

AWARD NUMBER: W81XWH-18-1-0316

TITLE: Investigation of Metabolism in Hypertrophic Cardiomyopathy

PRINCIPAL INVESTIGATOR: Maria Roselle Abraham, MBBS

CONTRACTING ORGANIZATION: University of California San Francisco

REPORT DATE: OCT 2020

TYPE OF REPORT: Final Progress Report

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 OCT 2020		2. REPORT TYPE Final technical		3. DATES COVERED 1JUL2018 - 30JUN2020	
4. TITLE AND SUBTITLE Investigation of Metabolism in Hypertrophic Cardiomyopathy				5a. CONTRACT NUMBER W81XWH-18-1-0316	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Maria Roselle Abraham E-Mail: Roselle.Abraham@ucsf.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Regents of the University of California, San Francisco 1855 Folsom Street, Ste 420 San Francisco, CA 94103-4241				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 In the Discovery Award Application, titled 'Investigation of metabolism in hypertrophic cardiomyopathy' (CDMRP Discovery Award, Log Number: PR171587), we proposed compilation of metabolite profiles (non-targeted metabolomics) from heart tissue and targeted quantitation of candidate plasma metabolite biomarkers at early stage of disease (5 weeks of age) in 2 mouse models of hypertrophic cardiomyopathy (R403Q-MyHC, R92W-TnT) and littermate control mice. Metabolomics was proposed 1) at rest and during dobutamine-stress (inotrope stimulation) at 5 weeks of age, and 2) following 6 weeks of 120 min/day of swim exercise (starting at 5 weeks of age) in the swim and age-matched non-swim group. we hypothesized 1) presence of differences in cardiac metabolism in early disease stage between mutants and age-matched controls, and that 2) acute increase in cardiac work (induced by dobutamine) or chronic increase in cardiac work (resulting from intense daily swimming) would result in mutation-specific changes in cardiac metabolic remodeling and plasma metabolite levels. Using non-targeted metabolomics that yielded >1000 annotated metabolites across 3 platforms (GC-TOF, HILIC-TripleTOF MS, CSH-QTOF MS/MS) we discovered allele-specific, marked differences in cardiac metabolism at baseline, and differences in the cardiac metabolic response to dobutamine in the mouse models at 5 weeks of age. Furthermore, the 2 mouse models also exhibited differences in cardiac metabolic remodeling following exercise training.					
15. SUBJECT TERMS Hypertrophic cardiomyopathy, metabolomics, dobutamine stimulation, exercise-induced metabolic remodeling					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	65	19b. TELEPHONE NUMBER (include area code)

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

In the Discovery Award Application, we proposed cardiac metabolic profiling in 2 mouse models (R403Q-MyHC, R92W-TnT) of hypertrophic cardiomyopathy (HCM) at the early disease stage (5 weeks of age), at baseline, following inotrope (dobutamine)-stress and following exercise-training. Non-targeted metabolomics yielded >1,000 annotated metabolites across the 3 metabolic platforms, namely, GC-TOF MS, HILIC-TripleTOF MS, CSH-QTOF MS/MS. We discovered allele-specific differences in cardiac metabolism at baseline, as well as differences in the cardiac metabolic response to acute inotrope (dobutamine)-stress and chronic exercise-training.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Hypertrophic cardiomyopathy, non-targeted metabolomics, dobutamine-stress, exercise-training, lipidomics, Krebs cycle metabolites, branched chain amino acids, mouse models

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aims: Metabolomics studies in 2 mouse models of HCM (R92W-TnT, R403Q-MyHC)

1. Non-targeted metabolomics in heart tissue at baseline and following inotrope (dobutamine)-stress at 5 weeks of age; and following 6 weeks of intense exercise-training starting at 5 weeks of age
2. Targeted metabolomics from plasma at baseline and following dobutamine-stress at 5 wks of age, and following 6 weeks of exercise training, starting at 5 weeks of age

Milestone 1: Mouse breeding for dobutamine ECHO and exercise- completed at 6 months

Milestone 2: Harvest hearts for dobutamine study: completed at 6 months

Milestone 3: Metabolomics of baseline study: completed at 15 months

Milestone 4: 6 weeks exercise study: completed at 18 months

Milestone 5: Harvest hearts and plasma for exercise study: Completed at 18 months

Milestone 6: Metabolomics analysis: Complete for baseline/dobutamine study; ongoing for exercise study

Milestone 7: Manuscript submission: Manuscript in preparation for baseline/dobutamine heart heart metabolomics study; Abstract presented in 2019.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

In the baseline/dobutamine study, non-targeted metabolomics yielded >1,000 annotated metabolites across the three platforms. Statistical analysis was performed using MetSolution, comparing each mutant with littermate control mice at rest and during dobutamine-stress. We used $p < 0.05$ after FDR correction to define statistically significant difference in metabolites between mutants and corresponding littermate controls.

GC-TOF: At **rest**, we found no statistically significant difference in metabolites between MyHC mutant/control mice. In contrast, 16 metabolites were significantly different, between TnT mutants/controls: they included 1) lower levels of Krebs cycle intermediates, α -ketoglutarate, succinic acid, fumaric acid (suggesting reduction of TCA flux/impairment of mitochondrial function), 2) higher levels of branched chain amino acids (BCAA), leucine/isoleucine, (suggesting reduced BCAA catabolism) and proline, 3) higher levels of nucleosides (inosine, uracil, guanosine). During **dobutamine stress**, MyHC mutants had significantly higher levels of glucose-1-P, when compared to littermate controls, suggesting activation of glycogenolysis by beta-adrenergic stimulation. Notably, we found no statistically significant differences in metabolites between TnT mutants/controls during dobutamine-stress.

Lipidomics: At **rest**, there was no difference in metabolites between MyHC mutants/controls, but 112 metabolites were significantly different between TnT mutants/controls. During **dobutamine-stress**, 25 metabolites were significantly different between TnT mutants/controls

Taken together, our metabolomics results at rest/dobutamine-stress in the 2 HCM mouse models indicate **allele-specific differences in metabolic remodeling and response to dobutamine at early disease stage**.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Professional development: This grant led to a gain of expertise in cardiac metabolism for the investigators. This was accomplished by participating in online seminars and individual study.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The results were presented at the Scientific meeting of the Metabolomics Association of North America (MANA, 2019) and resulted in an Early Career Award to Arpana Vaniya, PhD. We plan to disseminate our results at the International HCM Summit in October 2020, which is attended by physicians, researchers and HCM patients.

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report, since this is the final Report.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our study is the first of its kind demonstrating allele-specific differences in cardiac metabolism at early disease stage, using 2 mouse models of hypertrophic cardiomyopathy, that span the spectrum of human disease. This is also the first study that examined the effect of acute inotrope (dobutamine)-stress and chronic exercise-training on cardiac metabolism in hypertrophic cardiomyopathy. We discovered that dobutamine improved cardiac metabolism in R92W-TnT mutants, but had little effect on R403Q-MyHC-mutant mice. We are now investigating the mechanisms underlying metabolite differences between R92W-TnT mutants and littermate controls at baseline and following dobutamine-stress, but lack of differences between R403Q-MyHC mutants and littermate controls. The next step is to confirm differences in cardiac metabolism in human hypertrophic cardiomyopathy. Our results lead us to hypothesize that imaging cardiac metabolism may aid with preclinical diagnosis of hypertrophic and help distinguish hypertrophic cardiomyopathy from athlete heart resulting from intense exercise-training.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Our results of differences in Krebs cycle metabolites between TnT-mutant mice and controls at early disease stage, led to initiation of collaborations with Peder Larson PhD (Associate Professor in the UCSF Department of Radiology) and Matthew Merritt PhD (Associate Professor in the Department of Biochemistry and Molecular Biology at the University of Florida).

Dr. Larson is an expert on MRI technology development and has pioneered the creation of hyperpolarized MRI methods for animal and human studies. Dr. Larson’s team is working on hyperpolarized carbon-13 MRI technique development, cardiac imaging protocol development and data analysis methods for in vivo imaging of cardiac metabolism in humans. We have begun cardiac metabolism imaging in human volunteers, using hyperpolarized [1-¹³C]-pyruvate and [2-¹³C]pyruvate MRI.

Dr. Merritt has expertise in biochemistry and metabolic flux measurements. He will use hyperpolarized carbon-13 substrates for measuring acute metabolism as well as steady-state metabolism using isotopomer NMR, in mouse models of hypertrophic cardiomyopathy.

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

1) We experienced a 12 month delay in initiation of mouse swimming, which was related to difficulty receiving approval from the UCSF-IACUC committee for the mouse swimming protocol. Following UCSF-IACUC approval, DOD review and approval was needed prior to initiation of the project, which led to additional delays. Metabolomics analysis is now complete. The data is now being post-curated by the Fiehn Lab (West Coast Metabolomics Center).

2) Delay in performing plasma metabolomics analysis: We planned to use cardiac metabolite data to perform targeted plasma metabolomics. We are awaiting completion of the cardiac metabolite data analysis to enable selection of metabolites for plasma metabolomics analysis.

3) Delay in manuscript submission: We plan to submit 2 manuscripts describing our results 1) at baseline/dobutamine-stress, and 2) following exercise-training.

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

Not applicable

• **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Differences in microRNA-29 and Pro-fibrotic Gene Expression in Mouse and Human Hypertrophic Cardiomyopathy. Yamin Liu, Junaid Afzal, Styliani Vakrou, Gabriela V Greenland, C Conover Talbot Jr, Virginia B Hebl, Yufan Guan, Rehan Karmali, Jil C Tardiff, Leslie A Leinwand, Jeffrey E Olgin, Samarjit Das, Ryuya Fukunaga, M Roselle Abraham. Front Cardiovasc Med. 2019 Dec 17;6:170; PMID: 31921893; PMCID: PMC6928121; acknowledgement of federal support (yes)
2. One manuscript in preparation, will be submitted for Review in August, 2020

Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

- One conference paper that resulted in an Early Career Award (MANA, 2019) to Arpana Vaniya, PhD for the Abstract “Allele-specific differences in cardiac metabolism detected

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

These results led to investigation of cardiac metabolism in humans using a new imaging technique, namely hyperpolarized ¹³C-magnetic resonance imaging in collaboration with Dr. Peder Larson in the UCSF department of Radiology

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- physical collections;
- audio or video products;
- software;
- models;
- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: Junaid Afzal, MBBS, MS

Project Role: Research Specialist

Researcher Identifier: 0000-0002-2877-234X

Nearest person month worked: 2 months

Contribution to Project: Dr. Afzal developed the exercise-training model, performed echocardiography and metabolomics data analysis

Funding Support: UCSF Division of Cardiology Funds

Name: Yamin Liu, PhD

Project Role: Postdoctoral Fellow

Researcher Identifier:

Nearest person month worked: 7 months

Contribution to Project: Dr. Liu performed mouse genotyping, dobutamine-studies, mouse euthanasia, sample processing for metabolomics

Funding Support: UCSF Division of Cardiology Funds

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Organization Name: University of California, Davis

Location of Organization: California, United States

Facilities: Metabolomics studies were performed at the West Coast Metabolomics Center, at UC Davis

Collaboration: We collaborated with Dr. Oliver Fiehn’s lab; Arpana Vaniya PhD performed data curating, and Sili Fan PhD performed statistics on metabolomics data

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

1. Journal article copy
2. Abstract copy
3. Abraham CV



Differences in microRNA-29 and Pro-fibrotic Gene Expression in Mouse and Human Hypertrophic Cardiomyopathy

Yamin Liu^{1,2}, Junaid Afzal^{1,2}, Styliani Vakrou², Gabriela V. Greenland^{1,2}, C. Conover Talbot Jr.³, Virginia B. Hebl⁴, Yufan Guan², Rehan Karmali¹, Jil C. Tardiff⁵, Leslie A. Leinwand⁶, Jeffrey E. Olgin¹, Samarjit Das⁷, Ryuya Fukunaga^{8*} and M. Roselle Abraham^{1,2*}

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Cardiovascular Genetics and Systems
Medicine,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 06 June 2019

Accepted: 08 November 2019

Published: 17 December 2019

Citation:

Liu Y, Afzal J, Vakrou S, Greenland GV, Talbot CC Jr, Hebl VB, Guan Y, Karmali R, Tardiff JC, Leinwand LA, Olgin JE, Das S, Fukunaga R and Abraham MR (2019) Differences in microRNA-29 and Pro-fibrotic Gene Expression in Mouse and Human Hypertrophic Cardiomyopathy. *Front. Cardiovasc. Med.* 6:170. doi: 10.3389/fcvm.2019.00170

Background: Hypertrophic cardiomyopathy (HCM) is characterized by myocyte hypertrophy and fibrosis. Studies in two mouse models (R92W-TnT/R403Q-MyHC) at early HCM stage revealed upregulation of endothelin (ET1) signaling in both mutants, but TGF β signaling only in TnT mutants. Dysregulation of miR-29 expression has been implicated in cardiac fibrosis. But it is unknown whether expression of miR-29a/b/c and profibrotic genes is commonly regulated in mouse and human HCM.

Methods: In order to understand mechanisms underlying fibrosis in HCM, and examine similarities/differences in expression of miR-29a/b/c and several profibrotic genes in mouse and human HCM, we performed parallel studies in rat cardiac myocyte/fibroblast cultures, examined gene expression in two mouse models of (*non-obstructive*) HCM (R92W-TnT, R403Q-MyHC)/controls at early (5 weeks) and established (24 weeks) disease stage, and analyzed publicly available mRNA/miRNA expression data from *obstructive*-HCM patients undergoing septal myectomy/controls (unused donor hearts).

Results: Myocyte cultures: ET1 increased superoxide/H₂O₂, stimulated TGF β expression/secretion, and suppressed miR-29a expression in myocytes. The effect of ET1 on miR-29 and TGF β expression/secretion was antagonized by N-acetyl-cysteine, a reactive oxygen species scavenger. Fibroblast cultures: ET1 had no effect on pro-fibrotic gene expression in fibroblasts. TGF β 1/TGF β 2 suppressed miR-29a and increased collagen expression, which was abolished by miR-29a overexpression. Mouse and human HCM: Expression of miR-29a/b/c was lower, and *TGFB1*/collagen gene expression was higher in TnT mutant-LV at 5 and 24 weeks; no difference was observed in expression of these genes in MyHC mutant-LV and in human myectomy tissue. *TGFB2* expression was higher in LV of both mutant mice and human myectomy tissue. *ACE2*, a negative regulator of the renin-angiotensin-aldosterone system, was the most upregulated

transcript in human myectomy tissue. Pathway analysis predicted upregulation of the anti-hypertrophic/anti-fibrotic liver X receptor/retinoid X receptor (LXR/RXR) pathway only in human myectomy tissue.

Conclusions: Our *in vitro* studies suggest that activation of ET1 signaling in cardiac myocytes increases reactive oxygen species and stimulates TGF β secretion, which downregulates miR-29a and increases collagen in fibroblasts, thus contributing to fibrosis. Our gene expression studies in mouse and human HCM reveal allele-specific differences in miR-29 family/profibrotic gene expression in mouse HCM, and activation of anti-hypertrophic/anti-fibrotic genes and pathways in human HCM.

Keywords: hypertrophic cardiomyopathy, miR-29, TGF-beta, collagen, mouse, humans

INTRODUCTION

Hypertrophic cardiomyopathy (HCM), most frequently caused by mutations in sarcomeric protein genes, manifests as myocyte hypertrophy, myocyte disarray, fibrosis, and arteriolar remodeling (1). Expression of the pathologic hallmarks of HCM vary with disease stage and underlying mutation. Our studies comparing two mouse models of HCM (R92W-TnT, R403Q-MyHC) with mutations in cardiac troponin T (cTnT) (2, 3) and myosin heavy chain (MyHC) genes (4, 5), at an early stage of disease (5 weeks of age), revealed differences in expression of *TGFB1/3*, microRNA-29 (miR-29), collagen genes, and redox environment in the two mutant mouse lines (6). Dysregulation of cardiac miR-29 family (miR-29a/b/c) expression has been implicated in cardiac fibrosis in experimental models (7), and circulating miR-29a has been associated with cardiac fibrosis in HCM patients (8). But it is unknown whether miR-29 dysregulation varies with disease stage in HCM mouse models, and whether cardiac miR-29 expression is changed in human HCM (8). In order to address this question, we examined expression of *TGFB1-3*, miR-29a/b/c, and collagen genes in the left ventricle (LV) and left atrium (LA) of TnT/MyHC mutants and littermate control mice at 5 and 24 weeks of age, and analyzed publicly available gene (mRNA, miRNA) expression data from human myectomy samples and control subjects.

In our recently published study, Ingenuity Pathway analysis (IPA) of gene (mRNA) expression data in 5 weeks old mouse hearts (R92W-TnT, R403Q-MyHC and littermate controls) predicted upregulation of endothelin1 (ET1) and transforming growth factor-beta signaling only in TnT mutants, that demonstrated an oxidized redox environment (6). Prior studies have demonstrated upregulation of TGF β by mitochondrial reactive oxygen species (ROS) in lung fibroblasts (9), and by ET1 in cardiac myocytes (10). Additionally, ROS has been implicated as a mediator of ET1-induced cardiac hypertrophy (11, 12). We hypothesized that activation of ET1 signaling in cardiac myocytes increases ROS levels, which stimulates TGF β secretion by cardiac myocytes, and collagen expression in cardiac fibroblasts. We tested this hypothesis by assessing miR-29 and select pro-fibrotic gene expression in (1) cardiac myocytes/fibroblasts following ET1 treatment, and (2) in cardiac fibroblasts following ET1, TGF β 1, and TGF β 2 treatment. Our

results indicate that ET1 increases ROS and stimulates TGF β secretion by cardiac myocytes, which suppresses miR-29 and increases collagen expression in cardiac fibroblasts.

MATERIALS AND METHODS

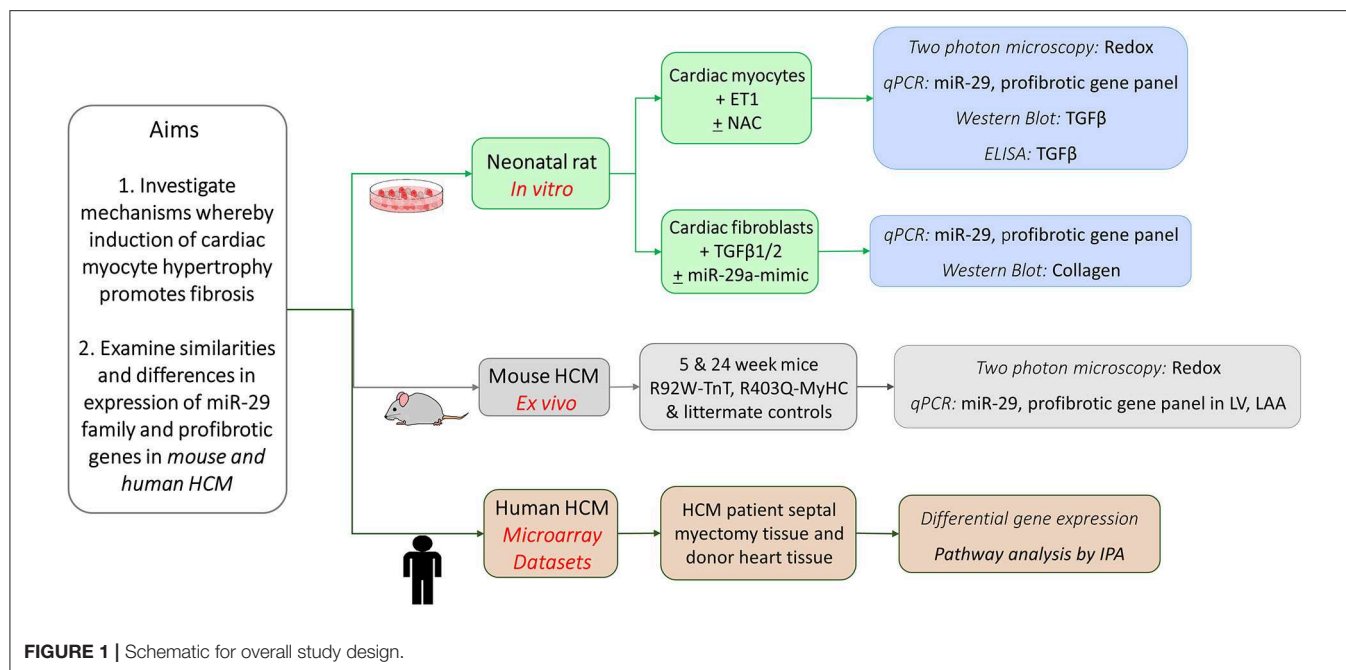
Study Design

We performed studies in cardiac myocytes and cardiac fibroblasts derived from neonatal rat hearts, examined redox/gene expression in two mouse models of HCM/littermate controls at early and established disease stage, and analyzed publicly available gene expression data from HCM patients undergoing myectomy and controls, with a focus on TGF β , miR-29 family, and its profibrotic target genes (Figure 1).

