3 and AXIN2, a WNT feedback inhibitor, over time after CHIR99021 mesoderm induction in wt and knockdown.*

<u>ZIC3 loss shifts developing mesoderm towards left sided:</u> The central goal of this proposal was to measure a difference in cell chirality in response to *ZIC3* knockdown. Because cardiomyocyte gene expression differences in response to *ZIC3* loss was essentially not significant, we focused our efforts on iPSC and the differentiation of those cells into lateral plate mesoderm. This parallels development as the failure of proper left right patterning which underlies the genesis of heterotaxy and visceral situs occurs during the correct specification of left and right sided mesoderm. Gene set enrichment analysis showed an upregulation of WNT signaling with knockdown of *ZIC3*. *NODAL* is the main driver of left sided mesoderm and eventual expression of *PITX2*, the regulator of organogenesis on the left side of the developing embryo, we further explored the genetic imprint of left and right side in ZIC3, specifically in response to WNT stimulation as well as NODAL stimulation. A time course of WNT activation demonstrated that *ZIC3* knockdown in iPSC's led to increased expression in *NODAL* and decreased expression of *BMP4* (Fig. 4A) showing a predominantly left sided mesodermal signature. Interestingly, ZIC3 itself was also expressed in response to WNT activation, perhaps representing a feedback loop mechanism for WNT signaling. As NODAL is a secreted factor that goes on to stimulate the expression of itself and is critical for left side specification, we also tested the knockdown of *ZIC3* in NODAL stimulated iPSC's. Similarly, ZIC3 knockdown increased *NODAL* and *PITX2* levels in response to NODAL ligand stimulation while BMP4 levels were attenuated (Fig 4B), further confirming that knockdown of ZIC3 potentiates left sided NODAL signaling and PITX2c driven left sided fate. Together, this indicates that ZIC3 controls the balance of left and right sided fate and its loss leads to abnormal left sided specification and aberrant axis formation.

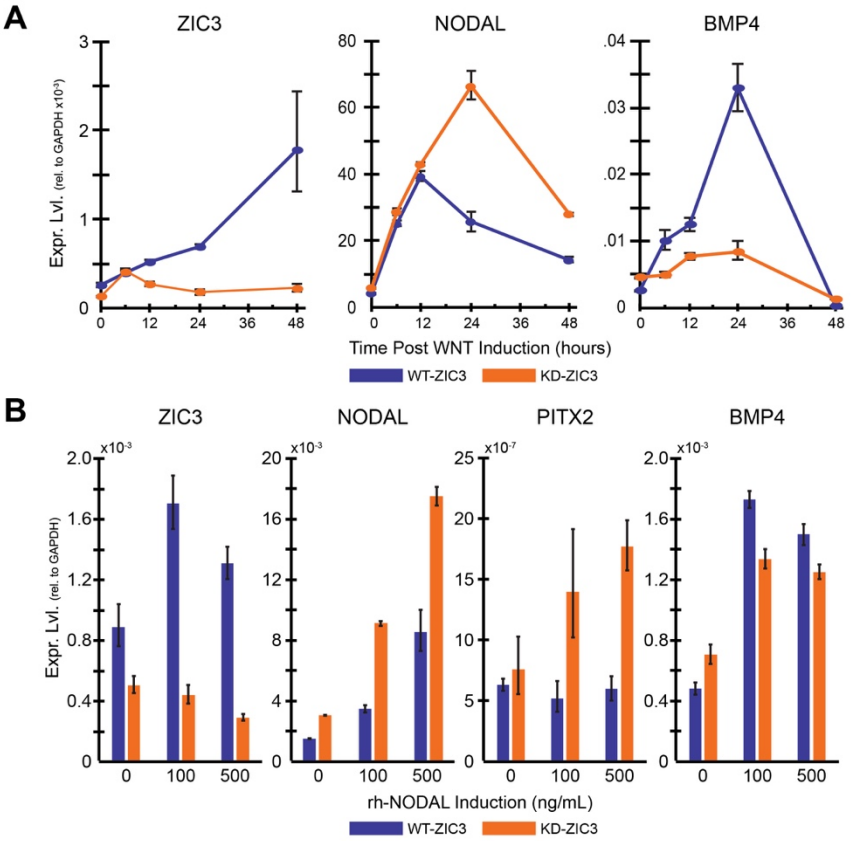


***Figure 4**: A) WT and ZIC3 knockdown iPSC exposed to WNT activator CHIR99021 at time zero. RTPCR over 48 hours demonstrate increased NODAL and decreased BMP4 in ZIC3 knockdown. B) WT and ZIC3 knockdown iPSC exposed for 24 hours to recombinant NODAL. RTPCR of 100 and 500ng of NODAL demonstrate increased NODAL and PITX2 expression and decreased BMP4 expression in knockdown ZIC3 iPSC. N=3 for each data point. Error bars are STD.*

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?
Nothing to report. This is the final report.

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. We report that knockdown of ZIC3 pushes iPSC derived mesoderm towards left sided gene expression, potentiating nodal signaling. Thus we were successful in modeling left right asymmetry and the consequence of ZIC3 loss on that axis. 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
During this initial 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.

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.

Cardiomyocyte gene expression was relatively similar between ZIC3 wild type and knockdown states and as such we decided to look for left and right sided identification of mesodermal cells given this is the developmental aberration underlying heterotaxy. We were successful in being able to identify increased left sided gene expression in response to ZIC3 loss in mesoderm derived from iPSC, thus indicating a potential mechanism whereby the left and right sided asymmetry is abrogated in response to ZIC3 loss leading to heterotaxy.

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
As stated above, during the initial reporting period (previously reported) we added minor recruitment to our human subjects protocol. Note that despite this addition, no minors were actually recruited as we were able to fill our prespecified recruitment of 4 patients with adult patients.

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:	1.2
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?
No changes since the last reporting period.

What other organizations were involved as partners?
Nothing to report

SPECIAL REPORTING REQUIREMENTS

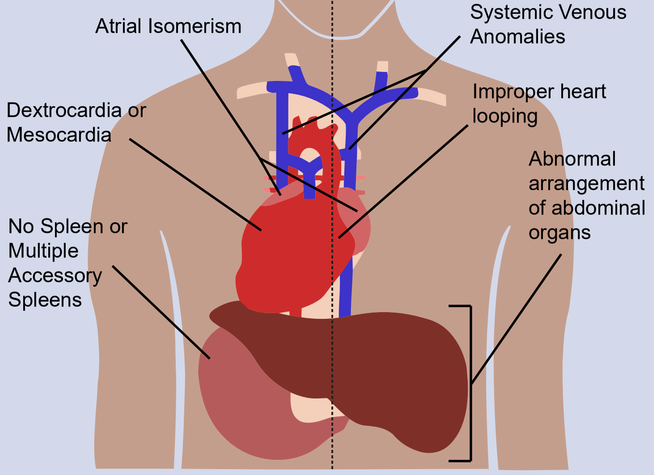
COLLABORATIVE AWARDS

Nothing to report

QUAD CHARTS

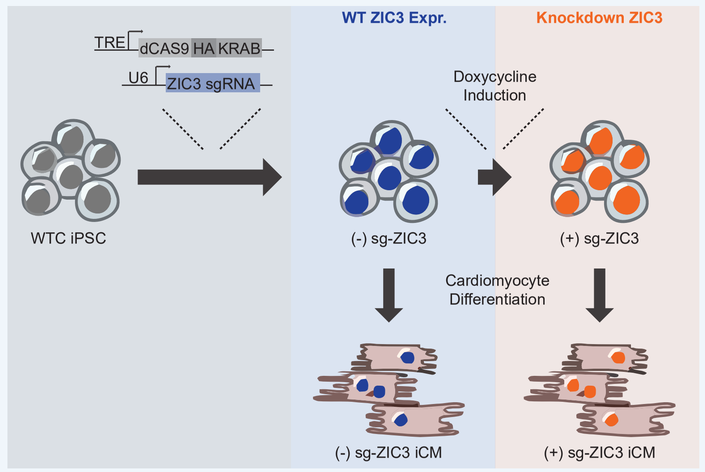
Nothing to report

APPENDICES



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.

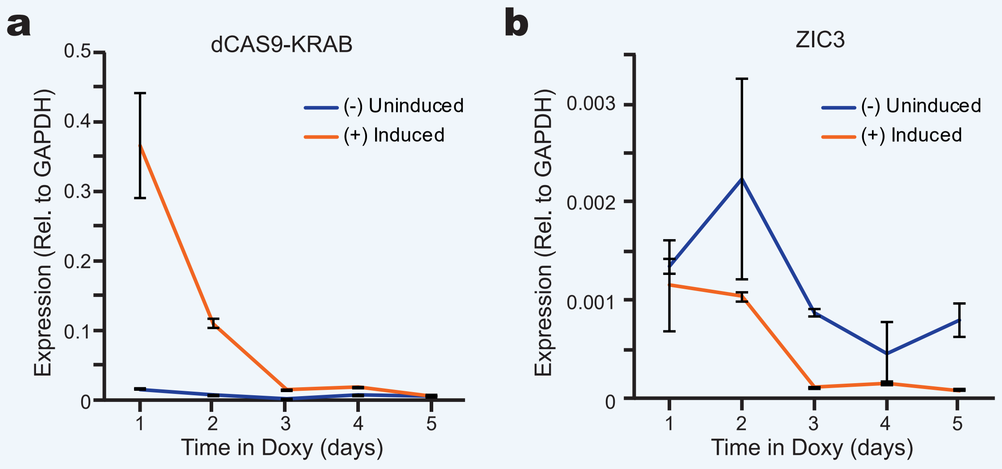
Research Design



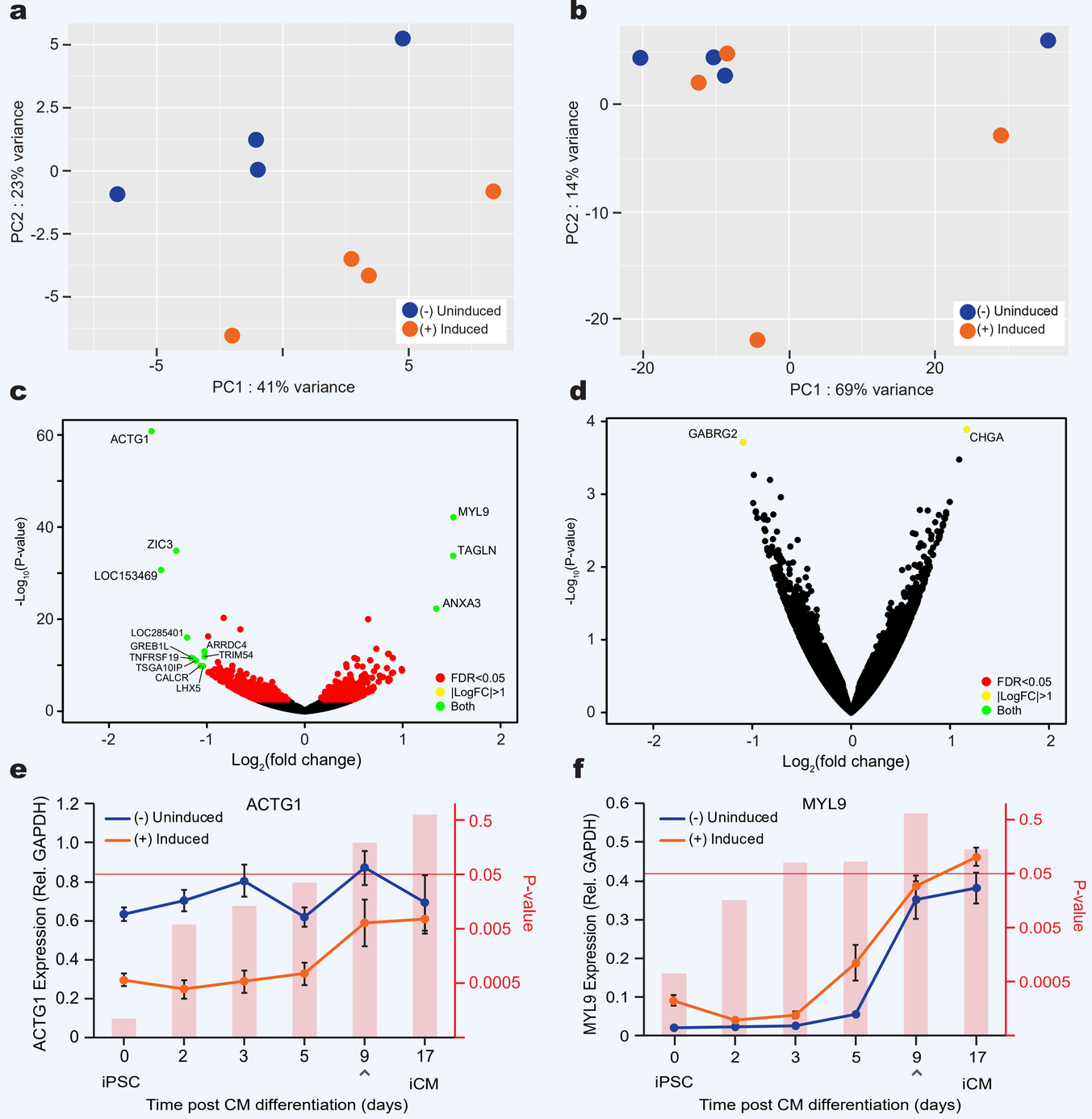
We transduced the commercially available pHAGE-TRE-dCas9-KRAB construct into WTC10 iPSCs. This construct expresses the CRISPRi recombinant protein dCas9-KRAB under the control of a doxycycline inducible tetracycline responsive promoter. dCas9-KRAB is a human codon-optimized, catalytically inactive version 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.

