Signal Transduction Mechanisms for Lysophosphatidic Acid Mediated Cardiac Differentiation of P19 Stem Cells

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ABSTRACT

The role of endogenous molecules in facilitating stem cell differentiation into cardiomyocytes is yet to be fully understood. SPC and S1P, common biolipids, promote cardiac differentiation of mesenchymal stem cells and cardiac progenitor cells, however, the same potential of closely related lysophosphatidic acid (LPA) has only recently become evident. The initial cardio-protection offered by elevated LPA levels in response to acute myocardial infarction and the ability of this biolipid to mediate other cellular fates served as a rationale to investigate the ability of LPA to mediate the cardiac differentiation of the murine P19 teratocarcinoma cell line and further examine the role of signalling molecules critical to lineage commitment.

All experiments were carried out using P19 stem cells, cultured in supplemented alpha-minimal essential medium. Cells were aggregated into embryoid bodies in the presence of 5μM LPA in non-tissue grade Petri dishes over the course of 4 days to commence the differentiation process. Inhibitors were added 60 minutes before LPA while control cells were cultured in medium only. Embryoid bodies were transferred to 6-well tissue culture grade plates and cultured for a further 6 days. Cardiac differentiation was assessed by examining the expression of ventricular myosin light chain (MLC1v) by western blot and the role of LPA receptors 1-4, PKC, PI3K, MAPKs, and NF-κB were determined by examining the changes in this expression in the presence of selective inhibitors. The induction and regulation of GATA4, MEF2C, ATF-2, JNK, and YAP was also determined by western blotting. The activity and regulation of transcription factors, AP-1 and NF-κB, and the MAPKs was determined using ELISA kits.

LPA induced the differentiation of P19 cells into cardiomyocytes most effectively when used at a concentration of 5μM as evidenced by the expression of MLC1v on day 10 of the differentiation process. Inhibition of LPA receptor 4 (0.1mg/mL Suramin), LPA receptors 1/3 (20μM Ki16425), LPA receptor 2 (7.5nM H2L5186303), PKC (10μM BIM-1), PI3K (20μM LY294002), ERK (20μM PD98059), JNK (10μM SP600125), and NF-κB (0.01nM CAY10470) blocked LPA induced expression of MLC1v. GATA4, MEF2C, pcJun, pJunD, and $pATF_2$ expression increased in a time-dependent manner peaking at day 10 in LPA treated cells. GATA4 and pcJun expression was suppressed by all the inhibitors whereas MEF2C expression was unaffected by CAY10470, pJunD expression was unaffected by H2L5186303, pATF2 and NF-κB expression was unaffected by LY294002, but the latter was enhanced by Suramin. JNK was transiently phosphorylated in all cells whereas YAP was dephosphorylated 24-48 hours after EB formation in LPA treated cells and were both affected by Ki16425 and partially by H2L5186303 treatment.

In conclusion, the studies carried out in this thesis have shown that LPA mediates the cardiac differentiation of P19 cells through LPA receptor 2, partially through receptors 1/3, and possibly through receptor 4. Conceivably downstream of these receptors, PKC, PI3K, MAPK, and NF-κB signalling pathways converge on the regulation of cardiac-specific transcription factors GATA4 and MEF2C along with ubiquitous transcription factor AP-1. JNK signalling is initiated through LPA receptors 1/3 and partially through receptor 2 to commence the cardiac program however the role of JNK and YAP in the proliferation of aggregating EBs is yet to be entirely established.

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Abbreviations

αMEM	Alpha-minimum essential medium
AC	Adenylate Cyclase
Akt	Protein Kinase B
ANOVA	Analysis of Variance
AP-1	Activator Protein 1
APC	Adenomatous Polyposis Coli
ASK	Apoptosis Stimulating Kinase
ATF2	Activating Transcription Factor 2
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BIM-1	Bisindolylmaleimide-1
BM	Bone Marrow
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CVD	Cardiovascular Disease
DAG	1, 2-diacyglycerol
DDW	Double Distilled Water
DKK1	Dickkopf-related protein 1
DLK	Dual Leucine zipper bearing Kinase
DMSO	Dimethyl sulfoxide
EB	Embryoid body
ECC	Embryonal Carcinoma Cell
Edg	Endothelial differentiation gene
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinase
ESC	Embryonic Stem Cell
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FHF	First Heart Field
FoxO	Forkhead box O
Gab1	Grb2-associated binding protein 1
GF	Growth Factor
GPCR	G protein-coupled receptor
G-Protein	Guanine nucleotide-binding protein
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen Synthase Kinase 3
HAND	Heart and neural crest derivatives expressed proteins
HEPES	4-(2-hydroxyethy)-1-peperazine ethane sulphonic acid

HRP	Horse Radish Peroxidase
ICM	Inner Cell Mass
IgG	Immunoglobulin
IP_3	Inositol (1,4,5) tris-phosphate
iPSC	Induced Pluripotent Stem Cell
Isl1	Insulin gene enhancer protein1
JNK	c-Jun N-terminal kinase
JSAP	JNK/Stress activated protein kinase associated protein
kDa	Kilo Daltons
Klf-4	Kruppel-like factor-4
LATS	Large tumour suppressor
LCAT	Lecithin cholesterol acyltransferase
LIF	Leukaemia Inhibitory Factor
LP	Lysophospholipid
LPA	Lysophosphatidic Acid
LPAR	Lysophosphatidic Acid Receptor
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPL	Lipoprotein Lipase
LPS	Lysophosphatidylserine
LVEF	Left ventricular ejection fraction
LVEDV	Left ventricular end diastolic volume
LVESV	Left ventricular end systolic volume
MAG	Monoacyl glycerol
MAPK	Mitogen Activated Protein Kinase
MAPKAPK	MAPK Activated Protein Kinase
MAPKK/MKK/MEK	Mitogen Activated Protein Kinase Kinase
MAPKKK/MEKK	Mitogen Activated Protein Kinase Kinase Kinase
MEF	Mouse Embryonic Fibroblast
MEF2	Myocyte Enhancer Factor 2
mEpiSC	Mouse Epiblast Stem Cell
MESP1	Mesoderm posterior 1
MGAT	Monoacyl glycerophosphate acyltransferase
MHC	Myosin heavy chain
MI	Myocardial Infarction
MK2, 3, 5	MAPK-activated protein kinase 2/3/5
MLC	Myosin Light Chain
MLK3	Mixed lineage kinase 3
MNK	MAPK interacting kinase
MOB	Mps One Binder
MSK	Mitogen and stress activated kinase
MST	Mammalian Ste20-like
MTT	(3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyl-tetrazolium bromide
NFAT	Nuclear Factor of Activated T cells

NFκB	Nuclear Factor kappa B
NKX2.5	NK2 homeobox 5
Oct3/4	Octamer-binding protein-3/4
PA	Phosphatidic Acid
PBS	Phosphate Buffered Saline
PI	Phosphoinositide
PI3K	Phosphatidylinositol-3 Phosphate Kinase
PIP_2	Phosphatidylinositol (1, 4) bis-phosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein Kinase C
PKG	Protein kinase G
PL	Phospholipid
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenyl Methyl Sulfonyl Fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PSC	Pluripotent Stem Cell
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene fluoride
RSK	Ribosomal S6 protein kinases
SAV	Salvador
S.E.M	Standard Error Mean
S1P	Sphingosine 1-phosphate
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH2	Src Homology Domain 2
SHF	Second Heart Field
SM	Skeletal Myoblasts
SMAD	Similar to Mothers Against Decapentaplegic
SOS	Son Of Sevenless
Sox2	Sex determining region Y-box 2
SPC	Sphingosylphosphorylcholine
SRE	Serum Response Element
SSEA	Stage specific embryonic antigen
STAT	Signal Transducers and Activators of Transcription
TAK	Transforming growth factor-β-activated kinase
TBS	Tris buffered saline
TBS-T	Tris buffered saline - Tween
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGF	Transforming growth factor
Wnt	Wingless-related integration site
YAP	Yes-associated protein

Chapter 1

Introduction

Overview

Cardiovascular diseases (CVDs) are amongst the leading cause of death worldwide and become more prevalent with age. Amongst all CVDs, the manifestation of myocardial infarction (MI) is the most devastating to the cardiomyocyte population. Injury to the human heart due to sudden damage, disease, or age-related degeneration is traditionally treated in a palliative manner. In contrast to current treatments, cell therapy aims to restore functional cardiomyocytes. Cell therapy harnesses the unique potential of stem cells to generate multi-lineage cells. The past twenty years have been dedicated to evaluating cardiac repair by several sources of stem cells. There is growing evidence that the introduction of multipotent stem cells into the damaged human heart does not restore functional cardiomyocytes but offer improvement, albeit limited, via unknown paracrine mechanisms. Another approach is the directed differentiation of stem cells into a cardiac fate before introduction into the damaged myocardium, the accomplishment of which is largely dependent on the thorough understanding of the signalling transduction mechanisms responsible for driving the production of cardiomyocytes. *In vitro* manipulation of the signalling pathways that control cardiac development *in vivo* have allowed for more efficient directed differentiation, however, the complexity of these signalling networks and the role of endogenous molecules in initiating the program of cardiac differentiation remains poorly described.

1.1 Myocardial infarction

Myocardial infarction (MI) is brought on by both genetic and environmental factors such as diet, exercise, stress, and smoking, and can be further modified by age, gender, obesity, hypertension, and diabetes. MI is most often caused by the build-up of plaque within the artery. Over time the plaque may either block the artery or rupture, facilitating thrombus formation thereby, blocking the flow of oxygen-rich blood to the heart. Cardiac physiology is rapidly altered in this ischemic environment, and physiological stresses induce multi-level adaptive responses. As reviewed by Webster (2009), a decrease in the mitochondrial ATP is initially compensated by increased production of glycolytic ATP but later leads to lactate accumulation and a decrease in cytosolic pH. Ultimately, the excessive depletion in ATP results in cell necrosis, initiated in the central zone of the infarcted area, and further damage is caused by ischemia related calcium overload, generation of reactive oxygen species, and the activation of inflammatory and cytokine signalling pathways. Apoptosis resulting from reperfusion of the myocardium can lead to secondary damage (Yellon & Hausenloy, 2007). Ischaemic events, such as MI, are the most devastating to the cardiomyocyte population where upwards of a billion cardiomyocytes are lost (Laflamme & Murry, 2005), resulting in the formation of fibrotic scar tissue and left ventricular remodelling (Sutton & Sharpe, 2000), diminishing the pumping capacity of the heart.

1.1.1 Current treatments

Endogenous repair mechanisms cannot restore the number of cardiomyocytes that are lost during an ischaemic event. Pharmaceutical interventions either at the onset of MI or prescribed as aftercare including aspirin, statins, nitrates, β-blockers, and fibrinolytic drugs may attenuate the symptoms but do not address the loss of cardiomyocytes (Sutton & Sharpe, 2000). Surgical interventions such as balloon

angioplasty and coronary stents also fail in this regard. Currently, the only treatment that restores lost cardiomyocytes is a heart transplant. Aside from the potential risk of organ rejection, this treatment is not a viable option on a large scale. In 2015 the World Health Organization reported over 17 million deaths worldwide were attributed to CVDs with the majority being due to MI, therefore, there is an immediate need for an alternative treatment with global availability.

1.2 Cell therapy

Experimental and clinical studies continue to focus on alternative therapies to address cardiomyocyte regeneration including stimulating the proliferation of existing cardiomyocytes, although terminally differentiated cardiomyocytes are resistant to cell cycle re-entry, and cell therapy. Cell therapy is the introduction of cells into the damaged myocardium, directly or indirectly, that are either not terminally differentiated or differentiated into cardiomyocytes *in vitro*. The former, known as stem cells, can differentiate into more mature cell types while maintaining the ability to undergo self-renewal. The differentiation capacity of stem cells varies depending on the source and potency of the cells. Several sources of multipotent stem cells for cardiac repair have been evaluated in preclinical and clinical settings and select studies are summarised in table 1.0 and 1.1 respectively. Preclinical and clinical trials have demonstrated that bone marrow mononuclear cells and mesenchymal cells are safe for cardiac cell therapy whereas skeletal myoblasts, although promising in preclinical studies with an increase in LVEF, result in higher incidences of arrhythmia in clinical trials. Amongst all cell types, bone marrow derived stem cells represent the greatest number evaluated in clinical trials. A meta-analysis of 50 publications including 2500 patients collectively concluded that although there is a moderate ~4% increase in LVEF, patients that have received cell therapy had lower mortality, reoccurrence of MI, and

rehospitalization for heart failure (Zimmet et al., 2012; Jeevanantham et al., 2012). A limited number of clinical trials have evaluated cardiac stem cells but a meta-analysis of 80 preclinical publications including over 1950 animals collectively by Zwetsloot *et al.* (2016) concluded that LVEF increased by ~12% and ~5% in small and large animals respectively compared to placebo groups. The effects of cell therapy for cardiac regeneration have been modest but a fair comparison between trials, both preclinical and clinical, is challenging due to the variation in cell isolation, delivery, dose, and patient cohort but it is evident that the beneficial outcomes of cell therapy may be due to paracrine effects rather than direct tissue regeneration.

Table 1.0. Preclinical studies of cell therapy for cardiac regeneration

Cell type	Study	Host	Dose/Route	Follow up	Outcome
Skeletal Myoblasts	Suzuki *et al.* (2001)	Rat	$1x10^6$ cells Intracoronary	4 weeks	↓Mortality
	Chachques *et al.* (2004b)	Sheep	$70x10^6$ cells Intramyocardial	3 months	↑LVEF ↓LV remodelling
	He *et al.* (2005)	Dog	$270\text{-}830x10^6$ cells Intramyocardial	10 weeks	↑LVEF ↓LV remodelling
	Farahmand *et al.* (2008)	Rat	$5x10^6$ cells Intramyocardial	30 days	↑LVFS ↓LV remodelling
Bone Marrow Stem Cells	Tomita *et al.* (1999)	Rat	$1x10^6$ cells Intramyocardial	3 weeks	↓LV remodelling ↑Angiogenesis CD+
	Bel *et al.* (2003)	Sheep	422 $x10^6$ cells Intramyocardial	2 months	↔LVEF ↔LV remodelling CD-
	Waksman *et al.* (2004)	Pig	24 $x10^6$ cells Intramyocardial	4 weeks	↓Infarct size ↑Angiogenesis
Mesenchymal Cells	Nagaya *et al.* (2005)	Rat	$5x10^6$ cells Intramyocardial	4 weeks	↑Angiogenesis ↓Fibrosis CD+
	Silva *et al.* (2005)	Dog	$100x10^6$ cells Intramyocardial	30 days	↑LVEF Neovascularization+
	Liu *et al.* (2008)	Rat	$1x10^6$ cells Intramyocardial	4 weeks	↓Infarct size ↓LV remodelling ↑LVEF ↓Fibrosis ↑Angiogenesis CD+
	Mazo *et al.* (2008)	Rat	$1x10^6$ cells Intramyocardial	3 months	↓Infarct size ↑LVEF ↓Fibrosis Neovascularization+
	Li *et al.* (2009)	Rat	$3x10^6$ cells Intramyocardial	4 weeks	↑LVEF ↓Fibrosis
	Schuler *et al.* (2009)	Pig	$200x10^6$ cells Intramyocardial	24 weeks	↑LVEF ↓Infarct size
	Mazo *et al.* (2010)	Rat	$1x10^6$ cells Intramyocardial	4 weeks	↑LVEF ↓Fibrosis ↑Angiogenesis
Cardiac Stem Cells	Rota *et al.* (2008) (cKit+ cells)	Rat	$4x10^4$ cells Intramyocardial	2 weeks	↑LVEF ↓Fibrosis ↓LV remodelling Cardiac regeneration+
	Johnston *et al.* (2009)	Pig	$10x10^6$ cells Intracoronary	8 weeks	↓Infarct size ↓LV remodelling Cardiac regeneration+
	Tang *et al.* (2010) (cKit+ cells)	Rat	$4x10^4$ cells Intracoronary	35 days	↑LVEF ↓Fibrosis ↓LV remodelling Cardiac regeneration+
	Lee *et al.* (2011) (cardiospheres)	Pig	$1x10^6$ cells Intracoronary	8 weeks	↑LVEF ↓LV remodelling
	Bolli *et al.* (2013) (cKit+ cells)	Pig	$5x10^5$ cells Intracoronary	31 days	↑LVEF ↓Fibrosis

↓ decreased, ↑ increased, ↔ no change, + observed, - not observed, CD – Cardiac Differentiation, LV – Left Ventricular, LVEF – Left ventricular ejection fraction, LVFS – Left ventricular fractional shortening. Amended from Bolli et al., 2013.

Table 1.1. Clinical studies of cell therapy for cardiac regeneration

Cell type	Name	Patients	Follow up	Dose/Route	Outcome
Skeletal Myoblasts	Menasche *et al.* (2001)	1	5 months	800x10^6 cells Intramyocardial during CABG	↑Wall motion and perfusion on PET
	MAGIC	97	6 months	400-800x10^6 cells Surgical injection during CABG	↔LVEF ↓LVESV ↓LVEDV ↑Arrhythmia
	SEISMIC	40	6 months	150-800x10^6 cells Intramyocardial	↔LVEF ↑Arrhythmia
	CAUSMIC	23	12 months	3-600x10^6 cells Intramyocardial	↑LVEF ↑Arrhythmia
Bone Marrow Mononuclear Stem cells	BOOST	60	60 months	24.6x10^8 cells Intracoronary	↑LVESV ↑LVEDV
	ASTAMI	100	36 months	80x10^6 cells Intracoronary	↔LVEF ↔infarct size
	REPAIR-AMI	204	24 months	200x10^6 cells Intracoronary	↑LVEF ↓LVESV
	TOPCARE-AMI	59	60 months	5.5x10^6 cells Intracoronary	↑LVEF ↓LVESV ↓LVEDV
	SWISS-AMI	200	12 months	140-160x10^6 cells Intracoronary	↔LVEF ↓infarct size ↓LVESV ↓LVEDV
	REGENERATE-AMI	100	12 months	59.8x10^6 cells Intracoronary	↑LVEF ↓infarct size
Mesenchymal Stem Cells	Prochymal	60	12 months	30-300x10^6 cells Intravenous infusion	↑LVEF
	POSEIDON	31	13 months	20-200x10^6 cells Intramyocardial	↑LVEF ↓infarct size
	PROMETHEUS	9	18 months	20-200x10^6 cells Intramyocardial	↑LVEF ↓infarct size
	PRECISE	27	18 months	42 x10^6 cells Intramyocardial	↔LVEF ↓infarct size
Cardiac Stem Cells	CADUCEUS	25	12 months	12.5-25x10^6 cells Intracoronary	↔LVEF ↔LVESV ↔LVEDV ↓infarct size
	SCIPIO	23	12 months	1x10^6 cells Intracoronary	↑LVEF ↓infarct size

↓ decreased, ↑ increased, ↔ no change, LVEF – Left ventricular ejection fraction, LVESV – Left ventricular end systolic volume, LVEDV – Left ventricular end diastolic volume. Amended from Cahill, Choudhury, and Riley (2017) and Hao, Wang, and Wang (2017).

1.2.1 Multipotent stem cells

The organ or tissue in which multipotent stem cells reside influences both their differentiation capacity; limited to maturing into cell types of that organ or tissue, and the rate of self-renewal. Several sources of multipotent stem cells for cardiac repair have been evaluated in clinical trials but with limited success. Aside from identifying the most appropriate cell type, dose, and method of transplantation, the other hurdles to be addressed include the poor survival of transplanted cells and further differentiation and integration into the myocardium with proper electrophysiological coupling.

1.2.1.1 Skeletal myoblasts

The use of multipotent stem cells as a means of reversing the damage ensued by MI was triggered by studies using a murine model demonstrating that skeletal myoblasts (SMs) engrafted the heart (Koh *et al*., 1993) and promoted cardiac repair (Taylor *et al*., 1998). SMs differentiate in response to muscle injury and show resistance to ischaemia (Chachques *et al.,* 2004a). The ease of *in vitro* expansion and autologous availability translated quickly into clinical trials (Menasche *et al.,* 2001) after success was achieved in both small and large animal models (Farahmand *et al.,* 2008; He *et al.,* 2005; Pouly *et al.,* 2004). However, trans-differentiation into cardiomyocytes or integration into the myocardium has not been seen (Menasche *et al.,* 2008; Reinecke *et al.,* 2002). Furthermore, compound procedures including coronary artery bypass grafting make it difficult to distinguish between effects of revascularization and cell treatment. Higher incidence of arrhythmia is also documented that may be caused by the myofibers derived from the transplanted SMs failing to electromechanically couple with the host myocytes posing a challenge for using this cell source for therapeutic purposes (Menasche *et al.,* 2003; Pagani *et al.,* 2003).

1.2.1.2 Bone marrow

In the late 1990's, investigators demonstrated reasonable repair, involving migration and differentiation of stem cells, after injury in muscle, the liver, and the brain. Orlic *et al.* (2001a, 2001b) found that direct transplantation of autologous bone marrow (BM) into the damaged myocardium of mice displayed the same repair. Numerous clinical trials have now evaluated BM mononuclear cells for cardiac myopathy with different cell dose, delivery route, follow up interval, and the number of patients. Evidence of BM stem cell plasticity *in vivo* (Assmus *et al.,* 2002; Perin *et al.,* 2003; Tse *et al.,* 2003) is lacking, and only a handful of clinical trials have provided long-term results. The ASTAMI (Beitnes *et al.,* 2009; Beitnes *et al.,* 2011; Lunde *et al.,* 2006), BOOST (Meyer *et al.,* 2006; Wollert *et al.,* 2004), and REPAIR-AMI (Assmus *et al.,* 2010) trials showed little to no improvement after 3-5 years although the latter reported reduction in infarct size and increased wall thickening of the infarcted region at a 2 year follow up. Of the several dozen trials, none have shown significant improvement in all the outcomes assessed (reduction in infarct size, change in LVESV and LVEDV, and improved LVEF) (Jeevanantham *et al.,* 2012). However, no major adverse events were reported showcasing the safety of this treatment (Sanganalmath & Bolli, 2013).

1.2.1.3 Mesenchymal stem cells

In the 1950's, it was revealed that bone marrow had two residing cell populations, namely hematopoietic and mesenchymal, responsible for the formation of all blood cells, and bone, cartilage, and fat cells respectively. Adipose tissue is another source of mesenchymal stem cells and are easily obtained through minimally invasive procedures such as liposuction (Lindroos, Suuronen, & Miettinen, 2011). Mesenchymal stem cells differentiate into multiple cell types including cardiomyocytes (Planat-Benard *et al.,* 2004; Toma *et al.,* 2002). The effects of mesenchymal stem

cells, isolated from both the bone marrow and adipose tissue, have been evaluated in several clinical trials (Hare *et al.,*2009; Hare *et al.,* 2012; Karantalis *et al.,* 2014) after observing improvement in cardiac function in animal models (Liu *et al.,* 2008; Mazo *et al.,* 2008; Mazo et al., 2010; Schuleri *et al.,* 2009). The PRECISE trial has shown modest effects with adipose derived cells (Perin *et al.,* 2014). However, the emerging consensus is that the therapeutic effect achieved by these cells may be due to paracrine effects and their ability to evade the immune system (Aggarwal & Pittenger, 2005).

1.2.1.4 Cardiac stem cells

The mammalian heart, earlier thought to be a post-mitotic organ composed of terminally differentiated cardiomyocytes, has recently shown a little intrinsic rate of cell turnover (Ali *et al.,* 2014; Kajstura *et al.,* 2010; Senyo *et al.,* 2013). Bergmann *et al.* (2009) elucidated with carbon dating that over an average human lifespan half the cardiomyocyte population is replaced with approximately 1% turnover per year in the early 20's to less than 0.5% turnover per year by the age of 75. Beltrami *et al.* (2001) also suggested that limited cell division occurred in ischaemic hearts. Debate exists over the attribution to this turnover by the proliferation of existing cardiomyocytes or by the differentiation of a progenitor population. A minuscule progenitor cell population within the heart exists (Chong, Forte, & Harvey, 2014) and cells capable of differentiating into cardiomyocytes have been isolated from myocardial biopsies (Smith *et al.,* 2007). Reported populations include c-Kit^{+} (Beltrami *et al.,* 2003), side populations (Martin *et al.,* 2004), cardiosphere derived cells (Messina *et al.,* 2004), and Sca1^{+} (Oh *et al.,* 2003), however, no Sca1^{+} homolog in humans has been described. Cardiospheres form because of free-floating cardiac progenitor cell aggregation and express c-kit in the inner cells although in limited numbers.

Improvement of heart function in animal models in respect to MI has been documented with treatment of cells derived from cardiospheres. However, the CADUCEUS trial (Makkar *et al.,* 2012) evaluating the use of cardiosphere derived cells demonstrated improvement in regional function only (Malliaras *et al.,* 2014). The SCIPIO trial (Bolli *et al.,* 2011) showed promise with an increase in LVEF using c-kit^{+} cells however the authenticity of cardiac progenitor cell identity is under debate (Sultana *et al.,* 2015).

1.2.2 Pluripotent stem cells

Pluripotent stem cells (PSCs) have the greatest differentiation potential, forming mature cell types derived from all three germ layers, and although pluripotency *in vivo* is a transient developmental stage, it can be captured or induced *in vitro* in defined growth conditions. PSCs can be propagated in culture for long periods, however, cannot be used in cell therapy without prior differentiation into specific cell types. Inadequate differentiation risks teratoma formation after treatment and this has been one of the greatest challenges facing the use of PSCs in cardiac repair. Additionally, guided *in vitro* differentiation of PSCs towards a specific cell fate through the manipulation of the growth conditions has highlighted the differences in PSC characteristics and the culture conditions required to maintain pluripotency amongst species and the source of cells. A brief review of these differences is offered in table 1.2.

Table 1.2. Pluripotent stem cell characteristics

	mECCs	mESCs	miPSCs	mEpiScs	hESCs	hiPSCs
Origin	Teratoma	ICM	Somatic	Late Epiblast	ICM	Somatic
Teratoma formation	YES	YES	YES	YES	YES	YES
Chimera/germ line contribution	YES	YES	YES	NO	ND	ND
Culture Conditions	LIF, FBS	LIF, BMP4	LIF	FGF2, A	FGF2, A, MEF CM	FGF2, A, MEF CM
Morphology	Domed	Domed	Domed	Flat	Flat	Flat
Pluripotency state	Naïve	Naïve	Naïve	Primed	ND	ND
Pluripotency Factors	O-S-N-K	O-S-N-K	O-S-N-K	O-S-N	O-S-N	O-S-N
Response to LIF	SR-P	SR-P	SR-P	None	None	None
Response to FGF2	D	D	D	SR-P	SR-P	SR-P
Response to BMP	SR-P	SR-P	SR-P	D	D	D
Response to 2i	SR-P	SR-P	SR-P	D and CD	D and CD	D and CD
SSEA	1	1	1		3 and 4	3 and 4

2i - 2 inhibitor system, A – Activin, BMP - bone morphogenic protein, CD - cell death, D - Differentiation, FBS - foetal bovine serum, FGF - fibroblast growth factor, ICM - inner cell mass, K - Klf, LIF - leukaemia inhibitory factor, mECCs - mouse embryonic carcinoma cells, MEF CM - mouse embryonic fibroblast conditioned medium, m/hESCS - mouse/human embryonic stem cells, mEpiSCs - mouse epiblast stem cells, m/hiPSCs - mouse/human induced pluripotent stem cells, N - Nanog, ND - not determined, O - Oct4, P - Pluripotency, S - Sox2, SR - self renewal. Amended from Bieberich and Wang, 2013.

1.2.2.1 Sources of pluripotent stem cells

The establishment of pluripotent cells in culture was propelled by researchers exploring teratocarcinoma. In the 1960's, Kleinsmith and Pierce, demonstrated cells derived from a teratocarcinoma tumour, and ectopically transplanted, gave rise to specialized cells representative of the three germ layers. The cells were termed as embryonal carcinoma cells (ECCs) and exhibited *in vitro* characteristics that now define stem cells: the potential to self-renew without undergoing senescence and to mature into multiple cell types. Chimera formation resulted from the incorporation of these cells into a blastocyst. Additional findings showing the similarities between the early differentiation of cultured ECCs and the *in vivo* inner cell mass (ICM) led to the isolation of cells from the ICM of a pre-implantation blastocyst without an intermediate teratocarcinoma stage and termed embryonic stem cells (ESCs) (Evans & Kaufman, 1981; Martin, 1981). Maintaining the pluripotency of these cells *in vitro* required the addition of leukaemia inhibitory factor (LIF) to the culture medium. Cells from mouse epiblast post-implantation (mEpiSCs) have also been isolated with naïve and primed pluripotency states displayed by mESCs and mEpiSCs respectively (Nicholas & Smith, 2009).

Human pluripotent stem cells were derived by Thomson and colleagues in 1998. The ethical concern stemming from the origin of these cells was by-passed with the advent of the induced pluripotent stem cell (iPSC) technology. In 2006, Takahashi and Yamanaka, successfully reverted terminally differentiated mouse fibroblasts into a pluripotent state using four pluripotent genes; Oct4, Sox2, c-Myc, and Klf4. A year later the same was accomplished in human fibroblasts (Takahashi *et al.,* 2007). Concern over the use of c-Myc and viral vectors in inducing pluripotency in somatic cells have since pushed researchers to create other combinations of genes, such as Oct3/4,

SOX2, Lin28, and Nanog (Yu *et al.,* 2007), and non- integrating methods using episomal DNAs (Okita *et al.,* 2011; Yu *et al.,* 2009), synthetic mRNAs (Warren *et al.,* 2010), and Sendai virus (Fusaki *et al.,* 2009) amongst others (Jia *et al.,* 2010; Kim *et al.,* 2009; Lin *et al.,* 2008; Miyoshi *et al.,* 2011; Stadtfeld *et al.,*2008; Woltjen *et al.,* 2009) to generate iPSCs.

1.3 Signal transduction

A cells decision to proliferate, migrate, differentiate, or undergo cell death, is dependent on the cues provided by the environment including neighbouring cells, hormones, growth factors, and physiological and chemical stress. Extracellular signals are transmitted into the cell most often via cell surface receptors and intracellular signalling pathways carry the message into the nucleus to alter gene expression through the regulation of transcription factors.

1.3.1 Transcription factors

Gene expression is regulated by the binding of transcription factors to regulatory regions in DNA, in enhancing or silencing regions, to activate or repress activity. This is a highly regulated process and frequently requires the binding of multiple activator or repressor transcription factors in a specific order or number. There are several families of transcription factors including helix-turn-helix, zinc finger, helix-loop-helix, basic protein-leucine zipper, and β-sheet motifs. Dimerization, phosphorylation, and ubiquitination are some of the regulatory mechanisms that allow versatility in transcription factor activity which is furthered by cooperation with other transcription factors and co-activators. Transcription factors can be ubiquitously expressed or confined to specific tissues and cell types. Transcription factors specific to *in vivo* mesoderm and cardiac development have been identified and are frequently used as

markers to assess *in vitro* cardiac differentiation. One of the most extensively studied is the GATA family of transcription factors which are members of the zinc finger family and divided into two subfamilies; GATA1-3 regulate hematopoietic stem cells and GATA4-6 regulate endoderm and mesoderm development (Arceci *et al.,* 1993; Kelley *et al.,* 1993; Laverriere *et al.,* 1994; Molkentin *et al.,* 2000; Morrisey *et al.,* 1996; Morrisey *et al.,* 1997; Suzuki *et al.,* 1996). Of the six GATA members, GATA4 is a critical regulator of cardio-myogenesis and is one of the earliest expressed transcription factors in cardiac progenitor cells. The diversity of cardiac genes regulated by GATA4 includes cardiac troponin I and C (Bhavsar *et al.,* 2000; Di Lisi *et al.,* 1998; Ip *et al.,* 1994; Murphy *et al.,* 1997), myosin heavy chain (Molkentin, Kalvakolanu, & Markhan, 1994), myosin light chain (McGrew *et al.,* 1996), and atrial natriuretic factor (Grepin *et al.,* 1994) amongst others (Cheng *et al.,* 1999; Nicholas & Philipson, 1999; Rivkees *et al.,* 1999). Regulation by GATA4 and differentiation of cardiac progenitor cells occurs through the interaction of several other transcription factor families including myocyte enhancer factor 2 (MEF2), NK2 transcription factor related locus 5 (NKX2.5), T-box (Tbx), and heart and neural crest derivatives (Hand) (Dodou *et al.,* 2004; Garg *et al.,* 2003; Lien *et al.,* 1999; Maitra *et al.,* 2008; Searcy *et al.,* 1998; Zeisberg *et al.,* 2005). Other co-factors include ubiquitous transcription factors such as activator protein 1 (AP-1).

AP-1 consists of dimers formed by members of the Jun and Fos protein families (Angel & Karin, 1991; Vogt & Bos, 1990). The Jun family includes c-Jun, JunB, and JunD and the Fos family includes c-Fos, FosB, and Fos-related antigen 1/2 (Fra1/2). The dimer composition of these families dictates which genes are regulated (Hai & Curran, 1991; Kouzarides & Ziff, 1988; Smeal *et al.,* 1989). The complete upstream regulatory network of these transcription factors is currently unknown however many intracellular

transduction pathways have been evaluated and implicated in both *in vivo* and *in vitro* cardiac differentiation models including the mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), and SMAD (Pandur, 2005; Rose, Force, & Wang, 2010).

1.3.2 Intracellular signalling kinases

1.3.2.1 MAPK

Mitogen-activated protein kinase (MAPK) catalytic activity relies on the phosphorylation of their activation loop and forms a three-tier kinase cascade involving the sequential activation of MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPKs. MAPKKKs are Ser/Thr kinases frequently activated through interaction with small G proteins such as Ras/Rho. MAPKKKs including TAK1, ASK1, TAO, MLK2, Raf, and DLK, act on specific MAPKKs. Activation of a specific MEK further phosphorylates and activates a MAPK subfamily through dual phosphorylation on Thr and Try residues within a conserved Thr-X-Tyr motif. MAPKs are subdivided into three kinase subfamilies: extracellular signal-regulated kinases (ERK), p38, and c-jun N-terminal kinases (JNK).

ERK, divided into ERK1/2 and ERK 3-5, was the first of the mammalian MAPKs to be characterized. ERK can be activated by multiple stimuli including growth factors, lipids, cytokines, and osmotic stress through the Ras/Raf pathway acting on MEK1-2 (Cooper *et al.,* 1982; Kazlauskas & Cooper, 1988; Raman, Chen, & Cobb, 2007; Ray & Sturgill, 1988; Shaul & Seger, 2007). The p38 MAPK has four isoforms; α, β, γ, and δ (Cuadrado & Nebreda, 2010; Han *et al.,* 1994; Lee *et al.,* 1994; Rouse *et al.,* 1994). The α and β isoforms are more ubiquitously expressed with α expression being higher between the two (Jiang et al., 1996). The p38 subfamily is more responsive to stress stimuli and inflammatory cytokines including hypoxia and ischaemia and can be

activated by the recruitment of TRAF by tumour necrosis factor alpha and interleukin 1 or by small G proteins Rac/Cdc42 (Bagrodia *et al.,* 1995; Bradley & Pober, 2001; Goldsmith & Dhanasekaran, 2007). Both pathways act on MAPKKKs that further stimulates MKK3 and 6. The former shows more selectivity in p38 isoform activation whereas MKK6 activates all isoforms (Derijard et al., 1995; Han *et al.,* 1996; Stein *et al.,* 1996). There are three JNK genes with ten splice variations; JNK1 has splice variations of JNK1α1/2 and JNK1β1/2, JNK2 has spice variations of JNK2α1/2 and JNK2β1/2, and JNK3 has splice variations of JNK3α1/2 (Derijard *et al.,* 1994; Gupta *et al.,* 1996; Kyriakis *et al.,* 1994). JNK1 and 2 are ubiquitously expressed whereas JNK3 is more restricted but is expressed in myocytes (Bode & Dong, 2007). As reviewed by Bogoyevitch *et al.* (2010) the JNKs can be activated by a plethora of stimuli including cellular stresses such as oxidative stress, DNA and protein synthesis inhibitors, heat shock, and UV irradiation and by growth factors, serum, and GPCR ligands. MAPKKs, MKK4 and 7, activate the JNKs (Lawler *et al.,* 1998). The MAPKs regulate a vast number of substrates independently while other targets are mutually regulated such as AP-1 or MAPK activated protein kinases (MAPKAPKs); MK2/3 and 5, MNKs, MSKs, and RSKs (Arthur, 2008; Buxade, Parra-Palau & Proud, 2008; Carriere *et al.,* 2008; Gaestel, 2008; Perander, Keyse, & Seternes, 2008; Ronkina, Kotlyarov, & Gaestel, 2008; Roux & Blenis, 2004).

1.3.2.2 PI3K

PI3K is sub-divided into three classes: class I PI3Ks, (further divided into IA and IB), are heterodimers of regulatory (p85, p55, or p101) and catalytic subunits (p110α, β, δ, and γ) as is the class III Vsp34 enzyme. Class II are monomeric proteins without adapter subunits (PI3K-C2α/β/γ). PI3Ks are activated by both receptor tyrosine kinases (IA) and GPCRs (IB) along with Ras and generate phosphatidylinositol (3,4,5)-

triphosphate (PIP_3) through the phosphorylation of phosphoinositide (PI) (Aoyagi & Matsui, 2011; Crackower *et al.,* 2002; Maehama & Dixon, 1998).

1.3.2.3 PKC

Protein kinase C (PKC) consists of three subfamilies and several isozymes: classical PKCs consist of α, β1/2, and γ isozymes. Novel PKCs include μ, δ, ε, η, and θ isozymes and atypical PKCs are formed of ζ, ι, and λ isozymes (Musashi, Ota, & Shiroshita, 2000; Newton, 2003; Nishizuka, 1995). Classic isozymes contain domains for both DAG and anionic lipid binding in a calcium-dependent manner with a strong preference for PIP_2. Novel isozymes are not sensitive to calcium and bind DAG with higher affinity than classic isozymes (Giorgione *et al.,* 2006; Nalefski & Newton, 2001). Atypical isozymes differ in the sense that their function is controlled by protein-protein interactions and not DAG, calcium, or phorbol esters.

1.3.3 Extracellular Targets

Intracellular signalling pathways, highlighted above, are typically initiated by the binding of a ligand to a cell surface receptor. Several classes of receptors are known to exist on a cells surface, with some, such as the ligand-gated ion channel receptors, limited to specific cell types, or present in most cells, such as the G-protein coupled receptors and tyrosine kinase receptors. Cooper (2000) has discussed the major classes of cell surface receptors which are summarised below.

1.3.3.1 Cell surface receptors

G-protein coupled receptors (GPCRs) are formed of a single polypeptide containing seven regions, three intracellular loops, and three extracellular loops. The N-terminus contains glycosylation sites located on the extracellular side, and the C-terminus contains sites for phosphorylation located on the intracellular side. Conformational changes occur after a ligand is bound on the extracellular side, mediating events

through both heterotrimeric guanine nucleotide binding proteins (G proteins) and non-G proteins. The activation of heterotrimeric G proteins causes the dissociation from the receptor and its subunits (α and βγ). The α subunit and the βγ complex then individually interact with specific intracellular targets. Several hundred GPCRs have been identified and different Gα-proteins, such as $G\alpha_{i}$, $G\alpha_{S}$, $G\alpha_{12/13}$, and $G\alpha_{q}$, associate with different receptors. Several enzyme-coupled receptors are also found on the cell surface including receptor protein tyrosine kinases. These receptors are formed of a single transmembrane α helix with both an extracellular and intracellular domain for ligand binding and with protein-tyrosine kinase (PTK) activity respectively. Upon ligand binding, dimerization and autophosphorylation of the receptor occurs. Recruitment of specific enzymes to the membrane by protein domains that bind specifically to phospho-tyrosine containing peptides such as SH2 domains in turn recruit and activate further proteins. A group of receptors also phosphorylate other residues for instance serine/threonine instead of tyrosine in their substrate enzymes. Ligand binding to these receptors leads to the hetero-dimerization of two distinct polypeptide chains that cross-phosphorylate one another. Cytokine receptors function similarly but do not possess an intracellular domain with catalytic activity. Dimerization of these receptors upon ligand binding phosphorylates non-receptor protein-tyrosine kinases which in turn phosphorylate the receptor.

1.3.3.2 Ligands

Growth factors (GFs) are secreted peptides that control cellular responses through specific binding of transmembrane receptors triggering intracellular secondary messengers. Groups of GFs are evolutionary conserved and can be divided into families and super-families based on amino acid sequence similarity and shared structural folds respectively.

Fibroblast growth factors

Members of the fibroblast growth factor (FGF) family regulate various cellular responses including growth and cell survival by binding to four tyrosine kinase FGF receptors (FGFRs) which have been extensively reviewed by Ornitz and Itoh (2015). FGFs are found in nearly all tissues and function in tissue maintenance and repair in the adult and are vital in early embryonic development. Currently, seven subfamilies of secreted FGFs are known. The endocrine FGF15/19 subfamily is comprised of FGF15/19, 21 and 23. The intracellular FGF11 subfamily is comprised of FGF11-14. The remaining five subfamilies are paracrine FGFs: the FGF1 subfamily is comprised of FGF1 and 2, the FGF4 subfamily is comprised of FGF4-6, the FGF7 subfamily is comprised of FGF3, 7, 10, and 22, the FGF8 subfamily is comprised of FGF 8, 17-18, and the FGF9 subfamily is comprised of FGF9, 16 and 20.

Activation of FGFRs by the binding of a specific FGF triggers the phosphorylation of adaptor proteins such as FGFR substrate 2α (FRS2α) initiating the activation of intracellular signalling pathways. The growth factor receptor-bound 2 (GRB2) membrane anchored adaptor protein is bound by active FRS2α and further recruits either SOS or GAB1. Recruitment of SOS to the plasma membrane by GRB2 activates the G protein Ras leading to the activation of the three-tiered MAPK cascade. Recruitment of GAB1 leads to the activation of PI3K and phosphorylation of PI in the plasma membrane. The synthesis of PIP_3 causes the translocation of AKT to the plasma membrane where it is phosphorylated by PKD1. AKT regulates both proteins and transcription factors such as GSK3 and FoxO respectively. The FGFR1 requires the phosphorylation of six tyrosine residues to activate the RAS-MAPK and PI3K-AKT pathways and the phosphorylation of a further two residues to activate the PLCγ and

STAT pathways. Activated PLCγ leads to the production of IP_3 and DAG which increase intracellular calcium and activate PKC respectively.

Transforming Growth Factor-β superfamily

Over forty members of the transforming growth factor-β superfamily (TGF-β) are known and regulate a variety of cellular responses including development, apoptosis, and inflammation (Beyer *et al.,* 2013). Bone morphogenetic proteins (BMPs), Nodal, and Activin are some of the subfamilies of the TGF-β superfamily. Ligands of this family can form both homo- or heterodimers and activate receptors with serine/threonine activity. TGF-β receptors are divided into type I and type II receptors with the type I receptor being phosphorylated by the type II receptor prompting activation of Smad proteins (Smad1-8). Smad 1-5, and 8 translocate into the nucleus whereas Smad 6 and 7 are inhibitory proteins. Non-Smad signalling pathways are also activated by ligand bound TGF-β receptors including RAS-MAPK, PI3K-AKT, and TAK (de Caestecker, 2004).

As reviewed by Bragdon *et al.* (2011) the largest subgroup of the TGF-β superfamily are the BMPs with over twenty members and further subdivided into four groups: BMP2/4, BMP5-8, BMP9-10, and BMP12-14. Eight BMP receptors have been identified: five type I, ALK1 (Acvrl1), ALK2 (ActRI), ALK3 (BRIa), ALK4 (ActRIb) and ALK6 (BRIb), and three type II receptors, BRII, ActRIIa, and ActRIIb. BMPs regulate cell survival, migration, and differentiation and activate several Smad independent pathways such as the MAPK pathway and the transcription factor NF-κB through the activation of the BRIa complexes along with other pathways including PKA/C/D and PI3K through the activation of other complexes.

Wnt – Secreted signalling proteins

Approximately 20 mammalian Wnt proteins are currently known that regulate cell survival, migration, proliferation, and cell fate through the binding and activation of Frizzled receptors. The transduction of Wnt signals is mediated by canonical and non-canonical pathways. Canonical Wnt pathways are activated by Wnt1, 2, 3A, 8A/B/C, and 10A/B whereas non-canonical Wnt pathways are activated by Wnt 4, 5A/B, 6, 7A/B and 11 (Parikh *et al.,* 2015). Canonical Wnt signalling results in the stabilization and accumulation of β-catenin in the nucleus by the inactivation of the multiprotein complex composed of APC, Axin, and GSK3β mediated by dishevelled (Dvl) proteins. Dvl proteins, when activated by the binding of non-canonical Wnt members to Frizzled receptors, activate RhoA and Rac resulting in the activation of downstream ROCK and JNK that modulate the activity of transcription factors such as ATF. Non-canonical Wnt signalling is β-catenin independent and is also mediated via the activation of G proteins resulting in the increase of calcium, in turn, activating calcium-dependent PKC (Komiya & Habas, 2008).

1.3.4 *In vivo* signalling associated with cardiomyocyte production

Proper development of the heart requires the induction of mesoderm formation and specification into cardiac mesoderm with a sophisticated program of migration, proliferation, and further differentiation of four distinct progenitors; the first heart field, the second heart field, the proepicardial organ, and the cardiac neural crest. The spatiotemporal activity of the extracellular signalling families discussed above regulates these processes.

Wnt3a, BMP4, and Nodal gradients established in the epiblast are critical for mesendoderm patterning and lineage specification. Early decisions between mesoderm, neuroectoderm, and extra-embryonic fates are affected by BMP signalling

(Parikh et al., 2015) whereas canonical Wnt signalling is crucial in the formation of the anterior/posterior axis, primitive streak and node, and mesoderm (Huelsken *et al.,* 2000; Liu et al., 1999). Nodal signalling is involved in the formation of the anterior mesoderm and endoderm (Beck *et al.,* 2002; Shen, 2007).

During gastrulation, the T-box Brachyury, initially expressed in the mesoderm, node, notochordal plate, and notochord, undergoes upregulation by canonical Wnt and further synergy with BMP and FGF8 signalling promote a mesodermal fate (Evans *et al.,* 2012; Paige *et al.,* 2015; Parikh *et al.,* 2015; Schultheiss, Burch, & Lassar, 1997). Canonical Wnt inhibitors, DKK1 and Crescent, are secreted by the adjacent endoderm whereas BMP antagonists, Noggin and Chordin, are emitted by the notochord to further mesoderm specification (Kwon *et al.,* 2007; Marvin *et al.,* 2001; Pandur *et al.,* 2002; Schneider & Mercola, 2001; Tzahor & Lasser, 2001). As the cells migrate through the primitive streak, Brachyury is downregulated, and the upregulation of T-box Eomes activates MESP1 (Costello *et al.,* 2011; David *et al.,* 2011; van den Ameele et al. 2012). MESP1 expressing cardiac progenitors arising from the anterior lateral mesoderm migrate to the cranial and cranio-lateral regions of the embryo forming the first heart field (FHF). The fusion of bilateral FHFs forms the cardiac crescent, the first morphological sign of heart development (Harvey, 2002). The earliest known cardiac-specific transcription factors expressed in the cardiac crescent include members of the Gata, Nkx, MEF2, Hand, and Tbx families. MESP1 is partly responsible for the activation of GATA4/6, Tbx20, Hand2, and Nkx2.5 (Bondue *et al.,* 2008), while BMP also induces activation of GATA4, MEF2c, and Nkx2.5 (Klaus *et al.,* 2012; Lien *et al.,* 2002) with cardiac neural crest cells later regulating FGF8 in the endoderm to further induce Nkx2.5 and MEF2 in cells exposed to BMP (Alsan & Schultheiss, 2002). Cells of the first heart field undergo rapid differentiation and proliferation forming the linear

heart tube and a gradient in Tbx5 is established, with higher levels observed posteriorly (Bruneau *et al.,* 1999).

Earlier belief was that all cardiomyocytes originated from the cardiac crescent until the discovery of a second source of cells in 2001, the pharyngeal mesoderm-derived second heart field (SHF) progenitors (Kelly, Brown, & Buckingham, 2001; Mjaatvedt *et al.,* 2001; Waldo *et al.,* 2001). Development of SHF progenitors has been linked to the suppression of canonical Wnt signalling by non-canonical Wnt5A and Wnt11 (Cohen *et al.,* 2012). The proliferation of both cell populations occurs, but the FHF cells differentiate quickly whereas a delay in differentiation occurs in cells of the SHF, which undergo differentiation as they contribute to the elongation of the heart tube at both the venous and atrial poles (Buckingham, Meilhac, & Zaffran, 2005; Paige *et al.,* 2015; Rochais, Mesbah, & Kelly, 2009). BMP signalling and inhibition of FGF may influence the switch from proliferating to differentiating cells (Hami *et al.,* 2011; Hutson *et al.,* 2010). The FHF contributes to the left ventricle and minimally to the atria while the anterior SHF adds to the right ventricle and outflow tract, and posterior SHF contributes to atria and inflow myocardium. Further proliferation and myocardial subpopulation specification with septation and valve development eventually form the four-chambered heart (Paige *et al.,* 2015).

1.3.5 *In vitro* cardiomyocyte production

The first pluripotent stem cell lines were established in culture over half a century ago with efficient differentiation of ECCs being dependent on the formation of three-dimensional aggregates in suspension called embryoid bodies (EBs). Two commonly used protocols for initiating the formation of EBs include allowing a definite number of cells to self-aggregate in non-adherent dishes for a period (e.g. 4 days) before being transferred to cell culture grade dishes to grow in monolayers or cultured using the hanging drop method. The latter allows for better control of EB size as the EBs aggregate in a droplet of medium suspended from the lid of a culture dish for a short period (e.g. 2 days). The EBs are collected and further cultured in suspension for several days before being plated on gelatin-coated culture dishes (Burridge *et al.,* 2007; Doetschman *et al.,* 1985; Ng *et al.,* 2005).

The use of an extensive variety of growth factors, small molecules, and organic chemicals during spontaneous EB formation has allowed several research groups to derive differentiated cardiomyocytes from PSCs with variable efficiency (Kawai *et al.,* 2004; Laflamme *et al.,* 2007; McBurney *et al.,* 1982; Ren *et al.,* 2011; Tran *et al.,* 2009; Ueno *et al.,* 2007). Inhibiting DNA methylation, using 5-azacytidine (Choi *et al.,* 2004; Yang *et al.,* 2009), or using hormones, such as oxytocin (Fathi *et al.,* 2009; Paquin *et al.,* 2002), have also been shown to persuade cells to commit to a cardiac fate. Highlighted by developmental studies, the critical role of the anterior endoderm in the cardiac induction of the mesoderm *in vivo* was applied to *in vitro* differentiation by co-culturing PSCs with visceral endoderm-like cells (END-2) (Mummery *et al.,* 1991).

Further dissection of the signal transduction mechanisms that regulate cardiac development *in vivo* has allowed researchers to mimic and modulate key factors to

further enhance *in vitro* cardiac differentiation (Kattman *et al.,* 2011; Lian *et al.,* 2012). The importance of Wnt signalling with timed activation and inhibition of canonical and non-canonical pathways in initiating cardiomyocyte differentiation (Gessert & Kuhl, 2010; Tzahor, 2007) has been studied in both mouse and human ESCs. Temporal modulation of Wnt in which canonical signalling is initially enhanced and then suppressed results in high yields of cardiomyocytes from several hESCs lines (Lian *et al.,* 2012; Mehta *et al.,* 2014). Canonical Wnt signalling is required by both mECCs (Nakamura et al. 2003) and hESCs (Paige *et al.,* 2010) to differentiate into cardiomyocytes whereas mESCs lack mesodermal precursors in which Wnt/β-catenin activity is suppressed by soluble DKK1 (Lindsley *et al.,* 2006). Temporary addition of both BMP4 and Activin A also induce cardiac mesoderm formation in both mouse and human cell lines with greater cardiomyocyte differentiation achieved using additional factors including FGF2, VEGFA, and DKK1 in combination with the inhibition of Nodal or TGFβ-R2 (Kattman et al., 2011; Laflamme *et al.,* 2007; Yang et al., 2008). The differentiation efficiency of human PSCs using the methods described above are summarised in table 1.3. In addition to deriving cardiomyocytes from stem cells, fibroblasts have also been reprogrammed into cardiomyocytes without an intermediate pluripotent stage. As reviewed by Chen and Qian (2015), several investigators have achieved this with varying efficiency using cardiac-specific transcription factors including a combination of GATA4, MEF2C, and Tbx5.

Table 1.3. Efficiency of established protocols for cardiac differentiation from stem cells

Method	Differentiation Efficiency %
Spontaneous differentiation by EB formation	**<10%**
END-2 method	**20-25%**
Guided differentiation method using Activin A and BMP4	**>30%**
Guided differentiation method using Activin A, BMP4, bFGF, VEGF, and DKK-1	**40-50%**

Amended from Rajala, Pekkanen-Mattila and Aalto-Setala, 2011.

1.3.6 Markers for *in vitro* cardiac differentiation

The progression of cardiac differentiation attained *in vitro* is measured by the expression of proteins, discussed earlier, at different stages and is summarised in Figure 1.0. Mesoderm lineages are confirmed by the early presence of Brachyury or Eomes and markers including MESP1/2 are used to confirm early cardiac differentiation. The differentiation cascade can be further followed by the expression of transcription factors including GATA4, MEF2c, and NKX2.5. Immature cardiomyocytes often display spontaneous beating and confirmed by the expression of cardiac-specific structural proteins including atrial and ventricular myosin. Cardiac troponin T and I, the former a subunit of the troponin complex responsible for the regulation of muscle contraction in response to changes in calcium ion concentrations, are also used as markers to evaluate cardiac differentiation as are connexin proteins. Atrial and ventricular connexin proteins, Cx43 and Cx40 respectively, are commonly used. A pure population of cardiomyocytes or a specific subset of cardiomyocytes has not been achieved, however, purification methods have been developed including the isolation of cells, from a heterogenous pool, that express differences in glucose versus lactate metabolism (Tohyama *et al.,* 2013) and the expression of signal regulatory protein alpha (Dubois *et al.,* 2011).

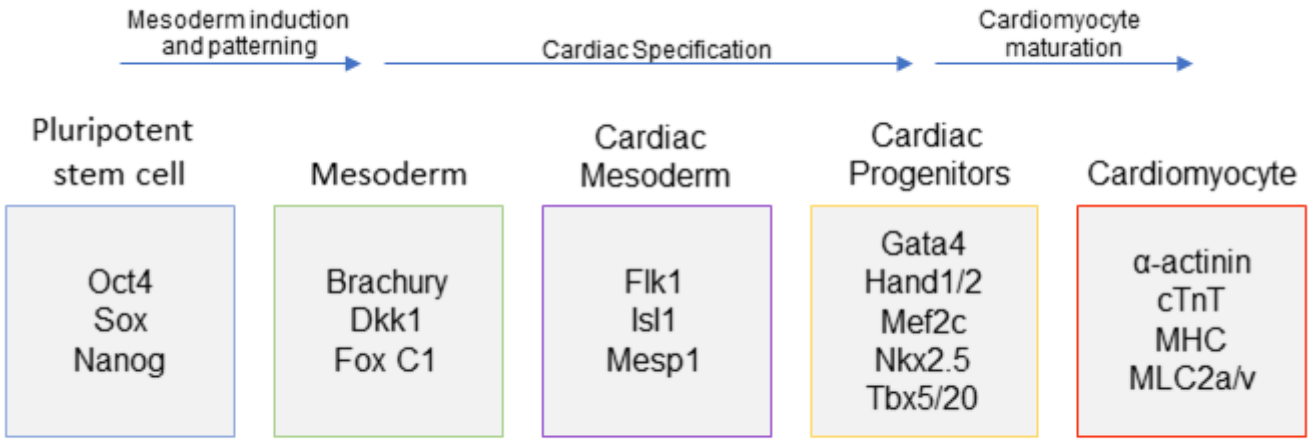

Figure 1.0. Markers for sequential steps required for obtaining cardiomyocytes from pluripotent stem cells
Image amended from Rajala, Pekkanen-Mattila and Aalto-Setala, 2011.
cTnT – Cardiac muscle troponin T, Dkk1 – Dickkopf-1, Flk1 – Fetal liver kinase 1, Fox C1 – Forkhead Box C1, Hand – heart and neural crest derivatives expressed proteins 1/2, Isl1 – Insulin gene enhancer protein 1, Mef2c – Myocyte enhancer factor 2c, Mesp1 – Mesoderm posterior 1, MHC – Myosin heavy chain, MLC2a/v – Myosin light chain 2 atrial/ventricular, Nkx2.5 - NK2 homeobox 5, Oct4 – Octamer binding protein 4, Sox – Sex determining region Y, Tbx5/20 – Tbox 5/20.

1.4 LPA

Wnt, FGF, and TGF-β signalling converge on the regulation of the MAPKs, PKC, and PI3K amongst others to mediate their actions. Lysophospholipids, a group of bioactive lipids, are also reported to regulate the same pathways via GPCRs to mediate cellular events including proliferation, apoptosis, differentiation, migration, and survival (Choi *et al.,* 2010). Sphingosylphosphorylcholine (SPC), sphingosine 1-phosphate (S1P), and lysophosphatidic acid (LPA) comprise this group of lipids and their role during cellular differentiation and development has recently become evident.

1.4.1 LPA characteristics

Lysophosphatidic acid (LPA) is a small ubiquitous phospholipid typically ester-linked to an acyl chain of various length and saturation, and these different chemical forms are present both extracellularly and intracellularly. Derived from a triglyceride backbone, LPA has low solubility in aqueous solution; therefore, water-soluble carrier proteins such as albumin or gelosin are typically bound to LPA (Eichholtz *et al.,* 1993; Goetzl *et al.,* 2000; Osborne & Stainier, 2003; Yatomi *et al.,* 2000). LPA concentrations of serum and plasma vary (Aoki, 2004), with the concentration being much lower in plasma under physiological conditions but increasing significantly following an infarction (Chen *et al.,* 2003).

1.4.2 LPA synthesis

The availability of precursor metabolites and catalytic enzyme expression regulate the production of LPA. Intracellular LPA, a precursor for glycerolipid synthesis, is produced enzymatically from the mitochondria and endoplasmic reticulum by either glycerol 3-phosphate being acylated by glycerophosphate acyltransferase or by degrading LPA into monoacylglycerol by monoacylglycerol phosphate acyltransferase which is re-

phosphorylated by monoacylglycerol kinase (Pages *et al.,* 2001). Other pathways derive LPA from phosphatidic acid produced from phospholipids by the hydrolysis of fatty acid at either the sn-1 or sn-2 position of phosphatidic acid by phospholipase A1 or A2 respectively (Aoki, Inoue, & Okudaira, 2008). Extracellular LPA is produced from lysophospholipids (LP) derived from phospholipids. LPs, lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS), can be enzymatically processed to produce LPA. Autotaxin, with lysophospholipase D (lysoPLD) activity, is responsible for the conversion of LPC, the major source of LPA (Aoki, Inoue, & Okudaira, 2008; Tanaka *et al.,* 2006; Watanabe *et al.,* 2007a). Sequential actions of lecithin-cholesterol acyltransferase (LCAT) and lysoPLD converting phosphatidylcholine to LPC also generate LPA. Enzymes secreted from activated platelets causes upregulation of LPA, during blood coagulation, by acting on plasma phospholipids (Aoki *et al.,* 2002; Baker *et al.,* 2001; Tokumara *et al.,* 2002; Umezu-Goto *et al.,* 2002; Watanabe *et al.,* 2007b).

1.4.3 LPA receptors

LPA influences developmental, physiological, and pathological processes through GPCR and PPARγ signalling but also functions as a direct intracellular messenger. Extracellular LPA confers its bioactive effects through at least eight GPCRs, LPA receptors (LPAR) 1-8, with the first identified in 1996 (Hecht *et al.,* 1996). LPARs 1-3 are members of the endothelial differentiation gene (EDG) family whereas the other receptors are members of the purinoreceptor family.

LPARs classified as members of the EDG family have a more ubiquitous expression whereas receptors identified as members of the P2Y cluster of GPCRs exhibit limited expression (Meyer zu Heringdorf & Jakobs, 2007). LPA receptors of both families

couple to $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ proteins allowing a diverse range of biological functions to be controlled.

1.4.3.1 EDG family

LPAR1 (Hecht *et al.,* 1996) is found in several human and mouse tissues including the heart, brain, GI tract, and reproductive organs (An *et al.,* 1998; Contos *et al.,* 2000; Dancs *et al.,* 2017; Ohuchi *et al.,* 2008; Yang, Ishii, & Chun, 2002). This receptor couples with $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ to alter a range of cellular responses including cell-cell contact through SRE, cell proliferation, survival, migration, calcium mobilization, cytoskeletal changes, and adenylyl cyclase (AC) inhibition through Rho, PLC, Akt, and MAPK signalling (Fukushima *et al.,* 2001; Meyer zu Heringdorf & Jakobs, 2007; Pebay, Bonder, & Pitson, 2007). LPAR2 is found in several mouse tissues, including both embryonic and neonatal brain tissues, and human tissues such as the thymus and spleen (An *et al.,* 1998; Ohuchi *et al.,* 2008). This receptor also couples with $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ to regulate cell proliferation and survival, through Rho, Ras, Rac, PLC, DAG, PI3K, and MAPK signalling (Fukushima *et al.,* 2001; Ishii *et al.,* 2000; Meyer zu Heringdorf & Jakobs, 2007). LPAR3 is found in heart and brain tissue amongst others in both humans and mice (Im *et al.,* 2000; McGiffert *et al.,* 2002; Ohuchi *et al.,* 2008; Ye *et al.,* 2005). LPAR3 couples to $G\alpha_i$, and $G\alpha_q$, with a preference for 2-acyl LPA unsaturated fatty acids, and calcium mobilization and AC modulation are mediated via PLC and MAPK signalling (Fukushima *et al.,* 2001; Meyer zu Heringdorf & Jakobs, 2007).

1.4.3.2 P2Y family

LPAR4 is found in the heart, thymus, and ovaries, amongst other tissues, of both mice and humans (Noguchi, Ishii, & Shimizu, 2003; Ohuchi *et al.,* 2008). This receptor couples to $G\alpha_s$ and $G\alpha_{12/13}$ to modulate cAMP accumulation and calcium mobilization.

LPAR4 also has suppressive effects on both cell motility and migration (Ishii, Noguchi, & Yanagida, 2009; Lee *et al*., 2007; Yanagida & Ishii, 2011). LPAR5 is found in many mouse tissues (Dancs *et al.,* 2017; Kotarsky *et al.,* 2006; Lee *et al.,* 2006; Oh *et al.,* 2008; Ohuchi *et al.,* 2008) but is limited to the heart, spleen, small intestine, liver, platelets, colon, placenta, and mast cells in humans (Amisten *et al.,* 2008; Lundequist & Boyce, 2011) and acts through $G\alpha_{12/13}$ signalling to regulate stress fibre formation (Lee *et al.,* 2006) and possibly $G\alpha_q$ to increase intracellular calcium (Yung, Stoddard, & Chun, 2014). LPAR6 is found in the heart, brain, lung, and kidney tissues amongst others (Lee *et al.,* 2009; Pasternack et al., 2008; Yukiura *et al.,* 2015) and acts through $G\alpha_{12/13}$ signalling (Lee *et al.,* 2009; Yanagida *et al.,* 2009). LPAR7 is found in both skin and hair, and LPAR8 is found in the uterus, placenta, prostate, brain, lung, and skeletal muscle (Murakami *et al.,* 2008; Pasternack *et al.,* 2008; Tabata *et al.,* 2007).

LPA also signals independently of GPCRs through peroxisome proliferator-activated receptor gamma (PPARγ) (McIntyre *et al.,* 2003). This receptor has shown to play a role in several diseases including atherosclerosis, diabetes, and cancer (Lehmann et al, 1995; Li *et al.,* 2000; Sarraf et al., 1998) as it regulates cell proliferation and apoptosis but also mediates inflammation and lipid and glucose homeostasis (Elstner *et al.,* 1998; Evans, 2005; Lehrke & Lazar, 2005; Mueller *et al.,* 1998; Ricote & Glass, 2007; Tigyi, 2010). PPARγ has shown to be activated by unsaturated LPA species only (Tsukahara *et al.,* 2006). LPA synthesis and signalling are summarised in Figure 1.1.

1.4.3.3 LPA receptor antagonists

Most of the commercially available LPAR antagonists target the EDG family and frequently lack selectivity between receptors 1-3 or target enzymes, such as ATX, that regulate LPA production (Yung, Stoddard, & Chun, 2014). Amongst the available

LPAR1-3 antagonists, Ki16425 is the most established and has been used to disrupt LPA signalling in various stem cells. Ki16425 targets LPAR1 and 3 with an IC_{50} of 0.34μM, 0.93μM, but also targets LPAR2 with an IC_{50} of 6.5μM (Ohta *et al.,* 2003). In contrast, the less established, H2L5186303 targets LPAR2 with much higher selectivity than LPAR 3 and 1 with an IC_{50} of 8.9, 1230, and 27534nM, respectively (Fells *et al.,* 2008). Currently, highly selective antagonists for the P2Y family of LPA receptors have not been described. The P2Y family of LPA receptors, notably receptor 4, have traditionally been targeted with broad spectrum antagonists, such as Suramin. Aside from targeting P2Y receptors Suramin also inhibits the binding of several extracellular targets (Coffrey *et al.,* 1987; Kathir *et al.,* 2006; Smolich *et al.,* 1993) and promotes the differentiation of mESCs towards a sinus node phenotype (Wiese *et al.,* 2011).

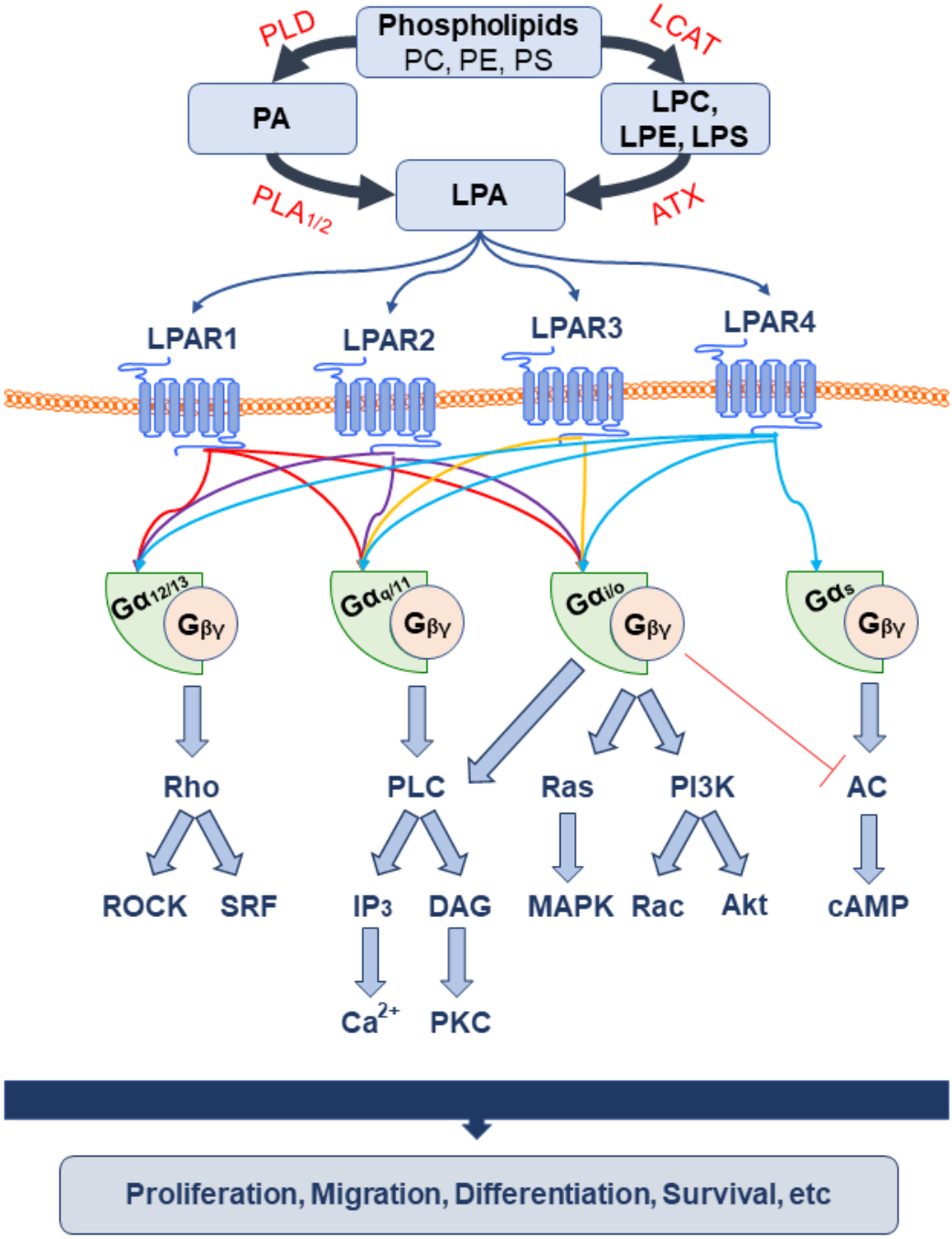

Figure 1.1. Summary of LPA synthesis and signalling
Image amended from Yung, Stoddard, and Chun, 2014.
AC – Adenylate Cyclase, ATX - Autotaxin, Ca^{2+}- Calcium, cAMP – cyclic adenosine monophosphate, DAG – 1, 2, diacyglyerol, IP_3 – Inositol (1,4,5) triphosphate, LCAT – Lecithin cholesterol acyltransferase, LPA – Lysophosphatidic acid, LPAR – LPA receptor, LPC - Lysophosphatidylcholine, LPE - Lysophosphatidylethanolamine, LPS - Lysophosphatidylserine, MAPK – Mitogen Activated Protein Kinase, PA – Phosphatidic acid, PC - Phosphatidylcholine, PE - Phosphatidylethanolamine, PI3K - Phosphatidylinositol-3-kinase, PKC – Protein Kinase C, PLA - Phospholipase, PLC – Phospholipase C, PLD – Phospholipase D, PS - Phosphatidylserine, ROCK - Rho-associated protein kinase, SRF – Serum Response Factor.

1.4.4 LPA in vascular biology

LPA exerts both protective and damaging effects on the cardiovascular system (Chen *et al.,* 2006a). Damage to myocytes during a heart attack occurs primarily due to ischaemia and hypoxia. LPA is elevated in atherosclerotic lesions and after acute myocardial infarction but how LPA confers a protective role in the early stages of each event is elusive. However, through Gi-coupled signalling, activation of downstream pathways, PI3K/AKT and ERK, elevated levels of LPA have been shown to increase myocyte (Karliner *et al.,* 2001), and mesenchymal stem cell (Chen *et al.,* 2008a) survival from hypoxia-induced apoptosis, whereas activation of downstream pathways Rho, PI3K/AKT, and NF-κB contribute to the hypertrophic response (Chen *et al.,* 2008b; Hilal-Dandan *et al.,* 2004). $CD34^+$ cells derived from umbilical cord blood treated with LPA also exhibit higher survival under ischaemic conditions through activation of PPARγ mediated ERK/AKT signalling (Kostic *et al.,* 2015). LPA also regulates myocyte contractibility (Cremers *et al.,* 2003), increased protein synthesis in cultured cardiomyocytes (Goetzl *et al.,* 2000), and increased LPL activity (Pulinikunnil *et al.,* 2005), however, topical application of LPA to the arterial wall prompts neointima formation associated with atherosclerosis (Zhang *et al.,* 2004).

1.4.5 LPA in stem cell differentiation

LPARs are expressed in both pluripotent and multipotent stem cells. Bone marrow and mesenchymal stem cells of human and murine origin express LPAR1-3 whereas distinct expression levels for LPAR1-6 are found in PSCs between species. Both hESCs and hiPSCs express higher levels of LPAR2, 3, and 4 whereas mESCs express higher levels of LPAR1, 2, 4-6 and miPSCs express higher levels of LPAR1 and 6 (Kleger et al 2011, Pebay, Bonder, & Pitson, 2007).

LPA induces the differentiation of multiple cell types into different lineages including mesenchymal stem cell differentiation into myofibroblasts (Jeon *et al.,* 2008, Tang *et al.,* 2014) and osteoblasts (Liu *et al.,* 2010), neuroblast differentiation (Fukushima *et al.,* 2007), neural stem cell differentiation into neuroglial and cholinergic committed neurons (Cui & Qiao, 2006; Cui & Qiao, 2007), and $CD34^+$ myeloid differentiation (Evseenko *et al.,* 2013). While the role of LPA in cardiac differentiation is not yet established, other similar bioactive lipids such as SPC and S1P both promote the differentiation of $Sca1^+$ cardiac stem cells and umbilical cord mesenchymal stem cells respectively into cardiomyocytes (Li *et al.,* 2016; Zhao *et al.,* 2011).

In addition to regulating pathways critical to cardiac differentiation discussed above, LPA modulates the Hippo pathway in both human ESCs and iPSCs. The Hippo pathway acts on the Yes-associated protein (YAP) and is fundamental in development but also a key regulator of stem cell pluripotency and differentiation (Lian *et al.,* 2010; Meng, Moroishi, & Guan, 2016; Qin *et al.,* 2016; Zhou *et al.,* 2015a).

1.5 Aim of the thesis

Our group has demonstrated that LPA may act as an effective molecule for regulating the differentiation of P19 stem cells, a murine embryonal carcinoma cell line, into cardiomyocytes (Pradmod, 2017). This study reaffirmed this ability of LPA and examined the role of LPA receptors and downstream signalling molecules associated with the differentiation of stem cells into cardiomyocytes. Furthermore, the regulation of both cardiac specific and ubiquitous transcription factors has also been investigated.

Chapter 2

Materials & Methods

2.1 Cell culture

Mouse P19 embryonal carcinoma cells (ATCC CRL-1825) were used to carry out all experiments and were cultured in complete alpha-minimum essential medium (complete αMEM) containing 10% heat-inactivated foetal bovine serum with penicillin (100units/mL) and streptomycin (100μg/mL) and maintained in a humidified 5% CO_2 atmosphere at 37˚C. The cells were sub-cultured every 48 hours (cells ~70% confluent) using 0.05% trypsin-EDTA.

All cell culture reagents were purchased from Gibco (Life Technologies) and warmed to 37˚C using a water bath before use. All centrifuge steps were carried out at 4˚C at 1000rpm for 5 minutes unless otherwise specified.

2.1.1 Recovery of P19 cells

P19 cells at passage 2, cryopreserved in 5% DMSO, were received from ATCC. For removal of the cryopreservative, cells were rapidly thawed by repeated pipetting of 1mL complete αMEM into the vial. The suspension was then transferred to a 15mL falcon tube containing 8mL complete αMEM and centrifuged. The medium was removed by aspiration, and the pellet was separated in 1mL of complete αMEM and added to a T25 flask containing 6mL of complete αMEM. The complete αMEM was changed every 48 hours until the cells were ~70% confluent. The pattern of cell growth during this period is shown in Figure 2.0.

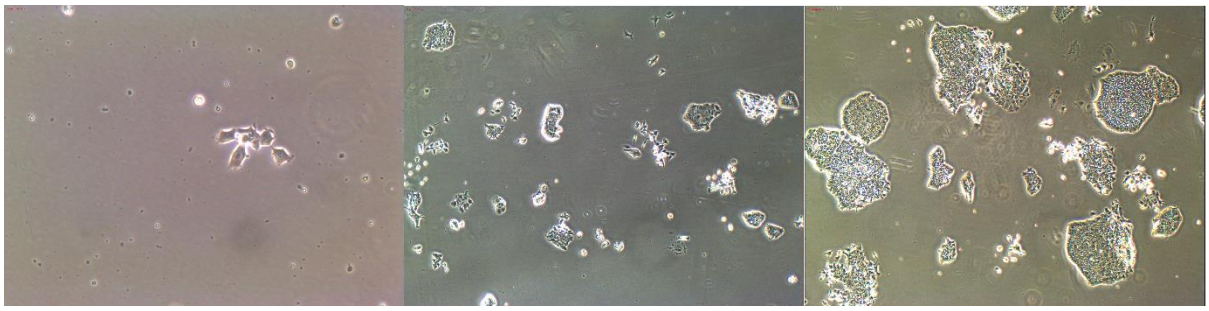

Figure 2.0. Growth of recovered P19 cells
Cells were recovered and cultured as described in section 2.1.1 with the observation of growth after 24 (left), 72 (middle), and 96 (right) hours post recovery. Images are taken at 200x, 40x and 40x, respectively, using the inverted Olympus light microscope.

2.1.2 Culturing of P19 cells

The cells were maintained in exponential growth and sub-cultured by aspirating the medium and washing the cells twice with 1x PBS. 1ml of 0.05% trypsin-EDTA was used to detach the cells with 4mL of complete αMEM being added to stop the action of trypsin. The suspension was transferred to a 15mL falcon tube and centrifuged. The medium was aspirated and the pellet was resuspended in 5mL of complete αMEM. When sub-culturing cells from a T25 to a T75 flask, 1mL of the cell suspension was added to a T75 flask containing 12mL of αMEM. For future studies, the cell pellet was cryopreserved in 1mL of freezing medium containing 80% FBS, 10% glycerol, and 10% complete αMEM. The cryovials were stored in a Mr. Frosty for 24 hours at -80˚C followed by storage in liquid nitrogen (-196˚C). The cells recovered thereafter were not centrifuged and were directly added to a T25 containing 6mL of complete αMEM as DMSO was not used as a cryopreservative.

2.2 Differentiation Protocol

2.2.1 Preparation of lysophosphatidic acid

LPA (Oleoyl-L-α-lysophosphatidic acid sodium salt) was purchased from Sigma (UK) and prepared in a 5mM stock solution dissolved in 1x PBS containing 0.01% fatty acid-free BSA. Aliquots of 10µL - 100µL were protected from light and stored at -20˚C. The final concentration of 5µM LPA was achieved by dilution in complete αMEM.

2.2.2 Formation of embryoid bodies

The differentiation model used required the formation of embryoid bodies (EBs) with an initial seeding density of $3.7x10^5$ cells into bacterial grade 50mm Petri dishes with a total volume of 5mL complete αMEM. The cell count was determined using the Countess electronic cell counter. After separating the pellet in 5mL of complete αMEM,

20µL of the cell suspension was mixed with an equal volume of trypan blue and added to each of the two chambers of the cell countess slide. The volume of cell suspension containing $3.7x10^5$ cells was determined from the average number of viable cells/mL as shown below.

Chamber **A**:	Chamber **B**:
Total cells: $2.0X10^6$/ml.	Total cells: $2.6X10^6$/ml
Live cells: $1.8X10^6$/ml	Live cells: $2.3X\ 10^6$/ml
Viability: 90%	Viability: 88%

Average live cells $= \frac{1.8x10^6+2.3x10^6}{2} = 2.05x10^6\ \frac{cells}{mL}$

Volume of cell suspension required for plating = $\frac{3.7x10^5 Cells}{2.05x10^6 Cells/mL}$ = 0.180mL.

Each Petri dish was plated with 0.180mL of cell suspension with the addition of 4.82mL of complete αMEM.

2.2.3 Cardiac differentiation of P19 cells

Differentiation was induced by supplementing the medium with 1% DMSO (positive control) or with LPA at the time of seeding. When used, EBs were incubated with LPA receptor antagonists or kinase-specific inhibitors for 60 minutes before the addition of LPA. An additional 2mL of complete αMEM was added 48 hours after seeding. The differentiation process was continued by transferring the EBs to a 15mL falcon tube 96 hours after seeding and allowed to aggregate at the bottom of the tube before gently aspirating the medium. 12mL of complete αMEM was added, and the EBs were gently separated into a single EB suspension by repeated pipetting. 2mL of the suspension was added to each well of a 6-well tissue culture grade plate, and the medium was replaced every 48 hours. The differentiation process is summarised in Figure 2.1.

2.2.4 Preparation of adherent cell cultures for ELISA analysis

ELISA kits were used to study the early phosphorylation of specific kinases in the presence of LPA. Cells that were approximately 70% confluent were trypsinized and pelleted as described in section 2.1.2. The number of viable cells per mL was determined using the Countess cell counter as described in section 2.2.2 The cell suspension was diluted to obtain 100µL of cell suspension containing $1.5x10^4$ cells. Each well of a 96-well plate was seeded with 100µL of cell suspension containing $1.5x10^4$ cells and incubated for 48 hours. Cells were then treated with LPA, diluted in serum-free αMEM, for 1, 5, 15, 30, 60 or 180 minutes. The cells were fixed and assayed as specified by the manufacturer's instructions for each ELISA kit. The preparation of adherent cell cultures is summarised in Figure 2.2.

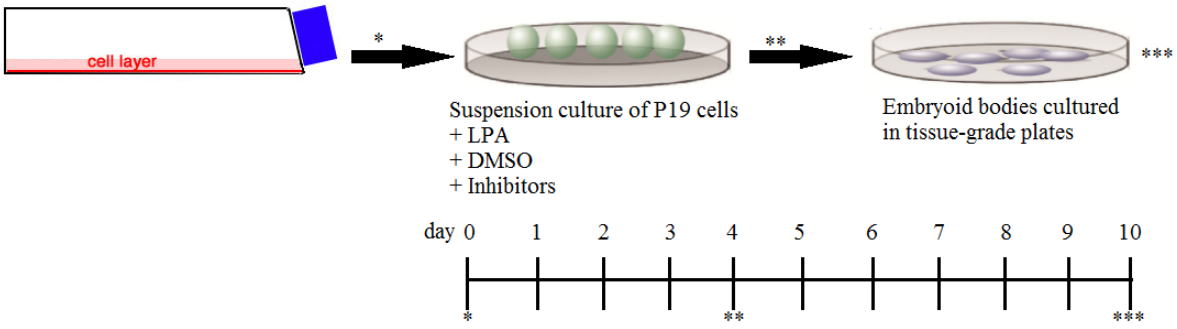

Figure 2.1. The differentiation of P19 cells into cardiomyocytes by the spontaneous formation of embryoid bodies in the presence of LPA

P19 cells were cultured in a T75 flask until approximately 70% confluent and seeded (*) into non-tissue culture grade microbiological Petri dishes in the presence or absence of 5μM LPA over the course of four days (**). The embryoid bodies were cultured in tissue culture grade 6-well plates for further differentiation and lysates were generated on day 10 of the differentiation process (***).

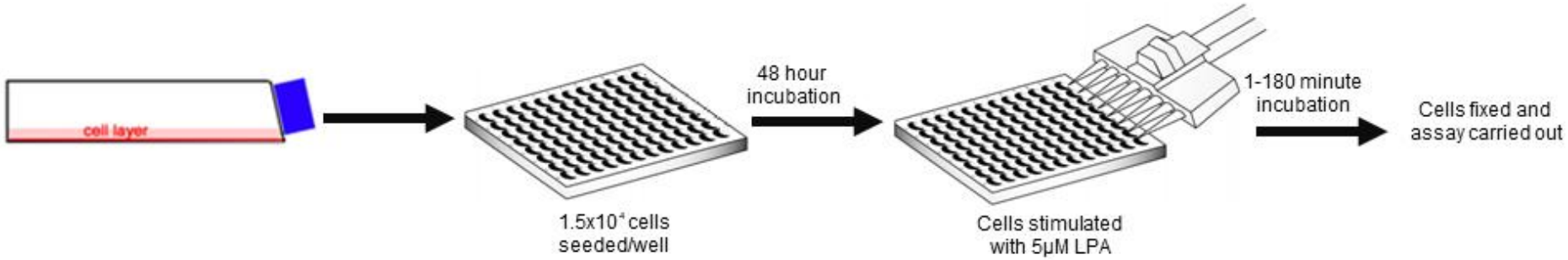

Figure 2.2. Preparation of adherent cell cultures for ELISA analysis
P19 cells were cultured in a T75 flask until approximately 70% confluent and seeded into each well of a 96 well at a density of $1.5x10^4$ cells. After an incubation period of 48 hours, the cells were stimulated with 5μM LPA, diluted in serum free αMEM, for 1-180 minutes. The cells were fixed and the ELISA was performed according to the manufacturer's instructions.

2.3 Protein quantification

2.3.1 Cell lysis for the detection of MLC1v

Lysates were generated on day 10 of the differentiation process. The 6-well plates were placed on ice, and after discarding the medium, each well was washed twice with cold 1X PBS followed by the addition of cold lysis buffer (10mM Tris-HCl, 1% SDS, pH 7.4). The lysed cells were gathered using a cell scraper and transferred to a chilled Eppendorf. The lysates were processed by ultra-sonication for 90 seconds with 30-second intervals and centrifuged for 20 minutes at 13000rpm. The supernatant was transferred to a chilled Eppendorf and stored at -80˚C until used.

2.3.2 Cell lysis for the detection of total and phosphorylated proteins

Lysates were generated at experiment-specific time points. For lysates generated on days 1-4 of the differentiation process, the EBs were transferred into 15mL falcon tubes and allowed to settle at the bottom before removing the medium. Cells were washed with 500µL of cold 1x PBS containing phosphatase and protease inhibitor cocktail (Sigma) at dilutions of 1:100 and 1:200 respectively and transferred to chilled 1.5mL Eppendorf tubes. Tubes were centrifuged for 15 seconds at maximum speed in a microcentrifuge after which the PBS was removed and replaced with 1x RIPA buffer (Cell Signaling Technology). 1mM PMSF was added to the 1x RIPA buffer immediately before use. The tubes were kept on ice for 5 minutes followed by high-speed vortex for 15 seconds and sonicated for 30 seconds in ice water. The lysates were then centrifuged at 13000rpm for 20 minutes at 4˚C. The supernatant was transferred to a chilled Eppendorf tube and stored at -80˚C until analysed. For lysates generated on days 6-10 of the differentiation process, each well of a 6-well plate was first washed with 1x PBS as described above followed by the addition of 1x RIPA buffer directly to the well. The plate was chilled on ice for 5 minutes, and the lysed cells were

scrapped using a cell scraper and transferred into a pre-chilled Eppendorf tube and processed in the same way as described above.

2.3.3 The bicinchoninic acid assay (BCA)

The Peirce BCA Protein Assay Kit was used to determine the total concentration of protein in the lysates. A stock of bovine serum albumin (BSA) was prepared at a concentration of 10mg/mL in double distilled water (DDW). Standards and a 96 well plate were prepared as shown in table 2.0 and 2.1 respectively. The plate was incubated for 60 minutes on an orbital shaker at room temperature. A reading at 620nm was taken using the Labsystems Multiskan ascent or CLARIOstar plate reader. A protein standard curve was generated by plotting absorbance against protein concentration, and the amount of protein in the samples was calculated using the equation y=mx+b.

Table 2.0. Preparation of BSA standards

BSA (10mg/mL)	Double Distilled Water	Final Concentration (µg/µL)
0µL	1000µL	0
20µL	980µL	0.2
40µL	960µL	0.4
60µL	940µL	0.6
100µL	900µL	1
200µL	800µL	2
300µL	700µL	3
400µL	600µL	4

Table 2.1. Preparation of a 96-well plate for protein quantification by BCA

Well	Double Distilled Water	Standard	Lysate	Lysis Buffer (1x)	BCA Solution
Control	5µL	-	-	5µL	100µL
Standard	-	5µL	-	5µL	100µL
Sample	5µL	-	5µL	-	100µL

2.4 Protein analysis by western blot

20µg of protein was mixed with an equal volume of 2x sample buffer and heated at 95˚C for 5 minutes. Proteins were separated by 12% SDS-PAGE with a current of 20mA and 25mA per gel, as the samples migrated through the stacking gel and resolving gel respectively, followed by transfer to a PVDF membrane with the Thermo G2 fast blotter using the pre-programmed method for mixed weight protein. The PVDF membrane was soaked in 100% methanol for 15 seconds and then in double distilled water for 2 minutes before transfer. Membranes were blocked with 5% non-fat milk for 60 minutes at room temperature on a rocking platform. Primary antibody, anti-myosin light chain 3 (Abcam), was used at a dilution of 1:500 in blocking buffer with an overnight incubation at 4˚C with gentle agitation. The membrane was washed with TBS-T 3 times for 5 minutes each followed by a 60-minute incubation with agitation at room temperature with the secondary antibody, anti-mouse IgG, HRP-linked (Cell Signaling Technology), used at a dilution of 1:4500 in blocking buffer. The membrane was washed with TBS-T 3 times for 5 minutes each, and the Thermo ECL Reagent was used for protein detection with the Thermo myECL Imager. The compositions of the buffers can be found in tables 2.2 to 2.4. Expression of the protein of interest was normalised against the expression of the housekeeping protein β-actin (Cell Signaling Technology), used at a concentration of 1:10000 dilution in blocking buffer during secondary antibody incubation, in all experiments. The Restore Western Blot Stripping Buffer (Thermo Fisher) was used to detect other proteins of interest on the same membrane by incubating the blots for 20 minutes at room temperature followed by blocking as described earlier.

Table 2.2. Preparation of 2x sample buffer	
Reagent	2x Sample Buffer (10mL)
Tris-HCl 0.5M pH 6.8	2500µL
Glycerol	2000µL
10% SDS	4000µL
1% Bromophenol Blue	400µL
2M Dithiothreitol	100µL
Double distilled water	1000µL
pH	6.8
Storage	Room Temperature

Table 2.3. Preparation of resolving and stacking gel for SDS-PAGE		
Reagents	12% Resolving Gel (5mL)	Stacking Gel (2mL)
DDW	1655µL	1220µL
30% Acrylamide	1995µL	260µL
10% Ammonium persulfate	50µL	10µL
10% SDS	50µL	20µL
TEMED	3µL	2µL
Tris-HCL **1.5M pH 8.8** **0.5M pH 6.8**	 1250µL -	 - 500µL

Table 2.4. Preparation of buffers			
Reagent	Electrophoresis Buffer	Transfer Buffer	TBS
Tris-Base	250mM	480mM	200mM
Glycine	1.92M	390mM	-
SDS	35mM	0.375%	-
NaCl	-	-	1.37M
pH	-	8.3	7.6
Storage	Room Temperature	4°C	4°C
Concentration	10x	10x	10x

Buffers were diluted to 1x in DDW before use.
For TBS-T; 0.01% Tween-20 was added to TBS before use.
For blocking buffer; 5% non-fat milk was added to TBS-T before use.
For transfer buffer; 20% methanol was added before use.

2.5 Cell viability assay

The MTT assay was used to determine the toxicity of the inhibitors used. $10x10^4$ cells were seeded into each well of a 96-well plate. Once the cells were 60% confluent, the medium was removed and replaced with medium containing the inhibitor for 24 hours after which it was replaced with 200µL of medium containing 0.1mg of MTT for 4 hours. The MTT was discarded, and 100µL of isopropanol was added to each well. The plate was incubated at room temperature on an orbital shaker for 10 minutes. The plate was read at 540nm using the Labsystems Multiskan Ascent plate reader.

2.6 Mycoplasma testing

Mycoplasma testing was routinely carried out using the MycoAlert mycoplasma detection kit purchased from Lonza. P19 cells were cultured in a T75 flask containing 12mL complete αMEM for 48 hours, and the cell supernatant was collected during passage of the cells. The complete αMEM was removed and transferred to a 15mL falcon tube and centrifuged for 5 minutes at 1500rpm. 100µL of the cleared supernatant was transferred to a 96 well clear bottom white polystyrene microplate and incubated at room temperature for 5 minutes with 100µL MycoAlert reagent. The luminescence was measured using the Promega Glomax multi-detection system followed by the addition of 100µL of MycoAlert substrate to the sample and incubated for a further 10 minutes at room temperature. Luminescence was measured again and reading 1 was divided by reading 2. A ratio of <0.9 was negative for mycoplasma whereas a ratio >1.2 was positive. Testing for mycoplasma was repeated 24 hours later if a ratio between 0.9-1.2 was calculated. Testing was carried out more frequently when cells exhibited changes in growth, morphology, or response to the positive

control (1% DMSO) and were not responsive to troubleshooting such as changes in serum, reagents, and cell culture conditions.

2.7 Statistical analysis

Densitometry was done using Image Studio Lite, and statistical analysis was done using GraphPad Prism 7. Statistical comparisons were performed by one-way and two-way analysis of variance (ANOVA) with Dunnett's or Bonferroni post hoc test respectively. A value of $p<0.05$ was significant. The data are expressed as means ± S.E.M of at least three independent experiments unless otherwise noted.

Chapter 3

Establishment of the cardiac differentiation model
&
The role of LPA receptors and the PKC/PI3K pathway in LPA mediated cardiac differentiation of P19 cells

3.1 Introduction

The murine P19 teratocarcinoma cell line (McBurney & Rogers, 1982) can be maintained in culture without the addition of LIF or a feeder layer and has been extensively studied as a model system for cardiomyogenic differentiation (Bradley *et al.,* 1984; van der Hayden & Defize, 2003). During spontaneous EB formation, the organic solvent, dimethyl sulfoxide (DMSO) (Edwards, Harris, & McBurney, 1983; McBurney *et al.,* 1982), has been widely used and led to invaluable insight into the mechanisms and signalling pathways that promote the differentiation to a cardiac fate (van der Hayden & Defize, 2003). Little was, however, known about the role endogenous biomolecules might play in generating cardiomyocytes from stem cells until recently when Pramod (2017) demonstrated that LPA has the potential to regulate the differentiation of P19 cells into cardiomyocytes. This occurred in a manner comparable to that of DMSO and established the upregulation of LPA receptors (LPARs) 1-4 in differentiating P19 cells. LPARs 1-4 signal through coupling with $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12/13}$ proteins that regulate kinases including PI3K and PKC. The requirement of PI3K signalling in LPA mediated cell survival (Weiner & Chun, 1999) and hypertrophy (Chen *et al.,* 2008b) and LPA mediated PKC activation in the presence of DAG, PS, and calcium (Sando & Chertihin, 1996) have been demonstrated and Pramod (2017) has also suggested that they may also regulate LPA induced differentiation of P19 cells to cardiomyocytes. The requirement of canonical Wnt signalling in DMSO treated P19CL6 cells was established by Nakamura *et al.* (2003) and Naito *et al.* (2005) demonstrated the importance of PI3K/AKT pathway signalling in maintaining canonical Wnt activity in these cells and as a required pathway for the further expression of GATA4 and NKX2.5 (Naito *et al.,* 2003). Whether

this pathway also mediates effects on LPA in generating cardiomyocytes from P19 stem cells remain to be established.

Stimulation with insulin or insulin-like growth factors also prompts ESC differentiation towards a cardiac fate mediated in part by PI3K signalling (Engels *et al.,* 2014). Bekhite *et al.* (2011) reported inhibition of the PI3K pathway impaired cardiac differentiation of ESCs and similar results were demonstrated by D'Amico *et al.* (2016) using H9C2 cells. PKC upregulation has been studied for its role in ischemia, hypertrophy, and heart failure (Gray, Karliner, & Mochly-Rosen, 1997; Mizukima *et al.,* 2000; Muth *et al.,* 2001; Rouet-Benzineb *et al.,* 1996), however, the combinatory effect of PKC isoforms makes it difficult to assess the precise role of PKC in *in vitro* cardiac differentiation, but Zhou, Quann, and Gallicano (2003) identified that the upregulation of the novel PKCε in co-operation with the downregulation of classic PKCβ and atypical PKCζ are critical in differentiating cardiomyocytes from ESCs. Further inhibition of both PKCβ and ζ in combination with PKG also enhances cardiac differentiation (Mobley *et al.,* 2010). Other studies have shown while PKCζ is unchanged during cardiac differentiation of ESCs, PKCα, β, δ, and ε increase in nuclear expression (Ventura *et al.,* 2003) however PKCα, ε, and ζ translocate to the cytosolic compartment in DMSO treated P19 cells (Xu *et al.,* 1999). Bekhite *et al.* (2011) found little effect of PKC inhibition on cardiac differentiation of ESCs but studies within the group have found that inhibition of PKC abolishes DMSO induced cardiac differentiation in P19 cells (Humphrey, 2009).

In this study, we aimed to validate the model developed by Pramod (2017) and confirm that LPA does indeed drive P19 stem cells down a cardiac lineage. In addition, the studies on LPARs conducted by Pramod were extended to establish the role of LPAR2 in the differentiation process induced by LPA as this was not previously investigated.

3.2 Materials and Methods

3.2.1 Determining the effect of LPA on P19 cells

Differentiation of P19 cells was carried out as specified in chapter 2 section 2.2. EBs were seeded in medium containing 1, 5, 10, or 20μM LPA and lysates were generated on days 6, 8, 10, and 16 of the differentiation process as described in chapter 2 section 2.3.1. Protein quantification and western blotting were performed as set out in chapter 2 section 2.3.3 and section 2.4 respectively. Cell viability in the presence of 1, 5, 10, 20, and 50μM LPA was determined using the MTT assay as described in chapter 2 section 2.5. Statistical analysis was carried out as described in chapter 2 section 2.7.

3.2.2 Determining the role of LPA receptors in LPA mediated cardiac differentiation of P19 cells

Differentiation of P19 cells was carried out as specified in chapter 2 section 2.2. The LPA receptor 4 antagonist, Suramin, was purchased from Sigma and prepared in a 1.43mg/mL stock solution dissolved in water. The antagonists of LPA receptor 1/3 and 2, Ki16425 and H2L5186303, were purchased from Tocris and prepared in a 10mM and 1mM stock solution, respectively, dissolved in DMSO. Aliquots were protected from light and stored at -20˚C until used. Working concentrations were obtained by dilution in complete αMEM on the day of use. Lysates were generated on day 10 of the differentiation process as described in chapter 2 section 2.3.1. Protein quantification and western blotting were performed as set out in chapter 2 section 2.3.3 and section 2.4 respectively. Cell viability in the presence of varying concentrations of each antagonist was determined using the MTT assay as described in chapter 2 section 2.5. Statistical analysis was done as described in chapter 2 section 2.7.

3.2.3 Determining the role of PKC and PI3K in LPA mediated cardiac differentiation of P19 cells

Differentiation of P19 cells was carried out as specified in chapter 2 section 2.2. In solution inhibitors for both PKC and PI3K were purchased from Merck Chemicals. The PKC inhibitor, Bisindolylmaleimide-1 (BIM-1), was received, dissolved in DMSO, at a concentration of 1mg/mL and the PI3K inhibitor, LY294002, was received, dissolved in DMSO, at a concentration of 10mM. Aliquots were protected from light and stored at -20˚C until use. Working concentrations were obtained by dilution in complete αMEM on the day of use. Lysates were generated on day 10 of the differentiation process as described in chapter 2 section 2.3.1. Protein quantification and western blotting were performed as set out in chapter 2 section 2.3.3 and section 2.4 respectively. Cell viability in the presence of varying concentrations of each inhibitor was determined using the MTT assay as described in chapter 2 section 2.5. Statistical analysis was done as described in chapter 2 section 2.7.

3.3 Results

3.3.1 LPA induced differentiation of P19 cells into cardiomyocytes

The number of cardiomyocytes derived spontaneously by the formation of EBs is very low but have been remarkably enhanced using chemical inducers. In establishing whether LPA is capable of inducing P19 cells into cardiomyocytes, the following studies were carried out.

Firstly, tolerance to LPA (1, 5, 10, 20, and 50μM) by P19 cells was established. The lowest concentration of 1μM showed an increase in MTT metabolism of 17% compared to the control. Concentrations of 5-20μM LPA were also well tolerated by the cells and showed no significant differences in MTT metabolism when compared to the control. Cytotoxicity was observed at the highest concentration tested, 50μM, where viability decreased by 30% compared to the control (Figure 3.0).

To examine the effects of LPA on the differentiation of cardiomyocytes, EBs were seeded in the absence and presence of LPA (1, 5, 10, 20μM). 1% DMSO was also used in parallel studies as a positive control. As evidenced by the expression of the cardiac-specific marker MLC1v, cardiac differentiation was induced by all the tested concentrations of LPA compared to untreated cells, but maximum induction was achieved with 5μM LPA, and the effect was comparable to that seen with 1% DMSO on day 10. Induction by concentrations of 1, 10 and 20μM LPA was approximately 20-30% less than that seen by 5μM LPA (Figure 3.1A).

As shown in Figure 3.1B, expression of MLC1v was sustained until day 16 of the differentiation process in cells treated with LPA compared to untreated cells. No significant difference between the lower concentrations of LPA was observed,

however, induction with the highest concentration of 20μM was approximately 20% less than the other concentrations of LPA tested.

Next, to establish whether the expression of MLC1v induced by LPA was time-dependent, EBs were treated with 5μM LPA, as maximum induction was achieved at this concentration. As shown in Figure 3.2, on days 6 and 8, MLC1v expression was near absent in cells seeded with or without LPA, establishing day 10 as the earliest expression of MLC1v in LPA treated cells.

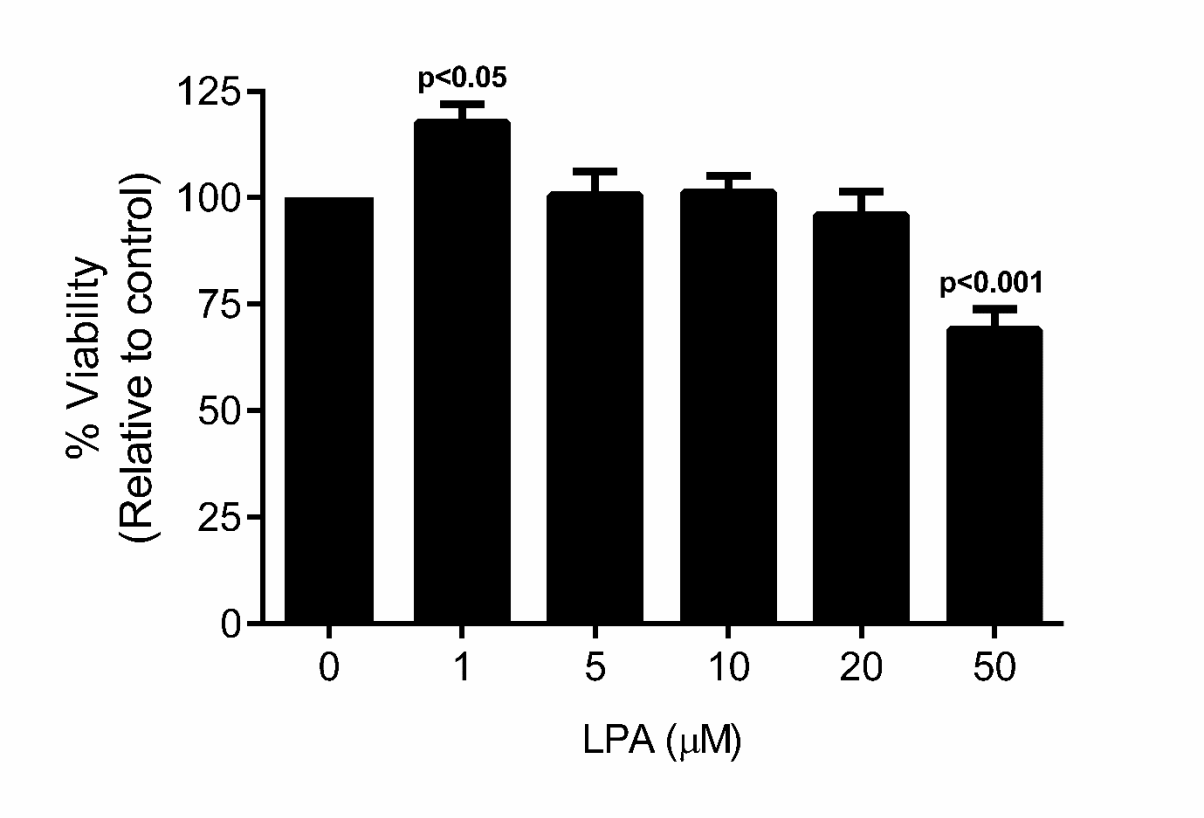

Figure 3.0. The viability of P19 cells in the presence of LPA
P19 cells were cultured in a 96 well plate until 60% confluent and incubated for 24 hours with medium alone or with increasing concentrations of LPA. Cells were incubated with medium containing 0.1mg MTT for a further 4 hours before assessing viability as described in chapter 2 section 2.5. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

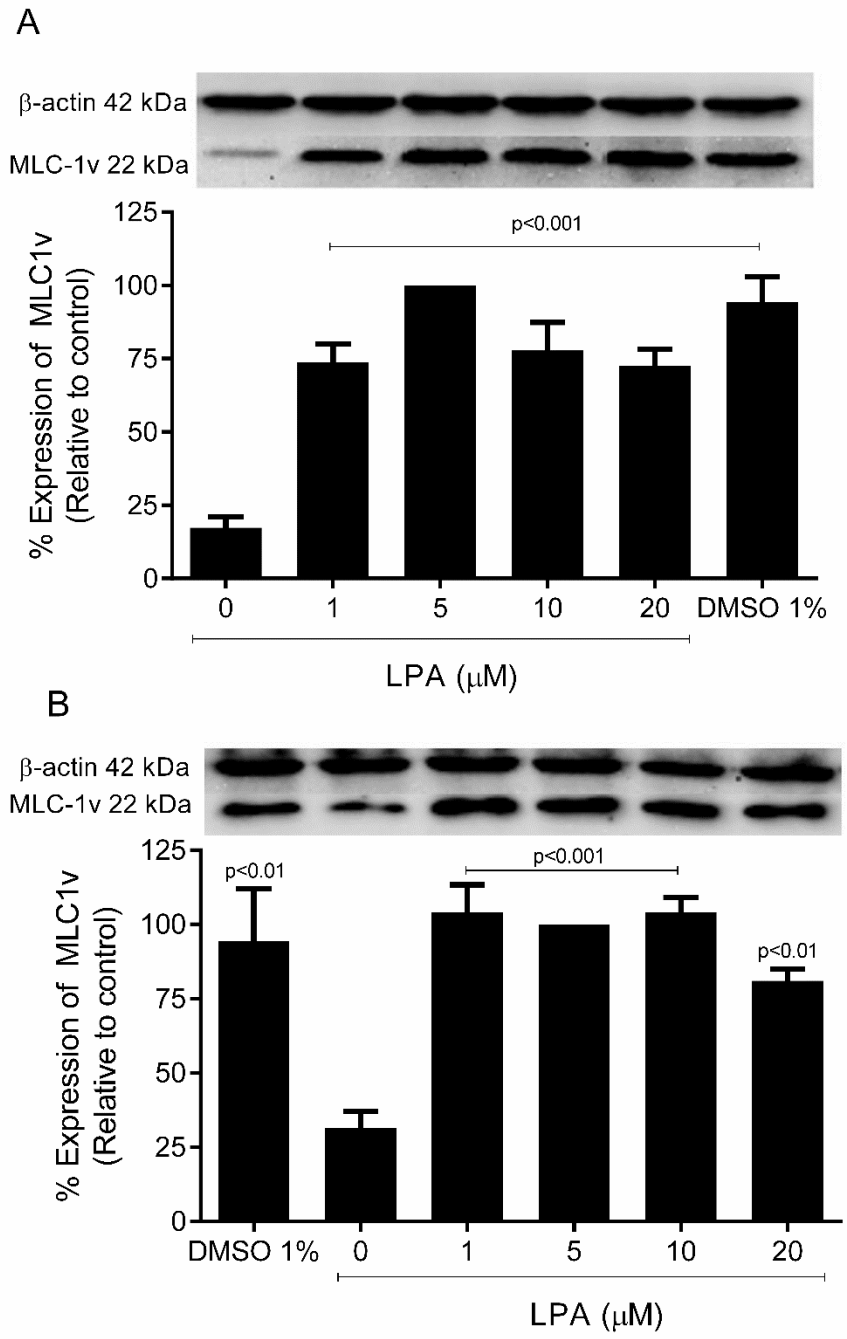

Figure 3.1. LPA induces the expression of MLC1v in P19 cells
P19 cells were seeded in the presence of LPA, 1% DMSO, or without an inducing agent in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 or 12 days. Lysates were generated on days 10(A) and 16(B) of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 6 experiments.

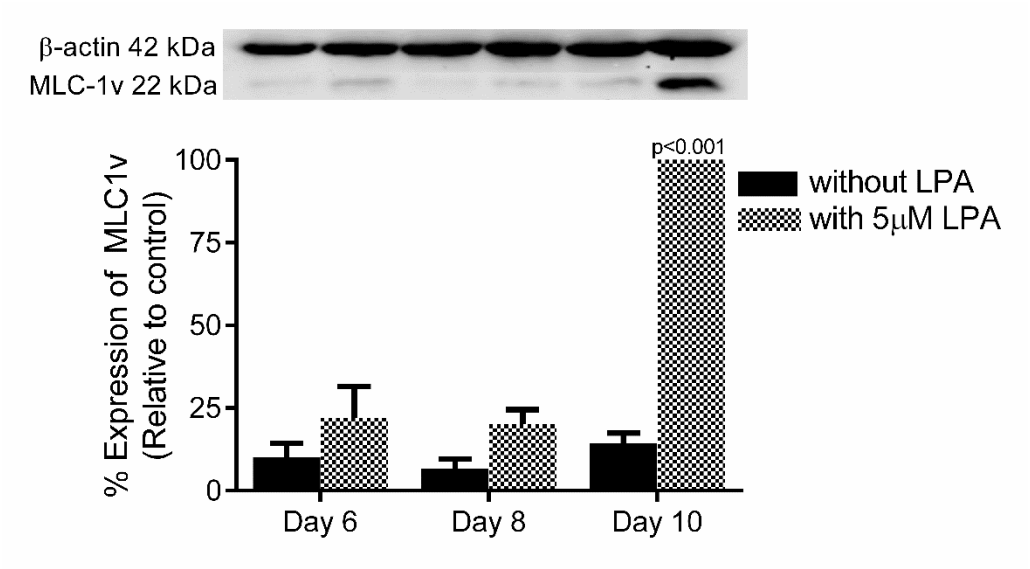

Figure 3.2. LPA induces the expression of MLC1v in P19 cells in a time-dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on days 6, 8, and 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

3.3.2 LPA receptors 1-4 mediate cardiac differentiation in LPA treated P19 cells

The concentrations used for each receptor antagonist were determined to be non-cytotoxic using the MTT assay. Ki16425 (Figure 3.3A) and H2L5186303 (Figure 3.3B) were well tolerated by P19 cells even at high concentrations whereas concentrations of 1 and 2mg/mL of Suramin (Figure 3.3C) indicated cytotoxicity.

To establish the importance of these receptors in LPA mediated cardiac differentiation, EBs were seeded in the presence of LPA receptor 1/3, 2, and 4 antagonists, Ki16425, H2L5186303, and Suramin respectively for 60 minutes before the addition of 5μM LPA. Treatment with each of the antagonists showed inhibition of MLC1v on day 10 in a concentration-dependent manner. Ki16425 reduced MLC1v expression by ~15, 35, and 85% using concentrations of 1, 10 and 20μM (Figure 3.4) whereas H2L5186303 showed a reduction of ~25, 60, and 80% using a concentration of 1, 5, and 7.5nM compared to cells treated with 5μM LPA alone (Figure 3.5). Differentiation was not induced in cells treated with Ki16425 or H2L5186303 alone.

Treatment with Suramin at 0.01, 0.05, and 0.1mg/mL concentrations decreased MLC1v expression by ~25, 40, and 90% respectively compared to cells treated with 5μM LPA alone. However, at a concentration of 0.01mg/mL Suramin alone induced significant differentiation when compared to untreated cells. Compared to treatment with LPA alone, Suramin alone at 0.01mg/mL showed a mean difference of 15% in MLC1v expression (Figure 3.6).

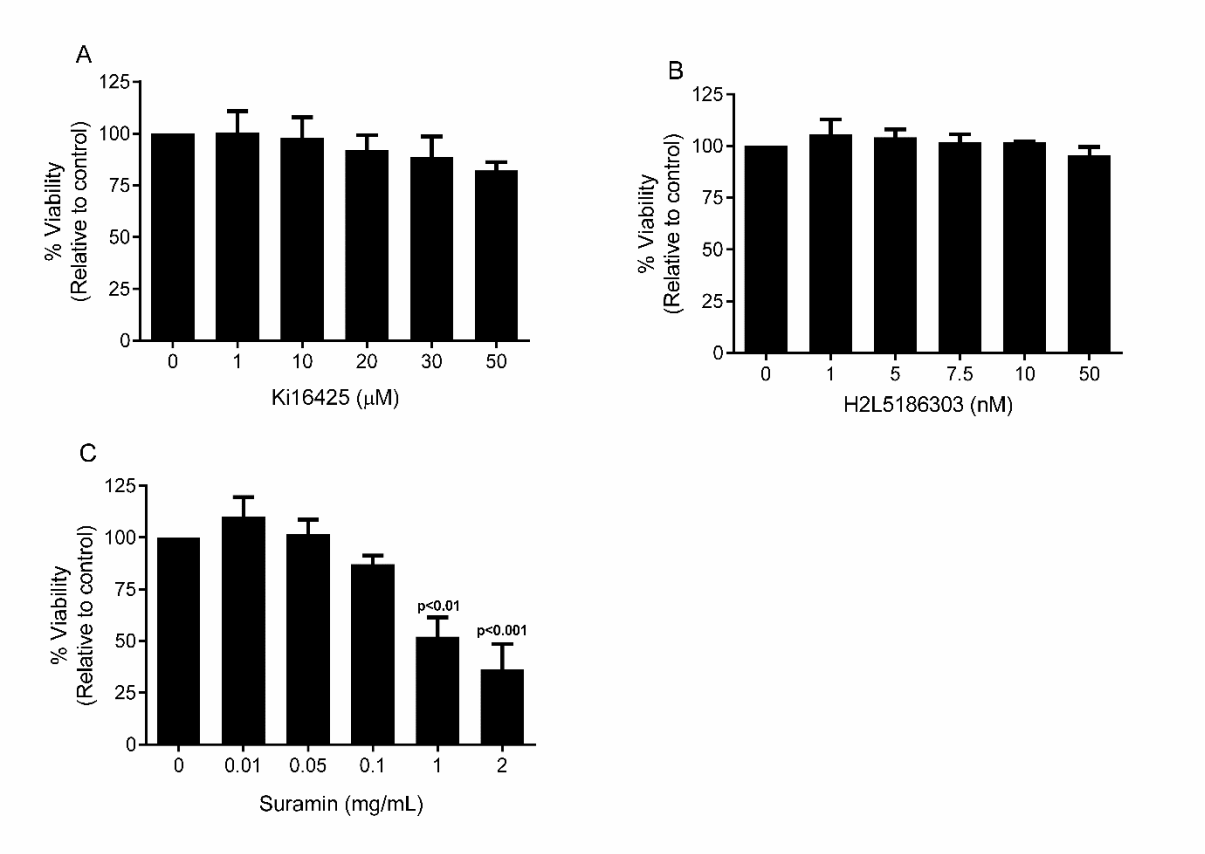

Figure 3.3. The viability of P19 cells in the presence LPA receptor antagonists
P19 cells were cultured in a 96 well plate until 60% confluent and incubated for 24 hours with medium alone or with increasing concentrations of Ki16425 (A), H2L5186303 (B), or Suramin (C). Cells were incubated with medium containing 0.1mg MTT for a further 4 hours before assessing viability as described in chapter 2 section 2.5. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

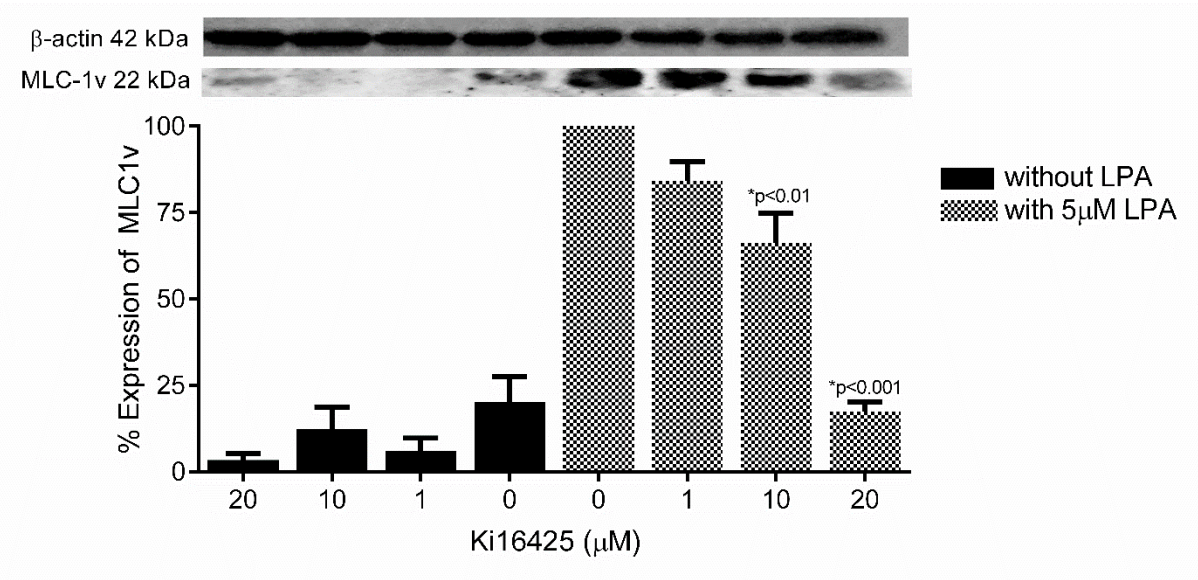

Figure 3.4. Ki16425 blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with Ki16425 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

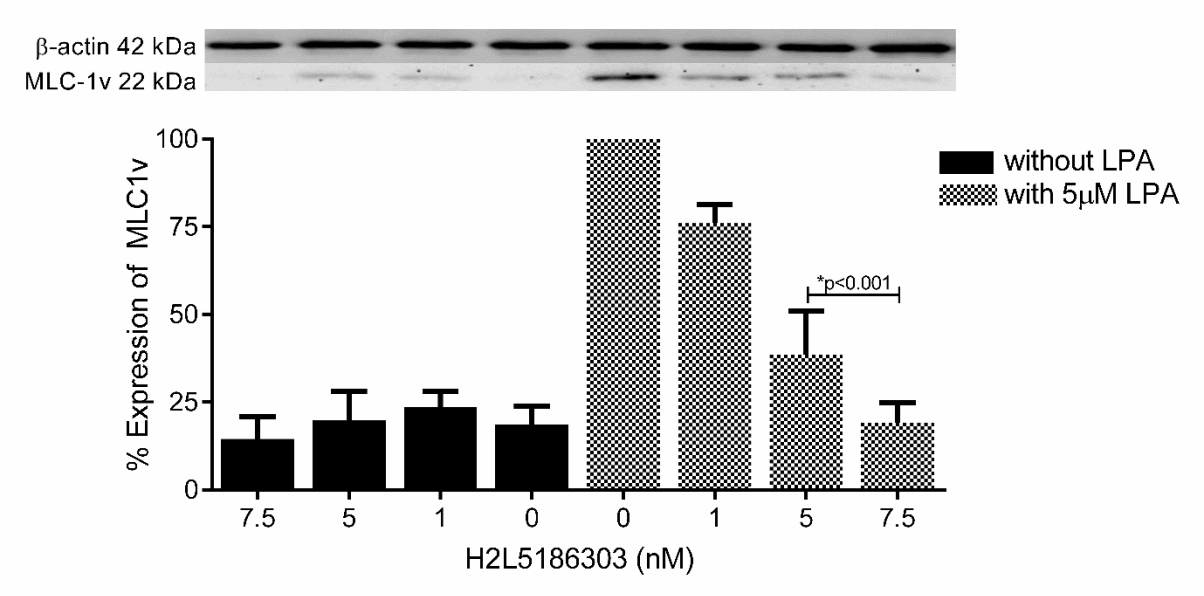

Figure 3.5. H2L5186303 blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with H2L5186303 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

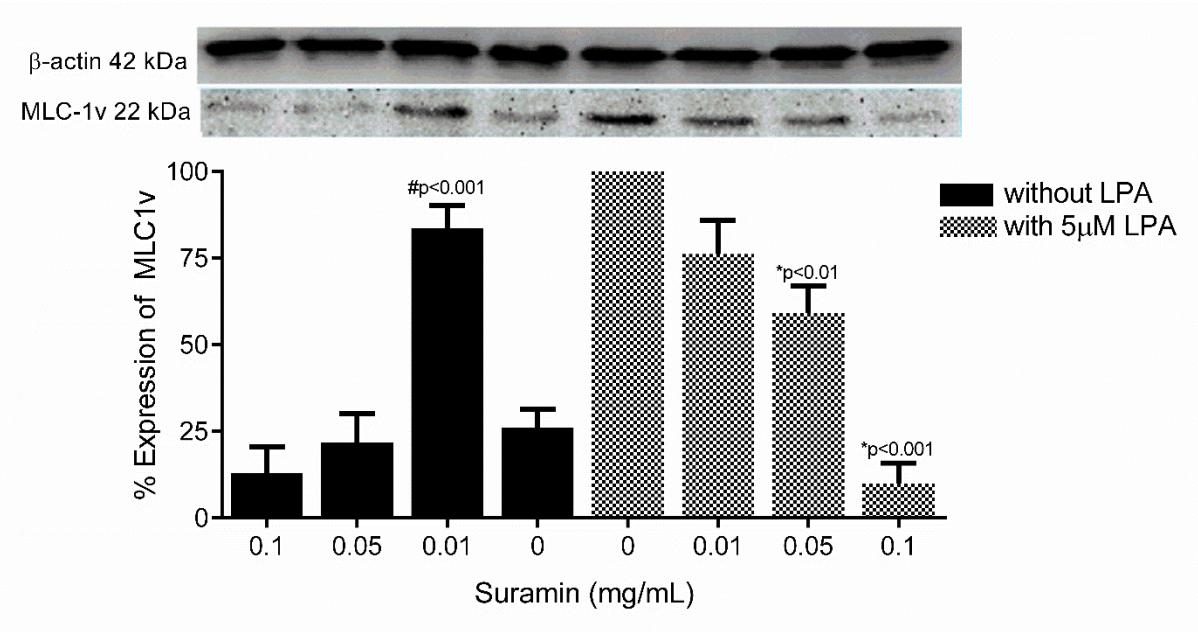

Figure 3.6. Suramin blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with Suramin for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone. #represents significance relative to without LPA treatment.

3.3.3 PKC and PI3K signalling mediate cardiac differentiation in LPA treated P19 cells

To establish whether PKC or PI3K was necessary to mediate the effects of LPA in cardiomyocyte differentiation, P19 cells were incubated during the EB forming stage with either BIM-1 or LY294002, inhibitors of PKC and PI3K respectively, for 60 minutes before the addition of 5μM LPA. The concentrations used for each inhibitor were determined to be non-cytotoxic using the MTT assay. BIM-1 was well tolerated by P19 cells (Figure 3.7A), and LY294002 displayed reduced viability at 30μM and higher (Figure 3.7B).

On day 10, neither of the inhibitors induced differentiation when used alone, and concentration-dependent inhibition of MLC1v was seen in cells treated with 5μM LPA following inhibitor treatment. 5μM BIM-1 treatment reduced MLC1v expression by half and abolished expression when treated with 10μM (Figure 3.8) compared to the treatment of 5μM LPA alone. LY294002 treatment of 1 and 10μM reduced expression by approximately 10%, and 40%, respectively, and abolished expression with 20μM treatment (Figure 3.9) compared to the treatment with 5μM LPA alone.

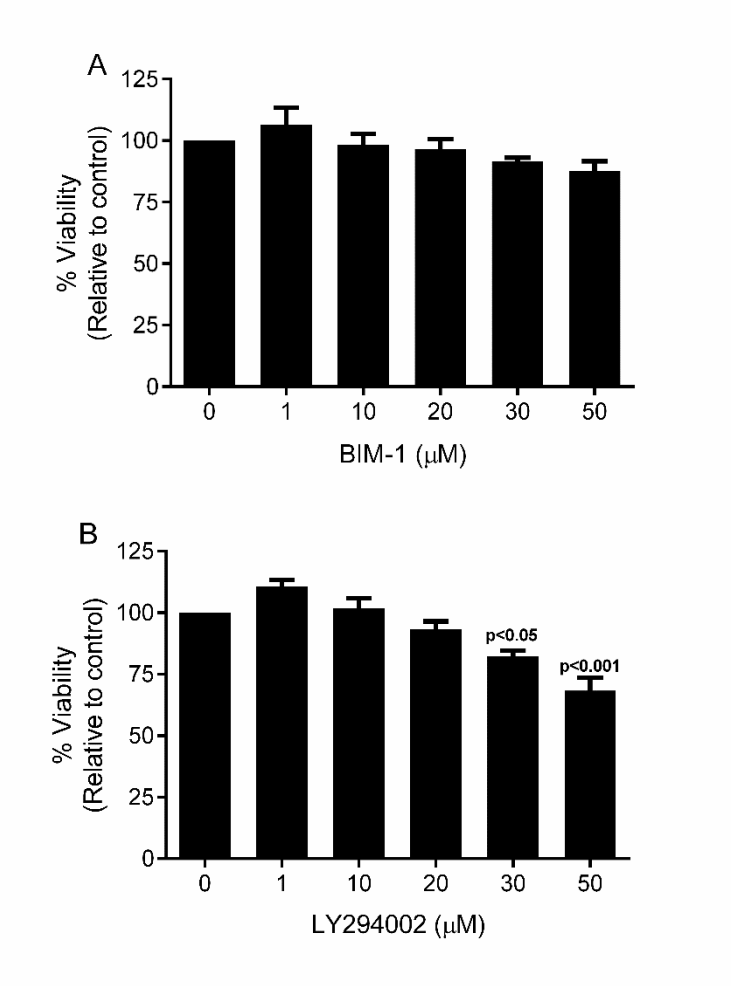

Figure 3.7. The viability of P19 cells in the presence of BIM-1 and LY294002
P19 cells were cultured in a 96 well plate until 60% confluent and incubated for 24 hours with medium alone or with increasing concentrations of BIM-1 (A), or LY294002 (B). Cells were incubated with medium containing 0.1mg MTT for a further 4 hours before assessing viability as described in chapter 2 section 2.5. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

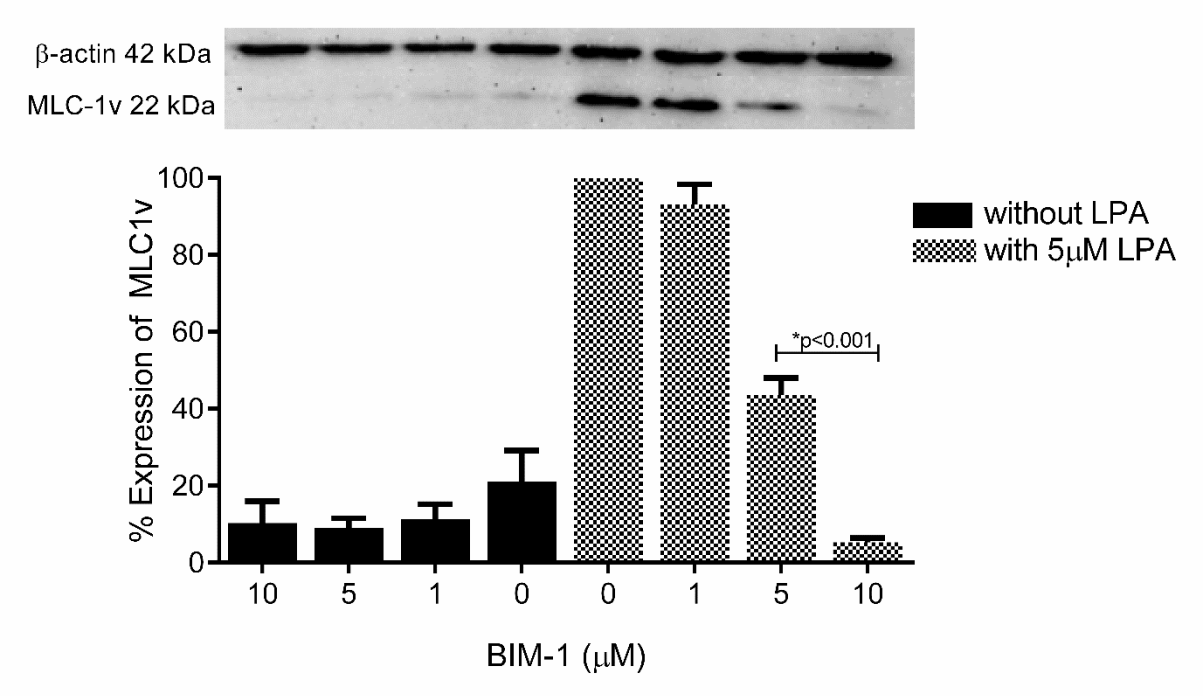

Figure 3.8. BIM-1 blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with BIM-1 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

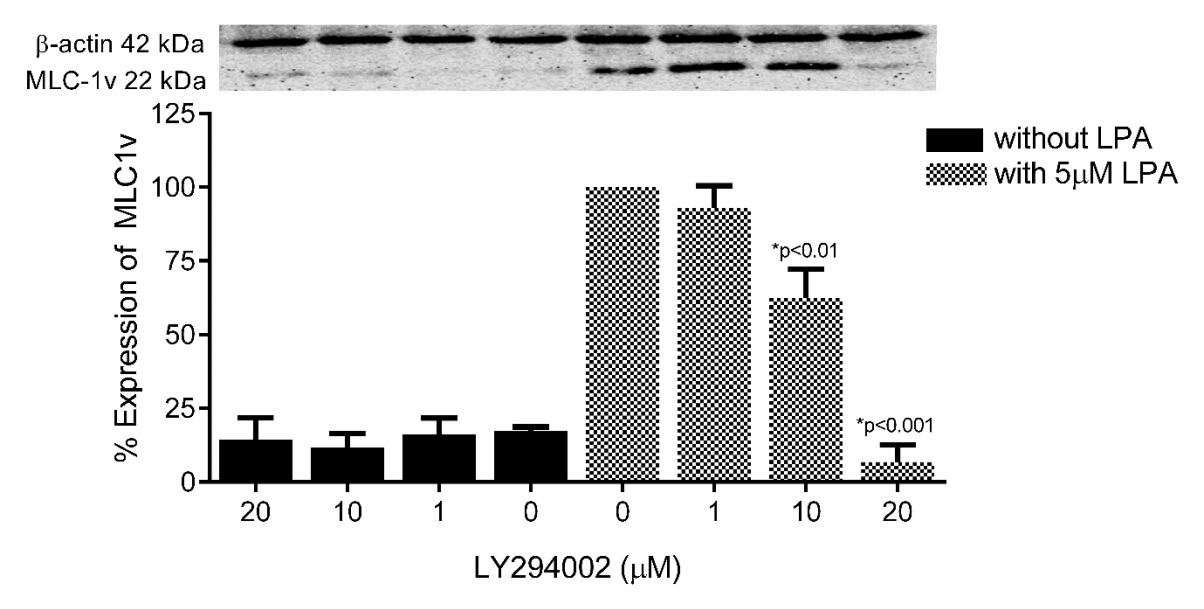

Figure 3.9. LY294002 blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with LY294002 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

3.4 Discussion

Several models are used to decipher the mechanisms mediating differentiation of stem cells into cardiomyocytes. Of these, the murine P19 teratocarcinoma cell line (McBurney & Rogers, 1982) has been extensively used for nearly 36 years and has established a foundation of signalling transduction mechanisms, although not entirely elucidated, that are involved in cardiac differentiation (van der Heyden & Defize, 2003). P19 cells are grown in culture medium containing foetal bovine or calf serum and maintained in an undifferentiated state, without the addition of leukaemia inhibitory factor (LIF) or a feeder layer, for prolonged period whereas culture in suspension initiates differentiation into multiple cell types (Edwards, Harris, & McBurney, 1983; McBurney *et al.,* 1982). Although the differentiation efficacy of these cells to develop into cardiomyocytes during spontaneous EB formation is low, the relatively simple culture conditions permit exaggeration or manipulation of differentiation. Changes in the culture medium by the addition of various molecules allows easy evaluation of the effects on differentiation, not only in comparison to DMSO but also to determine if signalling transduction mechanisms are conserved in regulating cardiac differentiation. In this regard, we evaluated the ability of a biolipid, LPA, in mediating cardiac differentiation of P19 cells.

Cardiac differentiation of P19 cells (Rudnicki *et al.,* 1990; Rudnicki *et al*., 1989) in response to DMSO treatment is verified by the expression of several cardiac-specific proteins, including MHC and MLC, and with the occurrence of beating cells. Our initial studies assessed the effect of non-cytotoxic concentrations of 1, 5, 10, and 20μM LPA on cardiac differentiation compared to 1% DMSO. Findings determined that all concentrations induce differentiation as evidenced by the expression of cardiac-specific ventricular myosin light chain (MLC1v) in a manner comparable to DMSO.

Consistent with the findings of Pramod (2017), maximum induction was achieved with 5μM LPA at day 10 that was sustained until day 16. The expression of MLC1v in LPA treated cells was time-dependent and the earliest expression of MLC1v, determined to be day 10, corresponded to the earliest time point in which beating cells were routinely observed in both LPA and DMSO treated cells.

The ability of LPA to induce cardiac differentiation may reflect the properties possessed by bioactive lipids as other closely related lipids, including S1P and SPC, have been reported to differentiate various cell lines into cardiomyocytes (Li *et al.,* 2016; Zhao *et al.,* 2011). A concentration of 5μM LPA is within range of commonly used concentrations of LPA for *in vitro* studies (Evseenko *et al.,* 2013; Fukushima *et al.,* 2007; Liu *et al.,* 2010; Shumay *et al.,* 2007) but also within the physiological concentrations found under normal conditions (Gaits *et al.,* 1997; Yung, Stoddard, & Chun, 2014). However, it is important to note that experiments were conducted in the presence of foetal bovine serum (FBS) containing culture medium. LPA and other lipids are constituents of FBS and vary in each batch. The concentration of LPA that resulted in enhanced cardiac differentiation of P19 cells was in addition to the unidentified concentration present in FBS. Very little cardiac differentiation was however observed in cells cultured without additional LPA treatment, suggesting that if LPA was present in FBS, it was at concentrations not sufficient to induce differentiation.

Although beating cells were routinely observed in LPA treated cells, this was not uniform in the well or amongst experiments. Obtaining a homogenous population of a specific cardiomyocyte sub-type is a challenge in deriving cardiomyocytes from pluripotent cells. Our studies used only the ventricular MLC marker to verify cardiac differentiation, and the existence of other cell types was not addressed.

LPA acts via G-protein coupled receptors (GPCRs) to control multiple cell processes. LPA receptors are present in pluripotent and multipotent stem cells of both murine and human origin (Pebay, Bonder, & Pitson, 2007). The effect of LPA on differentiation through receptors 1 and 3 has been shown in neural stem cells to oligodendrocytes (Cui & Qiao, 2007). Liu *et al.* (2010) revealed that LPA receptor 1 signalling was required for osteoblastic differentiation of human mesenchymal stem cells and this process was negatively regulated by LPA receptor 4. The role of LPA receptor 1 in stimulating neuronal differentiation of neuroblasts (Fukushima *et al.,* 2007) and myofibroblastic differentiation of mesenchymal stem cells (Tang *et al.,* 2014) is also known whereas opposing roles for LPA receptor 2 and 3 in differentiating K562 cells have been shown (Ho *et al.,* 2015; Lin *et al.,* 2016).

We next evaluated the role of LPA receptors 1-4 in mediating the action of LPA in differentiating P19 cells into cardiomyocytes. Pharmacological inhibitors were used to antagonise the action of LPA at receptors 1-3. Ki16425 selectively inhibits LPA receptor 1 and 3 with a slightly higher affinity for the former (Ohta *et al.,* 2003) and H2L5186303 selectively inhibits LPA receptor 2 (Fells *et al.,* 2008). Suramin is a non-metallic compound that inhibits many cellular enzymes, growth factors, and cell surface receptors. It is a broad-spectrum antagonist of P2Y purinergic receptors and was used to antagonise LPA receptor 4 (Beindl *et al.,* 1996; Charlton *et al.,* 1996).

Using RT-PCR, Pramod (2017) identified the expression of LPAR1-4 in P19 cells and found mRNA levels for LPARs 1-4 were upregulated in LPA treated P19 cells. A 1.25 and 1-fold increase was observed in mRNA levels for LPAR1 and 3/4 respectively whereas LPAR2 had a 3-fold increase compared to untreated cells. Pramod (2017) further established the role of LPARs 1/3 and 4 using receptor antagonists Ki16425 (1-50μM) and Suramin (0.001-0.5mg/mL) respectively which decreased MLC1v in a

concentration dependent manner. These findings were replicated in this study using the same receptor antagonists within the same concentration range.

LPA was unable to induce the same differentiation response in the presence of antagonists for LPA receptors 1-4 and this effect was concentration dependent for each antagonist used. Ki16425 decreased the expression of MLC1-v with 10μM treatment, but this effect was far more prominent with 20μM treatment. Other studies have used 10μM Ki16425 (Fukushima *et al.,* 2007; Li *et al.,* 2017; Liu *et al.,* 2010; Wu *et al.,* 2015) but treatment with 5μM and 20μM are also reported (Evseenko *et al.,* 2013; Tsutsumi *et al.,* 2015). Ki16425 has an IC_{50} of 0.34μM, 0.93μM, and 6.5μM for LPAR 1, 3, and 2 respectively, therefore, it is important to highlight the possible inhibition of all three receptors at the concentrations used in this study. Inhibition of each receptor individually would be required to establish whether the effects observed are due to compounded inhibition of the receptors or if cardiac differentiation can be abolished by inhibition of either receptor.

Pramod (2017) identified that mRNA levels for LPAR2 had the greatest fold increase in LPA treated cells, however, did not establish a role for LPAR2. To address this, we have now demonstrated that treatment with 7.5nM H2L5186303 for 60 minutes before the addition of LPA abolishes differentiation. The concentration range used is consistent with at least one other study by McArthur *et al.* (2015) whereas Wu *et al.* (2015) used higher concentrations of 1μM. H2L5186303 has an IC_{50} of 8.9, 1230, and 27354nM for LPA receptors 2, 3, and 1. As we used concentrations of 1-7.5nM in our studies, we can conclude that LPA receptor 2 is required for LPA mediated cardiac differentiation of P19 cells.

Suramin blocked LPA induced differentiation in a concentration-dependent manner, however, also induced expression of MLC1-v with a treatment of 0.01mg/mL alone. This may be due to the ability of Suramin to inhibit the binding of FGF, TGF-β, and Wnt proteins (Coffrey *et al.,* 1987; Kathir *et al.,* 2006; Smolich *et al.,* 1993). Modulation of these proteins in the culture medium alone has shown to impact cardiac differentiation (Laflamme *et al.,* 2007; Lian *et al.,* 2012; Mehta *et al.,* 2014; Monzen *et al.,* 1999; Yang *et al.,* 2008). Suramin has also been shown to differentiate cells into a sinus node phenotype (Wiese *et al.,* 2011). In this study, mesendoderm and cardiac-specific transcription factors were initially downregulated and later sinus node markers were upregulated. An established and selective LPA receptor 4 antagonist was not available at the time these studies were initiated, and as Suramin is a broad-spectrum antagonist, we can only conclude that LPA receptor 4 may be involved in LPA mediated cardiac differentiation of P19 cells.

We next set out to confirm the role of PKC and PI3K in mediating the actions of LPA in differentiating P19 cells into cardiomyocytes as established by Pramod (2017) wherein BIM-1 (0.1-10μM) and LY294002 (1-20μM) were used. The PKC inhibitor, BIM-1, is selective for PKCα, β, γ, δ, and ε isozymes with an IC_{50} of 10nM but may also inhibit GSK3/GSK3β and PKA at concentrations 30 and 200-fold higher respectively (Toullec *et al.,* 1991). The PI3K inhibitor, LY294002, is selective for PI3K in the order of α > δ > β with an IC_{50} of 0.5μM, 0.57μM, and 0.97μM respectively (Vlahos *et al.,* 1994).

Consistent with the data produced by Pramod (2017), BIM-1 inhibited the response produced by LPA in a concentration-dependent manner. Identifying the exact role of PKC in cardiac differentiation has been difficult as PKC isoforms have opposing and combinatory effects resulting in contradicting studies (Bekhite *et al.,* 2011; Ventura *et*

al., 2003; Xu *et al.,* 1999; Zhou *et al.,* 2003). Our results are consistent with other studies concluding that PKC was essential for cardiomyocyte differentiation from mESCs or circulating progenitor cells (Koyanagi *et al.,* 2005; Ronca *et al.,* 2009). Previous studies within our group also achieved concentration-dependent disruption of DMSO induced cardiac differentiation of P19 cells within the same concentration range of 1-10μM BIM-1 (Humphrey, 2009).

Similar to the data produced by Pramod (2017), LY294002 also inhibited the response produced by LPA in a concentration-dependent manner. The importance of PI3K signalling in cardiomyocyte differentiation has been documented by several groups (Bekhite *et al.,* 2011; Klinz *et al.,* 1999; Naito *et al.,* 2005; Sauer *et al.,* 2000). 20μM LY294002 abolished differentiation in our model which is consistent with the study by Naito *et al.* (2005) in which the use of 20μM LY294002 disrupted DMSO induced cardiac differentiation of P19CL6 cells.

In conclusion, the data generated in this study is consistent with that of Pramod (2017) in which LPA induced cardiac differentiation of P19 cells, most effectively when used at a concentration of 5μM. The effect is mediated through LPAR1/3 and 4, and both PKC and PI3K signalling pathways. We further established the critical role of LPAR2 in this study which was previously unknown. Although treatment with Ki16425 decreased the expression of MLC1v in LPA treated cells, the selectivity of the antagonist merits further assessment to establish the critical receptor in this experimental model. Similarly, the requirement of LPAR4 requires further evaluation as Suramin is a broad-spectrum antagonist.

Chapter 4

The role and regulation of the MAPKs in LPA mediated cardiac differentiation of P19 cells

4.1 Introduction

Response to signals from both intra- and extra-cellular stimuli including physical or chemical stress creates a portfolio of diverse cellular events mediated by the MAPKs. Cross-talk and feedback amongst MAPKs are partially owing to shared upstream activators and downstream targets but have been individually studied for their role in proliferation, survival, apoptosis, and differentiation (Binetruy *et al.,* 2007; Rose, Force, & Wang, 2010; Wada & Penninger, 2004; Wang, 2007). MAPKs are capable of signalling independently and are targets of several identified regulators of cardiac lineage specification in ESCs.

Activation of the ERK pathway by FGF effects many cellular processes including differentiation. ESCs isolated from $fgfr^{+/-}$ mice formed beating myocytes which was blocked with the inhibition of MEK1/2 but not MEK1 alone (Dell'Era *et al.,* 2003). Other growth factors such as VEGF have also been shown to mediate cardiac differentiation of mESCs in an ERK-dependent manner (Chen *et al.,* 2006b). Inhibition of the ERK pathway has also been reported to maintain ESC pluripotency (Force & Woodgett, 2009). Findings in P19CL6 cells have been inconclusive as studies have shown no role for ERK in cardiac differentiation (Davidson & Morange, 2000) or a partial role in the presence of DMSO (Eriksson & Leppa, 2002). However, Pramod (2017) identified ERK as a critical signalling kinase in LPA induced cardiac differentiation of P19 cells.

In differentiating P19 cells, the proliferative capacity of cells has been attributed mainly to JNK in the presence of DMSO (Eriksson & Leppa, 2002). This was challenged by Xu & Davis (2010) by demonstrating the requirement of JNK for ESC differentiation and not proliferation with other studies reporting similar findings (Byun *et al.,* 2013; Yao *et al.,* 2014). For the differentiation of ESCs, JNK1 and 2 is required for

establishing mesodermal and ectodermal lineages (Xu & Davis, 2010). JNK can be activated by numerous signals and factors including Wnt and TGF-β signalling. Wnt11 induction of the cardiac fate has reportedly been prevented with the inhibition of the JNK pathway (Chen *et al.,* 2015). Impaired cardiac differentiation was also observed in JSAP null ESCs with a decrease in NKX2.5 activity (Sato *et al.,* 2005). There are approximately 90 well-validated targets of JNK, and several have been identified as regulators of cardiomyocyte differentiation of ESCs including, but not limited to, β-catenin, ATF, Jun proteins, and YAP (Zeke *et al.,* 2016).

A study by Aouadi *et al.* (2006) suggested p38 activity may be critical in the promotion of a cardiac fate over a neural and is supported by p38 inhibition in DMSO treated P19 cells abolishing cardiomyocyte differentiation (Eriksson & Leppa, 2002). p38 may be acting through BMP activated TAK1 signalling which has been shown to regulate multiple transcription factors, including cardiac specific transcription factors, GATA4 and MEF2c, and ATF-2 (Hanafuse *et al.,* 1999; Monzen *et al.,* 2001; Sano *et al.,* 1999). In contrast, Pramod (2017) found inhibiting p38 did not impact LPA induced cardiac differentiation of P19 cells.

Pramod (2017) recently identified the role of ERK and p38 in LPA induced cardiac differentiation of P19 cells. We extended these studies to further establish the role of JNK which was previously not investigated. Furthermore, we aimed to determine the regulation of the three MAPKs by LPA receptors 1-4 and PKC/PI3K.

4.2 Materials and Methods

4.2.1 Determining the role of MAPKs in LPA mediated cardiac differentiation of P19 cells

Differentiation of P19 cells was carried out as specified in chapter 2 section 2.2. Inhibitors for ERK, p38, and JNK were purchased from Merck Chemicals. The ERK inhibitor, PD98059, was received in DMSO at a concentration of 5mg/mL. The p38 inhibitor, SB203580, was received in DMSO at a concentration of 1mg/mL and the JNK inhibitor, SP600125, was received in DMSO at a concentration of 50mM. Aliquots were protected from light and stored at -20˚C until use. Working concentrations were obtained by dilution in complete αMEM on the day of use. Lysates were generated on day 10 of the differentiation process as described in chapter 2 section 2.3.1. Protein quantification and western blotting were performed as set out in chapter 2 section 2.3.3 and section 2.4 respectively. Cell viability in the presence of varying concentrations of each inhibitor was determined using the MTT assay as described in chapter 2 section 2.5. Statistical analysis was done as described in chapter 2 section 2.7.

4.2.2 Determining the effect of 20μM SP600125 on the aggregation and proliferation of EBs

Differentiation was carried out as described in chapter 2 section 2.2 with the following modifications: EBs were seeded in the presence or absence of 5μM LPA with 20μM SP600125 treatment at the same time, 24 hours, or 48 hours after initial seeding. The lysates were generated at day 10 as described in chapter 2 section 2.3.1. Protein quantification was carried out as specified in chapter 2 section 2.3.3. Statistical analysis was done as described in chapter 2 section 2.7.

4.2.3 Determining the phosphorylation of JNK during EB formation

EBs were formed as described in chapter 2 section 2.2 and seeded in the absence or presence of 5μM LPA for 18, 24, 48 and 72 hours. Lysates were generated at 18, 24, 48, and 72 hours as described in chapter 2 section 2.3.2 and protein quantification was carried out as described in chapter 2 section 2.3.3. Western blot was carried out as specified in chapter 2 section 2.4 with the following modifications: Blocking buffer was made with 5% BSA in TBS-T, phospho-SAPK/JNK (Thr183/Tyr185) mouse primary antibody (Cell Signaling Technology) was used at a dilution of 1:3000, and SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

4.2.4 Determining the effect of delayed inhibition of the JNK pathway in differentiating P19 cells

Differentiation was carried out as described in chapter 2 section 2.2 with the following modifications: EBs were seeded in the presence of 5μM LPA with 10μM SP600125 treatment at the same time, 1, 3, 24, or 48 hours after initial seeding, or with 20μM SP600125 treatment 48 hours after initial seeding. Lysates were generated on day 10 as described in chapter 2 section 2.3.1. Protein quantification was done as set out in chapter 2 section 2.3.3 followed by western blotting as described in chapter 2 section 2.4. Statistical analysis was done as described in chapter 2 section 2.7.

4.2.5 Determining the regulation of JNK by LPA receptors

The regulation of JNK by LPA receptors 1-4 during EB formation was determined by forming EBs as described in chapter 2 section 2.2. EBs were seeded in the absence or presence of LPA after a 60-minute pre-treatment with LPA receptor antagonists, Suramin (LPAR4), Ki16425 (LPAR1 and 3), and H2L5186303 (LPAR2) or the JNK inhibitor, SP600125. Lysates were generated 48 hours after initial seeding as

described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as described in chapter 2 section 2.3.3 and section 2.4 with the following modifications: blocking buffer was made with 5% BSA in TBS-T, phospho-SAPK/JNK (Thr183/Tyr185) mouse primary antibody (Cell Signaling Technology) was used at a dilution of 1:3000, and SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

4.2.6 Determining the phosphorylation of MAPKs by LPA

A cell-based ELISA kit (Sigma: product RAB0352) was used for detecting phosphorylated and total ERK, p38, and JNK in LPA treated cells. Buffers were supplied with the kit and prepared according to the manufacturer instructions. P19 cells were prepared for the ELISA analysis as described in chapter 2 section 2.2.4. After treatment with LPA the medium was removed, and wells were washed three times with 1x wash buffer, and fixing solution was added. The plate was incubated for 20 minutes at room temperature with gentle shaking and washed three times. Quenching buffer was added and the plate was incubated for 20 minutes without shaking followed by four washes. Wells were then incubated with 50µL of the appropriate primary antibody and incubated for 2 hours at room temperature with gentle shaking. The plate was washed four times followed by incubation with 50µL/well of secondary antibody for 1 hour at room temperature. The plate was washed four times before the addition of TMB substrate reagent. The plate was incubated for 30 minutes in the dark with gentle shaking after which stop solution was added, and the plate was read at 450nm using the CLARIOstar plate reader. As multiple wash steps were carried out, resulting in the possible loss of cells, crystal violet solution was used to estimate the relative number of cells in each well. After reading the plate, cells were

washed twice with the buffer supplied and twice with 1x PBS. The plate was air dried for 10 minutes and 100µL of crystal violet solution was added to each well and left to incubate at room temperature for 30 minutes. The plate was washed using 1x PBS for 5 minutes, repeated three times, before incubation with 1% SDS solution for 1 hour with gentle shaking. The absorbance was read at 595nm using the CLARIOstar plate reader. The MAPK phospho- and total signals were then normalized for cell number and data expressed as a percentage of non-treated cells (basal expression). Statistical analysis was done as described in chapter 2 section 2.7.

4.2.7 Determining the cross-talk between MAPKs, and the regulation of MAPKs by LPA receptors 1-4, PKC, and PI3K

A cell-based ELISA kit (Sigma: product RAB0352) was used to determine the effects of the inhibition of LPA receptors 1-4, PI3K, and PKC on the phosphorylation of ERK, p38, and JNK in the presence of LPA. Compensation by other MAPKs when a single subfamily was inhibited was also determined. Antagonists for LPA receptor 1/3 (20µM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) were prepared in serum-free αMEM as were inhibitors for PI3K (20µM LY294002), PKC (10µM BIM-1), ERK (20µM PD98059), p38 (10µM SB203580) and JNK (10µM SP600125). Cells were prepared as described in chapter 2 section 2.2.4 but were treated with the antagonists for 60 minutes before stimulation with 5µM LPA, prepared in serum-free αMEM, for 15 minutes. The steps carried out next are the same as specified above in section 4.2.6. The MAPK phospho- signals were then normalized for cell number and data expressed as a percentage of LPA treated cells. As limited reagents were available, the treatments were repeated twice and presented as mean data.

4.3 Results

4.3.1 ERK and JNK signalling mediate cardiac differentiation in LPA treated P19 cells

To establish whether any of the MAPK subgroups were required in mediating LPA induced cardiomyocyte differentiation of P19 cells, the series of studies described were carried out.

The concentrations used to inhibit each of the MAPKs were determined to be non-cytotoxic using the MTT assay (Figure 4.0). Inhibition of MLC1v was achieved using 20μM of the ERK inhibitor, PD98059 (Figure 4.1), whereas concentrations of 1-10μM of the p38 inhibitor, SB203580, did not affect the expression of MLC1v in LPA treated cells (Figure 4.2). Neither inhibitor induced the expression of MLC1v in cells when used alone. The JNK inhibitor, SP600125, decreased MLC1v by a third when used at a concentration of 1μM followed by 5μM LPA treatment and 10μM SP600125 abolished the expression of MLC1v in the same conditions (Figure 4.3). Treatment with 20μM of SP600125 in the absence or presence of LPA resulted in significantly less protein being extracted from lysates generated at day 10 even though cytotoxicity was not observed when determined using the MTT assay (Figure 4.0C). The EBs treated with 20μM SP600125 displayed disrupted aggregation, and the aggregates did not demonstrate growth. Upon transfer to 6-well plates, the surviving EB's also failed to adhere.

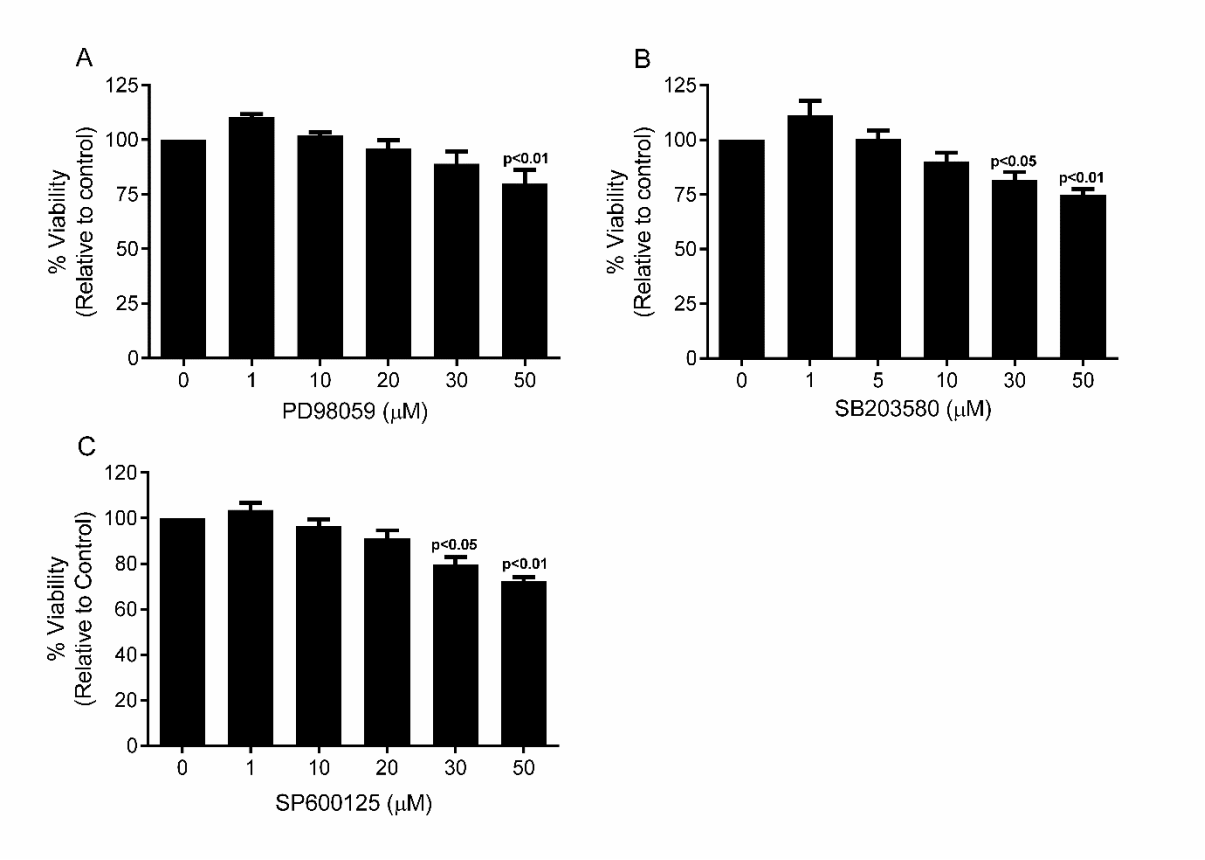

Figure 4.0. The viability of P19 cells in the presence of MAPK inhibitors
P19 cells were cultured in a 96 well plate until 60% confluent and incubated for 24 hours with medium alone or with increasing concentrations of PD98059 (A), SB203580 (B), or SP600125 (C). Cells were incubated with medium containing 0.1mg MTT for a further 4 hours before assessing viability as described in chapter 2 section 2.5. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

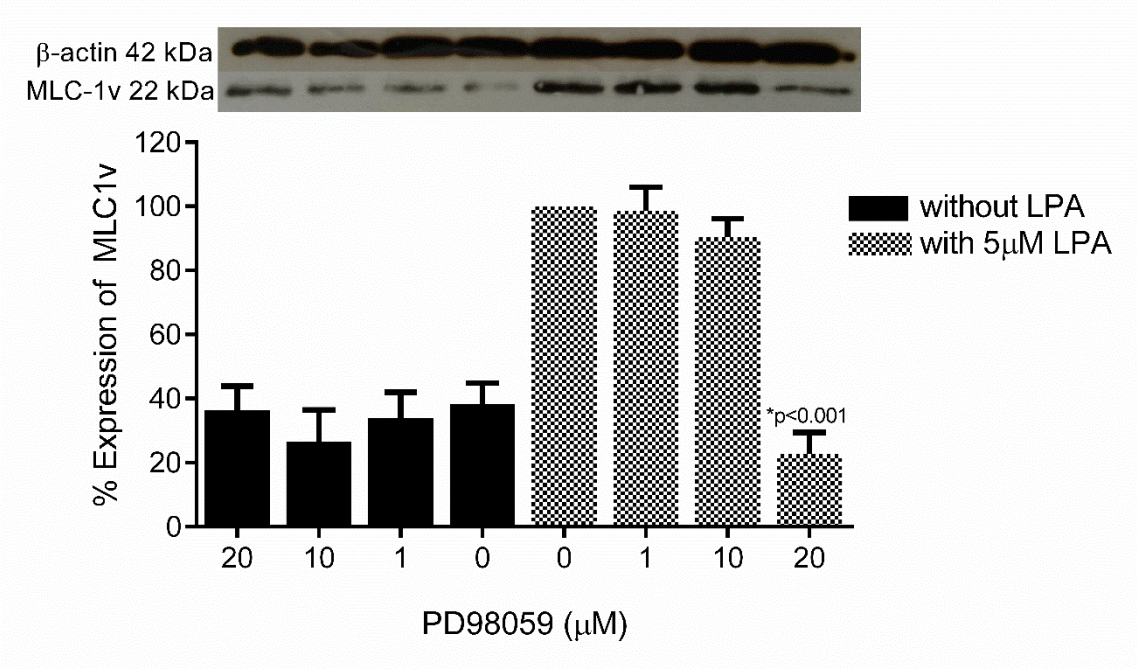

Figure 4.1. 20μM PD98059 blocks LPA induced MLC1v expression

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with PD98059 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

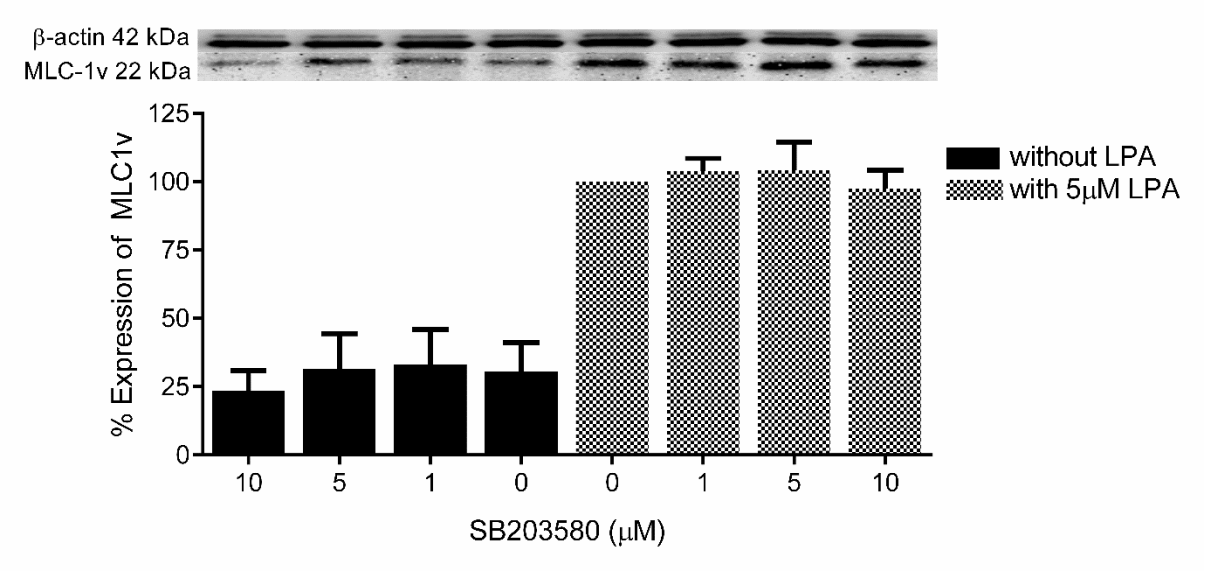

Figure 4.2. SB203580 does not block LPA induced MLC1v expression
P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with SB203580 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

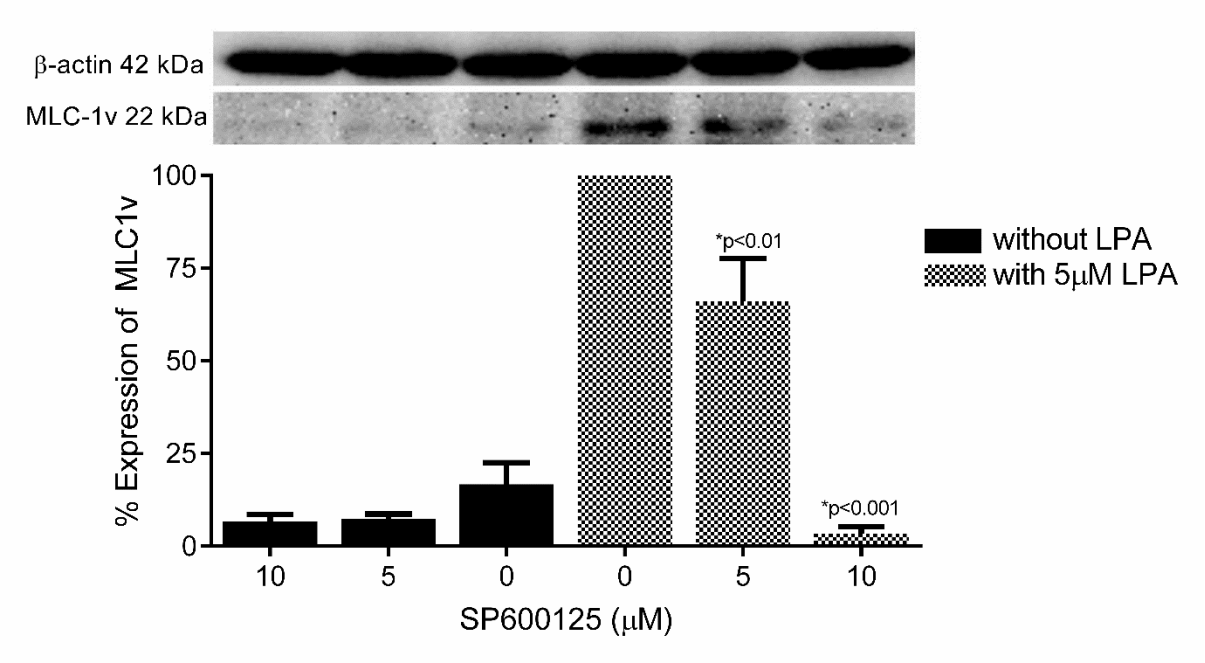

Figure 4.3. SP600125 blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with SP600125 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

4.3.2 The time-dependent effect of 20μM SP600125 on the aggregation and size of EBs

From the observations and results above, we next set out to determine if delayed inhibition of JNK using 20μM SP600125 would yield a similar effect. EBs were seeded in the absence or presence of 5μM LPA with the addition of 20μM SP600125 at the time of seeding, 24 hours, or 48 hours after initial seeding. Photographs were taken 96 hours after initial seeding (Figure 4.4A).

The cells seeded without (Figure 4.4A1) or with 5μM LPA (Figure 4.4A2) exhibited normal EB aggregation, growth, adhered once transferred to 6-well plates, and resulted in normal protein content of lysates generated on day 10 (Figure 4.4B). Cells treated with 20μM SP600125 at the same time as 5μM LPA (Figure 4.4A4) or without LPA (Figure 4.4A3) exhibited disrupted EB aggregation and growth and did not adhere once transferred to 6-well plates which resulted in low protein content of lysates generated on day 10. The addition of 20μM SP600125 24 hours after initial seeding without (Figure 4.4A5) or with 5μM LPA (Figure 4.4A6) yielded similar results but the EBs were slightly larger. Treatment with 20μM SP600125 48 hours after initial seeding without (Figure 4.4A7) or with 5μM LPA (Figure 4.4A8) yielded results similar to treatment with lower concentrations of SP600125 and the EBs exhibited normal aggregation, growth, and adhered once transferred to 6-well plates. Protein quantification of lysates generated at day 10 showed that treatment with 20μM SP600125 after 48 hours of initial seeding was comparable to EBs seeded in the absence of SP600125 with or without LPA treatment (Figure 4.4B).

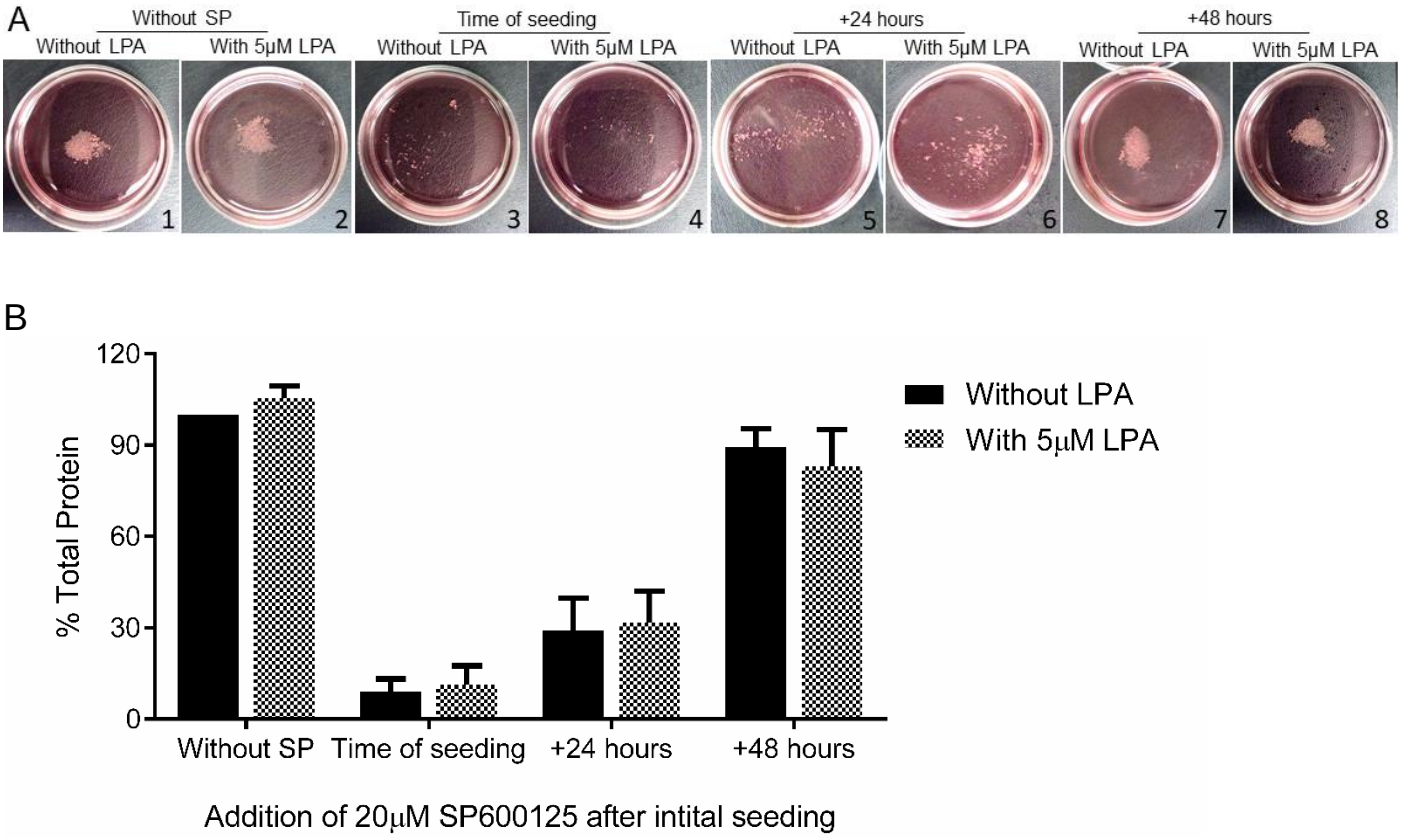

Figure 4.4. Time-dependent effects of 20μM SP600125 on aggregating P19 cells
P19 cells were seeded in the absence or presence of 5μM LPA with a 20μM SP600125 treatment at the same time, 24 hours, or 48 hours after initial seeding in non-tissue grade Petri dishes for 4 days. Photographs (A) were taken and EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for protein quantification using the BCA assay (B) as specified in chapter 2 section 2.3.3. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

4.3.3 Phosphorylation of JNK during EB formation

The above experiments were followed by studies examining the phosphorylation of JNK (p46 and p54) during EB formation.

As seen in Figure 4.5A, phosphorylation of p46 was gradual, peaking at 48 hours, and comparable in both LPA treated and untreated cells. Phosphorylation of both groups decreased at 72 hours to levels slightly less than at 18 hours. Phosphorylation of p54 (Figure 4.5B) was barely detectable at 18 hours but steeply increased at 24 hours and was maintained at 48 hours. A sharp decrease at 72 hours was comparable to phosphorylation at 18 hours suggesting a transient activation between 24 and 48 hours. No significant difference in phosphorylation between LPA treated and untreated cells was observed in any of the lysates generated.

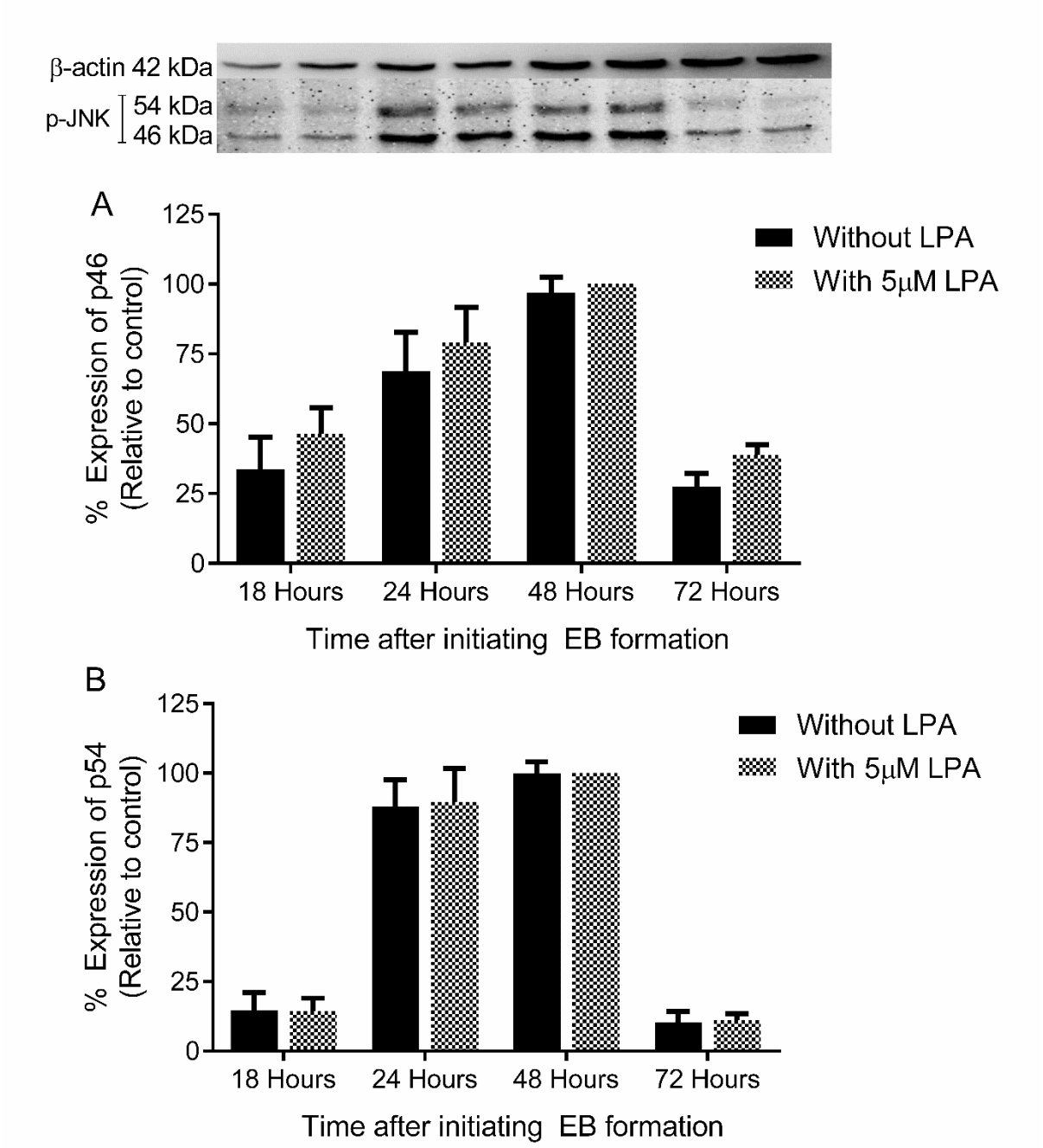

Figure 4.5. Phosphorylation of JNK during EB formation of P19 cells
P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 18, 24, 48 and 72 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-SAPK/JNK (Thr183/Tyr185) determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

4.3.4 The effect of delayed inhibition of the JNK pathway on the expression of MLC1v

From the experiment above, we next set to establish the effect of delayed inhibition of the JNK pathway in differentiating P19 cells.

Differentiation into cardiomyocytes was inhibited as evidenced by the abolishment of MLC1v expression at day 10 with the addition of 10μM SP600125 at the same time as 5μM LPA and 1, 3, 24, or 48 hours after initial seeding. Although, treatment with both 10μM and 20μM SP600125 48 hours after LPA treatment inhibited the expression of MLC1v, a greater reduction in MLC1v was seen with 20μM SP600125 treatment (Figure 4.6)

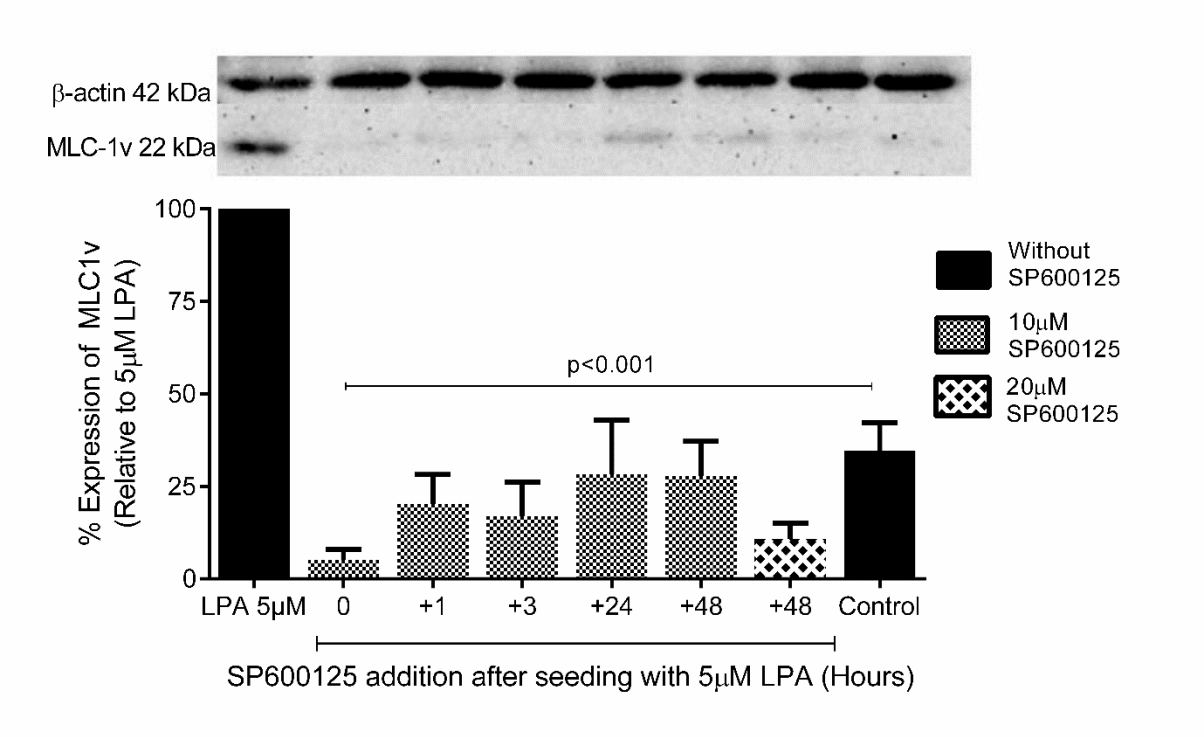

Figure 4.6. Delayed inhibition of the JNK pathway blocks LPA induced MLC1v expression

P19 cells were seeded in the presence of 5μM LPA in non-tissue grade Petri dishes. Addition of 10μM SP600125 was concurrent with LPA treatment or 1, 3, 24, or 48 hours after initial seeding. Treatment with 20μM SP600125 was 48 hours after initial seeding. Cells were cultured in suspension for 4 days and were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

4.3.5 LPA receptor mediated regulation of JNK

As seen in Figure 4.7, in EBs collected at 48 hours after initial seeding, the phosphorylation of both p54 and p46 JNK did not significantly differ with LPA treatment. In the presence of SP600125, 10μM of the inhibitor affected p46 JNK phosphorylation more drastically than p54 JNK, both of which were significantly inhibited irrespective of LPA treatment as did 20μM SP600125 treatment but affected both p46 and p54 JNK phosphorylation comparably. No change in JNK phosphorylation was detected with LPA receptor antagonist treatment alone. LPAR4 inhibition by 0.1mg/mL Suramin did not affect JNK phosphorylation whereas LPAR2 inhibition by 7.5nM H2L5186303 decreased both p46 and p54 mean phosphorylation by approximately 40% compared to LPA treatment alone. Phosphorylation of JNK was most drastically affected by LPAR1/3 inhibition by 20μM Ki16425, resulting in a mean decrease of both p46 and p54 by approximately 80% compared to LPA treatment alone.

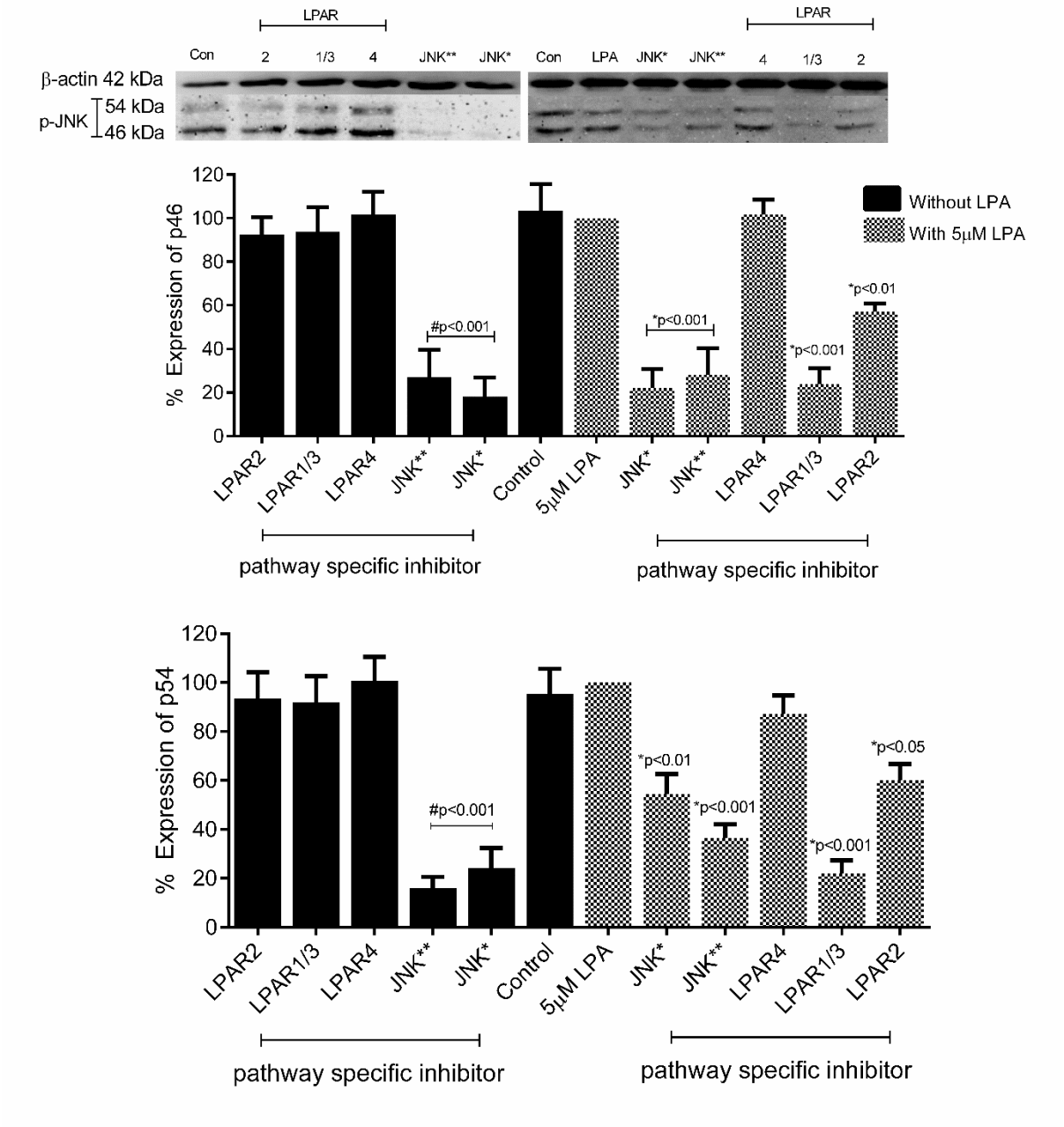

Figure 4.7. Ki16425 and H2L5186303 block JNK phosphorylation in LPA treated cells

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with LPA receptor 1/3 (20μM Ki16425), receptor 2 (7.5nM H2L5186303), receptor 4 (0.1mg/mL Suramin) antagonist, or JNK inhibitor (*10μM or **20μM SP600125) for 60 minutes in non-tissue grade Petri dishes for 48 hours. Lysates were generated using 1x RIPA buffer as described in chapter 2, section 2.3.2, for expression of phospho-SAPK/JNK (Thr183/Tyr185) by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone. #represents significance relative to without LPA treatment.

4.3.6 Phosphorylation of the MAPKs by LPA

A cell-based ELISA was used to investigate the effects of LPA treatment on early phosphorylation of the MAPKs. Basal expression was determined by untreated cells, marked at time 0. Change in expression by LPA treatment over the course of 180 minutes was compared to this. Total protein expression was unchanged in cells treated with LPA over the course of 180 minutes for all three MAPKs. Phosphorylation of each MAPK occurred within the first minute but was not significantly elevated for the first 15 minutes. A steep decrease in phosphorylated ERK was observed at 30 minutes that was sustained until 180 minutes (Figure 4.8). A reduction in p38 phosphorylation was also observed at 180 minutes (Figure 4.9) whereas a considerable decrease in phosphorylation was seen at 60 minutes in JNK with a further reduction at 180 minutes (Figure 4.10).

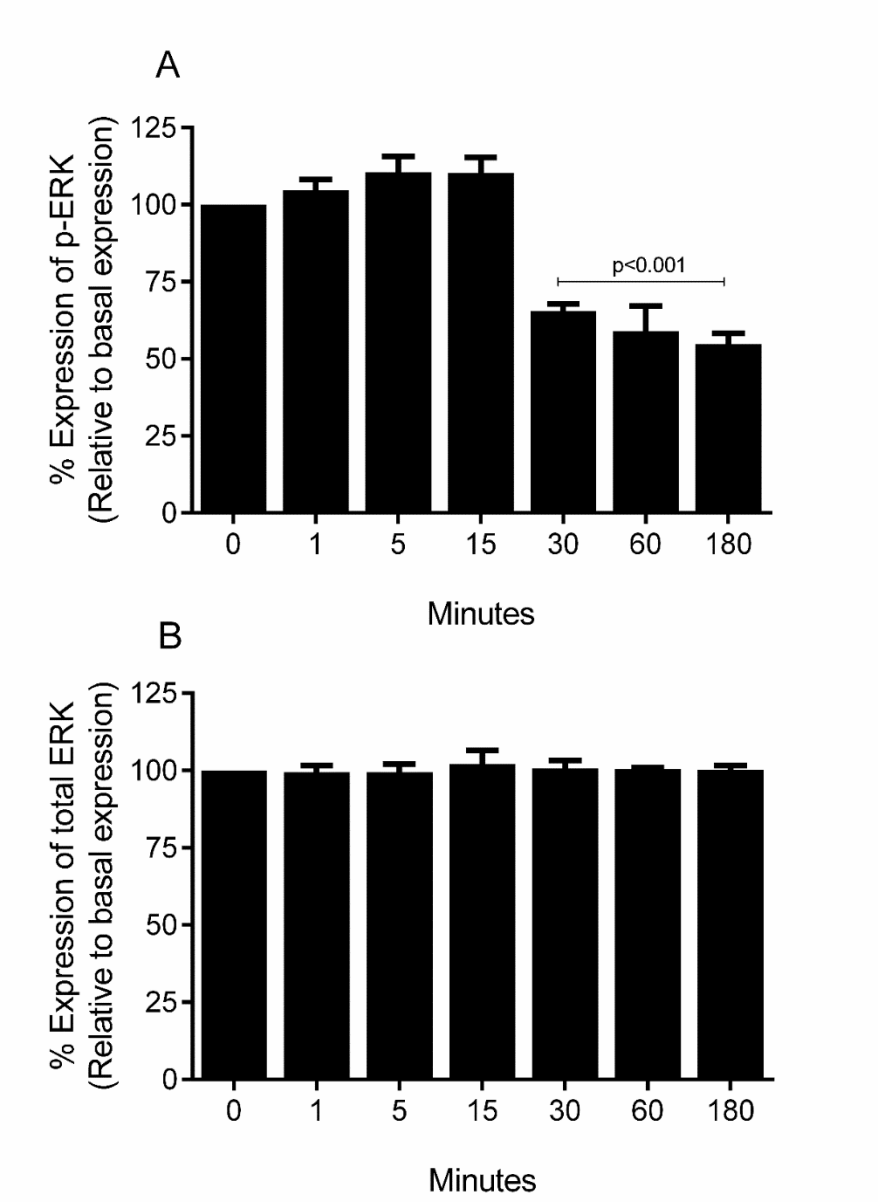

Figure 4.8. Phosphorylation of ERK by LPA in adherent P19 cells
A cell-based ELISA kit (Sigma) was used for detecting phosphorylated and total ERK, in LPA (5μM) treated cells. 100μL of cell suspension containing $1.5x10^4$ cells was seeded into each well of a 96-well plate and incubated for 48 hours. LPA was diluted in serum-free αMEM, and the cells were treated for 1, 5, 15, 30, 60 or 180 minutes. The assay was carried out as described in section 4.2.6. The ERK phospho- (A) and total (B) signals were normalised for cell number and data expressed as a percentage of non-treated cells (time 0). Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

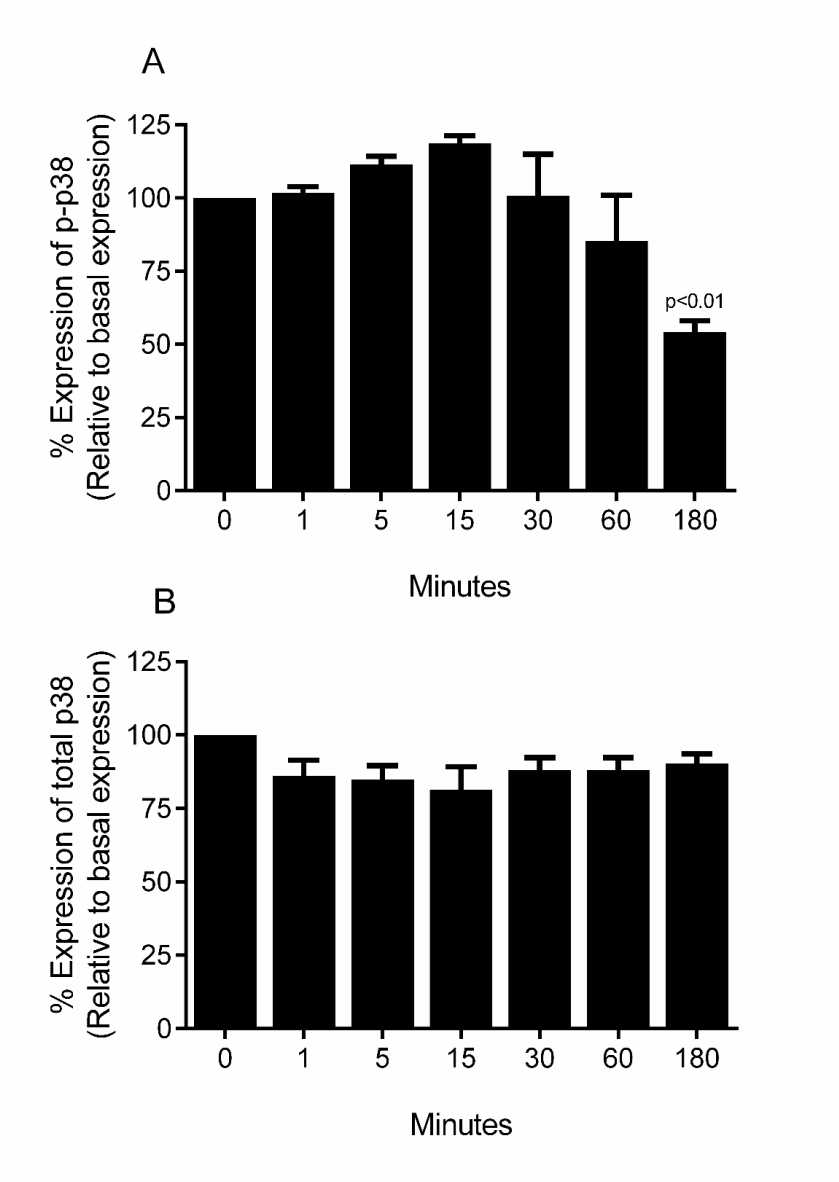

Figure 4.9. Phosphorylation of p38 by LPA in adherent P19 cells
A cell-based ELISA kit (Sigma) was used for detecting phosphorylated and total p38, in LPA (5µM) treated cells. 100µL of cell suspension containing $1.5x10^4$ cells was seeded into each well of a 96-well plate and incubated for 48 hours. LPA was diluted in serum-free αMEM, and the cells were treated for 1, 5, 15, 30, 60 or 180 minutes. The assay was carried out as described in section 4.2.6. The p38 phospho- (A) and total (B) signals were normalised for cell number and data expressed as a percentage of non-treated cells (time 0). Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

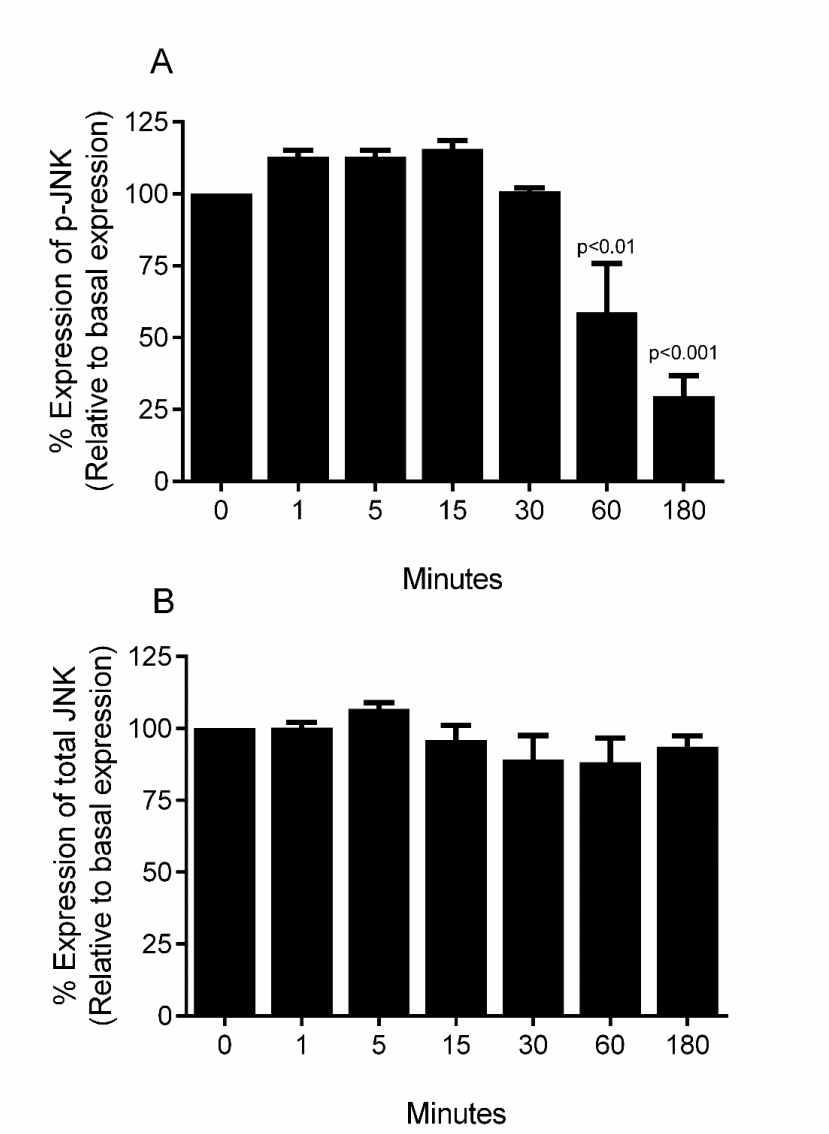

Figure 4.10. Phosphorylation of JNK by LPA in adherent P19 cells
A cell-based ELISA kit (Sigma) was used for detecting phosphorylated and total JNK, in LPA (5µM) treated cells. 100µL of cell suspension containing $1.5x10^4$ cells was seeded into each well of a 96-well plate and incubated for 48 hours. LPA was diluted in serum-free αMEM, and the cells were treated for 1, 5, 15, 30, 60 or 180 minutes. The assay was carried out as described in section 4.2.6. The JNK phospho- (A) and total (B) signals were normalised for cell number and data expressed as a percentage of non-treated cells (time 0). Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

4.3.7 Regulation of the MAPKs in LPA treated cells by LPA receptors 1-4, PKC, and PI3K

For further investigation into whether there is cross-talk amongst MAPK or regulation by PKC, PI3K and LPA receptors 1-4, an ELISA kit was used. Basal expression was determined by stimulating the cells with 5μM LPA for 15 minutes and change in expression by LPA treatment for 15 minutes after an initial 60-minute incubation with the inhibitor was compared to this. As limited reagent was available, the treatments were repeated twice and presented as mean data.

Phosphorylation of ERK appeared to be upregulated with the inhibition of the p38 pathway whereas a minuscule decrease was observed with LPA receptor inhibition and inhibition of the JNK pathway. No change was seen in ERK phosphorylation with the inhibition of the PKC and PI3K kinases (Figure 4.11A).

p38 phosphorylation appeared to be upregulated with LPA receptor 1-4 inhibition as with PKC. No noticeable difference in p38 phosphorylation was observed by the inhibition of PI3K, ERK, or JNK (Figure 4.11B).

JNK phosphorylation did not appear to be affected by LPA receptor 4 and 1/3 inhibition, but a slight decrease in phosphorylation with the inhibition of LPA receptor 2 was detected. Inhibition of the PKC kinase showed an increase in JNK phosphorylation and inhibition of the PI3K kinase showed no change. A slight increase in JNK phosphorylation was detected upon inhibition of the ERK pathway; this result was more prominent with p38 inhibition (Figure 4.11C)

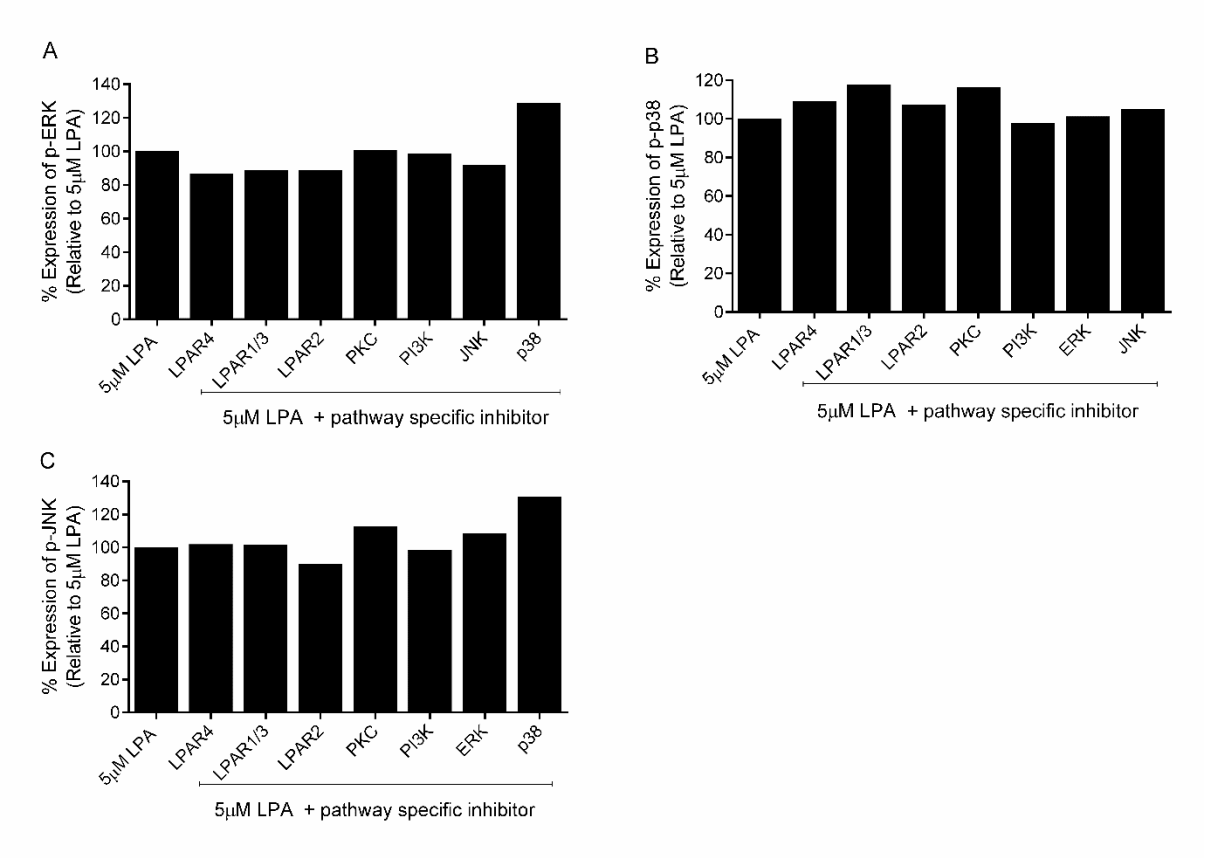

Figure 4.11. Regulation of the MAPKs in LPA treated cells

A cell-based ELISA kit (Sigma) was used to determine the regulation and cross-talk between MAPK subgroups. 100μL of cell suspension containing $1.5x10^4$ cells was seeded into each well of a 96-well plate and incubated for 48 hours. Basal expression was determined by stimulating the cells with 5μM LPA, prepared in serum-free αMEM, for 15 minutes. Changes in basal expression after an initial 60-minute incubation with the inhibitors for LPA receptors (LPAR 1/3 antagonized with 20μM Ki16425, LPAR 4 antagonized with 0.1mg/mL Suramin, LPAR2 antagonized with 7.5nM H2L5186303) or kinase inhibitors (ERK pathway inhibited with 20μM PD98059, p38 pathway inhibited with 10μM SB203580, JNK pathway inhibited with 10μM SP600125, PI3K inhibited with 20μM LY294002, PKC inhibited with 10μM BIM-1) followed by stimulation with LPA for 15 minutes was compared to basal expression. The assay was carried out as described in section 4.2.7. The phospho-signals for ERK (A), p38 (B), and JNK (C) were normalised for cell number and the data expressed as a percentage of basal expression. As limited wells were available, the treatments were repeated twice and presented as mean data.

4.4 Discussion

The mitogen-activated kinases are activated by multiple stimuli and receptor families including GPCRs. LPA signalling, via the MAPKs, regulates proliferation, survival, migration, and differentiation (Du *et al.,* 2010; Hayashi *et al.,* 2001; Kostic *et al.,* 2015; Malchinkhuu *et al.,* 2005; Schuck *et al.,* 2003; Sorensen *et al.,* 2003). The requirement of the MAPKs for LPA signal transduction and evidence of PKC and PI3K regulation of the MAPKs in other models, in conjunction with our findings highlighted in chapter 3, prompted us to extend our studies to examine the involvement of the MAPKs in LPA mediated cardiac differentiation of P19 cells.

Commercially available and widely used inhibitors were used to determine the role of each MAPK subfamily. ERK inhibition was achieved using PD98059, a selective inhibitor of MEK1 (Dudley *et al.,* 1995). Inhibition of p38 was achieved using SB203580, a selective inhibitor of p38α and β with slightly more affinity for the former. SB203580 has an IC_{50} of 0.6μM but also shown to inhibit Akt at 10-fold higher concentrations (Cuenda *et al.,* 1995; Lali *et al.,* 2000). JNK inhibition was achieved using SP600125, a selective inhibitor for JNK1> JNK2>JNK3 with an IC_{50} of 40nM, 40nM, and 90nM respectively but also MKK4 at 10-fold higher concentrations and MKK3/6, Akt, and PKCα at 25-fold higher concentrations (Bennett *et al.,* 2001).

Pramod (2017) established the role of ERK and p38 using 1-20μM of PD98059 and 1-10μM of SB203580 respectively and found while ERK was a required signalling target of LPA, inhibition of p38 did not result in the decrease of MLC1v in LPA treated cells. The current thesis has confirmed that LPA induced cardiac differentiation of P19 cells could be inhibited with the treatment of 20μM PD98059. The importance of ERK in cardiac differentiation is supported by several recent studies (Abbey and Seshagiri,

2013; Qian *et al.,* 2012; Wang *et al.,* 2017; Wu *et al.,* 2013), however, studies in P19 cells have not fully supported this (Davidson & Morange, 2000; Eriksson & Leppa, 2002). Dell'Era *et al.* (2003) shed light on this contradiction by treating $fgfr^{+/-}$ EBs with both PD98059 and U0126, the latter is an inhibitor of both MEK1 and MEK2. Only inhibition of MEK1/2 inhibited cardiac differentiation whereas inhibition of MEK1 alone did not. PD98059 is highly selective for MEK1, and the concentration of 20μM used in our studies is supported by reports inhibiting the ERK pathway to evaluate the effect on cardiac differentiation (Ronca *et al.,* 2009; Wu *et al.,* 2013).

Inhibition of p38 by 1, 5, and 10μM SB203580 did not affect LPA induced cardiac differentiation of P19 cells. This observation contradicts several reports that have implicated p38 in cardiac differentiation (Aoudi *et al.,* 2006; Ding *et al.,* 2008; Eriksson & Leppa, 2002; Li *et al.,* 2006). However, others have suggested that the use of SB203580 at concentrations of less than 10μM promotes cardiac differentiation of hESCs whereas higher concentrations oppose this (Graichen *et al.,* 2008; Kempf *et al.,* 2011; Xu *et al.,* 2008). Davidson and Morange (2000) and Gaur *et al.* (2010) also reported that effects are dependent on the stage of development in which p38 is inhibited.

The expression of MLC1v was abolished by treatment of 10μM SP600125 before LPA treatment. Earlier studies implicated JNK in regulating proliferation (Eriksson & Leppa, 2002) or differentiation by association to noncanonical Wnt signalling (Pandur *et al.,* 2002). However, recent reports indicate that JNK signalling is involved in cardiac differentiation induced by different stimuli and in multiple cell types (Li *et al.,* 2016; Ou *et al.,* 2016; Shi *et al.,* 2017; Tanwar *et al.,* 2014; Wu *et al.,* 2013). This is supported by the negative regulation of JNK on pluripotent genes, OCT4 and Klf4 (Byun *et al.,*

2013; Yao *et al.,* 2014). SPC, a closely related lipid to LPA, also induced differentiation into cardiomyocytes through JNK signalling (Li *et al.,* 2016). Supportive of our findings, both Li *et al.* (2016) and Tanwar *et al.* (2014) reported inhibition of cardiac differentiation upon 10μM SP600125 treatment.

Treatment with 20μM SP600125 60 minutes before LPA treatment resulted in the formation of smaller EB aggregates that were unable to adhere once transferred to tissue grade 6-well plates. The same was observed in EBs seeded with the inhibitor without LPA. Cytotoxicity was not reported at this concentration. Therefore, we furthered our investigation to determine if the effect was time-dependent. EBs were seeded in the absence or presence of 5μM LPA and 20μM SP600125 treatment was administered at the same time or delayed by 24 and 48 hours. Treatment at the same time or 24 hours after initial seeding yielded similar results although some cells appeared to adhere after transfer in the latter. Treatment 48 hours after initial seeding resulted in normal EB aggregation and cells adhered once transferred to tissue grade 6-well plates.

Our observations are supported by many studies as JNK is known to regulate development and cell cycle progression. Nakaya *et al.* (2009) observed defective cytokinesis, in the context of neurogenesis, of P19 clone #3 cells that was SP600125 concentration dependent; 2.5, 5, 10, and 20μM SP600125 concentrations were used. Zhou *et al.* (2013) reported that increasing concentrations of 5, 10, 15, 20, and 25μM SP600125 resulted in smaller and smaller sized ESC colonies. The same study also found that culturing mESCs for 24 hours followed by treatment with 25μM SP600125 arrested over 60% of the cells in the G2/M phase. Ou *et al.* (2016) highlighted the requirement of JNK in cardiac differentiation of miPSCs but also found that 20μM

SP600125 treatment significantly affected proliferation of these cells. In the context of neurogenesis, Tiwari *et al.* (2011) demonstrated that the addition of SP600125 in the early stages of differentiation resulted in cell death, however, apoptosis was not apparent when differentiated neurons were treated with SP600125.

In our studies, the effect of SP600125 was found to be time and dose-dependent, as highlighted above. Therefore, we next set out to determine the phosphorylation of JNK in aggregating EBs. The earliest collection of EBs was achievable 18 hours after initial seeding. Attempts were made to collect EBs 1-15 hours after initial seeding, however, the size of pelleted cells was too small making protein extraction difficult, and consistency could not be assured. The number of cells initially seeded could have been increased however this requires further optimisation as seeding density influences gene expression patterns (Chen *et al.,* 2015).

We examined JNK phosphorylation in EBs seeded in the absence and presence of 5μM LPA for 18, 24, 48, and 72 hours and found a transient phosphorylation of JNK in both groups between 24 and 48 hours. The phosphorylation of p46 was more gradual between 18-48 hours whereas p54 phosphorylation was steep and sustained at 24 and 48 hours. Both p46 and p54 phosphorylation was decreased at 72 hours. From these findings, we next set out to determine if disruption of JNK signalling during the period of transient phosphorylation inhibited cardiac differentiation. Our previous findings demonstrated that pre-treatment of EBs for 60 minutes with 10μM SP600125 was sufficient to block differentiation, however, whether JNK activation is a biphasic event, as JNK activation in EBs cultured for less than 18 hours was not determined, is unknown, therefore, we cultured EBs in the presence of 5μM LPA for 1 and 3 hours before the addition of 10μM SP600125. EBs were also cultured in the presence of LPA

for 24 and 48 hours before the addition of 10μM SP600125. It was determined from findings discussed above that treatment with 20μM SP600125 48 hours after initial seeding restored proliferation, however, the effect of this concentration on differentiation was unknown. Therefore, we treated EBs with 20μM SP600125 48 hours after LPA treatment. Treatment of SP600125 1, 3, 24, or 48 hours after LPA treatment disrupted cardiac differentiation suggesting that commitment to the cardiac lineage in LPA treated cells occurs post 48 hours. Treatment of 20μM SP600125 also inhibited cardiac differentiation more strongly than 10μM SP600125. Taken together these results suggest that the gene program for proliferation that is regulated by JNK is switched off half way through EB formation, as evidenced by the normal growth in EBs treated with 20μM SP600125 48 hours after initial seeding, whereas the gene program for cardiac differentiation that is regulated by JNK occurs post 48 hours.

Xu and Davis (2010) demonstrated something similar in ESCs derived from JNK deficient mice. They showed that compound $JNK1^{-/-}$ and $JNK2^{-/-}$ ES cells formed EBs but did not increase in number during culture suggesting that JNK is necessary for the proliferation of cells that have initiated a differentiation program. They further examined the effect of compound $JNK1^{-/-}$ and $JNK2^{-/-}$ ES cells on mesodermal lineage genes and found that Brachyury is poorly expressed in these cells as are cardiac progenitor genes, GATA4 and MEF2c, and MHC and MLC genes.

From the results above, we next set out to determine if 10μM and 20μM SP600125 were affecting p46 and p54 phosphorylation differently. We also furthered our studies to investigate the regulation of p46 and p54 by Ki16425, H2L5186303, and Suramin. EBs were seeded in the absence or presence of 5μM LPA after a 60-minute pre-treatment with SP600125 (10μM and 20μM), Ki16425 (20μM), H2L5186303 (7.5nM),

and Suramin (0.1mg/mL). Phosphorylation of p46 and p54 JNK in the presence of SP600125 revealed that 10μM of the inhibitor effected p46 JNK phosphorylation more drastically than p54 JNK, but both were significantly inhibited irrespective of LPA treatment as did 20μM SP600125 treatment but affected both p46 and p54 JNK phosphorylation comparably. No change in JNK phosphorylation was detected with LPA receptor antagonist treatment alone. LPAR4 inhibition by 0.1mg/mL Suramin did not affect JNK phosphorylation whereas LPAR2 inhibition by 7.5nM H2L5186303 decreased both p46 and p54 mean phosphorylation by approximately 40% compared to LPA treatment alone. The phosphorylation of JNK was most drastically affected by LPAR1/3 inhibition by 20μM Ki16425, resulting in a mean decrease of both p46 and p54 by approximately 80% compared to LPA treatment alone. A decrease in JNK signalling by interrupting LPAR2 (Saatian *et al.,* 2006), LPAR1 and LPAR2 (Contos *et al.,* 2002) and LPAR1 using Ki16425 (Iyoda *et al.,* 2012) is also seen in studies evaluating other cellular processes.

We evaluated the activation of each MAPK subfamily upon stimulation with LPA over the course of 180 minutes using the MAPK ELISA kit (Sigma). Cells were plated in 96 well plates and cultured for 48 hours to allow serum depletion. Cells were then stimulated with 5μM LPA, prepared in serum-free αMEM. Phosphorylated and total ERK, p38, and JNK was measured after 1, 5, 15, 30, 60, and 180 minutes. LPA did not cause a significant spike in the phosphorylation of any of the kinases, but an upward trend for each was seen for 15-30 minutes followed by a decrease at 30 and 60 minutes for ERK and p38/JNK respectively. Studies within the group have identified that EB formation is required for cardiac differentiation induction by LPA. It is possible that in EBs the upward trend initially seen in the phosphorylation of the kinases would continue, but as the cells were in adherent culture, LPA does not maintain their activity.

It is also possible that serum is required for the sustained activation of the MAPKs upon LPA treatment.

We next evaluated the regulation of the MAPKs by LPA receptors 1-4 and PKC/PI3K, as the importance of these kinases was highlighted in chapter 3. We also investigated the possibility of cross-talk between the MAPKs. We stimulated the cells with LPA for 15 minutes, where maximum activity was achieved as evidenced from the study above, after a 60-minute pre-treatment with inhibitors specific to the targets mentioned earlier. LPA and the inhibitors were prepared in serum-free αMEM and treatment was after cells were cultured for 48 hours in 96 well plates. The experiments were repeated twice as limited reagents were available.

Phosphorylation of ERK was unchanged (within 2% range) by the inhibition of PKC and PI3K by BIM-1 and LY294002 respectively. A minor decrease of 8-14% was seen in cells treated with Ki16425 (LPAR 1/3 antagonist), H2L5186303 (LPAR 2 antagonist), Suramin (LPAR 4 antagonist), and SP600125 (JNK inhibitor) whereas an increase of 30% occurred in cells treated with SB203580 (p38 inhibitor).

Phosphorylation of p38 was unchanged (within 5% range) by the inhibition of ERK by PD98059, JNK by SP600125, and PI3K by LY294002. Treatment with Suramin (LPAR 4 antagonist) and Ki16425 (LPAR 1/3 antagonist) increased phosphorylation by approximately 10% whereas targeting LPAR2 with H2L5186303 and PKC with BIM-1 resulted in a 16-17% increase.

Phosphorylation of JNK was unchanged (within 2% range) with the inhibition of PI3K by LY294002, LPAR4 by Suramin and LPAR1/3 by Ki16425. Phosphorylation increased by 8-12% in response to the inhibition of ERK by PD98059 and PKC by

BIM-1, and a 30% increase was seen in cells treated with SB203580 (p38 inhibitor). Inhibition of LPAR2 by H2L5186303 decreased phosphorylation by 10%.

In conclusion, LPA mediated cardiac differentiation of P19 cells requires both ERK and JNK signalling. JNK signalling is regulated by LPA receptors 1/3 and partially through LPA receptor 2. Disruption in JNK signalling using 20μM SP600125 suggests that early JNK signalling is required for the proper aggregation and growth of EBs whereas late signalling is required for LPA induced cardiac differentiation. However, the latter was also achieved with the treatment of 10μM SP600125. Therefore, the selectivity of SP600125 when used at a concentration of 20μM requires further evaluation.

Chapter 5

The role and regulation of ubiquitous transcription factors in LPA mediated cardiac differentiation of P19 cells

5.1 Introduction

Activator protein 1 (AP-1) is involved in cell regulation, apoptosis, development, and differentiation (Hess, Angel, & Schorpp-Kistner, 2004). It also impacts on gene expression through the dimerization of members of the Jun and Fos families. Members of the Jun family form both homo- and heterodimers with other Jun and Fos members whereas members of the Fos family do not form dimers with other Fos proteins (Deng & Karin, 1993; Whitmarsh & Davis, 1996). Disruption in the c-Jun protein is embryonic lethal at E12.5 due to defects in the heart outflow tract (Eferl *et al.,* 1999) and disruption in the JunB protein is embryonic lethal at E8.5-10 due to defects in extra-embryonic tissues (Schorpp-Kistner *et al.,* 1999). Fra-1 is also embryonic lethal at E9.5 (Schreiber et a., 2000) whereas Fra-2 knockout results in inadequate cartilage and spinal development (Karreth *et al.,* 2004). Disruption in JunD results in cardiac hypertrophy (Ricci *et al.,* 2005), while disruption in c-Fos affects bone development (Johnson, Spiegelman, & Papaioannou, 1992). AP-1 has been reported to be vital for the cardiac differentiation of P19 cells in the presence of DMSO (Eriksson & Leppa, 2002) and in icariin induced cardiac differentiation of mESCs (Wo *et al.,* 2008). Other transcription factors such as ATF also dimerize with Jun and Fos proteins (Angel & Karin, 1991; Fuchs & Ronai, 1999; Mechta-Grigoriou, Gerald, & Yaniv, 2001; Vogt & Bos, 1990). ATF and c-Jun are well-studied substrates of JNK as is c-Fos as an ERK substrate, however, other transcription factors and signalling pathways also converge on the regulation of AP-1 including NF-κB (Kracht, 2007; Whitmarsh & Davis, 1996).

The ubiquitous NF-κB transcription factor (Ghosh & Hayden, 2012) contains the Rel homology domain and is subdivided into five subunits, p100/p52, p105/p50, RelA (p65), RelB, and c-Rel. These subunits produce homo- and hetero-dimers forming inactive or active NF-κB complexes with different DNA binding affinities. IκB proteins

bind and retain NF-κB in the cytoplasm preventing translocation into the nucleus. Translocation can be mediated by events such as proteasomal degradation of IκB proteins or DNA damage. p38 is known to regulate NF-κB (Wagley *et al.,* 2013) and the most active p65 subunit has also been shown to be phosphorylated by the MAPK activated protein kinases, RSK1 and MSK1/2, amongst other non-MAPK mediated kinases (Buss *et al.,* 2004a; Buss *et al.,* 2004b; Schmitz *et al.,* 2004; Viatour *et al.,* 2005).

In this study, we aim to investigate the activation and regulation of AP-1 in differentiating P19 cells. Both the MAPKs and NF-κB are critical regulators of AP-1, and the importance of MAPK signalling in our model has already been established in chapter 4. Therefore, the role and regulation of NF-κB will also be evaluated here.

5.2 Materials and Methods

5.2.1 Determining the role of NF-κB in LPA mediated cardiac differentiation of P19 cells

Differentiation of P19 cells was carried out as specified in chapter 2 section 2.2. The inhibitor for NF-κB, CAY10470, was purchased from Merck Chemicals and received in a DMSO solution at a concentration of 10mM. Aliquots were protected from light and stored at -20˚C until use. Working concentrations were obtained by dilution in complete αMEM on the day of use. Lysates were generated on day 10 of the differentiation process as described in chapter 2 section 2.3.1. Protein quantification and western blotting were performed as set out in chapter 2 section 2.3.3 and section 2.4 respectively. Cell viability in the presence of varying concentrations of the inhibitor was determined using the MTT assay as described in chapter 2 section 2.5. Statistical analysis was done as described in chapter 2 section 2.7.

5.2.2 Determining the phosphorylation of NF-κB by LPA

The differentiation process was carried out as described in chapter 2 section 2.2. Lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process as described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as described in chapter 2 section 2.3.3 and section 2.4 respectively with the following modifications: the membrane was blocked using 1x TBS-T containing 5% BSA. The phospho-NF-κB p65 (Ser536) (Cell Signaling Technology) primary antibody was used at a dilution of 1:1000 and the anti-rabbit IgG, an HRP-linked secondary antibody was used at a dilution of 1/4000. SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

5.2.3 Determining the early phosphorylation of NF-κB by LPA in adherent P19 cells

The FACE NF-κB p65 Profiler Kit was purchased from Active Motif (product 48400) to detect the phosphorylation of NF-κB p65 at serine residues 536 and 468, and total p65 in LPA treated cells. Buffers were supplied with the kit and prepared according to the manufacturer instructions. P19 cells were prepared for analysis as described in chapter 2 section 2.2.4. After treatment with LPA the cells were fixed with 4% formaldehyde for 20 minutes at room temperature, washed 3 times with wash buffer for 5 minutes with gentle shaking, followed by incubation with quenching buffer for 20 minutes at room temperature. The wells were washed twice, incubated with blocking buffer for 1 hour, and washed twice before overnight incubation at 4˚C with primary antibody. The wells were washed three times before adding the secondary antibody for 1 hour and then washed three times with wash buffer and twice with 1x PBS. A chemiluminescent working solution was added to each well and read using the CCD camera equipped Thermo myECL Imager. As multiple wash steps were carried out, resulting in the possible loss of cells, crystal violet solution was used to estimate the relative number of cells in each well. After reading the plate, cells were washed twice with the buffer supplied and twice with 1x PBS. The plate was air dried for 10 minutes, and 100µL of crystal violet solution was added to each well and left to incubate at room temperature for 30 minutes. Each plate was washed using 1x PBS for 5 minutes, repeated three times, before incubation with 1% SDS solution for 1 hour with gentle shaking. The absorbance was read at 595nm using the CLARIOstar plate reader. The total and phospho- NF-κB signals were then normalised for cell number and data expressed as a percentage of non-treated cells (basal expression). Statistical analysis was done as described in chapter 2 section 2.7.

5.2.4 Determining the regulation of NF-κB in LPA treated cells by LPA receptors, MAPKs, PKC and PI3K

The FACE NF-κB p65 Profiler kit (Active Motif: product 48400) was used to determine the effects of LPA receptor, MAPKs, PKC, and PI3K inhibition on the phosphorylation of NF-κB in the presence of LPA. Antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) were prepared in serum-free αMEM as were inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), and JNK (10μM SP600125). P19 cells were prepared for analysis as described in chapter 2 section 2.2.4. but were treated with the antagonists for 60 minutes before stimulation with 5μM LPA, prepared in serum-free αMEM, for 15 minutes. The steps carried out next were the same as specified in section 5.2.3. The NF-κB phospho- signals were then normalised for cell number and data expressed as a percentage of LPA only treated cells. Statistical analysis was done as described in chapter 2 section 2.7.

5.2.5 Determining the phosphorylation of AP-1 subunits by LPA

The TransAM AP-1 family transcription factor assay kit was purchased from Active Motif (product 44296) to evaluate the activation of AP-1 subunits, c-Jun, JunD, FosB, and c-Fos. Buffers were supplied with the kit and prepared according to the manufacturer instructions.

5.2.5.1 Preparation of nuclear extract

Cardiac differentiation was carried out as described in chapter 2 section 2.2 and lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process. For the generation of lysates on days 1-4 of the differentiation process, EBs were transferred to falcon tubes as described in chapter 2 section 2.2.3 with the following modifications: cold 1x PBS containing phosphatase and protease inhibitor cocktails

(Sigma) (1:100 and 1:200 respectively) was added to the tubes instead of complete αMEM. The cells were transferred to a pre-chilled 1.5mL Eppendorf and centrifuged for 15 seconds at 13000rpm. The PBS was removed, and 150µL-350µL of hypotonic buffer (20mM Hepes pH 7.5, 5mM NaF, 0.1mM EDTA, and 10µM Na_2MoO_4) was added. The cells were gently broken into a single cell suspension by repeated pipetting and allowed to swell on ice for 15 minutes. Nonidet P-40 (final concentration of 0.5%) was added to the tube and vortexed for 10 seconds. The cells were then centrifuged for 60 seconds at 4˚C. The cytoplasmic fraction (supernatant) was removed, and the pellet was resuspended in 50µL of complete lysis buffer (provided with the kit). The tube was rocked on ice for 30 minutes and then centrifuged for 15 minutes at 13000rpm at 4˚C. The nuclear fraction (supernatant) was transferred to a chilled tube and stored at -80˚C until required. Lysates generated on days 6, 8, and 10 of the differentiation process were washed with 1x PBS containing phosphatase and protease inhibitors, and the hypotonic buffer was added to each well. The cells were scraped using a cell scraper and transferred to a pre-chilled Eppendorf before the addition of Nonidet P-40 and processed as described above.

5.2.5.2 Protein quantification

The Bradford-based assay was used to determine the total concentration of protein in the nuclear lysates. The Bradford reagent was made by first dissolving 100mg of Coomassie brilliant blue G-250 in 50mL of 95% ethanol followed by the addition of 100mL of 85% w/v orthophosphoric acid. The final volume was made to 1L using double distilled water. The reagent was filtered using Whatman filter paper and stored at room temperature. BSA standards were prepared as set out in table 2.0. A 96 well plate was used to carry out the assay. 300µL of Bradford reagent was added to 10µL of each standard or unknown sample and mixed for 30 seconds using a plate shaker

followed by a 10-minute incubation at room temperature. Absorbance was read at 595nm using the CLARIOstar plate reader. A standard curve was prepared by plotting the average of each BSA standard measurement against the concentration in µg/µL, and the protein concentration of each unknown sample was determined using the standard curve.

5.2.5.3 AP-1 assay

All buffers and antibodies were supplied with the kit and prepared according to the manufacturer's instructions. 30µL of complete binding buffer was added to each well followed by 20µg of sample diluted in 20µL of complete lysis buffer and incubated for 60 minutes at room temperature with mild agitation. The K-562 nuclear extract was provided with the kit and used as a positive control. The wells were washed three times with wash buffer, and 100µL of the primary antibody was added to each well. The plate was incubated for 60 minutes at room temperature, and each well was washed three times using wash buffer. 100µL of secondary antibody was added to each well and incubated for 60 minutes at room temperature. Wells were washed four times with wash buffer and then incubated with developing solution until the positive control wells turned dark blue. The plate was protected from light during this incubation. 100µL of stop solution was added to each well, and the absorbance was read at 450nm using the CLARIOstar plate reader. Statistical analysis was done as described in chapter 2 section 2.7.

5.2.6 Determining the regulation of AP-1 subunits in LPA treated cells by LPAR1-4, MAPKs, PKC, PI3K, and NF-κB.

The TransAM AP-1 family transcription factor assay kit (Active Motif: product 44296) was used to determine the role of LPA receptors, MAPKs, PKC, PI3K, and NF-κB inhibition on the activation of AP-1 subunits in the presence of LPA. Differentiation was

carried out as described in chapter 2 section 2.2 in the presence of antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) for 60 minutes prior to treatment with 5μM LPA. Preparation of nuclear extracts on day 10 of the differentiation process was completed as described in section 4.2.5.1. Protein quantification and the AP-1 assay were carried out as described in section 4.2.5.2 and 4.2.5.3 respectively. Statistical analysis was done as described in chapter 2 section 2.7.

5.2.7 Determining the phosphorylation of ATF_2 by LPA

The differentiation process was carried out as described in chapter 2 section 2.2. Lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process as described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as described in chapter 2 section 2.3.3 and 2.4 respectively with the following modifications: the membrane was blocked using 1x TBS-T containing 5% BSA. The phospho-ATF_2 (Thr71) primary antibody (Cell Signaling Technology) was used at a dilution of 1:1000, the anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at a dilution of 1:4000, and SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

5.2.8 Determining the regulation of ATF_2 in LPA treated cells by LPAR1-4, MAPKs, PKC, PI3K, and NF-κB.

The differentiation process was carried out as described in chapter 2 section 2.2. Cells were seeded in the presence of antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM

LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) for 60 minutes before treatment with 5μM LPA. Lysates were generated on day 10 of the differentiation process as described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as described in chapter 2 section 2.3.3 and section 2.4 respectively with the following modifications: the membrane was blocked using 1x TBS-T containing 5% BSA. The phospho-ATF_2 (Thr71) primary antibody (Cell Signaling Technology) was used at a dilution of 1:1000, the anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at a dilution of 1:4000, and SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

5.3 Results

5.3.1 NF-κB signalling mediates cardiac differentiation in LPA treated P19 cells

To establish whether LPA was acting through the NF-κB transcription factor in mediating cardiac differentiation of P19 cells, NF-κB was inhibited during EB formation using CAY10470. No cytotoxicity was reported with the concentrations used. A higher concentration of 1nM, not used in experimental studies, showed a slight decrease in the viability of P19 cells (Figure 5.0). In lysates generated on day 10, the inhibitor of NF-κB did not induce differentiation when used alone (Figure 5.1). When used in conjunction with LPA treatment, concentrations of 0.01nM and 0.05nM of CAY10470 returned MLC1v expression to basal level while the lowest concentration of 0.001nM had no significant effect

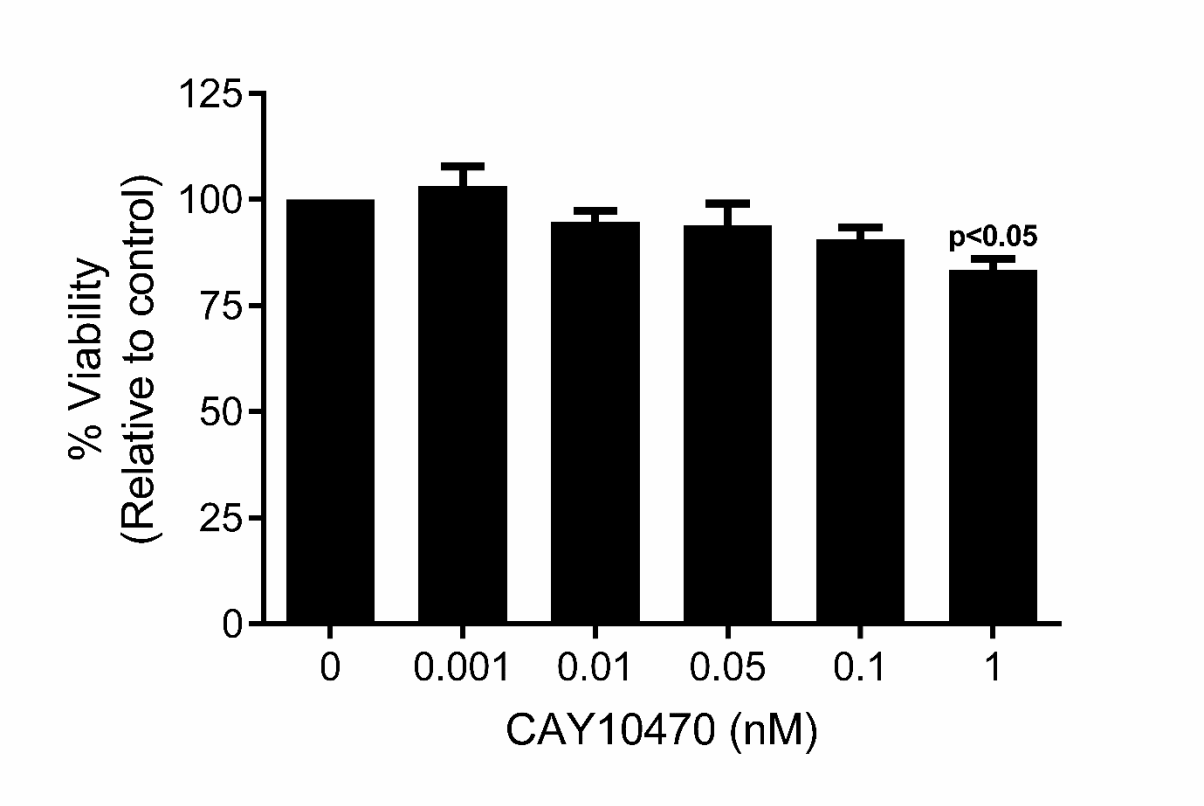

Figure 5.0. The viability of P19 cells in the presence of CAY10470
P19 cells were cultured in a 96 well plate until 60% confluent and incubated for 24 hours with medium alone or with increasing concentrations of CAY10470. Cells were incubated with medium containing 0.1mg MTT for a further 4 hours before assessing viability as described in chapter 2 section 2.5. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

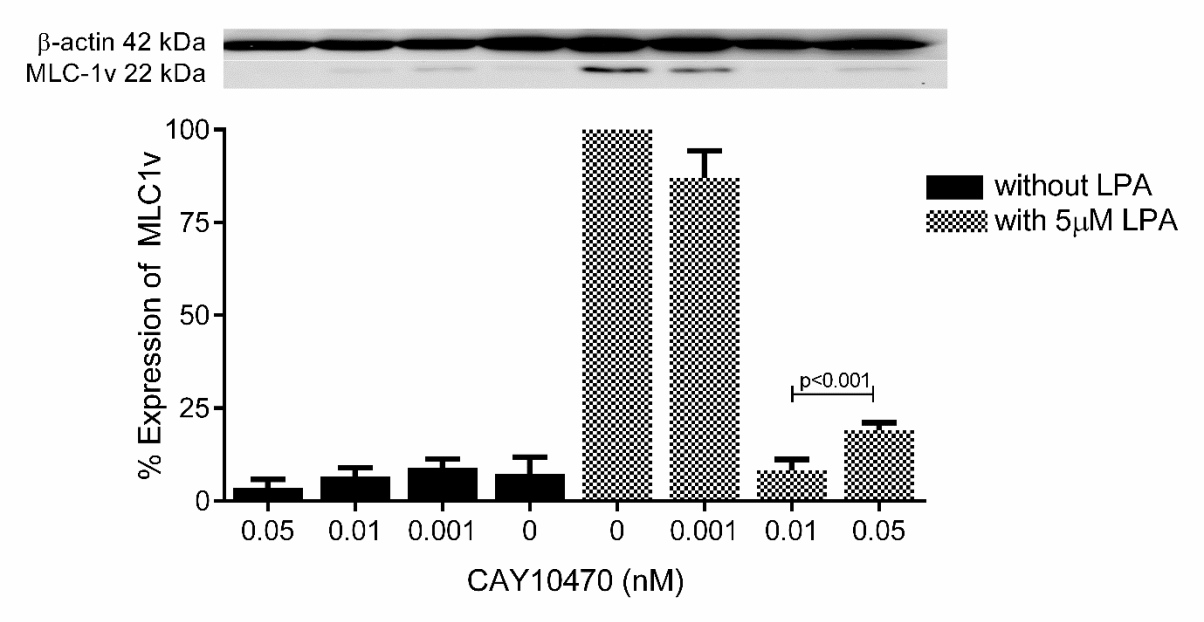

Figure 5.1. CAY10470 blocks LPA induced MLC1v expression in a concentration dependent manner

P19 cells were seeded in the absence or presence of 5μM LPA after pre-treatment with CAY10470 for 60 minutes in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process for expression of MLC1v determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments. *represents significance relative to LPA treatment alone.

5.3.2 Phosphorylation of NF-κB by LPA

To determine whether the phosphorylation of NF-κB by LPA is an early or late event the following studies were carried out. Lysates were generated on days 1, 2, 3, 4, 6, 8, and 10 of the differentiation process and as seen in Figure 5.2, NF-κB phosphorylation at the serine 536 residue was detectable on days 1 and 2 in both LPA treated and untreated cells and undetected afterwards. No difference in expression between LPA treated and untreated cells was seen throughout the differentiation process.

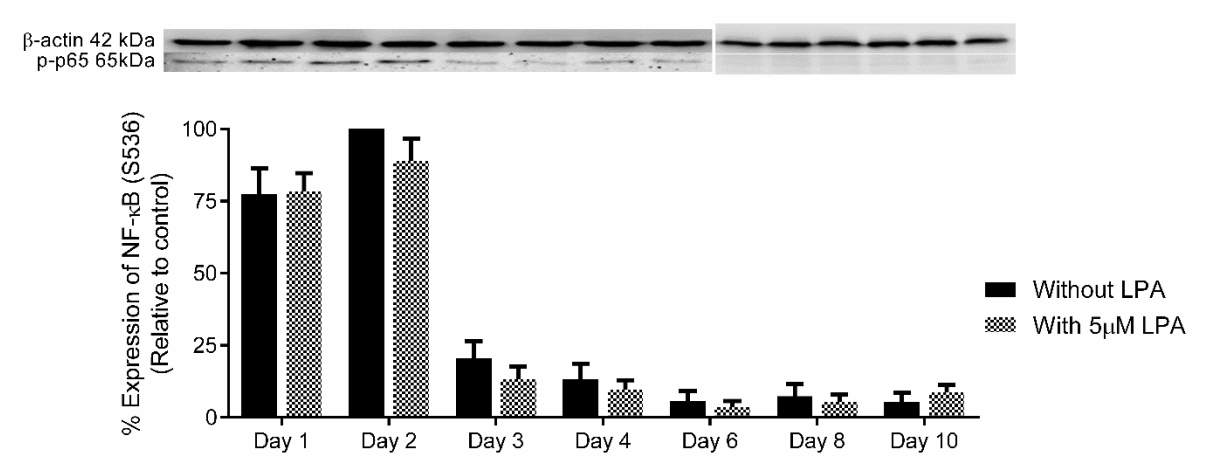

Figure 5.2. p65 at serine 536 is phosphorylated during early EB formation
P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-NF-κB p65 (Ser536) determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.3 Early phosphorylation of NF-κB by LPA

As very early activation of NF-κB at the serine 536 residue was detected in the study above, we next set out to determine if LPA activates NF-κB at serine 536 and 468 within 180 minutes of LPA stimulation. Early activation of NF-κB at both serine 536 and serine 468 residues by LPA was determined using the FACE NF-κB kit. As seen in Figure 5.3A and B, stimulation with 5μM LPA for up to 180 minutes did not significantly change the activation of NF-κB at either serine residue. Total NF-κB activity was also stable over the course of 180 minutes (Figure 5.3C). Although there was no significant change upon LPA stimulation, there was higher basal activity at the serine 468 residue compared to the serine 536 residue as seen in Figure 5.3D.

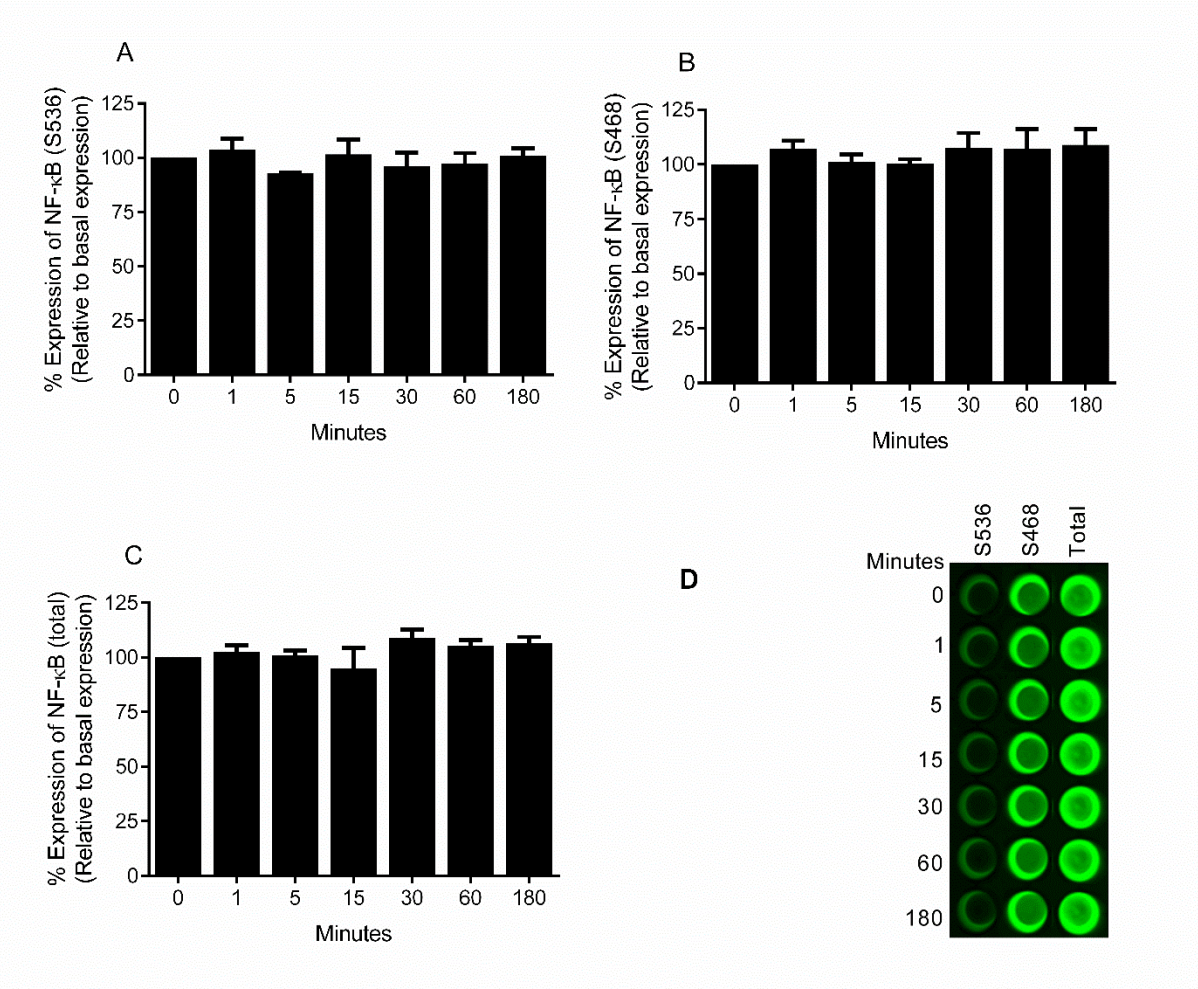

Figure 5.3. Effect of LPA on NF-κB activation in adherent P19 cells
A cell-based ELISA kit (Active Motif) was used for detecting phosphorylated and total NF-κB in LPA treated cells. 100µL of cell suspension containing $1.5x10^4$ cells was seeded into each well of a 96-well plate and incubated for 48 hours. LPA was diluted in serum-free αMEM, and the cells were treated for 1, 5, 15, 30, 60 or 180 minutes. The assay was carried out as described in section 5.2.3. Chemiluminescence was read using the CCD camera equipped Thermo myECL Imager (D). The NF-κB activation signal at Ser536 (A), Ser468 (B), or total (C), was normalised for cell number and data expressed as a percentage of basal expression (time 0). Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.4 The regulation of NF-κB in LPA treated cells by LPAR1-4, MAPKs, PKC and PI3K.

LPA receptors and specific kinases were inhibited by treating the cells with antagonists 60 minutes before stimulation with 5μM LPA for 15 minutes. As seen in Figure 5.4, the LPA receptor 4 antagonist, Suramin, increased the expression of both serine 536 (A) and serine 468 (B) residues compared to LPA treatment alone. The serine 536 residue was more sensitive to this inhibition and showed a much greater increase than the serine 468 residue which was evident but not significant. Inhibition of LPA receptors 1/3 and 2 using Ki16425 and H2L5186303 respectively resulted in approximately 30% decrease in mean phosphorylation at serine 468 (Figure 5.4B) but was without significant effect on serine 536 (Figure 5.4A). The same was observed with PD98059 and BIM-1 treatment. Inhibition of PI3K using the inhibitor LY294002 had no significant effect on either residue. The greatest effect at both residues was observed with JNK inhibition, reducing phosphorylation to ~50%. Expression of total NF-κB (Figure 5.4C) was unchanged by treatment with the inhibitors and LPA receptor antagonists.

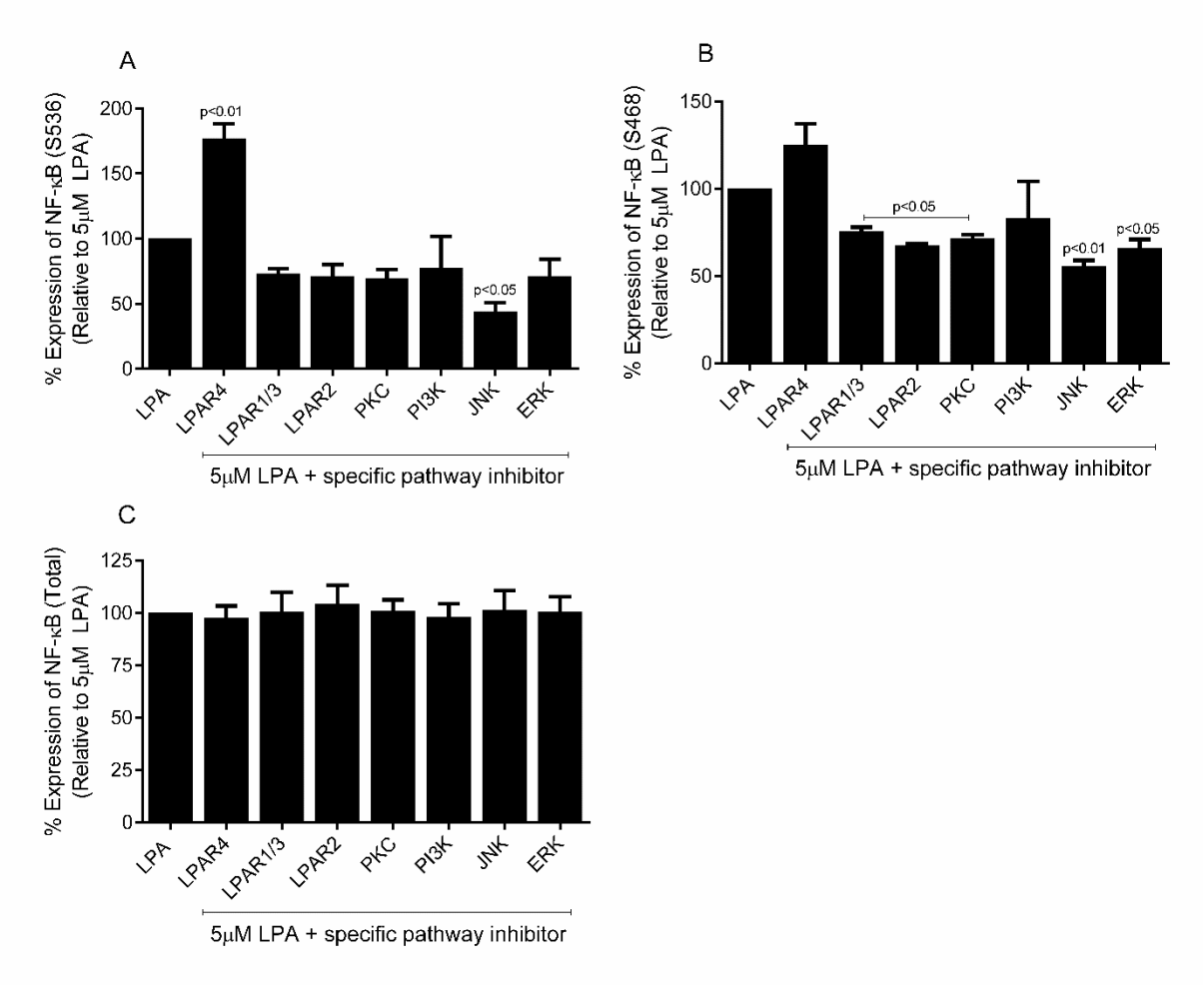

Figure 5.4. Regulation of NF-κB in LPA treated P19 cells

A cell-based ELISA kit (Active Motif) was used to determine the regulation of phosphorylated and total NF-κB in LPA treated cells. 100μL of cell suspension containing $1.5x10^4$ cells was seeded into each well of a 96-well plate and incubated for 48 hours. Basal expression was determined by stimulating the cells with 5μM LPA, prepared in serum-free αMEM, for 15 minutes. Changes in basal expression after an initial 60-minute incubation with the inhibitors for LPA receptors (LPAR 1/3 antagonized with 20μM Ki16425, LPAR 4 antagonized with 0.1mg/mL Suramin, LPAR2 antagonized with 7.5nM H2L5186303) or kinase inhibitors (ERK pathway inhibited with 20μM PD98059, JNK pathway inhibited with 10μM SP600125, PI3K inhibited with 20μM LY294002, PKC inhibited with 10μM BIM-1) followed by stimulation with LPA for 15 minutes was compared to basal expression. The assay was carried out as described in section 5.2.4. The NF-κB activation signal at Ser536 (A), Ser468 (B), or total (C) was normalised for cell number and data expressed as a percentage of basal expression. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.5 Phosphorylation of AP-1 subunits by LPA

To determine the activation of c-Fos, JunD, FosB, and c-Jun in LPA treated cells, the TransAm AP-1 family ELISA kit was used.

The phosphorylation of c-Fos (Figure 5.5) was relatively low and unchanged in cells seeded with or without 5μM LPA during EB formation. Maximum expression was seen on day 10 in both treated and untreated cells. Similarly, the phosphorylation of FosB (Figure 5.6) was also unchanged in cells seeded with or without 5μM LPA during EB formation. Expression in both treated and untreated cells was near constant until day 6 with a sustained peak on day 8 and 10. Although not statistically different, the untreated cells showed a higher FosB expression from day 6 to 10. JunD expression (Figure 5.6) was also relatively unchanged between treated and untreated cells from days 1-4. An increase in JunD phosphorylation in LPA treated cells was seen on day 6 and onwards compared to the untreated cells with a significant difference seen on day 10. Maximum activity of JunD in LPA treated cells was also seen on day 10. Phosphorylation of c-Jun (Figure 5.7) was also comparable in both treated and untreated cells on days 1-4. On day 6 a spike in cJun activity in both untreated and treated cells was seen with the latter being slightly greater. This decreased on day 8, and the decrease continued to day 10 in untreated cells but significantly increased in LPA treated cells.

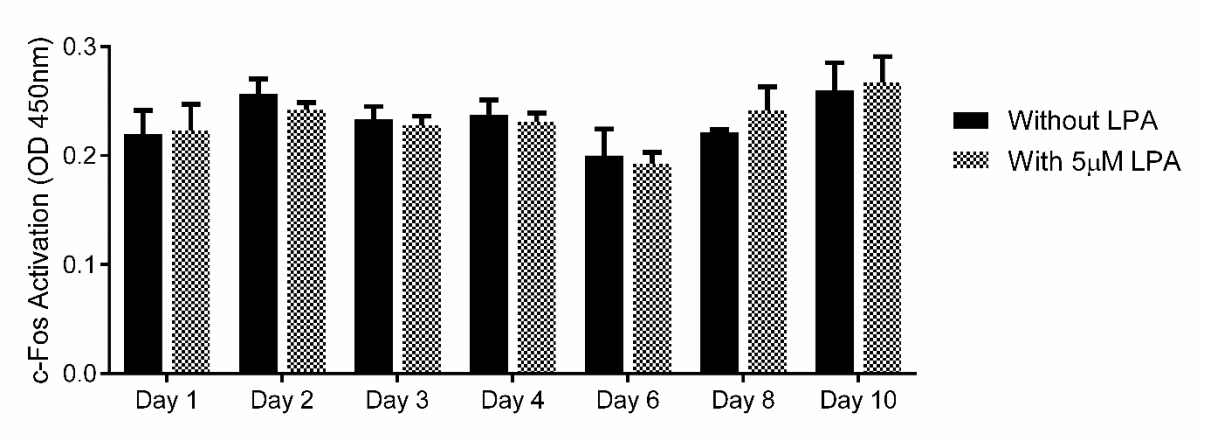

Figure 5.5. The effect of LPA on the activation of c-Fos

P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process and activation of c-Fos was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.5. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

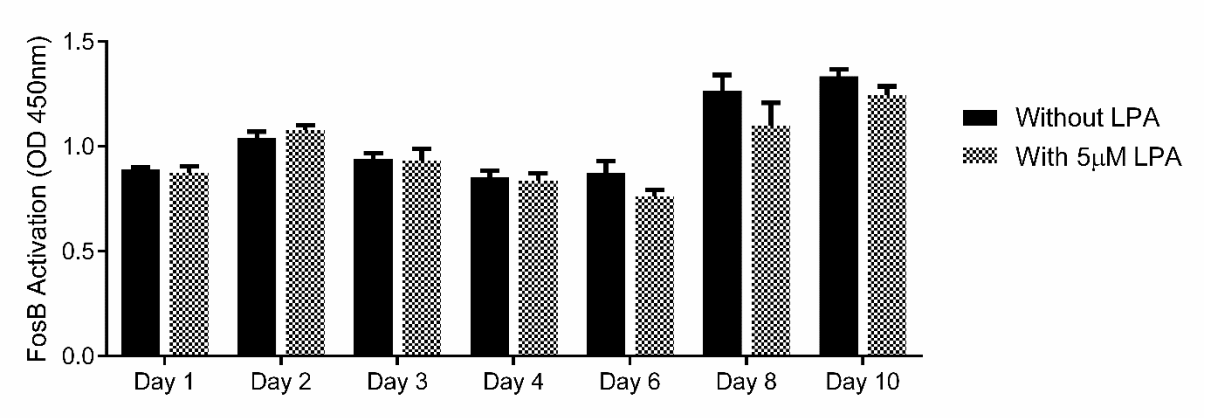

Figure 5.6. The effect of LPA on the activation of FosB

P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process and activation of FosB was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.5. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test ($\alpha=0.05$). The data represent the means ± S.E.M. of 3 experiments.

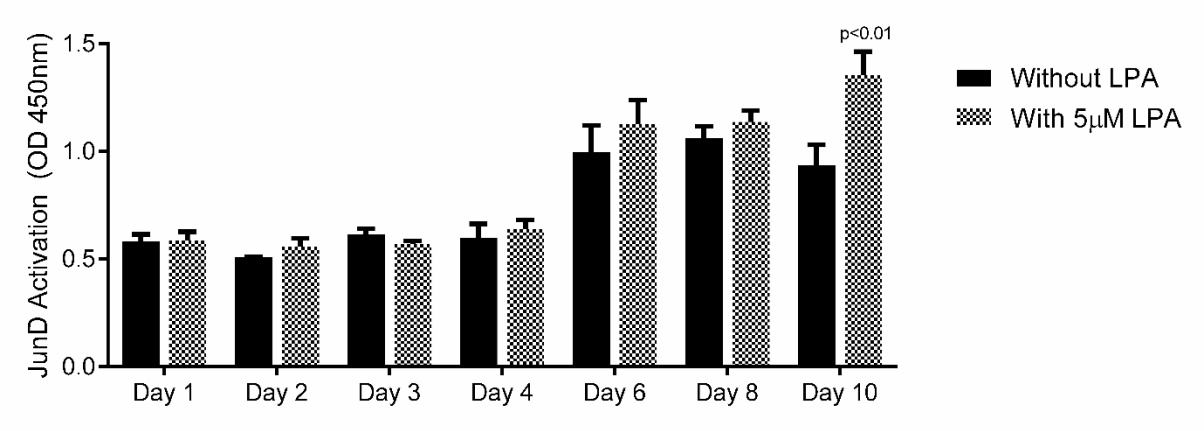

Figure 5.7. The effect of LPA on the activation of JunD

P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process and activation of JunD was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.5. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

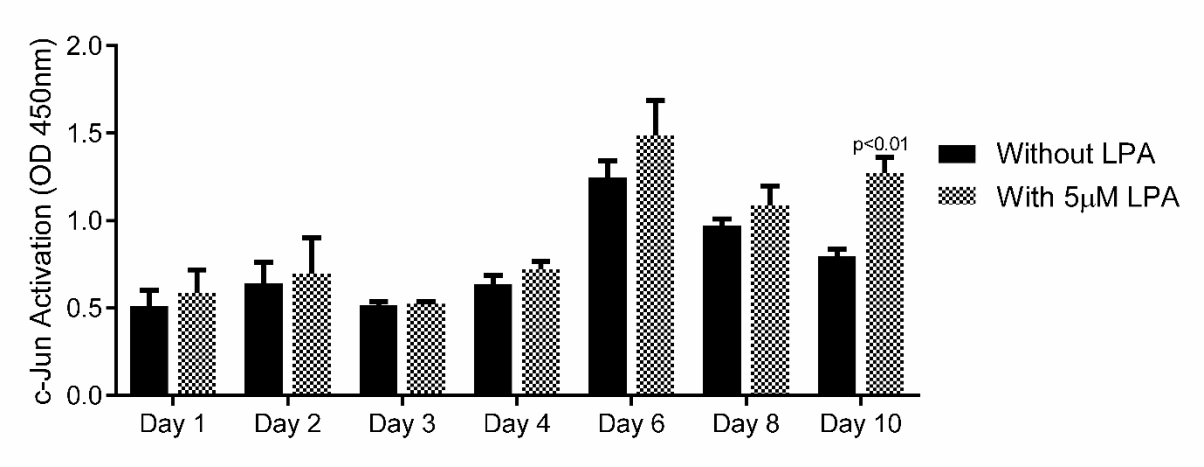

Figure 5.8. The effect of LPA on the activation of c-Jun

P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process and activation of c-Jun was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.5. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.6 Regulation of AP-1 subunits in LPA treated cells by LPAR1-4, MAPKs, PKC, PI3K, and NF-κB

5.3.6.1 c-Fos

As seen in Figure 5.9, inhibition of LPA receptor 4, 1/3, and 2 using Suramin, Ki16425, and H2L5186303 respectively did not significantly affect the activation of c-Fos. Inhibition of the PKC and PI3K kinases by BIM-1 and LY294002 respectively also did not affect the activation of c-Fos. The AP-1 subunits are well-established targets of the MAPKs and NF-κB, however, inhibition of JNK, ERK, and NF-κB by SP600125, PD98059, and CAY10470 respectively did not change the activity of c-Fos.

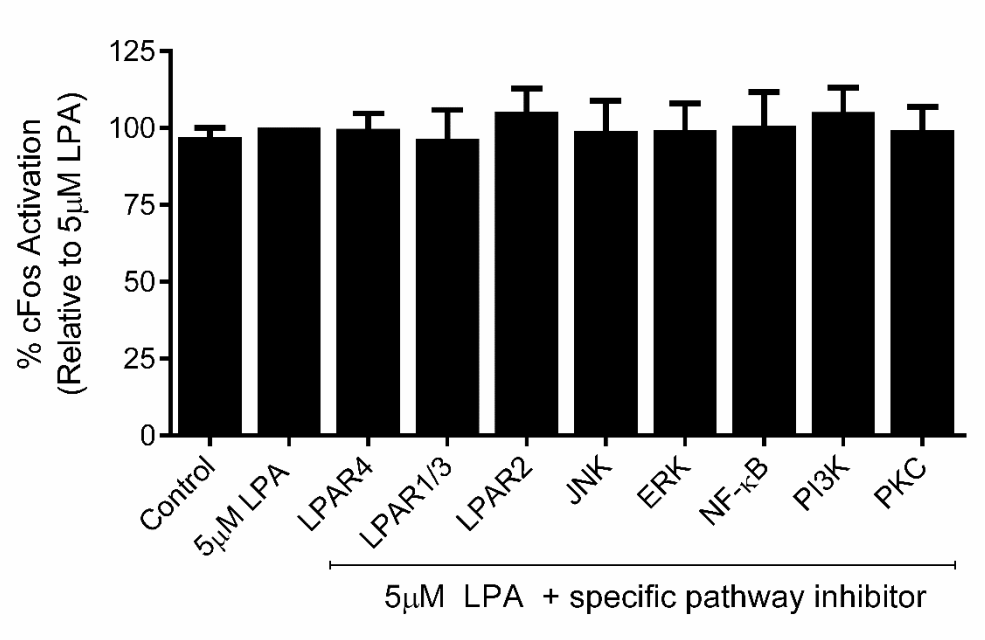

Figure 5.9. Regulation of c-Fos in LPA treated P19 cells
P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 10 of the differentiation process, and activation of c-Fos was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.6. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.6.2 FosB

As seen in Figure 5.10, inhibition of LPA receptor 4, 1/3, and 2 using Suramin, Ki16425, and H2L5186303 respectively did not significantly affect the activation of FosB. Inhibition of the PKC kinase by BIM-1 also did not change the activation of FosB. However, inhibition of PI3K by LY294002 significantly reduced the activity of FosB as did the inhibition of ERK by PD98059. Inhibition of JNK with SP600125 also left the activation of FosB unchanged. A slight decrease in FosB activation was observed with the inhibition of NF-κB by CAY10470, but this was not significant.

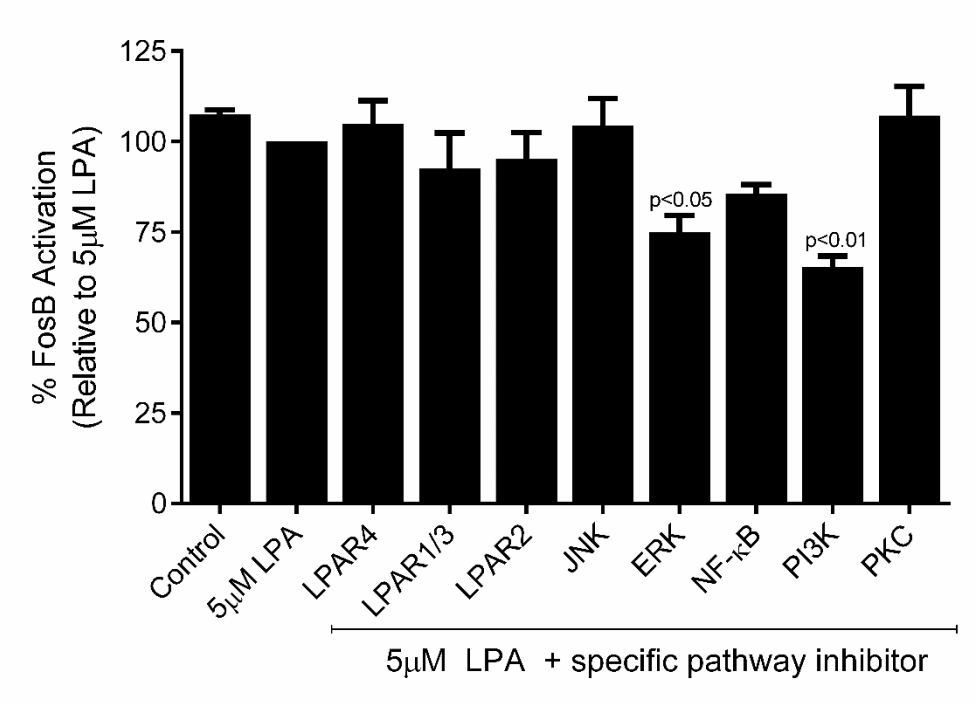

Figure 5.10. Regulation of FosB in LPA treated P19 cells

P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 10 of the differentiation process, and activation of FosB was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.6. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.6.3 JunD

As seen in Figure 5.11, inhibition of LPA receptor 4, and 1/3 using Suramin, and Ki16425 respectively noticeably decreased the activation of JunD as did the inhibition of JNK by SP600125 and PKC by BIM-1. A further decrease was seen with the inhibition of PI3K by LY294002 and NF-κB by CAY10470. The greatest decrease in the activation of JunD was achieved with the inhibition of ERK by PD98059. Inhibition of LRA receptor 2 using H2L5186303 was the only treatment that left JunD activation unchanged compared to LPA treatment alone.

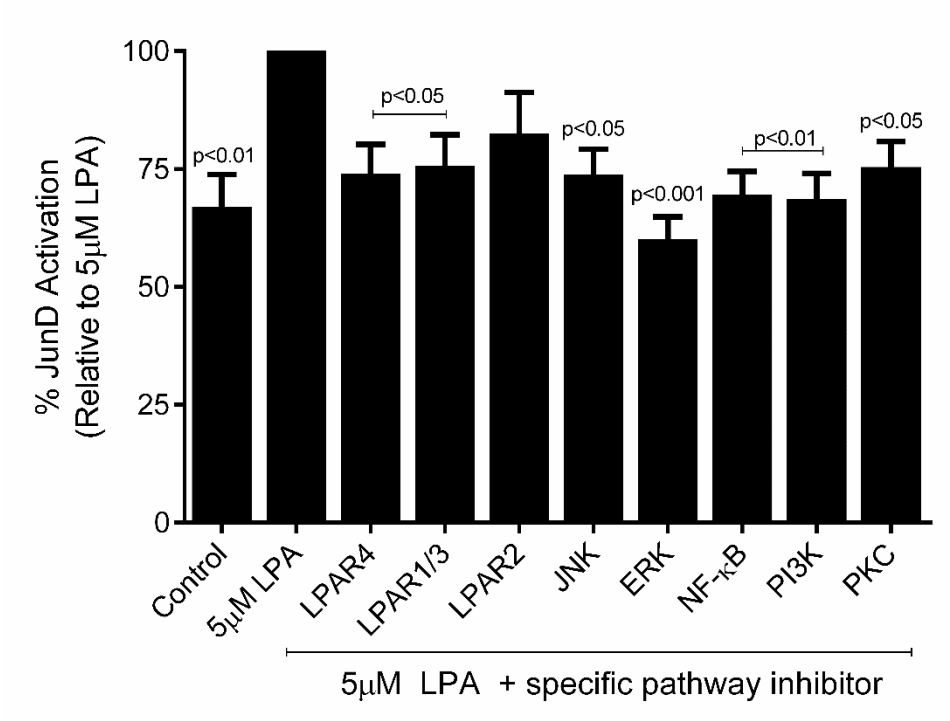

Figure 5.11. Regulation of JunD in LPA treated P19 cells

P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 10 of the differentiation process, and activation of JunD was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.6. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.6.4 cJun

As seen in Figure 5.12, each treatment inhibiting a specific kinase or LPA receptor significantly affected the activation of c-Jun compared to treatment with LPA alone. The decrease in cJun activation with the inhibition of LPA receptor 4, 1/3, and 2 was comparable amongst the group with the mean decrease between 25-35% when compared to LPA treatment alone. Inhibition of JNK also decreased cJun activation by a similar margin. A decrease of approximately 40% in the mean activation of cJun was achieved with the inhibition of PKC, PI3K, and NF-κB whereas the activation of cJun was reduced to ~50% with the inhibition of ERK.

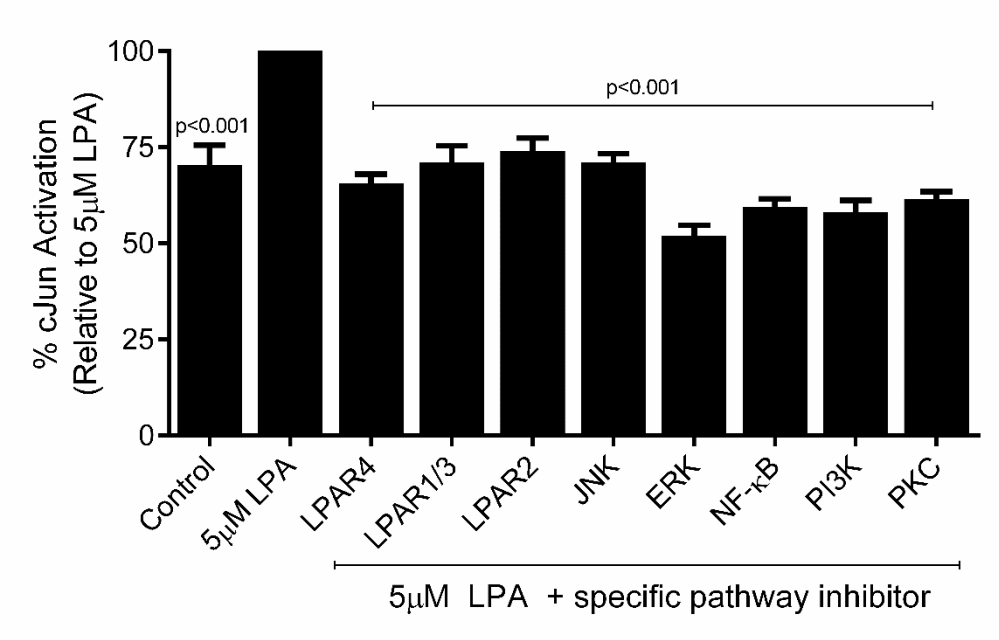

Figure 5.12. Regulation of c-Jun in LPA treated P19 cells

P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Nuclear lysates were generated on day 10 of the differentiation process, and activation of c-Jun was determined using the TransAM AP-1 family transcription factor assay kit (Active Motif) as described in section 5.2.6. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.7 Phosphorylation of ATF_2 by LPA

The expression of $pATF_2$ steadily increased in both LPA treated and untreated cells from day 1 until day 4 with comparable expression. Expression of $pATF_2$ plateaued from day 4 until 8 in both treated and untreated cells with slightly greater $pATF_2$ expression in LPA treated cells on day 4 and 6. On day 10 the expression of $pATF_2$ in untreated cells remained relatively the same as days 4-8 however the expression of $pATF_2$ approximately doubled in LPA treated cells (Figure 5.13).

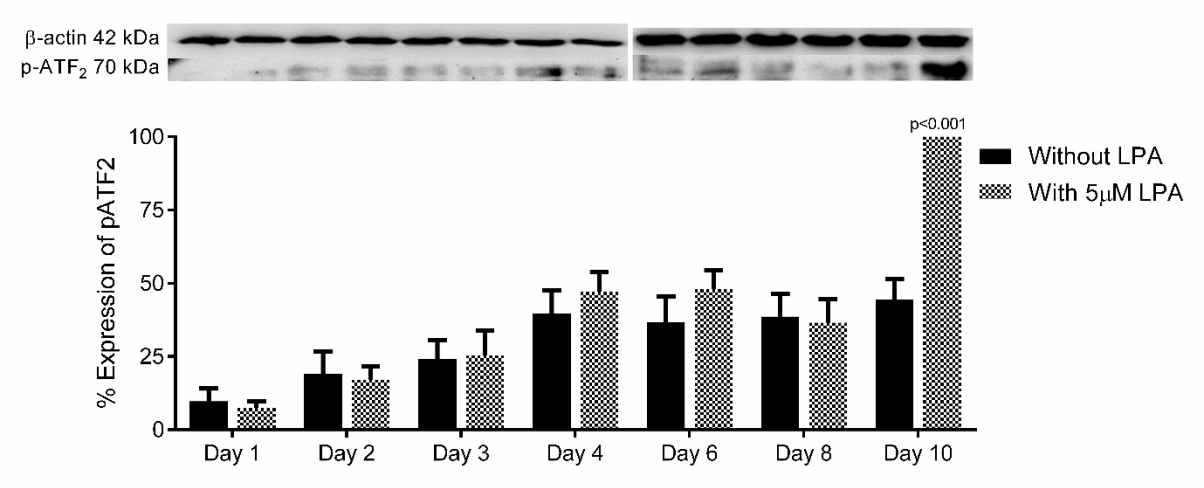

Figure 5.13. The effect of LPA on the expression of pATF2.
P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 1, 2, 3, 4, 6, 8, and 10 of the differentiation process using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-ATF2 (Thr71) determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.3.8 The regulation of $pATF_2$ in LPA treated cells by LPAR1-4, MAPKs, PKC, PI3K, and NF-κB.

As seen in Figure 5.14, each treatment inhibiting a specific kinase or LPA receptor significantly affected the phosphorylation of $pATF_2$ compared to treatment with LPA alone. The decrease in phosphorylated $pATF_2$ with the inhibition of LPA receptor 4, 1/3, and 2 was comparable amongst the group with the mean decrease of approximately 80% when compared to LPA treatment alone. Inhibition of the MAPKs, JNK and ERK, reduced the mean expression of phosphorylated $pATF_2$ by approximately 75% and 50% respectively. Inhibition of PKC also reduced mean expression by approximately 80% whereas inhibition of PI3K had no significant effect on the expression of phosphorylated $pATF_2$. A decrease of approximately two thirds in the expression of phosphorylated $pATF_2$ was achieved with the inhibition of NF-κB.

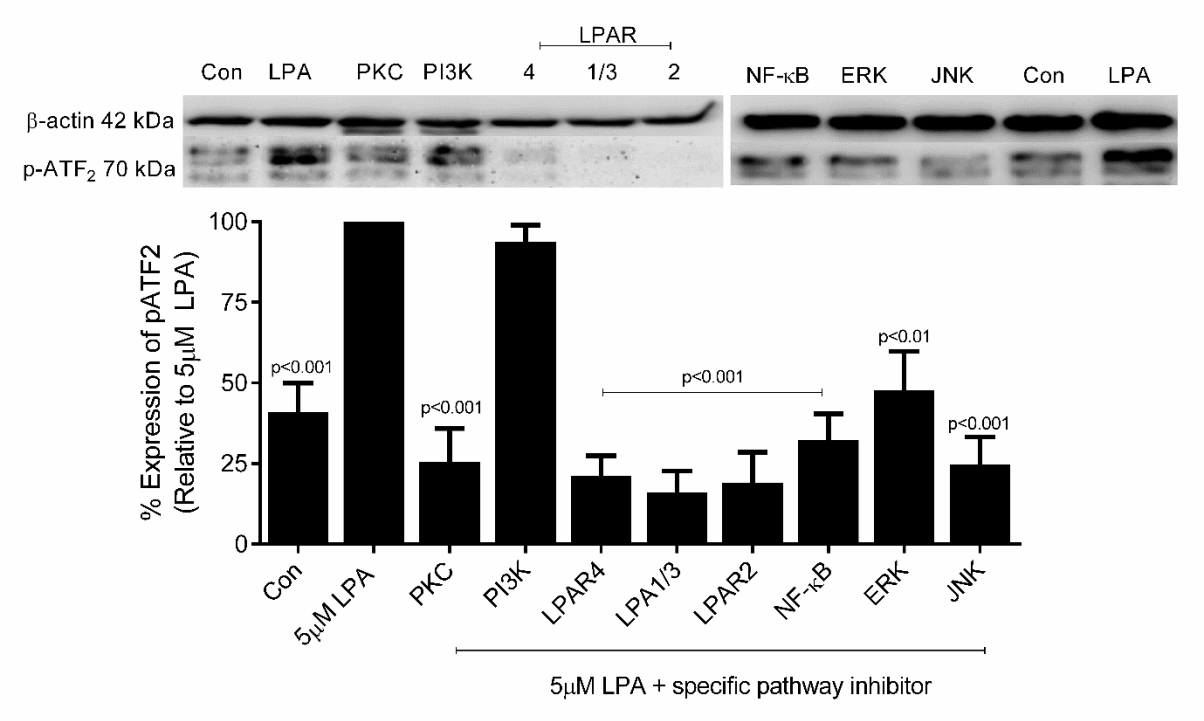

Figure 5.14. Regulation of pATF$_2$ in LPA treated P19 cells

P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-ATF$_2$ (Thr71) determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

5.4 Discussion

Little is known about the role of NF-κB in heart development or in the differentiation of stem cells into cardiomyocytes, but it has a cardioprotective ability during acute hypoxia and reperfusion injury (Misra *et al.,* 2003; Mustapha *et al.,* 2000). NF-κB activation in cardiomyocytes is also necessary for regeneration after injury in the zebrafish (Karra *et al.,* 2015). Although NF-κB subunits p50 and p65 are present throughout myocyte development (Norman, Yacoub, & Barton, 1998), studies in stem cells have been contradicting. NF-κB activity has been reported to maintain pluripotency of ihPSCs (Takase *et al.,* 2013) and hESCs (Armstrong *et al.,* 2006) while studies in mESCs suggest the up-regulation of NF-κB in differentiating cells (Kim *et al.,* 2008; Torres & Watt, 2008).

We evaluated the role of NF-κB in mediating the actions of LPA in P19 cells by blocking NF-κB activity using CAY10470 (Tobe *et al.,* 2003). This inhibitor has an IC_{50} of 7-11nM and has previously been used in the low nM range in P19 cells (Wagley *et al.,* 2013), and hiPSC derived neurons (Nekrasov *et al.,* 2016). Possible mechanisms for NF-κB inactivation by this inhibitor include indirect inhibition by blocking store-operated calcium entry (Choi *et al.,* 2006) or directly by blocking IκB-α phosphorylation (Bernard & Gallo, 2010).

Inhibition of NF-κB using 0.01nM of the inhibitor effectively blocked LPA induced expression of MLC1v on day 10 of differentiation implying that NF-κB is necessary in our model. We furthered examined the phosphorylation profile of p65 at serine 536 on days 1, 2, 3, 4, 6, 8, and 10 of the differentiation process in response to LPA and found that p65 phosphorylation occurs in both untreated and untreated cells only for the first 2 days. We next used the FACE NF-κB kit to examine the phosphorylation of p65 at

serine 536 and 468 in response to LPA over the course of 180 minutes to determine if NF-κB activation by LPA was an immediate event. Our findings indicate that, although activity is higher at serine 468, p65 activity does not increase in response to LPA. As discussed in chapter 4, this may be due to the adherent culture conditions in which the assay is conducted. We also evaluated the regulation of p65 using the same kit.

The LPA receptor 4 antagonist, Suramin, increased the expression of both serine 468 and 536 residues compared to LPA treatment alone. The serine 536 residue was more sensitive to this inhibition and showed a much greater increase than the serine 468 residue which was evident but not significant. Aside from blocking P2Y receptors, Suramin is an agonist of ryanodine receptors (Emmick *et al.,* 1994; Hohenegger *et al.,* 1996; Sitsapesan & Williams, 1996). These receptors activate NF-κB by mediating calcium release (Valdes *et al.,* 2007) and are expressed in iPSCs exposed to DMSO to initiate cardiac differentiation (Iglesias-García *et al.,* 2015).

Inhibition of LPA receptors 1/3 and 2 using the Ki16425 and H2L5186303 antagonists respectively resulted in approximately 30% decrease in phosphorylation at serine 468 but was without significant effect at serine 536. The same was observed with PD98059 and BIM-1 treatment. In the context of cardiac hypertrophy, LPAR 1/3 and ERK have been implicated in the induction of the NF-κB pathway (Chen *et al.,* 2008b). LPA also requires PKC signalling to induce NF-κB signalling in MEFs (Klemm *et al.,* 2007; Sun *et al.,* 2009), bronchial epithelial cells (Cummings *et al.,* 2004), and ovarian cancer cells (Mahanivong *et al.,* 2008), whereas PI3K signalling is dispensable (Chen *et al.,* 2008b; Klemm *et al.,* 2007) and is consistent with our results where inhibition of PI3K using the inhibitor LY294002 had no significant effect on either residue. The greatest effect at both residues was observed with JNK inhibition, reducing phosphorylation to

half. IκB proteins bind and retain NF-κB in the cytoplasm preventing translocation into the nucleus. Several kinase cascades including PKC, PKA, MAPKs, and Raf, can activate NF-κB for translocation into the nucleus (Valen, Yan, & Hansson, 2001). The upstream kinase TAK1 has been shown to regulate JNK and NF-κB, both of which cross-talk and converge on the regulation of AP-1 (Kracht, 2007).

The effects of AP-1 signalling are multi-faceted and span multiple cellular processes. This is achieved by different compositions of dimerization of Jun and Fos, and with other transcription factors including ATF2. AP-1 also acts as a co-factor for other transcription factors including GATA4 (Martin *et al.,* 2012; Schroder *et al.,* 2006; Suzuki *et al.,* 1999). c-Jun lacking murine embryos expressing the second heart field (SHF) marker Isl1 are reported to display multiple defects in cardiac development (Zhang *et al.,* 2013) supported by earlier findings of Eferl *et al.* (1999) and a study by Jahangiri *et al.* (2016) that implicated Fos-like antigen 2 in the transition of SHF progenitors to cardiomyocytes. AP-1 has been reported to be vital for the cardiac differentiation of P19 cells in the presence of DMSO (Eriksson & Leppa, 2002) and in icariin induced cardiac differentiation of mESCs (Wo *et al.,* 2008). Recently, Liu *et al.* (2017) also suggested Fos and Jun play important roles in cardiac differentiation by profiling both hiPSC and hESC cell lines during various stages of differentiation.

Eriksson and Leppa (2002) examined the induction of AP-1 in P19 cells stimulated by DMSO and reported the upregulation of c-Jun and JunD during differentiation whereas c-Fos remained steady and that this activity was necessary for cardiac differentiation of P19 cells. LPA induces AP-1 (Cook, Aziz, & McMahon, 1999; Marinissen *et al.,* 2004) and, in our experimental model, JunD and c-Jun were also upregulated in differentiating cells while c-Fos and FosB were steadily expressed in both treated and untreated cells on days 1-6 with an increasing trend on days 8 and 10. Our studies

were not extended to examine the necessity of AP-1 activity in LPA induced cardiac differentiation of P19 cells but initiated studies to understand the regulation of the AP-1 subunits in this model.

As c-Fos expression was barely detectable compared to other AP-1 subunits in response to LPA stimulation, it is reasonable that phosphorylation levels of c-Fos were unaffected by treatment with antagonists for LPA receptors 1-4, the MAPKs, PKC, PI3K, and NF-κB. FosB phosphorylation was decreased only with PD98059 and LY294002. Phosphorylated levels of JunD were brought to basal by the inhibition of LPAR4, LPAR1/3, JNK, PKC, PI3K, and NF-κB, but inhibition of the ERK pathway reduced levels to below basal. Phosphorylated levels of c-Jun were similarly brought to basal with the inhibition of LPAR4, LPAR1/3, but also LPAR2. Inhibition of NF-κB, PI3K, and PKC reduced levels to below basal. Of the MAPKs, inhibition of JNK returned phosphorylated levels to basal and inhibition of ERK had the greatest effect amongst all pathways targeted.

AP-1 forms complexes with ATF2 and is essential to cardiac differentiation in P19CL6 cells (Eriksson & Leppa, 2002; Monzen *et al.,* 2001). In our studies, the expression of phosphorylated ATF2 steadily increased during EB formation in both LPA treated and untreated cells and was sustained until day 10 in untreated cells but doubled in LPA treated cells on day 10. Phosphorylation of ATF2 was negatively affected by the inhibition of LPAR1-4 in a comparable manner, reducing the expression to approximately 20%. Inhibition of PKC, NF-κB, and JNK was slightly less effective and decreased phosphorylation by two thirds whereas inhibition of ERK only reduced expression by half. Inhibition of PI3K was without effect. Multiple pathways converge on ATF2 regulation as it is a target of SMAD and TAK1 pathways in TGF-β signalling (Sano *et al.,*1999). In mECCs, the overexpression of Noggin antagonized cardiac

differentiation in the presence of DMSO which was rescued by the addition of BMP to the culture medium as did the downstream targets of BMP signalling, SMAD 1/4 (Monzen *et al.,* 2001), and TAK1 (Monzen *et al.,* 1999).

In conclusion, NF-κB signalling is required in LPA mediated cardiac differentiation of P19 cells however p65 phosphorylation at serine 468 and 536 was unchanged in response to LPA treatment therefore further evaluation of other phosphorylation sites and subunits is required. Although the role of AP-1 in our model is yet to be identified, AP-1 subunits, c-Jun and JunD, and ATF2 were found to be regulated by NF-κB along with LPAR4, 1/3, PKC, JNK, and ERK. Inhibition of PI3K did not affect ATF2 whereas inhibition of LPAR2 did not affect JunD.

Chapter 6

The regulation of cardiac specific transcription factors in LPA mediated cardiac differentiation of P19 cells

6.1 Introduction

A complex gene regulatory network is involved in the development of the heart fields and their specification. GATA4, NKX2.5, and MEF2C are expressed in cardiac precursor cells and act in combination with several cofactors to regulate cardiac specification (Bruneau, 2013; Dodou *et al.,* 2004; Paige *et al.,* 2015; Roche, Czubryt, & Wigle, 2013). The precise regulation of these factors is elusive, and several compensatory mechanisms identified further complicate the endeavour. GATA4 activity has been suggested to be the earliest amongst several of the transcription factors, and MEF2C and NKX2.5 contain GATA sites in their enhancer region. These proteins are frequently used to assess differentiation into a cardiac fate and overexpression of GATA4, NKX2.5, or MEF2c in aggregated P19 cells induces cardiac differentiation without the addition of other factors. Overexpression of one of these factors result in the upregulation of the other two factors whereas upregulation is not seen in dominant negative GATA4 or NKX2.5 cells, and cardiac differentiation is arrested demonstrating a positive regulatory loop (Grepin, Nemer, & Nemer, 1997; Jamali *et al.,* 2001; Skerjanc et al., 1998).

Upstream signalling of these transcription factors is not well defined. MAPKs, ERK and p38, have both been implicated in their regulation and this has further been evidenced in P19 cells overexpressing noggin, a natural BMP antagonist. BMP signalling via TAK1 and possible downstream MAPK may regulate cardiac specific transcription factors as the combined overexpression of GATA4 and NKX2.5 or TAK1 (Monzen *et al.,* 1999) alone rescues the cardiac phenotype. Involvement of p38 MAPK in MEF2c nuclear localisation has also been reported (Han *et al.,* 1997; Hernandez-Torres *et al.,* 2008; Zhao et al., 1999). NKX2.5 was shown to be a direct target of the

JNK substrate NFAT and regulates NKX2.5 during cardiac differentiation with GATA4 co-ordination (Chen & Cao, 2009).

In this study, we aim to determine the expression profile of GATA, MEF2C, and NKX2.5 during LPA mediated cardiac differentiation. We further aim to evaluate the regulation of these factors by LPAR1-4, PI3K, PKC, NF-κB, and the MAPKs, as they have been found to be critical in our experimental model.

6.2 Materials and Methods

6.2.1 Determining the effect of LPA treatment on the expression of cardiac-specific transcription factors

The differentiation process was carried out as described in chapter 2 section 2.2. Lysates were generated on days 6, 8, and 10, and protein quantification was carried out as described in chapter 2 section 2.3.2 and 2.3.3 respectively. Western blot was carried out as outlined in chapter 2 section 2.4 with the following modifications. The GATA-4 and the MEF2C (Cell Signaling Technology) primary antibodies were used at a dilution of 1:1000. The anti-NKX2.5 (Abcam) primary antibody was used at dilutions of 1:1000, 1:2500, and 1:5000 in both 5% non-fat milk blocking buffer and 5% BSA blocking buffer. The anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at a dilution of 1:4000 for the detection of GATA4 and MEF2C and at dilutions of 1:4000 and 1:6000 for the detection of NKX2.5 Statistical analysis was done as described in chapter 2 section 2.7.

6.2.2 Determining the regulation of cardiac-specific transcription factors in LPA treated cells by LPA receptors 1-4, MAPKs, PKC, PI3K, and NF-κB

The differentiation process was carried out as described in chapter 2 section 2.2. EBs were seeded in the presence of antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) for 60 minutes before treatment with 5μM LPA. Lysates were generated on day 10 of the differentiation process as described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as described in chapter 2 section 2.3.3 and 2.4 respectively with the following modifications: The GATA-4 and the MEF2C (Cell Signaling Technology) primary antibodies were used at a dilution of 1:1000. The anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at a dilution of 1:4000. Statistical analysis was done as described in chapter 2 section 2.7.

6.3 Results

6.3.1 Expression of cardiac-specific transcription factors in LPA treated cells.

Lysates were generated on days 6, 8, and 10 of the differentiation process. The expression of both GATA4 (Figure 6.0) and MEF2C (Figure 6.1) was below 25% in both untreated and treated cells on day 6. GATA4 expression remained near this basal expression on days 8 and 10 in untreated cells, but the expression in LPA treated cells increased substantially on day 8 and was sustained until day 10. In comparison, the increase in MEF2C expression was time-dependent in LPA treated cells with a maximum expression on day 10. The untreated cells exhibited an unchanged basal expression from days 6-10.

NKX2.5 expression could not be determined as further optimisation with the primary antibody was required. The anti-NKX2.5 primary antibody was used at dilutions of 1:1000, 1:2500, and 1:5000 in both 5% non-fat milk blocking buffer and 5% BSA blocking buffer. A dilution of 1:1000 of primary antibody and 1:4000 dilution of the anti-rabbit IgG, HRP-linked secondary antibody is shown in Figure 6.2A. Too many unspecific bands were obtained, and a further dilution of 1:2500 of the primary antibody with the previously used dilution of secondary antibody was attempted (B). A further dilution of 1:5000 of primary antibody and 1:6000 dilution of the anti-rabbit IgG, HRP-linked secondary antibody is shown in Figure 6.2C.

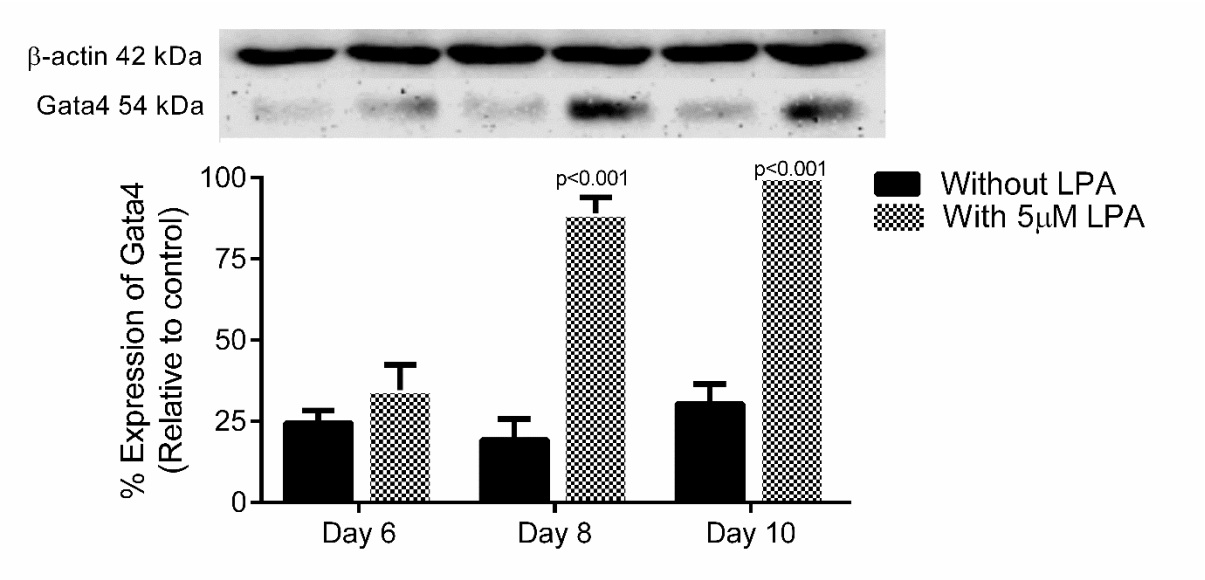

Figure 6.0. LPA induces GATA4 expression in a time-dependent manner
P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in tissue grade 6-well plates for a further 6 days. Lysates were generated on days 6, 8, and 10 of the differentiation process for expression of GATA4 determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

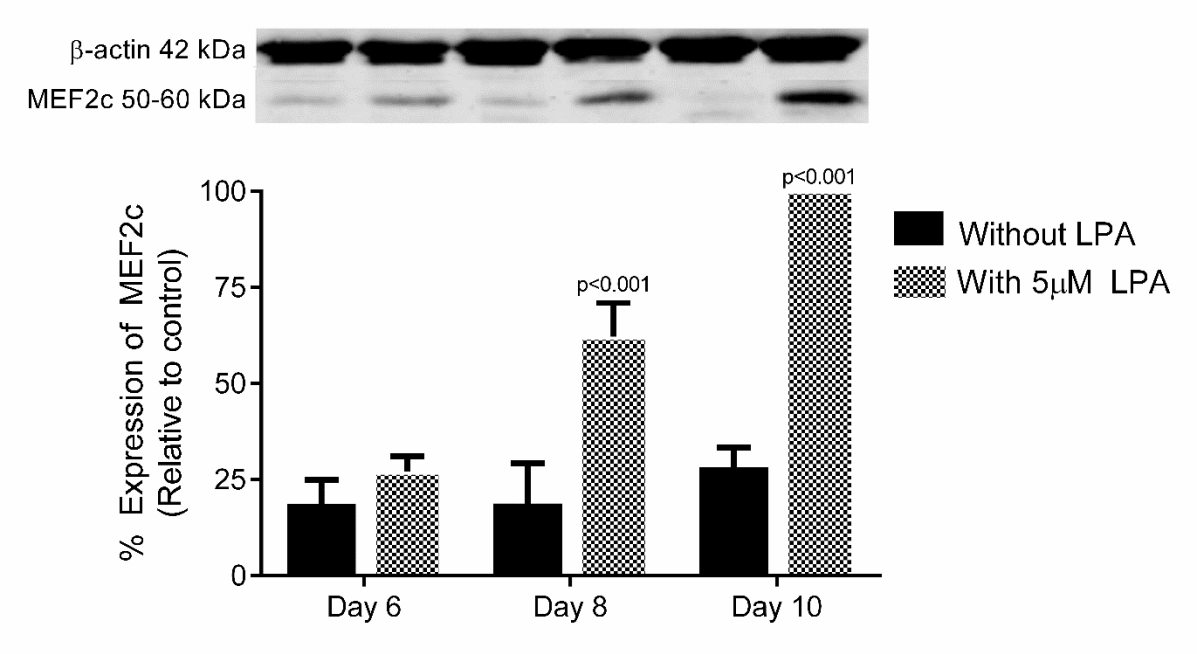

Figure 6.1. LPA induces MEF2C expression in a time-dependent manner
P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in tissue grade 6-well plates for a further 6 days. Lysates were generated on days 6, 8, and 10 of the differentiation process for expression of MEF2C determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

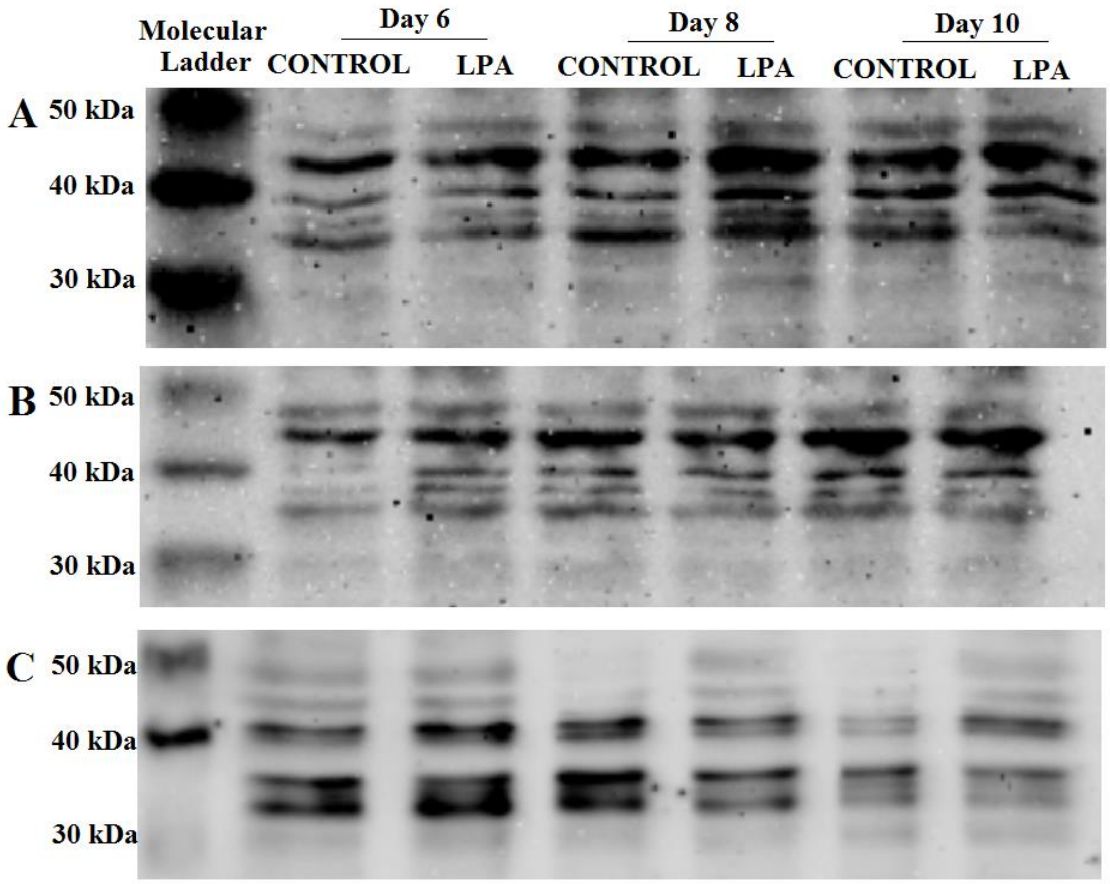

Figure 6.2. Optimization of the NKX2.5 antibody

P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in tissue grade 6-well plates for a further 6 days. Lysates were generated on days 6, 8, and 10 of the differentiation process for expression of NKX2.5 determined by western blot. Optimization of the primary antibody was attempted using a dilution of 1/1000 (A) and 1/2500 (B) with a 1/4000 dilution of the anti-rabbit IgG, HRP-linked secondary antibody and with a dilution of 1/5000 and 1/6000 for the primary and secondary antibody (C).

6.3.2 The regulation of cardiac specific transcription factors in LPA treated cells by LPA receptors 1-4, MAPKs, PKC, PI3K, and NF-κB

6.3.2.1 GATA4

From the study above, maximum GATA4 expression was observed on day 10 of the time points analysed, therefore, we next set out to determine if the inhibition of the LPA receptors or specific kinases changed this expression.

As seen in Figure 6.3, the expression of GATA4 was reduced by the inhibition of each of the LPA receptors. Inhibition of receptor 4 by Suramin was the most effective and reduced levels to below basal. Inhibition of receptor 1/3 and 2 by Ki16425 and H2L5186303 respectively were similar, with a mean expression of approximately 25% compared to cells treated with only LPA (5μM). Inhibition of PKC by BIM-1 also reduced expression levels to below basal while inhibition of PI3K by LY294002 showed a mean decrease of approximately 30%. Inhibition of JNK by SP600125 reduced the mean expression of GATA4 by over 80%. The decrease in GATA4 expression by the inhibition of ERK by PD98059, although significant, was less effective than JNK. Like ERK, the inhibition of NF-κB also reduced GATA4 expression by half compared to cells treated with LPA alone.

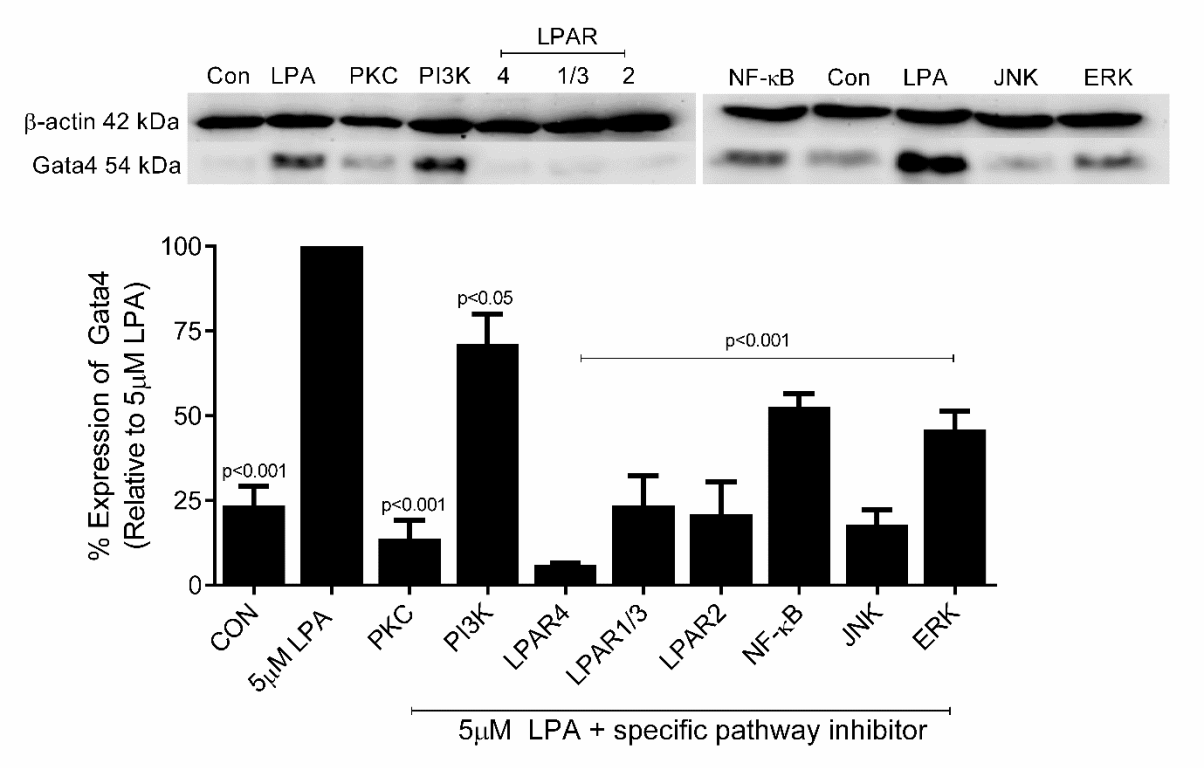

Figure 6.3. Regulation of GATA4 in LPA treated P19 cells
P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of GATA4 determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

6.3.2.2 MEF2C

From the study above, maximum MEF2C expression was observed on day 10 of the time points analysed, therefore, we next set out to determine if the inhibition of the LPA receptors or specific kinases changed this expression.

As seen in Figure 6.4, the expression of MEF2c was reduced by the inhibition of LPA receptor 4, 1/3, and 2 by approximately 65%, 80% and 65% respectively compared to LPA treatment alone. Inhibition of PKC and PI3K also significantly decreased the expression of MEF2c with BIM-1 reducing levels to basal and LY294002 reducing expression by approximately 40%. The inhibition of JNK also brought expression levels back to basal whereas inhibition of ERK caused 50% less expression compared to LPA treatment alone. Inhibition of NF-κB did not significantly change the expression of MEF2C.

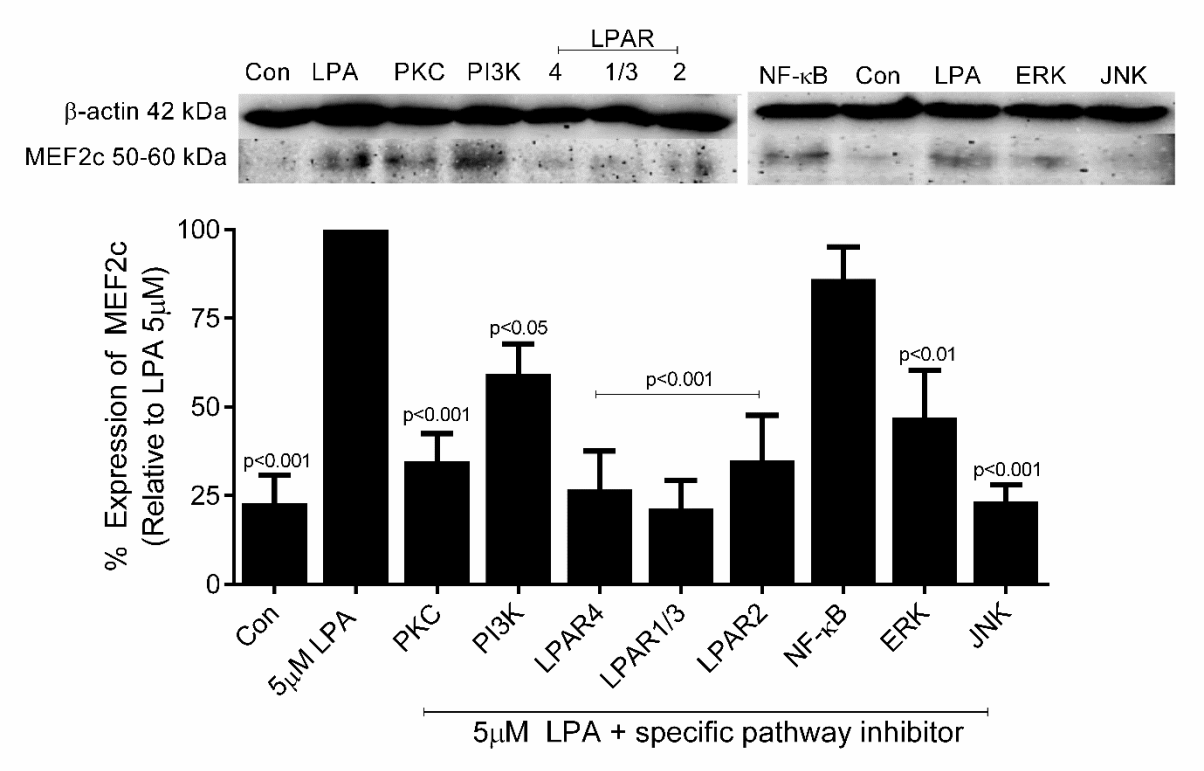

Figure 6.4. Regulation of MEF2C in LPA treated P19 cells
P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and inhibitors for PI3K (20μM LY294002), PKC (10μM BIM-1), ERK (20μM PD98059), JNK (10μM SP600125) and NF-κB (0.01nM CAY10470) in non-tissue grade Petri dishes for 4 days. EBs were then transferred and cultured in 6-well tissue grade plates for a further 6 days. Lysates were generated on day 10 of the differentiation process using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of MEF2C determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

6.4 Discussion

The expression of cardiac-specific transcription factors is used to validate the commencement of the cardiac program. However, the *in vitro* regulation of these factors is not entirely known, but transcription factors typically have multiple levels of control and often work in conjunction with other cell type dependent and independent transcription factors.

The expression of GATA4, MEF2C, and NKX2.5 in P19 cells exposed to 1% DMSO is typically evident after cells have been transferred to tissue culture plates (Harada *et al.,* 2008; Yilbas *et al.,* 2014). Therefore, we evaluated the expression of these factors in response to LPA treatment on day 6, 8, and 10 of the differentiation process. The expression of GATA4 in untreated cells remained relatively stable through days 6-10 with less than 30% expression. A similar expression was seen in LPA treated cells on day 6, with a dramatic increase in expression observed on day 8 which was sustained on day 10. The expression of MEF2C in untreated cells also remained stable on days 6, 8 and 10 with less than 30%. MEF2C expression in LPA treated cells on day 6 was comparable to the expression in untreated cells and gradually increased until day 10.

As maximum expression of GATA4 and MEF2C was observed on day 10 of the time points analysed, we next sought out to determine if treatment with kinase-specific inhibitors altered this expression. Inhibition of LPAR4 by Suramin abolished the expression of GATA4 and MEF2C whereas inhibition of LPAR2 by H2L5186303 and inhibition of LPAR1/3 by Ki16425 brought the expression of both to the basal level. These findings correlate to findings discussed in chapter 3 where treatment with Suramin (0.1mg/mL) resulted in the MLC1v expression being below that of basal

expression and treatment with H2L5186303 (7.5nM) and Ki16425 (20μM) returned levels of MLC1v to basal.

Inhibition of PKC by BIM-1 inhibited GATA4 and MEF2C expression by 85% and 65% respectively. PKC mediated GATA4 regulation has been observed by groups studying signalling in the context of cardiac hypertrophy (Wang *et al.,* 2005) however, in the context of cardiac differentiation, PKC regulation of GATA4 has not been well supported (Gallagher *et al.,* 2014).

Treatment with LY294002 had marginal effects on GATA4 and MEF2C, with approximately 30% and 40% reduction in expression respectively. Bekhite *et al.* (2011) have provided data suggesting inhibition of PI3K inhibits GATA4 expression when induced by VEGF. Naito *et al.* (2003) also demonstrated that 20μM LY294002 inhibited GATA4 expression in P19 cells. Tamir and Bengal (2000) reported MEF2C regulation by PI3K.

Inhibition of NF-κB decreased GATA4 expression by 50% whereas little effect was seen on the expression of MEF2C. Using a zebrafish model to study cardiac regeneration, Karra *et al.* (2015) found NF-κB activity was responsible for the induction of GATA4 regulatory sequences and that over-expression of GATA4, with NF-κB inhibition, was not enough to restore the regeneration program suggesting multifactorial regulation by NF-κB. Treatment with ERK inhibitor, PD98059, also decreased the expression of GATA4 and MEF2c by half which is line with the results of Ling *et al.* (2017) who used 50μM PD98059 to block the upregulation of GATA4 and MEF2C observed by the inhibition of miR155-3p.

JNK inhibition by SP600125 returned expression of GATA4 and MEF2C to the basal level. These findings are supported by the work of Geng, Liu, and Chen (2014) who

used BMP9 to induce cardiac differentiation and the expression of both GATA4 and MEF2C was negatively affected by JNK inhibition.

The same evaluation was attempted for NKX2.5 however further optimisation of the primary antibody is needed. All primary antibodies used were purchased from Cell Signaling Technology other than MLC1v and anti-NKX2.5. The predicted band size is 34kDa, but on the data sheet accompanying the product, the band shown was not in this range, therefore, making it more difficult to identify the appropriate band on the blots generated in our studies. Currently, Cell Signaling Technology does not have an anti-NKX2.5 antibody that reacts with mouse samples, therefore, the antibody from Abcam needs to be further optimised or sourced from elsewhere to accurately determine the expression profile of NKX2.5 using western blotting. Other studies have indicated that NKX2.5 expression is in line with the expression of GATA4 and MEF2C or lags slightly (Grepin, Nemer, & Nemer, 1997; Harada *et al.,* 2008; Naito *et al.,* 2003; Yilbas *et al.,* 2014).

In conclusion, GATA4 and MEF2C expression is upregulated on day 8 and 10 in response to LPA treatment. This expression is inhibited by LPAR1-4 antagonists which correspond to the effect of these antagonists on MLC1v expression. PKC inhibition also decreased GATA4 and MEF2C expression to basal whereas PI3K inhibition resulted in 30-40% decrease in GATA4 and MEF2C expression. NF-κB and ERK inhibition resulted in a 50% loss of GATA4 expression while only the latter had a similar effect of MEF2C expression. JNK inhibition abolished both GATA4 and MEF2C expression.

Chapter 7

The regulation of the Hippo pathway in LPA mediated cardiac differentiation of P19 cells

7.1 Introduction

Members of the Hippo pathway were individually identified earlier, but the pathway was pieced and studied together in the Drosophila only in the mid-1990's and the mammalian homologs of the core proteins comprising the pathways have since been identified (Del Re, 2014; Meng, Moroishi, & Guan, 2016). The mammalian hippo pathway contains mammalian Ste20-like 1 and 2 (MST1/2) and large tumour suppressor 1 and 2 (LATS1/2) Ser/Thr proteins that require co-activator regulatory proteins, Salvador homolog 1 (SAV1) and Mps One binder 1 (MOB1). MST1/2, activated by SAV1, phosphorylates both LATS1/2 and MOB1 (Callus, Verhagen, & Vaux, 2006; Chan *et al.,* 2005; Praskova, Xia, & Avruch, 2008). Active MOB1 increases the binding affinity to LATS1/2 resulting in full activation. The Yes-associated protein (YAP) is a transcriptional modulator that is retained in the cytoplasm when phosphorylated by MOB1 bound LATS1/2 (Huang *et al.,*2005). YAP was initially recognised to regulate proliferation, but several other roles have since emerged that are both cell and tissue-specific (Dong *et al.,* 2007; Zhao *et al.,* 2008; Zhao *et al.,* 2010). The earliest cell fate decision during embryonic development involves YAP, and co-activated TEAD (Nishioka *et al.,* 2009), and later regulates organ size, including the heart, and cardiomyocyte proliferation (Zhou *et al.,* 2015b). YAP has been shown to regulate other transcription factors including Smads (Ferrigno *et al.,* 2002) and Tbx5 (Murakami *et al.,* 2005). Yu *et al.* (2012) found that YAP was phosphorylated following serum starvation but rapidly dephosphorylated upon serum addition in multiple cell lines. They discovered that LPA, found in serum, inhibits LATS1/2, acting in parallel with MST1/2, thereby allowing YAP to translocate into the nucleus.

In this study, we aimed to evaluate the effect of LPA treatment on YAP phosphorylation and further determine the regulation of YAP and MOB1 by LPAR1-4 and JNK.

7.2 Materials and Methods

7.2.1 Determining the expression of phosphorylated YAP and MOB in LPA treated cells

The differentiation process was carried out as described in chapter 2 section 2.2 and lysates were generated 18, 24, 48 and 72 hours after initial seeding as described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as specified in chapter 2 section 2.3.3 and 2.4 respectively with the following modifications: Blocking buffer was made with 5% BSA in TBS-T, and Phospho-YAP (Ser397), Phospho-YAP (Ser127), and Phospho-MOB1 (Thr35) primary antibodies (Cell Signaling Technology) were used at a dilution of 1:1000 and anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at dilution of 1:4000. SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

7.2.2 Determining the regulation of YAP and MOB1 in LPA treated cells by JNK and LPAR1-4

The differentiation process was carried out as described in chapter 2 section 2.2. EBs were seeded in the presence of antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) and JNK (10μM and 20μM SP600125) for 60 minutes before treatment with 5μM LPA. Lysates were generated 24 and 48 hours after initial seeding as described in chapter 2 section 2.3.2 for analysis of phosphorylated MOB1 and phosphorylated YAP respectively. Protein quantification and western blot were carried out as specified in chapter 2 section 2.3.3 and 2.4 respectively with the following modifications: Blocking buffer was made with 5% BSA in TBS-T, and Phospho-YAP (Ser397), Phospho-YAP (Ser127), and Phospho-MOB1 (Thr35) primary antibodies (Cell Signaling Technology) were used at a dilution of

1:1000 and anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at dilution of 1:4000. SuperSignal West Dura Substrate (Thermo Fisher) was used for protein detection. Statistical analysis was done as described in chapter 2 section 2.7.

7.2.3 Determining the expression of Hippo pathway members in LPA treated cells

The differentiation process was carried out as described in chapter 2 section 2.2 and lysates were generated 18, 24, 48 and 72 hours after initial seeding as described in chapter 2 section 2.3.2. Protein quantification and western blot were carried out as specified in chapter 2 section 2.3.3 and 2.4 respectively with the following modifications: LATS1, MOB1, MST1, MST2, SAV1, and TAZ primary antibodies (Cell Signaling Technology) were used at a dilution of 1:1000 and anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling Technology) was used at dilution of 1:4000.

7.3 Results

7.3.1 The effect of LPA on phosphorylated YAP and MOB

EBs were seeded in the absence or presence of 5μM LPA and lysates were collected 18, 24, 48, and 72 hours after initial seeding. YAP was comparably phosphorylated at serine residue 127 (Figure 7.0) in both treated and untreated cells 18 hours after initial seeding. In untreated cells, this was sustained for 48 hours but radically decreased in LPA treated cells at 24 and 48 hours whereas phosphorylation was barely detectable at 72 hours in both groups. A similar trend was observed at the serine 397 residue (Figure 7.1). The untreated cells maintained phosphorylated YAP for 48 hours and then rapidly decreased at 72 hours whereas phosphorylation gradually decreased in LPA treated cells. The difference between untreated and treated cells at 24 hours, although significant, was not as drastic at serine 397 as serine 127 however, results were comparable at 48 and 72 hours.

Moderate phosphorylation of MOB1 (Figure 7.2) was seen at 18 hours and was comparable between untreated and treated cells. Phosphorylation decreased in LPA treated cells but increased in untreated cells at 24 hours. Both groups showed a comparable decrease in phosphorylation at 48 hours and 72 hours with a minuscule increase at the latter time point.

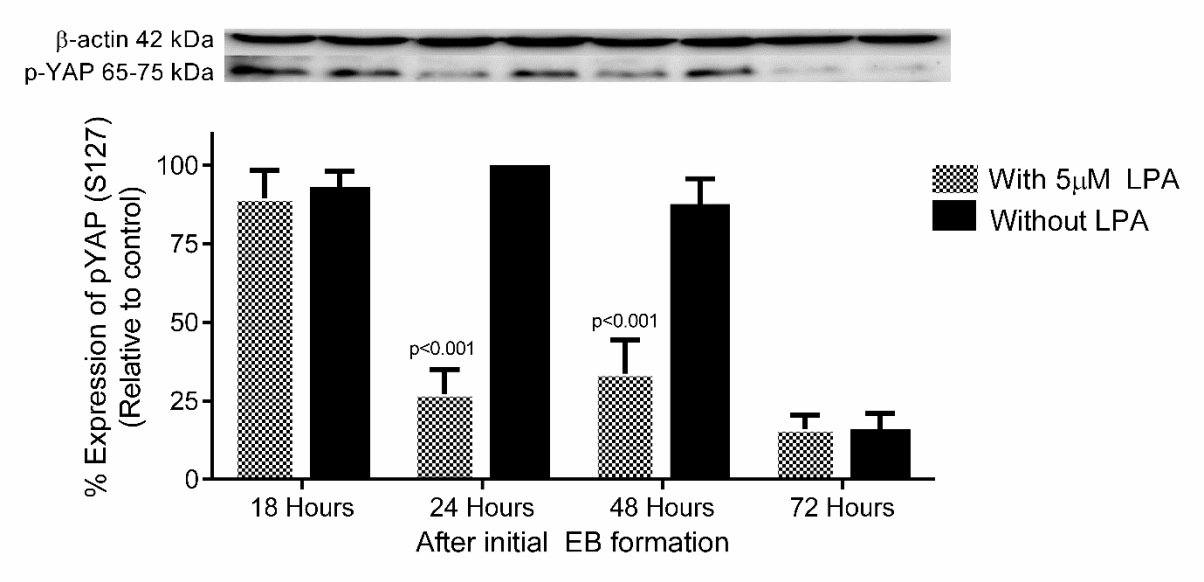

Figure 7.0. The effect of LPA treatment on p-YAP (Ser127) in P19 cells
The P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 18, 24, 48 and 72 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-YAP (Ser127) determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

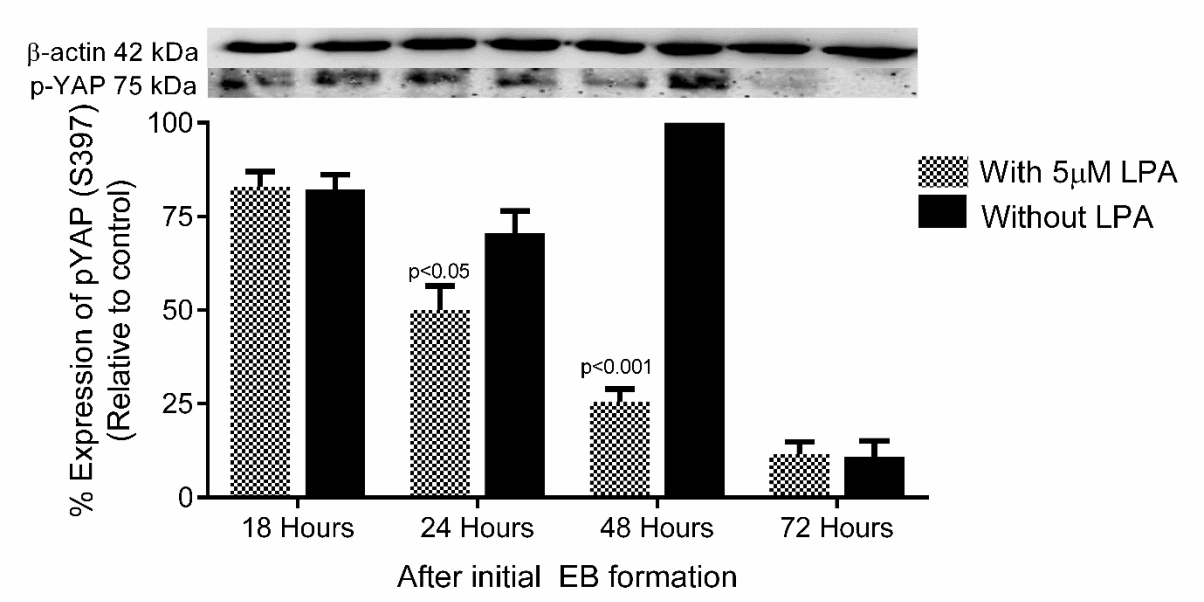

Figure 7.1. The effect of LPA treatment on p-YAP (Ser397) in P19 cells
The P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 18, 24, 48 and 72 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-YAP (Ser397) determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

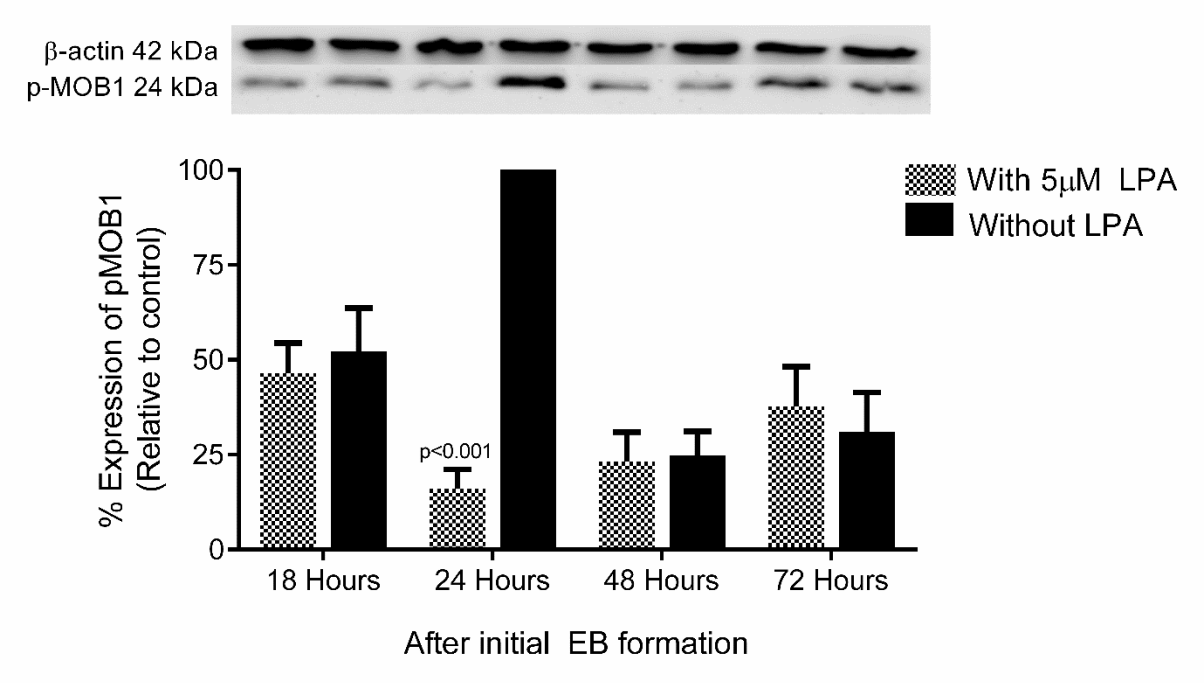

Figure 7.2. The effect of LPA treatment on p-MOB1 in P19 cells
The P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes for 18, 24, 48 and 72 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-MOB1 determined by western blot. Statistical comparisons were performed by two-way ANOVA with Bonferroni post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

7.3.2 Regulation of YAP and MOB in LPA treated cells by JNK and LPAR1-4

From the studies above, the difference in YAP activity at serine 127 and serine 397 between LPA treated and untreated cells was greatest at 48 hours after initial seeding, therefore, this time point was used to determine changes in activity by the presence of LPAR antagonists or JNK inhibition. YAP serine 127 phosphorylation (Figure 7.3) was maintained in the untreated cells and cells treated with 10 or 20μM SP600125. Phosphorylation decreased in LPA treated cells and was unaffected by inhibition of LPAR4 by 0.1mg/mL Suramin treatment. Inhibition of LPAR2 by 7.5nM H2L5186303 also had minimal effect on phosphorylation whereas inhibition of LPAR1/3 by 20μM Ki16425 caused a significant increase in phosphorylation compared to LPA treatment alone. Untreated and SP600125 (10 and 20μM) treated cells displayed the same increased phosphorylation at serine 397 (Figure 7.4). 5μM LPA treatment alone and with Suramin also exhibited similarly decreased phosphorylation at serine 397 as serine 127. LPAR1/3 inhibition by Ki16425 significantly increased phosphorylation, and unlike at serine 127, serine 397 phosphorylation was maintained with the inhibition of LPAR2 by H2L5186303 although not as predominantly as with Ki16425 treatment.

Unlike YAP, the greatest difference in MOB activity between LPA treated and untreated cells was 24 hours after initial seeding, therefore, this time point was used to determine changes in activity by the presence of LPAR antagonists or JNK inhibition. MOB phosphorylation (Figure 7.5) at 24 hours in response to LPAR1-4 and JNK inhibition was similar to YAP serine 397. Untreated and cells treated with SP600125 (10 or 20μM) maintained phosphorylation whereas LPA treatment alone and with Suramin decreased phosphorylation. Inhibition of LPAR2 and LPA1/3 by H2L5186303 and Ki16425 respectively increased phosphorylation compared to LPA treatment alone, however, phosphorylation with treatment using the latter was higher in comparison.

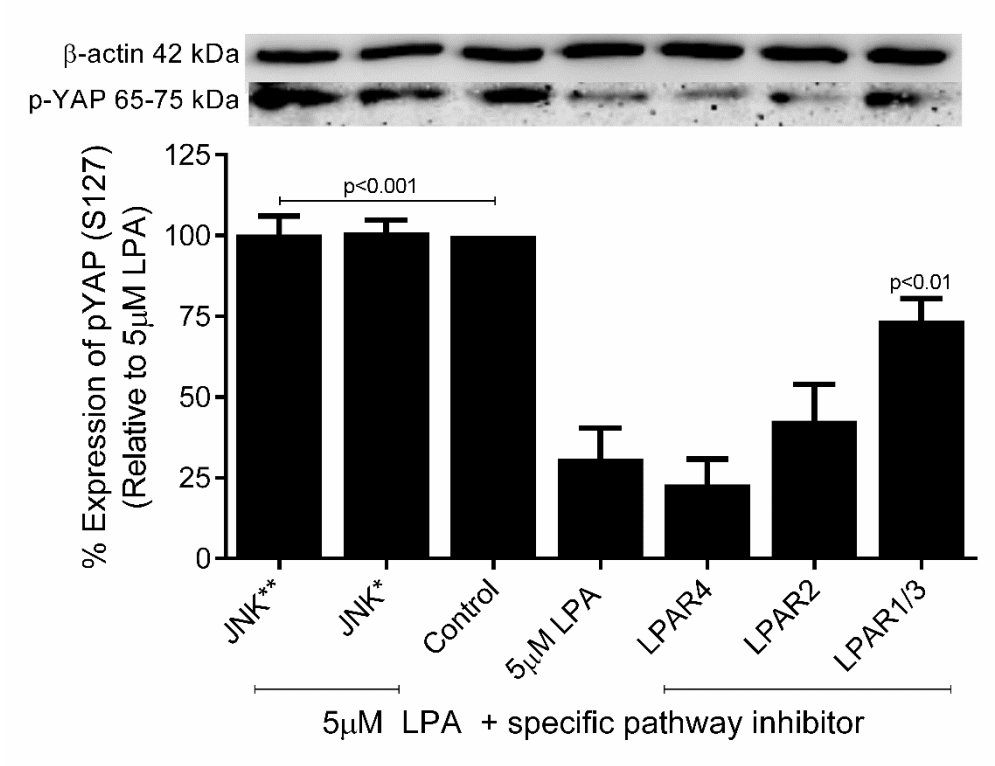

Figure 7.3. Regulation of p-YAP (Ser127) in LPA treated P19 cells
P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) or JNK inhibitor (*10μM or **20μM SP600125) in non-tissue grade Petri dishes for 48 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-YAP (Ser127) determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

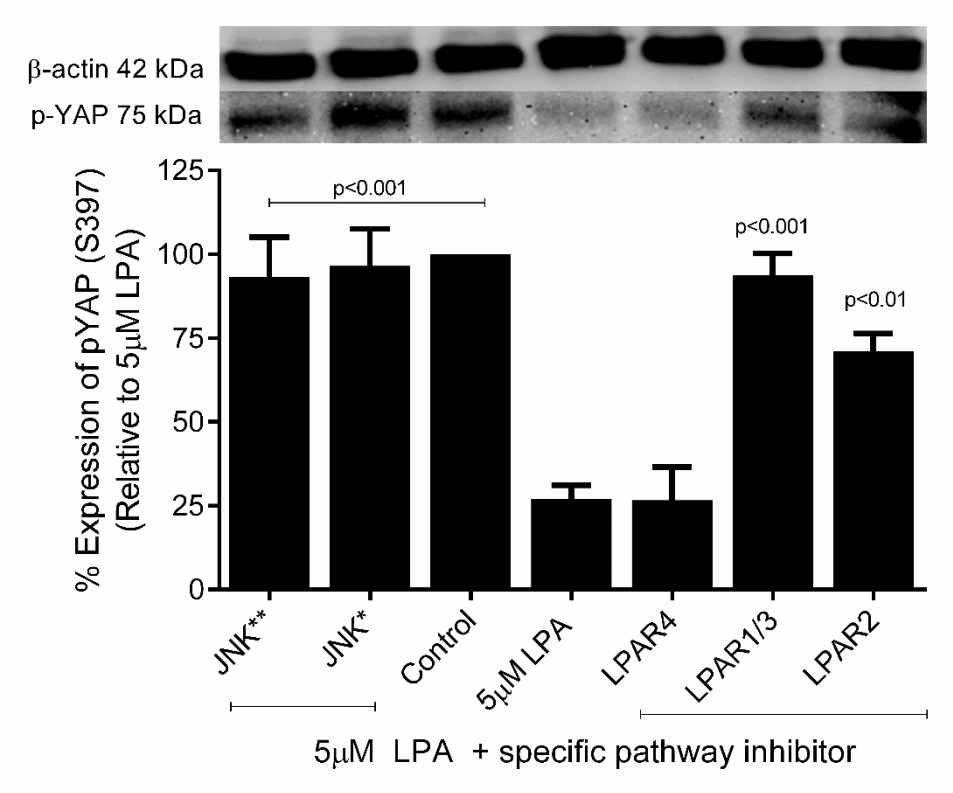

Figure 7.4. Regulation of p-YAP (Ser397) in LPA treated P19 cells
P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) or JNK inhibitor (*10μM or **20μM SP600125) in non-tissue grade Petri dishes for 48 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-YAP (Ser397) determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

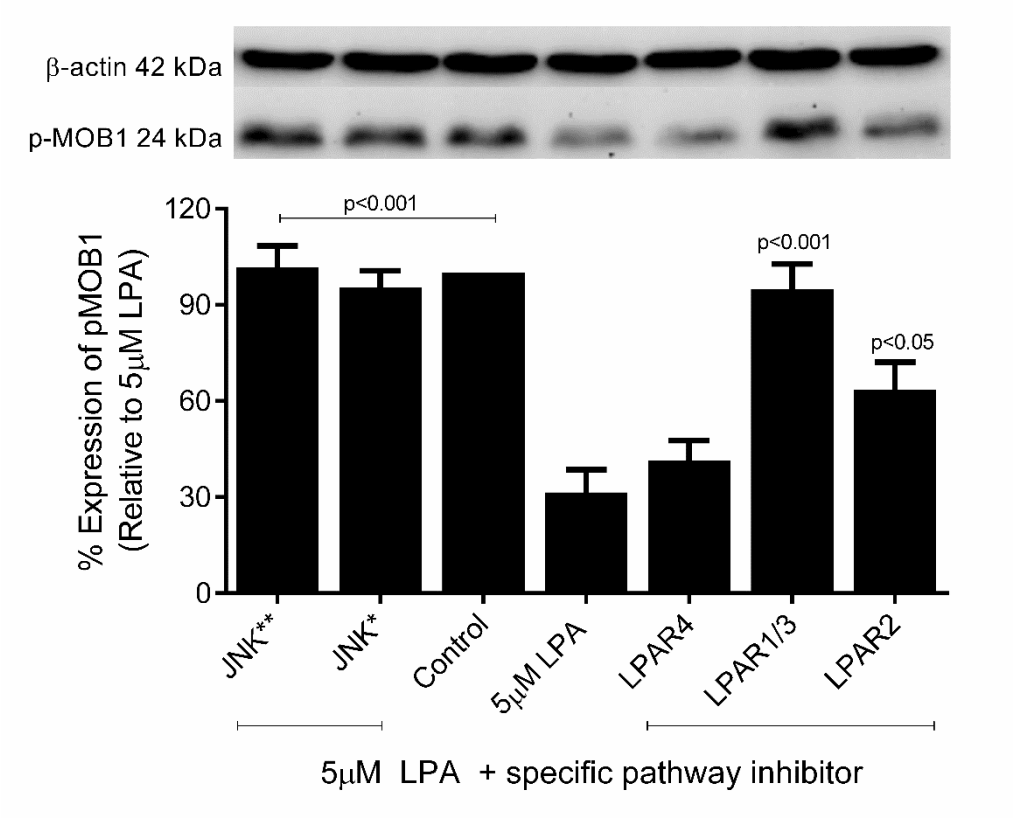

Figure 7.5. Regulation of p-MOB1 in LPA treated P19 cells

P19 cells were seeded in the presence of 5μM LPA after a 60-minute pre-treatment with antagonists for LPA receptor 1/3 (20μM Ki16425), 2 (7.5nM H2L5186303), and 4 (0.1mg/mL Suramin) or JNK inhibitor (*10μM or **20μM SP600125) in non-tissue grade Petri dishes for 24 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of phospho-MOB1 determined by western blot. Statistical comparisons were performed by one-way ANOVA with Dunnett's post hoc test (α=0.05). The data represent the means ± S.E.M. of 3 experiments.

7.3.3 Expression of Hippo pathway members

The total protein expressed for each member of the Hippo cascade was also determined at 18, 24, 48, and 72 hours after EB formation with and without LPA treatment. LATS1, SAV1, MOB1, and MST1 were unchanged at the time points assessed however MST2 was undetectable. Total TAZ was also unchanged throughout, but a decrease was observed in LPA treated cells after 72 hours. Experiments conducted to determine the changes in TAZ expression were preliminary and need to be further expanded to verify this trend and significance (Figure 7.6).

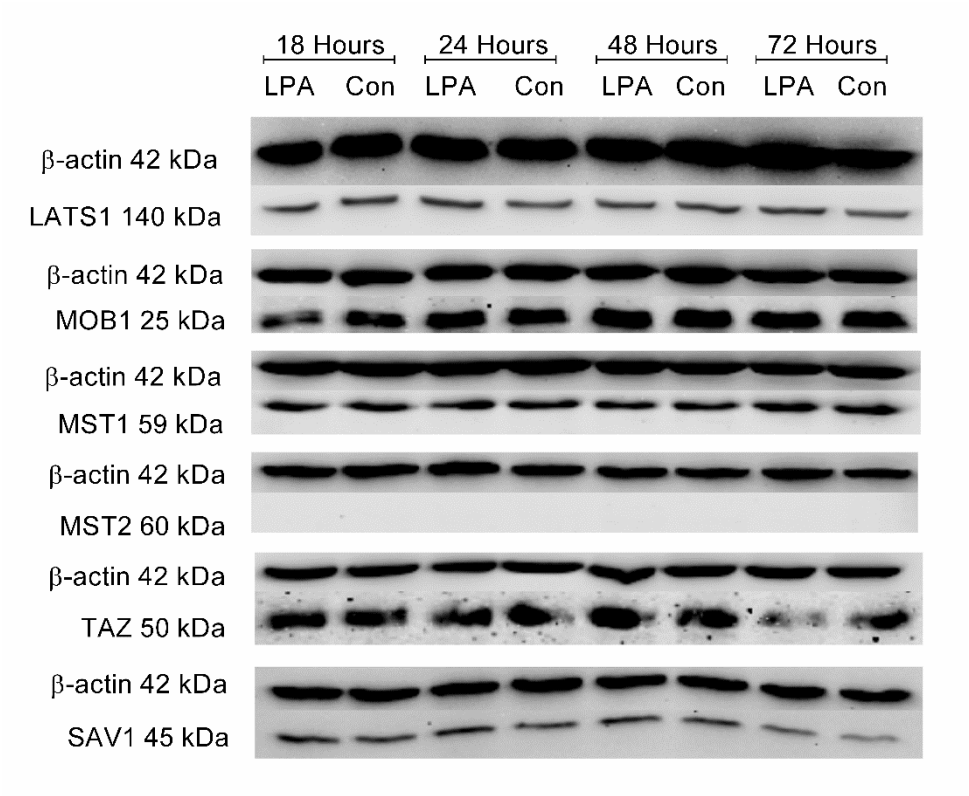

Figure 7.6. The effect of LPA treatment on kinases of the Hippo cascade
The P19 cells were seeded in the absence or presence of 5μM LPA in non-tissue grade Petri dishes and cultured in suspension for 18, 24, 48 and 72 hours. Lysates were generated using 1x RIPA buffer, as described in chapter 2 section 2.3.2, for expression of LATS1, MOB1, MST1, MST2, SAV1, and TAZ as determined by western blot. The data represent 3 individual experiments except for the latter, which represents 2 experiments.

7.4 Discussion

Soon after birth, the heart grows by increased cardiomyocyte size instead of cardiomyocyte proliferation (Ahuja, Sdek, & MacLellan, 2007; Li *et al.,* 1996; Maillet, van Berlo, & Molkentin, 2013). The loss of mitotic potential in cardiomyocytes prevents endogenous cardiac repair post-ischaemic injury. Efforts to identify cell cycle regulators capable of promoting cardiomyocyte proliferation have demonstrated a key role for the Hippo pathway. Evolutionarily conserved from the Drosophila to human, the Hippo pathway controls organ size including that of the heart (Zhou *et al.,* 2015b).

The main effector of the Hippo pathway is the Yes-associated protein (YAP), typically regulated through MOB activated LATS. In addition to regulating organ size, this kinase cascade is involved in early embryonic development, stem cell pluripotency, and differentiation (Aylon *et al.,* 2014; Li *et al.,* 2013; Posfai & Rossant, 2016).

Phosphorylation of YAP at serine 127 or 381 by LATS tags the protein for cytoplasmic sequestration by binding to 14-3-3 and ubiquitination dependent degradation respectively. In 2012, two independent groups identified S1P and LPA responsible for YAP activation (Miller *et al.,* 2012; Yu *et al.,* 2012). Yu *et al.* (2012) demonstrated rapid, although transient, de-phosphorylation of YAP, at serine 127 and 381, and nuclear localisation of YAP in response to stimulation by serum after serum starvation in several different cell lines. Furthermore, the serum component driving the nuclear localisation of YAP was found to be LPA, and this effect was unchanged by growth factors including FGF, EGF, insulin, and PDGF, or inhibitors for MEK, PI3K, mTOR, and p38.

Our findings, consistent with those of Yu *et al.* (2012), suggest YAP has a transient window of de-phosphorylation between 24-48 hours after commencing the differentiation process in the presence of 5μM LPA. We determined the

phosphorylation profile of YAP at serine residues 127 and 397 during EB formation in LPA treated and untreated cells. As reported by Zhao *et al.* (2010) the serine 397 residue of the YAP protein isoform 1 corresponds to the serine 381 residue of the YAP protein isoform 2. EBs collected 18 hours after initial seeding exhibited high phosphorylation at both serine residues irrespective of treatment. Phosphorylation was maintained in untreated cells for 48 hours and decreased rapidly at 72 hours at both sites. In LPA treated cells, phosphorylation at serine residue 127 decreased rapidly much earlier, at 24 hours, to below 30% compared to untreated cells whereas phosphorylation dropped by less than 50% at serine 397 at the same time point but dropped further at 48 hours to 25% with stable expression at serine 127. Expression levels were below 20% after 72 hours at both sites and equivalent in LPA treated and untreated cells. Further investigation is required to evaluate the possibility of YAP degradation. This was initiated by looking at changes in total YAP expression, however, time constraints did not allow for full optimisation of the antibody. Studies were also undertaken to determine the cellular localisation of YAP in LPA treated and untreated cells. This was to establish the involvement of the Hippo pathway in LPA induced cardiac differentiation of P19 cells. However, due to prolonged mycoplasma contamination, time constraints did not permit for these studies to be repeated after the contamination had been identified and resolved.

LPA is a ligand for multiple GPCRs initiating a complex signalling network that is difficult to fully decipher as the expression of GPCRs is cell-type specific and bound to an array of Gα proteins. Multiple Gα subunits, in the order of 12/13>q>i/o, have been identified to be potent inhibitors of the Hippo pathway and de-phosphorylation of YAP has been known to occur through LPA receptors 1 and 3 relayed via Rho

GTPases to inhibit LATS whereas Gs signalling induces YAP phosphorylation (Cai and Xu, 2013; Jeong *et al.,* 2013; Yu *et al.,* 2012).

In line with these studies, our finding also suggests that LPA receptors 1 and 3 are largely responsible for regulating YAP activity. Treatment with Ki16425 abolished YAP de-phosphorylation by LPA, with a greater effect at serine 397. However, our findings also suggest a partial role for LPA receptor 2. As with Ki16425, treatment with H2L5186303, had a greater effect at serine 397, increasing phosphorylation to approximately 70%, compared to LPA treatment alone but only approximately 40% at serine 127. Treatment with Suramin yielded no change in expression compared to LPA treatment alone possibly because LPA receptor 4 utilises Gs signalling (Lee *et al.,* 2007).

JNK and YAP are well-defined regulators of multiple cellular processes, and JNK activation of YAP (Grusche *et al.,* 2011; Shaw *et al.,* 2010; Staley & Irvine, 2010; Sun & Irvine, 2011; Sun & Irvine, 2013) is context dependent. JNK mediated regulation of YAP occurs upon mechanical stress and promotes the proliferation of epithelial cells through inactivation of LATS1 by LIMD1 (Codelia, Sun, & Irvine, 2014). In the context of UV irradiation, JNK directly phosphorylates YAP, further promoting UV induced apoptosis (Tomlinson *et al.,* 2010). Aside from JNK, other kinases critical to cardiac differentiation also regulate YAP including Wnt/β-catenin, PKC, and ERK (Heallen *et al.,* 2011; Imajo *et al.,* 2012; Konsavage *et al.,* 2012; Tomlinson *et al.,* 2010; Wang *et al.,* 2013).

The expression profile of pYAP in P19 cells exposed to LPA discussed above, correlated to the phosphorylation of JNK, discusses in chapter 4. We found interruption of the JNK pathway using SP600125 disrupted cardiac differentiation and proliferation

in a dose-dependent manner. As YAP has been implicated in both differentiation and proliferation and found to be activated by LPA and JNK we furthered our investigation to determine the effect of SP600125, both 10μM and 20μM, on YAP activation at both serine 127 and 397 residues in EBs treated with 5μM LPA for 48 hours. We found that Inhibition of JNK maintained phosphorylation levels equivalent to untreated cells at both serine residues irrespective of the concentration of SP600125 used. As insufficient EB proliferation is seen with 20μM SP600125 treatment within 48 hours of EB seeding, and equivalent phosphorylation of YAP was observed irrespective of the concentration of SP600125 as stated above, it is unlikely that YAP alone is involved in JNK mediated proliferation of EBs however, it is important to investigate YAP activity in the presence of 20μM SP600125 prior to 48 hours and the effect of JNK inhibition on other kinases implicated in EB proliferation which was not done here due to time constraints. YAP may be involved in regulating JNK mediated cardiac differentiation of P19 cells in the presence of LPA. YAP phosphorylation was not determined between 48 and 72 hours, however if YAP phosphorylation in untreated cells was sustained for several hours after the 48-hour time point examined, it may be possible that the lack of cardiac differentiation, observed with SP600125 treatment 48 hours after initial seeding, is due to continued YAP phosphorylation in untreated cells and de-phosphorylation of YAP in LPA treated cells that is disrupted upon JNK inhibition.

Hippo-dependent phosphorylation of YAP requires the full activation of LATS by the binding of phosphorylated MOB and studies have indicated LATS modulation by LPA but also de-phosphorylation of MOB by LPA in HSG cells (Hwang *et al.,* 2014). In P19 cells we found that MOB1 is moderately phosphorylated 18 hours after EB formation irrespective of LPA treatment but quickly decreased in LPA treated cells at 24 hours to less than 20% whereas expression in untreated cells doubled. Expression in LPA

treated cells was sustained near 30% thereafter with an equivalent expression observed in untreated cells at 48 and 72 hours. De-phosphorylation of MOB1 in P19 cells 48 hours after initial seeding irrespective of LPA treatment may indicate sustained MOB1 phosphorylation is not required in untreated cells for YAP phosphorylation 48 hours after EB formation or that dual regulatory mechanisms exist for YAP regulation.

The difference in MOB1 phosphorylation between LPA treated and untreated cells was most evident 24 hours after initial seeding. This expression profile was dramatically changed in the presence of JNK inhibitor, SP600125, and LPAR1/3 antagonist, Ki16425, compared to LPA treatment alone, both of which returned phosphorylation levels to near maximum. Treatment with Suramin was without effect whereas treatment with H2L5186303 increased phosphorylation expression to approximately 60% compared to LPA treatment alone.

In conclusion, the data discussed above suggests that LPAR1/3, and partially LPAR2 mediated JNK signalling targets the Hippo kinase, MOB1, for de-phosphorylation of YAP in the presence of LPA. However, sustained de-phosphorylation of MOB1 may not be required for prolonged YAP de-phosphorylation implying that multiple regulatory mechanisms may act on YAP in this context. To confirm this, other phosphorylation sites of MOB1 and MOB2 would need to be examined. The cellular localisation of both YAP and MOB1 also require investigation to determine the involvement of the Hippo pathway in LPA induced cardiac differentiation of P19 cells.

Chapter 8

General Discussion

Cardiovascular diseases are a major cause of morbidity and mortality around the globe, and the increasing awareness of stem cell research is linked to the hope of reversing the debilitating damage ensued by events such as myocardial infarction. The use of multipotent stem cells is being evaluated in multiple clinical trials, and thus far, improvement in cardiac function has not been substantial, therefore, there is growing interest in directed differentiation of stem cells for cardiac regeneration.

The role of endogenous molecules in facilitating stem cell differentiation into cardiomyocytes is yet to be fully understood. SPC and S1P, common biolipids, promote differentiation of mesenchymal stem cells and cardiac progenitor cells to cardiomyocytes (Li *et al.,* 2016; Zhao *et al.,* 2011). However, little was known about the same potential of closely related LPA until recently (Pramod, 2017).

The initial cardio-protection offered by elevated LPA levels in response to acute myocardial infarction (Chen *et al.,* 2003) and the ability of this biolipid to mediate cardiac differentiation (Pramod, 2017) served as a rationale to investigate the signal transduction mechanisms mediating LPA induced cardiac differentiation of the murine P19 teratocarcinoma cell line. The mechanisms of cardiac differentiation have been widely studied in this cell line typically in response to DMSO.

8.1 The Differentiation Protocol

Differentiation of P19 stem cells into cell types representative of the three germ layers occurs when cultured in suspension through the spontaneous formation of EBs (Edwards, Harris, & McBurney, 1983; McBurney & Rogers, 1982; McBurney *et al.,* 1982). The number of cardiomyocytes derived this way is very low but can be enhanced with manipulation of the culture medium. In our experimental model, LPA induced cardiac differentiation of P19 cells in a manner comparable to DMSO. This was achieved using LPA at physiologically relevant concentrations with maximal

induction attained with 5μM as previously shown by Pramod (2017). Differentiation was assessed by examining the expression of the ventricular myosin light chain protein (MLC1v). Beating clusters of cells were routinely observed on day 10 of the differentiation process, however, this was not uniform, and the presence of other cell types was not determined. Further studies were conducted to examine the role of signalling molecules critical to lineage commitment including the PI3K and PKC pathway (chapter 3), the MAPK pathway (chapter 4), NF-κB pathway (chapter 5), and their regulation of transcription factors, AP-1 (chapter 5), GATA4, and MEF2C (chapter 6). Furthermore, preliminary studies were conducted investigating the regulation of YAP in differentiating stem cells (chapter 7). The major findings of this thesis are summarised in Figure 8.0 and further discussed below.

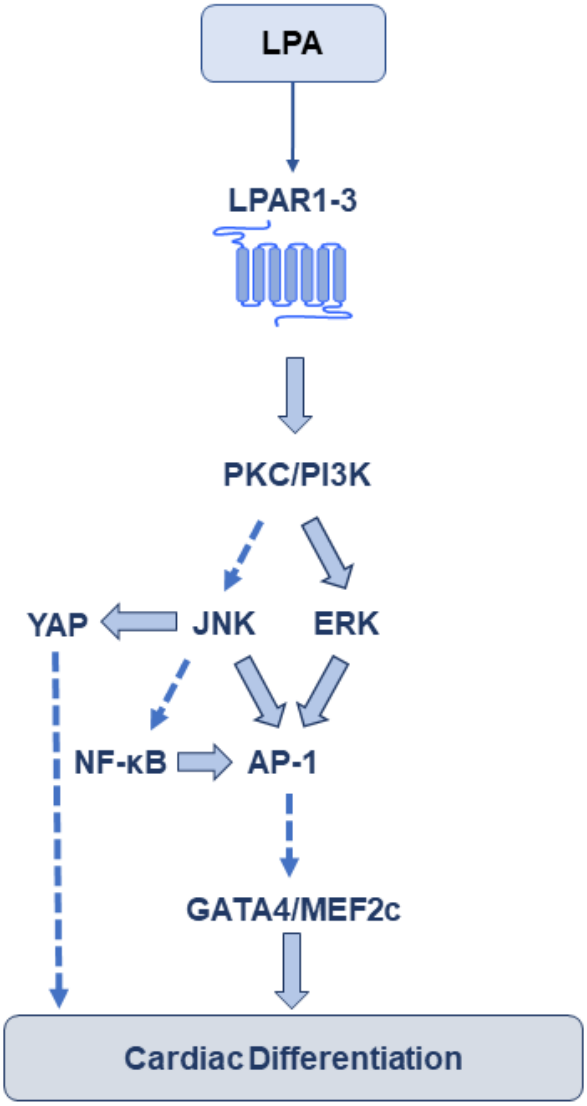

Figure 8.0. Signal transduction mechanisms for LPA induced cardiac differentiation of P19 stem cells.
LPA induces cardiac differentiation through LPA receptor 2 and partially through LPA receptor 1/3 signalling. Downstream to these receptors, the PKC and PI3K pathways converge on the regulation of ERK and possibly JNK. The MAPKs converge on the regulation of the ubiquitous transcription factors, AP-1/ATF2, which are also regulated by NF-κB signalling, which itself may be regulated by the JNK pathway. ERK, JNK, and NF-κB converge on the regulation of cardiac specific transcription factors, GATA4 and MEF2C, however, whether the AP-1 complex lies upstream and acts as a co-activator of these cardiac specific transcription factors is currently unknown. JNK regulates the Hippo pathway in this experimental model but the cellular localisation and role of YAP in LPA induced cardiac differentiation is yet to be determined.

8.2 LPA receptors

After confirming the suitability of the differentiation protocol followed by Pramod (2017) we examined the roles of LPARs 1-4 in LPA induced cardiac differentiation. Pramod (2017) found LPAR 1/3 and 4 to be involved using Ki16425 and Suramin, respectively, to antagonise the actions of these receptors. Ki16425 is selective for LPARs 1 and 3 at low concentrations but targets LPAR2 with an IC_{50} of 6.5μM. Pramod (2017) and studies carried out in this thesis have used 10μM or greater to target LPAR1/3, therefore, it is important to highlight that there is a possibility that the results obtained are due to LPAR2 inhibition. The current thesis determined the role of LPAR2 using H2L5186303, a highly selective antagonist, in LPA mediated cardiac differentiation. LPAR2 is a critical receptor in this experimental model and its inhibition blocks LPA induced MLC1v expression but in addition also blocks the phosphorylation of JNK which was found to be a critical signalling molecule. Inhibition of JNK phosphorylation was, however, more profound with Ki16425 treatment. The same effects were observed when investigating the regulation of YAP and MOB. These results suggest that LPAR1/3 either independently or in conjunction with LPAR2 regulate critical kinases and, therefore, merit further investigation into the precise role of LPAR1 and 3. Pramod (2017) established a role of LPAR4 using Suramin and this was confirmed in the current thesis. Suramin is a broad-spectrum antagonist and has a multitude of off-target effects including promoting differentiation of stem cells into sinus node cells and inhibiting the binding of various key extracellular targets. Although, studies completed in this thesis provide preliminary indication for the involvement of LPAR4, in the absence of a selective established pharmacological inhibitor, for confirmatory studies, siRNA may be a useful tool to explore the role of LPAR4. Therefore, we can

conclude that LPAR2 is a critical receptor and LPAR1/3 are partially involved and LPAR4 may possibly be involved in LPA induced cardiac differentiation.

8.3 The JNK pathway

JNK signalling was determined to be necessary for our differentiation model and regulated through LPA receptors 1-3, however, the specific regulation by LPAR1/3 requires further investigation as discussed above. Phosphorylation of JNK was transient during EB formation irrespective of LPA treatment. The use of 10μM SP600125, a JNK inhibitor, inhibited LPA induced MLC1v expression whereas 20μM SP600125 impacted the proliferation capacity of the aggregating EBs. The inhibition of LPAR 1/3 and 2 did not impact on proliferation suggesting the JNK isoforms regulated are the same as those targeted by 10μM SP600125 and that additional JNK isoforms or kinases are targeted by 20μM SP600125. Inhibition of specific JNK isoforms, perhaps using siRNA, may establish isoform specific roles during various stages of EB formation and rule out the possibility of non-selective kinase inhibition by SP600125.

The program of cardiac differentiation initiated by LPA may also involve the regulation of late genes by JNK as inhibition of the pathway 48 hours after initial EB seeding in the presence of LPA, using 10μM or 20μM SP600125, abolished the expression of MLC1v. Whether JNK signalling is required during early EB formation solely for proliferation, irrespective of an inducing agent, requires further investigation. For this, we initiated studies evaluating the regulation of the Hippo pathway, as it is implicated in the proliferation of several cell types and a target of both LPA and JNK. Phosphorylation of YAP at serine 127 and 397 was reduced in LPA treated cells during early EB formation and was found to be regulated by JNK. No difference was seen

between treatment of 10μM or 20μM SP600125 suggesting YAP is not required for proliferation in this context. The role of YAP was not identified in this study. To establish YAP as a critical signalling molecule in LPA induced cardiac differentiation, it is important to determine its cellular localisation and the effect its deletion would have on differentiation. Studies were initiated to determine the cellular localisation of YAP in LPA treated and untreated cells during EB formation using the Life Technologies NE-PER extraction kit. The results were, however, inconclusive as mycoplasma contaminations disrupted the programme of studies and time constraints did not allow for the experiments to be completed after the contamination had been resolved.

8.4 The NF-κB Pathway

LPA induced MLC1v expression was blocked by the inhibition of NF-κB, using CAY10470, during EB formation. Exploring the expression profile of NF-κB during LPA induced cardiac differentiation revealed that NF-κB is phosphorylated at serine 536 in both LPA treated and untreated EBs after an 18-hour incubation. Earlier time points could not be examined as the size of the pelleted EBs was very small making protein extraction difficult and consistency could not be assured. Although, due to time constraints, an ELISA kit was used to determine the changes in early NF-κB phosphorylation at serine 468 and 536 upon LPA treatment in adherent cells, it may be possible to study the changes in NF-κB phosphorylation at earlier time points in EBs if the initial seeding density is increased or if identical treatments are cultured and pooled together resulting in a larger EB pellet and easier protein extraction. Changes in early NF-κB phosphorylation at serine 468 or 536 were not evident upon LPA treatment in adherent cells. NF-κB phosphorylation at serine 536 and 468 was found to be mediated by JNK signalling, but, regulation at only serine 468 may be initiated through LPA receptors 1-3 as inhibition of these receptors did not affect serine 536.

Phosphorylation of the latter has been suggested to be a negative regulator of NF-κB activity (Pradere *et al.,* 2016), and further investigation into the possibility of negative regulation by JNK dependent phosphorylation at this residue or possible cross-talk through AP-1 is required. It is important to mention that previous studies within the research group have found that treatment with LPA without an EB formation stage does not induce cardiac differentiation. It is possible that the signalling cascade leading to and from NF-κB phosphorylation is different in these culturing conditions.

8.5 Transcription Factors

Pramod (2017) determined that LPA induced expression of MLC1v was blocked by the inhibition of PKC, PI3K, or ERK, and further found that both PI3K and PKC converged on the regulation of ERK in LPA induced cardiac differentiation. While only the former was confirmed in the current thesis, we further established the regulation of AP-1/ATF2 by PI3K, PKC, and ERK. JunD and cJun were found to be regulated by PI3K/PKC-ERK whereas only FosB was regulated in a PI3K-ERK manner and ATF2 was regulated by PKC-ERK. JNK and NF-κB were also found to regulate cJun, JunD, and ATF2. Although critical signalling molecules converge on the regulation of AP-1/ATF2, the necessity of AP-1 in LPA mediated cardiac differentiation of P19 cells requires evaluation before concluding the regulation by critical kinases as anything more than observational.

PKC, MAPKs, and NF-κB signalling converged on the regulation of GATA4, however, PI3K inhibition by LY294002 was shown to target AP-1 subunits. The combinatorial regulation by GATA4-AP-1 complexes have been suggested by several groups (Martin *et al.,* 2012; Schroder *et al.,* 2006; Suzuki *et al.,* 1999) and perhaps PI3K regulates the differentiation into cardiomyocytes through modulating the GATA4 co-factor AP-1 rather than GATA4 directly. Zeidler *et al.* (2016) also suggested that both AP-1 and

GATA4 may synergistically regulate genes during cardiac maturation after analysing the co-occurrence of several transcription factors during specific stages of heart muscle development. The role of AP-1 in differentiating P19 cells was not directly investigated in this study although it would be interesting to identify GATA4 co-factor activity, if any, in LPA mediated cardiac differentiation.

Aside from NF-κB signalling, all pathways converged on the regulation of MEF2C. As FosB regulation was found to be mediated through the NF-κB independent PI3K-ERK pathway, it may be possible that this pathway regulates MEF2C. The necessity of dual regulation for full MEF2C activity is unknown as inhibiting PKC and JNK signalling also affected MEF2C expression. The possibility of JNK regulation by PKC and PI3K are yet to be established in this experimental model, although, it may be possible that MEF2C is regulated in a PI3K-ERK and PKC-JNK manner.

8.6 Summary and Conclusion

The studies carried out in this thesis have shown that LPA mediates the cardiac differentiation of P19 cells through LPAR2 and partially through LPAR 1/3 and possibly through receptor 4. The inhibition of PKC, PI3K, JNK, ERK, or NF-κB signalling blocks LPA induced MLC1v expression. JNK is a critical signalling molecule downstream of LPARs 1-3 along with ERK. Conceivably, PKC and PI3K may regulate JNK as they have shown to converge on the ERK pathway. Further downstream ERK, JNK, along with NF-κB converge on the regulation of AP-1/ATF2, and GATA4/MEF2C. Whether the AP-1 complex lies upstream of the examined cardiac specific transcription factors or if there is any co-factor activity between AP-1/ATF2 and GATA4/MEF2C is yet to be established. The regulation of YAP was found to be mediated by JNK, however, the role of YAP in the proliferation or cardiac differentiation of P19 cells requires further investigation.

Although these results are encouraging, the evaluation of LPA as a mediator of cardiac differentiation is in its infancy. The actions of LPA must be reproducible in more clinically relevant stem cells types. LPA accumulates after the onset of an acute myocardial infarction and has shown to protect stem cells against hypoxic injury. Evaluating the profile of LPA receptors in the healthy and ischaemic heart followed by examining the effects of both overexpression and knockdown of each receptor may provide insight into a potential additional pharmacological avenue for the use of LPA as a strategy to alleviate some of the detrimental effects, enhance survival and differentiation of the transplanted stem cells, or enhance the activity of resident cardiac progenitor stem cells. Prolonged LPA accumulation, however, can have detrimental effects. Therefore, identifying the ideal window of LPA activity would be vital. For this, *in vivo* studies utilizing molecular imaging and labelling techniques may prove useful as it is beyond the scope of conventional dish-based methods to assess the dynamic cellular responses to the microenvironment and stimuli during an acute myocardial infarction.

Chapter 9

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