REGULATION OF CARDIAC FIBROSIS BY CLASS I HISTONE DEACETYLASES

by

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REGULATION OF CARDIAC FIBROSIS BY CLASS I HISTONE DEACETYLASES

Thesis directed by Associate Professor Timothy McKinsey

**Rationale:** Pan-Histone Deacetylase (HDAC) inhibitors are efficacious in animal models of left ventricular (LV) heart failure; however, the specific enzymes and mechanism(s) of action are not well defined.

**Objective:** These studies were completed using selective small molecule HDAC inhibitors to expose the mechanism(s) through which anti-fibrotic effects of HDAC inhibitors occur.

**Methods and Results:** To elucidate the roles of specific HDAC isoforms, the relative ability of pan- and isoform-selective HDAC inhibitors to block cardiac fibrosis was assessed in the mouse angiotensin II (Ang II) infusion model. Two-week Ang II osmotic mini-pumps (1.5 μg/kgBW/min) were subcutaneously implanted into male C57Bl/6J mice; pan- and class-specific small molecule inhibitors were given via daily Intraperitoneal injections. LV fibrosis was assessed using picrosirius red histological staining. Both pan- and class I HDAC inhibition blocked the development of cardiac fibrosis, leading to the conclusion that cardiac fibrosis occurs through a class I dependent mechanism. Remarkably, Class I HDAC inhibition did not reduce initial immune cell recruitment or inflammation, as assessed by flow cytometry and qPCR. Additional **in vivo** and **in vitro** studies confirmed multiple effects of class I HDAC inhibition on primary cardiac fibroblasts and circulating fibrocytes, both known as key effector cells of fibrosis. Class I HDAC inhibition blocked both cardiac fibroblast proliferation and differentiation of fibrocytes. Reduction of fibrosis was also associated with an increase in the population and suppressive function of anti-inflammatory T regulatory cells (T\text{reg}s), which have previously been shown to have anti-fibrotic capacity.
Conclusions and Future Directions: These data suggests that class I HDAC inhibition prevents the development of cardiac fibrosis through effects on cardiac fibroblasts and recruited fibrocytes. Additional anti-fibrotic effects may be due to increases in the suppressive function of T regs. Notably, effects do not appear to be dependent on a broadly anti-inflammatory mechanism. Additional experiments have been proposed to discern the importance of these differing cell populations, as well to uncover the molecular pathways responsible for the anti-fibrotic effect of class I HDAC inhibition.

The form and content of this abstract are approved. I recommend its publication.

Approved: Timothy McKinsey
DEDICATION

This work is dedicated to my husband, Evan Williams, and parents, Peter and Teresa Ross; their love and support through all of the ups, downs, and unexpected turns has been and will always be appreciated.
ACKNOWLEDGMENTS

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<td>αSMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzymes</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Ang I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ARVFs</td>
<td>Adult rat ventricular fibroblasts</td>
</tr>
<tr>
<td>AT₁</td>
<td>Angiotensin II Receptor Type 1</td>
</tr>
<tr>
<td>BCP</td>
<td>1-bromo-3-chloropropane</td>
</tr>
<tr>
<td>CCTSI</td>
<td>Colorado Clinical and Translational Sciences Institute</td>
</tr>
<tr>
<td>CDK4/6</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CDKi</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Col I</td>
<td>Collagen I</td>
</tr>
<tr>
<td>COMIRB</td>
<td>Colorado Multiple Institutional Review Board</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DEREG</td>
<td>Depletion of T regulatory cells</td>
</tr>
<tr>
<td>DPAH</td>
<td>2,2′-dipyridylamine</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDACi</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>HFpEF</td>
<td>Heart failure with preserved ejection fraction</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICF</td>
<td>Informed consent form</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Median lethal dose</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activating protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactant protein-1</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteases</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NRVFs</td>
<td>Neonatal rat ventricular fibroblasts</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>ROCK-1</td>
<td>Rho-associated, coiled-coil containing protein kinase 1</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase quantitative PCR</td>
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<tr>
<td>SAP</td>
<td>Serum Amyloid P</td>
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<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sHF</td>
<td>Systolic heart failure</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>Transforming growth factor beta-1</td>
</tr>
<tr>
<td>T_{reg}</td>
<td>T regulatory cell</td>
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<tr>
<td>TSA</td>
<td>Trichostatin A</td>
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<td>TubA</td>
<td>Tubastatin A</td>
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CHAPTER I
INTRODUCTION

Overview and Translational Relevance

Cardiac fibrosis is a difficult-to-treat feature of pathological cardiac remodeling. In the setting of both myocardial infarctions and heart failure (HF), fibrotic tissue within ventricles reduces compliance of the tissue. The reduction in tissue elasticity impedes the ability of the ventricles to relax and can lead to diastolic dysfunction, a component of diastolic heart failure, or heart failure with preserved ejection fraction (HFpEF). Despite the prevalence and clinical impacts of fibrotic disease in the heart and other organs, adequate anti-fibrotic pharmacological therapies are not currently available.

Interstitial fibrosis in the ventricles is characterized by the build-up of collagen I (Col I) between myocytes in the myocardium. In addition to mechanical stiffness that results from interstitial fibrosis, fibrotic remodeling of tissue structure can cause disruption of electrical signaling and hypoxia in the tissue (1;2). The fibrotic process includes proliferation and migration of resident cardiac fibroblasts, and their differentiation into alpha smooth muscle actin (αSMA)-expressing myofibroblasts (3;4). Myofibroblasts secrete matrix metalloproteases (MMPs) and collagen, resulting in remodeling of the extracellular matrix (ECM) and a net increase in collagen deposition. Additionally, recent work has demonstrated that infiltration of the LV by pro-fibrotic monocyte-derived fibrocytes and inflammation are critical to the development of fibrotic disease (5-7). These data suggest that pharmacological strategies designed to impact resident fibroblasts, disrupt recruitment or differentiation of pro-fibrogenic fibrocytes, and resolve inflammation will be beneficial for the treatment of HFpEF.

HDACs are classically thought of as enzymes that deacetylate histone tails,
increasing the interaction between histones and their surrounding DNA, resulting in reduced gene transcription. Strikingly, recent work has demonstrated a much wider function for these enzymes, which are now known to deacetylate more than 4,500 nuclear and cytosolic proteins (8). While HDAC inhibitors were originally identified as potential chemotherapeutics, recent investigations have demonstrated that these compounds are also efficacious in pre-clinical models of heart failure (9-12). Eleven classical, zinc-dependent HDACs have been identified in humans, and have been subdivided into groups: class I (1, 2, 3, and 8), class IIa (4, 5, 6, and 9), class IIb (6 and 10), and class IV (11); class III HDACs (Sirtuins) require NAD$^+$ rather than zinc as a cofactor, and are not affected by the inhibitors used in these experiments.

In the clinic, cardiac remodeling occurs over time, often in the setting of chronic hypertension (13). Thus, we have adopted a well-known mouse model of hypertension-induced cardiac remodeling that causes fibrosis to develop over several weeks following the implantation of angiotensin II (Ang II) osmotic mini-pumps. Current studies have demonstrated that pan-HDAC inhibitors are efficacious in animal models of left ventricular (LV) heart failure and cardiac remodeling, however, the specific enzymes and mechanism(s) of action are not well defined. To elucidate the roles of specific HDAC isoforms in cardiac fibrosis, we tested the relative ability of pan- and isoform-selective HDAC inhibitors to block cardiac fibrosis in (Ang II) infusion model.

Both the pan-inhibitor Scriptaid and the class-I specific inhibitor MGCD0103 (MGCD) dramatically suppressed Ang II-mediated cardiac fibrosis, leading to the novel conclusion that class I HDACs regulate cardiac fibrosis. While class I inhibition did not reduce early inflammatory mediators or immune cell infiltration three days after Ang II infusion, in vitro studies demonstrated that Class I HDAC inhibition reduced the
proliferation of resident fibroblasts and blocked the differentiation of pro-fibrotic fibrocytes. Significantly, class I HDAC inhibition reduced the population of pro-fibrotic fibrocytes both in circulation as well as the heart; this novel finding has important translational relevancy in the pathology and treatment of many human diseases (14).

In addition to the noted effects on fibroblasts and fibrocytes, the relative population of T_{reg} increased in the blood of animals treated with MGCD. *In vitro* suppressive function of these cells was also demonstrated to increase. These changes may also contribute to the anti-fibrotic effects of this class I HDAC inhibitor, as adoptive transfer of these cells into mice infused with Ang II results in a decrease in fibrosis; the mechanism of this effect is not clear, but may be related to reduction in reactive oxygen species (ROS), production of IL-10, and their suppressive activity on other immune cell populations.

**Background Information**

**Heart Failure with Preserved Ejection Fraction**

One goal of the McKinsey laboratory is to identify potential therapeutic targets for patients with heart failure with preserved ejection fraction (HFpEF), as adequate pharmacological treatments have not yet been identified. It has been hypothesized that anti-fibrotic and anti-hypertrophic agents may be efficacious in this population. Patients suffering from HFpEF, also referred to as diastolic heart failure, tend to be older, female, and frequently have a history of hypertension (15). While systolic function in these patients is generally preserved, HFpEF patients report a similar symptom burden and reduction in their standard of living as compared to patients with systolic heart failure (sHF). Over the last two decades, sHF patients have seen clinical benefits through pharmacological management; unfortunately, large clinical trials using standard-of-care
sHF medications including angiotensin receptor antagonists and digoxin (16-18), have not demonstrated an increase in lifespan in patients with HFP EF (18-20). Additionally, an epidemiological study conducted at the Mayo Clinic recently found that the prevalence of HFP EF had increased from 38 to 54% of HF patients at hospital discharge over a 15-year period (21), underscoring the public health need for the development of effective therapies for these patients.

**Pathophysiology of HFP EF**

The pathophysiology of HFP EF is a current topic of investigation; important components are thought to be cardiac hypertrophy and fibrosis and resulting remodeling of the myocardium (22;23). As the left ventricular wall thickens and becomes more fibrotic, the left ventricle (LV) becomes less compliant and is less able to relax and fill during diastole (13). In the clinic, cardiac remodeling associated with HFP EF occurs slowly over time and has often been shown to be related to chronic hypertension (13).

Using the Ang II mouse model, we can examine molecular mechanisms and screen small molecules in a timely and cost-effective manner. Preliminary data previously generated by our laboratory indicated that pan-HDAC inhibition blocks development of cardiac fibrosis. *In vivo* studies using the Ang II-infusion model were conducted in order to determine the effect of class-specific HDAC inhibition on cardiac fibrosis.

**Histone Deacetylases**

Research exploring the molecular mechanisms of heart failure has uncovered important roles of HDACs in cardiac remodeling (10;12;24-26), contractility (27;28), autophagy (26), and energy metabolism (29). Additionally, several groups have determined beneficial effects of pan-HDAC inhibitors in animal models of cardiac disease (10;12;24-26). Due to the dysregulation of HDACs in a variety of pathophysiological
processes, pharmaceutical companies have placed intense focus on development of class and isoform-specific drug-like small molecule HDAC inhibitors. Through collaborations with the University of Colorado School of Pharmacy, industry, and other academic institutions, we are fortunate to have access to a number of both class and isoform-specific HDAC inhibitors. Using the Ang II infusion model, we have determined that selective inhibition of class I HDACs efficiently blocks interstitial cardiac fibrosis (see below). Importantly, anti-fibrotic activity was not observed with inhibitors of class IIa or IIb HDACs. The primary goal of my work is to begin to define the roles of class I HDACs in the development of fibrosis.

**Cardiac Fibrosis**

Fibrosis can develop interstitially between myocytes, often an effect of long-term hypertension or aortic stenosis, or can replace tissue lost due to myocyte death, often in the setting of hypoxic conditions such as ischemic heart disease. Cardiac fibrosis has long been thought to be dependent on changes in extracellular matrix (ECM) production and activation of resident tissue fibroblasts (Fig. 1) (3;30;31). Recently, others have demonstrated that immune cells and chronic inflammation play a critical role in the development of cardiac fibrosis (6;32;33) (illustrated in the model in Fig. 1). As resident tissue cells and inflammation play necessary roles in the development of cardiac fibrosis, we hypothesized that the positive effect of class I HDAC inhibition could be due to actions that affected either of these systems, and as such, theorized that it was critical to explore inhibitor effects on local tissue fibroblasts as well as circulating immune cells.
Figure 1: Model depicting current opinions on the development of cardiac fibrosis. This process is thought to be dependent on activation of tissue fibroblasts, recruitment of immune cells such as monocytes (Mo), and the continued presence of inflammation over time. Fibroblasts have been shown to migrate, proliferate, and transdifferentiate to α-smooth muscle actin-expressing myofibroblastic phenotype. Monocytes are recruited to the tissue by MCP-1, and can differentiate into macrophages (Ma) as well as pro-fibrotic immune cells known as fibrocytes (F).
Resident Tissue Cells

In response to pro-fibrotic stimuli, fibroblasts in culture have been shown to proliferate, migrate, increase production of extracellular matrix proteins (such as collagen), and transdifferentiate into alpha-smooth muscle actin- (αSMA) and fibronectin-positive myofibroblasts (3) which produce increased Col I and decreased matrix metalloproteinase 1 (MMP1). Tissue flexibility decreases as increased extracellular matrix is deposited and as tension from the development of αSMA stress fibers in myofibroblasts increases. The cytokine transforming growth factor beta 1 (TGF-β1) has been shown to have an especially important role in cardiac fibroblasts adopting this pro-fibrotic myofibroblastic phenotype; for example, primary cardiac fibroblasts exposed to TGF-β1 in vitro increase αSMA expression. Importantly, investigators have found that ablation of TGF- β1 signaling blocks the development of cardiac fibrosis (34;35). Ang II increases TGF- β1 signal, while also acting as a mitogen for cardiac fibroblasts.

Inflammation and Immune Cell Recruitment

Under conditions of cardiac stress, cardiac fibroblasts and myofibroblasts (3;4), along with resident mast cells (36), secrete chemokines and cytokines which in turn recruit immune cells into the interstitial space, promoting inflammation. Recent evidence has demonstrated the crucial role of the immune system in the development of cardiac fibrosis (6;22;37); for example, monocyte chemotactic protein-1 (MCP-1) knock-out mice infused with Ang II had greatly reduced development of cardiac fibrosis (6). Interestingly, the authors hypothesized that the effects were due to a reduction in the recruitment of a profibrotic, bone-marrow derived fibroblast-precursor population (fibrocytes) (14). By targeting the recruitment or differentiation of these cells, it may be possible to target the development and progression of cardiac fibrosis. Intriguingly, in an ischemia-reperfusion
mouse model of ischemic cardiac disease, injection of the mice with Serum Amyloid P, a serum protein shown to block fibrocyte differentiation, prevented the development of both cardiac fibrosis and systolic dysfunction (33).

**Fibrocytes**

Fibrocytes are spindle-shaped bone-marrow derived cells which express leukocyte and mesenchymal markers; these cells have recently been identified as key effector cells of chronic inflammation and fibrosis (14). Fibrocytes express surface markers such as CD45 (leukocyte common antigen) as well as circulating progenitor cell markers such as the surface glycoprotein CD34. As CD14+ human monocytes isolated from peripheral blood mononuclear cells (PBMCs) are able to differentiate into fibrocytes (38;39), it is thought that fibrocytes are monocyte derived. Fibrocytes can be found in tissue as well as in circulation, but are thought to lose leukocyte and precursor cell markers over time as they differentiate in tissue (40). In addition to gaining myofibroblastic traits such as the expression of αSMA, these cells may be capable of reprogramming resident tissue fibroblasts to become more or less pro-fibrogenic (41). MCP-1 as well as stromal cell-derived factor-1 (SDF-1) are thought to be key signaling molecules in the recruitment and homing of these cells (6;38), and TGF-β1 appears to have a key role both in their expression of αSMA (38) and the ability of the cells to reprogram tissue fibroblasts into a pro-fibrotic phenotype (41). To our knowledge, our work to positively identify fibrocytes in cardiac tissue is among the first (42). Importantly, recent work has demonstrated increases in this population of cells in the circulation of patients with hypertensive heart disease (43).
Angiotensin-Induced Hypertension and Chronic Inflammation

The renin-aldosterone system is a critical regulator of blood pressure *in vivo*. Under stimulation, the liver increases production of angiotensinogen. Angiotensinogen can be cleaved to angiotensin I (Ang I) by renin that is released by pressure sensing juxtaglomerular cells in the kidney. Ang I can then be converted to Ang II by Angiotensin Converting Enzyme (ACE) (44). Ang II works on the AT1 receptor, a Gαq-coupled receptor, to stimulate phospholipase C and the generation of lipid signals such as IP$_3$ and DAG, increasing intracellular calcium and reactive oxygen species (ROS). This results in the contraction of smooth muscle cells lining arteries and arterioles, reducing the volume capacity of these blood vessels. Increased Ang II *in vivo* stimulates the production of aldosterone, leading to salt retention by the kidney which increases blood volume; concomitant increases in vessel contraction and blood volume lead to hypertension. Additionally, Ang II has direct actions on many other types of cells, including endothelial cells and fibroblasts; for example, ROS is believed to be an important mediator of inflammation caused by high levels of Ang II (45). Chronic inflammation has been established as critical in the development of fibrosis (46), leading us to hypothesize that increases in resolution of inflammation may halt the development of fibrosis. Importantly, recent investigations have also confirmed increased inflammation in patients with HFpEF (47).

Role of Regulatory T Cells

Regulatory T cells (T$_{reg}$) are known to have immune suppressive functions, and have been shown to serve critical roles in many human diseases through regulation of inflammation and polarization of immune cells. A recent report demonstrated that
circulating T_{regs} are reduced in patients with HFpEF, implicating this type of cell as a potential target for therapy (48). Consistent with this notion, adoptive transfer of T_{regs} into Ang II-infused mice reduced the development of cardiac fibrosis (49;50). Furthermore, recent investigations have demonstrated that IL-10, an important anti-inflammatory cytokine secreted by T_{regs}, is capable of both blocking and reversing cardiac dysfunction in murine models of cardiac remodeling (51). It has been found that pan-HDAC inhibitors are able to increase activity and numbers of T_{regs} in animal models (51); however, the identity of the specific HDAC(s) involved is currently an area of debate (52-56). While regulation of T_{regs} is an important area of investigation (57;58), no pharmacological therapies to specifically increase the number of T_{regs} in human patients are available; such a drug is hypothesized to have wide clinical benefits, from treatment of rheumatoid arthritis to prevention of organ rejection (57). We hypothesize that increased numbers or suppressive action of T_{regs} will reduce chronic inflammation associated with the development of cardiac fibrosis.

**Summary and Goals**

Goals of this thesis work included: (i) expanding the scientific understanding of cellular, immunological, and molecular mechanisms of cardiac fibrosis, (ii) clarifying reasoning for the potential use of class I HDAC inhibitors for HFpEF and hypertensive heart disease, (iii) exploring mechanisms of HDAC regulation of T_{regs}, and, (iv) generating further understanding of the role of circulating immune cells in cardiac disease. Additionally, we have developed a protocol for obtaining a sufficient number of leukocytes for multiple analyses with flow cytometry using single murine hearts, and have potentially identified a new class of pharmacological compounds capable of modulating both fibrocytes and T_{regs}, which have wide implications in human disease.
CHAPTER II

INHIBITION OF CLASS I HDACs BLOCKS CARDIAC FIBROSIS BY SUPPRESSING RESIDENT FIBROBLASTS AND BONE MARROW-DERIVED FIBROCYTES

Results

Class I Inhibition Blocks the Development of Cardiac Fibrosis

To assess the role of specific classes of HDACs in cardiac fibrosis, a panel of class-selective HDAC inhibitors (Figure 2A) was administered to ten week-old mice subcutaneously implanted with Ang II releasing mini-osmotic pumps. The panel included the pan-HDAC inhibitor Scriptaid, the class I HDAC inhibitor MGCD, class IIa inhibitor DPAH, and HDAC6 inhibitor Tubustatin A (TubA) (59). All compounds were given via IP injection at 10 mg/kgBW. Dosing began the day of pump implantation and continued throughout the two-week period (Figure 2B). Body weight was monitored daily. At the end of the study, mice were anesthetized and blood pressure was monitored using a catheter inserted through the carotid artery. Mice infused with Ang II experienced a decrease in body weight (Figure 2C) and an increase in mean systemic blood pressure (Figure 2D). After sacrifice, total heart weight, LV weight, and tibia length were collected; no significant increase in HW/tibia length or LV weight/tibia length was noted (data not shown); hypertrophy is known to be variable in two-week Ang II model in the C57Bl/6J mouse strain.

1Data presented in this chapter is in preparation for submission for publication to Circulation Research. As additional data are being collected for results involving T\textsubscript{reg} cells, these results have been separated and presented in Chapter III.
Figure 2: Panel of Pan- and Class Specific Histone Deacetylase (HDAC) Inhibitors were used in an Ang II-induced Model of Cardiac Fibrosis. A, Structures of Scriptaid, MGCD0103, DPAH, and Tubastatin A (TubA). B, Scriptaid, DPAH, TubA were dosed IP at 10 mg/kgBW daily. Due to the slow pharmacodynamics and long half-life of MGCD, dosing with this class I inhibitor was alternated every other day with vehicle injections. Dosing began at the time of pump implantation in the two-week Ang II studies. C, Animals infused with Ang II decreased in body weight over the two-week study compared to control animals. D, Mean systemic blood pressure increased with Ang II infusion, and was not effected by any of the compounds tested. *p<0.05; ANOVA with Tukey post-hoc testing. Error bars show standard error of the mean (SEM).
The upper half of the LV, above the papillary muscles, was fixed and sectioned for histology staining. Picrosirius red staining was completed to assess the development of fibrosis. Representative images of staining are shown in Figure 3A. Blinded analysis of 18 images/LV followed by quantification of picrosirius red stain confirmed Ang II infused animals treated with vehicle had an increase in interstitial fibrosis. Both Scriptaid and MGCD prevented the development of cardiac fibrosis, leading us to the novel conclusion that the process is dependent on Class I HDACs (Figure 3B). Importantly, these compounds were able to prevent the development of fibrosis without reducing blood pressure, as measured by carotid catheterization at sacrifice.

**Class I HDAC Inhibition Does Not Block Immune Cell Recruitment**

Recent work has demonstrated that inflammation and the subsequent recruitment of leukocytes is a necessary step in the development of fibrosis (6;33;60). As pan-HDAC inhibitors have been shown to be broadly anti-inflammatory (61;62), we hypothesized the reduction in fibrosis was due to a reduction in inflammation and immune cell recruitment. As investigations using the Ang II model have previously reported the largest changes in immune cell recruitment and inflammatory signaling several days after Ang II pump implantation (5;6), flow cytometry was used to assess immune cell recruitment to the heart three days after pump implantation. As expected, total leukocyte (CD45^+ cells) infiltration in hearts of animals exposed to Ang II was increased three days after pump implantation. Surprisingly, leukocyte infiltration was not reduced in the animals treated with MGCD (Figure 4A and 4B).
Figure 3

A

Sham + Vehicle  Ang II + Vehicle  Ang II + Scriptaid

Ang II + MGCD0103  Ang II + DPAH  Ang II + Tubastatin A

B

LV Interstitial Fibrosis

Collagen Fraction

Ang II:  –  +  +  +  +  +
Treatment:  Veh  Veh  Script  MGCD  DPAH  Tub A

Figure 3: Class I HDAC inhibition prevented the development of cardiac fibrosis. A, Representative images LV sections stained with picrosirius red for interstitial collagen. B, Quantification of picrosirius red staining was completed by determining the average stained pixels per total pixels in images of the myocardium using AxioVision software (18 investigator-blinded images used per animal) (n=7-8/group). *p<0.05; ANOVA with Tukey post-hoc testing. Error bars show standard error of the mean (SEM).
Use of flow cytometry allowed for the resolution of multiple populations of immune cells in the ventricles of mice. Analysis relying on surface markers using fluorochrome-conjugated antibodies confirmed that both the monocyte/macrophage (CD45^CD11b^-Ly6G^) and neutrophil (CD45^CD11b^-Ly6G^) populations had increased in Ang II-infused mice but were not reduced in MGCD treated animals (Figures 4-E). Analysis of cytokine message using reverse-transcriptase quantitative PCR (RT-qPCR) revealed class I HDAC inhibition did not reduce message of monocyte chemotactant protein-1 (MCP-1) in the left ventricle (Figure 11F). Of note, HDAC activity assays confirmed that class I activity had selectively been blocked in the LV of mice treated with MGCD (Figure 5). These results suggest that class I HDAC inhibition blocks the development of cardiac fibrosis without reducing inflammation.

**Class I HDAC Inhibition Arrests Cardiac Fibroblasts in G1 Phase of the Cell Cycle**

These data led to the hypothesis that the anti-fibrotic effects of class I HDAC inhibitor are dependent on inhibitor impacts in effector cells of fibrosis. Initially, inhibitor effects on primary rat ventricular fibroblasts were investigated in vitro. To investigate effects on cell cycle progression, fibroblasts were serum-starved for synchronization in G0/G1 of the cell cycle, and refed with full-serum media in the presence of vehicle (DMSO) or HDAC-inhibitor in order to reinitiate cell cycle progression. Cell cycle progression was assessed by measuring propium iodide (PI) staining in fixed cells 32 hours after refreshing with full-serum media; as cells move from 2n to 4n during S phase, PI fluorescence increases.
Figure 4: Class I HDAC inhibition did not reduce leukocyte recruitment. A, Representative graphs of CD45+ cells from live ventricular single cell suspensions surface labeled with fluorophore conjugated antibodies three days after pump implantation. Cells shown have been gated by FSC/SSC to include cells in the expected range of live cells to exclude debris, and show 200,000 FSC/SSC-gated events. B, Quantification of the population of leukocytes, defined as CD45+ cells, n=8/group. C, Populations of monocytes/macrophages and neutrophils were visualized using CD45+ gated cells. Monocytes/macrophages were defined as CD45+CD11b+Ly6G− and neutrophils were defined as CD45+CD11b+Ly6G+. Graphs show distribution of total CD45+ events collected (10,000 CD45+ events collected per samples). D, Relative populations of monocytes/macrophages in mouse ventricles three-days after pump implantation. E, Relative populations of neutrophils in mouse ventricles three-days after pump implantation. (n=8/group). *=p<0.05; ANOVA with Tukey post-hoc testing. Error bars show standard error of the mean (SEM).
Figure 5

Class I HDAC Activity

Class IIa HDAC Activity

Class IIb HDAC Activity

Figure 5: Class I HDACs were successfully inhibited in the LV of animals treated with MGCD. Enzymatic activity assays using protein lysates of LV from animals treated during the three-day Ang II implantation experiments demonstrated that MGCD selectively inhibited class I HDACs. *=p<0.05; ANOVA with Tukey post-hoc testing. Error bars show standard error of the mean (SEM).
After 32 hours, more fibroblasts could be seen in phases S and G2/M of the cell cycle. Initial studies completed with pan-HDAC inhibitor trichostatin A (TSA) revealed that treatment with this pan-HDAC inhibitor blocked primary neonatal rat ventricular fibroblasts (NRVF) in G0/G1 of the cell cycle (Figure 6A and 6B).

Experiments using a panel of class-selective HDAC inhibitors including 300 nM TSA (pan-), 1 μM MGCD (class I), 10 μM DPAH (class IIa), and 1 μM Tub A (HDAC 6i), confirmed that this arrest was class I HDAC dependent (Figure 6C and 6D). HDAC activity assays were completed in live NRVFs to confirm class-specific inhibition at the doses used in this panel (Figure 10). Experiments repeated in adult rat ventricular fibroblasts (ARVF) verified that this was not dependent on developmental stage of cardiac fibroblasts (Figure 6E). Additionally, consistent blockade in G0/G1 was noted after stimulation with fibroblast mitogens Ang II (10 μM) and endothelin-1 (ET-1; 25 nM) (Figure 7). Additional studies completed with isoform-selective HDAC inhibitors MS-275 (HDAC1/2), BRD2283 (HDAC1/2), and BRD3308 (HDAC3) indicated that cell cycle arrest is likely dependent on HDAC1/2 rather than HDAC3 (Figure 8).

While a number of groups have demonstrated that HDAC inhibitors block cell cycle progression in cancer (63;64), this effect has not been previously reported in cardiac fibroblasts. Several groups have shown this effect to be dependent on the upregulation of cyclin-dependent kinase inhibitors (CDKi) p21 and p27 in HDACi treated cells. These CDKis block the action of the cyclin-dependent kinase 4/6 (CDK4/6) and cyclin D1 complex, preventing phosphorylation of Retinoblastoma protein (Rb) and blocking the cells in G1 of the cell cycle (Figure 9A).
Figure 6: Inhibition of Class I HDACs arrests cell cycle progression in cardiac fibroblasts. A, Treatment with a pan-HDAC inhibitor increases the proportion of cells in G₀/G₁ of the cell cycle over time. B, Representative histograms of propidium iodide (PI) staining after synchronization and stimulation of neonatal rats ventricular fibroblasts (NRVF). C, Cell cycle arrest occurred with both pan- and Class I HDAC inhibitors. D, Quantification of cells outside of G₀/G₁ with a panel of HDAC inhibitors confirmed both TSA and MGCD blocked cell cycle progression as well as serum-free media; statistically significant changes from serum stimulate cells treated with vehicle are indicated. E, Similar results in adult rat ventricular fibroblasts confirmed that results were not development dependent. *=p<0.05, **=p<0.01, ***=p<0.001; Two-way ANOVA with Bonferroni post-test used for A. ANOVA with Tukey post-hoc testing used for D. Error bars show standard error of the mean (SEM).
Figure 7: Inhibition of Class I HDACs arrests cell cycle progression in cardiac fibroblasts. Cell cycle arrest in NRVFs was also noted after 32 hours of stimulation with Ang II (10 μM) and ET-1 (25nM).
Figure 8: Cell cycle arrest is dependent on inhibition of HDAC1/HDAC2. A, Histogram demonstrating propidium iodide staining in synchronized cells stimulated with full serum media in the presence of HDACi or vehicle (DMSO). Treatment with pan-inhibitor Scriptaid blocked cell cycle progression of NRVFs in G0/G1. Two HDAC1/2 specific inhibitors (BA-60 and BRD 2283) blocked cell cycle progression in G0/G1 in a dose dependent fashion. HDAC3 inhibitor BRD 3308 did not block cell cycle progression. B, Quantification of flow cytometric results. *=p<0.05, **=p<0.01, ***=p<0.001; ANOVA with Dunnett’s Multiple Comparison post-test used to identify statistically significant changes from serum-stimulate vehicle-treated control.
Figure 9: Inhibition of Class I HDACs arrests cell cycle progression in cardiac fibroblasts. A, Cyclin-dependent kinase inhibitors p15, p16, p18, p21, p27, and p57 inhibit the activity of the Cyclin D1/Cyclin-dependent Kinase 4/6 complex, preventing the phosphorylation of Rb; upregulation of cyclin-dependent kinase inhibitors has been shown to block cells in G0/G1 of the cell cycle. B, Immunoblot demonstrating that Rb is not phosphorylated in MGCD treated NRVFs (32 hours). Cyclin D1 remains elevated, and no changes are seen in p21 or p27, cyclin-dependent kinase inhibitors that have been shown to be increased by pan-HDAC inhibitors in some cell lines. C-G, Quantitative reverse transcriptase PCR (qRT-PCR) indicated an increase in p15 and p57. H, Increased protein of p15 was seen in MGCD treated NRVFs (32 hours). Adequate commercially available antibody for rat p57 was not available. I, Data indicated that cell cycle arrest was likely due to increases in p15 and p57.
Immunoblot analysis showed that cyclin D1 was present but that Rb was not phosphorylated in MGCD treated cells, confirming that NRVFs are blocked in G1 of the cell cycle. However, neither p21 nor p27 were upregulated after class I HDAC inhibition in NRVFs (Figure 9B). As recent data has demonstrated that class I HDACs can regulate the transcription of other CDKIs (65), we completed RT-qPCR to examine changes in transcription of additional cyclin-dependent kinase inhibitors p15, p16, p18, p19, and p57 using pooled samples of mRNA. Data indicated that mRNA transcript of p15 increases and the mRNA transcript of p57 was rescued after 32 hour treatment with MGCD (Figures 9C-G), as compared to vehicle-treated serum-stimulated controls. Immunoblotting confirmed that protein for p15 increased in MGCD-treated samples (Figure 9H). Unfortunately, an adequate antibody for rat p57 was not commercially available. Based on these data, class I HDAC inhibition blocks cell cycle progression in NRVFs through increasing expression of CDKis p15 and p57 (Figure 9I). All class specific inhibitors were used at doses demonstrated to be class specific in cardiac fibroblasts (Fig. 10).

**Class I HDAC Inhibition Reduces Fibrocyte Recruitment and Differentiation**

To address the effect of class I HDAC inhibitors on fibrocytes, 3-day mini-osmotic pumps were used to infuse Ang II into mice. Right and left ventricles were enzymatically digested to produce a single-cell suspension, and cells were labeled with fluorochrome-conjugated antibodies for surface antigens. Cells were then fixed and permeabilized to allow for antibody labeling of intracellular mesencymal markers Col I and αSMA. This staining protocol allowed for the identification of CD45+CD34+ cells that were also Col I+ or αSMA+, accepted markers for fibrocytes (14).
Figure 10: Inhibitor specificity in live neonatal rat ventricular fibroblasts (NRVFs). Compound specificity for HDAC class I, IIa, and IIb was assessed in live NRVFs pretreated with HDAC inhibitors for 24 hours; enzyme activity was assessed using a fluorometric assay where class specific deacetylation events increase fluorescence. IC$_{50}$ values were calculated using Graphpad Prism. Pan-inhibitor TSA inhibited class I, IIa, and IIb HDACs, while class-specific compounds MGCD (class I), DPAH (class IIa), and TubA (class IIb) showed specificity at the doses used \textit{in vitro}.
As expected, the population of fibrocytes increased in Ang II-infused mice (5;6); importantly, the population was significantly reduced in both circulation and ventricles of mice treated with MGCD (Figure 11A, 11B, 11E, and Figure 12; IgG controls shown in Figure 14). These data suggest the novel finding that a pharmacological inhibitor may be able to selectively effect this pro-fibrotic population of bone-marrow derived immune cells.

To further identify actions in fibrocytes, peripheral blood mononuclear cells (PBMCs) were isolated from human blood. Cells were allowed to adhere and mature for five days in full serum media before being stimulated with serum-free IL-4 and IL-13 (Figure 13A). Five days after stimulation, cells were harvested, fixed, and permeabilized for labeling with biotinylated anti-Col I in order to identify fibrocytes. Relative populations of Col I cells were captured using flow cytometry. Serum Amyloid P (SAP), a known inhibitor of fibrocyte differentiation, was used as a positive control. As expected, a sub-population of cells was Col I+ five days after stimulation in vehicle treated cells. The population of Col I cells was reduced in cells treated with both SAP (1 μg/ml) and 250 nM MGCD (Figure 13B-C). This supports the novel conclusion that class I HDACs are required for fibrocyte differentiation.

**Methods and Materials**

**Experimental Animals**

All animal experiments were conducted in accordance with the National Institutes Health ‘Guide for the Care and Use of Laboratory Animals’, and were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. The day prior to all studies, the mice were weighed and randomized into groups according to ranked weight distribution.
Figure 11: Population of fibrocytes was reduced in mice treated with a class I HDAC inhibitor. A, Representative graphs of fibrocytes identified in single cell suspensions of murine ventricles, identified as CD45$^+$CD34$^+$ and αSMA$^+$ or Col I$^+$ (20,000 FSC/SSC-gated cells shown in top graph, 200,000 FSC/SSC-gated cells shown in lower graph). B, Cardiac fibrocytes increased after 3-days of Ang II infusion, and were decreased by administration of MGCD (n=3-4/group). C, Representative images of cardiac monocytes, identified as CD45$^+$CD11b$^-$/Ly6G$^-$ F4/80$^-$ (graph shows all CD45$^+$CD11b$^+$Ly6G$^-$ cells). D, Monocytes, thought to be precursor cells of fibrocytes, were not reduced in animals treated with MGCD (n=8/group). E, Circulating fibrocytes increased after 3-days of Ang II infusion, and were decreased by administration of MGCD (n=4/group). F, Monocyte chemotactic protein-1 (MCP-1) expression was not reduced in animals treated with MGCD. *p<0.05; ANOVA with Tukey post-hoc testing. Error bars show standard error of the mean (SEM).
Figure 12: Inhibition of Class I HDACs reduced circulating fibrocytes. A, Representative graphs of circulating fibrocytes, defined as CD45⁺CD34⁺ and αSMA⁺ or Col I⁺ cells, from mice infused with Ang II over three days (n=4/group). Quantification is shown in Figure 11E.
Figure 13: Inhibition of Class I HDACs arrests cell cycle progression in cardiac fibroblasts. A, Circulating white blood cells were purified from blood and cultured for 5 days prior to stimulation with IL-4 and IL-13 (5 days). Col I+ cells were identified using flow cytometry. B, Adherent and non-adherent cells were collected, fixed, and permeabilized prior to labeling for Col I; Col I positive cells were reduced both by serum amyloid P (SAP) and MGCD. C, Representative graphs of Col I staining. Plots each show 10,000 events. * = p<0.05; ANOVA with Tukey post-hoc testing. Error bars show standard error of the mean (SEM).
Figure 14: Intracellular staining controls for flow cytometry

A, Intracellular IgG controls of fixed and permeabilized CD45^+CD34^+ cells identified in single cell suspensions of pooled murine ventricles.

B, Intracellular IgG control of fixed and permeabilized human white blood cells grown in vitro and stimulated for fibrocyte differentiation. Baseline represents harvested before stimulation with IL-4 and IL-13.
Anesthesia was induced with isoflurane (3% for induction, 1.5% for maintenance; 100% O<sub>2</sub>) and body temperature was maintained at 37°C. Once a plane of anesthesia was reached, the upper back of the animals was shaved and the surgical area prepped with 1% providone followed by 70% ethanol. A small, 1 cm, incision was made through the dermal layer between the shoulder blades of the mouse. Forceps were used to expand a sub-dermal pocket, and prepared osmotic mini-pumps (Alzet) were inserted subcutaneously. Incision was closed with 1-2 surgical staples. Ten week-old male C57Bl/6J mice were infused with 1.5 μg/kgBW/min Ang II for three days or two weeks; 84 mice were used in the noted studies. For the two-week studies, beginning at the time of pump implantation, mice were given daily intraperitoneal (IP) injections (all at 10 mg/kg) of either vehicle (50:50 DMSO:PEG-300), scriptaid (pan-HDAC inhibitor), DPAH (class IIa inhibitor), or a Tubastatin A (class IIb inhibitor); MGCD (class I inhibitor), a slow acting benzamide with a long half-life, was administered every other day. In three day studies, MGCD0103 was administered every second day beginning 18 hours prior to pump implantation (due to its slow action and the short duration of the study). Animals were sacrificed 20 hrs post-compound dosing unless otherwise indicated. Mice presented no health concerns associated with compound treatments. Animals were monitored daily for evidence of paleness in eyes, nose or skin, which are the most common signs of hematological toxicities. Mice were alert and conducted normal activities such as eating, drinking and grooming.

**Hemodynamic Measurements**

After two weeks, hemodynamic data was collected via carotid catheterization (Scisense). For analyses, animals were anesthetized using 2% isoflurane and body temperature was maintained at 37°C.
Tissue Procurement and Processing

After hemodynamic recordings, mice were sacrificed by exsanguination and hearts were immediately excised and perfused with ice-cold saline. RV was dissected from LV by cutting along the septum and the outer wall of the LV. LV was sectioned at the papillary muscles; the lower half was sectioned and immediately flash frozen for subsequent biochemical and gene expression analyses, while the upper half was fixed overnight in paraformaldehyde (PFA) before being transferred to 70% ethanol for storage prior to placement in paraffin block. Total RNA from LV was isolated using flash frozen tissue biopsies in Trizol (Sigma) using a Bullet Blender homogenizer (Next Advance). Protein from LV was isolated using flash frozen tissue biopsies in (PBS + 300 mM NaCl+ 0.5% Triton-X) with phosphatase and protease inhibitors (Thermo Scientific) using a Bullet Blender homogenizer (Next Advance).

Histological Analysis

Sectioned tissue was rehydrated and collagen was stained using picrosirius red (Chromaview, Richard-Allen Scientific) before sections were dehydrated and mounted for imaging. All histological analyses were carried out in a blinded manner using an Axiovert 200 inverted microscope with a digital camera equipped with AxioVision imaging software (Zeiss, Germany). Quantification of picrosirius red staining was completed by determining the average stained pixels$^2$ per total pixels$^2$ in images of the myocardium (18 images used per animal).

Flow Cytometry Using Cardiac Tissue and Blood

Digestion method was modified from (66) based on optimization. Briefly, hearts were manually sliced into several smaller pieces in HBSS/30 mM Taurine/10 mM HEPES, and placed in HBSS/30 mM Taurine/10 mM HEPES + 0.1% collagenase II +
0.5 ug/ml DNase and incubated at 37°C for 5 minutes. After incubation, tissue was manually disrupted by pipetting up and down with a trimmed 1 ml pipet; incubation and disruption steps were completed 5 times or until all tissue had been disassociated. Between incubation steps the digestion buffer was removed and the cells in suspension were placed into ice cold stop buffer (HBSS/30 mM Taurine/10 mM HEPES + 20% FBS). Cells were filtered through a 70 μm filter and washed twice in HBSS prior to staining for analysis using a BS Cantos II flow cytometer. Cells were pelleted and resuspended in FACS wash (PBS/1% BSA/0.1% Sodium azide) containing a 1:100 dilution of each of the fluorescently-conjugated antibodies including anti-CD45-V500 (BD), anti-CD11b-APC (eBioscience), anti-Ly6G-APC Cy7 (BD), anti-CD34-PE (BD), and anti-F4/80-PerCP Cy5.5 (eBioscience); surface staining was completed for 20 minutes at 4°C. Cells were washed prior to resuspension for FACS analysis if only surface labels were required. Intracellular stains were completed after washing and fixing surface stained cells; note minimal surface labels were completed for cells with intracellular labeled (typically CD45 and Cd11b only). Cells were fixed and permeabilized in Fix/Perm Buffer (Biolgend) before being stained with intracellular markers of interest (20 minutes at 4°C; Col I (Rockland) used at 1:100, and αSMA (Sigma) used at 1:250) in Permeabilization Buffer (Biolegend). Col I is biotin-conjugated, therefore, these cells were washed and underwent an additional staining step using fluorochrome-conjugated streptavidin (BD). Cells with intracellular staining were not labeled with anti-Ly6G or anti-F4/80.

Blood was collected in the presence of 500 mM EDTA from the vena cava of mice at the time of sacrifice. Surface antigens of interest were labeled with fluorochrome conjugated antibodies, as listed above. Red blood cells were osmotically lysed with Red Blood Cell Lysis Buffer (BD). Cells were fixed in Fix/Perm buffer (Biolegend) for 1 hour
before being washed and labeled with intracellular markers. Antibodies were used at concentrations reported in tissue, except for anti-αSMA-FITC (Sigma), which was used at 1:100. Data was collected and visualized for all flow cytometry experiments using FACSDiva (BD).

**Adult and Neonatal Cardiac Fibroblast Isolation**

Neonatal rat ventricular fibroblasts (NRVFs) were isolated from myocyte preparations from Sprague-Dawley rats pups (post-partum 1-2 days) as previously described (67). Adult rat ventricular fibroblasts (ARVFs) were collected by Langendorff-perfusion method from female Sprague-Dawley rats (67). Both NRVFs and ARVFs were cultured in DMEM with 20% FBS (in the presence of 100 U/ml Penicillin, 100 U/ml Streptomycin, and 29.2 μg/ml L-Glutamine) and split in 1:3 ratios; cells were used at passage two for all experiments. All cell culture supplies are purchased from Cellgro (Mediatech, Inc) unless otherwise noted.

**HDAC Activity Assay**

Cell based HDAC activity assay was completed using adherent cells in a 96-well plate (10,000 cells/well) and tissue lysate (lysate prepared as above, 60 μg/well) as previously reported (67). Briefly, for the concentration curve, cells were pre-dosed with increasing semi-log scale concentrations of compound 24 hours prior to assay. Cells/lysate was incubated in the presence of class specific 1 mM ε-N-acylated lysine derivatized on the carboxyl group with 7-amino-4-methylcoumarin (AMC) (67). After 2 hours, stop buffer (PBS+1.5 Triton-X, 3 mM TSA 0.75ug/ul) was added and the reaction was incubated for an additional 20 minutes; deacetylated substrate was available for cleavage by trypsin. Background fluorescence was subtracted and fluorescence values for compound dose response curves were calculated. In cardiac fibroblasts, GraphPad
Prism was used to calculate the IC\textsubscript{50} values for each compound and HDAC class (Fig 5A and 5B). All compounds demonstrated class-specificity as expected at the doses being used in all \textit{in vitro} experiments.

\textbf{Cell Cycle Analysis}

NRVF\textsubscript{s} were passaged at a 1:6 ratio 24 hours prior to serum deprivation in full serum media, and refreshed with serum starvation media for 18 hours in order to synchronize the cells in G\textsubscript{0}/G\textsubscript{1} (DMEM + 0.1\% Nutridoma Supplement (Roche) in the presence of 100 U/ml Penicillin, 100 U/ml Streptomycin, and 29.2 μg/ml L-Glutamine). Cells were then re-fed with either full serum media in the presence of either vehicle (DMSO) or inhibitor (concentrations as noted), and collected at at timepoints as required per experiment protocol. Samples of cells after 18 hours of serum starvation were collected as 0 hr control samples. Cell cycle analysis was completed by washing NRVFs in cold PBS followed by a brief 1 minute trypsinization. Cells were then collected and washed twice in cold PBS. Pelleted cells were fixed with the addition of ice cold 70\% ethanol with gentle shaking. All samples were kept at -20° C for less than one week prior to staining. Just prior to analysis by flow cytometry, samples were allowed to sit on ice for 30 minutes before being washed once with cold PBS. An equal amount of staining solution (50 μg/ml propium iodide (Sigma) and 100 μg/ml RNase, (Qiagen)) was added to each to sample to stain DNA. Samples were processed with a BD Cantos II Flow Cytometer (5000 FSC/SSC-gated cells were captured per sample).

\textbf{Immunoblotting}

Protein samples from cultured cells were collected in lysis buffer (PBS + 300 mM NaCl+ 0.5\% Triton-X) with phosphatase and protease inhibitors (Thermo Scientific). If not prepared and used the same day, protein samples were flash frozen, and defrosted
just prior to use. Protein lysates were sonicated for 5 seconds and kept on ice prior to centrifugation to remove debris. Protein concentrations were measured using BCA assay (Thermo Scientific) according to the manufacturer's instructions. Samples were prepared using lamelli buffer, and standardized amounts of protein were run on a 10% SDS-PAGE gel with Spectra ladder (Thermo Scientific). Gels were transferred onto a nitrocellulose membrane using standard laboratory protocols. Membrane was blocked with 5% milk in TBST buffer for 1 hour. Membrane was incubated at 4°C overnight using 1:1000 concentrations of primary antibody unless otherwise noted. Antibodies were commercially purchased as noted: α-tubulin (Santa Cruz), anti-phosphorylated Rb (Cell Signaling), anti-p15 (Cell Signaling), anti-Cyclin D1 (Cell Cycling), anti-p21 (Abcam), and anti-p27 (Cell Signaling). HRP-conjugated secondary antibodies (Southern Biotech) were used at a concentration of 1:2000.

**Gene Expression Analysis**

mRNA was purified from cells lysed in Trizol (Sigma) or from homogenized tissue (as noted above) followed by 1-bromo-3-chloropropane (BCP) phase separation of nucleic acids. The nucleic acid aqueous phase was collected, and RNA was quantified using a nano drop spectrophotometer, and standardized amounts of RNA were used to generate cDNA using a Verso kit (Thermo Scientific). For reverse transcription quantitative PCR (RT-qPCR), aliquots of cDNA were pooled and standards were made by serial dilutions (1:5). Samples were run per manufacturer's enzyme cycling recommendations on an Applied Biosciences StepOne using DyNamo Flash SYBR Green-Rox (RT-qPCR; Thermo Scientific). Primer sets were designed and run with 18s as a housekeeping gene. Relative copies of message of interest were normalized to 18s. Primers used are listed: mouse/rat 18s (F: 5'-GCCGCTAGAGGTAAATTCTTG-3', R:
5'-CTTTGCCTCTGGTCCGTCTT-3'), mouse MCP-1 (F: 5'-GCTCAGCCAGATGCAGT
TAACGC-3', R: TGGGGTCAGCACAGACCTCTCTCTCTCTCTCTCAGA-3'), rat p15 (F: 5'-ATCCCAACGCCGTCAAC-3', R: ATCATGCACAGGTCTGGTG-3'), rat p16 (F: 5'-TGCAGATAGACTAGCCAGGG-3', R: 5'-AGAGCTGCCACTTTTGACG-3'), rat p18 (F: 5'-GAACCTGCCCTTGTGCAAC-3', R: TCCCCCAACCCCATTCGCCTC-3'), rat p19 (F: TGGGGTCAGCACAGACCTCTCTCTCTCTCTCAGA-3', R: 5'-TTTGGTACAAGTAACCCA-3'), and rat p57 (F: 5'-GGAGCAGACGAGAATCAG-3', R: 5'-TTGCACGACCCCTTGTCCAGTCC-3'; IDT).

In Vitro Fibrocyte Differentiation Assay

Human white blood cells were provided by the Stenmark lab, and were collected according to a COMIRB approved protocol. Red blood cells were lysed using Red Blood Cell Lysis Buffer (BD), and cells were washed in PBS + 0.1% FBS two times prior to plating in DMEM +20% FBS +PGS. After 5 days, adherent cells were washed in PBS, and stimulated with serum free media with or without IL-4 (10 ng/ml), IL-13 (10 ng/ml) in supplemented serum-free media (DMEM+10 uM HEPES, Pen/Strep/L-Glut, non-essential amino acids, 1:1000 nutridoma, 10ng/ml IL-4, 10 ng/ml IL-13(PEPROTECH)). Cells were differentiated in the presence of vehicle (DMSO), MGCD (250 nM), and SAP (1 μg/ml). Samples of cells before and after stimulation were collected and used for flow cytometric analysis. Cells were fixed in FoxP3 Fixation/Permeabilization buffer for one hour (Biolegend), and incubated with biotinylated anti-Col I (Rockland) followed by PerCP labeled streptavidin. Pooled IgG controls and unstained samples were used to set gates for flow cytometry.

Statistical Analysis

Results are expressed as mean ± SEM if not otherwise noted. Analysis was completed by ANOVA followed by post-hoc testing (Tukey’s test was used for post-hoc
analysis unless otherwise noted); statistical analysis was completed using GraphPad Prism software. Statistical significance (α defined as 0.05) is reported as applicable.
CHAPTER III
CLASS I HDAC INHIBITION INCREASES THE RELATIVE POPULATION AND SUPPRESSIVE ACTIVITIES OF T REGULATORY CELLS

Results

Previous research has indicated that the T\textsubscript{reg} population is reduced in patients with HFpEF (14). Researchers have found that adoptive transfer of isolated T\textsubscript{regs} from healthy mice into mice with Ang II infusion pumps of T\textsubscript{regs} reduces cardiac fibrosis, potentially through regulation of oxidative stress and inflammation (49;50). Our preliminary data indicate that circulating T\textsubscript{reg} numbers increased both three and seven days after Ang II mini-pump implantation and IP dosing of MGCD (Fig 15A; T\textsubscript{regs} identified as CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} by flow cytometry).

\textit{In vitro} experiments completed by collaborators in the Hancock laboratory (University of Pennsylvania) demonstrated that MGCD treated T\textsubscript{regs} were able to significantly suppress proliferation of effector T cells at a 10 nM dose (Fig. 15B), as assessed by surface-labeling using carboxyfluorescein succinimidyl ester (CFSE) fluorescence in proliferating T helper cells. Additionally, mice treated with MGCD had decreased MCP-1 expression that correlated with relative populations of circulating T\textsubscript{regs} (Fig. 16).

These data suggest that treatment with MGCD may increase the anti-inflammatory population and function of T\textsubscript{regs}. We hypothesize that the effects on T\textsubscript{regs} may act to increase the anti-fibrotic effects of class I HDAC inhibition, perhaps acting synergistically with the inhibitor’s effects on cardiac fibroblasts and bone marrow-derived fibrocytes. Additional experiments have been proposed in the discussion portion of this.
thesis in order to delineate the importance of these findings in relation to the anti-fibrotic effects of the class I HDAC inhibitor.

**Methods and Materials**

**Flow Cytometry of Circulating T\textsubscript{regs}**

Blood was collected from the vena cava of mice in the Ang II infusion studies at the time of sacrifice, as noted in the previous chapter. Cells were fixed and antibody labeled as described in Chapter II. All antibodies were used at a concentration of 1:100. Surface antigens of interest were labeled with fluorochrome conjugated antibodies to CD45 (V500, BD), CD3 (PB, Biolegend), CD4 (FITC, eBioscience), CD8 (APC-H7, BD), and CD25 (APC, eBioscience). Red blood cells were osmotically lysed and cells were washed in FACS buffer. Cells were fixed in FoxP3 Fix/Perm buffer (Biolegend) for 1 hour before being washed and labeled with PE conjugated anti-FoxP3.

**T Effector Cell Suppression Assay**

T effector cell suppression assay was completed by collaborators at the University of Pennsylvania. Active T\textsubscript{regs} suppress the growth of effector T cells (T\textsubscript{effs}) in the presence of activated antigen presenting cells (APCs). Briefly, CD4\textsuperscript{+}CD25\textsuperscript{+} T cells (T\textsubscript{eff}), antigen presenting cells (APCs), and CD4\textsuperscript{+}CD25\textsuperscript{+} T cells (T\textsubscript{reg}) were purified using magnetic bead columns from pooled mouse lymph nodes and spleens. Carboxyfluorescein succinimidyl ester (CFSE) labeled T\textsubscript{eff} cells were stimulated for 72 hrs with anti-CD3 (0.5 \(\mu\)g/ml) in the presence of irradiated APCs and vehicle or MGCD (10 nM, based on optimization). Proliferation of T\textsubscript{eff} cells was measured through reduction of CFSE fluorescence during clonal expansion using flow cytometry.
Figure 15: Inhibition of class I HDAC increased the population and suppressive capacity of regulatory T cells. 

A, Class I HDAC inhibition increases circulating regulatory T cells in Ang II-infused animals compared to animals treated with vehicle alone (vehicle or compound given via daily IP injection; dose 10 mg/kg). Three day study: n=4/group; Seven day study: n=3/group. *P < 0.05 vs. AngII + vehicle. 

B, Pilot study suggested class I HDAC inhibition increases regulatory T cell population in the heart of Ang II treated animals compared to vehicle alone (n=1/group). Follow-up studies have been proposed in aim 1 of this proposal. 

C, Murine Tregs treated with a 10 nM dose of MGCD in vitro were more able to suppress the proliferation of T effs (as assessed by CFSE proliferation assay, where CFSE fluorescence is reduced with clonal expansion).
Figure 16: Reduction of MCP-1 correlates with relative population of T regulatory cells in animals treated with MGCD. Increase in the proportion of T_{reg}s seen after treatment with a Class I HDACi for three days correlated with decreases in expression of MCP-1, indicating that these cells may have strong anti-inflammatory effects in this model. Three day study: n=4/group; R²= 0.982, P < 0.009 by linear regression.
CHAPTER IV

PITFALLS AND FUTURE DIRECTIONS

Cardiac Fibroblasts

Current studies have not yet confirmed the molecular mechanism of class I HDAC inhibition for cell cycle arrest of cardiac fibroblasts. Current data suggests that this arrest is dependent on transcriptional upregulation of p15 and p57. In order to confirm if this is the case, I would propose using short hairpin RNA of p15 and p57 in an adenoviral system. NRVFs would be infected with adenovirus containing shRNA for p15, p57, p15/p57, and scrambled control. Cell cycle progression experiments would then be repeated; if cell cycle arrest was no longer seen with any of these conditions, it could be concluded that cell cycle arrest by HDACi is dependent on CDKi regulation. To further delineate HDACi regulation of CDKis as sufficient for blocking the activity of the Cyclin D1/CDK4/6 complex, in vitro kinase assays would be performed. Cyclin D1 and/or CDK4 would be immunoprecipitated from lysed cells and kinase activity tested using radiolabeled GST-Rb, a downstream Cyclin D1 dependent kinase.

Data generated from the above experiments would confirm a role for CDKis on cell cycle inhibition in response to class I HDACi. To delineate that canonical epigenetic regulation of a cyclin-dependent kinase inhibitor is responsible for the arrest of cardiac fibroblasts, I propose that we complete chromatin immunoprecipitation experiments (ChIP) examining the CDKi promoter. We expect that ChIP analysis will lead to the confirmation that class I HDAC inhibitor in cardiac fibroblasts block the deacetylation of histones in the promoter region of CDKis p15 and p57 in response to class I HDACi. It is possible that the promoter region with which the enzyme is interacting has not been
identified, and that qPCR with our designed primers will not be successful. If this is the case, we will consider using a ChIP-Seq approach after IP of HDAC1/2.

Fibrocytes

Reviews of the literature demonstrate that molecular work on fibrocyte differentiation is in its early stages; for example, differentiation is still assessed by morphological phenotype and expression of broad markers (for example, CD45 and Col I). At this time, more specific surface markers or transcription factors are not known. To date, studies have demonstrated that monocyte to fibrocyte differentiation is blocked with serum amyloid P (SAP). SAP is able to prevent fibrocyte differentiation through its actions on the Fc-gamma receptors FcRγ and FcγRI (68;69), although the downstream molecular mechanism(s) of action remain largely undefined. Data presented in this thesis are the first to demonstrate that HDACi block fibrocyte differentiation in vitro and impact fibrocyte populations in vivo. Additional work is needed to address how class I HDAC inhibitors are blocking cell differentiation, as well as to confirm that the effect is not due to selective cell death. Thus, I would propose the following goals for future experiments:

A) Characterize of the effects of class I HDAC inhibition on monocyte to fibrocyte differentiation and cell death.

B) Define of the molecular role of Class I HDACs in monocyte to fibrocyte differentiation.

Aim A

Peripheral blood mononuclear cells (PBMCs) will be collected from the buffy coat of human blood using a Ficoll gradient, and allowed to adhere for five days in full-serum DMEM. Cell differentiation assays will be completed after removing CD2 and CD19 (B
and T cells) using a bead purification kit. After bead purification, cells will be replated for 24 hours before undergoing stimulation with IL-4 and IL-13 in the presence of 250 nM MGCD or vehicle. Additionally, isoform selective inhibitors MS-275 (HDAC1/2), BRD 2283 (HDAC1/2), and BRD 3308 (HDAC3) will be used in order to identify if fibrocyte differentiation is dependent on HDAC1/2 or HDAC3. Adherent and non-adherent cells will be collected; live cells will be used for annexin-PI staining to address cell death, while similar numbers of cells will be fixed and permeabilized to allow for staining with CD45 and Col I to examine differentiation of the population. Assays will be assessed by flow cytometry on a BD Cantos II. Dose response curve using MGCD at 10 nM, 30 nM, 100 nM, 300 nM, 1000 nM, and 3000 nM will be completed in order to calculate an ED\text{50} and LD\text{50}.

Based on previous experiments, we expect that MGCD will block fibrocyte differentiation, and we expect to be able to calculate an ED\text{50} value for MGCD. As heterogeneous populations of immune cells are present in the method currently being used, it is possible we will need to use a more advanced method of cell selection, such as positive selection of CD14\text{+} or CD34\text{+} populations using magnetic bead columns. Additionally, trypsinization can damage surface markers; if this is noted, we will optimize for another method of retrieving live cells (for example, icing the cells, or using a different enzyme to release the cells from the plate).

**Aim B**

Currently, pathways involved in fibrocyte differentiation are not well defined. Literature reviews have shown that mitogen-activated protein (MAP) kinase signaling and Rho Kinase-1 (ROCK-1) signaling are necessary for the differentiation of fibrocytes (70-72); for example, sustained phosphorylation of ERK has been shown to be necessary in
monocyte to fibrocyte differentiation (72). Interestingly, a recent study noted regulation of ROCK-1 by another class I HDAC inhibitor (MS-275) in breast cancer cells. Other projects in our laboratory have revealed that HDAC inhibition regulates kinases through epigenetic regulation of phosphatases or post-translational protein modification (publications pending). We hypothesize that class I HDAC inhibition regulates MAP kinase or ROCK-1 kinase pathway activity. In order to address these hypotheses, we will complete the following experiments:

(i) Immunoblot analysis to determine the phosphorylation status of MAP kinases ERK, p38, and JNK.

(ii) Human qPCR array (SABiosciences) to determine if downstream signaling of MAPKs are affected, and

(iii) Immunoblot analysis of ROCK-1 phosphorylation and kinase activity. Kinase activity will be assessed by immunoblotting for phosphorylated Myosin Light Chain (MLC) of myosin II, a downstream target of ROCK-1.

If one of these initial pathway experiments is successful, we will identify the molecular role of class I HDACs in fibrocytes.

Immunoblot analysis will be completed using protein lysates from harvested human fibrocytes differentiated in the presence of IL-4 and Il-13, and will allow us to determine if any of these critical pathways have been affected in MGCD treated cells. Blots will be probed for total and phosphorylated ERK, p38, JNK, ROCK-1, and Myosin Light Chain (MLC) of myosin II (downstream target of ROCK-1; (73)).

In order to determine if signaling downstream of MAP kinases has been affected (for example, phosphorylation and subsequent activation of a transcription factor), we will pool mRNA from 5-8 biological samples. Pooled mRNA from non-differentiated,
differentiated, and MGCD treated cells will be used to produce cDNA for use on SABiosciences human MAP kinase signaling PCR arrays. In addition to providing information about message changes to MAP kinases and scaffolding proteins, this array will provide information about downstream transcription factors and their activity. The PCR array will be completed by the same method noted in the preliminary methods and materials.

Additionally, the target gene array will allow us to determine if MAP kinase signaling remains intact, or if a transcription factor downstream of one of these kinases has been affected. Based on significant changes in MAP kinase signaling that we have noted in other cell lines treated with a class I HDAC inhibitor (data unpublished), we hypothesize that changes to fibrocyte differentiation will be regulated through an epigenetically inducible phosphatase such as Dusp5. If changes in signaling are noted at the phosphorylation level, we will complete a human-specific phosphatase array (SABioscience) to identify a potential target. While we hope to see changes in one of the above signaling pathways, we acknowledge the possibility that class I HDAC inhibition is affecting monocyte to fibrocyte differentiation through a different mechanism.

Of note, working with human peripheral white blood cells has proved a challenge during the optimizing phase. In our hands, MGCD appears to facilitate the differentiation of a long, thin, Col I- cell type that appears to form ‘web-like’ patterns. Based on morphology, it has been hypothesized that these may be early neuronal-type cells. We plan to complete experiments to confirm the identity of these cells. Based on preliminary work, the appearance of this cell type is likely a HDAC1/2-dependent effect, while the reduction of fibrocyte differentiation may be an HDAC3-dependent effect. Additional experiments are needed to confirm these preliminary findings.
T Regulatory Cells

Current experiments do not delineate the role of specific cell types in the anti-fibrotic effects noted. In order to determine if effects of class I HDAC inhibition are dependent on T\textsubscript{regs}, we plan to conduct experiments to ablate T\textsubscript{regs} \textit{in vivo} prior to Ang II pump implantation. These critical experiments will be approached through two avenues, ablation through opsonization of T\textsubscript{regs} using an antibody for CD25 (resulting in T\textsubscript{reg} destruction by natural killer cells), and through a genetically modified mouse model that allows for specific destruction of cells with high FoxP3 activity. Briefly, male C57B/6J mice will be treated with 100μg anti-CD25 (clone PC61, eBioscience) via IP injection 1 day prior to Ang II pump implantation (Alzet) and every 7 days following (58). Mice will be divided into 6 groups: Control/Veh, Control/CD25/Veh, Ang II/Veh, Ang II/CD25/Veh, Ang II/MGCD, Ang II/CD25/MGCD (n=5/group). Mice will be dosed daily with compound or vehicle for 14 days. While ablation with anti-CD25 is commonly used, in some cases T\textsubscript{regs} are not completely ablated from tissue and lymph nodes.

Results of the antibody ablation experiment will be confirmed in the DEREG (DEpletion of T REGulatory Cells) mouse; in this mouse, the diphtheria receptor is expressed under the control of the FoxP3 promoter (74). DEREG mice will be treated with 1 μg (IP) diphtheria toxin 3 days prior to Ang II pump implantation, which will selectively kill T\textsubscript{regs} expressing FoxP3 (model will be conducted in collaboration with the Eltzschig group). Hemodynamics will be captured just prior to sacrifice; tissue and blood samples will then be collected for biochemical/molecular studies. Histology staining using picrosirius red will be used to quantify the development of fibrosis.

I hypothesize that fibrosis will be increased in the Ang II-infused mice with ablation of T\textsubscript{regs}, and that the anti-fibrotic effects of MGCD may be reduced in animals.
without T\textsubscript{regs}. Using both ablation models will assist us in making a strong conclusion about the role of T\textsubscript{regs} in cardiac fibrosis. If anti-fibrotic effects of class I HDAC inhibition are unchanged in the absence of T\textsubscript{regs}, this would suggest that the inhibitor’s direct effects on fibrocytes and cardiac fibroblast were sufficient to block the development of cardiac fibrosis.

Additionally, the mechanism through which MGCD increases the suppressive function of T\textsubscript{regs} is not understood. In the future, we plan to determine if regulation of FoxP3 activity is the mechanism(s) by which class I HDAC inhibition enhances T\textsubscript{reg} numbers and function, as this transcription factor is highly enriched in T\textsubscript{regs}, and its stability and activity is very important for both T\textsubscript{reg} differentiation and immune suppressive function (75). Experiments would seek to address possible mechanisms of FoxP3 regulation by class I HDAC inhibition (76), including increased FoxP3 activity, increased acetylation of FoxP3 (which serves to increase protein stability), or epigenetic regulation of a known acetylation site on histone H4 of the foxP3 locus. Proposed experiments include: (i) luciferase-reporter assays for FoxP3 activity, (ii) assays to determine acetylation status of FoxP3, and (iii) chromatin immunoprecipitation (ChIP) assays.

FoxP3 activity will be assessed through its ability to act as a transcriptional repressor. FoxP3 decreases luciferase production when bound to the 8 forkhead-binding sites upstream of a luciferase reporter (vector 8xFK1tk-luc) (56). Murine CD4\textsuperscript{+}CD25\textsuperscript{+} T cells (T\textsubscript{reg}) from control or MGCD-treated mice (Ang II-infused, vehicle or MGCD given IP for 7d) will be purified on MACS columns (Mitenyi Biotec) from pooled mouse lymph nodes and spleens (n=3/group) (54); please note FoxP3 is not used as a marker during isolation of live T\textsubscript{regs}, since intracellular staining requires fixation. Cells will be transfected
through electroporation (77) with 8xFK1tk-luc and *Renilla* luciferase control vector (55) using an Amaxa mouse T cell Nucleofector Kit (Lonza) (78). FoxP3 activity will be assessed through the reduction in firefly luciferase activity normalized to the *Renilla* luciferase transfection control using a dual luciferase activity assay (Promega) three days after transfection.

Acetylated FoxP3 is less likely to be polyubiquitinated and degraded by the proteasome, resulting in dramatically increased FoxP3 protein stability (55). FoxP3 will be immunoprecipitated (IP) from lysates of T\textsubscript{regs} isolated by the method outlined in 2a(i). Protein concentration will be quantified, and standardized quantities will be immunoprecipitated with rabbit anti-FoxP3 antibody. Immunoprecipitates will be denatured, run on an SDS-PAGE gel and immunoblotted with mouse anti-acetyl-lysine. Blots will be stripped and reprobed with mouse anti-FoxP3 to confirm equal levels of immunoprecipitation. Signals will be captured with an Alpha Innotech FluorChem HD2 imager and quantified using ImageJ.

Chromatin from T\textsubscript{regs} will be collected, sheared, and subjected to immunoprecipitation with a panel of antibodies recognizing distinct histone marks (e.g., acetyl-H3K4 or methyl-H3K9). Immunoprecipitates will be incorporated into PCR reactions containing primers that span the previously described regulatory regions in the *foxP3* promoter (76). Control experiments will include input without immunoprecipitation, and samples immunoprecipitated with an antibody that does not recognize histones.

If class I HDACs regulate acetylation of FoxP3, we would expect increased acetylation of FoxP3 in cells isolated from animals treated with MGCD. It is also possible that the acetylation status of the protein is unchanged, but that elevated levels of FoxP3 will be present due to epigenetic regulation of the *foxp3* promoter by MGCD. If
completion of this aim determines that suppressive function is independent of FoxP3 levels or activity, we plan to design experiments to address other mechanisms of \( T_{\text{reg}} \) suppressive function, including relative production of anti-inflammatory cytokines such as IL-10 and levels of surface receptors such as Galectin-1 and CTLA-4 (79), as these targets may be epigenically regulated by class I HDACs.

**Discussion**

Importantly, these data describe *in vivo* efficacy of selective, class-specific HDAC inhibitors in a model of fibrotic remodeling in the heart. To our knowledge, this report (upon publication) will be the first study to demonstrate efficacy of class I HDAC inhibitors in a preclinical model of cardiac fibrosis. Data presented in this thesis demonstrated that the class I HDAC inhibitor MGCD0103 reduced Ang II-induced cardiac fibrosis in a manner that correlated to inhibition of cardiac fibroblast proliferation, decreased fibrocyte recruitment and differentiation, and increased relative \( T_{\text{reg}} \) populations and activity (Fig. 17). Of note, we demonstrated that while fibrotic remodeling was prevented, Ang-II induced hypertension was not impacted by Class I HDAC inhibition, demonstrating that prevention of cardiac fibrosis was independent of blood pressure. As MGCD0103 (mocetinostat) has completed early stage clinical trials for cancer and is tolerated in humans, these data highlight the translatable potential of isoform-selective HDACi in cardiac remodeling and HFP EF.

Cardiac fibroblasts are well-known effector cells of fibrosis. In response to profibrotic stimuli, fibroblasts in culture have been shown to proliferate, migrate, increase production of extracellular matrix proteins (such as collagen), and transdifferentiate into an alpha-smooth muscle actin- (αSMA) and fibronectin-positive myofibroblasts (3;4;31;46). Pharmacological targeting of fibroblast activation has been suggested
Based preliminary data, we hypothesize that class I HDAC inhibition blocks the development of fibrosis through effects on both circulating and resident tissue cells.

Figure 17: Model of anti-fibrotic activity of class I HDAC inhibition. Based preliminary data, we hypothesize that class I HDAC inhibition blocks the development of fibrosis through effects on both circulating and resident tissue cells.
as a therapeutic target in the development of compounds that block or reduce fibrosis. A number of signals, including Ang II and ET-1, have been shown to stimulate fibroblast proliferation in vitro (31;80). As pan- and class I HDAC inhibitors are known inhibitors of cancer and smooth muscle cell proliferation (63-65;81), experiments were completed to determine if HDAC inhibition also blocked cell cycle proliferation in cardiac fibroblasts. Subsequently, we demonstrated that class I HDAC inhibitors completely arrest cardiac fibroblasts in G1 of the cell cycle, likely through the epigenetic regulation of CDKis p15 and p57, as demonstrated up the upregulation of p15 (message and protein) and p57 (message) in the presence of a class I HDAC inhibitor.

Recent evidence has demonstrated a critical role of the immune system in the development of cardiac fibrosis (6;22;37); for example, monocyte chemotactic protein-1 (MCP-1) knock-out mice infused with Ang II had greatly reduced development of cardiac fibrosis (6). Interestingly, the authors hypothesized that the effects were due to a reduction in the recruitment of a profibrotic, bone-marrow derived fibroblast-precursor population known as fibrocytes (14). Fibrocytes are spindle-shaped bone-marrow derived cells which express leukocyte and mesenchymal markers; these cells have recently been identified as key effector cells of chronic inflammation and fibrosis (14). Fibrocytes can be found in tissue as well as in circulation, but lose leukocyte and precursor cell markers, such as CD45 and CD34, over time as they differentiate in tissue (40). MCP-1 and stromal cell-derived factor-1 (SDF-1) are thought to be key signaling molecules in the recruitment and homing of these cells (6;38). Importantly, treatment with a class I HDAC inhibitor did not blunt MCP-1 expression or monocyte recruitment in our mouse model of fibrosis.
As CD14⁺ human monocytes isolated from peripheral blood mononuclear cells (PBMCs) are able to differentiate into fibrocytes (38;39); it is therefore thought that fibrocytes are monocyte derived. However, fibrocyte populations were decreased in response to class I HDAC inhibition in the blood and heart of Ang II-infused mice. These data suggest that MGCD is not effecting recruitment of precursor monocytes to the heart, but differentiation of monocytes to fibrocytes.

By targeting the recruitment or differentiation of these cells, it may be possible to target the development and progression of cardiac fibrosis. Intriguingly, in an ischemia-reperfusion mouse model of ischemic cardiac disease, injection of the mice with Serum Amyloid P, a serum protein shown to block fibrocyte differentiation, prevented the development of both cardiac fibrosis and systolic dysfunction (33) without decreasing overall immune cell recruitment. To our knowledge, the work presented in this thesis is the first demonstrating that a small molecule inhibitor may selectively target fibrocytes without effecting initial inflammation in vivo. Unfortunately, single specific surface markers and transcription factors have not been identified in this cell population to date, preventing experiments confirming that inhibitor effects on fibrocytes are sufficient for the prevention of fibrosis. If such proteins are identified, experiments manipulating this population of cells could be completed using antibody opsonization or genetic models of deletion.

While fibrocytes are currently under intense investigation due to the role in many human diseases, much remains unknown about pathways involved in their differentiation. As a rare population of cells among circulating PBMCs, basic experiments using standard biochemical and molecular techniques have remained challenging. In the future, we hope to further optimize the isolation of these cells in order to delineate the
molecular role of class I HDACs in these cells. Additionally, our current findings in regards to regulatory T cells are interesting and warrant further pursuit. Inbalances of both Treg and fibrocytes have been noted in patients with hypertensive heart disease and HFpEF (43;48), supporting the rationale for translational studies using class I HDAC inhibitors in rodent models of diastolic heart failure.

Each year, more than 600,000 cases of heart failure (HF) are diagnosed in the United States, adding to the more than five million adults with this condition; costs of care are estimated at $34.8 billion per year (19). About half of these patients are diagnosed with heart failure with preserved ejection fraction (HFpEF), or diastolic heart failure; pharmacological therapies to treat HFpEF are not currently available. This work contributes to a rationale to test class I HDAC inhibitors in models of diastolic dysfunction.

We would like to emphasize the significant novel findings that class I HDAC inhibition prevents the development of cardiac fibrosis and the differentiation of fibrocytes. We feel that the differentiation of fibrocytes is an especially important finding as these cells have been shown to have pathological roles in many diseases (14) including asthma(82;83), rheumatoid arthritis (84), pulmonary fibrosis (85;86), acute lung injury (87), long term organ rejection (88), and metastatic cancer (89;90). Additionally, it is of note that fibrotic disease is a leading cause of death worldwide, and no pharmacological therapies are currently available (91). As fibrocytes have been shown to play an important role in the development of fibrosis in many tissues, we hypothesize that class I HDAC inhibition may be clinically beneficial as an anti-fibrotic therapy.
Clinical and Translational Fellowship

During my time as a graduate student, I was awarded a pre-doctoral fellowship with the Colorado Clinical and Translational Sciences Institute (CCTSI). As part of this fellowship, I participated in clinical shadowing with a physician mentor, completed coursework aimed at understanding human disease and clinical studies, and eventually drafted a protocol and consent form for use in a clinical study. Additionally, I participated in monthly discussions and the annual Translational Science Meeting, which took place at the Mayo Clinic in May 2012. I very much enjoyed working with Dr. Peter Buttrick, and especially appreciated our meetings to discuss both my clinical questions and career direction.

Summary of Clinical Experiences

My clinical experiences occurred in various portions of the cardiology department at University Hospital. Through participation in this program, I was able to shadow Cardiology Rounds, Heart Failure Rounds, echocardiographers, physicians in the Catheter Lab, and physicians caring for patients in the outpatient clinic. Hospital rounds greatly expanded my clinical understanding of heart disease and treatment – many of the patients discussed had complicated histories and multiple comorbidities and medications, which is much different than single indication animal studies that I have conducted. Given the basic studies I was completing, it was especially interesting to learn about patients who were developing or were currently in various types of heart failure. For example, I learned that anterior wall myocardial infarctions can lead to both ventricular tachycardia and heart failure. Dr. Buttrick was especially helpful in meeting with me to discuss questions I had about the care and disease progression in these
patients, as well as what the current standard of care and where additional research is especially useful.

The McKinsey lab currently runs the mouse hemodynamic facility, and staff members regularly complete echocardiograms of mice and rats. During my time in the clinic, I was able to shadow echocardiographers while they worked with human patients. This time greatly increased my understanding both echocardiography and heart structure. It was especially interesting to have had the opportunity to observed echocardiographs of diseased adult hearts as well as congenital defects in neonatal infants. Some of the infants had quite complex changes in their cardiac physiology; this was of particular interest as our laboratory collaborates with a pediatric cardiologist who is interested in hypoplastic left heart syndrome.

While shadowing echocardiographers, I was also able to watch cardiac stress tests, and learn when they are appropriate and what they are good at detecting. I learned that stress tests are a sensitive way of testing for intermediate level problems, and are useful for calculating risk for patients with indications such as unstable angina. I also learned contraindications for stress testing, which included possibility of current myocardial infarction, significant valvular disease, and a test whose results were unlike to change patient management (for example, in a patient that was 97 years old). Use of microbubble injections allowed for the development of an even better understanding of normal and abnormal blood flow through the heart. Usefully, I learned that the sensitivity of imaging is about 80% by echocardiogram, 60% by EKG, and 90% by stress test.

I was also fortunate to observe catheter procedures. The procedures allowed for excellent visualization of the coronary arteries and plaques, which was extremely
interesting to see. It was exciting to learn how stents and angioplasty balloons are used in patients.

As a graduate student, we do not typically have the ability to interact with patients. During this program, I was able to shadow physicians and interact with patients who were currently in heart failure. In particular, I remember interactions with several patients that I met during this training. One patient was nearly my own age, and had been diagnosed with noncompaction cardiomyopathy, and was waiting for a heart transplant. Another patient had previously been transplanted, and was in for a check-up visit. A third needed a transplant but did not qualify for health coverage due to citizenship issues. Between all of my discussion with these and other patients, most of them mentioned changes in their quality of life, something that we aren’t able to consider very well during animal studies. This emphasized the importance of having good quality of life assessments for use during clinical trials, and for developing good endpoints for clinical studies. All of the patients were interested in hearing about what was being done to help patients in the future, and were very interested in quick bits of information about projects in the laboratory and how basic and translational research can lead to improvements in care.

As my research project progressed, it was clear that we would need access to populations of immune and progenitor cells. As this population is such a small percentage of circulating cells, the current standard of practice for researching this population of cells is to use circulating white blood cells from human blood. While a collaborator initially provided some of these cells, our lab needed to develop a clinical protocol to obtain samples of these cells in a more consistent manner. Due to my previous experiences in clinical research and participation in this program, we decided to
draft a protocol, consent form, and study documents in order to request small blood
donations from healthy patients. For brevity, basic study information including
inclusion/exclusion criteria is noted below; additional documents are available upon
request. Application should be submitted for expedited COMIRB approval shortly. We
plan to use the samples to complete future studies on the role of HDACs in fibrocytes,
and hypothesize that this research could lead to important rationales for the use of class
I HDAC inhibitors in fibrotic diseases in the future. Using cells from multiple subjects
should help us to confirm that the results are applicable to the population rather than a
sub-group of patients.

**Brief Study Overview**

The primary goal of this work is to define the molecular roles of class I HDACs in
differentiation of fibrocytes. This goal will expand scientific understanding of molecular
pathways involved in the development of fibrosis, and provide reasoning for the potential
future use of class I HDAC inhibitors for the treatment of cardiac fibrosis and HFpEF.
Isolation of these cells from human blood is currently the best and accepted method of
completing differentiation method using peripheral blood mononuclear cells.

Study plans to generate pre-clinical data that may provide reasoning for using
class I HDAC inhibitors in the future treatment of cardiac fibrosis. No clinical outcomes
will be measured as this protocol has no potential health benefits for study patients. Data
generated from *in vitro* studies using cells obtained from subjects will not be paired with
health information provided by the study subjects. We will not compare genetic
sequences from cells from different study subjects.

Study criteria will include the following inclusion criteria: (1) healthy adult over
110 lbs and (2) comfortable with blood draws. Study exclusion criteria will include: (1)
age > 18 years, (2) prisoners or other persons unable to give consent, (3) pregnant women, (4) known infection with blood borne pathogens such as HIV or Hepatitis, (5) known anemia, (6) currently using medications for the management of a chronic disease or condition (low dose aspirin and birth control allowed), (7) acute illness (such as the flu or a respiratory infection), (8) and illegal drug use.

Study participation will last up to three years. If the study ends, participation will be discontinued. Study visits will be scheduled every 2-12 weeks. No more than 200 ml of blood, or a little less than a cup, will be drawn in an 8 week period. If more than 12 weeks passes between visits, subjects will be reconsented to the study and assigned a new subject number. If subjects wish to stop participating in the study, they may withdraw from the study at any time.

**Summary and Impact**

In summary, preliminary data suggests that class I HDAC inhibitor MGCD suppresses the development of cardiac fibrosis through a mechanism that is associated with decreased cardiac infiltration and decreased differentiation of pro-fibrotic monocyte-derived fibrocytes, increased anti-inflammatory T\textsubscript{reg} numbers, and decreased proliferation of resident fibroblasts (Fig. 11). As no adequate therapies are currently available for the management of HFpEF, there is a crucial need for the development of safe and effective treatments; this need is likely to increase due to an aging population. In order to progress towards this translational goal, in the future we plan to test MGCD in a rodent model of diastolic dysfunction, the Dahl Salt-Sensitive Rat. These rats develop hypertension followed by diastolic dysfunction over several months when fed a high salt diet. Serial echocardiograms will be used in this model to assess changes in diastolic function in both prevention and regression studies.

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This proposal seeks to identify the molecular mechanism through which class I HDAC inhibition is preventing the development of cardiac fibrosis, and plans to identify roles in the regulation of resident and circulating pro-fibrotic cells. Under the supervision of my mentor, I plan to complete this project over the next three years, which is reflected in the timeline listed below. We have established key collaborations with experts in the fibrocyte and T_{reg} field that are located within the same building as our own laboratory, and from whom I have received training. Continued training with these groups will allow me to develop competencies in new techniques as well as promote the efficient completion of these aims that will lead to peer-reviewed publications and presentations at national/international meetings. I am excited about the therapeutic potentials being investigated in this proposal, and am eager to continue my work on this project.
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