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14. ABSTRACT

Human hearts are unable to self-repair due to their very limited endogenous regenerative capacity. Thus, mortality rates of heart failure are extremely high. Indeed, pediatric heart failure (PHF) is the leading non-trauma related cause of death for infant, child, or adolescent in the United States. Many children with PHF are treated by inserting a pump known as a Left Ventricular Assist Device (LVAD) into the heart to help circulate blood. However, most patients don't response to LVAD and require heart transplantation. Unfortunately, heart transplantation is severely limited by the scarcity of donor hearts. Obviously, it has great clinical value if we could find a way to predict if the PHF patient response to LVAD treatment, to help with clinical care and physicians decisions on whether heart transplantation will be needed. Our study will develop methods based on gene expression signatures for predicting PHF patients are LVAD treatment responders or non-responders. Moreover, identification of biomarkers in blood samples will provide a novel non-invasive method to understand if the heart is improving on LVAD treatment. Importantly, if we could find a way to facilitate endogenous cardiac regeneration, then survival rates of PHF would greatly improve. Our study will use cutting edge techniques to gain important insight of molecular mechanisms that stimulate endogenous cardiac regeneration, and develop potential novel therapeutic approaches.

15. SUBJECT TERMS

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Introduction

Congenital Heart Diseases and Pediatric Heart Failure

Congenital heart diseases (CHDs) is the most common birth defect that affects 10 out of every 1000 newborns (Hoffman et al., 2004; Tennant et al., 2010), and is a tremendous health burden. Severe CHDs often lead to pediatric heart failure (PHF), in which the heart has poor pumping function leading to organ failure and death at an early age. Thus, CHDs caused PHF is a leading cause of death and a devastating condition in an infant, child, or adolescent. It is estimated that 12,000 to 35,000 children under 19 years old in the United States are affected by PHF each year (Hsu and Pearson, 2009). In addition, modern medical advances have increased survival rates for pediatric CHD patients resulting in more patients surviving into adulthood. A significant number of adult CHD patients have a dramatically increased heart failure risk (Gurvitz et al., 2016; Ntiloudi et al., 2016). PHF is also highly costly to treat. There were nearly 14,000 PHF hospitalizations in 2006 with >$135,000 per admission in the United States, with aggregate charges exceeding $1.8 billion (Burns et al., 2014). Treatments of PHF such as Left Ventricular Assist Device (LVAD) implantation and heart transplantation have obvious value, but new approaches are still urgently required.

