AWARD NUMBER: W81XWH-17-1-0163

TITLE: Human Cardiac Microtissues to Study Sex-Dependent Genetic Determinants of Heart Failure

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REPORT DATE: March 2019

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE
March 2019

2. REPORT TYPE
Final

3. DATES COVERED
15 May 2017 - 14 Nov 2018

4. TITLE AND SUBTITLE
Human Cardiac Microtissues to Study Sex-Dependent Genetic Determinants of Heart Failure

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0163

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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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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
Heart failure with preserved ejection fraction (HFPEF) is a disorder with high morbidity and mortality. Unique among cardiovascular conditions, HFPEF affects women predominately more than men, but mechanisms for this sex dimorphism in HFPEF prevalence are incompletely understood. Sex-based dimorphisms have been studied mostly in non-human model systems, but are limited by incomplete recapitulation of human sex-based gene regulation and gene-environment interactions. Thus, there is a critical unmet need to develop in vitro human model systems to interrogate how sex regulates HFPEF pathogenesis and cardiac function broadly. The overarching goal of this project is to apply a cardiac microtissue assay to study sex dimorphisms in cardiac function. In Aim 1, we have generated cardiac microtissues from male and female induced cardiomyocytes (iCMs) differentiated from induced pluripotent stem cells (iPSCs) to study sex-based regulation of cardiac function. We quantified contractile function and gene expression analyses in these models. In Aim 2, we have produced the first FHL1 knockout iPS model using CRISPR/Cas9. We characterized the role of FHL1 in cardiomyocyte and cardiac tissue function using single cell and cardiac microtissues assays. Insights from this study have illuminated potential sex-dependent genetic mechanisms of HFPEF.

15. SUBJECT TERMS
Heart failure; cardiomyopathy; X chromosome; sex dimorphism; contractility; engineered heart tissue; cardiology

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

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1. Introduction

Heart failure with preserved ejection fraction (HFPEF) is a disorder with high morbidity and mortality. Unique among cardiovascular conditions, HFPEF affects women predominately more than men, but mechanisms for this sex dimorphism in HFPEF prevalence are incompletely understood. Sex-based dimorphisms have been studied mostly in non-human model systems, but are limited by incomplete recapitulation of human sex-based gene regulation and gene-environment interactions. Thus, there is a critical unmet need to develop in vitro human model systems to interrogate how sex regulates HFPEF pathogenesis and cardiac function broadly. The overarching goal of this project is to apply a cardiac microtissue assay to study sex dimorphisms in cardiac function. In Aim 1, we will study cardiac microtissues generated from male and female induced cardiomyocytes (iCMs) differentiated from induced pluripotent stem cells (iPSCs) to study sex-based regulation of cardiac function. In Aim 2, we will study the role of FHL1 by generating an FHL1 knockout human induced pluripotent stem cell model and characterize using cardiac microtissues assays.

2. Keywords

Heart failure; cardiomyopathy; X chromosome; sex dimorphism; contractility; engineered heart tissue; cardiology

3. Accomplishments

What were the major goals of the project?

Major goals of the project:
Aim 1: Create HFPEF cardiac microtissue (CMT) models using sex-specific human iPS-CMs and three conditions that induce HFPEF in patients (isoproterenol, angiotensin and increased mechanical load)

1A: Create CMTs from three pairs of male and female iPS-CMs, and measure contractility parameters in HFPEF conditions.
i. Create CMTs from pair 1 of male (M1) and female (F1) iPS-CMs – We have collected and validated set 1 of male and female human iPSCs and differentiated to iCMs. We have generated cardiac microtissues and measured contractility parameters in seven F1 and ten M1 samples.
ii. Create CMTs from pair 2 of male (M2) and female (F2) iPS-CMs- We have collected and validated set 2 of male and female human iPSCs and differentiated to iCMs. We have generated cardiac microtissues and measured contractility parameters in eight F2 samples.
iii. Create CMTs from pair 3 of male (M3) and female (F3) iPS-CMs- We have collected and validated set 3 of male and female human iPSCs and differentiated to iCMs.
iv. Computational analysis of CMT contractility assays – We have imaged and computationally analyzed all generated cardiac microtissue assays from i-iii.
Milestone (8 months): Goal analysis of ~120 CMTs analyzed for contractility parameters
Achieved: 50%

1B: Structural analysis of sex-specific CMTs using immunostaining
i. Immunostain CMTs for cardiomyocyte, fibroblast and extracellular composition – We have fixed and immunostained 30 cardiac microtissues from Aim 1A. We have performed confocal imaging.
ii. Computational analysis of imaging results-we processed 20 confocal images for tissue structure.
Milestone (12 months): Structural analysis of ~120 CMTs
Achieved: 20%

1C: Expression analysis of heart failure-associated genes by quantitative PCR. We have collected biological triplicates of male and female cardiomyocyte RNA samples. We have assessed RNA quality and have generated cDNA libraries.
Milestone (anticipated, month 14): qPCR analysis of seven gene transcripts across six iCM lines

Achieved: 10%

Aim 2: Determine the role of sex dimorphisms in FHL1 expression in HFPEF CMT assays.

2A: Create iPS lines with allelic series of FHL1 mutations using CRISPR/Cas9

i. Genome editing of iPS lines to achieve FHL1 allelic series of clones. We have generated six control and nine FHL knockout human iPSC lines using CRISPR/Cas9. We have differentiated these iPSC lines to iCMs and validated FHL1 expression analysis.

ii. Sequencing confirmation of mutations- We have Sanger sequenced 96 iPSC lines to assess FHL1 genotypes.

Milestone (8 months): Create isogenic iPS model of FHL1 deficiency

Achieved: 100%

2B: CMT assays of allelic series of FHL1 mutations

i. Measure contractility parameters in three HFPEF conditions for FHL1 allelic series of CMTs- We have measured CMT function in 15 FHL1 deficient and 15 control cardiac microtissues assays.

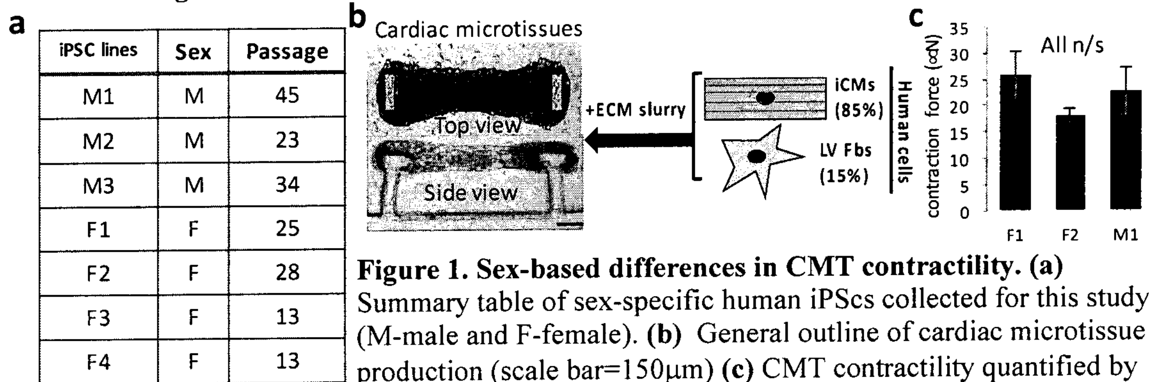
ii. Structural analysis of CMTs by immunostaining cardiomyocyte, fibroblast and extracellular components- We have fixed and immunostained 15 FHL1 deficient and 15 control cardiac microtissues assays.

iii. Gene expression analysis by quantitative PCR for heart-failure associated genes. We have collected biological triplicates of three control and six FHL1 deficient iCMs for transcript analysis.