Studies in cardiac myocytes and fibroblasts obtained from healthy neonatal rat hearts were used to examine the effect of ET1 and redox modulation of cardiac myocytes on TGF β signaling in cardiac myocytes, and collagen expression in cardiac fibroblasts. We examined miR-29a/b/c and profibrotic gene expression in the left ventricle (LV) and left atrial appendage (LA appendage) in 2 HCM mouse models because of the high incidence of cardiac fibrosis and ventricular/atrial arrhythmias in HCM patients. These two mouse models of sarcomeric HCM (R92W-TnT, R403Q-MyHC) are based on mutations that lead to sudden cardiac death at a young age in the setting of fibrosis/mild hypertrophy (13, 14), and significant LV hypertrophy/fibrosis/heart failure requiring heart transplantation in middle age (15, 16). Since obtaining endomyocardial biopsies from HCM patients for research is not feasible, we analyzed publicly available microarray datasets from HCM patients undergoing myectomy (compared to tissue obtained from unused donor hearts/controls) to assess whether expression of *TGFB1-3*, miR-29a/b/c, and its profibrotic target genes is dysregulated in human HCM.

Myocyte and Fibroblast Isolation From Neonatal Rat Hearts

Cardiac myocytes and cardiac fibroblasts were isolated from neonatal rat hearts as described previously (17). Briefly hearts from newborn Sprague Dawley rats (2–3 days old) were quickly excised, minced and digested with trypsin (at 4°C for 16–20 h) followed by collagenase type 2 (37°C for 30–45 min), following



which cell suspensions were centrifuged at $100\times g$ for 5 min. The pellet was re-suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin (Pen/Strep), and transferred to a flask for 1 h for preferential attachment of fibroblasts. After 1 h, cardiac myocytes were plated in six well plates, at a density of 6×10^5 per well. Fibroblasts adherent to the flask were cultured in DMEM containing 4.5 g/L D-glucose, 10% FBS, 100 U/mL Pen/Strep for the first 24 h. Fibroblasts were expanded in DMEM containing 1 g/L D-glucose, 10% FBS, 100 U/mL Pen/Strep. Fibroblasts were allowed to proliferate until they were 70% confluent; fibroblasts were passaged twice prior to studies.

Cell Treatment and miR-mimic Transfection Cardiac Myocytes

Neonatal rat cardiac myocytes were cultured in DMEM containing 4.5 g/L D-glucose, 10% FBS, 100 U/mL Pen/Strep for the first 24 h. Dead cells were removed by rinsing in PBS, and culture medium was changed to 45% DMEM (1 g/L D-glucose), 45% Medium 199, 10% FBS, 100 U/mL Pen/Strep for 24 h. Subsequently, cells were cultured in medium containing 49% DMEM (1 g/L D-glucose), 49% Medium 199, 2% FBS, 100 U/mL Pen/Strep for 12 h. On day 3 following initial plating, culture medium was changed to serum-free medium containing 50% DMEM (1 g/L D-glucose), 50% Medium 199, 100 U/mL Pen/Strep for 12 h prior to initiation of cell treatment with Endothelin1 (ET1) (11, 18, 19). We selected ET1 because it had the most pronounced effect on gene expression (ANP, BNP, TGFβ1-3, CTGF, and miR-29), when compared to Angiotensin II and Isoproterenol (data not shown).

Immunostaining was performed to confirm lack of fibroblast contamination of cardiac myocyte cultures

(**Supplemental Figure 1**). For immunostaining, cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature, washed with PBS and incubated with blocking solution for 1 h. Vimentin antibody (1:200; cat. no. D21H3; Cell Signaling Technology, Inc., Danvers, MA, USA) was added overnight at 4°C , followed by secondary antibody [Alexa-488 goat anti-rabbit IgG (H + L), 1:2,000; Cat.#A32731, Thermo Fisher Scientific] for 1 h. Hoechst (1:2,000; cat. no. H3569; Thermo Fisher Scientific, USA) was used to label nuclei. Slides were imaged using a Nikon confocal microscope with 20X objective.

We studied the following conditions: (1) Control, (2) ET1 treatment (ET1; 100 nM; Sigma) (20), (3) ET1 + N-acetyl-l-cysteine (NAC, 3.5 mM; Sigma). ET1 and NAC were added for 24 h; cell viability was confirmed prior to cell harvesting for gene and protein expression studies.

Cardiac Fibroblasts

Neonatal rat cardiac fibroblasts were expanded in DMEM containing 1 g/L D-glucose, 10% FBS, 100 U/mL Pen/Strep. Fibroblasts were plated in six well-plates at a density of 0.5×10^6 cells per well, and cultured in DMEM containing 10% FBS for 24 h. Medium was subsequently changed to DMEM containing 2% FBS for 24 h. Medium was changed to serum-free DMEM for 8 h prior to treatment with ET1/TGFβ/miR-mimic (21, 22).

The miR-29a-mimic (Cat.#4464066) and control mimic (Control-mimic; Cat.#4464058) were purchased from Life Technologies, Inc. For miR-29a overexpression studies, cardiac fibroblasts were transfected with miR-29a-mimic (5 nM) or control-mimic (5 nM) for 6 h using Lipofectamine[®] RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer instructions. Culture medium was changed to serum-free DMEM prior to treatment with TGFβ1 or TGFβ2.

We studied the following conditions: (1) Control, (2) TGFβ1 (2 ng/mL; R&D Systems, Wiesbaden, Germany), (3) TGFβ2 (1 ng/mL; R&D Systems, Wiesbaden, Germany), (4) TGFβ1 + miR-29a-mimic, (5) TGFβ1 + control-mimic, (6) TGFβ2 + miR-29a-mimic; and (7) TGFβ2 + control-mimic, for 24 h. Cell viability was confirmed by microscopy prior to harvesting for gene and protein expression studies.

HCM Mouse Models

The investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH 8th Edition, 2011), and was approved and monitored by the UCSF Laboratory Animal Resource Center. We studied two mouse models of sarcomeric HCM that have been well-characterized, namely the R403Q mutation in the α-myosin heavy chain gene (MyHC; *MYH6* gene) and the R92W mutation in the cardiac troponin T gene (TnT; *TNNT2* gene), that were kindly provided by Leinwand and Tardiff, respectively (3, 4). Mice were weaned and genotyped at the age of 4 weeks using PCR-amplified tail DNA. We studied male mice (mutants and littermate controls) at 5 and 24 weeks of age (redox studies, qPCR), and female mice at 5 weeks of age (qPCR of mouse LV tissue).

Adult Mouse Myocyte Isolation

Adult cardiac myocytes were dissociated as described previously (6). Briefly, mice were administered 100 IU heparin 10 min prior to euthanasia by cervical dislocation. Hearts were rapidly excised, cannulated via the aorta, and perfused in the Langendorff mode with a constant perfusion pressure of 80 mmHg. Hearts were perfused for 10 min using Ca²⁺-free Tyrode containing (in mM) NaCl (120), KCl (5.4), NaH₂PO₄ (1.2), NaHCO₃ (20), MgCl₂ (1.6), glucose (1 mg/ml), 2, 3-butanedione monoxime (BDM, 1 mg/ml), taurine (0.628 mg/ml), 0.9 mg/ml collagenase type 2 (Worthington, 299 U/mg), and gassed with 95% O₂-5% CO₂. The heart was then cut into small pieces and gently agitated, allowing myocytes to be dispersed in Ca²⁺-free Tyrode containing BSA (5 mg/L) for 10 min. Dispersed myocytes were filtered through a 150 μm mesh and gently centrifuged at 500 rpm for 30 s. Myocytes were then suspended in Tyrode containing gradually increasing amounts of Ca²⁺ (0.125–1 mM Ca²⁺) and stored in 1 mM Ca²⁺-containing Tyrode for microscopy studies.

RNA Isolation and Polymerase Chain Reaction

Total RNA from mouse LV, left atrial (LA) appendage, rat cardiac myocyte cultures and rat cardiac fibroblast cultures was extracted using an RNA isolation Kit (Life Technologies) according to the manufacturer's instructions. RNA (1 μg of each sample) was reverse-transcribed into cDNA using cDNA Reverse Transcription kit (Applied Biosystems).

mRNA

Real-time RT-PCR for mRNA was performed using the TaqMan assay on a QuantStudio 7 Flex Real-Time PCR System (ThermoFisher, Inc.). Real-time RT-PCR was performed in

duplicate, and samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. We tested the following genes involved in cardiac fibrosis. Profibrotic targets of miR-29, identified by TargetScan: collagen genes (*COL1A1*, *COL1A2*, and *COL3A1*) and elastin (*ELN*); connective tissue growth factor (*CTGF*); transforming growth factor-beta isoforms (*TGFβ1-3*); transforming growth factor-beta receptors (*TGFβR1-2*). Additionally, we assessed genes involved in ROS scavenging [superoxide dismutase2 (*SOD2*), catalase (*CAT*)] and modulators of cardiac fibrosis [atrial natriuretic peptide (*ANP*), brain natriuretic peptide (*BNP*)].

miRNA

The real-time RT-PCR, TaqMan microRNA assay kit (Applied Biosystems) was used to quantify expression of mature miR-29a, miR-29b, and miR-29c in heart tissue, cardiac myocytes and cardiac fibroblasts. Real-time RT-PCR was performed in duplicate, and samples were normalized to U6 expression.

Western Blot

Cardiac myocytes and fibroblasts were lysed in radio-immunoprecipitation assay lysis buffer (RIPA, Cat.#9806, Cell Signaling Technology) in the presence of protease inhibitors. Protein concentration was quantified using the bicinchoninic acid assay kit (Thermo Fisher Scientific). Denatured proteins (20 μg) from the samples were run on 4–12% NuPAGETM Bis-Tris Gel (Thermo Fisher Scientific), following which proteins were transferred to polyvinylidene difluoride membranes. The membranes were subsequently blocked using 5% BSA for 1 h at room temperature, and incubated with TGFβ (Cat.#3711; 1:2,000; Cell Signaling) or collagen I (Cat.#ab233080; 1:3,000; Abcam) primary antibody, overnight at 4°C, washed several times, and then incubated with GAPDH (Cat.#5174; 1:20,000; Cell Signaling Technology) for 1 h at room temperature. Subsequently, a horseradish peroxidase-linked anti-rabbit IgG secondary antibody (Cat. #NA9340; 1:20,000; GE healthcare, Inc.) was incubated for 1 h at room temperature. An enhanced chemi-luminescence reagent (SuperSignalTM West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific) was used to visualize the protein bands. Semi-quantification of protein was conducted by comparison against the GAPDH bands using Image J software (version 1.48, National Institutes of Health, Bethesda).

Quantification of TGFβ2 Secretion by ELISA

Release of TGFβ2 protein from cardiac myocytes into serum-free culture medium was determined using the Quantikine colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), according to the manufacturer's instructions. ELISA was performed in duplicate. Absorbance at 450 nm was measured using a BioTek Synergy 2 multi-mode microplate reader. Protein concentration was calculated using a standard curve, and expressed as pg/10⁶ cells.

Measurement of Reactive Oxygen Species and Cellular Redox Status

Rat Cardiac Myocyte Cultures

Neonatal rat cardiac myocytes were incubated with ET1 in the presence or absence of N-acetyl cysteine (NAC, 3.5 mM) for 24 h prior to imaging. Production of ROS in cultured cardiac myocytes was quantified using the cell-permeant fluorescence probes CM-H₂DCFDA (DCF; Cat.#C6827, Thermo Fisher) and MitoSOX Red (Cat.#M36008), using an Olympus FV1000 MP microscope and 20X water immersion lens as described previously (23). Beating cardiac myocytes were incubated with DCF (5 μ M) and MitoSOX Red (5 μ M) for 30 min at 37°C, washed with PBS, prior to imaging at 37°C. We used an excitation wavelength of 800 nm. Emitted light was collected by three photomultiplier tubes fitted with bandpass filters. 512 $\times$ 512 pixel images were collected and image analysis was performed using Image J (NIH, <http://rsb.info.nih.gov/ij/>). Signal quantification was performed by drawing a region of interest (ROI) and calculating mean fluorescence intensity which was normalized to fluorescent area. DCF and Mitosox fluorescence was calculated from 10 randomly selected regions from three biological replicates (~300 cardiac myocytes).

Isolated Mouse Cardiac Myocytes

Cellular redox status was examined in freshly isolated, non-paced, adult mouse cardiac myocytes, as described previously (6). Experiments were performed at 37°C in a thermostatically controlled flow chamber mounted on the stage of an upright

microscope (Nikon E600FN) attached to a multi-photon laser scanning system with excitation at 740 nm. Cells were suspended in Tyrode solution, pH 7.4, containing (in mM), NaCl (140), KCl (5), MgCl₂ (1), HEPES (10), CaCl₂ (1), and glucose (10). Monochlorobimane (MCB, 50 μ M, blue λ_{em} 480 $\pm$ 20 nm) was loaded for 20 min on the stage of the microscope at 37°C to monitor reduced glutathione (GSH). Autofluorescence of NAD(P)H, namely total fluorescence collected at <490 nm was monitored separately. The acquired signal was calibrated by the addition of KCN (1 mM) for maximum reduction of existing NAD(P)H, followed by the addition of FCCP (5 μ M) for maximum oxidation of NAD(P)H. Image analysis was performed using Image J software.

Analysis of Gene Expression Data From HCM Patients

Gene expression data from publicly available microarray datasets, GSE36961 (mRNA) and GSE36946 (miRNA) was downloaded from the NCBI GEO database, and analyzed for differential gene expression. The microarray datasets were obtained from ventricular septal tissue of HCM patients undergoing myectomy at the Mayo Clinic (Rochester, MN), and from age/sex-matched donor hearts (control subjects) from the Sydney Heart Bank (24, 25). Information on HCM patient genotype/phenotype and control subjects was obtained from the publicly available Master's Thesis by Hebl (24).

Briefly, the raw data underwent quality control analysis. A microarray batch effect was noted in the data and adjusted

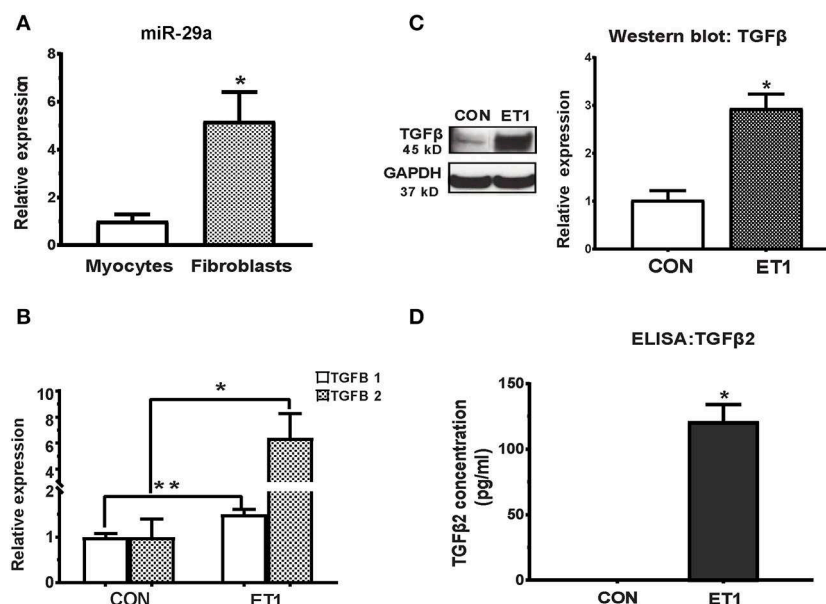


FIGURE 2 | ET1 induced TGF-beta expression in neonatal rat cardiac myocyte cultures. **(A)** Real-time RT-PCR indicated 5-fold higher expression of miR-29a in cultured rat cardiac fibroblasts, when compared to rat cardiac myocytes. **(B)** ET1 increased *TGFβ1/2* expression in cardiac myocytes. **(C)** ET1 increased TGFβ expression in cardiac myocytes. Image shows representative blot from one experiment and bar graph summarizes band intensity data from three experiments (data is expressed as mean $\pm$ S.D.; $n = 3$; two tailed Student's *t*-test was used to determine significance; * $P < 0.05$). **(D)** TGFβ2 secretion was estimated by ELISA. ET1 (100 nM) stimulated TGFβ2 secretion by cardiac myocytes. **[(A,B,D)** CON, control; ET1, Endothelin1 (100 nM) treatment for 24 h. All data are expressed as mean $\pm$ S.D.; $n = 3$ for each condition; each experiment was repeated three times. Two tailed Student's *t*-test was used to determine significance; ** $P < 0.01$, * $P < 0.05$].

for in the subsequent expression analysis. Data from 1 HCM patient that proved to be an outlier by principle component analysis, was excluded from further analysis. The fluorescence signal values were $\log_2$ converted and quantile normalized.

Statistical Analysis

All results are expressed as mean $\pm$ S.D. unless otherwise noted. Two tailed Student's *t*-test was used to compare data from each mutant mouse to its respective littermate control, and to compare expression of select genes from human myectomy samples with control subjects. Experiments requiring comparison of ≥ 3 conditions were analyzed by ANOVA and Tukey's test. Graphs were generated using Graphpad Prism 7.04 software. For Ingenuity pathway analysis, mRNA data from 105 HCM patient samples was compared to 39 controls by ANOVA using the Partek Genomics Suite 7.0 platform. Differential expression was reported as fold change, and its statistical significance as *P*-value. Gene identifiers were updated to current HGNC/NCBI nomenclature. A *P* < 0.05 was considered statistically significant.

RESULTS

Gene Expression in Rat Cardiac Myocyte and Fibroblast Cultures

Cultured Rat Cardiac Fibroblasts Express Higher miR-29a Levels Than Cardiac Myocytes

The miR-29 family, consisting of miR-29a, miR-29b, and miR-29c has been demonstrated to play an important role in cardiac (7), pulmonary (26), and renal (27) fibrosis. We found that expression of miR-29a was 5-fold higher in

cultured rat cardiac fibroblasts when compared to cultured rat cardiac myocytes (**Figure 2A**). Furthermore, miR-29b and miR-29c expression was very low in rat cardiac myocytes when compared to miR-29a (data not shown), which led us to focus on miR-29a in our cell studies. Of note, low expression of miR-29b/c when compared to miR-29a, has been described previously in rat hearts (GEO: GSE62883, GSE56300).

ET1 Increases ROS and Stimulates TGF β Secretion by Cardiac Myocytes

Fibrosis is a common pathologic and imaging feature in HCM (15, 28). In our recent study of MyHC and TnT mutant mouse hearts, before the onset of myocyte hypertrophy/fibrosis, pathway analysis of mRNA-seq data predicted up-regulation of ET1 signaling in both mutants (6). Since mutant sarcomeric proteins are expressed only in cardiac myocytes (not cardiac fibroblasts), and fibroblasts are the main effectors of cardiac fibrosis, we hypothesized involvement of a paracrine mechanism, namely TGF β secretion by myocytes, in generation of ET1-mediated collagen deposition/fibrosis. We performed studies separately in neonatal rat cardiac myocyte and fibroblast cultures to test our hypothesis.

Treatment of cardiac myocytes with ET1 (100 nM) induced upregulation of *TGFB1-3*, *CTGF*, and suppression of miR-29a (**Figure 2B** and **Table 1**), but did not change expression of redox genes, *SOD2* and *CAT* (**Table 1**). ET1 also increased TGF β protein expression (**Figure 2C**) and stimulated TGF β secretion by cardiac myocytes (**Figure 2D**).

