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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. 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 respond 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 accurately predict whether PHF patients respond to LVAD treatment. This would ease the decision of physicians on whether heart transplantation is needed. Accordingly, identification of biomarkers in PHF patient blood samples would provide a novel non-invasive method to determine whether heart function improves 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 stimulating 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 govern cardiac regeneration in order to develop novel PHF therapy approaches.

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Introduction

Congenital Heart Disease and Pediatric Heart Failure

Congenital heart disease (CHD), the most common birth defect, affects 1 of every 100 newborns(Hoffman et al., 2004; Tennant et al., 2010) and is the leading cause of newborn death and illness worldwide. Severe CHDs often lead to pediatric heart failure (PHF), the leading cause of death in infants, children, or adolescents, affecting estimated 12,000 to 35,000 children under 19 years old in the United States each year (Hsu and Pearson, 2009). In 2012, it was reported that there are roughly 11,000-14,000 PHF hospitalizations per year (Rossano et al., 2012). PHF treatment costs have increased dramatically and recent estimates indicate that the inpatient costs alone average $70,000 per admission in the United States (Nandi and Rossano, 2015), with aggregate charges eclipsing $1 billion (Nandi and Rossano, 2015). This represents a mere fraction of the total costs associated with PHF treatment. Although the number of pediatric CHD patients surviving into adulthood has increased due to modern medical advances, CHD survivors have a dramatically increased risk of heart failure as adults (Gurvitz et al., 2016; Ntiloudi et al., 2016).

The Hippo signaling pathway, cardiac regeneration and congenital heart disease

The Martin lab pioneered investigations into the Hippo Signaling Pathway (HSP), a fundamental organ size control pathway, which we first reported as a key inhibitor of cardiomyocyte proliferation during development to limit heart size (Heallen et al., 2011). As an organ size control pathway, the HSP is, by definition, a pathway that senses cell composition and tissue architecture of an organ. As shown in **Fig. 1**, the mammalian core HSP components include the Ste20 kinases *Mst1* and *Mst2*. Mst kinases, complexed with the Salvador (Salv) scaffold protein, phosphorylate the Large Tumor Suppressor Homolog (*Lats*) kinases which, in turn, phosphorylate Yap and Taz, two related transcriptional co-activators. Yap/Taz are downstream HSP effectors that partner with transcription factors, such as Tead, in the nucleus to regulate gene expression. Upon phosphorylation, Yap and Taz are excluded from the nucleus and rendered transcriptionally inactive. Other signals, such as mechanical signaling, also regulate Yap subcellular localization (**Fig. 1**). The HSP acts as a stop signal to inhibit the activity of Yap and Taz and inhibit tissue proliferation. We have recently discovered that Yap activates the inflammatory response in cardiac fibroblasts (CFs), indicating that HSP also inhibits cardiac inflammation (Xiao et al., 2019).

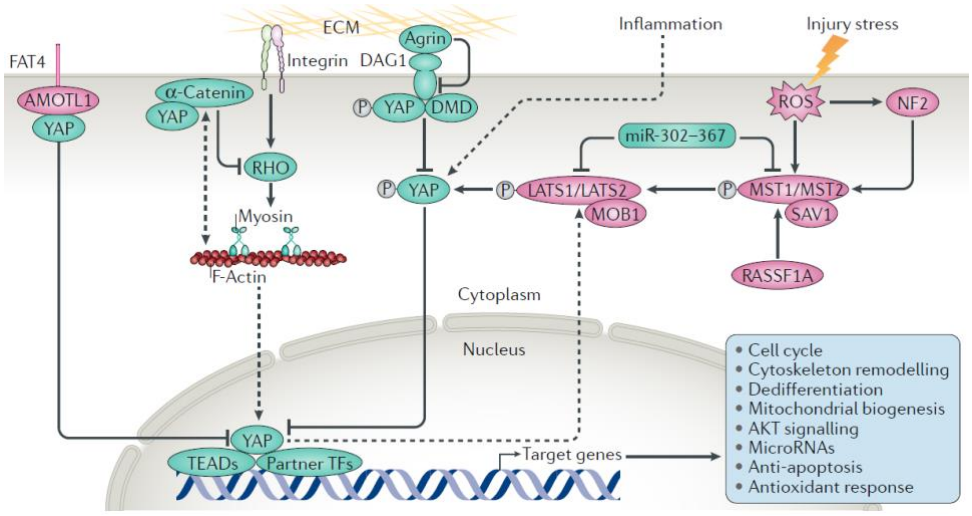

Figure 1. Overview of the Hippo Signaling Pathway. In the HSP, the Yap/Taz effectors are regulated by both the Hippo kinase cascade and the actin cytoskeleton (Wang et al, 2018). ECM, extracellular matrix.

Our initial experiments investigating the HSP in response to tissue injury were performed in postnatal mice. The postnatal mammalian heart has a vanishingly small capacity to repair following injury and can endogenously replace approximately 1% of cardiomyocytes (CMs) per year. Preclinical studies in cardiac injury model mice reveal that surgical resection of the cardiac apex within the first six days of life triggers a major cardiac regeneration response, whereas resection performed at postnatal day (P) 7 or later produces fibrotic scarring at the injury site, indicating that cardiac regeneration potential is permanently lost after the P7 stage (Porrello et al., 2011). In non-regenerative P8 mice, we performed apex resection in control hearts and hearts that were HSP-deficient in CMs. Serial sectioning at 21 days post-resection revealed that control hearts displayed severe scarring as expected while HSP-deficient CMs efficiently regenerated as evidenced by significantly reduced scar sizes and improved function (Heallen et al., 2013). We also evaluated regeneration in adult hearts following left anterior descending coronary artery obstruction (LAD-O), which models acute-MI injury, and observed that in contrast to controls, HSP-deficient hearts regenerated efficiently (Heallen et al., 2013). CMs in HSP-deficient hearts re-entered the cell cycle, underwent cytokinesis, ultimately leading to elevated CM proliferation, enhanced cardiac regeneration and functional recovery (Heallen et al., 2013; Morikawa et al., 2015; Tao et al., 2016).

Cardiac injury in any context severely disrupts cellular composition, tissue architecture, and induces 1) fibrosis and 2) an immune response that activates resident macrophages, which release pro-inflammatory cytokines to propagate the inflammatory response across the heart. We hypothesize that the pro-inflammatory signaling, if it can be understood in depth, is a target for directed therapies that limit tissue injury. Damaged cardiac tissue consists of dead CMs, activated CFs, extracellular matrix (ECM), and immune cells. Under normal cardiac homeostasis conditions, CFs are at rest. Following injury, resting CFs are activated and infiltrate the damaged area to promote fibrosis. We recently deleted HSP components in adult mouse resting CFs and observed that the HSP functions as the guardian of the resting CF cell state. Importantly, Yap directly activates inflammatory genes (Xiao et al., 2019).

Our recent progress investigating the HSP in the heart

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., 2017), two *Dev Cell* papers (Monroe et al., 2019; Xiao et al., 2018) and one *Genes Dev* paper (Xiao et al., 2019). We were invited to review Hippo signaling in cardiac regeneration in different journals including *Nature Reviews Cardiology*, *Circ 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 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., 2017). 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., 2017). This collaborative work was published in the same issue of *Nature* where another group demonstrated that the 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 signaling 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 indicate that Yap directly activates genes that encode pro-inflammatory factors and those that regulate myofibroblast cell identity. 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 included in this report. *For the no-cost extension reporting period of 09/01/2020-08/31/2021, we have continued with our analyses and are currently revising a manuscript that has arisen from this DOD award study (running title: "Integrated multiomic characterization of congenital heart disease").*

Keywords

Hippo signaling, cardiac regeneration, single-cell RNA sequencing (scRNA-seq), spatial transcriptomics (ST), hypoplastic left heart syndrome (HLHS).

Accomplishments

During the funding period of this grant, we made two important discoveries arising from our original hypotheses. In our **first major discovery**, we found that the HSP in CMs is an inhibitor of endogenous CM renewal and cardiac regeneration. In adult human HF patients, we found that HSP activity was maladaptively upregulated (Leach et al., 2017). Importantly, HSP inactivation in CMs in a failing heart reversed systolic heart failure in mice revealing the HSP to be a *bona fide* target to treat heart disease (Leach et al., 2017). By inhibiting the HSP using gene therapy, after heart failure was established, we showed that cell composition in the heart was returned to a more normal state with improved function indicating that tissue architecture of the failing heart can be rebuilt using gene therapy(Leach et al., 2017). These experiments were performed in mice, and *we have recently repeated the findings using gene therapy in a pig model of ischemic heart disease (Liu et al., 2021)*. In a **second major discovery**, we found that the HSP was required to maintain the resting cardiac fibroblast (CF) phenotype to prevent a spontaneous cell state transition to an activated myofibroblast (Leask, 2010). Moreover, the HSP mutant

myofibroblasts expressed multiple signaling molecules resulting in inflammatory activation of surrounding CFs and macrophages with a resulting feed forward induction of inflammation and fibrosis. This indicates that HSP responds to environmental inputs to maintain physiologic levels of Yap activity in the CF nucleus. When HSP is removed in CFs there is an increase of nuclear Yap and increased expression of Yap targets which included genes that induce the innate immune response, such as *Ccl2* and *Csf1*, and the expansion and recruitment of macrophages into the heart resulting in increased cardiac inflammation (**Fig. 2**) (Xiao et al., 2019).

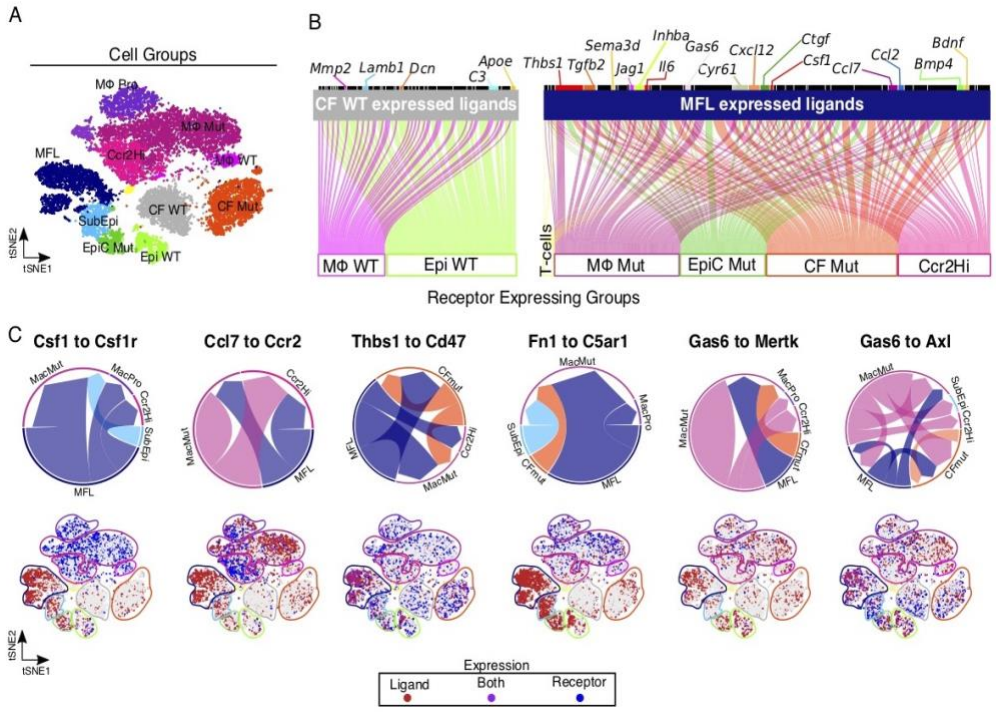

Figure 2. Representation of signaling between CF and inflammatory cells *Lats1/2* CF loss of function in adult mice. A) UMAP plot showing cell types, B) ligand interaction map, C) feature plots and signaling interaction maps.

We hypothesize that the HSP is important in human CHD pathogenesis, a notion that is strongly supported by a recent multi-laboratory, consortium study in human patients using exome sequencing of trios to find *de novo* mutations in CHD patients (Homsy et al., 2015). These extensive analyses combined whole exome sequencing with predictive modeling of mutation frequencies in over 1200 CHD patients to identify *de novo* spontaneously arising mutations. Twenty-one genes with multiple damaging *de novo* mutations were uncovered from these analyses. These mutations are prevalent in CHD phenotypes such as left ventricular outflow tract obstruction, conotruncal defects, and heterotaxy. Remarkably, 9 of these 21 genes have direct connections to HSP (**Table I**). Among these HSP interacting genes are Yap direct targets, including *Fbn1*, *Zeb2*, and *Jag1* (**Table I**). Other genes in this list encode for proteins that are directly Yap interactors, such as Cad, Chd4, Smad2, and Ahnak

Other recent work in mice, using a forward genetics approach, uncovered a mouse model for hypoplastic left heart syndrome (HLHS) (Liu et al., 2017). Using whole exome sequencing in the mouse mutants and multi-hit gene analyses by overlaying 1) HLHS mouse mutations and 2) LOF variants occurring in two or more HLHS human subjects they developed a list of HLHS genes. As illustrated in **Table I**, these HLHS genes interact with Hippo signaling at various points in the pathway, serving as upstream regulators of the core Hippo complex to being YAP/TAZ transcriptional targets.

Based on our published and preliminary data, as well as findings from the literature, our overriding hypothesis is that damaged tissue architecture can be reverted to a more normal three-dimensional architecture and cellular composition to improve heart function. The observation that inhibiting the HSP in adult CMs repairs the heart can be generalized to CHD patients including HLHS patients. We propose that in the future it will be possible to treat genetically complex disease, such as HLHS and other CHDs, with gene therapy targeting the HSP and other pathways. Our ongoing studies are aimed at identifying novel treatment targets and biomarkers for CHD and PHF with a focus on the HSP based on our preliminary data and published studies (Deshmukh et al., 2019; Heallen et al., 2019; Leach et al., 2017; Li et al., 2015; Monroe et al., 2019; Morikawa et al., 2017; Wang et al., 2018; Xiao et al., 2019; Xiao et al., 2018). We are using cutting edge single cell genomics and spatial transcriptomics approaches to investigate CHD to obtain an in depth understanding of HLHS and other CHDs. Our experiments will uncover the principles of disrupted tissue architecture with aberrant cell composition

Gene	Interaction with Hippo	Reference
HLHS HSP interacting genes (Liu et al., 2017)		
Ctbp2	TEAD4 interactor	(Zhang et al., 2018)
Ctnna3	Yap interactor	(Li et al., 2015)
Dchs2	upstream Hippo signaling pathway activator	(Pan, 2010)
Enah	YAP direct target gene	(Morikawa et al., 2015)
Rbpj	YAP direct target gene (Notch pathway)	(Slemmons et al., 2017)
Raptor	regulates YAP protein stability and activity	(Yao et al., 2019)
Vgll3	TEAD4 interactor	(Figeac et al., 2019)
Wnt5a	YAP/TAZ signaling	(Park et al., 2015)
Human CHD HSP interacting genes (Homsy et al., 2015)		
Ahnak	YAP interactor	(Enzo et al., 2015)
Cad	YAP interactor	(Enzo et al., 2015)
Chd4	YAP interactor	(Cotton et al., 2017)
Fbn1	YAP direct target gene	(Moya et al., 2019)
Jag1	YAP direct target gene	(Tschaharganeh et al., 2013)
Notch1	Regulates YAP/Tead expression	(Li et al., 2012)
Ptpn11	Activity regulated by YAP/TAZ	(Tsutsumi et al., 2013)
Smad2	YAP interactor	(Pefani et al., 2016)
Zeb2	YAP direct target gene	(Gao et al., 2014)

TABLE I: Hippo Signaling Pathway in CHD

Patient Population

We worked with Dr. Adachi's group at Texas Children's Hospital (TCH) to obtain myocardial samples for profiling experiments. The congenital heart clinical practice at TCH is one of the largest in the world in terms of volume, variety, and complexity of CHD cases. For each patient sample, we collected clinically relevant data, including demographics (age, gender, any available exome sequencing), underlying cardiac condition(s), primary diagnoses, medications, and laboratory data, from the

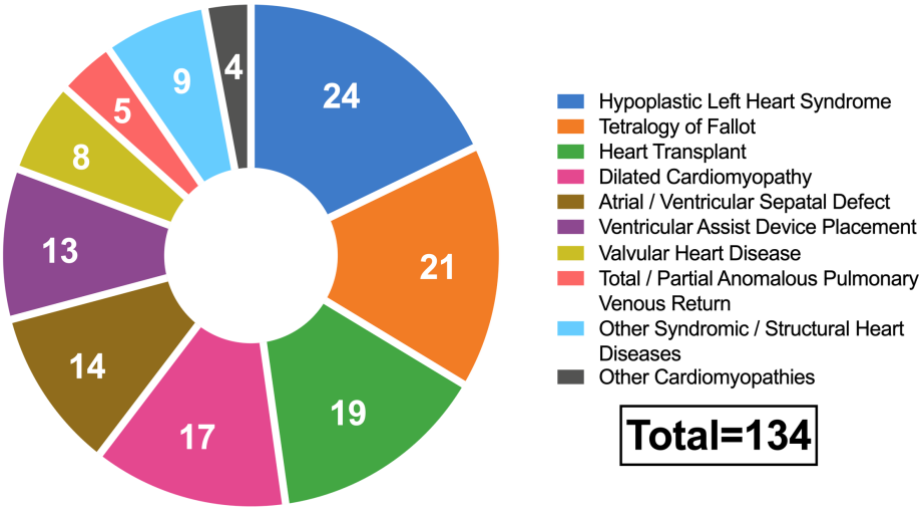

Figure 3. Distribution of pediatric heart disease diagnoses in the study. 134 paired cardiac and peripheral blood samples have been freshly collected directly from the operating room. These samples represent a wide variety of pediatric heart diseases, classified here into larger diagnostic categories. The number of samples in each diagnostic category is labeled directly on the chart in white.

medical record. We collected, both peripheral blood and cardiac tissue, from a total of 134 TCH patients and we continue to collect samples.

To ensure the highest possible quality for our experiments, we coordinate with the TCH surgical team prior to surgery to collect and flash-freeze these samples directly from the operating room as the tissue is harvested. As samples are collected, we record the cardiac anatomical region that was harvested, how tissues and blood were processed and the experiment(s) that have been performed for each sample. In addition, we run a series of quality control procedures to ensure sample integrity.

In addition to frozen tissue samples, we have fixed a portion of the tissue in formalin for immunofluorescence, imaging mass cytometry (large throughput IF using heavy metals to image multiple markers) and RNA-scope to validate our sequencing data. We have been able to collect from a large variety of CHD patients (collected samples are summarized in **Fig. 3**). Our cohort now includes a large number of patients with HLHS, dilated cardiomyopathy (DCM), tetralogy of Fallot (TOF), and cardiac septal defects. 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 large vessels.

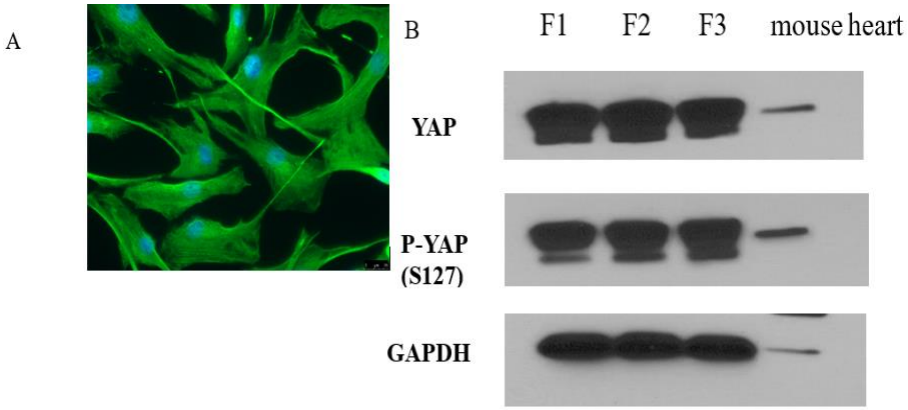

Figure 4. Human ventricular fibroblasts express Yap. We used an established collagenase digestion protocol to isolate human ventricular fibroblasts. A. Immunofluorescent staining of fibroblasts using the fibroblast-specific marker vimentin (*green*). B. Western blotting of whole cell lysates reveal that human fibroblasts isolated from three pediatric hearts (F1, F2, F3) express YAP and inactivated phosphorylated Yap (P-Yap).

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 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). These 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 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 (**Fig. 4**).

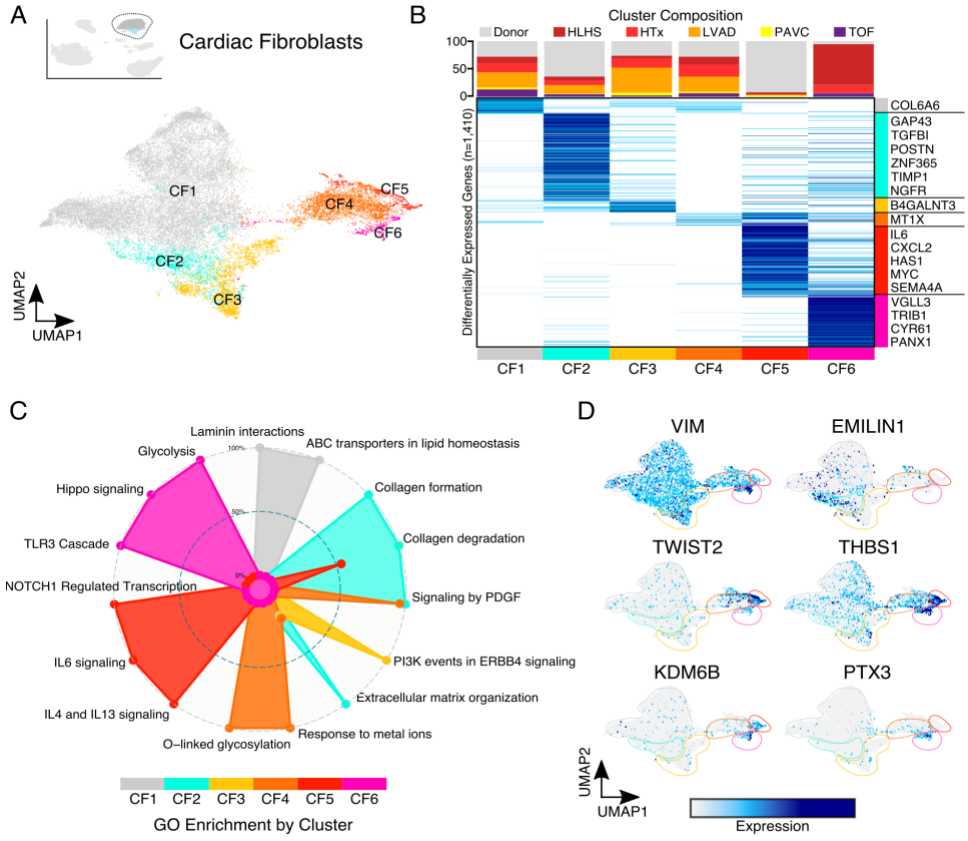

Figure 5. Cardiac Fibroblast Profiles in CHD: (A) Uniform Manifold Approximation and Projection (UMAP) embedding of CF reiterative clustering. (B) Top, cluster composition plot indicating percentage of cells from each diagnosis. Bottom, average expression heatmap with representative genes shown. (C) Gene Ontology radar plot with GO categories. Plot scaled by -log10(p-value). (D) Feature plots for CF genes.

Single-Cell Genomics

Normal tissue function and development requires interactions between different cell types in all organs including the heart. An ideal way to investigate cell state and intercellular interactions in a complex organ is single cell genomics, such as single cell RNA-seq (scRNA-seq) or single nuclear RNA-seq (snRNA-seq), combined with spatial transcriptomics. Spatial transcriptomics provides essential spatial information that is lost in scRNA-seq and snRNA-seq (Vickovic et al., 2019). During this funding period, we used snRNA-seq to discover a unique CF cell state, cluster CF6, which characterizes HLHS hearts. Importantly, CF6 expresses high confidence Yap target genes including *Vgll3*, *Cyr61*

and *Ptx3* indicating that the HSP is reduced in CF6 (**Fig. 5**). The CF6 gene signature also reveals clear signs of inflammation with expression of genes such as *Ptx3*, *Thbs1*, *Trib1*, and *Panx1* (**Fig. 5**). Of the inflammatory genes, *Thbs1* and *Ptx3* are also direct Yap target genes (Xiao et al., 2019). Moreover, there are also metabolic changes in CF6 with an elevated glycolytic metabolic profile that may further potentiate Yap transcriptional activity (Enzo et al., 2015) (**Fig. 5**).

Inflammation has both beneficial and pathologic effects in all organs including the heart. For example, in the mammalian heart, there is a short cardiac regenerative period in the immediate postnatal period where inflammation plays a pro-regenerative function (Forte et al., 2018). It is conceivable that manipulation of the inflammatory response can be used to treat HLHS. Especially given the fundamental nature of the inflammatory response in many different diseases, it is also feasible to adaptively manipulate inflammation in other CHDs (Kotas and Medzhitov, 2015). In our recently published work, we deleted the HSP in adult mouse resting cardiac fibroblasts (CF), the main sensor of cardiac damage (Johansen and Molkentin, 2019; Xiao et al., 2019). Our findings in the mouse model indicate that the HSP is the guardian of the resting CF cell state and, in addition, pro-inflammatory genes, which we also observed in HLHS patient, are Yap direct targets through enhancer promoter chromatin looping (Xiao et al., 2019). Moreover, using computation approaches to predict ligand-receptor interactions, we found that intercellular signaling was greatly increased in the HSP mutant hearts in which we deleted *Lats1/2* in CFs (Xiao et al., 2019) (**Fig. 2**). In particular, we observed a large increase in signaling between CFs and macro phages in *Lats1/2* mutant hearts (**Fig. 2**).

Spatial Transcriptomics

While sc-RNA-seq and snRNA-seq can profile the transcriptional expression of thousands of genes per cell (Vieth et al., 2019) and characterizes a broad array of cell-types, it fails to preserve the important spatial information of cells within the organ (Stuart and Satija, 2019). In order to uncover the spatially varying principles of tissue architecture, cell composition, and cell-cell communication, strategies that maintain spatial information are essential. Spatial transcriptomics (ST) uses high throughput methods to sample RNA from individual cells while maintaining the spatial information for each cell. ST provides essential genome-wide, spatially resolved and unbiased gene expression data on tissue sections (Salmen et al., 2018; Stahl et al., 2016; Vickovic et al., 2019). Moreover, ST is ideally suited to analyze complex diseases like CHD, with disrupted cell composition and abnormal tissue architecture. The tremendous regional diversity in the transcriptional landscape and cellular composition of cardiac tissue, which is particularly important in a pathologic context such as CHD, can be studied using ST. Indeed, ST has been used to study spatial gene expression in preclinical contexts including the mouse olfactory bulb (MOB) and the human fetal heart supporting its feasibility in the clinical context of CHD (Asp et al., 2019; Eng et al., 2019).

As noted, ST complements scRNA-seq by quantitatively capturing cellular gene expression values in their native environment from a standard-size tissue section, as has been shown in human fetal heart tissue (Asp et al., 2019). However, current ST protocols profile the transcriptional expression of only about half as many genes as scRNA-seq resulting in sparse data sets that can make it difficult to accurately identify cell-types in ST datasets. In particular, when the marker genes of different cell-types are absent in an ST dataset, it is unclear if one can assign correct types to the cells in that dataset potentially leading to inaccurate conclusions from an ST data analysis. To address this shortcoming, we developed a pipeline to combine ST with scRNA-seq datasets.

Computational approaches to analyze spatial transcriptomics datasets: proof of principle

As proof of principle, we developed a pipeline called STANN (Spatial Transcriptomics Cell Types Assignment Using Neural Networks), a neural network that learns from an scRNA-seq dataset to assign cell-types to the cells of an ST dataset (manuscript submitted). We developed STANN to integrate ST with scRNA-seq, in part using our published data from a previous Martin lab paper from mouse MOB (Tepe et al., 2018). MOB was chosen because of the availability of high-quality ST data with the seq-FISH-plus ST platform and the well-defined tissue architecture of the MOB (Eng et al., 2019). Current ST platforms profile relatively fewer genes than scRNA-Seq assays and integrating the two data sources uncover more accurate biological information about the tissue of interest including all types of CHD. In preliminary studies, we found that cell-type identification in

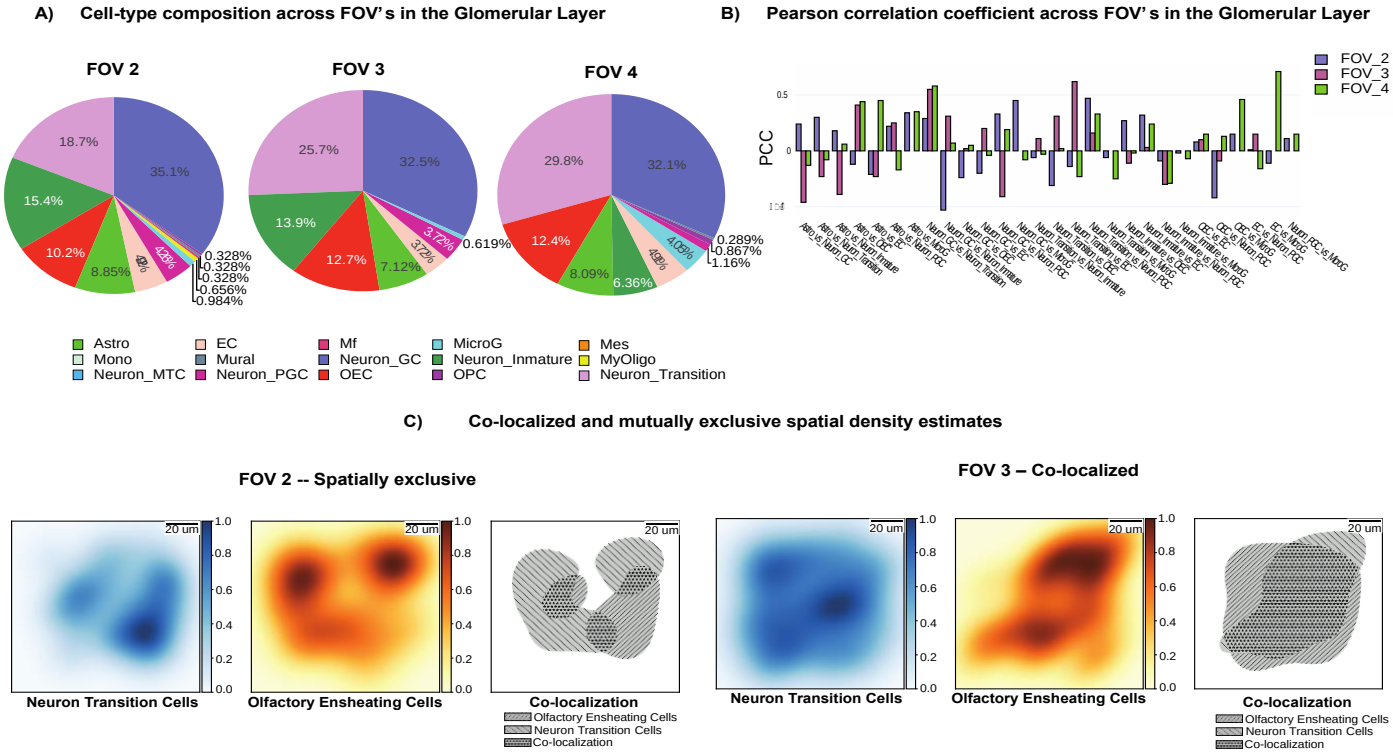

Figure 6. a) Varying composition of cell-types across different Fields of View (FOV) in the Glomerular Layer. b) Pearson Correlation Coefficient scores of FOVs mapping Glomerular Layer. c) Kernel density maps of Neuronal Transition cells vs Olfactory Ensheathing cells in two different FOVs, which resolve the GCL layer, showing distinct co-localization patterns

the ST dataset is significantly more accurate when the ST dataset is integrated with scRNA-seq dataset using STANN (**Fig. 6**). Conventional data integration methods (Stuart and Satija, 2019) fail to consider the large margin of difference in depth between datasets and often give inaccurate biological conclusions. Our pilot study on ST and scRNA-seq datasets from MOB (Eng et al., 2019) showed that current state of the art approaches (Butler et al., 2018) to integrate ST and scRNA-seq data is prone to predicting wrong cell-types in ST data. This failure of the current approaches, which our approach rectifies, in assigning cell-types is problematic for downstream analyses, since all downstream analyses depend on proper cell-type assignment. To overcome this significant shortcoming of current approaches, we developed STANN, an accurate statistical machine learning model, to integrate ST and scRNA-seq datasets (shown below).

A deep neural network to integrate ST and scRNA-seq data: STANN is a deep neural network that integrates ST and scRNA-seq datasets by carefully accounting for the differences between the two datasets. The model first learns correct cell types for the cells in an scRNA-seq dataset from the expression of only those genes that are profiled in both the ST and the scRNA-seq datasets. We then use this model to predict the cell-type for every cell in the ST data. In our pilot analysis on data from MOB from the Martin lab (Tepe et al., 2018), this method had remarkably high accuracy for all cell-types including the rare cell-types in MOB (accuracy on validation data: 0.95).

Identifying pairs of co-localized cell-types in ST data: Following methods for co-localization analysis in microscopy (McDonald and Dunn, 2013), we developed a statistical method to reveal the co-localization patterns between different cell-types in an ST data. In our pilot study on MOB data, we found that the co-localization patterns between a pair of cell-types can change significantly across the tissue. We hypothesize that capturing this variation in cell-type co-localization patterns is critical for a complete understanding of the tissue architecture and function in CHD and will provide important new insight that will lead to new therapies.

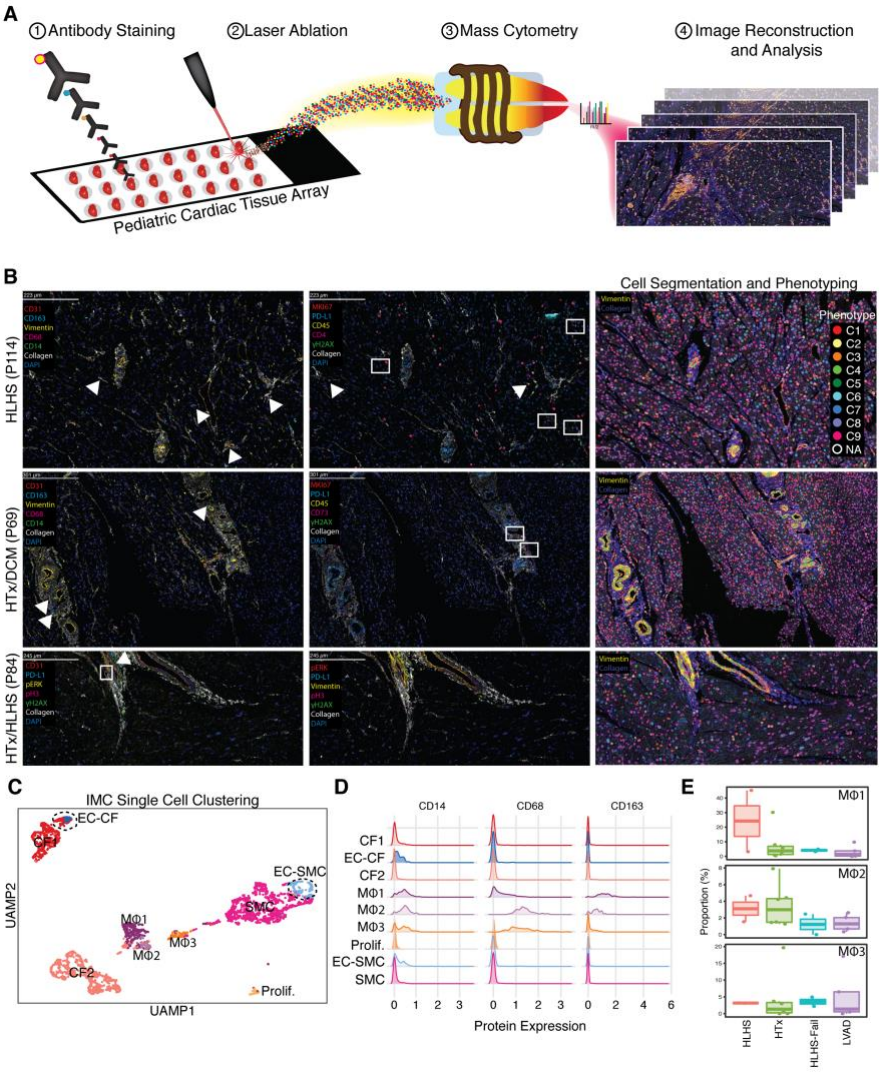

Figure 7. Mass Cytometry (CyTOF) analysis of CHD patients. A. Diagram of workflow. B. Spatial reconstruction of single labeled cells (arrowheads). C, D. UMAP clustering and cell density clustering reveals the distinct cell types in the CHD samples. E. Box plots of cell type proportions in different CHD samples.

Identifying spatial variation in cell-to-cell communication mechanisms: We recently developed a statistical method to identify how cell-to-cell communication mechanisms between a pair of cell-types, as represented by receptor-ligand co-expression patterns, change across different regions of a tissue (**Fig. 6**). We further identify the genes having regulatory relationships (Van de Sande et al., 2020) with these receptor-ligand pairs. By comparing these spatially localized, receptor-ligand centric gene regulatory networks, our method identified the different mechanisms of receptor-ligand activation that changes concomitantly with cell-type co-localization patterns across the tissue. These computational methods, combined with functional genetic manipulations, will provide a powerful approach to investigate our hypotheses in the

context of CHD. We will use mass cytometry (CyTOF) as a method to validate our transcriptomics data since this is an approach that looks at protein expression in medium throughput platform (**Fig. 7**). The value of this is that we can see protein expression which is powerful in terms of understanding pathophysiology and it is unbiased allowing us to look at tissue protein expression in novel ways.

Impact

CHD is the most common birth defect that approximately 1% babies are born with, affecting 40,000 newborns per year in the United States. CHD is also the leading cause of newborn death or illness worldwide. Despite a steady increase in the number of surviving CHD patients due to successful treatment early in life, adult CHD patients have significantly higher death rates compared with the non-CHD population. Given these reasons, there is a considerable clinical need to develop novel treatment targets and biomarkers for CHD. We have therefore made this the main focus of the studies in this research period. These studies encompass the development of regenerative medicine approaches, implementing novel models with an established phenotype, and research to improve the understanding of the causes of CHD defects. We are utilizing an innovative combination of high-throughput and cutting-edge methodologies to analyze human samples and mouse models. In addition, the proposed studies will be conducted in a collaboration across several laboratories using a combination of multidisciplinary expertise and physical resources to ensure successful and timely completion.

Due to our studies, we now have a deeper understanding of the molecular mechanisms that govern endogenous cardiac regenerative potential and functional recovery and have candidate therapeutic targets for the diagnostics and treatment of CHDs. In the course of this PRMRP research award, we discovered that inactivation of genes in the HSP reverses cardiac pathology such as heart failure in mice. HLHS is a lethal CHD, and to date, has no definitive treatment option. We have made important new discoveries that the HSP is reduced in cardiac fibroblasts of HLHS patients and plan to 1) define the spatial distribution of inflamed cardiac fibroblasts with reduced HSP in HLHS and 2) determine whether HSP is involved in the pathophysiology of HLHS. Multiple surgeries are required soon after a baby with HLHS is born and surviving infants with HLHS may suffer lifelong complications. In many cases, infants with HLHS require a heart transplant, which is restricted by the lack of donor hearts. Those who are lucky to receive a heart transplant will need to take medications for their whole life in to prevent their body from rejecting the transplanted heart. In the short term, our studies have revealed the feasibility of manipulating the inflammatory response to treat HLHS. Our data will also directly benefit the counseling and treatment of HLHS and will help to develop new therapies for HLHS in the short term. Importantly, the inflammatory response has shared significance among several different types of diseases, raising the intriguing possibility of manipulating it to treat other types of CHDs. For the long term, our studies have provided important insights for the diagnostics and treatment of other types of CHDs.

Changes/Problems

Given the quick development of new technologies and encouraging results from our recent studies, we either changed or replaced selected research methods proposed in this DOD award with more sophisticated and novel technologies including single-cell RNA sequencing (scRNA-seq), single nuclear RNA-seq (snRNA-seq), STANN (Spatial Transcriptomics Cell Types Assignment Using Neural Networks), and mass cytometry (CyTOF). Taken our recently published studies and featured results described above, we decided to pursue the proposed studies in this DOD award employing these technologies. These 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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