Milestone (16 months): Functional analysis of FHL1 dosage in CMT function and structure.

Achieved: 100%

What was accomplished under these goals?



iPSC lines	Sex	Passage
M1	M	45
M2	M	23
M3	M	34
F1	F	25
F2	F	28
F3	F	13
F4	F	13

Figure 1. Sex-based differences in CMT contractility. (a) Summary table of sex-specific human iPSCs collected for this study (M-male and F-female). **(b)** General outline of cardiac microtissue production (scale bar=150μm) **(c)** CMT contractility quantified by cantilever displacement and assessed for three lines (n>5 CMTs each)

1) *Major activities*: To date, we have collected seven iPSc lines (3 male and 4 female) and confirmed differentiation to the cardiomyocyte lineage with high efficiency (Figure 1a). We have generated cardiac microtissue models of three lines and quantified contractility parameters (Figure 1b, c). We have engineered nine iPSC clones with FHL1 knockout mutations and six clones for isogenic controls using clustered regularly interspaced short palindromic repeat (CRISPR) technology. We have differentiated FHL1 knockout and control iPSCs to iCMs with high efficiency and characterized cellular and microtissue contractility function. We have also performed additional computational analyses of sex-based gene expression changes in human cardiac samples from GTEX data and from iCM data generated by our lab. Finally, we generated FHL1 knockout and control cardiomyocytes and performed transcritpome analysis using RNA-sequencing and computational analysis.

2) *Specific objectives*: The specific objective in this reporting period was to assess sex-based differences in cardiac microtissue function, identify sex-based differences in gene expression and determine the role of FHL1 in iCM and cardiac microtissue function as FHL1 exhibits sex dimorphism in gene expression levels.

3) _Significant results:_ To date, we have collected seven iPSC lines from three males and four females to identify lines that have high propensity for iCM differentiation. We have next generated cardiac microtissues from three of these iPSC lines to study sex-based differences in cardiac tissue function. We have also completed additional computational analysis of gene expression data sets from human heart samples obtained as part of GTEX consortium and in our series of iCM models. Through additional validation, we have a more comprehensive understanding of sex-based dimorphisms in gene expression, especially those genes with conserved expression between iCMs and in vivo cardiac samples. We also have identified limitations in the utilization of human iPSCs for studying sex dimorphisms. Finally, we have also utilized CRISPR technology to study the role of FHL1 in iCM and cardiac microtissue function. We have generated the first FHL1 knockout iPSC models nad have identified phenotypes associated with FHL1 loss. Significant and preliminary results from this project period are as follows:

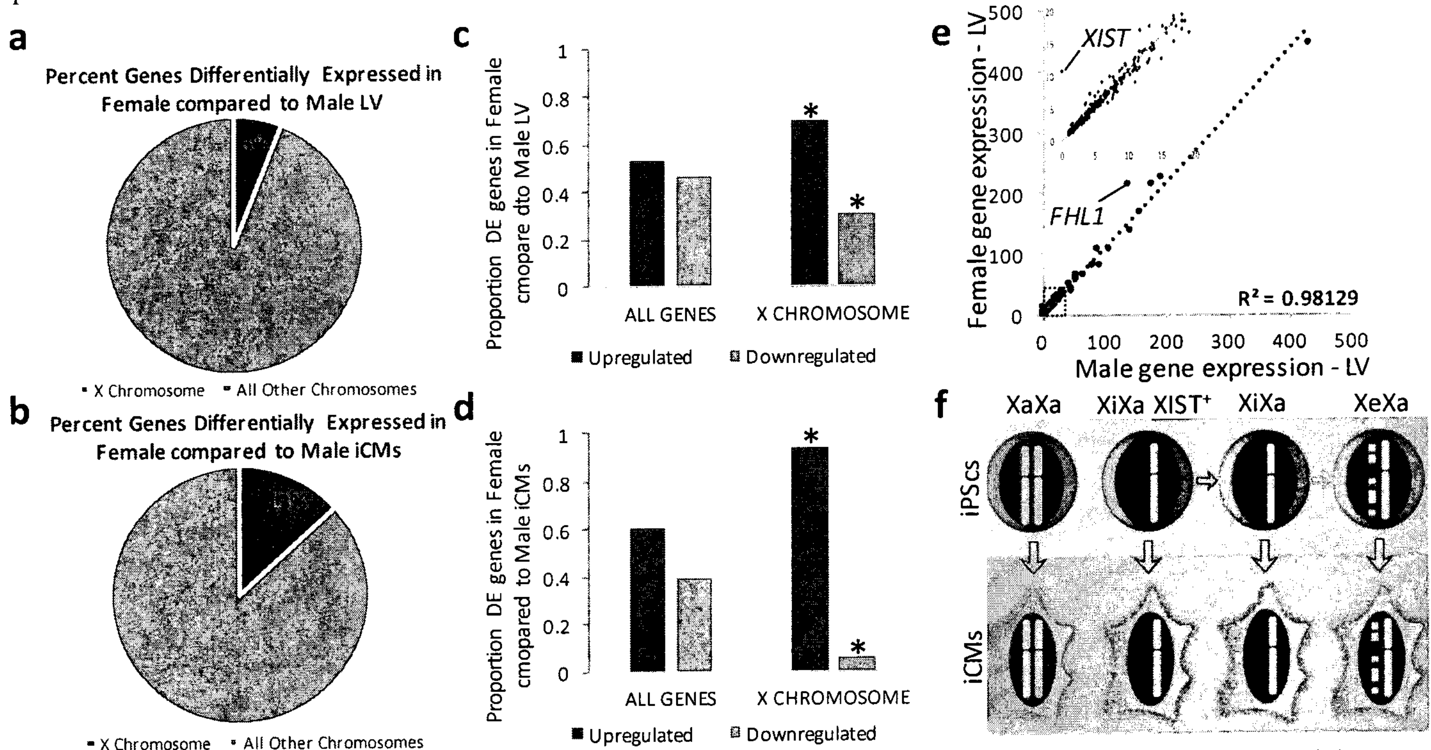
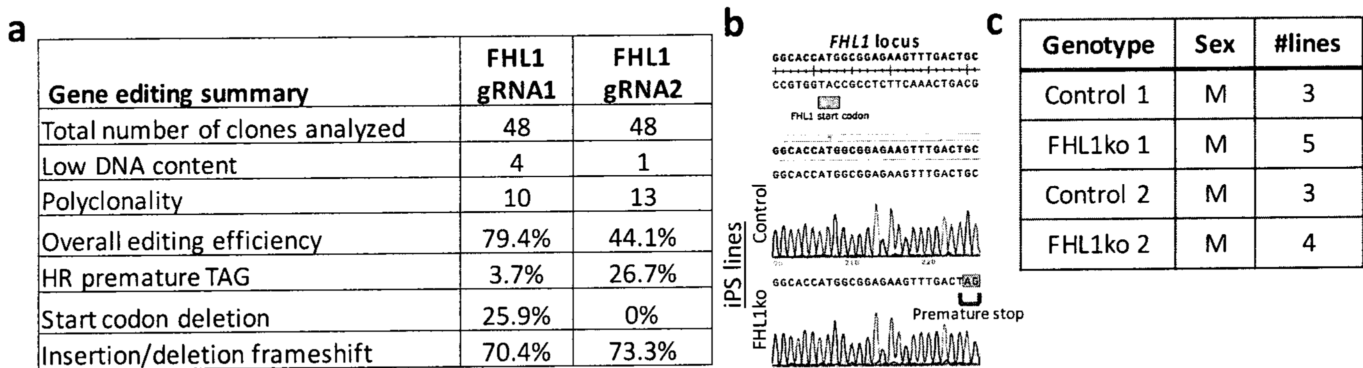


Figure 2. Summary of sex-based gene expression analysis in GTex and iCM data. (a) Sex-based differential gene expression analysis (FDR<0.1) of transcripts located on the X chromosome (blue) compared to autosomes (orange) and expressed in human cardiac left ventricle (LV) and **(b)** iCM lines. **(c)** Upregulated and downregulated gene expression based on chromosome location from LV and **(d)** iCMs (*p<0.001). **(e)** Female compared to male gene expression levels from LV tissue (note: *XIST* and *FHL1* are more highly expressed in females). **(f)** Human iPScs have four states of X chromosome activation (Xa-active (yellow), Xi-inactive (red), XIST+-coated with XIST (green), Xe-eroded)(adapted from Patel *et al.* PMID#27989715).

1. **Generation and phenotyping of sex-specific iCM and cardiac microtissue models.** As shown in Figure 1, we have collected seven iPSC lines from healthy males and females. We have differentiated iPSCs to iCMs, and generated cardiac microtissues from two females and one male. After generating cardiac microtissues, we found non-significant differences in contractile function at baseline. Because of low cardiomyocyte differentiation rates, we could not produce additional lines for cardiac microtissue testing to this point. We are currently optimizing cardiomyocyte differentiation methods to address this challenge prior to the next reporting period.

2. **Sex-based dimorphisms in iCM gene expression are confounded by iPSC X chromosome erosion.** As shown in Figure 2, we have analyzed differential gene expression in females compared to males from iCM and human heart datasets (left ventricle (LV) expression from GTEX). To our surprise, we identified that a disproportionate fraction of genes encoded on the X chromosome are upregulated in female iCMs compared to male iCMs when compared to LV samples (Figure 2a-d). We were surprised to identify that many of the differentially-expressed iCM genes were not shared in LV samples. However, FHL1 (focus of Aim 2) is upregulated in both datasets (Figure 2e), which further supports the role of FHL1 dosage in sex-based changes in cardiac function. During the last year, several research studies have reported that both human embryonic stem cells and iPScs are prone to unpredictable X chromosome leak, which could explain the increased expression of X chromosome genes in our female iCM lines. Several mechanisms including loss of XIST expression and epigenetic erosion have been reported (Figure 2f). Because of this confounding variable, we have paused additional experiments in Aim 1 until we can verify the status of our iPSC models.



a

Gene editing summary	FHL1 gRNA1	FHL1 gRNA2
Total number of clones analyzed	48	48
Low DNA content	4	1
Polyclonality	10	13
Overall editing efficiency	79.4%	44.1%
HR premature TAG	3.7%	26.7%
Start codon deletion	25.9%	0%
Insertion/deletion frameshift	70.4%	73.3%

b

c

Genotype	Sex	#lines
Control 1	M	3
FHL1ko 1	M	5
Control 2	M	3
FHL1ko 2	M	4

Figure 3. Summary of sex-based gene expression analysis in GTex and iCM data (a) Summary table of sex-specific human iPScs collected for this study (M-male and F-female). **(b)** General outline of cardiac microtissue production (scale bar=150μm) **(c)** CMT contractility quantified by cantilever displacement and assessed for three

3. **Generation of FHL1 knockout iPSC models to study the role of FHL1 in cardiac microtissue function.** As shown in Figure 3a, we have generated an FHL1 knockout iPSC model using CRISPR genome engineering technology. In total, we produced 96 iPSC clones, of which we obtained nine FHL1 knockout alleles that are sequence-verified and do not express FHL1 protein (Figure 3b, c). We have generated iCMs from these iPSC lines and studied iCM structure and cardiac microtissue contractility. We are excited to report that FHL1 knockout iCMs have dramatically reduced cell size and contractile function in cardiac microtissue assays (Figure 4). In sum, we studied 100 FHL1 knockout iCMs and 100 controls to assess changes in cell structure, and we generated 30 cardiac microtissues. Our leading hypothesis related to the deficit in contractility is due to diminished hypertrophic signaling, which is downstream of FHL1 function. To understand how FHL1 regulates the cardiomyocyte transcriptome, we performed RNA-sequencing analysis of biological triplicates of control and FHL1ko cells (Figure 5a-c). Our study identified that FHL1 knock-out results in down-regulation of 393 and upregulation of 233 gene transcripts; thus FHL1 acts as a transcriptional activator. We anlayzed differential expression by Ingenuity pathway analysis (IPA) to identify genetic networks regulated by FHL1. IPA identified downregulated pathways including SRF, insulin, MEF2C and RAF, and upregulated pathways including mifepristone and HEY2 (Figure 5d). RAF is a component of MAPK/ERK signaling that is known to be regulated by FHL1, but the other pathways identified are not previously known to be FHL1-dependent.

Stated goals not met: We have successfully collected and generated a series of male and female iPSCs that we differentiated to iCMs for further investigation. We successfully generated cardiac microtissues from male and female iCMs and studied contractile parameters by these assays. Through additional computational analysis by our group of iCM and LV (GTEX)

gene expression and through new insights from published studies on X chromosome regulation in iPSCs and ESCs, we previously halted using iPSC models to study sex dimorphisms in gene expression (Aim 1). This is because the X chromosome in female iPSCs is not reliably silenced in accord with in vivo X chromosome regulation. In Aim 2, we have successfully generated FHL1 knockout iPSC models and generated cardiac microtissues to study contractile function. We have also completed transcriptome analysis. We are meeting our goals for all components of Aim 2. Indeed, FHL1 is one of the X chromosome genes that is increased in females compared to males in both in vitro and in vivo LV samples (Figure 2e), and therefore we will continue our efforts to understand its role in sex dimorphism in HFPEF.

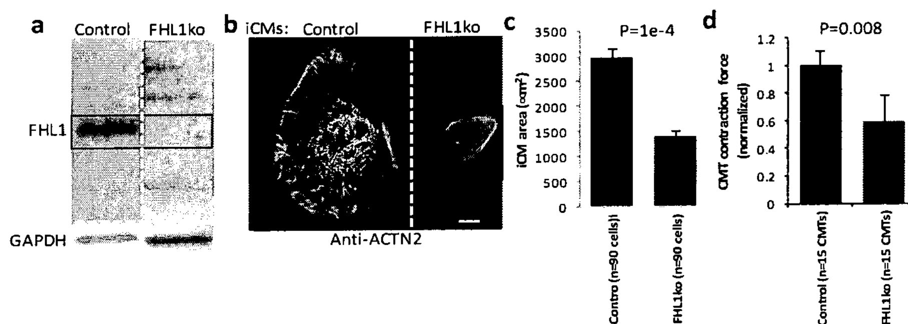


Figure 4. Characterization of FHL1 knockout iPSC models. (a) Immunoblot of iCM lysates from FHL1 knockout and controls probed with antibodies to FHL1 and GAPDH. **(b)** Representative confocal image of control and FHL1 knockout iCMs stained with antibodies to cardiac alpha actinin (ACTN2)(scale=10μm) **(c)** iCM cell area analysis of control and FHL1 knockout iCMs. **(d)** iCM cell area analysis of control and FHL1 knockout iCMs. (D)

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

Yu-Sheng Chen and I meet every week for one-on-one mentoring sessions to discuss experimental results, progress and data interpretation.

How were the results disseminated to communities of interest?

We have presented this data to the UConn Health Department of Cell Biology quarterly meetings. We have also presented this to the UConn Health Department of Medicine Grand Rounds in May 2018. We are presenting these data to "Work-In-Progress" meetings at The Jackson Laboratory. We are currently writing a manuscript to summarize the FHL1 modeling portion of this project (Aim 2). We are on schedule to submit a manuscript by end of summer 2019.

