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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 an 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 assist blood circulation. However, most patients don't response to LVAD treatment and require heart transplantation. Unfortunately, transplantation is severely limited by the scarcity of donor hearts. Hence, an unmet clinical need is to determine how to predict PHF patient response to LVAD treatment. This would ease the decision of physicians on whether heart transplantation is needed. Identification of biomarkers in blood samples would provide a novel non-invasive method to determine whether the heart is improving upon LVAD treatment. Hence, our study is aimed at developing gene expression signature-based methods that predict whether PHF patients respond favorably to LVAD treatment. Our studies are also aimed at facilitating endogenous cardiac regeneration with the goal of significantly improving PHF survival rates. As such, we will employ cutting-edge techniques to determine the molecular mechanisms that stimulate endogenous cardiac regeneration in order to develop novel therapeutic approaches.

15. SUBJECT TERMS

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Introduction

Congenital Heart Disease and Pediatric Heart Failure

Congenital heart disease (CHD) is the most common birth defect which affects 10 out of every 1000 newborns (Hoffman et al., 2004; Tennant et al., 2010) and poses a tremendous health burden. Severe CHD often produces pediatric heart failure (PHF), in which the heart has poor pumping function, leading to organ failure and death at an early age. Hence, CHD-related PHF is a devastating condition and leading cause of death 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). Notably, modern medical advances have increased survival rates for pediatric CHD patients, allowing them to reach adulthood. However, a significant number of adult CHD patients have a dramatically increased heart failure risk (Gurvitz et al., 2016; Ntiloudi et al., 2016). In addition, PHF is exceedingly expensive 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). PHF treatments such as Left Ventricular Assist Device (LVAD) implantation and heart transplantation have obvious value, but new approaches are still urgently required.

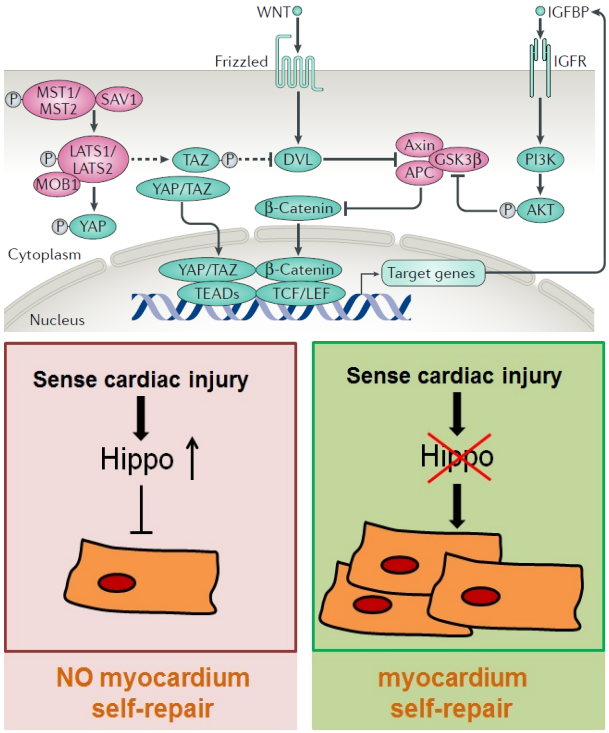

Figure.Int Overview of Hippo Signaling Pathway and its function in myocardial repair. In Hippo pathway, the Yap/Taz effectors are regulated by both the Hippo kinases and also the actin cytoskeleton. In normal hearts, Hippo signaling senses cardiac injury and represses cardiomyocyte repair. Inhibiting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

Hippo Signaling and Cardiomyocyte Regeneration

Hippo signaling, an ancient organ size control pathway, is a kinase cascade that inhibits cardiomyocyte proliferation during development (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 complex with the Salvador (Salv) scaffold protein and phosphorylate the Large Tumor Suppressor Homolog (Lats) kinases. Lats kinases, in turn, phosphorylate Yap and Taz, two related transcriptional co-activators that are major Hippo signaling effectors that partner with transcription factors, such as Tead, to regulate gene expression. Upon phosphorylation, Yap and Taz are sequestered from the nucleus and rendered transcriptionally inactive (**Figure. Int**). Hippo signaling cross talks with the canonical Wnt-β-catenin pathway and other signals in the heart (**Figure. Int**), which we recently reviewed (Wang et al., 2018).

We were the first to report that Hippo signaling inhibits cardiomyocyte proliferation during development by inhibiting Wnt signaling (Heallen et al., 2011). The human heart has minimal ability to repair itself after injury and endogenous signals can replace a mere

1% of heart muscle cells per year. In mice, resection of the cardiac apex in the first week of life triggers cardiac regeneration while resections performed at postnatal day (P) 7 or later yields permanent fibrosis and scarring (Porrello et al., 2011). We performed apex resection at the non-regenerative P8 stage in control and Hippo-deficient hearts and evaluation 21 days-post resection via serial sectioning revealed severe scarring of control hearts (Heallen et al., 2013). In contrast, resected Hippo-deficient hearts efficiently regenerated myocardium and exhibited reduced scarring. We also evaluated regeneration in the adult heart using a left anterior descending coronary artery obstruction (LAD-O) myocardial infarction (MI) model in which the left anterior coronary artery is tied off with a suture. Based on histology and functional analysis by echocardiography, Hippo deficient hearts regenerated efficiently (Heallen et al., 2013). Our recent study indicated that Hippo-deficient adult mouse cardiomyocytes re-enter the cell cycle and undergo cytokinesis. Taken together, our findings reveal that Hippo is an endogenous signal that represses cardiomyocyte proliferation and renewal (Heallen et al., 2013; Morikawa et al., 2015; Tao et al., 2016).

Our Recent progress of Studying Hippo Signaling in the Heart

To date, we have published a number of key discoveries during this DOD award revealing the roles of Hippo signaling in cardiac development and regeneration, including three *Nature* papers (Bassat et al., 2017a; Leach et al., 2017; Morikawa et al., 2017b),two *Dev Cell* papers (Monroe et al., 2019; Xiao et al., 2018) and one *Genes Dev* paper (Xiao et al., 2019). We have been invited to review Hippo signaling in cardiac regeneration in different journals including *Nature reveiws cardiology*, *Cir Res, Curr Opin Cell Biol,* and *Wiley Interdiscip Rev Dev Biol* (Deshmukh et al., 2019; Heallen et al., 2019; Liu and Martin, 2019; Wang et al., 2018)

We recently uncovered a direct connection between the dystrophin glycoprotein complex (DGC) and Hippo-Yap signaling. This study published in *Nature* revealed that Yap interacts directly with the DGC component Dystroglycan 1 (DAG1) to inhibit cardiomyocyte proliferation (Morikawa et al., 2017a). This interaction required Hippo -mediated phosphorylation of Yap, indicating a tight physiologic connection between the Hippo pathway and the DGC in cardiomyocyte homeostasis. We demonstrated that removing the Hippo pathway component *Salv* in the X-chromosome-linked muscular dystrophy *(mdx)* mutant mouse background suppressed the induction of stress-induced heart failure (Morikawa et al., 2017a). This collaborative work was published in the same issue of *Nature* where another group demonstrated that the extracellular matrix (ECM) component Agrin binds the DGC, allowing Yap to be released into the nucleus to enhance cardiomyocyte proliferation (Bassat et al., 2017b). Together, our findings indicate that the DGC senses changes in the ECM to control Yap subcellular localization. In addition, we made the exciting discovery that Hippo deletion protected against heart failure in hearts with established heart failure (Leach et al., 2017). We also recently used single-cell RNA sequencing (scRNA-seq) to uncover a critical role of Hippo signalling in the epicardium, a tissue that contains essential noncardiomyocyte progenitors that give rise to epicardial-derived cells (EPDCs) (Xiao et al., 2018). EPDCs contribute to the primary support cells of the heart such as 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 in which *Lats1* and *Lats2* are deleted exhibit fibroblast differentiation arrest (Xiao et al., 2018).

We recently created a mouse model that conditionally overexpresses active YAP (YAP5SA) and found that YAP5SA expression in adult cardiomyocytes induces chromatin to adopt a primitive and fetal-like transcriptional state, which promotes proliferation of adult cardiomyocytes (Monroe et al., 2019). In addition to cardiomyocytes, other cardiac cell types

such as cardiac fibroblasts are involved in cardiac injury and repair. Upon injury, cardiac fibroblasts can transit through multiple cell states, including resting and activated cardiac fibroblasts, as well as myofibroblasts. In our most recent published study, we found that in adult resting cardiac fibroblasts, deletion of *Lats1 and Lats2* initiates spontaneous and self-perpetuating fibrosis(Xiao et al., 2019). Notably, this cell state transition was exacerbated by MI. As indicated by our scRNA-seq data, *Lats1/ Lats2* deficient cardiac fibroblasts spontaneously transition into a myofibroblast cell state. Our integrated genome-wide analysis of Yap chromatin occupancy incdicate that Yap directly activates genes that encode pro-inflammatory factors and those that regulate myofibroblast cell identity and. These data reveal that *Lats1/ Lats2* maintain homeostasis of resting cardiac fibroblasts through restricting a Yap-dependent injury response (Xiao et al., 2019).

Encouraged by these findings and our recent success using cutting-edge technologies such as scRNA-seq, we have modified our research strategy of this DOD award to include additional similar technologies and expanded our studies to examine other non-cardiomyocyte cell types such as cardiac fibroblasts. We have obtained very promising preliminary data that is includied in this report. In addition, we are currently preparing a manuscript that has arisen from this DOD award study.

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 proposed to investigate the genetic mechanisms of PHF caused by CHD by using the most sophisticated approaches available. This study is aimed at improving the clinical management of PHF patients with LVAD support. To this end, we are uncovering the molecular mechanisms that underlie endogenous cardiac regeneration as a means to advance novel PHF therapy development.

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

Over the past year, we have continued the collection of fresh patient samples, both peripheral blood and cardiac tissue, from the large pediatric heart disease population at Texas Children's Hospital. We collected these samples directly from the operating room as the tissue was harvested to ensure the highest quality possible for downstream transcriptomic analyses. To date, we have collected paired heart tissue and peripheral blood samples from a total of 114 patients. We have also expanded the scope of the diagnoses that we were able to biobank, previously focusing solely on patients undergoing LVAD placement and heart transplants to now collecting from a large variety of pediatric heart diseases (**Figure 1**). Our cohort now includes a large number of patients with congenital heart diseases, such as

hypoplastic left heart syndrome (HLHS), tetralogy of Fallot (TOF), and various septal defects.

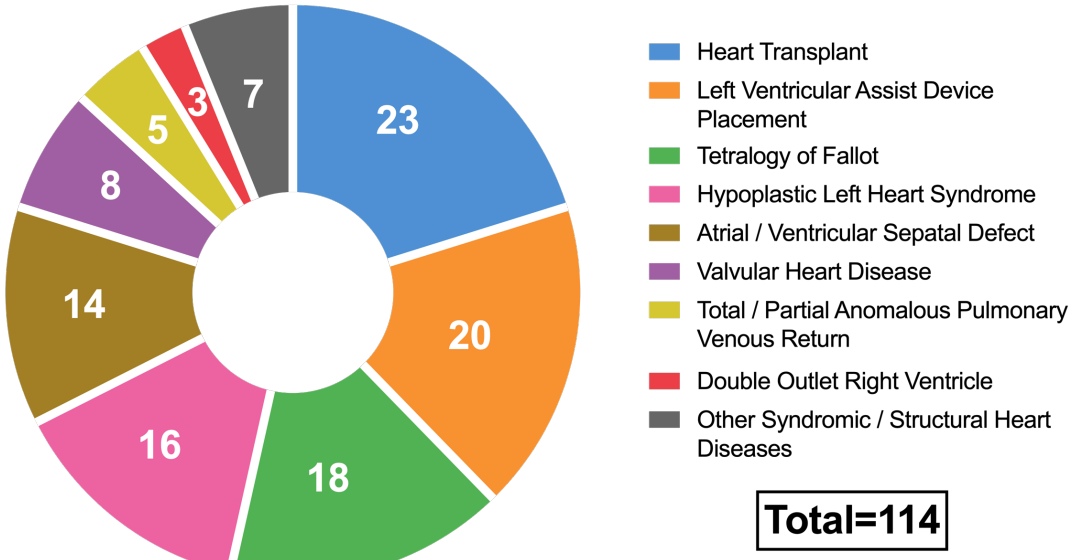

Figure 1. Distribution of pediatric heart disease diagnoses in the study. 114 paired cardiac and peripheral blood samples have been freshly collected directly from the operating room. These samples represent a wide variety of pediatric heart diseases, classified here into larger diagnostic categories. The number of samples in each diagnostic category is labeled directly on the chart in white.

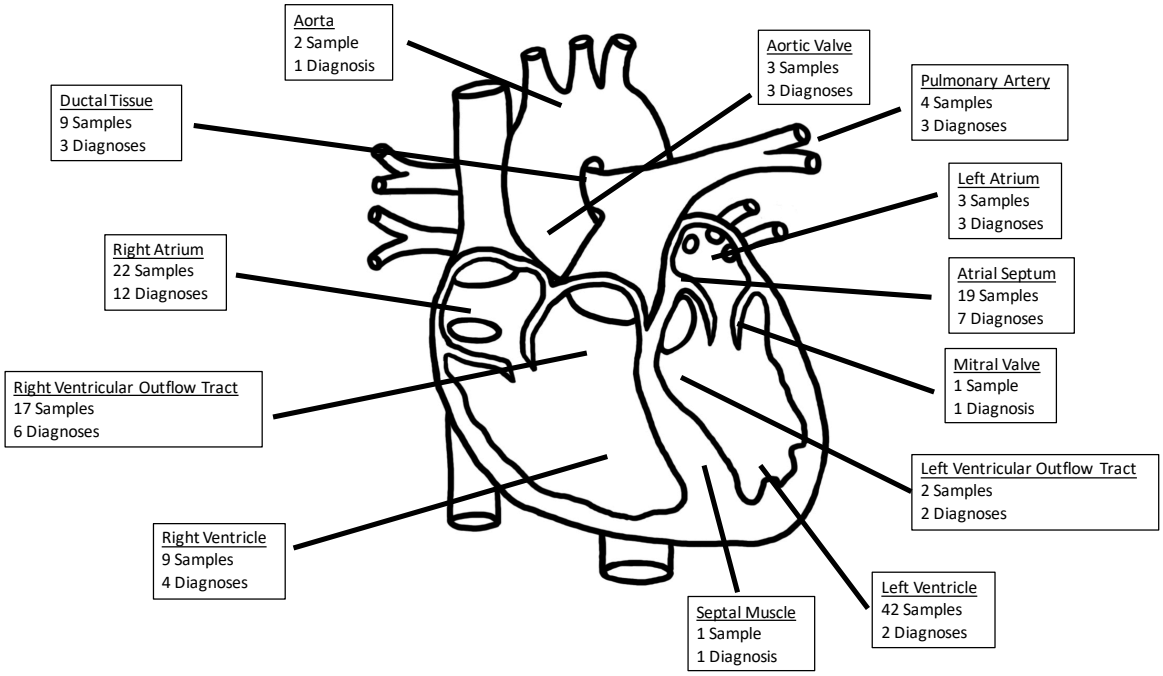

Figure 2. Anatomic representation of cardiac tissue collected. Each surgical procedure offers different opportunities to recover cardiac tissue that we use in our research program. Many tissues in the heart are included in our biobank, the majority of which come from a variety of different disease contexts.

Collecting from this large variety of patients has also provided us the opportunity to collect cardiac tissue from various anatomic locations within the heart. Our collection currently includes samples from all four chambers of the heart, multiple heart valves, and other local vessels (**Figure 2**). Each of these structures have been previously demonstrated to have unique transcriptional signatures, leading to our hypothesis that they have unique transcriptional responses after injury.

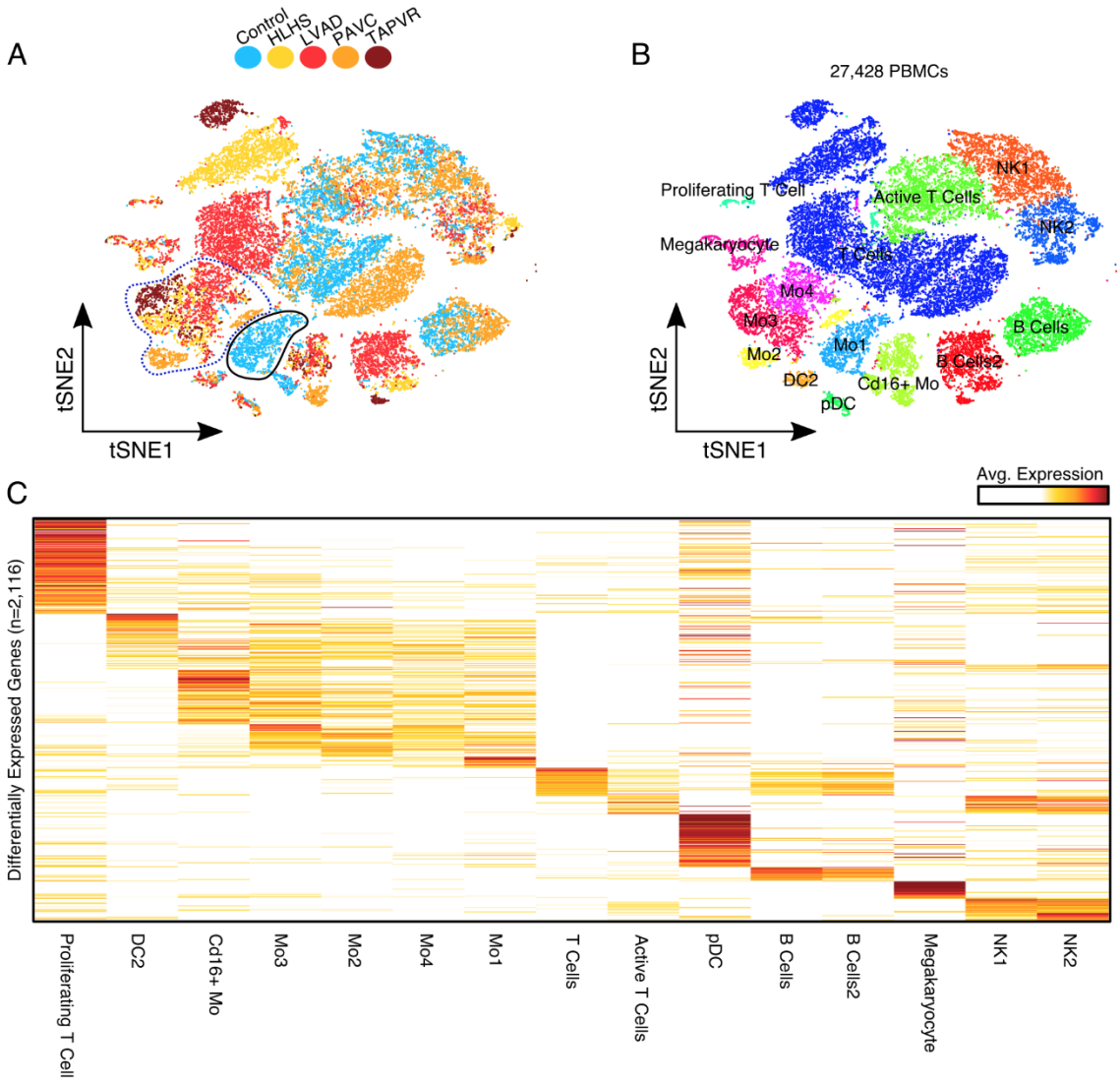

Figure 3. Single cell RNA-seq profiling of immune cells in pediatric heart failure and congenital heart disease. A) tSNE plot of scRNA-seq data collected from CHD and pediatric heart failure blood samples. Control, healthy non-heart failure adult; HLHS, hypoplastic left heart syndrome; LVAD, pediatric 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).

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 from whole blood of pediatric patients suffering from congenital heart disease and heart failure (**Figure 3**). These include 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, and 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 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 performed with t-SNE. After computational processing was completed, we observed a total of 27,428 single cell transcriptomes (**Figure 3A**). Importantly, we identified all known major 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.

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

scRNA-seq is difficult to perform on heart tissue which is due, in part, to the large size and fragility of cardiomyocytes. To overcome the complications associated with fluorescently activated cell sorting and microfluidic platforms, we chose to employ split-

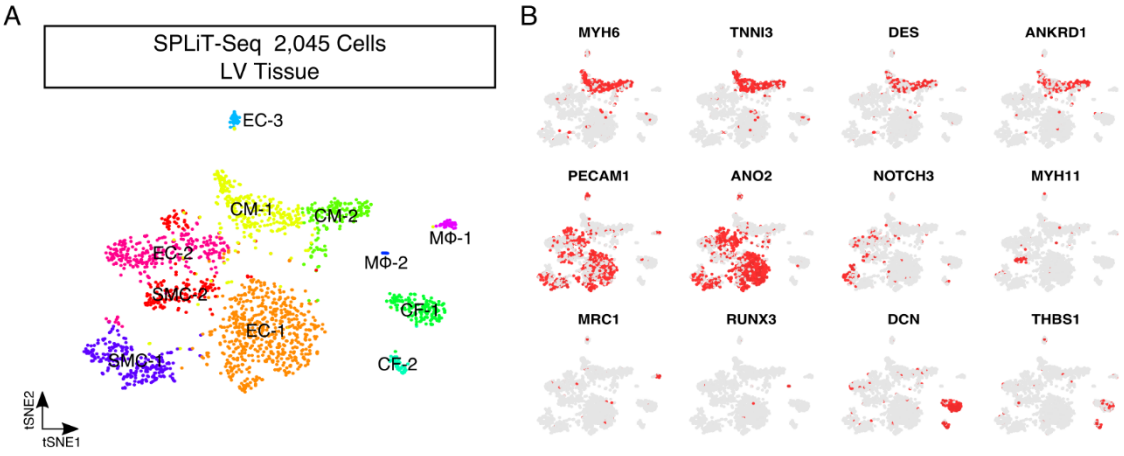

Figure 4. Profiling heart tissue at single-cell resolution with SPLiT-seq. A) tSNE plot of SPLiT-seq showing graph-based clustering results and cluster identities. This experiment was carried out on a sample derived from a pediatric heart failure patient, pre-LVAD placement. CM, cardiomyocyte; EC, endothelial 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.

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 and tissue was collected prior to LVAD placement (**Figure 4**). Following 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 observed considerable heterogeneity among the various cardiac tissue constituents, including 2 clusters of each MΦs, CMs, and CFs. For future studies, the large scale deployment of this technology on cardiac tissue will allow us to fully characterize the inflammatory status and composition of pediatric heart failure and CHD.

Epigenetic Landscape Profiling of Cardiac Cells

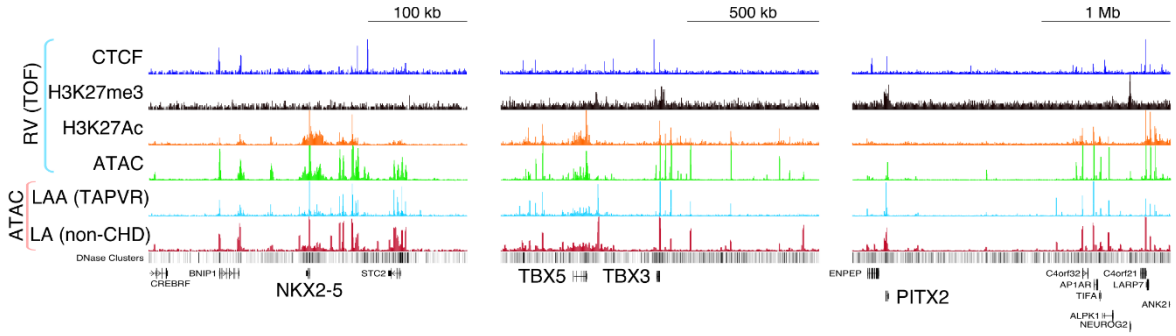

Figure 5. Epigenomic characterization of congenital heart disease. A) Representative genome browser tracks for CUT&RUN (CTCF, H3K27me3, and H3K27Ac), and ATAC-seq. Top 4 tracks are all derived from the RV tissue of the same patient.

To determine the epigenetic landscapes that underpin the transcriptional readout observed in cardiac cells, we used 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 an antibody-based recruitment of micrococcal nuclease (Skene and Henikoff, 2017). We elected to conduct this experiment in a cardiomyocyte-specific manner by isolating cardiomyocyte nuclei via PCM1 enrichment (Gilsbach et al., 2014; Preissl et al., 2015). Using CUT&RUN, we profiled CTCF repressor occupancy and interrogated enhancers and heterochromatin using H3K27ac and H3K27me3 respectively as markers. 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 TOF, TAPVR, and healthy adult left atrial tissue (LA) samples. Gene loci associated with active cardiomyocyte like *NKX2.5* were highly accessible and possessed active histone mark signatures commensurate with an absence of heterochromatin (**Figure 5**, left). Moreover, we could determine 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 a similar 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 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.

Isolation of Cardiac Fibroblasts from Pediatric Heart Tissues

Cardiac fibroblasts make up the majority of cells in the heart and have an important role in the synthesis and degradation of the extracellular matrix (ECM) (Camelliti et al., 2005). In response to certain injuries, activated fibroblasts secrete excess ECM, leading to pathological fibrosis and heart failure (Leask, 2010). Our recent study published in *Genes Dev* paper revealed that Hippo signaling plays an essential role in cell state transitions of cardiac fibroblasts (Xiao et al., 2019). Hippo deletion in adult resting cardiac fibroblasts initiates self-sustaining fibrosis. Importantly, Hippo signaling maintains homeostasis of resting cardiac fibroblasts by restricting a Yap-dependent injury response (Xiao et al., 2019).

Our recent findings lead us to consider inhibiting fibroblast activation as a potential therapeutic strategy for treating heart failure. Effective anti-fibrosis therapies are currently not available, and the mechanisms underlying fibroblast regulation in the context of heart failure are poorly understood, especially in pediatric patients. As fibroblast functions are tissue-specific (Zeisberg and Kalluri, 2013; Zhang et al., 2019), it is important to isolate cardiac fibroblasts from ventricular tissue of human hearts to study how they contribute to cardiac fibrosis. In 2019, we isolated human ventricular fibroblasts from pediatric heart tissues of 4 patients receiving heart transplantation and 1 patient with left ventricular assistant device (LVAD). We froze ventricular fibroblasts after one passage for future *in vitro* studies. Our lab has demonstrated that activated YAP is critical to cardiomyocyte proliferation (Monroe et al., 2019). Our preliminary data indicate that human ventricular fibroblasts express YAP in this line.

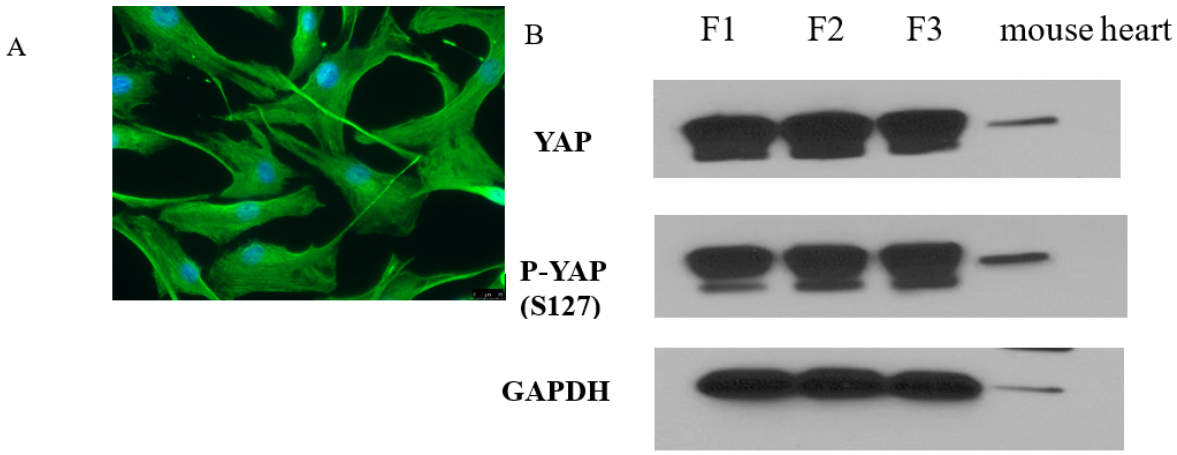

Figure 6. Human ventricular fibroblasts express Yap. We used an established collagenase digestion protocol to isolate human ventricular fibroblasts. A. Immunofluorescent staining of fibroblasts using the fibroblast-specific marker vimentin (*green*). B. Western blotting of whole cell lysates reveal that human fibroblasts isolated from three pediatric hearts (F1, F2, F3) express YAP and inactivated phosphorylated Yap (P-Yap).

Impact

CHDs cause PHF which is highly lethal and the costs to treat are exorbitant. 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 pose obvious limitations and new approaches are urgently required. To this end, findings from our study should significantly contribute to the clinical management of PHF patients with LVAD support and help physicians' decisions for transplantation. Notably, our efforts will uncover the molecular mechanism(s) that drive endogenous cardiac regeneration and functional recovery ability, which is needed to develop novel PHF therapies.

Changes/Problems

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

Products, Inventions, Patent Applications, and/or Licenses

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

Participants & Other Collaborating Organizations

The LVAD support program at Texas Children's Hospital (TCH) is one of the busiest in the world, and more LVAD implantations are performed there than any other pediatric center worldwide. We closely collaborate with TCH investigators and obtain myocardial samples at the time of LVAD placement, at the time of orthotopic heart transplantation, as well as collecting peripheral blood samples at these timepoints. One of our co-Investigators recently relocated to Department of Pediatrics in the McGovern Medical School of The University of Texas Health Science Center at Houston (UTH). UTH is located nearby to Baylor College of Medicine, which is convenient for our continued collaboration.

Special Reporting Requirements

No

Appendices

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