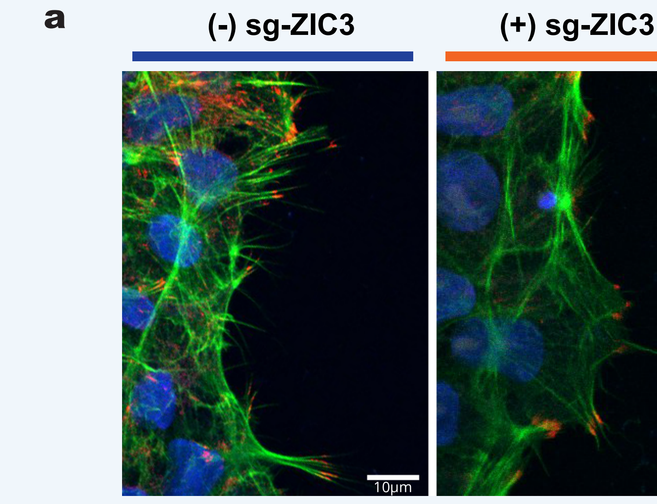


RNA sequencing was used to generate a gene expression profile of *ZIC3* loss in iPSCs and iCMs. We applied DESeq2 to identify differentially expressed genes between induced and uninduced cells. Principal component analysis (PCA) was then used to explore the broad gene expression differences in iPSCs **(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

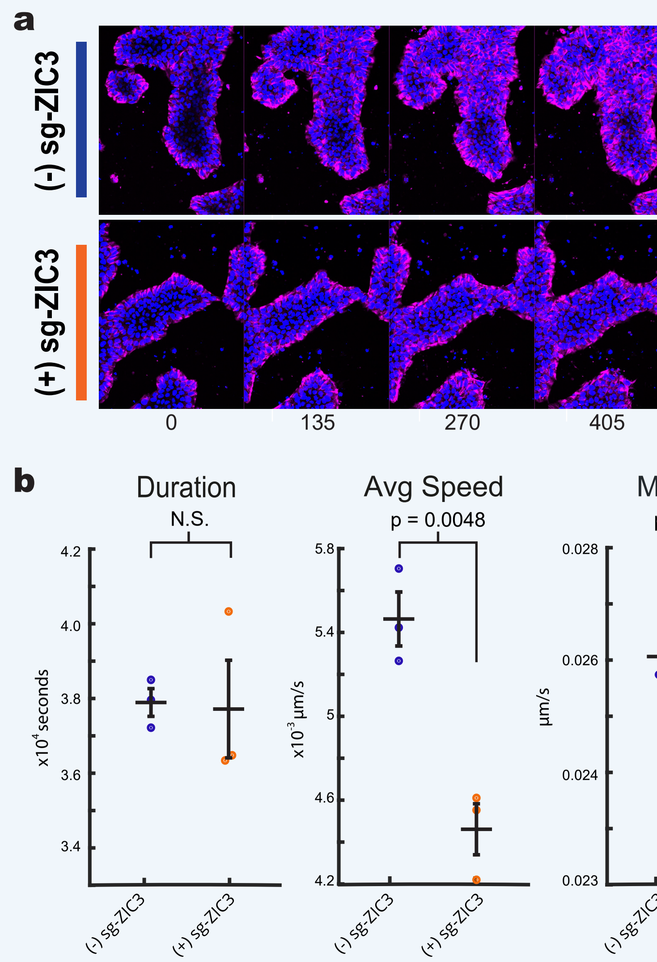
Molecular and Cellular Functions		Physiological System Development and Function	
Name	p-value	Name	p-value
1. Cellular Movement	1.55E-10	Organismal Survival	8.52E-11
2. Cell-to-Cell Signaling and Interaction	7.10E-10	Cardiovascular System Development	4.17E-10
3. Cellular Assembly and Organization	7.10E-10	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.



The effect of *ZIC3* knockdown on the actin cytosk
microscopy. Induced and uninduced sg-ZIC3 iPS
(Paxillin-red), filamentous actin (Phalloidin-gree
images showed ZIC3 knockdown in iPSCs reduc
well as focal adhesions at the edges of the cell c
protrusions found at the leading edge of migrati
hesion abilities. Therefore, the reduction of filo
that ZIC3 knockdown may impede cellular move

ZIC3 knockdown in iPSC



To further explore functional differences cause
cent imaging of iPSCs stained with SiR-Actin (Ma
sulting timelapses demonstrated visible reduct
pared to uninduced over 16 hours. Subsequent
the movement of individual cells through nuclei
cation of migration parameters demonstrated a
speed, distance, and displacement in induced iP
ance with differential gene expression analysis,
pacts iPSC cytoskeleton and cell mobility.

Concl

In this study, we demonstrate that human iPSCs
differentiation technologies, can be used to mec
nings of heterotaxy. Our model allowed us to de
cies in iPSC mobility and differences in cellular
nism of *ZIC3*-mediated heterotaxy. Importantly,
in human iPSCs and subsequent cardiomyocyte
ploring forms of congenital heart disease at a m

We gratefully acknowledge funding for this work by the Department of Defense Discovery Award W81XWH-17-1-0112 and the NIH Awards (HL076485, EB002520)