BMI1 MEDIATES CHROMATIN REMODELING AND PATHOLOGICAL FIBROSIS FOR CARDIAC REPAIR AFTER MYOCARDIAL INJURY

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ABSTRACT

Myocardial injury leads to scar formation and pathological fibrosis that has a significant impact on the development and progression of cardiac disease. Increasing evidence suggests alteration in the chromatin landscape of cells can exacerbate the extracellular matrix deposition and enhance disease progression. Chromatin alterations and fibrosis mediate several cardiac cellular changes, including scar formation, DNA damage, collagen deposition, and increased TGFβ expression which are all disease-driving mechanisms during heart failure. Targeting epigenetic dependent fibrosis pathways is thus a promising strategy for the prevention and treatment after myocardial injury. The polycomb complex protein Bmi1, an epigenetic regulator, is associated with numerous biological functions including mediating DNA damage, cellular fate, and proliferation. However, there is currently a lack of understanding on how Bmi1 mediated epigenetic modifications affect adult heart function after injury. It was previously determined that Bmi1 modulates the epigenetic landscape of cardiac stem cells that mediates various molecular processes during a stress condition. In the present study, using a Bmi1 global and fibroblast specific knockout model, cardiac function was assessed through echocardiography using adult mice following cardiac injury. The loss of Bmi1 caused a significant decrease in heart function after injury, which was associated with increased fibrosis and DNA damage. Specifically, we found that the adult cardiac fibroblasts, isolated from the Bmi1 knockout model, had increased expression of pro-fibrotic genes including TGFβ, αSMA, and Collagen1a1. Through multiomic
sequencing, we found significant changes in the pathological fibrotic signaling pathways of TGFβ, specifically with SMAD3 chromatin accessibility with the loss of Bmi1 epigenetic regulation. Concluding, Bmi1 epigenetic regulation mediates repair during pathological challenge by regulating adult cardiac fibroblasts and pathological fibrosis after cardiac injury.
DEDICATION

I dedicate this thesis to family, without whom none of this would be possible. I thank my parents, Lisa and Michael Kraus, for the continuous support, motivation, and encouragement. You have both led as an unwavering example of kindness, courage, and strength in all aspects of life. I also thank my sister, Alyssa, who has been and will always be my moral compass and best friend. I can never thank you all enough for your unconditional love.
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1. INTRODUCTION

1.1 Global Impact of Cardiovascular Disease

Cardiovascular diseases (CVDs) have been the leading causes of death worldwide for many years, making it a devastating and increasing concern across the globe. Worldwide, CVDs cause about 18.6 million deaths annually.\textsuperscript{1,2} CVDs encompass a broad range of phenotypes, including a variety of problems associated with the heart muscle, blood flow, and electromechanical signaling in the heart.\textsuperscript{3} Most commonly, CVDs result in a heart attack or heart failure, which is due to a lack of blood flow to or from the heart or body.\textsuperscript{4,3} The total CVD death toll is more than all respiratory diseases, cancers, and HIV combined. Therefore, making CVDs a monumental issue that desperately needs to be addressed.\textsuperscript{2}

1.1.1 Public Health Concerns and Initiatives

Due to the unrelenting effect of CVDs, public health initiatives have become a major source of awareness for the disease and its risk factors. CVDs are often deemed a “silent killer” because the risk factors of CVDs can go undetected for long periods of time, leading to irreversible damage and seemingly sudden life-threatening cardiac events.\textsuperscript{5} Because of these silent risk factors, public health initiatives were set forth to spread awareness about the risk factors to provide better prevention to the general public.

More recently, the incorporation of the social determinants of health have been set forth to provide a more individual and patient-centered approach to CVD treatment plans. There is no single steadfast way to treat CVD, so understanding the variations in socioeconomic status, educational backgrounds, genetics, and lifestyles within the
population is necessary to provide an inclusive treatment for CVD to individual patients.6

Due to the high mortality rate associated with CVD, new measures and studies have been set forth to help provide public awareness as well as target research regarding heart health. Since heart failure (HF) is the fastest growing cardiovascular condition worldwide7 and HF progression can vary based on sex, age, comorbidities, and environment, treatment and prevention is extremely complicated. This results in a large financial and healthcare burden globally,7 which desperately needs to be addressed.

Currently, the Centers for Disease Control (CDC) has enacted a Public Health Action Plan specifically created to prevent and educate about heart disease.8 The three main goals of this action plan include: incorporating cumulative knowledge and research of the disease, national partnerships to support disease prevention, and action and recognition by health professionals in regard to CVD as an epidemic.8

Every ten years, the Office of Disease Prevention and Health Promotion (ODPHP) sets goals and objectives based on current data and research. In the Healthy People 2030 goals set forth by the ODPHP, improvements in cardiovascular health in adults is a high priority objective, listed as HDS-01.9 Based on national data and guidelines constructed by the National Health and Nutrition Examination Survey and the CDC, cardiovascular health is given a score between 1.0 and 7.0, with 7.0 being the best health score possible. As of 2016, the mean cardiovascular health score for adults was 3.2, with the target goal to be 3.5 by 2030. This would be a 10% improvement from the baseline set in 2020.10 There is still a long way to go to improve cardiovascular health; however, with a combination of public awareness and translationally relevant research, these improvements can be made.
1.1.2 Heart Failure and Myocardial Infarction

There are many types of CVD, with heart failure being a major concern and causes of death in the United States. Heart failure, also known as congestive heart failure, is specifically when the heart does not provide enough blood for the heart or organs to function properly. Approximately 6 million adults in the United States have heart failure, which illustrates the scope and severity of this disease. Importantly, heart failure can lead to many downstream complications, including pulmonary hypertension, heart valve issues, and cardiac arrest.

Another example of CVD is a heart attack or myocardial infarction (MI), which occurs when blood flow is blocked, part of the heart does not receive enough oxygen, and the heart muscle cannot function properly. This is often due to a plaque deposit in the arteries, called atherosclerosis. When this blockage to the heart cuts off oxygen and blood supply, the muscle cannot function, which results in irreversible tissue damage, increased scarring, and cellular death in the heart. After MI, this increase in scarring or fibrosis is often thought to be irreversible and lead to lifelong damage; however, more recently, reversing/restricting this scar damage has been a major and pivotal research topic in the cardiovascular field. As mentioned previously, there is no cure for any form of CVD so preventing heart failure and heart attacks is limited to reducing the risk factors associated with the disease.

1.1.3 Current and Past Therapies and Treatments for CVD

Over the last several years, most therapies have been utilized to reopen blocked arteries and restore blood flow to an injured or damaged heart. However, with
optimizing blood flow and pressure, the damage due to ischemic heart tissue cannot be repaired.\textsuperscript{18}\textsuperscript{19} During ischemic injury or after MI, the damaged section of the heart, usually the left ventricle undergoes remodeling and becomes dilated due to enhanced scar tissue formation.\textsuperscript{20} This change in heart structure causes an increase in wall stress, issues with contractility, and overall dysfunctional cardiac cells. It was discovered that after injury, the angiotensin signaling aids in hemodynamic function, which is a focus of many therapies.\textsuperscript{21} But the overall stress on the heart results in issues in electromechanical signaling and contraction within the heart muscle and cells.\textsuperscript{22}

There is currently no cure for CVD; however, a combination of reducing risk factors via medication and lifestyle changes are often the main treatments.\textsuperscript{23,24} Some of these medications and early cardiovascular therapies were the diuretics, which aided in removing excess salt and water through fluid regulation.\textsuperscript{25} Vasodilators were a pivotal drug treatment discovered in the mid 19\textsuperscript{th} century but more popularly used in the United States in the 1960s. Vasodilators are used to prevent the constriction of the blood vessels, specifically decreasing blood pressure.\textsuperscript{26} Some common direct acting vasodilators include minoxidil, nitrates, and hydralazine.\textsuperscript{26} Next, the neurohormonal antagonists use the renin, angiotensin, aldosterone systems to again help with blood pressure regulation.\textsuperscript{27} Specifically, the angiotensin-converting enzyme (ACE) inhibitors are a major neurohormonal antagonists, with lisinopril and captopril being main examples. There are also angiotensin receptor blockers, mineralocorticoid receptor antagonists, and beta blockers drugs proven to help control blood pressure.\textsuperscript{28} The vasodilators and neurohormonal antagonists drugs changed the cardiovascular field and treated hypertension better than any therapy. However, these medications were only treating risk
factors for CVD.

More recently, the use of stem cell therapy has been suggested to restrict infarct area with modest functional improvement.\textsuperscript{29} Unfortunately, there were and are still many roadblocks to surpass with stem cell therapy including engraftment, timing, immune responses, and efficacy of stem cell treatments in a failing heart.\textsuperscript{29}

Because of the potential of stem cells, the reduction of scar size and scar formation after a heart attack has been of interest. This involves ablating mechanisms in the heart commonly associated with scar formation or fibrosis in cells which are already present in the heart specifically during injury or stress. Specifically, the cortical bone stem cells (CBSCs) have been of interest due to their repair mechanisms and wound healing properties in the heart.\textsuperscript{30} It was found that these CBSCs have enhanced changes and functions due to the epigenetic regulator Bmi1.\textsuperscript{31} This epigenetic regulator effected the CBSCs proliferation, survival, and epigenetic signaling.\textsuperscript{31}

This idea is a part of the age of epigenetics and cardiac reprogramming in pre-existing cells in an injured heart.\textsuperscript{32} The main goal of which is to optimize features within pre-existing cells in the heart such as transcription factors, epigenetic related genes, and stem cell like factors to change the phenotype of these pathological cells to become more reparative and functional after injury.\textsuperscript{33} Cardiac reprogramming of cells have used the concepts of epigenetics as well to change the phenotype and functionality of many cardiac cells, with an emphasis on cardiac fibroblasts.\textsuperscript{33,34} However, epigenetics has the potential to optimize the therapeutic effects from many of these therapies and utilize the pre-existing cells in the heart to reverse cardiac damage caused by injury.
1.2 Bmi1 Polycomb Regulation

A significant epigenetic regulator and the main premise of this research centers around Bmi1, a proto-oncogene and polycomb ring finger domain. Originally discovered in leukemia, it has been a major topic in the cancer biology field. More recently, the role of Bmi1 in stem cells have proven to be vital for proliferation and cell death signaling making it an interesting target for preventing or regulating mechanism in cardiac injury. Bmi1 also regulates stem cell survival and therefore cardiac homeostasis. However, the epigenetic mechanism and role in the adult heart have yet to be fully understood.

1.2.1 Epigenetic Role of Bmi1 and the Polycomb Repressive Complex 1

Bmi1 epigenetic regulation is strongly associated with the Polycomb Repressive Complex 1 (PRC1). This complex has been directly related to various mechanisms of homeostasis including cellular proliferation as well as cellular death. The complex is also conserved across most species making it a prolific and influential signaling complex.

The PRC1 is composed of chromobox proteins (CBX), RYBP, RING, and the main regulator, Bmi1. The Bmi1 protein within this complex is known to ubiquitinate histone 2 at lysine 119. Historically, this is known to be a repressive modification, causing further compaction of the chromatin, often leading to repressed gene transcription. However, as more information is being uncovered about PRC1 and Bmi1, the function of this histone modification is much more diverse, with strong effects on proliferation, cell death, ROS production, and cell cycle regulation.

The PRC1 has a sister complex, PRC2, both of which are known to be repressive in nature. As previously mentioned, the PRC1 ubiquitinates histone 2A, while the PRC2
acylated H3 on lysine 27. These complexes have a unique functionality and interaction. Once thought to be dependent on each other, it is now understood of their individual role in epigenetic regulation, specifically that Bmi1 and the PRC1 are not always dependent on the methylation by PRC2. Additionally, the PRC2 has been well studied in cancer, and has a new emphasis on cardiac cell and disease development, while the PRC1 has not. With a deeper understanding of the PRC2, it is important to assess the roles in the PRC1 as they can both be independent epigenetic modifiers with influential changes in epigenetic profiles.

1.2.2 Current Understanding of Bmi1 in the Heart

Due to the influential role Bmi1 plays in tumorigenesis, it has been suggested as a potential therapeutic target for heart failure. It has been debated on the prevalence of Bmi1 in the adult heart. Interestingly, Bmi1 has been well studied in stem cells, with an overall role of regulating cardiac injury and repair mechanisms vital for maintaining heart function. For example, an initial study found that Bmi1 positive cardiac progenitor cells help repair the heart following cardiac injury. The Bmi1 positive progenitor cells were found to contribute to the endothelial and smooth muscle cell lineages in the heart. Song et al. further studied the Bmi1 gene in stem cells and found that having high expression level of Bmi1 led to the ability to differentiate into smooth muscle cells and cardiomyocytes in vitro which would greatly enhance cardiac healing and cardiac function if confirmed in vivo. They also found that the lack of Bmi1 contributed to the dysregulation of proliferation and differentiation of these cardiac cells. However, the role in various adult cardiac cell populations is still debated.

It was determined that Bmi1 positive endothelial cells were necessary for the
neovascularization process during cardiac injury.\textsuperscript{47} During this study, it was found that Bmi1 expression was low in adult cardiomyocytes, which sparked the controversy about the total Bmi1 expression in the heart.\textsuperscript{47,36} An important study found that Bmi1 was necessary during injury, specifically with the regulation of the PTEN-PI3K/AKT-mTOR pathways. Bmi1 was strongly associated with cardiac proliferation and migration during ischemic heart failure, which had not been shown previously.\textsuperscript{48}

Because of the role of Bmi1 in stem cell mechanisms and injury models in the heart, Bmi1 has been suggested as a novel therapeutic target through direct cardiac reprogramming. Zhou \textit{et al.} contributed to this theory by assessing the role of Bmi1 in induced cardiomyocytes. They found that Bmi1 inhibits the reprogramming through the epigenetic regulation of the histone ubiquitination which affected cardiac gene expression.\textsuperscript{34} This suggested Bmi1 and its epigenetic regulation as a barrier in cardiac cells, similar to its role in tumorigenesis. A recent study assessed an anti-cancer drug labeled PTC-209, which is a known inhibitor of Bmi1. The pre-treatment of cardiac cells with the drug allowed for direct cardiac reprogramming of cardiac fibroblasts into inducible cardiomyocytes.\textsuperscript{49} Taken together, these studies have provided novel information on Bmi1 regulation in reprogramming, with an emphasis on stem cell regulation. However, it is still unknown the regulatory role of Bmi1 in the adult heart following injury.

\subsection*{1.2.3 How Bmi1 Functions in Cardiac Fibrosis and DNA Damage Mechanisms}

To further understand the regulatory role of Bmi1 in cardiac injury, the mechanisms of fibrosis, ROS production, and cell death due to changes in Bmi1 expression must be
understood. As mentioned, most of our knowledge of Bmi1 regulation of ROS production is in a stem cell mechanism.\textsuperscript{47,39} A study did assess Bmi1 oxidative stress regulation in the heart, but highlighted its main role in aging mechanisms. The study determined that increased oxidative stress due to age could be regulated by changes in Bmi1 expression.\textsuperscript{50} As mentioned, the role of Bmi1 in fibrosis has mostly been connected to cardiac reprogramming,\textsuperscript{48,34,49} so there is still a need to understand the direct role of Bmi1 in adult cardiac fibroblasts before and after cardiac injury in regards to cell death and DNA damage.

1.2.4 Bmi1 as a Target for Reducing Fibrotic Mechanisms in Response to Cardiac Fibrosis

Taken altogether, Bmi1 is an influential epigenetic regulator but there is currently a lack of understanding regarding the role of Bmi1 in the adult heart before and after injury. We know that Bmi1 expression is vital for cell development, differentiation, and proliferation in cardiac stem cells. It has been determined that Bmi1 plays a major role in repair mechanisms, cell survival mechanisms, and cell proliferation regulation. This has been connected to cardiac stem cells, cardiomyocytes, and endothelial cells as described above, but we still need to uncover how this affects injury, scar formation, and adult cardiac fibroblasts.

1.3 What is Epigenetic Regulation?

Epigenetics was first documented in 1942 by Conrad Waddington, an embryologist who notices developmental changes not directly associated with the DNA sequences.\textsuperscript{51} The word “epigenetics” stems from the Greek root “epi” which translates to “upon” or “on.” So, the loose definition of epigenetic modification is a change on top of the DNA.
In more detail, epigenetic mechanisms involve changes to the condensed DNA or chromatin, which is tightly wrapped around histones to conserve space and energy within a cell. These histones can be epigenetically modified by adding or removing a modification like an acetylation or phosphorylation to the tail of a histone. This epigenetic modification therefore affects the accessibility of the genes around histones. By altering these histone tails, the chromatin becomes more or less compacted, changing the accessibility of the chromatin, therefore altering potential gene expression. Epigenetic modifiers are often enzymes with the ability to add or remove modifications to these histone tails. The modifiers are classified as either writers, readers, or erasers.

1.3.1 Epigenetics and Cardiovascular Disease

As previously stated, targeting epigenetics within cells has been of interest since the discovery around 1942. Since then, epigenetic changes have been strongly associated with the development and evolution of various cellular systems across organisms due to these small conserved changes that have a profound effect on gene expression and phenotype. All of which make targeting epigenetics and interesting source for mediating disease progression.

Epigenetic changes and modifications were originally associated with mechanisms of development. So, the role of epigenetic modifications in CVDs is relatively new and has been argued. For example, it has been supported that changes in the epigenetic profile can lead to the progression of CVD. However, it has also been supported that the risk factors of CVDs can alter the epigenetic profile and therefore lead to the progression of CVD as well. Additionally, the root source of epigenetic changes, whether it be hereditary, environmental, or both, is still being uncovered. Throughout this research,
the changes in epigenetics mechanism and how that effects the progression of CVD phenotypes will be addressed.

Changes in epigenetic mechanisms have been associated with heart failure progression and increased injury to the heart. Due to the highly regulated mechanisms during cardiac remodeling, the epigenetic landscape has been assessed to see changes that occur during stress on the heart.59 A major finding suggested that epigenetic regulation is vital for different cardiac cell lineage commitments in CVDs, meaning that these epigenetic regulators play a key role in the development and homeostasis of different cardiac cells within the heart.60 Most of the epigenetic regulation in the heart has been associated with histone deacetylation and histone methylation; however, there are many other modifications and epigenetic pathways that have yet to be uncovered.

1.3.2 Current Role of Epigenetic Regulators in Cardiac Fibrosis

During cardiac injury or stress, the heart undergoes remodeling which directly connects to cardiac fibrotic pathways. Fibroblasts often become activated or differentiated during injury and are responsible for extracellular matrix deposition, leading to scar formation and stiffening of the heart.61 Understanding the role of fibroblast differentiation into the pathological fibroblasts or myofibroblasts has been strongly connected to epigenetics.53 There are epigenetic modifications associated with the regulation of the pro-fibrotic pathways, with an emphasis on cardiac fibroblast lineage development.53 Cardiac fibroblasts have a unique mesenchymal based phenotype, an ability to proliferate, and an altered gene expression during stress that make them a key target for epigenetic regulation in the heart.62 For example, by targeting these epigenetic mechanisms associated with fibroblasts differentiation and fate specification, it
is hypothesized that we could find a therapeutic potential in the heart through epigenetic regulation of fibrosis to limit the aggressive and irreversible damage following cardiac injury.\textsuperscript{53,59,63}

Due to the increased interest in cardiac remodeling and epigenetics, the regulation of these epigenetic modifications and cardiac fibrosis has been of interest. Targeting fibroblasts and their signaling molecules involved in scar formation has been suggested and studied using small molecule inhibitors of epigenetic regulators, such as histone deacetylases (HDACs) and histone acetyltransferases (HATs).\textsuperscript{64,65} Additionally, more recent studies have assessed the role of HATs in regulating TGFβ signaling, specifically by downregulating the epigenetic modification by MYST1. This downregulation attenuated the autophagy related signaling associated with TGFβ through SMAD3 pathways, indicating a potential therapeutic regulation with epigenetics and pathological fibrosis.\textsuperscript{66}

1.3.3 Epigenetic Regulation of Apoptosis and DNA Damage in the Heart

Changes in epigenetic regulation have also been strongly associated with the various cardiac dysfunction associated with aging, cell death, DNA damage, and reactive oxygen species (ROS) signaling.\textsuperscript{67} A major contributor to this dysfunction is attributed to epigenetic changes in oxidative stress. This is mainly associated with ROS molecules or free radicals with one or more unpaired electrons, which can be produced in a variety of enzyme based reactions.\textsuperscript{68} An increased accumulation of ROS species can lead to dangerous oxidative stress and injury leading to increased cell death.\textsuperscript{68} Specifically, through epigenetic regulation of histone modifications, the chromatin accessibility can be altered, which has been shown to directly affect the transcription of vital ROS regulators,
including the NADPH oxidase (NOX) pathway. Additionally, the histone modifications and epigenetic marks can directly modulate the oxidation and reduction pathway of ROS molecules to exacerbate cell death and disrupt homeostasis.\textsuperscript{69}

Changes in epigenetic regulation have also been associated with increased cardiovascular toxicity, which can lead to detrimental ROS production and cell death.\textsuperscript{70} In regards to the cardiovascular system, this has often been described through mitochondrial dysfunction.\textsuperscript{71} More recently, research has been connecting epigenetic regulation in cardiac injury models, but mostly through the lens of revascularization.\textsuperscript{72} There is currently a lack of understanding of the direct role of epigenetic modification in changing ROS production and the effect on specific cardiac cell survival and function before and after cardiac injury.

With increases in ROS production and a dysregulation in homeostasis due to changes in epigenetic regulation, there is often a drastic increase in DNA damage. Some of the original functions of epigenetic regulators were focused on DNA repair and DNA damage mechanisms in cancer.\textsuperscript{73} In cardiovascular disease, changes in epigenetic regulators have been shown to alter DNA interacting proteins, and therefore enhance disease states because of increased double strand breaks in DNA.\textsuperscript{74} Epigenetic changes have also been suggested as potential markers for certain disease due to these drastic changes in ROS production and DNA. Epigenetics have been labeled as biomarkers for disease because they can indicate increases in DNA damage.\textsuperscript{75}

1.4 Role of Fibrosis and Activated Fibroblasts in the Heart

Cardiac fibroblasts (CFs) are a unique cell type due to their ability to proliferate and create a connective network, or extracellular matrix, within the heart.\textsuperscript{76,77} They can also
have a diverse phenotype which has made them a target for various types of medical
research, specifically because their phenotype can be altered during injury or stress. They
are vital for maintaining homeostasis within the heart as well as many other organs which
makes them a noteworthy cell type to study.\textsuperscript{78}

The heart consists of four major cell types including endothelial cells, smooth muscle
cells, cardiomyocytes, and cardiac fibroblasts (CFs).\textsuperscript{79} The most abundant cell type in the
heart is the endothelial cells, with CFs consisting of about 10\% of the total cells in the
heart. However, it is thought that CFs make up about 27-50\% of the total cells in a
murine ventricle, an important location for proper heart function.\textsuperscript{76} All of these cells are
important for heart function; nevertheless, the CFs have a specific role in the composition
and integrity of the heart. These CFs have a mesenchymal origin that is known for for
spindle-like cytoplasmic branches that stretch out to create a network, which leads to the
connective matrix production and composition.\textsuperscript{78}

CFs play a vital role in the extracellular matrix composition (ECM). This ECM is
necessary for most of the cellular regulation as it creates a dynamic microenvironment
within the heart for signaling molecules, crosstalk between cells, and regulation of the
entire organ.\textsuperscript{80} Resident fibroblasts are designed to help with structural integrity and
mechanical function of a normal healthy heart. Specifically, through ECM deposition,
CFs build a collagen network that helps maintain normal heart function.\textsuperscript{76} However,
activated adult cardiac fibroblasts have enhanced function after injury, like after a MI.
These activated fibroblasts play an essential role in regulating the damage to the heart
through mediating cell death, scarring, and remodeling mechanisms.
1.4.1 Activated Fibroblasts after Injury to the Heart

The activated CFs are a major component of cardiac remodeling, which occurs after or during damage to the heart. After cardiac injury, there is a dramatic increase in fibrosis or the deposition of the ECM.\textsuperscript{81} This is often associated with the infarcted area of a heart, usually within the ventricles. An increase in fibrosis or activated fibroblasts causes an increase in collagen, an increase in heart stiffness, and a decrease in heart function.\textsuperscript{82} The activated fibroblasts, often referred to as myofibroblasts, have increased pro-inflammatory responses, increased secretion of ECM proteins, increased proliferation, and enhanced migration, all of which are vital for wound healing and scar formation after injury. Based on previous scRNA sequencing studies, it is believed that the activated fibroblasts can be categorized into three groups.\textsuperscript{83} The first is the non-proliferative fibroblasts that are most active three days after injury and thought to be the most similar to a quiescent fibroblasts. The second group is the fully differentiated myofibroblasts that have a high expression for ECM proteins and are associated with cardiac remodeling via scar formation. Finally, the third group has enhanced anti-fibrotic gene expression that is believed to be connected to repair processes that is not fully understood yet.\textsuperscript{83} Altogether, the differences between fibroblast populations are indicative of the heterogeneity within CFs, more specifically changes in phenotype and function for fibroblasts at different stages of cardiac injury. Understanding these mechanisms, differences, and changes is of great interest as certain populations could be targeted to prevent adverse cardiac remodeling.\textsuperscript{84}

1.4.2 Targeting Fibrosis in the Heart

Targeting pathological fibrosis and activated fibroblast is potentially a novel
mechanism for the treatment after MI, due to the increase in ECM deposition by the activated fibroblast pathways after damage or stress in the heart.\textsuperscript{85} Since the activated fibroblasts play a pivotal role in cardiac remodeling pathways through increased ECM deposition, migration, and proliferation, targeting their pathways and signaling molecules, such as collagens, have been suggested for understanding cardiac remodeling. Specifically, targeting the Transforming Growth Factor-β1 (TGF-β1) has been a main molecule of interest in fibrosis.\textsuperscript{86} The TGF-β family has been associated with the transition of quiescent fibroblasts to activated fibroblasts.\textsuperscript{84,87} There are many downstream targets of the TGF-β pathways; specifically, the SMAD pathway has been strongly associated with myofibroblast transdifferentiation.\textsuperscript{87}

The SMAD-dependent fibrotic pathway is a part of a larger phosphorylation signaling cascade that results in an increase in expression of pro-fibrotic genes.\textsuperscript{88} The TGFβ cytokine is the main activator of this pathways, binding with its receptor on the cell membrane. The binding activates the SMAD transcription factors, SMAD2 and SMAD3, which are known to regulate many collagens and other pro-fibrotic genes.\textsuperscript{88} In cardiac fibrosis, this canonical pathway is essential for fibroblast proliferation, differentiation, and production of the ECM.\textsuperscript{89} So, the dysregulation in this pathway could greatly alter the heart after injury.

Other upstream targets have been considered with the activated fibrotic pathways, with a large emphasis on epigenetics. Changes to the compact DNA accessibility or histone tails by epigenetic regulators have been shown to play a major role in cardiac fibrosis. Changes to these histones have been shown to affect the activation of vital genes in the fibrotic pathways, specifically changing fibroblast proliferation, differentiation, and
Targeting epigenetic changes or epigenetic regulators is a relatively novel approach to cardiac fibrosis, which requires further understanding.

1.4.3 Fibroblast Heterogeneity in the Heart

A hallmark feature of all fibroblasts, including CFs, is their heterogeneity. Fibroblasts heterogeneity was suggested due to their variation in lineage, phenotype, and plasticity. Because of the production of the ECM, several markers have been used to label fibroblasts. Some of the main markers include α-smooth muscle actin (SMA), fibroblast activation protein (FAP), platelet-derived growth factor receptor (PDGFR) α, and fibroblast-specific protein 1 (FSP1). However, the fibroblast phenotype is dynamic, especially during injury or stress conditions, which leads to changes in gene expression. Heterogeneity in fibroblasts is often described as stratified due to the location and function of the fibroblasts. This tissue state, regional variation, microenvironment, and cell state are all necessary components to understand the stratification of fibroblasts in the heart. The tissue state often is concern with the overall function of the tissue at that point in time, such as a development or regeneration of the heart tissue. The regional variation or heterogeneity can vary within the organ, often connected to different section of the heart such as the ventricle, myocardium, or endothelium. The variation within the microenvironment is especially important for cardiac fibroblasts as the ECM and signal interactions can vary between cells. Finally, the cell state is specific to function of a specific cell, whether is it undergoing a stage of the cell cycle, cell death, activation, etc., all of which result in various phenotypes and functions of a fibroblast cell. This breakdown of fibroblast stratification is necessary in understanding heterogeneity in fibroblasts because of their diverse functions and variations in regard to the systemic senescence.
changes that occur in the heart.

Due to the potential variation in fibroblasts, there is a need to further understand this heterogeneity in cardiac fibroblasts. Muhl et al. started to uncover and label this dynamic through single cells RNA (scRNA) sequencing of fibroblasts from the heart, skeletal muscle, intestine, and bladder. The scRNA sequencing identified fibroblast subtypes by more than just one marker and provided a new groundwork for understanding fibroblast heterogeneity. Despite this innovative and detailed classification of the subtleties of fibroblasts, there is still a lack of understanding regarding the changes that occur to these fibroblasts after cardiac specific injury. Uncovering details of this heterogeneity would help address changes that occur due to scarring and remodeling because of various activated CFs.

1.5 Scientific Premise and Novelty of Work

Ischemic injury to the adult myocardium is characterized by increased cardiac fibrosis leading to cardiac structure and function impairment. Recent data suggest that epigenetic regulation plays a role in post-injury cardiac repair, yet there is limited understanding of epigenetic regulation on cardiac fibrosis and DNA damage with Bmi1. Therefore, the significance of this thesis is to understand the mediating effects of epigenetic regulation by Bmi1 after cardiac injury in the modulation of activated fibroblasts and homeostasis in the adult heart. Overall, the goal of this thesis is to establish Bmi1 as a regulator for cardiac fibrotic signaling after cardiac injury in the adult heart. Additionally, we aim to understand the mechanism behind epigenetic regulation by Bmi1 in fibroblasts and its therapeutic potential in cardiac repair after injury.
1.5.1 Specific Aims and Hypothesis

This thesis hypothesizes that Bmi1 epigenetic regulation is a necessary regulator of pro-fibrotic mechanisms in the adult heart after injury as shown in Figure 1. The hypothesis is addressed in this thesis through the specific aims of this study, which include (1) determining the role of Bmi1 in cardiac cells and the adult heart, (2) defining the role of Bmi1 on heart function after injury, and (3) elucidating the regulatory function of Bmi1 epigenetic mechanisms on cardiac fibrosis, specifically regarding cell death and collagen deposition relative to cardiac function after injury. These aims are addressed using both *in vivo* and *ex vivo* methods by assessing heart function in a global and fibroblast specific mouse model as well as with isolated adult cardiac fibroblasts.

![Figure 1. Schematic of Hypothesis](image)

The use of novel multiomic sequencing is also used to provide a new perspective on the epigenetic profile and regulation in the heart after injury.

1.5.2 Major Conclusions

From these studies, it was determined that Bmi1 is highly expressed in Cortical Bone Stem Cells (CBSCs), a major stem cell type. With the loss of Bmi1 in these CBSCs, there
was a significant increase in cell death and an alteration in epigenetic mechanisms. This led to the more detailed exploration of Bmi1 in the adult heart. There was a significant increase in Bmi1 expression in the adult heart after injury. Using a global knockout of Bmi1, we confirmed a decrease in heart function with the loss of Bmi1 after cardiac injury. This was correlated with an increase in fibrosis, cell death, and DNA damage in the adult heart directly connected to the loss of Bmi1 epigenetic regulation. When Bmi1 was specifically lost in adult cardiac fibroblasts, we saw the same decrease in heart function and increase in fibrosis and DNA damage. The use of multiomic sequencing provided a new outlook on the epigenetic role of Bmi1 in fibroblast populations, as well as the total heart. Further exploration into the Bmi1 mechanism in the heart concluded a direct dysregulation of the SMAD-dependent fibrosis pathways with the loss of Bmi1. Specifically, the loss of Bmi1 epigenetic regulation after cardiac injury caused an increase in SMAD3 binding leading to increased pathological fibrosis. Taken together, this provided a novel understanding of Bmi1 in the adult heart, demonstrating Bmi1 epigenetic ubiquitination as a mechanism for regulating fibrotic homeostasis in adult cardiac fibroblast after injury, specifically in epigenetic signaling of SMAD-dependent fibrotic mechanisms.
2. METHODS

2.1 In Vivo Murine Studies

All animals were housed in Temple University’s Lewis Katz School of Medicine’s Animal Care Facility, an AAALAC accredited facility. C57BL/6J, Bmi1 fl/fl, Rosa Cre/Cre, and Periostin Cre/Cre animals were purchased from Jackson Laboratories (Bar Harbor, ME). All housing, breeding, surgical, and study interventions employed in the current report were approved by Temple University’s Lewis Katz School of Medicine’s Institutional Animal Care and Use Committee (IACUC).

2.1.1 Tamoxifen Administration

To knockout the Bmi1 gene in both the global (Bmi1/Rosa) and the fibroblast specific (Bmi1/Periostin) model, tamoxifen (Sigma, T5648) was administered as an intraperitoneal injection at 75mg/kg for 14 days. The tamoxifen was dissolved in 100% ethanol and diluted in corn oil at a concentration of 10mg/ml stock and stored for up to two weeks at 4°C. The last tamoxifen injection was administered 3 days before MI or TAC surgery. To confirm the knockout, the hearts were removed for cell isolations or tissue collection up to 8 weeks post injury.

2.1.2 Myocardial Infarction and Transaortic Constriction

Male and female mice (8 to 12 weeks of age) were anesthetized with 0.5-2% isoflurane and a transthoracic incision was made to expose the chest cavity. For myocardial infarction, the left anterior descending artery (LAD) was permanently occluded as previously described. For myocardial infarction surgeries, permanent ligation of the LAD was confirmed by changes in myocardial pigmentation downstream.
of the occlusion site. The sham control mice received the same procedure without the ligation. Animal respiration rate and temperature were controlled throughout the surgical procedure and post-operation recovery. Diet gels were placed on the floor of the cage to allow for easy access to food and water, *ad libitum*. Animal pain and distress were monitored throughout the experimental design.

Male and female mice (8 to 12 weeks of age) were anesthetized with 0.5-2% isoflurane and an upper partial sternotomy was performed to expose the transverse aorta for the transaortic constriction. Following previously described methods, the transverse aortic arch was ligated between the brachiocephalic artery and the left common carotid artery. The sham control mice received the same procedure without constriction. Animal respiration rate and temperature were controlled throughout the surgical procedure and post-operation recovery. Diet gels were placed on the floor of the cage to allow for easy access to food and water, *ad libitum*. Animal pain and distress were monitored throughout the experimental design.

2.1.3 Assessment of Cardiac Function

To identify the functional capacity of the heart pre- and post-MI/post-TAC, transthoracic echocardiography was performed using a Vevo2100 ultrasound system with MS400 transducer (VisualSonics; Toronto, Canada) at baseline, 3 days, 1-week, 2-weeks, 3 weeks, 4-weeks, 6 weeks, and 8 weeks post-MI and post-TAC. Animals were anesthetized using 0.5-2% isoflurane and then placed on a heated elevated surface platform to maintain body temperature. B-Mode and M-Mode images were collected and analyzed using the VevoLab v3.2.5 analysis software to determine ejection fraction (EF), fractional shortening (FS), end diastolic left ventricular mass (EDLVM), and end systolic
left ventricular mass (ESLVM) as previously described. Heart rate and temperature were standardized throughout image collection.

2.2 Ex Vivo Assessments Organ Assessments

Total organs were isolated from 8–12-week-old adult C57BL/6J mice and experimental mice including Bmi1/Rosa and Bmi1/Periostin. Organs including the heart, spleen, kidney, liver, and lung were isolated and weighed from all experimental and control mice for further western blot, PCR, or staining analysis as described below.

2.2.1 Quantification of Infarcted Hearts

At the time of death, 4-week post-MI, or 8 weeks post TAC, hearts were excised, weighed, and perfused with PBS followed by formalin fixation, tissue processing, parafilm embedment, and sectioning for histochemistry. To determine the prevalence of infarcted tissue, Picrosirius staining was performed using the Abcam kit (ab246832). Infarct sizes and fibrosis were quantified using densitometry and color threshold packages in ImageJ.

2.2.2 Immunohistochemistry

At the time of death, 4-week post-MI, or 8 weeks post TAC, hearts were excised, weighed, and perfused with PBS followed by formalin fixation, tissue processing, parafilm embedment, and sectioning for histochemistry. To determine DNA damage, the same treatments were assessed using the λH2AX antibody (Abcam, ab11174), alpha smooth muscle actin (Millipore Sigma, A5228), and DAPI (EDM Millipore, 268298). To determine cell death the TUNEL assay was performed following the Click-it TUNEL kit (ThermoFisher, C10617) on hearts fixed two days after injury.
2.3 Adult Cardiac Fibroblast Isolation

Adult cardiac fibroblasts (ACFs) were isolated from the ventricular myocardium of 8–12-week-old adult C57BL/6J mice and experimental mice include Bmi1/Rosa and Bmi1/Periostin. Briefly, ventricles were cut and washed with sterile 1X HBSS, and cut into fine pieces in digestion buffer (HBSS buffer, 100 U/mL of collagenase II, 2.5% trypsin) and transferred to a 50 mL sterile Falcon tube for digestion. The supernatant from digested tissue was then centrifuged, and pellets were combined and cultured in adult cardiac fibroblast media consisting of filtered DMEM/F12, 10%FBS, 100 U/mL pen/strep, 20 mM L-glutamine, and 0.1 mM 2-mercaptoethanol, on collagen coated plates for 3 days until confluency.

2.3.1 Cortical Bone Stem Cell Isolation

The Cortical Bone derived Stem Cells (CBSCs) were isolated from the tibias and femurs of C57BL/6J mice as previously described. Briefly, the tibias and femurs were flushed to remove all the bone marrow and then digested in collagenase at 37C. The digested cells were washed and plated in CBSC media until colonies of CBSCs appeared. The cells were characterized for CBSC markers and expanded for experiments.

2.3.2 Neonatal Rat Ventricular Myocyte Isolation

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old rat pups as described previously and cultured in F-10 medium (Gibco) supplemented with 10% fetal bovine serum. The RNA and protein were isolated from cells after plating.

2.3.3 Hypoxia Condition

The CBSCs and NRVMs were plated in culture in their appropriated media conditions as previously described. The culture cells were placed in a hypoxia chamber
with 5% oxygen for 48 hours without disruption. The RNA and protein were isolated from the cells after the 48 hours of hypoxia.

Additionally, the CBSCs and NRVMs were exposed to a 600uM concentration of hydrogen peroxide in their corresponding media for 48 hours. The RNA and protein were isolated from the cells after the 48 hours.

2.3.4 Lentivirus Overexpression and Knockdown of Bmi1

Using the isolated wildtype adult cardiac fibroblasts (ACF) and cortical bone stem cells (CBSCs) in culture, the lentivirus from Vector Labs for Bmi1 overexpression, Bmi1 knockdown, and the control “Scramble” were used following Vector Labs instructions. All lentiviruses are a shRNA vector. Briefly, in 6-well plates of 50,000 ACFs or CBSCs per well, a MOI of 20 was used for each virus, which was determined using the given titer from Vector Labs. The lentivirus was added to 1 mL of ACF or CBSC media respectively and let set overnight. The next day, the wells were washed and ACF or CBSC media was added. After 72 hours, the ACFs or CBSCs were used for experimentation.

2.3.5 Proliferation Assays

For cell counting, the CBSCs transduced with the Bmi1 or Scramble lentivirus were manually counted for 6 consecutive days. To measure cellular proliferation the CyQUANT Assay from ThermoFisher (C35011) was used following the manufacturer’s instructions on CBSCs that were treated with the Bmi1 or Scramble Lentivirus previously described. Fluorescence was measured on a spectrometer at Excitation/Emission of 508/527 nm.

For the MTT Assay, the MTT Assay Kit from Abcam (ab211091) was used to
measure cellular metabolism and proliferation following the manufacturer’s instructions on CBSCs that were treated with the Bmi1 or Scramble lentivirus previously described.

2.3.6 Cell Death and Apoptosis Assays

To measure single cell DNA damage, Bmi1 and Scramble lentivirus CBSCs were plated on glass microscope slides with low melt agarose to perform the Comet Assay. The cells were lysed in a buffer solution for 1 hour at 4C. The lysed cells on glass slides were placed in a gel electrophoresis box with 1xTAE and run at 18V for 1 hour. The slides were stained with SYBR Safe and kept in the dark for viewing under a confocal microscope at 20x objective. The images were analyzed using the OpenComet feature on Image J.

Using FACS sorting, the Bmi1 and Scramble lentivirus CBSCs were exposed to 600uM hydrogen peroxide for 12 hours. The cells were stained and sorted on the LSR-II for Annexin V (Invitrogen, A13201) and DAPI (EDM Millipore, 268298) to measure apoptosis and necrosis, respectively. The sort was analyzed using FlowJo software.

2.3.7 Cell Cycle Assays

Using FACS sorting, the Bmi1 and Scramble lentivirus CBSCs were exposed to serum starvation media for 24 hours and then treated with 30 μmol/L hydrogen peroxide for 3 hours the following day. Cell death was confirmed by visualizing the cells under a light microscope before collection. Data were acquired with the BD fluorescence activated cell sorting on the LSR-II and analyzed by FlowJo or fluorescence activated cell sorting Diva software (BD Biosciences).

2.3.8 Histone Modification Assays

RNA isolated from Bmi1 and Scramble lentivirus CBSCs, as described below, was
sent to Northwestern for ModSpec analysis. Proteomics histone analyses were performed by the Northwestern Proteomics Core Facility.

2.3.9 Western Blotting

To identify the role of with or without Bmi1 on activated pathological fibrosis, western blotting analysis was used to quantify Bmi1 expression as well as β-actin, as previously described. Briefly, protein concentrations were quantified by BCA, and samples were normalized to protein content and loaded into Mini-PROTEAN TGX Gels (Bio-rad, 4561096). Protein content was transferred onto nitrocellulose membranes via wet transfer and subsequently immersed in blocking buffer (Li-Cor Biosciences, 927-50000) for 30 minutes. Primary antibodies against Bmi1 (1:100, Abcam, ab85688), SMAD2 (1:100, Invitrogen, 51-1300), SMAD3 (1:100, Invitrogen, PA5-34774), SMAD4 (1:100, Abcam, ab236321), β-actin (1:500, Santa Cruz, 517582), H3 (1:100, rabbit polyclonal, Cell Signaling, catalog 9715S), Trimethylated H3 at Lysine 27 (1:500, rabbit polyclonal, Abcam, catalog ab195477), Acetylated Histone 3 (1:500, rabbit polyclonal, EMD Millipore, catalog 06-599) and GAPDH (IRDye 680RD/1:1000, Li-Cor,926-68072) expression were diluted in blocking buffer and incubated at 4°C overnight. Secondary light-sensitive conjugated IRDye antibodies were used to assess all primary antibodies (IRDye 800CW/1:1000, Li-Cor,926-32213). Secondary antibodies were diluted in 1x TBST and incubated for 1 hour, in the dark, at ambient temperature. Membranes were visualized using a Li-COR Odyssey Crx (Li-Cor Biosciences, Lincoln, NE). Densitometry analysis was performed using Image Studio v4.0 (Li-Cor) analysis software.
2.3.10 Real-Time Quantitative Polymerase Chain Reaction (PCR)

To quantify Bmi1 expression from experimental CBSCs, hearts, and fibroblasts, RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104) following the protocol provided by the manufacturer. Single-stranded cDNA was synthesized using 300ng of purified RNA in accordance with the protocol provided by the manufacturer (Fisher Scientific, 43-688-14). Real Time Polymerase chain reactions with SYBR green (95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute) were performed using the CFX96 Real-Time Systems (Bio-Rad, CA) to acquire transcript expression and amplification cycle numbers. Each PCR reaction contained 0.15μM forward and reverse primers designed against all genes of interest, identified below, Table 1.
Table 1. List of primer sequences employed in RTqPCR

<table>
<thead>
<tr>
<th>Primer Sequence (5’-&gt;3’)</th>
<th>Primer ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGCAGATTGGATCGGAAAG</td>
<td>Bmi1 Forward</td>
</tr>
<tr>
<td>GCATCACAGCTATTGCTGCT</td>
<td>Bmi1 Reverse</td>
</tr>
<tr>
<td>CTTCGCTGGTGATGATGCTTC</td>
<td>Alpha Smooth Muscle Actin Forward</td>
</tr>
<tr>
<td>GTTGGTGATGATGCCGTGTT</td>
<td>Alpha Smooth Muscle Actin Reverse</td>
</tr>
<tr>
<td>GCAGTAACCTCGTGCTATGC</td>
<td>Collagen 1a1 Forward</td>
</tr>
<tr>
<td>TCGTACTGATCCCCGATTGCA</td>
<td>Collagen 1a1 Reverse</td>
</tr>
<tr>
<td>CGAGGCCCTTGACTATCAGTC</td>
<td>E2F Forward</td>
</tr>
<tr>
<td>AGGTCCCCAAGTCACAGTC</td>
<td>E2F Reverse</td>
</tr>
<tr>
<td>GTCAAGCTGCTGGGGAATG</td>
<td>CTGF Forward</td>
</tr>
<tr>
<td>GGGCCAAATGTGTCTTCCAG</td>
<td>CTGF Reverse</td>
</tr>
<tr>
<td>AGCGAAGAGCTACAGGAAG</td>
<td>CCNB1 Forward</td>
</tr>
<tr>
<td>TTCACCTCTGTTTCAACA</td>
<td>CCNB1 Reverse</td>
</tr>
<tr>
<td>TCACCTGAGTTGTACGGCAG</td>
<td>TGF Beta Forward</td>
</tr>
<tr>
<td>TCGAAAGGCCTGTATTCCGT</td>
<td>TGF Beta Reverse</td>
</tr>
<tr>
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<td>GAPDH Forward</td>
</tr>
<tr>
<td>CTTCCACCATTTTTCTTACGGGA</td>
<td>GAPDH Reverse</td>
</tr>
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<tr>
<td>GAGCCACCATACTGAAGGC</td>
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</tr>
<tr>
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<td>Wee Forward</td>
</tr>
<tr>
<td>TACGCCTCTCTCTTCCACAG</td>
<td>Wee Reverse</td>
</tr>
</tbody>
</table>

2.4 Molecular Epigenetic Assessments

2.4.1 Simple ChIP Chromatin Immunoprecipitation

Using samples from the Bmi1/Rosa mouse hearts from all conditions as well as the lentiviral treated fibroblasts, the Chromatin Immunoprecipitation Magnetic Bead Kit from Cell Signaling Technologies (9003) was used following the manufacturer’s instructions. Briefly, the samples were crosslinked before chromatin digestion. The crosslinked chromatin preparation was used for immunoprecipitation of the chromatin complexes and eluted using Antibody/Protein G Magnetic Beads. The purified samples

29
were analyzed using PCR as described previously.

2.4.2 Multiomic Sequencing: ATAC and scRNA Sequencing

Using the Bmi1/Rosa mouse model, total heart samples were digested to isolate nuclei following the protocol designed by 10xGenomics entitled “Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing” to perform and create Chromium Single Cell Multiome ATAC + Gene Expression bundle data set. We isolated 8000 nuclei/ul from three combined total hearts samples, from four conditions including, the wildtype, Bmi1 −/−, MI with Bmi1 +/+, and MI with Bmi1 −/− from 1 week after surgery. The Center for Applied Genomics Core at the Children’s Hospital of Pennsylvania performed the multiomic sequencing which included the creation of single cell RNA sequencing and ATAC sequencing libraries. They also provided the bioinformatics analysis.

2.5 Statistical Analysis

All data is reported as mean ± SEM. Statistical comparison between multiple treatment groups was determined by 1-way ANOVA and 2-way ANOVA using the Tukey’s multiple comparison test, Brown-Forsythe test, and Bartlett’s test using the PRISM software. Statistical comparison between singular treatment groups was assessed using the unpaired student’s T-Test and a F-test to compare variances, using the PRISM software. A p-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 9.0.
3. RESULTS

3.1 Bmi1 Expression is Increased in Cardiac Stem Cells

Because of the role Bmi1 plays in development and differentiation, we explored the expression of Bmi1 in various cardiac related stem cells. Through protein and RNA isolations, we found a significant increase in Bmi1 protein (Fig. 2A) and RNA (Fig. 2B) expression in Cortical Bone Stem Cells (CBSCs) compared to Mesenchymal Stem Cells (MSCs) and Cardiac Derived Stem Cells (CDCs) (p value 0.026 and 0.011 for Western blotting and p value 0.0003 and 0.031 for PCR).

![Figure 2](image_url). Bmi1 is overexpressed in CBSCs. (A) Western blot analysis of protein from cortical bone stem cells (CBSC), mesenchymal stem cell (MSC), and cardiac-derived stem cells (CDC) expressing Bmi1 and GAPDH (p values for CBSC vs. MSC = 0.026, p value CBSC vs. CDC = 0.011, n = 4; One way ANOVA); (B) PCR analysis of Bmi1 expression from the RNA from the three stem cell types in A (p values for CBCS vs. MSC p = 0.0003 and CBSC vs. CDC 0.031, n = 3; One way ANOVA).

3.2 The Loss of Bmi1 in CBSCs Causes Dysregulation of Proliferation

Based on the increased Bmi1 expression in CBSCs, a lentivirus to knockdown Bmi1 in CBSCs was used to see what functional changes occurred. Initially, there was a significant decrease in Bmi1 protein expression (Fig. 3A). With the loss of Bmi1, there was a decrease in CBSC proliferation as shown by the images of CBSCs in Figure 3B and by cell count changes in Figure 3C (p < 0.0001 for last 3 days). To further explore
the role of proliferation capacity we performed a CyQuant assay (Fig. 3D) which demonstrated a decrease in proliferation at Day 3, 4, and 5 (p = 0.0002, p < 0.0001, p<0.0001, respectively) with the loss of Bmi1. Using an MTT assay (Fig. 3E), we saw a significant decrease in metabolic activity at Day 4 in CBSCs lacking Bmi1 (p<0.0001).

Figure 3. Bmi1 knockdown causes dysregulation of proliferation of CBSCs. (A) Western blot representative image of the control and the Bmi1 knockdown relative to GAPDH; (B) representative images of Bmi1 knockdown using shBmi1 lentivirus and the control using shScramble in CBSCs grown over 4 days; (C) cell count of the Bmi1 knockdown and control over 5 days (p value for Day 4 p < 0.0001 Day 5 p < 0.0001, n = 3; One way ANOVA); (C,D) Graph of the CyQuant assay comparing Bmi1 knockdown and control CBSC DNA content (p value for Day 3 0.0002, Day 4 < 0.0001, and Day 5 < 0.0001, n = 3; One way ANOVA); and (E) graph of MTT assay comparing Bmi1 knockdown to control CBSCs (p value for Day 4 < 0.0001, n = 3; One way ANOVA). * p < 0.05, **** p < 0.0001.

3.3 The Loss of Bmi1 in CBSCs Causes an Increase in Cell Death

To further explore the loss of Bmi1 on CBSCs, comet assays were performed as visualized by Figure 4A. There was an increase in comet length (p < 0.0001), tail DNA% (p < 0.0001), and Olive Moment (p < 0.0001) with the loss of Bmi1, all of which signify an increase in DNA damage in these cells (Fig. 4B). The cells were also stained for
Annexin V and DAPI to measure apoptosis and necrosis using FACS sorting. The CBSCs with the knockdown for Bmi1 and the Scramble control were also treated with hydrogen peroxide to simulate stress and therefore DNA damage. Without Bmi1, 67.9% of the stained CBSCs population was double positive population for both Annexin V and DAPI compared to the control at 10.7% of the population, indicating an increase in necrotic and apoptotic populations without Bmi1 (Figure 4C).

**Figure 4.** Bmi1 knockdown reduces survival and causes DNA damage in CBSCs. (A) representative images of the comet assay in the Bmi1KD CBSCs and the control CBSCs; (B) comet assay box plot analysis from the Bmi1 KD and the control CBSCs measuring Tail DNA Percent (p value < 0.0001), comet length (p value < 0.0001), and olive moment (p value < 0.0001 (n = 3); Unpaired student’s T-test; (C) flow cytometry gating strategy to determine apoptotic population. Dead cells were included in the analysis, and cells were gated on mCherry to ensure that only successfully infected cells were included in analysis. Percent of cells stained with AnnexinV positive and DAPI negative staining indicating apoptosis and necrosis (n = 3).

### 3.4 The Loss of Bmi1 Causes Changes in Cell Cycle Regulation of CBSCs

Due to the changes in proliferation and cell death, the loss of Bmi1 in CBSCs on cell cycle regulation was further examined. We found using FACS sorting (Fig 5A) the loss of Bmi1 in CBSCs resulted in a significant difference in cell cycle progression. For
example, there were less Bmi1 knockdown CBSCs in the G1 stage (p value 0.007); and more in the G2 phase (p value 0.003) (Figure 5B). Using RNA isolated from the CBSCs, we found significant changes a few cell cycle related genes. There was a significant decrease in the cell cycle inhibitor WEE (p value 0.001) without Bmi1. There was also a significant decrease in the tumor suppressor gene, E2F (p value 0.023). Finally, there was an increase in cyclin B (CCNB1) (p value 0.041) in the Bmi1 knockdown, which is directly connected to the cell cycle transition of the G2 to M phase (Figure 5C).

**Figure 5.** Bmi1 knockdown causes cell cycle arrest in CBSCs. (A) representative flow cytometry plots showing the cell cycle profile of serum-starved Bmi1 knockdown or control CBSCs; (B) graph showing the relative populations of G1, S, and G2 populations of CBSCs transduced with shBmi1 or shScramble (p value 0.007 for G1 and p value 0.003 for G2, n = 3; One way ANOVA); (C) the relative fold change (RFC) of PCR analysis shows that Bmi1 knockdown causes a decrease in cyclin B (p value 0.041), increase in WEE (p value 0.001), and an increase in E2F (p value 0.023, n = 3; One way ANOVA). * p < 0.05, ** p < 0.01
3.5 Epigenetic Changes with the Loss of Bmi1 in CBSCs

Using isolated protein from the control and Bmi1 knockdown CBSCs, western blotting was performed to assess changes in histone modifications. Without Bmi1, the CBSCs had a significant decrease in the total histone 3 expression (p value 0.006) as well as the polycomb dependent trimethylation at histone 3 lysine 27 epigenetic mark (p value 0.050) (Fig 6A). There was no significance for the acetylation of histone 3. To further explore these epigenetic changes, ModSpec analysis was used to assess the epigenetic mechanism without Bmi1 as shown in Figure 6B. Interestingly, the biggest difference in histone 3 was with the trimethylation of histone 3 on lysine 27, further confirming the loss of Bmi1 polycomb epigenetic regulation results in a loss of polycomb epigenetic modifications.
**Figure 6.** Bmi1 knockdown inhibits differentiation and modifies the epigenetic regulation in CBSCs. (A) Western blot analysis of histone 3 (p value 0.006), trimethylated histone 3 (p value 0.050), and acetylated histone 3 in CBSCs with and without Bmi1 using GAPDH for control (n = 4; Unpaired Student’s T-test); (B) ModSpec data of Bmi1 KD versus control CBSCs for the percent of histone modification on H3K27 (n = 3); * p < 0.05, ** p < 0.01

A schematic of this proposed epigenetic mechanism is detailed in Figure 7. It summarized the loss of Bmi1 epigenetic regulation in CBSCs causes a decrease or inhibition of histone 3 trimethylation at lysine 27 which further causes a decrease in cell survival due to increase DNA damage. This DNA damage corresponds with an arrest of the cell cycle and a decrease in CBSC proliferation.

**Figure 7.** Schematic representation of the mechanism describing the loss of Bmi1 in CBSCs.

### 3.6 Bmi1 Expression in Cardiac Cells and the Adult Heart

Due to the drastic changes seen in the cardiac stem cells, it was necessary to further understand the role Bmi1 plays in the adult heart and other cardiac cells. Neonatal Rat Ventricular Myocytes (NRVMs) were used because they are an ideal model for cardiac
The NRVMs isolated and placed in normal NRVM media as well as in an hypoxic condition using an hypoxia chamber with 5% oxygen. The cells were isolated for RNA and Bmi1 expression was assessed. It was determined that the Bmi1 expression was significantly higher under hypoxic conditions compared to the normal NRVM media condition (Fig. 8A) (p <0.0001). To determine the expression of Bmi1 in the total adult heart, wildtype C57BL/6J mice underwent a MI or sham surgery and the total heart was isolated up to 14 days post MI. The sham controls were used for comparison. Using RNA isolated from the hearts, it was determined that Bmi1 expression was significantly increased after myocardial injury, with the highest increase in Bmi1 expression at 5 days post-MI (Fig. 8B) (p value=0.001). All of which provides a new understanding of the increase in Bmi1 expression after injury or stress, representing that the upregulation of Bmi1 would indicate to its necessity and importance after myocardial damage, which had yet to be explored.

Figure 8. Bmi1 expression in cardiac cells and the adult heart. (A) RNA isolated from NRVM in normal NRVM media and hypoxic conditions (p <0.0001; Unpaired Student’s T-test). (B) RNA isolated from wildtype adult hearts up to 14 days after injury as well as the equivalent sham controls (p value 0.001 and p value 0.0390) (n=4; One way ANOVA).
3.7 Bmi1 Knockout Mouse Model in the Adult Heart

Due to the increase in Bmi1 expression after stress to cardiac cells and increase after cardiac injury, a global knockout model was created to assess the role of Bmi1 in the heart after injury as shown in the schematic in Figure 9A. The knockout model was confirmed using protein isolated from the hearts (Fig 9B) (p value <0.001). Interestingly, the heart weight relative to the tibia length was noticeably increased in the Bmi1 knockout mice four weeks after injury compared to the control injury model (Fig. 9C) (p value 0.0058).

3.8 The Cardiac Cell Populations with the Loss of Bmi1 after Injury

Using a multiomic approach, the nuclei from the total heart of the knockout and control samples after cardiac injury were analyzed for scRNA and ATAC sequencing one week after injury. Through UMAP cluster analysis, it was found that the fibroblasts population (dark yellow) was significantly different between the samples, with a larger fibroblast population in the injury model without Bmi1 (Fig. 10A and B). More specifically, with Bmi1 after injury the fibroblast population accounts for 23.9% of the
total cell population, but without Bmi1 after injury the fibroblasts account for 29.7% of the population (Fig. 10 C and D).

So, the fibroblast population is larger without Bmi1 after injury. To understand this population more, expression blots demonstrated an increase in the Platelet Derived Growth Factor Receptor Alpha (PDGFRα) expression in the fibroblasts without Bmi1 after injury (Fig. 11B) compared to the control (Fig. 11A) as indicated by the arrows, thus providing a specific expression of the larger population lacking Bmi1 after injury.
The Loss of Bmi1 Causes a Decrease in Heart Function

Due to the changes seen in the sequencing, the heart function was further explored following cardiac injury. Using longitudinal velocity (top row), and speckle tracing (middle row), and echocardiography analysis (bottom row), it was determined that the heart function was significantly reduced after injury with the loss of Bmi1 (Fig. 12). The ejection fraction and fractional shortening of Bmi1 knockout were significantly decreased.
up to four weeks after injury (p value 0.038 and p value 0.046) respectively). The end systolic and end diastolic left ventricular masses were both significantly increased without Bmi1 four weeks following injury (p value 0.002 and p. value 0.009 respectively). So, without Bmi1 in the heart following MI, there is a decrease in heart function and increase in heart size.

**Figure 12.** Heart function analysis for global Bmi1 knockout 4 weeks after myocardial infarction. (A) Longitudinal Velocity at 4 weeks after MI. (B). Speckle tracing at 4 weeks after MI. (C). Ejection Fraction, Fractional Shortening, End Systolic Left Ventricular Mass (ESLVM), and End Diastolic Left Ventricular Mass (EDLVM) at four weeks following MI (p value 0.0038, 0.0046, 0.003, 0.009 respectively comparing MI with Bmi1 +/- to MI with Bmi1 -/- at 4 weeks) (n=5); Two Way ANOVA.

After isolating the hearts from these conditions, the total hearts were used for ChIP sequencing or embedded for immunohistochemistry. Using the RNA for ChIP PCR, we found that the loss of Bmi1 significantly reduced the H2A K119 ubiquitin mark associated with Bmi1 epigenetic regulation, but the total histone 3 mark was unchanged (Fig. 13A) (p value <0.0001). Further confirming that Bmi1 epigenetic regulation in the heart relies on the histone 2A ubiquitination at lysine 119. Through staining, the loss of
Bmi1 causes an increase in DNA damage as measured using the γH2Ax stain in Figure 13B (p value 0.032). Finally, the hearts were also stained for Picrosirius staining to assess fibrosis. With the loss of Bmi1 there was a significant increase in the fibrotic area with the loss of Bmi1 after injury (Fig. 13C) (p value 0.031).
3.10 Loss of Bmi1 Causes an Increase in the Activated Cardiac Fibroblast Phenotype

Because of the drastic increase in fibrosis with the loss of Bmi1, the adult cardiac fibroblasts (ACFs) were isolated from this model as described in Figure 14A. The RNA was isolated from these p0 ACFs. With the loss of Bmi1, confirmed in Figure 14B (p value 0.016), we also saw an increase in pro-fibrotic genes, including Collagen1a1 (p value 0.001), CTGF (p value 0.023), Smooth Muscle Actin (p value 0.006), and TGFβ expression (p value 0.003) (Fig. 14C). So, with the loss of Bmi1, there is a decrease in heart function, an increase in fibrosis, and an increase the pro-fibrotic fibroblast phenotype.
Figure 14. Loss of Bmi1 causes increase in pro-fibrotic phenotype. (A) Schematic of fibroblast isolation. (B) qPCR of ACFs isolated from each condition assessing Bmi1 expression (p value 0.016 for MI with Bmi1 +/+ vs MI with Bmi1 -/-; n=3, One way ANOVA). (C) qPCR analysis of ACF RNA on profibrotic genes including Collagen1a1, CTGF, Smooth Muscle Actin, and TGFβ (p value 0.001 (n=4), 0.023 (n=4), 0.006 (n=5), 0.003 (n=4) respectively for MI with Bmi1 +/+ vs MI with Bmi1 -/-; One way ANOVA).

3.11 Fibroblast Specific Loss of Bmi1 Causes Decrease in Heart Function

Because there was a significant increase in fibrosis with the loss of Bmi1 after injury and a significant increase in the pro-fibrotic phenotype in isolated fibroblasts we sought out to explore what is specifically happening to these fibroblasts with the loss of Bmi1. To do this, an adult fibroblast specific Bmi1 knockout model (Periostin/Bmi1) was created and received a transaortic constriction (TAC) to simulate injury to the heart (Fig. 15A). Like previously, all organs were isolated including the heart. Protein was isolated from the heart to confirm the knockout in the fibroblasts (Fig. 15B) (p value 0.001).

Heart function was assessed in this fibroblast specific model using speckle tracing (top row), longitudinal velocity (middle row), and heart function parameters (bottom row).
row) as shown previously. With the fibroblast specific knockout of Bmi1 following cardiac injury there was a similar decrease in heart function as measured by ejection fraction (p value 0.010) (Fig. 16). This study was carried out up to 8 weeks following the TAC surgery to further understand the long-term effect of the loss of Bmi1. Additionally, there was an increase in the heart size as measured by the ESLVM (p value 0.036) and the EDLVM (p value 0.019) (Fig. 16). This was the same trend as seen in the global Bmi1 knockout, further confirming the influential role the loss of Bmi1 plays on heart function, specifically in the adult cardiac fibroblasts.

**Figure 16.** Heart function analysis for fibroblasts specific Bmi1 knockout 8 weeks after transaortic constriction. (A) Representative images of speckle tracing of the controls and injury models 8 weeks following TAC surgery. (B) Representative images of longitudinal velocity of the controls and injury models 8 weeks following TAC surgery. (C) Ejection Fraction (p value 0.010 at 8 weeks comparing TAC with Bmi1/Post +/+ versus TAC with Bmi1/Post -/-), ESLVM (p value 0.036 at 8 weeks comparing TAC with Bmi1/Post +/+ versus TAC with Bmi1/Post -/-), and EDLVM (p value 0.019 at 8 weeks comparing TAC with Bmi1/Post +/+ versus TAC with Bmi1/Post -/-) up to 8 weeks following injury with or without Bmi1; Two-way ANOVA. n=4
Moreover, with the loss of Bmi1 in the fibroblasts, there was a similar increase in heart weight without Bmi1 after injury (Fig. 17A) (p value 0.043). Using the Picrosirius Red Stain, there was also an increase in the fibrotic area without Bmi1 following injury (Fig. 17B) (p value 0.037). And finally, using the γH2AX stain, there was also an increase in DNA damage without Bmi1 following injury (Fig. 17C) (p value 0.001). So, like with the loss of Bmi1 globally, when Bmi1 was lost in adult cardiac fibroblasts there was more fibrosis and DNA damage indicating to the specific and significant effect of this epigenetic signaling after cardiac injury.

**Figure 17.** Loss of Bmi1 in fibroblasts causes increase in fibrosis and DNA damage. (E) Heart weight from all samples 8 weeks after TAC (p value 0.043; One way ANOVA). (F) Picrosirius Red Stain from total hearts from control and injury model 8 weeks after TAC (p value 0.037 for TAC with Bmi1/Post +/- versus TAC with Bmi1/Post -/-; One way ANOVA). (G). Immunohistochemistry of the γH2AX stain, DAPI, and alpha smooth muscle actin from total heart samples at 8 weeks after TAC (p value 0.001 for TAC with Bmi1/Post +/- versus TAC with Bmi1/Post -/-; One way ANOVA). n=3.
3.12 Loss of Bmi1 in Fibroblasts Causes Dysregulation of SMAD-dependent Pathways

A.

**Figure 18.** Enrichment plots from the scRNA sequencing profiles with or without Bmi1 after injury. Comparing total heart samples from injury hearts with Bmi1 versus inured hearts without Bmi1. n=3

Due to the overall changes in heart function and changes in fibroblasts, it was necessary to uncover the mechanism that was affected by this loss of Bmi1 epigenetic regulation. First, using scRNA sequencing comparing the total heart from the MI with

**Figure 19.** ATAC and motif analysis in hearts with or without Bmi1 after injury. (A). ATAC sequencing between hearts that received MI with (blue) or without Bmi1 (red). (B) Motif analysis between the two groups. n=3
Bmi1+/+ versus the MI with Bmi1-/-, the enrichment plots were explored to assess which pathways were significantly affected by the loss of Bmi1 after injury. It was determined...
that both the SMAD signaling transduction and the extracellular matrix structure pathways were top hits as shown in Figure 18.

Additionally, ATAC sequencing was performed on the total heart from MI with Bmi1+/+ versus the MI with Bmi1-/- and found changes in chromatin accessibility. Specifically, it was determined that SMAD3 binding and TGFβ receptor binding was altered with the loss of Bmi1 after injury as well (Fig. 19A). The peak analysis showed the open chromatin regions. With the loss of Bmi1, we observed the biggest changes at the SMAD3 locus on chromosome 9 (Fig. 19A left) and the TGFBR3 locus on chromosome 13 (Fig. 19A right). At these two loci, the cis regulatory elements are the most different, indicating that with the loss of Bmi1 epigenetic regulation, the chromatin accessibility is altered specifically at SMAD3 and TGFBR3. Motif analysis indicated that SMAD3 was a significant transcription site with the changes in Bmi1 epigenetic regulation (Fig. 19B).

To further assess this binding, ACFs with Bmi1 knockdown had increased SMAD3 binding indicated in the ChIP sequencing shown in Figure 20A. The Bmi1 overexpression in ACF had significantly less of the SMAD3 binding indicating that Bmi1 regulates SMAD3 in fibroblasts. To further examine this, protein expression of SMAD3 was measured in hearts after cardiac injury with or without Bmi1 and found that the loss of Bmi1 after injury causes an increase in SMAD3 expression (Fig. 20B). So, epigenetic regulation by Bmi1 inhibits excessive SMAD3 signaling by regulating the chromatin accessibility to the SMAD3 loci and therefore inhibits downstream transcription of the SMAD dependent fibrotic signaling proteins, inhibiting enhanced pathological fibrosis as shown in schematic in Figure 21.
Figure 21. Schematic overview of the loss of Bmi1 in adult cardiac fibroblasts.
4. DISCUSSION

Our findings demonstrate a novel role of Bmi1 epigenetic regulation in the adult heart following injury. Specifically, our data links Bmi1 to a SMAD-dependent fibrotic signaling mechanism in adult cardiac fibroblasts. Without Bmi1 epigenetic regulation, there is an increase in fibrosis and an overall decrease in heart function following cardiac injury that is associated with SMAD3 fibroblast activation. Overall, we saw an increase in the fibroblast population, an increase in collagen gene expressions, and an increase in PDGFRα expression with the loss of Bmi1. This led us to explore the epigenetic role of Bmi1 in the adult heart after injury. We found a decrease in heart function and an increase in heart size with the loss of Bmi1 after injury. This was associated with an increase in fibrosis and DNA damage in the heart. Isolated adult cardiac fibroblasts (ACFs) from these treatments saw an increase in pathological fibrotic gene expression (αSMA, TGFβ, collagen). We also started to see changes in SMAD-dependent signaling. In the fibroblasts specific Bmi1 knockout model, there was a similar decrease in heart function and increase in heart size that parallels the global knockout. However, this model provided a more detailed understanding of Bmi1 in fibroblasts after injury. A deeper investigation into the signaling and sequencing revealed significant changes in the SMAD3 binding and transcription with the loss of Bmi1. Without Bmi1, there was an increase in SMAD3 expression and an increase in SMAD3 binding, indicating a regulatory mechanism of Bmi1 and SMAD-dependent fibrotic signaling that has not been reported previously. Overall, these studies provided a novel insight into the epigenetic role of Bmi1 in maintaining cardiac fibroblast homeostasis after cardiac injury.
4.1 Bmi1 is a Key Feature of CBSCs

To reach these conclusions, we first had to understand the role of Bmi1 in various cardiac stem cells. Cardiac stem cells have been widely studied due to their potential for cardiac regeneration and wound healing; however, engraftment and negative immune responses have been difficult barriers to overcome. Because of the potential for beneficial regeneration, differentiation, and healing, cardiac stem cells like factors been a focus of direct cardiac reprogramming in pre-existing cells, to avoid issues of stem cell integration. Direct cardiac reprogramming is a potentially powerful clinical way to change cardiac fibroblasts found in the dying scar tissue of the heart and reprogram them into functional cardiomyocytes. This potential new therapeutic is only possible due to the developments made using stem cells and epigenetic mechanisms.

We explored the epigenetic regulation of various cardiac stem cells to see the basic cellular changes that would occur. Using Cardiac Derived Cells (CDCs), Mesenchymal Stem Cells (MSCs), and Cortical Bone Derived Stem Cells (CBSCs), we found that the epigenetic regulator, Bmi1, was most highly expressed in the CBSCs. The CBSCs were an interesting target due to their enhanced ability to repair ischemic injury. To further explore the role of Bmi1 in the CBSCs, Bmi1 was knocked down. It was determined the loss of Bmi1 led to a decrease in proliferation, an increase in cell death, and a loss of epigenetic regulation. All taken together, this connected Bmi1 to the survival and reparative abilities of these CBSCs, thus providing an important connection to Bmi1 and the potential repair mechanism in the heart.
4.2 Bmi1 Expression is Necessary in the Adult Heart

As mentioned, the role of stem cells in cardiac wound healing has major barriers. So, it was vital to explore the epigenetic role of Bmi1 in the adult heart, specifically in the pre-existing cardiac cells. The role of Bmi1 in the adult heart had yet to be fully uncovered, with most literature focused on development or aging. So, by using cardiac cell lines such as fibroblasts and neonatal rat ventricular myocytes, their protein and RNA indicated an increase in Bmi1 expression in these cells specifically after stress. To examine this more translationally, wildtype mice underwent a myocardial infarction (MI) or sham control surgery. The hearts isolated from these mice found that Bmi1 expression was the highest in the hearts around one week after injury confirming the role of Bmi1 in the adult heart during stress.

This finding was vital to the field due to the previous lack of understanding of Bmi1 in the adult heart. Originally discovered in cancer, the role of Bmi1 was severely lacking in cardiac biology. We have now shown that Bmi1 is present in the heart and various cardiac cells. However, there is a specific increase in Bmi1 expression in the heart after injury or stress which could connect to its potential role in cardiac healing and cardiac cell regeneration.

4.3 The Loss of Bmi1 in the Adult Heart After Injury Leads to a Decrease in Function

Since Bmi1 is increased in the heart during injury or stress, it was necessary to uncover what happens when Bmi1 is lost in the adult heart under these conditions. Before this research, there has not been a Bmi1 knockout model in the adult heart. However, previous studies have shown that the loss of Bmi1 in other systems have shown an
increase in aging, infertility, and loss of “stemness” in various stem cells. With this and our previous findings, the loss of Bmi1 in the heart after injury was a necessary next step.

The loss of Bmi1 was studied for up to four weeks following injury to the myocardium in adult mouse hearts. Initially, there was a drastic increase in heart weight with the loss of Bmi1 after injury. This was paralleled by the increase in heart size as measured by echocardiography. With the loss of Bmi1, there was also a decrease in heart function at four weeks post MI. So, without Bmi1 there was an increase in heart size and decrease in heart function, providing a novel role of Bmi1 in cardiac biology and concluding that Bmi1 is necessary to maintain heart function following stress or injury.

More exploration of these hearts revealed there was also an increase in the fibrotic area of the heart. So, with a decrease in the heart function, there is an increase in scarring. Additionally, there was an increase in DNA damage in cardiac cells. Interestingly, this was explored in other research, specifically in a renal model and found similar results. Specifically, the authors discovered that changes in hypoxia induced Bmi1 expression was connected to epithelial cell transitions and renal fibrosis. Like what was shown here and with more research being conducted, the use of Bmi1 as a cardiovascular therapy has been suggested for similar fibrosis related therapeutics. This research further defines the role of Bmi1 in the heart and provides more information regarding the loss of Bmi1 after injury. Interestingly, a previous study explored the epigenetic loss of Bmi1 using a lentivirus transduction and found a regulatory effect in the PTEN-PI3K/Akt-mTOR pathway. Specifically, Yang et al. found that Bmi1 promoted ischemic related fibrosis, which is contradictory to our findings and as well as others. It is
important to note Bmi1 close connection to cardiac fibrosis, but the potential difference in various stages and types of pathological fibrosis.

4.4 The Loss of Bmi1 in Adult Cardiac Fibroblasts Causes a Pro-Fibrotic Phenotype

Because of the major changes seen in heart function and fibrosis, the adult cardiac fibroblasts were of great interest regarding Bmi1. Epigenetic regulation of fibroblasts has been suggested in the past,\textsuperscript{115} and Bmi1 epigenetic regulation specifically has been addressed in various fibrosis models.\textsuperscript{108} In pulmonary hypertension, Bmi1 was found to help alleviate fibroblast senescence through ROS related signaling.\textsuperscript{116} In the heart, most of the work with Bmi1 and fibroblasts has been studied in direct cardiac reprogramming.\textsuperscript{34,49} These published studies described Bmi1 as a barrier for the transition of activated fibroblasts to functional inducible cardiomyocytes and has been suggested to be due to the epigenetic regulation and fate specific role Bmi1 plays in the fibroblast phenotype.\textsuperscript{34} However, cardiac reprogramming has many obstacles to overcome, with timing and dosage being a major hurdle. In this study, the loss of Bmi1 epigenetic regulation was shown to drive the profibrotic phenotype in the damaged heart and highlight more details about the epigenetic changes that occur in these cardiac cells.

The loss of Bmi1 had a profound effect on the activated fibroblasts. These ACFs isolated from the damaged hearts had a significant increase in many profibrotic genes, including TGFβ, Collagen1a1, and αSMA. The loss of Bmi1 also caused substantial changes to the epigenetic signaling, with a loss of the histone 2A ubiquitin mark associated with Bmi1. With the changes in the fibroblasts, it was necessary to further explore the effect of Bmi1 specifically in fibroblasts on the total heart.
4.5 The Fibroblast Specific Loss of Bmi1 on the Adult Heart Leads to a Decrease in Heart Function

Previously, an adult cardiac fibroblast specific loss of Bmi1 in a mouse model had yet to be explored. Here, for the first time, we used a Periostin specific Bmi1 knockout mouse model to explore the role of Bmi1 in fibroblasts on the adult heart function. With the loss of Bmi1 in ACFs, there was a substantial decrease in heart function following injury. Alone, this provides a novel role of Bmi1 in cardiac fibroblasts that was previously not understood. However, with continued exploration, it was found that Bmi1 plays an important role in regulating ACFs in the heart, specifically under stress or after injury.

Importantly, the loss of Bmi1 affected the size and weight of the heart. Fibroblast populations have been notoriously complicated to account for in the heart due to the novel understanding of the heterogeneity. Fibroblast populations vary before and after injury with specific populations being present at different stages of development and injury. We have suggested that the epigenetic regulation by Bmi1 dictates potential changes in these fibroblasts’ populations. With the loss of Bmi1, we saw changes in heart function, but we also documented changes in the activated fibroblast phenotype. The RNA sequencing data suggested that the fibroblasts population was larger in number relative to the total number of cardiac cells with the loss of Bmi1 and that this larger population expressed more of the PDGFRα fibroblast marker. This marker is a known fibroblast marker, specifically for fibroblasts survival, growth, and maintenance. Interestingly, there have been connections to Bmi1 regulation and PDGFRα signaling in cancer regulation in mediating cellular renewal and differentiation. Here, we
showed that the loss of Bmi1 appears to affect the composition of the activated fibroblasts population in the heart after injury, leading to the sequential decrease in heart function that was previously unknown.

4.6 Bmi1 Signaling and SMAD-Dependent Fibrosis

The role of Bmi1 signaling in the heart has also not been completely understood. With the major changes found in the heart function and in the fibroblast’s phenotype, the activated fibroblast signaling mechanism was further explored. Using multiomic sequencing, the SMAD-dependent signaling was a major target found in the enrichment profiles as well as the ATAC sequencing chromatin accessibility profiles. The SMAD3 locus was a specific binding site in the sequencing that appeared to have the biggest alterations with the loss of Bmi1. A deeper examination revealed that without Bmi1 there was often an increase in the SMAD3 expression, indicating that the Bmi1 has a connection to downstream SMAD-dependent fibrotic signaling.

The Bmi1 epigenetic signaling has a close connection to the TGFβ and SMAD fibrotic signaling mechanism. Bmi1 has been shown to inhibit these profibrotic signaling cascades to regulate pathological fibrosis in cancers.121,122,123 This research demonstrates the similar role Bmi1 plays in cardiac fibroblasts and cardiac function. The loss of Bmi1 causes a dysregulation in this SMAD signaling, increasing the pro-fibrotic response in the ACFs. Concluding, the loss of Bmi1 led to a larger pathological fibroblast population, increased fibrosis, and decreased overall heart function, which has not been shown previously.

4.7 Limitations

A few limitations of the study include the overall effect of the global Bmi1 knockout
mouse model on other organs and cells. We did see changes in the spleen and T cell population from the global knockout of Bmi1 in the sequencing data which could indicate an effect on the immune response in the heart. Additionally, the sequencing data assessed changes from the global mouse model and not the fibroblast specific mouse model. Finally, the global model assessed heart function following myocardial infarction while the fibroblasts specific model assessed heart function following transaortic constriction. This research only assessed changes in the adult cardiac fibroblasts, it would be of interest to see how the loss of Bmi1 globally and in fibroblasts affects other cardiac cell types.

4.8 Future Directions

With all these major conclusions of Bmi1 in the heart, there are many details to continue to understand. An important next step would be to uncover the regulatory role of the Polycomb Repressive Complex (PRC1) in the heart. It is well established that Bmi1 ubiquitinates histone 2A; however, how Bmi1 functions within the PRC1 is still debated. The intricacies of this complex and how other proteins interact with Bmi1 are still unknown. The PRC1 is closely associated with the PRC2 and understanding the interaction between the two complexes in the heart would also be a novel and important mechanism to understand to elucidate the epigenetic role of the polycomb regulation in heart failure.

Finally, the field is just beginning to understand the details and usage of fibroblast heterogeneity. It is vital to document and explore these populations before and after injury to better understand the regulation in the heart after cardiac injury. Uncovering how epigenetics further dictate these populations would provide an important
understanding of remodeling and wound healing.
5. CONCLUSION

Epigenetic regulation of heart failure mechanisms is a novel and vital approach to creating better therapeutics for a devasting and massive disease. With no current cure for heart failure, there is a desperate need for new clinical approaches in cardiac biology. Here, epigenetic regulation by Bmi1 is shown to play a major role in regulating heart failure mechanisms.

Bmi1, a ubiquitinating epigenetic regulator, is upregulated in many important repair and survival states. Bmi1 was shown to be upregulated in stem cells necessary for cardiac wound healing compared to other stem cells. Bmi1 is also present in the heart; however, it is the most highly expressed in a damaged heart. Stress and injury correlate to an increase in Bmi1 expression, concluding the necessity of Bmi1 in cardiac damage regulation.

To understand the role of Bmi1 in injury, the global and fibroblast specific loss of Bmi1 was examined in adult murine hearts. Without Bmi1, there was a drastic increase in heart size and a decrease in heart function. This matched the increase in fibrosis and increase in DNA damage without Bmi1 after injury; all of which helped provide insight into the regulatory role Bmi1 plays in maintaining heart function and cardiac cell homeostasis.

With an emphasis on the fibrotic phenotype, the cell specific loss of Bmi1 further confirmed the role of Bmi1 plays in adult cardiac fibroblasts. There was a similar decrease in heart function and increase in heart size without Bmi1 in the fibroblasts after injury. A deeper analysis revealed this was connected to a larger activated fibroblasts
population and more activated pathological fibrotic signaling. Specifically, the loss of Bmi1 was connected to an increase in the SMAD-dependent fibrotic signaling mechanisms, with SMAD3 being a major binding site for Bmi1 epigenetic regulation. Overall, it was determined that Bmi1 is necessary for maintaining the fibroblast population, specifically for regulating the pro-fibrotic mechanism during heart failure.

These results provide a novel understanding of epigenetic regulation in the adult heart after injury. Understanding this regulation provides a foundation for Bmi1 as a clinical regulator for heart failure and a potential resource for regulating scar formation and cardiac repair mechanisms.
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