ENGINEERING STEM CELL RESPONSES USING OXIDATIVE STRESS AND NOTCH LIGAND CONTAINING HYDROGELS

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ENGINEERING STEM CELL RESPONSES USING OXIDATIVE STRESS AND NOTCH LIGAND CONTAINING HYDROGELS

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Gratefully dedicated to my parents

For giving me wings to pursue my dreams and roots to come back to

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LIST OF ABBREVIATIONS

AHA	American Heart Association
MI	Myocardial Infarction
CDC	Cardiosphere-Derived Cell
CSC	Cardiac Stem Cell
CPC	Cardiac Progenitor Cell
MSC	Mesenchymal Stem Cell
HSC	Hematopoietic stem cell
EB	Embryoid Body
CEC	Cardiac Endothelial cell
Sca1	Stem Cell Antigen
MDR1	Multidrug Resistance Protein 1
SAP	Self-Assembling peptide
NICD	Notch Intracellular Domain
RBP-JK	Recombinant signal binding protein1- for J-k
TACE	TNFα converting enzyme
H_2O_2	Hydrogen Peroxide
GOX	Glucose Oxidase
ROS	Reactive Oxygen Species
ECM	Extracellular matrix
PLGA	Poly Lactic acid-Glycolic Acid
PEG	Polyethylene glycol
PDGF	Platelet-Derived Growth Factor
SCF	Stem Cell Factor

HGF	Hepatocyte growth factor
LV	Left Ventricle
CABG	Coronary Artery Bypass Grafting
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
ANOVA	Analysis of Variance

SUMMARY

Heart failure is the leading cause of death worldwide. In 2013, the American Heart Association estimated that one American will die of cardiovascular disease every 39 seconds. While heart transplantation is the most viable treatment option, the limited availability of donor hearts has necessitated the search for treatment alternatives such as the use of adult stem cells for cardiac repair and regeneration. Following myocardial infarction (MI), the inflammatory cardiac microenvironment, limited survival of stem/progenitor cells, myocardial scarring and fibrosis affect cardiac regeneration. This dissertation examines adult stem cell based approaches for cardiac regeneration by studying the effect of i) H_2O_2- mediated oxidative stress on mesenchymal stem cells, ii) Notch1 activation in cardiac progenitor cells using a self-assembling peptide hydrogel containing the Notch1 ligand mimic RJ in vitro and functional consequences in a rat model of MI.

Levels of oxidative stress are elevated in the infarcted heart. Delivery of mesenchymal stem cells (MSCs) to the infarcted heart has resulted in modest improvements in function. Hence, we sought to determine if oxidative stress can regulate MSC gene expression by subjecting MSCs to varying levels of oxidative stress (through H_2O_2 or Glucose oxidase). We found that H_2O_2 and Glucose oxidase regulate cardiogenic gene expression in MSCs through Notch1 signaling.

Notch1 signaling plays an important role in cardiac development, stem cell survival and differentiation. Mechanical force exerted by ligand bound to Notch1

receptor is essential for activation of Notch1 signaling. So we designed a 3D self-assembling peptide hydrogel that incorporated a peptide mimic of the Notch1 ligand Jagged1 (RJ). Utilizing a reporter cell line, we demonstrate the synergistic effect of hydrogel stiffness and presence of Notch1 ligand RJ on Notch1 activation. We also found that hydrogel stiffness dependent Notch1 activation differentially regulates cardiogenic gene expression in rat cardiac progenitor cells (CPCs). Culture of CPCs in RJ-containing hydrogels also exerted paracrine effects on endothelial cells and myocytes in vitro. In rats subjected to MI, intramyocardial delivery of CPCS in 2RJ hydrogels promoted cardiac repair through increased ejection fraction, stroke work and decreased fibrosis on day 21 after treatment.

Through these approaches, the central hypothesis that modulation of stem cell response using cues such as oxidative stress and activation of Notch1 signaling can improve functional outcome following myocardial infarction has been studied.

CHAPTER 1

INTRODUCTION

Cardiovascular disease is a global healthcare burden. Apart from heart transplantation, pharmacological interventions, cardiac- assisted devices and lifestyle modifications have improved the quality of life of people with cardiovascular disease. Although these treatments maintain cardiac function, new therapies that promote cardiac repair and regeneration are needed. These include enhancement of endogenous myocyte and progenitor cell responses as well as transplantation of culture expanded autologous progenitor cells in suitable cell delivery vehicles.

1.1 MOTIVATION

Cardiovascular disease is the leading cause of global morbidity and mortality. The AHA estimates that 1 in 3 Americans will suffer from cardiovascular disease during their lifetime (1). Although heart transplantation is the most viable treatment option, limited availability of donor hearts has necessitated the need for treatment alternatives. Several strategies aimed at improving acute cardiac function by utilizing pharmacological inhibitors and implanted devices have met with moderate success. However, improving cardiac function through myocardial regeneration by triggering the resident cardiac cells or adult tissue resident stem cells needs to be further investigated and developed as a potential clinical treatment. Studies have demonstrated different routes for delivery of stem/progenitor cells to specific organs by implantation on novel scaffolds ranging from

synthetic to biologically inspired materials. Though these studies have shown modest to significant functional improvements, there is a need for better understanding of the precise molecular pathways responsible for the observed functional benefits. Furthermore, the microenvironment in which the stem cells are implanted holds great promise as a source of cues to direct survival, proliferation and differentiation of stem cells. This is especially important as clinical trials using bone marrow-derived mesenchymal stem cells (MSCs) and heart-derived cardiac progenitor cells (CPCs) demonstrate modest functional improvements. However, these outcomes can be improved by promoting cardiac retention and differentiation of implanted progenitor cells to contribute directly to the regenerative process. The myocardial environment following an infarction has elevated levels of reactive oxygen species (ROS) that may greatly influence the cardiogenic differentiation of endogenous or implanted progenitor cells. Many adult cells also alter their Notch signaling during disease states (2, 3), and no link has been made between oxidative stress and Notch signaling to date. The motivation for this study stems from the increased survival rates in patients following infarction who require cardiac therapies that promote sustained myocardial repair and regeneration. Moreover, the improvements in function following administration of CPCs in Phase I clinical trials in patients with cardiac disease provides further impetus to find ways to engineer, deliver and retain the CPCs in the infarcted heart and direct stem cell fate in vivo.

1.2 SPECIFIC AIMS

Heart failure due to myocardial infarction (MI) is a major source of global morbidity and mortality. Inadequate blood supply to the heart followed by death of cardiac myocytes in the ischemic region of the heart leads to MI. While reperfusion and transplantation surgeries have been successful in treating patients with MI to a significant extent, the use of stem cells to regenerate the infarcted heart is a promising emerging alternative. Stem cells of different origins have been shown to improve cardiac function in experimental models of cardiovascular diseases (4-6). Adult stem cells are an attractive therapeutic approach due to the ease of isolation and culture expansion as well as fewer ethical issues and lessened teratoma fears compared to embryonic sources. Adult bone marrow-derived mesenchymal stem cells (MSCs) and heart-derived cardiac progenitor cells (CPCs) are multipotent, undifferentiated tissue-resident cells. Injection of MSCs or CPCs into infarcted hearts has resulted in cardiogenic differentiation and acute functional improvements (7-11)}. However, the mechanism by which the implanted cells improve function in the infarcted heart is still not fully understood.

Pathophysiological levels of oxidative stress elicit stem cell specific responses (12). In addition to redox levels, stem cell differentiation is also strongly influenced by Notch signaling (13). Notch knockouts are embryonic lethal due to blood vessel and heart defects (14). While, the effects of Notch signaling on stem cell differentiation are well studied, the interplay between oxidative stress and Notch signaling is unknown. Phase I clinical trials of cardiac progenitor cell therapy in patients with cardiovascular diseases have demonstrated promising outcomes (15, 16). Moreover, Notch signaling has been shown to precede cardiac regeneration in zebrafish and regulate CPC function (10, 17).

Based on these scientific advancements, this dissertation focusses on developing an adult stem cell based therapy for myocardial infarction (Figure 1).

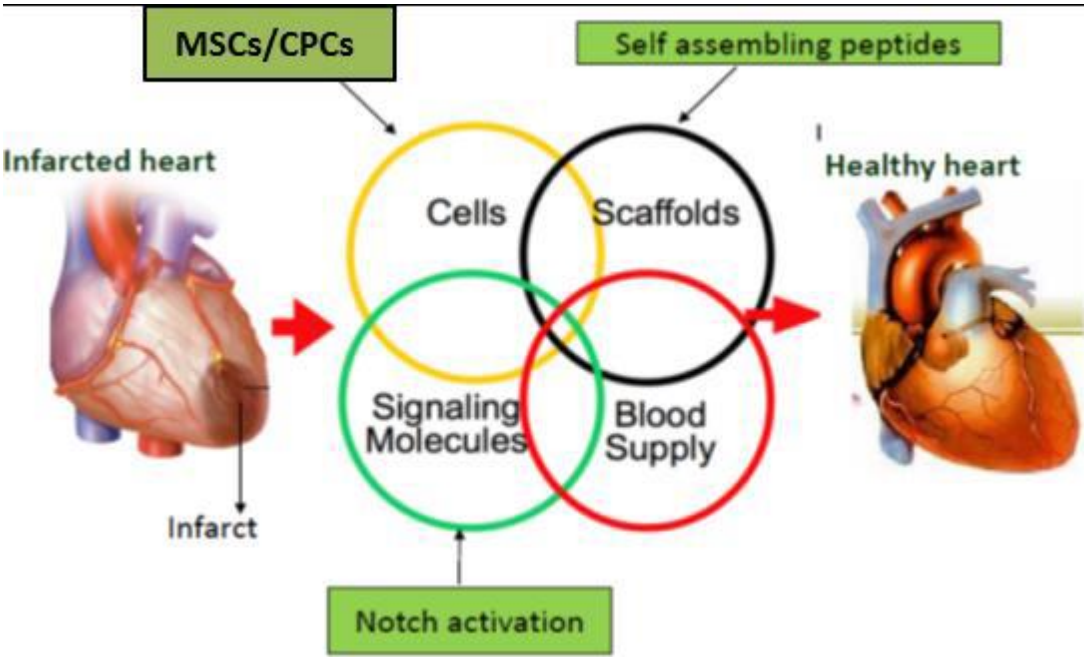

Figure 1: Schematic representing overall goal of the dissertation study.

Specific Aim 1: Investigate the effect of H_2O_2 and Notch1 signaling on cardiogenic differentiation of MSCs and CPCs.

We hypothesized that adult stem cell differentiation is redox-sensitive and the balance between redox signaling and Notch1 activation modulates stem cell gene expression. Rat bone marrow-derived MSCs or heart-derived CPCs were treated with a range of H_2O_2 concentrations (0.1-100μM) for 1 week or Glucose oxidase (GOX, 0-5mU/ml) for 48h. The effect of H_2O_2 or GOX- mediated oxidative stress on cardiogenic gene expression and Notch1 signaling was examined by qPCR, western blotting and flow cytometry.

Specific Aim 2: Determine the effect of hydrogel-immobilized Notch1 ligand Jagged1 on Notch1 activation in CPCs.

We hypothesized that hydrogel stiffness and the peptide mimic of the Notch1 ligand Jagged1, RJ could modulate Notch1 signaling. The effect of hydrogel stiffness and ligand RJ on Notch1 activation was studied using a Notch1 reporter system. CPCs were also cultured in a self-assembling peptide hydrogel containing RJ and the effect on cardiogenic gene expression was examined in vitro.

Specific Aim 3: Use self-assembling peptide hydrogels containing RJ to direct CPC function in vivo following an infarction.

We hypothesized that delivery of CPCs in a bioactive hydrogel containing a ligand to activate Notch1 signaling could lead to improvements in cardiac function after myocardial infarction. The effect of intramyocardial delivery of CPCs in empty, scrambled or RJ-containing self-assembling peptide nanofiber hydrogels was investigated through pressure-volume hemodynamics and whole-animal imaging.

The results from these studies have demonstrated i) how oxidative stress and Notch1 activation regulate cardiogenic gene expression in MSCs either separately or synergistically and ii) a new method to direct CPC function in a hydrogel containing signals for Notch1 activation. These results may have broad clinical applications in cardiac repair and/or regeneration.

CHAPTER 2

BACKGROUND

2.1 MYOCARDIAL INFARCTION

The heart is the first organ to develop during embryogenesis and plays a central role during both development and adulthood, because it is required for pumping nutrients and oxygen to all organs. However, partial or complete loss of blood supply due to occlusion of the coronary artery leads to myocardial infarction and the subsequent loss of ventricular function ultimately causes heart failure. To maintain pump function, the viable myocardium undergoes ventricular dilation and wall thinning by side-to-side slippage of myocytes. This leads to infarct expansion from the apex with extensive myocyte death in the infarct area and myocyte hypertrophy in non-infarcted regions along with increased wall stress (18). These changes that result in alterations of existing structures after MI constitute cardiac remodeling. The long term consequences of these structural changes also depend on the extent of fibrosis. Acute inflammation following MI increases local TGFβ that promotes collagen synthesis by fibroblasts (19). Although acute reparative fibrosis that results in scar formation stabilizes the infarct, over time the stiffened ventricular wall impairs efficient pump function. Hence, morphological alternations that support acute remodeling may improve function, but over time, scar formation and comorbidities lead to end stage heart failure and death (20).

The American Heart Association estimates that one person will die of cardiovascular disease every 39 seconds in the United States (1). With heart disease being the leading cause of death worldwide and the limited availability of donor hearts

for transplantation, there is a strong need to develop alternative therapies ranging from assisted heart devices, small molecule drugs to stem cell based cardiac repair for improving cardiac function.

2.2 OXIDATIVE STRESS IN THE HEART

Our preliminary data and extensive published studies demonstrate significant increases in level of reactive oxygen species (ROS) almost immediately following acute myocardial infarction (MI) (21). While the source of these radicals is unclear (invading inflammatory cells, resident cells), it is evident that elevated levels of oxidative stress have deleterious effects on the cardiovascular system and play a prominent role in the pathophysiology of heart failure (22). Converging lines of evidence for involvement of ROS in cardiac remodeling and failure include: 1) studies demonstrating an association between increased oxidative stress and heart failure (23), 2) levels of endogenous scavengers such as catalase and superoxide dismutase are downregulated following infarction (24); 3) antioxidant based interventional treatments suggest a role for ROS in MI (25) and 4) genetically modified animal models have provided direct molecular evidence for the role of ROS in mediating post-MI remodeling in mice (26, 27). These studies demonstrate that reduction of oxidative stress in the ischemic heart leads to reduced apoptosis, scar formation, and inflammatory molecule release, all of which may modulate the efficacy of delivered therapeutics.

2.2.1 Influence of oxidative stress on stem cells

The levels of reactive oxygen species (ROS) modulate the balance between physiological and pathophysiological cardiac microenvironment at the infarct site. Potential sources of ROS in the myocardium include the NADPH oxidases of fibroblasts and infiltrating inflammatory cells, as well as the myocytes themselves. Ischemic injury further elevates ROS production in these cells which influences differentiation of endogenous or implanted stem cells at the infarct. Physiological H_2O_2 levels increase cardiogenic differentiation of embryonic stem (ES) cells, while pathophysiological levels induce apoptosis in ES-derived myocytes (28). Moreover, the effect of oxidative stress on stem cells is varied ranging from promotion of neural stem cell differentiation (12), to inhibition of MSC adhesion to the infarcted myocardium (29).

2.3 CARDIAC REGENERATION

The long standing paradigm that the adult mammalian heart is a post-mitotic organ has been challenged by identification of cardiomyocyte proliferation and resident cardiac stem cells in the heart (30-32). However, the limited regenerative capacity of the human heart is evident when compared to lower organisms such as the zebrafish and newt in which resection of ~20% the heart results in complete regeneration within 2 months. By a process of dedifferentiation of cells adjacent to the injury followed by cell proliferation, new myocytes are formed that integrate functionally with the pre-existing myocardium along with minimal evidence of injury (33). Mice can also regenerate the heart after injury during embryonic development and 1 week after birth by mechanisms similar to newts and zebrafish (34). The therapeutic challenge to promote such regeneration in humans after infarction is compounded by the loss of >1billion (~25% of the total) myocytes and extensive scar formation (35). Hence, development of therapies

aimed at delivering exogenous stem cells or promoting endogenous stem cell responses with long-term tissue integration will be critical to achieve cardiac regeneration in humans.

2.3.1 Mesenchymal stem cells for cardiac regeneration

There is ample evidence of the beneficial effects of bone-marrow derived mesenchymal stem cells (MSCs) for treating myocardial infarction in small and large animal studies (36-38). Whereas MSCs are capable of differentiation to multiple lineages, when delivered to the heart after acute MI, cardiogenic (all cell types in the myocardium) differentiation was noted (39). Additionally, several studies have shown improvements in cardiac function following MI by mobilizing endogenous MSCs via injection of GM-CSF and other stem cell mobilizing factors (40). Finally, early human clinical trials have demonstrated that bone marrow cell transplantation in patients is safe with modest, yet significant, improvements in cardiac performance after MSC administration (41, 42). Despite potential advantages in the use of bone marrow derived stem cells to treat heart failure, patients who received autologous bone marrow cells had <5% increase in ejection fraction (43-46). However, parameters indicative of long term improvement such as infarct remodeling and exercise capacity showed positive improvement at 6 month follow up (47). Interestingly, severity of infarction correlated with maximum therapeutic benefit (46). Evidence from studies in animal models suggest the predominant mechanism by which bone marrow derived cells exert modest beneficial effects to be through paracrine factors and fusion with myocytes rather than actual transdifferentiation (48). It is interesting to note that these studies had positive data with

very small retention of administered cells, and cardiogenic differentiation of the remaining population is estimated to be quite low with the predominant effect thought to be paracrine.

2.3.2 Cardiac progenitor cells for cardiac regeneration

Myocardial infarction and other cardiovascular diseases have poor prognosis due to lack of sufficient endogenous regeneration in the heart. However, in the past few years, the identification of stem/progenitor cell populations in small niches within the heart with surface expression of c-Kit, Sca1 (Stem cell Antigen 1) and MDR1 (multidrug resistance protein 1) (49). Beltrami et al isolated Lin^{-} $cKit^{+}$ cells from the heart of adult mice which were clonogenic, self-renewing and differentiated into cardiac lineages (31). Oh and colleagues isolated $Sca1^{+}$ CPCs from adult mice with cardiogenic potential (50). A side population of cells that efflux Hoechst dye and express the ATP-binding cassette transporter Abcg2 has also been identified. These cells proliferate, differentiate and adopt cardiac cell fates (51). Cardiosphere-derived cells (CDCs) are obtained by culturing cells from heart tissue in suspension cultures. These CDCs are a heterogenous cell population with expression of cKit by cells in the core and expression of endothelial genes Flk1 and CD31 by cells in the periphery (52). Although these progenitor cells differ in the cellular and phenotypic characteristics, the potential for use in cardiac cell therapy has prompted extensive studies in animal models. Reports indicate the ability of CPCs to regenerate the myocardium and improve cardiac function in rat and canine models of MI (31, 53). Isolation of $c\text{-}Kit^{+}$ cardiac stem cells from humans has promoted the extensive study of these cells for myocardial regeneration (32). Taken together, the different stem cells present in the heart could differentiate in vivo to endothelial cells,

vascular smooth muscle cells, cardiomyocytes or combination of all three cell types that constitute the heart.

Based on the promising results from the pre-clinical studies discussed above and to address the critical need for alternate treatments for patients with heart failure, the safety and efficacy of the progenitor cells (CPCs, CDCs) have been tested in Phase I clinical trials. Moreover, the presence of CPCs in the heart, the expression of certain early cardiogenic genes, and the ability to differentiate into cells of the cardiogenic lineage in vitro and in vivo makes CPCs a more suitable cell type for cardiac regeneration than other adult stem cell sources (54). The SCIPIO (Stem Cell Infusion in Patients with Ischemic Cardiomyopathy) is a Phase I, randomized, open-label trial utilizing autologous cKit+ cardiac stem cells (CSCs) from patients undergoing coronary artery bypass grafting (CABG) for ischemic cardiomyopathy (15). The trial achieved the primary goal of demonstrating safety and feasibility of the procedure. Following introacoronary administration of CSCs, a remarkable improvement in both global and regional cardiac function was observed in patients at 4 and 12 months following CSC infusion. In the randomised CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction) trial, intracoronary delivery of autologous CDCs to patient with left ventricle dysfunction resulted in decreased infarct size, increased viable mass and improved regional function at 12 months following treatment (16). These Phase I trials demonstrate safety and efficacy of progenitor cell therapy and merit further clinical trials.

2.4 NOTCH SIGNALING IN CARDIAC DEVELOPMENT AND STEM CELLS

2.4.1 Mechanism of Notch signaling

In 1917, Thomas Morgan in The American Naturalist described a mutant *Drosophila melanogaster* strain with serrated wing ends called Notch. In the late 1980s, two groups independently identified the Notch gene (55, 56). Notch signaling is an evolutionarily conserved intercellular communication pathway that regulates diverse cellular processes, ranging from cell fate decision, differentiation, and proliferation to apoptosis (57). In mammals, the Notch signaling pathway consists of 4 Notch transmembrane receptors (Notch1-4) and the DSL (Delta-Serrate-Lag2) family of ligands composed of Jagged 1,2 and Delta 1,3 and 4. The Notch receptor has an extracellular domain composed of 29-36 EGF repeats followed by a linker domain. The Notch intracellular domain (NICD) is composed of a trans-membrane domain, nuclear localization sequences, RAM domain for interaction with RBP-Jk, ankyrin repeats for interaction with other proteins and a PEST sequence for degradation (58). The DSL ligands also contain several EGF repeats for binding to the Notch receptor. After translation, the receptor undergoes fucosylation in the endoplasmic reticulum and Golgi apparatus. This determines the specificity of binding to different ligands and is presented as a dimer on the cell surface through non-covalent linkages (59). Transactivation of the Notch receptor by adjacent cell surface bound ligands of the Jagged and Delta family leads to downstream activation while cis-activation of Notch by binding of ligands on the same cell leads to ligand-receptor sequestration in the cytoplasm. Release of the NICD requires 3 sequential cleavages of the receptor: the 1st mediated by metalloprotease

TNFα-converting enzyme (TACE/ADAM17) followed by two cleavages by γ-secretase complex composed of presenilins and nicastrin. Cleavage leads to nuclear translocation of the NICD. The NICD binds and converts the co-repressor RBP-JK (recombinant signal binding protein1- for J-k) into a transcriptional activator. This leads to canonical activation of the direct Notch targets of the Hes/Hey family of basic helix-loop-helix transcription factors as shown in Figure 2.

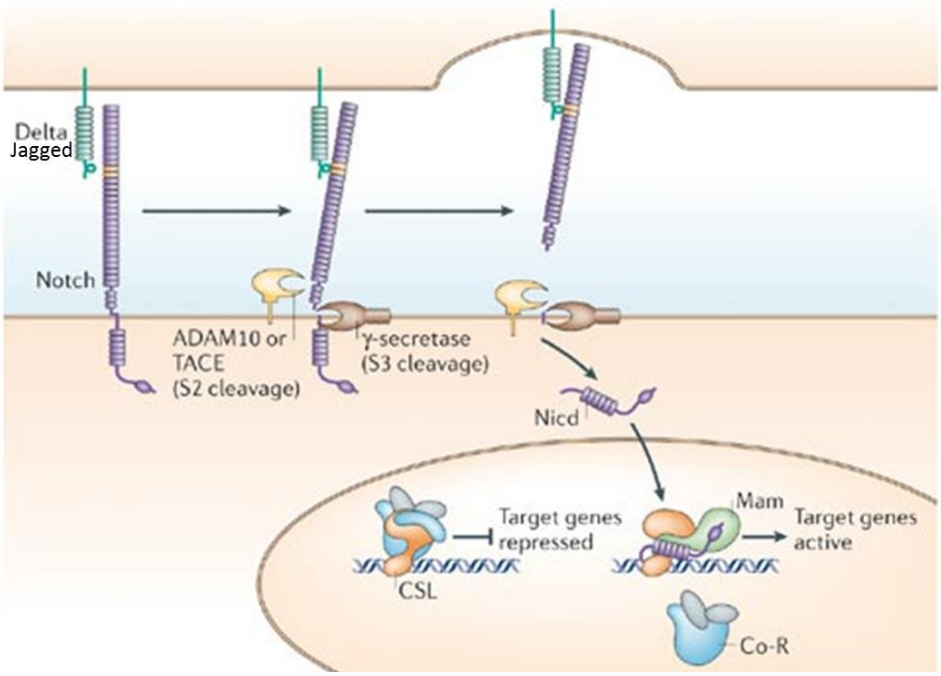

Figure 2: Schematic representation of Notch signaling. Binding of the Delta/Jagged family of ligands to the Notch receptor on the signal receiving cell leads to release and translocation of the Notch intracellular domain (NICD) into the nucleus. NICD mediates transcription of Notch responsive genes. Adapted from (58).