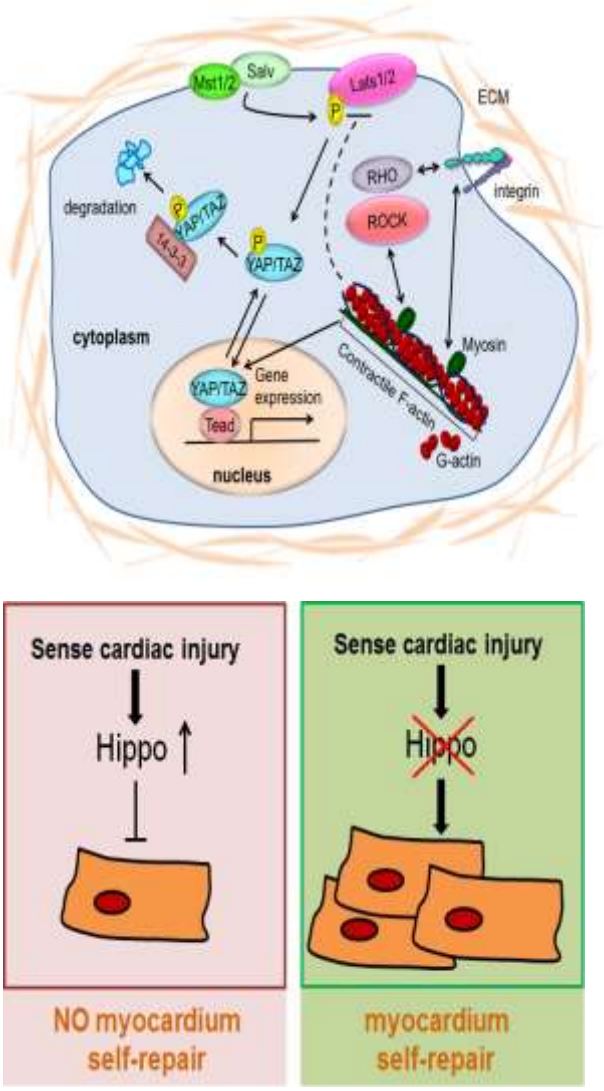

Figure.Int Overview of Hippo Signaling Pathway and its function in myocardial repair. In Hippo pathway, theYap/Taz effectors are regulated by both the Hippo kinases and also the actin cytoskeleton. In normal hearts, Hippo signaling senses cardiac injury and represses cardiomyocyte repair. Inhibiting Hippo signaling promotes cardiomyocyte repair.

Hippo Signaling and Cardiomyocyte Regeneration

Hippo signaling, an ancient organ size control pathway, is a kinase cascade that inhibits developing cardiomyocyte proliferation (Heallen et al., 2011). The mammalian core Hippo signaling components include the Ste20 kinases *Mst1* and *Mst2* that are orthologous to the Drosophila Hippo kinase. Mst kinases, complexed with the Salvador (Salv) scaffold protein, phosphorylate the Large Tumor Suppressor Homolog (Lats) kinases. Lats kinases, in turn, phosphorylate Yap and Taz, two related transcriptional co-activators that are the most downstream Hippo signaling components and partner with transcription factors, such as Tead, to regulate gene expression.

Upon phosphorylation, Yap and Taz are excluded from the nucleus and rendered transcriptionally inactive (**Figure. Int**). Mechanical signaling also regulates Yap subcellular localization (**Figure. Int**).

We previously were the first to report that Hippo signaling inhibits developing cardiomyocyte proliferation by inhibiting Wnt signaling. The human heart has minimal ability to repair itself after injury and the endogenous heart can replace only about 1% of heart muscle cells per year. In mice, resection of the cardiac apex in the first six days of life results in cardiac regeneration while resections performed at postnatal day (P) 7 and later results in fibrosis and scarring(Porrello et al., 2011). We performed apex resection of uniform size at the normally non-regenerative P8 in control and Hippo-deficient hearts. Evaluation of 21 day post resection hearts by serial sectioning revealed severe scarring of control hearts (Heallen et al., 2013). In contrast, resected Hippo-deficient hearts efficiently regenerated the myocardium with reduced scar size. We evaluated regeneration in the adult heart using the left anterior descending coronary artery obstruction (LADO) assay in which the left anterior coronary artery is tied off with a suture. The Hippo deficient hearts were able to regenerate the heart efficiently based on histology and functional analysis by echocardiography(Heallen et al., 2013). Our recent study indicated that Hippo-deficient adult mouse cardiomyocytes re-enter the cell cycle and undergo cytokinesis. Hippo deficiency enhanced cardiomyocyte regeneration and Hippo mutant cardiomyocytes had elevated proliferation (Heallen et al., 2013; Morikawa et al., 2015; Tao et al., 2016).

Our Recent progress of Studying Hippo Signaling in Heart Regeneration

During the first year of this DOD award, we have published a number of important discoveries revealing the roles of Hippo signaling in cardiac regeneration, including three *Nature* papers and one *Dev Cell* paper. We recently discovered a direct connection between the dystrophin glycoprotein complex (DGC) and Hippo-Yap signaling. Our recent study published in *Nature* indicated that Yap interacts directly with the DGC component Dystroglycan 1 (DAG1) to inhibit cardiomyocyte proliferation (Morikawa et al., 2017). The Yap-DAG1 interaction required Hippo pathway phosphorylation of Yap indicating a tight physiologic connection between the Hippo pathway and the DGC in cardiomyocyte homeostasis. We showed that removing Hippo pathway component *Salvador* (*Salv*) in the X-chromosome-linked muscular dystrophy *(mdx)* mutant mouse background suppressed the induction of stress-induced heart failure (Morikawa et al., 2017). Our collaborative work published in the same issue of *Nature* showed that the extracellular matrix (ECM) component, Agrin, binds to the DGC causing Yap to be released into the cardiomyocyte nucleus to enhance proliferation (Bassat et al., 2017). Together, our findings indicate that the DGC senses changes in the ECM to control Yap subcellular localization. Importantly, we have obtained remarkable findings that Hippo deletion protected against heart failure in hearts with established heart failure (Leach et al., 2017). Recently we have used single-cell RNA sequencing (scRNA-seq) to uncover a critical role of Hippo signalling in epicardium that contains essential noncardiomyocyte progenitors giving rise to epicardial-derived cells (EPDCs) (Xiao et al., 2018). EPDCs contribute to the primary supporting cells of the heart like vascular smooth muscle cells and fibroblasts, which are important for myocardial and coronary vascular development (Katz et al., 2012; Wessels and Perez-Pomares, 2004). Our scRNA-seq data showed that epicardial cells with deletion of *Lats1* and *Lats2* had fibroblast differentiation arrest (Xiao et al., 2018). Importantly, encouraged by the success of our recent studies using the cutting-edge technologies such as scRNA-seq, we have modified our research strategies of this DOD award by using more cutting-edge technologies.

