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14. ABSTRACT

In the first year of this project, we were able to evaluate loss of function mutations for gata4, gata5, and gata6, in both zebrafish and human ESC models. We were surprised to find that gata4 null mutations were tolerated in zebrafish, while the gata5 and gata6 mutations phenocopied previous analyses including a small truncated heart tube for gata6 mutants. We used western blotting to demonstrate that the gata4 mutation is null. Most strikingly, aged mutant adults showed a severe cardiomyopathy. The heart is enlarged at least two-fold in size. We are currently characterizing hearts by histology to determine if the gata4 mutants may be a model for human dilated cardiomyopathy or some other specific disorder. This is an exciting result that supports our underlying hypothesis that the mutants can be used to model human congenital cardiac disease. In addition, we found a striking defect in cardiogenesis in the human GATA6 null mutant ESCs. We correlated this with a sharp increase in expression of RALDH2, suggesting that GATA6 normally restricts retinoid signaling important for cardiac differentiation.

15. SUBJECT TERMS
Cardiomyopathy, hESCs, hiPSCs, directed differentiation, congenital heart disease, zebrafish

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INTRODUCTION:

The overall goal of the project is to discover mechanisms that cause congenital cardiomyopathy. As a model system, we are using animals (zebrafish) and human stem cells that carry defined mutations in the Gata4, Gata5, or Gata6 genes (Gata456), which have been associated with patients that suffer from congenital cardiomyopathy. **Our overall hypothesis** is that by manipulating Gata456 with combinatorial, temporal, and spatial specificity, we will identify important regulators and modifiers of lineage specification, tissue morphogenesis, and cell differentiation that are deregulated and thereby cause congenital cardiomyopathies related to human GATA456 gene variants. The genes and pathways we discover will provide relevant therapeutic targets to treat congenital heart disease and potentially even morbidity in adult patients that had been treated successfully by surgery. We are using zebrafish with defined mutants as an animal model and human pluripotent cells to translate to human lineages. One important goal is to find genes and pathways that interact with GATA factors that might explain why the human mutations are haploinsufficient.

1. KEYWORDS:

Cardiomyopathy; Genetics; Mutations; Zebrafish; Embryonic Stem Cells; Induced Pluripotent Stem Cells; Progenitors; Differentiation

2. ACCOMPLISHMENTS:

What were the major goals of the project?

For Specific Aim 1 (Identify the function and downstream regulatory programs for individual and combinations of Gata456 during zebrafish cardiogenesis).

Major Goal 1: Compare cardiac phenotype in wildtype and mutant gata4, gata5, gata6, gata45, gata46, and gata456 embryos at defined stages of cardiogenesis (1-18 months). Completed in 24 months.

Major Goal 2: Profile isolated cardiac cells in wildtype and mutant strains of Gata456 zebrafish mutants (6-24 months). Completed by 36 months.

Major Goal 3: Define temporal and tissue specificity for each Gata456 gene during zebrafish cardiogenesis (1-36 months). Partially completed in 36 months (50%).

For Specific Aim 2 (Identify the function and downstream regulatory programs for individual and combinations of GATA456 during human cardiogenesis from hESCs).

Major Goal 1: Compare cardiac phenotypes in wildtype and mutant GATA4, GATA5, GATA6, GATA45, GATA46, and GATA456 hESC lines at defined stages of cardiogenesis (1-24 months). Partially completed in 36 months (75%).

Major Goal 2: Define the capacity of each human GATA456 gene to direct cardiogenesis (1-24 months). Partially completed in 36 months (75%).

Major Goal 3: Carry out analogous differentiation and phenotyping assays using patient-derived hiPSCs carrying defined mutations in GATA456 (1-24 months). Completed in 24 months.

For Specific Aim 3 (Discover interacting pathways of Gata456 relevant to human congenital heart disease).

Major Goal 1: Carry out chemical screening platform using the GATA6+/- and/or GATA6-/- hESC lines (1-24 months). Partially completed in 36 months (50%).

Major Goal 2: Decode the mechanism of the hit compounds (focused on 1-3 top candidates (6-36 months). Partially completed in 36 months (25%).

Major Goal 3: Validate the identified interacting signaling pathway (18-36 months). Partially completed in 36 months (25%).

What was accomplished under these goals?