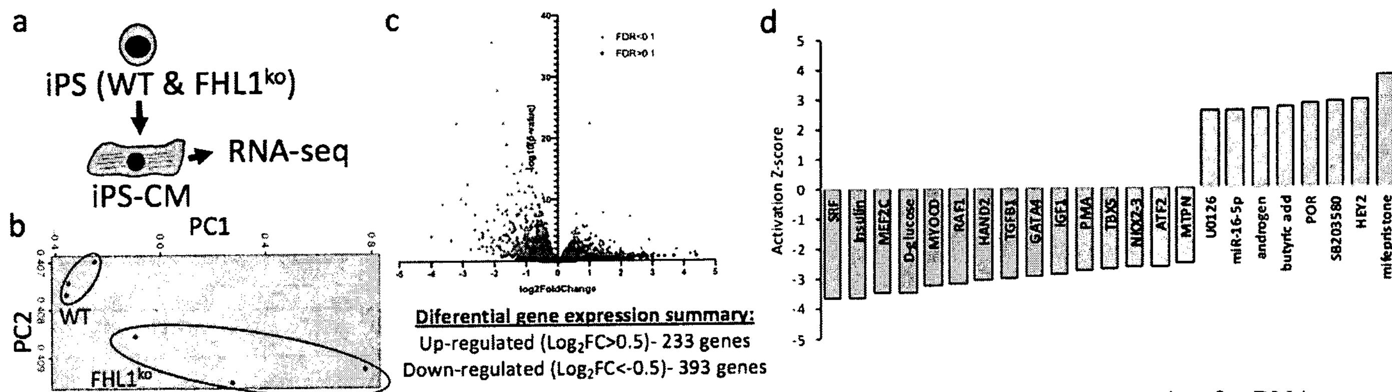


Figure 5. Transcriptome analysis of FHL1ko cardiomyocytes. (a) Overview of sample generation for RNA-sequencing. **(b)** Principle component (PC) analysis of RNA-sequencing data derived from three biological replicates of control (WT) and FHL1^{ko} cardiomyocytes. **(c)** Volcano plot of RNA-sequencing data analyzed by DESeq2. FHL1 knockout results in differential expression of 233 upregulated and 393 downregulated genes. **(d)** Pathway analysis by IPA of differentially expressed transcripts identifies FHL1-activated and inhibited pathways.

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

Not-applicable.

4. Impact

In accord with recent scientific studies, our study has confirmed that the X chromosome activation status in human iPSCs is unreliable, and the use of sex-specific iPSCs for studying heart disease is limited without significant specialized assessment of inactivation status. This is because unlike human cells within an individual, human cells in cell culture have increased expression especially from the X chromosome because of "expression leak." Despite this limitation, our study does confirm that the FHL1 gene is more highly expressed in female hearts compared to males, and we have generated the first FHL1 knockout human stem cell model that can be used to identify the role of FHL1 in heart function broadly. This is a unique resource to the biomedical field, and will inform how FHL1 dosage affects heart function as female hearts have higher FHL1 levels compared to males. We have produced cardiomyocytes from human stem cell models and characterized the role of FHL1 in the regulation of cellular hypertrophy, signaling and gene expression. We have paired these studies with cardiac microtissues models that read-out contractile function in a biomimetic context. We believe these studies provide new insights into the role of FHL1 in cardiomyocyte function, especially relevant to women's heart disease given that FHL1. This study also impacts the fields of tissue engineering, cardiology and human genetics as FHL1 mutations cause human myopathies.

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants and Other Collaborating Organizations

Name:	Dr. Travis Hinson
Project Role:	*PD/PI*
Nearest person month worked:	1
Contribution to Project:	*Dr. Hinson has managed the project, directing both the experiments and analyses completed by Chen*

Name:	*Yu-sheng Chen*
Project Role:	*Post-doctoral fellow*
Nearest person month worked:	6
Contribution to Project:	*Dr. Chen has generated data, interpreted results and compiled figures for this proposal.*

8. Special Reporting Requirements

Not-applicable

9. Appendices

Not-applicable