REGULATION OF STEM CELL DIFFERENTIATION INTO CARDIOMYOCYTES BY LYSOPHOSPHATIDIC ACID

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Abstract

The mechanisms that regulate the differentiation of stem cells (SCs) into cardiomyocytes are still unclear and the role of endogenous molecules on this process remains unexplored. One such molecule is the bioactive phospholipid lysophosphatidic acid (LPA) which accumulates in the myocardium following acute infarction and exerts multiple biological functions, including the regulation of cell growth and differentiation as well as cell survival (Tigyi et al., 2003; Sengupta, et al., 2004). Experiments were therefore carried out in this thesis to reveal whether LPA can induce the differentiation of stem cells into cardiomyocytes and to identify the signalling mechanisms that mediate this effect.

All experiments were carried out in the mouse P19 carcinoma stem cell line. Treatments with LPA in the absence and presence of various pharmacological compounds were conducted in embryoid bodies (EBs) formed from the P19 cells in sterile Petri dishes over 4 days. The EBs were subsequently transferred into 6-well cell culture plates and cultured for specific time points. Lysates were generated and subjected to western blotting for expression of cardiac-specific myosin light chain -1v (MLC-1v). To look at the expression of LPA receptors (LPAR1-LPAR5) experiments were carried out by RT-PCR using specific primers for each LPA receptor and the role of the latter in mediated responses to LPA were examined in the presence of the LPAR 1/3 antagonist, Ki16425, or the LPAR 4 receptor blocker suramin. In addition, experiments were carried out investigating the role of Gαi and specific signalling pathways that may be involved in the differentiation of P19 cells. These were carried out

using potent inhibitors/antagonists of Gαi inhibitor (Pertussis toxin), PI3K inhibitor (LY294002), Akt inhibitor (Akt inhibitor XIII), PKC inhibitor (Bisindolylmaleimide I BIM-I), ROCK inhibitor (Y-27632), p38-MAPK inhibitor (SB203580) and ERK1/2 inhibitor (PD98059). Further experiments were carried out to establish whether the presence of LPA results in the phosphorylation of the targeted kinases. These studies were however limited to Akt, p38 MAPK and ERK1/2.

Incubation of cells with LPA resulted in the differentiation of P19 cells into cardiomyocytes as reflected by the induction of MLC-1v. The latter increased significantly above basal in a time-dependent manner, reaching a maximum 10 days after plating EBs in 6-well plates. The induction of MLC-1v was more pronounced in cells incubated with 5 μM LPA at 6 days but showed little concentration differences at day 12. RT-PCR analysis confirmed the expression of LPA receptors 1 to 4 but not 5. Pre-incubating cells with suramin and Ki16425 concentration-dependently inhibited MLC-1v expression with 0.05 mg/ml and 10 μM respectively, virtually abolishing the expression of MLC-1v. Additionally, inhibitors of LPAR1/3 and LPAR4 receptors and all the signalling inhibitors except SB203580 abolished the phosphorylation of ERK1/2. Similarly, p38 MAPK activation was completely abolished by LPAR1/3 and LPAR4 receptor antagonists, Interestingly, only LY294002 (5 μM) and Y27632 (10 μM) abolished the LPA induced activation of p38 MAPK while SB203580, BIM-I, Akt inhibitor XIII and PD95080 caused no significant changes to the phosphorylation of p38 MAPK.

In conclusion, the studies carried out in this thesis have shown that LPA can induce P19 stem cells to differentiate into cardiomyocytes and they are linked to the well characterised LPA receptors (LPAR1/3 and 4). These receptors are coupled to downstream signalling pathways of which those involving the ROCK, PI3K, PKC and/or Akt may be critical, and may converge on ERK1/2. Inhibition of any of these pathways has the potential to suppress differentiation. In contrast, signalling leading to p38 activation may potentially suppress differentiation but this needs further clarification.

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Abbreviations

ACTB	Beta actin
ADP	Adenonosine diphosphate
AGPAT	1-acylglycerol 3-phosphate acyltransferase
ANF	Atrial Natriuretic Factor
ATX	Autaxin
Akt	Protein Kinase B
APS	Ammonium persulfate
α-MEM	alpha Minimum Essential Medium
APS	Ammonium persulfate
ASCs	Adult stem cells
BCA	Bicinchoninic Acid
B2M	Beta 2 microglobulin
BMP	Bone morphogenic protein
BSA	Bovine Serum Albumin
CABG	oronary artery bypass grafting
cAMP	cyclic adenosine monophosphate
CANX	Calnexin
cDNA	complementary Deoxyribo Nucleic Acid
cdx2	Caudal type homeobox2 transcription factor
C-EtOH	Control ethanol

(c) PKC	conventional PKC
CO_2	Carbon dioxide
COX1	Cyclooxygenase pathway
CREB1	cAMP respose element-binding protein
Ct	Threshold cycle number
cTnC	cardiac troponin C
cTnI	cardiac troponin I
CYC	Cyclophilin
DAG	Di-acyl-glycerol
DDW	Double Distilled Water
DHAP	Dihydroxy acetone phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Efficiency of amplification
EBs	Embryoid bodies
ECCs	Embryonic carcinoma cells
ECL	Enhanced chemiluminescence
ECP	Eosinophil cationic protein
Edg	Endothelial differentiation gene
EDTA	Ethylene Diamine Tetra Acetic Acid

EGF	Epidermal growth factor
EGCs	Embryonic germ cells
END-2	Visceral Endoderm like cells
ERKs	Extracellular signal Regulated Kinases
ESCs	Embryonic stem cells
ESC-CM	Embryonic stem cells derived cardiomyocytes
FBS	Foetal Bovine Serum
FGF-4	Fibroblast growth factor
FHF	First Heart Field
Fz proteins	Frizzled proteins
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Gbp2	Growth factor binding protein 2
GDP	Guanosine diphosphate
GPAT	Glycerophosphate acyltransferase
G3P	Glycerol-3-phosphate
GPCRs	G protein-coupled receptors
GTP	Guanosine triphosphate
HAND	Heart and neural crest derivatives expressed proteins
hESC-CM	Human embryonic stem cells derived cardiomyocytes
HGF	Hepatocyte growth factor

HKG	Housekeeping gene
ICM	Inner cell mass
IgG	Immunoglobulin
IP3	Inositol (1,4,5)tris-phosphate
iPSCs	Induced Pluripotent Stem cells
iPSCs-CM	Induced pluripotent stem cells derived cardiomyocytes
Isl1	Insulin gene enhancer protein1
JNK	c-Jun N-terminal Kinases
kDa	Kilo daltons
Klf-4	Kruppel-like factor-4
Km	Affinity constant (half maximal saturation constant)
KOSM	Klf4, Oct3/4, Sox2, and c-Myc transcription fators
LDL	Low Density Lipoprotein
LIF	Leukaemia Inhibitory Factor
LPA	Lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferases
LPP	Lipid phosphate phospho- hydrolase
LPAR1	Lysophosphatidic acid receptor
LRP5/6	Low density lipoprotein receptor-related protein 5/6
LVEF	Left ventricular ejection fraction

mA	milliamp
MAG	Monoacyl glycerol
MAGT	Monoacyl glycerophosphate acyltranferase
MAPK	Mitogen Activated Protein Kinases
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MAPKKKK	MAP kinase kinase kinase kinase
MEK	Mitogen-activated ERK Kinase
MEF	Mouse Embryonic Fibroblast
MEF2	Myocyte Enhancer Factor 2
MEF2A	Myocyte Enhancer Factor 2A
mESCs	Mouse embryonic stem cells
MESP1	Mesoderm posterior 1
MHC	Myosin heavy chain
MI	Myocardial infarction
MLC	Myosin light chain
MLC-1v	Myosin light chain-1v
mRNA	messenger Ribonucleic Acid
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MSC-CM	Mesenchymal stem cells derived cardiomyocytes
MSK	MAPK/SAPK-activated kinase
Nanog	Tir *nan Og*, a mythical celtic land of the ever young
NFAT	Nuclear factor of activated T-cell
NF-κB	Nuclear Factor- κB
(n) PKC	novel PKC
NPC	Neural progenitor cells
Nppa	Natriuretic peptide precursor type A
Oct3/4	Octamer-binding protein-3/4
PAP	Phosphatidicacid phosphatase
p38 MAPK	p38 Mitogen activated protein kinase
PBS	Phosphate Buffered Saline
PcG	Polycomb group
PCI	Precutaneous coronary interventions
PCP pathway	Planar cell polarity pathway
PCR	Polymerase Chain Reaction
PDK1	3-Phosphoinositide-Dependent Protein Kinase 1
PDK2	3-Phosphoinositide-Dedendent Protein Kinase 2
PG	Prostaglandin
PGCs	Primordial germ cells

PGR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphoinositol (1,4) bis phosphate
PIP_3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PKA	Protein kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PL	Phospholipids
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLC	Phospho lipase C
PLD	Phospholipase D
PPARγ	Peroxisome proliferator-activated receptor gamma
PTX	Pertussis toxin
PVDF	Polyvinyldene difluoride
PS	Penicillin/Streptomycin
PTEN	Phosphatase and tensin homolog
P2Y	Purinergic receptor
Q-PCR	Qualitative or Real time Polymerase Chain Reaction
RPM	Revolutions per minutes

RNA	Ribonucleic acid
RPL13A	Ribosomal protein L13a
ROCK	Rho associated kinase
RT	Reverse transcriptase
RTKs	Receptor tyrosine kinases
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCs	Stem cells
S.D.	Standard deviation
S.E.M.	Standard Error Mean
SD	Serum deprivation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
SH-2	Src Homology Domain 2
SHF	Second Heart Field
SHP2	Sh-2-Containing Tyrosine Phosphatase
Sox2	Sex determining region Y-box 2
S1P	Sphingosine 1-phosphate
SPC	Sphingosylphosphorylcholine
(s) PKC	atypical PKC
STAT3	Signal activator and transducer of transcription 3

Taq Polymerase	Thermus aquaticus Polymerase
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBS-T	Tris Buffer Saline-Tween
Tbx	T box transcription factor
TEMED	NNN'N'-Tetramethylethylenediamine
TG	Triglyceride
TGF	Transforming growth factor
TXA2	Thromboxane A2
UBC	Ubiquitin C
UTF1	Undifferentiated embryonic cell transcription factor 1
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cells
VZ	Ventricularzone
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

Chapter 1

Introduction

1.1 Coronary heart disease

Cardiovascular diseases such as coronary artery disease or atherosclerosis remain the leading cause of mortality all over the world. The primary risk factors that lead to the impairment of the heart and vascular systems are obesity, diabetes mellitus, smoking, and additionally age, gender, sedentary lifestyle and heredity (Brekke *et al*., 2014; Kurian *et al.,* 2007). The physiological and biochemical changes induced by these factors may lead to myocardial infarction precipitated by myocardial ischemia following the development of coronary artery disease or atherosclerosis.

1.1.1 Pathophysiology of atherosclerosis

Atherosclerosis is characterised by plaque formation and obstruction of coronary arteries. Atheroma formation results from the subendothelial accumulation of lipids, mainly triglycerides and low-density lipoprotein (LDL) in the arterial walls along with circulating monocytes that lead to the formation of fatty streaks. Further, smooth muscle cells migrate and proliferate in the intimae in response to chemokines leading to neointima formation. This continued process of lipid accumulation and cellular changes can lead to stenosis of the vessels. The atheromatous tissues can also undergo rupture with platelet plugs, leading to occlusion of the coronary arteries. This leads to myocardial ischemia and subsequently myocardial infarction (MI).

1.1.2 Myocardial Infarction (MI)

Myocardial infarction is a major cause of death arising from an atherosclerotic plaque. It is due to reduced blood and oxygen supply to the heart muscles leading to necrosis of the portion of myocardium that is poorly perfused with blood. Clinically, MI is defined by persistent ST segment elevation on an electrocardiograph, which is characteristic of the

ischemic symptoms. The major causes are occlusions of the blood vessels due to atheroma formation subsequently leading to narrowing and hardening of the coronary arteries and aortic stenosis. Other causes include sudden immense need of blood supply to the heart as in the case of haemorrhage, shock and severe physical exertion. The other major risk factors which can lead to MI are diabetes mellitus, hypertension, hyperlipidaemia, smoking, gender (particularly male) and familial. It is observed that these risk factors double the incidents of MI (Ross *et al*., 1999).

1.1.3 Treatments

The preliminary strategies for treating coronary artery disease are to restore normal blood flow through the coronary arteries and to recover the myocardium (Anderson *et al*., 2007; Krumholz *et al*., 2008). This can be achieved using pharmacological interventions, for instance, by administration of antiplatelet agents such as aspirin. Aspirin inhibits the cyclooxygenase pathway (COX1) irreversibly and thus prevents the formation of thromboxane A_2 and the activation of platelet aggregation. Fibrinolytic drugs like streptokinase and reteplase stimulate the production of plasminogen that disintegrate fibrin. Statins such as pravastatin and atorvastatin are also recommended for patients with MI. These statins competitively inhibits the HMG CoA reductase, the rate-limiting enzyme for cholesterol biosynthesis. In doing so, these drugs reduce the production of the very low-density lipoprotein (VLDL), the precursors for low-density lipoprotein (LDL). As a result, they cause increased LDL receptor expression and lead to a decrease in the level of LDL in the circulation (Cannon *et al*., 2004). However, none of these pharmacological interventions actually cures the disease state but rather regulate the consequence and symptoms of the disease.

An alternative to pharmacological intervention is heart transplant especially where the heart is infarcted resulting in a high risk of mortality. This approach, by its nature, also has several limitations including cost and immune rejection as well as limited donor availability due to increased young population with heart disease along with an increasing population of an elderly group with end stage heart failure. Thus, there is a concerted effort to develop strategies that will overcome these hurdles and result in the successful regeneration of the myocardium. In this regard, stem cell therapy has become very prominent as a potential tool in translational medicine for regenerating the human heart.

1.2 Stem cells (SCs)

Stem cells (SCs) are unspecialised cells capable of undergoing self-renewal without going into senescence but can be induced to become specialised cells (Evans *et al*., 1981). Thus, SCs have the capacity to maintain and repair tissues within the body. Depending on the potency to differentiate into specialised cells, stem cells are categorized into totipotent, pluripotent, multipotent and oligopotent.

Totipotent cells derived from the early divisions of the zygote have the potential to give rise to virtually all cells of the body. Pluripotent cells are primarily present in the inner cell mass at the blastocyst stage of the embryo and have the capacity to differentiate into cells and tissues of the three primary germ layers namely ectoderm, mesoderm, and endoderm. Multipotent cells are characterised by the potential to differentiate into several but limited cell types as in the case of haematopoietic stem cells, which can give rise to different blood cells. Unipotent stem cells can give rise to only a specific cell lineage.

Depending on their origin, stem cells can be divided into two major groups, namely adult or somatic stem cell and embryonic stem cells (ESCs).

1.2.1 Stem cell types

1.2.1.1 Adult stem cells or Tissue-restricted stem cells

Adult stem cells (ASCs) are multipotent and maintain tissue repair that restore dying cells in tissues. Adult stem cells have been isolated from various organs including blood, bone marrow, muscle, skin, liver, adipose tissue, heart, brain (Laugwitz *et al*., 2008; Mackay *et al*., 1998; Schmelzer *et al*., 2007). Hematopoietic stem cells, mesenchymal stem cells (MSCs), umbilical cord stem cells and olfactory mucosal stem cells are few examples of ASCs. Studies have shown the potential of ASCs to differentiate into other types of cells. For example Human MSCs could be differentiated into smooth muscle cells (Bajpai *et al*., 2012), to osteoblasts (Dieudonne *et al*., 2013) and to cardiomyocytes (Toma *et al*., 2002).

1.2.1.2 Embryonic stem cells (ESCs)

Embryonic stem cells are pluripotent stem cells derived from the inner cell mass of pre-implanted blastocyst (3-5 day) comprising of 3 structural components namely trophoblast, blastocoel and inner cell mass (Evans *et al*., 1981; Martin, 1981). Trophoblasts are the cells covering the blastocyst, which gives rise to the formation of extra-embryonic tissues: placenta and umbilical cord. The hollow cavity is called the blastocoel carrying the inner cell mass. Other forms of ESCs are embryonic carcinoma cells (ECCs) derived from malignant germ cell tumours called teratocarcinoma (Martin, 1975). Embryonic germ cells (EGCs) originate from primordial germ cells (PGCs) in the gonadal ridge. During normal embryogenesis PGCs give rise to the germ cells, sperm or egg (Shamblott *et al*., 1998).

Embryonic stem cells being pluripotent have high potential to differentiate into various lineages include cardiomyocytes. However, there has been much success achieving this *in vitro* than *in vivo* especially in relation to translation medicine. One of the limitations with ESCs is the fact that they have a tendency to form teratoma *in vivo*. Moreover, there are ethical as well as immunogenicity issues that limit the exploitation of these cells in full. Thus, alternative sources of stem cells are required and this has led to the generation of induced pluripotent stem cells (iPSCs) which gets around, at least, the ethical and immunogenic concerns (Hentze *et al*., 2009; Ida, 2008).

1.2.1.3 Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells are derived from non-pluripotent adult somatic cells by inducing the expression of specific transcription factors and pluripotency markers specifically octamer-binding protein-3/4 (Oct3/4), sex determining region Y-box 2 (Sox2), Kruppel-like factor-4 (Klf4) and c-Myc (Takahashi *et al*., 2006). Alternatively, a combination of Oct4, Sox2, Lin28 and Nanog could also be used to induce pluripotency in human cells (Liao *et al*., 2008; Okita *et al*., 2008). Currently researchers have successfully differentiated iPSCs to neuronal cells, astrocytes (Juopperi *et al*., 2012; Yuan *et al*., 2013) cardiomyocytes and hepatocytes (Di Pasquale *et al*., 2013; Miki *et al*., 2013). The most important advantage of iPSC technology is that it does not involve ethical issues associated with ESCs, and only requires the consent of the patient. In addition, the patient phenotype could be greatly correlated and therefore iPSCs could standout better in generating cells for specific disease condition which are not possible with ESCs. It is notable that the iPSCs from each individual are typical and unique and differ from individuals with the same genetic disorder (Ellis *et al*., 2011).