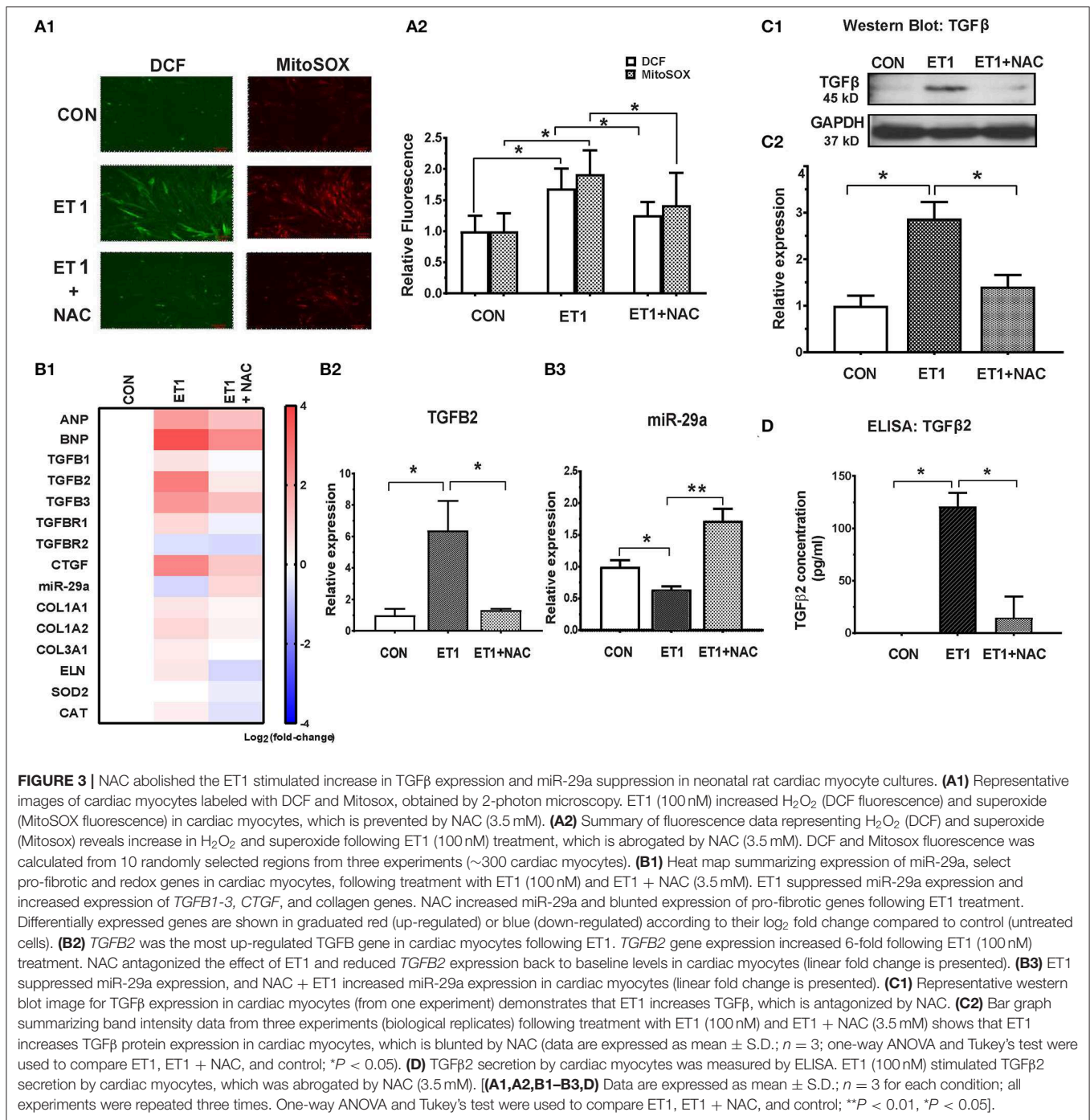
Two photon microscopy demonstrated increase in superoxide and hydrogen peroxide (H₂O₂) levels in cardiac myocytes

TABLE 1 | Effects of ET1, ET1+NAC on gene expression in cardiac myocyte cultures.

Genes	ET1		ET1 + NAC	
	FC	<i>P</i> -value vs. con	FC	<i>P</i> -value vs. ET1
Natriuretic peptide A (<i>ANP</i>)	4.1	0.01	2.4	0.02
Natriuretic peptide B (<i>BNP</i>)	12.4	0.005	5.2	0.006
Transforming growth factor, beta 1 (<i>TGFB1</i>)	1.5	0.004	0.9	0.006
Transforming growth factor, beta 2 (<i>TGFB2</i>)	6.4	0.03	1.3	0.04
Transforming growth factor, beta 3 (<i>TGFB3</i>)	4.3	0.0001	2.4	0.001
Transforming growth factor, beta receptor 1 (<i>TGFB1R</i>)	1.6	0.004	0.8	0.01
Transforming growth factor, beta receptor 2 (<i>TGFB2R</i>)	0.7	0.1	0.6	0.8
Connective tissue growth factor (<i>CTGF</i>)	5.6	0.002	2.1	0.005
MicroRNA-29a (miR-29a)	0.6	0.01	1.7	0.006
Collagen type I alpha 1 chain (<i>COL1A1</i>)	1.4	0.005	1.1	0.009
Collagen type I alpha 2 chain (<i>COL1A2</i>)	1.7	0.0009	1.1	0.0008
Collagen type III alpha 1 chain (<i>COL3A1</i>)	1.3	0.03	1.0	0.03
Elastin (<i>ELN</i>)	1.4	0.03	0.6	0.004
Superoxide dismutase 2 (<i>SOD2</i>)	0.9	0.9	0.8	0.3
Catalase (<i>CAT</i>)	1.2	0.6	0.7	0.1

Expression of miR-29a, select pro-fibrotic and redox genes in cardiac myocytes, following treatment with ET1 (100 nM) and ET1+NAC (3.5 mM).

Data are expressed as linear fold change (FC) compared to control or ET1; *n* = 3 (biological replicates) for each condition; all experiments were repeated three times. One-way ANOVA and Tukey's test were used to compare ET1, ET1+NAC, and control.



following ET1 treatment (Figure 3A). Treatment with the ROS scavenger, N-acetyl-cysteine (NAC), a therapy predicted to be effective by IPA, only in 5 week-old TnT mutants (6), reduced cellular superoxide/H₂O₂, reduced expression of pro-fibrotic genes (*TGFB1-3*, *CTGF*, *COL1A1*, *COL1A2*, and *COL3A1*), and up-regulated miR-29a expression in cardiac myocytes (Figures 3B1–3 and Table 1). NAC also antagonized the ET1-induced increase in TGFβ expression (Figure 3C) and secretion by cardiac myocytes (Figure 3D).

TGFβ-Induced Collagen Expression in Cardiac Fibroblasts Is Prevented by a miR-29a-mimic

Since fibroblasts are the main effectors of cardiac fibrosis, we investigated the effect of ET1 and TGFβ on cardiac fibroblasts. ET1 did not change expression of *TGFB1-3*, miR-29a or its pro-fibrotic target genes in cardiac fibroblasts (Supplemental Figures 2A–C). In contrast, treatment with TGFβ1 (2 ng/ml) or TGFβ2 (1 ng/ml) downregulated miR-29a, upregulated *CTGF* and miR-29 targets (*COL1A1*,

COL1A2, *COL3A1*, and *ELN*) in cardiac fibroblasts (Table 2, Figures 4A1,2, Supplemental Figures 3A,B). Notably, a miR-29a-mimic prevented upregulation of collagen genes (*COL1A1*, *COL1A2*, and *COL3A1*), elastin, and collagen expression in cardiac fibroblasts, following TGFβ1 and TGFβ2 treatment (Table 2, Figures 4A–C, Supplemental Figures 3A,B) highlighting the importance of miR-29a as a mediator of collagen deposition/fibrosis (27).

Taken together, our *in vitro* studies indicate that ET1 increases ROS and induces TGFβ secretion by cardiac myocytes, which downregulates miR-29a and de-represses its pro-fibrotic target genes in cardiac fibroblasts, leading to an increase in collagen expression in fibroblasts (Figure 4D).

Redox Status in Mouse HCM

Allele-Specific Differences in Cardiac Myocyte Redox Status in HCM Mice

Since cellular redox status influenced TGFβ secretion by cardiac myocytes in our *in vitro* studies, we investigated cellular redox status in cardiac myocytes isolated from mutant and littermate control mouse hearts at 5 and 24 weeks of age. We performed two photon microscopy to assess reduced glutathione (GSH) and the NAD(P)H pool in isolated cardiac myocytes (Figure 5). Myocytes were labeled with monochlorobimane (MCB) to measure reduced glutathione (GSH), and cellular auto-fluorescence was used as an indicator of NAD(P)H.

TnT mutant myocytes had evidence of an oxidized redox environment, characterized by lower GSH, and NAD(P)H pool levels, when compared to littermate controls, at 5 weeks of age (Figures 5A,B). In contrast, MyHC mutant myocytes demonstrated a reduced redox environment, characterized by higher GSH and NAD(P)H pool at 5 weeks of age (6)—(Figures 5A,B).

At 24 weeks of age, both mutants demonstrated an oxidized redox environment. TnT mutants showed markedly lower GSH levels and a trend toward lower NAD(P)H pool levels ($p = 0.05$) when compared to littermate controls, whereas MyHC

mutants demonstrated lower GSH and NAD(P)H pool levels, when compared to littermate controls (Figures 5C,D).

Taken together, our results indicate allele-specific, and disease stage-specific changes in cellular redox status in the two HCM mouse models.

Gene Expression in Mouse and Human HCM

Allele-Specific Differences in Expression of miR-29a/b/c and Pro-fibrotic Genes in Left Ventricle of HCM Mice

We studied two mouse models of sarcomeric HCM with mutations that lead to cardiac phenotypes that span the spectrum of human HCM, namely malignant ventricular arrhythmias (R92W-TnT) (2, 3) and heart failure (R403Q-βMyHC) (4, 5, 16). The R403Q mutation in the *human* β-myosin motor domain, influences myosin-ATPase activity and leads to LV hypertrophy, severe heart failure (requiring heart transplantation), and/or arrhythmias (5, 29). In contrast, the R92W mutation in cTnT confers increased Ca^{2+} sensitivity to muscle fiber contraction, and predisposes to cardiac fibrosis and ventricular arrhythmias/sudden cardiac death in young individuals, in the absence of significant hypertrophy (30).

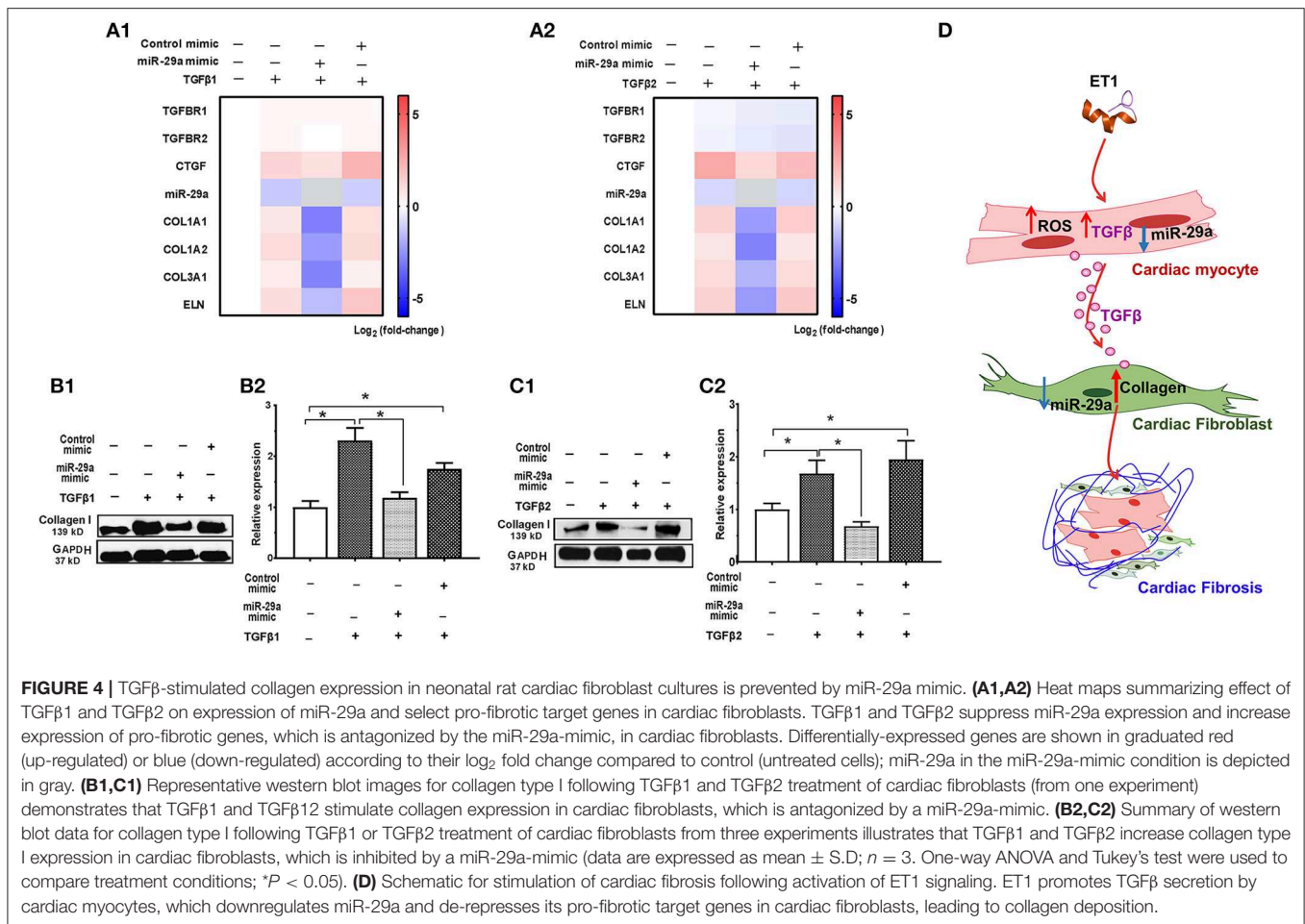
Our recent study comparing molecular phenotypes in 5 weeks old R92W-TnT and R403Q-MyHC mice with their respective littermate controls, demonstrated reduction in miR-29 expression only in mutant TnT whole hearts (6). However, it is unknown whether these differences are driven by gene expression in the LA and/or LV, and whether they persist later in disease course. In order to address these questions, we isolated the LV and LA appendage from each mouse heart, and assessed expression of *TGFβ1-3*, miR-29a/b/c, and select target genes in these two chambers of mutant and littermate control mice at 5 and 24 weeks of age.

We observed upregulation of *TGFβ2* and *CTGF* expression in the LV of both mutant mice at 5 and 24 weeks of age, but *TGFβ1* gene expression was increased in TnT mutant-LV, but not MyHC mutant-LV, at both time points (Figures 6A,B

TABLE 2 | Effects of TGFβ1, TGFβ2 and miR-29a mimic on gene expression in cardiac fibroblast cultures.

Genes	TGFβ1		TGFβ1 + miR-29a mimic		TGFβ1 + control mimic		TGFβ2		TGFβ2 + miR-29a mimic		TGFβ2 + control mimic	
	FC	P vs. con	FC	P vs. TGFβ1	FC	P vs. con	FC	P vs. con	FC	P vs. TGFβ2	FC	P vs. con
<i>TGFβR1</i>	1.1	0.12	1.1	0.7	1.1	0.1	0.8	0.4	0.7	0.2	0.7	0.1
<i>TGFβR2</i>	1.1	0.3	1.0	0.3	1.2	0.2	0.8	0.2	0.6	0.3	0.7	0.07
<i>CTGF</i>	2.3	0.002	1.8	0.09	4.8	0.02	6.5	3.2E-05	2.2	0.002	4.1	0.01
miR-29a	0.4	0.02	NA	NA	0.4	0.02	0.5	0.03	NA	NA	0.5	0.006
<i>COL1A1</i>	1.6	0.01	0.1	0.004	1.9	0.003	2.4	0.01	0.2	0.005	2.8	0.003
<i>COL1A2</i>	2.0	0.03	0.2	0.01	2.1	0.001	1.7	0.01	0.1	0.0007	1.6	0.02
<i>COL3A1</i>	1.5	0.02	0.1	0.004	1.3	0.01	2.0	0.01	0.3	0.0007	2.0	0.009
<i>ELN</i>	1.9	0.01	0.3	0.003	3.2	0.02	2.6	0.01	0.1	0.01	3.0	0.01

Expression of miR-29a, select pro-fibrotic genes in cardiac fibroblasts, following treatment with TGFβ1 (2 ng/mL) or TGFβ2 (1 ng/mL) with/without miR-29a mimic. Data are expressed as linear fold change (FC); $n = 3$ for each condition; all experiments were repeated 3 times. One-way ANOVA and Tukey's test were used to compare TGFβ1/2, TGFβ1/2 + miR29a-mimic, TGFβ1/2 + control-mimic and control. *TGFβR1*, Transforming growth factor, beta receptor 1; *TGFβR2*, Transforming growth factor, beta receptor 2; *CTGF*, Connective tissue growth factor; miR-29a, microRNA 29a; *COL1A1*, Collagen type I alpha 1 chain; *COL1A2*, Collagen type I alpha 2 chain; *ELN*, Elastin.



and Table 3). Furthermore, miR-29a/b/c was downregulated, and its pro-fibrotic target genes (*COL1A1*, *Col1A2*, *COL3A1*, and *ELN*) were upregulated in TnT mutant-LV, but not MyHC mutant-LV at both time points (Figures 6A,B and Supplemental Figure 4).

Gene Expression in Left Atrial Appendage of HCM Mice

We did not find a statistically significant difference in the expression of *TGFβ1-3*, miR-29a/b/c or its target genes in LA appendages from TnT and MyHC mutants compared to respective littermate control mice at 5 and 24 weeks of age (Supplemental Figure 5).

Human Data: Gene Expression in Septal Myectomy Tissue Compared to Donor Heart Tissue (Controls)

We analyzed publicly available microarray data for mRNAs (GSE36961) and miRNAs (GSE36946) from ventricular septal tissue, obtained at the time of surgical myectomy from HCM patients, and control subjects (24, 31). Donor heart tissue (LV free wall or LV side of the interventricular septum) from the Sydney Heart Bank (24) was used as controls. Age of HCM patients ranged from 9 to 78 years. Patients had a severe cardiac HCM phenotype, with maximum LV wall thickness of $2.2 \pm$

0.7 cm, and LV gradients of 71.5 ± 47.4 mmHg (24). Genotyping was performed in 100/107 HCM patients undergoing myectomy, revealing pathogenic variants in MYH7 (*n* = 17), MYBPC3 (*n* = 24), TNNT2 (*n* = 4), TNNC1 (*n* = 1), TPM1 (*n* = 2), MYL2 (*n* = 3), and MYH6 (*n* = 1) (24); no causal mutation was identified in the remainder (*n* = 48). Figure 7A illustrates the age and sex distribution of HCM patients and control subjects. We excluded 1 HCM patient based on the results of our principle component analysis (PCA), leading to inclusion of 105 patients/39 controls from the mRNA dataset, and 106 patients/20 controls from the miRNA datasets (Figure 7B) in our analysis.