2.4.2 Regulation of Notch signaling

As Notch signaling plays a critical role in several developmental processes, multiple levels of regulation exist such as the amount of receptors and ligand present on the cell surface which in turn depends on ligand/receptor endocytosis, ubiquitination, degradation and recycling (60). Moreover, soluble ligands can act as signal antagonists (61). Notch is regulated by lateral inhibition wherein assumption of a particular fate by a cell inhibits assumption of the same fate by adjacent cells. Notch activation also leads to dosage dependent effects. For example, low levels of Notch activation promote neural stem cell growth while high levels cause growth arrest (62). Apart from canonical signaling, the effects of Notch signaling can also be amplified or attenuated by interaction with other signaling pathways such as Wnt, TGFb and JAK/STAT (63).

2.4.3 Notch in cardiac development

Notch signaling during development regulates cell fate and tissue formation. In the developing heart, Notch signaling plays a critical role in atrioventricular canal formation, epithelial-to-mesenchymal transition, valve formation and ventricle and outflow tract development (64). Notch1 and Notch2 are the predominant receptors in the developing heart while Notch3 and Notch4 are present in smooth muscle and endothelium respectively (65). Loss- or gain- of function of Notch1 and the ligands and spontaneous mutations in humans indicate the important role for Notch in cardiac development. Deregulation of Notch signaling in humans leads to congential birth defects such as Alagille syndrome, ventricular hypertrophy, septal defects and valve calcification (66).

2.4.4 Effects of Notch signaling

Apart from regulating normal development and damage-induced regeneration, Notch signaling has also been found to promote cardiomyocyte survival (67). Notch signaling also regulates stem cell fate during asymmetric cell division. The daughter cell with active notch signaling remains undifferentiated while the other daughter cell adopts a committed fate. This occurs through Numb, a Notch antagonist, asymmetrically inherited by daughter cells (58). Notch signaling has also been identified to precede heart regeneration in zebrafish (17). Notch activation has been shown to promote cardiac gene expression in human circulating endothelial progenitor cells through Wnt5a (68). In mice subjected to myocardial infarction, activation of Notch signaling in cardiomyocytes in the border zone exerts a cardioprotective role in improving cardiac function (67). Differentiation of bone marrow-derived MSCs into cardiomyocytes is increased by Jagged1 mediated Notch signaling (8). Notch promotes cardiac cell fate, function and gene expression in CPCs (10) while attenuating cardiac differentiation of embryonic stem cells (69). In Xenopus, Notch exerts inhibitory effects on cardiac differentiation (70). In the hematopoietic stem cell (HSC) niche, inductive Notch signaling between HSC and osteoblasts maintains the HSCs in an undifferentiated state (71). The differential response of various stem cells to Notch activation underscores the need to characterize the stem cell- specific responses which can be modulated under pathological conditions.

2.5 HYDROGELS FOR CARDIAC CELL THERAPY

A healthy myocardium is a contractile tissue composed of cardiomyocytes, endothelial and smooth muscle cells. Efficient pump function requires the interaction of

these cells with the extracellular matrix (ECM) which provides stimuli for growth of cardiac cells, differentiation and migration of stem/progenitor cells. However, myocardial infarction damages this organized tissue with extensive cell death, loss of contractility and formation of stiff fibrotic scar. A variety of biomaterials have been developed to address these changes that occur after MI. For cardiac applications, biomaterials need to developed with i) mechanical properties similar to the myocardium, ii) incorporation of signals for growth, proliferation and differentiation, iii) controlled degradation into non-toxic metabolites, and iv) limited foreign-body reaction and host-inflammatory response. By providing mechanical support to the infarcted heart, biomaterials can increase wall thickness and decrease adverse remodeling after MI.

When the mechanical properties of the biomaterial are similar to the myocardium, more pronounced effects have been observed. For example, an injectable alginate hydrogel has been shown to increase myocardial mass and decreased end diastolic volume (72). In a similar study, injection of a polyethylene glycol (PEG) hydrogel into infarcted rat hearts showed that the improvement in function seen at 4 weeks was lost at 3 months suggesting that improvements in function by providing mechanical support can only provide temporary benefits (73). For sustained improvement in cardiac function, injection of biomaterials with cells has been explored. For cell delivery, the biomaterial should provide an environment for cell survival that does not interfere with integration and mechanical coupling to the host myocardium.

2.5.1 Types of hydrogels

Drug and cell delivery systems could be modulated to serve as instructive microenvironments in regenerative medicine. Towards this end, several synthetic

biomaterials have been developed to mimic the natural extracellular matrix (ECM) for therapeutic use. These include synthetic polymers, decellularized ECM, self assembling polymers and cell responsive hydrogels with varied applications. Several biomaterials such as matrigel, PLGA, PEG, alginate and self-assembling peptides have been used as cell delivery vehicles to regenerate the myocardium. Advances in biomaterial design and fabrication processes have led to tunable hydrogels that can incorporate instructive cues, deliver cells/growth factors and regulate endogenous or implanted stem cell responses in the heart.

Matrigel is a heterogenous mixture of soluble basement membrane proteins secreted by mouse tumor cells which polymerizes into a hydrogel at 37°C. Embryonic stem cells injected in matrigel to the infarcted heart have shown improvement in function (74). However, being obtained from mouse tumor makes these hydrogels unsuitable for clinical application. Collagen is an abundant ECM protein present in the heart and other tissues. Collagen production by fibroblasts is increased after infarction in the scar region. Being present in the heart makes collagen a nontoxic, non-immunogenic and biodegradable biomaterial. Collagen gels used to deliver MSCs to the pericardial space improves function and retention in pigs (75). Tissue patches of collagen gels containing endothelial progenitor cells applied on the infarcted LV wall in rats results in improved cardiac function through increased vascular density (76).

Fibrin is a protein that forms a FDA approved fibrin glue when fibrinogen is mixed with thrombin (77). Application of fibrin glue containing MSCs as an epicardial patch on the heart improves function after MI through increased cell retention and angiogenesis (78). It is also used as a vehicle to deliver proteins and plasmids through

catheters to the heart. Decellularized extracellular matrix isolated from whole animal hearts can be milled into a powder and used as a hydrogel for cell therapy. It has also been shown to promote cardiac gene expression in rat cardiac progenitor cells in vitro (79). Poly Lactic acid-glycolic acid (PLGA) is a FDA-approved polymer used in tissue engineering scaffolds and microparticle formulations. As the chemical formulation process could affect proteins and cells, it has been used as a substrate for microprinting defined geometries that promote stem cell differentiation. For example, MSCs form elongated cell shape with myogenic gene expression on PLGA scaffolds with 20μm fibronectin strips (80). PEG-based hydrogels have been used to deliver growth factors to the infarcted rat heart with improvements in function (81). Apart from these hydrogels, microparticles such as polyketals and dendrimers have been used as siRNA delivery vehicles that have also resulted in improved cardiac function following MI in rodents (82, 83). The development of self-assembling peptide hydrogels has led to its use as a cell, drug and growth factor delivery vehicle for regenerative medicine applications as discussed below.

2.5.2 Self-assembling peptide hydrogels

Self-assembling peptides are composed of alternating hydrophilic (arginine or aspartic acid) and hydrophobic (alanine or lysine) amino acids with the peptide sequence H_2N-RARADADARARADADA-OH. Due to the charges on the hydrophilic groups when the peptides are aligned, a stable antiparallel β-sheet is formed that at neutral pH polymerizes into a hydrogel (84). The peptides self-assemble into nanofibers (7–20 nm in diameter) with >99% water content at physiological pH and osmolarity. The peptide hydrogel has

been shown to be non-inflammatory and non-immunogenic (85, 86). The peptide sequence does not contain any cell recognizable structures and is considered to be a non-instructive hydrogel. However, incorporation of the adhesion motif RGD (arginine–glycine–aspartic acid) in the self-assembling peptide improves survival of MSCs and promotes MSC differentiation into cardiomyocytes (87). Furthermore, the 3D hydrogel environment also promotes attachment, proliferation and differentiation of different cell types: neuronal cells (88), endothelial cells (89), hepatocytes (90), chondrocytes (91), osteoblasts (92) and progenitor cells (54, 93). During solid-phase synthesis, the peptides can be functionalized with ligands and adhesive motifs at the C-terminal to provide signaling cues that promote cell retention in the scaffold (85, 94). The self assembling peptides can be functionalized with i) short motifs for cell adhesion to modulate human adipose stem cell behavior (95), ii) VEGF to promote cardiac repair following infarction in pigs (86) and iii) growth factors through biotin-streptavidin linkages for use in cell therapy (54).

The hydrogels have also been used for cartilage repair, axon regeneration, treatment of bone defects and myocardial infarction (96). In a pig model of MI, delivery of bone marrow mononuclear cells in a self-assembling peptide hydrogel improves retention, promotes vascular and endothelial differentiation and augments cardiac function 4 weeks after intramyocardial transplantation (97). Furthermore, selective differentiation of stem cells can be achieved through incorporation of cell specific adhesion signals in the hydrogels. Inclusion of the neurite-promoting laminin epitope peptide (isoleucine-lysine-valine-alanine-valine) in the hydrogel directs differentiation of neural progenitors into neurons and not astrocytes (98, 99). Work by Nickoloff et al., has

shown that a 20 amino acid long peptide mimicking the active site of the human Jagged1 ligand, RJ can activate Notch1 signaling in keratinocytes (100). RJ can be incorporated into self-assembling hydrogels with tunable mechanical properties to regulate Notch signaling and stem cell function. After synthesis, the peptides can be purified by high pressure liquid chromatography on reverse-phase columns and the presence of a correct composition verified by amino acid analysis.

CHAPTER 3

OXIDATIVE STRESS MEDIATED CARDIOGENIC GENE EXPRESSION IN MESENCHYMAL STEM CELLS

3.1 INTRODUCTION

Accumulating lines of evidence indicate beneficial effects of bone-marrow derived mesenchymal stem cells (MSCs) for treating MI in small and large animal studies (101). Whereas MSCs are capable of differentiating into multiple lineages (102), upon delivery to the heart after acute MI, cardiogenic (all cell types in the myocardium) differentiation was noted (103). Additionally, several studies have shown improvements in cardiac function following MI either by endogenous bone marrow (7) or by augmenting this endogenous MSC response via injection of GM-CSF and other stem cell mobilizing factors (40). Finally, early human clinical trials have demonstrated modest, yet significant, improvements in cardiac performance after MSC administration (42, 104).

Extensive published studies demonstrate significant increases in reactive oxygen species (ROS) almost immediately following an acute MI. Elevated levels of ROS have deleterious effects on the cardiovascular system and are critical in the pathophysiology of heart failure. Potential sources of ROS in the myocardium include the NADPH oxidases of fibroblasts and infiltrating inflammatory cells, as well as the myocytes themselves (105). Ischemic injury further elevates ROS production in these cells which may influence differentiation of endogenous or implanted stem cells at the infarct site. While effects of ROS on cardiac cell death, remodeling, and function are well studied, stem and

progenitor cells that could be used for potential regeneration have both adaptive and maladaptive responses to oxidative stress. For example, acute bursts of ROS to embryonic stem cells (ESCs) in culture facilitate differentiation toward the cardiomyocyte phenotype, while chronic exposure to H_2O_2 inhibits differentiation (106). Although the effect of ROS on certain stem cell types is fairly established, the exact signaling pathways regulated by ROS especially in the cardiogenic differentiation of stem cells is under intense investigation. Thus, pinpointing the exact signals modulated by ROS leading to alterations in MSC differentiation is of great therapeutic interest.

One of the major signaling pathways involved in stem cell differentiation is the Notch signaling pathway. Notch signaling is an evolutionarily conserved intercellular communication pathway that regulates diverse cellular processes, ranging from cell fate decision, differentiation, and proliferation to apoptosis. Activation of the Notch receptor by adjacent cell surface bound ligands of the Jagged and Delta family leads to proteolytic cleavage and nuclear translocation of the Notch intracellular domain (NICD) and subsequent transcriptional regulation of target genes, leading to maintenance of cells in an uncommitted state or induction of cell-type specific differentiation (107). Notch signaling promotes early cardiac development (64) and has also been identified to precede heart regeneration in zebrafish (17). Furthermore, certain mutations in Notch ligands or receptors are associated with embryonic lethality in mice (14). Apart from regulating normal development and damage-induced repair, Notch signaling has also been found to promote cardiomyocyte survival (67). Notch activation has been shown to promote cardiac gene expression in circulating endothelial progenitor cells (68), bone

marrow-derived MSCs (11) and cardiac progenitors (10) while attenuating cardiac differentiation of embryonic stem cells (69).

While, the effects of Notch signaling on different cell types are well studied, its regulation by oxidative stress is unknown. We therefore sought to determine the role of oxidative stress on cardiogenic gene expression in MSCs and whether Notch signaling plays a role in directing differentiation of MSCs in the setting of elevated local H_2O_2 levels following MI. Our data suggest that H_2O_2 or glucose oxidase mediated oxidative stress promotes cardiogenic differentiation in adult stem/progenitor cells through upregulation of Notch1 signaling, possibly involving Wnt11.

3.2 RESULTS

Mesenchymal stem cell characterization

Mesenchymal stem cells (MSCs) had a spindle shaped, fibroblast-like morphology and expressed common mesenchymal cell surface markers, c-Kit, CD73, CD90 and CD105, with low expression of the hematopoietic marker CD45 and no expression of CD34 (**Figure 3.1A**). To determine the multipotent trilineage differentiation capacity, the MSCs were cultured for 21 days in media that promotes differentiation into adipogenic, osteogenic and chondrogenic lineages. As shown in **Figure 3.1B**, the MSCs differentiated into the three lineages as demonstrated by staining for Oil Red O, Alizarin Red and Toluidine blue respectively. Moreover, treatment with 100μM H_2O_2 for 1 week did not induce trilineage differentiation indicating that the MSCs are multipotent but H_2O_2 treatment does not promote differentiation into adipogenic, osteogenic, or chondrogenic lineage.

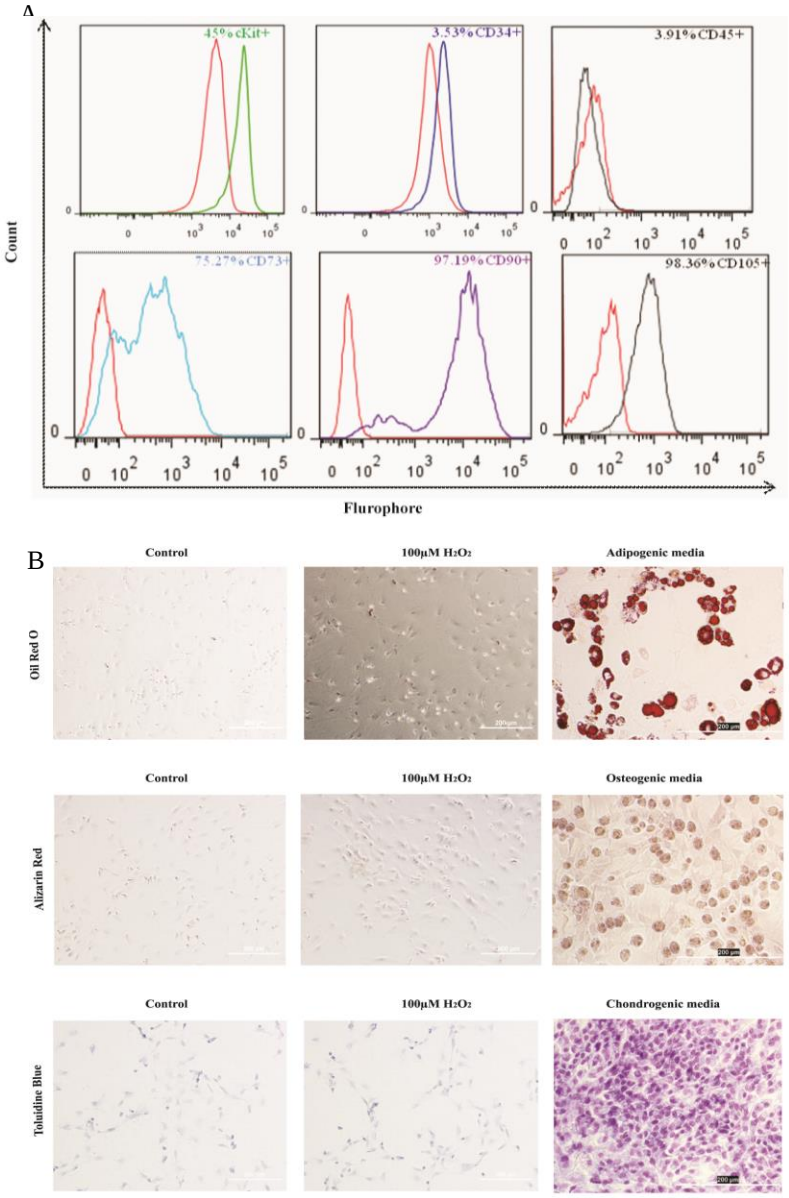

Figure 3.1. Characterization of Mesenchymal stem cells. (**A**) The MSCs isolated from rat bone marrow were culture expanded and % +ve cells expressing c-Kit, CD34, CD45, CD73, CD90 and CD105 was determined by flow analysis. (**B**) The trilineage differentiation potential of the mesenchymal stem cells (MSCs) cultured on adipogenic, osteogenic and chondrogenic media or ±100 µM H_2O_2 was assessed by Oil Red O, Alizarin Red and Toluidine blue staining, respectively. Scale bar = 200 µm.

Oxidative stress regulates early cardiogenic gene expression in MSCs

To determine whether H_2O_2 or Glucose oxidase (GOX)-mediated oxidative stress regulates endothelial gene expression, bone marrow-derived MSCs were cultured in media containing hydrogen peroxide (H_2O_2; 0-100μM) for 1 week or Glucose oxidase (GOX; 0-5 mU/ml) for 48hrs. The mRNA expression of VEGF receptor Flt1, vWF, and PECAM1 following exposure to H_2O_2 or GOX was quantified by qRT-PCR. While there was no effect due to lower levels of H_2O_2 (0.1-10μM), high levels (100μM) significantly increased the expression of Flt1 by 2-fold ($p<0.05$, **Figure 3.2A**), vWF by almost 2-fold ($p<0.05$, **Figure 3.2B**) and PECAM1 by 3-fold ($p<0.01$, **Figure 3.2C**). In MSCs treated with GOX for 48 hrs, no change in gene expression was seen at the lower doses (0.01-2.5 mU/ml). However at 5mU/ml, a significant increase in expression of Flt1 by 400- fold ($p<0.05$, **Figure 3.2D**), vWF by 150- fold ($p<0.05$, **Figure 3.2E**) and PECAM1 by 20-fold ($p<0.01$, **Figure 3.2F**) was observed.

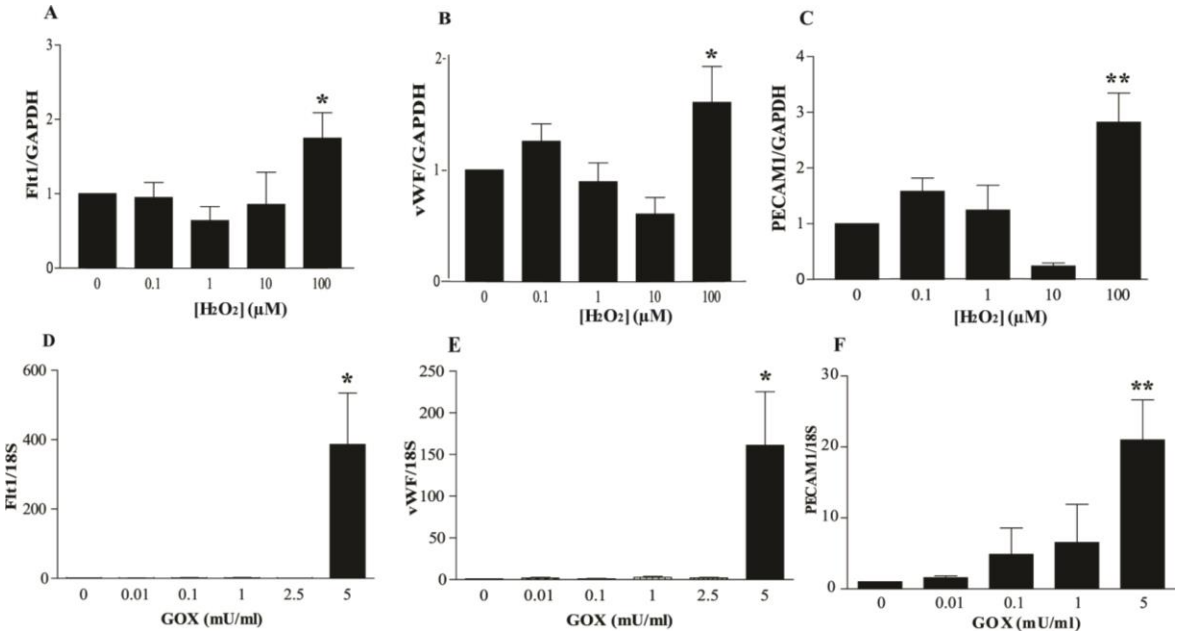

Figure 3.2. Oxidative stress promotes endothelial gene expression in MSCs. MSCs were treated with either H_2O_2 for 1 week (0-100µM; **A-C**) or GOX for 48h (0-5mU/ml; **D-F**). Expression of the endothelial markers Flt1 (**A,D**), vWF (**B,E**) and PECAM1 (**C,F**) was determined by qRT-PCR. Values are mean ± SEM after normalizing gene expression to GAPDH (A-C) or 18S (D-F) expression. For A-C, $n \geq 4$ and D-F, $n \geq 6$. * $p<0.05$ and ** $p<0.01$ when compared to control (0µM H_2O_2 or 0mU/ml GOX) by one-way ANOVA followed by Dunnett's post test.

To determine whether H_2O_2 or GOX-mediated oxidative stress regulates cardiac gene expression, we measured levels of early cardiac markers nkx2-5 and α-myosin heavy chain (αMHC). While treatment of MSCs with low levels of H_2O_2 (0.1-10μM) had no effect on αMHC levels, treatment with 100μM H_2O_2 significantly increased expression by 2-fold ($p<0.05$, **Figure 3.3A**) compared with time-matched, untreated controls. To confirm with another early cardiac marker, nkx2-5 gene expression was measured. As shown in **Figure 3.3B**, the expression of nkx2-5 increased by nearly 2-fold ($p<0.05$) in 100μM H_2O_2 treated MSCs. In MSCs treated with GOX for 48 hrs, no change in gene expression was seen at the lower doses (0.01-2.5 mU/ml). In MSCs treated with 5mU/ml GOX, a 30- fold increase in expression of αMHC ($p<0.001$, **Figure 3.3C**) was observed along with a75- fold increase in nkx2-5 expression ($p<0.05$, **Figure 3.3D**).

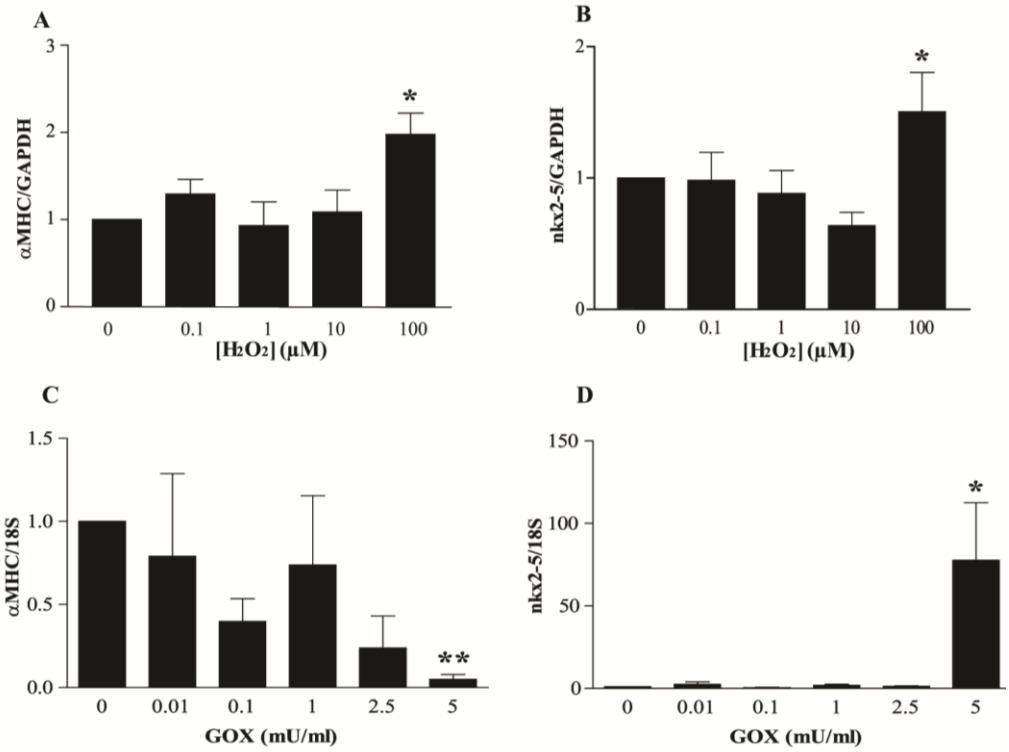

Figure 3.3. Oxidative stress regulates cardiac gene expression in MSCs. MSCs were treated with either $H_2O_2$for 1 week (0-100μM; **A,B**) or GOX for 48h (0-5mU/ml; **C,D**). Expression of the cardiac markers αMHC (**A,C**) and nkx2-5 (**B,D**) was determined by qRT-PCR. Values are mean ± SEM after normalizing gene expression to GAPDH (A,B) or 18S (C,D) expression. For A and B, $n \geq 4$; C and D, $n \geq 5$. * $p<0.05$ and *** $p<0.001$ when compared to control (0μM H_2O_2 or 0mU/ml GOX) by one-way ANOVA followed by Dunnett's post test.

To identify whether H_2O_2 regulated expression of smooth muscle markers, the levels of the early smooth muscle markers-smooth muscle α-actin (sm α-actin) and the calponin-related protein sm22α were examined. Treatment with 100μM H_2O_2 significantly decreased expression of smα-actin by 3-fold ($p<0.01$, **Figure3.4A**) and sm22α expression by 2-fold ($p<0.01$, **Figure 3.4B**). Similarly, the expression of vimentin, an intermediate filament protein characteristic of fibroblasts, was significantly decreased by 1.5-fold in both 1μM ($p<0.05$) and 100μM H_2O_2-treated MSCs ($p<0.01$) as shown in **Figure 3.4C**. No change in expression of sm α-actin, sm22α, and vimentin was observed in MSCs treated with GOX (0-5mU/ml) as in **Figure 3.4D-F**.

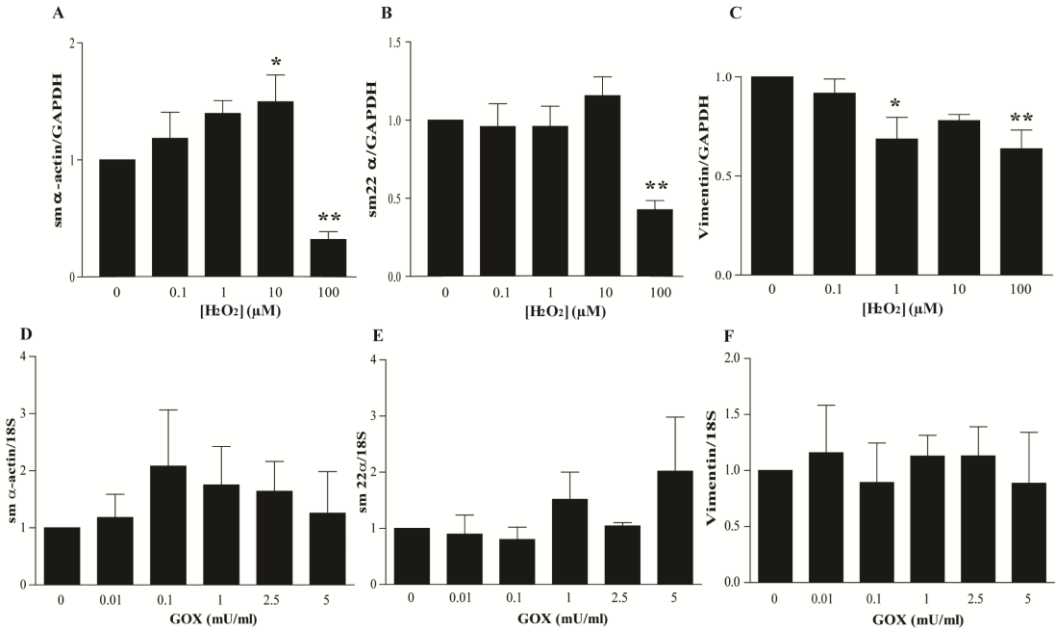

Figure 3.4. Oxidative stress differentially modulates smooth muscle and fibroblast gene expression in MSCs. MSCs were treated with either H_2O_2 for 1 week (0-100μM; A-C) or GOX for 48h (0-5mU/ml; D-F). Expression of the smooth muscle markers sm α-actin (**A,D**), sm22α (**B,E**) and the fibroblast marker Vimentin (**C,F**) was determined by qRT-PCR. Values are mean ± SEM after normalizing gene expression to GAPDH expression. For A-C,n≥3; and D-F, n=6. * $p<0.05$ and ** $p<0.01$ when compared to control (0μM H_2O_2or 0mU/ml GOX) by one-way ANOVA followed by Dunnett's post test.

To verify if the changes in cardiogenic gene expression translated to changes in protein expression, levels of the cardiac marker α-MHC, endothelial marker Flt1and smooth muscle α-actin were determined by flow analysis (**Figure 3.5A**). As shown in the grouped data in **Figure 3.5B**, GOX induced a 4-fold increase in cardiac α-MHC

expressing cells (left; $p<0.05$), a 1.4-fold change in Flt1 expressing cells (middle; $p=0.07$), and a 1.5- fold decrease in sm α-actin expressing cells (right; $p<0.05$).

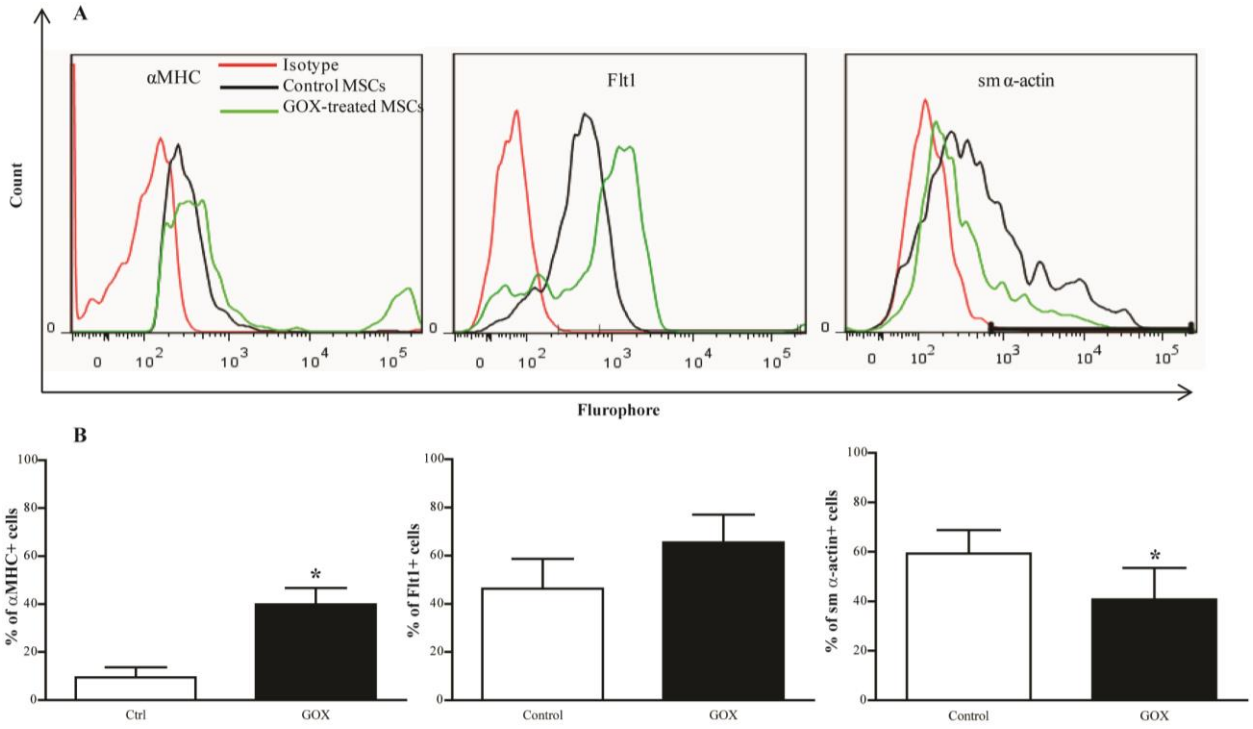

Figure 3.5. Quantification of GOX-mediated cardiogenic protein expression in MSCs. MSCs were treated with GOX (5mU/ml) for 48h. Expression of the cardiac proteins (αMHC, Flt1 and sm α-actin) was determined by flow cytometry (**A**) and quantified in **B (**mean±SEM with n≥3. * $p<0.05$ vs control by Student's paired t-test).

Oxidative stress induces Notch1 signaling

As the Notch signaling pathway has been shown to be critical for cardiovascular development and differentiation, we analyzed the expression levels of the cell surface receptor Notch1 and its ligand Jagged1 in H_2O_2 and GOX treated MSCs. High levels of H_2O_2 (100μM) augmented Notch1 gene expression by1.5-fold after 1week and significantly increased Notch intracellular domain (NICD) cleavage ($p<0.05$ for gene,

p<0.01 for NICD cleavage, **Figure 3.6 A&B**), while decreasing Jagged1 expression by about 1.5-fold (p<0.05, **Figure 3.6F**). Moreover, the expression of the downstream targets of Notch1, Hes5 was increased by up-to 2-fold in MSCs treated with 100µM of H_2O_2 (p<0.05, **Figure 3.6D**) and Hey1 by 1.5 fold (p<0.01, **Figure 3.6E**).While no change in gene expression was observed in MSCs treated with 0-2.5mU/ml GOX, a significant increase in expression of Notch1 (15-fold, p<0.05, **Figure 3.6C**), Hes5 (150-fold, p<0.05, **Figure 3.6G**), Hey1 (150-fold, p<0.01, **Figure 3.6H**) and a significant decrease in expression of Jag1 (p<0.05, **Figure 3.6I**) was observed in MSCs treated with 5mU/ml GOX.

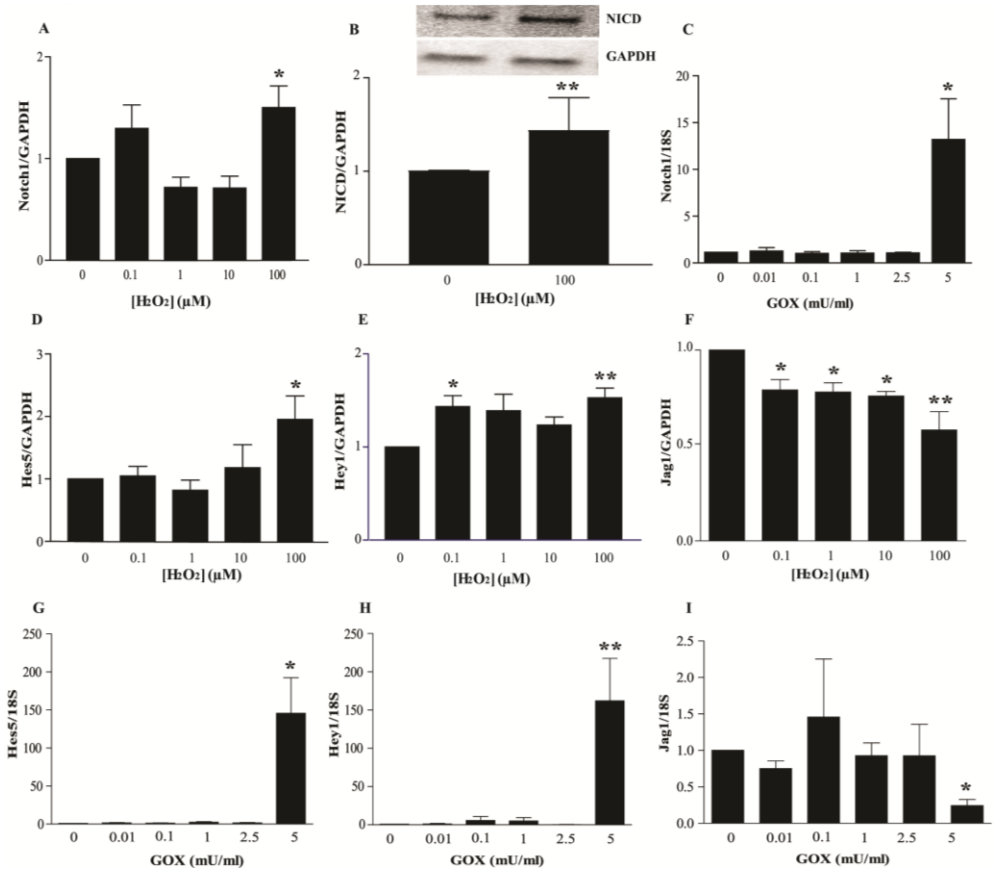

Figure 3.6. Oxidative stress increases expression of Notch1 and its downstream targets. MSCs were treated with either H_2O_2 for 1 week (0-100μM; **A,B,D-F**) or GOX for 48h (0-5mU/ml; **C,G-I**). The mRNA expression levels of Notch1 (**A,C**), Hes5 (**D,G**), Hey1 (**E,H**) and the ligand Jagged1 (**F,I**) was determined by qRT-PCR. (**B**) Quantification of expression of activated Notch1 (NICD) with representative Western blot image. Values are mean±SEM after normalizing gene expression and Western blots to GAPDH (A,B,D-F) or 18S (C,G-I) expression and protein, respectively. For A,B,D-F,n≥4; C,G-I,n≥6; * $p<0.05$ and ** $p<0.01$ when compared to control (0μM H_2O_2 or 0mU/ml GOX) by one-way ANOVA followed by Dunnett's post test and Student's unpaired t-test for B.

To determine the Notch1 related genes that were regulated in MSCs treated with 5mU/ml of GOX, a PCR array was performed. The data was analyzed and grouped based on known associated function of the gene. As shown in **Figure 3.7A**, treatment of MSCs with 5mU/ml of GOX resulted in an increase in expression of i) Wnt11(5-fold) and its receptor Fzd3 (3-fold), ii) genes involved in cell adhesion and proliferation (Cd44, Ccnd1, Cflar; ~3fold), iii) ligands for Notch1 (Dll1, Dll4; 3-5fold), iv) components of γ-secretase complex involved in Notch1 processing (Psen1, Mfng, Lfng, hr, Neurl; 3-15 fold) and iv) downstream target of Notch1 (Hes5; 5-fold). As Wnt11 is a known downstream target of Notch1, the expression of Wnt11 was validated in MSCs treated with GOX (0 or 5mU/ml). As shown in **Figure 3.7B**, a significant increase in Wnt11 expression was observed in MSCs treated with 5mU/ml GOX. The results were also validated using cardiac progenitor cells where a similar significant increase was observed (**Figure 3.7B**, bottom).

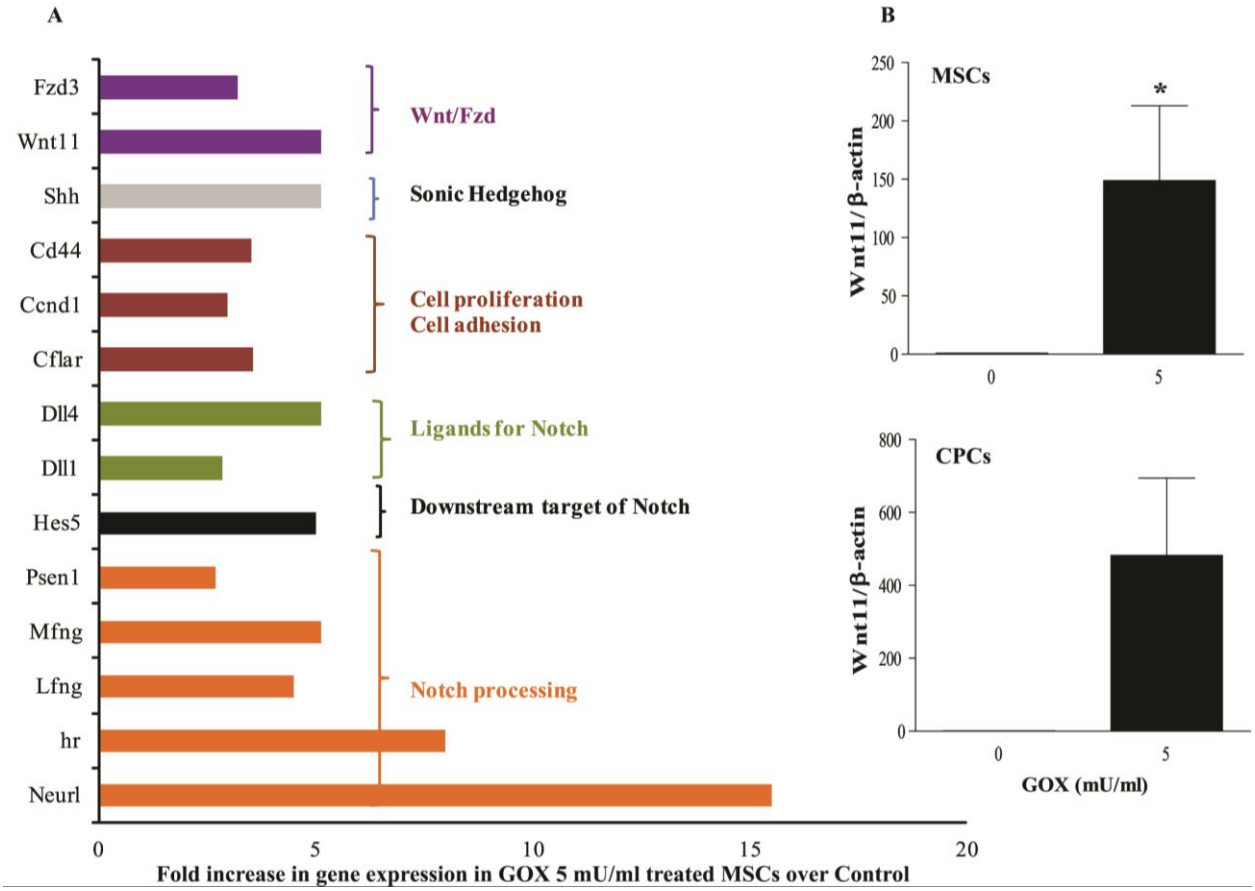

Figure 3.7. PCR array analysis of Notch1 related genes. Gene expression of Notch1 related genes in MSCs treated with GOX (0 or 5mU/ml) was analyzed using a PCR array. (**A**) Fold increase in gene expression in 5mU/ml GOX treated MSCs over control cells was normalized to β-actin. (**B**) Validation of increase in Wnt11 expression upon 5mU/ml GOX treatment in MSCs (top, $p<0.05$) and CPCs (bottom, $p=0.08$).

To determine if the effects of oxidative stress mediated by H_2O_2 are specific for MSCs, we subjected adult cardiac progenitor cells to GOX treatment. Interestingly, an increase in expression of Notch1 by 15-fold ($p<0.001$) and Hes5 by 175- fold ($p<0.05$)

along with changes in cardiogenic gene expression similar to the response of MSCs was observed (**Figure 3.8**).

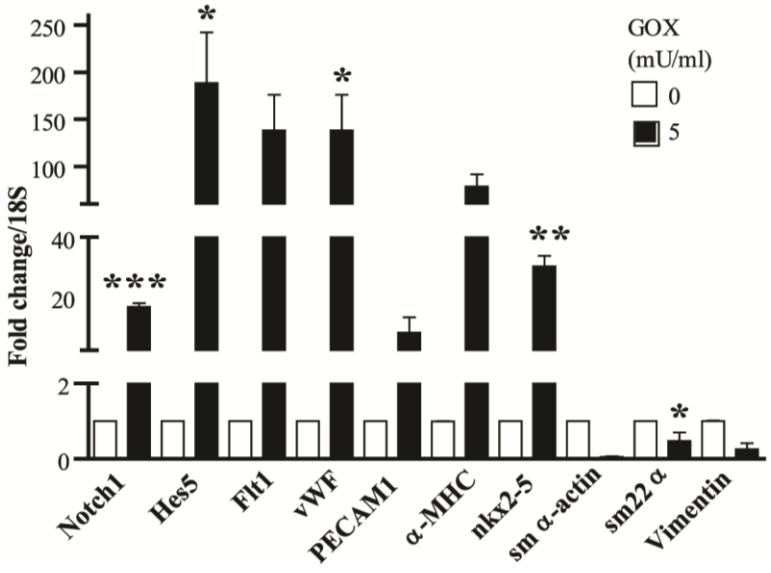

Figure 3.8. Oxidative stress regulates cardiogenic gene expression in cardiac progenitor cells. Cardiogenic gene expression in cardiac progenitor cells treated with GOX (0 or 5mU/ml) was determined by qRT-PCR. An increase in expression of Notch1 ($p<0.001$), Hes5 ($p<0.05$), flt1 ($p=0.08$), vWF ($p<0.05$), nkx2-5 ($p<0.01$), sm22α ($p<0.05$) was observed. Values are mean±SEM after normalizing gene expression to 18S expression (n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control (0mU/ml GOX) by Student's unpaired t-test).

Oxidative stress regulates cardiogenic gene expression through Notch1

To determine the link between increased Notch1 gene expression and activity by H_2O_2 treatment and cardiogenic gene regulation, MSCs were treated with a γ-secretase inhibitor IX DAPT (10μM) for 1 week with or without 100μM H_2O_2. γ-secretase is

required for the release of the active Notch1 intracellular domain (NICD) (58) and thus its inhibition blocks Notch1 activation. To demonstrate that DAPT inhibits Notch1 activation, MSCs were treated with DAPT (10μM) for 1 week with or without 100μM H_2O_2 and protein levels of NICD were measured by Western blotting. Knockdown of Notch1 expression using siRNA was also attempted. MSCs were transfected with siRNA using oligofectamine as it resulted in the highest transfection efficiency when compared to other commercially available reagents (**Figure 3.9A**). As shown in **Figure 3.9B**, siNotch1 significantly decreased mRNA expression of Notch1 ($p<0.05$) when compared to untreated or siNC treated MSCs. Furthermore, siNotch1 decreased the GOX induced increase in Notch1 expression comparable to untreated cells while the siNC had no effect (**Figure 3.9C**). As shown in **Figure 3.9D**, DAPT decreases NICD expression in presence of 100μM H_2O_2.

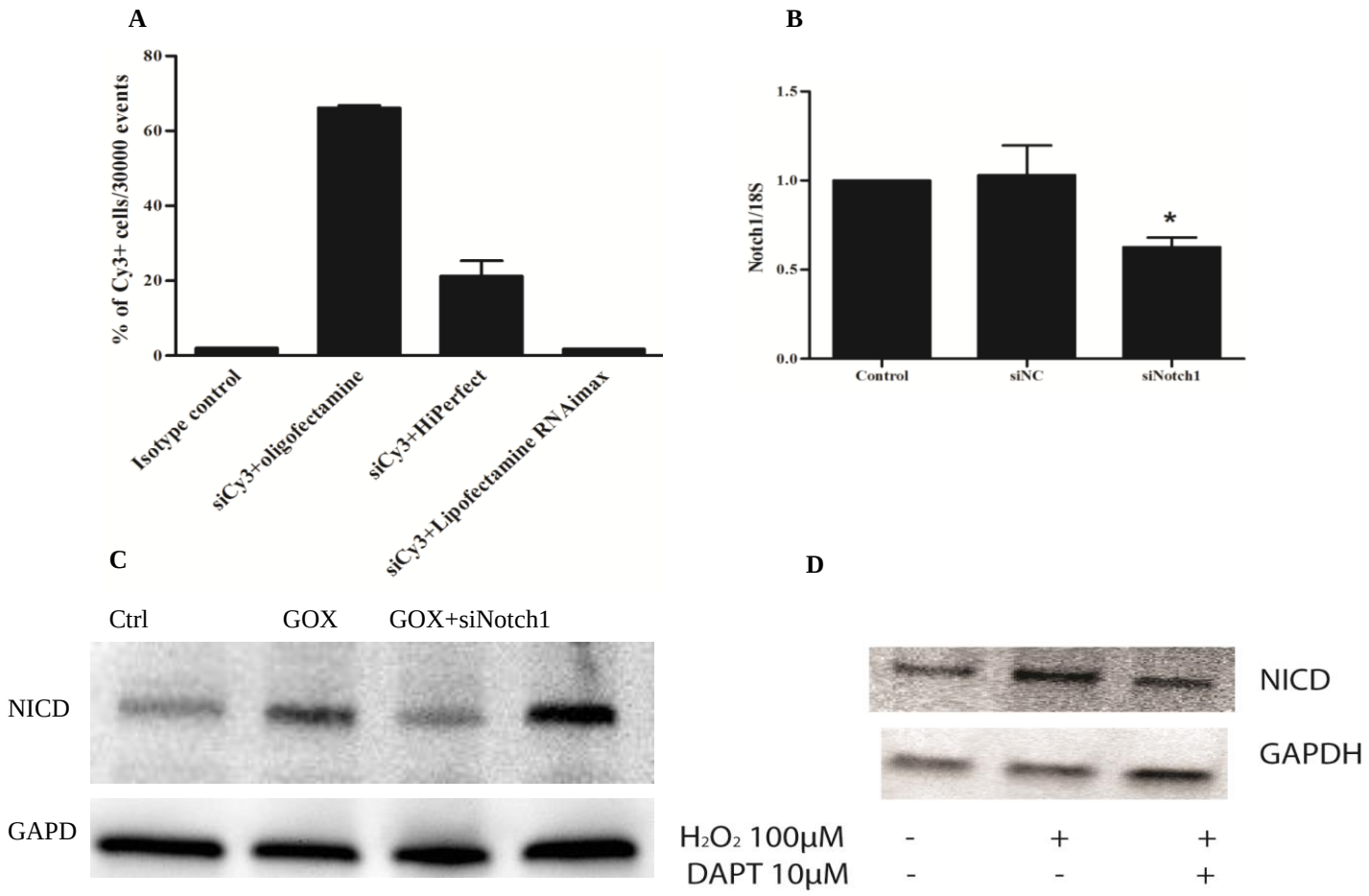

Figure 3.9. Notch1 knockdown using siRNA and DAPT. (**A**) MSCs were transfected with mock siRNA labeled with Cy3 for 24h and transfection efficiency of commercially available reagents Oligofectamine, HiPerfect and Lipofectamine RNAimax was evaluated. Oligofectamine with the highest transfection efficiency was chosen for all siRNA studies. (**B**) MSCs were transfected with negative control siRNA (siNC) or siNotch1 for 48h. The mRNA expression of Notch1 is significantly decreased with siNotch1 transfection when compared to transfection reagent or siNC treated MSCs. (**C**) MSCs were transfected with 25nM of negative control siRNA (siNC) or siNotch1 for 48h in presence of 5mU/ml GOX. The increase in protein levels of the Notch1 Intracellular

Domain (NICD) by GOX are decreased by addition of siNotch1 while levels are unaltered by siNC. (**D**) Representative Western blot for NICD (upper) and GAPDH (lower) in MSCs treated with vehicle, 100μM H_2O_2, or 100μM H_2O_2 + 10μM DAPT respectively (mean±SEM, * $p<0.05$, $n\geq5$ by One-way ANOVA followed by Dunnett's post test).

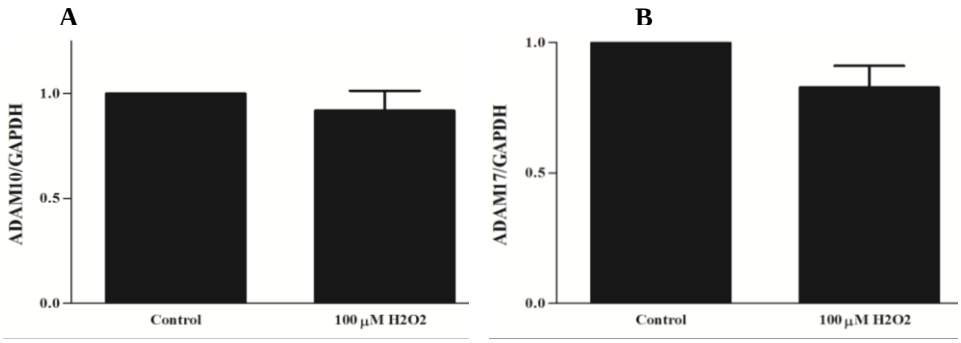

Figure 3.10. H_2O_2 does not alter mRNA expression of ADAMs 10 and 17. MSCs were treated with 100μM H_2O_2 for 1 week and mRNA expression of (**A**) ADAM10 and (**B**) ADAM17 was measured by real time qPCR (mean±SEM, n=10 by Student's unpaired t-test).

ADAM10 and ADAM17 are mettaloproteases are involved in Notch1 processing, Treatment with H_2O_2 did not affect the expression or activity of ADAM17 (**Figure 3.10 A** and **B**). Inhibition of Notch1 signaling by DAPT for 1 week had a small but non-significant decrease on basal αMHC expression, but prevented the increase seen in 100μM H_2O_2 treated cells ($p<0.05$ vs. H_2O_2 alone; **Figure 3.11A**). A similar effect of DAPT was observed in the expression pattern of the endothelial marker Flt1, where Notch1 inhibition prevented the increase in Flt1 expression by 100μM H_2O_2 ($p<0.05$vs

H_2O_2 alone, **Figure 3.11B**). Interestingly, inhibition of basal Notch1 signaling by DAPT decreased expression of sm α-actin by 4-fold ($p<0.001$, **Figure 3.11C**) and sm22α by 3-fold ($p<0.001$, **Figure 3.11D**). This decrease was further augmented by the combined treatment with 100μM H_2O_2 and DAPT with a 10-fold and 6-fold decrease in expression of smooth muscle α-actin and sm22α, respectively ($p<0.001$vs H_2O_2 alone, **Figures 3.11C** and **D**).

To determine if glucose oxidase mediated changes in cardiogenic gene expression through Notch1 signaling, MSCs were treated with 5mU/ml GOX in presence of a negative control siRNA (siNC) or siNotch1 for 48h. As shown in **Figures 3.11E-H**, addition of siNotch1 did not clearly demonstrate dependence of GOX-mediated gene expression changes on Notch1.

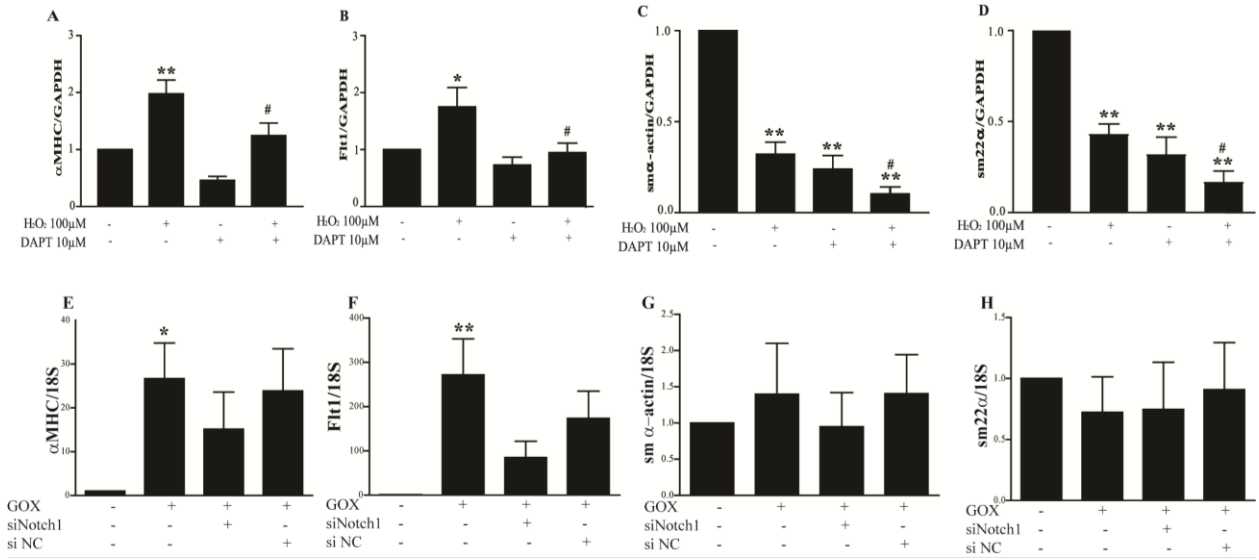

Figure 3.11. Oxidative stress regulates cardiogenic gene expression in MSCs through Notch1 dependent and independent manner. MSCs were treated with 100μM H_2O_2 ± Notch1 inhibitor DAPT (**A-D**) or with 5mU/ml GOX± siNotch1 (**E-**

H).Expression of the cardiac marker αMHC (**A,E**), endothelial marker Flt1 (**B,F**), and the smooth muscle markers sm α-actin (**C,G**) and sm22α (**D,H**) were determined by qRT-PCR. Values are mean±SEM after normalizing gene expression to GAPDH (**A-D**) or 18S (**E-H**) expression. $n \geq 4$, *$p<0.05$ and **$p<0.01$ compared to control (0μM H_2O_2), or #$p<0.05$ compared to 100μM H_2O_2 by one-way ANOVA followed by Bonferroni's post test.

3.3 DISCUSSION

Previous studies have shown that transplantation of MSCs in the heart following MI leads to small but significant functional improvements (7). Understanding the molecular mechanisms by which MSCs promote cardiac function, especially in the oxidative microenvironment following MI will greatly aid in improving efficacy of stem cell-based therapies. Following myocardial infarction, elevated levels of reactive oxygen species (ROS) have been found at the infarct site (106) suggesting that ROS such as H_2O_2 might influence the differentiation and function of implanted MSCs. As substantial amounts of ROS have been found in the area at risk after MI (29), and as ROS have been identified to play a critical role in the differentiation of other stem cell types (108, 109), we chose to study the effect of H_2O_2 on MSC differentiation in vitro. Here we show that MSCs that were subjected to pulses of pathophysiological levels of H_2O_2 for 1 week or continuous H_2O_2 produced by oxidation of glucose in the extracellular media by Glucose oxidase (GOX) for 48h increased the expression of early cardiac- and endothelial- genes with decreased expression of early smooth muscle genes.

Although only a 2-fold increase in cardiac markers is observed with H_2O_2 treatment, addition of GOX results in a more robust increase of 30-fold for α-MHC and 75-fold for nkx2-5 which are comparable to neonatal rat ventricular cardiomyocytes with 40- fold and 100- fold higher expression of α-MHC and nkx2-5 respectively when compared to untreated MSCs. These results were in agreement with those reported for human embryonic stem cells (ESCs) (109). Similar results were obtained when adult heart resident cardiac progenitor cells (CPCs) were treated with GOX. These data demonstrate redox-sensitive alteration in cardiogenic gene expression in MSCs and CPCs.

Our results also demonstrate that only high levels of exogenous H_2O_2 (100μM) and high concentrations of GOX (5mU/ml) were able to regulate expression of Notch1 and cardiogenic genes. We believe this very narrow threshold effect may be due to a combination of factors such as presence of basal H_2O_2 and constitutive expression of antioxidant enzymes by cells. Unpublished data from our laboratory demonstrate basal H_2O_2 levels of 1μM in cultured stem cells as measured by electron paramagnetic spin resonance. Recent reports demonstrate many stem cells including MSCs contain higher levels of antioxidants (110, 111). We measured H_2O_2 levels in stem cells following addition of 100μM H_2O_2 and found that the concentration reduced to 12μM, within an hour, indicating rapid scavenging of exogenous oxidants. Finally, recent data from human MSCs determined higher levels of catalase and glutathione peroxidase, with no changes in superoxide dismutase compared with other stem cells and fully differentiated cells (111). In that study, there was a threshold response with human MSCs demonstrating almost 80% survival at 4mM H_2O_2, falling to <10% at 8mM. Our studies

indicate robust survival at 100μM H_2O_2 and 5mU/ml GOX with cellular responses, but higher concentrations to be potentially cytotoxic. Taken together, these data demonstrate that many cells, especially stem and progenitor, have threshold responses with small windows of dose-responses.

Next, we investigated whether H_2O_2 regulates any signaling pathway involved in stem cell differentiation. One of the signaling pathways that greatly influences stem cell differentiation is the Notch signaling pathway (112). Therefore, we investigated whether interplay existed between H_2O_2 and Notch1 signaling pathways. Interestingly, mRNA level of Notch1 as well as proteolytic cleavage of the Notch1 intracellular domain (NICD) was upregulated by treatment with either 100μM H_2O_2or GOX suggesting that high levels of H_2O_2 affect expression of both the mRNA and protein activity of Notch1. Although only a 1.5-fold increase in mRNA and 2-fold increase in NICD protein is observed, reports suggest that very small changes in Notch1 activation are sufficient to induce Notch1 signaling (107). Furthermore, this increase in Notch1 also significantly increased mRNA expression of downstream targets of Notch1. As MSCs were pulsed with $H_2O_2$for 1 week, discontinuous oxidative stress resulted in small fold changes in cardiac gene expression. While these changes may not represent true differentiation, it suggests that H_2O_2 levels influence cardiac gene expression in MSCs.

Although upregulation of cardiac and endothelial genes by Notch1 signaling may appear to be counterintuitive given the role of Notch signaling in suppressing cardiomyogenesis in ESCs (69), upregulation of nkx2-5 and vWF is consistent with reports indicating involvement of Notch1 signaling in regulating these genes in cardiac progenitor cells and bone marrow stromal cells respectively (10, 113). Our observation

that treatment with 100μM H_2O_2or 5mU/ml GOX decreased expression of the Notch1 ligand Jagged1 in MSCs is consistent with previous reports of an inverse relationship between expression levels of Notch1 and Jagged 1 in other cell types (114). As we observed changes in cardiogenic gene expression at the mRNA level, we sought to determine if these changes translated correspondingly at the protein level. Flow analysis of GOX-treated MSCs indicated that a small number of MSCs have high expression of α-MHC along with increase in Flt1 expression and decreased sm α-actin expression. These results suggest that GOX treatment increases the frequency α-MHC and Flt1 positive cells while decreasing sm α-actin+ cells.

The mechanism of upregulation of Notch1 activation by H_2O_2may be due to activation of enzymes involved in Notch1 cleavage and processing. It is possible that H_2O_2may increase Notch1 activation via γ-secretase activation as H_2O_2 mediated increase in γ-secretase activation has been demonstrated in the pathogenesis of Alzheimer's disease (115). Pharmacological inhibition of γ-secretase activity using DAPT inhibits Notch1 activation in different stem cells (113, 116). Therefore, to determine whether H_2O_2 regulates cardiogenic gene expression in MSCs through Notch1 signaling, we blocked Notch1 activation daily using DAPT and analyzed expression of the different cardiogenic markers in presence and absence of 100μM H_2O_2. Among the markers analyzed, the increase in expression of the high affinity VEGF receptor Flt1 and the cardiac marker α-MHC observed with 100μM H_2O_2 was abrogated by co-treatment with DAPT indicating that H_2O_2 regulates Flt1 and α-MHC expression through Notch1 signaling. We attempted knockdown of Notch1 using siRNA, however, this was not successful as siRNAs that significantly reduced Notch1 gene expression greatly reduced

cell survival over the 1 week of treatment. To determine if Glucose oxidase (GOX) mediated acute changes in cardiogenic gene expression through Notch1 signaling, MSCs were transfected with siNotch1 along with GOX. Treatment with siNotch1 showed a strong trend towards decreasing the GOX-mediated increase in α-MHC and Flt1 while no effect was observed on smooth muscle gene expression by addition of GOX ± siNotch1. The lack of changes in ADAM 10/17 expression on H_2O_2-treatment suggests the importance of the γ-secretase component of this pathway. Furthermore, treatment with GOX increased expression of enzymes involved in processing of Notch1 and Jagged1 such as *Mfng*, *Lfng* and *Neurl* indicating that H_2O_2 influences both notch1 cleavage and processing enzymes.

Expression of smooth muscle markers decreased significantly upon treatment with 100μM H_2O_2. Inhibition of Notch1 also decreased basal expression of smooth muscle markers, keeping with prior findings (117). Interestingly, co-treatment of MSCs with H_2O_2 and DAPT resulted in a further decrease in smooth muscle markers. This indicates that H_2O_2 decreases smooth muscle gene expression through a parallel pathway, and that activation of Notch serves as a compensatory mechanism to stabilize smooth muscle gene expression. Finally, expression of vimentin was also decreased by both H_2O_2 and GOX treatment. While vimentin is expressed in many cell types, it is most prevalent in fibroblasts and thought to be a partial marker of fibroblastic lineage (118) and lower expression could lead to decreased fibrosis.

To understand the mechanism by which oxidative stress mediated by GOX resulted in robust increases in cardiogenic gene expression, the expression of Notch1-related genes in GOX treated MSCs was analyzed by PCR array. Interestingly, Wnt11

expression was increased in GOX treated MSCs. Wnt11 signaling has been shown to promote cardiomyogenic differentiation of human endothelial progenitor cells and mouse marrow mononuclear cells (119, 120). Moreover, Wnt signaling has been identified as a downstream target of Notch1 that regulates expression of cardiac transcription factors during mouse cardiogenesis and is essential for cardiac development (121, 122). MSCs overexpressing Wnt11 have been shown to be cardioprotective following oxidative stress in rats through increased cardiac gene expression and release of paracrine factors (123, 124).

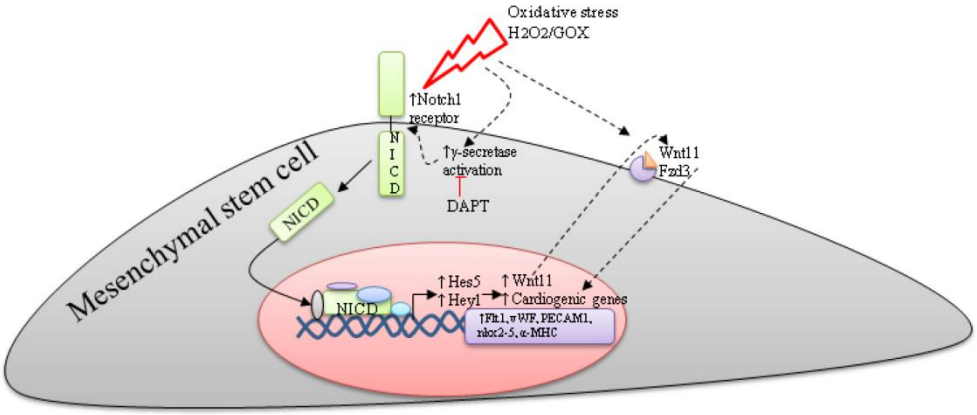

Figure 3.12. Proposed model of H_2O_2/GOX mediated induction of cardiogenic gene expression in MSCs.

Of note, MSCs used in this study were a heterogenous mix of cells present in the adult rat bone marrow. It is unclear whether there is one particular lineage in the heterogenous mix that is most responsible for these changes or whether Notch1 is activated in all these cell types. Though published literature suggests that all these cells

express Notch (10, 71), the optimal cell type needs to be determined. To our knowledge, this study demonstrates for the first time two important findings in the field of stem cell therapy. Oxidative stress in rat bone marrow derived MSCs and heart derived CPCs i) regulates expression of selected cardiac, endothelial, and smooth muscle genes and ii) promotes Notch1 signaling and downstream Wnt11 activation. Our current working model is depicted in **Figure 3.12** and summarizes the findings of our study. Given that cardiogenic gene expression of 2 adult progenitor types (MSCs and CPCs) was induced by high levels of H_2O_2/GOX via Notch1 signaling, this may represent an important conserved response. As both of these cell types are in clinical trials, this study may have implications in developing adult stem/progenitor cell-based therapies.

CHAPTER 4

HYDROGEL-DEPENDENT NOTCH1 ACTIVATION MODULATES CARDIAC PROGENITOR CELL FUNCTION

4.1 INTRODUCTION

Heart failure is the leading cause of death worldwide and myocardial infarction (MI) is the predominant cause (1). While heart transplantation is the most viable treatment, limited availability of donor hearts, complications from immunosuppression, and possibility of graft failure have necessitated the search for treatment alternatives. New emphasis has been placed on tissue engineering approaches to treat MI including delivery of growth factors and/or stem cells via direct injection, with natural scaffolds such as gelatin and alginate or with artificial self-assembling peptide scaffolds (125, 126). Evidence for myocyte renewal in human hearts and the existence of cardiac progenitor cells (CPCs) in niches in the post natal heart have consequently led to cell-based therapeutic strategies (31, 127). These cells have been identified based on expression of cell surface markers (c-Kit, Sca1), ability to efflux dyes, or form spheroids in culture. Success in clinical trials based on CPC therapy for cardiovascular diseases (15, 16) and the inherent ability of CPCs to differentiate into cardiovascular cell types (31) has established CPCs as a clinically relevant cell source for cardiac therapy.

However, the reparative capacity of different CPCs is dependent on the myocardial environment, activation of specific signaling pathways, and sustained

retention in the infarcted heart. While VEGF, IGF-1 and Notch signaling promote CPC mediated cardiac repair, Wnt activation exerts anti-proliferative effects (10, 128-130). Studies have demonstrated the effect of incorporating such signals into natural or synthetic scaffolds to promote cardiac regeneration (54, 81). Injectable self-assembling peptide (SAP) hydrogels are composed of 16 amino acid long peptides that assemble into 3D microenvironments under physiological conditions (85). SAP hydrogels have been used for delivery of growth factors and stem cells in vivo and as a 3D system to study stem cell/biomaterial interactions and direct stem cell responses. Self-assembling peptides (SAP) promote cell-cell interactions (85) that are essential for progenitor cell survival and differentiation in the hydrogel. Studies have demonstrated the effect of incorporating growth factors and other bioactive signals in the hydrogel on improving stem/progenitor cell function in the infarcted heart upon transplantation. For example, SAPs functionalized with homing and adhesion motifs have been shown to regulate human adipose stem cell behavior in vitro (95). Delivery of VEGF in the SAPs has also been shown to improve cardiac function following infarction in rats and pigs through increased angiogenesis (86). The SAP used in this study $(RARADADA)_2$ has hydrophilic and hydrophobic residues with alternating charges that allows for self-assembly into a hydrogel under physiologic pH and osmolarity. Along with providing a 3D environment for stem cells, these hydrogels can also be precisely modified with bioactive motifs to deliver cues for regeneration. This is important as stem cells require spatially and temporally defined signals to exert their beneficial effects.

Notch signaling is an evolutionarily conserved cell-cell communication pathway where the binding of the ligand Jagged1 on the signal sending cell to the Notch1 receptor

on the signal receiving cell activates downstream signaling events that regulates gene expression in several cell types (58). Deletion of Notch, the ligands or the downstream effectors is embryonic lethal due to cardiovascular defects suggesting the importance of Notch signaling in early cardiac development (131). Further, Notch signaling has been shown to precede cardiac regeneration in zebrafish (17). Mechanistically, the external force generated by ligand endocytosis on binding to the Notch1 receptor is required for Notch1 activation in the signal receiving cell (132). To achieve this force generation, we immobilized a peptide mimic of the Notch1 ligand Jagged1 (RJ) in the 3D hydrogel. The peptide RJ used in this study has been shown to promotes differentiation of keratinocyte and monocyte derived dendritic cells by activating Notch1 signaling (100, 133). However, the effect of activating Notch1 signaling using a peptide mimic of Jagged1 in progenitor cells for cardiac regeneration has not been investigated. Therefore, we designed a SAP hydrogel that incorporated the Jagged1 peptide RJ to enhance cardiac differentiation of CPCs. We then investigated the effect of Notch1 activation on cardiogenic gene expression, growth factor production and cardiac repair through in vitro and in vivo studies.

4.2 RESULTS

Hydrogel characterization

Mechanical testing of Young's modulus of the different hydrogels using atomic force microscopy showed that addition of either the scrambled or RJ peptide to the SAP did not change the Young's modulus in comparison with the unmodified hydrogels at both 1 and 2% w/v. The average Young's modulus of all the 1% and 2% hydrogels was 500Pa and 1800Pa respectively (**Fig. 4.1A**). Evaluation of hydrogel swelling ratio demonstrated that the 1% hydrogels had a significantly higher swelling ratio than the corresponding 2% hydrogels indicating a more loosely linked peptide network at lower stiffness (n=3, $p<0.05$, **Fig. 4.1B**). There was no significant effect of addition of either scrambled (RS) or RJ peptide.

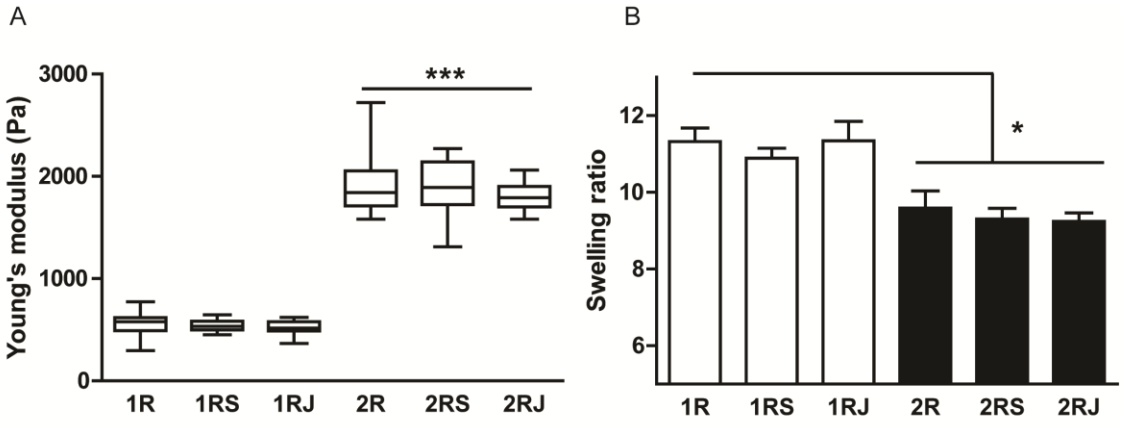

Figure 4.1: Mechanical characterization of the hydrogels. (A) Atomic Force Microscopy measurements of the Young's modulus of the hydrogels. The average Young's modulus of the 1% hydrogels (1R, 1RS and 1RJ) is 500Pa and the 2% hydrogels (2R, 2RS, 2RJ) is 1800Pa. *** $p<0.001$, Student's t-test. (B) Swelling ratio of the

hydrogels determined at 24h following gellation. All the 1% hydrogels had a significantly higher swelling ratio than the corresponding 2% hydrogels indicating a more loosely cross-linked network. n=3, * $p<0.05$, Student's t-test.

RJ-mediated Notch1 activation is stiffness dependent

CHO cells with Notch1-responsive YFP expression were cultured in 96 well plates with media containing 20 or 40μM of R, RS or RJ for 48h.

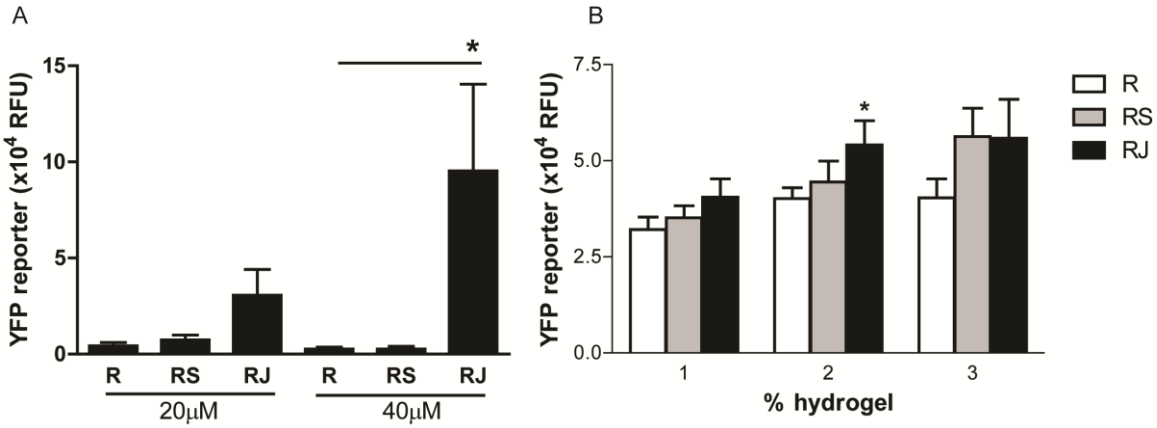

Figure 4.2: Notch1 activation in 3D is stiffness dependent. (A) CHO cells with Notch1 responsive YFP expression were cultured in 2D with media containing the peptides (R, RS and RJ) at 20 and 40μM for 48h. A significant increase in Notch1 activation was observed on treatment with 40μM RJ, n=3, * $p<0.05$, one-way ANOVA. (B) CHO cells were cultured in 3D in 1-3% w/v hydrogels containing R, RS or RJ for 48h. A significant increase in Notch1 activation was observed on increasing % hydrogel (open bars) and presence of RJ at 2% had the highest levels of Notch1 activation (middle black bar), n=7, * $p<0.05$, two-way ANOVA.

A significant increase in YFP expression was observed on treatment with RJ at 40 µM compared to all other treatment groups ($p<0.05$, n=3, **Fig. 4.2A**). These cells were also cultured in 3D in 1, 2 or 3% w/v hydrogels composed of R, RS or RJ for 48h. A significant increase in YFP expression was observed with increasing hydrogel stiffness in the absence of any ligand (n=7, $p<0.01$, open bars, **Fig. 4.2B**). Interestingly, the presence of RJ in 2% hydrogels resulted in maximum Notch1 activation ($p<0.05$, black bar, **Fig. 2B**). Further increase in stiffness to 3% w/v did not promote Notch1 activation.

Stiffness dependent Notch1 activation differentially regulates CPC gene expression

CPCs were clonally expanded and characterized as described in (79). Flow analysis demonstrated >96% expression of the Notch1 receptor on CPCs, >90% expression of the stem cell receptor c-Kit, and the cardiac transcription factors nkx2-5 and gata4 **(Figure 4.3).**

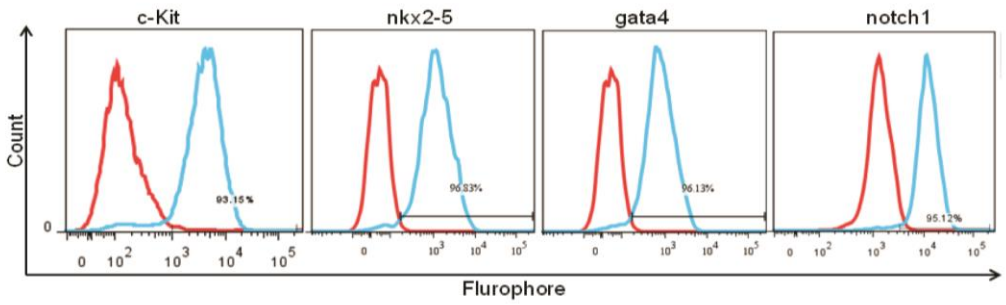

Figure 4.3: Characterization of CPCs. Representative histogram of cell surface expression of c-Kit, nkx2-5, gata4 and Notch1 on CPCs.

For in vitro studies, CPCs were embedded in 1 or 2% (w/v) SAP hydrogels (1R, 1RS, 1RJ or 2R, 2RS, 2RJ) and cultured for 48h. Culture in 1% hydrogels with RJ resulted in a significant increase in Hey1, a downstream target of Notch1 signaling ($p<0.05$, n=4; **Fig. 4.4A**). A significant increase in expression of the endothelial genes Flt1 ($p<0.05$, n=5) and vWF ($p<0.05$, n=9; **Fig. 4.4B and C**), and smooth muscle genes sm22α ($p<0.05$, n=7) and sm α-actin ($p<0.05$, n=7; **Fig. 4.4D and E**). No change in expression of cardiac genes, nkx2-5, MEF2C and GATA4 was observed (n=4, **Fig. 4.4 F-H**).

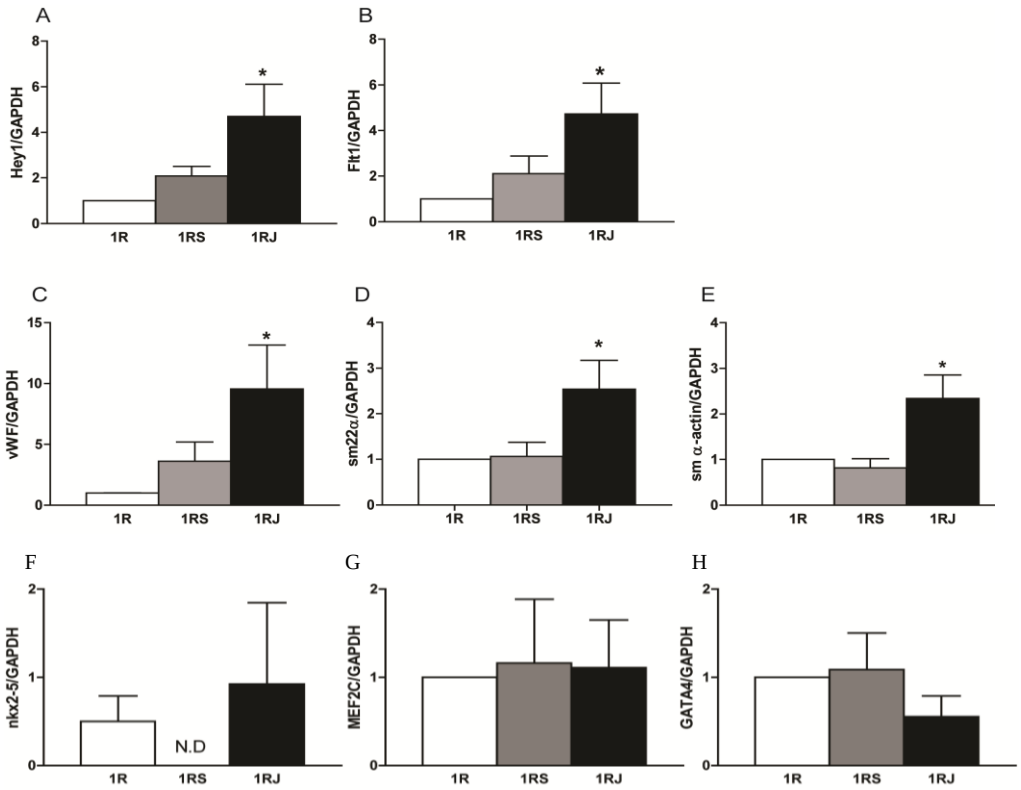

Figure 4.4: Culture of CPCs in 1% hydrogels with RJ activates Notch1 signaling and promotes endothelial and smooth muscle gene expression. CPCs were cultured in 1% hydrogels (R, RS, RJ) for 48h and expression of (A) Notch1 downstream target,

Hey1, (B) VEGF receptor1 Flt1, (C) vWF, (D) sm22α, (E) sm α-actin, (F) nkx2-5, (G) MEF2C, and (H) GATA4 was measured by qPCR. n=4-9, * $p<0.05$ vs 1R.

Culture of CPCs in 2RJ hydrogels increased expression of Hey1, a downstream target of Notch1 signaling ($p<0.05$, n=4, **Fig. 4.5A**). Interestingly, culture in 2% hydrogels with RJ promoted expression of the cardiac genes nkx2-5 ($p<0.001$, n=5), mef2c ($p<0.05$, n=8) and gata4 ($p<0.05$, n=6; **Fig. 4.5B-D**) while no changes in expression of the endothelial or smooth muscle genes was observed (n=5-7, **Fig. 4.5E-H**). No significant differences were observed in gene expression between CPCs in control and scrambled hydrogels.

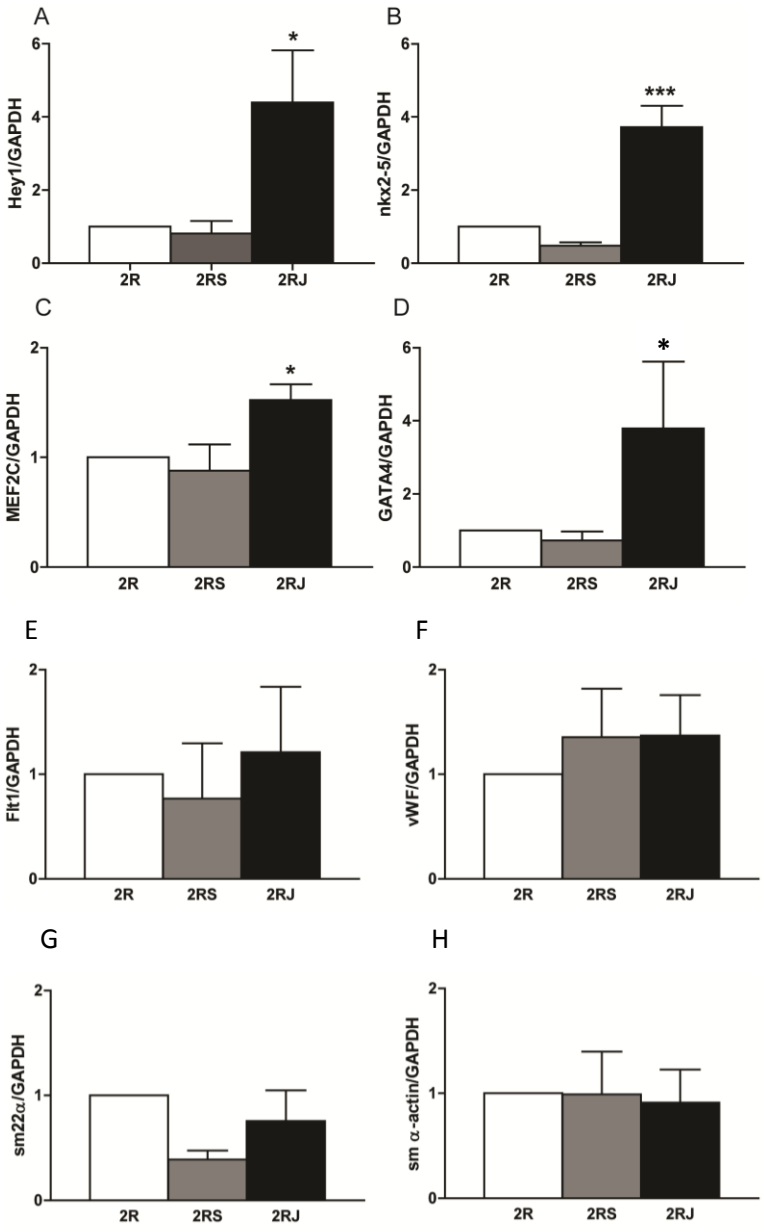

Figure 4.5: Culture of CPCs in 2% hydrogels with RJ activates Notch1 signaling and promotes cardiac gene expression. CPCs were cultured in 2% hydrogels (R, RS, RJ) for 48h and expression of (A) Notch1 downstream target, Hey1, (B) nkx2-5, (C) MEF2C, (D) GATA4, (E) VEGF receptor1 Flt1, (F) vWF, (G) sm22α and (H) sm α-actin was measured by qPCR. n=4-8, * $p<0.05$, *** $p<0.001$ vs 2R.

Low stiffness hydrogels promote endothelial gene expression in mouse embryoid bodies

Mouse embryoid body-derived cells were cultured in low stiffness (1%) hydrogels for 48h. A significant increase in expression of the VEGF receptor Flk1 ($p<0.05$), cell surface adhesion molecule PECAM1 ($p<0.001$) and smooth muscle myosin heavy chain Myh11 ($p<0.01$) was observed in cells cultured in 1% hydrogels containing RJ (n=3, **Fig.4.6 A-C**). No expression of the cardiac marker Troponin T was observed. Culture in 2% hydrogels containing RJ showed a trend towards increased cTnT expression (data not shown).

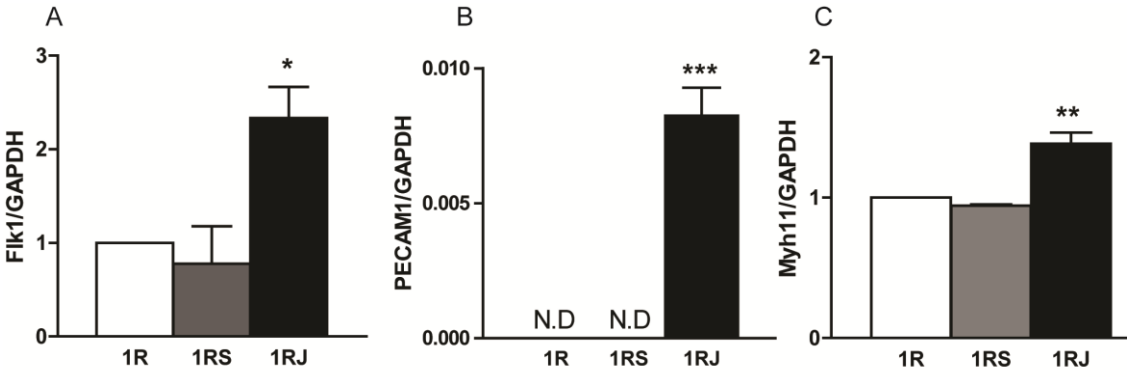

Figure 4.6: Long term culture of mouse embryoid bodies (EBs) in 1% hydrogels with RJ promotes endothelial and smooth muscle gene expression. Mouse EBs were cultured in 1% hydrogels (R, RS, RJ) for 1 week and expression of (A) VEGF receptor2 Flk1, (B) PECAM1, and (C) smooth muscle Myosin Heavy chain Myh11 was measured by qPCR. n=3, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs 1R.

RJ-mediated Notch1 activation augments growth factor production in CPCs

Conditioned media was obtained from CPCs cultured for 48h in 1R, 1RS, 1RJ, 2R, 2RS and 2RJ hydrogels. The secreted growth factors in the conditioned media were analyzed by an ELISA array. No change in levels of VEGF, GM-CSF, IP10, bFGF and IGF1 were observed between CPCs in 1R and 1RJ hydrogels (n=6, $p>0.05$). However, culture in 1RJ hydrogels increased expression of SCF ($p<0.01$), IL6 ($p<0.05$) and PDGF ($p=0.08$, n=6, **Fig 4.7 A-H**).

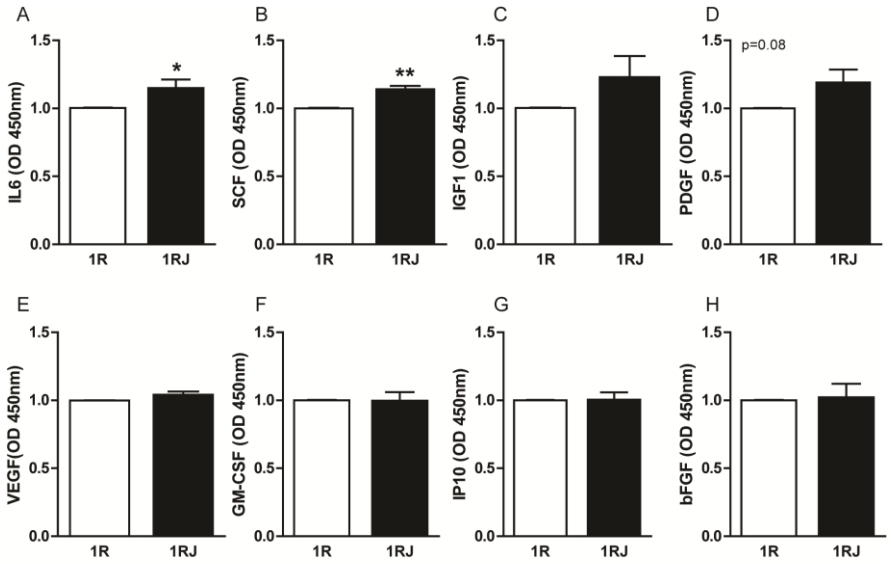

Figure 4.7: Angiogenic growth factor expression in CPC conditoned media. CPCs were cultured in 1R and 1RJ hydrogels for 48h and the secreted growth factors, (A) IL6, (B) SCF, (C) IGF1, (D) PDGF, (E) VEGF, (F) GM-CSF, (G) IP10 and (H) bFGF, present in the conditioned media were analyzed by an ELISA array. Higher levels of IL6, SCF and PDGF were observed in media from CPCs in 1RJ hydrogels compared with 1R hydrogels. * $p<0.05$, ** $p<0.01$, n=6, Student's t-test.

To validate and determine the exact quantities of growth factors secreted, a growth factor-specific ELISA was performed. As shown in **Figure 4.8A**, CPCs cultured in 1RJ hydrogels had a 43% increase in Platelet derived growth factor-BB (PDGF) production compared to 1R gels ($p<0.05$, **Fig. 4.8A**). Less than 3pg of PDGF was present in the conditioned media from CPCs in 2R and 2RJ hydrogels (**Fig. 4.8A**). The effect of this conditioned media on tube formation by rat cardiac endothelial cells (CECs) was analyzed. As shown in **Figure 4.8B**, CECs formed more tubes and interconnections between cells when cultured in 1RJ conditioned media. A significant increase in tube length was observed in CECs in 1RJ media when compared to 1R and 1RS ($n=3$, $p<0.05$, **Fig. 4.8C**). The amount of stem cell factor (SCF) in conditioned media obtained from CPCs in 1R, 1RJ, 2R, and 2RJ hydrogels was determined by a SCF-specific ELISA. As shown in **Figure 4.8D,** a 6% increase in SCF production was observed in 1RJ conditioned media compared to 1R ($n=10$, ns). A 13% increase in SCF was observed in 2RJ conditioned media compared to 2R ($n=5$, $p<0.05$ vs 2R, # $p<0.001$ vs 1R).

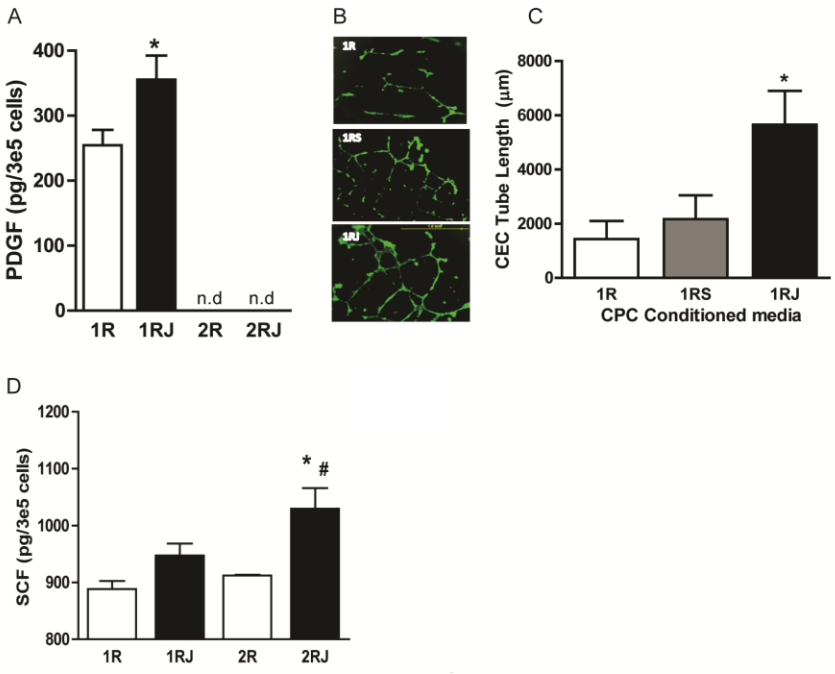

Figure 4.8: Paracrine effects of Notch1 activated CPCs is stiffness dependent. (A) Higher PDGF levels present in conditioned media obtained from CPCs cultured for 48h in 1RJ compared to 1R hydrogels. * $p<0.05$, n=6, Student's t-test. (B) Representative images of tube formation by rat cardiac endothelial cells (CECs) cultured in conditioned media obtained from 1R (top), 1RS (middle) and 1RJ (bottom). Scale bar 1mm. (C) Quantification of CEC tube length. A 3-fold increase in tube length is observed in CECs cultured in conditioned media obtained from CPCs cultured in 1RJ hydrogels. n=3, * $p<0.05$ vs 1R. (D) Higher SCF levels present in conditioned media obtained from CPCs cultured for 48h in 2RJ compared to all other hydrogels. * $p<0.05$ vs 2R, # $p<0.05$ vs 1R, n=5-10, One-way ANOVA.

As Notch1 signaling is known to promote cell proliferation, CPCs were cultured in 1R, 1RS, 1RJ, 2R, 2RS and 2RJ hydrogels for 24 hours and cell proliferation was quantified by the extent of EdU incorporated DNA in cells by click-iT EdU assay. As shown in **Figure 4.9A,** CPCs cultured in 2RJ hydrogel had a significant increase in EdU incorporated DNA indicative of proliferation compared to all other groups (n=3-5, $p<0.05$ vs 2RS and 1RJ; $p<0.01$ vs 2R; $p<0.001$ vs 1R). The effect of conditioned media (2R, 2RS and 2RJ) on rat primary cardiomyocytes and H9C2 myoblast proliferation was analyzed using click-iT EdU assay.

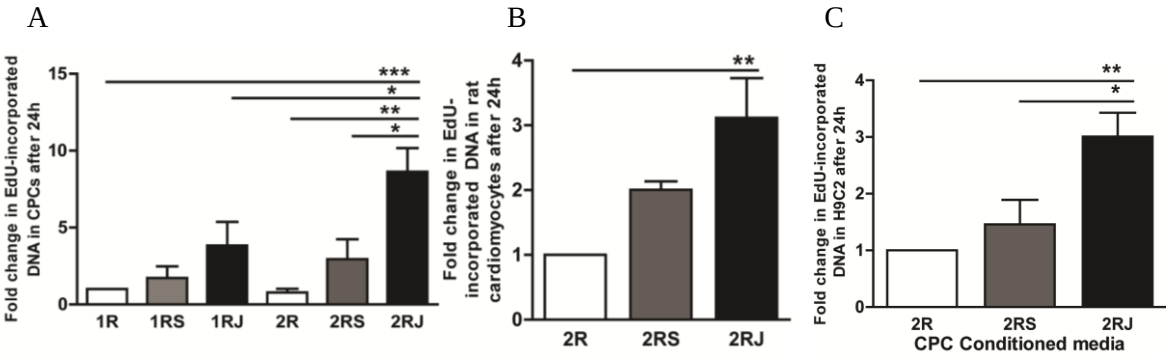

Figure 4.9: Proliferative effects of Notch1 activated CPCs is stiffness dependent. (A) Increased EdU-incorporated DNA present in CPCs cultured in 2RJ compared to all other hydrogels. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, n=3-5, One-way ANOVA. Increased EdU-incorporated DNA present in rat primary adult cardiomyocytes (B, ** $p<0.01$ vs 2R, n=4) and H9C2 myoblasts (C, * $p<0.05$ vs 2RS, ** $p<0.01$ vs 2R, n=4) cultured in 2RJ compared to all other hydrogels. One-way ANOVA.

As shown in **Figure 4.9B**, a significant increase in EdU incorporation is observed in primary adult cardiomyocytes cultured in 2RJ CPC conditioned media (n=5, $p<0.01$ vs 2R). A similar result was observed in H9C2 myoblasts (**Fig. 4.9C**, n=4, $p<0.05$ vs 2RS, $p<0.01$ vs 2R).

Hydrogel concentration differentially regulates key signaling pathway target genes

Expression levels of several signal transduction pathway targets in CPCs cultured in 1R or 2R hydrogels were analyzed by qPCR array. CPCs in 1R hydrogels had higher expression of proinflammatory genes such as TNFα, TRAIL and IFNγ, MMP7 and CyclinD2 compared to CPCs in 2R hydrogels. The downstream targets of Notch1- Hes5 and Heyl were also increased indicative of Notch1 activation (**Fig. 4.10A**).

Culture of CPCs in higher concentration hydrogel (2R) resulted in increase of the Notch1 target Hes1, the Notch1 ligand Jagged1 and γ-secretase interacting gene Herpud1 (**Fig. 4.10B**). Higher expression of i) antioxidant genes - glutathione reductase (Gsr), NAD(P)H dehydrogenase quinone 1 (Nqo1), Sequestosome 1(Sqstm1) and carbonic anhydrase 9 (Car), ii) PPAR targets - Glut1, Fatty acid transporter member 4 (Slc27a4) and long chain acyl-CoA synthetases- members 4 and 5(Acsl4, Acsl5) iii) Bone morphogenetic protein 2 (BMP2), iv) Wnt ligands- Wnt5A and Wnt1 inducible signaling pathway protein 1 (Wisp1), v) TGFβ targets -Cyclin-dependent kinase inhibitor 1B (Cdkn1b) and epithelial membrane protein 1 (Emp1), vi) NFκB target macrophage colony stimulating factor 1 (M-CSF) and vii) JAK/STAT inhibitor- Suppressor of cytokine signaling 3 (Socs3) and JAK/STAT target myeloid cell leukemia sequence 1 (Mcl1) was detected.

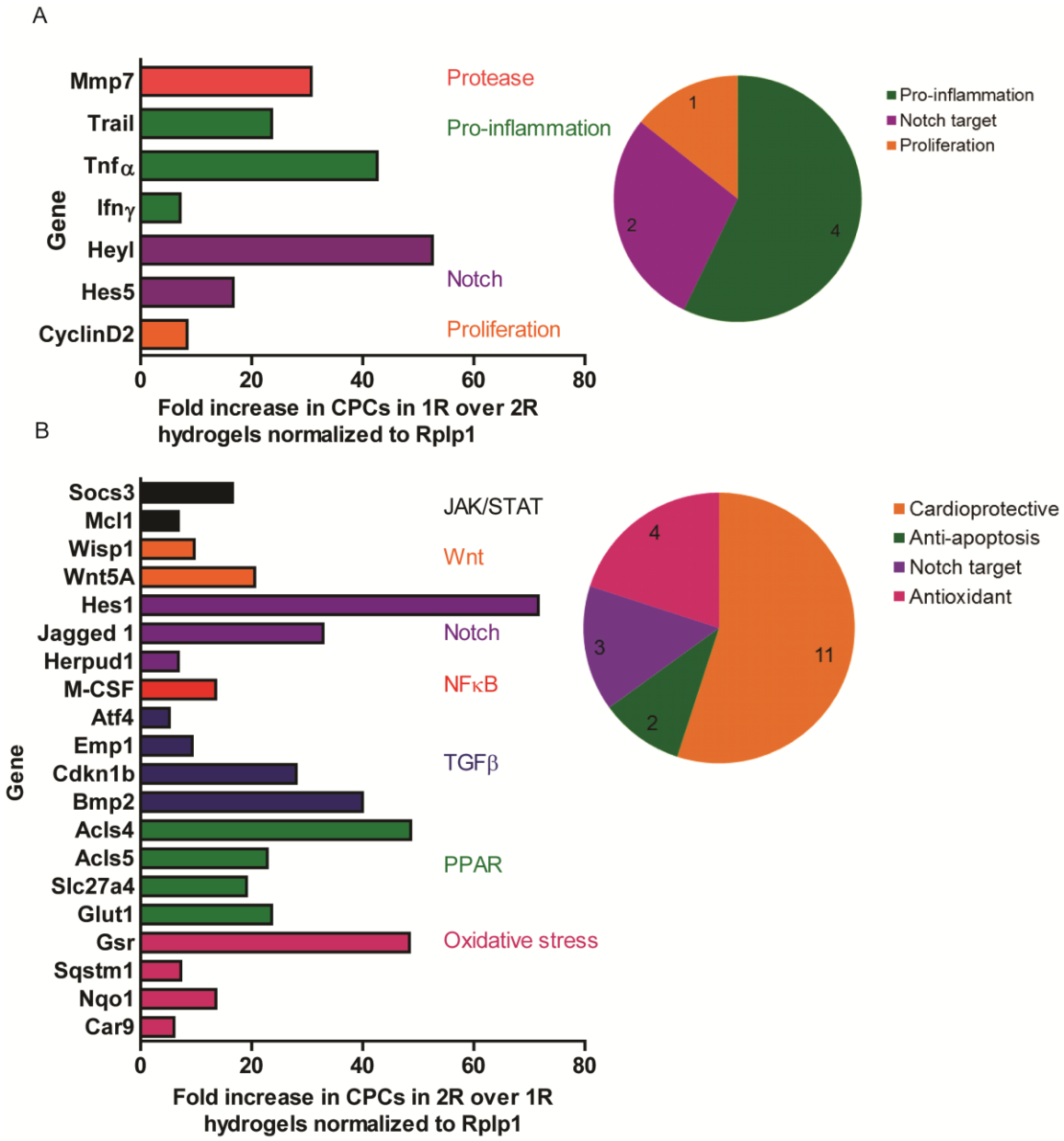

Figure 4.10: Hydrogel concentration differentially regulates gene expression in CPCs. (A) Higher pro-inflammatory gene expression in CPCs cultured in 1R hydrogels compared to 2R. (B) Increased cardioprotective gene expression in CPCs cultured in 2R hydrogels compared to 1R. The pie charts indicate the number of genes and known associated function.

4.2.6 CPC delivery in RJ hydrogels improves cardiac retention

To analyze the extent of myocardial retention of the CPCs, DiR-labeled CPCs in SAP hydrogels (2R, 2RS, and 2RJ) were injected in 3 border zones around the infarct. A time course in vivo imaging of the rats was performed to determine the fluorescent intensity in the hearts as readout of cardiac retention. As shown in **Figure 4.11A**, CPCs in 2RJ hydrogels had a significantly higher % retention compared to CPCs in 2R and 2RS hydrogels until day 7 ($n=5$ per group, $p<0.05$ vs 2R at days 1,2,3 and 4; $p<0.05$ vs 2RS at days 4 and 7, two-way ANOVA). A similar trend was observed until day 14. Representative images of each treatment group are shown in **Figure 4.11B.**

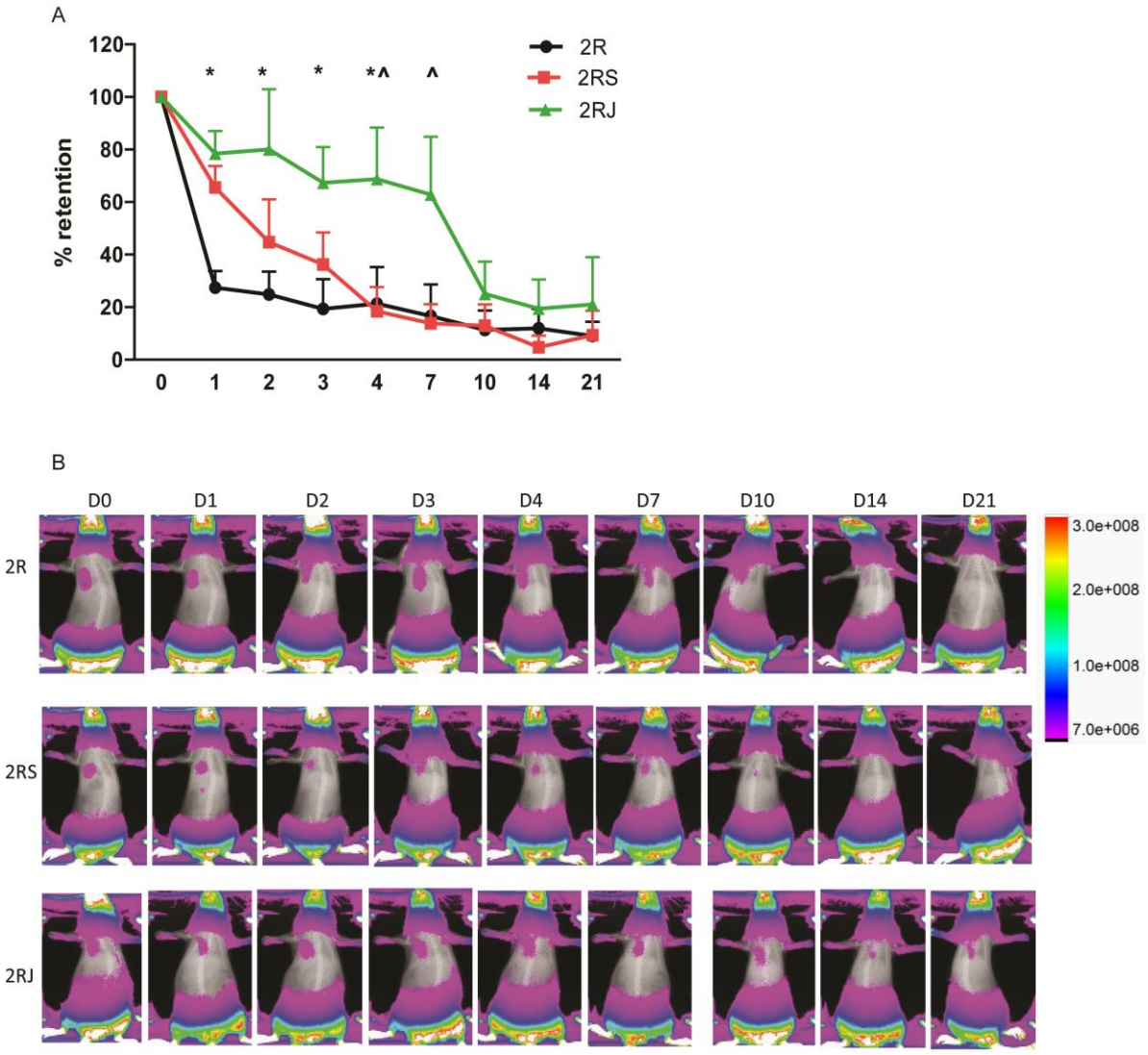

Figure 4.11: Improved myocardial retention of CPCs in 2RJ hydrogels. (A) Quantification of myocardial retention over time. Rats with intramyocardial injection of DiR labeled CPCs were imaged on days 0,1,2,3,4,7,10,14 and 21. Increased retention of implanted CPCs is observed in 2RJ hydrogels compared to CPCs in 2R and 2RS. * $p<0.05$ vs 2R and ^ $p<0.05$ vs 2RS, n=5 per group, Two-way ANOVA. (B) Representative images of each group over time. DiR-CPCs in 2R (top), in 2RS (middle) and in 2RJ (bottom).

CPC delivery in RJ hydrogels promotes cardiac repair

The functional consequences of CPC implantation in 2R, 2RS or 2RJ hydrogels was investigated in rats subjected to experimental myocardial infarction. Male CPCs in 2% hydrogels containing empty, scrambled or Jag1 peptide (2R, 2RS, or 2RJ) were injected intramyocardially (i.m.) in 3 border zones in female rats following myocardial infarction. Cardiac function was evaluated 21 days later by echocardiography and invasive pressure-volume hemodynamic measurements. Ischemia/Reperfusion (IR) for 30minutes significantly decreased ejection fraction of the hearts compared to sham operated animals. Treatment with CPCs in 2RJ hydrogels significantly improved ejection function comparable to levels in sham operated animals as assessed by invasive hemodynamic analysis (**Fig. 4.12A**). No improvement in function was observed in animals treated with CPCs in empty (2R) or scrambled (2RS) hydrogels indicating the importance of Notch1 activation in improving function following infarction. A trend for improvement was observed in rats treated with CPCs in 2RJ hydrogels for the cardiac contractility indicator ±dP/dt (**Fig. 4.12B**). Among other parameters of ventricular function, the significant decrease in stroke work, stroke volume and cardiac output following IR was reversed in rats treated with CPCs in 2RJ hydrogels (**Fig. 4.12C, F and G**). End systolic volume (ESV), an indicator of cardiac contractility was elevated following IR and treatment with CPCs in 2RJ decreased it to levels comparable to sham operated rats (**Fig. 4.12D**). No change in end diastolic volume (EDV) was observed (**Fig. 4.12E**).

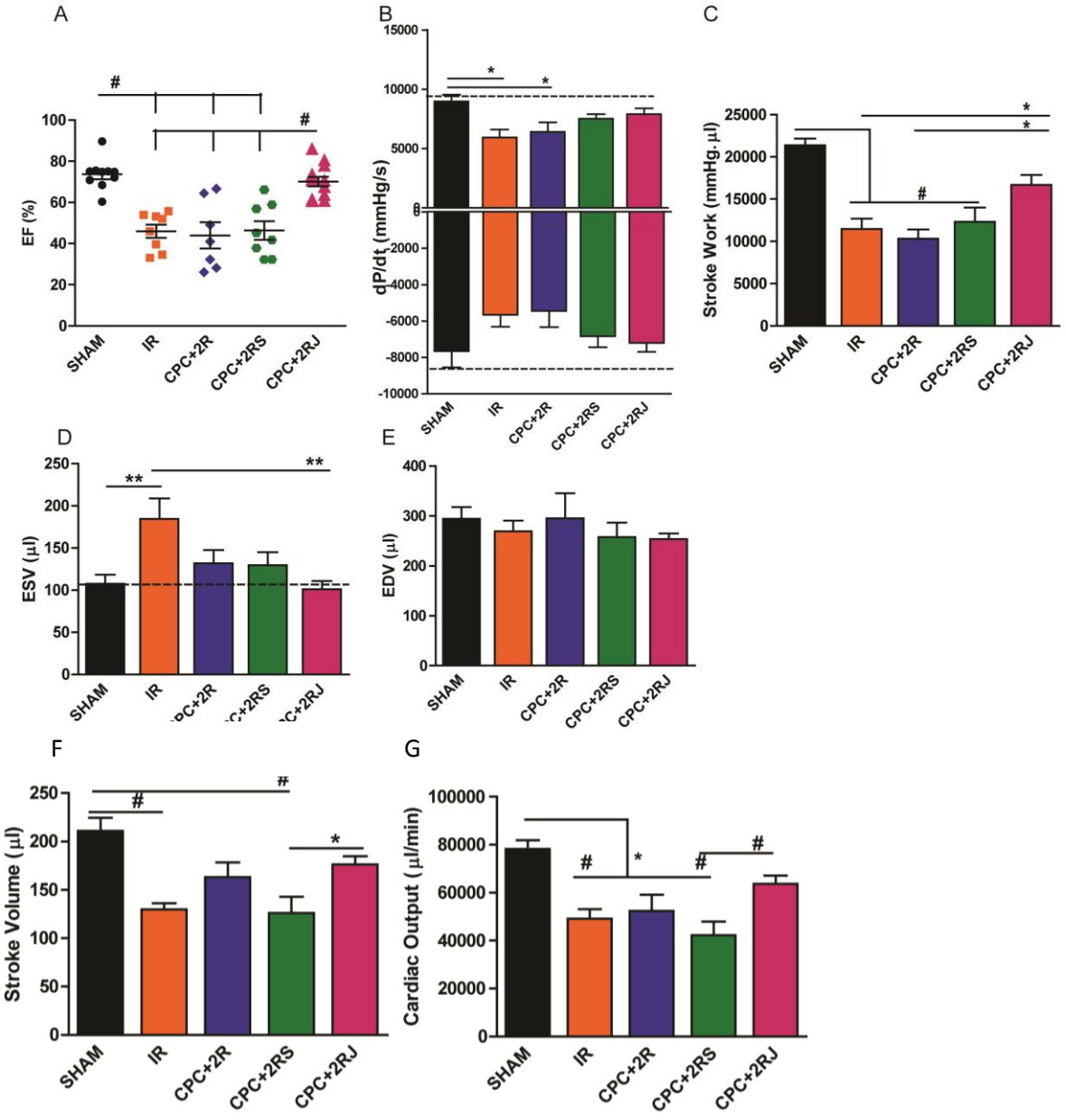

Figure 4.12: Delivery of CPCs in 2RJ hydrogel improves cardiac function following MI. (A) Pressure Volume Hemodynamic measurements of (A) ejection fraction (EF%), (B) dP/dT, (C) Stroke Work, (D) End Systolic Volume (ESV), (E) End Diastolic Volume (EDV), (F) stroke volume and (G) cardiac output in rats treated with CPCs in 2RJ hydrogels. n=10-12, * $p<0.05$, **$p<0.01$, # $p<0.001$, One-way ANOVA and Tukey's post test.

To evaluate the potential mechanism behind the observed improvement in function, Picosirius Red staining of heart sections was performed. Infarction resulted in a significant increase in cardiac fibrosis. In comparison, treatment with CPCs in 2RJ hydrogel resulted in a significant decrease in fibrosis ($p<0.05$, $n\geq5$, **Fig. 4.13A,** representative images in **Fig. 4.13B).**

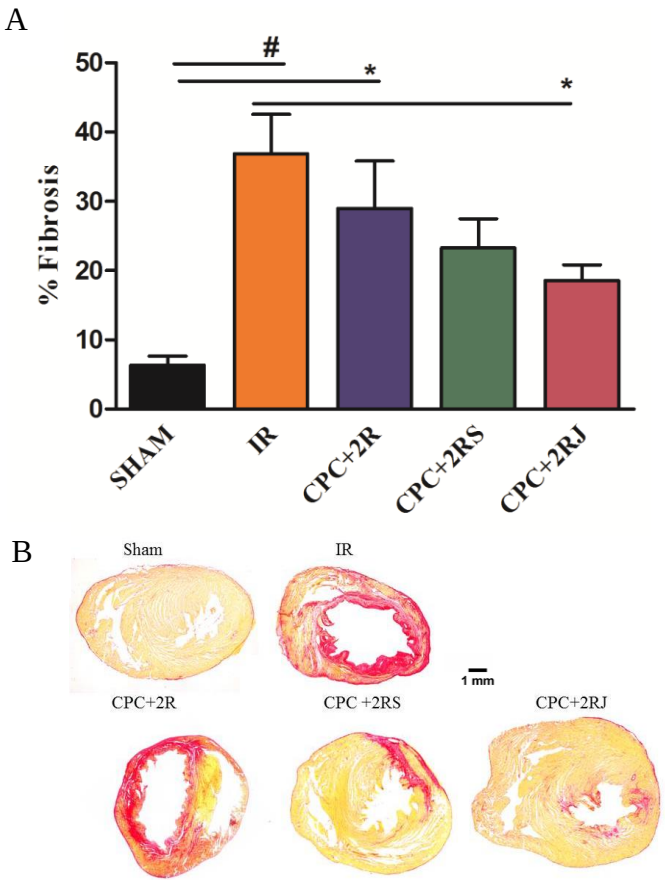

Figure 4.13: Delivery of CPCs in 2RJ hydrogel decreases fibrosis. (A) Quantification of % fibrosis in rat hearts on day 21 shows a significant increase in fibrosis following 30min of ischemia-reperfusion (orange bar). Administration of CPCs in 2RJ attenuated this increase. * $p<0.05$, and # $p<0.001$ vs IR. $n\geq5$ per group. (B) Representative images of heart sections stained with Pico-Sirius Red. Scale bar 1mm.

4.3 DISCUSSION

Cardiac progenitor cells (CPCs) can be easily obtained from autologous sources and have shown promising outcomes in Phase I clinical trials in patients with cardiovascular disease. However, lack of myocardial retention and specific soluble cues impedes successful regeneration. Thus, hydrogel scaffolds that mimic cardiac tissue could provide an environment with appropriate mechanical and chemical cues to augment CPC function. Stem cell function is regulated by growth factors, cytokines, cell-cell interactions and mechanical stimuli such as substrate stiffness, rigidity, shear stress, stretch and topography (134). Stem cells are sensitive to mechanical properties of the environment such as stiffness of native tissue which varies from kilopascals to hundreds of megapascals throughout the human body. Substrate stiffness has been shown to regulate specification of adult stem cells into different lineages depending on correlation with native tissue stiffness. For example, Engler et al have shown that culture of MSCs on substrates with stiffness similar to brain, muscle and bone leads to differentiation into neural, myogenic and osteogenic phenotype, respectively (135). Endothelial differentiation of cardiac precursor cells cultured on substrates with rigidity matching native cardiac tissue has also been reported (136). Substrate stiffness has also been shown to affect human pluripotent stem cell derived cardiomyocyte contraction (137). These studies demonstrate control of stem cell fate and function through mechanical properties of the culture environment. Here, we demonstrate for the first time that hydrogel-stiffness activates Notch1 signaling and regulates CPC gene expression and function in vivo.

Several natural and synthetic materials have been investigated for use as cardiac scaffolds such as fibrin, collagen, PLGA, gelatin and hyaluronic acid (138). However, local inflammation, toxic degradation products and large pore sizes have limited their widespread use (139). Previous studies have shown that self-assembling peptide (SAP) hydrogels can be used to deliver growth factors (86, 140), cytokines (141) and cardiac stem cells (142) to the infarcted heart. Moreover, the stiffness of these hydrogels and the concentration of the bioactive factor can be tuned to provide hydrogels with well-regulated mechanical and biochemical properties. These SAP hydrogels also recruit host endothelial cells and cardiomyocytes into the myocardium and promote neovascularization (94). Implantation of CPCs in Insulin-like growth factor 1 (IGF1) containing SAP hydrogels has also demonstrated improved cardiac function following infarction in rats (54). However, the precise mechanism by which cells are retained in the hydrogel is not clearly understood. RADA16-II used in this study has no known cell adhesion motifs; so physical entrapment within the gel and appropriate pore size that allows for nutrient diffusion are plausible explanations for cell retention (85). Moreover, the SAP hydrogels can be easily synthesized, have low immunogenicity (141), support cell survival and allow for addition of bioactive motifs (such as RJ), thus providing a platform for incorporation of cell adhesion motifs and varying peptide concentrations in future studies.

The development of biomaterials in which signaling pathways critical for CPC function such as Notch1 can be incorporated is of great importance since CPCs are endogenously present in niches of defined composition (143, 144) and exert their reparative effects depending on environmental cues following injury, aging or disease

(31, 145-147). The current work demonstrates that RJ-mediated Notch1 activation significantly influences cardiogenic gene expression and cardiac repair. In vitro 3D culture of CPCs on RJ containing 1 or 2% hydrogels increased expression of Hey1, a downstream target of Notch1. Interestingly, culture on lower stiffness 1% RJ hydrogels resulted in increased endothelial and smooth muscle gene expression. However, higher stiffness 2% RJ hydrogels promoted cardiac gene expression indicating the synergistic effect of hydrogel stiffness and Notch1 activation on cardiogenic gene expression in CPCs. Recent studies have demonstrated the reliance of Notch activation on ligand-mediated pulling force exerted on the notch extracellular domain (132). This mechanical force leads to γ-secretase mediated cleavage and nuclear translocation of the NICD. Such mechanotransduction-mediated notch activation has been attempted in this study using RJ containing hydrogels of varying stiffness. These results also suggest that in vitro 3D culture in 1RJ or 2RJ hydrogels could pre-commit the CPCs towards the endothelial/smooth muscle or cardiac lineage respectively before in vivo delivery. This is critical as previous studies suggest that the fibrotic infarct environment is less compliant and delivery of stem cells in a hydrogel provides an environment amenable to remodeling and repair (135, 148).

Paracrine signaling could play a pivotal role in stem cell therapy as release of soluble factors by the transplanted cells can augment cardiac repair (149). We examined the conditioned media obtained from in vitro 3D culture of CPCs for secreted factors. We observed the secretion of PDGF and SCF in the media of CPCs in 1RJ and 2RJ hydrogels respectively. While the role of PDGF in promoting angiogenesis and SCF in regulating CSC migration is well known (150, 151), SCF has also been shown to promote

endothelial cell tube formation (152). The conditioned media obtained from CPCs cultured in 1RJ hydrogels promoted cardiac endothelial cell tube formation indicating paracrine angiogenic benefits. Matsuura et al have reported beneficial effects of CPC sheet transplantation in a mouse model of MI due to secreted factor- mediated angiogenesis. Specifically, array analysis showed increased secretion of VCAM1, IL6, and IL4 among others with VCAM1 identified to be critical for cardiomyocyte survival and function (153). Proteomic analysis of cardiosphere-derived cell secretome has also identified key factors that are differentially secreted compared to cardiomyocytes (154). These results suggest that differential soluble factor secretion by CPCs cultured in RJ hydrogels is stiffness dependent. The increase in cardioprotective gene expression in CPCs in 2R hydrogels compared to 1R hydrogels and the increased pro-inflammatory gene expression in CPCs in 1R hydrogels led to the use of the 2R hydrogels in the in vivo studies.

Notch1 signaling mediates several cellular processes such as cardiac embryogenesis, tissue regeneration, stem/progenitor cell proliferation and survival. Studies have shown that the effect of Notch1 activation is stem cell specific. ESC differentiation requires Notch1 inhibition while Notch1 activation promotes cardiac gene expression in MSCs and CPCs (10, 68, 69). Interestingly, mouse embryoid bodies cultured in the 1RJ hydrogels for 1 week had increased endothelial and smooth muscle gene expression indicating that 3D Notch1 activation results in a similar stiffness-dependent gene expression in different stem/progenitor cells. As Notch1 signaling has been shown to regulate stem cell proliferation, we examined CPC proliferation in the hydrogels. Culture in 2RJ hydrogels increased the extent of EdU incorporated DNA in

the CPCs suggesting improved proliferative capacity on 3D culture in 2RJ hydrogels. As culture in 2RJ hydrogels increased cardiac gene expression, we examined if the conditioned media increased cardiomyocyte proliferation. Culture in 2RJ conditioned media increased adult rat cardiomyocyte proliferation. Taken together, these results suggest that CPCs cultured in Notch1-activated hydrogels secrete growth factors that can exert paracrine benefits on cardiomyocytes and endothelial cells.

Next, we examined if the positive effects of CPC cultured in 2RJ hydrogels translated to improvements in cardiac retention and function in a rat model of myocardial infarction. Delivery of CPCs in 2RJ hydrogels led to sustained retention of ~80% of the implanted cells in the myocardium for 1 week. Such high level of acute retention is significant as Terrovitis et al report less than 20% retention of transplanted cardiac-derived stem cells one week following administration in a rat myocardial infarction model (155). As low engraftment of implanted stem cells in the heart impedes cardiac regeneration and repair, delivery in 2RJ hydrogels provides an environment for successful CPC engraftment. However, as DiR signal loss over time and uptake of the lipophilic dye by other host cells could occur, CPC retention in the myocardium needs to be verified by approaches based on identifying the Y-chromosome+ CPCs in the heart tissue sections.

As shown in figure 4.12, ejection fraction recovered to sham-operated levels in rats treated with CPCs in 2RJ hydrogels whereas all other treatment groups had persistently decreased ejection fraction. We also assessed other metrics indicative of functional improvement through pressure-volume hemodynamics. Measures of cardiac contractility such as stroke work and end systolic volume (ESV) were preferentially

improved in rats that received CPCs in 2RJ hydrogels. New data from human clinical trials support the reparative potential of stem cell therapy for heart failure. Moreover, c-Kit^{+} CPCs can be easily isolated from cardiac biopsies even from patients with advanced heart failure (156). Hence, the improvement in cardiac function, contractility and retention in the CPC in 2RJ group could translate into positive clinical outcomes in patients. These studies need to be performed in large animal models of MI to be predictive of effects in humans.

Taken together, we have shown that Notch1 activation is stiffness dependent, which differentially regulates cardiogenic gene expression in CPCs, and that delivery of CPCs in 2RJ hydrogels improves acute myocardial retention and hemodynamic function after MI. Intramyocardial injection of CPCs in 2RJ hydrogels could be an efficient and robust cell delivery strategy for myocardial repair.

CHAPTER 5

NOTCH ACTIVATING HYDROGELS ATTENUATE CARDIAC DYSFUNCTION

5.1 INTRODUCTION

Myocardial infarction (MI) is a leading cause of death worldwide (1). Occlusion of the coronary artery leads to MI characterized by local ischemia, myocyte apoptosis, fibrotic scar and irreversible tissue damage which progresses over time to heart failure. While current treatments such as pharmacological intervention and assistive device implantation salvage the injured heart acutely, heart transplantation is the most viable treatment option for patients with end-stage heart failure. However, limited availability of donor hearts and side-effects of transplant-associated immunosuppression in patients with comorbidities has created the need for treatment alternatives. Early infarct expansion leading to increased regional stress has been identified as a key factor that accelerates cardiac remodeling with poor prognosis (157). Use of ventricular restraints that limit cardiac expansion and provide mechanical support in large animal models of MI has shown limited global remodeling (158). However, the invasive surgical procedure required has limited clinical application. To circumvent this, our group and others have investigated the use of injectable cell-free biomaterials to support cardiac function following MI by serving as tissue bulking agents and cell/growth factor delivery vehicles (72, 81, 159-162).

Hydrogels are a class of biomaterials composed of hydrophilic polymers of natural or synthetic origin characterized by high water content that can provide support to

the damaged myocardium following MI. A tissue compatible hydrogel is characterized by the ability to promote specific ligand-receptor interactions, host cell migration, minimal host inflammatory or foreign body response, controlled biofactor release and tunable degradation kinetics. Hydrogels with diverse physical properties have been used in a variety of cardiac applications ranging from controlled delivery of drugs and small molecules to growth factors and stem cells (163). For use in the infarct environment, hydrogels with chemical and mechanical properties of native tissue are needed for successful cardiac regeneration.

Self-assembling peptides (SAP) are a class of hydrogels composed of alternating hydrophilic and hydrophobic aminoacids that self-assemble at physiologic pH and osmolarity to form a hydrogel. Two well-studied types of SAPs are RADA16-I (AcN-$(RADARADA)_2$-CNH_2) and RADA16-II (AcN-$(RARADADA)_2$ -CNH_2) (164). RADA16-II has been used in this study. To attenuate the viable myocardium lost after MI, cardioprotective cues have been delivered through SAP to the infarcted heart. For example, using streptavidin-biotin conjugation system with SAP, sustained delivery of IGF-1 to the infarcted rodent heart has resulted in improved cardiomyocyte survival (160). Sustained PDGF delivery in SAP hydrogels has led to improved cardiac function and limited adverse remodeling after MI (126). Delivery of VEGF in SAP hydrogels to the infarcted rat and pig heart following MI has promoted tissue repair and restored cardiac function (86). Dual delivery of PDGF and FGF in SAP hydrogels improved cardiac function of the infarcted rat heart compared to either growth factor alone (140). These studies demonstrate the potential of SAPs to serve as instructive biomaterials for cardiac applications.

Notch1 signaling is known to play a critical role in cardiac development and in the survival, cardiogenic lineage commitment, and differentiation of cardiac stem/progenitor cells (10, 58, 64). These varied critical roles played by Notch signaling have led to research on identifying ways to activate and control Notch signaling in vitro and in vivo. This has been achieved through soluble Notch ligands, over-expression of the active Notch intracellular domain in cells, ligand immobilization on tissue culture polystyrene surfaces and development of biomaterials with immobilized notch ligands. While soluble Delta/Jagged ligands also allow for ligand-receptor interaction, the lack of ligand-mediated receptor endocytosis may antagonize downstream signal activation (132). As overexpression of the receptors or ligands does not represent clinically translatable systems, ligand immobilization strategies have been attempted for controlled induction of Notch signaling.

In this study, we functionalized self-assembling peptide (SAP) hydrogels with a peptide mimic of the Notch1 ligand Jagged1 (RJ) to evaluate the effect of hydrogel-mediated Notch activation in the myocardium in a rat model of myocardial infarction. Endogenous progenitor cells and other resident cells are known to migrate to the infarct site due to the signals present in the infarct and in response to the presence of SAP (94, 165, 166). Moreover, following an infarction, an increase in levels of Notch activation along with increases in downstream targets and corresponding ligand expression has been observed (67). So we sought to include a ligand to promote Notch signaling in the host cells and this study demonstrates the potential therapeutic benefit of functionalizing SAP hydrogels with the peptide mimic of the Notch1 ligand Jagged1, RJ, for hydrogel based cardiac repair.

5.2 RESULTS

Delivery of 2RJ hydrogel improves cardiac function

The functional consequences of implantation of 2R or 2RJ hydrogels were investigated in rats subjected to experimental myocardial infarction. Two percent hydrogels containing empty or Jag1 peptide (2R or 2RJ) were injected intramyocardially (i.m.) in 3 border zones in female rats following myocardial infarction. Cardiac function was evaluated 21 days later by echocardiography and invasive pressure-volume hemodynamic measurements. Ischemia/Reperfusion for 30 minutes significantly decreased ejection fraction of the hearts when compared to sham operated animals. Treatment with 2RJ hydrogels significantly improved ejection function comparable to levels in sham operated animals as assessed by invasive hemodynamic analysis (**Fig. 5.1A**). No improvement in function was observed in animals treated with empty (2R) hydrogel indicating the importance of Notch1 activation in improving function following infarction. Improvement was also observed in rats treated with 2RJ hydrogels for the cardiac contractility indicator +dP/dt (**Fig. 5.1B**). Among the other parameters of ventricular function, the significant decrease in stroke work, stroke volume and cardiac output following IR was reversed in rats treated with 2RJ hydrogels (**Fig. 5.1C, D and E**). End systolic volume (ESV), an indicator of cardiac contractility was elevated following IR and treatment with 2RJ hydrogel decreased it to levels comparable to sham operated rats (**Fig. 5.1F**). No change in end diastolic volume (EDV) was observed (**Fig. 5.1G**). The cardiac compliance was also restored to sham levels on treatment with 2RJ hydrogels (**Fig. 5.1H**).

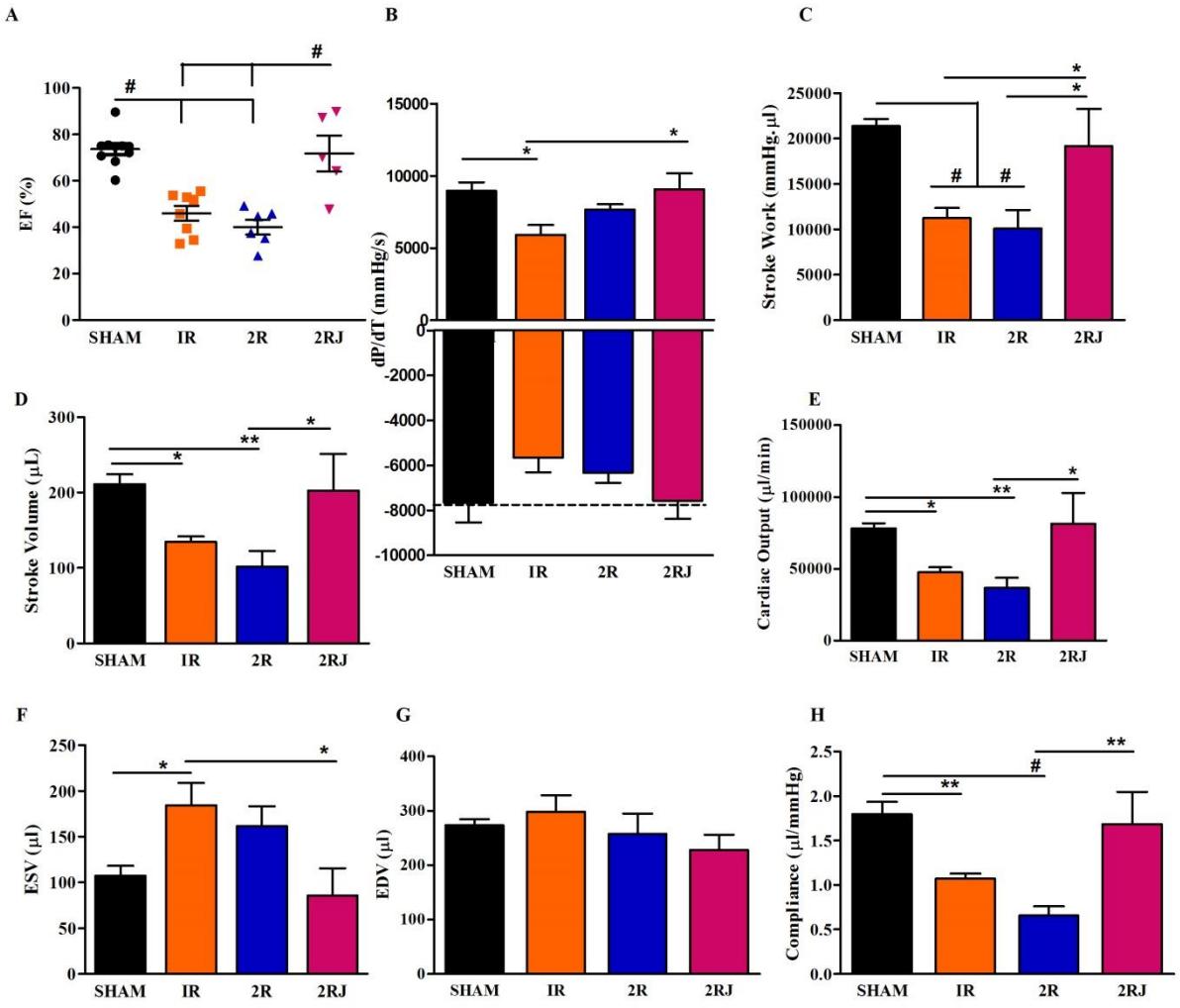

Figure 5.1: Delivery of 2RJ hydrogel improves cardiac function following MI.

Pressure-volume hemodynamic measurements of (A) ejection fraction (EF%), (B) ±dP/dT, (C) Stroke Work, (D) Stroke Volume, (E) Cardiac Output, (F) End Systolic Volume (ESV), (G) End Diastolic Volume (EDV), and (H) compliance. n≥5/group, * p<0.05, **p<0.01, # p<0.001, One-way ANOVA and Tukey's post test.

To evaluate a potential mechanism behind the observed improvement in function, Picosirius Red staining of heart sections was performed. Infarction resulted in a significant increase in cardiac fibrosis. In comparison, treatment with 2RJ hydrogel resulted in a significant decrease in fibrosis ($p<0.05$, $n=5$, **Fig. 5.2A**). Representative images of heart sections stained with Pico-Sirius Red is shown in **Fig 5.2B**

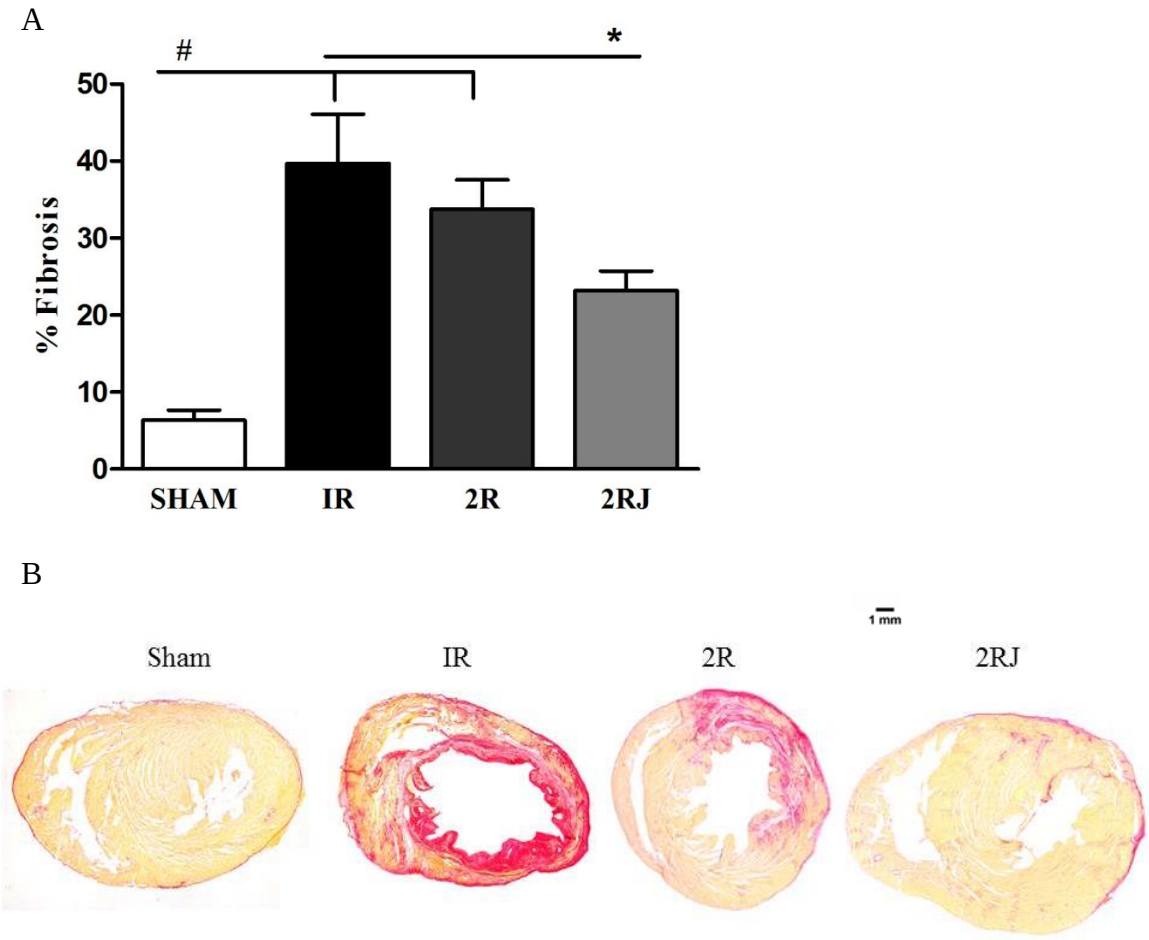

Figure 5.2: Delivery of 2RJ hydrogel decreases fibrosis. (A) Quantification of % fibrosis in rat hearts on day 21 shows a significant increase in fibrosis following 30min of ischemia-reperfusion (black bar). Administration of 2RJ hydrogel attenuated this

increase. * $p<0.05$, and # $p<0.001$ vs IR. n=5 per group. (B) Representative images of heart sections stained with Pico-Sirius Red. Scale bar 1mm.

5.3 DISCUSSION

Delivery of cell-free hydrogel therapy has resulted in improvement in cardiac function in animal models of MI through improved mechanical support and beneficial effects of delivered bioactive cargo (167). Interest in these approaches is high due to relative ease of clinical translation, high reproducibility and potential for off-the-shelf availability. As activation of Notch signaling known to occur following MI has been shown to be cardioprotective, our objective was to augment Notch signaling in the host myocardium using a cell-free SAP hydrogel containing the Notch ligand RJ. As shown in figure 5.1, ejection fraction recovered to sham-operated levels in rats treated with 2RJ hydrogels whereas treatment with 2R hydrogel had persistently decreased ejection fraction comparable to untreated infarcted rats. We also assessed other metrics indicative of functional improvement through pressure-volume hemodynamics. Measures of cardiac contractility such as stroke work and end systolic volume (ESV) were preferentially improved in rats that received 2RJ hydrogels. These results demonstrate that hydrogel immobilized Notch1 ligand improves cardiac function in a rat model of MI.

These results are in agreement with other studies that have examined the effect of Notch activation in the infarcted heart. Specifically, activation of Notch signaling in cardiac progenitor cells (CPCs) is known to promote cardiomyogenic differentiation of CPCs (10). Activation of Notch signaling by hepatocyte growth factor (HGF) in the

border zone after infarction is known to be cardioprotective by promoting cardiomyocyte survival, cell cycle reentry and improving cardiac function (67). Recent studies on Notch activation using immobilized ligands show that substrates immobilized with full length Jagged-1/Fc receptor chimeric protein activate Notch signaling in a ligand density dependent manner (168). Such systems have also been used to direct differentiation of hematopoietic progenitors into T-cells using microbeads functionalized with the Notch ligand Dll4 (169).

Delivery of 2RJ hydrogel also decreased fibrosis significantly compared to untreated infarcted rat hearts. Recently, the role of Notch activation in regulating fibrotic response of the heart has been investigated. Studies demonstrate the inhibitory effect of Notch activation on cardiac fibroblast-myofibroblast transformation which is critical for initiation of fibrosis (170). Moreover, Notch signaling has been shown to decrease cardiac fibrosis and promote cardiac progenitor cell proliferation in a mouse model of cardiac pressure overload (171). Taken together, Notch activation plays a vital role in regulating the fibrotic and regenerative responses of the infarcted heart and delivery of 2RJ hydrogels could modulate these responses.