Presently, there are different methods used to develop iPSCs, which include a non-viral integrating vector method, deletion after integration, DNA free methods and chemical induction method. A full description of each of these protocols is beyond the scope of this thesis but worth pointing out that the non-viral integration method has been developed using viral vectors like adenovirus, sendai virus and non-viral vectors like episomal vector/ plasmid vectors and expression plasmids. However, among these the sendai viral incorporation has been reported to produce high efficiency in generating iPSCs (Zhou *et al*., 2013). This method prevented the incorporation of any of the viral gene into the target and minimised mutagenesis and changing the prospective to differentiate the cells (Shi *et al*., 2008). Currently, researchers are focused on increasing the efficiency of the reprogramming process and are exploiting techniques such as mRNA and reprogramming proteins to produce high efficiency in generating iPSCs when compared to the viral vector method (Zhou *et al*., 2013). Lately, at least one study has reported that the efficiency of reprogramming was improved close to 100% by the reduction of the Mbd3, a Nucleosome Remodelling Deacetylase complex subunit that is considered to directly interact with Klf4, Oct3/4, Sox2, and c-Myc (KOSM) transcription factors (Luo *et al*., 2013).

Researchers have also discovered chemicals that increase the efficiency in generating iPSCs include compounds such as SB431412, PD0325901 and Thiazovivin. These compounds increase the reprogramming efficiency and furthermore shorten the time for the whole process (Lin *et al*., 2009). Valproic acid/HDAC inhibitor and 616452/ Transforming growth factor β (TGF-β) inhibitors have also been shown to increase efficiency, even when

Oct4 alone was used to reprogram the cells (Masuda *et al*., 2013). Another potential approach reported, is the use of micro RNA to improve reprogramming efficiency of iPSCs. Micro RNAs have also been shown to regulate the differentiation in multiple cell systems and after differentiation of ESCs miR-302 has been reported to be down regulated. In addition the efficiency of reprogramming was increased when iPSCs were induced with miR-302 along with KOSM transcription factors (Anokye-Danso *et al*., 2012). There are reports suggesting that miR-155 acts as an inducer in the differentiation of iPSCs into hematopoietic progenitor cells (Vitaloni *et al*., 2013). Interestingly, another study reports that the generation of iPSCs was completely blocked when miR-302/367 were knocked out in human fibroblasts (Zhang *et al*., 2013).

1.2.2 Stem cell pluripotency

The regulatory mechanisms that control the maintenance of pluripotency include chromatin stability, transcription factors, cell cycles, extracellular signalling molecules and micro RNA circuits (Sokol *et al*., 2011; Tsubouchi *et al*., 2013; Wan *et al*., 2013.). In ESCs the chromatin is likely to be trimethylated on histone 3 (Lysine 4) and is acetylated on histone 4. These areas are more associated with trithorax and the polycomb-group (PcG) proteins that regulate pluripotent gene expression, thus maintaining the pluripotency of ESCs. Trimethylation of histone 4 at Lysine 27 (inactivation) and its association with PcG promotes lineage specific gene expression and thus lineage commitment (Ringrose, 2007). Interestingly, transcription factors like Sox2, Nanog, and Oct3/4, bring about repression at histone 4 trimethylation. As explained above, stem cells pluripotency is sustained by mutual interactions of transcription factors Oct-3/4, Sox2 and Nanog. Oct-3/4 attaches to AGTCAAAT octamer sequence of the target gene and belongs to a POU family of

transcription factors. Oct-3/4 is expressed in the early embryo but is down regulated during formation of ectoderm and endoderm (Brehm *et al*., 1998). In the inner cell mass (ICM), Oct-3/4 forms a complex with cdx2 transcription factor to initiate specific lineage commitment. Studies also suggest that the levels of Oct-4 is vital as repression of this transcription factor initiates differentiation to trophectoderm whereas its over expression can lead to extra-embryonic endoderm formation (Niwa *et al*., 2000). Sox2 is expressed in the ICM epiblast and is later confined to neural tube (Avilion *et al*., 2003). It is a SRY-related HMG box gene member and its over expression directs stem cells to differentiate into neuronal precursors. Oct-4 and Sox2 can regulate pluripotency and gene expression by binding to adjacent sites on regions of the promoters of the pluripotent genes.

Oct-3/4 and Sox2 are reported to induce the expression of several genes including fibroblast growth factor (FGF)-4, undifferentiated embryonic cell transcription factor 1 (UTF1) and Nanog (Boyer *et al*., 2005; Yuan *et al*., 1995). In addition, it is observed that at some stage during induction of differentiation there is a down regulation of Oct3/4, Sox2 and UTF1. These changes also denote negative regulation caused by Oct-3/4 and Sox2 on the expression of UTF1 (Boyer *et al*., 2005; Nishimoto *et al*., 1999). Nanog in mouse is expressed in ICM and early epiblast but embryo failed to form an epiblast when this gene was deficient (Mitsui *et al*., 2003). Evidence also indicates that Nanog null Mouse embryonic stem cells (mESCs) are directed to differentiation in the presence of Leukaemia Inhibitory Factor (LIF) but it's overexpression helps ES cell to self-renew even in the absence of LIF (Chambers, 2004). From the above reports, it is suggested that these 3

transcription factors have to be jointly synchronized to maintain stem cell self-renewal and pluripotency.

Cell cycle is yet another important crucial point that decides cell fate. ESCs at the epiblast stage mostly exist in the S1 phase with less G1 phase. This is unlike somatic cells where G1 is the predominating phase and determines the fate of cells to proliferate, differentiate, senesce or undergo apoptosis (Burdon *et al*., 2002). ESCs are therefore maintained in their self-renewal and pluripotency state due to their maintenance in the S1 rather than the G1 phase.

1.2.3 Stem cells for heart diseases

Numerous studies in human and animal models have demonstrated the potential of exploiting stem cells in regenerative medicine and potentially in developing stem cells-derived cardiomyocytes (Iglesias-Garcia *et al*., 2013; Pfister *et al*., 2014). This is demonstrated by recent studies reporting transplantation of human embryonic stem cells derived cardiomyocytes, (hESC-CM) improved myocardial function in the ischemic heart. The above data shows the therapeutic potentials of stem cells in regenerating the infarcted heart.

Similarly, other studies have also suggested the role of stem cells in regenerating the infarcted myocardium (Caspi *et al*., 2007). This study in infarcted rat showed that the grafting of pre-differentiated hESC-CM improved cardiac functioning and prevented teratoma formation. Embryonic stem cells derived cardiomyocytes (ESC-CM) and mesenchymal stem

cells derived cardiomyocytes (MSC-CM) have revealed predictable morphology and action potential similar to the rhythmic contractions of normal cardiomyocytes (Antonitsis *et al*., 2008; Labovsky *et al*., 2010; Reppel *et al*., 2005). A recent report has shown that aggregates of contracting hESC-CM improved myocardial function in infarcted hearts of rat however; in contrast, another study has shown that injection of hESC-CM in rat heart did not show any significant improvement in left ventricular function in a chronic myocardial model (Fernandes *et al*., 2010; Moon *et al*., 2013). Encouragingly though, iPSC-CM have been shown to successfully improve myocardial functioning in infarcted rat hearts. The iPSC-CM derived from H9c2 cells also appears to induce neovascularisation in the infarcted mouse heart (Miki *et al*., 2012; Singla *et al*., 2011).

There are several reports of clinical trials with different types of stem cells. The study with autologous adipose derived regenerative stem cells improved the left ventricular function of patients with no alternative treatment option (Perin *et al*., 2014). Previous studies conducted looking at the role of autologous bone marrow stem cells in acute myocardial infarction reported that the condition was significantly improved but the mode of administration and dose has to be optimised for reproducibility (Martin-Rendon *et al*., 2008). In addition, the intramyocardial injection of $CD34^+$ cells in phase1 and IIa trials in intractable angina patients showed improvement (Losordo *et al*., 2007). However, the delivery of bone marrow mononuclear cells administered through trans endocardial injection did not show improvement in patients with chronic heart failure (Perin *et al*., 2012). In contrast, intramyocardial injection of bone marrow stem cells have been shown to improve

angiogenesis, exercise time and left ventricular ejection fraction (LVEF) in other trials (Briguori *et al*., 2006; Tse *et al*., 2007).

Despite some of the current limitations, it is becoming evident that stem cell therapy holds promise for regenerative medicine with excellent potential to provide an alternative therapeutic approach for the infarcted heart. However, there are still many hurdles to overcome, if therapeutic outcomes are to be successful. These obstacles include viability and integrity of cells, proper delivery routes, electromechanical integrity, and understanding the cellular mechanisms that regulate differentiation (Harding, 2014).

1.2.4 Mechanisms that regulate the generation of cardiomyocytes from stem cells.

Currently cardiomyocytes could be generated from co-culture with mouse visceral Endoderm like cells (END-2), through embryoid body (EB) formation, with growth and intrinsic factors as well as high density monolayer plating. The co-culture method with END-2 shows low efficiency, but this can be improved with low concentrations of foetal bovine serum (FBS), L-ascorbic acid and growth factors. The more common technique of EB formation is a more reliable method. However, generating cardiomyocytes from iPSCs appears more promising, especially as they can be generated from patient specific cells and can be induced to differentiate into cardiomyocytes.

The potency of stem cells and progenitor cells to differentiate into diverse cell types has been widely documented and reports suggest much promise in regenerative medicine.

However, to direct the differentiation of these cells to a particular lineage and to exploit these for cell based therapy there is need for clear understanding of the cellular mechanisms that mediate their differentiation. Interestingly, the regulatory molecules involved in the differentiation of stem cells to cardiomyocytes are identical to those expressed/activated during the process of vertebral heart development. Moreover, the mechanisms mediating the differentiation of stem cells to cardiomyocytes are comparable to those associated with the embryonic development of the heart, the primary organ formed during embryonic development. Zygote formed from the fertilized egg, transform into a blastocyst comprising the inner cell mass. During the gastrulation, the primitive streak is formed and the inner cell mass, forming the epiblastic cells migrate towards the anterio-posterior direction of the primitive streak to form three distinct germ layers. The mesodermal cells further develop into lateral plate mesoderm, intermediate and paraxial. The Lateral plate mesoderm further forming the splanchnic mesoderm moves anteriorly and this group of mesodermal progenitor cells are called First Heart Field (FHF). These cells differentiate and meets at the midline to form the cardiac crescent forming primary heart tube (van den Berg *et al*., 2009). The progenitor cells that are sequentially added to the FHF with undifferentiating cells but showing higher rate of proliferation are called Second Heart Field (SHF). The heart tubes are further elongated and form chambers, valves, and septa (Evans *et al*., 2010; van den Berg *et al*., 2009).

The main genes that regulate cardiac development and encode the structural and/or regulatory proteins in the heart are Brachyury T, mesoderm posterior 1 (MESP1), GATA family, Homeobox Nkx2.5, Myocyte Enhancer Factor 2 (MEF2), Insulin gene enhancer

protein1 (Isl1), Heart and neural crest derivatives expressed proteins (HAND1/2) and T-box. MESP1 has been recognised as a crucial gene, which acts as a preliminary marker for cardiac progenitor cell commitment and fate, and is activated by Brachyury(T) during mesoderm formation (Bondue *et al*., 2008; David *et al*., 2011; Herrmann, 1992). MESP1 further up regulate major cardiac progenitor genes like GATA4, Nkx2.5, MEF2C and HAND2 by downregulating the genes for maintaining pluripotency namely Oct4, Nanog and Sox2.

Cardiac differentiation is initiated by the mesodermal induction of cells with a high level of expression of Brachyury T, which causes the induction of specific cardiac progenitor marker, mesoderm posterior 1 (MESP1). An increased expression of Brachyury T was used to confirm the differentiation of P19CL6 cells into cardiomyocytes by eosinophil cationic protein (ECP) (Jin *et al*., 2012). MESP1, the key regulator further activates cardiac specific genes including MEF2C, Isl1, T box transcription factor 5 (Tbx5), HAND1/2, GATA4, and Nkx2.5 and proteins like Atrial Natriuretic Factor (ANF), MLC-2a, MLC-2v, Myosin heavy chain (MHC) and troponin T (Jin *et al*., 2012). This induction of cardiac lineage commitment causes the inhibition or downregulation of pluripotency genes (Bondue *et al*., 2008; Wu, 2008).

GATA4 participates in the terminal differentiation of P19 cells into cardiomyocytes (Grepin *et al*., 1997). Studies have shown the direct action of GATA4 is mainly by acting on the promoter regions of specific cardiac genes namely α-MHC, cardiac troponin I (cTnI), cardiac troponin C (cTnC), ANP and MLC (Charron *et al*., 1999a; Charron *et al*., 1999b; McGrew *et al*., 1996). In P19 cells and ESCs it was shown that the nuclear factor of activated T-cell

(NFAT) regulates the action of Nkx2.5 by the coordinated action with GATA4 during cardiogenesis (Chen *et al*., 2009). Other transcriptional genes required for the normal heart development include the MEF2C family, Isl1, and HAND1/2. GATA4 gene is essential in heart morphogenesis and myogenesis in mouse and results suggests that a coordinated action of Nkx2.5 and MEF2C is necessary for the formation of the ventricle (Lin *et al*., 1997; Vincentz *et al*., 2008). The overexpression of MESP1 leads to the up regulation of GATA4, Nkx2.5 and MEF2C (David *et al*., 2011). T box transcription factor 5 (Tbx5) is a critical transcription factor during cardiac development. Essentially, during morphogenesis, it is expressed in the heart tube and its mutation lead to septal defect in heart in the case of Holt-Oram Syndrome. Tbx5 is closely associated with Nkx2.5 during cardiac differentiation. Previous studies suggest they act cooperatively by being both closely attached to the promoter region of cardiac-specific natriuretic peptide precursor type A (Nppa) (Basson *et al*., 1997; Hiroi *et al*., 2001; Horb *et al*., 1999). HAND1 and 2, which belong to the Twist family of proteins, also play critical roles in early cardiogenesis and within the development of the heart, facilitating maturation of ventricular cardiomyocytes and mesothelial cells formed in the epicardium. During looping of the heart tube, the expressions of HAND genes are elevated and HAND1 specifically on left ventricular outer curvature and HAND2 on outflow tract, and right ventricle (Barnes *et al*., 2010).

The major extracellular signalling molecules involved in regulating differentiation include Wnt, FGF, TGF superfamily (Bone morphogenetic proteins, TGF-β, Nodal), Notch, and Hedgehog signalling proteins (Klaus *et al*., 2007; Li *et al*., 2010; Taha *et al*., 2008; Wang *et al*., 2006; Yu *et al*., 2011). Studies have indicated that Wnt signalling activates formation of

lateral mesoderm, morphogenesis of cardiac cushion and valve formation (Hurlstone *et al*., 2003; Ueno *et al*., 2007). Similarly, in mESCs Wnt signalling decreased contraction in EBs and led to a reduction in differentiation (Liu *et al*., 1999; Naito *et al*., 2006). There are three major pathways for Wnt signalling, a canonical and two non-canonical pathways namely, the planar cell polarity pathway (PCP pathway) and the Ca^{2+}/Wnt pathway. Canonical Wnt signalling or the Wnt/β-catenin pathway is activated when Wnt binds to the Frizzled proteins (Fz proteins) and interacts with its co-factor low-density lipoprotein receptor-related protein 5/6(LRP5/6) to form a complex. The PCP pathway, in contrast, does not involve β-catenin and co-factors, however the Wnt/Fz proteins activates DSH and stimulate the small G-protein Rho and Rac subsequently stimulating Rho-associated kinase (ROCK) and c-Jun N-terminal kinase (JNK), respectively (Endo *et al*., 2008). Bone morphogenic protein (BMP) signalling is also crucial in each of the stages of cardiomyocytes formations, namely precardioblast stage, cardioblast stage and cardiomyocytes formation stage. Similar to Wnt signalling, BMP signalling has been reported to play a pivotal role in cardiogenesis and stem cell differentiation. In addition BMP additionally plays a roles in self-renewal, cell proliferation, migration, and apoptosis (Monzen *et al*., 2002). Studies have also indicated the role of BMP signalling in the formation of first heart field and second heart field (Klaus *et al*., 2007). There are a multitude of other signalling pathways and molecules implicated for cardiac lineage commitment which may be directed by the various extracellular stimuli and these are further discussed below (Chapter 4).

1.2.5 Clinically relevant inducers of stem cell differentiation

One of the other challenge facing the field of translational stem cell research is the fact that although differentiation has been induced and successfully, this has been achieved using agents which may have limited clinical applications. Most of the experimental studies carried out to date have used agents such as growth factors including Epidermal growth factor (EGF), FGF-4, Hepatocyte growth factor (HGF), (Mummery *et al*., 1993) organic solvents like dimethyl sulphoxide (Skerjanc, 1999) hormones such as Oxytocin (Paquin *et al*., 2002) or chemicals such as 5-azacytidine (Choi *et al*., 2004). There is therefore a need to identify more suitable and preferably endogenous molecules that may be more clinically relevant in regenerative medicine. In this regard, our research has focused on identifying endogenous mediators that have a potential to regulate the differentiation of stem cells into cardiomyocytes. One such molecule is the lysophosphatidic acid.

1.3 Lysophosphatidic acid (LPA)

Lysophosphatidic acid is a derivative of phospholipids with the molecular formula $C_{21}H_{41}O_7P$ and has a molecular mass of 436.52 kDa. It is considered as an important bioactive mediator modulating various biological effects depending on its cellular targets (Moolenaar *et al*., 1997). Lysophosphatidic acid is a simple glycerophospholipid, which is exceptionally water soluble and exists in two 1-alkyl- and alkenyl-ether forms (Anliker *et al*., 2004).The most commonly used in experiments and commercially available LPA is an unsaturated form called oleoyl-LPA or 18:1 LPA (Figure 1). LPA is soluble in water as it has a free hydroxyl and phosphate moiety (Jalink *et al.*, 1990; Xie *et al.*, 2002; Xie *et al.*, 2002). It was first identified as the Darmstoff's active ingredient (smooth muscle-stimulating

substance) that is present in serum. Biological fluids such as saliva, aqueous humour, seminal and follicular fluids and malignant effusions from several cancers, including ovarian cancer ascites fluid also contains LPA (Hama *et al.*, 2002; Liliom *et al*., 1998; Tokumura *et al*., 1999; Xu *et al.*, 1995; Xu *et al*., 1998).

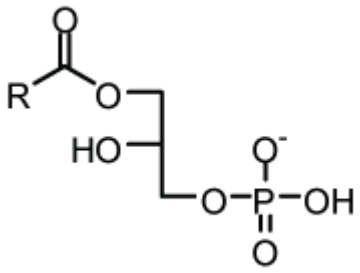

Figure 1. Lysophosphatidic acid (1-acyl-sn-glycerol-3-phosphate) (LPA), M.F-C21H41O7P

Lysophosphatidic acid carries a phosphate group, a fatty acid chain and glycerol moiety. There is a hydroxyl group at the sn-2 (or sn-1) position, a phosphate group at the sn-3 position, and a fatty acid chain at the sn-1 (or sn-2) position (Hopper *et al.*, 1999). In body fluids, LPA can exist as a long chain of saturated or unsaturated fatty acid linked to glycerol moiety with acyl or alkyl groups (Xiao *et al.*, 2000). However, LPA with unsaturated fatty acid linkage is more biologically active than the LPA linked to saturated fatty acid (Yoshida *et al*., 2003). The selectivity of the functional relationship with cell receptors depends on the information on the structure. The fatty acid components in LPA are different from the acyl groups of LPA produced from platelets in serum and plasma (Sano *et al*., 2002). The normal concentration of LPA in serum ranges between 1 and 5 μM. Interestingly, the LPA generated in the circulation by activated platelets ranges between 2 and 20 μM (Baker *et al*., 2002; Eichholtz *et al.*, 1993). Production of LPA due to increased cellular activations are triggered by several stimuli and agonists during the process of inflammation, blood clotting, or wound healing which cause an increase in local LPA production and activity.

1.3.1 Metabolism and transport of lysophosphatidic acid

Lysophosphatidic acid synthesis takes place intracellularly and extracellularly by specific enzymes. Studies have shown that LPA is the key transitional product in the intracellular synthesis of lipids namely triglyceride (TG) and phospholipids (PL) in all cells (Gerrard *et al.*, 1989; Mauco *et al.*, 1978; Pages *et al.*, 2001). It exists in a saturated (16:0 and 18:0) and as an unsaturated (16:1, 18:1,18:2, and 20:4) form (Eichholtz *et al*., 1993). In the endoplasmic reticulum and in mitochondria, glycerophosphate acyltransferase (GPAT) catalyses the synthesis of LPA by means of acylation of glycerol-3-phosphate (G3P). In mitochondria, monoacyl glycerol (MAG) through the activities of monoacyl glycerophosphate

acyltranferase (MAGT) is converted to phosphatidic acid (Vancura *et al.*, 1992).

Alternatively, in mitochondria and microsomes LPA is also synthesised from MAG by MAG-kinase, which is involved in the production of phosphatidyl inositol synthesis (Gerrard *et al.*, 1989; Pages *et al*., 2001). Di-acyl-glycerol (DAG) processed to LPA via the phosphorylation and hydrolysis of the long fatty acid chain. Phosphorylation of DAG is catalysed by DAG-kinase or an agonist stimulated phospholipase D (PLD) resulting in phosphatic acid (PA).

Glycerol-3-phosphate ⟶ 1- acyl-sn-glycerol-3-phosphate ⟶ Phosphatidic acid

In peroxisomes in pancreatic islets under high glucose concentration, LPA is synthesised by the reduction of dihydroxy acetone phosphate (DHAP) which is converted to acyl DHAP with the help of an acyltranferase. This, reductase enzyme acts on the latter to produce phosphatidic acid. This pathway predominately takes place in the peroxisomes (Dunlop *et al.*, 1985).

Dihydroxy acetone phosphate (DHAP) ⟶ Acyl DHAP ⟶ Phosphatidic acid

Other major intracellular enzymes which are involved in the production of LPA include phospholipase D (PLD), phospholipase A1 (PLA1) and phospholipase A2 (PLA2) (Mauco *et al*., 1978). Phospholipase A1 and phospholipase A2 produce LPA by acting on phosphatidic acid. Another major pathway for LPA synthesis is the plasma enzyme LysoPLD, autotaxin (ATX) from lysophosphatidylcholine. Autotaxin is a nucleotide pyrophosphatase/phosphodiesterase family member and is present in cerebrospinal

and seminal fluids. Reports suggest that the level of ATX is noticeably increased in patients with B-cell follicular lymphomas, Hodgkin-lymphomas and haematological malignancies (Masuda *et al*., 2008). There are correlations between these conditions with elevated LPA concentration in plasma however the cause of this elevation is not yet fully understood (Valet *et al*., 1998). Plasma LPA is mostly found bound to albumin and protected from enzymatic digestion by phospholipases (Tigyi *et al.*, 1992). To elicit the biological activities in serum and to prolong its physiological half-life in the circulatory system it is important that LPA be bound to albumin. Intracellularly, it is transported by binding fatty acid binding protein (Thumser *et al.*, 1994). Another mode of transport is binding to gelsolin, an actin-binding protein that is a key regulator of actin filament assembly and disassembly and by means of lipoproteins (Meerschaert*et al.*, 1998). The enzymes lipid phosphate phosphohydrolases (LPPs) which include LPP1/PAP2A, LPP3/PAP2B, and LPP2/PAP2C are the main enzymes involved in the maintenance of LPA homeostasis. These membrane bound LPPs dephosphorylate LPA to monoacylglycerol and PA. The nuclear phosphohydrolase which is yet to be identified also contributes to LPA degradation (Baker *et al*., 2002). Report suggests that an LPA specific lysophospholipase (80kDa), purified from rat brain, can also catabolise LPA by hydrolysing the acyl chain from the glycerol backbone. This group of lysophpospholipases has high affinity towards 1-oleoyl and 1-stereoyl rather than 1-palmitoyl and 1-myristyl-LPA.

The third enzyme known as 1-acylglycerol 3-phosphate acyltransferase (AGPAT)/ lysophosphatidic acid acyltransferases (LPAAT) catalyses the esterification of the sn-2

position of LPA to form PA. Here, Acyl-COA acts as a substrate for the reaction (Yuki *et al.*, 2009; Koeberle *et al.*). So far, five family members of LPAAT have been identified; LPAATβ/1-AGPAT 1,LPAATβ/1-AGPAT 2), LPAATγ/1-AGPAT 3, LPAATδ/1-AGPAT 4, and LPAATε/1-AGPAT 5. Of these, LPAATα shows a higher affinity towards LPA over the other members of the family (Aguado *et al.*, 1998).

1.3.2 Biological effects of LPA

1.3.2.1 Role of LPA in the vascular system

Previous studies suggest the influence of LPA in vascular development and formation of the vasculature (Smyth *et al*., 2008; Teo *et al*., 2009). The main functions regarding the vascular system are vasculogenesis, angiogenesis, which also includes proliferation and migration, vascular maturation and blood-brain barrier formation (Choi *et al*., 2010).

The studies conducted in autaxin (ATX) deficient mice showed high vascular defects in yolk sac and embryo which were embryonically lethal and were deficient of allantois and neural tube formation, suggesting that ATX is the major source for LPA production and shows the observed pathological effects in the ATX null mice (Tanaka *et al*., 2006). LPA may also cause activation of platelets during vascular injury and bind to integrins on the vessel walls. This subsequently leads to thrombus formation initiated by serotonin and thromboxane A2 (TXA2), reducing blood flow. LPA also leads to proliferation of vascular smooth muscle cells (VSMC) (Gennero *et al*., 1999). The above mentioned role on cell proliferation along with the migratory action of cells stimulated by LPA has beneficial effects in wound healing suggesting that LPA has some potential beneficial properties in the body. LPA is also reported to be a cardio protective molecule under hypoxia or hypoxia/serum deprived

induced apoptosis in mesenchymal stem cells and in cardiomyocytes (Chen *et al*., 2008; Karliner, 2004).

1.3.2.2 Role of LPA in the Neuronal system

The neuronal system is the major reservoir for LPA receptors. LPAR1 on the ventricular zone (VZ) of cortical neurons has been cloned. Further research has demonstrated the effects of LPA on neuroblasts includes cell proliferation (Chun *et al*., 2002) morphological changes such as cellular and nuclear rounding (Fukushima *et al*., 2002) and alteration in the ionic conductance which contributes to cortical neurogenesis (Dubin *et al*., 2010). Most of the LPA receptors are present in astrocytes, neurons, Schwann cells, oligodendrocytes and microglia (Noguchi *et al*., 2009). Other major roles of LPA in neuronal system include growth cone collapse, neurite retraction, cerebral cortex growth and folding, cell adhesion, anti-apoptotic actions in Schwann cells and migration (Tigyi *et al*., 1996; Zheng *et al*., 2004). LPAR1 is involved in the proliferation and differentiation of neural progenitor cells (NPC) and cultures of neurospheres. However, LPAR4 has also been reported to induce differentiation of immortalised hippocampal progenitor cells (Rhee *et al*., 2006). These roles of LPA on the neuronal system show the significant involvement of LPA in neuronal disorders like schizophrenia and neurological pain through LPAR1 (Inoue *et al*., 2004).

1.3.2.3 Role of LPA in Cancers

LPA has been shown to have major roles in pro-tumorigenic activity. Reported studies indicated the effects of LPAR1, LPAR2 and LPAR3 receptor mediate proliferation of tumours. These are mainly in the ovarian and gynaecological as well as lung cancers and little is known about the involvement in prostate cancer, mesothelioma, and glioma. The

elevation of LPA concentration in the plasma and ascitic fluid is yet to be explained. In ovarian cancer cells, LPA has been reported to induce cell survival, invasion, migration, proliferation that involves activation of metalloproteinase and vascular endothelial growth factors. These are reported to be mediated through LPAR2 receptor. LPA mediates angiogenesis, which further influence the neovascularisation of the tumours. Microvesicles released during apoptosis or malignant ascites contributing to high LPA production as in case of cancer patients (Mills *et al*., 2003).

1.3.2.4 Role of LPA in the Reproductive system

LPA mediated cellular signalliing plays diverse role in mammalian reproduction and reported to regulate spermatogenesis, development/implantation/spacing of embryo, fertilization, pregnancy maintenance (Hama *et al*., 2007; Sordelli *et al*., 2012; Ye *et al*., 2010). A previous study has also shown that LPA signalling is crucial for blastocyst formation in vertebrates (Kobayashi *et al*., 1994). A role for LPA in the reproductive system is linked to its production and release in the endometrium and in addition, LPA receptors are expressed in the endometrium. Moreover, LPA modulates prostaglandin (PG) synthesis and maintain early pregnancy in bovine species. This is mediated through LPAR3 that is expressed in the bovine endometrium. (Woclawek-Potocka *et al*., 2009). It has also been reported that the presence of LPA receptors on the mouse blastocyst during embryogenesis partially helps in calcium signalling mediated effects on autocrine and paracrine signals (Liu *et al*., 2004). Similarly, LPA has an important role in the transactivation mechanism through heparin binding EGF-like growth factor, triggering the differentiation of mouse blastocyst (Armant, 2005).

1.3.3 LPA receptors

Previous studies have indicated that LPA- stimulated biological activities are coupled through G proteins. The heterotrimeric G protein is composed of a α subunit and a $\beta\gamma$ complex. The inactive G protein bound to the receptor on binding of ligand undergoes conformational change and becomes active and dissociates from the receptor. Thus the equilibrium changes from the inactive G protein to the active state (Rubenstein *et al*., 2006). They undergo activation to actively combine the stimulated receptors to the target (Figure 2). Under basal condition the $\beta\gamma$ subunit are in combination with α subunit. On association with the activated receptor an exchange of guanosine diphosphate (GDP) with guanosine triphosphate (GTP) occurs on the α subunit, and the GTP-bound α subunit detaches from the stimulated receptor and the $\beta\gamma$ subunits. These α and $\beta\gamma$ subunits initiate diverse signaling pathway. The reassociation of α and $\beta\gamma$ subunits terminates the signaling pathway (Cabrera-Vera *et al*., 2003).

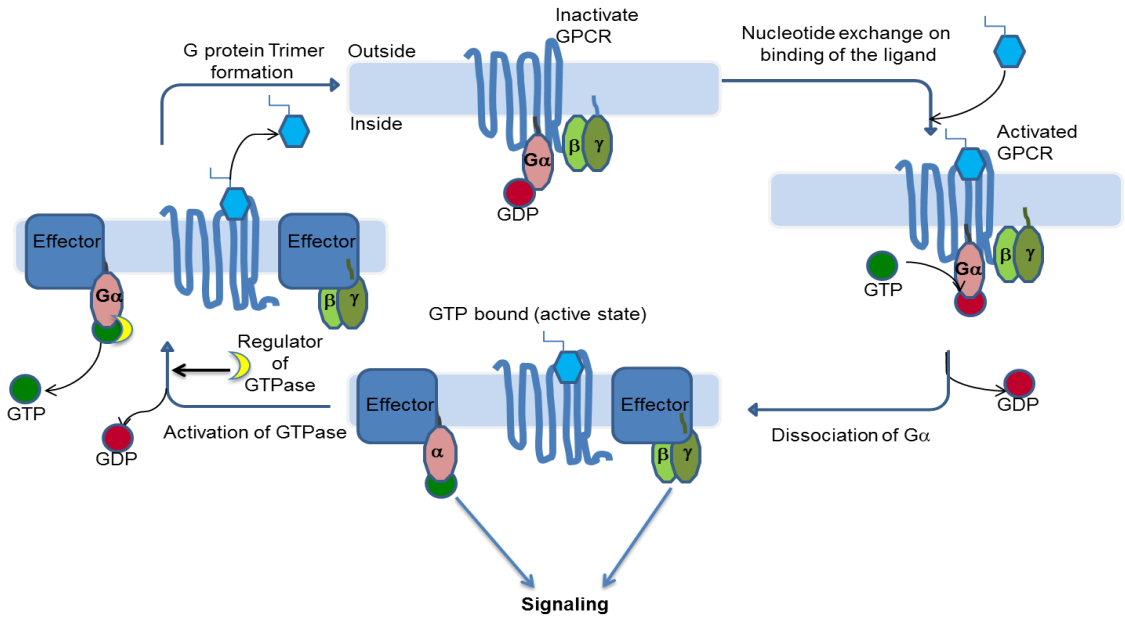

Figure 2. The process of Intracellular activation of GPCRs and confirmation changes. The inactive G protein bound to the receptor on binding of ligand undergoes conformational change and becomes active and dissociates from the receptor. Thus the equilibrium changes from the inactive G protein to active state and this causes the stimulation of downstream signalling events mediated through G protein subunits, α and $\beta\gamma$.

There are three main G-protein-mediated signaling pathways, mediated by four sub-classes of G-proteins distinguished from each other by sequence homology ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$). LPA activated G_q stimulates Protein kinases C (PKC) and increase in Ca^{2+} through phospho lipase C (PLC). Some of the key effects of $G\alpha_{i/o}$ include inhibition of adenylate cyclase (Sunahara *et al*., 1996); activation of mitogen activated protein kinase signalling; stimulation of phosphoinositide 3-kinases (PI3Ks), Ras and then Akt signalling through which they show mitogenic and anti-apoptotic effects cells (Hu *et al*., 2005). Similar effects may be mediated through ERKs pathways in fibroblasts (Fang *et al*., 2000). However, PI3K/Akt and ERKs have been reported to be involved in similar effects including cell survival in systems such as rat Schwann cells, murine hepato cell line, intestinal epithelial cells (Fang *et al*., 2000; Sautin *et al*., 2001).

$G\alpha_q$ is another subunit of heterotrimeric G protein that stimulates PLC. During this process, PLC cleaves the phosphatidyl inositol bisphosphate into diacyl glycerol and inositol triphosphate (IP3). On dissociation, IP3 remains in the cytosol and bind to the IP3 receptors to trigger the release of calcium from endoplasmic reticulum, however DAG stays bound to the membrane. The released calcium along with DAG induces PKC triggered signalling events.

$G\alpha_{12/13}$ is another subunit of G protein, which, through Guanine Nucleotide Exchange factors, regulates various biological activities. $G\alpha_{12/13}$ share 45% amino acid sequence identity with the remaining alpha subunits and 67% amino acid sequence identity among each other (Harhammer *et al*., 1996; Strathmann *et al*., 1991). The major function of $G\alpha_s$ is to activate

cAMP dependent pathway by activating adenylate cyclase. Intracellularly, two major enzymes; adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase regulate the level of cAMP. The three main targets of cAMPs are protein kinase A (PKA), guanine nucleotide exchange factors (GEF), and cyclic nucleotide gated ion channels. PKA plays important roles in glycogen metabolism, and inactivates PLC by phosphorylation. PKA also phosphorylates tyrosine phosphatase and activates MAPK. In addition, it is also reported to phosphorylate Rynodine receptors (Yano *et al*., 2003) and can modulate ion channel permeability by reducing the activity of Raf and Rho (Sassone-Corsi, 2012).

The role of $G\alpha_i$ has been examined with a toxin from clostridium botulinum pertussis toxin (PTX), which acts as a $G\alpha_i$ un-coupler. Pertussis toxin ADP-ribosylates the carboxyl terminal sites of most of the $G\alpha_i/G\alpha_o$ family, thereby inhibiting their interaction with the receptor, thus uncoupling the receptor from the G-protein.

As previously stated lysophosphatidic acid is a biologically important phospholipid present in various tissues and has a wide range of biological activities. Studies revealed that LPA binds to specific receptors to mediate its intracellular biological effects (Jalink *et al*., 1995). LPA binding protein was identified with the help of photo affinity study (van der Bend *et al*., 1992). LPA was reported as a ligand for ventricularzone-1(VZ-1) receptor first identified and isolated from mouse complementary deoxyribonucleic acid (cDNA). This was further identified as endothelial differentiation gene, a human homologue of VZ-1 (Hecht *et al*., 1996). LPAR1-3 are members of the endothelial differentiation gene (Edg) family (Mutoh *et al*., 2008; Virag *et al*., 2003). All these LPA receptors belong to the family of seven

transmembrane spanning G protein-coupled receptors (GPCRs) and share 45-56% overall amino acid identity. Recently used nomenclature for LPA receptors are LPAR1 (Edg-2), LPAR2 (Edg-4), and LPAR3 (Edg-7) with the guidelines of the International Union of Pharmacology (Contos *et al*., 2000; Contos*et al*., 2000; Contos *et al*., 2000). The newly identified receptors includes LPAR4/P2Y9/GPR23, LPAR5/GPR92 (Lee *et al.*, 2007), and LPAR6 (GPR87)(Ye, 2008). These receptors are coupled to G-proteins, i.e, $G\alpha_{12/13}$, $G\alpha_{i/o}$, $G\alpha_{q}$, and probably other G proteins except $G\alpha_{s}$ (Contos *et al*., 2000) which have already been described above.

1.3.3.1 LPAR1/ Edg2

In mouse, LPAR1/ Edg2 gene is localised at centromeric region of chromosome 4 consisting of multiple exons and introns. It is a 41 kDa protein consisting of 364 amino acids. In the central nervous system, it is highly localised in the neo-ventricular zone (VZ) and meninges during embryogenesis and expression of LPAR1 continues through the VZ disappears during cortical neurogenesis. In-situ hybridisation studies have reported the expression of LPAR1 in oligodendrocytes and Schwann Cells (Weiner *et al*., 1998). In addition, LPAR1 mediates its biological effects through the interaction with $G\alpha_{i/o}$, $G\alpha_{12/13}$ and $G\alpha_{11/q}$ followed by signalling mechanisms which include inhibition of adenylyl cyclase (Hecht *et al*., 1996), Ca^{2+} signalling, phospholipase C activation (Hofer *et al*., 1998) activation of the serum response element (Contos *et al*., 1998) and stimulation of Rho signalling (Arimura *et al*., 1998).

1.3.3.2 LPAR2/ Edg4

LPAR2/ Edg4 gene is located at chromosome 8 in mouse, in humans (chromosomal locus 19p12) and shares 60% homology towards LPAR1. It has a molecular mass of 39kDa with

348 amino acid residues (Contos *et al*., *et al*., 2000). Similar to LPAR1, its biological activities are mediated through $G\alpha_{i/o}$, $G\alpha_{11/q}$, and $G\alpha_{12/13}$ and have multifunctional activities. Unlike LPAR1, LPAR2 is present in the brain during embryogenesis but disappears within 2 weeks after birth. In mouse, LPAR2 is highly expressed in uterus, kidney, and testis but expression of LPAR2 receptors is moderately present in lung and much lower in heart, stomach, spleen and thymus. In human tissues, high expression of LPAR2 is detected in testis and leukocytes, with moderate expression found in prostate, spleen, thymus, and pancreas. This highlights the targets and the potential of LPAR2 gene and its oncogenic role in addition to its role in cell migration and survival (Goetzl *et al*., 1999; Zheng *et al*., 2001).

1.3.3.3 LPAR3/ Edg-7

Another edg family member of the LPA receptors is the LPAR3/ Edg-7. Human forms of LPAR3 consist of 353 amino acid residues and 40.1 kDa of estimated molecular weight (Bandoh *et al*., 1999). It is predominantly present in prostate, heart, testis, pancreas, lung, ovary and brain in human. In mouse it is mostly present in kidney, small intestine, heart, stomach, thymus, brain, lung, spleen, testis (Contos *et al*., 2002; Kingsbury *et al*., 2003; Sardar *et al*., 2002). Reports suggest that the major actions of LPAR3 are mediated through $G\alpha_{i/o}$, $G\alpha_{11/q}$ and shows lower response towards saturated LPA than LPA bound to unsaturated fatty acids (Bandoh *et al*., 1999).

1.3.3.4 LPAR4/P2y9

LPAR4/P2y9 is an integral G-protein-coupled receptor which has high affinity towards 1-oleoyl LPA with a Kd value of 45 nM and sharing 20-24% amino acid sequence identity towards Edg receptor family (Noguchi *et al*., 2003). LPAR4 shows a closer identity towards

the P2Y receptor than Edg family (20-24 %) (Yanagida *et al*.,2007). Its affinity towards the LPA analogues is shown in the order of 18:1- > 18:0- >16:0- > 14:0- > 1-alkyl- > 1-alkenyl-LPA (Noguchi *et al*., 2003). In humans, LPAR4 is ubiquitously present with the highest expression in the ovary. The main reported effects are induction of Ca^{2+} mobilisation, cAMP accumulation, cell rounding, and stress fibre formation. These reported effects are mediated through G-proteins mainly $G_{\alpha s}$, $G_{\alpha q11}$ and $G\alpha_{12/13}$. However, LPAR4 shows suppressive effect on cell motility, cell migration in B103 cells and invasion of colon cancers (Lee *et al*., 2008).

1.3.3.5 LPAR5/GPR92

In human, the LPAR5 gene is located on chromosome 12p13.31 and encodes a 41-kDa protein consisting of 372 amino acids, sharing 35% homology with LPAR4. It has been reported to be well expressed in tissues like the heart, skin, spleen, small intestine, stomach, thymus, liver, embryonic brain and embryonic stem cells, B cells, intestinal CD8+ lymphocytes, and platelets (Kotarsky *et al*., 2006). The major biological activities mediated through LPAR5 include LPA-induced neurite retraction, stress fibre formation mediated through $G\alpha_{12/13}$, increasing intracellular calcium through $G\alpha_q$ and increasing cAMP levels. Recently identified lipid-derived molecules (farnesyl pyrophosphate and *N*arachidonylglycin) were characterised as LPAR5 ligands (Yoon *et al*., 2008).

Other recently identified receptors which can mediate the biological effects of LPA are LPAR6/GPR87, LPAR7/p2Y5, and LPAR8/p2Y10 (Murakami *et al*., 2008; Pasternack *et al*., 2008). LPAR6/GPR87 is mainly expressed in brain, skeletal muscle, and the reproductive organs. LPAR7/p2y5 is found in the skin and hair. P2Y5 was identified as

a critical mediator for human hair growth and is a causal gene of a rare familial form of human hair loss (Pasternack *et al*., 2008). LPAR8/p2y10 has been found in the uterus, prostate, brain, lung, placenta, and skeletal muscle (Murakami *et al*., 2008).

Non-GPCR includes intracellular receptor, peroxisome proliferator-activated receptor gamma (PPARγ) which is a potent ligand for serum lipids like LPA. It shows highest response towards the unsaturated LPA species and plays an important role in cell proliferation (Hurst-Kennedy *et al*., 2009) apoptosis (McIntyre *et al*., 2003). These properties explain the role of this receptor in diseases like atherosclerosis, diabetes and cancer.

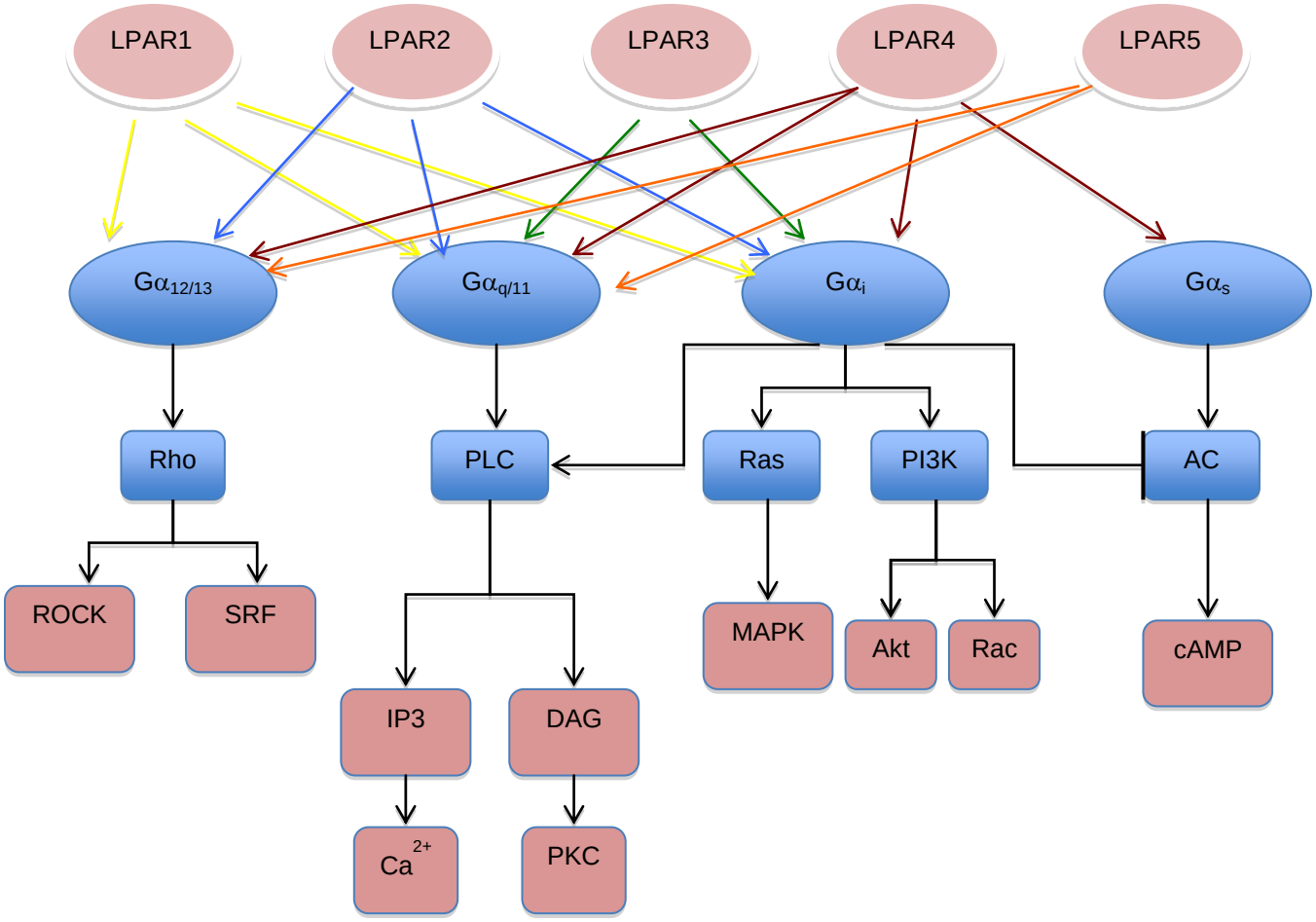

Figure 3. Lysophosphatidic acid (LPA) receptors induced signalling mechanisms

The figure elucidates the main signalling events mediated by LPA1-5 receptors, coupled through G-proteins. The major downstream signalling molecules involved are MAPKs, PI3K, PKC and ROCK pathways.

1.4 G Protein Coupled Receptors (GPCRs) and the downstream signalling mediated by Lysophosphatidic Acid

As already indicated above, GPCRs trigger various biological activities in cellular systems and these are mainly by the involvement of major signalling molecules such as PI3K, PKC, ROCK, and through small GTPase activating the downstream of Akt or MEK pathways. The detailed overview of signalling events briefed below.

Phosphoinositide 3-kinases are mostly present in all cells and regulate cellular responses like proliferation, growth, migration, differentiation, survival, apoptosis and even cell cycle. These are mainly activated via heterotrimeric G Protein subunits $G\alpha_i$, $G\alpha_q$ and $G_{\beta\gamma}$. PI3Ks are subdivided into 3 subclasses. Class I is a heterodimeric protein having a catalytic and regulatory subunit. It is also further divided into IA and IB subtypes. Class IA PI3Ks have a p85 regulatory and p110 catalytic subunits. Class III are heterodimeric enzymes with a catalytic and regulatory site but class II PI3Ks are monomeric proteins with very little known about these enzymes (Elis *et al*., 2008). Receptor tyrosine kinases (RTKs) (Class I PI3K) and GPCRs activates their downstream target, class IB PI3Ks. PI3K catalyses the formation of phosphatidylinositol (3, 4, 5)-trisphosphate (PIP_3) by transfer of a gamma phosphate of ATP to the 3'-OH position of the inositol ring of membrane bound phosphatidylinositol (4, 5)-bisphosphate (PIP_2). Further, this activates a downstream enzyme, Akt or protein kinase B (PKB) that is a serine/threonine protein kinase. This is mediated by phosphatidylinositol dependent protein kinases PDK-1 and PDK-2 on the plasma membrane by which the Akt enzyme becomes completely active. PDK1 catalyses the phosphorylation of Akt in the domain active loop at threonine 308 and PDK2 phosphorylates at serine 473. PI3K/Akt plays a vital role in activating crucial effector molecules including the induction of c-myc and Bcl-2,

phosphorylation of 4E-BP1 and activation of translation initiation factor and regulation of anti-apoptosis. PDK1 has been reported to phosphorylates Protein Kinase C (novel, atypical), p70 s6 kinase. The de-phosphorylation and degradation of PIP3 are mediated by tensin homolog deleted on phosphatase and tensin homologue (PTEN) and Src homology 2-containing inositol 5-phosphatase (SHIP) removing 3-phosphate from PIP3 to generate PI (4, 5) P2 and PI (3, 4) P2 respectively. PI3K has been reported to be involved in differentiation of various cells such as trophoblastic cells, osteoblasts, chondrocytes, embryonic stem cells (Kent*et al*., 2010). In addition, PI3K plays a crucial role in differentiation of stem cells into cardiomyocytes (Fujita *et al*., 2004; Kent *et al*., 2010; Naito *et al*., 2003; Sauer *et al*., 1999).

Protein kinase C belongs to a family of protein kinase enzymes that acts by phosphorylation of hydroxyl groups on serine and threonine residues in the proteins. They play significant roles in cellular mechanisms like proliferation, differentiation, and apoptosis. These are mainly activated by high concentrations of diacyl glycerol, which is produced by the action of PLC and Ca^{2+}. PKC activation through heterotrimeric G Protein is mainly through $G\alpha_i$/ $G\alpha_q$. The PKC family is sub-divided into conventional (c)PKC, novel (n)PKC and atypical (s)PKC depending on their requirement of second messengers. Conventional PKCs are α, β_1, β_2 and γ isoforms, which require Ca^{2+}, DAG and a phospholipid (phosphatidyl serine) for their activation. Novel PKCs contain δ, ε, η and theta isoforms and require only DAG for its activation and atypical PKC do not respond to either DAG or Ca^{2+}. Structures of PKCs consist of a regulatory site and a catalytic site. The regulatory site carries a C1, C2, C3 and C4 domains. The C1 domain has high affinity to bind to DAG and phorbol esters. This is functional in conventional and novel isoforms of PKCs, but not in the atypical isoforms. The

C2 domains acts as a Ca^{2+} sensor which is present in both (c)PKC and (n)PKC but it is functional only in (c)PKC. The catalytic site of PKC consists of the N terminal at the β sheet and c terminal at the α-helix. The enzyme with two lobes forms a cleft that is also the site for ATP, substrate and pseudo-substrate. The activation of PKC is regulated by phosphorylation at specific sites. (c)PKC and (n)PKC have three phosphorylation sites called activation loop, the turn motif and the hydrophobic motif but the atypical PKC has only activation loop and turn motif phosphorylation sites. First, PDK-1 mediated phosphorylation occurs at the activation loop of conventional, novel and atypical PKC, which is followed by the auto phosphorylation at threonine 450 in the turn motif and serine 660 in the hydrophobic motif (Newton, 2003). This event is PIP3 independent; however the PDK-1 driven Akt activation is PIP_3 dependent (Sonnenburg *et al*., 2001). Previous reports suggest the importance of PKC in the differentiation of various cell types including mESCs, human dental pulp stem cells, bone marrow cells (Kiraly *et al*., 2009; Li *et al*., 2009).

Other importantly, signalling actions are channelled through multiple signalling pathways including the mitogen activated protein kinases (MAPKs). MAPKs are activated by sequential phosphorylation of MAP Kinase Kinase Kinase, MAP Kinase Kinase. However, previous reports have indicated that this could be accelerated either by activation or inhibition of these MAPK families (Gaur *et al.* 2010; Li *et al.*, 2002). The MAPKs family include extracellular signal regulated kinases (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK).c-Jun N-terminal kinase (JNK), these are further discussed in detail (Chapter 4).

Aim of the project

Evidence over the past decade does suggest that LPA does induce differentiation of neuronal and mesenchymal stem cells (Wang *et al.*, 2006; Cui *et al.*, 2007). Interestingly, reports have also suggested the importance of LPA during the development of embryo to blastocysts/gastrula and it has been reported to act as a cardio protective molecule under hypoxic condition and protects MSC against hypoxia/ serum deprived (SD)-induces apoptosis, (Chen *et al.*, 2008; Karliner 2004). LPA also plays diverse physiological roles in cellular system as discussed above and include regulation of cell proliferation, differentiation, chemotaxis as well as acting as an anti-apoptotic agent and accumulates in the myocardium after an acute infarction. Most of its effects are mediated via signalling molecules (discussed above) which also play a critical role in the lineage commitment of stem cells. Hence we hypothesise that LPA could be a potential endogenous molecule that regulates stem cell differentiation into cardiomyocytes, exerting its effects through distinct LPA receptors linked to specific downstream signalling pathways. This study has therefore investigated the effect of LPA on the ability of the murine P19 stem cells to differentiate into cardiomyocytes and further examined the role of the different LPA receptors and the downstream signalling mechanisms associated with the commitment of the P19 cells into a cardiac lineage.

Chapter 2

Materials and methods

2.1 Resuscitation and culture of P19 stem cell from frozen stock

P19 mouse embryonal carcinoma stem cells (P19 stem cells) (passage 7) were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, UK). One ml of frozen P19 stem cells was quickly thawed in 15 ml falcon tube, resuspended in 9ml of fresh complete culture medium containing alpha Minimum Essential Medium (α-MEM) supplemented with 10% foetal bovine serum (FBS), 100units/ml penicillin/100μg/ml streptomycin (all from Invitrogen, UK). The tubes, containing the cells, were centrifuged at 1000 RPM at 4^{o}C for 5 minutes. The medium was then aspirated and the pellet was separated. The cells were then resuspended in 7 ml of medium and dissociated into single cells by repeated pipetting in a T25 flask. Further, the cells were cultured in a cell culture incubator at 37^{o}C, 95% air and 5% CO_2.

On day 2, cells in the T25 flask were observed under a microscope for normal growth. On day 3, the spent media was replaced with fresh medium. The cells were subsequently trypsinised and sub-cultured when they were approximately 60-70 % confluent. Following growth, cells were harvested and the resulting cell suspension approximately divided into five parts (1:5 splitting) and further sub-cultured for future investigations in this thesis.

2.1.1 Routine cell culture

All P19 cells were routinely cultured in falcon flasks with complete medium maintained at 37^{o}C, 95% air and 5% CO_2. Cells at 60-70% confluence were sub-cultured by removing the spent media from the flask and were washed 3 times with warm (37^{o}C) 1X phosphate buffer saline (PBS). 1.5 ml of warm 1% of trypsin solution (Invitrogen-UK) was added into each flask and vigorously tapped. 8.5 ml of fresh complete medium was added to inactivate

the trypsin in the flask and 1 ml of the re-suspended cells was then added to a new T-75 flask with 14 ml of fresh medium and cultured in an incubator.

2.1.2 Cryopreservation of cells

When necessary, cells were stored for future use by trypsinizng cells as above and spun at 1000 RPM for 5 minutes at 4^{o}C. The supernatant was aspirated and the pellet re-suspended in 1 ml of freezing medium containing 90% FBS and 10% glycerol (Invitrogen, UK). The cell suspension was aliquoted in cryovials (cells from one T-75 flask were frozen in 2 cryovials i.e. splitting ratio of 1:2) and placed in a Thermo Scientific NALGENE® Mr. Frosty (i.e. a controlled rate freezing container) (Fisher Scientific, UK) for freezing overnight at -80^{o}C. The next day, the vials were transferred into liquid nitrogen for long-term storage.

2.2 Embryoid body (EB) formation and differentiation into cardiomyocytes

2.2.1 Cell counting and plating for EB formation

To initiate differentiation, cells have to form 3 dimensional structures called embryoid body (EBs). For this, cell monolayers were treated with 1% trypsin to form a single-cell suspension. The pellet was resuspended in full medium. 20μl of the cell suspension was mixed with equal volume of trypan blue and placed in the Countess electronic cell counter chamber. The concentration of viable cells (cells/ml) to be plated from the cell suspension was determined in the following example:

Viable cells in the cell suspension (1 ml) = $3.1X\ 10^6$

Desired concentration of cells/ Petri dish = $3.7X10^5$

$$\text{Volume of cell suspension (x) plated} = \frac{\text{Desired concentration of cells (i.e. } 3.7\ X10^5 \text{ cells)}}{\text{Viable cells in the cell suspension (1 ml) } (3.1X\ 10^6)}$$

Hence, the volume of cell suspension (x) containing $3.7X\ 10^5$ cells was calculated.

119.4μl of cell suspension was plated into non-tissue culture grade microbiological Petri dishes with 4880.6 μl of full medium.

2.3 Initiation of P19 cell differentiation with 1% DMSO

Semi confluent P19 cells ($3.7x10^5$) were seeded into ultra-low attachment petri dishes containing full medium and left to form EBs over 4 days (Figure 4) before plating into normal 6-well cell culture dishes for differentiation on the fifth day. Initially, to establish a differentiation model, confluent monolayer were trypsinised and treated with 1% DMSO during the EB formation phase. Embryoid bodies plated into normal cell culture dishes were allowed to attach and grow into monolayers, changing the medium every 2 days. The day of plating the EBs into cell culture dishes was designated as day 0. Whole cell lysates were generated at days 2, 4, 6, 8, 10 and 12. In parallel, studies were also conducted to look at the effects of seeding density of EBs on efficiency of differentiation. Embryoid bodies were formed from a predetermined number of P19 cells ($3.7\ X\ 10^5$

cells/petri dish) in the presence of 1% DMSO. The EBs were then resuspended in 12 ml fresh complete culture medium. Various volumes from 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 ml of the resuspended EBs were plated into 6 well tissue culture plates. Whole cell lysates were generated at day 6 after plating into cell culture dish. Lysates were analysed for their total protein content (Section 2.7) and subsequently subjected to western blot analysis (section 2.8).

2.4 Initiation of P19 cell differentiation with LPA

To induce differentiation with LPA, the culture medium was supplemented with 18:1 LPA purchased from Sigma Aldrich and dissolved in 1X PBS containing 0.1% fatty acid free bovine serum albumin. LPA solutions was added at concentrations of 1μM, 5μM, 10μM or 25μM immediately before the cells were seeded into the non-tissue culture grade microbiological Petri dishes and prior to EB formation. In parallel studies 1% DMSO was used in place of LPA as a positive control for the induction of P19 cells into cardiomyocytes.

Cell lysates were generated at different time points at days 6 and 12 after plating into cell culture dish. Whole cell lysates were also generated for time course studies at days, 2, 6, 10 and 14 days after plating into cell culture dish. Lysates were analysed for their total protein content (Section 2.7) and subsequently subjected to western blot analysis (Section 2.8) for the cardiac-specific MLC-1v.

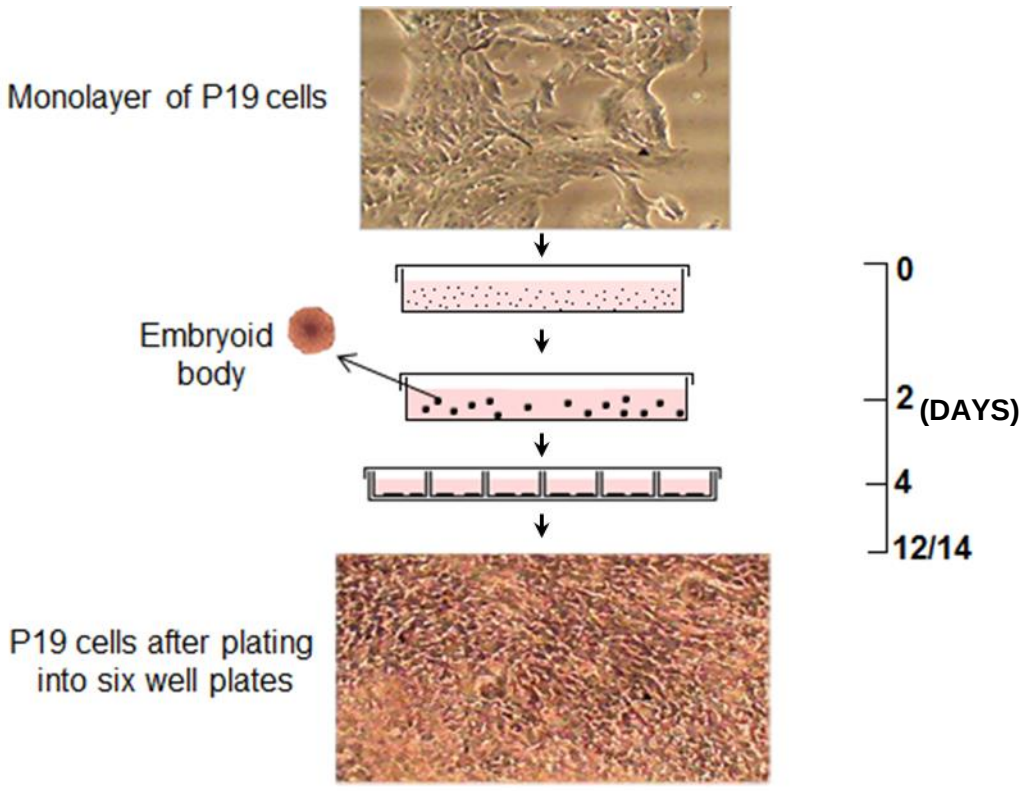

Figure 4. Induction of differentiation of P19 stem cells into cardiomyocytes.

Embryoid bodies were formed from P19 stem cell in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and LPA or 1% DMSO for four days. The EBs were transferred into standard cell culture dishes and cultured over a period of up to 12 or 14 days. For the study carried out for the time course on the induction of differentiation, Lysates were generated at different time points (days- 2, 6, 10 and 14) and for the concentration dependent studies lysates were generated on day 6 and 12 as required.

2.5 Effects of receptor/signalling inhibitors on P19 stem cell differentiation into cardiomyocytes induced by LPA

Cells ($3.7x10^5$) were seeded into ultra-low attachment petri dishes containing complete medium and allowed to form EBs over 4 days as described above. Where an inhibitor for a receptor or signalling was used, cells were treated with the inhibitor for 60 min before the addition of 5μM LPA. When used, pertussis toxin (PTX; 100 and 200 ng/ml) cells were incubated for 24 hours before the addition of LPA. Inhibitors were kept in the medium throughout the EBs formation stage. On day 5, EBs were transferred into 6 well cell culture dishes containing full medium which was changed every 2 days. Monolayers of cells were lysed on day 6 after plating in the 6 well plates for the detection of MLC-1v by western blot analysis.

The inhibitors used were Ki16425 (1, 10 and 50 μM) purchased from Cambridge Biosciences (Cambridge, UK), suramin (Sigma Aldrich, UK), Y-27632 (1, 5 and 10 μM; Calbiochem, UK), SB203580 (1 and 10 μM), LY294002 (1, 2, 5, 10 and 20 μM), Akt inhibitor XIII (0.1, 0.5 and 1.0 μM) and bisindolylmaleimide I (0.1, 0.5, 1.0, 5.0 and 10 μM) from Merck Chemicals Ltd, UK.

2.6 Time course of phosphorylation of Akt, p38 MAPK and ERK1/2 and effects of inhibitors on phosphorylation induced by 5μM LPA

For time course study, P19 cells were seeded into petri dishes at a density of $2X10^4$ cells/ml in complete growth medium and placed in the incubator for normal growth. When cells were 70% confluent, the culture medium was removed. The cells were then supplemented with low serum containing growth medium (1% FBS) and serum starved for a period of 24 hours.

Cells were eventually either incubated in fresh medium alone or in medium containing 5μM LPA for a time point of 0, 1, 3, 5, 10, 15, 30 or 60 minutes. In parallel dishes, cells were pre-treated with either receptor or specific signalling inhibitors for 60 minutes before LPA treatment for selected time points. Each P35mm petri dish was lysed on ice with the (1X) lysis buffer (Section 2.7) containing 1:100 dilution of a phosphatase inhibitor cocktail (Sigma Aldrich, UK). The lysates were then stored at -20^{o}C until analysed by western blotting using kinase-specific primary monoclonal antibodies. Antibodies for phospho-Akt (Ser473), phospho-p38 MAPK and phospho-ERK1/2 were purchased from Cell Signaling Technology, (UK).

2.7 Cell lysis and quantification of proteins

Cell monolayers were washed 3 times with 1X ice-cold PBS and lysed by adding 350μl of ice-cold lysis buffer (2 mM Tris-HCl, pH 7.4, 1% SDS). Lysates were transferred into 1.5 ml micro-centrifuge tubes and heated at 95^{o}C for 5 minutes followed by sonication 3 times, 30s each cycle. Cell lyses for phospho-proteins were generated using 1X lysis buffer containing phosphatase inhibitor (1:100). Lysates were then centrifuged and the supernatant was used for analysis. Total cell protein was colorimetrically quantified using the bicinchoninic acid (BCA) assay reagent (Smith *et al.*, 1985). The peptide bonds of proteins reduces cupric (Cu^{2+}) ions to cuprous (Cu^{+}) ions, which further chelates with two molecules of bicinchoninic acid forming a purple colour complex measured at 620 nm. A protein standard was prepared with bovine serum albumin (BSA) by dissolving 10mg/ml of BSA in double distilled water. This was then diluted as shown in Table 1 below to give the required concentration range of standard proteins.

Table 1. Preparation of working BSA standards

BSA from stock10mg/ml (µl)	Double distilled water (DDW) (µl)	Final concentration µg/µl
0	1000	0
25	975	0.25
50	950	0.5
100	900	1.0
200	800	2.0
300	700	3.0
400	600	4.0
500	500	5.0
600	400	6.0
700	300	7.0

The volumes of 10 mg/ml BSA as indicated in **Table.1** were diluted with the appropriate volumes of double distilled water to give the final concentrations indicated. 5µl of each concentration was used to set up the standard curve as described below.

A 96 well plate was set up as follows:

a. Control wells: 5μl double distilled water (DDW) + 5μl lysis buffer + 100μl BCA.
b. Standards: 5μl standards + 5μl lysis buffer + 100μl BCA.
c. Sample wells: 5μl DDW + 5μl lysate + 100μl BCA.

The plates were incubated at room temperature for 45 minutes on a shaker and the absorbance determined by using a Multiskan II plate reader (Ascent, BCA protocol) set at a wavelength of 620 nm. Protein concentration for each sample was determined from the BSA standard curve. A sample standard curve is shown in Figure 5 below. A standard curve is constructed using the absorbance reading of the series of standards against the known concentration of the standards. The concentration of protein in samples was determined using the formula y= mx+c.

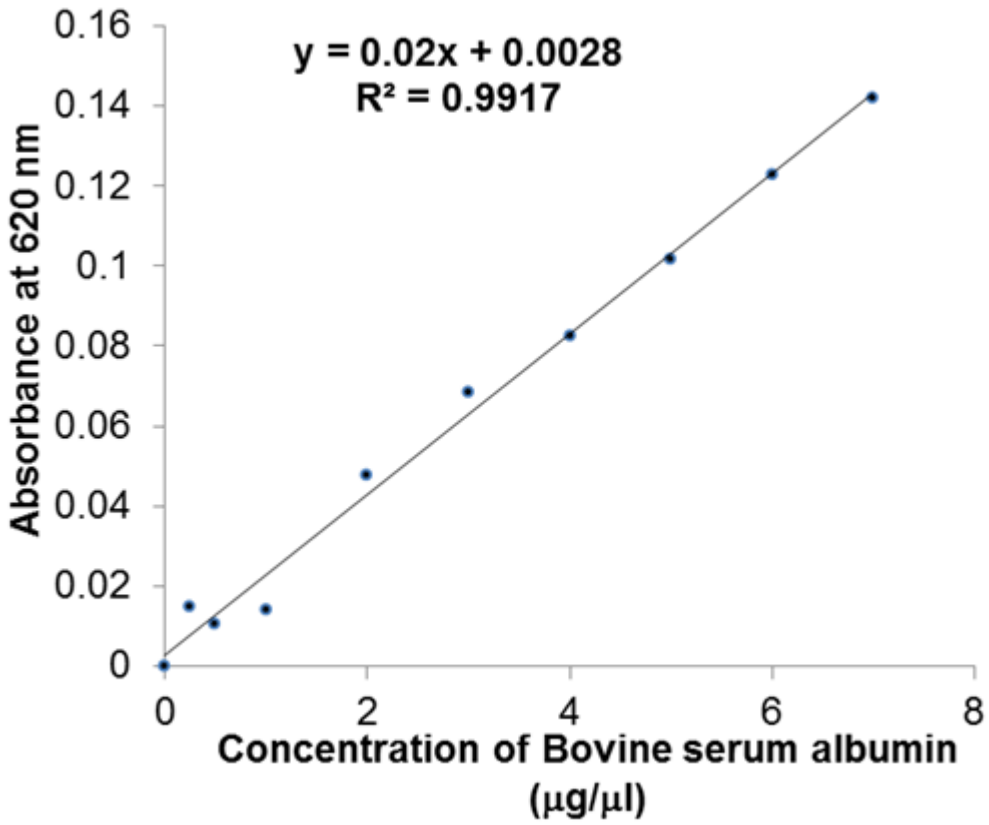

Figure 5. BCA Protein standard curve

The quantification of protein for western blot analysis was determined using the BCA assay. The standard curve was constructed by plotting known concentrations (ranging from 0-5 µg/µl) on x- axis against the absorbance of the standards on y-axis. Concentration of protein in the sample was then calculated using this standard curve with the slope and the formula y=mx+c.

2.8 Western Blotting

Protein lysates were separated and resolved using sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). This comprised of cell lysates preparations, separation of lysates proteins by gel electrophoresis, transfer of proteins from gel to a membrane, immunoblotting and detection of protein band using enhanced chemiluminescence.

2.8.1 Sample preparation and gel preparation

A known volume of cell lysates for loading 25 μg of protein was mixed with equal volume of 2X sample buffer containing SDS (4%), glycerol (10%), β-mercaptoethanol (2%) and bromophenol blue 0.006%), Tris-HCl, pH6.8 (250 mM). The gel electrophoresis was carried out on a Bio-Rad Mini-PROTEAN II casting stand (Bio-Rad, UK). The resolving gel (12 %) was prepared for two gels as follows: double distilled water (3.31 ml), 30% acrylamide/bisacrylamide (3.99 ml), 1.5M Tris-HCl, pH8.8 (2.5 ml), 10% sodium dodecyl sulphate (SDS) (0.1 ml), 10% ammonium persulfate (APS) (0.1 ml) and N, N, N′, N′-tetramethylethylenediamine (TEMED) (6μl). The solution was mixed well and 4 ml was added between the glass plates for polymerisation. A thin layer of iso-butanol was layered on top to prevent air contact and to allow the formation of a uniform layer. After polymerisation of the resolving gel the layer of iso-butanol was removed, rinsed with distilled water and dried well. The 8% stacking gel was prepared as follows: ultra-pure water (5 ml), 30% acrylamide/0.8% bisacrylamide solution (1.3ml), Tris-HCl (0.5M; pH 6.8) (2.5 ml), 10% SDS (0.1ml), of 10% ammonium persulfate (0.05ml) and TEMED (0.01ml). The stacking solution was mixed well and added on top of the resolving buffer. 1 mm Teflon comb was placed in the gel to form loading wells for the samples. This was kept for polymerisation for 30 minutes. The comb was removed after the polymerisation and the gel was placed in the electrophoresis

tank with 1% tank buffer (0.025M Tris, 0.192M glycine and 0.1% SDS). The samples mixed with 2X sample buffer were boiled at 95^{o}C for 3-5 minutes and 25μg of protein was loaded into each well along with 5μl of molecular weight ladder in the last well. The electrophoresis was run at 20mA/gel for stacking the samples and changed to 25mA/gel for resolving. The electrophoresis was performed at a voltage of 220 V until the tracking dye reached the bottom of the resolving gel.

2.8.2 Transfer of proteins from gel to polyvinylidene difluoride PVDF membrane

The proteins were transferred from the gel to the polyvinylidene difluoride (PVDF) membrane in a semi-dry transfer cell (Trans-Blot SD, Bio-Rad) using 1X transfer buffer consisting of 48mM Tris base (pH 7.5), 39mM glycine, 0.0375% SDS and 20% methanol. The PVDF membrane was treated with methanol for 30 seconds followed by washing with distilled water. This removes the hydrophobicity of the membrane. The gels were then removed from the electrophoresis tank and placed on top of the PVDF membrane. These were sandwiched between two filter papers soaked in transfer buffer. The transfer of proteins was carried out for 20 min at 25 volts.

2.8.3 Blocking of membrane and detection of protein bands by enhanced chemiluminescence (ECL)

Membranes were blocked for one hour at room temperature or overnight at 4^{o}C in blocking buffer to prevent non-specific binding of the antibody. For this, 100ml of blocking buffer was prepared with 10ml of 10X Tris Buffer Saline-Tween (TBS-T) washing buffer (100 mM NaCl, 10 mM Tris (pH 7.4)), 0.1% (v/v) of Tween-20 was added on the day to the 1X diluted buffer, 5% (w/v) of non-fat skimmed milk was also added to the blocking buffer just prior to use. Membranes were then probed with the primary antibody which was a mouse

primary monoclonal antibody against MLC-1v (1:500; ab680, Abcam, Cambridge, UK). The secondary antibody used was a horseradish peroxidase conjugated mouse mAb against β-actin (1:5000; Sigma Aldrich, Dorset, UK). At the end of the incubation period with primary antibody, the membrane was washed at least 3 times with Tris buffer saline-tween 20 (TBS-T) before being incubated with the goat-anti mouse horseradish peroxidase conjugated secondary antibody (1:5000; Cell Signalling Technology, UK) for 1 h followed by 3x washes with TBS-T. All antibody dilutions were in blocking buffer. The bands were visualised using the ECL reagent. For this, equal volumes of ECL reagent 1 (5ml of 100 mM Tris pH8.5 + 25μl of 90 mM p-coumaric acids + 50 μl of 250 mM luminol) and reagent 2 (5ml of 100 mM Tris pH8.5 + 3μl of 30 % H_2O_2) were mixed, and applied over the membrane for 1 minute. Excess ECL solution was drained off and the membrane covered with cling film avoiding trapping air bubbles. An autoradiography hyperfilm (Amersham, UK) was then placed over the membrane and left in the cassette for the desired time. The film was immediately developed using Kodak DEKTOLTM developer and fixed with Kodak UNIDIXTM fixer. The protein bands were then quantified using image-j densitometry.

2.9 Cell viability assay

The cell viability assay also called the3-[4, 5-Dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay was performed to establish the cytotoxicity of any drug used in the study. Metabolically active cells reduce MTT to insoluble formazan crystal that was measured colorimetrically at 540 nm. This enzymatic conversion of MTT was used as an index of cell viability (Slater *et al.*, 1963). Cells were seeded in 96-well plates and treated with a range of concentrations of the different drugs in full medium for 24 hours. 20μL of MTT solution (5mg/mL) was added to each well and incubated for 4 hours. The medium

was removed and formazane crystals were dissolved in 100μl iso- propanol and absorbance was read at 540nm on a Multiskan II plate reader (Ascent, MTT protocol). Viability was expressed as a percentage of control untreated cells.

2.10 Extraction of RNA and RT-PCR study

To establish the expression profile of LPA receptors in P19 cells, transcripts of the receptors were analysed using quantitative PCR (qPCR). In these studies, controls and LPA (5μM) treated cells in T75 flasks were incubated with 2 ml of the RNA STAT60 reagent and the cells lysed by repetitive pipetting. Further extraction of RNA, removal of genomic DNA contamination and the reverse transcription of the RNA to cDNA were carried out as described below. Changes in mRNA levels for each receptor was normalised by amplification of a reference housekeeping gene (HKG), which is essential for the relative quantification of cDNA in each target genes in individual reactions.

Total RNA was extracted from a confluent layer of cells in a T-25 flask using the RNA STAT60 reagent (Amsbio, UK) as per manufacturer's instructions. The protocol was as follows: 2 ml of the RNA STAT60 reagent was added to a monolayer of P19 cells and homogenised by repetitive pipetting. The lysates were further incubated for 5 min for complete dissociation of nucleoprotein. Following the addition of 0.4 ml of chloroform (equivalent to 0.2:1.0 ml, chloroform/lysates) lysates and the contents were vigorously mixed, allowed to stand for an additional 5 minutes and then centrifuged at 12,000 RPM for 15 minutes at 4^{o}C. Total RNA was precipitated from the top aqueous layer by the addition of 0.5 ml of isopropanol followed by incubation at room temperature for 5 min

before a final centrifugation step at 12,000 rpm for 15minutes at 4^{o}C. The resulting white pellet of RNA was washed 3 times with 1 ml of 75% ethanol with vortexing and centrifuging each time before air- d r y i n g the pellet in a fume cupboard. The pellet was then dissolved in 50μl of DNAse free water. The RNA was quantified spectrophotometrically at 260nm. The purity of the RNA was determined from the 260/280 ratio which was expected to be >1.8 for the samples to be considered suitable for analysis. The quality of the RNA was further evaluated with RNA electrophoresis visualizing sharp and non-smearing bands for the two intact ribosomal bands of 28s and 18s with the intensity ratio of 2:1 respectively.

To remove genomic DNA contamination, RNA was next subjected to RNase-free DNase 1 treatment using Ambion Turbo DNA-free kit. Next, 1μg of total RNA was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Qualitative detection of LPA receptor expression was carried out by standard PCR. The amplification conditions were as follow: 10 min at 95^{o}C; then 35 cycles of 15 s at 95^{o}C; 1 min at 60^{o} C; and 10 sec at 72^{o}C followed by dissociation at 58^{o}C to 95^{o}C. Primers for LPA receptors1 to 5 were designed using fast PCR, purchased from Primerdesign Ltd, (UK).

The expression of the LPA receptors was then confirmed by running 2% agarose gel electrophoresis in 1X TBE buffer (10X Tris borate EDTA (TBE) buffer- 89mM Tris-HCL pH 7.8, 89 mM borate, 2mM EDTA). The weighed agarose was dissolved in 100ml of 1X TBE buffer by heating in a microwave for 2-3 minutes. The content was allowed to cool and added 0.50 μl of Ethidium Bromide and mixed it by swirling. This is them poured into the two sides

tapped electrophoresis tray, then placed the comb and the gel was left to set for 30 minutes. The gel was then placed in an electrophoresis tank with sufficient volume of 1X TBE buffer to cover the top layer of the gel and removed the comb. Each sample 10 μl each were then loaded into the well along with respective negative controls (nuclease free water) and added 2 μl of the DNA ladder (50bp) (Life Technology, UK). The Electrophoresis was carried out for around 60-90 minutes at 100 V until it reaches towards the end of the gel making sure not to run off the gel.

Table 2. LPA Receptor Primers

Receptors	Forward strand	Reverse strand
LPA1	5^{I}- TCTTG TTATTGCTGGATGTG-3^{I}	3^{I}-CGGTAGGAGTAGATGATGG-5^{I}
LPA2	5^{I}- TAATAACAGCGGCAAGGA-3^{I}	3^{I}-AATACCAGCACACTGACT -5^{I}
LPA3	5^{I}- TTCTACAACAGGAGCAACA-3^{I}	3^{I}- CAATGACCAGGGAGTTAGA-5^{I}
LPA4	5^{I}- CCACCACTAATGTCAACAAT-3^{I}	3^{I}-CTAAGCACCACAGAAGAAC-5^{I}
LPA5	5^{I}- GCCAATCTCATCATCTTCC-3^{I}	3^{I}-GCAGCACCATTATCATCAG-5^{I}

The PCR amplicon were run on a 2% agarose gel and visualised with ethidium bromide for 30 minutes. To determine which housekeeping gene would be used, experiments were initially conducted to establish the stability of eight potential candidates: ribosomal protein L13a (RPL13A), ubiquitin C (UBC), beta 2 microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (ACTB), cyclophilin (CYC), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ) and Calnexin (CANX). Total RNA was isolated from cultured P19 cells at day 0 and day 6 from untreated and 5μM LPA treated samples. The RNA was subjected to quantitative RT- PCR as described above and the threshold cycle number (Ct) values used in the Genorm software to identify the most stable gene. Further, the changes in the expression of LPA receptors were quantified using the selected housekeeping gene.

Calculation of relative gene expression levels.

The Ct was noted for both target gene and housekeeping gene in each reaction.

Relative quantification was then assessed using the following method:

∆Ct target = Ct control – Ct sample

∆Ct Reference = Ct control – Ct sample

Where:

∆Ct target= differences between Ct values of LPAR gene

∆Ct Reference= differences between Ct values of Housekeeping gene (CANX)

Ct Sample = threshold cycle of LPA treated cells

Ct control = threshold cycle of untreated cells

Determination of the efficiency of amplification (E)

To determine the efficiency of amplification in each cycle, the slope from the standard curves were used to determine efficiency as shown in the equation below:

$$E = 10^{(-1/slope)} - 1$$

Ideally this must be 100%, indicating that for every cycle the quantity of product doubled (E=2). Very often, however an efficiency of ≥ 90% is accepted and considered adequate. Once calculated the efficiency was used to determine the fold change in the expression using following equation as shown below:

$$\text{Ratio} = \frac{(\text{E target})^{\Delta Ct\ \text{Target (control-sample)}}}{(\text{E Reference})^{\Delta Ct\ \text{Reference (control-sample)}}}$$

(Pfaffl, 2001).

2.11 Statistical Evaluation

All data are the means ± SE of at least 3 experiments. Statistical significance was performed using one-way ANOVA with post-hoc Dunnett's test comparing with the control or untreated cells. Statistical differences were determined by one-way ANOVA and significance indicated with *$p<0.05$, **$p<0.01$, ***$p<0.05$. Repeated Measure Anova was carried out in experiment were it showed no statistical significance with one-way Anova.

Chapter 3

Results

Chapter 3.I

Establishment of differentiation model and characterization of LPA receptors

3.1.1 Development and characterization of P19 stem cell differentiation model

The first objective of the thesis was to establish the differentiation model that could be used throughout the studies. The steps taken to achieve these objectives are outlined as below.

3.1.1.1 Establishing embryoid bodies from monolayers of p19 cells

To generate embryoid bodies (EBs), monolayers of P19 cells in culture (Figure 6) were routinely trypsinised and seeded in non-tissue culture grade Petri dishes in complete cell culture medium. Under this condition, the cells formed aggregates to form EBs in suspension. This occurred within a few hours with the EBs growing in size over time but this was highly variable (Figure 7).

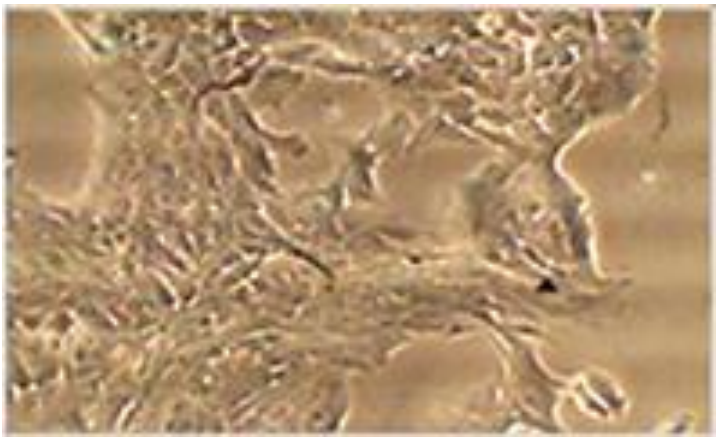

Figure 6. Regular growth pattern of P19 stem cells in culture.

P19 cells were cultured in complete medium containing α-minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum, penicillin (100units/ml) and streptomycin (100μg/ml) in falcon-T75 flasks and were maintained at 37^{o}C, 95% air and 5% CO_2. The photograph was taken under 100X magnification using an Olympus inverted microscope 2 days after plating cells.

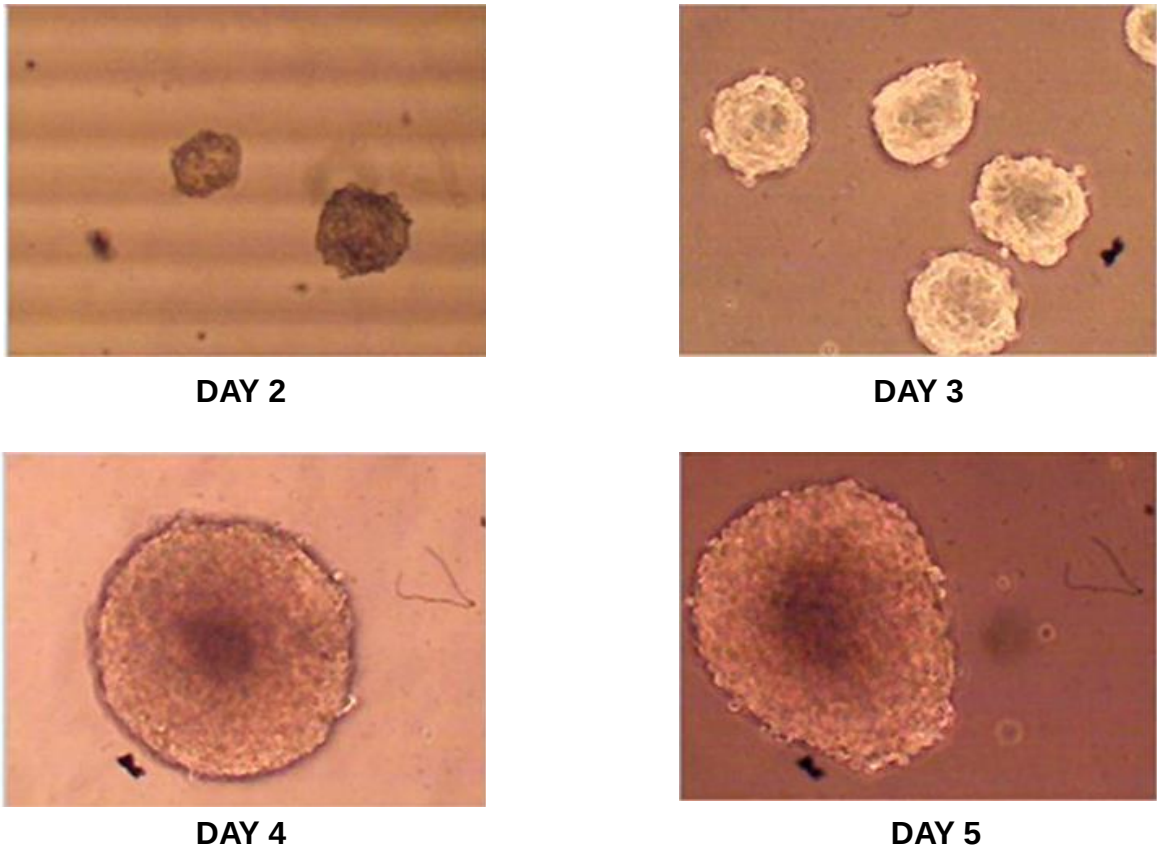

Figure 7. Formation of embryoid bodies from P19 stem cells in culture.

Confluent monolayers of P19 cells were trypsinised and seeded 3.7 X 10^5 cells in non-tissue culture grade microbiological Petri dishes in complete cell culture medium consisting of α-minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum, penicillin (100units/ml) and streptomycin (100μg/ml) and were maintained at 37^{o}C, 95% air and 5% CO_2. The photographs were taken under 100X magnification using an Olympus inverted microscope and are representative of EBs routinely formed.

3.1.1.2 Differentiating p19 cells into cardiomyocytes using DMSO

To ensure that P19 cells used in the studies could be differentiated, a preliminary experiment was conducted using DMSO, an agent known to induce stem cells differentiation (McBurney *et al.*, 1982). The induction of P19 cells to differentiate into cardiomyocytes was initiated by treating cells with 1% DMSO during the EB forming stage. Lysates were generated at 2, 4, 6, 8, 10 and 12 days after plating the EBs in the tissue culture grade plastics. Each lysate was subjected to western blotting for the cardiac-specific MLC-1v protein expression. In addition to detecting the latter, cells were also routinely viewed under an Olympus inverted microscope at 100x to look for clusters of beating cells which was taken as evidence of the generation of cardiomyocytes from the stem cells. These were often but not consistently seen in some monolayers treated with DMSO where groups of cells appear to have a uniform rhythm and contracted uniformly with each other. Such clusters are indicated by the broken circles (Figure 8).

In addition to the beating cells, DMSO also induced a time-dependent increase in the expression of MLC-1v which was evident at day 4, increasing to a peak at day 8 to 10 but declining by day 12 (Figure 9). This confirms that DMSO induced the differentiation of P19 cells into cardiomyocytes and these cells could then be used for investigating whether LPA can direct their differentiation into cardiac muscle cells.

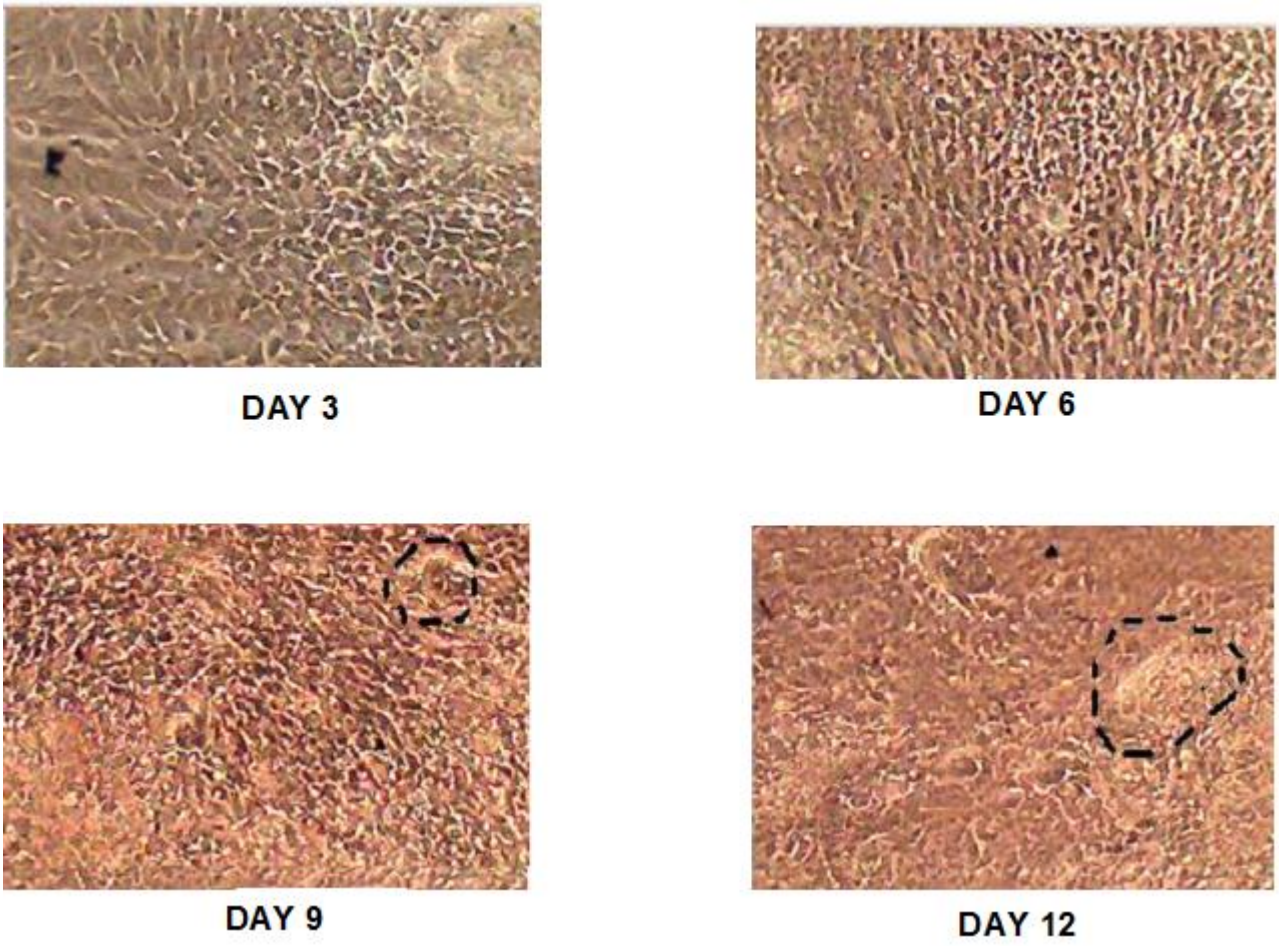

Figure 8. DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes.

Confluent monolayer of P19 stem cell were trypsinised and seeded at a density of 3.7 X 10^5 cells per dish in non-tissue culture grade microbiological Petri dishes. 1 % DMSO was added and embryoid bodies (EBs) allowed to form over a 4 day period. The EBs were then transferred into 6 well plates **i**n DMSO-free complete culture medium. The photographs were taken under 100X magnification using an inverted Olympus microscope on the days shown. The broken circles represents where clusters of beating cells were identified following DMSO treatment.

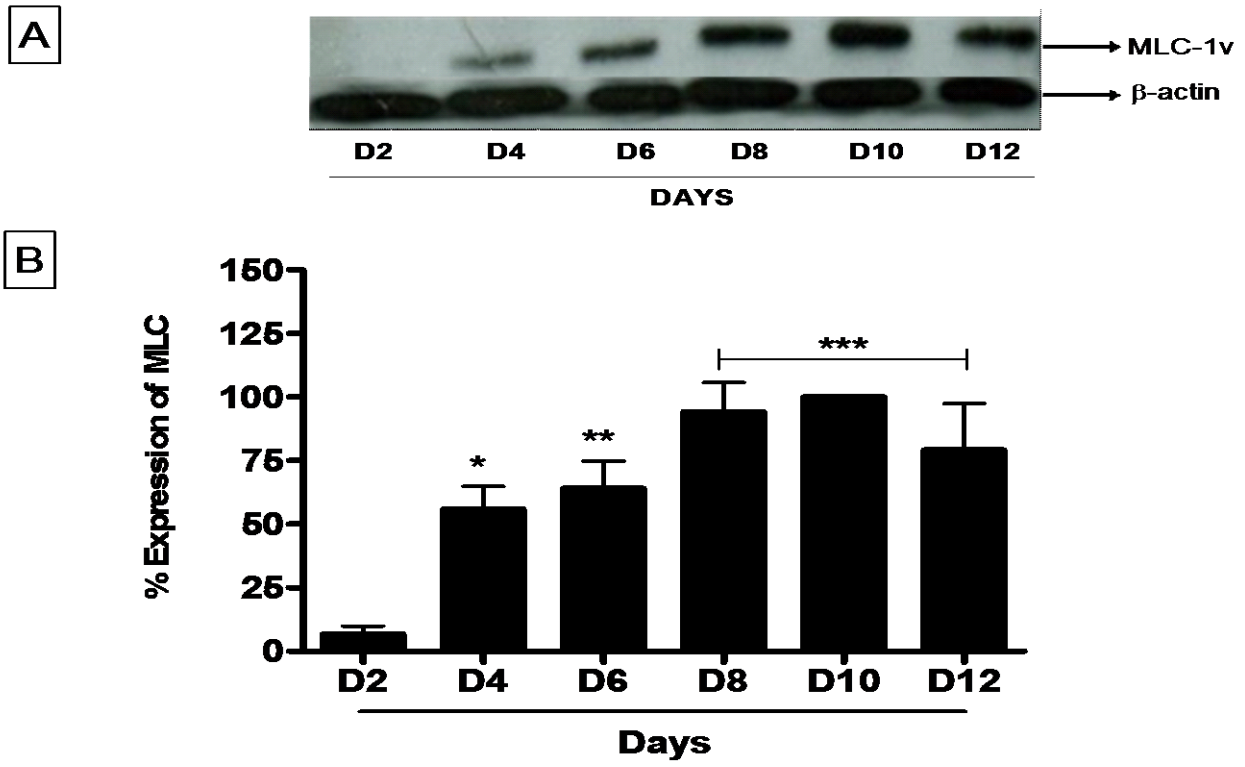

Figure 9. DMSO induced differentiation of P19 cells into cardiomyocytes.

P19 cells were treated with 1% DMSO during the EB formation stage and subsequently plated in tissue culture grade plastics as described in the Methods (section 2.3). Whole cell lysates were generated at the time points indicated on the graph and subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least 3 independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data is transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the day 2 sample.

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3.1.1.3 Effect of embryoid body seeding density on the differentiation of P19 cells into cardiomyocytes

In order to establish whether the seeding density of EB determined the efficiency of differentiation of P19 cells, EBs were formed from a predetermined number of P19 cells (3.7 X 10^5 cells/petri dish) in the presence of 1% DMSO and subsequently resuspended in 12 ml fresh complete culture medium. Various volumes from 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 ml of the resuspended EBs were plated into 6 well tissue culture plates. The medium was changed on alternative days and lysates generated on day 6. The expression of the cardiac-specific MLC-1v protein was determined by western blotting. Figure 10 shows that 1.5, 2.0 and 2.5 ml of the resuspended EBs were efficiently induced to differentiate into cardiomyocytes. In all subsequent studies, P19 cells were seeded at the same density of 3.7 X 10^5 cells/petri dish and 2 ml of the resuspended EBs plated for the differentiation phase of the experiments.

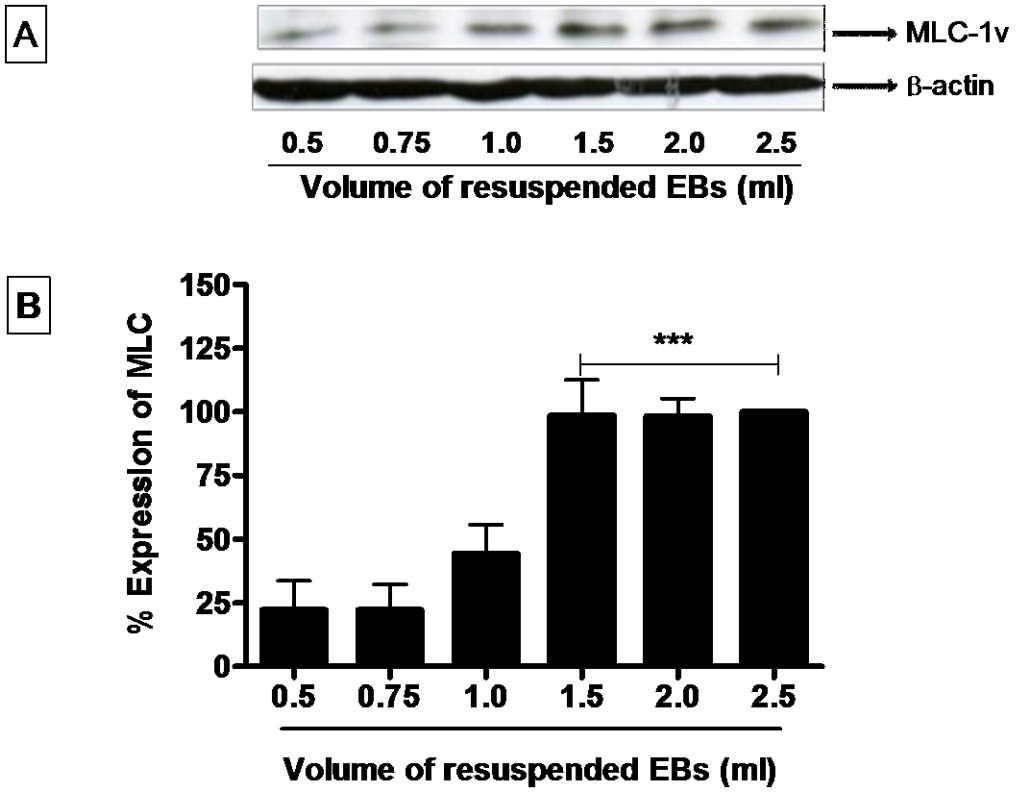


Figure 10. Effects of embryoid body seeding density on the differentiation of P19 cells into cardiomyocytes.

P19 cells were treated with 1% DMSO during the EB formation phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the in the Methods (section 2.3). Whole Cell lysates were generated after 6 days and subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data *** $p<0.001$ when compared to the 0.5ml suspension samples.

3.1.1.4 Effects of LPA on cell viability

Before examining the effects of LPA on differentiation, it was important to establish whether it would be cytotoxic at the concentration range to be used in the studies. P19 cells were therefore treated with LPA at 1, 5, 10, 25 and 50μM as described in the methods. As shown in Figure 11, there was no statistically significant difference in viability between the untreated cells and even the highest concentration of LPA (i.e. 50μM) used suggesting that the compound was not cytotoxic to the cells.

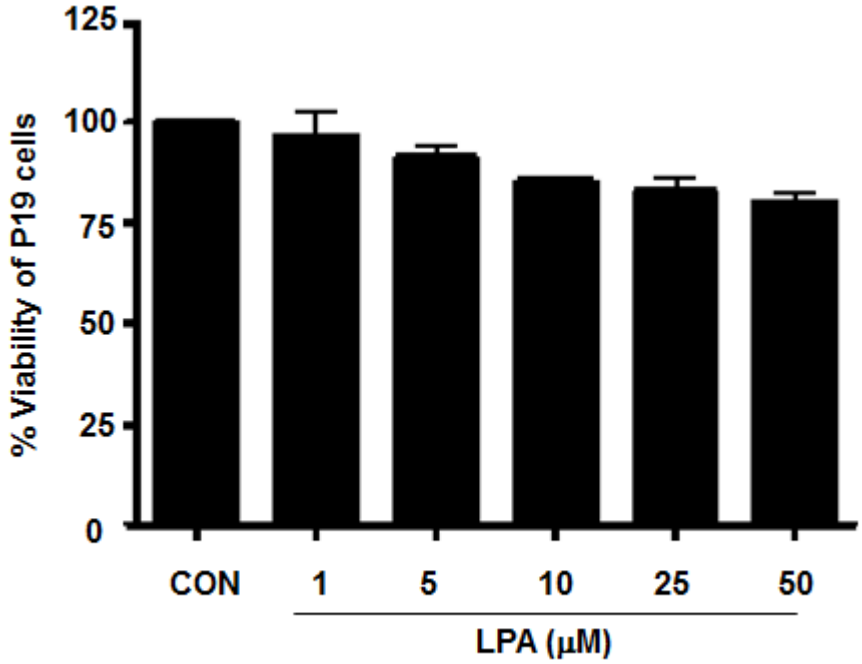


Figure 11. Viability of P19 cells in the presence of various concentration of LPA.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of LPA indicated on the graph for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. $P>0.05$ confirmed there was no significant difference when compared to non-treated control.

3.1.1.5 LPA induced differentiation of P19 cells into cardiomyocytes

i. Concentration-dependent effect of LPA.

P19 cells were treated with increasing concentration of LPA (1μM, 5μM, 10μM and 25μM) at the EB forming stage. The EBs were subsequently plated into 6-well tissue culture plates on day 5. The cells were allowed to grow, changing the medium on alternative days and cells were lysed for western blot analysis 6 and 12 days after plating.

As shown in Figure 12, at day 6 LPA induced cardiac differentiation at concentrations of 1, 5 and 10μM with 5μM giving the maximum induction as evidenced by the expression of the cardiac-specific marker MLC-1v. At 25 μM, LPA failed to induce significant induction of MLC-1v but it is not clear why this is especially as the cytotoxicity studies showed that LPA was relatively well tolerated even at concentrations of up to 50 μM. Interestingly, the induction of MLC-1v produced by 5 μM LPA was higher than that caused by DMSO which was used as a positive control. On day 12, all four LPA concentrations tested were able to induce cardiac differentiation to the same extent with no difference between concentrations (Figure 13).

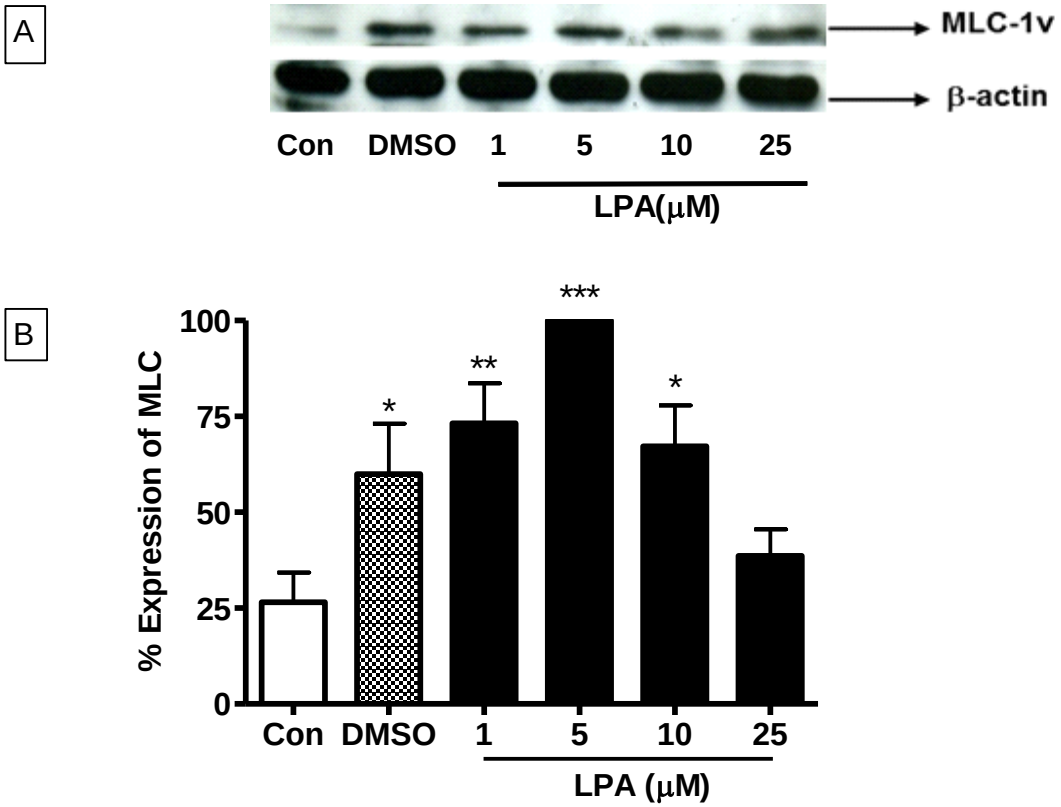


Figure 12. LPA induced differentiation of P19 cells into cardiomyocytes

P19 cells were treated with varying concentrations of LPA or 1% DMSO during the EB formation stage. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.4). Whole Cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data is transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data.*$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the untreated control samples.

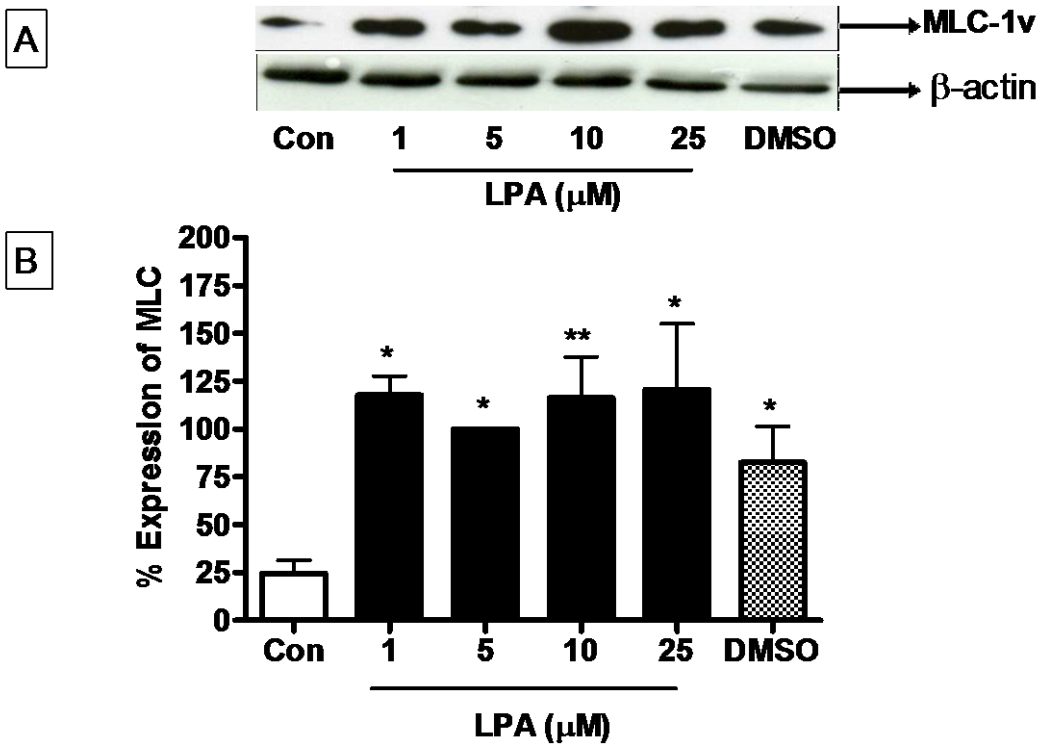


Figure 13. LPA induced cardiac differentiation of P19 cells.