Expression of Genes Involved in Cardiac Fibrosis

Since fibrosis is a prominent feature in the hypertrophied interventricular septum, we examined several genes involved in cardiac fibrosis. *TGFβ2* and *TGFβ3* were significantly upregulated, and *TGFβR2* was downregulated in HCM, but there was no difference in expression of *CTGF*, *TGFβ1*, miR-29a/b/c and its pro-fibrotic targets (collagen genes, elastin), in myectomy samples compared to donor heart tissue (Figures 8A,B). Next, we examined expression of renin-angiotensin-aldosterone system (RAAS) genes. The angiotensin converting enzyme 2 (*ACE2*) gene, a

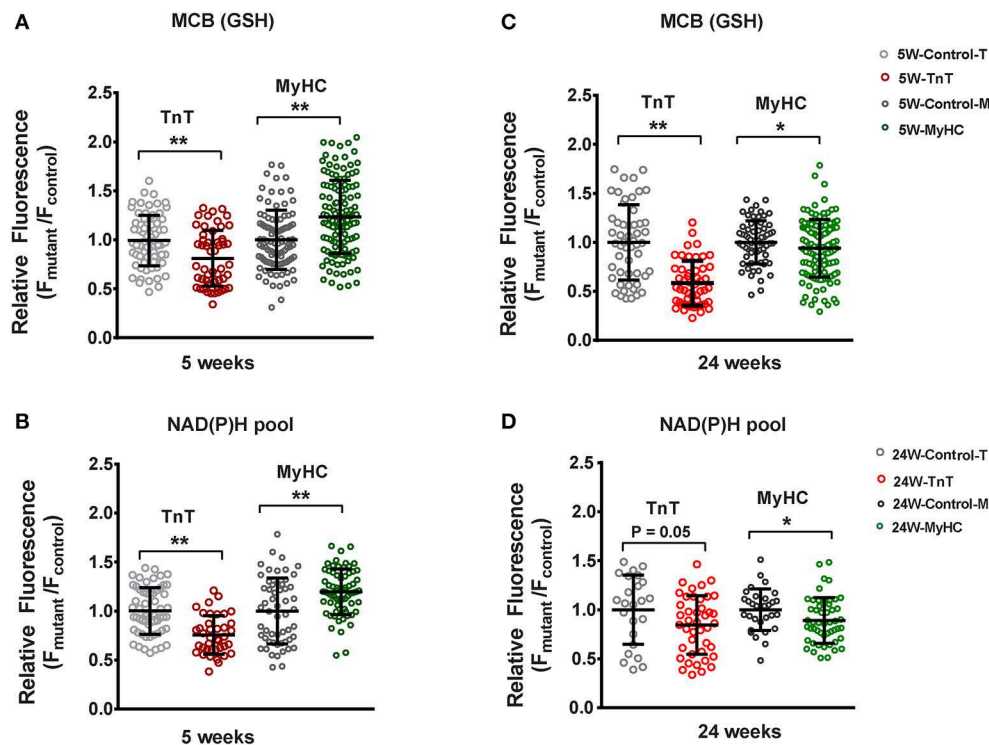


FIGURE 5 | Two photon microscopy to assess redox status in mouse cardiac myocytes at 5 and 24 weeks of age. Cardiac myocytes were labeled with monochlorobimane (MCB; 50 μ M, blue λ_{em} 480 $\pm$ 20 nm) at 37°C, to monitor reduced glutathione (GSH). Data from mutant myocytes is presented as fluorescence units normalized to littermate control myocyte data. NAD(P)H was assessed in non-labeled cells by measuring autofluorescence (total fluorescence collected at <490 nm). Potassium cyanide (KCN; 1 mM) and carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 5 μ M) were used to calibrate the NAD(P)H signal, permitting estimation of the NAD(P)H pool. All fluorescence recordings were obtained from non-paced cardiac myocytes. **(A)** TnT mutant myocytes had lower GSH (MCB signal), and MyHC mutant myocytes had higher GSH when compared to respective littermate control myocytes at 5 weeks of age. **(B)** NAD(P)H pool was lower in TnT mutant myocytes, and higher in MyHC mutant myocytes, when compared to respective littermate control myocytes at 5 weeks of age. **(C)** At 24 weeks of age, GSH (MCB signal) was lower in both mutant myocytes when compared to respective littermate control myocytes. **(D)** At 24 weeks of age, only MyHC mutant myocytes had statistically lower NAD(P)H pool levels, when compared to littermate control myocytes; a trend toward lower NAD(P)H pool levels were seen in TnT mutant myocytes when compared to littermate control myocytes ($p = 0.05$) (control-T, TnT-littermate control; TnT, TnT-mutant; Control-M, MyHC-littermate control; MyHC, MyHC-mutant) [Data are expressed as mean $\pm$ S.D. We present results from three mouse hearts (~20–40 myocytes per animal) in each mutant and control group. The two-tailed unpaired Student's t -test was used to compare TnT mutant myocytes and MyHC mutant myocytes with respective littermate control myocytes; * $P < 0.05$; ** $P < 0.01$].

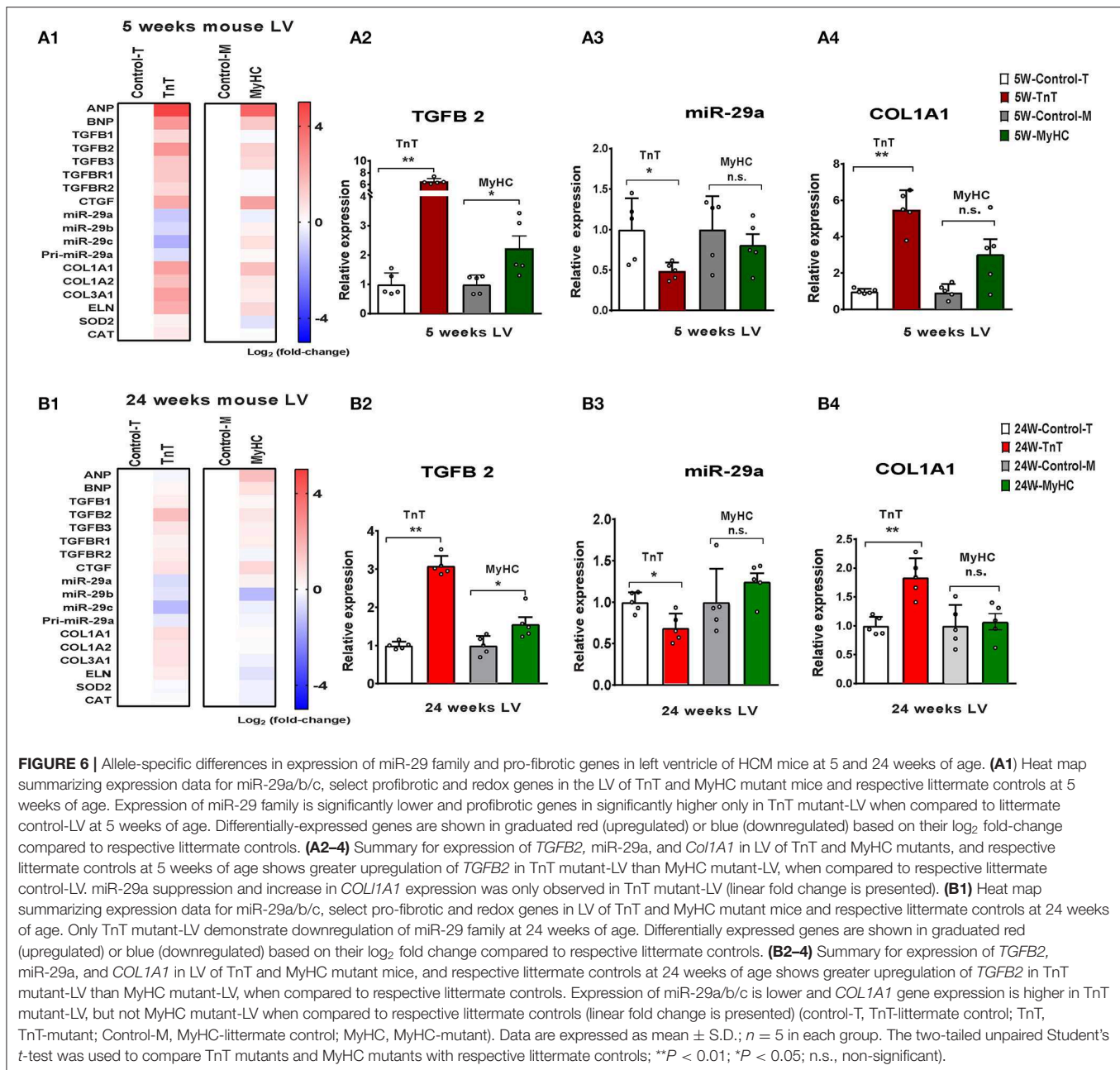
negative regulator of RAAS (32) was the most upregulated transcript in the HCM patient dataset (Table 4). Other members of the RAAS system, namely renin-binding protein (*RENBP*), renin receptor mannose-6-phosphate/insulin-like growth factor 2 receptor (*IGF2R*), angiotensinogen (*AGT*) and angiotensin II receptor type 1 (*AGTR1*) were significantly downregulated (Table 4), suggesting activation of compensatory anti-hypertrophic/anti-fibrotic pathways in human obstructive HCM.

We observed lower expression of *SOD2* and higher expression of catalase in myectomy tissue when compared to donor heart tissue. Furthermore, α MYHC, *SERCA2a*, and *GLUT1* were downregulated, and *ANP* was upregulated, compared to donor heart tissue (Figure 8A), confirming activation of the fetal gene program (33) in human obstructive HCM.

Signaling Pathways

We used Ingenuity Pathway Analysis (IPA, Qiagen) to predict dysregulated signaling pathways and upstream transcriptional regulators using the mRNA dataset. The red and blue dots in the volcano plot reflect the gene values used for IPA (Figure 9A). We used a $\log_2$ fold change cutoff of 2SD (standard deviations) up or down, which corresponds to a linear fold change of ~ 1.37 , for IPA.

Pathway analysis predicted activation of the liver X receptor/retinoid X receptor (LXR/RXR) pathway ($Z = 2.0$; $P = 0.00006$; Figure 9B), and downregulation of pro-hypertrophic ET1 signaling ($Z = -1.1$; $P = 0.007$), TGF β signaling ($Z = -2.2$; $P = 0.4$), and cardiac hypertrophy signaling ($Z = -1.5$; $P = 0.5$) in human HCM hearts compared to control hearts (data not shown).



Upstream Transcriptional Regulators

We used IPA to predict transcription factors that were up-regulated and down-regulated in human myectomy tissue, when compared to donor heart tissue. Transcription factors involved in development (SOX2, NKX2-3, and DACH1), inflammation (ZFP36), metabolism (SIRT1, FOXA2), and fibrosis-suppression (SMAD7) were predicted to be upregulated ($Z > 2$) in HCM patients, when compared to controls (**Figure 9C**). Transcription factors involved in development/immune response (STAT3), metabolism/mitochondrial function (CREB1), cellular stress response/fibrosis (NUPR1), and cellular response to hypoxia (HIF1) were predicted to be most downregulated ($Z > -4$)

in human myectomy samples, when compared to donor heart tissue (**Figure 9C**).

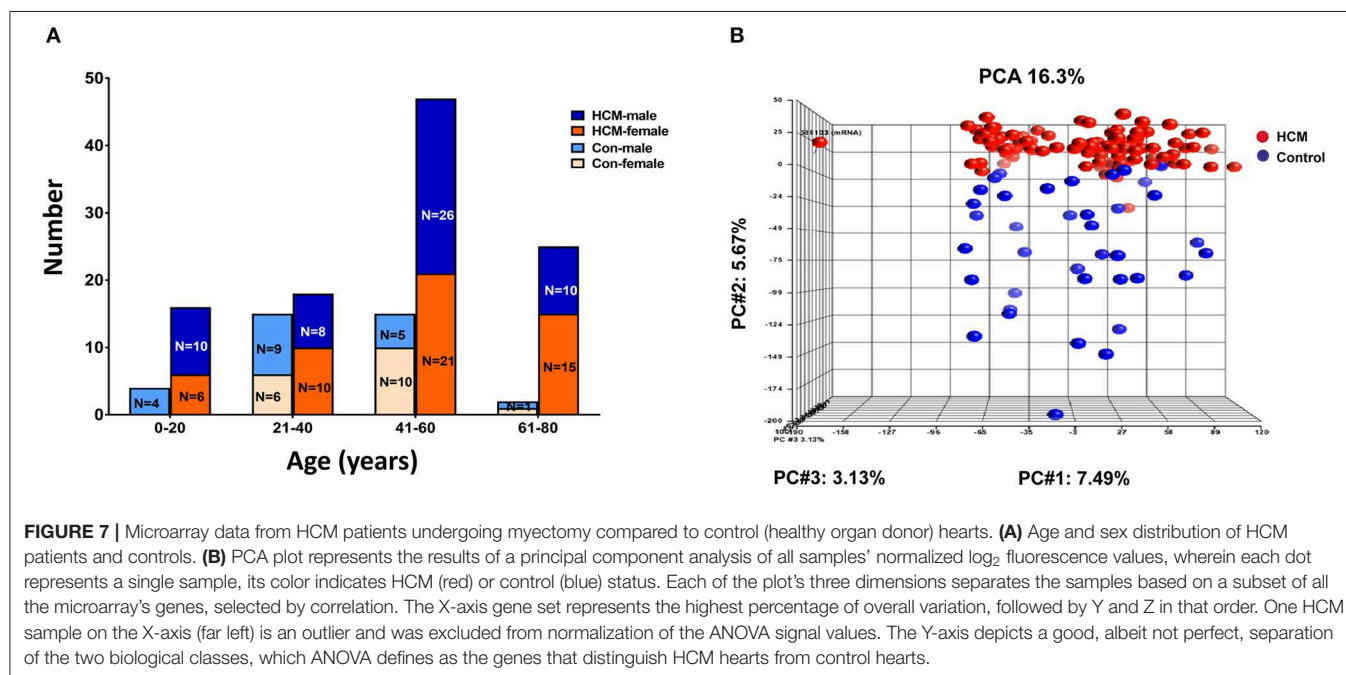
DISCUSSION

In this study, we demonstrate marked differences in expression of pro-fibrotic genes/pathways in mouse and human HCM. Gene expression analysis revealed activation of anti-hypertrophic and antifibrotic genes/pathways selectively in human myectomy tissue, which is novel. Our results in cardiac myocytes and fibroblasts provide insights into mechanisms whereby hypertrophy induction in cardiac myocytes promotes collagen

TABLE 3 | Gene expression in mutant mouse-LV compared to littermate control-LV at 5 and 24 weeks of age.

Genes	5 week male mice LV				24 week male mice LV			
	TnT		MyHC		TnT		MyHC	
	FC	P-value vs. con	FC	P-value vs. con	FC	P-value vs. con	FC	P-value vs. con
<i>ANP</i>	29	0.04	16.3	0.01	0.9	0.3	3.1	7.4E-05
<i>BNP</i>	6.2	1.1E-05	2.7	0.1	1.2	0.1	1.7	0.004
<i>TGFB1</i>	2.0	0.001	0.9	0.7	1.3	0.004	1.2	0.4
<i>TGFB2</i>	6.5	6.4E-08	2.2	0.04	3.1	9.6E-06	1.6	0.03
<i>TGFB3</i>	2.7	1.0E-05	1.9	0.1	1.6	0.002	1.3	0.01
<i>TGFB1</i>	2.6	8.4E-05	0.9	0.8	1.3	0.04	1.3	0.07
<i>TGFB2</i>	2.0	2.2E-06	1.0	0.9	1.4	0.06	0.9	0.2
<i>CTGF</i>	4.4	0.0004	5.2	0.001	1.6	0.0003	2.0	0.002
miR-29a	0.5	0.03	0.8	0.4	0.6	0.01	1.2	0.2
miR-29b	0.6	0.03	1.2	0.5	0.7	0.03	0.4	0.06
miR-29c	0.3	0.04	1.7	0.04	0.4	0.004	0.8	0.3
Pri-miR-29a	0.6	3.5E-05	1.1	0.7	0.8	0.03	0.9	0.4
<i>COL1A1</i>	5.5	0.0006	3.0	0.06	1.8	0.002	1.1	0.7
<i>COL1A2</i>	3.1	1E-05	1.5	0.2	1.6	0.004	1.0	0.9
<i>COL3A1</i>	5.5	0.0002	1.4	0.2	1.6	0.0002	0.8	0.5
<i>ELN</i>	4.2	4.1E-07	2.0	0.2	1.4	0.02	0.7	0.06
<i>SOD2</i>	1.3	0.06	0.7	0.06	0.9	0.4	0.8	0.1
<i>CAT</i>	1.4	0.2	1.0	0.7	1.0	0.8	0.8	0.9

Expression data for miR-29a/b/c, select profibrotic and redox genes in the LV of TnT and MyHC mutant mice and respective littermate controls at 5 and 24 weeks of age. Data are expressed as mean $\pm$ S.D.; $n = 5$ in each group. The 2-tailed unpaired Student's *t* test was used to compare TnT mutants and MyHC mutants with respective littermate controls. ANP, Natriuretic peptide A; BNP, Natriuretic peptide B; TGFB1, Transforming growth factor, beta 1; TGFB2, Transforming growth factor, beta 2; TGFB3, Transforming growth factor, beta 3; TGFB1, Transforming growth factor, beta receptor 1; TGFB2, Transforming growth factor, beta receptor 2; CTGF, Connective tissue growth factor; miR-29a, microRNA-29a; COL1A1, Collagen type I alpha 1 chain; COL1A2, Collagen type I alpha 2 chain; ELN, Elastin.



expression in fibroblasts. We confirmed allele-specific differences in expression of *TGFB*, miR-29 family, and its profibrotic target genes at both early and established disease stage in two mouse models of sarcomeric HCM. Our studies

of redox and gene expression suggest that allele-specific differences in redox and signaling pathways could contribute to allele-specific differences in upregulation of profibrotic genes in mouse HCM.

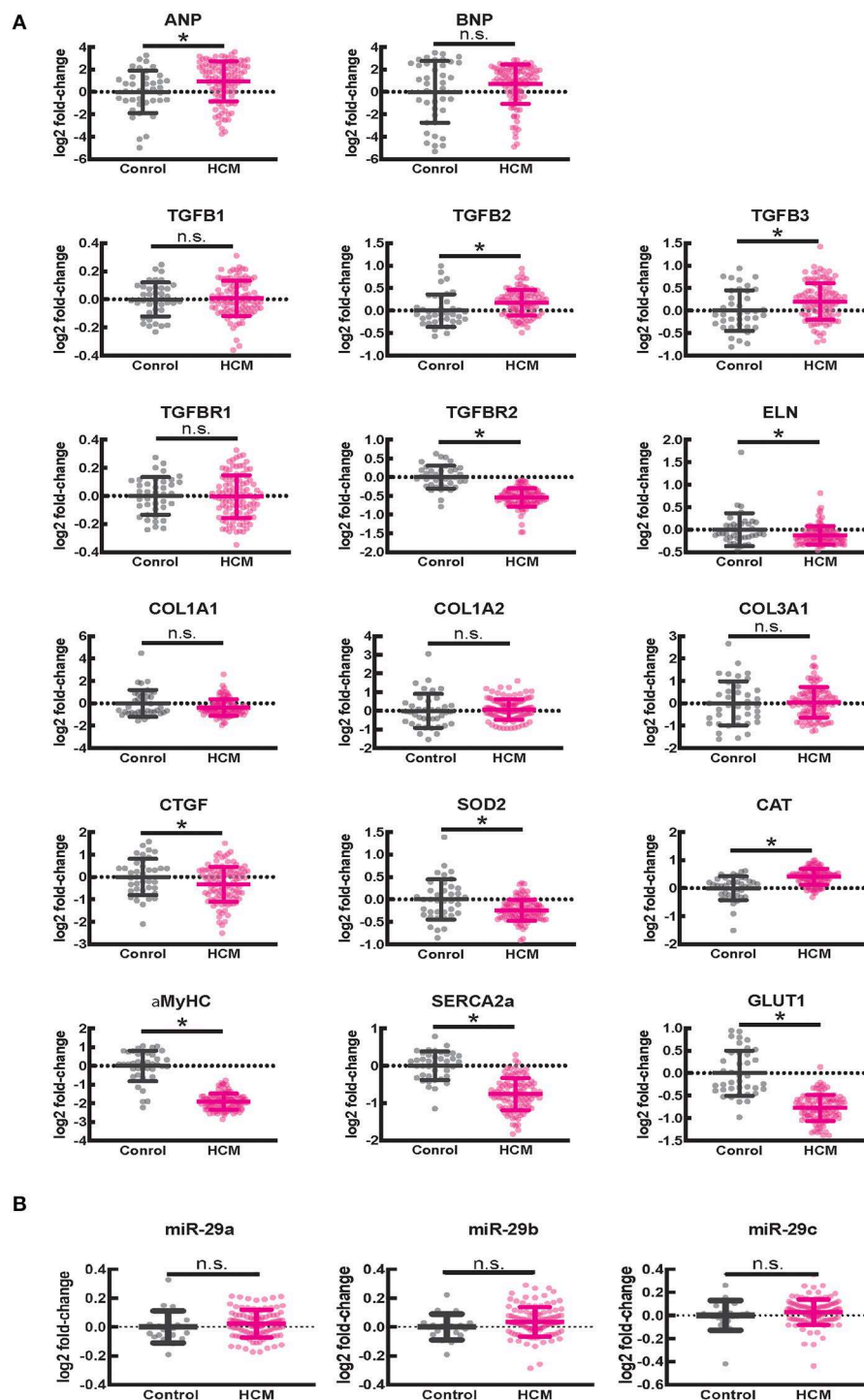


FIGURE 8 | Gene expression in human ventricular septal tissue of HCM patients undergoing myectomy compared with control (healthy organ donor) hearts. **(A)** Summary of mRNA data (select pro-fibrotic and redox genes) from 105 HCM patients compared to data from 39 controls shows upregulation of *TGFB2* and *TGFB3* and downregulation of *TGFB2R1*, *ELN*, *α MYHC*, *SERCA2a*, and *GLUT1* genes. **(B)** Summary of data for miR-29a/b/c from 106 HCM patients compared with data from 20 controls shows no difference in expression of miR-29a/b/c between HCM patients and controls. * $P < 0.05$.