1) Major activities.
We completed extensive phenotyping of the single and combined gata456 mutant zebrafish embryos. The adult *gata4* mutant cardiomyopathy was analyzed in detail, particularly with respect to morphology and fat deposition. Mutant hESCs were evaluated fully for differentiation potential, leading to a focus on *GATA6* mutants that fail to generate cardiomyocytes. Focus turned to potential downstream genes and signaling pathways that are responsible. Similar experiments were carried out using hiPSCs harboring a *GATA6* mutation, compared to our heterozygous mutant hESCs. We optimized our capacity to generate floxed alleles in the zebrafish model, and established key Cre driver lines for myocardium and endoderm.

2) Specific objectives.
One major goal was to determine if mutations cause similar or distinct cardiomyopathies. A major objective was to determine if combinations of mutations were additive or synergistic. We also wanted to know if mutations in the zebrafish and human system cause the same or different phenotypes, as it will help us interpret how to translate results to patients. We were surprised that the zebrafish gata4 mutation was tolerated during embryogenesis, which led us to characterize in

more detail the phenotype of adult fish with gata4 mutations, including cardiac function. We sought to determine if the iPSCs behaved more like heterozygous or null ESCs in the differentiation. A clear objective is to find the key downstream target genes that mediate function of GATA456.

Key outcomes.
The goals for Aim1 were completed and led to important new insights. We successfully generated targeted deletions to disrupt each *gata4/5/6* gene in zebrafish and analyzed cardiac phenotypes in single, double, and triple mutants. The analysis confirmed that loss of *gata5* causes *cardia bifida* and validated functional redundancies for *gata5/6* in cardiac precursor specification. Surprisingly, we discovered that *gata4* is dispensable for early zebrafish development, while loss of one *gata4* allele can suppress the *bifid* phenotype of the *gata5* mutant. We discovered that the *gata4* mutants eventually develop an age-dependent cardiomyopathy. By combining combinations of mutant alleles, we showed that cardiac specification depends primarily on an overall dosage of *gata4/5/6* alleles rather than a specific gene. We also identified a specific role for *gata6* in controlling ventricle morphogenesis through regulation of the both the first and second heart field, while loss of both *gata4/6* eliminates the ventricle. Thus, different developmental programs are dependent on total dosage, certain pairs, or specific *gata4/5/6* genes during embryonic cardiogenesis.

We successfully generated a floxed allele of gata6 and are currently validating a floxed allele of gata5. These will be important tools for ongoing studies to evaluate temporal and lineage-specific roles for these key cardiac transcription factors.

Regarding Aim 2, we used cardiac directed differentiation with human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) as a platform to study GATA6 function. *GATA6*$^{-/-}$ hESCs failed to generate cardiomyocytes or cardiac progenitors during cardiac-directed differentiation. The expression of markers for cardiac mesoderm were markedly reduced in *GATA6*$^{-/-}$ cells compared to controls, indicating a defect in mesoderm patterning. Transcript profiling by RNA-seq at the mesoderm patterning stage revealed reduced expression of BMP4 responsive gene sets, suggesting that GATA6 functions to promote SMAD transcriptional activity during this stage. In addition, we identified *LGR5*, encoding a GPCR associated with WNT signaling as a putative key downstream target of GATA6. In contrast to the homozygous mutants, *GATA6*$^{+/-}$ hESCs generated cardiac progenitors and cardiomyocytes but did so less efficiently than wildtype controls. Analysis of an iPSC line containing a heterozygous mutation in *GATA6* (c.1071delG) derived from a patient with CHD had similar defects in cardiomyocyte differentiation efficiency, suggesting that it can be used to study *in vitro* the human disease phenotype. Together, the data provide evidence for a regulatory function for GATA6 during human cardiac mesoderm patterning and describes a system for examining *GATA6* haploinsufficiency *in vitro*.

Regarding Aim 3, we identified, based on RNA-seq data of differentiating mutant cells compared to wildtype cells, two key developmental signaling pathways that are candidates for mediating GATA6 function, namely those pathways controlled by retinoic acid and TGF-beta. Experiments are ongoing to validate these and define how they interact with GATA-dependent functions.

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.

4. IMPACT:

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

We developed a number of research tools that will be of interest to the wider scientific community, including defined mutants, especially conditional mutants. We also generated many datasets from phenotyping these mutant animals and cell lines. All of these tools will be made available to other scientists as our results become published in the literature. Our discovery of a gata4 age-dependent cardiomyopathy in zebrafish may lead to new insight in adult cardiomyopathy. We are particularly excited by the observation of a fatty heart phenotype, which could provide a new animal model for this disease.

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.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No changes.

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

We encountered technical delays generating conditioinal alleles but were able to overcome these and successfully generate the floxed alleles. This set us back somewhat in the timeline, but we are moving ahead with the analysis using these valuable new tools.

Changes that had a significant impact on expenditures

Nothing to Report

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

No changes.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

No changes.

Significant changes in use of biohazards and/or select agents

N/A

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. Sam, J., Mercer, E.J., Torregroza, I., Banks, K.M., and Evans, T. Specificity, Redundancy, and Dosage Thresholds among *gata4/5/6* Genes during Zebrafish Cardiogenesis. *Biology Open.* 9, 2020. bio053611. PMCID: PMC7327998. YES.
2. Badieyan, Z.S., Banks, K.M, Lacko, L.A., Hurtado, R., Schwartz, R.E., Chen, S., and Evans, T. A Hormone Cocktail that Enhances hESC-derived Cardiomyocyte Maturation. In revision. YES.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Invited seminars:

Stanford University, Palo Alto, CA. Sept. 2019
Stony Brook University, Stony Brook, NY. Oct. 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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Note: person-months listed below have been rounded to the nearest whole number.

Todd Evans, Principal Investigator
Researcher Identifier: TEVANS
Nearest person-month worked: 2

Contribution to Project: Dr. Evans supervised all experimental work, analyzed data and provided constant consult and advice to the investigators on the project.

Funding Support: R35HL135778, 3R01DK119667-02S1, R01GM129380, NYSTEM, American Heart Association, Bill & Melinda Gates Foundation

Brandoch Cook, Assistant Professor: "No Change"

Katherine Zollo, Research Technician: "No Change"

Fabrice Jaffre, Instructor:
Nearest person-month worked: 3

Contribution to Project: Dr. Jaffre is an expert in using hPSCs for disease modeling. He assists the team in optimizing differentiation assays using our hESC and hiPSC models. He also led the team studying the role of specific RAF1 mutations to model human congenital heart disease in vitro.

Funding Support: R35HL135778

Joseph Bisson, Postdoc
Nearest person-month worked: 7

Contribution to Project: Dr. Bisson has focused effort primarily on the GATA6 mutant hESCs and has discovered alterations in the Wnt signaling pathway. He has also helped to generate and validate additional mutant cell lines.

Yi-Fan Lin, Postdoc
Nearest person-month worked: 3

Contribution to Project: Dr. Lin is investigating alterations in mitochontrial metabolism in the context of defined mutations.

Funding Support: NYSTEM

Ingrid Torregroza, Research Associate
Nearest person-month worked: 5

Contribution to Project:	Ms. Torregroza has functioned as laboratory manager to help coordinate research supplies and data collection. She is also working to generate and validate conditional zebrafish mutants.
Funding Support:	NYSTEM and Tri-Institutional Stem Cell Initiative

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

For Todd Evans, PI

Ended since last reporting of this DOD contract:

Research Award (Evans, Todd, PI) NCE 08/01/14-12/31/19
Tri-Institutional Stem Cell Initiative
"Molecular control of primitive versus definitive endoderm fates from stem cells".
Role: PI

New since activation of this DOD contract:

F32 HL152575-01 (Bisson, Joseph, PI) 06/01/20-05/31/23
National Heart, Lung, Blood institute
"Defining the downstream genetic networks regulated by GATA6 during human cardiogenesis using iPSC and hESC models"
Role: Mentor

3R01DK119667-02S1 (Chen, Shuibing, PI) 06/01/20-05/31/22
NIH/NIDDK
"Metallothionein 1E as a Central Regulator of Human Pancreatic Beta Cell Function and Survival"
Role: Co-I

BMGF INV-018723 07/22/20-6/30/21
Bill & Melinda Gates Foundation
"COVID-19 CTA: Compound screening using organoid model"
Role: Co-I

For David Christini, co-PI

Ended since last reporting of this DOD contract:
CU MOU 190509-01 (Knuth, Barbara, PI) 11/01/17-10/31/19
Cornell University – Ithaca
"Coalition for Next Generation Life Sciences Doctoral Alumni and Postdoctoral Career Outcomes"
Role: subaward PI

Ended effort allocation since last reporting of this DOD contract:
R35HL135778 (Evans) 1/10/17 – 12/31/19
NIH/NHLBI
"A GATA456 pipeline of discovery"
Role: Co-Inv

*** *Dr. David Christini left Weill Cornell Medicine May 2020*

What other organizations were involved as partners?

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: