Action of SNAIL1 Protein is Critical for Fibrosis

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by

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# Table of Contents

List of Figures .......................................................................................................................... iii
List of Tables ............................................................................................................................. v
Acknowledgements ................................................................................................................ vi
Abstract of the Dissertation .................................................................................................. viii
Chapter 1: Introduction ....................................................................................................... 1
Chapter 2: Role of SNAIL1 in cardiac fibrosis ................................................................. 16
  Introduction ....................................................................................................................... 17
  Results .............................................................................................................................. 20
  Discussion ....................................................................................................................... 28
  Figures ............................................................................................................................. 32
Chapter 3: SNAIL1 reporter and knockout mouse models ............................................ 74
  Introduction ................................................................................................................... 75
  Results ........................................................................................................................... 78
  Discussion ..................................................................................................................... 87
  Figures ........................................................................................................................... 90
Chapter 4: Conclusions and Future Directions ............................................................... 116
Chapter 5: Materials and Methods ................................................................................ 124
Chapter 6: References ...................................................................................................... 133
Chapter 7: Appendix .......................................................................................................... 149
List of Figures

Figure 2.1: SNAIL1-CBR signal after myocardial infarction by Left Anterior Descending Artery Occlusion ............................................................................................................................................32

Figure 2.2: SNAIL1-CBR signal after myocardial infarction by Ischemia-Reperfusion ..........34

Figure 2.3: Fibrosis gene expression after cardiac injury ..................................................................................................................36

Figure 2.4: SNAIL1-CBR signal in Angiotensin II induced fibrosis model .................38

Figure 2.5: Immunofluorescence staining of SNAIL1 and cardiac cell type markers in the ventricular infarct zone of LAD surgery mice .................................................................40

Figure 2.6: Immunofluorescence staining of SNAIL1 and cardiac cell type markers in the remote (non-infarct zone) of LAD surgery mice ................................................................................44

Figure 2.7: Verification of cardiac fibroblasts isolated from uninjured SNAIL1-CBR hearts .....45

Figure 2.8: Pro-fibrotic factors increase SNAIL1-CBR level in primary cardiac fibroblasts .....47

Figure 2.9: Verification of SNAIL1 deletion in Snail1f/f cardiac fibroblasts ..................49

Figure 2.10: Effect of SNAIL1 on proliferation and migration during wound healing .......51

Figure 2.11: Characterization of immortalized Snail1f/f; Rosa LSL tdTomaro cardiac fibroblasts ........................................................................................................................................53

Figure 2.12: SNAIL1 effect on αSMA expression in cardiac fibroblasts .......................55

Figure 2.13: Snail1 deletion effects pro-fibrotic cytokines and collagen remodeling proteins ....57

Figure 2.14: Effect of Snail1 knockout on extracellular matrix gene expression in cardiac fibroblasts .......................................................................................................................................59

Figure 2.15: Effect of Snail1 deletion on collagen deposition by cardiac fibroblasts .........61

Figure 2.16: Effect of collagen on Snail1 stability .............................................................63

Figure 2.17: DDR2 knockour effect on SNAIL1 stability in cardiac fibroblasts plated on 3D collagen I matrix ..........................................................65

Figure 2.18: miR29 binding site analysis – predictive analysis ........................................67

Figure 2.19: Possible mechanisms of action of SNAIL1 in cardiac fibrosis .................72
Figure 3.1: Creating Snail1-LacZ reporter mouse model
Figure 3.2: Snail1-IRES-YFP reporter mouse model
Figure 3.3: SNAIL1-CBR reporter model
Figure 3.4: SNAIL1 overexpression in cardiomyocytes in-vivo
Figure 3.5: Col1α1-Cre-ERT² characterization in the heart
Figure 3.6: Effect of SNAIL1 knockout on cardiac fibrosis post myocardial infarction by LAD ligation in Snail1²f/f; Col1α1CreERT²; Rosa LSL tdTomato mice
Figure 3.7: CAG-Cre-ER characterization in the heart
Figure 3.8: RosaCreERT² characterization in the heart
Figure 3.9: αSMACre characterization in the heart
Figure 3.10: FSP1-Cre characterization in the heart
Figure 3.11: Effect of SNAIL1 knockout on cardiac fibrosis post myocardial infarction by LAD ligation
Figure 3.12: Characterization of FSP+ cells in the heart 7 days after LAD ligation
List of Tables

Table 2.1: SNAIL1 expression in the cardiac cell types in the infarcted zone………………..42
Table 2.2: Promoter analysis of mir29 family………………………………………………...69
Table 2.3: Promoter analysis of collagen I and III……………………………………….70
Table 2.4: 3’UTR region of collagen I and III…………………………………………....71
Table 3.1: SNAIL1 reporter mice…………………………………………………………...98
Table 3.2: Characterization of Cre strains to target cardiac fibroblasts…………………..115
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Hypoxic injury to the heart causes cardiac fibrosis leading to cardiac dysfunction and heart failure. SNAIL1 is a zinc finger transcription factor which has been implicated in fibrosis following organ injury. To investigate the role of SNAIL1 in cardiac fibrosis, we used an endogenous SNAIL1 bioluminescence reporter mice, and SNAIL1 knockout mouse models. Here we report that SNAIL1 is expressed in the infarcted myocardium, especially in the myofibroblasts. Utilizing cardiac fibroblasts in-vitro we demonstrate that pro-fibrotic factors and collagen increases SNAIL1 in cardiac fibroblasts. By knocking out SNAIL1 in cardiac fibroblasts in-vitro, we demonstrate that SNAIL1 is required for adoption of myofibroblast fate, collagen 1 expression and fibrotic genes expression. Taken together the data suggests that SNAIL1 expression is induced in the cardiac fibroblasts after injury which causes adoption of a myofibroblast phenotype and a fibrotic scar formation. The collagen deposition in the scar can maintain the elevated SNAIL1 expression in the myofibroblasts and help to propagate fibrosis.
Chapter 1 - Introduction
Significance

Cardiovascular diseases including hypertension, myocardial infarction, heart failure and congenital heart defects affect over 82 million Americans\(^1\). Heart failure is an end stage condition of almost all cardiovascular diseases and annually about 0.7 million people are diagnosed with heart failure in America\(^2\). Cardiovascular diseases and cancer are the two leading causes of death in the western world and cardiovascular diseases account for one out of three deaths in the United States, claiming more lives than cancer, lower respiratory diseases and accidents, making it the leading causes of death in the Unites States\(^1\). The annual cost of cardiovascular diseases in the United States in 2015 is estimated to be $565 billion and is expected to increase by 100% in the next 15 years to about 1.1 trillion in 2030\(^2\). The high economic burden can be attributed to high disease prevalence, costly therapies and hospital stay. About 69% of the treatment cost is attributed to hospitalizations and 18% for drugs\(^3\). Cardiovascular diseases represent a huge economic burden on the healthcare system. Therefore, a better understanding of the mechanisms of cardiovascular diseases development and progression will lead to better diagnostics, drugs, reduced hospitalizations and ultimately reduce morbidity and mortality.

Fibrosis – Wound healing gone awry

Injury and wounding to a healthy organ results in healing or repair of the injury. It is the body’s natural response to injury with the goal of repairing and restoring the function of the injured organ. The wound healing process requires an orchestrated interplay between various cell types like epithelial cells, fibroblasts, and immune cells as well as structural proteins of the
extracellular matrix and a multitude of cytokines like Transforming Growth Factorβ, Platelet derived growth factor etc. Wound healing begins immediately after injury and is a dynamic process that involves many distinct but overlapping phases. Immediately after the injury, a fibrin clot forms at the wound to prevent blood loss and creates a temporary matrix for the cells involved in wound healing to anchor and/or pass to the injury site. The fibrin clot contains important signaling molecules like TGFβ, PDGF etc. which are important for recruiting and activating cells which lead to wound healing. After the wound is stabilized, the inflammatory phase begins. Macrophages, neutrophils and lymphocytes migrate into the injury site. The major function of the neutrophils is to protect against bacterial infection and clear cellular debris.

Tissue macrophages clear the expanded neutrophils and damaged tissue thereby controlling and limiting the extent of inflammation. The proliferation and migration phase occurs next. This involves proliferation and activation of fibroblasts, keratinocytes and the induction of angiogenesis. T-lymphocytes and bone marrow derived cells can also migrate to the wound site. Following this, the reparative and remodeling phase follows. Granulation tissue is created at the injured site. To do so fibroblasts present at the wound are activated and become αSMA expressing myofibroblasts. Myofibroblasts generate the extracellular matrix at sites of repair (i.e., granulation tissue), remodel the ECM, and promote wound contraction, a process in which the wound edges move towards the center. Next, re-epithelialization occurs as cells move over the granulation tissue and close the wound.

During the wound healing process, the acute inflammation and wound healing mechanism can become chronic which can lead to excessive accumulation of ECM components and fibrotic scar formation. Fibrosis occurs when the rate of ECM deposition by the myofibroblasts exceeds the rate of ECM degradation and turnover. The fibrotic ECM mainly
comprises of collagens I and III and is non-contractile. Persistent fibrosis leads to interference of the normal tissue functions, which over time can lead to organ failure\textsuperscript{11}.

**Cardiac fibrosis**

Cardiac injury leads to extensive repair and also structural and functional remodeling. This can include cardiomyocyte hypertrophy and extracellular matrix deposition, i.e. cardiac fibrosis\textsuperscript{12}. Cardiac fibrosis results in both systolic and diastolic dysfunction of the heart and also causes increased arrhythmias due to decreased coupling of fibroblasts and myofibroblasts\textsuperscript{13}. The increased arrhythmias are correlated with sudden cardiac death\textsuperscript{14}. A study found that 3\% increase in fibrosis tissue is associated with 50\% increase in adverse cardiac event risks\textsuperscript{15}.

Myocardial fibrosis is classified into two types; reactive fibrosis and replacement fibrosis\textsuperscript{16}. Reactive fibrosis is similar to fibrosis occurring in other organs. In the heart, reactive fibrosis causes extracellular matrix deposition in the perivascular spaces and cardiac interstitium. Cardiomyocyte hypertrophy occurs in reactive fibrosis but there is no loss of cardiomyocytes. Pressure overload, renin-angiotensin II-aldosterone signaling, endothelin can cause reactive fibrosis\textsuperscript{17}. In replacement fibrosis, the extracellular matrix deposition occurs at the site of cardiomyocyte loss. For example, during myocardial infarction, a blockage in an artery supplying blood to the heart causes hypoxia in the region supplied by the artery due to loss of blood flow. This leads to cardiomyocyte death that is replaced by scar tissue comprising mainly of collagens.

Wound healing following myocardial infarction leads to the three distinct but overlapping phases of wound healing i.e. inflammatory phase, proliferative phase and maturation phase\textsuperscript{18}. In
the mouse heart, the inflammatory phase occurs between 3-72 hours post injury and is characterized by an influx of leukocytes. Between 2-7 days, the immune response begins to subside and fibrous tissue is deposited and neo-vascularization in the scar takes place. From day 7-21 days post infarction, the more matrix is laid down and the fibroblasts begin to clear leaving behind the fibrotic scar\textsuperscript{18}.

The cardiac fibroblasts are the major player in the cardiac fibrosis process\textsuperscript{19}. In the normal, uninjured heart, cardiac fibroblasts have distinct functions like maintaining the extracellular matrix homeostasis, production of growth factors and cytokines, homeostasis of cardiac blood vessels and cardio electrophysiology by coupling myocytes and propagating electrical signals\textsuperscript{20}. In response to hypoxic injury, the cardiac fibroblasts accumulate in the infarcted myocardium, become activated and adopt a myofibroblast phenotype. The myofibroblasts are characterized by the expression of alpha smooth muscle actin (\(\alpha\text{SMA}\)) and thus are contractile cells. The myofibroblasts are activated by pro-fibrotic cytokines in the infarct area and are responsible for secreting the extracellular matrix and the ECM remodeling enzymes such as growth factors and proteases. In the heart, the markers for cardiac fibroblasts are the collagen receptor Discoidin domain receptor 2 (DDR2), fibroblast specific protein 1 (FSP1) and the intermediate filament vimentin and are distinguished from myofibroblasts by the expression of \(\alpha\text{SMA}\) and periostin\textsuperscript{19,20}. However, these distinctions may not be accurate as the fibroblasts / myofibroblasts might not be a static population of cells. Their heterogeneity might be a result of their different origins and also the surrounding environment.

The origin of myofibroblasts in the infarct zone is an area of active investigation. Since the cardiac fibroblasts are the major cell type in the heart by numbers and even outnumber cardiomyocytes and are sensitive to circulating pro-fibrotic factors, thus making them the likely
source of the myofibroblasts in the injured sites\textsuperscript{21,22}. However, there are other sources of myofibroblasts in the infarcted area. It is estimated that ~30\% of the myofibroblasts arise from the endothelial cells by a process called endothelial to mesenchymal transition (EndMT)\textsuperscript{23}. The perivascular cells, surrounding blood vessels, have been shown to give rise to collagen producing myofibroblasts in dermal scarring and kidney fibrosis\textsuperscript{24,25}. However, this has not been demonstrated in the heart due to lack of a pericyte specific lineage tracing tool in the heart\textsuperscript{26}. Bone marrow derived progenitor cells and circulating fibrocytes have been shown to be present in the infarcted myocardium and can give rise to 25-60\% of the fibroblasts\textsuperscript{27}. However, this population declines during later scar maturation phases\textsuperscript{28,29}. Thus it would be fair to assume that the cardiac fibroblasts in the infarcted myocardium are derived from multiple sources but whether they are functionally similar, in addition to being phenotypically similar remains to be seen.

The main structural proteins in the fibrotic scar secreted by the myofibroblasts are fibrillar collagens i.e. collagens I and III. In the mature scar about 85\% of the collagen is collagen I and about 11\% collagen III. Collagen I is associated with thick fibers which are more tensile and are resistant to stretch and deformation. Collagen III is associated with thin fibers which confer more resilience. In the infarct zone, collagen III is first synthesized. As the scar matures, the collagen III is replaced by collagen I, thereby increasing the collagen I/III ratio and increased resistance to distension with more tensile strength\textsuperscript{30}. Periostin is another matricellular protein that is secreted by the cardiac myofibroblasts in the infarct zone. Periostin has been suggested to regulate collagen fibrillogenesis by allowing collagen cross linking \textsuperscript{31,32} and also serves as a ligand for avb3 and avb5 integrins\textsuperscript{33}. 
In addition to secreting the ECM components, the myofibroblasts actively remodel the ECM. Myofibroblasts secrete lysyl oxidases, various matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP’s) during cardiac fibrosis. In patients with myocardial infarction MMP2, MMP9, TIMP1, TNFα and IL6 were elevated as compared to healthy controls. In mice, deletion of MMP2 and MMP9 have a better outcome, measured as less death due to cardiac (left ventricle) rupture and reduced LV dilation and dysfunction.

**TGFβ – multipotent pro-fibrotic cytokine**

In response to cardiac injury, a variety of cytokines, chemokines and mitogens are secreted in the infarcted area. These include transforming growth factor β, platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), fibroblast growth factor family (FGF’s), interleukins 1, 6, 8 and many others. These signaling molecules act through different receptors and signal transduction pathways, although many of these signaling pathways share some convergence distally (e.g. activation of MAP kinases). All of these factors act on the cardiac fibroblasts and can have multiple and overlapping effects, such as activation of fibroblast migration, proliferation, collagen synthesis, matrix degradation etc. In addition to these secreted molecules, the physical or mechanical properties of the extracellular matrix can also have pro-fibrotic signals. Matrix stiffness is associated with wound healing and fibrosis. For example, cardiac fibroblasts secrete collagen III and these fibers might be regulated by mechanical stretching. Collagens, periostin, fibronectin and other ECM components can also activate the cardiac fibroblasts, through the action of the collagen receptor Discoidin domain receptor (DDR2) or integrin activation and signaling.
TGFβ is a multifunctional cytokine and central mediator in cardiac fibrosis and remodeling. There is a marked increase in TGFβ production and activation following myocardial infarction. The metalloproteinases MMP2 and MMP9, which are secreted by cardiac fibroblasts, can convert latent TGFβ present in the myocardium to active TGFβ in the ECM\textsuperscript{40, 41, 42}. Additionally, Thrombospondin I and avb6 integrin signaling can activate TGFβ\textsuperscript{43,44}. Active TGFβ binds to its receptors which are type I and type II transmembrane serine-threonine kinase receptors. Binding of TGFβ activates receptors through transphosphorylation of the receptors. The active receptors now phosphorylate various downstream signaling effector molecules like SMAD2/3 (activating SMAD) or SMAD6/7 (inhibitory SMAD’s) and thus affect a variety of cellular signaling cascades depending on the context\textsuperscript{42}.

TGFβ induction has shown to enhance ECM deposition by mesenchymal cells by expressing ECM genes and suppressing genes involved in ECM degradation (for example: MMP’s)\textsuperscript{45,46}. The importance of TGFβ in wound healing and fibrosis is demonstrated by the severely impaired wound healing and collagen deposition in mice deficient in TGFβ\textsuperscript{1,47, 48}. The induction of collagen by TGFβ is an immediate-early effect, under the control of SMAD3 transcription factors\textsuperscript{46}. Fibroblasts deficient in Smad3 gene fail to upregulate type I collagen and CTGF in response to TGFβ. Interestingly, fibronectin gene induction is independent of SMAD but requires JNK MAP kinase cascade\textsuperscript{49}. SMADs 3 and 4 have been shown to bind to the Col1a2 promoter at the SMAD recognition motif CAGACA directly\textsuperscript{50}. Col1a1 promoter lacks SMAD recognition element, but SMAD2 forms a complex with Sp1 and binds to the Col1a1 promoter at the -162 to -142 regions and activates Col1a1 gene transcription\textsuperscript{51,52}. The induction of collagen I in mesangial cells with TGFβ treatment required RAS/MEK/ERK MAP kinase cascade but not
p38 MAP kinase cascade. However, in dermal fibroblasts, p38 MAP kinase cascade, but not RAS/MEK/ERK MAP kinase cascade is required to upregulate collagen I gene transcription. CTGF gene induction by TGFβ stimulation requires the RAS/MEK/ERK MAP kinase cascade.