Keywords

Hippo signaling, cardiac regeneration, single-cell RNA sequencing (scRNA-seq), split-pool ligation-based transcriptome sequencing (SPLiT-seq), Cleavage Under Targets and Release Using Nuclease (CUT&RUN),

Accomplishments

With the long term goal of developing new therapies for PHF patients, we have proposed to investigate the genetic mechanisms of PHF caused by CHDs using the most sophisticated genetic approaches available. Our study will significantly contribute to the clinical management of PHF patients with LVAD support. Notably, our studies will uncover the molecular mechanisms of endogenous cardiac regenerative potential and lead to essential new insights that will be required to develop new PHF therapies.

Successful Collection of Fresh Samples from Patients with Various Pediatric Heart Disease Diagnoses.

Over the past year we have been collecting fresh patient samples, both peripheral blood and cardiac tissue, from the large pediatric heart disease population at Texas Children's Hospital (TCH). We are able to collect these samples directly from the operating room as the tissue is harvested to ensure the highest possible quality for our downstream transcriptional output analyses. Currently we have collected paired samples, heart tissue and peripheral blood, from a total of 73 patients. We also have expanded the scope of the diagnoses we are able to biobank, previously focusing solely on patients undergoing left ventricular assist device (LVAD) placement and heart transplants, to now collecting from a

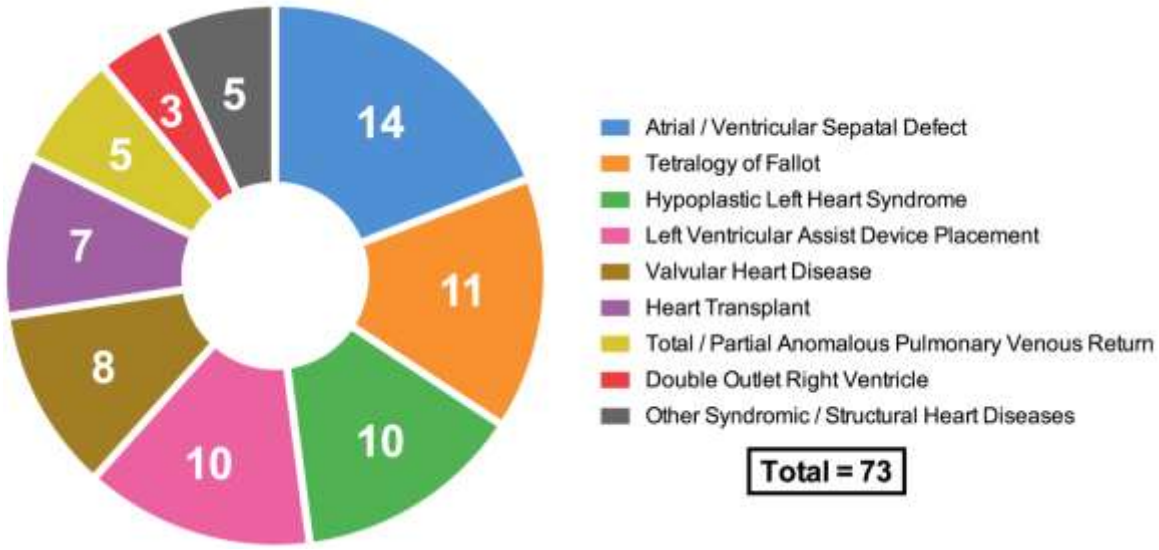

Figure 1. Distribution of pediatric heart disease diagnoses in the study. 73 paired cardiac and peripheral blood samples have been freshly collected directly from the operating room. These samples represent a wide variety of pediatric heart diseases, classified here into larger diagnostic categories. The number of samples in each diagnostic category is labeled directly on the chart in white.

large variety of pediatric heart diseases (**Figure 1**). Our cohort now includes large numbers of patients with congenital heart diseases such as hypoplastic left heart syndrome (HLHS), tetralogy of Fallot (TOF), and various septal defects.

Collecting from this large variety of cases has also given us the opportunity to collect cardiac tissue from various anatomic locations within the heart. Currently our collection includes samples from all four chambers of the heart, multiple heart valves, and other local vessels (**Figure 2**). Each of these locations have been demonstrated to have unique transcriptional signatures, lead us to believe that they also likely have unique transcriptional responses after injury.

Single cell RNA-seq profiling of immune Cells in pediatric heart failure and congenital heart disease.

We performed single cell RNA-seq (scRNA-seq) on peripheral blood mononuclear cells (PBMCs) isolated, via density gradient centrifugation of whole blood, from pediatric patients suffering from congenital heart disease and heart failure (**Figure 3**). Patients with HLHS, partial atrioventricular canal defect (PAVC), total anomalous pulmonary venous return (TAPVR), and a patient suffering heart failure necessitating the implantation of an LVAD. Blood was collected prior to corrective surgery. For control PBMCs, we collected blood from healthy adult donors. All scRNA-seq experiments were carried out using a high-throughput droplet based microfluidic capture technology, namely the 10X Genomics chromium instrument. After Illumina Paired-end sequencing, batch effects were corrected, principle components analysis (PCA) was performed, and significant PCs were used as input for graph-based clustering. 2-dimensional visualization of the multi-dimensional data set was done with t-SNE. After all computational processing was completed, we observed a total of 27,428 single cell transcriptomes (**Figure 3A**). Importantly, we identified all known major

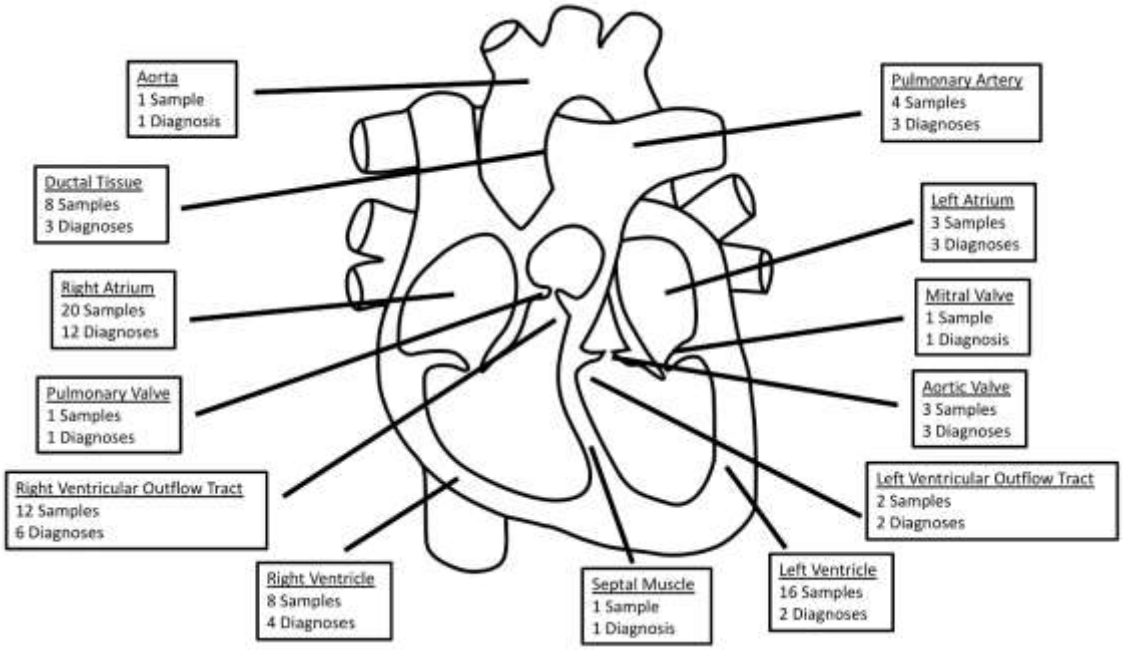

Figure 2. Anatomic representation of cardiac tissue collected. Each surgical procedure offers different opportunities to recovery cardiac tissue that we can use in our research program. Many anatomic locations in the heart are represented in our biobank, with a majority of locations also having specimens from a variety of different disease contexts.

immune cell types, including T-cells, B-cells, monocytes, plasmacytoid dendritic cells (pDCs), natural killer cells (NK), and megakaryocytes (**Figure 3B,** and **1C**). Strikingly, we noticed that many immune cell clusters were distinguishable from our control PBMCs. In particular, we found that the monocyte and NK cell clusters displayed a profound phenotypic expansion in patients with CHD and heart failure. In the future, we aim to isolate both monocytes as well as NK cells to further characterize and validate the observed changes in their respective transcriptional states.

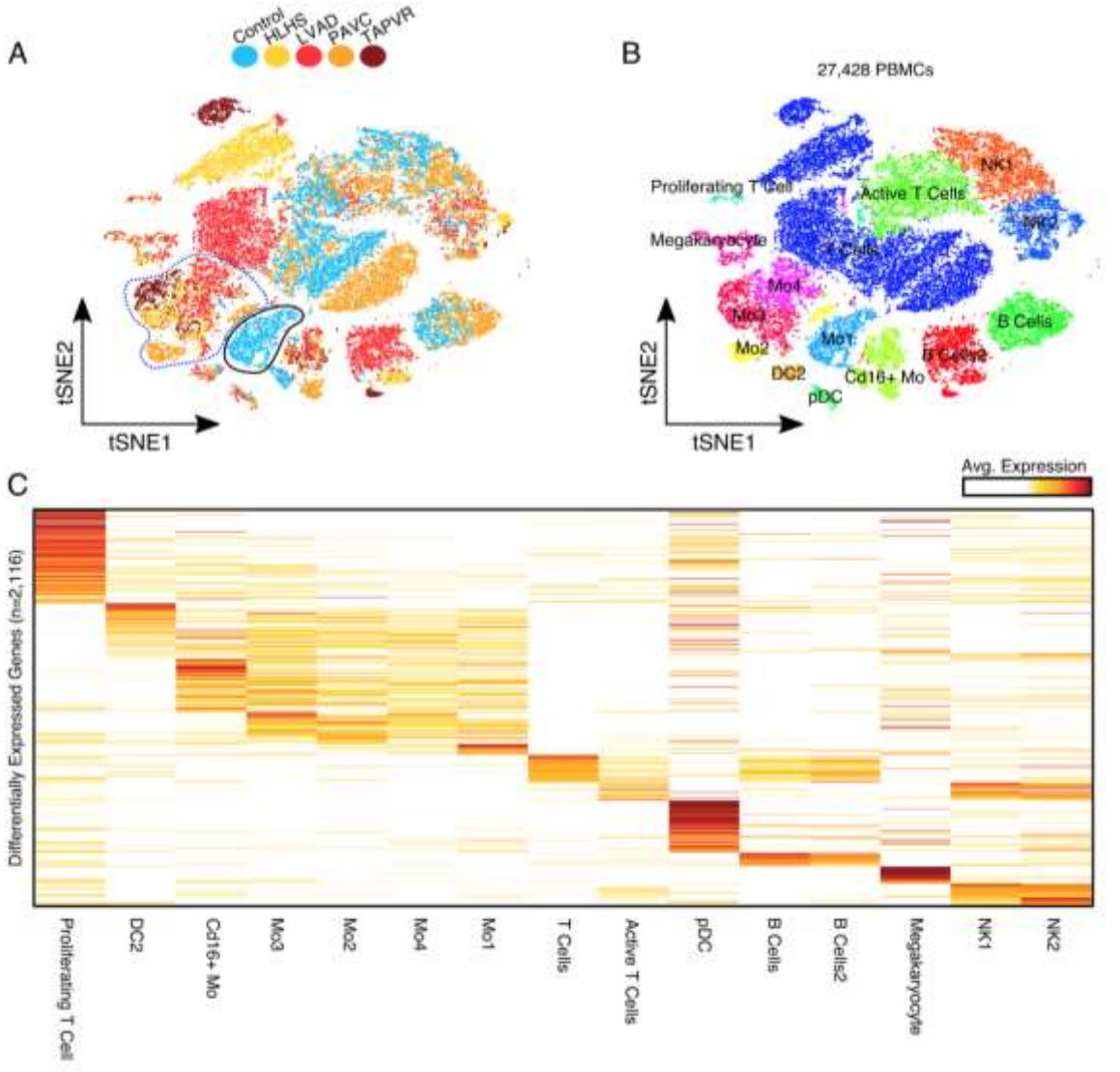

Figure 3. Single cell RNA-seq profiling of immune Cells in pediatric heart failure and congenital heart disease. A) tSNE plot of scRNA-seq data collected from CHD and pediatric heart failure blood samples. Control, healthy non-heart failure adult; HLHS, hypoplastic left heart syndrome; LVAD, pediatric heart failure pre-LVAD placement; PAVC, partial atrioventricular canal defect; TAPVR, total anomalous pulmonary venous return. B) tSNE plot showing the graph-based clustering results. Mo, monocyte; DC, dendritic cell; NK, natural killer cells; pDC, plasmacytoid dendritic cells. C) Heatmap showing the average expression for each cluster of the top differentially expressed genes (n=2,116).

Split-pool ligation-based transcriptome sequencing using left ventricular tissue from pediatric heart failure patient

scRNA-seq is difficult to perform on heart tissue which is, in part, due to the large size and fragility of cardiomyocytes. To overcome the complications associated with fluorescently activated cell sorting (FACS), and microfluidic platforms, we decided to employ split-pool ligation-based transcriptome sequencing (SPLiT-seq), a method that labels the cellular origin of RNA through combinatorial barcoding in a 96 well format (Rosenberg et al., 2018). We performed SPLiT-seq on dissociated left ventricular tissue derived from a pediatric patient in heart failure, tissue was collected prior to LVAD placement (**Figure 4**). After sequencing and computational analysis we were able to capture 2,045 single cell transcriptomes. Importantly, we were able to identify all major cardiac cell types, including cardiomyocytes (CMs), endothelial cells (ECs), cardiac fibroblasts (CFs), macrophages (MΦ), and smooth muscle cells (SMCs) (**Figure 4A**, and **4B**).

Overall, we were able to uncover considerable heterogeneity among the carious cardiac tissue constituents, including 2 cluster of MΦs, 2 clusters of cardiomyocytes, and 2 clusters of CFs. In the future, the deployment of this technology on cardiac tissue on a large scale will allow us to fully characterize the inflammatory status and composition of pediatric heart failure and CHD.

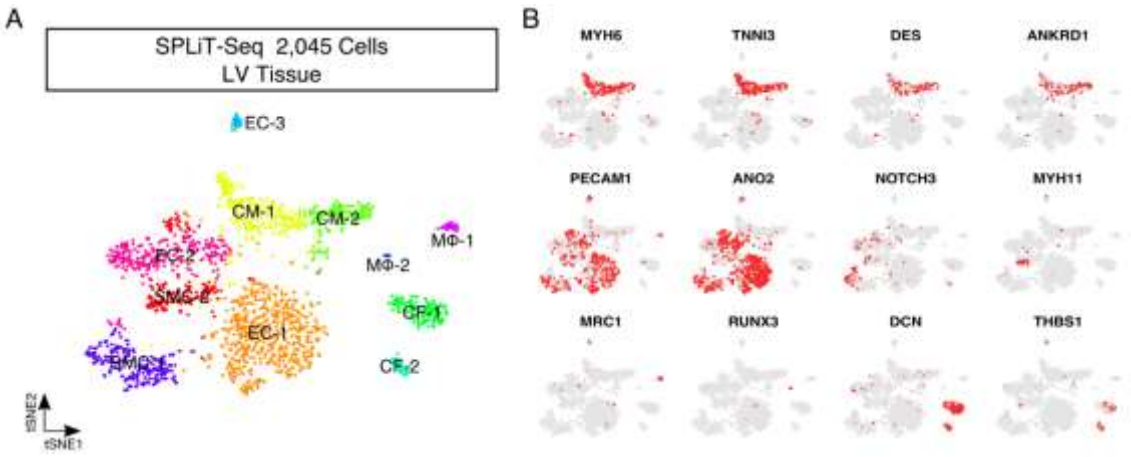

Figure 4. Profiling heart tissue at single-cell resolution with SPLiT-seq. A) tSNE plot of SPLiT-seq showing the graph-based clustering results and cluster identities. Experiment was carried out on sample derived from a pediatric heart failure sample, pre-LVAD placement. CM, cardiomyocyte; EC, endothelial cell; CF, cardiac fibroblast; MΦ, macrophage; SMC, smooth muscle cell. B) Feature plots showing gene expression (red) for indicated genes embedded across tSNE shown in panel A.

Determine the Epigenetic Landscapes of Cardiac Cells using Cleavage Under Targets and Release Using Nuclease (CUT&RUN)

To determine the epigenetic landscapes underpinning the transcription observed in cardiac cells, we wanted to use un-biased and targeted methods for epigenome profiling. Firstly, we performed Cleavage Under Targets and Release Using Nuclease (CUT&RUN), a protein-DNA interaction mapping methodology that relies on the antibody-based recruitment of micrococcal nuclease (Skene and Henikoff, 2017). We elected to conduct this

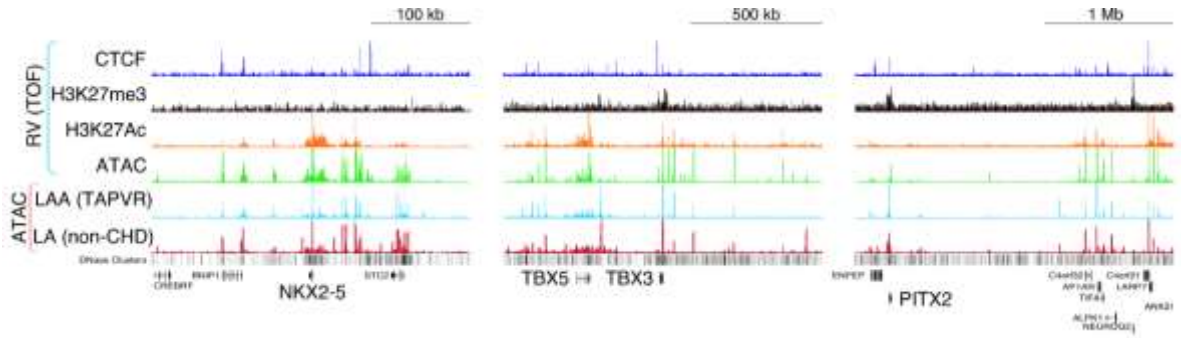

Figure 5. Epigenomic characterization of congenital heart disease. A) Representative genome browser tracks for CUT&RUN (CTCF, H3K27me3, and H3K27Ac), and ATAC-seq. Top 4 tracks are all derived from the RV tissue of the same patient.

experiment in a cardiomyocyte-specific manner by isolating cardiomyocyte nuclei via PCM1 enrichment (Gilsbach et al., 2014; Preissl et al., 2015). Specifically, we profiled CTCF CUT&RUN and then interrogated enhancers with H3K27ac CUT&RUN, and heterochromatin with H3K27me3 CUT&RUN. Further, we unbiasedly assessed chromatin accessibility using an optimized version of the Assay for Transposase-Accessible Chromatin using sequencing (ATAC) (Corces et al., 2017). We used tissue obtained from Tetralogy of Fallot (TOF), TAPVR, and healthy adult left atrial tissue (LA) samples. Active cardiomyocyte identity gene loci like *NKX2.5* were highly accessible and possessed active histone mark signatures commensurate with a dearth of heterochromatin (**Figure 5**, left). Moreover, we could demonstrate cardiac region specificity from this data. LA and left atrial appendage (LAA) CMs both possessed a highly accessible ATAC-seq peak in a *TBX5* enhancer region, whereas this accessible region was lost in RV tissue marked with H3K27me3 and devoid of H3K27Ac marks (**Figure 5,** middle). Moreover, we noticed the same pattern when looking at the known LA CM marker gene *PITX2* (**Figure 5,** right). Thus, we were able to perform high resolution epigenomic profiling on pediatric cardiac tissue. Looking forward, we hope to combine this data with the peripheral blood scRNA-seq findings to develop a more comprehensive understanding of the gene regulatory networks active in the diseased and failing pediatric myocardium and the inflammatory response in PBMCs. We aim to extend this analysis to subsets of PBMCs, like monocytes.

Inactivate Dchs1 in mouse hearts and determine the influence on Hippo signaling activity and cardiomyocyte proliferation.

Our previous and unpublished studies have suggested that Hippo signaling has a great potential for therapeutic manipulation in heart failure. In our recent published *Nature* paper, we have shown that genetically removing Hippo signaling has benefits in failing mouse hearts (Leach et al., 2017). Our preliminary data indicate that *Dchs1*, a known upstream Hippo pathway activator, expression is reduced at the transcriptional level in the post-LVAD samples of pediatric heart failure patients, suggesting that Hippo signaling is reduced in pediatric failing hearts that improve after LVAD unloading. In kidney, *Dchs1* is important in stroma and cap mesenchymal cross talk(Mao et al., 2015). In the heart, it is unknown how *Dchs1* functions other than having a role in mitral valve prolapse (Durst et al., 2015). To gain insight into the role of *Dchs1* in cardiomyocyte regeneration and heart function, we are using mouse models to determine the influence of Dchs1 on Hippo signaling activity and heart function. In our DOD award application, we have shown the preliminary data that Yap

was preferentially nuclear in *Dchs1* germline null mutant hearts compared to control hearts. Importantly, *Dchs1* mutants act similarly to Hippo pathway mutants.

Because that the *Dchs1* germline mutants die at birth, we have obtained the $Dchs1^{flox}$ mice generated by Dr. Ken Irvine from Jackson lab (Stock No: 018851), and crossed it to $Myh6^{creert}$ cre transgenic to inactivate *Dchs1* in cardiomyocytes to dissect *Dchs1* function in cardiomyocytes. Currently, the $Dchs1^{flox}$ mice are breeding to $Myh6^{creert}$ to inactivate *Dchs1* in cardiomyocytes.

Impact

CHDs caused PHF that is highly lethal and also highly costly to treat. There were nearly 14,000 PHF hospitalizations in 2006 with >$135,000 per admission in the United States, with aggregate charges exceeding $1.8 billion (Burns et al., 2014). Treatments of PHF such as LVAD implantation and heart transplantation have obvious value, but new approaches are still urgently required. Our study will significantly contribute to the clinical management of PHF patients with LVAD support and help physicians' decisions for transplantation. Notably, our study will uncover the molecular mechanism of endogenous cardiac regenerative potential and functional recovery ability, which will help to develop new therapy for PHF.

Changes/Problems

Because of the quick development of new technologies and success of our recent studies, we have changed or replaced selected research methods proposed in this DOD award with the most cutting-edge technologies including single-cell RNA sequencing (scRNA-seq), split-pool ligation-based transcriptome sequencing (SPLiT-seq), Cleavage Under Targets and Release Using Nuclease (CUT&RUN). With the success of our recently published studies and promising preliminary studies described above, we are very confident to investigate the proposed study in this DOD award using new technologies. The changes do not change the specific aims or the budget.

Products, Inventions, Patent Applications, and/or Licenses

Up to date, this DOD award has not led to any products, invenstions, patent applications or licenses.

Participants & Other Collaborating Organizations

LVAD support program at Texas Children's Hospital (TCH) is one of the busiest in the world and performs more LVAD implantations than any other pediatric center worldwide. We closely collaborate with TCH investigators and obtained myocardial samples at time of LVAD placement and then at the time of orthotopic heart transplantation, as well as peripheral blood samples meanwhile.

Special Reporting Requirements

No

Appendices

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