Human HCM

Hypertrophic cardiomyopathy is classified as non-obstructive, labile obstructive or obstructive, based on pressure gradients

obtained by Doppler-echocardiography, at the level of the LV outflow tract and mid-LV cavity. Non-obstructive HCM is characterized by peak gradient <30 mm Hg at rest and stress,

TABLE 4 | Expression of RAAS genes in human myectomy tissue from HCM patients compared with control (healthy donor) hearts.

Gene	FC	Adjusted P-value
Angiotensin converting enzyme 2 (<i>ACE2</i>)	3.5	2.2E-24
Renin-binding protein (<i>RENBP</i>)	1.1	5.7E-05
Insulin-like growth factor 2 receptor (<i>IGF2R</i>)	0.8	1.1E-03
Angiotensinogen (<i>AGT</i>)	0.8	1.2E-04
Angiotensin II receptor type 1 (<i>AGTR1</i>)	0.8	2.0E-13
Angiotensin converting enzyme (<i>ACE</i>)	1.0	0.09
Angiotensin II receptor type 2 (<i>AGTR2</i>)	1.0	0.9
Angiotensin II receptor associated protein (<i>AGTRAP</i>)	0.9	0.4
Renin (<i>REN</i>)	0.9	0.9
Aldosterone synthase (<i>CYP11B2</i>)	1.0	0.4
(Pro)renin receptor (<i>ATP6AP2</i>)	0.9	0.7
Cathepsin D (<i>CTSD</i>)	0.9	0.5
Chymase A (<i>CMA1</i>)	0.9	0.5

labile-obstructive HCM by peak gradient <30 mm Hg at rest, ≥30 mm Hg with stress, and obstructive HCM with peak gradient ≥30 mm Hg at rest and stress (34). Symptomatic HCM patients with obstructive HCM are referred for myectomy to relieve LV obstruction.

The ventricular septum is usually the most hypertrophied region and is a frequent location for late gadolinium enhancement, reflecting replacement fibrosis, by cardiac magnetic resonance imaging (35). Septal myectomy samples often demonstrate both replacement and interstitial fibrosis (36) by histopathology. To our surprise, analysis of gene expression in the excised hypertrophied ventricular septum revealed no difference in expression of *CTGF*, *TGFB1*, miR-29a/b/c, and its pro-fibrotic target genes (collagen genes, elastin) between HCM patients and control subjects. Notably, IPA predicted downregulation of pro-fibrotic/pro-hypertrophic TGFβ/ET1 signaling and several RAAS genes, along with marked upregulation of the LXR/RXR pathway, which is anti-fibrotic, and anti-hypertrophic. These results suggest that other mechanisms such as ischemia (30), cell death, and inflammation could underlie the fibrosis observed in the hypertrophied ventricular septum in human HCM. Interestingly, we did not find differences in expression of RAAS genes, and IPA did not predict upregulation of the LXR/RXR pathway in HCM mouse models that lack LV outflow tract obstruction at early and late disease stage (data not shown). These results suggest that LXR/RXR pathway activation may be a compensatory response (37) to sustained LV obstruction, which is characteristic of obstructive HCM.

The liver X receptor (LXR) and retinoic acid receptor (RXR) are nuclear receptors that form obligate heterodimers and bind to LXR response elements (LXREs) in the nucleus (38). In the heart, LXR expression has been reported to be 10- to 15-fold higher in non-myocytes (fibroblasts, endothelial cells) when compared to

cardiac myocytes (39). RXR is involved in cardiac development and LXR has been implicated in the adaptive metabolic response to hypertrophic stress (40). Studies in mouse models reveal that LXR regulate cell survival through ROS inhibition, antagonize TGFβ-mediated collagen synthesis, promote angiogenesis, and suppress pro-inflammatory NFκB signaling, which are all cardio-protective (39, 41, 42). But very little is known about the role of LXR/RXR pathway in the genesis of the cardiac HCM phenotype.

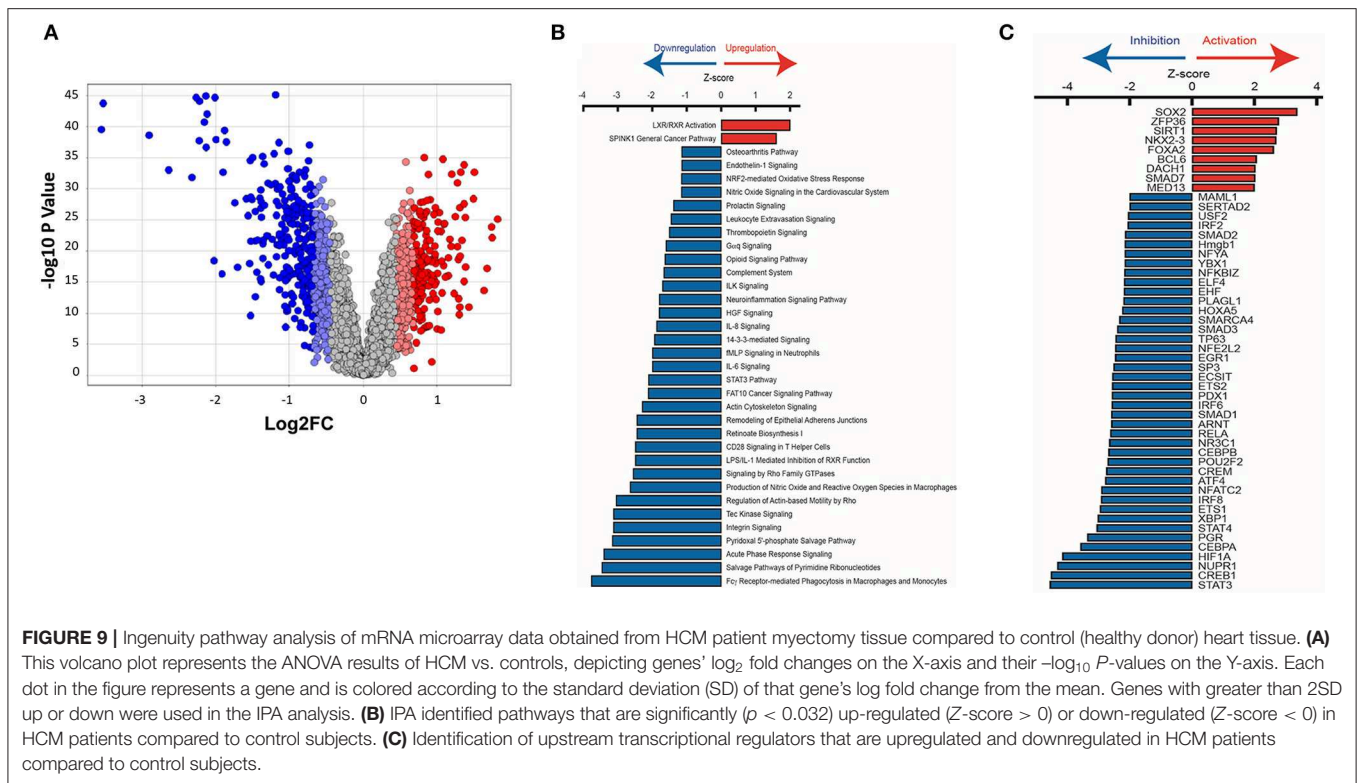
Differential Expression of TGF-Beta Isoforms

Fibrosis is frequent in HCM patients, and contributes to arrhythmias and heart failure (27, 43, 44). *TGFB1* was up-regulated in TnT mutant-LV and ET1-treated cardiac myocytes, but not in human myectomy tissue or MyHC mutant-LV. The TGF-beta isoform upregulated in both mouse and human HCM, as well as ET1-treated cardiac myocytes, was *TGFB2*. Differential expression of TGFB isoforms has also been reported in DCM mice (45), but the mechanisms underlying differential regulation of *TGFB1* and *TGFB2* genes, and the implications of these differences on HCM pathophysiology is unknown. Our *in vitro* studies of gene and collagen expression reveal that both TGFβ1 and TGFβ2 suppress miR-29a, de-repress its profibrotic target genes, and stimulate collagen expression in cardiac fibroblasts.

Association Between miR-29 and Cardiac Remodeling

A previous study of 41 HCM patients revealed significant increases in plasma levels of miR-29a and a positive association with cardiac hypertrophy and fibrosis, assessed by cardiac magnetic resonance imaging (8). This result was surprising, because tissue fibrosis has been associated with suppression of miR-29 expression, which should have resulted in a negative association between miR-29 and cardiac fibrosis. These clinical results could be explained by a recent study that performed over-expression and knockdown of miR-29 family in cardiac myocytes (46). Sassi et al. found that increasing miR-29 family in cardiac myocytes led to cardiac hypertrophy, and inhibition or genetic deficiency of miR-29 family protected against cardiac hypertrophy and fibrosis in a mouse model of chronic cardiac pressure overload. The authors proposed that de-repression of Wnt signaling underlies the pro-hypertrophic and pro-fibrotic effects of increased levels of miR-29 family in cardiac myocytes (46).

In our study, miR-29 family expression was lower in TnT mutant-LV at 5 and 24 weeks of age, but no difference was observed in MyHC mutant-LV at these two time points. Interestingly, TnT mutant-LV mass has been reported to be lower than controls at both time points (47), which may contribute to lower cardiac miR-29 family levels. We found no difference in expression of miR-29a/b/c and its pro-fibrotic target genes in ventricular septal tissue obtained from patients with obstructive HCM undergoing myectomy for relief of LV outflow tract/mid-cavity obstruction. The discrepancy between



our result and the clinical plasma-miRNA study which reported a positive association between miR-29a and cardiac hypertrophy and fibrosis, may be due to differences in cardiac physiology. The mean value for the LV outflow tract gradient, which is used to determine presence of LV outflow tract obstruction, was 18 ± 21 mmHg, suggesting that most of the patients in the clinical plasma-miRNA study had non-obstructive HCM (8). In contrast, the HCM patients in our study had obstructive HCM which results in chronic increase in left ventricular afterload. Interestingly, Sassi et al. reported dynamic regulation of miR-29 family expression following transverse aortic constriction (TAC), which increases left ventricular afterload (46). They found a prominent increase in cardiac myocyte expression of miR-29 in the first 48 h after TAC surgery, and downregulation thereafter, which could help explain our results in myectomy tissue. It is also possible that circulating miRNAs may not correlate with miRNA expression in tissues like the heart. Investigation of plasma miR-29 family levels in conjunction with gene expression studies in patients undergoing myectomy would be helpful to define the relationship between cardiac expression and plasma levels of the miR-29 family.

Allele-Specific and Disease Stage-Specific Differences in miR-29a/b/c and Pro-fibrotic Gene Expression

We observed 5-fold higher levels of miR-29a in cultured rat cardiac fibroblasts, when compared to rat cardiac myocytes,

which is similar to the results from a previous study (7). In contrast, Sassi et al. found that miR-29 family levels were 6-fold lower in freshly isolated cardiac fibroblasts, when compared to cardiac myocytes (46), and increased with cell culture. A likely explanation for this difference is that long term culture of cardiac fibroblasts on stiff substrates like tissue culture plastic or glass coverslips activates them to myofibroblasts (48).

In our *in vitro* study that employed cultured rat cardiac fibroblasts and myocytes, the pro-hypertrophic cytokine ET1 increased superoxide and H_2O_2 levels, suppressed miR-29a/b/c expression, and induced TGF β secretion by cardiac myocytes. Interestingly, ET1 was predicted (by IPA) to be upregulated in *both* mutants at 5 weeks of age (6), but TGF β signaling was predicted to be upregulated only in TnT mutants. Furthermore, miR-29a/b/c was suppressed only in TnT mutants who also demonstrated an oxidized redox environment at 5 weeks of age. In contrast, MyHC mutants exhibited a reduced redox environment and demonstrated similar miR-29 expression as littermate controls at 5 weeks of age. Based on our *in vitro* results of ET1 increasing cellular ROS levels and *TGFBI-3* expression, we speculate that TnT mutants that demonstrate an oxidized redox environment are more susceptible to ET1-stimulated activation of TGF β signaling, which suppresses miR-29 and leads to increase in collagen gene expression/cardiac fibrosis. Our hypothesis is supported by identification of the antioxidant NAC, as a potential therapy by IPA, in TnT mutants, but not MyHC mutants at 5 weeks of age (6).

In addition to TGF β signaling, hedgehog, NF κ B signaling, C-Myc, CCAAT/enhancer-binding protein- α (CEBPA), and ROS have been demonstrated to regulate miR-29 expression (49–51). Our study did not detect a difference in miR-29a/b/c expression between human myectomy tissue and donor heart tissue, which was used as controls. Based on results of a prior study of HCM patients which suggested that obstructive HCM is associated with greater oxidative stress than non-obstructive HCM (52), we expected higher myocardial miR-29 levels in human myectomy tissue (51). The mechanism underlying our result could be downregulation ($Z > -3$) of CEBPA, an inducer of miR-29 expression, in human myectomy samples. We speculate that the net effect of oxidative stress, CEBPA downregulation and lack of activation of TGF β signaling, could have resulted in no difference in miR-29 family/target gene expression between HCM patients and controls, observed in our study.

LIMITATIONS

We focused on the miR-29/TGF β signaling pathway, and did not investigate the LXR-RXR pathway in HCM mouse models or *in vitro*. We limited our proof-of-principle studies to two mouse models of HCM, in order to examine allele-specific and stage-specific differences in redox and gene expression. Studies in additional experimental models (53) would be helpful to ascertain whether HCM pathophysiology is gene-specific or mutation-specific. We used publicly available microarray data, and did not have access to human myectomy tissue to perform qPCR or Western blot for confirmation of IPA predictions. Since human myectomy tissue was obtained from the septum, gene expression in this region may not be representative of the whole heart. Furthermore, the contribution of cardiac myocytes, fibroblasts, and endothelial cells to differences in gene expression observed in human heart tissue could not be assessed because RNA was obtained from homogenized heart tissue, without cell dissociation. Lastly, we obtained genotype information for the myectomy population from the publicly available Master's Thesis by Hebl (24), but did not have access to genotype data from individual patients. Hence we were unable to examine associations between HCM patient genotype and gene expression, in this study.

CONCLUSION

In summary, we found marked differences in expression of *TGFB1*, miR-29 family and its pro-fibrotic target genes in two mouse models of sarcomeric HCM, and between mouse and human HCM. Only human myectomy tissue demonstrated upregulation of antihypertrophic/antifibrotic genes/pathways. Allele-specific differences in myocyte redox status and signaling pathways could contribute to the observed differences in expression of miR-29 family/profibrotic genes in the 2 mouse models of HCM, and differences in cardiac physiology could underlie predicted activation of compensatory anti-fibrotic/anti-hypertrophic signaling/genes selectively in human HCM. Parallel studies in mouse and human HCM are needed to define HCM

pathophysiology and identify therapies that prevent/regress the cardiac HCM phenotype in humans.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GSE36961 (mRNA) and GSE36946 (miRNA).

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH 8th Edition, 2011) and was approved and monitored by UCSF Laboratory Animal Resource Center.

AUTHOR CONTRIBUTIONS

YL, MA, and RF were responsible for overall study design and data analysis. YL designed, performed, and analyzed rat and mouse studies. RF and CT analyzed microarray data. JA performed two-photon studies in rat myocytes, and contributed to rat myocyte/fibroblast study design. SV performed two photon microscopy studies in isolated mouse cardiac myocytes. YG performed mouse myocyte isolation. YG and GG maintained the mouse colonies and performed mouse genotyping. RK assisted with rat and mouse studies. JT and LL provided transgenic mouse breeders for colony establishment. YL, RF, CT, MA, SD, VH, LL, JT, and JO were involved in manuscript preparation. All authors reviewed and approved the manuscript.

FUNDING

This work was funded by the Innovator Project Award (American Heart Association) and startup funds from the UCSF Division of Cardiology to MA. This work was also funded by the Assistant Secretary of Defense for Health Affairs endorsed by the Department of Defense, through the FY17 PRMRP Discovery under Award No. W81XWH-17-PRMRP-DA. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

ACKNOWLEDGMENTS

The authors would like to acknowledge the support of NIH R01 GM116841 and American Heart Association 15SDG23220028 to RF; NIH R01 GM29090 and HL117138 to LL; NIH R01 HL075619 and HL107046 to JT.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2019.00170/full#supplementary-material>

Supplemental Figure 1 | Vimentin immunofluorescence microscopy.

Immunostaining for vimentin demonstrates no fibroblast contamination of neonatal rat cardiac myocyte cultures. **(A1–A3)** Representative microscopy images of a neonatal rat cardiac fibroblast culture shows co-localization of Hoechst and vimentin-labeling. **(B1–B3)** Representative microscopy images of a neonatal rat cardiac myocyte culture shows nuclear labeling with Hoechst (blue), but no vimentin-positive cells.

Supplemental Figure 2 | ET1 has no effect on miR-29a and pro-fibrotic gene expression in cultured neonatal rat cardiac fibroblasts. **(A–C)** Summary of gene expression of *TGFB2*, *CTGF*, collagen genes, and miR-29a show no difference in expression between untreated (control) and fibroblasts treated with ET1 (100 nM for 24 h) [CON, control; ET1, endothelin1 (100 nM) treatment for 24 h. Data are expressed as mean $\pm$ S.D.; $n = 3$ (biological replicates); all experiments were repeated three times. Two tailed Student's *t*-test was used to determine significance; n.s., non-significant].

Supplemental Figure 3 | TGF β 1/2 stimulated collagen expression in neonatal rat cardiac fibroblast cultures is antagonized by miR-29-mimic. Cardiac fibroblasts were either treated with 5 nM concentration of either miR-29a-mimic or control-mimic for 6 h, prior to stimulation with TGF β 1 or TGF β 2 for 24 h. **(A)**

miR-29a-mimic suppressed TGF β 1-stimulated expression of profibrotic miR-29 targets, but not *CTGF*, which is not a miR-29 target gene (linear fold change is presented). **(B)** miR-29a-mimic suppressed TGF β 2-stimulated expression of profibrotic miR-29 targets, but not *CTGF* (linear fold change is presented) [data are expressed as mean $\pm$ S.D.; $n = 3$ (biological replicates) for each condition; all experiments were repeated three times. One-way ANOVA and Tukey's test were used to compare treatment conditions; * $P < 0.05$].

Supplemental Figure 4 | Allele-specific differences in expression of miR-29a, *ANP*, *TGFB1/2*, *COL1A1* in LV of female HCM mice/littermate controls at 5 weeks of age. **(A–E)** Allele-specific differences in expression of *ANP*, *TGFB1/2*, miR-29a, and *COL1A1* in female TnT and MyHC mutant mouse -LV and littermate control-LV at 5 weeks of age (Control-T, TnT-littermate control; TnT, TnT-mutant; Control-M, MyHC-littermate control; MyHC, MyHC-mutant) (data are expressed as mean $\pm$ S.D.; $n = 6$ in each group. The two-tailed unpaired Student's *t*-test was used to compare TnT mutants and MyHC mutants with respective littermate controls; * $P < 0.05$; n.s., non-significant).

Supplemental Figure 5 | Gene expression in left atrial appendage of HCM mice and littermate controls at 5 and 24 weeks of age. **(A,B)** No difference in expression of miR-29a/b/c, select pro-fibrotic and redox genes between left atrial appendage of mutant mice and littermate controls at 5 and 24 weeks of age (control-T, TnT-littermate control; TnT, TnT-mutant; Control-M, MyHC-littermate control; MyHC, MyHC-mutant) (data are expressed as mean $\pm$ S.D.; $n = 5$ for each group. The two-tailed unpaired Student's *t*-test was used to compare TnT mutants and MyHC mutants with respective littermate controls; n.s., non-significant).

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Conflict of Interest: LL is a stockholder and founder of MyoKardia, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Allele-specific differences in cardiac metabolism detected by comprehensive metabolomics in two mouse models of hypertrophic cardiomyopathy

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1. NIH West Coast Metabolomics Center, University of California, Davis

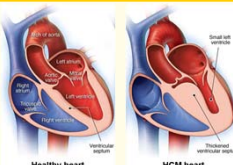
2. Hypertrophic Cardiomyopathy Center of Excellence, Division of Cardiology, University of California San Francisco



INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most frequent cardiac genetic disease.

- Estimated prevalence of 1:300-1:500 worldwide
- HCM is due to sarcomeric, cyto-structural, or mitochondrial mutations



Two mouse models of HCM in pre-hypertrophic stage (5 weeks of age) indicate allele-specific differences in mRNA/miRNA expression, cellular redox, and mitochondrial function.



R403Q-MyHC mice: arginine to glutamine mutation at amino acid 403 in the α -myosin heavy chain gene (MyHC).

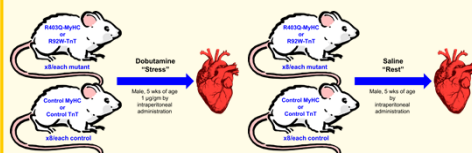


R92W-TnT mice: arginine to tryptophan mutation at amino acid 92 in cardiac troponin T gene (TnT).

Here we performed comprehensive metabolomics using both LC-MS and GC-MS to discover markers for pre-clinical HCM in HCM families and find therapeutic targets.

METHODS

STUDY DESIGN FOR STRESS & REST METABOLOMICS



64 whole hearts from male mice, 5 weeks old, were harvested from mice either under stress after inotropic stimulation by intraperitoneal dobutamine administration (left) or at rest (right).

SAMPLE EXTRACTION

Lipidomics (Complex Lipids) & HILIC (Biogenic Amines)

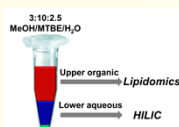
- Amount extracted: 5 mg
- Extraction solvent: 3:10:2.5 (v/v/v) MeOH/MTBE/H₂O
 - Upper organic phase: Lipidomics
 - Lower aqueous phase: HILIC

GC-TOF: Primary Metabolites

- Amount extracted: 4 mg
- Extraction solvent: 3:3:2 (v/v/v) ACN/IPA/H₂O

NAD(P)H Targeted Assay

- Amount extracted: 5 mg
- Extraction solvent: 3:1 (v/v) ACN with 50 mM ammonium acetate at pH 7.0



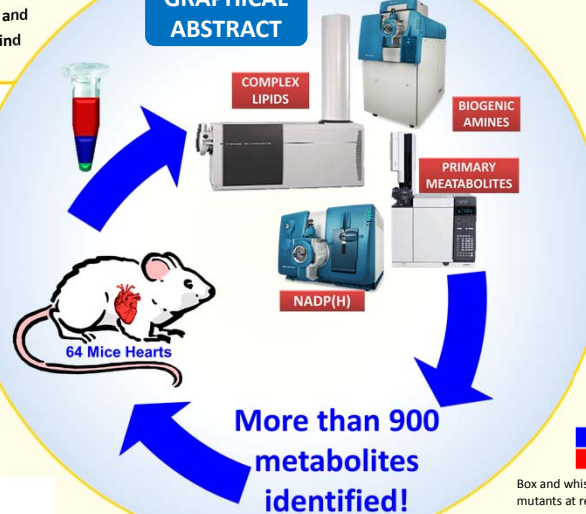
Combined extraction for lipidomics and biogenic amines using 3:10:2.5 (v/v/v) MeOH/MTBE/H₂O.

COMPREHENSIVE UNTARGETED & TARGETED METABOLOMICS

Platform	Metabolite Type	Type	Amount Extracted (mg)	Instrument	Ionization Mode	Column
Lipidomics	Complex lipids	Untargeted	5	Agilent 6530 Q-TOF + Agilent 1290 Infinity UHPLC	ESI (+)/(-)	Waters Acquity UPLC CSH C18 (100 × 2.1 mm; 1.7 μ m)
HILIC	Biogenic amines & other polar metabolites	Untargeted	5	SCIEX TripleTOF 6600 + Agilent 1290 Infinity UHPLC	ESI (+)	Waters Acquity UPLC BEH Amide (150 × 2.1 mm; 1.7 μ m)
GC-TOF	Primary metabolites	Untargeted	4	Leco Pegasus HT TOF + Agilent 7890A GC	EI 70 eV	Restek RTX-5Sil MS (95% dimethyl/5% diphenyl polysiloxane) (0.25 mm × 30 m; 0.25 μ m)
NAD(P)H	(5'->5')-dinucleotides	Targeted	5	SCIEX QTRAP 6500+ + Waters Acquity UPLC I-Class	ESI (-)	Agilent Polar 3 C18-A HPLC (2.0 × 150 mm, 3 μ m)

Summary of untargeted and targeted metabolomics for the analysis of mice hearts from MyHC & TnT mutant mice and littermate controls.

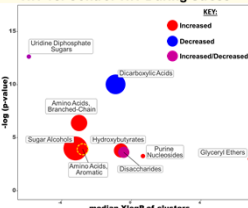
GRAPHICAL ABSTRACT



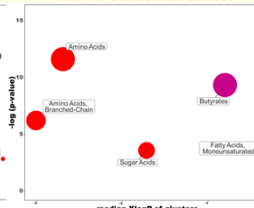
More than 900 metabolites identified!

RESULTS

TnT vs. Control TnT During Stress



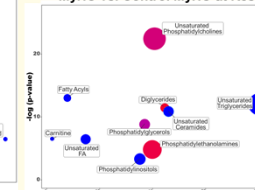
TnT vs. Control TnT at Rest



ChemRICH plots for primary metabolites.

Many compound clusters were only significant in TnT mutants compared to controls. No difference was found in MyHC mutant mice compared to controls. Branched-chain amino acids (BCAAs) assessed by HILIC, were higher in TnT mutants compared to controls during stress. BCAAs are essential nutrients and important for cell growth and stress response.

MyHC vs. Control MyHC at Rest



ChemRICH plots for complex lipids.

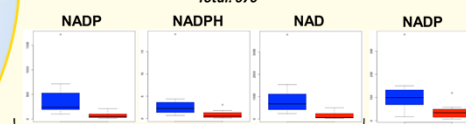
Carnitines were lower only in MyHC-mutants when compared to controls. These lipids are a strong indicator of mitochondrial function.

GC-TOF
Total:163

HILIC
Total: 165



Lipidomics
Total: 670



Box and whisker plots of target NAD(P)H metabolites. NADP, NADPH, and NAD were lower in TnT-mutants at rest. In contrast, only NADP was lower in MyHC-mutants at rest.

Comparison	MyHC vs. Control MyHC		TnT vs. Control TnT	
I.P. Admin.	Dobutamine	Saline	Dobutamine	Saline
Condition	Stress	Rest	Stress	Rest
Platforms	Number of significant metabolites (p-value < 0.05)			
Lipidomics	49	84	144	256
HILIC	9	8	18	39
NAD(P)H	--	1	--	3
GC-TOF	28	14	35	52

Summary of number of significant metabolites found in each platform in the comparison of mutant vs. control for each condition of either stress or rest. Statistics was performed by MetSolutions.

CONCLUSIONS

Three untargeted metabolomics assays from whole hearts of 2 HCM mouse models identified more than 900 metabolites.

Differences in cardiac metabolite levels in the two HCM mouse models at rest and stress in the preclinical stage of HCM suggest the possibility of using metabolomics for pre-clinical diagnosis in HCM families.

ACKNOWLEDGEMENTS

This study was funded by the NIH as pilot project in phase I of the Common Funds Metabolomics program and DOD Grant. Work continued into phase II of the West Coast Metabolomics Center, funded by NIH U2C ES030158.

University of California, San Francisco
CURRICULUM VITAE

Name: Maria Roselle Abraham, MBBS

Position: Associate Professor In Residence, Step 3
Medicine
School of Medicine

EDUCATION

1985 - 1992	Goa Medical College	MBBS	Medicine
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LICENSES, CERTIFICATION

1998	State of Minnesota Licence
1998	American Board of Internal Medicine Certification
2002	State of Maryland License (D60471)
2003	American Board of Medical Specialties (Cardiovascular Disease) Certification
2017	State of California License (C148336)

PRINCIPAL POSITIONS HELD

1992 - 1994	Goa Medical College	Resident	Medicine
1996 - 1996	Wake Forest University, Winston Salem, NC. Supervisor: William Hazzard	Intern	Medicine
1996 - 1998	Parkland Hospital, UT Southwestern, Dallas, Tx. Supervisor: Daniel Foster	Resident	Medicine
1998 - 1999	Mayo Clinic, Rochester, MN. Supervisors: Fellow Andre Terzic, Jamil Tajik		Basic Electrophysiology, Cardiology
1999 - 2001	Mayo Clinic, Rochester, MN. Supervisors: Fellow Andre Terzic, Jamil Tajik		Cardiology, Medicine
2001 - 2002	Mayo Clinic, Rochester, MN. Supervisor: Stephen Hammill	Fellow	Clinical Electrophysiology, Cardiology, Medicine

2002 - 2003	Johns Hopkins University School of Medicine, Baltimore, MD. Supervisor: Eduardo Marban	Research Associate	Cardiology, Medicine
2003 - 2016	Johns Hopkins University School of Medicine, Baltimore, MD	Assistant Professor	Cardiology, Medicine
2016 - 2017	Johns Hopkins University School of Medicine, Baltimore, MD	Associate Professor	Cardiology, Medicine
2017 - present	University of California, San Francisco	Associate Professor	Cardiology, Medicine

OTHER POSITIONS HELD CONCURRENTLY

2009 - 2017	Johns Hopkins University, Baltimore, MD	Adjunct Faculty	Institute of Computational Medicine, Biomedical Engineering
2015 - 2017	Johns Hopkins University School of Medicine, Baltimore, MD	Medical Director, Hypertrophic Cardiomyopathy Center of Excellence	Cardiology, Medicine
2017 - 2018	Johns Hopkins University School of Medicine, Baltimore, MD	Adjunct Associate Professor	Cardiology, Medicine
2017 - present	University of California San Francisco, San Francisco, CA	Director, Hypertrophic Cardiomyopathy Center of Excellence	Cardiology, Medicine

HONORS AND AWARDS

1986	Lady Reay Award in Physiology	University of Bombay
1988	Bazaz Memorial Award in Pathology	Goa Medical College
1990	Thali Memorial Award in Medicine	Goa Medical College
1990	Counto Award in Medicine	Goa Medical College
1998	Electrophysiology Research Award	Guidant Corporation
1999	Clinical Research Award	Mayo Foundation
2002	Career Development Award	American College of Cardiology
2004	Finalist, Melvin Marcus Young Investigator Award	American Heart Association

2005	Clinician Scientist Award	Johns Hopkins University School of Medicine
2007	Young Investigator Award Finalist (Marvin Chang, student)	Heart Rhythm Society
2007	Melvin Judkins Young Investigator Award (John Terrovitis, visiting faculty)	American Heart Association
2007	Provost Undergraduate Research Award / PURA (Linmiao Xu, undergraduate student)	Johns Hopkins University
2008	Sidney Blumenthal Young Investigator Award (Michael Bonios, postdoctoral fellow)	Cardiology Research Retreat, Johns Hopkins University
2008	Melvin Judkins Young Investigator Award Finalist (Michael Bonios, postdoctoral fellow)	American Heart Association
2010	Best Poster Abstract Finalist (Michael Bonios, postdoctoral fellow)	American College of Cardiology
2011	Young Investigator Award Finalist (Michael Bonios, postdoctoral fellow)	Hellenic Heart Failure Society
2011	Provost Undergraduate Research Award/ PURA (Patrick Armstrong, undergraduate student)	Johns Hopkins University
2011	Sidney Blumenthal Young Investigator Award finalist (Angel Chan, postdoctoral fellow)	Cardiology Research Retreat, Johns Hopkins University
2012	Melvin Judkins Young Investigator Finalist (Angel Chan, postdoctoral fellow)	American Heart Association
2015	Young Investigator Award (Junaid Afzal, postdoctoral fellow)	Heart Failure Society of America
2015	Young Investigator Award (Junaid Afzal, postdoctoral fellow)	Society of Nuclear Medicine
2016	Travel Award (Stella Vakrou, postdoctoral fellow)	European Society of Cardiology
2016	Travel Award (Mitsu Shah, graduate student)	American Society for Pharmacology and Experimental Therapeutics
2017	New Investigator Award (Stella Vakrou)	Heart Failure Society of America

KEYWORDS/AREAS OF INTEREST

1. Hypertrophic cardiomyopathy 2. Electrophysiology; ventricular arrhythmias and atrial fibrillation 3. Multi-modality molecular imaging 4. Stem cells for cardiac regeneration and as in vitro models of heart disease 5. Metabolism and redox. 6. Tissue engineering. 7. Precision Medicine in hypertrophic cardiomyopathy

CLINICAL ACTIVITIES

CLINICAL ACTIVITIES SUMMARY

1. Attending physician on inpatient Cardiology services at Parnassus (General Cardiology, CCU, Consults)
2. EKG reader
3. Attending in clinic and Hypertrophic Cardiomyopathy Center

CLINICAL SERVICES

2003 - 2007	Attending physician in the Cardiac Intensive Care Unity	6-8 weeks per year
2003 - 2007	Attending physician, Electrophysiology Clinic	half day per week
2008 - 2011	Attending physician, General Cardiology and Electrophysiology Clinic	3 half days per week
2011 - 2017	Stress ECG and Holter reader	Daily
2011 - 2017	Attending on inpatient General Cardiology and Consult services	12-20 weeks per year
2011 - 2017	Attending in Hypertrophic Cardiomyopathy and General Cardiology clinic	1-3 half days per week
2017 - present	Attending physician on Inpatient Cardiology Services (CCU and Consults)	8 weeks per year
2017 - present	Attending physician in General Cardiology Clinic and Hypertrophic Cardiomyopathy Center	1 half day per week

PROFESSIONAL ACTIVITIES

MEMBERSHIPS

- 1999 - 2017 American Heart Association, Member
- 1999 - 2017 NASPE/Heart Rhythm Society

SERVICE TO PROFESSIONAL ORGANIZATIONS

- None

SERVICE TO PROFESSIONAL PUBLICATIONS

2015 - present Circulation: Genomic and Precision Medicine, Editorial Board

INVITED PRESENTATIONS - INTERNATIONAL

2004	Cardiostim	Invited speaker
2006	Cardiostim	Invited speaker
2006	Japanese Heart Rhythm Society	Invited speaker
2011	Hellenic Heart Failure Society	Invited speaker

INVITED PRESENTATIONS - NATIONAL

2005	Heart Rhythm Society	Invited speaker
2005	Heart Failure Society of America	Invited speaker
2006	American Molecular Imaging Society	Invited speaker
2006	Society for Cardiac Computed Tomography	Invited speaker
2006	Mayo Clinic	Invited speaker
2007	Heart Rhythm Society	Invited speaker
2007	Women in Electrophysiology	Keynote speaker
2008	American College of Cardiology	Invited speaker
2008	Heart Rhythm Society	Invited speaker
2009	Women in Electrophysiology	Key note speaker
2009	American Society of Nuclear Cardiology	Invited speaker
2011	American College of Cardiology	Invited speaker

INVITED PRESENTATIONS - REGIONAL AND OTHER INVITED PRESENTATIONS

2006	Update on stem cells for state of Maryland judges	Speaker
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CONTINUING EDUCATION AND PROFESSIONAL DEVELOPMENT ACTIVITIES

None

GOVERNMENT AND OTHER PROFESSIONAL SERVICE

- None

UNIVERSITY AND PUBLIC SERVICE

SERVICE ACTIVITIES SUMMARY

None

UNIVERSITY SERVICE

UC SYSTEM AND MULTI-CAMPUS SERVICE

- N/A

UCSF CAMPUSWIDE

- N/A

SCHOOL OF MEDICINE

- N/A

SCHOOL OF DENTISTRY

- N/A

SCHOOL OF NURSING

- N/A

SCHOOL OF PHARMACY

- N/A

DEPARTMENTAL SERVICE

2018 - 2020	Faculty search committee for HS - Clinical Instructor	Member of Search Committee
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2019 - 2020	Faculty search committee for CVRI faculty	Member of Search Committee
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SERVICE AT OTHER UNIVERSITIES

2015 - 2017	Womens Task Force Committee, Department of Medicine	Johns Hopkins School of Medicine
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COMMUNITY AND PUBLIC SERVICE

- None

CONTRIBUTIONS TO DIVERSITY

CONTRIBUTIONS TO DIVERSITY

Mentor, Post Baccalaureate NIH Diversity Supplement to Kirubel Woldemichael

TEACHING AND MENTORING

TEACHING SUMMARY

Lecturer (1 lecture) in Systems Bioengineering I Course for Biomedical Engineering Undergraduates, Johns Hopkins University

FORMAL TEACHING

Not UCSF	Academic Yr	Course No. & Title	Teaching Contribution	School	Class Size
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Not UCSF	Academic Yr	Course No. & Title	Teaching Contribution	School	Class Size
X	2008 - 2017	Systems Bioengineering I	One lecture: Cell cycle, stem cells, potential for cardiac regeneration		>100

INFORMAL TEACHING

2000 - present I have taught Medical Residents, Cardiology fellows and medical students while attending on inpatient Cardiology services (Consults, CCU). I have used white board talks and teaching on rounds to help trainees integrate clinical history, lab and imaging data to understand disease pathophysiology, and develop patient specific treatment plans.

MENTORING SUMMARY

In addition to clinical mentorship and teaching on inpatient cardiology services, I have mentored several graduate students, medical students, undergraduate students and visiting faculty on basic, translational and clinical projects.

Since my move to UCSF in August 2017, I have mentored 3 postdoctoral fellows, 2 medical students, 2 visiting faculty, 1 undergraduate student and 2 medicine residents.

Mentees include Dr. Junaid Afzal (Research Specialist in my laboratory), Dr. Yamin Liu, (postdoctoral fellow in my laboratory), Dr. Gabriela Greenland (postdoctoral fellow in my laboratory), Dr. Daiyin Lu (postdoctoral fellow), Dr. Yueting Guo (visiting faculty in my laboratory), Ren LI (visiting faculty in my laboratory), Bahadir Simsek (visiting medical student in my laboratory), Rehal Karmali (visiting medical student in my laboratory), Leanne Peres de Silva (undergraduate student in my laboratory), Jacob Mayfield and Sebastian Rodriguez (Medicine Residents).

Dr. Afzal is focused on the investigation of metabolism and redox in hypertrophic cardiomyopathy (HCM), using mouse and cell (patient-specific cardiac myocytes derived from induced pluripotent stem cells) models of this disease. We are working on several projects describing the effect of acute inotropic stimulation and chronic exercise on metabolic remodeling in HCM.

I have mentored Dr. Liu in the investigation of crosstalk between cardiac myocytes and fibroblasts, to investigate mechanisms underlying fibrosis in HCM.

I have mentored Dr. Gabriela Greenland, a postdoctoral fellow in my laboratory whose projects involve MRI image analysis and EKG analyses in HCM patients.

I have mentored Bahadir Simsek, medical student from Turkey, who spent the summer in my lab, in performing mouse echocardiography, dobutamine stress-echocardiography, image analysis, review of the literature and manuscript-writing.

I have mentored Rehan Karmali in basic research - he spent 6 weeks in my lab in the summer of 2018, learning qPCR, histology, and will spend an additional 6 weeks during the winter break.

I have mentored 2 visiting faculty (Yueting Guo, Ren Li) in my laboratory in establishing exercise models of hypertrophic cardiomyopathy.

I have mentored 2 UCSF medicine residents in clinical research, specifically EKG analyses in HCM and 2 case reports/series.

PREDOCTORAL STUDENTS SUPERVISED OR MENTORED

Dates	Name	Program or School	Mentor Type	Role	Current Position
2002 - 2007	Marvin Chang	Undergraduate and Masters Program, Biomedical Engineering, Johns Hopkins University	Research/Scholarly Mentor, Co-Mentor/Clinical Mentor	Mentor, research supervisor	Cardiac Anesthesia Fellow, Massachusetts General Hospital, Boston, MA
2003 - 2012	Connie Chang	Undergraduate, Masters and PhD program, Biomedical Engineering, Johns Hopkins University	Research/Scholarly Mentor	Research mentor and supervisor	Cardiac Anesthesia Fellow, Cornell University, NY
2006 - 2007	Linmiao Xu	Undergraduate student, Biomedical Engineering, Johns Hopkins University	Research/Scholarly Mentor, Project Mentor	Research mentor and supervisor	Private sector

Dates	Name	Program or School	Mentor Type	Role	Current Position
2008 - 2010	Xiaoping Lin	PhD student, Tongji University School of Medicine, Shanghai, China	Project Mentor, Co-Mentor/Clinical Mentor	Research mentor and supervisor	Medical resident, Department of Cardiology, the Second Affiliated Hospital School of Medicine, Zhejiang University, Hangzhou, China
2011 - 2013	Junaid Afzal	Masters Program in Biotechnology, Johns Hopkins University	Research/Scholarly Mentor, Co-Mentor/Clinical Mentor	Research mentor and supervisor	Research Specialist, Division of Cardiology, UCSF
2013 - 2015	Mitsu Shah	Masters Program in Medical Research Technology, University of Maryland, Baltimore, MD	Research/Scholarly Mentor, Co-Mentor/Clinical Mentor	Research mentor and supervisor	PhD program, Howard University, Washington DC
2010 -	Xun Zhou	Medical student, Johns Hopkins University School of Medicine	Project Mentor	Project mentor	Surgery resident, Johns Hopkins School of Medicine
2010 - 2011	Patrick Armstrong	Undergraduate student, Biomedical Engineering, Johns Hopkins University	Research/Scholarly Mentor, Project Mentor	Research mentor and supervisor	Research Assistant Johane Laboratory, Boston Children's Hospital, MA.