In addition to induction of pro-fibrotic gene expression, TGFβ signaling has effects on cardiac fibrosis. TGFβ can be chemotactic for neutrophils and monocytes contributing to the migration of these cells into the infarcted region. Depending on the cytokine milieu, TGFβ can prevent migration of neutrophils across the endothelial cells. These different roles for TGFβ could explain the initial migration of neutrophils and leukocytes in the infarct area, which then subsides as the scar matures. TGFβ also can stimulate fibroblasts, including cardiac fibroblasts, into αSMA expressing myofibroblasts. Signaling through the Rho > Rock > MRTF A/B > SRF pathway by TGFβ stimulation is important for expression of αSMA in the myofibroblasts. In colonic myofibroblasts, it has been shown that Rho kinase is required for TGFβ induced αSMA and collagen expression\textsuperscript{53–55}.

MicroRNA 29 family has been shown to affect fibrosis in multiple organs, including heart, kidney and liver. TGFβ has been shown to suppress miRNA 29 during cardiac, renal fibrosis and hepatic fibrosis and this suppression is mediated by SMAD3\textsuperscript{56,57}. miRNA 29 has multiple functions during fibrosis. In the heart fibroblasts, renal tubular epithelial cells and hepatocytes, miR29 has been shown to downregulate expression of fibrosis genes like collagens I and III\textsuperscript{58}. In addition, TGFβ stimulation of myoblasts represses miR29 through the binding of SMAD3/YinYang1/EzH2 complex at the SBE/CAAT domain in the promoter if mir29\textsuperscript{59}. Interestingly, there is a Ebox sequence, just upstream of the SBE/CAAT domain\textsuperscript{59}. Inhibition of miR-29 expression by TGF-beta-Smad3 signaling could happen via SNAIL1 binding to this
Ebox sequence and its interaction with the SMAD3 complex at the SBE/CAAT domain. Thus repression of miR29 after stimulation of TGFβ results in expression of collagen genes in response to TGFβ signaling.

**SNAIL1 and its role in fibrosis**

Epithelial to Mesenchymal transition (EMT) is a morphogenetic cell transformation process where epithelial cells lose their polarity, epithelial gene expression and start expressing mesenchymal genes and become motile. Such EMT processes is necessary for normal development i.e. gastrulation and also in pathologies i.e. cancer cell motility. EMT can be triggered by a variety of signaling molecules and pathways, including TGFβ, all of which converge on a set of transcription factors called EMT inducers. The transcription factor SNAIL1 has been shown to be the master regulator of EMT gene expression. SNAIL1 induced EMT is critical for embryogenesis as SNAIL1 knockout mice fail to undergo gastrulation. During the cardiac development TGFβ induced EMT has been shown to be important for invasion into the cardiac jelly via SNAIL1 repression of VE-Cadherin.

Snail1 is a member of the Snail superfamily of zinc finger transcription repressors. The Snail superfamily is highly conserved from insects to mammals and plays a critical role in morphogenesis, especially mesoderm formation during gastrulation. The C-terminal region of the Snail family transcription factors contain four to six highly conserved zinc finger domains of C2H2 type. The zinc finger domains recognize and bind E-box sequences (CAGGTG). SNAIL1 repression activity is due to the SNAG domain in the amino terminal region via interaction of SNAIL1 with the co-repressor CtBP, polycomb complex 2, SIN3A/HDAC complex and Ajuba.
During the induction of EMT, SNAIL1 represses epithelial genes such as E-Cadherin, desmoplakin, cytokeratin 18, in addition to directly or indirectly upregulating mesenchymal genes such as vimentin and fibronectin. SNAIL1 has also been shown to actively modulate gene transcription by modulating heterochromatin regulation by interaction with LoxL2. Although, SNAIL1 is classically studied as a transcriptional repressor, certain modifications, such as acetylation of SNAIL1, causes it to function as a transcriptional activator. Acetylated SNAIL1 induced cytokine gene activation influences macrophage recruitment by tumor cells.

SNAIL1 is highly regulated as aberrant activation or upregulation of SNAIL1 might have unwanted consequences. For example, SNAIL1 has been observed in the nuclei of cells transitional cells, which are myofibroblasts derived from epithelial origin, in the fistulae from Crohns disease patients, suggesting a role for EMT in the etiology of Crohns disease. SNAIL1 is regulated at multiple levels including transcription, translation, post-transcriptional modification and protein stability. Stimulation with TGFβ can induce SNAIL1 gene transcription and once SNAIL1 is produced, it represses its own expression by binding to Ebox sequences in this own promoter. This transient upregulation of SNAIL1 is sufficient for induction of EMT in MCF10A mammary epithelial cells. Furthermore, at the transcriptional level, SNAIL1 is regulated by the action of microRNA’s during the induction of EMT. miRNA-34 and miRNA-30 have been shown to repress SNAIL1 by binding to 3’UTR of SNAIL1. Once Snail1 mRNA has been transcribed, SNAIL1 is phosphorylated at its Nuclear Localization Signal (NLS) (amino acids 132-143), is imported to the nucleus by the binding of CRM1 transporter. SNAIL1 is a highly unstable protein with a half-life of 25 minutes. The GSK3β can phosphorylate SNAIL1 at nuclear export site which triggers its translocation from nucleus to cytoplasm, followed by another phosphorylation by GSK3β in the nucleus. This dual phosphorylation targets SNAIL1
for degradation by the ubiquitin-proteasome pathway\textsuperscript{72,73}. Other E3 ligases like FBXL14 can target SNAIL1 for degradation and in response to hypoxia FBXL14 is down-regulated which ultimately increases SNAIL1 levels\textsuperscript{74}. Another E3 ligase FBXL5 can polyubiquitinate SNAIL1 affecting its stability and DNA binding capacity\textsuperscript{75}.

In addition to being involved in EMT events during normal development, SNAIL1 is also implicated in wound healing, pathological conditions such as fibrosis and cancer. SNAIL1 upregulation in mesenchymal cells (fibroblasts) has been shown to induce cell invasion and migration and disrupting SNAIL1 prevents fibroblasts from migrating into a wound site\textsuperscript{76}. SNAIL1 has been shown to be upregulated during fibrosis following organ injury. Using knockout mouse models, SNAIL1 has been shown to be important in hepatocytes for liver fibrosis following injury\textsuperscript{77}. In a kidney injury model, SNAIL1 induced endothelial to mesenchymal transition (EndMT) was shown to contribute to fibrosis\textsuperscript{78,79}. Recently, SNAIL1 expression was detected in cardiac fibroblasts and cardiac endothelial cells following ischemia reperfusion injury to the heart\textsuperscript{80}. SNAIL1 in endothelial cells was necessary for secretion of connective tissue growth factor that caused neighboring fibroblasts to adopt a myofibroblast phenotype\textsuperscript{81}. In the pancreas, SNAIL1 co-operates with KrasG12D to promote fibrosis via activation of stellate cells and increased phosphorylation of Smad2 and TGFβ\textsuperscript{82}.

Expression of SNAIL1 has been linked to a variety of cancers including breast cancer, bladder cancer, cervical cancer, colorectal cancer, pancreatic cancer etc. SNAIL1 expression in epithelial tumors induces EMT, invasion, migration and has been associated with cancer stemness and resistance to chemotherapy\textsuperscript{83,84}. High SNAIL1 expression in tumors often correlates with poor differentiation, increased migration, invasion and metastasis and prognosis\textsuperscript{84}. The cancer microenvironment is highly fibrotic and stiff due to excess ECM deposition and as
such might be tumor promoting via mechanical signals\textsuperscript{37,85–87}. Increasing evidence suggests that SNAIL1 expression in the cancer micro-environment can support metastasis. SNAIL1 expression in cancer associated fibroblasts (CAF’s) can increase extracellular matrix stiffness and anisotropic fiber alignment which allows cancer cells to migrate and invade\textsuperscript{88}. SNAIL1 expression has been observed in tumor stroma in multiple human cancers. For example, in tumors from colorectal cancer patients, SNAIL1 expression co-related with fibroblast markers like aSMA and fibroblast activated protein (FAP)\textsuperscript{89}. SNAIL1 expression in colon cancer CAF’s can be used as a prognostic marker\textsuperscript{90}.

**Fibrosis therapies**

Fibrosis following injury can occur in any organs and thus collectively, fibrosis is one of the largest group of diseases\textsuperscript{17}. Once established, fibrosis is hard to treat as removing the deposited scar tissue is not trivial. The main approach to treat fibrosis has been to suppress the fibrotic reaction, thus to prevent further ECM deposition. In the case of cardiac fibrosis following a trans-mural myocardial injury (infarction), the formation of a scar is necessary to close the wound created by dying cardiomyocytes. A failure of scar formation in the infarct area might lead to cardiac rupture. However, since the scar matures and becomes fibrotic, the therapies aimed to prevent cardiac fibrosis need to be directed toward making a ‘better scar’, i.e. have enough scar formation to prevent rupture at the infarcted area but prevent the excessive ECM deposition and preserve heart function.

Although fibrosis is a widespread disease, there aren't many approved therapies to prevent or treat fibrosis. There have been several approaches under investigation to prevent
excessive collagen deposition in fibrosis. As such, the signaling molecules and pathways involved in fibrosis like collagen synthesis, deposition and cross linking pathways, chemokines that recruit immune cells, conversion and recruitment of myofibroblasts, represent attractive targets to prevent fibrosis. Small molecule drugs like Pirfenidone which targets signaling molecules involved in fibrosis (TGFβ, TNF and IL10) and the multi-tyrosine kinase inhibitors like Nintedanib have been approved to treat idiopathic pulmonary fibrosis (IPF) and investigation is underway for treatment of these drugs for other fibrosis diseases. Some other examples of small molecule drugs that have been tested as anti-fibrotic agents are Losartan (AT1 receptor antagonist), Atrasentan (ETA receptor antagonist) and Rosiglitazone (oral). Several of these small molecule compounds are in advanced stages of clinical trials (phase III).

Interestingly, a majority of the molecule agents have been tested for pulmonary, skin, liver and kidney fibrosis. The only two small molecule agents tested in cardiac fibrosis in pre-clinical models are Distertide (P144) - a TGFβ inhibiting peptide and MMI-0100 - MK2 peptide inhibitor.

In addition to small molecule inhibition of fibrosis, there have been several biological inhibitors of fibrosis tested. For example, GC1008, a humanized antibody against TGFβ1, 2 and 3, and LY2382770, a humanized antibody against TGFβ1 are in clinical trials for treatment of fibrosis diseases. STX-100 an a6β6 integrin blocking antibody is being tested for idiopathic pulmonary fibrosis and GS-6624, a non-competitive allosteric antibody to LoxL2 is also being tested as an anti-fibrotic agent. Several microRNA’s that are involved in the fibrogenic pathway are under investigation as potential therapeutics by some companies like miRagen and Regulus Therapeutics. Recently, gene therapy approaches have been investigated to repair mutations in CFTR genes as therapy for cystic fibrosis. Another novel approach to treat cardiac fibrosis is to
genetically convert cardiac fibroblasts to cardiomyocytes. Using retroviral vectors expressing a defined set of transcription factors and genetic lineage tracing methods, cardiac fibroblasts were shown to be able to re-program into cardiomyocytes after myocardial infarction in mice\textsuperscript{93}. 
Chapter 2: Role of SNAIL1 in cardiac fibrosis
Introduction

Hypoxic (ischemic) injury to cells and organs such as occurs to the heart during myocardial infarction due to artery occlusion result in a wound healing process. Hypoxia causes death of cardiac cells and as a result the release of inflammatory mediators and subsequent inflammation in the injured region. This inflammatory response sets up a wound healing cascade which ultimately deposits fibrous scar tissue in the infarcted region. The scar consists of deposited extracellular matrix (ECM), and is the attempt to prevent rupture of the dead cardiac tissue. However, when the scarring reaction is persistent this can lead to excess deposition of extracellular matrix (ECM) and cardiac fibrosis, which leads to stiffening of the heart, reduced cardiac output (ventricle dysfunction), and heart failure. Therefore, understanding of the mechanisms for and regulation of cardiac fibrosis following injury could result in development of anti-fibrotic therapies to improve quality of life after myocardial infarction.

In the heart fibroblasts are the major cell type responsible for generating the fibrotic reaction after ischemic injury. In term of numbers, cardiac fibroblasts make up the majority of the cells in the heart, even outnumbering the cardiomyocytes. In response to ischemic injury, the number of cardiac fibroblasts at the injury site increase and become activated to myofibroblasts. The increased number of myofibroblasts in the scar area are derived from various sources that include: proliferation of resident cardiac fibroblasts, Endothelial to Mesenchymal Transition of endothelial cells, circulating fibrocytes, pericytes and inflammatory cells and bone marrow derived mesenchymal progenitor cells. These activated fibroblasts, or myofibroblasts, express the markers alpha smooth muscle actin (αSMA) and periostin and secrete pro-fibrotic and pro-inflammatory cytokines as well as ECM proteins and ECM
modifying enzymes in the scar area. The deposited ECM is mainly comprised of fibrillar collagens (collagens I and III). Periostin, secreted by myofibroblasts, regulates the alignment of the collagen I fibrils while secreted enzymes that cross-link collagen fibers (e.g., lysyl oxidases) can increase tissue stiffness.

A number of transcriptional regulators have been implicated as contributing to fibroblast to myofibroblast phenotypic switch. Their expression is activated by inflammatory cytokines released after hypoxic injury. For example, the serum response factors (SRF), Myocardin Related Transcription Factor A/B (MRTFA/B). Another critical mesenchymal cell transcriptional regulator is SNAIL1, and SNAIL1 has been shown to be important for organ fibrosis that develops following liver and kidney injury, as well as the fibrosis associated with some cancers. Recent studies have suggested that SNAIL1 expression in the heart is activated following ischemia. Increased SNAIL1 levels in endothelial cells following ischemia reperfusion cardiac injury causes secretion of connective tissue growth factor (CTGF) by endothelial cells, which in turn activates neighboring cardiac fibroblasts to form myofibroblasts. Whether SNAIL1 is actually necessary for cardiac fibrosis following ischemic injury, and if so in what cells in the heart and how, is unknown.

SNAIL1 is a critical mesenchymal cell fate regulator and a zinc finger transcriptional repressor, although a recent study suggests that under certain conditions and in the presence of specific co-factors it can act as a transcriptional activator. SNAIL1 protein level and function is regulated at the transcriptional and post-transcriptional levels by various chemical and mechanical extracellular stimuli that can be present in the hypoxia-injured heart. These include: TGFβ, PDGF, hypoxia and increased ECM stiffness. Using a SNAIL1 reporter mouse and immunohistochemical analyses we find that SNAIL1 levels are increased in heart fibroblasts.
following hypoxic injury. Through genetic manipulation of SNAIL1 expression in primary heart fibroblast cells \textit{ex vivo} we find that SNAIL1 is critical for the formation and function of myofibroblasts and their fibrotic response following TGF\(\beta\) stimulation.
Results

**SNAIL1 expression in the heart increases post myocardial infarction**

To determine whether SNAIL1 expression in the heart was induced in response to ischemic cardiac injury, we made use of a SNAIL1-Click Beetle Red (CBR) fusion bioluminescence reporter mouse (Figure 2.1A). In this mouse the CBR bioluminescent enzyme was inserted into the SNAIL1 gene, in frame, downstream of the third (terminal) exon of SNAIL1 so as to generate a SNAIL1-CBR fusion allele. Through this design, SNAIL1-CBR expression is under regulation of the endogenous SNAIL1 promoter.

Heterozygote SNAIL1-CBR/+ mice did not express SNAIL1-CBR in the uninjured normal heart (Figure 3.3C). To induce hypoxic cardiac injury, SNAIL1-CBR/+ mice were subjected to Left Anterior Descending (LAD) Artery ligation. Hearts were harvested and analyzed 7-days post-surgery. In sham treated mice there was minimal SNAIL1-CBR bioluminescence detected, but in SNAIL1-CBR/+ mice subjected to LAD ligation these was a 10-fold increase in bioluminescence signal (Figure 2.1C, quantified in 2.1D). SNAIL1-CBR signal was restricted to the ventricular infarct area (Figure 2.1C). SNAIL1 mRNA levels also increased in the hearts of mice exposed to hypoxic injury, as determined by Q-PCR of tissue from the ventricular region of infarcted and sham surgery control hearts (Figure 2.1E). Like the LAD ligated hearts, SNAIL1-CBR signal increased in hearts injured by ischemia reperfusion surgery, albeit to a lesser level than LAD surgery heart (Figure 2.2A, quantified in Figure 2.2B).

Fibrogenic gene expression (e.g., collagen I and III, CTGF, IL6, TGFβ and periostin) increased in the infarct region of LAD ligated hearts (Figure 2.3A) and IR hearts (Figure 2.3B). The increased SNAIL1-CBR levels were co-related with increased fibrosis as determined by
Masson’s trichrome staining and decreased ejection fraction of the heart, determined by echocardiogram (Figure 2.3 C and D).

In sum, following ischemic injury to the heart SNAIL1 mRNA and protein level increased in the infarct zone.

Myofibroblasts within the infarct zone express SNAIL1

To identify which cells within the infarct zone expressed SNAIL1, we performed co-immunostaining with different cardiac cell type markers and CD45 (infiltrating leukocytes). SNAIL1 staining was observed only in infarct zone and not the non-injured, remote zone (Figure 2.5 and 2.6). A significant number of αSMA (40%) and periostin (31%) positive myofibroblasts in the infract zone expressed SNAIL1. A small proportion (~10%) of CD45 positive leukocytes expressed SNAIL1 (Table 2.1). SNAIL1 expression was not detected in α-actinin positive cardiomyocytes within the infarct zone (Figure 2.5E). Contrary to previous reports we did not observe SNAIL1 staining in CD31 positive cells within the infarct zone. In sum, these results indicated that following cardiac ischemic injury the increase in SNAIL1 expression within the infarct zone was predominantly in activated myofibroblasts.

Pro-fibrotic factors increase SNAIL1 expression in cardiac fibroblasts.

When activated, such as following exposure to hypoxia, cardiac myofibroblasts secrete a variety of pro-fibrotic factors. To determine whether these pro-fibrotic factors influenced SNAIL1 expression in cardiac fibroblasts/myofibroblasts, we isolated primary cardiac fibroblasts...
from normal, uninfarcted SNAIL1-CBR/+ mice. Their fibroblastic origin was confirmed by intermediate filament vimentin staining and Discoidin Domain Receptor 2 (DDR2) western blot (Figure 2.7A and B). Since SNAIL1 expression is induced simply by exposing fibroblasts to serum, cardiac fibroblasts were first starved of serum for 4 hours prior to treating with pro-fibrotic factors. TGFβ, PDGF, CoCl₂ (hypoxia mimetic agent) and angiotensin II all increased SNAIL1-CBR bioluminescence signal (i.e., increased SNAIL1 protein level) (Figure 2.8A - D). In combination these factors did not exhibit any additive effect on SNAIL1-CBR bioluminescence value. (Figure 2.8E). The effect of most of these pro-fibrogenic factors upon SNAIL1 level was at the protein level, as only TGFβ treatment increased SNAIL1 mRNA (Figure 2.8F).

**SNAIL1 knockout cell line creation and verification**

To study the function of SNAIL1 in cardiac fibroblasts, we created a cell line to easily delete SNAIL1. To accomplish this, we isolated primary cardiac fibroblast from normal SNAIL1^{f/f}; ROSA-LSL-tdTomato mice. To delete SNAIL1, the cells were infected with either Adeno-LacZ (CTL) or Adeno-Cre (+ Cre) virus for 4 hours and then cultured. SNAIL1 deletion was verified by Q-PCR for SNAIL1 mRNA (Figure 2.9A) and western blot (Figure 2.9B). Infection with Adeno-Cre virus turned on the Rosa-LSL-tdTomato reporter (Figure 2.9C) and SNAIL1 immunostaining was performed on the cultured control or Adeno-Cre infected cardiac fibroblasts, in presence of PDGF (Figure 2.9 D). Furthermore, here was no significant difference in proliferation or migration during wound healing (scratch assay) of control versus SNAIL1 deleted cells. (Figure 2.10 A and B). After verification, the Snail1^{f/f}; Rosa-LSL-tdTomato
cardiac fibroblasts were immortalized by allowing them to undergo senescence and repeated pas- saging (~15 passages). The cells were sorted by FACS with PDGFRα+ antibody and only the high PDGFRα expressing cells were collected and used for further experiments. We further determined that SNAIL1 deletion does not affect signaling through the PDGF receptor.

**SNAIL1 is important for conversion of cardiac fibroblasts to activated myofibroblasts**

The conversion of fibroblasts to myofibroblasts is a crucial step for fibrosis to occur. SNAIL1 has been suggested to play a role in giving rise to myofibroblasts via EndMT process in kidney fibrosis. Whether SNAIL1 can is necessary for conversion of cardiac fibroblasts to myofibroblasts is unknown. To test this we used the immortalized cardiac fibroblasts isolated from SNAIL1^ff^, ROSA-LSL-tdTomato mice. SNAIL1 was deleted by infection of these cells with Adeno-Cre expressing viruses. Control cells were infected with Adeno-LacZ viruses. Cells were then activated by virtue of being plated on plastic (high stiffness). Very few (~10%) αSMA positive (i.e., myofibroblast) cells were detected in SNAIL1 deleted cells (Figure 2.12A, quantified in 2.12 B). In this experiment, the presence of Tomato positive cells is a surrogate marker for SNAIL1-deleted cells (i.e., both expression of Tomato and deletion of SNAIL1 are Cre responsive) and >95% of cells were tomato positive (Figure 2.12A). This was in stark contrast to the case with control cells. Control cells were tomato negative and SNAIL1 positive, and ~85% of cells were αSMA positive (Figure 2.12A, quantified in 2.12B).

The loss of αSMA positive myofibroblasts when SNAIL1 was deleted had functional consequences. SNAIL1-deleted cardiac fibroblasts (i.e., + Cre) were deficient in contracting collagen gels (Figure 2.12 C, quantified in 2.12 D). Furthermore, fibrotic gene expression (IL6,
TGFβ and CTGF) and collagen remodeling gene expression (periostin and lysyl oxidase L2) were blunted in SNAIL1-deleted fibroblasts exposed to TGFβ (Figure 2.13 A-F). Periostin protein level in SNAIL1-deleted fibroblasts was also decreased (Figure 2.13 G). Collectively, this suggested that SNAIL1 controls the adoption of the full myofibroblast phenotype by cardiac fibroblasts, at least following exposure to pro-fibrotic TGFβ and a stiff environment.

**SNAIL1 is required for ECM collagen production and deposition by cardiac fibroblasts/myofibroblasts**

Activated fibroblasts or myofibroblasts are the primary source of matrix secreted in zones of ischemic injury. To determine whether SNAIL1 was necessary for matrix deposition by myofibroblasts, we made use of the immortalized control and SNAIL1 knockout cells indicated above. When treated with TGFβ, Collagen I transcript expression increased in control cells, but not in SNAIL1 deleted cells (Figure 2.14 A). We did not observe any significant change in collagen 3 level or fibronectin, however (Figure 2.14 B). To assess ECM production by cardiac fibroblasts, ex vivo, we plated control and SNAIL1-deleted cells in the presence of ascorbic acid and PDGF for 7 days. Cells were removed and the cell free matrix stained with antibodies against collagen I or with Sirius red. Matrix produced by SNAIL1 deleted cells exhibited decreased collagen I staining, as determined by quantitative immunofluorescence (Figure 2.15 A, quantified in 2.15 B). However, if the cells were not extracted, there was punctate Collagen I immunofluorescence staining in the SNAIL1 knockout cells. (2.15C). In addition, the SNAIL1 knockout cells had decreased Sirius red staining (Figure 2.15 D), and decreased type I and type III collagen fibers birefringence of Sirius red sections (Figure 2.16 E, quantified in 2.16 F).
These results indicated that induced expression of SNAIL1 in cardiac myofibroblasts was necessary for efficient extracellular matrix deposition in response to pro-fibrotic TGFβ.

**Collagen I affects SNAIL1 via DDR2**

Excess fibrillar collagen (i.e. collagen I and III) deposition is a hallmark of cardiac fibrosis. The cardiac (myo)fibroblasts that are responsible for this matrix synthesis express the fibrillar collagen receptor, Discoidin Domain Receptor 2 (DDR2), a receptor tyrosine kinase. It has been shown in tumor cells, that DDR2 activation by collagen I can stabilize SNAIL1 via Erk signaling. Thus we asked whether cardiac fibroblasts can stabilize SNAIL1 in response to collagen. We plated SNAIL1-CBR/+ primary cardiac fibroblasts isolated from normal uninfarcted hearts on increasing concentration of collagen I gel for 4 hours and observed increased SNAIL1-CBR bioluminescence signal with increasing collagen concentration at 4 hours, as compared to baseline signal at time 0 (Figure 2.16 A). Furthermore, we determined that SNAIL1-CBR bioluminescence signal is elevated over time by plating the SNAIL1-CBR primary cardiac fibroblasts on plastic or 4mg/mL collagen gel (Figure 2.16 B). To determine whether the elevated SNAIL1 signal is via DDR2 receptor activation via collagen, we isolated cardiac fibroblasts from uninfarcted normal DDR2<sup>f/f</sup>; Rosa LSL tdTomato mouse. In this mouse, the exon 8 of DDR2 is flanked by LoxP sites and the tdTomato reporter is used to identify the cells that express Cre recombinase (surrogate for DDR2 knockout) (Figure 2.17 A). We infected these cardiac fibroblasts with Adeno LacZ (CTL) or Adeno-Cre (+Cre) to knockout DDR2. We observed >95% of the cells expressing tdTomato reporter after infecting with Adeno-Cre (Figure 2.17 B). Then the control or DDR2 knockout cells were plated on either plastic (NT) or on Collagen, treated with TGFβ for 2 hours and the TGFβ was washed out for 6 hours. The DDR2
knockout cells had decreased SNAIL1 protein levels, even in the normal unstimulated (NT) conditions. Furthermore, we isolated mRNA from control (AdLacZ) or DDR2 knockout cells (AdCre) plated on collagen for 4 hours. qPCR analysis showed no significant change in SNAIL1 or Collagen 3a1 mRNA in the control or DDR2 knockout cells. However, the Col1a1 transcript was reduced in the DDR2 knockout cells.

In sum, these results suggest a role of DDR2 in maintaining SNAIL1 protein levels in cardiac fibroblasts and the elevated SNAIL1 levels might be responsible for maintaining col1a1 transcript levels.

**Pro-fibrotic miR29 family might be regulated by SNAIL1 in response to TGFβ**

TGFβ treatment increases levels of Collagen I mRNA via the binding of SMAD2/3 to the Col1a1 promoter. We show that deletion of SNAIL1 decreases Col1a1 transcript levels, even in the presence of TGFβ stimulation. It is known that microRNA 29 expression is suppressed in the hearts of infarcted mice and that TGFβ exposure can mediate this response in cardiac fibroblasts. We wanted to determine whether SNAIL1, a transcriptional repressor, can increase collagen I transcript via mir29 repression. Firstly, to determine whether the collagen (and other fibrosis related) gene promoters have conserved E-box (CACCTG) binding sites for SNAIL1. Using a custom bioinformatics software we could not find E-box (i.e. SNAIL1 binding) sequences in the promoters of fibrillar collagens. Since E-Cadherin is negatively regulated by SNAIL1 binding to its promoter, we used E-Cadherin promoter to verify the software (Table 2.3). We then looked at the promoters of mir29 family to determine that all promoters of miR 29 a/b/c have conserved SNAIL1 binding E-box sequences (Table 2.2). Furthermore, using TargetScan 7.1 mouse, a
microRNA binding predicting software available online, we determined that the 3’UTR of collagen I and III mRNA have multiple conserved mir29 binding sites in their 3’UTR (Figure 2.18). Collagen 1a1 mRNA has 3 conserved binding sites and 2 of these sites had strong 8-mer sequence for miR29 family. Collagen 1a2 has 7-mer binding sequence and collagen 3a1 has one 8mer and one 7mer binding sites for mir29 family (Table 2.4).
Discussion

The wound healing response following a myocardial infarction (hypoxic injury) is necessary to form a scar in the infarcted region. Persistent deposition of extracellular matrix, predominantly collagens I and III, causes fibrosis in the infarct region, leading to decreased cardiac function. It is well appreciated that the cardiac myofibroblasts are responsible for collagen deposition in response to pro-fibrotic cytokines including TGFβ, PDGF and CTGF. SNAIL1, a zinc finger transcription factor, usually expressed in mesenchymal cells, is necessary for liver and kidney fibrosis in mice following injury using carbon tetrachloride and urinary ureter obstruction models respectively. In the heart, SNAIL1 has been shown to be expressed in cardiac fibroblasts and endothelial cells following Left Anterior Descending Artery Occlusion (LAD)\textsuperscript{80} and ischemia-reperfusion (IR)\textsuperscript{81} injury models, but whether SNAIL1 is necessary for fibrosis, especially in mesenchymal (non-epithelial) cells in the heart following injury and if so, the possible mechanism is not well understood.

The infarcted myocardium contains many cell lineages, including cardiac myofibroblasts, endothelial, circulating fibrocytes, bone marrow derived progenitor cells and monocytes\textsuperscript{26}. Similar to previously reported studies, we observe elevated SNAIL1 levels 7 days post hypoxic injury by Left Anterior Descending Artery Occlusion\textsuperscript{80} and show that SNAIL1 is expressed predominantly in myofibroblasts, as identified by αSMA and periostin staining, and a small proportion of CD45+ immune cells. We do not find SNAIL1 expression in endothelial cells as previously reported, albeit, our analysis is done on L.A.D surgery model rather than a IR surgery model)\textsuperscript{81}. It is likely that SNAIL1 is expressed in endothelial cells during early stages of wound healing, which might enable the endothelial cells to undergo endothelial to mesenchymal transition after
cardiac injury and contribute to increased number of myofibroblasts in the infarct area. The increase in SNAIL1 level correlates with increased SNAIL1 mRNA, collagen deposition in the infarct area and increased levels of pro-fibrotic cytokines. Interestingly, these pro-fibrotic cytokines can elevate SNAIL1 levels in cultured primary cardiac fibroblasts isolated from normal, uninjured hearts. Cardiac fibroblasts/myofibroblasts in the infarcted region express pro-fibrotic cytokines like TGFβ, CTGF and IL6 but SNAIL1-deleted cardiac fibroblasts do not increase pro-fibrotic cytokine gene expression in response to TGFβ and mechanical stress (culturing on high tension plastic tissue culture plates). This suggests that the presence of SNAIL1 in cardiac fibroblasts/myofibroblasts in the infarct region is responsible for increased the pro-fibrotic genes expression.

Collagens I and III are the primary collagens secreted by the cardiac fibroblasts/myofibroblasts in the infarcted myocardium in response to pro-fibrotic factors. SNAIL1-deleted cardiac fibroblasts have reduced collagen I mRNA expression and collagen deposition in response to TGFβ. Collagen III expression in response to TGFβ was unaffected by the absence of SNAIL1. This may reflect distinct regulation of Collagen I and III gene expression in cardiac fibroblasts, since collagen III but not collagen I in cardia fibroblasts is regulated by mechanical stretch signals. Additionally, SNAIL1 is necessary for extracellular collagen modifying and crosslinking genes expression, i.e. LoxL2 and periostin. This data suggests that SNAIL1 is important for collagen deposition and remodeling. Since increased collagen I deposition increases SNAIL1 levels in cardiac fibroblasts this suggests a feed forward loop whereby SNAIL1 causes increased collagen deposition, which in turn maintains elevated SNAIL1 in the cardiac fibroblasts, possibly through the collagen receptor DDR2 or mechanical signals through Integrin activation.
The cardiac fibroblasts adopt a myofibroblast fate during fibrosis and increase in number in the infarcted region. SNAIL1 expression is responsible for cell fate changes, for example, during Epithelial to Mesenchymal transition\textsuperscript{62} and during embryonic stem cell differentiation\textsuperscript{97}. Whether SNAIL1 is responsible for the transformation of cardiac fibroblast to myofibroblasts is unknown. We show that by knocking out SNAIL1, the cardiac fibroblasts do not express the myofibroblast marker $\alpha$SMA. It is likely that through activation of Rho kinase, the myocardin related transcription factor (MRTF), which can mediate TGF$\beta$ induced EMT\textsuperscript{98} and also $\alpha$SMA expression\textsuperscript{99}, might be either interacting / affecting the activity of SNAIL1 and vice versa.

TGF$\beta$ treatment of cardiac fibroblasts upregulates multiple genes including ECM genes (collagens), ECM modifiers (periostin, LoxL2) and other profibrotic cytokines (IL6, CTGF). SNAIL1 gene transcription and protein levels are also increased following TGF$\beta$ yet SNAIL1 is a transcriptional repressor. So how does SNAIL1 deletion, abrogate the elevation of the above mentioned TGF$\beta$, target genes? One possibility is that include that SNAIL1 is acetylated in cardiac fibroblasts under these conditions and as such now functions as a transcriptional (co)activator\textsuperscript{67}. Alternatively, TGF$\beta$ could repress microRNA 29 via SNAIL1 action, which then releases miR29’s inhibition of collagen I and III expression\textsuperscript{56}. SNAIL1 repression occurs by binding to E-box sequences in the promoter and indeed, the miR29b promoter has 2 conserved E-box sequences.

In sum, our data suggest that cardiac fibroblasts in the infarcted myocardium express SNAIL1 and adopt a myofibroblast phenotype. SNAIL1 contributes to fibrosis by allowing expression of pro-fibrotic cytokines and deposition and remodeling of the collagen matrix causing a scar. The pro-fibrotic cytokines and collagen I in turn maintain SNAIL1 levels in the
myofibroblasts in a feed-forward loop to sustain the fibrotic state. SNAIL1 suppresses expression of mir29 family which relieves the suppression of fibrosis genes by mir29.
Figure 2.1

A

Endogenous Promoter

E1 E2 E3 CBR Stop 3’UTR

B

day 0

Sham or LAD ligation I.R surgery

day 7

analyze

C

Sham L.A.D

D

BLI Fold Change

E

mRNA Fold Change

*
Figure 2.1:

**SNAIL1-CBR signal after myocardial infarction by Left Anterior Descending Artery Occlusion:**

(A) Schematic of SNAIL1-CBR bioluminescence mouse.

(B) Timeline and endpoint of experiment.

(C) Representative ex-vivo imaging of hearts of sham or LAD surgery SNAIL1-CBR/+ mouse.

(D) Bioluminescence fold change of ex-vivo hearts from sham (n=3) and LAD (n=4). Sham bioluminescence values were arbitrarily set to 1.

(E) QPCR for Snail1 transcript from isolated ventricles of sham and LAD infarcted mice. All values were normalized to GAPDH and fold change was compared to Sham which was arbitrarily set to 1.
Figure 2.2:

**SNAIL1-CBR signal after myocardial infarction by ischemia reperfusion.**

(A) Representative ex-vivo imaging of hearts of sham or I/R surgery SNAIL1-CBR/+ mouse.

(B) Bioluminescence fold change of ex-vivo hearts from sham (n=3) and LAD (n=4). Sham bioluminescence values were arbitrarily set to 1.

(C) QPCR for Snail1 transcript from isolated ventricles of sham and I/R infarcted mice. All values were normalized to GAPDH and fold change was compared to Sham which was arbitrarily set to 1.
Figure 2.3:

Fibrosis gene expression after cardiac injury

(A) QPCR for fibrosis genes from mRNA isolated from ventricles of sham or infarcted hearts by Left Anterior Descending Artery (LAD) occlusion.

(B) QPCR for fibrosis genes from mRNA isolated from ventricles of sham or infarcted hearts ischemia reperfusion (IR) surgery. All values done in triplicates. Representative of 2 mice in each experiment.

(C) Ejection fraction of the mice undergoing sham (n=5), LAD (n=6) or I/R (n=4) surgery 7 days after surgery. Ejection fraction was determined by echocardiogram.

(D) Trichrome staining for Collagen in sham, LAD and I/R fixed heart sections 7 days after surgery.
Figure 2.4

A

Implant pump
Angiotensin II or saline

0

14

Analyze

B

ROI 1=5949.5
ROI 2=7398.2

Saline
Angiotensin II

C

Ex-vivo hearts bioluminescence

BL units

0

1000

2000

3000

4000

5000

6000

7000

8000

9000

Saline
Angiotensin II

D

Trichrome Staining

Saline
Angiotensin II
Figure 2.4:

SNAIL1-CBR signal in Angiotensin II induced fibrosis model

(A) Timeline of experiment. Pumps with saline (control) or Angiotensin II (experimental) were implanted in the back of the mice for 14 days.

(B) Representative ex-vivo bioluminescence imaging of hearts from saline and Angiotensin II treated mice at 14 days.

(C) Quantification of bioluminescence units from B. There were 4 mice in saline and 3 in Angiotensin II treat groups respectively.

(D) Representative images of trichrome staining for collagen in saline and Angiotensin II treated mice.
Figure 2.5:

Immunofluorescence staining of SNAIL1 and cardiac cell type markers in the ventricular infarct zone of LAD surgery mice.

Cardiac cell type markers used:

(A) αSMA (alpha smooth muscle actin) – myofibroblast marker

(B) Periostin – myofibroblast marker,

(C) CD31 – endothelial cell marker,

(D) CD45 – leukocyte cell marker,

(E) α-actinin – cardiomyocyte marker.

Scale bars: 50 microns
Table 2.1

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<tr>
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<td>60 %</td>
</tr>
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<td>69 %</td>
</tr>
<tr>
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Figure 2.6:

Immunofluorescence staining of SNAIL1 and cardiac cell type markers in the remote (non-infarct zone) of LAD surgery mice.

Cardiac cell type markers used:

(A) αSMA (alpha smooth muscle actin) – myofibroblast marker

(B) Periostin – myofibroblast marker,

(C) CD31 – endothelial cell marker,

(D) CD45 – leukocyte cell marker,

(E) α-actinin – cardiomyocyte marker.

Scale bars: 50 microns
Figure 2.7

A

Control – No Primary

with antibody

DAPI
Vimentin

Cardiac Fibroblasts

B

Cardiomyocytes

Cardiac fibroblasts

DDR2
Snail1
Actin
Figure 2.7

Verification of cardiac fibroblasts isolated from uninjured SNAIL1-CBR hearts:

(A) Vimentin staining of SNAIL1-CBR primary cardiac fibroblasts

(B) Western blot showing DDR2 expression in SNAIL1-CBR cardiac fibroblasts and cardiomyocytes
**Figure 2.8:**

**Pro-fibrotic factors increase SNAIL1-CBR level in primary cardiac fibroblasts**

Primary fibroblasts were isolated from the hearts of un-infarcted, normal SNAIL1-CBR mice. Bioluminescence signal after treatment with (A) TGFβ (2 ng/μL), (B) PDGF (10 ng/μL), (C) Hypoxia (400 μM CoCl2) and (D) and Angiotensin II (1 μM) Representative data from one of 4 experiments.

(E) Additive effect of pro-fibrotic factors on SNAIL1-CBR bioluminescence signal. Representative data of 4 independent experiments.

(F) SNAIL1 mRNA fold change in primary cardiac fibroblasts 4 hours post stimulation with indicated factors. All values were normalized to GAPDH and compared to non-treated cells as control. Representative example from 3 experiments.
Figure 2.9:

Verification of SNAL1 deletion in Snail1 flox/flox cardiac fibroblasts.

(A) qPCR for SNAIL1 mRNA level in SNAIL1 flox/flox; ROSA-LSL-tdTomato cardiac fibroblasts, treated with Adeno-LacZ (CTL) or Adeno-Cre (+ Cre), with and without TGFβ stimulation.

(B) Western blotting for SNAIL1 in SNAIL1 flox/flox; ROSA-LSL-tdTomato cardiac fibroblasts, treated with Adeno-LacZ (CTL) or Adeno-Cre (+ Cre), with and without TGFβ stimulation

(C) tdTomato expression in Snail1 flox/flox; Rosa LSL tdTomato cardiac fibroblasts after Adeno LacZ (CTL) or Adeno-Cre (+Cre) infection

(D) Immunofluorescence staining for SNAIL1 infected with Adeno-LacZ (CTL) or Adeno-Cre (+ Cre), with PDGF (10ng/mL) stimulation.
Figure 2.10

A

Growth Curve

![Graph showing growth curve for LacZ and Cre](image)

B

Scratch Assay

![Images showing scratch assay for different conditions](images)
Figure 2.10:

Effect of SNAIL1 on proliferation and migration during wound healing

(A) Growth curve of control and Snail1 knockout (+Cre) cells.

(B) Scratch assay of control and SNAIL1 knockout cells (+Cre) with and without TGFβ stimulation.
Figure 2.11

A

B

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<tr>
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<td>-</td>
<td>+</td>
</tr>
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Figure 2.11.

Characterization of immortalized Snail1 f/f; Rosa LSL tdTomato cardiac fibroblasts.

(A) Live cell fluorescence activated cell sorting (FACS) of immortalized cardiac fibroblasts

(B) Analysis of signaling pathways after PDGF stimulation of PDGFRα+ fibroblasts infected with Adeno LacZ or Adeno Cre virus
Figure 2.12

A

CTL

+ Cre

B

% αSMA+ve cells

0%

20%

40%

60%

80%

100%

CTL

+ Cre

C

0 minutes

40 hours

D

% initial gel area at 40 h

0%

20%

40%

60%

80%

CTL

+ Cre
Figure 2.12

SNAIL1 effect on αSMA expression in cardiac fibroblasts.

(A) αSMA immunofluorescence of control (CTL) and SNAIL1-deleted (+ Cre) cardiac fibroblasts.

(B) Quantification of results in (A). 30 fields were counted in each group. Shown is a representative result of one of 3 independent experiments.

(C) Gel contraction assay of control (CTL) and SNAIL1-deleted (+ Cre) cardiac fibroblasts.

(D) Quantification of (C). Shown is pooled data from 2 independent experiments, done in triplicates.
Figure 2.13

Snail1 deletion effect pro-fibrotic cytokines and collagen remodeling proteins.

Relative fibrogenic gene mRNA expression after SNAIL1 deletion and TGFβ stimulation:

(A) IL6, (B) TGFβ, (C) CTGF
(D), LoxL2, (E) LoxL3 (F) LoxL1

Representative example of 3 separate experiments.

(G-H) Periostin mRNA levels after 2 hours and 3 days of TGFβ stimulation.

(I) Western blot analysis with periostin antibody of extracts from control (CTL) and SNAIL1-deleted cardiac fibroblasts. Representative example of one of two separate experiments.
Figure 2.14:

Effect of Snail1 knockout on extracellular matrix gene expression in cardiac fibroblasts:
mRNA levels of the following ECM and ECM modifying genes with and without TGFβ
stimulation (2ng/mL) for 2 hours of control (CTL) and SNAIL1 knockout cells (+Cre)

(A) Col1α1

(B) Col3α1

(C) Fibronectin

(D) MMP2

(E) MMP9

(F) MMP1.

Shown is a representative result of one of 4 independent experiments.
Figure 2.15

A: Cells extracted from matrix

B: Graph showing pixel counts for Collagen Iα1 with CTL and +Cre conditions.

C: Cells not removed from matrix

D: Sirius Red staining images for CTL and +Cre conditions.

E: Birefringence images for CTL and +Cre conditions.

F: Bar graphs showing pixel counts for Yellow (Type I Fibers) and Green (Type III Fibers) with CTL and +Cre conditions.
Figure 2.15

Effect of Snail1 deletion of collagen deposition by cardiac fibroblasts

(A) Collagen 1 immunofluorescence of cell free matrix deposited by control (CTL) and SNAIL1-deleted (+ Cre) cardiac fibroblasts. (n = 2).

(B) Quantification of results in (A).

(C) Collagen 1 immunofluorescence of matrix deposited by control (CTL) and SNAIL1-deleted (+ Cre) cardiac fibroblasts. (n = 2). Cells were not extracted for this staining experiment.

(D) Detection of Collagen 1 by Sirius Red Staining of cell free matrix deposited by control (CTL) and SNAIL1-deleted (+ Cre) cardiac fibroblasts. (n = 4).

(E) Birefringence imaging of Sirius red stained matrix produced by control (CTL) or SNAIL1-deleted (+ Cre) cardiac fibroblasts.

(F) Quantification of Birefringence imaging (n = 4, 20 images counted for each experiment).
Figure 2.16

A

Fold bioluminescence change

Collagen I concentration

B

Bioluminescence units

Time (hrs)
Figure 2.16

**Effect of collagen on Snail1 stability:**

(A) Relative fold change in SNAIL1-CBR bioluminescence intensity in cardiac fibroblasts exposed to increasing collagen I concentration. In each condition values at t=0 were arbitrarily set to equal 1.

(B) SNAIL1-CBR bioluminescence intensity in cardiac fibroblasts plated on 4mg/mL collagen I over increasing time. A representative example from 3 experiments is shown.

(C) Effect of cyclohexamide on SNAIL1 bioluminescence of SNAIL1CBR/+ cardiac fibroblasts plated on 4mg/mL of collagen.
Figure 2.17

A. Conditional DDR2 knockout allele - DDR2^{f/f}

Exon 8

B. tdTomato

Ad LacZ  tdTomato
Ad Cre

C. DDR2^{f/f}, Rosa LSL td Tomato

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D. qPCR from cells extracted from collagen gel

- AdLacZ
- AdCre

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Figure 2.17

**DDR2 knockout effect on SNAIL1 stability in cardiac fibroblasts plated on 3D collagen I matrix.**

(A) Schematic of the conditional DDR2 knockout allele.

(B) tdTomato expression in cardiac fibroblasts isolated from DDR2 f/f; Rosa LSL tdTomato mice and infected with Adeno-LacZ (Control) and Adeno Cre (DDR2 KO) virus.

(C) Western blot with indicated antibodies of control and DDR2 KO cardiac fibroblasts plated on 4mg/mL collagen I gel. (n=2)

(D) qPCR detection for SNAIL1 and Collagens in control and DDR2 KO cardiac fibroblasts plated on collagen (n=1)
Figure 2.18

miR29 binding site analysis – predictive analysis

(A) 3’UTR (untranslated region) of Collagen Ia1 mRNA. Binding site of miR-29 indicated in boxes.

(B) miR-29 binding site conservation across species on the 3’UTR of col1a1 including mouse and humans.

(C) 3’UTR (untranslated region) of Collagen 3a1 mRNA. Binding site of miR-29 indicated in boxes.

Data acquired from TargetScan Mouse v7.1
Table 2.2: Promoter analysis of mir29 family

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Table 2.3: Promoter analysis of collagen I and III

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Table 2.4: 3’UTR region of Collagen I and III

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Figure 2.19

Possible mechanisms of action of SNAIL1 in cardiac fibroblasts

(A) SNAIL1 expression in cardiac fibroblasts converts it into myofibroblasts which setup the fibrosis cascade in the heart.

(B) Possible role of miR29 regulation by SNAIL1 in response to TGFβ and its implication in promoting cardiac fibrosis.
Chapter 3: SNAIL1 reporter and knockout mouse models
**Introduction**

SNAIL1 is a zinc finger transcription factor involved in the process of Epithelial to Mesenchymal transition is highly regulated. The tight regulation of SNAIL1 is necessary as SNAIL1 had very important functions, including cell fate determination during normal development. For example, SNAIL1 is expression is necessary for gastrulation during embryogenesis, differentiation of embryonic stem cells and for normal wound healing. Aberrant or overexpression of SNAIL1 is implicated in multiple diseases like cancer and fibrosis.

Regulation of SNAIL1 levels occurs both at transcriptional and post translational level. SNAIL1 protein is unstable with a short half-life of 20 minutes and is constantly targeted for degradation in the cytoplasm by the GSK3B /Axin / βcatenin complex. SNAIL1 is also self-regulated at the gene level where increase in SNAIL1 can bind to E-box sequences in its own promoter and turn off transcription. Furthermore, SNAIL1 is also regulated at the post-transcriptional level by phosphorylation and acetylation and each of these modifications have different effect on SNAIL1 activity i.e. SNAIL1 can be a transcriptional co-repressor (phosphorylation) or co-activator (acetylation). Due to the dynamic and transient nature of SNAIL1, it is difficult to study endogenous SNAIL1 levels and functions in-vivo. Most of the experiments done to study SNAIL1 in-vivo have been done using qPCR and immunohistochemical methods which do not represent a complete picture of SNAIL1 expression pattern, activity or function.

SNAIL1 is a mesenchymal gene whose transcript is expressed at very low levels in both epithelial and mesenchymal cell types. In epithelial cells, SNAIL1 expression in response to EMT inducing signals (like TGFβ) causes the epithelial cells to undergo EMT by direct
repression of epithelial genes like E-Cadherin and upregulation of mesenchymal genes like vimentin. Induction of EMT reduces cell proliferation rates and increases motility of the cells. In mesenchymal cells, like fibroblasts, SNAIL1 expression is upregulated during wound healing process following injury.

Most of the experiments done in mouse models to study the effects of SNAIL1 deletion were done by global deletion of SNAIL1 or deletion in the epithelial cell compartment. Germline deletion of SNAIL1 causes a failure of the developing embryos to undergo gastrulation causing the mouse embryo’s to die at e6.5. Deletion of SNAIL1 in the mammary epithelial cells using a SNAIL1 floxed mouse crossed to MMTV-Cre (Mouse Mammary Tumor Virus), prevents the invasion of the mammary ductal epithelial cells into the mammary fat pad. Studies done to elucidate the role of SNAIL1 in fibrosis in the liver were carried out using Albumin-Cre, which is expressed in hepatocytes. In the kidney, Ksp1.3-Cre (Cadherin-16) was used to knock out SNAIL1 and its effect on fibrosis was determined. In both the studies, SNAIL1 deletion was caused a reduction in fibrosis following carbon tetrachloride and urinary ureter obstruction injury. In the heart, following cardiac injury, the cardiac (myo)fibroblasts increase in number in the infarcted area and are responsible for fibrosis by deposition of extracellular matrix. We have shown that in-vitro, SNAIL1 is necessary for conversion of fibroblasts into cardiac fibroblasts. However, the function of SNAIL1 in-vivo in these cardiac (myo)fibroblasts haven’t been determined yet.

In the following study, we attempted to determine the cellular identity if SNAIL1 expression at the organ and cellular level in the hearts using different SNAIL1 reporter mice. Using a reporter mouse, we determined the expression pattern of different mesenchymal Cre recombinase’s in the heart. We show that SNAIL1 deletion in the fibroblasts do not have any
effect on mouse development. Knocking out SNAIL1 in the mesenchymal cells suggests that SNAIL1 is at least partially responsible for fibrosis in the infarct zone in the heart.
Results

SNAIL1-LACZ reporter mouse model.

To determine the cellular identity of SNAIL1 expressing cells in the normal and infarcted heart, we decided to create a SNAIL1-LacZ reporter mouse. We obtained two SNAIL1-LacZ reporter Embryonic stem (ES) cells (denoted AE3 and AG11) from the international mouse consortium (KOMP) repository. The ES cells were in the BL/6 background. The SNAIL1 targeting allele is shown in Figure 3.1A. In these ES cells, one of the two alleles of SNAIL1 is replaced by knocking in a SNAIL1-LacZ reporter construct. Thus SNAIL1 expressing cells in this mouse would be identified using beta-galactosidase (X-Gal) staining. The ES cell lines expressed LacZ gene, determined by PCR (Figure 3.1B). Since SNAIL1 is expressed during ES cell differentiation, we induced ES cells to differentiate by plating them on tissue culture plated in absence of LIF (Leukemia Inhibitory Factor). By staining the ES cells undergoing differentiation with beta-galactosidase we observed LacZ staining in both the ES cell strains. However, when the ES cells were injected in the blastocyst and implanted in a pseudo-pregnant mouse, we obtained only 3 chimeric mice from the AG11 ES cell line. Out of these, 2 chimeric mice died soon after birth and from the remaining chimeric mouse, we did not obtain germline incorporation of the SNAIL1-LacZ allele. The AE3 ES cells injected mice did not bear any litter.
SNAIL1-IRES-YFP mouse characterization

SNAIL1-IRES-YFP mice were a kind gift from Dr. Xin Ye and Dr. Robert Weinberg (Whitehead institute). In this mouse, the third exon of SNAIL1 is fused to an IRES-YFP cassette (Figure 3.2 A). Thus any cell expressing SNAIL1 will be YFP positive. Since SNAIL1 is not fused to the YFP reporter, only the cellular identity of SNAIL1 expressing cells can be determined but YFP expression cannot be used to determine the levels of SNAIL1 or the subcellular localization. When normal uninfarcted hearts were obtained and stained using YFP antibody, we saw very few positive cells in the heart (Figure 3.2 B). In contrast, there were a significant number of YFP expressing cells (Figure 3.2C) When cardiac fibroblasts from the SNAIL1-IRES-YFP mouse was isolated and cultured in tissue culture dishes, >95% of the cells expressed YFP (Figure 3.2D). This suggested that during the cardiac fibroblast isolation process or by culturing of these SNAIL1-IRES-YFP fibroblasts on high tension tissue culture plates SNAIL1 is expressed in them. However, when the SNAIL1-IRES-YFP mice were subject to Left Anterior Descending Artery Occlusion, none of the mice survived beyond Day 2 post-surgery. In these infarcted mice we could not detect any YFP expression by immunofluorescence in tissue slices.

SNAIL1-CBR reporter mouse characterization

SNAIL1-CBR/TRE-SNAIL-Flag mice were generated by Dr. David Tran (University of Florida). In these mice, the third exon of SNAIL1 is fused to the bioluminescent enzyme Clic Beetle Red (Figure 3.3 A). The fusion allele was inserted in-frame in the endogenous SNAIL1 locus and driven by the endogenous Snail1 promoter. Thus the CBR bioluminescence is a direct
readout of the endogenous levels of SNAIL1 protein. The SNAIL1-CBR reporter allele also has a TRE-SNAIL1Flag construct, which can be used to temporally, and spatially overexpress flag tagged SNAIL1 (TRE-Tetracycline responsive element). Thus when the SNAIL1-CBR / TRE-SNAIL1-Flag mouse is crossed to a reverse tetracycline transactivator (rtTA) expressing mouse and doxycycline is administered to this mouse, SNAIL1-Flag will be overexpressed. Alternatively, if the SNAIL1-CBR / TRE-SNAIL1-Flag mouse is crossed to the tetracycline transactivator tTA expressing mouse, SNAIL1-Flag will be overexpressed in the cells expressing the tTA transgene and doxycycline administration will stop the expression of SNAIL1-Flag. The Flag tag can be used to distinguish endogenous v/s overexpressed SNAIL1.

The SNAIL1-CBR/ TRE-SNAIL1-Flag mice had normal patterns of SNAIL1 expression but mice homozygous for the SNAIL1-CBR/TRE-SNAIL1-Flag allele were not obtained. (SNAIL1-CBR/TRE-SNAIL1-Flag allele is referred to as SNAIL1-CBR/+ henceforth for brevity). Bioluminescence imaging of WT littermate or SNAIL1 CBR/+ mice showed that only the reporter mice had bioluminescence signal (Figure 3.3 B). When the organs from these mice were isolated, the heart had only minimal SNAIL1-CBR bioluminescence signal (Figure 3.3C). The CBR reporter is an enzyme and the output is light, single cell resolution can’t be obtained but since the SNAIL1- reporter fusion has a half-life, similar to the half-life of SNAIL1, the light emitted is directly correlated to SNAIL1-protein level. These mice were further used for infarction studies and for in-vitro experiment with isolated primary cardiac fibroblasts.
SNAIL1-Flag overexpression in the heart

SNAIL1 has been shown to be necessary in cardiac fibroblasts for fibrosis, but whether SNAIL1 is sufficient for fibrosis in the heart is unknown. To address this question, we crossed the SNAIL1-CBR/TRE-SNAIL1-Flag mouse to αMHC-rtTA expressing mouse. In this mouse, the reverse tetracycline transactivator (rtTA) is expressed in cardiomyocytes under the myosin heavy chain 6 promoter (αMHC). Thus in the presence of doxycycline, rtTA will get activated in the cardiomyocytes and turn on the TRE-SNAIL1-Flag, thereby overexpressing SNAIL1-Flag in the cardiomyocytes specifically. The timeframe of the doxycycline (DOX) administration is outlined in Figure 3.4B. The hearts of the control (-DOX) mice and experimental (+DOX) were collected after 4 weeks of doxycycline treatment. Flag immunohistochemistry showed nuclear SNAIL1 in cells, appeared to be cardiomyocytes (Figure 3.4 C). Protein lysates from 3 control and 3 experimental mice were used to detect Flag expression. (Figure 3.4D). However, by trichrome staining, excess collagen deposition was not observed in either control or experimental cohorts (Figure 3.4E). It is possible that 4 weeks of SNAIL1 overexpression is not sufficient for fibrosis or that SNAIL1 expression in cardiomyocytes does not cause fibrosis.

Col1α1-CreERT2 expression in the heart

After myocardial infarction, the transcript levels of Col1a1 increases in the infarcted ventricular region and in the cardiac fibroblasts (Figure 2.3 and 2.13). To knockout SNAIL1 in the heart, Col1a1-CreERT2 mice were obtained from JAX and crossed to SNAIL1fl/fl mice (Dr. Stephen Weiss, U.Michigan) and a Rosa-lox-stop-lox-tdTomato reporter (Figure 3.5A). Any cell that expresses Cre recombinase in response to tamoxifen should turn on tdTomato reporter i.e.
tdTomato reporter is a surrogate for SNAIL1 deletion. The time-frame of the Cre induction by tamoxifen and the infarction is shown in Figure 3.5B. Early induction of Cre by weaning time is necessary as the Col1a1 gene expression subsides in the adult mice (unless induced by injury). When cardiac fibroblasts from the tamoxifen induced mouse hearts at 6 weeks of age were isolated and cultured ex-vivo only 4% of the cells expressed the tdTomato reporter (Figure 3.5C). At 6 weeks of age, control Col1a1-CreERT²; Rosa LSL tdTomato mice without tamoxifen induction did not have tdTomato expression in any organ. In contrast, Col1a1-CreERT²; Rosa LSL tdTomato mice with tamoxifen induction showed tdTomato expression. Representative heart, skin and pancreas sections are shown (Figure 3.5D). When the Snail1⁻/⁻; Col1α1CreERT2; Rosa LSL tdTomato mice were infarcted by LAD ligation and analyzed after 7 days, there was an accumulation of tdTomato positive cells in the infarcted regions (Figure 3.6A). The Snail1⁻/⁻; Col1α1CreERT2; Rosa LSL tdTomato had reduced cardiac fibrosis in the ventricular regions as determined by mason's trichrome staining as compared to wild type (WT) infarcted hearts (Figure 3.6 C and D). A caveat to these observations is that the WT controls are not genetic controls (i.e. Snail1⁺/⁺; Col1α1CreERT2; Rosa LSL tdTomato).

**CAG-CreER and Rosa-CreERT2 (inducible global Cre recombinase) expression in the heart**

Alternative to using an inducible cardiac fibroblast specific Cre (Col1α1-CreERT2), we sought use of a global inducible Cre to knock out SNAIL1 in the entire mouse and study the effect of SNAIL1 deletion in the infarcted myocardium following LAD ligation surgery. Using an inducible Cre to knock out SNAIL1 in adult is necessary, as using a ubiquitous Cre
recombinase like Actin-Cre would cause the embryos to die in-utero, possibly due to failure of epithelial cells to undergo EMT and migration during gastrulation. Although SNAIL1 will potentially be deleted in the entire animal following tamoxifen exposure, we would be determining the effect of SNAIL1 deletion in all the cells that contribute to cardiac fibrosis (including Bone Marrow Derived progenitor cells) and not specifically (myo)fibroblasts.

Snail1<sup>ERT</sup>; CAG CreER; Rosa-LSL-mTmG mice were generated (Figure 3.7A). In this mouse, the inducible Cre recombinase (CreER) is driven by Chicken Actin Globin (CAG) promoter that is ubiquitously expressed. The mTmG reporter switched from membrane-tdTomato (i.e. red) at baseline to membrane-GFP (i.e. green) after tamoxifen treatment. To characterize the efficiency of the CAG CreER, we treated Snail1<sup>ERT</sup>; CAG CreER; Rosa-LSL-mTmG with tamoxifen and the non-tamoxifen treated mice were used as controls. The experimental timeline is outlined in figure 3.7 B. In the absence of induction by tamoxifen, we observed green cells (i.e. recombined reporter) in the tissue sections of the mice. The proportion of green cells increased after tamoxifen treatment of the mice (Figure 3.7C). Shown are representative sections for the heart and pancreas, before and after tamoxifen induction. The leakiness of either CAG CreER or the mTmG reporter in our hands would impede the proper interpretation of observations and hence further experiments were not carried out using this mouse.

We sought use of another global inducible Cre (Rosa-CreERT<sup>2</sup>) to delete SNAIL1 in the mice. We crossed Rosa-CreERT<sup>2</sup> mice to Snail1<sup>ERT</sup> and Rosa LSL tdTomato mice to obtain Snail1<sup>ERT</sup>; Rosa-CreERT<sup>2</sup>; Rosa LSL tdTomato (Figure 3.8 A). The experimental protocol is shown in figure 3.8 B. To characterize the efficiency of Rosa-CreERT2 activation we analyzed organs from Rosa-CreERT<sup>2</sup>; Rosa LSL tdTomato (control) and Rosa-CreERT<sup>2</sup>; Rosa LSL
tdTomato fed tamoxifen chow (experimental). The control mice (without tamoxifen) did not show any reporter activity, but the tamoxifen fed mice had high number of tdTomato positive cells in multiple organs (Figure 3.8C-H). In the heart, there were very few cells labeled with tdTomato (<1%). This suggested that either the Rosa locus is repressed in the heart or tamoxifen did not circulate to the heart efficiently. Due to the inefficiency of the RosaCreERT2 to label cardiac cells, further experiments were not carried out using this mouse. However, in a control experiment, when mammary glands from tamoxifen fed RosaCreERT2; Rosa-LSL-tdTomato (control) and Snail1<sup>fr</sup>; RosaCreERT2; Rosa-LSL-tdTomato (experimental) mice were analyzed, the control mice did not have invasion of the mammary epithelium into the mammary fat pad. This indicated that early SNAIL1 expression is necessary for proper development of the mammary gland.

**αSMA Cre characterization in the heart**

Since the (myo)fibroblasts in the infarct zone express αSMA, we used aSMA-Cre to target these cells in the heart. The rationale for using a non-inducible Cre is that since αSMA is expressed mainly in activated fibroblasts and not in epithelial cells and thus knocking out SNAIL1 using aSMA Cre would not result in embryonic lethality due to impaired gastrulation by epithelial cells. To test this hypothesis, we generated Snail1<sup>fr</sup>; aSMA Cre; Rosa LSL tdTomato mice (Figure 3.9 A). Indeed these mice made it to adulthood and they grow, breed and develop normally. We harvested hearts from uninfarcted aSMA Cre; Rosa LSL tdTomato (control) and Snail1<sup>fr</sup>; aSMA Cre; Rosa LSL tdTomato mice (experimental) mice. In hearts from both the mice we saw very few cells being labeled with the tdTomato reporter and several of these cells
had cardiomyocyte appearance (Figure 3.9 B). Due the low labeling of cells by aSMA-Cre in the heart before infarction, further infarction experiments were not performed using these mice.

**FSP1-Cre characterization in the heart**

Since Snail1 knockout using a non-inducible Cre in the heart (i.e. aSMA –Cre) led to viable mice, we decided to use FSP1-Cre to delete SNAIL1 in the cardiac fibroblasts in mice. FSP1 has been shown to be expressed in the infarcted myocardium using lineage tracing /reporter studies\textsuperscript{101}. We generated Snail1\textsuperscript{fl/fl}; FSP1-Cre; Rosa LSL tdTomato mice (Figure 3.10 A). Uninjured hearts from FSP1-Cre; Rosa LSL tdTomato (control) and Snail1\textsuperscript{fl/fl}; FSP1-Cre; Rosa LSL tdTomato (experimental – Snail1 KO) were analyzed for tdTomato reporter expression (Figure 3.10 B). We observed tdTomato expression in the heart and other organs but the identity of these cells count not be verified immunohistochemically in tissue sections (Figure 3.10 C-F). To determine whether SNAIL1 is deleted in FSP1cre (i.e. tdTomato + cells) we isolated cardiac fibroblasts from uninjured Snail1\textsuperscript{fl/fl}; FSP1-Cre; Rosa LSL tdTomato (Figure 3.10 G). About 23% of the cardiac fibroblasts were tdTomato positive and only a small fraction of these cells (< 3%) expressed SNAIL1. We subject these mice to myocardial infarction by LAD ligation and the experimental timeline is outlined in figure 3.10 B. The adult mice were infarcted and hearts were collected 1 week post-surgery. The Snail1\textsuperscript{fl/fl}; FSP1-Cre; Rosa LSL tdTomato mice hearts had decreased collagen staining by Masson’s trichrome staining in the ventricular infarcted region as compared to the wild type control mice. (Figure 3.11 A). The fibrosis amount was determined by color assignment of blue (collagen) and muscle (red) (Figure 3.11 B) and quantifying the ratio of blue/total area (Figure 3.11C). The remote zone of the WT control and infarcted mice did not have fibrosis and the heart valve in this region (arrows) was used a
positive control for trichrome staining (Figure 3.11D). Sections from the formalin fixed hearts from these mice showed increased tdTomato+ cells only in the infarct zone as compared to the remote zone (Figure 3.11E). Immunofluorescence staining of these hearts indicated that the tdTomato positive cells did not express SNAIL1 and these cells are vimentin positive (Figure 3.12A). To determine whether the tdTomato positive cells (i.e. SNAIL1 deleted cells) were myofibroblasts we performed immunofluorescence on 7 day infarcted Snail1^{f/+}; FSP1 Cre; Rosa LSL tdTomato (control) and Snail1^{f/f}; FSP1-Cre; Rosa LSL tdTomato (experimental) hearts with αSMA antibody. Interestingly, in both the control and experimental infarcted hearts, tdTomato positive cells were not αSMA positive indicating that αSMA positive myofibroblasts in the infarct zone are derived from a lineage, distinct from FSP1+ cells as described previously^{102}. 
Discussion

Snail1 is a zinc finger transcription factor that is expressed at very low levels in the cells and increase in SNAIL1 levels due to various stimuli can cause a cell fate change in the cells (i.e. EMT) or cause diseases like fibrosis. The half-life of SNAIL1 in cells is 20 minutes and it is degraded by the ubiquitin proteasome pathway. To further our understanding of SNAIL1 regulation, it is imperative to create a proper endogenous SNAIL1 reporter without overexpressing it.

We compared 3 endogenous SNAIL1 reporter in this study, each with its unique characteristic (Table 3.1). The first reporter was SNAIL1-LacZ and ES cells expressing this allele under the endogenous SNAIL1 promoter was obtained from KOMP (international knockout mouse consortium). One SNAIL1 allele was completely replaced by the LacZ reporter and thus the cells expressing the reporter would indicate that SNAIL1 promoter activation. This reporter would not be useful to determine post-transcriptional modification of SNAIL1 as the LacZ is not fused to SNAIL1. The attempt to create the Snail1-LacZ reporter mouse failed as germline transmission of SNAIL1 could not be achieved.

The second SNAIL1 reporter is SNAIL1-IRES-YFP mouse. The third exon of SNAIL1 is fused to an IRES-YFP reporter. In this mouse, the YFP fluorescence would indicate that SNAIL1 mRNA was transcribed, as the reporter is read by an IRES sequence off the SNAIL1 mRNA. This reporter is not suitable to study the subcellular localization or stability of SNAIL1. When the SNAIL1-IRES-YFP reporter mouse was subject to infarction, they all died one day after surgery.
The third SNAIL1 reporter mouse is SNAIL1-CBR. In this mouse, the third exon of SNAIL1 is fused to a bioluminescent enzyme Clic Beetle Red (CBR). Since it’s a fusion protein (SNAIL1-CBR) with the same half-life as SNAIL1 itself, the reporter is a direct readout of SNAIL1 expression. However, the reporter lacks cellular resolution as the reporter output is light. Homozygosity of the SNAIL1-CBR allele was never achieved in mice, however. In addition to the bioluminescence reporter, the SNAIL1-CBR mouse has a TRE-SNAIL1-Flag overexpression cassette that can be used to induce SNAIL1 expression in a temporal and spatially controlled manner. In cardiomyocytes, overexpression of SNAIL1 for 4 weeks was not sufficient to induce fibrosis. It is possible that the timeframe of 4 weeks of SNAIL1 induction was not sufficient or that SNAIL1 expression cardiomyocytes do not contribute to fibrosis. It would be interesting to determine whether forced overexpression of SNAIL1 in the cardiac fibroblasts is sufficient for fibrosis. This could be achieved by crossing the Col1a2-rtTA mouse to the SNAIL1-CBR/TRE-SNAIL1-Flag mouse and administering doxycycline.

Several cardiac fibroblast Cre recombinase mouse lines have been described but most of them lack specificity\textsuperscript{102}. In our hands we determined that the Col1a1-CreERT2 mouse might be a viable option to delete floxed genes in the cardiac fibroblasts. The collagen promoter is active only during early development and as such tamoxifen administration in these mice have to occur early (during weaning or earlier) to get enough labeling of cardiac fibroblasts in the adult mice. Such early tamoxifen treatment might interfere with normal growth and development of the mice and deleting SNAIL1 might enhance the development defect (if any). The other inducible cre’s i.e. Chicken Actin Globin CreER (CAG-CreER) or its reporter Rosa-LSL-mTmG was leaky in our hands. With RosaCreERT\textsuperscript{2} we didn’t achieve efficient labeling of enough cardiac cells (but
other organs were well labeled). These two inducible Cre lines (i.e. CAG-CreER and RosaCreERT²) mice might be useful in temporal deletion of genes in the whole animal.

We also characterized αSMA-Cre and FSP1 Cre, both reported to be expressed in the cardiac fibroblasts. We were able to obtain SNAIL1<sup>ff</sup> homozygous mice with either of these Cre lines. This finding is significant as the mice develop normally and did not die in-utero as has been observed with genetic deletion of SNAIL1.

αSMA Cre did not express widely in the uninfarcted hearts. Knocking out SNAIL1 using FSP1 Cre, we observed a decrease in cardiac fibrosis. However, this reduction cannot be attributed to cardiac myofibroblasts in the infarct zone as αSMA+ myofibroblasts and FSP1+ cells might belong to distinct lineages. It is possible that SNAIL1 deletion in the FSP+ cells either has a paracrine effect on the myofibroblasts ability to deposit collagen and/or causes a reduction in recruitment of fibrocytes or bone marrow derived cells to the infarct zone, which can explain the small reduction in fibrosis observed.

The two other Cre lines that can be tested further to target cardiac fibroblasts are Col1 α2-CreER and also periostin-Cre, both of which have been shown to be expressed in the cardiac fibroblasts in the infarcted zone post myocardial infarction.
Figure 3.1

A

WT allele

Endogenous Promoter
START
E1
E2
E3
3'UTR

Reporter allele

Endogenous Promoter
ATG
loxP
loxP
lacZ
pA
pA
neo

: Snail1-lacZ heterozygous reporter mouse

B

ES Cell differentiation assay

Snail1-LacZ ES cells Clone AE3
Snail1-LacZ ES cells Clone AG11

Brightfield Images
Blue=LacZ (X-gal staining)
Figure 3.1:

Creating Snail1-LacZ reporter mouse model:

(A) SNAIL1-LacZ targeting construct. One allele of SNAIL1 is replaced by the LacZ gene under the endogenous Snail1 promoter. Two embryonic stem cell (ES) clones AE3, AG11 carrying the SNAIL1-LacZ reporter were obtained

(B) ES cell differentiation assay for both ES cell clones, AG3, AG11, stained for LacZ (X-Gal). LacZ positive cells appear blue.
Figure 3.2

Snail1-IRES-YFP construct

B

Fixed Heart Sections

WT

DAPI
YFP

Snail1-IRES-YFP

DAPI
YFP

C

Fixed Pancreas Sections (+ve control)

DAPI
YFP

D

Isolated Cardiac Fibroblasts (Live cell imaging)

WT

YFP

Snail1-IRES-YFP

YFP
Figure 3.2:

Snail1-IRES-YFP reporter mouse model:

(A) Targeting construct of Snail1-IRES-YFP knock in allele. Mouse was a kind gift from Dr. Xin Ye (Whitehead Institute)

(B) Immunofluorescence staining with anti-GFP antibodies on FFPE normal, uninjured heart and pancreas sections from wild type littermate and SNAIL1-IRES-YFP mouse

(D) Live cell YFP fluorescence of cardiac fibroblasts isolated from wild type littermate and SNAIL1-IRES-YFP mouse
Figure 3.3:

SNAIL1-CBR reporter mouse.

(A) Targeting construct of SNAIL1-CBR mouse. The third (final) exon of SNAIL1 is fused to the Clic Beetle Red (CBR) bioluminescence enzyme and the whole construct is knocked in the endogenous SNAIL1 locus to generate SNAIL-CBR/+ reporter mice. The TRE-SNAIL1-Flag construct can be used to overexpress SNAIL1 in a temporally and spatially controlled manner.

(B) SNAIL1-CBR bioluminescence signal of normal WT littermate and SNAIL1-CBR/+ mice.

(C) SNAIL1-CBR bioluminescence signal of organs isolated from normal WT littermate and SNAIL1-CBR/+ mice.
Figure 3.4

A
Snail1 overexpression mouse Genotype:
αMHC-rtTA ; SNAIL1-CBR / TRE-SNAIL-FLAG

B

<table>
<thead>
<tr>
<th>AGE</th>
<th>P0</th>
<th>Postnatal P21</th>
<th>3 weeks</th>
<th>6 weeks</th>
<th>10 weeks</th>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Doxycycline (DOX) start → Continuous Doxycyclin → Analyze

C

FLAG - IHC

No DOX

4 weeks DOX

D

<table>
<thead>
<tr>
<th></th>
<th>No DOX</th>
<th>4 weeks DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E

Trichrome Staining

No DOX

4 weeks DOX
**Figure 3.4**

**SNAIL1 overexpression in the cardiomyocytes in-vivo**

(A) Genotype of the mouse used to induce overexpression of SNAIL1 in the cardiomyocytes.

(B) Timeline of doxycycline administration

(C) Immunohistochemistry for FLAG in the control (no doxycycline) and experimental (4 week’s doxycycline)

(D) Western blot for FLAG in control (no DOX) and experimental (4 weeks DOX). Each column represents one individual mouse (3 control, 3 experimental)

(E) Representative trichrome staining images for collagen in the control (no DOX) and experimental (4 weeks DOX). N=5 in each group.
Table 3.1: SNAIL1 reporter mice

<table>
<thead>
<tr>
<th>SNAIL1-reporter</th>
<th>Status</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail1-LacZ</td>
<td>Null. No viable SNAIL1-LacZ mouse was obtained from the ES cell injections</td>
<td>-</td>
</tr>
<tr>
<td>SNAIL1-IRES-YFP</td>
<td>Viable with no abnormal behavior noted</td>
<td>Mark expressing SNAIL1 with YFP. Cannot determine subcellular localization of SNAIL1 as YFP is not fused to SNAIL1</td>
</tr>
<tr>
<td>SNAIL1-CBR / TRE-SNAIL1-Flag</td>
<td>Viable as heterozygous mice. Homozygous mice not viable. No abnormal behavior noted</td>
<td>CBR reporter can be used to identify levels of SNAIL1 in organs or group of cells. Single cell resolution of bioluminescence imaging can be achieved in certain imaging systems. TRE-SNAIL1-Flag cassette can be used to overexpress SNAIL1 temporally and spatially by crossing mouse to rtTA or tTA expressing mouse and doxycyclin administration</td>
</tr>
</tbody>
</table>
Figure 3.5

Snail1 fibroblast knockout mouse Genotype:
Snail1 \( f/f \); Col1a1-CreERT2; Rosa LSL tdTomato

<table>
<thead>
<tr>
<th>AGE</th>
<th>Born P0</th>
<th>Wean P21</th>
<th>3 weeks</th>
<th>5 weeks</th>
<th>Tam induction</th>
<th>8 weeks</th>
<th>9 weeks</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isolated Cardiac Fibroblasts at 8 weeks – Live cells

No tamoxifen + Tamoxifen

Bright

tdTomato

Labeled cells

% tamoxifen cells (of total)

BASELINE analysis (8 weeks)

Heart

With Tamoxifen

Skin

DAPI tdTomato

Lungs

DAPI tdTomato

DAPI tdTomato

DAPI tdTomato
**Figure 3.5**

Col1α1-Cre-ERT² characterization in the heart

(A) Genotype of the mouse

(B) Experimental timeline

(C) Cardiac fibroblasts from control (no tamoxifen) and experimental (with tamoxifen) were isolated and imaged for tdTomato

(D) Quantification of tdTomato positive cells (of total cells) of cells shown in (figure 3.5C)

(E) Representative images of heart, skin and lungs of control (no tamoxifen) and experimental (with tamoxifen) at 8 weeks of age, before any infarction.
Figure 3.6

Effect of SNAIL1 knockout on cardiac fibrosis post myocardial infarction by LAD ligation in Snail1 $^{ff}$; Col1α1-Cre-ERT$^2$; Rosa LSL tdTomato mice

(A) Representative images of tdTomato expressing cells in the remote zone and infarct zone of the same heart, 7 days post LAD ligation.

(B) Representative images of Trichrome staining in WT and Snail1 KO mouse in the remote and infarct zone, 7 days post LAD ligation
Figure 3.7

A

Genotype:
Snail1\(^{f/+}\); CAG-CreER; Rosa LSL mTmG

B

<table>
<thead>
<tr>
<th>AGE</th>
<th>Born P0</th>
<th>Wean P21</th>
<th>3 weeks</th>
<th>5 weeks</th>
<th>8 weeks</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Tam induction</td>
<td>Tam washout</td>
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<td></td>
<td></td>
<td></td>
<td>Start Tamoxifen chow</td>
<td>End Tamoxifen chow</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Baseline organs Analysis / Infarction</td>
</tr>
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</table>

C

NO Tamoxifen

Tamoxifen

Heart

Pancreas
Figure 3.7

Cre-ERT2 characterization in the heart

(A) Genotype of the mouse

(B) Experimental timeline

(C) Representative images of heart and pancreas (no tamoxifen) and experimental (with tamoxifen) at 8 weeks of age
Figure 3.8

A

Genotype:
Snail\textsuperscript{1/}\textsuperscript{f/f}; Rosa-CreERT\textsuperscript{2}; Rosa LSL tdTomato

B

\begin{tabular}{|c|c|c|c|}
\hline
AGE & Born & Wean & 5 weeks & 6 weeks \\
\hline
P0 & P21 & 3weeks & & \\
\hline
\end{tabular}

\begin{itemize}
\item Start Tamoxifen chow
\item End Tamoxifen chow
\item Baseline organs Analysis
\end{itemize}

C

\textbf{Heart}

\begin{itemize}
\item DAPI
\item Tomato
\end{itemize}

D

\textbf{Kidney}

\begin{itemize}
\item DAPI
\item Tomato
\end{itemize}

E

\textbf{Liver}

\begin{itemize}
\item DAPI
\item Tomato
\end{itemize}

F

\textbf{Lungs}

\begin{itemize}
\item DAPI
\item Tomato
\end{itemize}

G

\textbf{Mammary Glands}

\begin{itemize}
\item DAPI
\item Tomato
\end{itemize}

H

\textbf{Pancreas}

\begin{itemize}
\item DAPI
\item Tomato
\end{itemize}

I

\textbf{Mammary gland whole mount at 8 weeks}

\begin{itemize}
\item WT
\item Snail\textsuperscript{1/}; RosaCreERT\textsuperscript{2} mouse + Tamoxifen
\end{itemize}
Figure 3.8

RosaCreERT2 characterization in the heart

(A) Genotype of the mouse

(B) Experimental timeline

(C-H) Representative images of heart, kidney, liver, lungs, mammary glands and pancreas from experimental at 6 weeks of age

(I) Mammary gland whole mount from a WT and Tamoxifen treated Snail1f/f; RosaCreERT2 mouse
Figure 3.9

A  Genotype:  
αSMA-Cre ; Rosa LSL tdTomato

B  AGE  
<table>
<thead>
<tr>
<th>Born</th>
<th>Wean</th>
<th>P0</th>
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<th>8 weeks</th>
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<tr>
<td></td>
<td>P0</td>
<td></td>
<td>Wean</td>
<td>8 weeks</td>
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Baseline organs
Analysis

C  
Heart

D  
Pancreas

E  
Liver

F  
Lungs
Figure 3.9

αSMA-Cre characterization in the heart

(A) Genotype of the mouse

(B) Experimental timeline

(C-F) Representative images of heart, pancreas, liver and lungs at 8 weeks of age showing tdTomato expression.
Figure 3.10

A. Snail1 fibroblast knockout mouse Genotype:
Snail1 $^{f/f}$; FSP1-Cre; Rosa LSL tdTomato

<table>
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<th>AGE</th>
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<th>Wean</th>
<th>P21</th>
<th>3 weeks</th>
<th>8 weeks</th>
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<tr>
<td></td>
<td>P0</td>
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</table>

- **C.** Heart
- **D.** Pancreas
- **E.** Liver
- **F.** Lungs

G. Snail1 $^{f/f}$; FSP Cre; Rosa LSL tdTomato
Cardiac fibroblasts (in-vitro)

H. tdTomato and Snail1

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<tr>
<td>Tomato</td>
<td>Tomato + Snail1</td>
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</table>
Figure 3.10

FSP-Cre characterization in the heart

(A) Genotype of the mouse

(B) Experimental timeline

(C-F) Representative images of heart, pancreas, liver and lungs at 8 weeks of age showing tdTomato expression.

(G) Snail1 and tdTomato co-staining in cardiac fibroblasts isolated from Snail1\textsuperscript{fl}; FSP1-Cre; Rosa-LSL-tdTomato mice.

(H) Quantification of tdTomato and Snail1 from (G)
Figure 3.12

A
Infarct Zone

WT

Snail1<sup>1<sup>st</sup></sup>
FSP1-Cre
tdTomato

Trichrome Staining

B
Color Assignment

C
Blue (collagen) / total area assigned (%)

WT
Snail1
KO

D
WT

Snail1<sup>1<sup>st</sup></sup> ; FSP1-Cre ; tdTomato

Verification of Trichrome Staining

E
Snail1<sup>1<sup>st</sup></sup> ; FSP1-Cre ; Rosa-LSL-tdTomato hearts – 7 Days LAD ligation

Remote zone

Infarct Area

Remote zone

Infarct Area

DAPI
Tomato

DAPI
Tomato

111
Figure 3.11

Effect of SNAIL.1 knockout on cardiac fibrosis post myocardial infarction by LAD ligation

(A) Representative trichrome staining for collagen in the infarct zone of WT and Snail1<sup>f/f</sup>; FSP1-Cre; Rosa-LSL-tdTomato mouse hearts 7 days after infarction

(B) Color assignment for images in A. Red is muscle and Blue is collagen

(C) Quantification of fibrosis from color assigned images in B

(D) Trichrome staining in the remote zone to verify trichrome staining (structures like valves and aorta express collagen)

(E) Representative images tdTomato+ cells in the remote zone and infarcted zone in Snail1<sup>f/f</sup>; FSP1-Cre; Rosa-LSL-tdTomato mice 7 days after infarction
Figure 3.12

A  Snail^{1fr}; FSP1-Cre; Rosa-LSL-tdTomato hearts – infarct zone – 7 Days LAD ligation

B  Snail^{1fr}; FSP1-Cre; Rosa-LSL-tdTomato (Control)  Snail^{1fr}; FSP1-Cre; Rosa-LSL-tdTomato (Snail1 KO)

Infarct Zone  Infarct Zone
Figure 3.12

**Characterization of FSP+ cells in the heart 7 days after LAD ligation**

(A) Co-immunofluorescence with Tomato+ cells (i.e. FSP1+ cells) in the infarct region with SNAIL1 and vimentin

(B) $\alpha$SMA staining in control (Snail1$^{+/+}$; FSP1-Cre; Rosa-LSL-tdTomato) and Snail1 KO hearts (Snail1$^{+/f}$; FSP1-Cre; Rosa-LSL-tdTomato) in the infarct region. $\alpha$SMA and tdTomato do not localize in either the control or SNAIL1 KO tissue sections.
Table 3.2: Characterization of Cre strains to label cardiac fibroblasts

<table>
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<tr>
<th>Cre strain</th>
<th>Infarction</th>
<th>Survival</th>
<th>Cardiac fibroblasts labeled?</th>
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<tr>
<td>Col1\textsuperscript{\textalpha}1-CreERT2</td>
<td>Yes</td>
<td>4/6</td>
<td>Baseline – Minimal Post Infarction – Yes</td>
</tr>
<tr>
<td>CAG-CreER</td>
<td>No</td>
<td>-</td>
<td>Yes. But cre and/or mTmG reporter expression leaky in our strain</td>
</tr>
<tr>
<td>Rosa-CreERT2</td>
<td>No</td>
<td>-</td>
<td>Very few cells labeled in the normal un-injured heart. Identity of these cells is not determined</td>
</tr>
<tr>
<td>\textalpha SMA Cre</td>
<td>No</td>
<td>-</td>
<td>Very few cells labeled in the normal un-injured heart. Identity of these cells is not determined.</td>
</tr>
<tr>
<td>FSP1-Cre</td>
<td>Yes</td>
<td>3/8</td>
<td>Many FSP1 expressing cells present in infarct zone but are not \textalpha SMA positive. Identity of these cells needs to be determined</td>
</tr>
</tbody>
</table>
Chapter 4: Conclusion and future direction
In summary, we show that SNAIL1 protein is not expressed in the normal un-infarcted hearts. SNAIL1 levels increase during hypoxic injury to the heart i.e. myocardial infarction. The expression of SNAIL1, as determined by SNAIL1-CBR bioluminescence, is restricted to the ventricular infarct zone and SNAIL1 expression is not observed in the remote zone of the heart or sham hearts. The SNAIL1-CBR signal from the heart in the whole animals is obscured by the thymus which expresses significant SNAIL1-CBR bioluminescence signal. The difference in SNAIL1 signal between sham and infarcted hearts became apparent when the hearts were imaged ex-vivo. The increased SNAIL1 levels in the ventricular infarct zone is positively correlated with fibrosis and expression of pro-fibrotic cytokines. SNAIL1 is expressed predominantly in the myofibroblasts in the heart which are marked by aSMA and periostin and to a smaller extent in CD45+ leukocytes.

Interestingly, only a fraction of the total myofibroblasts express SNAIL1. This could be because SNAIL1 expression is transient and not all the myofibroblasts are expressing SNAIL1 simultaneously. Moreover, SNAIL1 is an unstable protein with a half-life of 20 minutes and thus not all myofibroblasts don’t appear positive for SNAIL1 at the same instance. Myofibroblasts are heterogeneous as they can be derived from multiple sources and as such, not all myofibroblasts may express SNAIL1. Alternatively, there could be other fibrosis promoting transcription factors that are expressed in the other (non SNAIL1) expressing myofibroblasts. One of them could be Twist1, a basic helix-loop-helix transcription factor that has very similar functions to SNAIL1, and infact it can bind to E-box sequences. Twist 1 has been implicated in cardiac fibrosis as well\textsuperscript{80}.

The increase in SNAIL1-CBR bioluminescence signal in the heart after myocardial infarction can be attributed to increase in SNAIL1 protein level per individual myofibroblast and
collectively the increased number of myofibroblasts in the infarct zone contribute to the overall SNAIL1-CBR bioluminescence signal. The increase in SNAIL1-CBR signal also correlates to the magnitude of cardiac injury. SNAIL1-CBR levels were higher (10 fold over sham) in Left Anterior Descending artery (LAD) occlusion mice as compared to ischemia reperfusion (I.R) surgery (2 fold over sham). LAD surgery causes more injury to the heart as it is a permanent occlusion, whereas in the ischemia reperfusion surgery, the occlusion is for 30 minutes followed by the re-perfusion injury. The ischemia-reperfusion surgery has lesser inflammation due to surgery, as the 30 minute ligation is done a week after putting in the suture in the heart. In contrast, when fibrosis was induced by Angiotensin II infusion to the mouse hearts, we did not observe a significant increase in SNAIL1 bioluminescence. This could be because Angiotensin II causes a more systemic fibrosis in the heart over a longer period of time (2 weeks) as compared to the more rapid induction of fibrosis (1 week) by surgical methods i.e. myocardial infarction. In Angiotensin II infusion, the fibrosis is not localized to one region of the heart but rather is reactive fibrosis throughout the interstitial and perivascular spaces16.

It is an important finding that SNAIL1 is expressed in the myofibroblasts in the infarct zone. The myofibroblasts in the infarct zone can be derived from multiple sources including activation and proliferation of resident cardiac fibroblasts, Epithelial to Mesenchymal Transition of epicardial cells, Endothelial to Mesenchymal Transition of endothelial cells, pericytes, circulating fibrocytes and bone marrow derived progenitor cells26. It has been shown that SNAIL1 can cause cells to undergo EMT, EndMT and induce migration. Thus there is a possibility that during cardiac fibrosis, SNAIL1 is expressed in the epicardial, endothelial or other cells, which then can become myofibroblasts and/or migrate to the infarcted regions and
contribute to fibrosis. To address these questions, lineage tracing of the myofibroblasts from various sources can be performed.

Many pro-fibrotic factors are secreted in the infarcted myocardium following injury. These various factors signal through distinct pathways, but there is always cross-talk between them. These factors include TGFβ (TGFβ signaling pathway), PDGF (Jak-Stat pathway), Angiotensin II (GPCR [Gq] signaling pathway) and hypoxia (via Hif). Interestingly, all of these factors increased SNAIL1 bioluminescence (i.e., SNAIL1 protein level) in cultured SNAIL1-CBR cardiac fibroblasts. Out of these only TGFβ increased SNAIL1 mRNA levels. This suggests that during normal conditions, SNAIL1 mRNA in cardiac fibroblasts is maintained at a low level and the resulting SNAIL1 protein is kept at a low level via the GSK3β/Axin/βCatenin complex by targeting SNAIL1 for degradation by the ubiquitin proteasome pathway.

TGFβ is a potent pro-fibrotic cytokine which causes expression, synthesis, secretion and accumulation of extracellular matrix, collagen I in particular. The increase in collagen transcription is mediated through the binding of SMAD2/3 complex following TGFβ stimulation. Since TFGβ also increases SNAIL1 transcription, we asked whether SNAIL1 might affect collagen gene expression directly or indirectly. We have shown that SNAIL1 deletion in cardiac fibroblasts results in decreased Collagen I gene expression and subsequently decreased extracellular matrix deposition in-vitro. This finding suggests that the effect of SNAIL1 on collagen synthesis probably occurs at a post transcriptional level (REALLY???). Interestingly, SNAIL1 deletion in cardiac fibroblasts does not have an effect on other extracellular matrix gene mRNA levels like collagen III or fibronectin.
The cardiac fibroblasts adopt a αSMA expressing myofibroblast phenotype during cardiac fibrosis. Expression of αSMA can occur through activation of Rho > MRTF A/B > SRF pathway in cardiac fibroblasts. In our hands we determined that SNAIL1 deletion abrogates expression of αSMA in cardiac fibroblasts when cultured on a high stiffness environment (tissue culture plates). In essence, lack of SNAIL1 prevents the fate change of cardiac fibroblasts into myofibroblasts. This observation can add to the theme of SNAIL1 as a cell fate determining protein as SNAIL1 can induce epithelial cells to undergo a mesenchymal cell fate change and is also implicated during differentiation of embryonic stem cells into fibroblasts. In addition it is likely that SNAIL1 could exert its effect on collagen I transcript levels by repressing mir29, which is a repressor of collagen I mRNA and other pro-fibrotic mRNA.

In our studies we also show that the excess collagen deposited during fibrosis can increasing SNAIL1. Cardiac fibroblasts express the Discoidin domain receptor 2 (DDR2) and it has been shown that activation of DDR2 receptor via its ligand fibrillar collagen, can stabilize SNAIL1 post transcriptionally. It is possible that the collagen deposited in the fibrotic scar following cardiac injury, activates SNAIL1 via DDR2 receptor engagement, which in turn sustains more fibrosis secreting more collagen I, creating a feed forward loop.

We attempted to knockout SNAIL1 in the cardiac fibroblasts in-vivo using several different Cre recombinase mouse strains. Out of these only Col1a1-CreERT2 and FSP1 seemed promising. We observed a slight reduction in fibrosis (my trichrome staining) at 7 days post infarction, in SNAIL1 knockout mice using FSP1 Cre (Snail1^{ff}; FSP1 Cre; Rosa-LSL-tdTomato) as compared to wild type controls. However, FSP1 does not appear to be expressed in the αSMA+ myofibroblasts in the infarct zone. Further investigation is warranted in this study with the use of genetically control (i.e. FSP1 Cre; Rosa-LSL-tdTomato) mice.
Future directions

Several unanswered questions remain considering the role of SNAIL1 in cardiac fibrosis. There are several sources of cardiac myofibroblasts that accumulate in the infarct region of the heart. We have shown that SNAIL1 might be responsible for converting the cardiac fibroblasts into myofibroblasts \textit{in-vitro}. Preliminary in-vivo data using FSP1 and Col1a1-CreERT2 to knockout SNAIL1 suggests a role of SNAIL1 in cardiac fibroblasts to contribute to cardiac fibrosis. However, further investigation is warranted to determine this effect \textit{in-vivo} and periostin-Cre or Col1a2-CreERT2, which has been shown to be expressed in cardiac fibroblasts, can be used to determine this. Additionally, whether SNAIL1 expression is necessary in other cells types like endothelial cells, pericytes, fibrocytes or bone marrow derived progenitor cells to traffic to the infarction site and become myofibroblasts remains to be determined. This can be achieved by deleting SNAIL1 by using crossing Snail$^{Ef}$; Rosa LSL tdTomato mice to cell type specific Cre mice such as Tie-2Cre (endothelial); NG2-Cre (pericytes); Twist2-Cre (BMDC).

Whether SNAIL1 expression in cardiac fibroblasts is sufficient to induce cardiac fibrosis \textit{denovo} is unknown. To determine this, SNAIL1-CBR / TRE-SNAIL-Flag mouse can be crossed to Col1a2-rtTA mouse. When administered doxycycline, this mouse should overexpress SNAIL1 in the cardiac fibroblasts. However, since the collagen promoter active in the murine hearts during development, the doxycycline induced SNAIL1 expression has to be done during early development stages.

Our preliminary data suggests involvement of DDR2 signaling in cardiac fibroblasts to promote fibrosis. Involvement of DDR2 during Angiotensin II mediated cardiac fibrosis has been determined. Thus, during cardiac fibrosis, the excessive collagen deposition might
activate the collagen receptor DDR2 in the cardiac (myo)fibroblasts and this might stabilize
SNAIL1. Accumulation of SNAIL1 in these cells might cause a feed forward loop to synthesize
and secrete more collagen. The effect of DDR2 signaling on cardiac fibrosis can be determined
by subjecting DDR2 null mice to myocardial infarction. Alternatively DDR2 \(^{f/f}\) mice crossed to
cardiac fibroblast specific Cre can be used for this experiment. It is likely that lack of DDR2 may
not affect initial deposition of collagen in the infarct zone but rather have an effect during the
remodeling phase of the scar formation. These experiments need to be interpreted cautiously as
DDR2 null mice have altered heart structure and impaired cardiac fibroblast function\(^{105}\).

SNAIL1 affects collagen I deposition and is responsible for conversion of cardiac
fibroblasts to myofibroblasts. However, the precise molecular mechanisms that control these
pathways still needs to be determined. It is evident that the transcriptional control of collagen 1a1
and collagen 1a2 genes in response to TGF\(\beta\) stimulation is not equivalent. SMAD2/3/4 complex
regulates transcription of Col1a2 gene directly via binding to SMAD recognition element in its
promoter. Col1a1 gene does not have a SMAD binding site in its promoter, and SMAD2 requires
a co-factor Sp1 to bind to Col1a1 promoter and induce its transcription. We see that collagen 1a1
mRNA levels decrease in absence of SNAIL1, even with TGF\(\beta\) stimulation. Whether SNAIL1
affects Col1a1 gene transcription as a co-activator with SMAD2/SP1 or via repressing collagen
Ia1 translation via repression of mir29 family remains to be determined. It is likely that there
might be other mechanisms by which SNAIL1 might affect collagen gene transcription.
Therapeutic targeting of fibrosis

Although fibrosis is a widespread disease affecting multiple organs, there are very limited therapeutic options. Managing fibrosis in the heart following myocardial infarction is especially challenging, as the scar formation is necessary initially to prevent cardiac rupture. Ideally the therapy needs to allow for a better scar formation, which would prevent rupture but not propagate beyond that and allow the scar to heal overtime, thereby restoring cardiac function. Since SNAIL1 is involved in cardiac fibrosis, targeting SNAIL1 directly or pathways that increase SNAIL1 or the mechanisms by which SNAIL1 regulates cardiac fibrosis would represent attractive targets.

SNAIL1 expression in the heart increases only in the cardiac fibroblasts only in the infarct zone. Thus after fibrosis cascade is initiated in the heart following myocardial infarction and the formed scar has stabilized the wound, blocking SNAIL1 in the infarct area either via a siRNA delivery (retroviral or liposomal mediated carrier) or CRISPR/Cas9 mediated SNAIL1 gene excision could probably be used to reduce fibrosis. These approaches need more research and development to be actually viable. Even if SNAIL1 is suppressed/deleted in neighboring cardiomyocytes or other cells, it should not have serious effects as SNAIL1 is not expressed in these cells. Another more promising approach would be to block DDR2 signaling using DDR2 blocking antibodies or other methods. MicroRNA’s which affect collagen’s can also be a target.
Chapter 5: Materials and methods
Mice and animal husbandry

All mice were housed and experiments performed according to institutional guidelines. Production of SNAIL1-CBR/+ mice has been previously described\textsuperscript{62}. SNAIL1\textsuperscript{f/f} mice were provided by S. Weiss (U. Michigan)\textsuperscript{76}. All mice were on mixed genetic backgrounds. Experiments were carried out on 8-10 week old mice. All mice were used in compliance with the Washington University Institutional Animal Care and Use Committee under protocol #20150145. All mouse experiments were reviewed and approved by the Washington University Institutional Animal Care and Use Committee under protocol #20150145. Approximately 20 SNAIL1-CBR/+ mice and 20 SNAIL1\textsuperscript{f/f} mice were used for these experiments. 5 mice were kept per cage with 12 hour light / 12 hour dark cycle and standard rodent chow and water available \textit{ad libitum}. The mice were monitored everyday.

Mouse surgeries and tissue processing

Left Anterior Descending Artery (LAD) occlusion and Closed Chest Ischemia – Reperfusion (I/R) surgeries were performed at the Mouse Cardiovascular Phenotyping Core at Washington University, in compliance with the Washington University Institutional Animal Care and Use Committee under protocol #20150145. For Closed-Chest I/R surgery, the mice were surgically prepped and ventilated. The mice were taped to an ECG board (lead II) to measure S-T segment elevations during ischemia and reperfusion. After a midline incision and small “non-rib cutting” thoracotomy, the pericardium was gently dissected to visualize the coronary anatomy. An 8-0 polypropylene suture with a U-shaped tapered needle was passed under the LAD at a consistent level on the heart directly underneath the LA. The needle was then cut from the suture and the
two ends of the 8-0 suture was threaded through a 0.5mm piece of PE-10 tubing that had been previously soaked for 24 hours in 100% ethanol. The tubing formed a loose snare around the LAD. Each end of the suture was then threaded through the end of a size 3 Kalt suture needle and exteriorized through each side of the chest wall. The chest was closed with interrupted stitches. The ends of the exteriorized 8-0 suture was tucked under the skin and the skin closed.

The mice were removed from the respirator, kept warm and allowed to recover to full consciousness. After a recovery period of 1 week after initial instrumentation, the animals were reanesthetized under isoflurane (1.5% maintenance) but not mechanically ventilated and ONLY the skin above the chest wall was reopened. The 8-0 suture ends were cleared of all debris and carefully secured in small hemostats. Ligation of the LAD for 90 minutes was accomplished by gently pulling the hemostats apart and anchoring them until the S-T segment elevation appeared on the EKG. The EKG was constantly monitored throughout the occlusion period to ensure persistent ischemia. At the end of the 90 minutes, reperfusion was accomplished by releasing the hemostats, cutting the suture close to the chest wall, and releasing the tension. Reperfusion was confirmed by resolution of the S-T segment elevation. The skin was closed with suture and the animal was allowed to recover on a warmer for 1-7 days.

For Permanent LAD occlusion surgery, mice were anesthetized with Ketamine/Xylazine (100/10 mg/kg) and surgically prepped and ventilated on a heated magnetic stainless steel surgical board. After a midline incision and small “non-rib cutting” thoracotomy, the chest wall was retracted to better expose the left ventricle and the left main coronary artery system. The left anterior descending branch of the left coronary artery was then ligated with an 8-0 silk suture. This occlusion will be accomplished by passing a tapered needle modified to a U-shape, underneath the LAD at a consistent level on the heart directly underneath the left atrium and tying this suture
directly over the vessel. Mice were then hyperventilated at 150 beats/min until the chest was closed by purse string suture. The surgical incision was closed in two layers with an interrupted suture pattern. The animals were allowed to recover and kept warm on a heating pad throughout the procedure until extubation, return to sternal position, and normal activity. The mice were monitored twice daily for 7 days.

Hearts from the infarcted mice were harvested 7 days post infarction. Hearts were rinsed in PBS and placed in 10% neutral buffered formalin overnight at room temperature. The hearts were cut using a zivic heart slicer into infarcted region (distal to ligation) and remote region (proximal to ligation). The hearts sections were either placed in 70% ethanol for paraffin embedding or cryoprotected in 30% sucrose for embedding in Optimal Cutting Temperature (OCT) compound. For mRNA isolation, the left ventricle was dissected under a dissecting microscope and mRNA was extracted using a Qiagen mini RNA kit.

**Cardiac fibroblast isolation and cell culture**

Cardiac fibroblasts were isolated according to a modified cardiomyocyte isolation protocol. Hearts were obtained from mice after anesthetizing with isoflurane followed by cervical dislocation. The hearts were excised, placed in sterile PBS. In a tissue culture hood, hearts were finely minced with sterile scissors and transferred into a 50 ml falcon tube with 9 mL of Wittenberg Isolation Medium [NaCl (116 mM), KCl (5.4mM), MgCl2 (6.7mM), glucose (12mM), glutamine (2mM), NaHCO3 (3.5mM), KH2PO4 (1.5mM), Na2HCO3 (1mM), HEPES (21mM), commercial vitamin solution (1X), commercial amino acid solution (1X)] suppled
with trypsin (1X) and collagenase II (0.8 mg/mL). The minced tissue was placed on a rotator in a 37°C incubator for 15 minutes. The tubes were spun down for 5 minutes and the supernatant (extract 1) was discarded. The extraction process was repeated 4 times with the remaining tissue and the supernatants (extract 2-5) were pooled, spun down and plated in p100 tissue culture dishes in DMEM/F12 media with 10% Fetal Bovine Serum and 1% penicillin – streptomycin. The media was changed after 6 hours to allow cells to adhere and wash away the debris. Media was replaced every other day until cells were confluent (~5 days). Fibroblast identity was verified by vimentin and DDR2 expression.

**Bioluminescence imaging**

For live animal and *ex-vivo* heart imaging, the mice were injected with d-luciferin 10 minutes before imaging, anesthetized using isoflurane and imaged with the IVIS-100 instrument.

For live cell imaging, $8 \times 10^3$ SNAIL1-CBR/+ primary cardiac fibroblasts (passage 2 or 3) were plated in 96 well black well plates. After 20 hours, cells were washed with PBS, and serum free, phenol red-free medium added. After 4 hours of serum starvation, d-luciferin was added along with one of the following: TGFβ (2ng/mL), PDGF (10ng/mL), Angiotensin II (1 micromol/L), CoCl$_2$ (400uM). The plate was imaged every 15 minutes for 4 hours with IVIS 100 instrument at 37°C with ~5% O$_2$ flow the imaging chamber. For collagen I stimulation, the cells were serum starved and then plated on tissue culture plates coated with 60 uL of 1 - 4 mg/mL collagen I gel and imaged as described before.
**Cardiac fibroblast immortalization and SNAIL1 gene deletion**

Cardiac fibroblasts from hearts of SNAIL1\(^{f/f}\); ROSA-LSL-tdTomato mice were isolated and immortalized by allowing the cells to undergo cellular senescence i.e. \(\sim 15\) passages. The emergent cell line was sorted for PDGFR\(\alpha\) positive cells and cellular phenotype verified by vimentin expression.

To delete the SNAIL1 gene, \(1 \times 10^6\) SNAIL1\(^{f/f}\); ROSA-LSL-tdTomato cardiac fibroblasts were infected with either Adeno-LacZ (control, denoted CTL) or Adeno-Cre (experimental, denoted + Cre) viruses at an MOI of 50. The virus was removed after 4 hours, cells washed and used 48 hours post infection. The rate of infection was \(>98\%\) as determined by tdTomato expression. SNAIL1 gene deletion was verified by qPCR and western blotting. Cells were freshly infected for each experiment.

**qPCR Analyses**

500 ng of mRNA was used to synthesize cDNA using the Invitrogen Superscript II kit. qPCR was performed using 1uL of cDNA in a 20 uL reaction using the SyBr green reagents (Applied Biosystems) on StepOnePlus instrument (Applied Biosystems). The cT values were normalized to GAPDH and the fold change was calculated using the \(2^{-\Delta\Delta C_{T}}\) method.

**Western blotting**

Western blotting was done according to standard protocols. 50ug protein was loaded in each lane. The following antibodies were used: SNAIL1 (1:100 Cell Signaling), Periostin (1:500, gift
from Dr. Russell Norris, MUSC), β tubulin (1:10,000 Sigma), DDR2 (1:500, Cell Signaling) ERK (1:1000, Cell Signaling) phospho-ERK (1:1000, Cell Signaling), AKT(1:1000, Cell Signaling), phospho-AKT(1:1000, Cell Signaling ), STAT1 (1:1000, Cell Signaling), phospho-STAT1 (1:1000, Cell Signaling)

**Ex vivo Matrix deposition and analysis**

A modified scar-in-a-jar assay was used for matrix deposition by cardiac fibroblasts\textsuperscript{106}. \(1 \times 10^5\) control or SNAIL1 knockout cells were plated on sterile 100mm coverslips overnight. The cells were then treated everyday with fresh media containing 50 \(\mu\)g/mL ascorbic acid for 7 days, supplemented with 10 ng/\(\mu\)L PDGF every other day. The cells were removed by treating with prewarmed extraction buffer for 3-5 minutes (25 mmol/L Tris-HCl, pH 7.4; 150 mmol/L sodium chloride; 0.5% Triton X-100; and 20 mmol/L ammonium hydroxide).

For collagen I immunofluorescence (1:100, BD bioscience antibody), the matrix was fixed with 4% paraformaldehyde for 10 minutes. For Sirius red staining cells were fixed in bouins fixative for 20 minutes and washed in tap water. Staining was then performed according to standard protocol\textsuperscript{107}. Birefringence imaging was performed on the Sirius red stained sections under polarized light\textsuperscript{108}.

**Histology and immunostaining**

5 \(\mu\)m sections of paraffin or OCT embedded hearts were utilized for Masson’s Trichrome Staining (KY034, Diagnostic BioSystems) and immunofluorescence. Immunofluorescence was
performed according to standard protocol. The following antibodies were used: SNAIL1 (1:100, Cell Signaling), αSMA (1:300, Sigma), Periostin (1:100, Cell Signaling), CD31 (1:200, Abcam), CD45 (1:100, BD Biosciences), αactinin (1:100, Abcam). For SNAIL1 immunofluorescence, antigen retrieval was done in Nuclear Decloaker solution (CB911M, Biocare Medical) and TSA plus kit (Perkin Elmer) was used to amplify SNAIL1 signal.

**Gel Contraction Assay**

5x10⁴ CTL or Experimental (+Cre) cardiac fibroblasts were embedded in 2mg/mL of collagen I gel (Rat tail collagen 4mg/mL, 10%FBS and 23uL of NaOH /mL of collagen). 500uL of the cells-collagen was plated in 24 well plate in triplicate for each condition. The gel was solidified at 37°C for 30 minutes and the gel was released from the sidewall of the well by gently running a 10uL pipette tip. 1mL of DMEM/F12 media with 2% FBS and 1% Penicillin-Streptomycin was added on top. The gels were imaged at baseline and every 4 hours subsequently. The percent contraction was measured by comparing the initial image area to contracted image area by ImageJ software (surface area function). Data representative of 3 independent experiments.

**Image analysis**

Sirius Red stained images and birefringence images were analyzed with a custom image segmentation algorithm written in MatLab (The Mathworks, Inc.). Images were converted from the standard RGB color space to the CIE L*a*b* color space, which encodes perceived lightness and color differences well. Each pixel in the image was classified as positive (collagen I) or negative (background) based on its distance in CIE L*a*b* space to the closest member of an
empirically determined set of positive and negative training pixels. Positive signal was quantified as the fraction of positive pixels out of the total number of pixels in an image.

**Statistics**

Student’s t test was used to determine statistical significance. p values < 0.05 was considered statistically significant.
Chapter 6: References


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Chapter 7: Appendix
Abstract

SNAIL1, a zinc finger transcription factor, is considered the master regulator of Epithelial to Mesenchymal Transition. Induction of SNAIL1 in cancer cells induces EMT and confers invasive and migration properties to the cancer cells. SNAIL1 has also been observed to be expressed in the tumor stromal cells and is thought to actively remodel the extra cellular matrix (ECM), thereby increasing the invasive potential of the cancer cells. In this study, we determined that SNAIL1 expression in the Fibroblast Specific Protein 1 (FSP1) positive stromal cells does not play a role in MMTV-PyMT breast cancer metastasis to the lungs. However, a heterozygous knockout of SNAIL1 in the FSP1+ cells increases metastasis of the MMTV-PyMT breast cancer cells to the lungs.
**Introduction**

SNAIL1, a zinc finger transcription factor, highly regulated protein that is normally expressed at very low levels in normal epithelial cells\(^1\). Expression of SNAIL1 can cause epithelial cells to undergo a cell fate change called Epithelial to Mesenchymal transition (EMT) which confers invasion and migration properties to epithelial cells\(^2\). The transient expression of SNAIL1 and subsequent induction of EMT is critical for many developmental processes, like gastrulation, mammary duct formation by epithelial cell invasion into the mammary fat pad etc\(^3\). Cancer cells, which often arise from epithelial cells, express SNAIL1 and this is thought to contribute to the invasiveness and metastatic potential of cancer cells\(^3\). Knockout of SNAIL1 in mammary epithelial cells in MMTV-PyMT mouse tumor models decreases the incidence of lung metastasis\(^4\). In some studies, SNAIL1 has been shown to be expressed in the tumor-stroma interface, suggesting a possible role of SNAIL1 in the tumor stroma\(^5,6\) (appendix figure 1A). Recently, it was shown that SNAIL1 expression in the tumor stroma, and not tumor cells is predictive of survival in human breast cancer (appendix figure 1B)\(^7\). Whether SNAIL1 expression in the tumor stroma affects breast cancer metastasis in mouse tumor models is still unknown. In this study, we knocked out SNAIL1 in the Fibroblast Specific Protein1 (FSP1+) expressing cells in mice with a genetic MMTV-PyMT tumor. We did not observe any significant difference in the growth rate of tumors and the number of lung metastasis in the control and the SNAIL1 knockout in FSP1+ cells. Surprisingly, there was a significant increase in the lung metastasis nodules in SNAIL1 heterozygous mice. Moreover, the mammary tumors in the SNAIL1 heterozygous mice have a more variegated appearance and the lung metastasis are larger in size. Since SNAIL1 is self-regulated (i.e. SNAIL1 shuts down its own transcription)\(^8\), there is a possibility that the amount of SNAIL1 protein in a heterozygous mouse is not high.
enough to auto-regulate its own transcription. Alternatively, the half-life of SNAIL1 in a heterozygous cell could be doubled as the amount of SNAIL1 required to trigger self-regulation would take twice as long to saturate. These studies are preliminary and more investigation needs to be conducted with increased number of mice in the study.
Materials and Methods

Mammary gland whole mount and carmine alum staining

Mammary glands +4 from adult female virgin mice at 8 weeks of age was harvested and whole mount carmine alum staining to visualize ducts was performed as previously described\(^9\).

Histology and immunostaining

5 um sections of paraffin embedded mammary glands were utilized for immunofluorescence staining. Immunofluorescence was performed according to standard protocol. The following antibodies were used: SNAIL1 (1:100, Cell Signaling), \(\alpha\)SMA (1:300, Sigma), CD31 (1:200, Abcam), CD45 (1:100, BD Biosciences), K8 (1:50, DSHB), K14 (1:200, BioLegend), Vimentin (1:200, Abcam), tdTomato (1:200, Rockland). For SNAIL1 immunofluorescence, antigen retrieval was done in Nuclear Decloaker solution (CB911M, Biocare Medical) and TSA plus kit (Perkin Elmer) was used to amplify SNAIL1 signal.
Results

SNAIL1 knockout in the mammary stromal cells does not affect mammary gland development

To knockout SNAIL1 in the stromal cells, we crossed FSP1-Cre mice to SNAIL1\textsuperscript{f/f} mice and Cre recombinase reporter mice to generate SNAIL1\textsuperscript{f/f} FSP1-Cre; Rosa LSL tdTomato mice, henceforth referred as SNAIL1 KO mice (Appendix figure 2A). Any cell in these mice expressing FSP1-Cre will delete SNAIL and turn on the tdTomato reporter. The advantage of using the FSP1-Cre as opposed to an inducible stromal Cre (like Col1a2-CreERT2) is that Tamoxifen is not required to be administered to induce gene deletion, as tamoxifen might affect the mammary gland. Although the disadvantage of using FSP1-cre is that SNAIL1 deletion cannot be achieved in a temporally restricted manner.

The SNAIL1\textsuperscript{f/f} FSP1-Cre; Rosa LSL tdTomato mice were born in expected Mendelian ratios, bred normally and no gross defects were observed in them as compared to their wild type (SNAIL1\textsuperscript{f/f}; Rosa LSL tdTomato) littermate controls. The mammary glands from adult WT and SNAIL1 knockout appeared normal, however, the secondary and tertiary branching appeared to be increased in the SNAIL1 knockout mammary gland (Appendix figure 2B). In a control experiment, SNAIL1 was deleted in the entire mammary gland (and the entire animal) in a SNAIL1\textsuperscript{f/f}; Rosa-CreERT2; Rosa LSL tdTomato mouse by administering tamoxifen at 3 weeks of age for 2 weeks and tamoxifen wash out for additional 3 weeks. The mammary glands from these mice showed a defect in mammary gland development and invasion in the mammary fat pad (appendix figure 2C).
**FSP1+ is expressed in mammary gland lymph node and in mammary gland fibroblasts**

To determine where FSP1-Cre is expressed in the mammary gland, the entire #4 mammary gland from virgin adult female FSP1-cre; Rosa LSL tdTomato mice was excised and directly imaged without fixation for tdTomato. Under low magnification, bright tdTomato expression was expressed in the mammary lymph node. Under high magnification tdTomato expressing cells were seen surrounding in the mammary ducts and also distributed between the mammary fat cells. This is further verified by co-immunostaining for tdTomato and cell type specific markers for ductal epithelium (αSMA), luminal epithelium (K8), basal epithelium (K14) and fibroblast (Vimentin) (Appendix figure 4).

**SNAIL1 knockout in FSP1+ cells does not affect tumor growth rate and burden.**

To determine the effect of SNAIL1 deletion in the stromal cells on mammary tumor growth and metastasis, the SNAIL1^{ff}; FSP1-Cre; Rosa LSL tdTomato mice were crossed to MMTV-PyMT mice to generate SNAIL1 KO; MMTV-PyMT mice. Two groups of control mice were used:

1. SNAIL1^{+/-}; FSP1-Cre ; Rosa LSL tdTomato; MMTV-PyMT
2. SNAIL1^{ff}; FSP1-Cre ; Rosa LSL tdTomato; MMTV-PyMT

PyMT was chosen as it has high penetrance and relatively quick metastasis rate to lungs. There was no gross difference in tumor development, measured as a function of age to reach 2cm tumor, size of the biggest tumor and average tumor volume.
SNAIL1 gene dosage has an effect on breast cancer lung metastasis and gross tumor characteristics

Lungs harvested from WT SNAIL1 control and SNAIL1 KO tumor bearing mice at the end point (2cm tumor) did not show a significant difference in the number of metastasis per lobe. However, the SNAIL1 het tumor bearing mice, had a significantly large number of metastatic nodules per lobe. The primary tumors from these three groups of tumor bearing mice (SNAIL1 WT, SNAIL1 Heterozygous, and SNAIL1 KO) were scored for gross appearance. The WT and the SNAIL1 KO mice had predominantly rounded tumors whereas the SNAIL1 heterozygous knockout mice had tumors with a variegated (invasive) morphology.
Appendix Figure 1

A

![Images a and b with T and S labels](Franci 2006)

F

- **Stroma cells**
  - Cumulative survival
  - Snail1-negative
  - Snail1-positive
  - years: 0, 2, 4, 6, 8, 10
  - P = 0.001

- **Tumor cells**
  - Cumulative survival
  - years: 0, 2, 4, 6, 8, 10
  - P = 0.346

Appendix Figure 1:

A. Immunohistochemistry staining showing expression of SNAIL1 in tumor stroma interface. T=Tumor, S=Stroma, Brown staining = SNAIL1. Adapted from Franci et.al\textsuperscript{10}

B. Kaplan–Meier analysis showing SNAIL1 expression in stroma and not tumor is predictive of survival. Adapted from Stanisavljevic et al\textsuperscript{7}.
Appendix Figure 2

A.  **Snail1 fibroblast knockout mouse Genotype:**  
Snail1 \(^{+/+}\); FSP1-Cre; Rosa LSL tdTomato

B.  
- Mammary Glands – Whole mount
  - No defect in branching

![WT image]

![KO image]

C.  **Control complete SNAIL1 KO mammary gland whole mount**  
Genotype: Snail1 \(^{+/+}\); Rosa-CreERT2; Rosa LSL tdTomato  
Tamoxifen chow fed for 2 weeks at weaning. Analysis @ 8 weeks

![Control image]
Appendix Figure 2:

A. SNAIL1 knockout mouse phenotype

B. Mammary gland whole-mount from 8 week old WT and SNAIL1 knockout virgin adult female mice.

C. Mammary gland whole mount from an 8 week old SNAIL1 knockout mouse (Snail1<sup>fl/fl</sup>; RosaCreERT<sub>2</sub>; Rosa-LSL-tdTomato).
Appendix Figure 3:

A. tdTomato expression in a freshly isolated mammary gland from FSP1-Cre; Rosa LSL tdTomato mice. Each successive image is a zoomed in view.

B. Immunofluorescence detection of tdTomato using tdTomato antibody.
Appendix Figure 4:

Immunofluorescence of tdTomato and cell type specific markers in a FSP1-Cre; Rosa LSL tdTomato mammary gland isolated from an adult virgin female mouse.
Appendix Figure 5

A) Age to End Point

B) Volume of biggest tumor

C) Average Tumor Burden
Appendix figure 5

A. Time taken for tumor to each end point (2cm) in control (SNAIL1\textsuperscript{f/f}; MMTV-PyMT; Rosa-LSL-tdTomato), Snail1 KO (SNAIL1\textsuperscript{f/f}; MMTV-PyMT; FSP1-Cre Rosa-LSL-tdTomato) and Hets (SNAIL1\textsuperscript{f/+}; MMTV-PyMT; Rosa-LSL-tdTomato).

B. Volume of the biggest tumor in the above mentioned mice at end point.

C. Average tumor burden of the three groups of mice at end point of experiment.
Appendix Figure 6

A

Mets / 5 lobes

250
200
150
100
50
0

Control
Snail1 KO
Snail1 Hets

Genotype

B

Control
Snail1 KO
Snail1 Hets
Appendix figure 6:

A. Total number of lung mets, quantified from H&E stained sections of the 5 lung lobes, in control, SNAIL1 KO and Snail1Het tumor bearing mice analyzed at end point of experiment (2cm primary tumor). 3 slides were counted for each mouse at 200micron depth from surface.

B. Representative H&E images of lung mets at end point.
Appendix figure 7:

A. Representative images of bulk tumor from Control, Snail1 KO and Snail1 Het mice.

B. Quantification of gross tumor morphology into rounded and variegated phenotypes. Total number of round/variegated tumors / total tumors was used to determine the percentage of each type.
Discussion and future direction

Tumor stroma comprises of a large number of cell types including cancer associated fibroblasts, tumor associated macrophages, dendritic cells and different populations of T cells\textsuperscript{5}. There isn’t a good marker to target all stromal cells in the tumor microenvironment. Using FSP1-Cre to knockout SNAIL1 in cancer stromal cells, we show that SNAIL1 knockout in FSP1+ stromal cells does not affect tumor development, growth and metastasis. FSP1 is not expressed in all tumor stroma cells and it is possible that the FSP1+ cells do not contribute to breast cancer metastasis.

Surprisingly, in a small cohort of mice, we noticed that deleting only one copy of SNAIL1 in the FSP1+ cells significantly increases lung metastasis and changes the tumor characteristics and number and size of lung metastasis nodules. SNAIL1 is a transcription factor and has early and late target genes and also regulates its own transcription. When both copies of SNAIL1 is present, SNAIL1 protein increases, induces transcription of early and late genes and reaches a critical concentration in the cell to become degraded by the ubiquitin proteasome system. In a heterozygous condition, the level of SNAIL1 reaches its critical concentration in twice the amount of time. Thus SNAIL1 target genes could be expressed for twice the normal duration which could be responsible for the adverse effects on tumor development and metastasis. Alternatively, in the heterozygous condition, SNAIL1 protein level is maintained at half the critical concentration, at a level where it cannot shut down its own transcription (self-regulation). Although statistically significant, the observation with the SNAIL1 heterozygous mice is done on a small cohort and more mice need to be studied to make a definitive conclusion.
Future studies need to be designed to study the knockout of SNAIL1 in the multiple stromal cells in the tumor microenvironment. These studies could be completed by crossing the SNAIL1$^{fr}$ mice to stromal specific Cre mice like αSMA-Cre, Periostin Cre, Cola1-CreERT2, NG2-Cre etc. and a metastatic tumor model (like MMTV-PyMT). Proper controls, especially for the inducible Cre mice need to be considered.

Additionally, the effect of deleting SNAIL1 in the stromal cells in the metastatic site need to be determined. These can be done by tail-vein injections of tumor cells or implanting tumor cells in cleared mammary fat pad of stromal specific SNAIL1 knockout in mice and analyzing the metastasis rate and burden as compared to control (WT) mice.
References:


