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Role of GPCR Signaling in Cardiac Progenitor Cells

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by

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The Thesis of Gino Paul Chesini is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2012

DEDICATION

This thesis is dedicated to my parents and grandparents for their unconditional love and support, and to everyone who has provided me guidance. Thank you.

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LIST OF ABBREVIATIONS

AC	Adenyl Cyclase
Akt	Protein Kinase B
Ang II	Angiotensin II
CPCs	Cardiac Progenitor Cells
cAMP	Cyclic Adenosine Monophosphate
DAG	Diacylglycerol
Dex	Dexamethasone
GPCR	G-Protein Coupled Receptor
IP3	Inositol Triphosphate
ISO	Isoproterenol
LPA	Lysophosphatidic Acid
LPAR	Lysophosphatidic Acid Receptor
MI	Myocardial Infarction
PECAM1	Platelet endothelial cell adhesion molecule
Ptx	Pertussis toxin - Ptx
PE	Phenylephrine
PLC	Phospholipase C
PKA	Protein Kinase A
PKC	Protein Kinase C
PKD	Protein Kinase D
ROCK	Rho Kinase
Ser	Serum
SRE.L	Serum Response Element Luciferase Reporter
SRF	Serum Response Factor
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
Thr	Thrombin

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ABSTRACT OF THE THESIS

Role of GPCR Signaling in Cardiac Progenitor Cells

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Professor Joan Heller Brown, Chair
Professor Michael David, Co-Chair

Heart disease is the leading cause of mortality in developed nations and imposes a tremendous cost on society. The relatively recent discoveries that the heart is a postmitotic organ capable of self-renewal and contains a pool of multipotent c-kit$^+$ cardiac progenitor cells (CPCs) has opened doors for the development of new stem cell therapies. Our lab is interested in elucidating the ubiquitous roles of G-protein coupled receptor (GPCR) signaling on these endogenous cells. We were able to isolate c-kit$^+$ cells from adult mouse hearts that demonstrated stem cell like characteristics and express the cardiac lineage markers GATA4 and MEF2C. A Taqman array was used to determine that these cells express a distinct set of GPCRs

that differ from adult cardiomyocytes. Furthermore, the CPCs' receptor constellation is susceptible to receptor isotype switching following differentiation. Treatment with GPCR agonists such as Lysophosphatidic Acid (LPA) and Sphingosine-1-phosphate (S1P) induced proliferation in CPCs. Both S1P and LPA induce Rho dependent transcription as determined by the SRE.L luciferase reporter. In the CPCs, proliferation induced by S1P was determined to be Rho dependent while LPA is $G\alpha_i$ dependent. Addition of LPA potentiated dexamethasone induced differentiation of the CPCs towards a cardiac lineage based on changes in gene expression assayed by qPCR. However, addition of S1P failed to potentiate differentiation. Finally, cell death induced by oxidative stress was attenuated by S1P in CPCs. Our results demonstrate that S1P induces proliferation and survival possibly through Rho without effecting differentiation; while LPA induces proliferation and differentiation possibly through $G\alpha_i$.

Introduction

Heart Failure Background and significance

Since 1935 heart disease has remained the leading cause of death in the United States.[1] Heart failure, which is the fastest growing subclass of cardiovascular,diseases.[2,3] can be caused by chronic hypertension, myocardial infarction (MI), valvular stenosis, and coronary artery disease and costs the United States a staggering 39.2 billion dollars in medical treatment annually.[3] Heart failure is a progressive disease that causes the heart to lose its ability to sufficiently pump blood throughout the body and is characterized by increased cardiomyocyte death (via apoptosis and necrosis), fibrosis, ventricular dilation, relative ventricular wall thinning, and decreased cardiac output.[4,5] Cardiac hypertrophy is a compensatory mechanism that improves cardiac function in response to stress through the growth of the myocardium.[2] Physiological hypertrophy is a reversible process induced by pregnancy or exercise that is characterized by an increase in size and coronary vasculature.[5] On the other hand, pathological hypertrophy is caused by diseases that induce heart failure and is characterized by ventricular remodeling, an increased rate of myocyte death, and the re-expression of fetal genes such as Atrial Natriuretic peptide and β-myosin heavy chain which are normally not expressed in the adult organism.[2,5,6] Sustained pathological cardiac hypertrophy increases the risk of developing heart failure.[2,7]

Stem Cells

Heart transplants are an established treatment for heart failure that can extend the life of patients for up to five years, but the waitlist for donated organs is greater than the current supply.[8] Stem cell based therapies, which have been used for decades to treat leukemia,[9] may help alleviate the need for donor organs and provide new more successful methods of treatment. Stem cells are unspecialized cells with the capability of long-term self-renewal and ability to differentiate into specialized lineages[10] and are categorized by the source from which they derive into three categories: embryonic, induced pluripotent, and adult stem cells. Embryonic stem cells are totipotent cells derived from embryonic tissue that can differentiate into any cell type within an organism including extra-embryonic tissue.[10,11] Induced pluripotent stem cells (iPSCs) are fibroblast cells reprogrammed through retroviral transduction of the Oct4, Sox2, Klf4 and c-Myc genes[12] which can give rise to any cell type present in an adult organism. Adult stem cells, also known as somatic stem cells, are multipotent in the adult organism in tissue specific niches that give rise to a limited number of cell types.[10,11] The primary function of adult stem cells is to replenish and maintain the cells of the tissue in which they reside. They have been identified in the gastrointestinal tract,[13] the nervous system,[14] in bone marrow,[9,11] and most recently in the heart.[15] The discovery of endogenous stem cells and the heart's capacity for self-renewal made stem cell therapeutics a valid candidate for treatment of heart failure.

Traditionally the heart was thought to be a terminally differentiated post-mitotic organ incapable of cardiac myocyte regeneration. However, the accumulation of experimental evidence, such as the occurrence of natural cardiomyocyte

apoptosis,[16,17] telomere shortening of cardiomyocytes,[18] and histological images of myocyte replication[19-21] changed the perception of the heart to a dynamic self-renewing organ that undergoes continual cardiomyocyte turnover. After it was discovered that the heart contains replicating cardiac myocytes, studies of cardiac chimerism from sex-mismatched transplants[22-28] suggested that the origin of these mitotic myocytes may be from existing stem cell populations.

Endogenous adult multipotent cardiac progenitor cells (CPCs), positive for the stem cell lineage marker c-kit and negative for hematopoietic markers, were discovered in adult rat hearts.[15] These undifferentiated c-kit$^+$ CPCs were shown to express cardiac lineage transcription factors (Nkx2.5, GATA4, and MEF2C) *in vitro.*[15] Following differentiation *in vitro* caused by dexamethasone treatment, CPCs were shown to express cardiac myocyte, smooth muscle, and endothelial markers such as tropomyosin (cardiac), α-smooth muscle actin (smooth muscle), and Von Willebrands factor (endothelial), however, the cells failed to differentiate completely into beating cardiomyocytes.[15,29] In addition, it has been observed that these cells respond to cardiac injury *in vivo*. In mouse models, it was demonstrated that these endogenous c-kit$^+$ CPCs undergo population expansion following myocardial infarction (MI) along the border zones of the infarct region.[29] Even though these endogenous CPCs exist and localize in areas of damaged myocardium, the stem cells fail to regenerate damaged cardiac tissue in a diseased heart. However, exogenous administration of the c-kit$^+$ CPCs following MI in rats, led to a decrease in infarct size, an increase in ejection fraction, and an increase in ventricular wall thickness.[15] These findings demonstrate the ability of c-kit$^+$ CPCs to differentiate into functional cardiac

myocytes. Recently, preliminary results were published from the phase 1 SCIPIO clinical trial that administered 1million autologous c-kit$^+$ CPCs derived directly from the patients for the treatment of heart failure induced by ischemic heart disease.[30] These preliminary results mirrored the results of previously published animal studies and demonstrated an increase in ventricular wall thickness, improvement of left ventricular ejection fraction, and a reduction in infarct size in the groups treated with the c-kit$^+$ CPCs.[30] While these preliminary findings are extremely exciting, it takes 4-6 weeks from the point of surgical tissue isolation to reintroduce the 1 million autologous CPCs back into the patient.[30] Due to this time lag, patients who are suffering from a heart attack cannot receive immediate treatment with these stem cell therapies. With a better understanding of the endogenous signaling mechanisms that control proliferation, survival and differentiation of c-kit$^+$ CPCs new therapeutic treatments may be developed that enhance the performance of these cells and improve cardiac repair of the diseased heart.

G-Protein Coupled Receptor Signaling

It is estimated that there are about 200 GPCRs in the heart and several of these receptors have been shown to alter cardiac inotropy and chronotropy.[31,32] Besides affecting cardiac function, GPCRs have been implicated in hypertrophy, heart failure development, and protection against ischemic damage.[2,31,32] In fact, the most widely used pharmacology treatment for heart failure is to block GPCR signaling through the use of β-blockers and Angiotensin converting enzyme inhibitors that respectively block the chronic β-adrenergic/$G\alpha_s$ and Angiotensin II receptor/$G\alpha_q$ activity, can attribute to heart failure.[31,32]

The classical G-protein coupled receptor (GPCR) signal transduction pathway consists of a 7 transmembrane spanning receptor coupled to a heterotrimeric G-protein complex. The heterotrimeric complex is composed of three subunits: α, β, γ.[10,31-33] The three subunits each exist in multiple isoforms which play distinct roles in cellular signaling. Upon activation of GPCRs by an external ligand, the heterotrimeric G-protein complex becomes activated. The ligand bound receptor mediates the activation of the Gα subunit by facilitating the exchange of guanosine diphosphate (GDP) molecule for guanosine triphosphate (GTP) molecule. This binding causes a conformational change and the activation of the G-protein which can affect downstream targets and transduce extracellular signals to alter cellular function.[10,31-33] Small monomeric G-proteins are cytoplasmic proteins, dependent on GTP binding, which are involved in signal transduction pathways. The specificity of GPCR signaling depends primarily on the Gα subunit coupled to the receptor and the downstream effectors. Scheme 1 illustrates common downstream targets of the Gα isoforms $G\alpha_{s,}$ $G\alpha_{i,}$ $G\alpha_{q,}$ $G\alpha_{11,}$ and $G\alpha_{12/13.}$

GPCRs have been implicated in regulating cellular processes such as maintenance of pluripotency, differentiation, proliferation, survival, and migration[10,33] in various stem cells. The ubiquitous expression of GPCRs in the heart and the abundant roles in cellular processes make this family of receptors a good candidate for further investigation. GPCRs signaling is also readily manipulated with the use of known agonists and a wide range of antagonists such as the α and β adrenergic blockers, C3 exoenzyme Rho family inhibitor, and pertussis toxin $G\alpha_i$ inhibitor. In order to examine the role of GPCRs on c-kit$^+$ CPC function, experiments were

performed to determine the type of receptors present and the effects they have on cellular processes. An *in vitro* system was chosen because stem cells make up a small fraction of the total heart, and it is necessary to use isolated CPCs in order to manipulate and observe the cellular signaling mechanisms following agonist stimulation, inhibitor treatment, or gene knockdown by siRNA transfection.