Dates	Name	Program or School	Mentor Type	Role	Current Position
2010 - 2011	Matthew Kongkatong	Medical student, Pennsylvania Commonwealth University, PA	Research/Scholarly Mentor, Project Mentor	Project mentor	Medical resident, Pennsylvania Commonwealth University, PA.
2011 -	Vanessa Nelson	Undergraduate student, University of Maryland, Baltimore County	Project Mentor	Project mentor	Medical Student, St. James Medical School, St. James, Anguilla
2010 - 2012	Blaid Mbiyangandu	Research trainee (NIH diversity supplement)	Research/Scholarly Mentor, Co-Mentor/Clinical Mentor	Research mentor	Pharmacy student, Massachusetts College of Pharmacy and Health Sciences, Boston MA
2011 - 2012	Alex Hilser	Undergraduate, Rice University	Research/Scholarly Mentor	Research mentor	Military service
2011 - 2013	Kirubel Voldemichael	Research trainee (NIH diversity supplement)	Research/Scholarly Mentor, Project Mentor	Research mentor and supervisor	Medical Student, University of Wisconsin, Madison WI
2011 - 2013	Sophia Brown	Research trainee (NIH diversity supplement)	Research/Scholarly Mentor, Project Mentor	Research mentor and supervisor	Grant writer for Non-profit organization
2012 -	Clare Goggins	Undergraduate, Georgetown University	Research/Scholarly Mentor	Research Mentor	Medical Student., Georgetown University, Washington DC

Dates	Name	Program or School	Mentor Type	Role	Current Position
2012 -	David Hurtado de Mendoza Paz	Medical student, Cayetano Heredia University, Lima, Peru	Project Mentor	Research mentor	Medical Resident, Miller School of Medicine, University of Miami
2014 -	Roberto Pineda Reyes	Medical student, Cayetano Heredia University, Lima, Peru	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical resident, Cayetano Heredia University, Peru
2015 -	Benjamin Ostrander	Medical student, Johns Hopkins School of Medicine, Baltimore, MD	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical Student, Johns Hopkins University School of Medicine, Baltimore, MD
2016 - 2018	Nestor Enrique Vasquez Encinas	Medical student, Cayetano Heredia University, Lima, Peru	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical resident, UT Southwestern Medical Center, Dallas, TX, USA
2016 - 2018	Sanjay Sivalokanathan	Medical student, University College of London, UK	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical resident, University College of London, UK
2016 -	Isinsu Yilmaz	Medical student, Ankara University, Turkey	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical student. Ankara University, Turkey

Dates	Name	Program or School	Mentor Type	Role	Current Position
2016 -	Anil Tasci	Visting Medical student, Ankara University, Turkey	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical student, Ankara University, Turkey
2017 -	Waseem Abbas	Medical Student, Nishtar Medical College, Multan, Pakistan	Research/Scholarly Mentor, Project Mentor	Research Mentor	Medical Student, Nishtar Medical College, Multan, Pakistan
2017 - 2018	Shibani Kudchadkar	Medical Student, Jawarharlal Nehru Medical College, Belgaum, India	Research/Scholarly Mentor, Project Mentor, Career Mentor	Research mentor	Medical Student, Jawarharlal Nehru Medical College, Belgaum, India
2017 -	Erini Papas	Undergraduate, Johns Hopkins University	Research/Scholarly Mentor, Project Mentor	Research mentor	Undergraduate, Johns Hopkins University
2017 -	Joy Song	Undergraduate, Johns Hopkins University	Research/Scholarly Mentor, Project Mentor	Research mentor	Undergraduate, Johns Hopkins University
2017 -	Effrysini Kontari	Visiting Medical Student, University of Patras, Greece	Research/Scholarly Mentor, Project Mentor	Research Mentor	Medical Student, University of Patras, Greece
2017 -	Sagar Goyal	Medical Student, All India Institute of Medical Sciences, Delhi, India	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical Student, All India Institute of Medical Sciences, Delhi, India

Dates	Name	Program or School	Mentor Type	Role	Current Position
2017 - 2019	Bahadir Simsek	Medical Student, Istanbul Turkey	Research/Scholarly Mentor	Research mentor	Medical Student, Istanbul University, Cerrahpasa Medical Faculty
2018 - 2019	Rehan Karmali	Medical Student, Touro College of Osteopathic Medicine	Research/Scholarly Mentor, Project Mentor	Research mentor	Medical Student, Touro College of Osteopathic Medicine, Middletown, New York
2018 - 2019	Leanne Peres da Silva	Undergraduate, UC Berkeley	Research/Scholarly Mentor	Research mentor	Undergraduate, UC Berkeley
2019 - 2019	Jacob Mayfield	Internal Medicine Resident	Research/Scholarly Mentor	Research mentor	University of California, San Francisco

POSTDOCTORAL FELLOWS AND RESIDENTS MENTORED

Dates	Name	Fellow	Mentor Role	Faculty Role	Current Position
2007 - 2009	Michael Bonios	Post doctoral fellow	Research/Scholarly Mentor, Project Mentor	Research and project mentor	Attending physician Onassis Cardiac Surgery Center, Univ Utah
2011 - 2013	Angel Chan	Post doctoral fellow, T32 Training program	Research/Scholarly Mentor, Project Mentor	Research and project mentor	Advanced Imaging Fellow, Memorial Sloan Kettering Cancer Center, NY

Dates	Name	Fellow	Mentor Role	Faculty Role	Current Position
2013 - present	Junaid Afzal	Post doctoral fellow	Research/Scholarly Mentor, Project Mentor	Research and project mentor	Post doctoral fellow, UCSF
2013 - 2014	Yalda Soleimanifard	Post doctoral fellow			Internal Medicine Resident, Univ of Toledo
2011 - 2013	Sudip Saha	Medical Resident	Project Mentor	Project mentor	Cardiologist, Mid-Atlantic Permanente, Mclean, VA
2011 - 2014	Stella Vakrou	Post doctoral fellow	Research/Scholarly Mentor, Project Mentor	Research and project mentor	Cardiology Fellow, Laikos Hopsital, University of Athens, Greece
2013 - 2016	Yufan Guan	Post doctoral fellow	Research/Scholarly Mentor, Project Mentor	Research and project mentor	Cardiologist, Hebei Medical University, China
2016 - present	Gabriela Villarreal Romo Greenland	Post doctoral fellow	Research/Scholarly Mentor, Project Mentor	Research and project mentor	Postdoctoral fellow, UCSF
2017 - present	Sanjay Sivalokanathan	Medical Resident	Research/Scholarly Mentor, Project Mentor	Research and medical mentor	University College of London, UK
2017 - 2017	Harleen Chahil	Medical Resident	Research/Scholarly Mentor, Project Mentor	Research and medical mentor	Sinai Hospital of Baltimore, MD

VISITING FACULTY MENTORED

2005 - 2008	John Terrovitis, Assistant Professor and Assistant Chief of Cardiology (deceased 2014)	University of Athens, Greece
2013 - 2015	Mehmet Karakas, Assistant Professor, Cardiology	Mustafa Kemal University, Turkey
2013 - 2015	Hulya Yalcin, Assistant Professor, Nuclear Medicine/Radiology	Mustafa Kemal University, Turkey
2014 - present	Yamin Liu, Assistant Professor, Clinical Pharmacology	Zhongda Hospital, School of Medicine, Southeast University, Jiangsu, China
2015 - 2016	Zhao Li, Assistant Professor, Cardiology	The First Hospital of China Medical University, Shenyang, China.
2016 - 2017	Yuhui Luo, Associate Chief Physician, Vice director of Cardiology, Department of Cardiology	Department of Cardiology, Chongqing Zhongshan Hospital, Yu Zhong District Chongqing City, China
2017 - present	Dai-Yin Liu, Assistant Professor, Division of Cardiology	Division of Cardiology, Taipei Veterans General Hospital, Taipei, Taiwan
2018 - 2019	Yueting Guo, Physician and Director of Cardiology Department	Beijing Yanhua Hospital, Beijing, China
2018 - present	Ren Li, Chief Physician	Guang anmen Hospital, China Academy of Chinese Medical Science

RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AND CREATIVE ACTIVITIES SUMMARY

Research Program in Hypertrophic Cardiomyopathy (HCM): At UCSF I have established a research program in HCM with Basic/Translational and Clinical components, in collaboration with investigators in the department of Radiology and divisions of Vascular Surgery and Cardiology at UCSF, as well as the West Coast Metabolomics Center at UC Davis, and the Computer Science Department at the University of Delaware.

HCM is caused by sarcomeric protein mutations in 50-60% of mutations, with the genetic cause being unknown in the remainder 40-50% of patients. HCM is also characterized by phenotypic heterogeneity and variable penetrance. The pathologic hallmarks are cardiac hypertrophy, myocyte disarray, arteriolar remodeling. Importantly, the pathophysiology of HCM is complex as are the molecular mechanisms that underlie it. There is no consensus on whether a single or multiple mechanisms underlie genesis of the cardiac phenotype. Hence, I plan to develop non-genetic diagnostic tests and therapies based on disease pathophysiology.

Using a combination of functional studies and gene expression (RNA-seq, miR-Seq) in 2 mouse models of HCM that span the spectrum of human disease (sudden cardiac death and

heart failure) my lab has discovered mutation-specific differences in cellular redox, mitochondrial function, Ca²⁺ handling, gene expression and activation of (pro-fibrotic) TGF β signaling, in the early stage of disease, indicating differences in disease pathogenesis and the need for individualized therapies (JCI Insight, 2018)

Basic/Translational Research Program: I have established the infrastructure for investigation of metabolism/energetics/redox in mouse and cell models (fibroblasts and cardiac myocytes obtained by differentiation of induced pluripotent stem cells) at UCSF.

a) Investigation of metabolism in experimental models of HCM: We have two HCM mouse models that span the spectrum of human disease, namely heart failure and arrhythmias. We have established methods for a detailed investigation of redox, Ca²⁺, metabolism and energetics in isolated mitochondria and isolated myocytes. We are employing parallel studies in mouse models and iPSC-CMs expressing homologous mutations in genes encoding sarcomeric proteins (**co-clinical trial model**) to investigate disease mechanisms and test individualized therapies. The advantage of mouse models over iPSC-CMs is myocyte maturity and the ability to examine effects of physiologic and pathologic stressors on multiple scales (whole heart-myocyte-mitochondria-RNA/miR-protein-metabolites).

b) Regulation of cardiac fibrosis in HCM: Fibrosis (interstitial/replacement) is one of the pathologic hallmarks of HCM. Left ventricular fibrosis increases ventricular stiffness, reduces impulse conduction velocity, and thus predisposes to heart failure and sudden cardiac death. In the atrium, fibrosis predisposes to atrial fibrillation and stroke. But the mechanisms whereby mutant sarcomeric proteins lead to cardiac fibrosis has not been completely elucidated. Furthermore it is unknown whether human atrial and ventricular fibroblasts respond similarly to pro-fibrotic stimuli in the heart. In order to address these questions, we are examining effects of metabolic/redox modulation in human atrial and ventricular fibroblasts and establishing an in vitro model of arrhythmia to assess cross-talk between fibroblasts and human cardiac myocytes.

c) Mechanisms underlying microvascular dysfunction in HCM: A substantial proportion of HCM patients has angina and evidence of microvascular dysfunction of the coronary circulation, in the absence of epicardial coronary stenosis. But the mechanisms whereby mutant sarcomeric proteins predispose to arteriolar remodeling, and the molecular mechanisms underlying microvascular dysfunction are unknown. In order to answer this question, we are performing in vitro studies to investigate crosstalk between endothelial cells, iPSC-CMs and vascular smooth muscle cells. Additionally, we are isolating and characterizing exosomes secreted by endothelial cells, in collaboration with Dr. Robert Raffai at the UCSF-VA Medical Center to understand mechanisms underlying microvascular dysfunction in HCM patients.

Clinical Research Program: Multi-modality molecular cardiac imaging offers the possibility of deep phenotyping and longitudinal followup. I have established a collaboration with investigators (Peder Larson, PhD, Jing Liu, PhD, Karen Ordovas, MD, Miguel Pampaloni, MD, Youngho Seo, PhD) for multi-modality advanced cardiac imaging in HCM.

a) Metabolism Imaging: In collaboration with Drs Larson and Kurhanewicz in the UCSF department of Radiology, we are developing methods for combined hyperpolarized-¹³C-MRI and ³¹P-MRS imaging in mouse models and humans. In combination with metabolite studies (in collaboration with Dr. Oliver Fiehn at UC-Davis), our goal is to investigate metabolic remodeling in HCM for preclinical diagnosis, prognosis, individualization of therapies and assess response to therapy.

b) Left atrial imaging: In collaboration with Drs. Ordovasc and Liu in the UCSF-department of Radiology, we have established methods for non-invasive quantification of left atrial fibrosis and mechanics in HCM patients. The goal of our research is to use MRI and machine learning (collaborative project with Hagit Shatkay, PhD, at the University of Delaware) to predict atrial fibrillation and stroke in HCM. The ultimate goal is to identify patients at risk for developing left atrial fibrosis, using a combination of imaging and blood biomarkers (exosomes) for targeted therapies to prevent progression of fibrosis and atrial arrhythmias.

c) Perfusion imaging by $^{13}\text{NH}_3$ -PET and $^{13}\text{NH}_3$ -PET-MR: In collaboration with Drs. Pamaplioni and Seo in the UCSF department of Radiology, we are developing methods for non-invasive quantification of myocardial blood flow in HCM. The goal of this research is to use a combination of imaging and blood biomarkers (exosomes) to understand mechanisms underlying microvascular dysfunction in HCM and develop targeted therapies.

RESEARCH AWARDS - CURRENT

1.	PI	15 % effort	Abraham (PI)
	Department of Defence (CDMRP)	01/01/2018	06/30/2020
	Investigation of metabolism in hypertrophic cardiomyopathy		\$ 200,000 total
	Development of metabolism-based biomarkers for preclinical detection of hypertrophic cardiomyopathy. We propose compilation of metabolite profiles from heart tissue and targeted quantitation of candidate biomarkers in plasma in two mouse models of HCM and littermate controls		
	I generated the hypothesis and wrote the grant		
2.	Co-PI	0 % effort	Abraham, Larson, Merritt (PI)
	MagLab	01/01/2019	6/30/2020
	Hyperpolarized ^{13}C metabolism studies for preclinical detection of hypertrophic cardiomyopathy	\$ 5,000 direct/yr 1	\$ 5,000 total
	The goal of this proposal is to obtain ex vivo data of metabolic fluxes in mouse models of hypertrophic cardiomyopathy, using resources at the Maglab (https://nationalmaglab.org/)		
	I contributed to hypothesis generation, study design and grant-writing		

RESEARCH AWARDS - PAST

1.	Co-Investigator	2 % effort	Shatkay (PI)
	National Science Foundation, EAGER	01/01/2017	12/31/2017
	Toward learning of probabilistic predictive models for diagnosis and severity scoring of disease, from temporal multi-modal data	\$ 70,000 direct/yr 1	\$ 70,000 total

The proposal aimed to develop and build computational tools, utilizing both traditionally recorded time-series signals (ECG data) and image data (echocardiography data), for improved diagnosis and prediction of hypertrophic cardiomyopathy (HCM)

I contributed to study design and grant writing. My collaborator and I identified two areas where computational expertise would be useful to assist with for diagnosis and longitudinal follow up of HCM patients. Here, we focused on diagnosis of HCM by echocardiography (ECHO) and electrocardiography (ECG), which are widely available and relatively low cost.

1) Echocardiography: Since the location and pattern of hypertrophy can vary in HCM patients, automated identification and measurement of the maximally hypertrophied region of the ventricular septum in each patient is important for diagnosis and follow up of HCM patients. While echocardiography is widely available and low-cost when compared to magnetic resonance imaging (which has high spatial resolution), the images can be noisy and hence require a high level of expertise to read correctly and to obtain reliable measurements which are crucial for the diagnosis of HCM. The PIs worked together to computationally identify and annotate the endpoints of the thickest part of the interventricular septum using images that have been manually annotated by expert cardiologists with extensive experience in the diagnosis of HCM patients. 2) ECG is widely available and easy to perform as a screening test in large numbers of patients such as athletes, which motivated the PIs to use automated ECG analysis for early detection of HCM. My clinical studies reveal that ECGs may be useful to detect pro-arrhythmic electrophysiologic remodeling. Longitudinal automated ECG analysis could be useful to identify markers of response to therapy or disease progression and thus help with prognostication in HCM patients.

2.	Co-PI	5 % effort	Abraham, Larson (PI)
	RAP Team Science grant (UCSF)	01/01/2019	12/31/2019
	Hyperpolarized 13-C metabolic imaging for preclinical detection of hypertrophic cardiomyopathy	\$ 75,000 direct/yr 1	
	The goal of this grant is to develop methods for metabolic imaging in mouse models of hypertrophic cardiomyopathy		
	As Co-PI, I contributed to hypothesis generation, study design and grant-writing		
3.	PI	5 % effort % effort	Abraham (PI)
	American Heart Association, Innovative Research Grant	01/01/2017	12/31/2019
	Microfluidic 3-D printing of myocardial tissue constructs to examine the metabolic basis of hypertrophic cardiomyopathy	\$ 75,000 direct/yr 1	\$ 150,000 total

The overall objective of this proposal is to use a new technique microfluidic 3-D printing to engineer myocardial tissue constructs (MTCs) using cardiac myocytes obtained by differentiation of human induced pluripotent stem cells (hiPSC-CMs). We hypothesize that longitudinal/anisotropic alignment in three dimensions will promote sarcomere organization as well as metabolic and electrophysiologic maturation of hiPSC-CMs to the adult stage of cardiac development. Once established as an effective in vitro model, MTCs will be used in proof-of-principle experiments, to test whether reducing myofilament Ca²⁺ sensitivity prevents activation of hypertrophic signaling in hiPSC-CMs derived from a patient with hypertrophic cardiomyopathy (HCM).

I provided the biological question, contributed to grant writing and study design. My laboratory will perform the human IPSC culture, cardiac differentiation and collaborate with Dr. Benkoski on the design of myocardial tissue constructs (MCTs), with the initial goal of maximizing survival of encapsulated IPSC-CMs. We will use the MCTs to promote cardiac maturation and establish MCTs as in vitro models of human HCM. We have already optimized all the assays needed for the study and have established the importance of anisotropic culture for metabolic and redox maturation in human IPSC-CMs in our preliminary studies.

4.	PI	20 % effort	Abraham MR (PI)
JTB Foundation		11/01/2016	10/30/2017
Investigation of metabolism in HCM in mouse and man		\$ 70,000 direct/yr 1	\$ 70,000 total
We investigated the potential of a drug that reduces myofilament calcium sensitization to prevent derangements in cardiac metabolism, redox and arrhythmias, using human IPSC-CMs and 2 HCM mouse models.			
I wrote the grant application. All studies were performed in my laboratory.			
5. 14GRNT20370009	PI	15 % effort	Abraham MR (PI)
American Heart Association, Grant In Aid		07/01/2014	06/30/2016
Investigation of adult stem cell bioenergetics		\$ 77,000 direct/yr 1	\$ 154,000 total
We investigated the metabolic determinants of survival and proliferation of adult stem cells (cardiosphere-derived cells) and the link between energetics and adhesion. We tested the potential of metabolic modulation to improve viability of stem cells in suspension, improve stem cell engraftment and cardiac regeneration.			
My laboratory developed the methodology and generated the preliminary data for the application. I wrote the grant application; all studies were performed in my laboratory.			
6. RO1 HL092985	Co-PI	20 % effort	Abraham MR and Bengel (PI)
NIH/NHLBI		03/01/2010	12/31/2015
Imaging of the myocardial microenvironment to facilitate cardiac progenitor cell engraftment		\$ 250,000 direct/yr 1	\$ 1,250,000 total

Our goal was to develop molecular imaging strategies that optimize the benefit of cell therapy based on imaging of individual disease biology. The grant was based on the premise that molecular-targeted nuclear imaging prior to cell delivery can characterize an optimal biologic environment which is supportive of cell engraftment after delivery, and thus predictive of successful myocardial regeneration. This hypothesis was tested in 3 specific aims. Quantitative nuclear imaging techniques were employed to characterize both, myocardial environment, as well as stem cell engraftment in a rat model of myocardial infarction. Therapy outcome were defined by serial echocardiography and histologic workup.

