

**Determining the Role of ZEB1 and ZEB2 in Controlling
Fibroblast Activation**

by

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Abstract

Myocardial infarction (MI) is a leading cause of death in Canada. The activated fibroblast, also known as myofibroblast, is a key player in extracellular matrix production, remodeling, and fibrosis in the heart following a MI. Previous studies in our lab have shown that the transcription factor ZEB2 is expressed in activated cardiac fibroblasts and that the overexpression of ZEB2 upregulates the expression of the myofibroblast markers α -SMA, SMemb and ED-A fibronectin. The Zinc finger transcription factor family proteins ZEB1 and ZEB2 have crucial roles in embryonic development, angiogenesis, Epithelial-Mesenchymal transition (EMT), and Endothelial-Mesenchymal transition (EndoMT). This study was focused on determining the role of both ZEB1 and ZEB2 in cardiac fibroblast activation. We compared the protein levels of ZEB1 and ZEB2 in primary adult male and female rat cardiac fibroblast cells during the process of fibroblast activation *in vitro*. As well, we analyzed the effect of over-expression and knockdown of ZEB1 and ZEB2 in the mouse NIH3T3 embryonic fibroblast cell line. Our results show that both ZEB1 and ZEB2 are expressed in the activated fibroblasts with their expression being highest at 48 hours and then decreasing. We observed that ectopic expression of ZEB2 in both NIH3T3 cells and rat primary cardiac fibroblasts resulted in a corresponding decrease in endogenous ZEB1 expression. Furthermore, siRNA mediated knockdown of ZEB2 resulted in increased ZEB1 expression in cardiac fibroblasts and NIH3T3 cells. Similarly, ZEB1 knockdown resulted in increased ZEB2 expression. This shows that ZEB1 and ZEB2 may form a negative feedback loop to maintain an optimal level of their expression in cardiac fibroblasts. ZEB1 and ZEB2 may directly compensate for the loss of the other transcription factor in fibroblasts. Interestingly, despite these regulatory interactions, the expression of the myofibroblast markers, α -smooth muscle actin (α -SMA) and Embryonic smooth muscle heavy chain (SMemb), remain

ZEB1 and ZEB2. This study enhances our understanding of the complex interplay between ZEB1 and ZEB2 in cardiac fibroblast activation and provides new insight into the regulatory mechanism underlying this process. These findings could have significant implications to develop new therapeutic strategies for myocardial infarction and heart failure by targeting ZEB1 and ZEB2 to control fibroblast activation and fibrosis.

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Dedication

I dedicate this thesis to my precious son,

Janvik Jithin

Who was born during the course of this study.

My dear boy, this work is a reflection of the time I spent away from you in pursuit of my research, when you needed me the most. I am deeply grateful for the patience and understanding you have unknowingly shown. This achievement would not have been possible without your support.

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List of Abbreviations

ACE	Angiotensin converting enzyme
Ang-II	Angiotensin-II
ATLL	Adult tumor cell leukemia/ lymphoma
AV	Atrioventricular
AVT	Atrio-valve stenosis
BP	Blood pressure
BSA	Bovine serum albumin
BSC	Biosafety cabinet
CAD	Coronary artery diseases
CBD	Coactivator p300-pCAF binding domain
CF	Cardiac fibroblast
CID	CtBP interacting domain
CO	Carbon monoxide
CtBP	C-terminal binding protein
CVD	Cardiovascular diseases
CZF	C-terminal Zinc finger
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
DAMPs	Danger associated molecular patterns
DMEM	Dulbecco's Modified Eagle medium
ECG	Electrocardiogram
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix

ED-A FN	Extra domain-A fibronectin
EDV	End diastolic volume
EF	Ejection fraction
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
Endo-MT	Endothelial to mesenchymal transition
ESV	End systolic volume
FBS	Fetal bovine serum
FECD	Fuch's endothelial corneal dystrophies
FGF	Fibroblast growth factor
FN	Fibronectin
FSP-1	Fibroblast specific protein-1
HA	Hemagglutinin
HD	Homeodomain
HDAC	Histone deacetylase
HDL	High density lipoprotein
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HRP	Horseradish peroxidase
IL-1 β	Interleukin- 1 β
KGF	Keratinocyte growth factor
LAP	Latency associated protein

LDL	Low density lipoprotein
LGBTQ+	Lesbian, gay, bisexual, and transgender, queer and other gender, and Sexual minority population
LTBP	Latent TGF- β associated protein
LVHF	Left ventricular heart failure
MI	Myocardial Infarction
MMPs	Matrix metalloproteinases
MOWs	Mowat-Wilson syndrome
NIM	NuRD interacting motif
NK cells	Natural killer cells
NMD	Nonsense mediated m-RNA decay
NuRD	Nucleosome remodelling and histone deacetylation complex
NZF	N-terminal Zinc finger
PAGE	Polyacrylamide gel electrophoresis
PAS	Pulmonary artery stenosis
PDGF	Platelet derived growth factor
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
Postn	Periostin
PPCD	Posterior polymorphous dystrophy
PRCF	Primary rat cardiac fibroblast
RAS	Renin-Angiotensin system
RGD	Arginin-Glycine-Asparagine
ROS	Reactive oxygen species

RPM	Rotation per minute
RT	Room temperature
RVHF	Right ventricular heart failure
SBD	SMAD binding domain
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SMA	Smooth muscle actin
SMADs	Suppressor of mothers against decapentaplegic
SMEMB	Embryonic smooth muscle myosin
SOD	Superoxide dismutase
TBST	Tris buffer saline with Tween – 20
TCF 21	Transcription factor 21
TF	Transcription factor
TGF- β	Transforming growth factor- β
TIMPs	Tissue inhibitor metalloproteinases
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VICs	Valvular interstitial cells
WHS	Women's health study
XSMAD1	Xenopus SMAD1
ZEB1	Zinc finger E-box binding homeobox-1 gene
ZEB2	Zinc finger E-box binding homeobox-2 gene

Chapter I – Introduction

Cardiovascular diseases (CVD) are a leading cause of death and morbidity worldwide, causing an estimated 20.5 million deaths each year (Di Cesare et al., 2024) and lead to very large medical and financial burden for the patients, their families, and the health care systems (Savarese et al., 2022; Fry et al., 2016). CVDs are a group of diseases that affect the heart and blood vessels such as coronary artery disease (CAD), cerebrovascular disease, rheumatic heart disease and other conditions (Benjamin et al., 2018). Within these categories, four out of five CVD deaths occur due to either a myocardial infarction (MI) (heart attack) or a stroke (Pandey et al., 2023). The end stage of CVDs is heart failure (HF), and there is no comprehensive therapeutic solution currently available for HF, other than heart transplantation (Di Palo et al., 2024). The available medications for HF patients primarily focus on mitigating the impact of HF symptoms.

1.1 Heart Failure

Heart failure is a chronic condition, which occurs due to the inability of the heart to pump enough blood to meet the metabolic needs of the body. Based on the changes in pumping ability (systolic or diastolic HF) (Federmann & Hess, 1994), progression of the disease (chronic or acute HF) (Jones et al., 2020) and the specific affected heart chambers (left-sided, right -sided or biventricular HF) (Bosch et al., 2017; Frustaci et al., 1999; Rosenkranz et al., 2016), HF can be classified in different ways.

Ejection fraction (EF) is the measurement of the heart's ability to pump blood out of the left ventricle to the rest of the body. EF is expressed as the percentage of blood pumped out with each

contraction of the ventricle. A normal ejection fraction ranges between 55% to 70% (Redfield & Borlaug, 2023a). Systolic heart failure, also known as heart failure with reduced ejection fraction (HFrEF) occurs when the ventricle cannot contract sufficiently to pump enough blood (Murphy et al., 2020), leading to a reduction in the EF to less than 40%. HFrEF leads to an insufficient blood supply to meet the body's need, causing fatigue, shortness of breath and fluid retention. This condition begins with ventricular dilation and cardiac remodeling and is associated with weakened or damaged heart muscles due to coronary artery diseases or myocardial infarction (Murphy et al., 2020). Diastolic heart failure, also known as heart failure with preserved ejection fraction (HFpEF) (EF>50%), occurs due to the heart's inability to relax or fill with adequate amount of oxygenated blood during the diastolic phase (Redfield & Borlaug, 2023b). In HFpEF, the heart maintains its ability to contract and pump forcefully enough to preserve a normal EF. However, the defect lies in the diastolic phase, where the heart muscles stiffen, and left ventricle dilation is impaired, thereby reducing its capacity to fill with blood resulting in insufficient blood flow to the body organs and tissues when heart contracts. HFpEF will lead to higher pressure in the blood vessels in the heart and lungs, causing symptoms such as shortness of breath, fatigue, and leg swelling (Harper et al., 2018; Redfield & Borlaug, 2023b). The major cause of diastolic HF is the increased stiffness and thickness of the ventricles due to aging, fibrosis, high blood pressure or diabetes (Harper et al., 2018; Redfield & Borlaug, 2023b).

Heart failure can develop suddenly or gradually based on the risk factors associated with it. Acute heart failure normally occurs in persons, who have a family history of heart failure, and is characterized by sudden onset or rapid worsening of the symptoms (Deniau et al., 2023) and it mainly occurs after a heart attack or an arrhythmia. Arrhythmias are complications in the heart's electrical system that result in the heart beat irregularly, either beating too fast (tachycardia), or

too slow (bradycardia). These disruption in beating affect the heart function and leads to HF (Deniau et al., 2023). Chronic heart failure on the other hand occurs due to different risk conditions such as high blood pressure, aging, and diabetes and has a longer onset (Ramani et al., 2010).

The most common type of heart failure, resulting from CAD, MI, or prolonged high blood pressure, is left ventricular HF (LVHF) (Peteiro et al., 2011). In LVHF, the left ventricle no longer pumps sufficient blood to meet the body's demand and the blood will build up in the blood vessels that carry blood away from lungs (pulmonary veins). This buildup of blood will cause fluid build-up in the lungs, resulting in difficulty in breathing, coughing, and shortness of breath (Peteiro et al., 2011). Right ventricular heart failure (RVHF) occurs when the heart cannot pump sufficient deoxygenated blood from the right ventricle to the lungs to be oxygenated (Chizinga & Fares, 2018; Peteiro et al., 2011). This results in fluid build-up and increased pressure in the veins, causing fluid leakage to the surrounding tissues, such as the legs, genital areas and in the abdomen (Chizinga & Fares, 2018; Peteiro et al., 2011). Biventricular heart failure occurs when both sides of the heart are affected, and it shows similar symptoms as seen in both LVHF and RVHF (Natanzon & Kronzon, 2009).

CVDs and HF have many underlying risk factors, which are broadly divided into two groups: modifiable and non-modifiable risk factors.

1.1.1 Modifiable Risk Factors

Modifiable risk factors for CVDs can be controlled by lifestyle changes such as exercise and diet (Di Cesare et al., 2024). The major modifiable risk factors of CVDs are (Figure 2):

1.1.1.1 High Blood Cholesterol

Cholesterol is an important component of all of our cell membranes, and it is a precursor for the synthesis of steroid hormones (Zampelas & Magriplis, 2019). However, serum cholesterol is one of the most important risk factors for heart diseases (Mushenkova et al., 2021). Low-density lipoprotein (LDL), also referred to commonly as the “bad cholesterol” transports cholesterol throughout our body via the bloodstream and has a long circulating time in the blood (~3 days) (Cinoku et al., 2020; Mushenkova et al., 2021; Sukhorukov et al., 2016). Consequently, LDL-cholesterol is susceptible to oxidization, and the oxidized form of LDL (Ox-LDL) has a high binding affinity to the scavenger receptors found on macrophages (Mushenkova et al., 2021; Palinski et al., 1996; Sukhorukov et al., 2016). High blood levels of LDL can cause the accumulation of cholesterol in the macrophages located in the blood vessel wall (Cinoku et al., 2020; Sukhorukov et al., 2016). This buildup of cholesterol in turn, triggers an immune-inflammatory response, resulting in the formation of atherosclerotic plaques in the blood vessels (Cinoku et al., 2020; Palinski et al., 1996; Sukhorukov et al., 2016). Atherosclerosis in the coronary arteries causes angina (Manfredi et al., 2022) and cardiac arrhythmias (Goonasekara et al., 2010). The plaque can further develop as cholesterol, other debris, and foam cells, accumulate, narrowing the arterial lumen, thereby reducing the blood flow through the arteries (Kowara & Cudnoch-Jedrzejewska, 2021). Eventually, the fibrous cap, covering the plaque can rupture and this event leads to the formation of a blood clot, which can rapidly and completely block the blood flow and cause a heart attack (Herrington et al., 2016). If the blood clot lodges in the carotid arteries, it can block blood flow to the brain, causing a stroke.

Atherosclerotic Plaque Progression

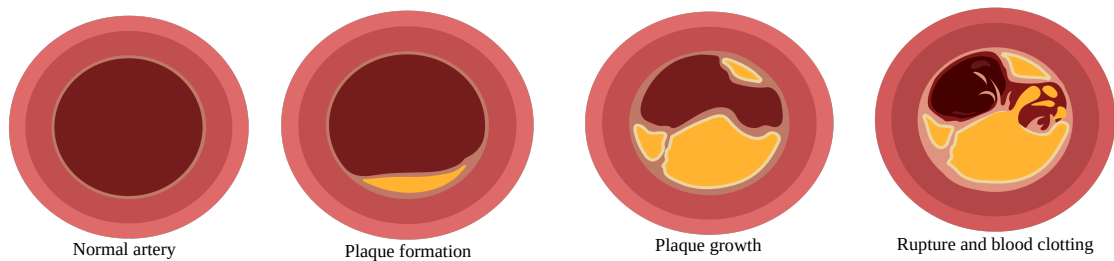


Figure 1: Atherosclerotic plaque formation and rupture: The formation of the plaque starts with the accumulation of the cholesterol in the arteries appearing as a yellowish fibrous mass. The plaque grows overtime through the accumulation of cellular debris and when the plaque ruptures, a blood clot will be formed that can completely block the blood flow through the artery leading to a myocardial infarction or a stroke (Created in <https://BioRender.com>).

1.1.1.2 High Blood Pressure

High blood pressure, also known as hypertension, is an important risk factor for myocardial infarction, and heart failure (Oh & Cho, 2020). Studies show that people with hypertension have a six-fold higher risk for myocardial infarction than the normotensive population (Levy, 1996). Hypertension is also associated with ventricular hypertrophy arrhythmias (McLenachan et al., 1987) and atrial fibrillation (Kannel et al., 1998). Normal healthy blood pressure is defined as a systolic pressure ≥ 120 mmHg and a diastolic pressure ≥ 80 mmHg (Wong, 2020). When the blood pressure exceeds these thresholds, it can damage the blood vessels and the major arteries carrying blood to different parts of our body by reducing their elasticity (Gonzalez-Guerra et al., 2021). This damage to the blood vessels accelerates atherosclerosis in the arteries (Gonzalez-Guerra et al., 2021). Hypertension also enhances the production of collagen fibers, which leads to the stiffening of the coronary arteries and thereby further elevation in the blood flow (Kim, 2023; Lee & Oh, 2010). Constant high blood pressure in the weakened arteries can lead to an aneurysm (bulging) of the arteries. These aneurysms can suddenly burst and cause bleeding inside the body, which is an acute life-threatening condition (Spittell, 1983). Hypertension also forces the heart to work harder to pump blood to the body, which causes the stiffening and enlargement of the left ventricle (Kahan & Bergfeldt, 2005). This condition is called left ventricular hypertrophy, and it is a risk factor for MI and heart failure (Kahan & Bergfeldt, 2005). Overall, untreated high blood pressure makes the heart muscle weaker or stiffer and less efficient as a pump, which can lead to heart failure.

1.1.1.3 Physical Inactivity and Obesity

Lack of physical activity results in a reduced energy consumption by our body leading to the accumulation of energy in the form of fat, which contribute to weight gain and obesity (Ogden et al., 2014). Obesity due to physical inactivity leads to increased fat deposition in the arteries, increased levels of triglycerides and LDL cholesterol and decreased HDL cholesterol levels, leading to high BP, atherosclerosis and MI (Gamage & Seneviratne, 2021; Grundy et al., 2019). In addition to physical inactivity, poor diet also plays a major role in the development of obesity and related CVD complications. Processed food, refined sugars, low fiber and high calorie diet along with sedentary lifestyle accelerate fat accumulation, inflammation and obesity (Welsh et al., 2024).

Obesity occurs due to an excess of caloric intake as compared to energy utilization by the body. The percentage of Canadians who are either overweight or obese is increasing dramatically. Both physical inactivity and obesity also increase the workload of the pancreatic islet cells, cause mitochondrial dysfunction, decrease/ impair the glucose metabolism. Together these effects cause elevated blood sugar levels in our body and lead to insulin resistance over time (Yaribeygi et al., 2021). Lack of exercise also causes increased oxidative stress and inflammation (Yaribeygi et al., 2021), which can damage endothelial cells and thereby disrupt vascular homeostasis (Tkacz et al., 2024). Obesity makes our heart pump blood more forcefully to meet the body's oxygen demand and leads to higher blood pressure (Hall et al., 2015). Overall, physical inactivity and obesity lead to the development of CADs and eventually to heart failure (Lavie et al., 2019).

1.1.1.4 Diabetes

Diabetes mellitus is a risk factor for CADs and HF (Fan, 2017). High blood sugar damages the blood vessels and nerves, accelerating cholesterol deposition in the arteries, thereby leading to atherosclerosis and MI (Dahl-Jørgensen et al., 2005; Orchard et al., 2006). High levels of sugar in the blood can non-enzymatically react with the proteins in the bloodstream, called glycation, forming advanced glycation end products (AGE). AGEs can accumulate in the blood vessels leading to stiffening of the vessels and reduction of elasticity (Twarda-Clapa et al., 2022), which in turn weakens the blood vessels, and damages vascular endothelium, impairing blood vessel dilation, which leads to a decrease in the blood flow and an increase in blood pressure, and elevates the risks of CVDs (Kaplovitch et al., 2020; Y. Li et al., 2023; van Kuijk et al., 2010). Diabetes also increases inflammation in the body and chronic inflammation promotes plaque build-up in the arteries (Orchard et al., 2006; van Kuijk et al., 2010). People with diabetes often have hyperlipidemia and decreased HDL levels, which also promotes atherosclerosis (Jia et al., 2018; van Kuijk et al., 2010). Obese people often have insulin resistance, which in turn causes Type 2 diabetes and CVDs (“Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults--The Evidence Report. National Institutes of Health,” 1998; Hardy et al., 2012). The reduced fibrinolysis and increased level of fibrinogen production present in diabetic patients increases the risk of clot formation in their arteries, leading to an increased risk of MI and stroke (Athysos et al., 2018; Katakami, 2018). Moreover, diabetic neuropathy in chronic diabetes patients can cause “silent cardiac attack” since these people have reduced sensitivity to pain. These silent MIs can lead to HF and even death among these people (Agashe & Petak, 2018; Vinik & Ziegler, 2007).

1.1.1.5 Smoking

Tobacco use is a major cause of CVDs and heart failure (Messner & Bernhard, 2014a). Cigarette smoking leads to the production of organic substances such as carbon monoxide (CO), nicotine, acetaldehyde, and formaldehyde (Ambrose & Barua, 2004; Messner & Bernhard, 2014b). CO has a higher affinity to hemoglobin than oxygen and binds with hemoglobin to form carboxyhemoglobin. This reaction reduces the amount of hemoglobin available to carry oxygen. Therefore, CO diminishes the oxygen carrying capacity of hemoglobin, leads to hypoxia, and thereby leads to other CADs and heart failure (Ambrose & Barua, 2004). In response to chronic hypoxia, our body adopts a process called compensatory polycythemia (increased erythropoiesis), which makes the blood more viscous and contribute to thrombosis in the blood vessels (Ambrose & Barua, 2004; Messner & Bernhard, 2014b). These changes in hemodynamics and thrombosis causes our heart to work harder to pump blood through the arteries, and it results in increased blood pressure (Ambrose & Barua, 2004; Klein, 2022b). The gas phase of cigarette smoke and tar, produces enormous amounts of free radicals, which can damage the vascular endothelium and accelerate atherosclerosis (Kaufmann et al., 2000; Klein, 2022a; Messner & Bernhard, 2014a; Pittilo, 2000). Patients who chronically smoke also show increased levels of cholesterol, LDL, and triglycerides (Clarke et al., 1986; Craig et al., 1989; Maeda et al., 2003) and a decreased levels of HDL in their blood (Maeda et al., 2003; Neufeld et al., 1997), which also enhances atherosclerosis (Bolorunduro et al., 2015; Klein, 2022a).

Nicotine contained in tobacco leads to the increased release of catecholamines which increases myocardial contractility and thereby increases heart rate (Benowitz & Gourlay, 1997; Messner & Bernhard, 2014b). The increased vasomotor tone contributes to the rupture of atherosclerotic plaque in the arteries and thereby leads to acute cardiac events such as MI and heart failure (Klein, 2022a; Messner & Bernhard, 2014b).

Non-Modifiable Risk Factors

Risk factors that cannot be controlled through lifestyle changes are referred to as non-modifiable risk factors (Di Cesare et al., 2024) (Figure 2). Some important non-modifiable risk factors of CVDs are:

1.1.2.1 Genetics/Family History

Many cardiac conditions, leading to MI or HF can be inherited ((Kathiresan & Srivastava, 2012). A study in hypercholesterolemia patients revealed a 5 kb deletion of the LDL- receptor gene, which eliminates several exons and thereby abolishes the function of the major cholesterol transport mechanism in the body (Lehrman et al., 1985). In some patients with hypertrophic cardiomyopathy, a mutation in β -cardiac myosin heavy chain was observed (Geisterfer- Lowrance et al., 1990). Marfan syndrome (FBN-1 gene mutation) (Dietz et al., 1991) and Mowat-Wilson syndrome (ZEB2 gene mutation) (Birkhoff et al., 2021) are the other examples of single gene mutation defects that affect heart function. Other cardiac disorders such as the long QT syndrome (Curran et al., 1995), severe hypercholesterolemia (Abifadel et al., 2003;

Brown & Goldstein, 1986; C. K. Garcia et al., 2001), familial hypobetalipoproteinemia (Cohen et al., 2006; Musunuru et al., 2010), hypertrophic cardiomyopathy (Seidman & Seidman, 2001), low and high blood pressure (Boyden et al., 2012; Geller et al., 1998; Lifton et al., 2001) on the other hand are caused by multiple genetic variants.

1.1.2.1 Sex

In biological terms sex is defined as the classification of male and female based on their reproductive anatomy. Male and female display differences in the structure and function of the cardiovascular system. A female heart is typically about three-quarters the size of that of a male heart featuring smaller chamber size, lower cardiac mass, reduced wall thickness and narrowed blood vessels compared to male (Molina & DiMaio, 2012, 2015). The difference in their cardiac structure makes males and females respond differently to symptoms, risks, and diagnostic methods (St Pierre et al., 2022). For a male, the onset of CVDs will be earlier in their life compared to female, because females are protected by estrogen hormone until menopause (Bajelan et al., 2019; Stanhewicz et al., 2018). Since female arteries are smaller, they are more prone to blood clots or plaque blockages and are more difficult to repair (Mehta et al., 2016; Schamroth Pravda et al., 2021). This makes them more vulnerable to MI and HF later in their life (Schamroth Pravda et al., 2021).

The symptoms of heart failure also vary between male and female. Males exhibit more “classic” symptoms of heart failure such as, shortness of breath, fatigue, fluid retention and irregular heartbeat. However, females suffering a MI often present with atypical symptoms such as nausea, weakness, headache, back pain, sleep disturbance and depression (DeVon et al., 2017;

Lichtman et al., 2018). Consequently, in females these symptoms are often under-diagnosed and misdiagnosed, leading to further complications in their condition stemming from delays in receiving appropriate treatment (DeVon et al., 2017).

The risk factors associated with CVDs are often seen in both male and female, but their prevalence and impact may vary between these groups. Specifically, some of the risk factors of CADs and heart failure are more potent in females as compared to males. For instance, pregnancy itself is a risk factor of CVDs. Gestational diabetes, gestational hypertension, (Gotsch et al., 2009; Kessous et al., 2013) and perinatal depression (Nicholson et al., 2016), all adversely affect the heart function and lead to the development of cardiac complications later in their life. Smoking (Huxley & Woodward, 2011), emotional stresses such as depression and anxiety (Nicholson et al., 2016), obesity (Wilson et al., 2002), menopause, oral contraceptive uses (Dou et al., 2023), hormone replacement therapy (Staniewicz et al., 2018), are more prevalent in women and have adverse impact on heart health. Additionally autoimmune (del Rincón et al., 2015) and inflammatory diseases such as lupus, scleroderma, rheumatoid arthritis (Kottlil & Mathur, 2022) are the other risk factors affecting female heart health. Also, females face heart disease risks as a result of chemotherapy treatments for ovarian, breast and uterine cancers (Kitson et al., 2018). In contrast, in males high blood cholesterol, diabetes, alcohol usage, high BP and physical inactivity are the major causes of heart diseases (Dunlay et al., 2009; George et al., 2015).

Also, male and female require different diagnostic and treatment procedures for heart diseases. However, there is a notable gap in the cardiac research field, in which far fewer studies include females or focus on females (Bosomworth & Khan, 2023; Thompson & Daugherty, 2017). For

instance, aspirin is a widely used drug for CAD to prevent the formation of arterial blood clots (Cofer et al., 2022). However, despite its efficacy in reducing CVDs in males, studies supporting its use in females remain inconsistent and controversial (Zheng & Roddick, 2019). For instance, the women's health study (WHS) initially suggested that aspirin could be beneficial for women, particularly in reducing the risks of stroke, heart attack and other CVDs (Cook et al., 2012). However, more recent studies raise concerns about the use of aspirin in women particularly in post-menopausal women with additional CVD risk factors such as high blood pressure, diabetes, or a family history of heart diseases (Garcia et al., 2016). A large cohort study done by WHS shows no significant reduction in major cardiac events such as MI with the use of aspirin (Cook et al., 2012). Furthermore, a study done by Dr. Fuchs and his team revealed that long term use of aspirin in women is associated with higher mortality rates compared to the women who did not use aspirin (Chan et al., 2007). It also increases the risk of gastrointestinal bleeding, ulcers and hemorrhagic stroke in older women (Huang et al., 2011). Despite these conflicting results, aspirin continues to be prescribed for CVDs prevention in women. The necessity for tailored diagnostic and treatment strategies and the limited scope of women cardiac health research and the lower participation of women in clinical trials, further complicates their cardiac health (Bosomworth & Khan, 2023; Thompson & Daugherty, 2017).

1.1.2.1 Gender

Gender is attributed to the social, cultural and psychological characteristics, roles and behaviors that a society assigns to a particular sex. It is different from the biological sex, which is associated with chromosomes, hormones and reproductive anatomy. It influences the CVD risk through difference in healthcare access, stress exposure, societal roles and behavioral patterns

(Zelco et al., 2023). The lesbian, gay, bisexual, transgender, queer and other gender, and sexual minority populations (LGBTQ+), face inequality across various fields including cardiovascular risk factors compared to their heterosexual peers (Caceres et al., 2020; Thompson & Daugherty, 2017) (*The Health of Lesbian, Gay, Bisexual, and Transgender People*, 2011). Even though there is evidence that LGBTQ+ community experience worse cardiovascular health related problems, they have received only limited attention (Caceres et al., 2017; Streed et al., 2017). Relative to the heterosexual people, LGBTQ + people experience unique challenges which affect their mental and physical health (Meyer, 2003). Structural factors including discriminatory laws and social norms constitute barriers to readily accessing the necessary resources and support (Meyer, 2003). These combined stresses result in the development of higher rates of depression and substance use which increases their risk of CVDs (Caceres et al., 2019; A. Klein & Golub, 2016; McCabe et al., 2019; Meyer, 2003; Streed et al., 2017). Also, studies indicate that there is higher rate of HIV infection among transgender women, which is associated with an increased risk for CVDs (Feinstein et al., 2019). Moreover, the gender affirming hormone therapies and surgeries raise the risk of CVDs and HF in the LGBTQ + community (Connelly et al., 2019; Defreyne et al., 2019; Maraka et al., 2017).

1.1.2.1 Age

Aging results in structural and functional changes in the heart, which can impact its efficiency and overall cardiovascular health. In an individual over 70 years of age, several key changes such as the left ventricular wall thickening, ventricular hypertrophy and atherosclerosis in the blood vessels occur which affects the heart's ability to pump blood effectively (Scholz et al., 1988). Aged people have increased levels of collagen deposition and fibrosis in their heart leading to

increased diastolic dysfunction and HF (Lakatta & Levy, 2003; Scholz et al., 1988). Aging increases vascular stiffness, and loss of elasticity of the blood vessels, which in turn increases the development of high blood pressure (Climie et al., 2023). Increased ventricular thickness and hypertension leads to the stiffening of the myocardium which in turn leads to functional changes in the heart including, changes in contractility, heart rate, end diastolic volume (EDV), and end systolic volume (ESV) (Aïdoud et al., 2023; Marengoni et al., 2011; Najjar et al., 2005). Aging also increases chronic low-grade inflammation, damages the blood vessels and leads to the development of atherosclerosis (Aïdoud et al., 2023; Climie et al., 2023). Metabolic changes among the aged people, including insulin resistance, changes in lipid profile, hyperlipidemia, increased risk of development of diabetes also increases the CVDs and HF risks (Aïdoud et al., 2023; Marengoni et al., 2011; Najjar et al., 2005; Strait & Lakatta, 2012).

Overall both non-modifiable risk factors and modifiable risk factors contribute to several CVD risks and can ultimately lead to death. While non-modifiable risk factors cannot be altered, changing lifestyle habits can manage modifiable risk factors and reduce the likelihood of CVD diseases (Figure:2).

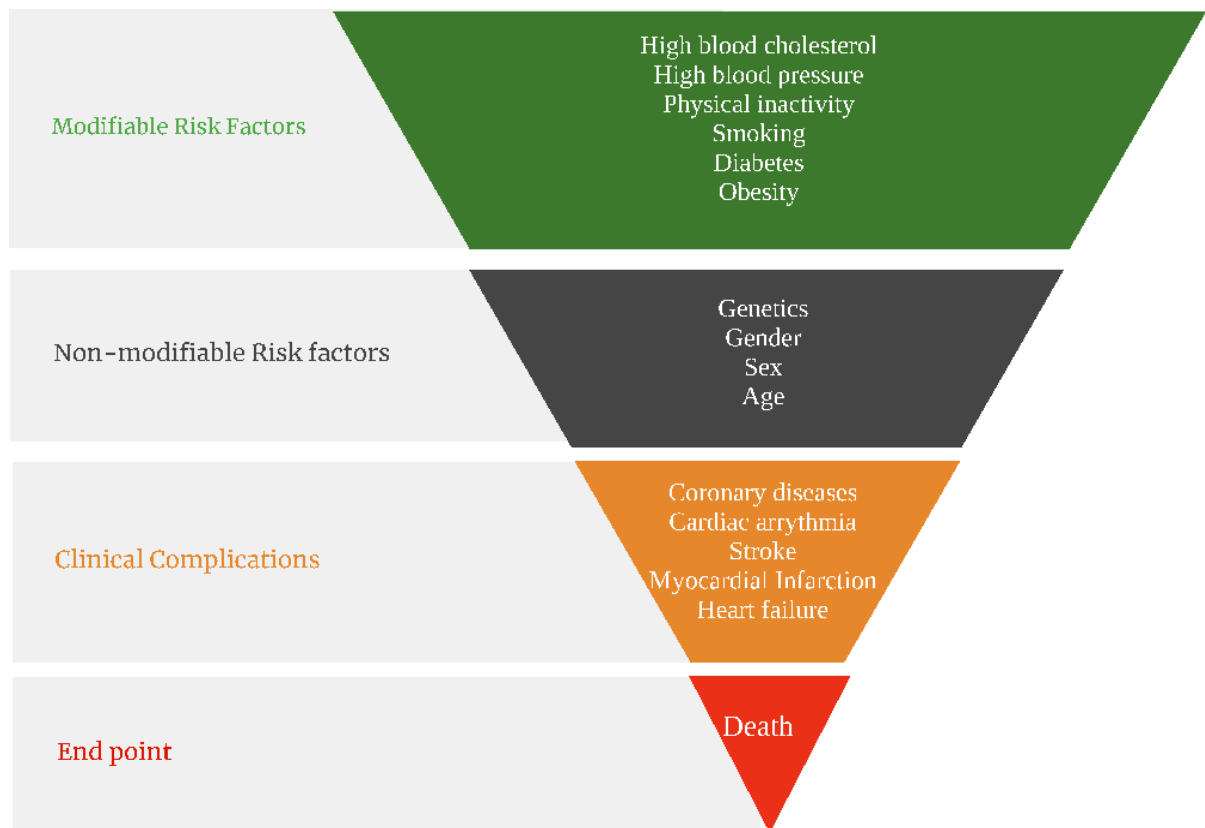


Figure 2: Schematic diagram of the risk factors associated with CVDs. The modifiable and non-modifiable risk factors of heart failure cause different CVD clinical complications (Created in <https://BioRender.com>).

According to a 2023 study, by the Heart & Stroke foundation of Canada, about 750,000 Canadians are currently living with HF and an estimated 100,000 Canadians are diagnosed with HF each year. This growing epidemic significantly challenges public health as the patients who are diagnosed with heart failure often die within 5 years (Savarese & Lund, 2017). Interestingly about 61% of HF occurs due to ischemic heart diseases, mainly as a result of MI and there is a critical need for efficient therapeutic solutions to address this escalating crisis in cardiovascular health

(Cahill & Kharbanda, 2017).

1.2 Myocardial Infarction (MI)

Atherosclerotic plaque rupture can cause platelets to clump and form clots, which can completely obstruct the blood flow in the vessel and lead to a MI (Figure 1). This blockage causes hypoxia in the myocardium, leading to the death of the heart muscle cells (Jenča et al., 2021; Mehta et al., 2016; Saleh & Ambrose, 2018). Once the heart muscles die, the damage is irreversible because cardiomyocytes are terminally differentiated cells and cannot regenerate (Jenča et al., 2021).

The classic symptoms of MI include sudden, crushing, or radiating chest pain along with substernal pain that may extend to the jaw, left arm, back and shoulder. The non-classic symptoms of MI are heartburn and shortness of breath (Lu et al., 2015a), nausea, vomiting with abdominal pain, pale cool skin, sweating, and anxiety (Lu et al., 2015a).

However, patients with diabetic neuropathy and women with MI may not be diagnosed effectively. Neuropathic patients may not sense pain leading to the occurrence of a “silent” heart attack (Agashe & Petak, 2018; Vinik & Ziegler, 2007), while women often experience more non-classic symptoms of MI such as nausea and tiredness, which can result in misdiagnosed or undiagnosed conditions (Mehta et al., 2016).

The diagnostic method of MI includes measuring serum cardiac markers (Troponin, creatine kinase), electrocardiogram (ECG) (ST- elevation/depression), echocardiography, coronary angiogram, and exercise stress test (Lu et al., 2015a; Saleh & Ambrose, 2018). The major treatment options of MI are coronary angioplasty and coronary artery bypass grafting, which

restore blood flow to the ischemic area. Patients are also given thrombolytics, aspirin, β -blockers, statins, and ACE inhibitors to reduce the stresses in the heart (Lu et al., 2015b; Mehta et al., 2016; Saleh & Ambrose, 2018). If MI goes undiagnosed or untreated, it can lead to myocardial fibrosis, heart failure and potentially death (Chalise et al., 2023a; Jenča et al., 2021).

My project primarily focuses on the mechanism of wound healing after MI, with particular emphasis on how the activation of fibroblast to myofibroblast is controlled. We aim to investigate the key proteins, and transcription factors that are activated during the cardiac fibroblast activation and differentiation processes in the context of MI induced cardiac injury. Understanding the precise mechanism by which the fibroblasts are activated and transformed into myofibroblasts will provide critical insight into the pathophysiology of post-MI wound repair. Ultimately, a deeper understanding of these processes will contribute to identifying potential therapeutic targets to improve post-MI cardiac function and modulate cardiac fibrosis.

1.3 Cardiac Fibroblasts

Cardiac fibroblast cells (CF) are a major cell population in the heart (Moore-Morris et al., 2015). They are connective tissue cells and have important roles in cardiac development and function (Souders et al., 2009c, 2009a). The primary role of CF is to secrete extracellular matrix (ECM) proteins in order to maintain the functional integrity of the heart. CF are the key players of wound healing and fibrosis in the heart after MI (Moore-Morris et al., 2015; Souders et al., 2009c; Tallquist & Molkentin, 2017a). The hypoxic tissue injury following MI, releases endogenous signals known as danger associated molecular patterns (DAMPs), which in turn activate the innate immune system to remove the dead cells and debris in the infarcted area (Bianchi, 2007a; Timmers et al., 2012a). These inflammatory immune cells also secrete and activate different chemokines, cytokines (tumor necrosis factor (TNF), interleukin-1 β (IL-1 β))

and growth factors such as fibroblast growth factor (FGF), transforming growth factor- β (TGF- β), platelet derived growth factor (PDGF) which promote the activation of fibroblasts from their quiescent stage (Dewald et al., 2004; Frangogiannis, 2007; Herskowitz et al., 1995). The activated fibroblast, also known as the myofibroblast, secretes different ECM proteins to repair the wound area, and these proteins contribute to collagen cross linking and scar formation which are essential for wound healing (Chalise et al., 2023b). However, abnormal activation or prolonged secretion of ECM proteins by the myofibroblasts leads to defective cardiac remodeling, fibrosis, and HF in the future (Czubryt, 2012a).

1.3.1 Phenotypes of Cardiac fibroblast

CF cells have traditionally be thought to exist in three different phenotypes, which are known as quiescent or homeostatic cardiac fibroblasts, myofibroblast and matrifibrocytes. Each type has distinct structural, functional, and molecular properties (Ivey & Tallquist, 2016).

1.3.1.1 Quiescent Cardiac Fibroblast

The quiescent CF, also known as homeostatic CF, are morphologically flat, spindle shaped cells and may have multiple projections radiating from the central cell body (Krenning et al., 2010; Tallquist, 2020). They are mesenchymal cells and maintain extracellular matrix (ECM) homeostasis and provide structural support to cardiomyocytes and blood vessels (Krenning et al., 2010; Souders et al., 2009b; Tallquist, 2020). These cell types maintain the balance of matrix proteins and regulate ECM turnover to support the structural integrity of the heart (Camelliti et al., 2005a; Eschenhagen, 2018; Ivey & Tallquist, 2016). They also respond to signals under

mechanical stress situations and maintain the baseline architecture of the heart (Camelliti et al., 2005a; Ivey & Tallquist, 2016). The quiescent cardiac fibroblasts are lowly contractile and are not actively proliferating (Ivey & Tallquist, 2016).

1.3.1.2 Activated Fibroblast

These are metabolically active fibroblast cells and structurally more elongated than the quiescent CF (Ivey & Tallquist, 2016; Souders et al., 2009c; Tallquist & Molkentin, 2017a). Under moderate stress situations, the quiescent fibroblasts convert into activated fibroblasts, and secrete focal adhesion molecules, and stress fiber proteins (Souders et al., 2009c; Tallquist & Molkentin, 2017a). The major molecular features of activated fibroblasts are the increased expression of the ECM proteins such as collagen type I, II, III, IV, fibronectin, intermediate level expression of the contractile protein α -smooth muscle actin (α -SMA) and increased integrin expression to interact with the cell ECM matrix (Moore-Morris et al., 2015; Souders et al., 2009b; Tallquist, 2020). They also upregulate the expression of growth factors such as TGF- β , PDGF and FGF, and IL-6, which in turn, helps to further activate inflammatory cells and myofibroblast under stress situations (Camelliti et al., 2005a; Moore-Morris et al., 2015; Tallquist, 2020). This intermediate phenotype is a transition phase of non-active fibroblast to become myofibroblast (Camelliti et al., 2005a; Moore-Morris et al., 2015).

1.1.1.1 Myofibroblast

Under persistent stress situations, the partially activated fibroblast will further convert into the highly active myofibroblast phenotype (Camelliti et al., 2005a; Ivey & Tallquist, 2016; Souders

et al., 2009c). These cells are morphologically enlarged and irregular shaped cells with contractile and proliferative properties (Souders et al., 2009c; Tallquist & Molkentin, 2017a). Myofibroblasts are rarely found in non-pathological conditions, and they are the primary cells responsible for wound healing and repair after tissue injury (Baudino et al., 2006; Plikus et al., 2021a; Tallquist & Molkentin, 2017a). Myofibroblasts produce high levels of ECM proteins, such as collagen type I and III, fibronectin (FN), ED-A fibronectin (ED-A-FN), α -smooth muscle actin (α -SMA) and embryonic smooth muscle myosin (SMemb), which helps for the wound contraction, and healing and scarring of the wound after MI (Alexanian et al., 2021; Camelliti et al., 2005a; Krenning et al., 2010). Three major conditions are necessary for the myofibroblast activation, which include TGF- β activation, production of the fibronectin splice-variant ED-A FN and a stressed/strained ECM by the interaction between the ECM and ED-A FN/integrin (Alexanian et al., 2021; C. Hall et al., 2021a; Herum et al., 2017). Also, myofibroblasts often express the plasminogen activator inhibitor-1 protein (PAI-1), which inhibits ECM degradation and promotes fibrosis (Offersen et al., 2003). In the heart, prolonged activation of myofibroblasts leads to excessive deposition of ECM proteins and thereby the stiffening and fibrosis (Plikus et al., 2021a; Tallquist, 2020; Tallquist & Molkentin, 2017b). The major functional properties of myofibroblast include contractility, increased rates of proliferation, and decreased migration (Plikus et al., 2021b; Tallquist & Molkentin, 2017b).

1.1.1.2 Matrifibrocytes

Matrifibrocytes are less contractile and proliferative compared to the myofibroblast and are primarily found in the stable scar (Fu et al., 2018a). They are mostly seen around day-10 post-MI, following the initial stages of wound healing and scar maturation (Fu et al., 2018a; C. Li et

al., 2022). In matrifibrocytes, the expression of genes associated with bone, cartilage and connective tissue are upregulated, while the levels of contractile and proliferative marker genes such as α -SMA are relatively lower as compared to myofibroblasts (Fu et al., 2018a; Herum et al., 2020). A recent study has shown that removing matrifibrocytes from the heart after MI leads to poor cardiac health, indicating the importance of this cell type in mediating wound healing, however, the precise function of this cell type in heart tissue repair is not fully understood (Herum et al., 2020).

1.1.2 Origin of Cardiac Fibroblast

Even though the cardiomyocytes contribute to ~ 70% of the total tissue volume, the non-myocytes cells of the heart, such as cardiac fibroblasts, endothelial cells, and smooth muscle account for approximately 70% of the total number of cells, depending on the organism (Brutsaert, 2003; Deb & Ubil, 2014; Deng et al., 2023). Fibroblasts are abundant cells in the heart and arise from several distinct sources during the development.

1.1.2.1 Developmental Origins of Cardiac Fibroblasts

Cell lineage tracing studies have shown that cardiac fibroblasts arise from two main mechanisms (Figure 3) during embryonic heart development.

- 1) epithelial to mesenchymal transition and (EMT).
- 2) endothelial to mesenchymal transition (EndoMT).

The epicardium, which is the outermost layer of the heart, is composed of epithelial cells and is the primary source of fibroblasts during development (Muñoz-Chápuli et al., 2001). These epicardial cells undergo a differentiation process known as EMT, in which they lose their epithelial characteristics following downregulation of epithelial genes such as E-Cadherin and transform into the mesenchymal fibroblast like cells (Muñoz-Chápuli et al., 2001; Pinto et al., 2016; Zhou et al., 2010). The signaling molecules, TGF- β , FGF, PDGF and the transcription factor TCF-21 (also known as epicardin) play regulatory roles in guiding epicardial progenitor cells to the fibroblast lineage (Acharya et al., 2012; Deng et al., 2023; Olivey et al., 2006). Epicardial derived fibroblast cells further differentiate into the sub-endocardium, coronary vasculature, atrioventricular (AV) valve and the fibrous skeleton of the heart during embryonic development (Muñoz-Chápuli et al., 2001; Zhou et al., 2010). Fate mapping studies in the mouse

heart show that 80-90% of the adult cardiac fibroblast are derived from the TCF-21 expressing epicardial progenitor cells (Pinto et al., 2016).

In addition to epicardial progenitor cells, a significant portion of cardiac fibroblast (10-20%) arise from the endothelial cells by EndoMT (de Lange et al., 2004). During this process, the endothelial cells in the endocardium differentiate into the fibroblast cells. EndoMT is regulated by signaling pathways such as TGF- β , Wnt and PDGF that activate the mesenchymal transcription factors Snail and Twist (Ali et al., 2014a; Armstrong & Bischoff, 2004; de Lange et al., 2004; Kisanuki et al., 2001). The fibroblasts derived from EndoMT function to form the cardiac cushions and valves, where they are called valvular interstitial cells (VICs) (Armstrong & Bischoff, 2004). These cells are also critical for the AV canal and interstitial septum development and the disruption of cardiac cells differentiation by EndoMT can leads to congenital heart defects (Armstrong & Bischoff, 2004; Kisanuki et al., 2001; Niessen et al., 2008).

Other progenitor populations, such as the neural crest cells and the multipotent progenitor cells derived from the bone marrow known as mesangioblasts also contribute to the fibroblast pool, but their contributions are not very well defined (Cossu & Bianco, 2003; Liyew et al., 2024).

1.1.2.2 Origin of Cardiac Fibroblast During Cardiac Pathology

Previously it was believed that during pathological conditions activated fibroblast could be derived from a variety of different sources such as epithelial cells, endothelial cells, fibrocytes, pericytes, bone marrow progenitor cells, monocytes, and smooth muscle cells (Ali et al., 2014b; Haudek et al., 2006; Ieronimakis et al., 2013; Kramann et al., 2015; Moore-Morris et al., 2014;

van Amerongen et al., 2008; Widyantoro et al., 2010; Zeisberg et al., 2007). However recent studies have demonstrated that the vast majority of the activated fibroblasts and myofibroblast present in the pathological myocardium are derived from the resident fibroblasts of embryonic endothelial and epicardial origins (Ali et al., 2014b; Kanisicak et al., 2016a; Moore-Morris et al., 2014; Ruiz-Villalba et al., 2015). A genetic lineage study in the MI mouse heart done by Kanisicak and colleagues, demonstrate that the TCF-21 positive fibroblast cells population, which are the resident fibroblasts, increased tenfold in fibrotic areas (Kanisicak et al., 2016a). The study also revealed that TCF-21 positive fibroblasts are the progenitor cells for approximately 70% of the periostin (Postn) expressing myofibroblasts in the MI heart (Kanisicak et al., 2016b, 2016a; Pinto et al., 2016). The study using a pressure overloaded mouse model of hypertension also showed similar results. Furthermore, this study also indicated that the myofibroblasts may revert to its original less active stage after injury resolution, which shows the plasticity of the fibroblast phenotype and the potential for therapies to reverse the process of fibroblast activation (Kanisicak et al., 2016b).

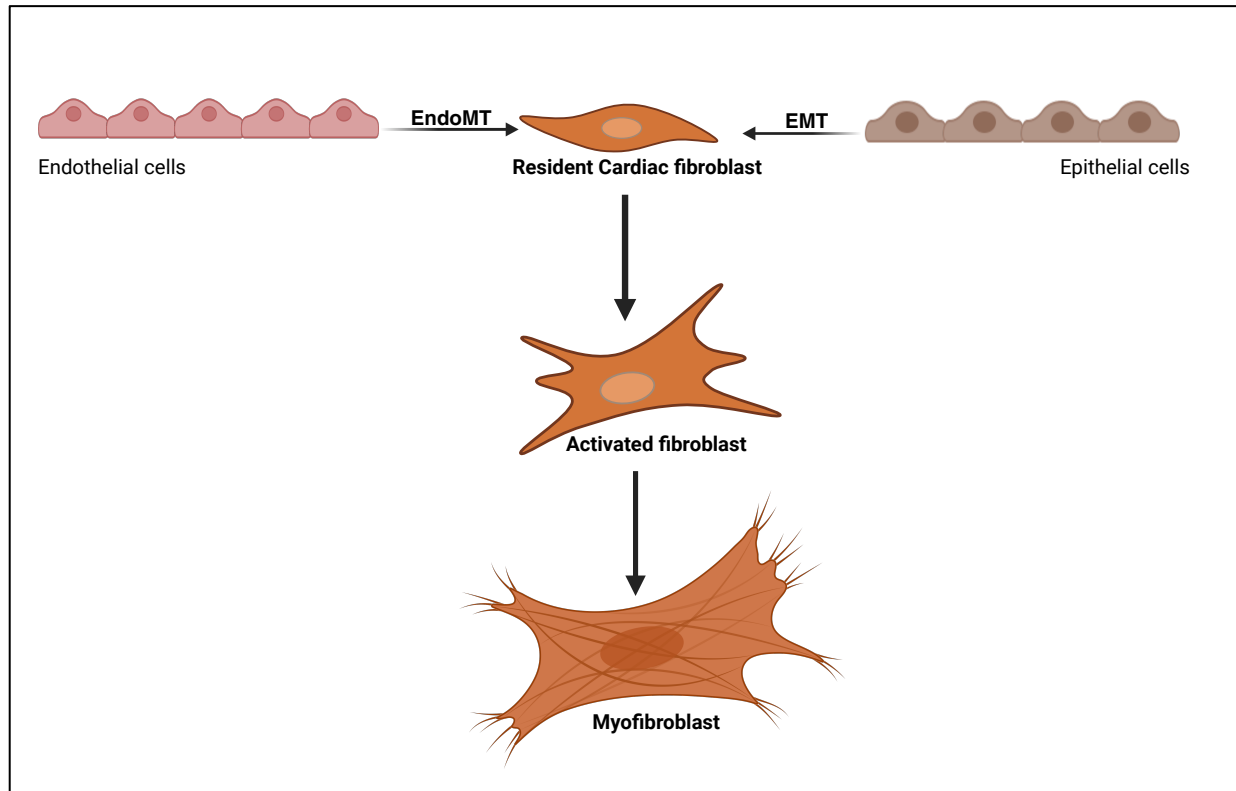


Figure 3: Origin of myofibroblast in the diseased heart. Recent lineage tracing studies have confirmed that the resident fibroblast cells derived from embryonic epicardial, and endothelial cells, are the progenitors of nearly all myofibroblasts in the diseased heart (Created in <https://BioRender.com>).

1.1.3 Molecular Diversity of Fibroblasts

The fibroblasts derived from epicardium and endothelium are important during development and pathogenesis and these fibroblasts often express markers such as α -SMA, TCF-21, Postn and collagen I (Kovacic et al., 2012; Thiery et al., 2009; Travers et al., 2016b). In response to injury, the resident fibroblast become activated and differentiate into myofibroblasts characterized by the expression of markers, α -SMA, postn, FSP-1, desmin and, vimentin (Kanisicak et al., 2016b, 2016a). Other than these progenitor cells, fibroblast also arise from different sources. Cell lineage and fluorescent reporter studies revealed that there are several additional contributors to its diversity (Pakshir et al., 2020a). In the eye, the keratinocytes are the major precursor of myofibroblast (Rocher et al., 2020; aWilson et al., 2017), while in the skin, the dermal fibroblast cells are the primary source (Jiang & Rinkevich, 2020; Lynch & Watt, 2018). The pericytes, which wrap the endothelial cells of the microvasculature, are the major contributors of myofibroblast in several organs including skin, muscle, lung, kidney, and liver where they are known as hepatic stellate cells (Di Carlo & Peduto, 2018; Pakshir et al., 2020b). In tumor tissues, the myofibroblast are largely derived from cancer associated fibroblasts (CAF) (Affo et al., 2017). However, in the brain the activated astrocytes and microglial cells differentiate and are involved in scar formation (Fernández-Klett & Priller, 2014; Pakshir et al., 2020b).

1.1.4 Functions of Cardiac Fibroblast

1.1.4.1 ECM Production and Maintenance

The primary function of CF is the production and maintenance of ECM. Cardiac fibroblasts

produce all the ECM components, including fibrous proteins, adhesion molecules and matrix glycoproteins and proteoglycans, that provide structural support and facilitate cell-matrix interactions in the myocardium (Kendall & Feghali-Bostwick, 2014a). Collagen type- I, a rope shaped triple stranded tertiary protein, is the most abundant protein in the mammalian body, is produced by the fibroblasts and provides rigidity, and tensile strength to the ECM and prevents it from overstretching (McKleroy et al., 2013; Singh et al., 2023). On the other hand, the elastin protein produced by the fibroblast, form highly unstructured elastic crosslinks and help the ECM stretch without breaking (Camelliti et al., 2005b). The other collagen subtypes produced by the fibroblast cells are also involved in the matrix formation. For example, the collagen type IV largely contributes to the composition of the basal membrane of the ECM (Kendall & Feghali- Bostwick, 2014a; McKleroy et al., 2013).

The adhesive proteins such as laminin and fibronectin, produced by the fibroblast, are mainly involved in facilitating the interaction between cells and ECM (Kendall & Feghali-Bostwick, 2014a; Tracy et al., 2016). Fibronectin contains an RGD tripeptide domain which helps it interact with the transmembrane adhesion protein integrin and this interaction helps for fibronectin filaments assembly (Zent & Guo, 2018a). The splice variant of fibronectin called ED-A fibronectin forms a positive feedback loop with TGF- β during stress stimulation (Benito-Jardón et al., 2017; Zent & Guo, 2018a). Laminin also binds to the integrin receptors to interact with the basal lamina, which is crucial for initiating various signaling pathways (Kendall & Feghali-Bostwick, 2014a; Pang et al., 2023).

Proteoglycans, glycosaminoglycans, and hyaluronic acid in the ECM, are also produced by the fibroblast cells (Kendall & Feghali-Bostwick, 2014a; Souders et al., 2009c). Proteoglycans fill

the space between structural proteins and help for cell-cell interactions and help aid in migration of immune cells, fibroblasts, myofibroblast through the ECM as well as migration of endothelial cells during angiogenesis (Ghatak et al., 2015; Kolesnikoff et al., 2022). They also play key roles in regulating chemical signals like growth factors and TGF- β (Ghatak et al., 2015; Kendall & Feghali-Bostwick, 2014b).

In addition to matrix production, fibroblast remodel the ECM by regulating its degradation. CF secrete proteases known as matrix metalloproteinases (MMP), which degrade the ECM components (Kendall & Feghali-Bostwick, 2014b; Lindsey, 2004) as well as the tissue inhibitor of metalloproteinases (TIMPS), which inhibits MMPs activity (Moore et al., 2012). Therefore, overall, the fibroblast maintains the normal homeostasis of the ECM.

1.1.4.2 Chemical and Mechanical Signaling

Fibroblasts are known to respond to a range of chemical and mechanical signals, which maintain cardiac homeostasis and regulate the heart's response to injury (Catalucci, 2008; Weber, 2004). The heart is constantly exposed to mechanical stretch, and its proper regulation is important for normal cardiac function (Holmes et al., 2005; Weber, 2004). Mechanical stretch induces fibroblast cells to produce more ECM proteins, growth factors and cytokines (van Wamel et al., 2001; Weber, 2004). It also triggers the fibroblast to secrete MMP to degrade ECM components, release TGF- β , allowing for tissue remodeling (van Wamel et al., 2001).

The factors secreted by fibroblasts are determined by the signals they receive, such as those induced by inflammation, hypoxia, or mechanical stress (Holmes et al., 2005). In response to

these stimuli, CF secrete a range of chemical signals such as cytokines and growth factors (Nian et al., 2004). CF also regulates cardiac function by activating angiotensin – II (Ang II) or TGF- β under specific stress conditions (Dostal & Baker, 1999; Eghbali et al., 1991). Ang II is the key effector molecule of the renin-angiotensin (RAS) system, which is important for regulating normal blood pressure and volume (Dostal & Baker, 1999; Kumar et al., 2008). TGF- β activation induces the synthesis of collagen, fibronectin, proteoglycans and increases cardiac fibroblast activation, proliferation, migration, and apoptosis (Chen et al., 2004; Eghbali et al., 1991; Lea, 2007a). Following cardiac injury or heart failure, the cardiac fibroblast also secretes pro-inflammatory interleukins such as IL-1 β , IL-6 and tumor necrosis factor- α (TNF- α) (Nian et al., 2004; Turner et al., 2007). IL-1 β stimulates cell migration (Long, 2001) and IL-6 is involved in regulating cardiac fibroblast- myocytes interactions (Banerjee et al., 2009).

1.1.4.3 Electrical Signaling

Cardiac fibroblasts directly influence the electrophysiological properties of the myocardium (Kamkin et al., 2003a). Unlike cardiomyocytes, cardiac fibroblasts are electrically “non-excitable” cells, and impede electrical signaling by separating cardiomyocytes and reducing regional coupling (Kohl et al., 1992; Yue et al., 2011). They interact with cardiomyocytes by gap junctions, ECM remodeling, and paracrine signaling and thus influence the electrical conduction of the heart (Camelliti et al., 2005c; Martins-Marques, 2021). By mechanotransduction or by electromechanical coupling with the cardiomyocytes, these cells can influence the electrical activity in the myocardium (Kamkin et al., 2003b). Mechanical forces can activate ion channels, such as Na⁺, K⁺, Ca²⁺ to allow the flux of ions into/out of cells. This process in turn can influence

fibroblast functions including ECM remodeling, cytokine secretion and even fibrosis, which further affect the electrical properties of the heart (Camelliti et al., 2005c; Hu & Sachs, 1997).

In summary, CF have a central role in maintaining the normal structural integrity and function of the heart by producing and remodeling the ECM. They produce the key ECM components such as collagen, elastin, fibronectin, and proteoglycans which provide structural support, facilitate cell- matrix interactions and contribute to tissue homeostasis. CF also secrete cytokines and growth factors, and MMPs in response to mechanical and chemical signals, which regulate the remodeling and repair of the cardiac tissues. They are also involved in regulating cardiac homeostasis by responding to several stress situations, which can trigger the activation of different profibrogenic molecules such as TGF- β and Ang II. Additionally, CF influences the heart's electrical properties by interacting with cardiomyocytes through gap junction and cardiac remodeling. These diverse roles of CF are essential for the normal functioning of the heart and its ability to adapt to injury or stress.

1.1.5 Fibroblasts in the Diseased Heart

The primary function of the fibroblast in the diseased heart is its key role in the wound healing process (Fan et al., 2012). After a MI, the fibroblasts will be activated to become myofibroblasts and they secrete ECM proteins to repair the wounded regions making collagen crosslinks and the formation of the scar (Chalise et al., 2023a; Fan et al., 2012; Vasquez et al., 2011). However, increased production of ECM proteins leads to myocardial stiffening, which affects the normal systolic and diastolic functions of the heart, leading to fibrosis and heart failure (Chalise et al., 2023b, 2023a).

Also, after MI, the electrical properties of fibroblast changes, which includes a shift in their action potential, increased membrane stiffness and increased sensitivity to mechanical stresses (Camelliti et al., 2005c; Ma et al., 2017a). If these fibroblasts couple with myocytes it could disrupt the normal electrical activity of the heart (Ma et al., 2017a; Pellman et al., 2016). Moreover, in the infarcted region, increased collagen and scar tissue can disrupt the connection between fibroblast and myocytes that could further interfere the electrical signaling and thereby contributing to the development of arrhythmias (Hall et al., 2021b; Souders et al., 2009c).

The disruption of ECM causes changes in the connection between cardiac cells and blood vessels, thereby weakening the heart structure and function (Díez et al., 2002). Studies have shown that the expression of ECM maintenance proteins TIMPs and MMPs is highly altered in the hypertrophic and MI heart, which disrupts the ECM integrity and leads to ECM remodeling, one of the key processes that leads to fibrosis and heart failure in the diseased heart (Laviades et al., 1998; Lindsay et al., 2002; Picard et al., 2006; Zervoudaki et al., 2003).

Overall, in the context of heart diseases, the fibroblast cells play a double-edged role. They are essential for wound healing and ECM maintenance; however, their excessive activation leads to cardiac remodeling, fibrosis, and reduced heart function (Chalise et al., 2023a; Czubryt, 2012a). Modulating cardiac fibroblast activation, regulating their response to injury, and controlling ECM remodeling are potential therapeutic strategies to prevent fibrosis and further heart failure.

1.2 The Wound Healing Processes

All of the tissues in our body respond to injury through a general wound-healing process, including inflammation, proliferation, and maturation phases (Czubryt, 2012a; Frangogiannis, 2012). However, the extent of tissue regeneration differs among organs with some able to fully regenerate the damaged tissues while others only heal partially, resulting in scar formation (Czubryt, 2012a; Richardson, 2018). When the damaged tissue is completely replaced by the original endogenous cells, complete tissue regeneration occurs in some organs resulting in healing with no sign of injury (Baddour et al., 2012). Skin, intestine, liver, and skeletal muscles fall under this category (Baddour et al., 2012; Poss, 2010). These organs have progenitor cells, which help for complete regeneration. For example, our skin has epidermal progenitor cells, which undergo regular division and differentiation into keratinocytes, which are essential for maintaining the protective barrier of the skin (Baddour et al., 2012). When an injury occurs, the progenitor cells rapidly proliferate and migrate to the epidermis, healing the superficial wound that only affects the outer layer of the skin, without scarring. However, deeper wounds, that affect the dermis area, will not be fully regenerated, which leads to scar formation (Lindsey & Becirovic-Agic, 2023; Poss, 2010). Following injury, the stem cells present in the intestine proliferate and develop into different cell types required for restoring the intestinal wall (Baddour et al., 2012; Lindsey & Becirovic-Agic, 2023). Similarly, in skeletal muscle, satellite cells (a type of stem cell) will be activated upon injury, and they differentiate into myoblasts, which fuse with the existing muscle fibers to repair and replace the damaged muscle cells (Lindsey & Becirovic-Agic, 2023; Poss, 2010). However, chronic damage to skeletal muscles can lead to fibrosis as seen in diseases such as muscular dystrophy. The liver, on the other hand, has a different wound- healing mechanism.

It doesn't rely on the stem cell population. The hepatocytes remaining in the injured liver proliferate and differentiate into healthy, replacement liver cells (Baddour et al., 2012; Lindsey & Becirovic-Agic, 2023).

The heart, kidney, and brain, on the other hand, have little or no endogenous regenerative capacity. The heart muscle cells, 'cardiomyocytes' are terminally differentiated cells, and have a very limited ability to re-enter into the cell cycle and proliferate (He et al., 2020). As a result, in the area where the cardiomyocyte loss occurs following injury, a scar will be formed. The remaining cardiomyocytes compensate for the lost cardiomyocytes by increasing in size through hypertrophy (Baddour et al., 2012; Czubryt, 2012a; Lindsey & Becirovic-Agic, 2023; Ma et al., 2017b). Likewise, the regenerative capability of the brain is also limited after an injury, because damaged neurons typically do not regenerate, leading to permanent loss of function in the affected area. Formation of a glial scar, which helps localizing the neuronal scar, creates an environment that is not favorable for the axonal growth, remyelination and integration of stem cells, thus limiting regeneration (Lindsey & Becirovic-Agic, 2023; Zamproni et al., 2021).

The kidney also has a limited ability to regenerate. Its' proximal tubular cells can proliferate and repair the damaged nephron segments; however, the glomerular and interstitial cells have a limited capacity for repair. Therefore, damage to these areas can lead to permanent loss of function of the kidney (Liu, 2011; Z. Zhang & Cai, 2016).

Although there are differences in the tissue regeneration capabilities of each organ, the dysregulation in the wound-healing process can lead to similar outcomes across different tissues (Lindsey & Becirovic-Agic, 2023). For effective tissue repair, all three phases of wound healing,

the inflammation, proliferation, and reparation should occur in a harmonious manner (Prabhu & Frangogiannis, 2016). In the inflammatory phase, it is crucial to remove the injured cells promptly while maintaining an appropriate level of inflammation. The inflammatory phase should resolve, and there should be a shift to an anti-inflammatory or reparative phenotype, called the proliferative phase. (Chalise et al., 2021; Czubryt, 2012a). In this phase, the fibroblast cells proliferate and differentiate to myofibroblasts and synthesize new extracellular matrix proteins to repair the wound area. They also activate the endothelial cells to establish revascularization in the affected area. If the inflammation phase is prolonged or vascularization is impaired, the repair process will be affected (Chalise et al., 2023a; Czubryt, 2012a). After the proliferative phase, the ECM protein collagen makes crosslinks in the wounded area to form a scar to prevent further damage. Insufficient deposition of ECM or limited crosslinking of collagen leads to the formation of a weak scar, however excessive ECM deposition leads to stiffening of the scar and fibrosis (Chalise et al., 2023a; Czubryt, 2012a; Li et al., 2023; Lindsey & Becirovic-Agic, 2023; Prabhu & Frangogiannis, 2016).

1.2.1 Skin Wound Healing: An Insight into Cardiac Wound Healing

Both skin and cardiac wound healing are complex processes that involve several similar stages yet differ significantly due to the unique characteristics of each tissue (Becirovic-Agic et al., 2022). The inflammatory and proliferative phases are very similar in both forms of wound healing. The major difference occurs in the reparative phase where the ECM deposition, collagen cross-linking, and scar revascularization occur (Lindsey & Becirovic-Agic, 2023; Richardson, 2018). In the skin, epidermal progenitor cells regenerate into keratinocytes, and the fibroblasts, smooth muscle cells, and endothelial cells are completely repopulated. Therefore, the skin wound

healing process restores the pre-injury state of the skin nearly completely (Chen et al., 2009). After MI, the cardiac fibroblast, smooth muscle cells, and epithelial cells can proliferate. However, since cardiomyocyte regeneration is negligible, it leads to scar formation that does not function as effectively as the original myocardial tissues. Nevertheless, this scar is essential for preventing complications such as left ventricular aneurysms and cardiac rupture (Chalise et al., 2023a; Lindsey & Becirovic-Agic, 2023).

Another major difference between skin and cardiac wound healing is the re-epithelialization process. In the skin, keratinocytes undergo cell division, and they migrate under the blood clots from wound edges to help close the gap (Pastar et al., 2014; Rodero & Khosrotehrani, 2010). Different growth factors such as keratinocyte growth factor (KGF), and epidermal growth factor (EGF), released by the fibroblasts and immune cells activate the keratinocytes, stimulating them to divide and migrate towards the wound area. When skin wounds become chronic, re-epithelialization becomes impaired (Lindsey & Becirovic-Agic, 2023; Pastar et al., 2014; Rodero & Khosrotehrani, 2010). In cardiac wound healing, a re-epithelialization process does not occur.

1.4.2 Phases of Cardiac wound Healing

1.4.2.1 Inflammatory Phase

The initial phase of wound healing starts immediately after an injury, and it begins with activation of clotting factors, platelet aggregation, blood vessel constriction, to control hemorrhage and form a temporary barrier to pathogens (Frangogiannis, 2012) (Figure 4). Tissue injury and cardiomyocyte death

both generate various endogenous signals, such as danger-associated molecular patterns (DAMPs), promoting the activation of the innate immune cells (Bianchi, 2007b; Timmers et al., 2012b). The neutrophils, macrophages, and lymphocytes will be recruited to the infarct site and mediate the activation and the secretion of the pro-inflammatory chemokines and cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), monocyte chemoattractant protein-1 (MCP-1) (Frangogiannis, 2008; Timmers et al., 2012b). Neutrophils are the first responders that migrate towards the wounded area. They release reactive oxygen species (ROS), and various enzymes such as lactoferrin, and superoxide dismutase (SOD) to fight invading pathogens and these neutrophils eventually undergo apoptosis (Czubryt, 2012a; Frangogiannis, 2008). Macrophages and lymphocytes arrive slightly later than neutrophils, however, they play crucial roles in clearing the dead cells, pathogens, and debris from the infarct zone by phagocytosis (Czubryt, 2012a). Macrophages also release cytokines and growth factors such as transforming TGF- β , PDGF, FGF, that regulate inflammation and initiate the transition from the inflammatory phase to the proliferative phase (Czubryt, 2012a; Timmers et al., 2012b). The inflammation phase starts minutes after a cardiac injury and usually lasts between one to two weeks. However, during chronic inflammation, the macrophages will persist in the infarct zone, and they trigger continued infiltration and proliferation of fibroblasts (Czubryt, 2012a).

1.4.2.2 Proliferative Phase

The proliferative phase is when fibroblasts convert to myofibroblasts and the transition of inflammatory macrophage M1 to the proliferative macrophage M2 (Figure 4). The fibroblasts play major roles in tissue repair and extracellular matrix (ECM) remodeling. They migrate to the infarct site by the actions of different signaling molecules such as growth factors, chemokines,

and cytokines, which were activated/released in the inflammatory phase (Baudino et al., 2006). These molecules also promote the proliferation and activation of fibroblast to the myofibroblast (Baudino et al., 2006; Czubryt, 2012a; Frangogiannis, 2008; Fu et al., 2018b). Once the fibroblast is activated to become a myofibroblast, it secretes elevated levels of extracellular matrix proteins such as collagen, fibronectin, elastin, and proteoglycans, which helps to maintain the integrity of the ECM by providing structural support and contribute to tissue strength and elasticity (Czubryt, 2012a; Hewitson et al., 1995a). Myofibroblasts also express integrins, which mediate the attachment of myofibroblast to the ECM and regulate cell migration and contraction (Faxon et al., 2002; Zent & Guo, 2018b). The pro-angiogenic factors, such as- TGF- β , FGF, PDGF and, vascular endothelial growth factor (VEGF) released at the wound site, stimulate endothelial cell proliferation and angiogenesis thereby increasing vascular supply to the tissues (Hanna & Frangogiannis, 2019; J. Li et al., 2003). Myofibroblasts also express the proteins, α -SMA and the embryonic form of smooth muscle myosin heavy chain (SMemb), which are involved in wound contraction and closure (Czubryt, 2012a; Frangogiannis, 2008).

1.4.2.3 Maturation Phase

The final stage of the wound healing process is the maturation phase, which is also known as the remodeling phase (Figure 4). Interestingly, both matrix degradation and new matrix formation occur in this stage. The ECM components present in the earlier stages of healing, including collagen type III and other components that were initially important for temporary support, will be degraded by MMPs (Becirovic-Agic et al., 2021; Yaguchi et al., 1998) and new ECM components, predominantly collagen type I, which provides greater strength and durability to the tissues will be synthesized and deposited by the myofibroblasts (Baudino et al., 2006;

Czubryt, 2012a). More collagen type I deposition occurs at this stage, and it is cross-linked in the wound area, which significantly increases the tensile strength of the tissues (Czubryt, 2012a). Wound closure is facilitated by the action of contractile proteins, α -SMA and SMemb along with collagen to contract the wound edges together and restore normal tissue strength (Czubryt, 2012a; Prabhu & Frangogiannis, 2016). Once their role in wound closure and ECM deposition is complete, myofibroblasts may die by apoptosis, or transition into the less active matrifibrocytes phenotype, allowing the tissue to transition to a more stable state (Prabhu & Frangogiannis, 2016; Tenkorang et al., 2019). The maturation phase can range from months to years, depending on the size and type of the wound, and individual factors such as age, nutrition, and personal health (Guo et al., 2012).

1.4.3 Defective Wound Healing

Prolongation of the inflammatory phase leads to the uncontrolled inflammation in the wound area. Persistent and excessive inflammation in the wound can damage the surrounding healthy myocardial tissues and prolong the reparative phases (Baudino et al., 2006; Czubryt, 2012a). The continued inflammation stimulus leads to the prolonged presence of myofibroblast cells within the infarct zone for many years and contributes to various forms of cardiac dysfunction. Excessive amounts of ECM proteins will accumulate in the infarct zone, which stiffens the myocardium and thereby impairs the contraction and relaxation of the heart (Czubryt, 2012a). This leads to the

formation of the fibrotic scar with reduced contractile properties, resulting in ventricular remodeling, reduced cardiac function, cardiac hypertrophy, and increased risk of fibrosis and heart failure (Czubryt, 2012b; Weil & Neelamegham, 2019). The death of terminally differentiated cardiomyocytes and the persistence of myofibroblast cause unusual electrical properties in the myocardium may eventually lead to arrhythmias (Czubryt, 2012a; Sharma et al., 2004).

The areas that are remote to the scar tissue also can experience various stresses due to hemodynamic changes post-MI, which leads to remodeling of the non-infarcted myocardium (Azevedo et al., 2016). The loss of contractile tissues in the infarct area decreases the overall pumping efficiency of the heart. As a result, the remaining myocardial tissues work harder to maintain an optimum cardiac output. This leads to increased wall stress in the non-infarcted areas, causing hypertrophy in later years (Leancă et al., 2022). Additionally, the scarred tissue stiffens, leading to altered pressure in the left ventricles. These elevated pressure dynamics thicken the non-infarcted myocardium further to adapt to handle the increased workload (Chalise et al., 2023b; Forrester et al., 1972). Along with the increased wall stress, scar tissue disrupts the normal blood flow dynamics within the heart, which leads to ischemia and further increases the workload for the remaining viable myocardium prompting further adaptation (Richardson et al., 2015). This will ultimately impact the overall function of the heart, increasing the risk of fibrosis and heart failure (Chalise et al., 2023b; Leancă et al., 2022).

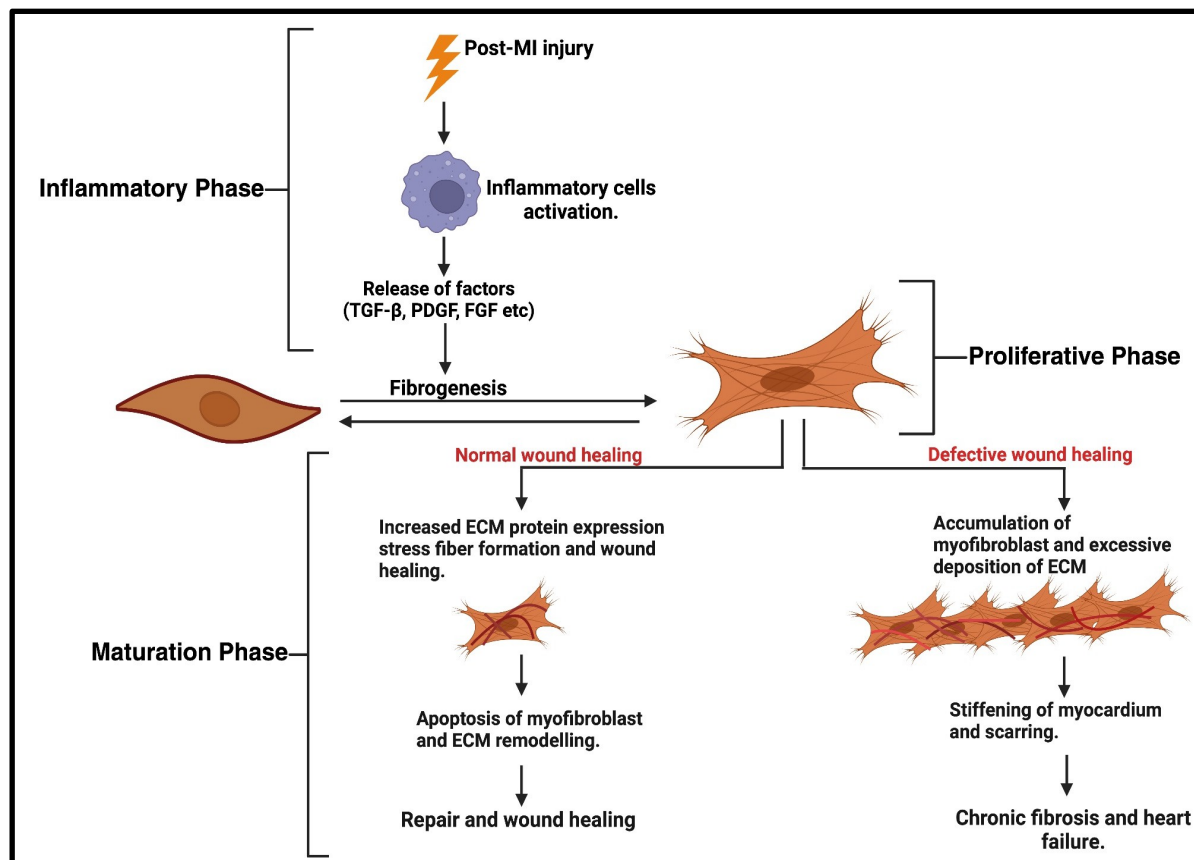


Figure 4: Stages of Cardiac wound healing. The inflammatory phase begins with the recruitment of immune cells and the secretion of growth factors, and subsequent removal of cellular debris followed by the proliferative phase which marks fibroblast activation and production of new ECM proteins to remodel the injured myocardium. Finally, in the maturation phase normally wound healing and repair will happen by collagen cross linking and scar formation while in defective wound healing situations, excessive amounts of proinflammatory cells as well as myofibroblasts will be activated. The myofibroblasts persist in the scar area, which causes increased deposition of ECM proteins. These changes lead to stiffening of myocardium, increases cardiac hypertrophy, fibrosis, and systolic and diastolic dysfunction, which can lead to heart failure. (Created in <https://BioRender.com>).

1.4.4 Classes of Cardiac Fibrosis

Cardiac fibrosis is associated with most myocardial diseases. Cardiac fibrosis is characterized by the enlargement of the cardiac interstitium due to the accumulation of ECM proteins and can arise from various pathological conditions such as myocardial infarction, chronic inflammation, and hypertension (Frangogiannis, 2021; Travers et al., 2016a). Even though fibrosis is a natural part of the wound healing process, excessive or improper fibrosis can lead to major cardiac functional impairments (Frangogiannis, 2021). There are three different classes of cardiac fibrosis: replacement fibrosis, interstitial fibrosis and perivascular fibrosis (Frangogiannis, 2021).

1.4.4.1 Replacement Fibrosis

As described above, the adult mammalian heart muscle cells have negligible regenerative capacity, therefore the heart relies on reparative process to heal through replacement of the dead cardiomyocytes with a collagen rich scar following different insults (Frangogiannis, 2019, 2021; Travers et al., 2016a). An example of replacement fibrosis is the fibrotic process following myocardial infarction, which is characterized by significant cell loss (Eijgenraam et al., 2020; Frangogiannis, 2019, 2021). The reparative myofibroblast activation after the sudden cardiomyocyte's death and inflammation in MI leads to ECM secretion and collagen cross linking leading to scar formation in the heart (Frangogiannis, 2019). As discussed in more detail in the previous section, replacement fibrosis is critical for wound healing after MI, however it also leads to heart failure complications in the future.

1.4.4.2 Interstitial Fibrosis

Interstitial fibrosis occurs when structural ECM proteins accumulate in between the cells without the loss of cardiomyocytes (Eijgenraam et al., 2020; Frangogiannis, 2019). Chronic conditions such as hypertension, inflammation, and aging can trigger myofibroblasts activation, thereby increasing the ECM production (Eijgenraam et al., 2020). This leads to the formation of excess fibrous connective tissue formation, and its deposition throughout the myocardium (Eijgenraam et al., 2020; Frangogiannis, 2019, 2021; Travers et al., 2016a). The hallmark of interstitial fibrosis is the widening of the endomysium and perimysium caused by the increased deposition of these structural ECM proteins (Eijgenraam et al., 2020; Travers et al., 2016a).

1.4.4.3 Perivascular Fibrosis

Increased deposition of connective tissues, and collagen around the coronary artery is known as perivascular fibrosis (Eijgenraam et al., 2020; Ytrehus et al., 2018). It is frequently observed in response to chronic stresses such as hypertension and atherosclerosis (Eijgenraam et al., 2020; Travers et al., 2016a). When the blood vessels are stressed, it triggers an inflammatory process, leading to myofibroblast activation in the surrounding tissues. These cells secrete excessive ECM proteins that stiffen the perivascular region (Frangogiannis, 2019, 2021). Therefore, perivascular fibrosis leads to impaired vascular function, increased resistance to blood flow and contributes to poor myocardial tissue perfusion (Eijgenraam et al., 2020; Ytrehus et al., 2018). Overtime, this decreased cardiac perfusion can exacerbate cardiac dysfunction and lead to heart failure.

1.4.5 Major Signaling Pathways in Cardiac Fibroblast Activation and Myofibroblast Regulation during Fibrosis

The wound healing process following, myocardial injury including the inflammation, proliferation, maturation phases, and the differentiation of fibroblast to other mesenchymal lineages are tightly regulated by numerous signaling mechanisms. A comprehensive understanding of these molecular processes driving fibrosis is crucial for developing novel therapeutic strategies aimed at improving the treatment of MI and HF. Among the numerous signaling pathways involved in fibroblast activation and wound healing process, both TGF- β and Angiotensin-II (Ang-II) pathways play particularly crucial roles. The other pathways such as PDGF (Gallini et al., 2016; Olson & Soriano, 2009), WNT (Gay et al., 2020; Plikus et al., 2017), and the DAMP signaling pathways, also contribute to this complex healing process and fibrosis. This section will mainly focus on the TGF- β and Ang-II pathways in detail due to their significant impact on cardiac remodeling and fibrosis.

1.4.5.1 The TGF- β -SMAD Pathway

The activation of TGF- β from its latent form is one of the key steps in the transition of the inflammatory phase to the reparative phases (Figure 5). TGF- β , has many different roles in the tissue repairing process. In normal conditions, it induces mesenchymal cells to produce ECM. However, under stress situations, such as MI, it plays important roles in the wound healing process. TGF- β helps to terminate the inflammatory phase by causing macrophage deactivation, and chemokine and cytokine suppression. TGF- β also converts fibroblast cells to

myofibroblasts and induces them to secrete ECM proteins, such as collagen and fibronectin (Bujak & Frangogiannis, 2007). The TGF- β and the Extra Domain- A containing fibronectin (ED-A fibronectin) are the ECM signaling mediators, that establish a negative feedback loop during the myofibroblast activation process (Zent & Guo, 2018c).

The TGF- β family is comprised of three isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. All three isoforms of TGF- β are markedly upregulated following MI, however the timing of their expression during wound healing phases as well as the specific cell types responsible for their secretion may vary (Bujak & Frangogiannis, 2007; Frangogiannis, 2017). For instance, among these three isoforms of TGF- β , TGF- β 1 is present abundantly in the heart and expressed highly in all three post-MI wound healing phases and has a major role in cardiac fibrosis. However, the other two isoforms of TGF- β , increase mainly during the maturation phases of wound healing (Frangogiannis, 2017). In normal conditions, TGF- β present in the ECM is bound to the latency associated protein (LAP) and the latent TGF- β associated protein (LTBP). This bound form of TGF- β is not biologically active (Robertson & Rifkin, 2016). Different physical, biological and physicochemical factors mediate the activation of latent TGF- β (Robertson & Rifkin, 2016b). The transmembrane receptor protein integrin and the proteases enzymes are well-known activators of TGF- β (Robertson & Rifkin, 2016b; Shi et al., 2011) and this activation involves a mechanism mediated by traction force. The LAP protein, bound to the TGF- β , contains an RGD (Arg-Gly-Asp) sequence which facilitate its binding with integrins on the cell surface on one end and the other end is connected to the ECM via the LTBP-1 (Annes et al., 2004; Dong et al., 2017). The tension between ECM and cells can induce the deformation of LAP-1, leading to the release of TGF- β 1 (Annes et al., 2004; Dong et al., 2017; Hinz, 2015). The pulling force generated by

the contraction of myofibroblast stress fibers can also accelerate this process of TGF- β release (Wipff et al., 2007). Various proteolytic enzymes such as MMP-14 (Matrix Metalloproteinase-14), MMP-2 (Matrix Metalloproteinase -2) and MMP-9 (Matrix Metalloproteinase-9) can also mediate the activation of TGF- β , by degrading the LAP-1 protein or by cleaving the LAP-1 and LTBP-1 bond (Zent & Guo, 2018b). Once activated, TGF- β binds to its receptor TGF- β receptor II. This binding mediates the autophosphorylation of TGF- β II and its dimerization with the TGF- β I receptor. The TGF- β II receptor further phosphorylates TGF- β I receptor, which in turn, phosphorylates and thereby induces the dimerization of the intracellular signaling receptor-regulated SMADs (R-SMAD), SMAD 2 and SMAD 3 (Hata & Chen, 2016; Khalil et al., 2017; Zent & Guo, 2018b). This enables R-SMADs to associate with the common mediator SMAD (Co-SMAD), SMAD 4 (Hata & Chen, 2016; Zent & Guo, 2018b). This process facilitates the formation of the heterotrimeric complex, comprising of two R-SMADs and one Co-SMAD and their translocation to the nucleus (Hata & Chen, 2016; Zent & Guo, 2018b). In the nucleus, SMADs interact with the promoter regions of the different pro-fibrogenic factors and regulate their gene expression by modulating transcription and influencing the epigenetic landscape (Hata & Chen, 2016; Wipff et al., 2007). Studies have indicated that cardiac fibroblast specific SMAD 2/3 deletion in mice suppresses the pro-fibrogenic gene program and ECM remodeling, emphasizing their critical role in cardiac fibrosis (Khalil et al., 2017; Zent & Guo, 2018b).

However, the repressive SMAD, known as SMAD-7, functions as an inhibitory regulator of the TGF- β -SMAD pathway. SMAD-7 competes with R-SMAD for binding to the TGF- β R1, thereby preventing the phosphorylation and activation of R-SMAD. This regulation helps to prevent the excessive activation of pro-fibrotic genes in the MI heart. It also limits the aberrant differentiation

of fibroblast to myofibroblasts (Leask & Abraham, 2004; Morikawa et al.). A recent study showed that loss of SMAD-7 in mouse models promotes fibrosis in the MI heart, highlighting its protective role in maintaining normal tissue remodelling and controlling fibrosis (Humeres et al., 2024).

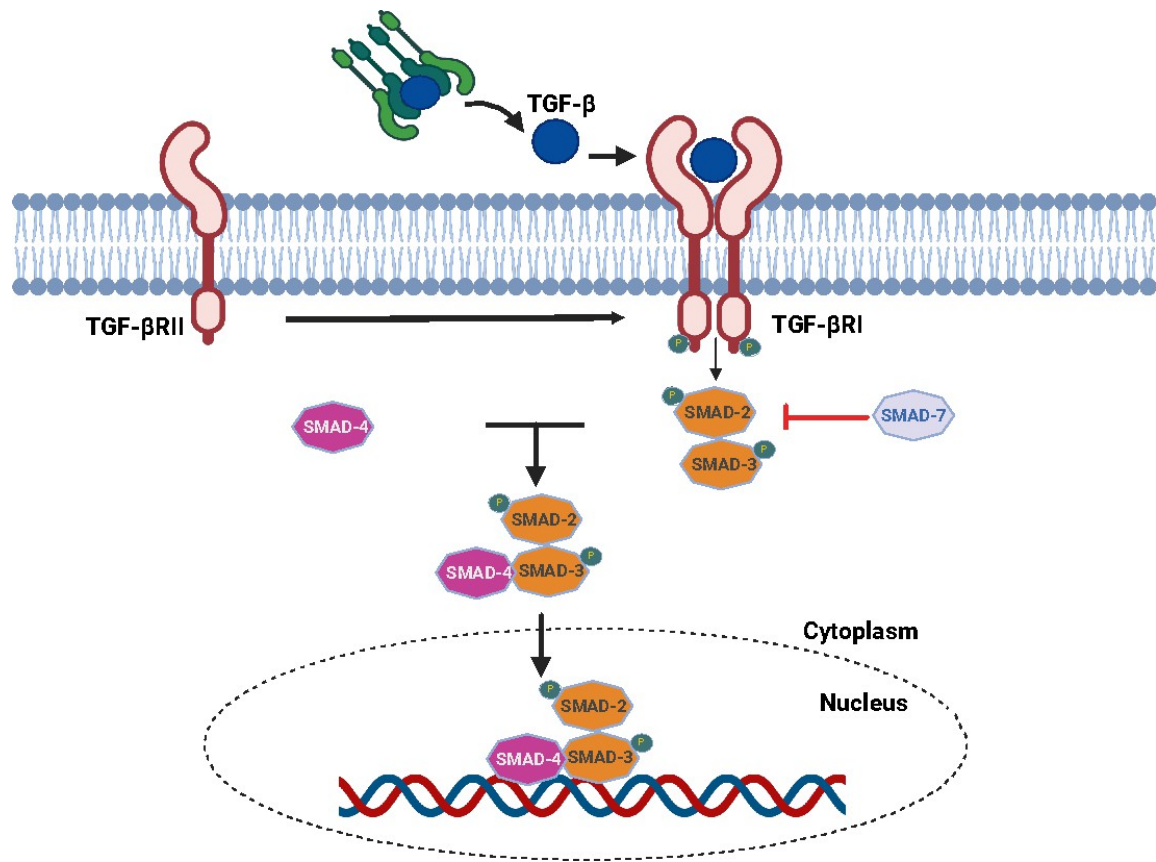


Figure 5: The TGF- β -SMAD pathway. In normal conditions, TGF- β is present in the ECM in its latent form, bound to the latency associated protein (LAP) and the latent TGF- β associated protein (LTBP). Different physical, biological, and physicochemical factors mediate the activation of TGF- β from its non-active form. Active TGF- β binds to the TGF- β receptor II, which in turn phosphorylates and recruits TGF- β receptor I. The TGF- β receptor II further undergoes autophosphorylation, and these two receptors form a heterodimer complex and phosphorylate the R-SMADs SMAD 2 and SMAD 3. The dimerized R-SMADs bind with the Co-SMAD, SMAD 4 and the heterotrimeric complex translocate to the nucleus, where this complex interacts with the promoter regions of different pro-fibrogenic genes such as collagen, fibronectin, MMP2 and ZEB2 and regulate their transcription. (Created in <https://BioRender.com>).

1.4.5.2 Angiotensin-II Pathway

Angiotensin – II (Ang-II) is the key downstream effector of the physiological actions of the Renin-Angiotensin system (RAS) which include regulating blood volume, maintaining blood pressure and stimulating aldosterone secretion. Ang-II levels increase under hemodynamic stresses such as that experienced during cardiac injury (Sanghi et al., 2005). In such cases, Ang-II is processed through the combined action of macrophages and cardiomyocytes. Macrophages express key enzymes such as angiotensinogen, renin and angiotensin converting enzyme (ACE) which convert Ang-I (an inactive precursor) to Ang-II (active hormone), contributing to inflammatory and fibrotic processes related to cardiovascular disease progression (Sadoshima & Izumo, 1993). Although, Ang-II acts on cardiomyocytes to modulate their function, its primary role in the myocardium involves activating paracrine signals on cardiac fibroblasts, thereby influencing their activation into myofibroblasts (Leask, 2007b; Lijnen et al., 2002). Ang-II act as an upstream enhancer of TGF- β signalling pathway, which in turn activate profibrotic genes and myofibroblasts and promote the synthesis of ECM components, contributing to cardiac remodelling following injury (Autelitano et al., 2003; Leask, 2007b; Lijnen et al., 2002). Recent studies suggest that Ang-II can activate the expression of the myofibroblast marker α -SMA. This effect of Ang-II will increase the contractile property of myofibroblast independent of TGF- β , in cardiac fibroblast cells. Ang-II also can directly induce myofibroblast differentiation through the activation of the MAPK pathway (Davis et al., 2012; Davis & Molkenin, 2014).

1.3 The ZEB2 and ZEB1 Transcription Factors

The **Zinc finger E-box binding homeobox-2 (ZEB2)** and **Zinc finger E-box binding homeobox-1 (ZEB1)** transcription factors (TF) are members of the Zinc finger family of proteins, characterized by their ability to bind to E-box motifs in the promoters of target genes (Birkhoff et al., 2021; Hill et al., 2013; Soen et al., 2018). They are paralogs that evolved from a common ancestral gene. Both ZEB1 and ZEB2 play crucial roles in embryonic development (Fardi et al., 2019; Gheldof et al., 2012a), epithelial to mesenchymal transition (Fardi et al., 2019; Goossens et al., 2011; Li et al., 2017), tissue regeneration (Fardi et al., 2019; J. Li et al., 2017), cancer metastasis (Brabletz & Brabletz, 2010; Li et al., 2017a). They also may have significant roles in cardiac remodeling and wound healing processes (Cunnington et al., 2014; Jahan et al., 2018). Since these two genes have arisen from a common ancestral gene through duplication, it is not surprising that they have evolved to perform different, though related functions within an organism (Postigo & Dean, 2000).

ZEB2 contains multiple functional domains, and functions as a DNA-binding transcription repressor. ZEB2 was first identified in a yeast two- hybrid screen where it was found to bind to the C-terminal MH2 domain of the *Xenopus* SMAD1 (XSMAD1) gene (Hegarty et al., 2015a; Verschueren et al., 1999). In the human genome, the ZEB2 gene is found on chromosome 2q22, and its 9-exons encode a protein of 1214 amino acids with a predicted molecular weight of 140 KDa (Birkhoff et al., 2021). ZEB2 interacts with activated SMADs, therefore also known as Smad interacting protein-1 (SIP-1) or Zfh1b (Grabitz & Duncan, 2012). It plays important roles in various biological processes including embryonic development (tissue morphogenesis, EMT, regulation of stem cell pluripotency) (Stryjewska et

al., 2017), neural development (specification of neural crest cells (multipotent stem cells in the neural tube)) (Birkhoff et al., 2021) and in cancer progression (tumor aggressiveness, chemoresistance, poor prognosis) (Li et al., 2017b) (Birkhoff et al., 2021; Fardi et al., 2019; Hegarty et al., 2015a). The ability of ZEB2 to modulate cell plasticity makes it an important regulator in both development and in disease.

ZEB1, previously known as TCF8, dEF1, and Zfhx1a, is also a multidomain transcription repressor found in mammals (Zhang et al., 2015). ZEB1 was first recognized in the chicken embryo as a repressor of the lens specific $\delta 1$ -crystallin enhancer (Funahashi et al., 1993; Zhang et al., 2015). The ZEB1 gene is found on human chromosome 10q22.3 and it encodes a protein of molecular weight of 115 KDa. Beyond its role in embryonic development and in EMT, ZEB1 is also involved in cancer invasion and tumorigenicity (Madany et al., 2018; Zhang et al., 2015). The detailed functions of both ZEB1 and ZEB2 will be discussed more comprehensively below.

1.3.1 Structure

Both ZEB1 and ZEB2 contain two highly conserved C2H2 type zinc finger domains, which show 88% and 93% of sequence identity between their N-zinc finger (NZF) and C-zinc finger (CZF) domains respectively (Birkhoff et al., 2021; Hegarty et al., 2015b; Madany et al., 2018) (Figure 6). They both possess a centrally located homeodomain (HD), which is less conserved between ZEB1 and ZEB2 (44%) and which is involved in protein-protein interactions rather than DNA binding (Birkhoff et al., 2021; Zhang et al., 2015). The N-terminal zinc finger cluster (NZF) consist of four zinc fingers, containing three CCHH- type and one CCHC- type zinc fingers and the C- terminal zinc finger cluster (CZF) includes three CCHH zinc fingers (Birkhoff et al.,

2021; Bruneel et al., 2020; Hegarty et al., 2015b). These zinc finger clusters can bind to the E-box (5'CANNTG) or E-box like DNA motifs in the promoters of target genes. All of the other domains in ZEB1 and ZEB2 share only a low level of sequence identity (30-40%) between them, which implies that although both of these TF have common DNA binding specificities, they may interact with different protein partners (Birkhoff et al., 2021; Hegarty et al., 2015a; Zhang et al., 2015). Both ZEB1 and ZEB2 can bind to the corepressor proteins called C-terminal binding protein 1 and 2 (CtBP1/2) through their CtBP interacting domain (CID). The CtBP protein recruits the corepressor, histone deacetylase 1 and 2 (HDAC 1/2) (Furusawa et al., 1999). The CtBP1/2 - ZEB1/2 interactions are also important for repressing E-Cadherin transcription during EMT. A SMAD binding domain (SBD) is present in both ZEB1 and ZEB2 and helps for the direct interaction of ZEB2- R-SMAD proteins, the downstream component of the TGF- β pathway (Gheldof et al., 2012b; Long et al., 2004). However, ZEB1 cannot interact with SMAD directly, since it lacks a tandem repeat of four amino acids (QXVX)₂, which is present in ZEB2 and crucial for R-SMAD binding (Conidi et al., 2013). Also, ZEB2 has a Nucleosome remodeling and histone deacetylation complex (NuRD) interacting motif (NIM) and two SUMOylation sites, which are absent in ZEB1 (Birkhoff et al., 2021). The NIM domain mediates the binding of the repressor protein HDAC to ZEB2 and the two conserved SUMO sites modify ZEB2 by Pc-2 mediated SUMOylating in these sites. This will disrupt the CID motif, thereby making ZEB2 a less efficient repressor. ZEB1 has an additional Coactivator P300-PCAF binding domain (P300/CBP associated factor, also known as lysine-acetyltransferase factor) (CBD), that helps for the binding of co-activator lysine-acetyltransferases to ZEB1. This helps ZEB1 function as a transcription activator as well (Birkhoff et al., 2021).

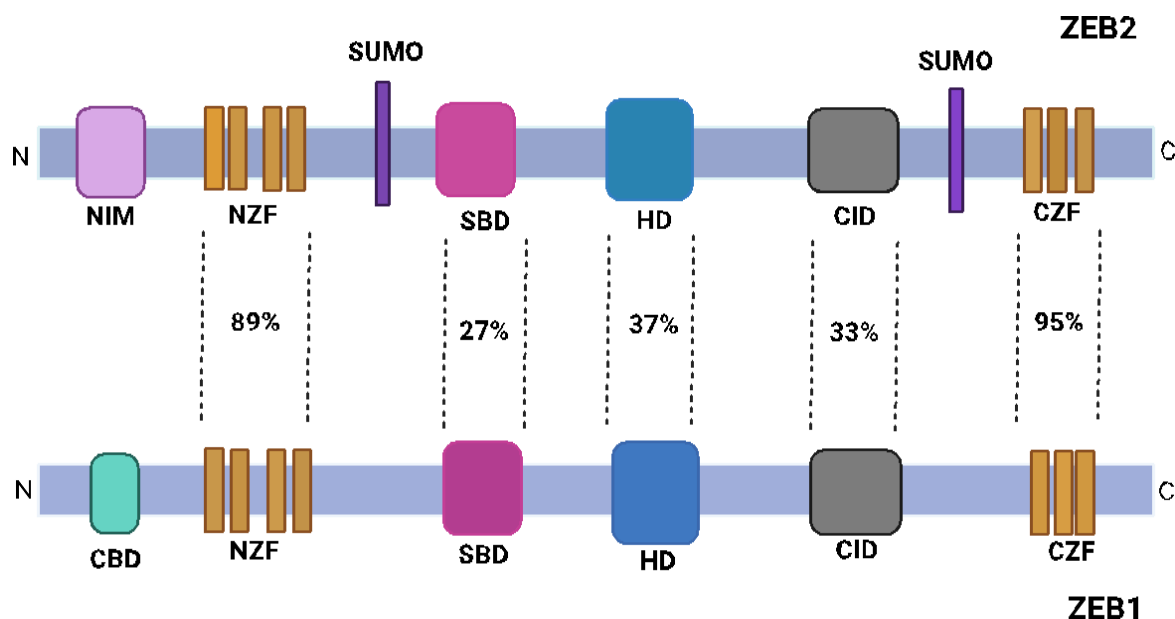


Figure 6: The Structure of Human ZEB1 and ZEB2 Transcription Factors. The two zinc finger domains are highly conserved between ZEB1 and ZEB2 (89% identical for NZF and 95% identical for CZF) and these domains are important for DNA binding. The Homeodomain (HD) has 45% sequence similarity between ZEB1 and ZEB2 and mediates protein-protein interaction. The other domains such as NIM: NuRD interacting domain, SBD: Smad binding domain, CID: C- terminal binding protein interacting domain, and CBD: Coactivator P300/PCAF binding domain are involved in either facilitating the transcription activation or repression of the ZEB target gene. (Created in <https://BioRender.com>).

1.3.2 ZEB1 and ZEB2-Roles in Human Body- EMT and in Development

In the human body, both ZEB1 and ZEB2 play important roles in EMT, enteric nervous system development and skeletal system development (Goossens et al., 2011; Krebs et al., 2017; Li et al., 2017). EMT is a crucial process in the developmental stages that involve gastrulation, neural crest formation and tissue morphogenesis (Krebs et al., 2017). Different molecular pathways activate the EMT process. For example, during gastrulation, the TGF- β /SMAD pathway is activated which in turn induces the activation of ZEB1 and ZEB2. Once these TFs are activated, they inhibit the transcription of the epithelial genes such as E-Cadherin, which is crucial for cell-cell adhesion and promote the transition of epithelial cells to mesenchymal cells by activating the transcription of mesenchymal genes such as N-Cadherin and Vimentin (Comijn et al., 2001; Konradi et al., 2014). This process is further complicated by the involvement of microRNAs such as miR200, which is an epithelial marker, and exerts a strong repressive effect on ZEB1/ZEB2 expression (Brabletz & Brabletz, 2010). TGF- β activation increases ZEB1/ZEB2 expression levels, which in turn represses the miR200 transcription and thereby further increases ZEB1/ZEB2 levels (Brabletz & Brabletz, 2010). Therefore, both ZEB1 and ZEB2 are crucial for the EMT process and are essential for early developmental phases such as gastrulation and organogenesis.

1.3.3 Roles in Cancer Cells

Even though EMT is a normal developmental process, it is also involved in wound healing, fibrosis and in cancer. Tumor cells can also exploit this process by undergoing a phenotypic switch to a more mesenchymal-like cell phenotype with increased motility, invasiveness, and resistance to apoptotic signals to enhance their ability to metastasize (Hill et al., 2013; Li et al., 2017a). An increase in ZEB1 during the EMT process, is closely linked to metastasis in many types of cancer including lung, colon, breast and pancreatic cancers and its inhibition can reduce the metastasis in adults (Hill et al., 2013; Li et al., 2017a). However, this phenomenon is not observed with other EMT genes such as *SNAIL* and *TWIST* (Krebs et al., 2017). In several different tissue types including, lung, breast, colon, gastric, brain, uterine and in pancreases, ZEB1 is shown to be associated with neoplastic transformation and tumor progression, invasion, and migration (Fardi et al., 2019). A study conducted in the breast cancer tissue cell line-MDA-MB-231 shows that ZEB1 knockdown downregulates the expression of more than 30 genes and upregulates the expression of more than 200 genes, many of which are from the epithelial E-cadherin gene family, tight junction genes, gap junction genes and cellular differentiation genes (Hugo et al., 2013; Liang et al., 2018). In some cancer types such as ductal adenocarcinoma, colorectal cancers and in uterine cancer, it was found that a high level of ZEB1 expression correlates with poor patient survival (Krebs et al., 2017; Madany et al., 2018; Zhang et al., 2015). The functional characteristics of ZEB1 in cancer cells is primarily related to its ability to repress E-cadherin, which is also known as a tumor suppressor gene (Bracken et al., 2008). ZEB1 also regulates the cell-cell adhesion and polarity genes by binding to their promoter region and repressing their activity, which is associated with tumor metastasis (Perez-Oquendo & Gibbons,

2022). Further, they can activate the expression of anti-apoptotic genes and repress the activation of pro-apoptotic genes which in total promotes chemotherapy resistance of the cancer cells (Perez-Oquendo & Gibbons, 2022).

Although a majority of studies highlight the roles of ZEB1 in cancer progression, there is evidence that ZEB1 may act as a tumor suppressor as well. For instance, in the adult tumor cell leukemia/lymphoma (ATLL), it has shown that the ZEB1 is often epigenetically silenced (Wu et al., 2018). Mice with ZEB1 mutation developed CD4 + T-cell lymphoma. *In vitro* studies in the ATLL cells with reduced ZEB1 expression show resistance to TGF- β , thereby increased cell growth. This suggests that evading TGF- β induced growth inhibition, by mutating ZEB1, is critical for ATLL progression (Madany et al., 2018). Similarly, in PC-3 prostate cancer cells, it was found that ZEB1 inhibition upregulates the expression of laminin-332, which is a pro-migratory molecule, thus increasing the migration and metastasis of these cells (Drake et al., 2010). Interestingly, in lung carcinomas ZEB1 shows dual effects. In KRAS-mutated lung cancer, ZEB1 acts as an oncogene. However, in the EGFR-mutated lung cancer cells, it acts as a tumor suppressor (Zhang et al., 2016). Overall, these findings uncover the complex roles of ZEB1 in different cancer types.

Similar to ZEB1, ZEB2 also plays significant roles in tumor progression and metastasis. Studies in the HCT116 colorectal cancer cells showed, ectopic expression of ZEB2 accelerates EMT and metastasis in the cancer cells in vitro and in vivo (Li et al., 2017a; Li et al., 2019). Overexpression of ZEB2 increased tumor growth and angiogenesis in the xenotransplantation models and silencing of ZEB2 expression using the shRNA reduced tumor growth and metastasis (Li et al.,

2017a). Studies in gastric cancer cells show that the circular RNA circTNIK, is expressed highly in the tumor cells as compared to its normal controls (Chen et al., 2023). This circular RNA functions as a sponge for different miRNAs such as miR135-5p, which in turn upregulate the ZEB2 expression in the gastric cancer cells (Chen et al., 2023). This increased level of ZEB2 enhances the aggressive behavior of cancer cells by increasing their proliferation, migration, and invasion (N. Chen et al., 2023). Another study conducted on the effect of knockdown of ZEB2 in the glioblastoma cell lines U87 and U373 showed cell cycle arrest in G₀/G₁ phases, increased apoptosis, and reduced cell migration (Safaei et al., 2021). Similarly, an increased apoptotic effect of ZEB2 knockdown was seen in the human myeloid leukemia HL-60 cells (Fardi et al., 2021). In colorectal tumors that are not treated, there exists a cell population which are quiescent, but have stem cell like properties (Francescangeli et al., 2020). These cell populations are called quiescent cancer stem cells (QCSC) (Francescangeli et al., 2020). These cells have high expression levels of ZEB2 and are resistant to chemotherapy. ZEB2 upregulates the expression of the anti-apoptotic proteins in these cells and thereby increases the chemoresistance (Francescangeli et al., 2020). Overall, ZEB2 is mostly implicated in chemoresistance and cancer cell migration in many cancer types.

1.5.4 Human Disorders Associated with Mutations in ZEB1 & ZEB2

Even though ZEB1 and ZEB2 are genetic paralogues and share sequence similarities, mutations in these transcription factors lead to different outcomes in humans. Haploinsufficient mutations in ZEB2 leads to the development of a rare (1:50,000 to 70,000 live births) congenital anomaly syndrome called Mowat-Wilson syndrome (MOWS), which is characterized by severe

intellectual disability, facial dimorphism, Hirschsprung disease (Birkhoff et al., 2021) and retinal coloboma (Wei et al., 2019). About 58% of the patients with MOWS, shows severe congenital heart defects such as tetralogy of Fallot, septal defects, pulmonary artery stenosis (PAS), and aortic valve stenosis (AVS) (Birkhoff et al., 2021). In severe cases of MOWS patients, a complete deletion of the ZEB2 gene was observed (19%). Also, nonsense mutation or small insertion or deletion mutations involving a frameshift were also seen in many of the MOWS patients (Dastot-Le Moal et al., 2007; Ivanovski et al., 2018). When these mutations occur, it leads to a premature stop codon, which results in nonsense mediated mRNA decay (NMD), a process by which the mutated mRNA is degraded before it can be translated to protein (Birkhoff et al., 2021; Lejeune, 2022). However, in patients with mild MOWs symptoms, missense mutation, in-frame deletions, and rare-frameshift mutations in ZEB2 have been identified (Garavelli et al., 2009; Ghoumid et al., 2013; Heinritz et al., 2006).

Mutation of the human ZEB1 gene results in two rare conditions, which are posterior polymorphous and Fuch's endothelial corneal dystrophies (PPCD and FECD respectively) (Chung et al., 2014a, 2014b). Both of these diseases affect the corneal endothelial layer development and leads to blurred and gradual vision loss. PPCD is caused by the ZEB1 haploinsufficiency, by the truncation mutation in ZEB1 which leads to reduced production of ZEB1 proteins and its impaired nuclear localization. In contrast, FECD occurs due to ZEB1 missense mutation (Chung et al., 2014a, 2014b).

1.5.5 Mouse Models of ZEB2 and ZEB1

Mouse models of ZEB1 and ZEB2 provide valuable insight into development and disease mechanisms. Studies on the ZEB2 null mice show embryonic lethality and specific deletion of ZEB2 decreases survival rate of the embryo (Higashi et al., 2002). As ZEB2 is an important transcription factor in neuronal development, significant absence of vagal neural crest cells, which are the key precursor cells of enteric nervous system, were observed in the ZEB2 knockout mice (Hegarty et al., 2015c; Watanabe et al., 2017). Loss of ZEB2 in mice also affects the cranial neural crest cells migration and differentiation, which results in serious craniofacial defects (Wei et al., 2019). ZEB2 is also involved in mouse neural, cortical interneurons and lens epithelial patterning, specification, and migration. However, the exact mechanism by which ZEB2 dysfunction leads to retinal coloboma, a congenital eye defect causing retinal gaps and vision impairment remain unclear (Wei et al., 2019). Conditional loss and gain of function of ZEB2 mouse models, shows inactivation of this TF in the hematopoietic lineage cells blocks the maturation of functional blood cells, and the development of natural killer cells (NK cells) (Wei et al., 2019), T cells and dendritic cells (Villani et al., 2017; Scott et al., 2016). This disruption causes problems in blood vessel development and function, leading to bleeding in the heart and head region (Villani et al., 2017; Scott et al., 2016). The embryonic lethality observed in ZEB1 KO mouse is primarily due to skeletal defects, because ZEB1 play crucial roles in the differentiation of mesenchymal stem cells into chondrocytes and osteoblasts (Soen et al., 2018; Xu et al., 2021). The mouse models with truncated ZEB1 gene lacking CZF domain shows a block in development of T-cell, B-cell, and NK cells in the hematopoietic system. (Miyoshi et al., 2006; Takagi et al., 1998). This will cause altered immune response and chronic inflammation,

leading to severe cardiovascular abnormalities (Miyoshi et al., 2006; Takagi et al., 1998).

However, a study conducted on MI mice revealed that ZEB2 is expressed in the injured cardiomyocytes and that the cardiomyocytes specific knockout of ZEB2 causes defects in the cardiac wound healing process (Gladka et al., 2021). They have also generated a cardiomyocyte specific ZEB2 overexpressing mouse and induced MI for 14 days and analysed molecular and functional properties of the heart (Gladka et al., 2021). They found that ZEB2 helped improve cardiac function after MI by reducing infarct size and reducing left ventricular internal diameter. It also accelerates angiogenesis in the infarct heart (Gladka et al., 2021). They also discovered that AAV-mediated ZEB2 delivery after 28-days of MI improved cardiac functions (Gladka et al., 2021). They repeated the same study in the hypoxic heart of mice after ischemia-reperfusion injury and found that ZEB2 overexpression reduced hypertrophy and contractile dysfunction that occurred after cardiac injury (Gladka et al., 2024). However, study on ZEB1 knockout mice revealed that ZEB1 null mice showed embryonic lethality and reducing ZEB1 expression in myeloid cells showed an increase in atherosclerosis (Martinez-Campanario et al., 2023).

1.5.6 Overlapping Functions of ZEB1 and ZEB2

In the hematopoietic stem cells and in multipotent progenitor cells both ZEB1 and ZEB2 are co-expressed in moderate level (Li et al., 2017; Soen et al., 2018). Studies in the hematopoietic cells suggest that these two TFs, acting as transcription repressors form a negative feedback loop through mutually repressing each other's transcription, allowing them to overlap in function and compensate for each other during hematopoietic stem cell differentiation (Soen et al., 2018). As

differentiation starts, ZEB1 and ZEB2 level oscillates, and the level of oscillation determines the differentiation fates (Postigo & Dean, 2000). This mutual regulation allows them to compensate for each other to maintain a balanced and controlled progression of the hematopoietic stem cell differentiation (Postigo & Dean, 2000). The overlapping roles and the mutual feedback between ZEB1 and ZEB2 are also seen in the nonhematopoietic cells as well such as melanocytes (Postigo & Dean, 2000), and skeletal muscles and mutation in a single ZEB gene in these tissues show little or no effects (Takagi et al., 1998).

Overall, both ZEB1 and ZEB2 have their own specific distinct roles and similar roles in development as well as in the diseased conditions. They are critical factors for different cellular processes including, EMT, hematopoiesis, neural development, and various disease progression. In the context of the heart, ZEB1 and ZEB2 are involved in vessel formation. Also, previous studies in our lab have shown that ZEB2 is involved in the activation of cardiac fibroblasts in the post-MI heart. Understanding the precise mechanism by which ZEB1 and ZEB2 influence the fibroblast activation and maintenance of the activated myofibroblast phenotype provide valuable insight into the molecular underpinning of fibrotic heart diseases.

CHAPTER II: RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 : Rationale

Despite the advancements in cardiac research and medicine, MI and cardiac fibrosis remain a major cause of death worldwide (Roger, 2021). Currently, there is no established anti-fibrotic therapy available, due to a limited understanding of the pathways that lead to MI and cardiac fibrosis (Czubryt, 2012b; Leask, 2010). Therefore, understanding the biochemical mechanisms that lead to persistent cardiac fibrosis is vital for achieving improved patient prognosis (Leask, 2010; Leask & Abraham, 2004).

Subsequent to MI, different growth factors such as TGF- β , FGF, and PDGF are activated and secreted into the myocardium (Leask, 2010). These growth factors mediate the process of fibroblast activation to a myofibroblast phenotype. The myofibroblast will migrate to the infarcted area and secrete ECM proteins which helps the wound healing and formation of the scar in the heart after a MI (Gabbiani et al., 1971). However, the prolonged activation of myofibroblasts will lead to the excessive accumulation of ECM proteins in the heart, and thereby stiffening of the myocardium and eventually fibrosis (Chalise et al., 2023b; Czubryt, 2012b; Gabbiani et al., 1971; Hewitson et al., 1995b).

Previous studies in our lab have shown that the transcription factor MEOX2 can inhibit fibroblast activation by downregulating the myofibroblast markers ED-A fibronectin and α -SMA (Cunnington et al., 2014). These studies also found that the transcription factor ZEB2, a known direct transcription repressor of MEOX2, is highly expressed in the myofibroblast (Jahan et al., 2018). Ectopic ZEB2 expression leads to the activation of primary rat cardiac myofibroblast by upregulating the expression of embryonic smooth muscle myosin heavy chain (SMEMB), ED-A fibronectin and α -SMA (Jahan et al., 2018). However, following ZEB2 knockdown using small interfering RNAs (siRNAs) for 48-hours, expression of these myofibroblast markers was not altered. These findings strongly suggest that other pro-fibrotic transcription factors, such as ZEB1 may also have compensatory roles in the activation of the myofibroblast phenotype. Both ZEB1 and ZEB2 may synergize together to promote the switch from a fibroblast to a myofibroblast phenotype.

2.2 : Hypothesis

I hypothesize that ZEB1 and ZEB2 are essential to establish the activated fibroblast phenotype. I also hypothesize that ZEB1 and ZEB2 form a negative feedback loop, by compensating for the loss of each other. The aim of my thesis is to determine the role of ZEB1 and ZEB2 in controlling the acquisition of the activated fibroblast phenotype *in vitro*.

2.3 : Specific Objectives

Objective 1: Compare the expression of ZEB1 and ZEB2 during fibroblast activation in primary rat cardiac fibroblasts.

Sub Hypothesis I: The expression of both ZEB1 and ZEB2 will increase during the activation of fibroblast to myofibroblast.

In order to accomplish this objective, we used primary rat cardiac fibroblast cells (PRCF) from adult male and female Sprague – Dawley rats. The stiffness of the tissue culture plates stimulated fibroblast activation. Cells were harvested at three different time points. The 24-hour harvest cells represent the quiescent cardiac fibroblasts, and the 48-hour cells represent the partially activated fibroblast, and the 96-hour cells are considered to be the fully activated myofibroblasts. Western blotting was used to determine ZEB1 and ZEB2 protein expression during the process of fibroblast activation. α -SMA was used as the myofibroblast marker and GAPDH served as the loading control for normalization.

To further investigate the role of ZEB2 in the cardiac fibroblast activation process, we transfected PRCF cells with ZEB2 siRNA and harvested the cells after 48-hrs. We evaluated the expression of ZEB1 in the ZEB2 knockdown cell lysates. This experiment sought to determine whether ZEB1 can compensate for ZEB2 loss during fibroblast to myofibroblast transition. These studies provide insight into the potential regulatory relationship between these two transcription factors during cardiac fibroblast activation.

Objective 2: Investigate the role of ZEB1 and ZEB2 in regulating the phenotype of mouse NIH3T3 cell line.

Sub Hypothesis II: Loss of both ZEB1 and ZEB2 is required to alter cardiac fibroblast activation as ZEB1 and ZEB2 negatively regulate each other's expression.

To address this objective, we performed overexpression and knockdown of ZEB2 and ZEB1 in the mouse embryonic NIH3T3 cell lines. For the knockdown studies, the cells were transfected with ZEB2, ZEB1 and controls (GAPDH and Non-Targeting) siRNAs and harvested after 48 hours. For the overexpression study, the cells were transfected with pcDNA3.1-ZEB2-HA, pcDNA3.1 empty and pCMV6-ZEB1 vectors and the cells were harvested after 48 hrs. To analyse the effect of knockdown and overexpression of ZEB1 and ZEB2 in the myofibroblast, we have utilized western blotting as well as immunofluorescence techniques. We determined the expression level of different myofibroblast markers, such as ZEB1, ZEB2, α -SMA and SMemb using western blotting.

This experimental approach provides insight into how ZEB1 and ZEB2 influence the activation of fibroblasts and their transition into myofibroblasts. Additionally, it will help to determine whether a negative feedback loop exists between ZEB1 and ZEB2 during fibroblast activation, which may result in one ZEB family member compensating for the loss of the other family member. Therefore, by investigating the interaction between ZEB1 and ZEB2, we aim to better understand the molecular mechanisms behind fibroblast activation.

Chapter III: Materials and Methods

3.1 : Primary Rat Cardiac Fibroblast (PRCF) Isolation and Cell Culture

3.1.1 : NIH3T3 Cell Line

NIH3T3 mouse embryonic cell line was purchased from ATCC (VA, USA) and cultured in Dulbecco's Modified Eagle medium (Gibco, Carlsbad, USA) (containing 25 mM glucose+ 4 mM L-glutamine + phenol red) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, USA) + 1% penicillin/ streptomycin (P/S) (10,000 U/mL, Gibco, Carlsbad, USA).

3.1.2 : PRCF Isolation and Culture

The experimental protocols for the use of adult rats were approved by the animal care committee of the University of Manitoba. These protocols adhere to the guidelines set forth by the Canadian Institute of Health Research and the Canadian Council of Animal Care. Protocol #20-062, which specifically addresses the isolation of PRCF was approved on January 1, 2021. PRCFs were obtained from both female and male Sprague-Dawley Rats (200-250 g weight). Before harvesting the heart, the adult rats were deeply anesthetized with 2-3% isoflurane by a member of the RO Burrell laboratory staff. The rat heart was collected in a sterile 10 cm petri plate and washed 3 times with 10 ml of warm (37°C) phosphate buffer saline (PBS) (Gibco, Carlsbad, USA) with 1% Penicillin/ Streptomycin (P/S) to remove the residual blood in the chambers. The ventricles were dissected from the remainder of the heart and dissected into small cubes. The ventricular

tissues were transferred to a fresh sterile dish and further minced into small pieces in 1 ml warm collagenase type II (Gibco Carlsbad, USA) solution. For a rat, approximately 250 g in size, a total of 60 ml, 280 units/ml of collagenase type II solution in serum-free DMEM/F12 media with 1% P/S is required. Once the tissue can pass through a 1 ml pipette tip, they were transferred to a 25 ml conical flask containing 15 ml collagenase type II solution (280 units/ml). The tissues were digested for 30 minutes in the 37°C at 5% CO₂ incubator by stirring the tissues using a magnetic stirrer bar at a spin setting of 6. The supernatant obtained from the first digestion (S1) was discarded since it contained blood, cell debris, epithelial and endothelial cells. Into the tissue remaining in the conical flask, another 20 ml of collagenase type II solution was added, and the tissue was digested for a second time at 37°C, 5% CO₂ incubator for 45 minutes spin setting of 7. The supernatant obtained from the second digestion (S2) was transferred to a 50 ml tube. For the final digestion, 20 ml of collagenase type II solution was added to the remaining cells in the conical flask and spun at speed 7 for another 45 minutes in the CO₂ incubator. To the supernatant S2, an equal volume of complete DMEM/F12 media (Gibco, Carlsbad, USA) was added and incubated for 5 minutes at room temperature (RT) to stop the collagenase digestion. After incubation the supernatant - S2 was centrifuged at RT, 5000 rpm for 5 minutes. The supernatant was discarded and 5 ml of ice-cold 1x Gey's solution (1.49 mM CaCl₂ + 4.96 mM KCl + 0.22 mM KH₂PO₄ + 1.03 mM MgCl₂·6H₂O + 0.284 mM MgSO₄·7H₂O + 0.14 mM NaCl + 2.70 mM NaHCO₃ + 0.845 mM Na₂HPO₄·7H₂O) was added to the pellet containing cells and tissue debris for washing. The solution was incubated on ice for 1 minute and centrifuged at 2000 RPM and RT for 5 minutes. Then the pellet was resuspended in 5 ml of DMEM/F12 complete media and plated in a 6 cm tissue culture plate. After the third digestion, the supernatant obtained was named S3 and subjected to the same treatment as S2. Two hours after plating S3, the media was changed on both the S2 and S3 plates after washing with 5 ml of warm

PBS containing 1% P/S. The cells were incubated in a 37°C, 5% CO₂ incubator, and harvested at three different time points- 24 hours, 48 hours, and 96 hours.

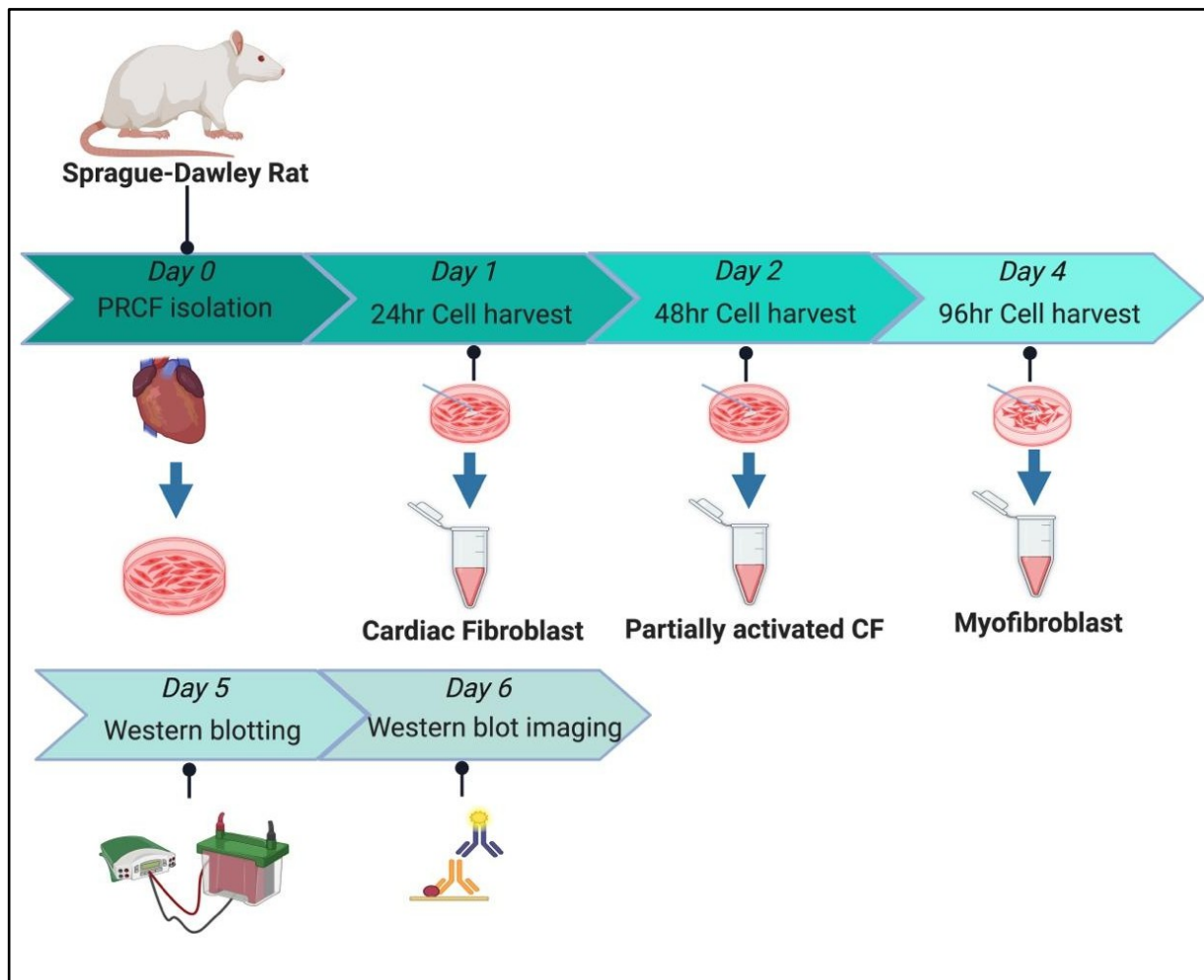


Figure 7: Overview of Culturing Primary Rat Cardiac Fibroblasts. Primary rat cardiac fibroblasts (PRCF) were isolated from male and female Sprague-Dawley rats on Day 0 and then cultured in the incubator at 37°C and 5% CO₂ in complete DMEM/F12 media. The cells were harvested at three different time points. At the time point 24 hours the cells were designated as Cardiac Fibroblasts (CF), at the 48-hour time point they were designated as partially activated CF and at the 96-hour time point, the cells were considered to be fully activated myofibroblasts. The expression of different proteins was analyzed by using western blot technique. (Created in <https://BioRender.com>).

3.2 : Lipofectamine 3000 Transfection – ZEB2 Overexpression Study

The NIH3T3 cells were grown in 6 cm petri plates in the DMEM complete media at 37°C, 5% CO₂ until they reached 70-80% confluency. Lipofectamine 3000 (ThermoFisher, Waltham, USA) was diluted in Opti-MEM™ media (Gibco, Massachusetts, USA) at a ratio of 1:16 in a 1.5 ml Eppendorf tube, labeled vial- A. In another 1.5 ml Eppendorf tube, labelled as Vial B, P3000 (ThermoFisher, Waltham, USA) reagent was diluted in Opti-MEM media at a ratio of 2:49 with 5 ng of expression vector (pcDNA3.1 Empty, pcDNA3.1 HA-ZEB2, and pCMV6-ZEB1 vectors were used for this study). The contents of vial B were transferred to vial A, mixed gently by flicking, and incubated for 20 minutes at RT in the biosafety cabinet (BSC). The master mix in the vial-B was added dropwise to the NIH3T3 cells and incubated at 37°C, 5% CO₂ incubator. The media was changed after 5 hours, and the cells were harvested 24 hours after transfection.

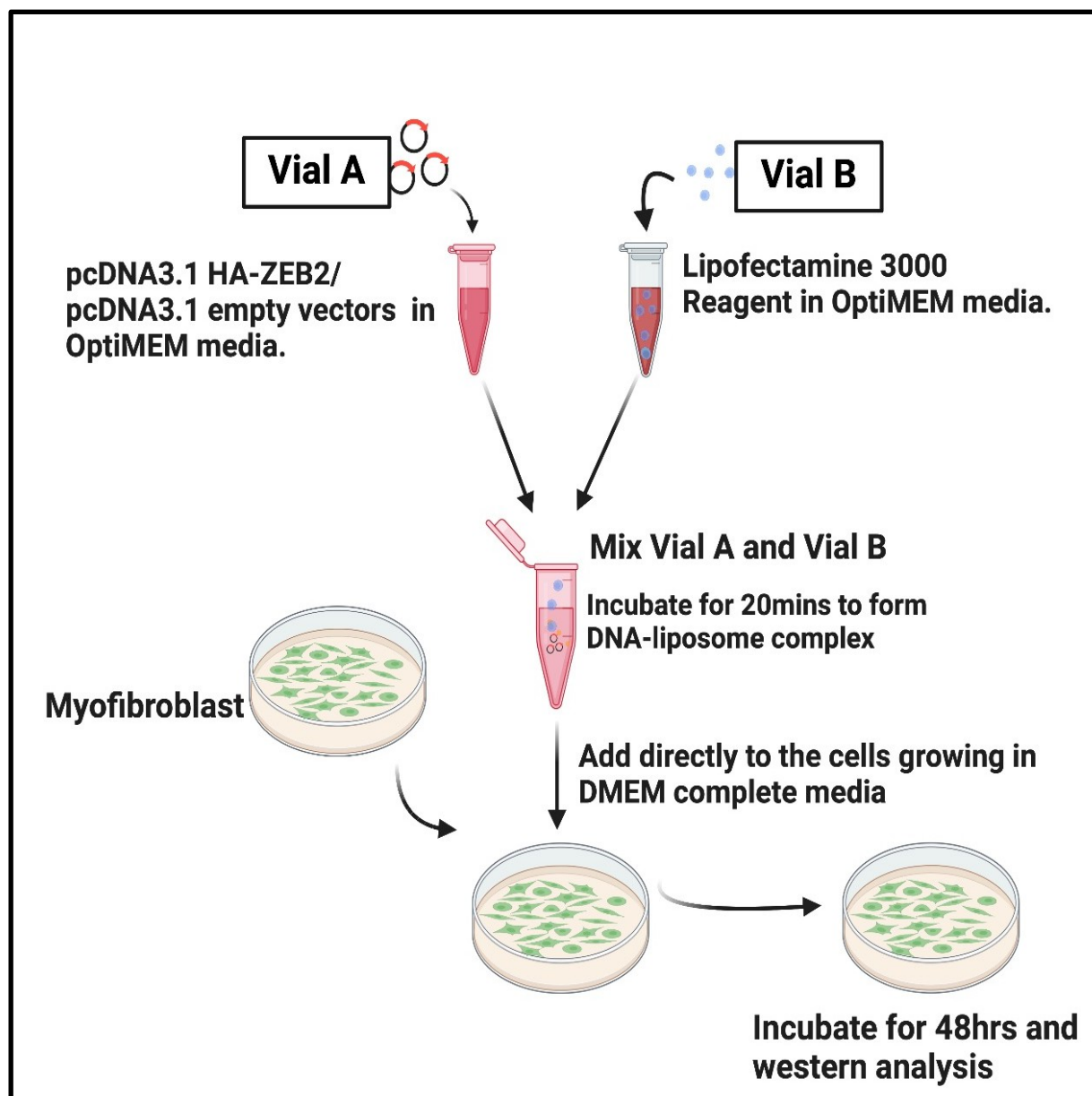


Figure 8: Ectopic Expression of ZEB2 in NIH3T3 Cells. The cells were cultured in DMEM complete media containing 10% FBS and 1% P/S and allowed to grow until they reached 50% confluency. Subsequently, they were transfected with pcDNA3.1 ZEB2-HA and pcDNA3.1-Empty vectors using the Lipofectamine 3000 reagent transfection method and harvested after 48 hours of incubation. The expression of different proteins was compared by western blotting.

3.3 RNAi Single Knockdown Study

One day before transfection, NIH3T3 cells were seeded at a density of 3.5×10^5 cells/well in 6 well tissue culture plates with DMEM + 10% FBS media without P/S. PRCFs were cultured in the DMEM F12 + 10% FBS media also without P/S. The cells were transfected the next day when they reached 60-70% confluency. For transfection, siRNAs were diluted in Opti-MEM reduced serum medium. The siRNAs used for this study include ZEB1-A, ZEB1-B, ZEB1-C, ZEB1-D, ZEB2-A, ZEB2-B, ZEB2-C, ZEB2-D, GAPDH (mouse and rat) and negative control (nontarget) siRNAs from Dharmacon, as well as ZEB2-E, ZEB2-F and additional GAPDH and nontarget siRNAs from Invitrogen. For each well in the six-well plate, 30 pmol of diluted siRNA in Opti-MEM media were prepared first. For the Dharmacon siRNA, which were stored as a 20 μ M stock, a 5 μ M of working siRNA solution was prepared by diluting the stock with 1x siRNA buffer (Dharmacon, Massachusetts, USA). In a 1.5 ml Eppendorf tube, 6 μ l of 5 μ M working solution (30 pmol) was added to 250 μ l of Opti-MEM media and labelled as vial-A. For the Invitrogen siRNAs, which were stored as a 100 μ M stock, a 20 μ M working solution was prepared by diluting it with the nuclease free water (Invitrogen, Waltham, USA). From this 1.5 μ l was (30 pmol) was added to 150 μ l of Opti-MEM media to dilute the RNiMax duplex. The solution was mixed gently by flicking.

In a second tube, labelled as Vial B, the diluted Lipofectamine RNAiMax (ThermoFisher, Waltham, USA) solution was prepared. For the Dharmacon siRNAs, 5 μ l of lipofectamine was mixed with 250 μ l of Opti-MEM, whereas for the Invitrogen siRNAs, 9 μ l of Lipofectamine was mixed with 150 μ l of Opti-MEM. The solutions from the two tubes (Vial A and Vial B) were

transferred into a single tube and mixed by gentle flicking. The siRNA – Lipofectamine master mix solution was incubated for 20 minutes at RT for the Dharmacon siRNA transfection and for 5 minutes for the invitrogen siRNA transfection. Meanwhile, the old media was replaced with fresh DMEM+10% FBS without antibiotic media. The siRNA-Lipofectamine master mix solution was then added to the cells dropwise. The cells were incubated at 37°C in CO₂ incubator and harvested the total cell lysate after 48 hours post-transfection.

3.4 : RNAi Double Knockdown Studies

In a similar approach to the single knockdown procedure, the NIH3T3 cells were seeded one day prior to the double knockdown experiment, in 6-well plates with DMEM media supplemented with 10% FBS, without antibiotics. The cells were counted and seeded at a density of 3.5×10^5 cells/well. The following day, RNAiMax transfection procedure was performed when the cells reached 60-80% confluency. For the double knockdown study, the most efficient ZEB1 and ZEB2 siRNAs from the single knockdown study were selected (ZEB1-C and ZEB2-F). Each siRNA was used at a concentration of 30 pmol, with 30 pmol of ZEB1-C and 30 pmol of ZEB2-F mixed in 150 µl of Opti-MEM media. GAPDH and non-targeting siRNAs were used as the positive and negative controls (Invitrogen, Waltham, USA) (30 pmol/well) respectively. In a separate tube, the Lipofectamine RNAiMax solution (Thermofisher, Waltham, USA) was diluted by mixing 9 µl of Lipofectamine RNAiMax with 150 µl of Opti-MEM media. The diluted siRNA mixture was then transferred to the diluted lipofectamine solution tube. The contents were gently mixed by flicking in the tube and incubated for 5 minutes at room temperature. Meanwhile, the old media was replaced with fresh DMEM+10% FBS without antibiotic media and after 5 minutes of

incubation, the transfection mix was added dropwise to the cells. The cells were incubated in 37°C, 5% CO₂ before harvesting.

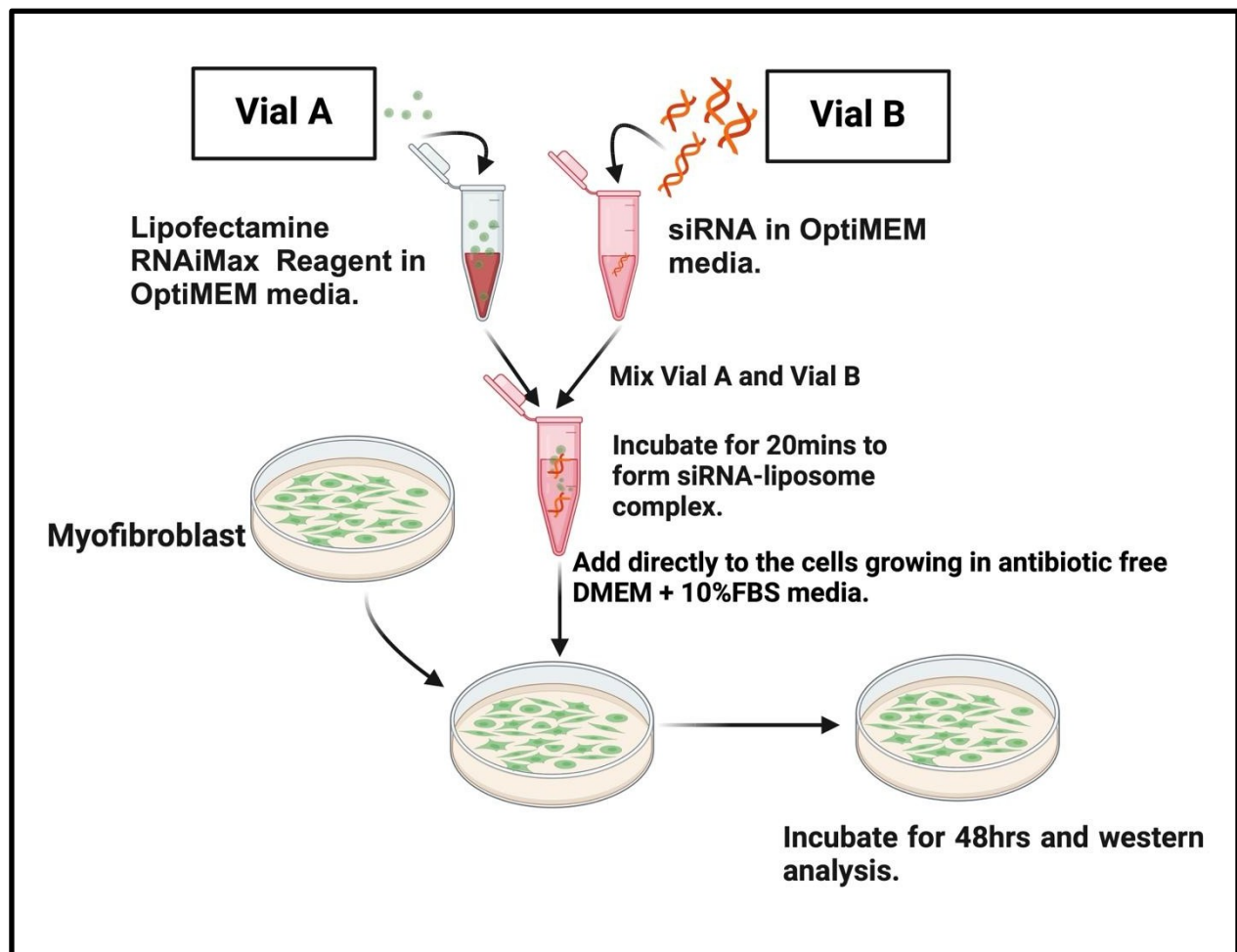


Figure 9: siRNA Mediated Knockdown of ZEB1 and ZEB2 in PRCF and in NIH3T3 Cell line. The cells were cultured in DMEM/F12 + 10% FBS media without antibiotic for PRCF cells or in DMEM + 10% FBS media without antibiotic for NIH3T3 cells and allowed to grow until they reached 50%-60% confluency. Following this, they were transfected with Nontarget, GAPDH, ZEB1 and ZEB2 siRNAs using the RNAiMax transfection method, and harvested after 48 hours of incubation. The expression of different proteins was compared by western blotting. (Created in <https://BioRender.com>).

siRNA	Host	Company	Catalogue Number/ Reference Number
ZEB1 A	Mouse	Dharmacon	Cat # J-051513-11-0005
ZEB1 B	Mouse	Dharmacon	Cat # J-051513-10-0005
ZEB1 C	Mouse	Dharmacon	Cat # J-051513-09-0005
ZEB1 D	Mouse	Dharmacon	Cat # J-051513-12-0005
ZEB2 A	Mouse	Dharmacon	Cat # J-059671-05-0005
ZEB2 B	Mouse	Dharmacon	Cat # J-059671-06-0005
ZEB2 C	Mouse	Dharmacon	Cat # J-059671-07-0005
ZEB2 D	Mouse	Dharmacon	Cat # J-059671-08-0005
ZEB2 E	Mouse	Invitrogen	Ref # 4390771 ID - s76956
ZEB2 F	Mouse	Invitrogen	Ref # 4390771 ID - s76954
Negative control	Mouse	Dharmacon	Cat # D-01810-10-05
Negative control	Mouse	Invitrogen	Ref # 4390843
GAPDH	Mouse	Invitrogen	Ref # 4390849
GAPDH	Mouse	Dharmacon	Cat # D-01830-30-05
GAPDH	Rat	Dharmacon	Cat # D-001830-20-05

Table 1: List of siRNAs Used for the Transfection Experiment.

3.5 : Preparation of Total Cell Lysate Harvest

After incubation, the cells were harvested at different time points. The plates were kept on ice and washed three times with 5 ml ice-cold PBS (without antibiotic). Between 100 µl- 300 µl radioimmunoprecipitation assay lysis (RIPA) buffer (Sigma-Aldrich, Missouri, USA) (50 mM, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NaDeoxycholate, 1% SDS, pH=7.4) with PierceTM protease and phosphatase inhibitor mini tablets, EDTA free (ThermoFisher, Massachusetts, USA) were added to the cells depending on cell confluency and incubated on ice for 20 minutes. Cells were then mechanically scraped using cell scrapers, and the lysates were centrifuged at 4°C, 12000 RPM for 10 minutes. The supernatants were transferred to fresh tubes, labeled, and stored in -80°C freezer for further use.

3.6 : DC Protein Assay

The concentrations of proteins in each cell lysate were quantified using detergent compatible (DC) protein assay. The S' solution was prepared by combining 1000 µl of reagent A (alkaline copper tartrate solution) (Bio-Rad, Hercules, USA) with 20 µl of reagent S (surfactant solution-dilute sodium lauryl sulfate) (Bio-Rad, Hercules, USA) and mixed by inverting the tubes 5-6 times. To a 96-well plate, 25 µl of S' solution was added in each well and 5 µl of protein standard/samples was added to the S' solution in the well. Finally, 200 µl of reagent B (dilute Folin reagent) (Bio-Rad, Hercules, USA) was added to each well and the plates were incubated at RT for 15 minutes. The optical density was measured using the plate reader and the concentrations were calculated using a standard protein curve.

3.7 : Western blot analysis

The samples for western blotting were prepared by mixing 15-30 µg of protein with loading dye, (which is made by adding 900 µl of 5X Laemmli buffer (Bio-Rad, California, USA), to 100 µl of β-mercaptoethanol (Sigma-Aldrich, Missouri, USA)) and autoclaved water to make a volume of 45 µl. The samples were denatured at 95°C for 5 minutes. SDS-PAGE was performed to resolve the proteins using 4-15% polyacrylamide Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad, California, USA) at 100 V for 1 hour. The gels were activated in the Chemidoc imaging system (Bio-Rad, California, USA), and the proteins were transferred to a 0.45 µM nitrocellulose membrane using semi-dry Trans-Blot Turbo system (Bio-Rad, California, USA) at 1.3 A, 25 V for 7 minutes. The membrane was imaged using the Chemidoc system to ensure the protein transfer and blocked with either 5% skimmed milk or 5% bovine serum albumin (BSA) (Sigma-Aldrich, Missouri, USA) in Tris buffered saline with Tween 20 (TBST) (Sigma-Aldrich, Missouri, USA) for 2 hours on a rocker at RT. The primary antibodies were diluted in their respective blocking buffer as outlined here: ZEB2 (Rabbit, Invitrogen, Waltham, USA) (1: 1000), (Rabbit, Sigma-Aldrich, Missouri, USA) (1:1000), ZEB1(Rabbit, Proteintech[→], Illinois, USA) (1:1000), α-SMA (Mouse, Invitrogen, Waltham, USA) (1: 3000), GAPDH (Rabbit, Cell signaling technology[®], Massachusetts, USA) (1: 2500), HA (Rabbit, Invitrogen, Waltham, USA) (1: 1000), FLAG (Mouse, Sigma-Aldrich, Missouri, USA) (1: 1000). The membrane was incubated for overnight in 5 ml of primary antibody at 4°C on a rocker. On the next day, the blot was washed 3 times with 25 ml of TBST with constant shaking for 10 minutes. The blots were then transferred to the secondary antibody, which is horseradish peroxidase (HRP) conjugated goat-anti-rabbit or goat-anti-mouse antibody (Cell signaling technology[®], Massachusetts, USA)

in the blocking buffer (1: 5000 dilution) and incubated for 1 hour in RT on rocker. The blots were washed again 3 times with 25 ml TBST on the rocker for 10 minutes at RT and the protein bands were developed using the western blotting enhanced chemiluminescence substrate (ECL) reagents (Bio-Rad, California, USA). The blots were imaged on the fluorescence and the chemiluminescence detection Chemidoc system (Bio-Rad, California, USA), and the intensity of each protein band were quantified by using Image J software (Bio-Rad, California, USA). The bands were first normalized either by single protein detection method (by comparing the intensity of the protein of our interest with the intensity of the loading control) or by total protein normalization method (by comparing the intensity of the protein of our interest with the intensity of the total protein). To normalize the data, the intensities of each test protein band were divided by the intensity of either the loading control (GAPDH) or the total protein. Finally, statistical analyses were conducted on the normalized data by using the GraphPad Prism 10 software (Dotmatics company).

Name	Dilution	Host	Clonality	Company	Cat Number/ Ref Number	Blocking Buffer
α -FLAG	1:1000	Mouse, IgG	Monoclonal	Sigma-Aldrich	Ref # 1804	BSA
α -GAPDH	1:3000	Rabbit, IgG	Polyclonal	Cell signalling technology	Ref # 5174S	BSA
α -HA	1:1000	Rabbit, IgG	Polyclonal	Invitrogen	Ref # 26183	BSA
α -SMA	1:3000	Mouse, IgG	Monoclonal	Invitrogen	Ref # 14-9760-82	BSA
α -ZEB1	1:2500	Rabbit, IgG	Polyclonal	Proteintech	Cat # 21544-1-AP	Milk
α -ZEB2	1:1000	Rabbit, IgG	Polyclonal	Sigma-Aldrich	Ref # SAB2108744	Milk
	1:1000	Rabbit, IgG	Polyclonal	Invitrogen	Ref # PA5-82094	BSA
α -SMemb	1:2000	Mouse, IgG	Monoclonal	ThermoFisher	Ref # MA5-27767	BSA
α -Mouse IgG	1:5000	Horse, IgG	Polyclonal	Cell signalling technology	Ref # 7076S	BSA/Milk
α -Rabbit IgG	1:5000	Goat, IgG	Polyclonal	Cell signalling technology	Ref # 7074S	BSA/Milk

Table 2: List of primary and secondary antibodies and their dilutions for western blotting.

3.8 : Immunofluorescence

After siRNA transfection, cells were grown for 48 hours. The cells were washed with 1 x PBS and fixed in 4% paraformaldehyde (PFA) for 12- 15 minutes at RT. The cells were again washed 3 times with 1x PBS and permeabilized with 0.1% Triton X in PBS for 5 minutes, solution was aspirated, and the cells were blocked with 2% BSA in 0.1% Triton X – 100/PBS for 1 hour at RT, rocker. The cells were incubated in immunofluorescent α -SMA primary antibody solution in the blocking buffer (Invitrogen, Massachusetts, USA, Ref # 14-9760-82) (1:200 dilution) for 2 hours in RT, rocker, and washed 3 times with blocking buffer for 5 minutes. The cells were then incubated in donkey anti-Mouse IgG Alexa fluorTM Plus 488 secondary antibody solution in blocking buffer (1:500 dilution) (Invitrogen, Massachusetts, USA, Ref # A32766) and incubated the cells in secondary antibody for 1 hour in the dark room. The cells were washed 3 times again with 1 x PBS for 5 minutes. The nuclei were stained using Hoechst 33342 (Thermofisher, Massachusetts, USA) 3 μ l + 100 ml PBS and incubated two minutes at RT, washed 3 more times with 1x PBS and imaged using a Nikon ti2 automatic fluorescent microscope.

To begin the imaging process, both the computer system and the microscope cameras were powered on. Once the system was up, launched the Nikon Ti12 software and selected the “NikonTi12/Acquisition mode” in the software. The immunostained plates were placed on the microscope stage aligning the region of interest for imaging. Focus was adjusted by using the joystick to ensure the targeted area was clearly visible. To capture the image the “prime-BSI express – FL” was enabled to fluorescent live imaging. Since the samples were stained with two fluorophores DAPI (Hoechst 33342) and GFP (donkey anti-Mouse IgG Alexa fluorTM Plus 488)

chose the “ND acquire” option in the software’s multichannel settings. This allows the imaging of both DAPI and Alexa-488 signals in two separate channels. The focus was adjusted by switching between DAPI and GFP channels to make sure both images were clear. Once focus was confirmed, fluorescent images of the cells were captured and saved in the designated folder for further analysis.

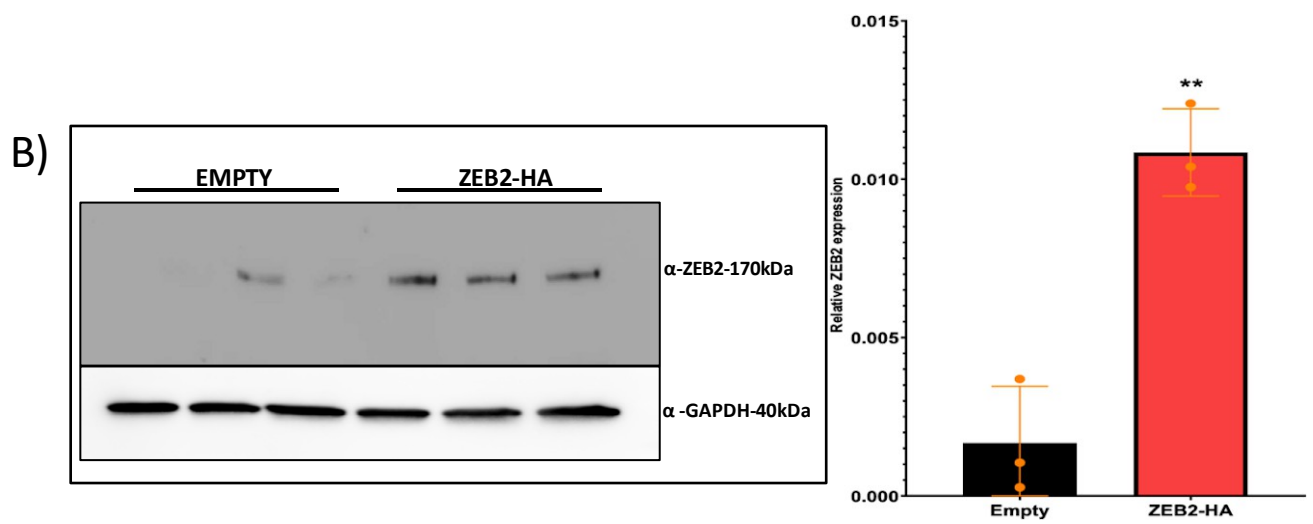
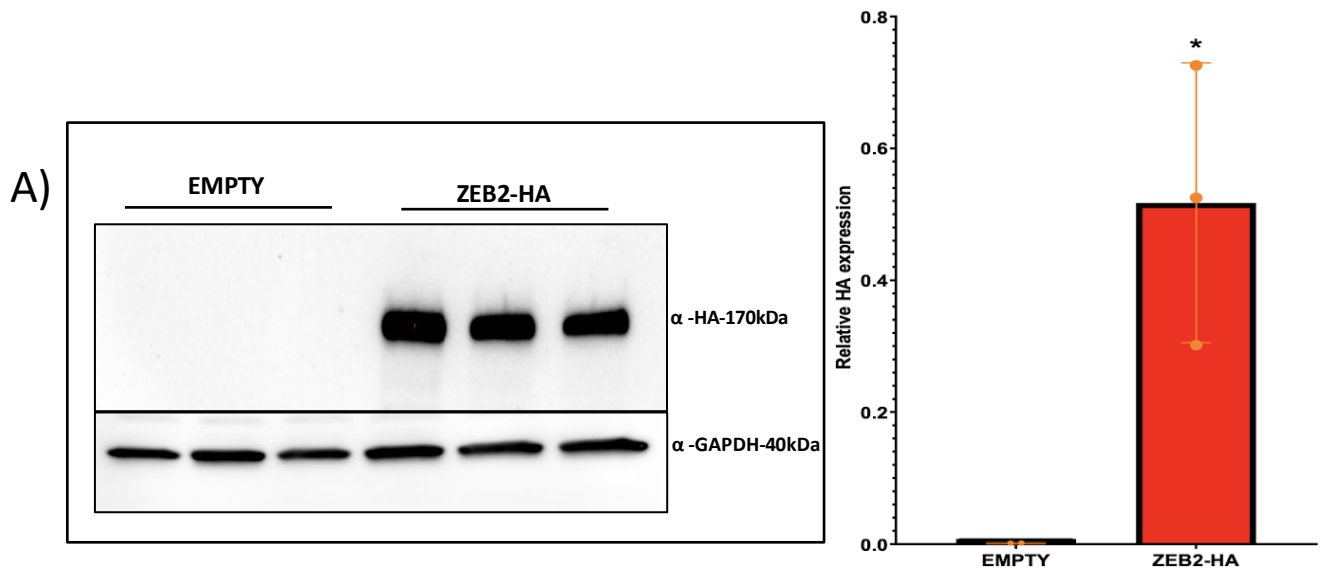
Chapter IV: Results

4.1 Both ZEB1 and ZEB2 Protein Expression is Increased During Cardiac Fibroblast Activation

The ZEB2 antibody was validated by transfecting NIH3T3 cells with a pcDNA3.1 plasmid encoding HA tagged mouse ZEB2. Empty pcDNA3.1 vector transfected NIH3T3 cells were used as the negative control. After 48 hrs, the cells were harvested, and western blotting was performed. The blots were probed with either anti-ZEB2 or anti-HA antibodies. The ZEB2 antibody detected a protein at approximately 171 KDa, which corresponds to the size detected by the HA antibody (Figure 11A and B).

To determine the potential roles of ZEB1 and ZEB2 during cardiac fibroblast activation, we first performed expression analysis of these transcription factors at 24-hr, 48-hr, and 96-hr time points in PRCF (Figure 11). Cardiac fibroblasts were isolated from both male and female Sprague-Dawley rats and were harvested at three different time points in tissue culture plates. The rigid plastic surface of standard tissue culture plate effectively induces the activation of cardiac fibroblast. The cells harvested at 24-hr time point are denoted as quiescent cardiac fibroblasts, those harvested at 48-hr are designated as partially activated cardiac fibroblast and the 96-hr harvested cells are considered to be the myofibroblasts. Western blotting results show that both ZEB1 and ZEB2 protein expression increase upon fibroblast activation (in 48-hr), and their levels decrease after the 48-hr time point in both male and female PRCFs (Figure 11). However, we observed differences in the expression pattern of these transcription factors. ZEB2 is not expressed in the quiescent male or female cardiac fibroblasts (24- hr), however, ZEB1 is

expressed in these cells. The 96-hr matured myofibroblast cells of female rats express both ZEB1 and ZEB2, however in the male rat myofibroblasts, the ZEB2 expression was almost non-detectable. Activation to the myofibroblast phenotype was confirmed by the drastic increase seen in α -SMA levels, which is a myofibroblast marker (Figure 11). This result suggests that both ZEB1 and ZEB2 may have regulatory roles in the activation of cardiac fibroblasts.



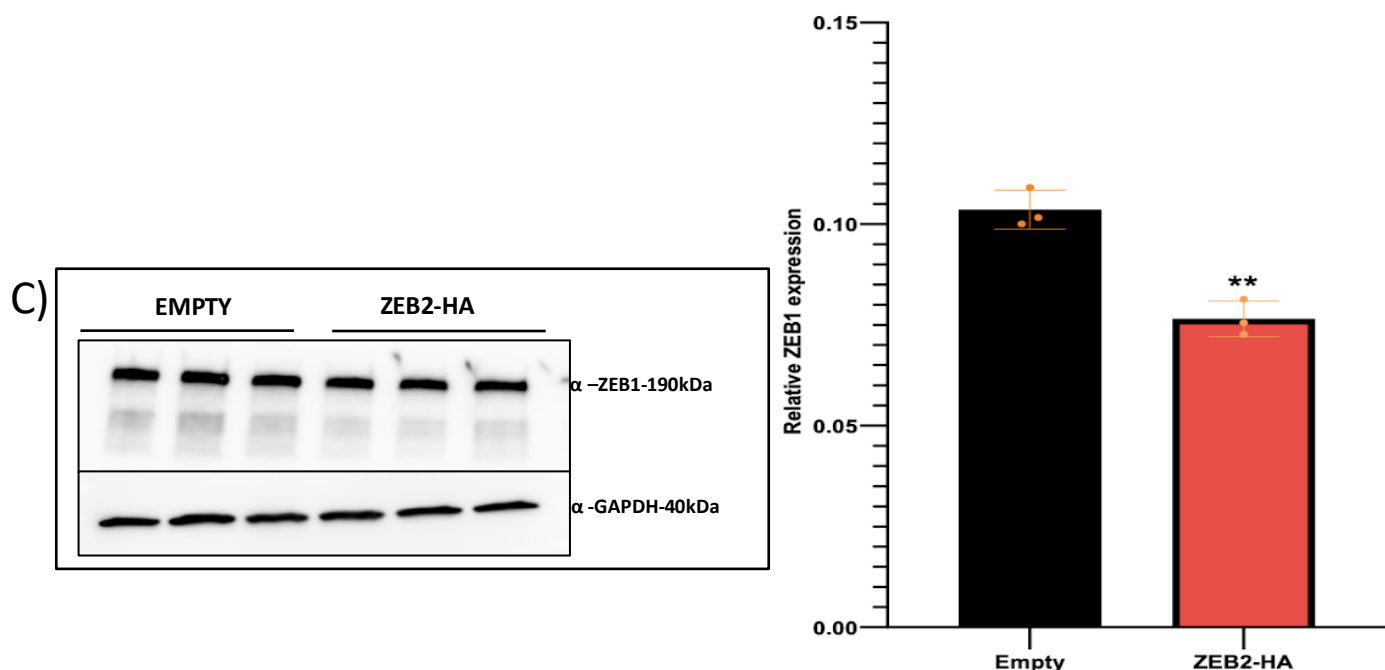


Figure 10: Validation of the anti-ZEB2 antibody and the ectopic expression analysis of ZEB2 in NIH3T3 cell line. Three biological samples of empty cell lysates and three biological samples of treated cell lysates were analyzed. GAPDH is used as the loading control. α -ZEB2, α -HA and α -GAPDH primary antibodies were used. A and B) Anti-ZEB2 and ant-HA both detected a band of 170 kDa. The molecular weight of ZEB1 is 190KDa. HA-ZEB2 protein was only detected in the test samples (ZEB2-HA) and the expression of ZEB2 is increased in the test lysates as compared to the empty vector control samples (Empty). ZEB1 expression decreased in the pcDNA3.1-HA-ZEB2 transfected cells as compared to the control lysates. The Mean +Standard deviation (SD) graph was plotted (N=3) relative to GAPDH and statistical significance (*, $p < 0.05$, **, $p < 0.01$) was determined relative to control empty vector transfection using unpaired two-tailed Student's t-tests.

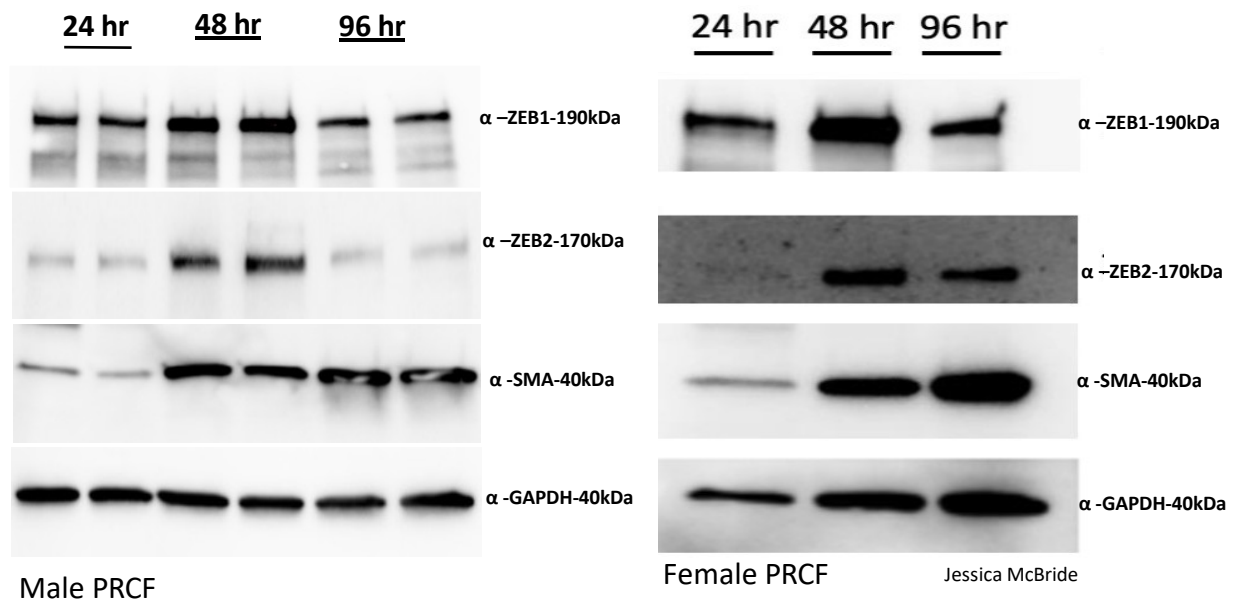


Figure 11: Expression analysis of ZEB1 & ZEB2 during PRCF activation *in vitro*. ZEB1, ZEB2 and α -smooth muscle actin (α -SMA) protein expression levels in male and female primary rat cardiac fibroblasts (PRCF). GAPDH is used as the loading control, as all the protein signals from the female PRCF cells are from a single blot, and similarly all the signals from the male PRCF are from a separate single blot. Both ZEB1 and ZEB2 expression were highest in the 48-hr time point and in both male and female PRCFs and both decreased after 48-hr. The ZEB2 expression is almost nil in the 24-hr samples from both male and female rats as well as the 96 hrs male rat myofibroblasts. However, it shows a higher level of expression in female fibroblast at 96 hrs. In contrast, the ZEB1 expression is notably higher than ZEB2 in male and female fibroblasts in 24hr and 96hr and it attains a peak level of expression at the 48hr time point. The α -SMA expression dramatically increased with time.

4.2 ZEB2 Knockdown in the PRCF Increases ZEB1 Expression

After evaluating the levels of ZEB1 and ZEB2 during cardiac fibroblast activation, we next aimed to find the role of these two transcription factors in the fibroblast activation process. To achieve this goal, we performed knockdown of ZEB2 in PRCFs. The cells were cultured to 60% confluency and transfected with non-targeting siRNA (negative control), GAPDH siRNA (positive control), and four ZEB2 siRNAs (named ZEB2 A, B, C and D). Following 48-hrs of transfection, the cells were harvested and the levels of ZEB2 protein were determined by using western blotting technique. Among the four siRNAs, two (ZEB2 A and B) effectively knocked down ZEB2 in which ZEB2 B achieved the largest reduction of ZEB2 expression (95%) (figure: 12). GAPDH (positive control) expression was also reduced in the GAPDH knockdown samples confirming that the transfections were successful (figure:12).

Interestingly, when we analysed ZEB1 protein expression in the ZEB2 knockdown samples, we observed an unexpected increase in ZEB1 expression level as compared to the nontargeting and GAPDH controls (figure:12). This suggests that ZEB2 may negatively regulate ZEB1 expression during cardiac fibroblast activation, indicating a potential regulatory relationship between these two transcription factors in this process.

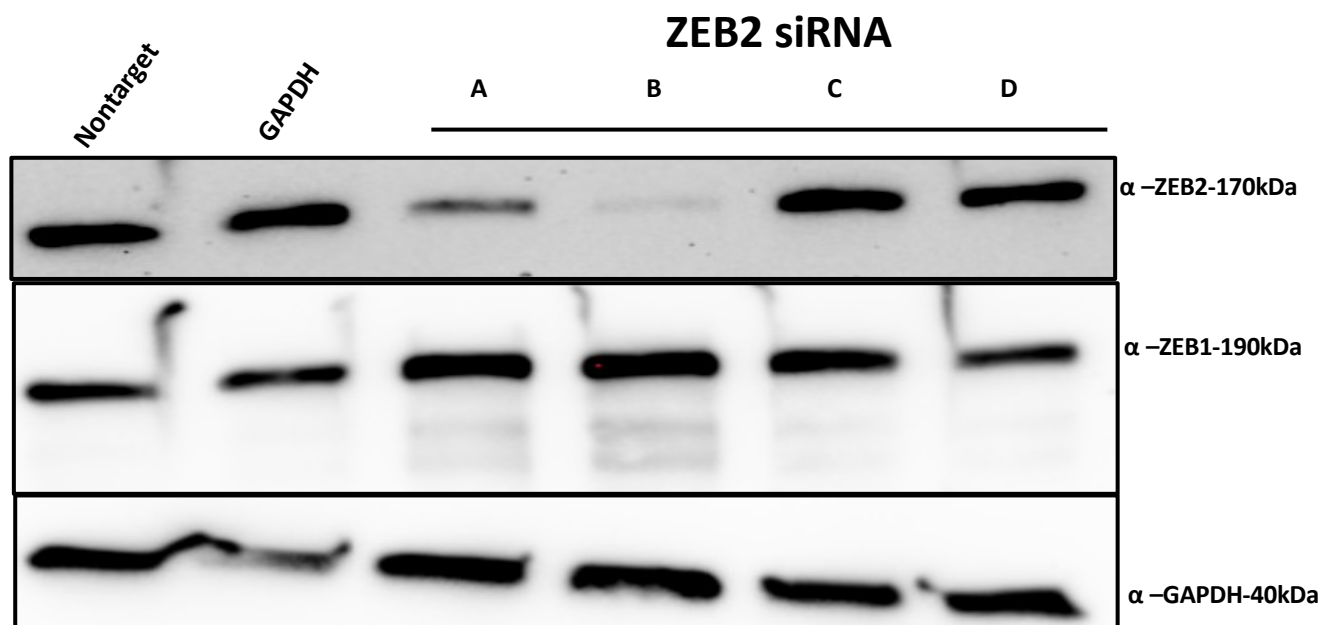


Figure 12: ZEB2 knockdown in primary rat cardiac fibroblast. Primary rat cardiac fibroblasts were transfected siRNAs targeting ZEB2 (ZEB2 A, B, C, and D siRNAs). GAPDH siRNA (positive control) and a non-targeting siRNA (Negative control) were transfected as well. Transfection with siRNAs A and B effectively knocked down ZEB2 protein, with siRNA B being the most effective. The expression of ZEB1 protein was increased upon ZEB2 knockdown by siRNA A and siRNA B. The expression of GAPDH protein was reduced in the GAPDH (positive control) siRNA treated samples.

4.3 ZEB2 Overexpression Decreases ZEB1 Protein Levels in NIH3T3 Cell

Lines

To confirm this potential regulatory relationship between ZEB1 and ZEB2, we overexpressed ZEB2 in NIH3T3 cells. NIH3T3 cells are a well-established mouse embryonic cell line, often used to study fibroblast activation and myofibroblast differentiation. While PRCFs are a more direct representation of fibroblasts in the hearts, NIH3T3 cell lines offer several advantages such as ease of culturing and availability, high reproducibility, easily genetically manipulated and high cellular plasticity, together these traits lead to them being extensively used for research purposes.

The NIH3T3 cells were seeded in DMEM complete media and were grown to 60% confluency. The transfection was performed using Lipofectamine 3000 transfection procedure. The controls were transfected with pcDNA3.1- empty plasmid vector and the experimental cells were transfected with pcDNA3.1 ZEB2-HA vector. The cells were cultured for 48-hrs and harvested to perform western blotting procedure to analyze the expression levels of HA, ZEB2 and ZEB1 in the ZEB2 knockdown samples.

The expression of HA was only observed in the test samples transfected with pcDNA3.1-ZEB2-HA vector, and not in the control samples transfected with the empty vector. The ZEB2-HA fusion protein was detected at approximately 170 KDa (figure:10 A), using an anti-ZEB2 antibody. This confirms that the transfection was successful. The expression of ZEB2 was seen both in control as well as in the test samples and it was increased in the samples which were transfected with the pcDNA3.1 ZEB2-HA vector (figure: 10 B). Notably, ZEB1 expression

decreased in the samples in which ZEB2 was overexpressed as compared to the controls (Figure 10 C). This further supports a potential negative regulatory relationship between ZEB1 and ZEB2 during fibroblast activation.

4.4 ZEB2 Knockdown Increases ZEB1 Expression in NIH3T3 Cell Line

To further investigate the role of ZEB2 in fibroblast activation, we performed a knockdown study of ZEB2 in NIH3T3 cell lines using six-different siRNAs and analysed the expression of α -SMA in these samples. Four out of these six siRNAs were purchased from Dharmacon (ZEB2 A, B C, and D) and two from Invitrogen (ZEB2 E and F). The cells were transfected with siRNAs, using RNAiMax transfection procedure, when the cells reached 60-80% confluency and were harvested 48-hrs post-transfection. The expression of ZEB2 was determined by using α -ZEB2 (Sigma) antibody and the results revealed that three of the six siRNAs significantly reduced the expression of ZEB2, with ZEB2 F being the most effective in knocking down ZEB2 (figure: 13). The GAPDH expression was reduced by 90% in the GAPDH knockdown samples, confirming the effectiveness of the transfection procedure.

However, despite the successful knockdown of the ZEB2 expression, the expression of α -SMA remained unchanged (figure: 13). In contrast, ZEB1 expression was increased in the ZEB2 knockdown sample (figure: 14), which may indicate the presence of the compensatory mechanism between ZEB1 and ZEB2. Together, these findings suggest that loss of ZEB2 alone may not be sufficient to modulate the myofibroblast markers, and that ZEB1 may compensate for the loss of ZEB2.

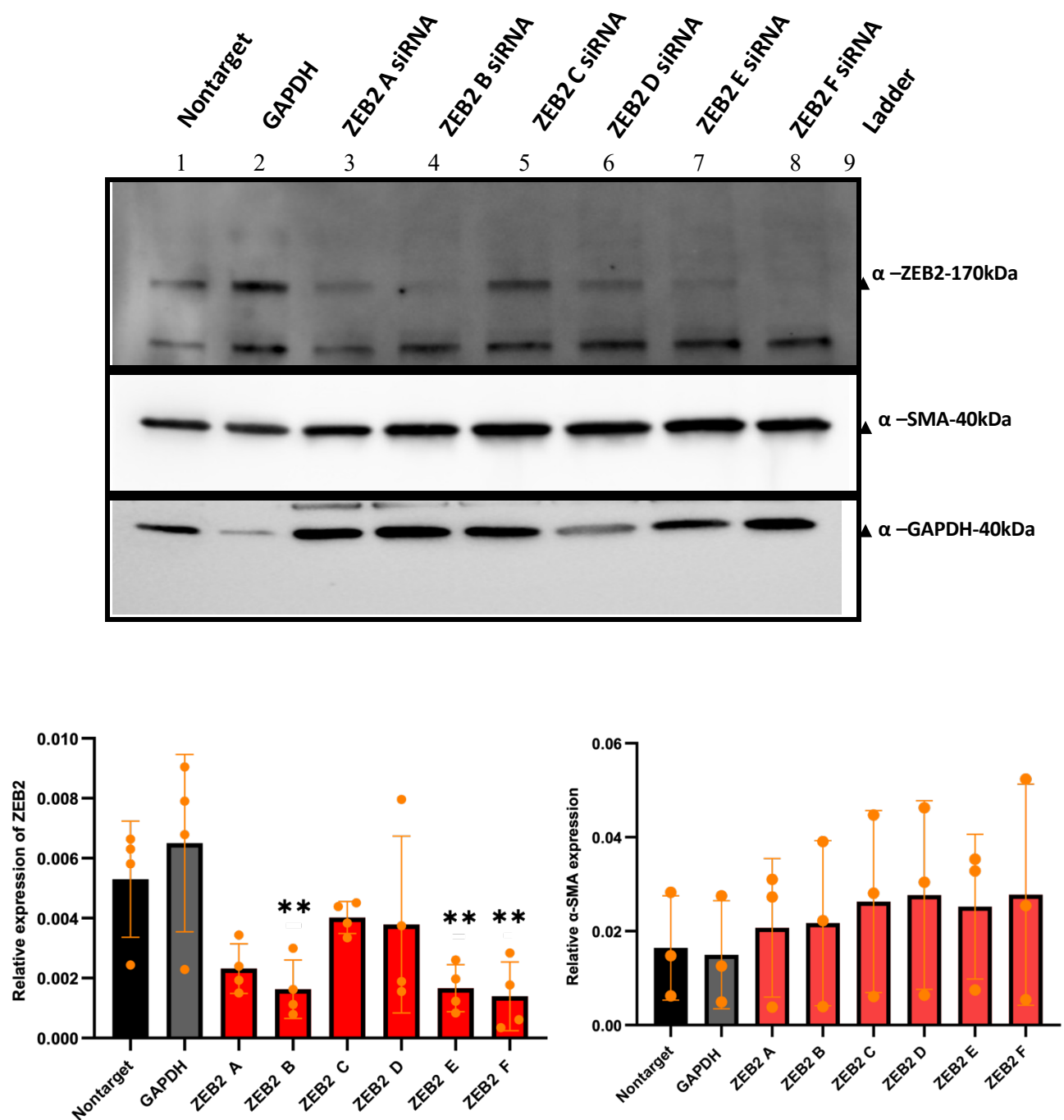


Figure 13: siRNA mediated knockdown of ZEB2 in NIH3T3 cells. Cells were transfected with ZEB2 A-F siRNAs. The cells treated with ZEB2 B, E and F siRNAs showed significantly reduced levels of ZEB2 after treatment. The myofibroblast marker α -SMA, however, showed no

significant change in expression following ZEB2 knockdown even in the ZEB2 F siRNA treated samples. GAPDH is used as the positive control for the knockdown procedure. The Mean + SD graph plotted relative to total protein (N=4) and statistical significance (**, $P < 0.002$) was determined relative to non-targeting control using 1-way ANOVA analysis followed by Dunnett's multiple comparison test.

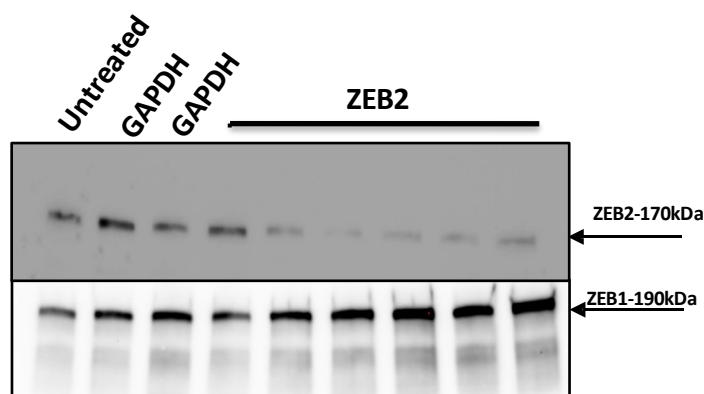


Figure 14: Preliminary Western blot analysis of ZEB1 regulation by ZEB2 in NIH3T3 cell line. After 48-hrs of transfection with ZEB2-specific siRNAs, NIH3T3 cells were harvested for analysis. The results demonstrate an increase in ZEB1 expression in all of the samples where ZEB2 was knocked down effectively, suggesting a potential regulatory role of ZEB2 in the modulation of ZEB1 levels.

4.5 ZEB1 Knockdown Increases ZEB2 Expression in NIH3T3 Cell Line

To explore the regulatory role of ZEB1 in fibroblast activation, we conducted knockdown analysis of ZEB1 in NIH3T3 cells. For this study, we employed a similar approach to ZEB2 knockdown, using four specific siRNAs targeting ZEB1 (named as ZEB1-A, B, C and D). The cells were transfected with ZEB1 siRNAs when they reached 60-80% confluency, followed by harvesting 48-hr post transfection. Western blot analysis was performed to assess the expression of ZEB1 and α -SMA. The knockdown efficiency was verified by using a ZEB1 specific antibody. The ZEB1 A, C and D siRNA treated samples show a 90% reduction in the expression of ZEB1 as compared to the control. In contrast, ZEB1 B siRNA did not reduce ZEB1 expression significantly (Figure 15). The α -SMA expression did not exhibit any significant change (Figure 15), similar to what was observed with ZEB2 knockdown. However, preliminary results suggest that there was an increase in ZEB2 expression in response to ZEB1 knockdown (Figure 16). This observation gives an insight into the presence of a potential negative feedback loop between ZEB1 and ZEB2.

In conclusion, both ZEB1 and ZEB2 single knockdown studies, did not show significant changes in α -SMA expression, indicating that neither loss of ZEB1 nor ZEB2 alone is sufficient to modulate myofibroblast differentiation in NIH3T3 cells. ZEB1 and ZEB2 may function in coordinated manner to regulate fibroblast activation and myofibroblast differentiation. The observed compensatory upregulation of ZEB1 upon ZEB2 knockdown and vice versa, further supports the interdependent relationship between these two transcription factors.

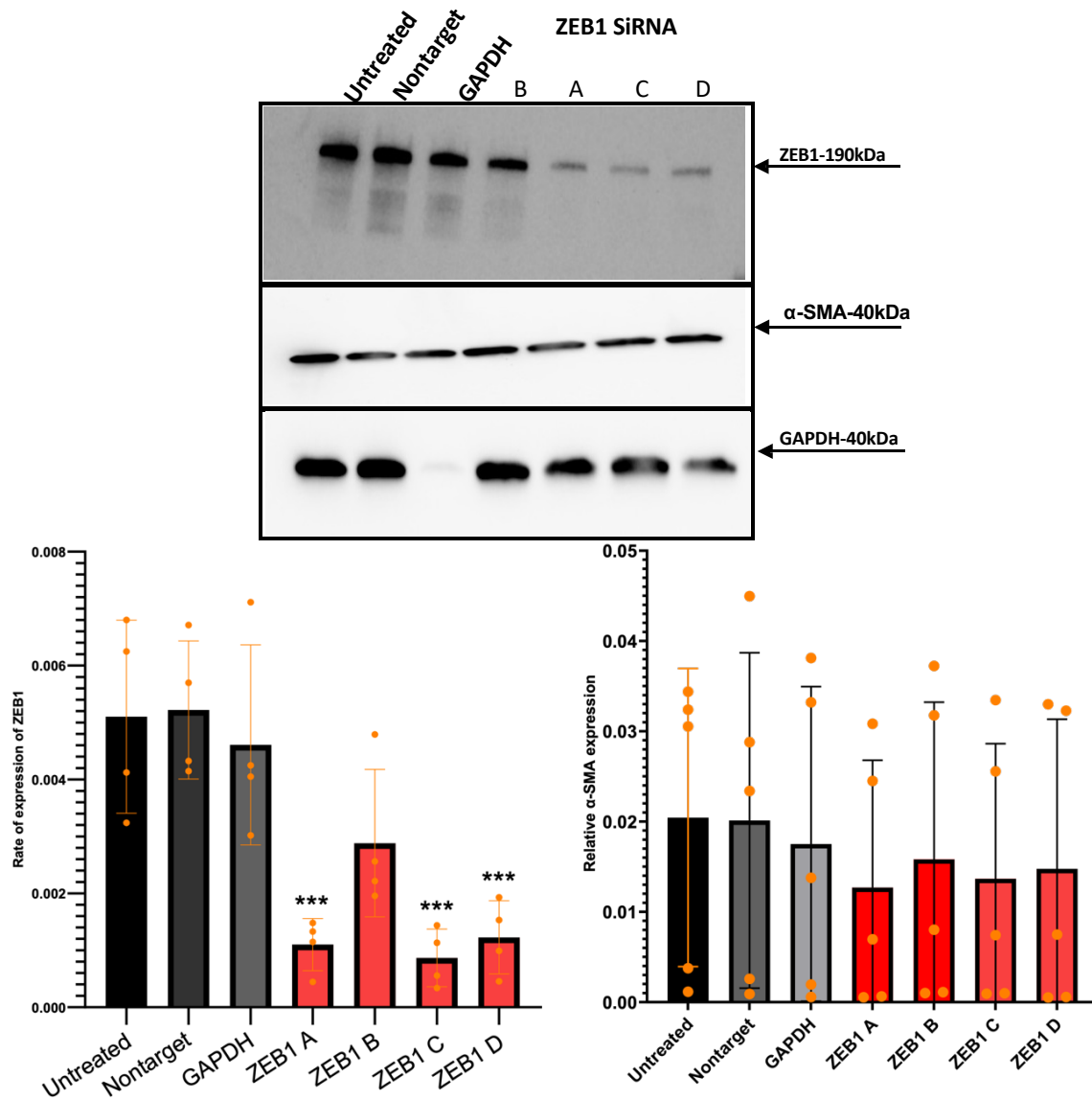


Figure 15: Western blot analysis of knockdown of ZEB1 in NIH3T3 cells. Cells were treated with ZEB1 A-D siRNAs. Transfection with ZEB1 A, C and D siRNAs significantly reduced ZEB1 levels. Levels of α -SMA, however, showed no significant change following ZEB1 knockdown. GAPDH is used as the positive control for the knockdown procedure. The Mean + SD graph plotted relative to total protein (N=4) and statistical significance (**, $P < 0.002$) was

determined relative to nontarget control using 1-way ANOVA analysis followed by Dunnett's multiple comparison test.

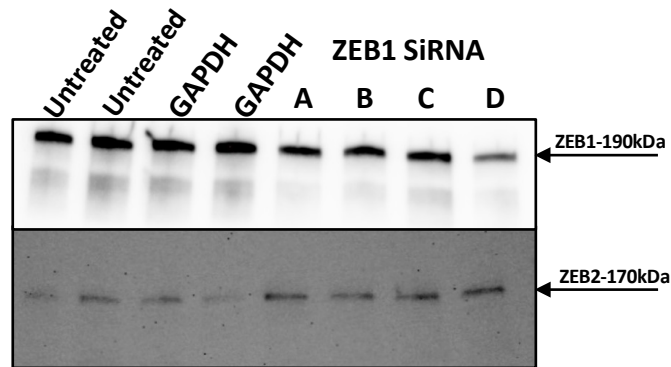


Figure 16: ZEB2 expression following ZEB1 knockdown in NIH3T3 cell line. After 48-hrs of transfection with ZEB1-specific siRNAs, NIH3T3 cells were harvested for analysis. The results demonstrate an increase in ZEB2 expression in all of the ZEB1 knockdown samples, suggesting the presence of a potential feedback loop between ZEB1 and ZEB2 during fibroblast activation.

4.6 Novel Confirmation of the ZEB1 - ZEB2 Direct Feedback Loop in NIH3T3 Cell Line

To confirm the compensatory relationship between ZEB1 and ZEB2, we repeated the single knockdown of ZEB1 and ZEB2, along with the double knockdown of both of these transcription factors. This approach was aimed to assess whether the combined loss of both transcription factors would alter the phenotype of the NIH3T3 cells. The NIH3T3 cells were transfected with non-targeting, GAPDH, ZEB1, ZEB2 and ZEB1+ZEB2 siRNAs. In the double knockdown treatment, an equal amount of both ZEB1 and ZEB2 specific siRNA were added, which was equal to the amounts used in the single knockdowns. The transfection was performed when the cells reached 60-80% confluency and the cells were harvested 48-hrs after transfection.

All transfections showed approximately 95% efficiency (Figure 17:1 A and 17.2 C). The western blotting results confirmed the existence of a negative feedback loop mechanism between ZEB1 and ZEB2. Specifically, ZEB1 expression was significantly elevated in the ZEB2 single knockdown samples, indicating the loss of ZEB2 leads to significant upregulation of ZEB1 (Figure 17.1 B). Similarly, ZEB2 expression was significantly increased in the ZEB1 knockdown samples (Figure 17.2 D), supporting the idea that ZEB1 is a negative regulator (or repressor) of ZEB2.

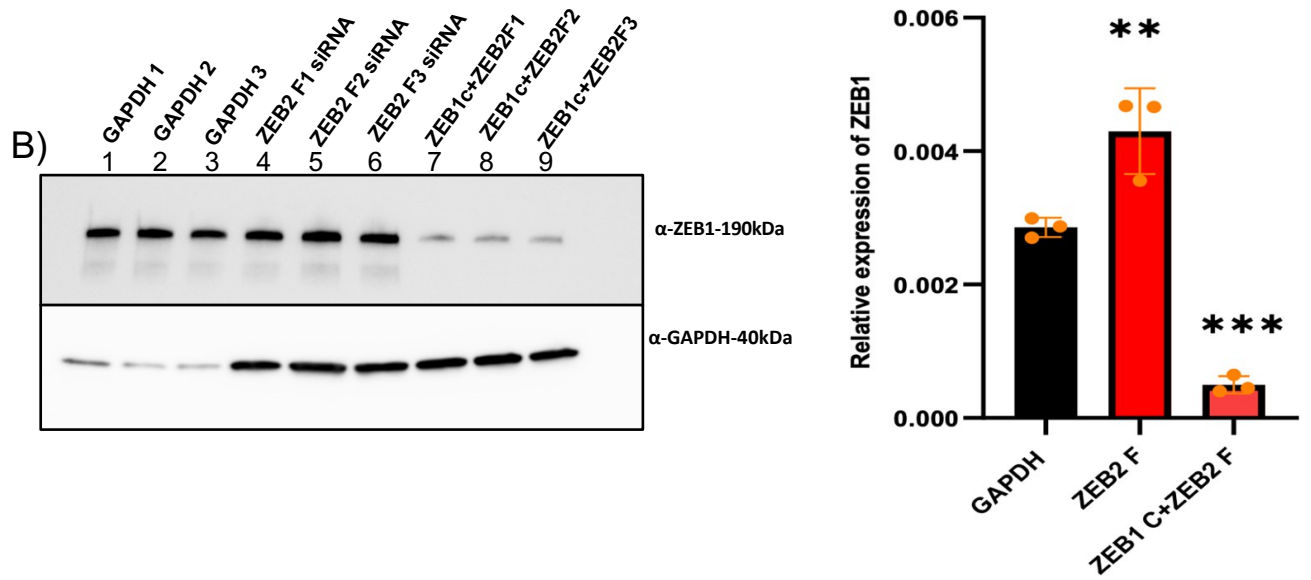
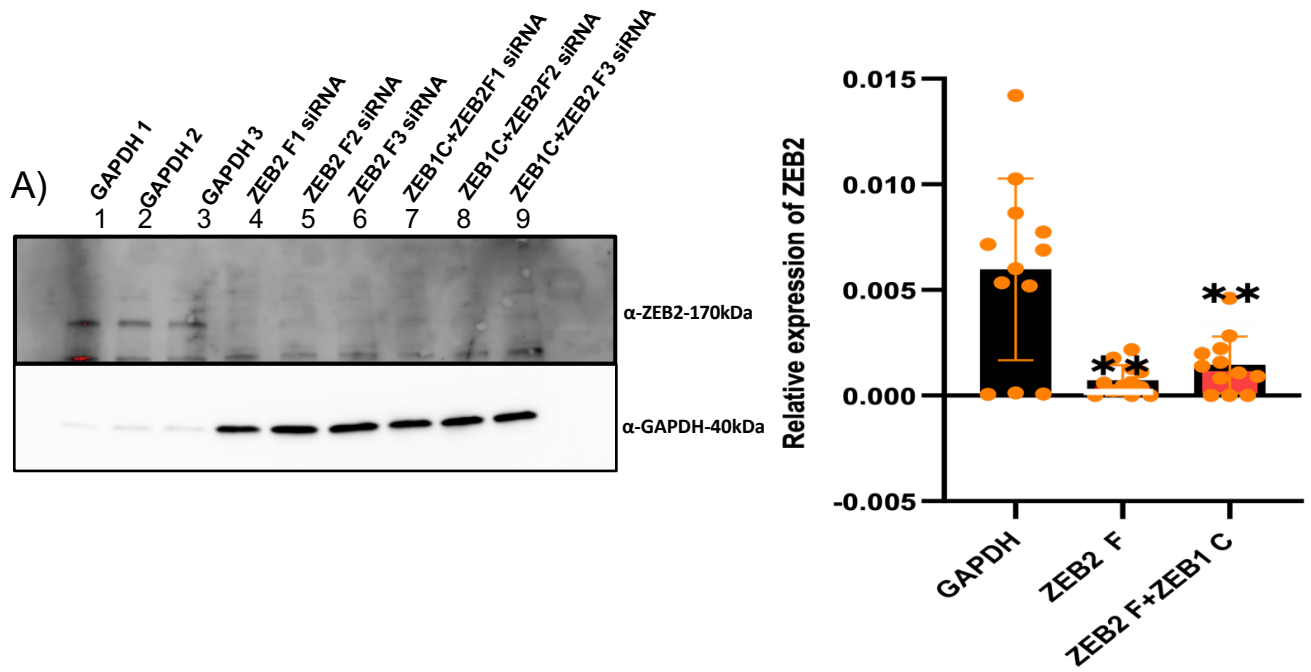


Figure 17.1: Loss of ZEB2 results in increased ZEB1 levels in NIH3T3 cells. Cells were transfected with ZEB2 F siRNA for single knockdown of ZEB2 and with a combination of ZEB2 F and ZEB1 C siRNAs for the double knockdown. Significant decrease in ZEB2 expression was seen in both the single and double knockdown samples. In contrast, the expression of ZEB1 increased in the ZEB2 single knockdown samples but was reduced in the double knockdown cells. GAPDH was used as the positive control, showing nearly complete reduction in the expression, indicating the high efficiency of the knockdown. The Mean + SD graph plotted relative to total protein (N=3) and statistical significance (**, $P \leq 0.002$) was determined relative to nontarget control using 1-way ANOVA analysis followed by Dunnett's multiple comparison test.

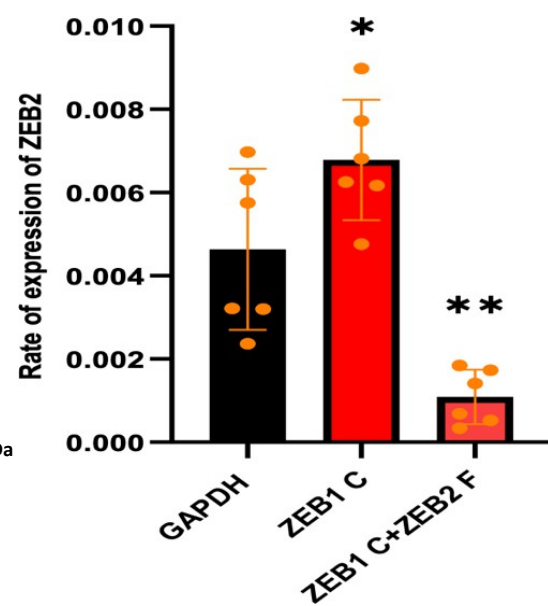
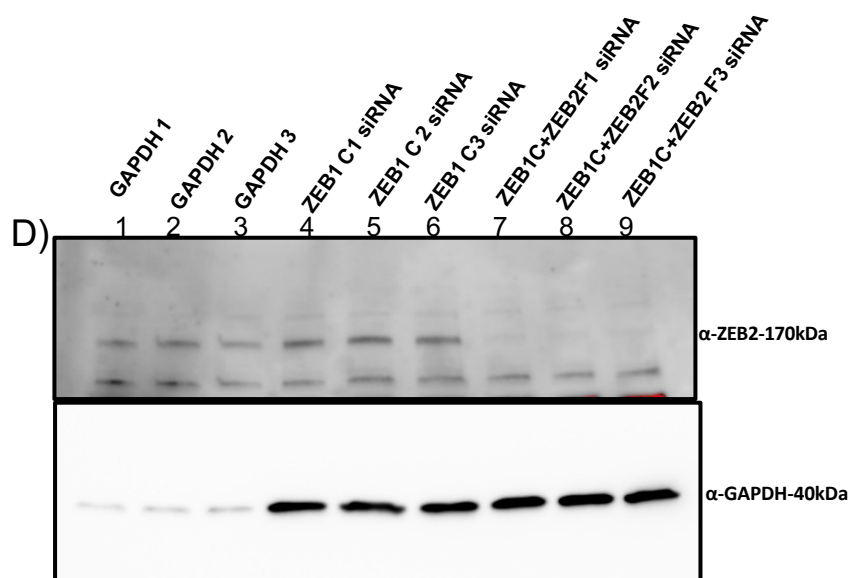
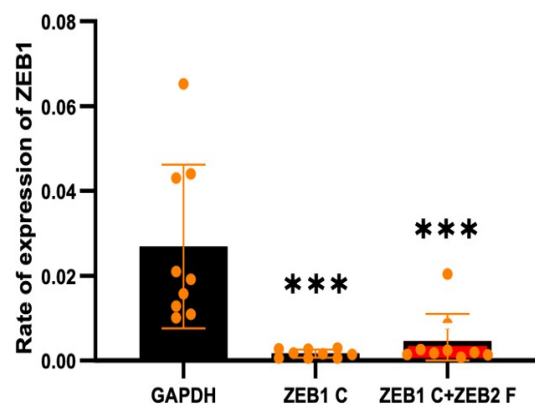
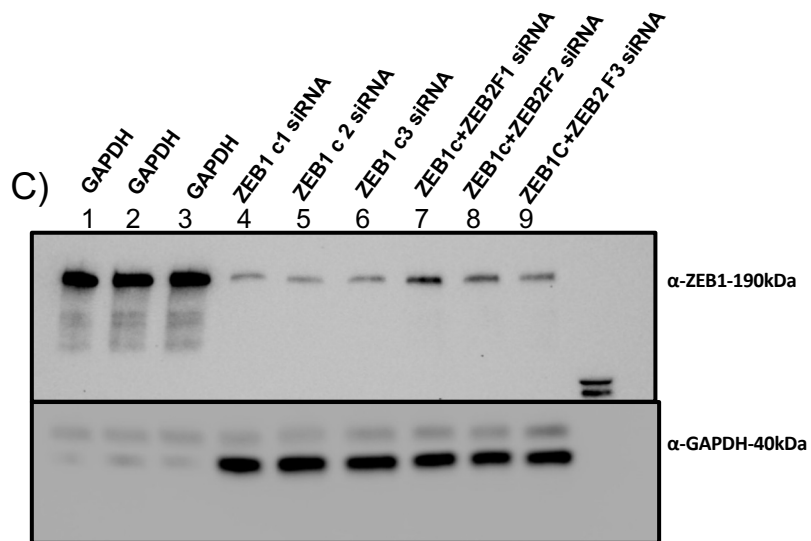


Figure: 17.2: Loss of ZEB1 results in increased ZEB2 expression in NIH3T3 cells. Cells were transfected with ZEB1 C siRNA for single knockdown of ZEB1 and with a combination of ZEB2 F and ZEB1 C siRNAs for the double knockdown. Significant decrease in ZEB1 expression was seen in both single and double knockdown samples. In contrast, the expression of ZEB2, increased in the ZEB1 single knockdown samples but was reduced in the double knockdown samples. GAPDH was used as the positive control, showing nearly complete reduction in the expression, indicating the high efficiency of the knockdown. The Mean + SD graph plotted relative to total protein (N=9) and statistical significance (**, $P < 0.002$) was determined relative to non-targeting control using 1-way ANOVA analysis followed by Dunnett's multiple comparison test.

4.6 Combined Knockdown of ZEB1 and ZEB2 Does Not Impact α -SMA Expression

The expression of α -SMA was further evaluated in ZEB1 and ZEB2 single knockdown and the combined knockdown samples. After 48-hrs of transfection with ZEB2 F and ZEB1 C siRNAs, NIH3T3 cells were harvested, and western blot analysis was performed to assess α -SMA levels. The results showed that despite the efficient double knockdown of ZEB1 and ZEB2, α -SMA expression was not significantly changed (Figure 18).

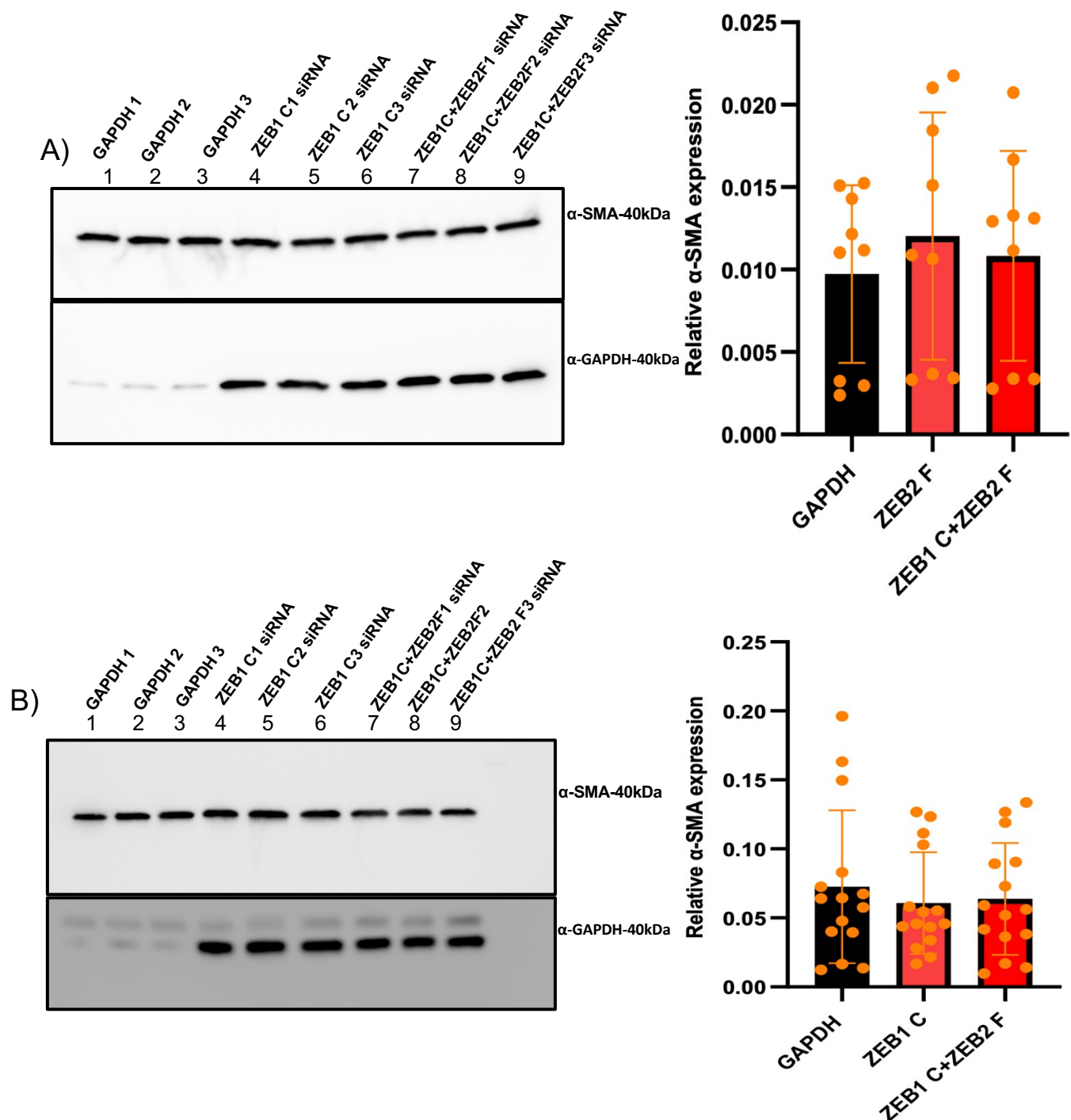


Figure 18: α -SMA expression was not altered by combined ZEB1 and ZEB2 knockdown.

Expression of α -SMA was investigated in the ZEB1 C, ZEB2 F single knockdown and ZEB1 C+

ZEB2 F double knockdown samples. Both single and double knockdowns of ZEB1 and ZEB2 had no significant effect on α -SMA expression. (A) ZEB1 knockdown, B) ZEB2 knockdown). The Mean + SD graph plotted relative to total protein (N=15) and statistical significance was analysed relative to nontarget control using 1-way ANOVA analysis followed by Dunnett's multiple comparison test. The result shows no significant change.

4.7 The Immunohistochemistry Analysis Shows Decreased Expression of α -SMA in the Single and Double Knockdown Samples

To further evaluate the relationship between ZEB1, ZEB2 and α -SMA at the cellular level, we performed immunostaining analysis. The cells were fixed with paraformaldehyde after 48-hrs of transfection with ZEB1 and ZEB2 siRNAs. Immunohistochemistry analyses were conducted using two different stains, DAPI for nuclear staining and fluorescent primary and secondary antibodies for α -SMA. The results show a trend towards a reduction in α -SMA expression following ZEB1 and ZEB2 single knockdown and double knockdown, with the highest reduction was seen in the double knockdown (Figure: 19).

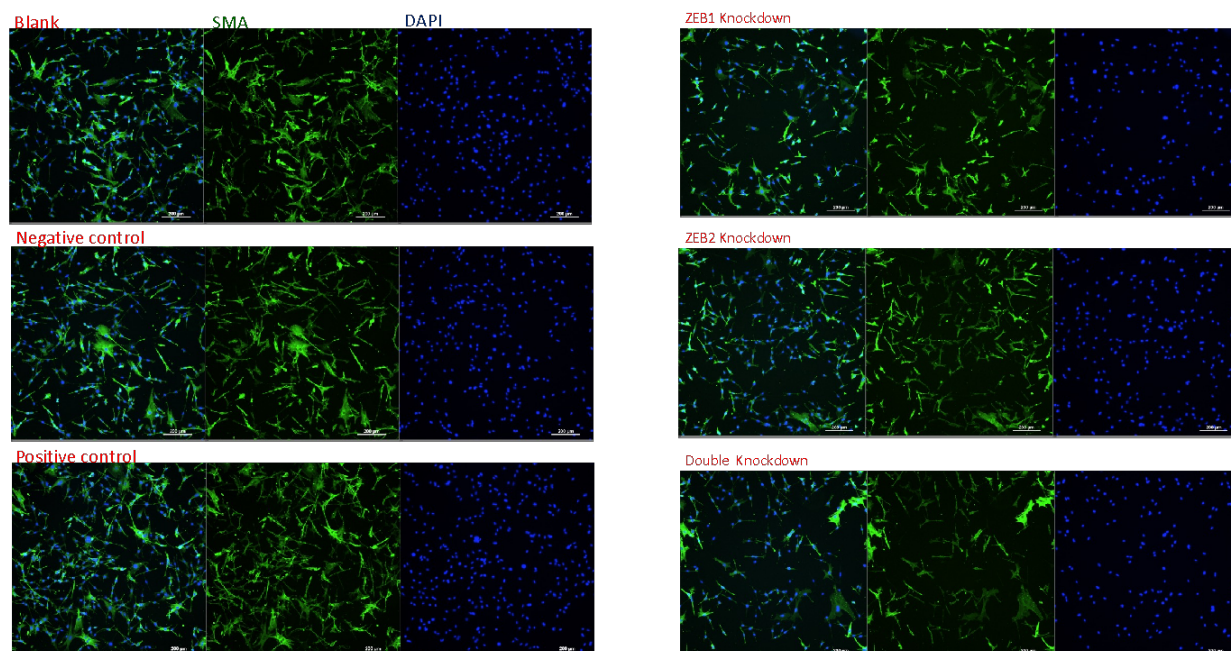


Figure 19: Immunohistochemistry analysis of α -SMA in ZEB1 and ZEB2 single and double knocked down NIH3T3 samples. ZEB2 and ZEB1 were knocked down with ZEB2 F and ZEB1 C siRNAs and the double knock down was performed by knocking down both ZEB1 and ZEB2 in the same well. GAPDH siRNA treated cells are the positive control. Compared to the three controls, ZEB1, ZEB2 and double knockdown samples showed an apparent decrease in the expression of α -SMA.

Chapter V: Discussion

In this study, we have shown for the first time that ZEB1 and ZEB2 form a negative feedback loop to maintain their expression level in fibroblasts. We have shown the expression of both ZEB1 and ZEB2 increased during fibroblast activation in male and female PRCFs, with the peak expression occurring at 48-hr time point. The siRNA mediated ZEB2 knockdown resulted in the increased expression of ZEB1 in both PRCFs and in NIH3T3 cells. Ectopic expression of ZEB2 in the NIH3T3 cell line decreased the endogenous expression ZEB1. Similarly, ZEB1 siRNA mediated knockdown in NIH3T3 cells, resulted in the increased expression of ZEB2, confirming the presence of a negative feedback loop between these transcription factors.

Previous studies in our lab have shown that the transcription factor ZEB2 is expressed in rat myofibroblasts and the overexpression of ZEB2 increased the expression levels of three myofibroblast markers α -SMA, SMemb and ED-A fibronectin (Jahan et al., 2018). However, the knockdown of ZEB2 for 48 hours did not impact expression of these myofibroblast markers (Jahan et al., 2018). Therefore, in this study we analysed the roles of both ZEB1 and ZEB2 in cardiac fibroblast activation and the effect of knocking down both ZEB1 and ZEB2.

We examined the expression of ZEB1 and ZEB2 protein during fibroblast activation. We harvested PRCFs at three different time points, (24-hr, 48-hr, and 96-hr), western blotting analysis was performed. We first validated the specificity of the ZEB2 polyclonal antibody, by western blot analysis using samples from NIH3T3 cells transfected with pcDNA3.1 HA-ZEB2

and pcDNA3.1-empty vectors. The ZEB2 antibody detected a protein at 171 KDa molecular weight, which matches the size detected by the HA antibody. Further, we observed that the anti-ZEB2 antibody detected additional lower molecular weight bands as well. siRNA mediated knockdown of ZEB2 using specific siRNAs, did not decrease the expression of these lower molecular weight bands, supporting their non-specific nature. Similar to ZEB2, the ZEB1 antibody detected a protein at the molecular weight observed in the literature but it also detected lower molecular weight bands. However, in this case the siRNA mediated knockdown of ZEB1 showed a correspondingly decreased in the intensity of the lower bands, confirming that this band is specific for ZEB1. The possible sources for the presence of a specific lower molecular weight band for ZEB1 include ZEB1 protein degradation, alternative splicing of ZEB1 mRNA, or post- translational modifications, which could include in the formation of a truncated protein.

The analysis of both ZEB1 and ZEB2 expression during the activation of cardiac fibroblast showed that these transcription factors were upregulated when fibroblasts are activated, reaching their highest level of expression at 48-hrs and then decreasing at 96-hrs in both male and female PRCFs. This temporal expression pattern suggests that the involvement of these two transcription factors is more prominent in the early stages of fibroblast activation, potentially facilitating the transition from a quiescent to an active fibroblast state. The similar pattern of expression of ZEB1 and ZEB2 indicate that these transcription factors may have an overlapping role in the regulation of cardiac fibroblast activation. However, there is significant difference in their expression in each time point, which indicates their distinct roles in fibroblast activation and differentiation. ZEB2 expression was nearly absent at the 24-hr time point in both male and female PRCFs. In contrast, the ZEB1 protein was present at all time points with highest expression at 48-hrs. Interestingly, while ZEB2 expression was almost absent in the 96-hr male PRCF, in the female

cells a significant amount of ZEB2 was detectable at 96-hrs. The observed decrease in the expression of these transcription factors at the 96-hr time point, and the dramatic increase and sustained high levels of α -SMA at the 96-hr time point suggest that the role of ZEB1 and ZEB2 is more critical in the early stages of cardiac fibroblast activation and differentiation to myofibroblast, and not in the maintenance of the mature myofibroblast phenotype. Therefore, the downregulation of ZEB1 and ZEB2 may thus indicate a transition from the initiation of fibroblast activation to a more mature myofibroblast phenotype, with other regulatory pathways supporting the fully activated myofibroblasts. Since ZEB1 and ZEB2 are present in the initial phase of fibroblast activation, their expression may be necessary for the proper activation of myofibroblast. Thus, targeting these transcription factors in fibroblasts, could block the myofibroblast activation. This strategy can be used to prevent excessive activation of the myofibroblast, which leads to fibrosis.

In our investigation of the roles of ZEB1 and ZEB2 in fibroblast activation, the siRNA mediated knockdown of ZEB2 in both PRCF and NIH3T3 cells resulted in a compensatory increase in ZEB1 levels. This finding suggests that ZEB2 may function as a negative regulator of ZEB1 during fibroblast activation. This finding was further confirmed by ectopic expression of ZEB2 in the NIH3T3 cells, which resulted in the downregulation of endogenous ZEB1 expression levels. This reciprocal regulation may function to maintain an optimal level ZEB1 and ZEB2 during fibroblast activation and myofibroblast differentiation. This finding also suggests a potential redundancy between ZEB1 and ZEB2 during fibroblast activation. Reciprocal relationship between ZEB1 and ZEB2 is an indication that these transcription factors have overlapping functions in the fibroblast activation, where downregulation of one protein may

trigger the upregulation of the other to maintain an optimum level of expression during the activation of myofibroblasts.

To evaluate whether this regulation is bidirectional, we also performed siRNA mediated ZEB1 knockdown in NIH3T3 cells. This study showed an increase in the expression levels of ZEB2 protein upon ZEB1 knockdown, confirming the presence of a negative feedback between these two transcription factors. This mechanism is essential to prevent either the overexpression or the under expression of either ZEB transcription factor. Potentially the dysregulation of ZEB1/ZEB2 expression could impair normal cardiac fibroblast activation.

Additionally, these findings emphasize that it is possible that ZEB1 and ZEB2 modulation may be important for the later stages of cardiac fibroblast activation as well, including myofibroblast maturation and function. Fibroblast cells may control the development and resolution of fibrosis by modulating the levels of ZEB1 and ZEB2, indicating these transcription factors may act as a potential therapeutic target for fibrotic diseases like heart failure. Therefore, further studies focusing how ZEB1 and ZEB2 interact during fibroblast activation and how their expression levels influence fibroblast behaviour, could provide new insight into the regulatory mechanism underlying fibrosis and other cardiac diseases.

During the course of our knockdown studies, several challenges emerged. Upon returning from my maternity leave, we observed a significant decline in the knockdown efficiency of ZEB1 and ZEB2 as compared to our initial studies. We also observed that the siRNA mediated GAPDH not resulting in an efficient knockdown as well. This decreased transfection efficiency resulted in

being unable to observe the negative feedback loop. This prompted a comprehensive review of our experimental protocols, cell culturing techniques and conditions.

The recurrent bacterial and fungal infections were addressed by implementing strict sterile techniques. To ensure the consistency of the cell confluency across all wells, used a hemocytometer to count cells before plating them for transfection. To further optimize the conditions, the cells were seeded, grown, and transfected in antibiotic free media, as antibiotics can interfere with transfection efficiency. Also, we revised our media change protocol. Initially we replaced the transfection mixtures containing media with antibiotic containing DMEM media 5-hrs post-transfection. However, in the modified approach, we left the transfection media until harvesting at 48-hrs. Finally, we switched to diluting the siRNAs and lipofectamine in Opti-MEM reduced serum medium rather than in DMEM medium. These changes successfully improved the consistency and efficiency of our knockdown results.

Despite using four different siRNAs for ZEB2 from Dharmacon, I was unable to achieve a significant knockdown, in contrast to my previous results. In response, we purchased two additional ZEB2 siRNAs from Invitrogen, but the results remained the same; we did not observe any knockdown after transfection using six siRNAs for ZEB2, as confirmed by Western blotting. To troubleshoot further, we decided to change the antibody we were using, switching from ProteinTech to the original antibody from Sigma. Initially, we were using the Sigma ZEB2 antibody in my first studies, which was validated for specificity. However, due to supplier availability, we had to switch to a different antibody from ProteinTech. Upon returning to the Sigma antibody, we observed significant knockdown in the samples treated with three of the

ZEB2 siRNAs. We discovered that the previous antibody was detecting a nonspecific band at the expected molecular weight of ZEB2.

With the ability to efficiently knockdown both ZEB2 and ZEB1 and detect the knockdown restored, we were able to validate the negative feedback loop that had been evident in the initial phase of my project. However, there is still great variability between Sigma ZEB2 antibodies with different reference numbers. Therefore, for my final studies, I opted to use a ZEB2 antibody from Thermo Fisher, which showed more consistent and reliable results.

Further, our western analysis revealed, there was not a significant change in α -SMA expression following both single and double knockdowns of ZEB1 and ZEB2 at 48-hrs as well as at the 72-hr time point. In contrast, the immunostaining results showed a trend towards a reduction in α -SMA expression following ZEB1 and ZEB2 single knockdown and double knockdown, with highest reduction in the double knockdown. Despite this trend, there was a great variability in the immunostaining results. The images revealed that while some cells exhibited a drastic reduction in α -SMA expression, others maintained high levels of the protein. This variability may either be due to incomplete knockdown in some cells, which could explain the lack of significant decrease in total α -SMA observed in the western blot analysis, as western blotting only provides an aggregate measurement of protein levels across the entire cell population. Another reason could be that factors, other than ZEB1 and ZEB2, control α -SMA expression during fibroblast activation.

While previous studies have shown that ZEB2 overexpression can increase the myofibroblast markers expression including α -SMA, our findings suggest that the regulation of α -SMA in fibroblast activation may not be dependent on ZEB1 and ZEB2 function. There might be other signaling pathways such as TGF- β , SMADs also participate in the regulation of this marker. Further studies including alternative regulatory mechanisms, different time points or different model organisms could help to better understand the role of ZEB1 and ZEB2 in the regulation of α -SMA during fibroblast activation. Finally, our study explored the impact of ZEB1 and ZEB2 in fibroblast activation. The long-term goal of this project is to identify potential therapeutic strategies for improving post-MI wound healing. The final goal of our studies is to discover a small molecular drug that specifically target ZEB1 and ZEB2 in the cardiac cells after proliferative phase of post-MI repair by using advanced delivery systems like nanoparticles to minimize systemic exposure and off-target effects.

Chapter VI: Conclusions

Cardiac fibrosis is an irreversible complication of most cardiovascular diseases including MI, which can eventually lead to heart failure. Even after the advancements in cardiovascular research and clinical care, specific treatment options for cardiac fibrosis and heart failure are limited. All current medicines available in the market, such as nutraceuticals, beta blockers, ACE inhibitors, thrombolytics, only treat the symptoms or risk factors that lead to fibrosis and heart failure, rather than focusing the underlying fibrotic process itself. Activation of the cardiac fibroblast is a critical phase in the post-MI wound healing process, and excessive cardiac fibroblast activation will lead to defective cardiac remodeling and fibrosis. This study reveals that both ZEB1 and ZEB2 expression increases during the initial stages of cardiac fibroblast activation, and then decreases after the conversion of fibroblast to myofibroblast, indicating both these transcription factors may have important roles in the initiation of fibroblast activation, and regulation of the post-MI wound healing process. We have shown for the first time that both ZEB1 and ZEB2 form a negative feedback loop, during fibroblast activation in the NIH3T3 cell line. This mechanism may regulate the myofibroblast differentiation, wound healing, and fibrosis. Western blotting results showed that α -SMA expression did not significantly change after ZEB1 and ZEB2 single knockdown or ZEB1 and ZEB2 combined double knockdown in NIH3T3 cells. These results demonstrate ZEB1 and ZEB2 are not be the only factors regulating α -SMA, there might be other signaling pathways also involved in this regulation during myofibroblasts activation.

Findings from this study, advance our current knowledge about the complex mechanism of fibroblast activation into myofibroblast and the interplay of ZEB1 and ZEB2 during this

activation process. Overall, this study enhances our understanding of cardiac fibroblast biology and give insights into the regulatory mechanisms that coordinate the fibroblast activation and open new strategies for therapeutics, targeting ZEB1 and ZEB2 negative feedback loop, there by modulating fibroblast activation and preventing excessive fibrosis.

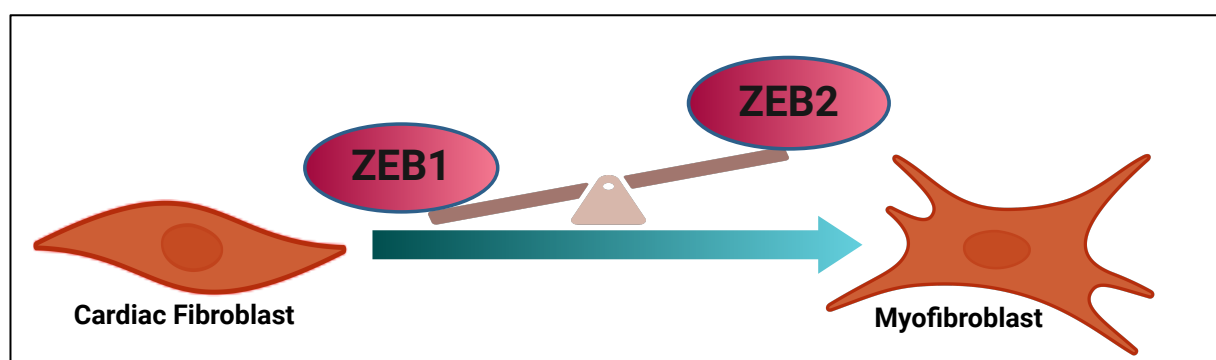


Figure 20: ZEB1-ZEB2 negative feedback loop during cardiac fibroblast activation. ZEB1 and ZEB2 may establish a negative feedback loop to regulate their expression levels during cardiac fibroblast activation, mutually compensating each other's loss. This reciprocal mechanism helps for the proper regulation of fibroblast activation and thereby controlling fibrosis. (Created in <https://BioRender.com>)

Chapter VII: Limitations and Future Directions

This study provides new insight into the complex regulatory mechanism between ZEB1 and ZEB2 during fibroblast activation and differentiation. However, the limitations of this study need to be mentioned. Even though the NIH3T3 cell line is a well-used model for fibroblast studies, the fibroblast behaviour varies between species and between tissues as well. Therefore, further validation of the negative feedback loop between ZEB1 and ZEB2 in primary rat cardiac fibroblasts or in human cardiac fibroblast needs to be performed to strengthen this conclusion. This study relied on performing in vitro studies of primary cells and a cell line. Therefore, future studies focused on cell specific knockout of ZEB1 and ZEB2 in the MI model mouse system could give more specific and clear mechanism of regulation between ZEB1/ZEB2 and fibroblast activation. The transfected cells were harvested at only 2 timepoints, 48 hrs and 72 hrs, which may reflect the full dynamic nature of fibroblast activation and differentiation. A more detailed knockdown analysis of the ZEB1 and ZEB2 in different time points, such as 24-hrs, 48-hrs, 72-hrs, and 96-hrs may give a better picture of its role in regulating myofibroblast markers not only α -SMA but also SMemb, ED-A fibronectin and Postn.

Analyzing the changes in the functional properties of myofibroblasts, such as proliferation, migration, contractility, and apoptosis upon ZEB1 and ZEB2 knockdown can also improve our understanding of the roles of these transcription factors in fibroblast activation. While our study, focused on the protein- level effect of ZEB1 and ZEB2, exploring their impacts at the transcriptional level would provide further insight into their regulatory roles in fibroblast activation. Also, further investigation of the upstream and downstream regulators of ZEB1 and

ZEB2 is crucial for identifying the mechanism of crosstalk between ZEB1/ZEB2 with other transcription factors and proteins during the course of activation of fibroblast to myofibroblast.

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