Lysophospholipid signaling

Our lab is interested in lysophospholipid signaling in particular that of sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA). Both S1P and LPA are present in high concentrations in serum and are known to mediate a wide variety of physiological and pathological processes, such as development, cancer, and the immune response.[34-37] LPA is generated by activity of a series of phospholipases (autotaxin) and is recognized by five LPA receptor (LPAR) isoforms.[37] These LPARs can couple promiscuously to G-proteins. LPARs 1 & 2 couple to $G\alpha_i$, $G\alpha_{12/13}$, and $G\alpha_q$.[37] LPAR 3 couples to $G\alpha_i$ and $G\alpha_q$ while LPAR 4 couples to $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_s$. The LPAR 5 couples to $G\alpha_q$ and $G\alpha_{12/13}$. S1P is a bioactive lipid paracrine factor generated by sphingosine kinase[38] that is up regulated during pathophysiological conditions[39,40] and carried by high density lipoprotein.[35] There are five S1P receptor (S1PR) isoforms. Cardiomyocytes express the S1PR 1, 2, and 3 with the S1PR1 being the predominant isoform expressed.[41] It has been demonstrated that in the heart S1PR1 is coupled solely to the $G\alpha_i$ subunit while S1PR2 and S1PR3 isoforms couple primarily to $G\alpha_{12/13}$ subunits.[42-44] S1PR 4 and 5 can couple to either $G\alpha_i$ or $G\alpha_{12/13}$ and $G\alpha_q$ subunits.[45-48]

We are interested in studying the roles of lysophospholipids which have been implicated in numerous cell processes[34-37] The CPCs' ability to proliferate and differentiate during pathological states will determine the degree of cardiac tissue repair. Following pathological stimuli, endogenous CPCs must proliferate and migrate to the site of injury while retaining the ability to differentiate into functional cells. It was previously documented that LPA caused proliferation in vascular smooth muscle cells[49] and inhibited differentiation of brown adipose precursor cells.[50] While studies with S1P demonstrated its ability to induce proliferation of satellite cells[51] and promote differentiation of myoblasts.[52,53] CPCs must also be able to survive harmful conditions present in pathological states such as hypoxia during a heart attack. Both S1P and LPA have been shown to have protective effects. In neonatal rat cardiac myocytes, LPA and S1P provided protection against hypoxia induced cell death.[54] S1P's protective effects were demonstrated to be dependent on S1PR1/$G\alpha_i$ signaling using an *in vitro* hypoxia experiment with adult mouse cardiomyocytes.[55] In contrast, our lab demonstrated that S1P mediates protection from ischemia/reperfusion injury in the mouse heart through the S1PRs 2 and 3, which couple to $G\alpha_{12/13}$ G-proteins,[42-44] by using an *in vivo* model with S1P receptor knockout mice.[56] The $G\alpha_{12/13}$ G-proteins, which couple to S1P and LPA receptors, are known activators of the small G-protein Rho.[34,57-60] RhoA activation has been linked to many cellular roles which include cytoskeletal remodeling, migration, proliferation, and protection.[34,61] We previously demonstrated that RhoA mediates protection against ischemia/reperfusion injury in the mouse heart[62] using RhoA transgenic mice. Also, the protective effect of S1P has recently been linked to RhoA activation (unpublished data). The importance of

lysophospholipid signaling and activation of RhoA in the cardiovascular system warrant the investigation of S1P and LPA effects on CPC function. A better understanding of GPCR signaling pathways in cardiac stem cells may hopefully lead to the development of more effective treatments for heart disease.

Materials & Methods

CPC Isolation

This protocol was adapted from Fransioli et al.[29] Cardiac c-kit$^+$ stem cells were isolated from 8-12 week old mice. Prior to isolation, all buffers (see Appendix) were made, and the water jacketed Langendorff apparatus was cleaned with 70% ethanol and ddH_2O and heated to 37°C. The speed of the peristaltic pump, attached to the apparatus, was adjusted so that a single drop of solution was expelled from the cannula every 3 seconds. Mice were euthanized by cervical dislocation and their hearts removed by cutting the aorta just below the aortic arch. The heart was placed in a 6cm dish filled with oxygenated Basic Buffer (Table 1) and quickly cleaned of excess connective tissue. The aorta was cannulated and secured with 6-0 suture, attached to the Langendorff apparatus, and perfused with oxygenated Basic Buffer for 2-4 minutes while submersed in a water jacked reservoir containing the perfusate. The perfusion buffer was replaced with a Collagenase II Solution and the heart was perfused with Digestion Buffer (Table 2). After the heart was digested for 8 to 10 minute and became soft to the touch it was removed from the cannula and placed in a 15mL conical tube containing 7mL of cold Incubation Buffer (Table 3). In a sterile hood the heart was plunged though a 100 micron filter into a conical tube and set on ice while a second heart that is age was prepared in the same manner.

The dissociated heart pellets were combined and resuspended in a single conical tube which was centrifuged for 1 minute at 100 relative centripetal force (rcf). The supernatant was collected and transferred through a Miltenyi Biotech 30μm filter into a new 15mL conical tube. The filtrate was centrifuged at 4°C for 10 minutes at

600 rcf. The supernatant was discarded and the cell pellet was resuspended in 80μL of Washing Buffer (Table 4) and 20μL of CD117 (Milteny Biotech Mitenyimicrobeads #130091224). The cells were nutated at 4°C for 20 minutes. Washing Buffer (1mL) was used to resuspend the cells before a 10 minute 600 rcf centrifugation at 4°C. The supernatant was discarded and the cells were resuspended in 500μL of cold Washing Buffer. Miltenyi Biotec MiniMACS sorting column was placed on a magnetic stand and activated with 500μL of Washing Buffer. The cell solution was passed over the activated filter, and the column washed three times with 500μL of cold Washing Buffer before the column was removed from the magnetic stand. The cells were eluted using 1mL of Washing Buffer. The elutant was centrifuged at 4°C for 10 minutes at 600 rcf. The supernatant was removed and the cell pellet was resuspended in 2mL of CPC Growth media (Table 5) and plated on a 35mm tissue culture dish.

CPC Culture

The CPC Growth media was changed on the CPCs five days after isolation and then changed twice a week. The stem cells were allowed to grow to 50-60% confluency and cell to cell contact was minimized by passaging the cells. Typically the cells were passaged from a 35mm dish to a single 6cm dish which was passaged to a single 10cm tissue culture dish. Once the 10cm dish reached 50-60% confluency it was split to three 10cm tissue culture dishes which were passaged to a single 15cm dishes. The CPCs are ready to use after the 15^{th} passage and are typically used up to the 30^{th} passage. In order to passage the stem cells the tissue culture dishes were washed with sterile PBS before adding 1mL of 0.25% Trypsin/EDTA to the cells. The

cells were removed from the dish and the Trypsin dissociation was halted by the addition of CPC Growth media. The cells were centrifuged for 6.5 minutes at 600 rcf before the supernatant was removed and the cells were resuspended in new growth media and plated on sterile TC dishes.

Differentiation

The CPCs were differentiated by treating the cells with Differentiation Media (Table 6) containing dexamethasone (10nM). The media was changed every three days. The length of differentiation and cell plating varied between experiments and is further explained in the appropriate section.

Immunofluorescence

Four thousand CPCs were plated on a chambered slide then differentiated for seven days. Following differentiation the media on the slides was removed and washed with cold phosphate buffered saline (PBS) solution before they were fixed with 4% Paraformaldehyde solution for 15 minutes. The cells were covered with PBS for storage at 4°C. Cells were permeablized and rehydrated in a 0.2% Triton-X solution made with PBS for 10 minutes. The slides were blocked with an Immunocytochemistry (IC) blocking solution (Table 7) for 15 minutes. Primary antibodies (Table 8) were diluted in the IC blocking solution and placed on the cells overnight at 4°C. The following day the slides were washed 3 times with 0.2% Triton-X on a rocker for 4 minutes. Secondary antibodies, conjugated to alexa fluorophores, were diluted in PBS (Table 8) and applied for 1 hour. Slides were washed three times

with 0.2% Triton-X solution they were mounted with Vectashield Hardset that contains DAPI.

RNA Isolation

For RNA isolation, CPCs were harvested in 1mL of Trizol and allowed to incubate at room temperature (RT) for 5 minutes. Chloroform (200µL) was added to the samples and mixed by shaking before allowing the samples to sit at RT for 2-5 minutes. Following the RT incubation the samples were centrifuged at 4°C for 10 minutes at 20,000 rcf. The clear supernatant phase on top was removed and placed in a clean microcentrifuge tube containing 500µL of isopropanol and allowed to incubate for 10 minutes at RT. The samples were centrifuged at 4°C for 15 minutes at 20,000 rcf. The isopropanol was removed leaving a RNA pellet. The pellet was rinsed with 500µL of 70% ethanol, made with nuclease free water. The samples were centrifuged at 4°C for 15 minutes at 20,000 rcf. The ethanol was removed and the pellet allowed to air dry for 30 minutes to 1 hour. To redissolve the RNA 25µL of nuclease free water was added to the pellet and heated at 55°C for 10 minutes.

RNA Concentration Measurements

RNA dilutions (1mL, 1:500 dilution) were made and the optical density at 260nm was measured using a quartz cuvette and a spectrometer. In order to determine the concentration of the RNA samples the OD readings and the dilution factor were inserted into the following equation to get the RNA concentration in ug/uL. OD 260 x Dilution factor x 0.04

cDNA Synthesis

For cDNA synthesis, RNA was converted to cDNA using the Verso cDNA synthesis kit from Thermo Scientific. RNA (1µg) was added to PCR tubes and the final volume was brought up to 11µL with nuclease free water. The tubes were heated to 70°C for 5 minutes and cooled to 4°C for at least 1 minute in a PCR machine before adding the components of the kit. Each sample received 4µL of the 5x synthesis buffer, 2µL of the dNTP mix, and 1µL of the random heaxamers, RT Enhancer, and verso enzyme mix supplied in the kit. The samples were heated to a temperature of 50°C for 50 minutes followed by 2 minutes at 95°C before being cooled to 4°C. Upon completion of the cycle the cDNA mixture was brought to a final volume of 40µL by the addition of 20µL of nuclease free water. The cDNA was then further diluted with nuclease free water in a 1:50 ratio.

Microarray

For GPCR determination, CPC cDNA was analyzed using the Taqman GPCR mouse array from Applied Biosystems 4378718 and read on the Applied Biosystems 7500 Fast Real-Time PCR System according to the manufacture's protocol.

Standard CPC Agonist Treatments

CPCs were plated and starved the following day (cell plating varies by experiment). Following 24 hours of starvation, cells were treated with various agonists. Agonist product information and treatment concentrations can be found in Table 9. For inhibitor studies, cells were pretreated prior to the addition of agonists

according to Table 8. The C3 Rho inhibitor required a five hour pretreatment and the $G\alpha_i$ inhibitor, Pertussis toxin (Ptx), required a 24 hour pretreatment.

Transfetion Protocol

CPCs were transfected with reporter plasmids or siRNA in order to study cellular signaling. OptiMEM from Gibco (31985-070) was used as the transfection media and a 1:3 ratio of nucleic acid material to transfection reagent was used when making the transfection solution. The amount of nucleic acid and transfection reagent product varies from each experiment and is specified in the preceding sections. For siRNA and transfection reagent product information refer to Table 10 & 11 respectively. Initially two stock solutions, a transfection reagent solution and nucleic acid solution, were made in equal volumes with a 2x concentration in OptiMEM. The two stock solutions were vortexed and allowed to equilibrate for 10 minutes. The nucleic acid and transfection reagent stock solutions were then combined in a 1:1 ratio to get a final 1x transfection solution. This transfection solution was allowed to equilibrate for 20 minutes to facilitate the interaction between the nucleic acid material and the transfection reagent before adding the media directly to the cells.

Luciferase Assay

For the Luciferase experiment, CPCs were plated on a 12 well plate with $2x10^4$ CPCs per well. The following day the cells were transected with 1ug of an SRE.L Luciferase reporter and 100ng of Renilla. Fugene 6 was used as the transfection reagent. The transfection media was removed 24hrs later and the cells were serum

starved. After a day of starvation the cells were pre-treated with C3 for five hours before a six hour agonist treatment (Table 9). Following agonist stimulation, luciferase was measured using the Promega Dual-Luciferase Reporter Assay according to the online protocol. The luciferase readings were normalized to the renilla readings, which served as an internal control for transfection efficiency.

siRNA Luciferase Assay

For the siRNA Luciferase experiment, CPCs ($2x10^4$) were plated on a 12 well plate. The cells were co-transfected with 1µg of an SRE.L Luciferase reporter, 100ng of Renilla, and 0.75µg of siRNA using Lipofectamine 2000 as the transfection reagent. After 4 hours the transfection media was removed to avoid cell death caused by siRNA and the media was replaced with CPC Growth media. Refer to Table 10 in the Appendix for siRNA product information. The CPCs were starved the next day for 24 hours and treated with agonists for 6 hours. The reporter activity was measured with the Promega Dual-Luciferase Reporter Assay.

Proliferation Assay

Proliferation was quantified using the NF CYQUANT proliferation assay kit (catalog #C35007) from invitrogen, which measures cell number based on fluorescent DNA. On a 48 well plate, $8x10^3$ CPCs were plated per well and the cells were serum starved for 24 hours the following day. After 24 hours of treatment, proliferation was measured according to the manufacturer's protocol. Briefly, 11 mL of 1x HBSS solution was made from a 5x stock and MQ water. Media from the cells was removed,

CYQUANT reagent (22µL) was added to the 1x HBSS, and 200µL of the reagent was added to each well. The cells were incubated for 30 minutes and measured for fluorescence.

siRNA Proliferation Assay

Proliferation was performed as described above with the addition of siRNA. CPCs were plated and transfected the next day with 0.05µg of siRNA using a 1:3 ratio of Dharmafect #1 as the transfection reagent. Following 24 hours after transfection, the media was removed and the CPCs were serum starved overnight before agonist addition. Proliferation was measured after 24 hours of agonist treatment. Refer to Table 10 in the Appendix for siRNA product information.

qPCR

mRNA levels were assessed using the Applied Biosystem 7500 Fast Real-Time PCR System. To evaluate gene expression, 10µL of the 2x Taqman Universal Master Mix II with UNG from Applied Biosystems (catalogue # 4440038) was added to each well of the qPCR plate along with 1µL of the primer from Applied Biosystems and 9µL of the 1:50 dilution of cDNA. The standard program was run and the data was analyzed using the C_T Method.[63] Gapdh levels were used as the internal control. See Table 12 for a list of the Applied Biosystems Taqman primers.

Dexamethasone Differentiation with Agonist treatments

CPCs were plated at $5x10^4$ cells per 6cm dish. The following day, the cells were starved overnight and treated with serum depleted differentiation media which contained normal concentrations of glutamine, penicillin-streptomycin, and dexamethasone with or without GPCR agonists. On the day of treatment, a set of untreated controls were frozen. After 3 days of differentiation, the cells were analyzed by qPCR for the presence of lineage markers and the fold expression was determined by comparing the normalized CT values to the undifferentiated set using the C_T Method.[63]

Cell Death Assay

CPCs were plated at $4x10^4$ cells per 6cm TC dish. The following day, the cells were starved overnight before treatment with or without 150μM H_2O_2 and 0.3μM S1P. Following 18 hours of treatment, the cells were harvested in freshly made lysis buffer previously described.[64] The cell extracts were nutated for 10-20 minute at 4°C and centrifuged at $2x10^4$ rcf for 10 minutes. Cell death was quantified using the Cell Death Detection Elisa^{PLUS} kit from Roche that measures cell death via a TUNNEL based assay. The cell supernatant (20μL) was added to the wells along with 80 μL of the immunoreagent prepared according to the protocol. The wells were covered in foil and placed on a shaker at RT for 3 hours. Meanwhile a bradford assay was done with the remainder of the cellular supernatants for quantitation purposes. After shaking, the wells were washed 3 times with 200μL of washing buffer provided in the kit. The remainder of the solution was aspirated following the final wash. ABST reagent was

prepared according to the kit's protocol and 100μL was added simultaneously to the wells using a multipippette. Absorbance was measured at 405 nm to quantify cell death. Fold changes were determined by normalizing absorbance to protein concentration (determined by Bradford Assay).

Statistical Analysis

All the data was normalized to an internal control if available. The normalized values were all compared to non-treated control group and expressed as relative fold changes. The average, standard deviation, and standard error of the mean were calculated from the experimental data. The student's two tailed t-test with equal variance done to determine significance. A probability ≤ 0.05 was considered significant.

Results

Isolation and characterization of c-kit$^+$ cardiac progenitor cells. CPCs were isolated from male FVBN mice (8 to 12 weeks of age) according to the protocol in Materials and Methods which utilizes affinity purification with magnetic antibodies for the c-kit stem cell marker. In order to evaluate the quality of the cells, immunofluorescent detection of the stem cell marker c-kit was determined. Undifferentiated CPCs and cells that were differentiated with a seven day dexamethasone treatment were stained for the c-kit marker. As expected, fluorescent detection of c-kit was observed on the undifferentiated cells (Figure 1a). Upon differentiation, loss of c-kit immunoreactivity was observed (Figure 1b). This decrease in c-kit expression is consistent with previous findings.[29] In order to confirm that the cells we isolated were multipotent, the CPCs were tested for the presence of cellular lineage markers. Differentiated CPCs were stained with Rhodamine-Phalloidin (Figure 2b, 2f, 2j) for cytoskeletal structure, and for the cardiac lineage markers MEF2C (Figure 2a) and GATA 4 (Figure 2e). The CPCs were determined to express both MEF2C and GATA4 through immunoflourescent detection. The secondary control slides showed the absence of staining. Our cell staining results were verified by the detection of GATA4 and MEF2C as well as the smooth muscle lineage marker, smooth muscle actin, and the endothelial marker, PECAM, through qPCR (data not shown). Thus experiments characterizing the isolated cells demonstrated that the CPCs are positive for the c-kit stem cell marker and express cellular lineage markers for cardiac, smooth muscle, and endothelial cell types.

Determination of G-protein coupled receptor expression in undifferentiated and differentiated c-kit$^+$ cardiac progenitor cells. In order to determine the constellation of GPCRs present on the CPCs, mRNA was isolated from undifferentiated and differentiated CPCs (Dex (10nM), 7 days). The mRNA levels were analyzed using a Taqman GPCR array from Applied Biosystems. The array confirmed the presence of 200 GPCRs. Figure 3 shows the relative expression of a select group of GPCRs (black bars) that were of interest to our lab and the receptor isotype switching and changes that occur following differentiation (red bars). The CPCs were positive for both β and α adrenergic receptors, PAR1 (Thrombin receptor), Angiotensin II receptor isoforms 1 and 2, Lysophosphatidic acid receptor isoforms 1 and 2, and Sphingosine-1-phosphate receptors isoforms 1, 2, and 3. Interestingly, the CPCs express the S1PR2 isoform predominately. This is in contrast to adult cardiomyocytes in which the S1PR1 is the highest isoform expressed followed by S1PR2 and S1PR3.[41] Also, CPCs express the Angiotensin II receptor isoforms with the AT2 receptor in a greater abundance then the AT1 receptor, while the AT1 receptor is the predominant isoform expressed in adult cardiomyocytes.[65] In adult cardiomyocytes, β adrenergic receptors are expressed in a greater abundance than the α adrenergic receptors,[65] but in CPCs the expression of adrenergic receptors is reversed. In addition, in undifferentiated CPCs the predominant β adrenergic receptor isoform is the β_2 receptor as opposed to the β_1 receptor which is predominant in adult cardiomyocytes.[31,65] Following differentiation the β_1 receptor becomes the predominant isoform. This data demonstrates that CPCs contain a distinct set of

GPCRs which differ from that of adult cardiomyocytes and have the ability to change relative isoform expression upon differentiation.

G-protein coupled receptor agonists induce proliferation in CPCs. Since GPCRs are highly expressed on CPCs, we determined whether a subset of GPCR agonists could stimulate proliferation. Phenylephrine (50μM), Lysophosphatidic acid (10μM), Isoproterenol (1μM), Sphingosine-1-phosphate (0.3μM), Thrombin (0.5U/mL), and Angiotensin II (1μM) all caused a significant increase in cellular proliferation following 24 hour stimulation as well as serum (20%) which was a positive control (Figure 4).

Sphingosine-1-phosphate activates Rho dependent transcription in cardiac progenitor cells. Our lab has shown that S1P activates RhoA in cardiomyocytes, we hypothesized that S1P activates Rho in CPCs. Since CPC cell number is insufficient to directly measure GTP-Rho by pull down assay we assessed RhoA activation indirectly in undifferentiated CPCs transfected with an SRE.L reporter. The SRE.L reporter is dependent on the activity of Rho and independent of ERK, SAPK/JNK, and MPK2/p38 signaling pathways.[66] Serum and S1P induce robust increases in SRE.L reporter activity (Figure 5) compared to the untreated control. The exoenzyme C3, which is a Rho specific inhibitor, was able to significantly decrease the reporter activity at baseline and following S1P or serum stimulation. Also, knockdown of RhoA by siRNA (Figure 6) attenuated SRE.L reporter activity in response to serum or

S1P (Figure 7). Taken together these experiments demonstrate the ability of S1P to activate Rho dependent transcription in CPCs.

Sphingosine-1-Phosphate mediated cardiac progenitor cell proliferation is dependent on Rho activation. Since Rho signaling has been previously implicated in cellular proliferation,[34] and S1P induced Rho dependent transcription in CPCs, we wanted determined whether S1P induced proliferation was dependent on Rho. Cells were treated with Rho activator (1U/mL), S1P (0.3μM), and serum (20%) with or without C3 (1μg/mL) or pertussis toxin (0.1μg/mL) pretreatments. We demonstrated that the Rho activator caused a significant increase in proliferation (Figure 8). Both serum and S1P induced proliferation was diminished by inhibition of Rho with C3, but was unaffected by $G\alpha_i$ inhibiton with pertussis toxin (Figure 8). To determine the S1P receptor subtype responsible for proliferation and confirm Rho's involvement, siRNA transfection was used to knockdown RhoA and the S1PR isoforms (Data not shown). Proliferation induced by S1P was inhibited by siRNA knockdown of RhoA, S1PR2, or S1PR3 however; knockdown of S1PR1 had no effect (Figure 9). Since the S1PRs 2 and 3 primarily couple to $G\alpha_{12/13}$, this data suggests that S1P induces proliferation in a $G\alpha_{12/13}$-Rho dependent manner.

Sphingosine-1-phosphate protects CPCs from apoptosis induced by oxidative stress. S1P has been previously shown to protect the heart from ischemia/reperfusion injury[56] and adult cardiomyocytes from hypoxic damage[55]. We determined whether S1P could protect CPCs from oxidative damage. Apoptosis was

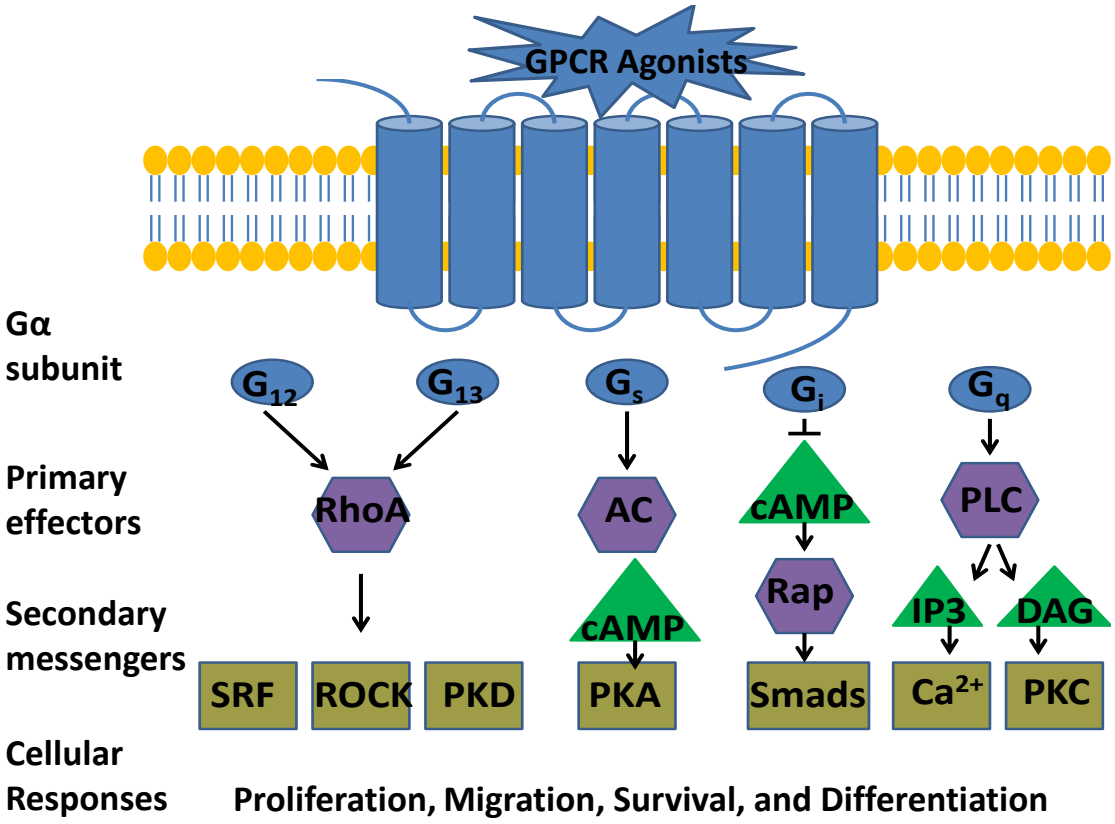
induced in CPCs by H_2O_2 (150μM) treatment in the presence or absence of S1P. We found that S1P treatment significantly attenuated cell death caused by oxidative stress (Figure 10). This data is similar to previous findings in cardiommyocytes and demonstrates that S1P protects CPCs from cell death.

Differentiation of c-kit$^+$ cardiac progenitor cells is not potentiated by Sphingosine-1-phosphate. GPCR signaling has previously been implicated in differentiation of stem cells[10,33] and S1P in particular has been shown to enhance cellular commitment in myoblasts.[52,53] To analyze S1P's effect on CPC differentiation, S1P was added (0.3μM) to serum free differentiation media containing dexamethasone over a three day time course. As demonstrated in Figure 11, the addition of S1P did not significantly change the expression of MEF2C (cardiac marker) or GATA6 (smooth muscle marker) when compared to CPCs treated with dexamethasone alone. However, S1P did significantly inhibit the expression of GATA4 (cardiac marker) induced by dexamethasone treatment. Our results suggest that S1P does not enhance differentiation and may even inhibit cardiac lineage expression.

Lysophosphatidic Acid induces Rho mediated transcription, but mediates proliferation through $G\alpha_i$ signaling. S1P mediated proliferation of CPCs was determined to be Rho dependent, thus we wanted to investigate if the GPCR agonist LPA, which has been shown to activate Rho, behaved in a similar manner. Using the SRE.L reporter system we confirmed that LPA, a known Rho activator, [34] induces Rho

dependent transcription that was significantly attenuated by C3 pretreatment (Figure 12A). Notably however, pretreatment with pertussis toxin ($G\alpha_i$ inhibitor) inhibited LPA induced proliferation in CPCs while C3 had no effect (Figure 12B). Thus LPA can activate Rho dependent transcriptional activity; however, unlike S1P, proliferation induced by LPA was not dependent on Rho, but rather on $G\alpha_i$ signaling.

Lysophosphatidic Acid increases differentiation induced expression of cardiac lineage markers. To determine whether LPA affected differentiation of CPCs, LPA was added to serum free differentiation media over a three day time course. Unlike S1P, LPA treatment caused a significant increase in the cardiac lineage marker expression of GATA4 and MEF2C when compared to dexamethasone (10nM) alone (Figure 13). Expression of GATA6, a smooth muscle marker, was not changed. Overall, addition of LPA potentiates differentiation-induced expression of cardiac lineage markers in CPCs without affecting smooth muscle marker expression.



Scheme1: G-protein coupled receptor signaling pathways. Extracellular agonists couple to the 7 transmembrane GPCR and activate the heterotrimeric G-protein complex. Five common Gα subunits are shown above along with intermediate signaling proteins and downstream kinases that control crucial cellular functions. A List of Abbreviations is available on page vi.

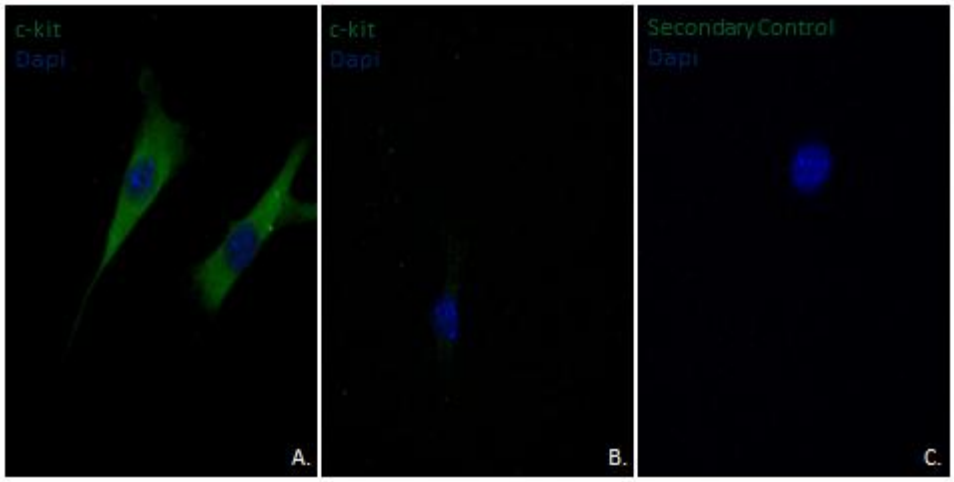


Figure 1: Detection of the stem cell marker c-kit on CPCs. CPCs were isolated from male FVBN mice (8 to 12 weeks of age) through magnetic bead sorting for the c-kit antigen as described in Materials and Methods section. CPCs between passage 15 and 30 were fixed with 4% paraformaldehyde and immunocytochemistry for c-kit antigen was performed. CPCs were differentiated with Dex (10nM) for 7 days to determine loss of the c-kit antigen. Images were stained for nuclei with dapi (blue) and c-kit (green) in undifferentiated CPCs (A.) and differentiated CPCs (B.). The secondary control (C.) did not express any nonspecific fluorescence. Pictures were taken using a 40x oil objective leans.

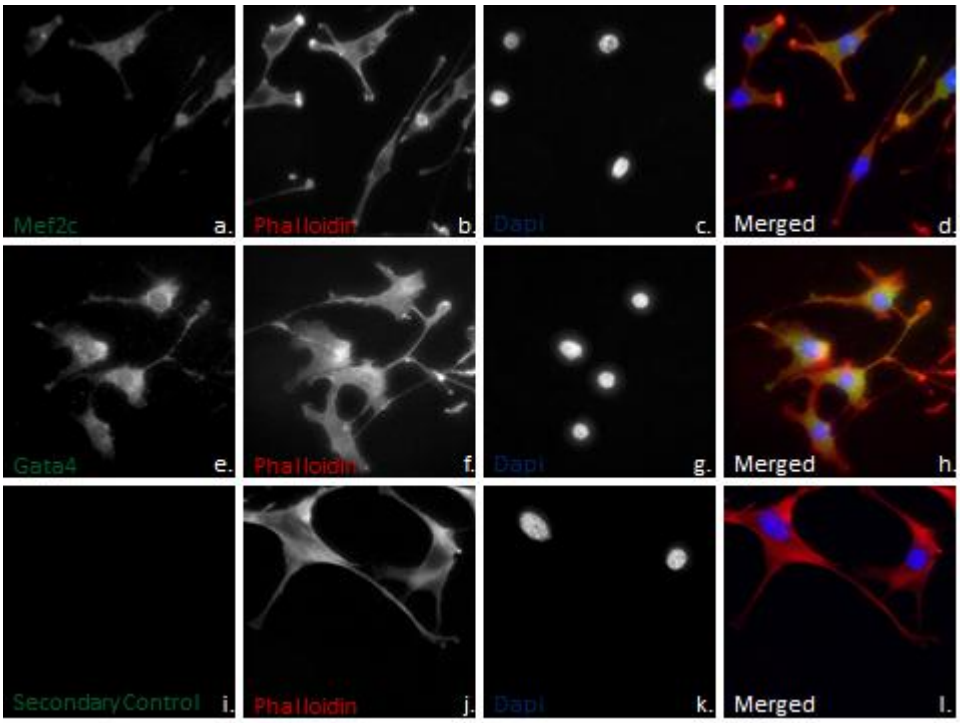


Figure 2: Detection of cardiac lineage markers on CPCs. CPCs between the passages 15 and 30 and positive for the c-kit stem cell marker were differentiated for 7 days with 10nM Dexamethasone. Following differentiation, the cells were fixed and stained with primary antibodies for the cardiac lineage markers MEF2C (a.) and GATA4 (b.). Phalloidin was used to stain the cytoskeletal structure (red) of the cardiac stem cells (b, f, j). Secondary control (i) was negative for nonspecific fluorescence. The nuclei (c, g, k) were stained with dapi (blue). The merged images are shown above (d, h, l). Pictures were taken using a 40x oil objective lens.

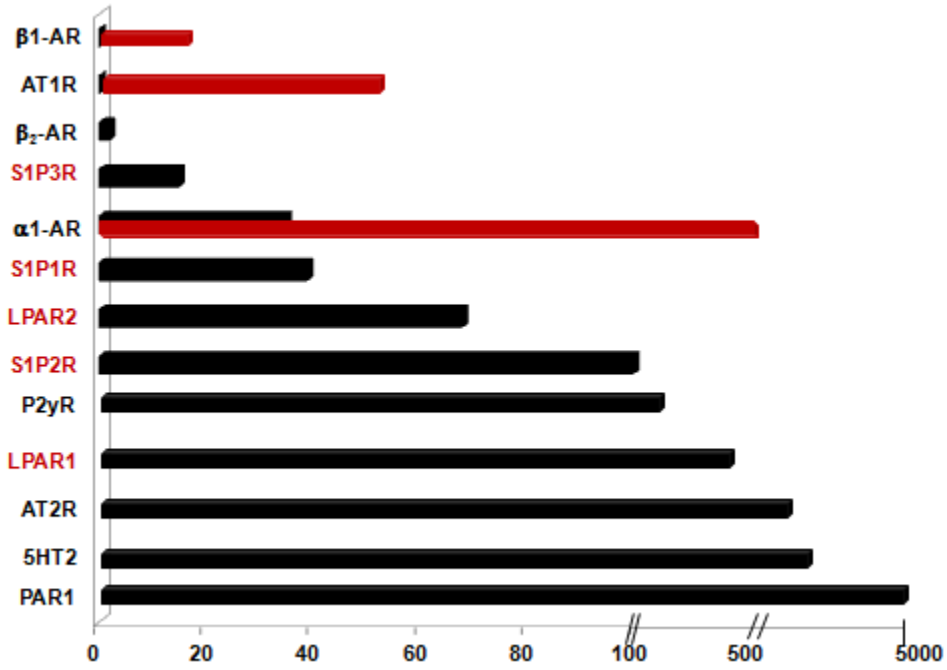


Figure 3: Expression of GPCRs on mouse cardiac progenitor cells. Analysis of GPCRs using a TaqMan Real Time-PCR array. Expression levels of selected GPCR in passage 15 cardiac progenitor cells (CPCs) relative to Gapdh are indicated above. Black bars represent baseline receptor expression of undifferentiated CPCs. Red bars are relative expression of receptors that dynamically change following differentiation of the CPCs (7 days dexamethasone treatment). The receptor names in red font are the lysophospholipid receptors that were of particular interest to our lab.

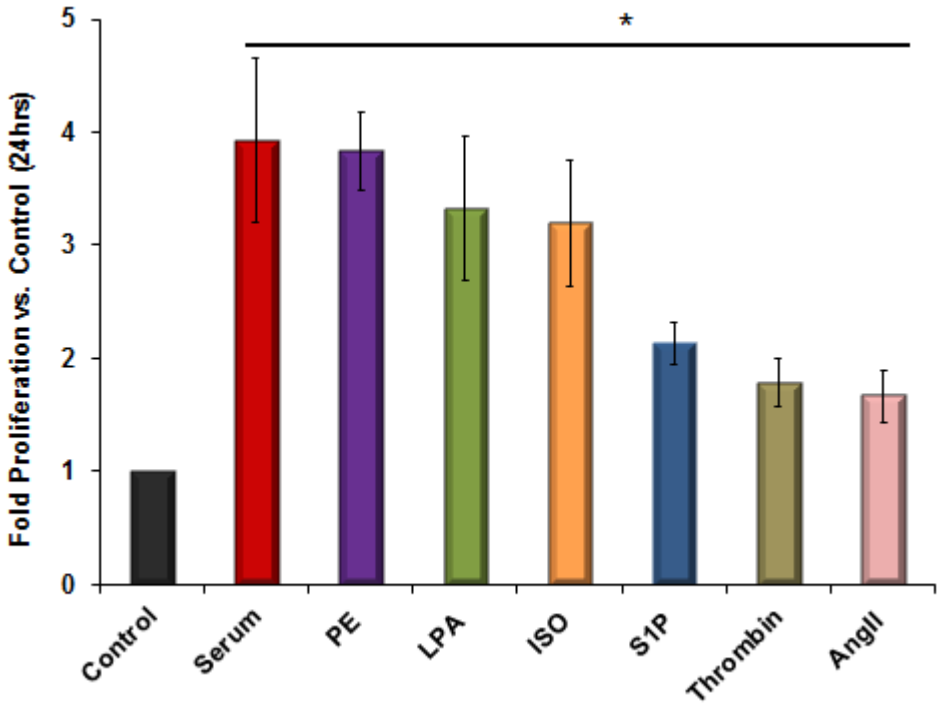


Figure 4: GPCR agonists induce CPC proliferation. CPCs were serum starved overnight and treated with serum (20%), PE (50μM), LPA (10μM), ISO (1μM), S1P (0.3μM), Thrombin (0.5U/mL), or Ang II (1μM) for 24 hours. Proliferation was measured by CyQUANT Assay. The graph represents 6 independent experiments. Significance was determined by the student's two tailed t-test with *$p \leq 0.05$ vs. control n=12

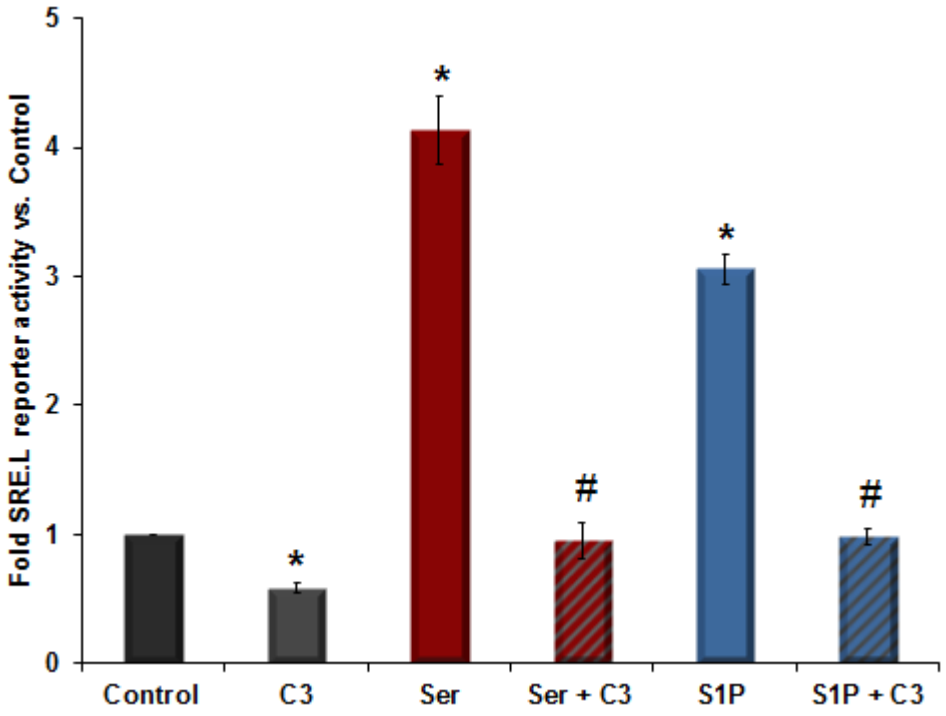


Figure 5: SIP activates Rho dependent transcriptional activity in CPCs. CPCs were transfected with a Rho specific SRE.L reporter construct and renilla plasmid. The following day, CPCs were serum starved overnight and treated for 6 hours with serum (20%) or S1P (0.3μM) with or without a 5 hour C3 pretreatment (1μg/mL). SRE.L reporter activity was determined by the Promega Dual-Luciferase Reporter Assay. Data represents two independent experiments n=3. Significance was determined by the student's two tailed t-test with *$p \leq 0.05$ vs. Control and #$p \leq 0.05$ compared to its respective agonist.

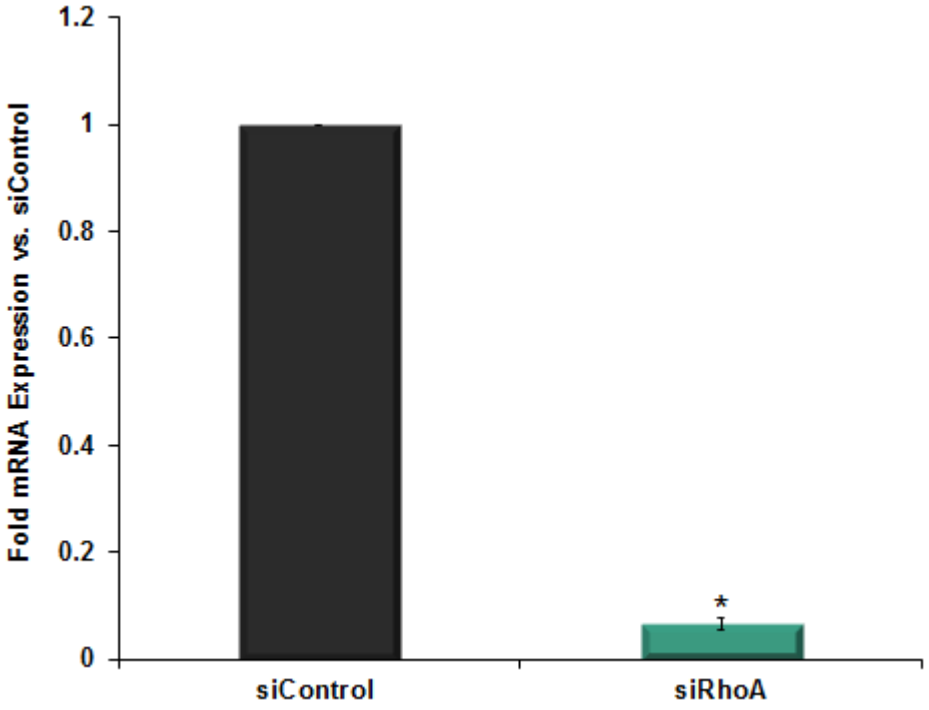


Figure 6: siRNA knockdown of RhoA. CPCs were transfected with either Control or RhoA siRNA using Dharmafect transfection reagent. The transfection media was removed the following day and RNA was isolated from the cells 48 hours post-transfection. RhoA levels were assessed by qPCR analysis. Significance was determined by the student's two tailed t-test with *$p \leq 0.05$ vs. siControl n=9

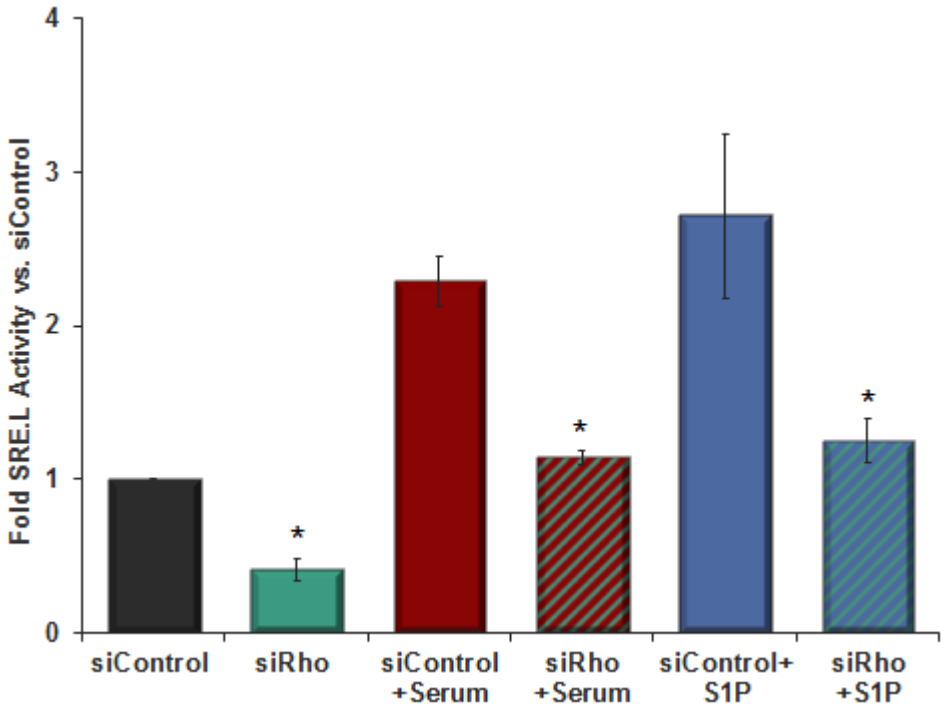


Figure 7: S1P induced SRE.L transcriptional activity is Rho dependent in CPCs. CPCs were cotransfected with the SRE.L reporter (100ng) and siRNA (0.75µg) using Lipofectamine 2000. The cells were starved overnight. Following 48 hour transfection, CPCs were treated with serum (20%) or S1P (0.3µM) for 6 hours. Luciferase activity was assessed with the Promega Dual-Luciferase Reporter Assay. Data represents one independent experiments with n=4. Significance was determined by the student's two-tailed t-test with *$p \leq 0.05$ vs. siControl under equivalent treatment conditions.

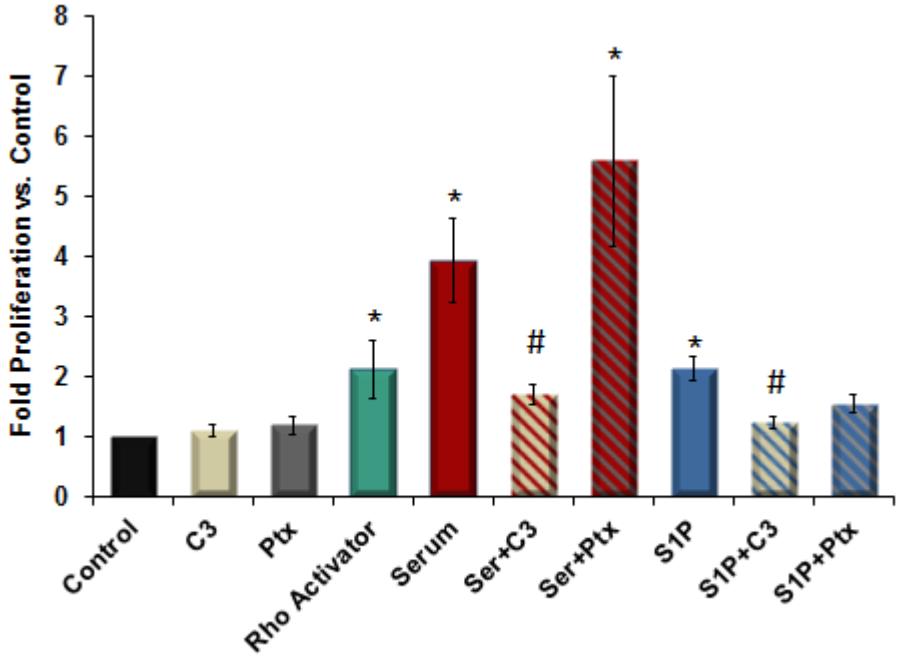


Figure 8: S1P and Serum induce CPC proliferation in a Rho dependent manner. Serum starved CPCs were treated with Rho Activator (1U/mL), serum (20%), or S1P (0.3µM) with or without C3 (1µg/mL, 5hr) or Ptx (0.1µg/mL, 24hr) pretreatment to inhibit Rho and $G\alpha_i$ signaling respectively. Proliferation was measured 24 hours after agonist treatment using CyQUANT assay. The graph represents 3 independent experiments. Significance was determined by student's two tailed t-test with *$p \leq 0.05$ vs. Control and #$p \leq 0.05$ vs. agonist n=6

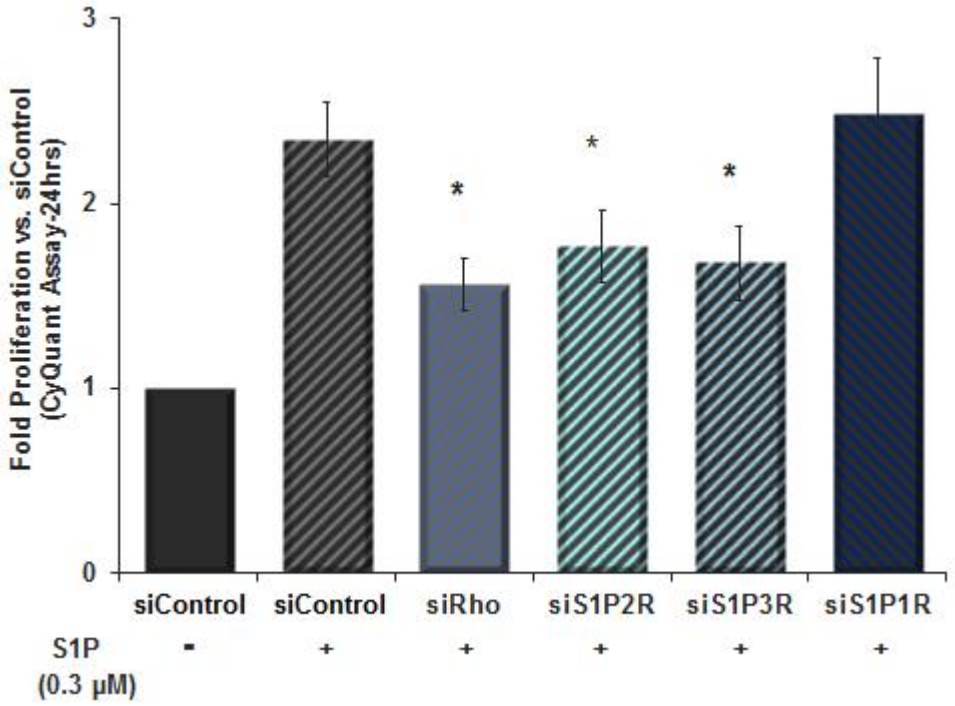


Figure 9: S1P mediated proliferation was blocked by knockdown of RhoA, S1PR2, or S1PR3. CPCs were transfected with siRNA (0.05µg). The transfection media was removed the following day and the CPCs were serum starved overnight before 24 hour treatment with or without S1P (0.3µM). Cell proliferation was measured using CyQuant assay and significance was determined by student's two tailed t-test with *$p \leq 0.05$ vs. siControl with S1P n=3

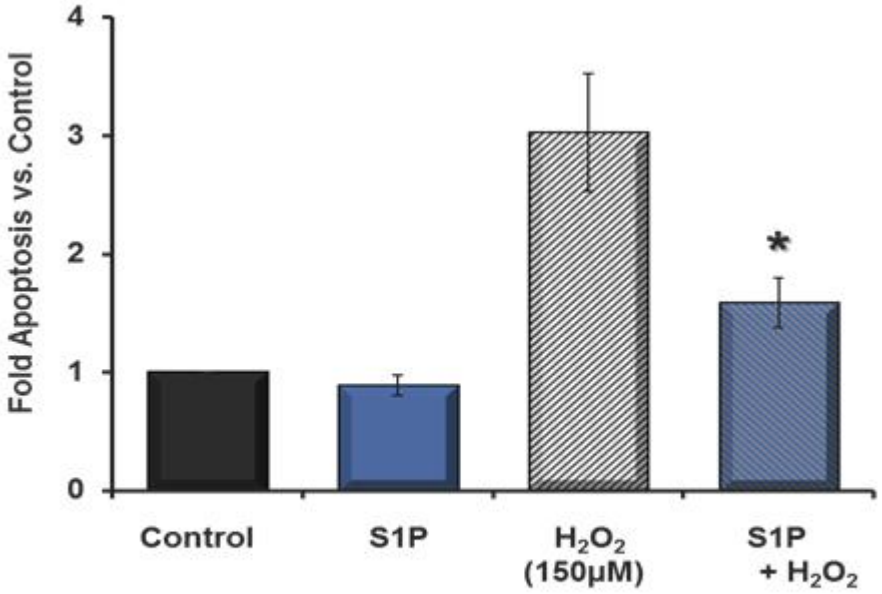


Figure 10: S1P treatment protects CPCs against cell death induced by oxidative stress. CPCs were serum starved for 24hrs and treated with S1P (0.3µM), hydrogen peroxide (H_2O_2, 150µM) or both for 18hrs and cell death was quantified by DNA fragmentation using an ELISA based POD assay. Fold apoptosis was compared to control cells. The graph represents 3 independent experiments. Significance was determined by the student's two tailed t-test with *$p \leq 0.05$ compared to H_2O_2 n=6

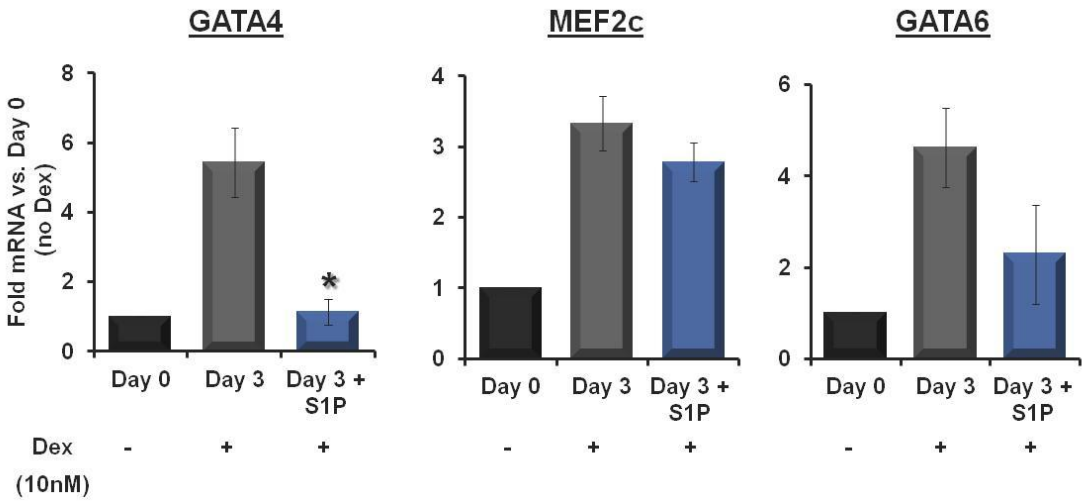


Figure 11: S1P treatment does not enhance CPC differentiation. CPCs were treated with Dexamethasone (Dex, 10nM) for 3 days to induce differentiation with or without S1P (0.3μM). RNA was isolated after 3 days of treatment and the expression of lineage markers for cardiac (GATA4, MEF2C) and smooth muscle (GATA6) differentiation was examined by TaqMan PCR. The graph represents 3 independent experiments with an n=3. Significance was determined by the student's two tailed t-test with *$p \leq 0.05$ vs. Day 3 (Dex).

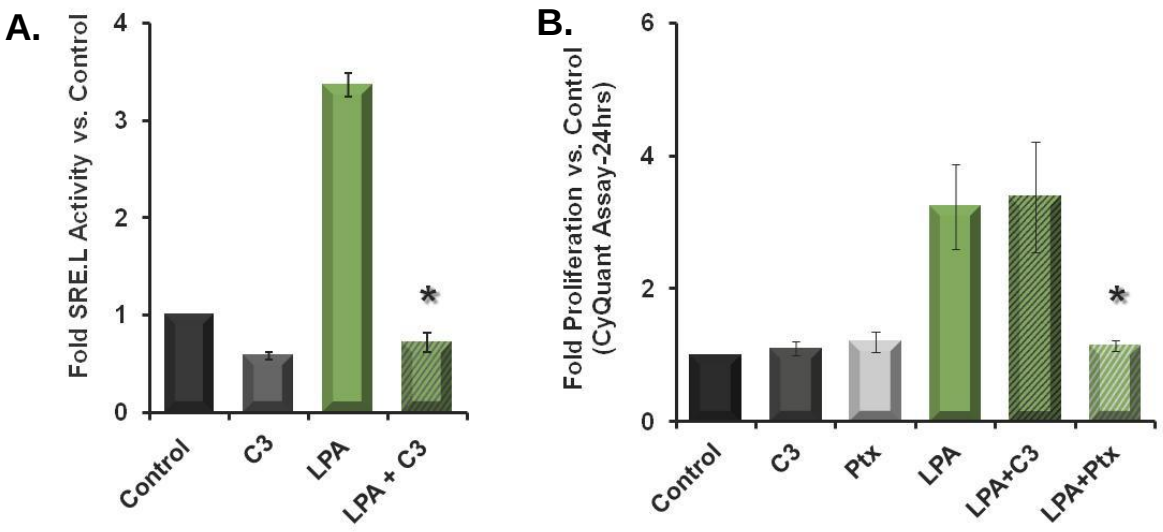


Figure 12: LPA induced proliferation is $G\alpha_i$ not Rho dependent. (A.) CPCs were transfected with a Rho specific SRE.L reporter construct and renilla plasmid. The following day, CPCs were serum starved and treated with LPA (10μM) for 6 hours with or without a 5 hour C3 pretreatment (1μg/mL). SRE.L reporter activity was determined with the Promega Dual-Luciferase Reporter Assay. Significance was determined by the student's two tailed t-test with *$p<0.05$ vs. Control n=3. (B.) CPCs were serum starved and treated with LPA (10μM) for 16 hours with or without C3 (1μg/mL, 5hr) or Ptx (0.1μg/mL, 24hr) pretreatment to inhibit Rho and $G\alpha_i$ signaling respectively. Proliferation was measured 24 hours after agonist treatment using CyQUANT assay. The graph represents 3 independent experiments. Significance was determined by the student's two tailed t-test with *$p\leq0.05$ vs. Control n=6.

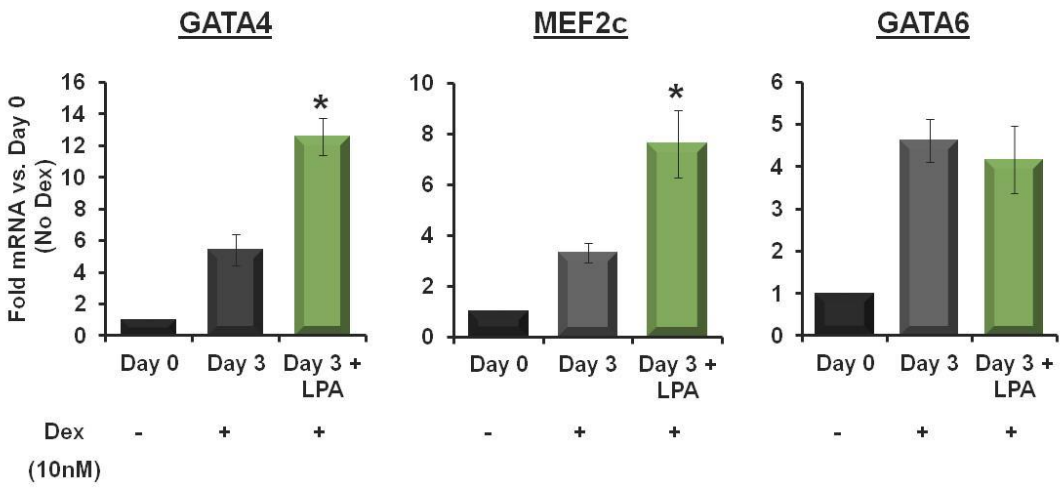


Figure 13: LPA increases differentiation of CPCs towards a cardiac lineage. CPCs were treated with Dexamethasone (Dex, 10nM) for 3 days to induce differentiation with or without LPA (10μM). RNA was isolated after 3 days of treatment and the expression of lineage markers for cardiac (GATA4, MEF2C) and smooth muscle (GATA6) differentiation was examined by TaqMan PCR. The graph represents 3 independent experiments with an n=3. Significance was determined by the student's two tailed t-test with *$p \leq 0.05$ vs. Day 3 (Dex).

Discussion

The discovery that the heart is a self-renewing organ that undergoes adult cardiomyocyte turnover and contains a population of endogenous multipotent c-kit^{+} cardiac stem cells has changed the field of cardiology tremendously. The success of the SCIPIO trial has demonstrated the extraordinary capacity of these CPCs to regenerate the heart. Our laboratory is interested in the role of G-protein coupled receptors which are known to mediate many physiological and pathological responses in the cardiac system such as development of heart failure, cardiac hypertrophy, cardiac contractility, and protection against ischemia/reperfusion injury.[2,31,32] GPCRs have also been implicated in the regulation of stem cell processes such as maintenance of pluripotency, differentiation, proliferation, survival, and migration,[10,33] however, how GPCR signaling affects CPCs is largely unknown.

CPCs were isolated from wild type FVBN using magnetic antibody sorting. We demonstrated that the undifferentiated cells were c-kit positive through immunofluorescent staining (Figure 1). A seven day differentiation time course with 10nM Dexamethosone resulted in an apparent decrease in c-kit fluorescence. A defining characteristic is the loss of the stem cell marker c-kit following differentiation. However, the cells do not differentiate completely *in vitro* and therefore retain a small level of c-kit expression. This decrease in c-kit expression determined by immunofluorescence agrees with the previous findings from Fransioli et al.[29]

In an effort to determine if our cells expressed markers of multiple cellular lineages, GATA4 and MEF2C (cardiac) were detected through immunocytochemistry

(Figure 2). These findings also correspond to previous studies which characterized c-kit$^+$ cardiac progenitor cells.[15,29] Additionally the lineage specific markers α-actinin, PECAM, and smooth muscle actin previously shown to be present in the endogenous c-kit$^+$ CPCs[15,29] were detected using qPCR (data not shown). These findings provide convincing evidence that the isolated cells are multipotent c-kit$^+$ cardiac progenitor cells.

In order to determine the complement of GPCRs expressed on CPCs, a GPCR array was used to detect the receptors present on undifferentiated versus differentiated CPCs. Among the GPCRs expressed on CPCs were receptors that recognize Thrombin, Angiotensin II, Lysophosphatidic acid, Sphingosine-1-phosphate, and adrenergic agonists (Figure 3). Interestingly, we have shown the relative expression of receptor isoforms on the CPCs differed significantly from the receptor constellation reported on adult cardiomyocytes. In CPCs, S1PR2 was the predominate S1P receptor as opposed to S1PR1 found primarily on adult cardiomyocytes.[35,41] Also, the Angiotensin receptor isoform expression and relative abundance of α and β adrenergic receptors also differ from cardiomyocytes. Additionally, the β2 receptor is the predominant β isoform on undifferentiated CPCs while the β1 receptor is the most highly expressed adrenergic receptor among adult myocytes. However, we have demonstrated that upon differentiation the stem cells experience a shift in β adrenergic receptor expression and the β1 receptor becomes the major β receptor in agreement with adult myocytes. The discovery that CPCs contain a distinct set of GPCR and have the ability to change relative isoform expression upon differentiation may have important implications. In the heart, GPCR agonists, unregulated in response to

cardiac injury, may promote cellular responses in CPCs such as proliferation, migration, and survival that are mediated through predominant receptor isoforms that switch upon differentiation. For example the use of beta blockers to attenuate chronic adrenergic signaling in the heart may actually prevent crucial stem cell functions controlled by the β2 adrenergic receptor such as proliferation.

Many GPCR agonists including adrenergic agonists,[67,68] Thrombin,[69] Angiotensin II,[70,71] LPA,[49] and S1P[51] have been demonstrated to effect proliferation on a wide range of cells which include cancer, satellite, and vascular smooth muscle cells. Similar to previous studies in other cell types, we have shown that GPCR agonists such as adrenergic agonists, Thrombin, Angiotensin II, LPA, and S1P all significantly induce proliferation in CPCs (Figure 4). Our data suggests that GPCR signaling may be an important mediator of the endogenous CPC expansion observed in response to cardiac injury. Since, the endogenous cells are unable to repair the heart, whereas additional administration of CPCs greatly improves cardiac function, manipulating GPCR signaling with pharmacological tools may increase the performance of these endogenous cells *in vivo*.

It is known that GPCRs can couple promiscuously to Gα proteins. Both S1P[34,72] and LPA[34,73] have previously been shown to activate Rho and our results confirmed that these agonists along with serum treatment, which served as a positive control, did indeed cause Rho dependent transcription of the SRE.L reporter. Additionally, the use of C3 toxin inhibited transcription in response to serum, S1P (Figure 5), and LPA (Figure 12A), which confirmed that the SRE.L activity was in fact dependent on Rho. siRNA experiments also established that knockdown of RhoA

specifically attenuates the increase in reporter plasmid transcription in response to serum and S1P (Figure 7). RhoA has been implicated as a crucial mediator of many cellular responses such as proliferation, survival, and migration. Interestingly, in CPCs the predominant S1PR isoform couples efficaciously to Rho GEFs. S1P is known to play an important role in cardiac pathology and it is possible that Rho signaling also regulates the cellular function of CPCs.

Through the use of the C3 cell permeable Rho inhibitor and siRNA, we have further demonstrated that S1P induced proliferation is Rho dependent and mediated through the S1P receptors 2 and 3 (Figure 8, and 9). Our data using Ptx ($G\alpha_i$ inhibitor) and by siRNA receptor knockdown indicate, that S1P proliferation is not dependent on $G\alpha_i$ or S1PR1 (Figure 8, and 9). Conversely, LPA mediated proliferation was shown to be dependent upon $G\alpha_i$ signaling and independent of Rho (Figure 12B). These findings agree with our array data in that the predominant sphingosine-1-phosphate receptor expressed, S1PR2, is known to couple to Rho GEFs $G\alpha_{12}$ and $G\alpha_{13}$[34,42-44,57-60] while the primary LPA receptor isoform, LPAR1, has been shown to couple to $G\alpha_i$.[37]

Our lab's previous work has demonstrated that S1P[56] and Rho[62] signaling has a protective effect against ischemia/reperfusion injury in the heart and recent unpublished data suggests that S1P mediates this protection through Rho. We confirmed that S1P prevented cell death (Figure 10) induced by oxidative stress in CPCs. We have yet to determine whether the protective effects of LPA, which has been demonstrated in adult cardiomyocytes[55], occurs in CPCs. Although S1P protects CPCs from oxidative damage we do not yet know whether this is dependent on Rho

signaling. Future experiments to elucidate the signaling pathway responsible for S1P and LPA protection are planned.

GPCR agonists have been previously implicated in differentiation of stem cells[10,33] however their effect on CPC differentiation was unknown. S1P has been shown to enhance myogenic differentiation in myoblasts[52] and formation of osteoblasts from myoblast,[53] while LPA inhibited differentiation in primary brown adipose tissue precursors.[50] Interestingly, S1P and LPA had the opposite effects on differentiation induced in CPCs. S1P inhibited expression of the cardiac lineage marker GATA4 and did not affect other lineage markers (Figure 11). In contrast, LPA increased the expression of GATA4 and MEF2C (cardiac markers) without changing the expression of the smooth muscle marker, GATA6 (Figure 13). Based on our findings regarding lysophospholipid mediated proliferation we know that S1P induces proliferation through Rho, but LPA's effects are $G\alpha_i$ dependent. This difference may account for the contrasting CPC differentiation results observed using S1P and LPA. Our data is preliminary and more experiments are needed to solidify the relationship between the roles of G-proteins in CPCs. It is possible that with a better understanding of the signaling that controls differentiation, the efficacy of CPC therapeutics may be improved.

Our findings have helped characterize roles of G-protein coupled receptors on c-kit$^+$ cardiac stem cells and implicated them as mediators of proliferation, differentiation, and cellular survival. These experiments just begin to reveal the role of GPCR signaling in CPCs. The different GPCR expression profiles on adult cardiomyocytes and CPCs are an interesting topic. Adrenergic and angiotensin II

signaling pathways play major roles in cardiac physiology and need to be further investigated in c-kit$^+$ cardiac progenitor cells. Our collaborators in the Sussman lab have shown that β-adrenergic signaling and isotype switching plays an important role in cellular survival. However, the roles of adrenergic signaling on c-kit$^+$ cardiac progenitor cells in differentiation and gene expression have not been previously studied and are of interest to us. The differences between S1P and LPA signaling need to be further elucidated. In order to determine the role of Rho signaling on CPC function, CPCs from cardiac specific Rho transgenic mice will be employed to confirm Rho's importance in proliferation, protection, and differentiation. CPCs from S1PR 1, 2, and 3 knockout mice will help to delineate the receptors responsible for S1P's effect on the CPC function. Lastly, the importance of GPCR signaling in CPC migration and commitment during injury will need to be investigated using our genetically modified mice. Previous mouse studies have shown that genetic manipulation of CPCs can lead to an increase in therapeutic efficacy of c-kit$^+$ CPC administration.[74] In the future, manipulating GPCR signaling may help to either improve the function of endogenous CPCs through pharmacological means or through the use of genetic alteration that increases efficacy of CPC administration to patients with heart failure. Hopefully, additional data will lead to the elucidation of the roles of G-protein coupled receptor signaling in c-kit$^+$ cardiac progenitor cells and help to better understand of stem cell function and improve therapeutic treatments.

Appendix A

Table of Buffers, Media, and Standard Agonist Treatments

Table 1: Basic Buffer (Good for 1 week)

Reagent	Source	F.W. or Stock Concentration	Quantity	Final Concentration
J-MEM	Sigma		11.2g	1x
HEPES	Calbiochem	283.3g	0.7g	0.0025M
Taurine	Sigma	125.15g/mol	1.25g	0.01M
Insulin (self-made aliquots)	Sigma-Aldrich I-5500	20 Units/mL	1mL	20Units/L
L-Glutamine	Gibco 25030-081	200mM	10mL	2mM
Penicillin-Streptomycin	GIBCO/ Invitrogen 15140-122	Penicillin 10,000U/mL Streptomycin 10,000ug/mL	10mL	100U/mL Pen 100ug/mL Strep
Gentamicin	Gibco 15750-060	50mg/mL	1mL	50µg/mL
Fungizone Amphotericin B	Gibco 15290-018	250mg/mL	10mL	2.5mg/mL

Add components to 900mL of double distilled water and titrate with 5M NaOH to a pH between 7.2-7.3. If the pH exceeds 7.3 during titration discard and start over. Filter sterilize the solution.

Table 2: Digestion Buffer (Made Day of Isolation)

Reagent	Source	F.W. or Stock Concentration	Quantity	Final Concentration
Collagenase Type II	Worthington Biochemical	310U/mg	100mg	320U/mL

Dissolve in 97mL of basic buffer then filter sterilize.

Table 3: Incubation Buffer (Good for 1 month)

Reagent	Source	F.W. or Stock Concentration	Quantity	Final Concentration
Albumin, from Bovine Serum	Sigma A8806-5G	N/A	0.25g	0.5%

Dissolve in 50mL of fresh basic buffer then titrate the solution with 1M NaOH between a pH of 7.2-7.3 before filter sterilization. If the pH exceeds 7.3 during titration discard and start over.

Table 4: Washing Buffer (Good for 1 month)

Reagent	Source	F.W. or Stock Concentration	Quantity	Final Concentration
Albumin, from Bovine Serum	Sigma A8806-5G	N/A	0.25g	0.5%

Dissolve in 50mL of sterile PBS from Gibco/Invitrogen then titrate the solution with 1M NaOH between a pH of 7.2-7.4 before filter sterilization. If the pH exceeds 7.4 during titration discard and start over.

Table 5: Growth media

Reagent	Source	F.W. or Stock Concentration	Quantity	Final Conc.
Leukemia Inhibitory Factor	Bio Pioneer Inc. sc-041-2	$1x10^7$ U/mL	50µL	500U
Insulin Transferrin Selenium	BioWhittaker 17-838Z	500x	1mL	1x
Epidermal Growth Factor (Self-made aliquots dissolved in 10mM Acetic Acid, 0.1% BSA)	Invitrogen PHG0311	200ug/mL	0.5mL	0.2ug/ mL
Basic Fibroblast Growth Factor (Self-made aliquots dissolved in 10mM Tris-HCl pH=8.5)	Peprotech 100-18B	100ug/mL	0.1mL	0.02ug/ mL
L-Glutamine	Gibco 25030-081	200mM	5mL	2mM
Penicillin-Streptomycin	Gibco 15140-122	Penicillin 10,000U/mL Streptomycin 10,000ug/mL	5mL	100U/ mL Pen 100ug/ mL Strep
Embryonic Stem Cell Grade Fetal Bovine Calf Serum	Gibco 10439-024	1x	50mL	10.00%

All the reagents except the serum were added to500mL of DMEM F12 media from Bio Whittaker catalogue # 12-719F. After the solution was filter sterilized the Embryonic Stem Cell Grade Fetal Bovine Calf Serum was added and the media was stored a 4°C.

Table 6: Differentiation Media

Reagent	Source	F.W. or Stock Concentration	Quantity/Dilution	Final Concentration
Embryonic Stem Cell Grade Fetal Bovine Calf Serum	Gibco 10439-024	1x	50mL	10.00%
L-Glutamine	Gibco 25030-081	200mM	5mL	2mM
Penicillin-Streptomycin	Gibco 15140-122	Penicillin 10,000U/mL Streptomycin 10,000ug/mL	5mL	100U/mL Pen 100ug/mL Strep
Dexamethasone		Self-Made 10µM	1:1000	10nM

All the reagents, except dexamethasone, were added to a 500mL bottle of MEM Alpha media from Gibco catalogue # 12561 and stored at 4° C. The Dexamethasone is added separately to the media right before treating the cells in order to preserve its activity.

Table 7: Immunocytochemistry (IC) Block

Reagent	Source	F.W. or Stock Concentration	Quantity/Dilution	Final Concentration
BSA, Fraction V	EMD Chemicals	N/A	5g	2%
Goat Serum	Sigma Aldrich	100%	25mL	10%
Triton-X	Sigma Aldrich	100%	250µL	0.1%

Table 8: Antibodies

Antigen	Product Source/Catalog #	Dilution
c-kit (primary)	Cell Signaling Technology #3074	1:25
MEF2C (primary)	Cell Signaling Technology #5030	1:100
Gata-4 (primary)	Santa Cruz Biotechnology sc-9053	1:25
Rhodamine Phalloidin (secondary)	Life Technologies R415	1:100
Alexa Fluor 488 Anti-Rabbit IgG (secondary)	Life Technologies A-11008	1:200

Table 9: Standard Agonist Treatments

Agonist (abbreviations)	Source	Final Concentration
Serum (Ser)	Gibco catalogue #10439-024	20%
Sphingosine-1-phosphate (S1P)	Avanti Polar Lipids catalogue #860492P	0.3µM
Lysophosphatidic Acid (LPA)	Avanti Polar Lipids catalogue #857130P	10µM
Thrombin (Thr)	Enzyme Research Laboratories catalogue # HT10029	0.5U/mL
Angiotensin II (Ang II)	Calbiochem catalogue #05-23-0101	1µM
Isoproterenol (ISO)	Sigma catalogue #I6504	1µM
Phenylephrine (PE)	Sigma catalogue #P6126	50µM
Rho Activator	Cytoskeleton catalogue #CN01	1U/mL
C3	Cytoskeleton catalogue #CT04	1µg/mL
Pertussis Toxin (Ptx)	Calbiochem catalogue #516560	0.1µg/mL

Table 10: siRNA List

Gene of Interest	Source
Negative Control	Qiagen catalogue # 1027281
RhoA	Bioneer catalogue # 1418633
S1PR1	Bioneer catalogue # 1353054
S1PR1	Bioneer catalogue # 1353055
S1PR1	Bioneer catalogue # 1353056
S1PR2	Bioneer catalogue # 1353096
S1PR2	Bioneer catalogue # 1353097
S1PR2	Bioneer catalogue # 1353098
S1PR3	Bioneer catalogue # 1353077
S1PR3	Bioneer catalogue # 1353078
S1PR3	Bioneer catalogue # 1353079

*Note: A siRNA stock solution for each of the S1P receptors was made by pooling the three siRNA products together. Each siRNA product was present in equal concentrations. These siRNA pools were used to knockdown the S1P receptors.

Table 11: Transfection Reagents

Reagent	Source	Assay
Fugene 6	Promega (catalogue #E2691)	Luciferase
Lipofectamine 2000	Invitrogen (catalogue #11668-019)	siRNA Luciferase
Dharmafect 1	Thermo Scientific (catalogue # T-2001-03)	siProliferation

Table 12: Applied Biosystems qPCR Primer List

Gene of Interest	Applied Biosystems primer #
Gapdh	Mm03302249_g1
RhoA	Mm0122806_g1
GATA4	Mm00484689_m1
MEF2C	Mm01340842_m1
GATA6	Mm00802636_m1

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