I wrote the grant in collaboration with Dr. Frank Bengel. My laboratory developed methods for genetic and radionuclide labeling of stem cells and in vivo imaging, in collaboration with Dr. Benjamin Tsui in the Johns Hopkins Division of Nuclear Medicine. My laboratory performed stem cell culture, synthesized lentiviruses, transduced stem cells, designed metabolic scaffolds and performed the in vivo SPECT and PET studies in rat models. Dr. Tsui designed the imaging protocols and his laboratory personnel performed image reconstruction of SPECT and PET images.

7.	Co-investigator	5% % effort	Lee S (PI)
	TEDCO	7/1/2013	6/30/2016
	Design of Highly Fluorinated Stem Cells for 19F MR Imaging in Cardiac Repair	\$ 100,000 direct/yr 1	\$ 200,000 total
	To develop translatable preclinical and clinical stem cell 19F MRI techniques based on a multidisciplinary research approach in the theranostic field.		
	I contributed to the methods section of the grant that pertained to assessment of functional effects of cell therapy by Echocardiography		
8.	ATIP 5UL1RR025005-05 PI	5% % effort	Abraham MR (PI)
	NIH- 5UL1RR025005-05	2/1/2012	1/31/2013
	Development of a hydrogel for medical applications	\$ 100,000 direct/yr 1	\$ 100,000 total
	The goal of the proposal was to develop an autologous, biodegradable, bioadhesive hydrogel that promoted rapid stem cell adhesion, reduced transplanted cell egress to the lungs, boosted stem cell engraftment and cardiac regeneration.		
	My laboratory designed metabolic, biodegradable and bioadhesive scaffolds to promote stem cell survival/proliferation, in vivo engraftment and cardiac repair in collaboration with an investigator in the Biomedical engineering department at Johns Hopkins. I wrote the grant, optimized all methods; all studies were performed by my laboratory.		
9.	Co-investigator	20% % effort	Abraham, TP (PI)
	NIH/NHLBI	2/1/2010	1/31/2013
	Development of a Hypertrophic Cardiomyopathy Consortium		
	To develop a world-wide consortium for patients with HCM, test novel diagnostic and prognostic tools		

I identified the clinical questions and collaborated on grant writing. I tested the ability of ECG to predict electrophysiologic remodeling in HCM patients, by combining ECG analysis with multi-modality clinical imaging in HCM patients.

10. BGIA 2520000	PI	15% % effort	Abraham MR (PI)
American Heart Association -BGIA		7/1/2009	6/30/2012
Role of integrins in cardiac derived progenitor cell engraftment			
To determine the role of integrin signaling and reactive oxygen species in cardiac progenitor cell engraftment			
My laboratory generated the preliminary data; all studies were performed in my laboratory. I wrote the grant.			
11.	PI	20% % effort	Abraham MR (PI)
TEDCO		4/1/2008	3/31/2010
Molecular engineering of human cardiac-derived stem cells and the myocardial micro-environment to improve cardiac regeneration		\$ 100,000 direct/yr 1	\$ 200,000 total
The goal of this proposal is to investigate the effects of hypoxic signaling on progenitor cell engraftment and optimize cell culture conditions for isolation of cardiac progenitor cells.			
I wrote the grant application. My laboratory generated preliminary data; all studies were performed in my laboratory			
12. RO1HL083109	Co-investigator	20% % effort	Marban E (PI)
NIH/NHLBI		3/1/2006	9/30/2007
Electrophysiology and Cell Biology of Cardiac Stem Cells.		\$ 250,000 direct/yr 1	\$ 1,25,000 total
The primary goal is to investigate electrophysiology, cell biology and proteomics of cardiosphere-derived cells			
I generated the specific aims and wrote the grant in collaboration with Dr. Marban. Additionally, I generated preliminary data for the grant application.			
13. U54HL081028 SCCT	Co-investigator, Project 1	25% % effort	Mabran E and Hare J (PI)
NIH/NHLBI		9/30/2005	8/31/2007
Cellular Cardiomyoplasty for Acute and Chronic Ischemic heart disease		\$ 436,803 direct/yr 1	\$ 1,747,213 total
The primary goals are to develop cellular cardiomyoplasty for the treatment of ischemic heart disease, both post-myocardial infarction left ventricular dysfunction as well as chronic ischemic cardiomyopathy			

I contributed to the Specific Aims and wrote Project 1 in collaboration with Dr. Marban. I developed methods to expand cardiosphere-derived cells which enabled generation of large numbers of cells for studies in large animal models and patients. I generated a large portion of the preliminary data for the grant and developed methods for optimizing stem cell engraftment.

14. 0535205N	PI	15% % effort	Abraham MR (PI)
American Heart Association - Scientist Development Grant 7/1/2005 6/30/2009			
Arrhythmogenesis of Myoblast Transplantation			
The overall aim of this proposal was to investigate the mechanisms of arrhythmogenesis in myoblast transplantation and to develop novel gene-based therapies for myoblast transplantation-related ventricular arrhythmias.			
I generated the hypothesis, preliminary data and wrote the grant. All studies were performed in my laboratory			
15.	Co-investigator, Project 1	20% % effort	Marban E (PI)
Donald W. Reynolds Foundation		7/1/2007	6/30/2011
Donald W. Reynolds Cardiovascular Clinical Research Center on Sudden Cardiac Death.			\$ 10,528,764 total
The primary goal is to develop new means to identify individuals at risk for SCD and to develop novel therapies to prevent SCD in those individuals. Project 1 is developing novel, biologically-based therapies to prevent arrhythmias and SCD in post-MI patients			
I contributed to writing project 1 of the grant in collaboration with Dr. Marban. I generated a large portion of the preliminary data, pertaining to the use of multi-modality imaging to track transplanted stem cells and promote cardiac regeneration, for the renewal application.			
16.	Associate Investigator	25% % effort	Marban E (PI)
Donald W. Reynolds Foundation		7/12003	6/30/2007
Donald W. Reynolds Cardiovascular Clinical Research Center on Sudden Cardiac Death.		\$ 5,226,626 direct/yr 1	\$ 20,906,607 total
The Johns Hopkins Reynolds Center is a broad, multidisciplinary approach to the problem of sudden cardiac death (SCD) associated with atherosclerosis. The primary goal is to develop new means to identify individuals at risk for SCD and to develop novel therapies to prevent SCD in those individuals. Project 1 is developing novel, biologically-based therapies to prevent arrhythmias and SCD in post-MI patients.			
Development of gene therapy to prevent arrhythmias and sudden cardiac death and multi-modality stem cell imaging.			

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4. Wu JC, **Abraham MR**, Kraitchman D. Perspectives on imaging cardiac stem cell therapy. *J of Nuclear Medicine*. 2010 May;51 Suppl 1:128S-136S.
5. Chan A, **Abraham MR**. SPECT and PET to optimize cardiac stem cell therapy. *J Nucl Cardiol*. 2012 Feb;19(1):118-125.
6. Vakrou S, **Abraham MR**. Hypertrophic cardiomyopathy: a heart in need of an energy bar? *Front Physiol*. 2014 Aug 19;5:309

BOOKS AND CHAPTERS

1. None

OTHER PUBLICATIONS

1. **Abraham MR**, Hare JM. Is skeletal myoblast transplantation proarrhythmic? The jury is still out. *Heart Rhythm*. 2006;3(4):462-463.
2. **Abraham MR**, Gerstenblith G. Preconditioning stem cells for cardiovascular disease: An important step forward. *Circulation Research*. 2007;100(4):447-449.
3. Miller PE, Czarny MJ, **Abraham MR**. A rare cause of chest pain and ventricular fibrillation. *JAMA Internal Med*. 2014;174(7):1173-1175.
4. Cruz D, Ahmed H, Gandapur Y, **Abraham MR**. *Propionibacterium acnes*: A treatable cause of constrictive pericarditis. *Case Rep Med*. 2015;2015:193272.

SIGNIFICANT PUBLICATIONS

1. Lu DY, Yalçin H, Sivalokanathan S, Greenland GV, Vasquez N, Yalçin F, Zhao M, Valenta I, Ganz P, Pampaloni MH, Zimmerman S, Schindler TH, Abraham TP, **Abraham MR**. Higher incidence of vasodilator-induced left ventricular cavity dilation by PET when compared to treadmill exercise-ECHO in hypertrophic cardiomyopathy. J Nucl Cardiol. 2018 Nov 19. doi: 10.1007/s12350-018-01521-x. PMID: 30456498

I designed this study as part of my goal to investigate the microvascular dysfunction leading to angina in patients with hypertrophic cardiomyopathy (HCM). We used clinical data generated at Johns Hopkins for the analyses. I have established collaborations with investigators at UCSF (Drs. Miguel Pampaloni and Youngho Seo in the department of Radiology), Dr. Peter Ganz (Cardiology, ZSFGH) and Dr. Robert Raffai (UCSF-VAMC) for investigate mechanisms underlying microvascular dysfunction in HCM.

2. Sivalokanathan S, Zghaib T, Greenland GV, Vasquez N, Kudchadkar SM, Kontari D, Lu DY, Dolores-Cerna K, Van der Geest RJ, Kamel IR, Olgin JE, Abraham TP, Nazarian S, Zimmerman SL, **Abraham MR**. Hypertrophic cardiomyopathy patients with paroxysmal atrial fibrillation have a high burden of left atrial fibrosis by cardiac magnetic resonance imaging. JACC Clin Electrophysiol. 2019 Mar;5(3):364-375. doi: 10.1016/j.jacep.2018.10.016. Epub 2018 Dec 26. PMID: 30898240

This pilot study was designed to non-invasively characterize the electrophysiologic substrate in HCM patients. We used clinical data generated at Johns Hopkins for the analysis. I have now established similar methods at UCSF, in collaboration with investigators in the department of Radiology (Drs. Karen Ordovasc, Jing Liu) and Cardiac Electrophysiology (Dr. Ed Gerstenfeld). The goal of this research is to use multi-modality imaging to predict and prevent atrial fibrillation in HCM patients.

3. Vakrou S, Fukunaga R, Foster DB, Sorensen L, Liu Y, Guan Y, Woldemichael K, Pineda-Reyes R, Liu T, Tardiff JC, Leinwand LA, Tocchetti CG, Abraham TP, O'Rourke B, Aon MA, **Abraham MR**. Allele-specific differences in transcriptome, miRNome, and mitochondrial function in two hypertrophic cardiomyopathy mouse models. JCI Insight. 2018 Mar 22;3(6). PMID: 29563334

I designed this multi-scale study to test the hypothesis that mutation-specific differences in pathophysiology underlie the cardiac phenotype in HCM. At UCSF, I have established methods for multi-scale investigation of metabolism, redox and calcium handling in human myocytes (obtained by cardiac differentiation of patient-specific induced pluripotent stem cells) and mouse myocytes (obtained from transgenic mouse models), and collaborations with investigators in the department of Radiology (Dr. Peder Larson, John Kurhanewicz) for in vivo imaging of metabolism (in mice and humans).

4. Corona-Villalobos CP, Saha S, Pozios I, Hurtado-de-Mendoza Paz D, Sorensen L, Gonzalez Cordoba J, Dolores-Cerna K, Kamel IR, Mormontoy Laurel W, Bluemke DA, Abraham TP, Zimmerman SL, **Abraham MR**. Exercise-QTc is associated with diffuse interstitial fibrosis reflected by lower approximated T1 relaxation time in hypertrophic cardiomyopathy patients. J Electrocardiol. 2017 Jul - Aug;50(4):484-490. doi: 10.1016/j.jelectrocard.2017.02.002. Epub 2017 Feb 12. PMID: 28292523

I designed this study as part of my effort to non-invasively characterize the electrophysiologic substrate for prediction of ventricular arrhythmias in HCM patients. We plan to incorporate clinical data, imaging data and digital EKG analysis in a machine learning model to identify HCM patients who are low and high risk for ventricular arrhythmias.

5. Bhattacharya M, Lu DY, Kudchadkar SM, Greenland GV, Lingamaneni P, Corona-Villalobos CP, Guan Y, Marine JE, Olgin JE, Zimmerman S, Abraham TP, Shatkay H, **Abraham MR**. Identifying Ventricular Arrhythmias and Their Predictors by Applying Machine Learning Methods to Electronic Health Records in Patients With Hypertrophic Cardiomyopathy (HCM-VAr-Risk Model). Am J Cardiol. 2019 May 15;123(10):1681-1689. doi: 10.1016/j.amjcard.2019.02.022. Epub 2019 Feb 27. PMID: 30952382

I collaborated with Dr. Hagit Shatkay, a computer scientist at the University of Delaware to develop machine learning models to predict fatal ventricular arrhythmias, atrial fibrillation and heart failure in patients with hypertrophic cardiomyopathy. We plan to test this model prospectively in multiple centers.

PATENTS ISSUED OR PENDING

1. Marban E, **Abraham MR**. Preventing Arrhythmias Associated with Cell Transplantation
2. Marban E, **Abraham MR**, Smith RR. Cardiac Stem Cells

CONFERENCE ABSTRACTS

1. **Abraham MR**, Henrikson C, Tung L, Marbán E. Mechanisms and potential pharmacologic and gene therapy strategies for myoblast transplant-associated ventricular arrhythmias: insights from a unique in vitro model. **Melvin Marcus Young Investigator Award Finalist; American Heart Association**, Nov 2005.
2. Terrovitis J, Kwok KF, Lautamäki R, Engles JM, Barth AS, Miake J, Leppo MK, Fox J, Seidel J, Wahl RL, Tsui B, Bengel F, Marbán E, **Abraham MR**. Ectopic expression of the sodium-iodide symporter enables imaging of transplanted cardiac stem cells in vivo, by SPECT or PET. **Melvin Judkins Young Investigator Award**; American Heart Association, Nov 2007.
3. Sasano T, **Abraham MR**, Chang KC, Ashikaga H, Mills KJ, Holt DP, Hilton J, Nekolla SG, Dong J, Lardo AC, Halperin H, Dannals RF, Marban E, Bengel FM. Sympathetic Dysinnervation of Viable Myocardium and the Substrate of Ventricular Tachycardia after Myocardial Infarction. **Young Investigator Award; International Society for Nuclear Cardiology**, March 2007.

4. Sasano T, **Abraham MR**, Chang KC, Dannals RF, Holt DP, Hilton J, Lardo AC, Osman NF, Marban E, Bengel FM. Myocardial sympathetic innervation and regional strain in a porcine model of chronic myocardial infarction. **Young Investigator Award Finalist; Society of Nuclear Medicine**, June 2007.
5. Bonios M, Terrovitis J, O'Rourke B, Fox J, Yu J, Engles JM, Higuchi T, Lautamäki R, Pomper M, Wahl RL, Tsui BM, Bengel FM, Marban E, **Abraham MR**. Myocardial substrate determines acute cardiac retention and lung biodistribution of intramyocardially injected cardiac-derived stem cells- A PET/CT experimental study. **Melvin Judkins Young Investigator Award Finalist**, AHA, Nov 2008.
6. Lautamäki R, Terrovitis J, Bonios M, Fox J, Engles JM, Yu J, Tsui BM, Marbán E, **Abraham MR**, Bengel FM. Perfusion defect size predicts engraftment but not early retention of intramyocardially injected cardiac-derived stem cells after acute myocardial infarction. **Young Investigator Award Finalist**, Society of Nuclear Medicine, June 2009.
7. Bonios M, Pinheiro A, Damiano V, Terrovitis J, Chang CY, Abraham TP, **Abraham MR**. Cellular cardiac resynchronization by cardiac-derived stem cell transplantation in an experimental model of myocardial infarction. **Finalist, Best Post Abstract, American College of Cardiology**, March 2010.
8. Chan A, Chang C, Lin X, Afzal J, Rittenbach A, Higuchi T, Yu J, Sun D, Wahl R, Pomper M, O'Rourke B, Tsui B, **Abraham MR**. SPECT-CT and bioluminescence imaging reveal the important contribution of cellular bioenergetics on the molecular imaging signal from stem cells expressing luciferase and the sodium-iodide symporter. **Melvin Judkins, Young Investigator Award Finalist**, AHA, 2012
9. Afzal JM, Chan A, Karakas MF, Woldemichael K, Vakrou S, Pomper MG, Tsui BM, **Abraham MR**. 1. Dual isotope SPECT-CT imaging using sodium iodide symporter (NIS) reveals improved energetics following cardiac transplantation of stem cells encapsulated in hydrogels. **Young Investigator Award**, Society of Nuclear Medicine meeting, June 2015.
10. Afzal JM, Chan A, Karakas MF, Woldemichael K, Vakrou S, O'Rourke B, **Abraham MR**. Rapid restoration of transplanted stem cell bioenergetics by metabolic scaffolds that promote cell adhesion and provide substrates. **Young Investigator Award**, Heart Failure Society of America, September 2015
11. Vakrou S, Fukunaga R, Guan Y, Aon M, Sorensen L, Woldemichael K, Tocchetti G, Pineda R, Foster DB, Abraham TP, O'Rourke B, **Abraham MR**. Allele-specific differences in gene expression and mitochondrial function are present at the pre-hypertrophic stage of hypertrophic cardiomyopathy: results from 2 mouse models. **Travel Award**, European Society of Cardiology, August 2016.
12. Vakrou S, Fukunaga R, Foster DB, Sorensen L, Guan Y, Aon M, O'Rourke B, **Abraham MR**. Differences in miRNome, transcriptome and mitochondrial function in 2 mouse models of hypertrophic cardiomyopathy at the prehypertrophic stage suggest need for precision medicine approach to treatment. **Finalist, New Investigator Award**, Heart Failure Society of America, September 2017.
13. Bhattacharya M, Lu DY, Lingamaneni P, Kudchadkar S, Villareal G, Sivalokanathan S, Villalobos PC, Zimmerman S, Abraham TP, **Abraham MR**, Shatkay H. Identifying ventricular arrhythmia cases and their predictors by applying machine learning methods to

electronic health records of hypertrophic cardiomyopathy patients. Circulation Vol 136, No. Suppl_1Abstract 15967, November 2017.

14. Prakosa A, Cartoski M, Boyle P, O Hara R, Pashakhanloo F, Ferrantini C, Cerbai E, Coppini R, Zimmerman S, **Abraham MR**, Maurizi N, Olivotto I, Trayanova N. Ventricular Arrhythmia (VA) in Hypertrophic Cardiomyopathy (HCM): Novel Computational Risk Stratification Using T1 Mapping, Late Gadolinium Enhancement MRI (LGE-MRI), and an HCM Specific Cell Model. Circulation Vol 136, No. Suppl_1Abstract 16889, November 2017.
15. Lu DY, Yalcin H, Sivalokanathan S, Greenland GV, Vasquez N, Schindler TH, Abraham TP, **Abraham MR**. Discordance in apparent left ventricular cavity dilation by vasodilator-PET and exercise-ECHO in hypertrophic cardiomyopathy: Potential mechanisms. **Alan S. Pearlman Research Award Winner**, American Society of Echocardiography, June 2018.
16. Lu DY, Ventoulis I, Liu H, Kudchadkar S, Greenland G, Vakrou S, Abraham TP, **Abraham MR**. Gender-specific clinical outcomes in patients with hypertrophic cardiomyopathy. Accepted for presentation at the Scientific Sessions of the American Heart Association, November 2018
17. Vasquez N, Ostrander BT, Lu DY, Ventoulis I, Haileselassie B, Goyal S, Vakrou S, Abraham TP, **Abraham MR**. Left atrial strain predicts adverse outcomes in hypertrophic cardiomyopathy. Accepted for presentation at the Scientific Sessions of the American Heart Association, November 2018.

ACADEMIC LEADERSHIP

Program building

Since moving to UCSF, I have established Clinical and Research programs in Hypertrophic Cardiomyopathy. I am the Director of the Hypertrophic Cardiomyopathy Program which was recently (Dec 2018) accredited as a Center of Excellence (HCM-COE). The goal of the HCM-COE is to provide comprehensive care and tailored therapies to HCM patients and their families, with the goal of preventing complications such as heart failure/sudden death and improving quality of life.

The Interdisciplinary HCM Research Program is geared towards developing a Precision Medicine approach to HCM diagnosis and treatment. Our goal is to understand disease pathogenesis using a combination of basic studies in cell/animal models (co-clinical trial approach), multi-modality imaging, blood biomarkers and Machine learning, to enable tailored therapies that prevent disease progression and aid in pre-clinical diagnosis in families with known and unknown causal HCM mutations.