However, to clearly delineate the effects of 2RJ hydrogel, the potential effects on endogenous CPCs, myocyte survival and neovascularization need to examined. As Notch activation has been shown to regulate the balance between fibrosis and regeneration, the extent of migration of fibroblasts and endogenous CPCs into the infarcted region merits investigation. Moreover, as delivery of SAP hydrogel has been shown to promote migration of endothelial and smooth muscle cells into the hydrogel (94), the extent of such migration, Notch activation in these cells and angiogenesis

requires further study. These studies also need to be performed in large animal models of MI to be predictive of effects in humans. The improvement in cardiac function and contractility coupled with decreased fibrosis in the 2RJ group could translate into positive clinical outcomes in patients. Intramyocardial injection of 2RJ hydrogels could be an efficient and robust hydrogel therapy for myocardial repair.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Heart failure is the leading cause of death worldwide. In 2013, the American Heart Association estimated that one American will die of cardiovascular disease every 39 seconds. While heart transplantation is the most viable treatment option, the limited availability of donor hearts has necessitated the search for treatment alternatives such as the use of adult stem cells for cardiac repair and regeneration. Following myocardial infarction (MI), the inflammatory cardiac microenvironment, limited survival of stem/progenitor cells, myocardial scarring and fibrosis affect cardiac regeneration. This dissertation examines adult stem cell based approaches for cardiac regeneration by studying the effect of i) H_2O_2- mediated oxidative stress on mesenchymal stem cells, ii) Notch1 activation in cardiac progenitor cells using a self-assembling peptide hydrogel containing the Notch1 ligand mimic RJ in vitro and functional consequences in a rat model of MI. Through these approaches, the central hypothesis that modulation of stem cell response using cues such as oxidative stress and activation of Notch1 signaling can improve functional outcome following myocardial infarction has been studied.

6.1 OXIDATIVE STRESS INFLUENCES MESENCHYMAL STEM CELLS

Chapter 3 describes how oxidative stress influences cardiogenic gene expression in mesenchymal stem cells (MSCs). Levels of oxidative stress are elevated in the infarcted heart. Delivery of MSCs to the infarcted heart has resulted in modest improvements in function. In this study, we sought to determine if oxidative stress can regulate MSC gene expression. MSCs were treated with either H_2O_2 for 1 week (0-

100μM) or with Glucose oxidase (GOX, 0-5 mU/ml) for 48h to represent pulsed and continuous exposure to oxidative stress respectively. We found a threshold effect of 100μM H_2O_2 or 5mU/ml GOX in increasing cardiac and endothelial gene expression in MSCs through Notch1 signaling. Similar results were obtained with another adult stem cell, cardiac progenitor cell (CPC).

Based on these results, pre-conditioning adult stem cells with oxidative stress stimuli could improve therapeutic benefit following transplantation in the infarcted heart through increased cardiac differentiation. To examine this possibility, work in the Davis lab has also focused on examining the effects of H_2O_2 preconditioned CPCs on cardiac function in a rat model of experimental infarction. Delivery of H_2O_2 preconditioned CPCs in self-assembling peptide (SAP) hydrogels to the infarcted rat heart led to improvement in function, decreased fibrosis, and increased capillary density. These results suggest that response to oxidative stress is conserved among the adult stem cell types- MSCs and CPCs and could be developed in a therapeutic pre-conditioning strategy. However, exposure of rat MSCs to H_2O_2 has been shown to decrease cell adhesion and spread which was overcome by co-treatment with the reactive oxygen species scavenger, N-acetyl cysteine (29). Although we did not observe changes in cell adhesion to tissue culture flasks, the dose and duration of treatment of stem cells with H_2O_2 needs to be optimized for in vivo applications. As more robust increases in cardiac gene expression were obtained on treatment of MSCs with GOX, MSCs over-expressing GOX can be generated and therapeutic efficacy tested in vivo. Recent studies in primary glial cells show that the continuous exposure to H_2O_2 results in more pronounced effects than pulses of H_2O_2 (172).

In vivo delivery of these cells will demonstrate the effect of continuous H_2O_2 generation on cardiac function. As high levels of H_2O_2 cause cell death, the extent of intracellular H_2O_2 generated in GOX-overexpressing cells needs to be determined and can be regulated under an inducible promoter. As SAP hydrogels containing RJ promote Notch1 signaling (described in Chapters 4 and 5), H_2O_2-preconditioned MSCs can be delivered in RJ containing SAP hydrogels to activate Notch1 signaling in MSCs and in the host cells that may infiltrate the hydrogel in a rat model of myocardial infarction. These studies will explore the potential of synergistic Notch activation on improving cardiac function following infarction.

6.2 NOTCH ACTIVATION IN CARDIAC PROGENITOR CELLS

Notch signaling plays an important role in cardiac development, stem cell survival and differentiation. Mechanical force exerted by ligand bound to Notch1 receptor is essential for activation of Notch1 signaling, thus we designed a 3D self-assembling peptide hydrogel that incorporated a peptide mimic of the Notch1 ligand Jagged1 (RJ) to engineer stem cell response to Notch1 signaling. As described in Chapter 4, utilizing a reporter cell line, we demonstrated a dynamic effect of hydrogel stiffness and presence of Notch1 ligand RJ on Notch1 activation. These results can be studied in more detail using force measurements of the ligand RJ- Notch1 interaction on substrates of varying stiffness (173). We also found that hydrogel stiffness-dependent Notch1 activation differentially regulated cardiogenic gene expression in rat CPCs. Culture of CPCs in RJ-containing hydrogels also exerted paracrine effects on endothelial cells and myocytes in vitro. However, as Notch signaling is involved in a multitude of cell developmental stages, determining the effect of Notch activation at a single set time point may not

reflect its actual temporal effect and additional time course experiments could be performed in vitro.

In rats subjected to MI, intramyocardial delivery of CPCs in 2RJ hydrogels promoted cardiac repair through increased ejection fraction, stroke work and decreased fibrosis on day 21 after treatment. The mechanism by which the CPCs in 2RJ hydrogels cause improvement in cardiac function needs to be elucidated. In previous studies, differentiation of CPCs into cardiac cells has been reported (10). To that end, immunohistochemistry of heart sections will show the origin of any newly formed cells by co-expression of Y-chromosome or Luciferase with proteins specific for cardiac, endothelial and smooth muscle cells. Other potential effects on vascularization, inflammatory cell infiltration, myocyte proliferation, endogenous CPC recruitment also need to be investigated. The differential effects of Notch1 activation in hydrogels of varying stiffness can be explored in vivo by transplanting cells in 1% hydrogels (1R, 1RS and 1RJ). The CPCs in hydrogels could also be transplanted 4 weeks after MI to represent a more clinical situation through double survival surgeries in rats. Currently, human CPCs have been successfully isolated from human heart explants and expanded in the Davis laboratory. Efforts are ongoing to extend the present study to examine the effect of SAP hydrogel mediated Notch1 activation on human CPCs in vitro and determine the effects in vivo in a nude rat model of myocardial infarction.

6.3 HYDROGELS FOR NOTCH ACTIVATION

As described in Chapter 5, delivery of a peptide mimic of the Notch1 ligand Jagged1, RJ in a 2% SAP hydrogel (2RJ) to the infarcted rat heart attenuated cardiac

dysfunction and decreased cardiac fibrosis. The SAP hydrogel was chosen to provide cues for Notch activation because i) mechanical properties of the SAP can be tuned to match the host myocardium, ii) the peptide can be reproducibly synthesized chemically, iii) the ligand density can be regulated and iv) acellular hydrogel systems are more clinically translatable. The mechanism by which the 2RJ hydrogels improve cardiac function following MI needs further investigation. Potential mechanisms to examine include extent of vascularization (capillary density, vessel formation), recruitment of endogenous CPCs, decreased apoptosis, increased survival of cardiomyocytes and decreased fibroblast activation.

Many acellular biomaterials improve cardiac function by providing mechanical support as tissue bulking agents. Both the 2R and 2RJ hydrogels have the same elastic moduli and swelling ratio. But the lack of functional improvement in 2R hydrogel-treated rats shows that mechanical support of the infarcted heart does not explain the observed results. To demonstrate the involvement of Notch signaling in the observed functional improvement, chemical Notch inhibitors such as DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) may be given to rats before hydrogel treatment. Also, Notch activation in host cells can be examined by immunohistochemistry for NICD. These experiments will clearly delineate the role of 2RJ hydrogels in activating Notch in the myocardium.

It is interesting to note that improvement in cardiac function was observed after intramyocardial injection of CPCs in 2RJ hydrogels or 2RJ hydrogels without CPCs. However, the mechanism by which both treatment groups improve function could be different. Improvement in function with CPCs in 2RJ hydrogels may result from cardiac

differentiation of transplanted cells and paracrine benefits of secreted growth factors. In contrast, the 2RJ hydrogels may augment the endogenous healing response by promoting proliferation of endogenous c-Kit^{+} CPCs and cardiomyocytes. These effects can be distinguished by long term follow up studies where presence of CPCs may be more beneficial.

Timing of hydrogel injection is also critical. In the current study, hydrogels were injected immediately post-MI. Reports suggest that effects of acellular hydrogels are most pronounced when injected immediately post-MI (167). Double survival studies in rat with hydrogel treatment after infarct stabilization may result in different outcomes. The current study can also be extended to a pig model of MI. SAP mediated delivery of VEGF to the infarcted pig heart did not lead to an inflammatory response (86). Such large animal studies could pave the way for clinical translation of 2RJ hydrogels to treat patients with MI.

6.4 PERSPECTIVE

The occurrence of 1 death every 39 seconds due to cardiovascular disease (CVD) provides a strong impetus to find treatment alternatives for patients with CVD. Adult stem cell therapy has emerged as a therapeutic option with promising results in pre-clinical and Phase I clinical trials. While extensive research has produced large advancements in the field, the type of stem cell, source, dose, route of delivery, time of injection and delivery of additional cell survival cues have to be clearly established for large scale clinical translation. This dissertation was an attempt at developing a stem cell based therapy for myocardial infarction.

APPENDIX A

EXPERIMENTAL METHODS

A.1 CELL CULTURE

Mesenchymal stem cell isolation-Mesenchymal stem cells were obtained from the femur and tibia of adult male Sprague-Dawley rats by Percoll density gradient centrifugation and adherence to tissue culture flasks. MSCs from passages 2 to 4 cultured on Mimimal Essential Media alpha (MEMα; Hyclone) supplemented with 20% Fetal Bovine Serum (Hyclone), L-Glutamine (Cellgro) and 100U Penicillin-Streptomycin (Invitrogen) were used.

Cardiac progenitor cell isolation- Cardiac progenitor cells (CPCs) were isolated from the hearts of adult male Sprague-Dawley rats by selection of c-Kit^{+} cells with anti-cKit antibody (H-300, Santa Cruz) coated magnetic beads (Dynal) as previously described (31).

Media and Cell culture: The mouse embryoid bodies were cultured in DMEM/F12 containing 10% FBS, 1X Penicillin-Sterptomycin and L-Glutamine. The CHO cells with Notch1 responsive YFP expression were cultured in Alpha MEM containing 10% FBS, 1X Penicillin-Sterptomycin, L-Glutamine, Zeocin (400 µg/ml), Blasticidin (10 µg/ml) and Geneticin (600 µg/ml). The rat cardiac endothelial cells were cultured in low glucose DMEM media (GIBCO) supplemented with 10% FBS, 50µg/mL endothelial cell growth supplement (Sigma E2759) and 1% 100X MEM Non-essential amino acids solution (GIBCO). The primary rat cardiomyocytes were isolated from rat hearts and cultured as described in (174).

A.2 STEM CELL CHARACTERIZATION

Characterization of MSCs and CPCs- The surface expression of c-Kit (H-300, SantaCruz), CD45 (Invitrogen), CD34 (sc-7324, SantaCruz), CD73 (#551123, BDPharmingen), CD90 (#554898, BDPharmingen) and CD105 (bs-0579R, Bioss) in MSCs and expression of c-Kit, the transcription factors nkx2-5 (sc-14033, SantaCruz) and gata4 (sc-9053, SantaCruz) and the Notch1 Extracellular domain (NECD, Millipore) in CPCs was determined by flow analysis using a FACSCalibur (Becton Dickinson). The isotypes of each antibody served as the negative control.

Trilineage differentiation of MSCs- To determine the trilineage differentiation capacity, the MSCs were cultured in adipogenic (Cat# SR811D250, Amsbio), osteogenic (Cat# SR417D250, Amsbio) or chondrogenic (Cat# SC00B5-2, VitroBiopharma) media for 21 days with media replenishment every 3 days. The MSCs were also cultured for 1 week ± 100μM H_2O_2. To demonstrate adipogenic differentiation, the cells were stained with 0.3% Oil Red O (Cat# O0625, Sigma) in isopropanol for 30min and rinsed with PBS. For osteogenic differentiation, the cells were stained with 1% Alizarin Red (Cat# 500-4, RiccaChem) for 15min. For chondrogenic differentiation, cells were stained with 0.5% Toluidine Blue O in PBS (Cat# 198161-5G, Sigma). The stained cells were imaged under a Phase contrast microscope (Olympus).

A.3 IN VITRO EXPERIMENTS

Induction of oxidative stress in MSCs and CPCs- To induce acute oxidative stress, MSCs or CPCs were cultured in serum-free media with Insulin/Transferrin/Selenium

(ITS) containing i) H_2O_2 (0-100μM; Fisher Scientific) for 1 week or ii) Glucose oxidase (0-5mU/ml; Sigma) for 48h. Media was replenished every day with fresh media with or without H_2O_2. MSC and CPC growth media contains 5.5mM and 10mM glucose respectively and addition of GOX results in continuous generation of H_2O_2.

3D culture of cells in hydrogels: The cells (CPC, CHO, mEB) were embedded in the different hydrogels (1R, 1RS, 1RJ, 2R, 2RS, 2RJ) with a 100μl total volume for 48h in Transwell cell culture insets (Millicell 0.4μm, PICM01250). The treatment media consisted of base media with ITS supplement (Cellgro 25-800-CR), 1X Penicillin-Sterptomycin and L-Glutamine.

Chemical inhibition of Notch signaling- MSCs were treated with a γ-secretase inhibitor IX DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine*t*-Butyl Ester, Calbiochem, 10μM) every day for 1 week to inhibit Notch1 activation in MSCs ± H_2O_2. On day 7, RNA and protein was harvested for subsequent qRT-PCR and Western Blotting respectively.

siRNA mediated knockdown of Notch1- To determine the optimal transfection reagent, MSCs were transfected with 100nM mock siRNA labeled with Cy3 for 48h using oligofectamine (Life Technologies), HiPerfect (Qiagen), or Lipofectamine RNAimax (Life Technologies) according to the manufacturer's instructions. The transfection efficiency was determined by flow analysis (FACSCalibur, Becton Dickinson) and fluorescent microscopy (Nikon). To knockdown Notch1 expression in MSCs, the cells were transfected with 25nM of either QIAgen Allstar Negative control siRNA (siNC) or QIA siNotch1 (cat# S101920730) with oligofectamine (Life Technologies) according to

the manufacturer's instructions. After 48h, RNA and protein was harvested for subsequent qRT-PCR and Western Blotting respectively.

Protein expression- MSCs were treated with or without 5mU/ml GOX for 48h. The protein expression of α-MHC (ab50967, abcam), Flt1 (ab32152, abcam) and smooth muscle α-actin (SAB250093, Sigma) was determined by flow analysis using a FACSCalibur (Becton Dickinson). Primary antibodies were used at 1:300 and appropriate secondary antibodies were used at 1:500 with isotype controls.

Measurement of NICD- Total protein was isolated from the organic phase of Trizol extraction. Equal amounts of protein were loaded on 4-15% SDS-PAGE gradient gel (Bio-Rad). Following transfer, the nitrocellulose membrane was probed with anti-Notch intracellular domain (Cell Signaling) antibody. A horseradish peroxidase conjugated goat anti-rabbit secondary antibody was used (Bio-Rad) and chemiluminiscent signals were obtained on a Kodak Imager Station 4000MM Pro (Carestream Molecular Imaging). NICD protein levels were normalized to GAPDH (Santa Cruz).

ELISA for In Vitro Growth factor Expression- CPCs were cultured in the different hydrogels (1R, 1RS, 1RJ, 2R, 2RS, 2RJ) for 48h. The conditioned media was screened for secreted growth factors using a custom array (Signosis, USA) according to the manufacturer's directions. Growth factors of interest were confirmed by specific ELISAs (SCF, PDGF; Signosis). All immunosorbent measures were analyzed based on a standard curve and normalized to initial cell number.

Tube formation- The rat cardiac endothelial cells were cultured in 1:1 CPC conditioned media and low serum endothelial growth media for 24h. The cells were then cultured on

Geltrex (Life Tech) for 8h and stained with calcein. The tube formation was imaged using a fluorescent microscope (Olympus) and tube length quantified using ImageJ.

A.4 GENE EXPRESSION AND PCR

as described in (175). Quantitative real time PCR was performed on a StepOne Plus real time PCR system (Applied Biosystems) using specific primers for the cardiogenic and Notch1 related genes (see Table 1). Gene expression data was normalized to GAPDH in the H_2O_2 treated MSCs and in all CPCs and to 18S in the GOX treated MSCs. GAPDH

Table 1: List of Primers

Gene	Forward Primer (5' to 3')	Reverse primer (5' to 3')
Flk1	GCCAATGAAGGGGAACTGAAGAC	TCTGACTGCTGGTGATGCTGTC
Flt1	CCACTTCTGTCTTGCCACACA	CCAACCAATTAAGACCTTCTG
vWF	CCCACCGGATGGCTAGGTATT	GAGGCGGATCTGTTTGAGGTT
α-MHC	AACGCCCAAGCCCACTTGAA	CATTGGCACGGACTGCGTCA
Troponin I	CCAGGAATCTGCAATCCCATT	CCGCATCGCTGCTCTCA
Gata4	ACCTGCTACAGCAGGGTTGGT	TTCTAGCACAACTGCAAGCATGGC
Nkx2-5	CAAGTGCTCTCCTGCTTTCC	GGCTTTGTCCAGCTCCACT
Sm α-actin	CCCAGATTCAGGAACAGCAT	GTTAGCAAGGTCGGATGCTC
Sm22α	AGCCAGTGAAGGTGCCTGAGAAC	TGCCCAAAGCCATTAGAGTCCTC
Notch1	CACCCATGACCACTACCCAGTT	CCTCGGACCAATCAGAGATGTT
Hey1	CCGCTTCGTGTTCGCCTGGT	TGCTGCCTGTGAGGGTGTCG
Hes5	ACCGCATCAACAGCAGCATT	AGGCTTTGCTGTGCTTCAGGT
Vimentin	CCCAGATTCAGGAACAGCAT	CACCTGTCTCCGGTATTCGT
FSP1	GAGGAGGCCCTGGATGTAAT	CTTCATTGTCCCTGTTGCTG
ADAM10	GCCTATGTCTTCACGGACCG	TGCCAGACCAAGAACACCATC
ADAM17	ACTCTGAGGACAGTTAACCAAACC	AGTAAAAGGAGCCAATACCACAAG
18S	TTCCTTACCTGGTTGATCCTGCCA	AGCGAGCGACCAAAGGAACCATAA
Mef2c	TaqMan MGB Rn01494046_n1	
Jagged1	TaqMan MGB Rn00569647_n1	
β-actin	TaqMan VIC MGB 4352340E	

is a gene involved in glucose metabolism and as addition of Glucose oxidase (GOX) alters the glucose levels in the media, 18S and not GAPDH was used as the house keeping gene for GOX studies.

PCR array- Based on the manufacturer's instructions, the Qiagen Rat Notch PCR Array PARN-059A was used to analyze gene expression in MSCs treated with or without 5mU/ml GOX.

A.5 HYDROGEL CHARACTERIZATION

Design of the hydrogel-ligand system- The self-assembling peptide RADA 16-II (H_2N-RARADADARARADADA-OH) was generated with a 7 glycine linker following the terminal alanine, followed by the active sequence of the Notch ligand Jagged-1 (H_2N-CDDYYYGFGCNKFCRPR-OH) to create the final peptide RJ shown in Figure A1. As a control, the Jagged1 portion was replaced with the scrambled sequence (H_2N-RCGPDCFDNYGRYKYCF-OH) to create the final RS peptide containing the same residues. RJ or RS was combined with RADA in a ratio of 1:10 to ensure the extra sequence does not interfere with nanofiber assembly.

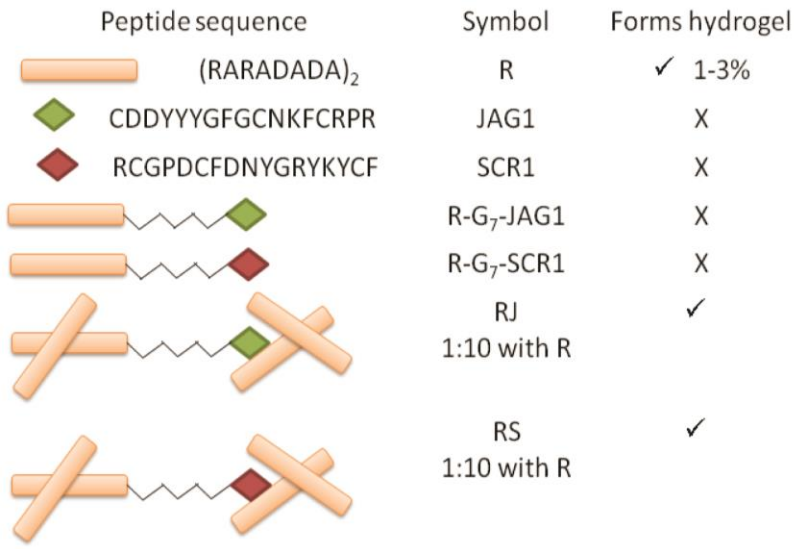

Figure A1: Schematic of the hydrogel-ligand system

AFM Modulus Testing: Using an MFP-3D-BIO atomic force microscope (Asylum Research; Santa Barbara, CA), samples were probed under fluid conditions using sucrose buffer. A 15µm bead tipped-silicon nitride cantilever (Bruker, Camarillo, CA) was used. Cantilever spring constants were measured prior to sample analysis using the thermal fluctuation method, with nominal values of 20-30 mN/m. The force-indentation curve was obtained for each measurement and then analyzed with a Hertzian model for a spherical tip (Wavemetrics, IgorPro) from which the Young's modulus was calculated. The sample Poisson's ratio was assumed as 0.5, and the deflection set point was 10 nN.

Swelling ratio: The hydrogels were created in a 100µl total volume and allowed to swell in sucrose solution. After 24h, the wet weight of the gels was measured. The gels were

then lyophilized and the dry weight noted. Swelling ratio was calculated as the (wet weight- dry weight)/wet weight.

A.6 IN VIVO EXPERIMENTS

In vivo imaging: Rats were subjected to 30minutes of Ischemia/Reperfusion (IR). CPCs were labeled with DiR as per the manufacturer's instructions (Life Technologies). About 1e6 DiR-labeled CPCs in the different hydrogels (2R, 2RS and 2RJ) were injected in 3 areas in the border zone surrounding the infarct (n=5/group). The rats were imaged on days 0, 1, 2, 3, 4, 7, 10, 14 and 21. The fluorescent intensity in the rat hearts due to the DiR- labeled CPCs was quantified as % retention (100% on day 0) over time.

Myocardial Infarction, Cell Injection, and Functional Evaluation

Myocardial infarction was performed in female adult Sprague Dawley rats (Charles River Laboratories) in a randomized double-blinded manner. Briefly, the rats were anaesthetized (1-3% isoflurane), intubated and heat exposed by separation of ribs. The left anterior descending (LAD) coronary artery was ligated for 30 minutes. During reperfusion, CPCs in hydrogels (40µl volume, 31G Ultra-Fine short syringe, BD) were injected into the myocardium at 3 border zones. Cardiac function was evaluated 21 days after treatment by echocardiography (Acuson Sequoia 512 with a 14 MHz transducer) and invasive pressure-volume hemodynamics (Millar Instruments) to assess the functional effects of each cell therapy. All functional evaluations were conducted and analyzed by investigators blinded to the animal's treatment group. The rats were euthanized and the hearts were excised for histological analysis.

A.7 HISTOLOGY

Picosirius Red staining

The hearts were fixed in 4% paraformaldehyde, dehydrated in ethanol, embedded in paraffin, and sectioned at 5µm thickness. The tissue sections were dewaxed in Histoclear followed by a series of washes in ethanol and stained with pico-sirius red solution for 1 hour (Sigma). The sections were washed in acidified water and ethanol and mounted with resinous medium (Histomount). Images of the entire heart section were taken at 2.5x magnification on a bright field microscope (Olympus) and tiled together using Adobe Photoshop. The % fibrosis was quantified using Image J as the ratio of fibrotic tissue (stained red) to total tissue.

A.8 STATISTICS

Statistical analysis- All data are expressed as mean $\pm$ SEM. To determine significance, either a One-way or Two-way analysis of variance (ANOVA) was done followed by the appropriate post-hoc test, or a Student's t-test was performed using GraphPad Prism5. A p-value <0.05 was considered significant.

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