P19 cells were treated with varying concentrations of LPA or 1% DMSO during the EB formation stage. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.4). Whole Cell lysates were generated after 12 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least six independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data is transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from six independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. *$p<0.05$, **$p<0.01$, when compared to the untreated control samples.

ii. Time-dependent induction of differentiation of P19 cells into cardiomyocytes by LPA.

From the above experiments, it was confirmed that LPA induced differentiation and 5μM was selected for time-course studies. The cells were treated with 5μM LPA during the EB formation stage and were subsequently plated into 6 well plates. Every alternative day, the medium was changed and lysates were collected and analysed for MLC-1v expression by western blotting. As shown in Figure 14 the treatment with LPA at 5μM induced differentiation of P19 cells from day 6 and was sustained until day 14 as evidenced by the expression of the cardiac-specific marker MLC-1v. In untreated cells, the basal expression of MLC-1v remained virtually the same from day 2 to 14.

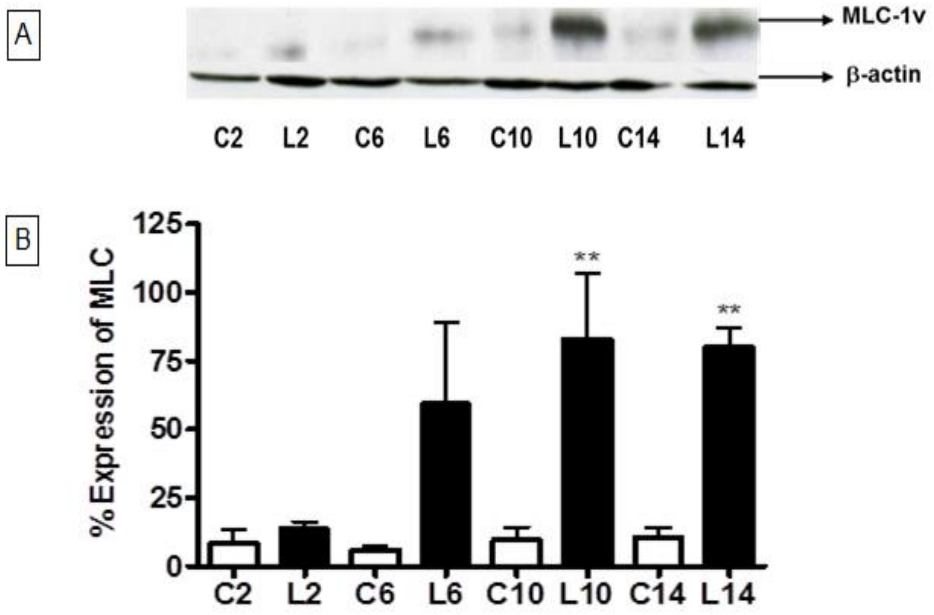


Figure 14. Time dependent induction of differentiation of P19 cells by LPA.

P19 cells were either incubated with culture medium alone control (C) or treated with 5 µM LPA (L) during the EB formation stage. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.4). Whole cell lysates were generated after 2, 6, 10 and 14 days, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data is transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. **p<0.01, when compared to the untreated control samples.

3.1.1.6 Identification of LPA receptors expressed in P19 cells.

Following the observations made and highlighted above (section 3.1.1.5), a series of experiments were next initiated to identify the particular LPA receptors expressed and associated with differentiation following induction by LPA. Total RNA was extracted from a monolayer of P19 cells, reverse transcribed, and amplified by PCR using specific primers for the different LPAR subtypes (LPAR1-5). Sybr-Green based q-PCR analysis showing amplification curves for each of the receptor types identified and the housekeeping gene are presented in Figure 15 (A to E). A dissociation curve depicting a single curve following q-PCR is suggestive of product specificity in our designed assay. As shown in Figures 15 and 16, LPAR-1,-2,-3, and -4 were detectable whereas LPAR5 was absent (Figure 15E) from the total RNA analysed, suggesting that LAPR5 is not expressed by P19 cells.

The products obtained from the RT-PCR are shown in Figure 16. The sizes of the amplicons detected were: LPAR1= 115 base pairs (bp), LPAR2 = 90 base pairs (bp), LPAR3 = 121 base pairs (bp), LPAR4= 157 base pairs (bp), LPAR5 = 142 base pairs (bp).

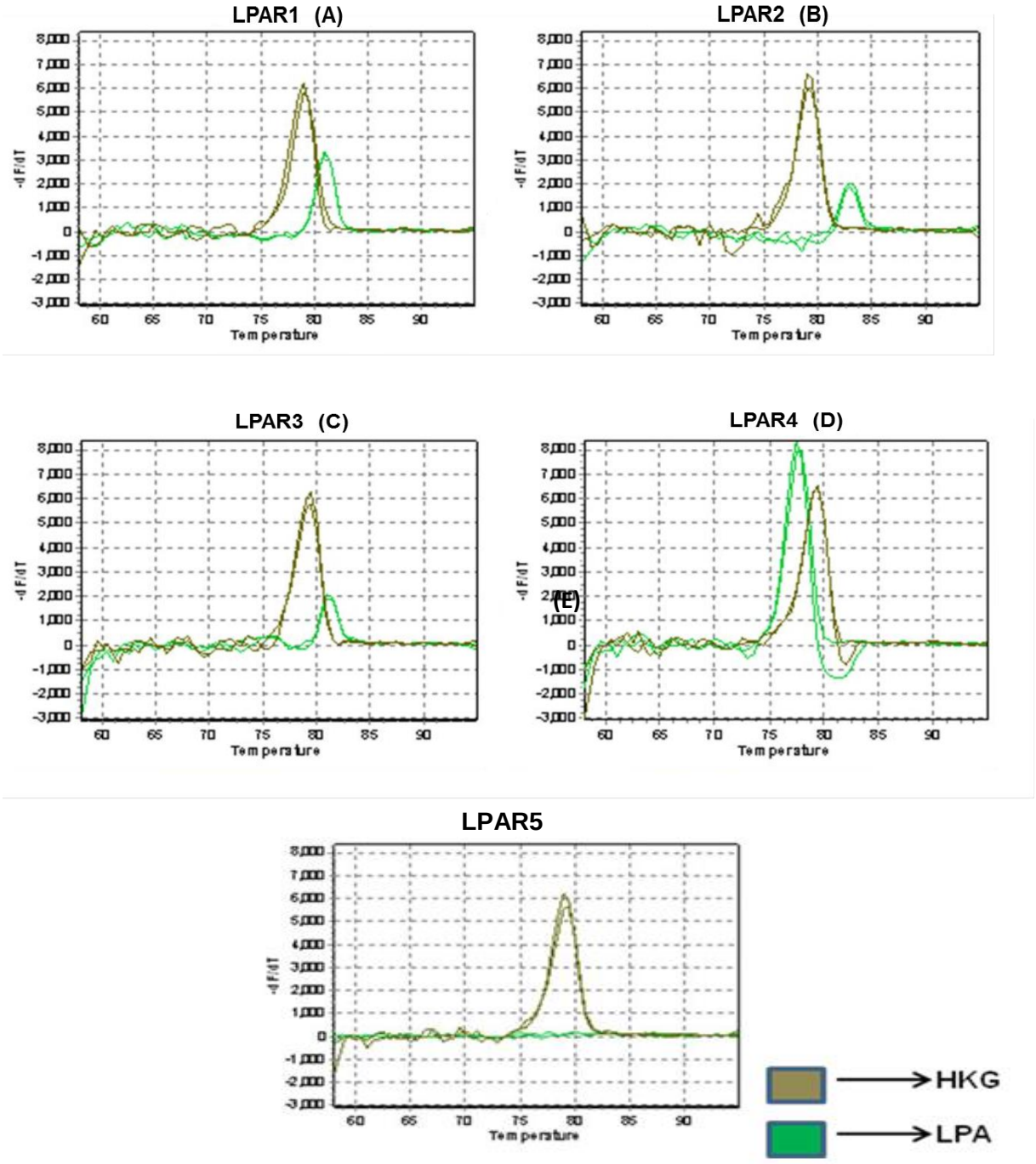


Figure 15. Lysophosphatidic acid receptor expression profile in control P19 cells

Total RNA isolated from cells was DNase treated, reversed transcribed using LPA receptor specific primers and subjected to PCR analysis using a Quantica Thermal Cycler as described in the Methods (section 2.10). Panels show dissociation curves for each receptor probed in control P19 cells and are representative of three individual experiments.

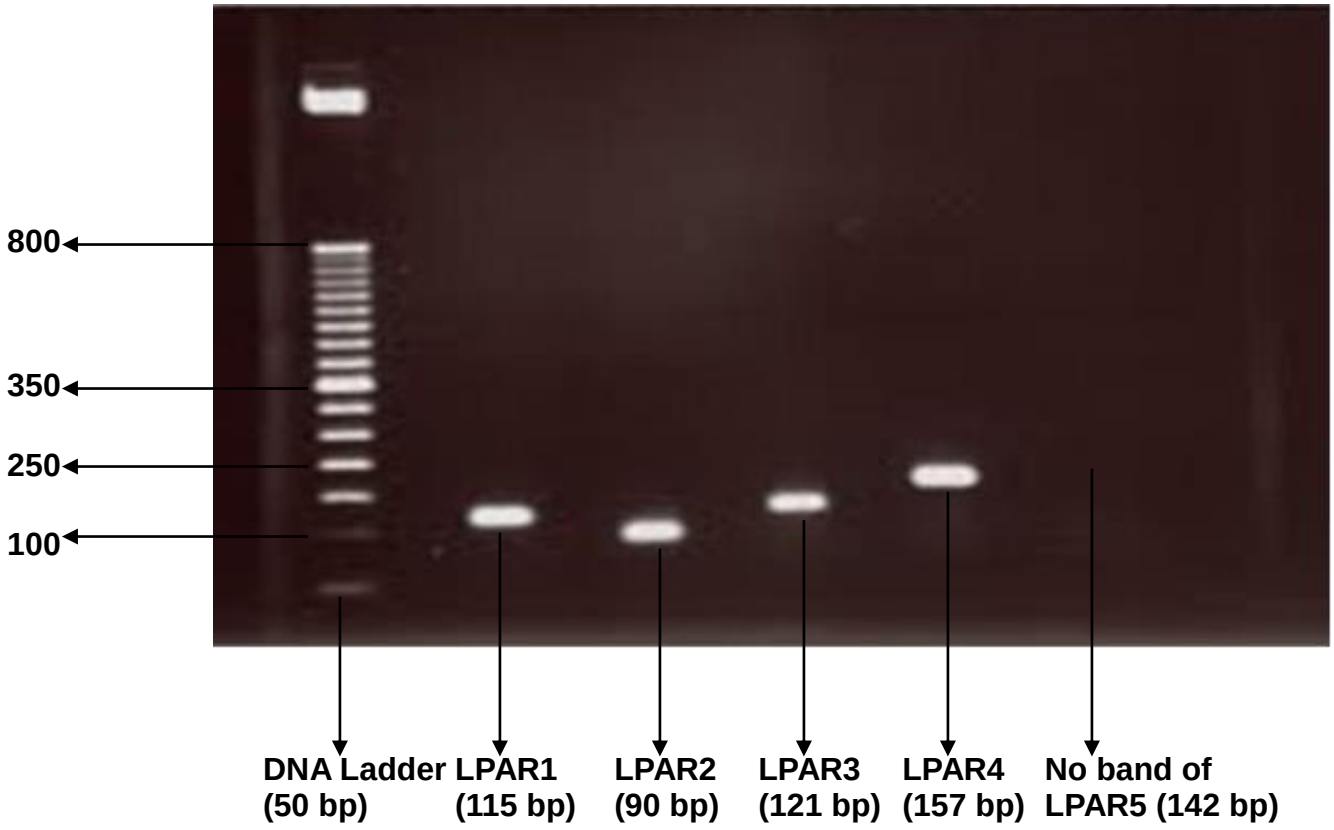


Figure 16. Lysophosphatidic acid receptor expression profile in control P19 cells.

Total RNA isolated from cells was DNase treated, reversed transcribed using LPA receptor specific primers and subjected to PCR analysis using a Quantica Thermal Cycler as described in the Methods (section 2.10). The respective PCR products were subjected to agarose gel electrophoresis as described in the Methods (section 2.10). The gel is representative of three independent experiments.

3.1.1.7 Expression profile of LPA receptors in differentiated P19 cells.

To establish whether the profile and/or levels of the receptors changed in differentiated cells, total RNA was isolated from controls and from LPA treated cells 6 days after plating EBs in the tissue culture grade 6-well plates. These were then subjected to quantitative RT-PCR and the levels of receptor transcripts detected normalised against the target housekeeping gene. The latter was initially determined by screening eight potential candidate 60s ribosomal protein L13a (RPL13A), ubiquitin C (UBC), beta-2-migroglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (ACTB), cyclophilin (CYC), tyrosin-3-monooxygenase/tryptophan-3-monooxygenase activation protein, zeta (YWHAZ) and calnexin (CANX) for stability under our experimental conditions. In these studies, comparison of Ct values using the Genorm software revealed that of the 8 housekeeping genes analysed, RPL13A was the least stable while CANX and YWHAZ were the most stable and the others lying in between (Figure 17). From this data, CANX was selected as the target housekeeping gene for normalising the levels of receptor transcripts detected. The standard and dissociation curves for each of the receptor analysed are shown in Figures 18, 19 and 20. All four LPA receptors (LPAR1-LPAR4) were present in differentiated P19 cells obtained 6 days after LPA treatment. Each of these appear to be significantly enhanced at least at the mRNA level when compared to the controls non-LPA treated cells analysed in parallel. More importantly, of the four receptor genes, the mRNA for LPAR2 appears to be much more significantly enhanced, increasing by approximately 3 fold when compared to levels in controls (Figure 21).

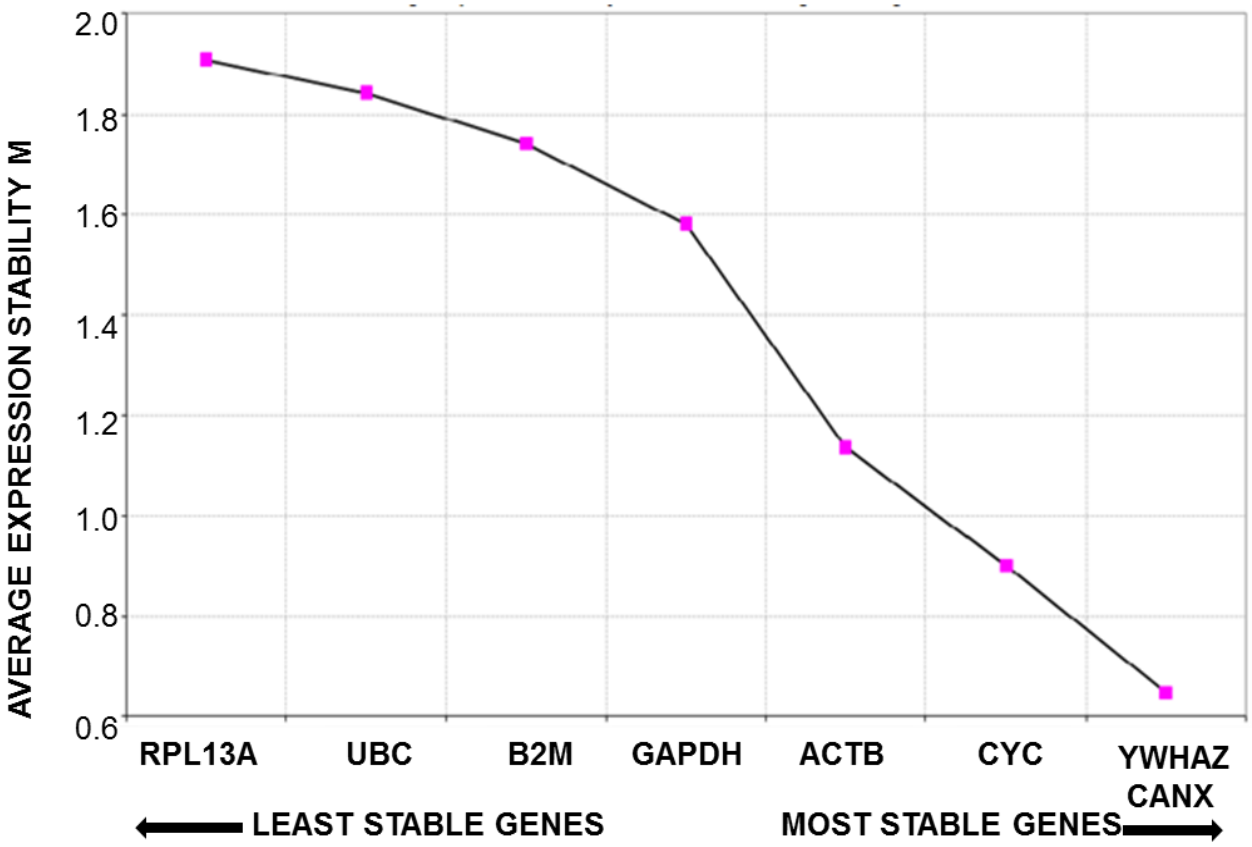


Figure 17. Stability of housekeeping gene.

Total RNA was isolated from cells and then DNase treated before reverse transcribing as described in methods using specific primers for HKGs RPL13A, UBC, B2M, GAPDH, ACTB, CYC, YWHAZ and CANX. The data is representative of two independent experiments.

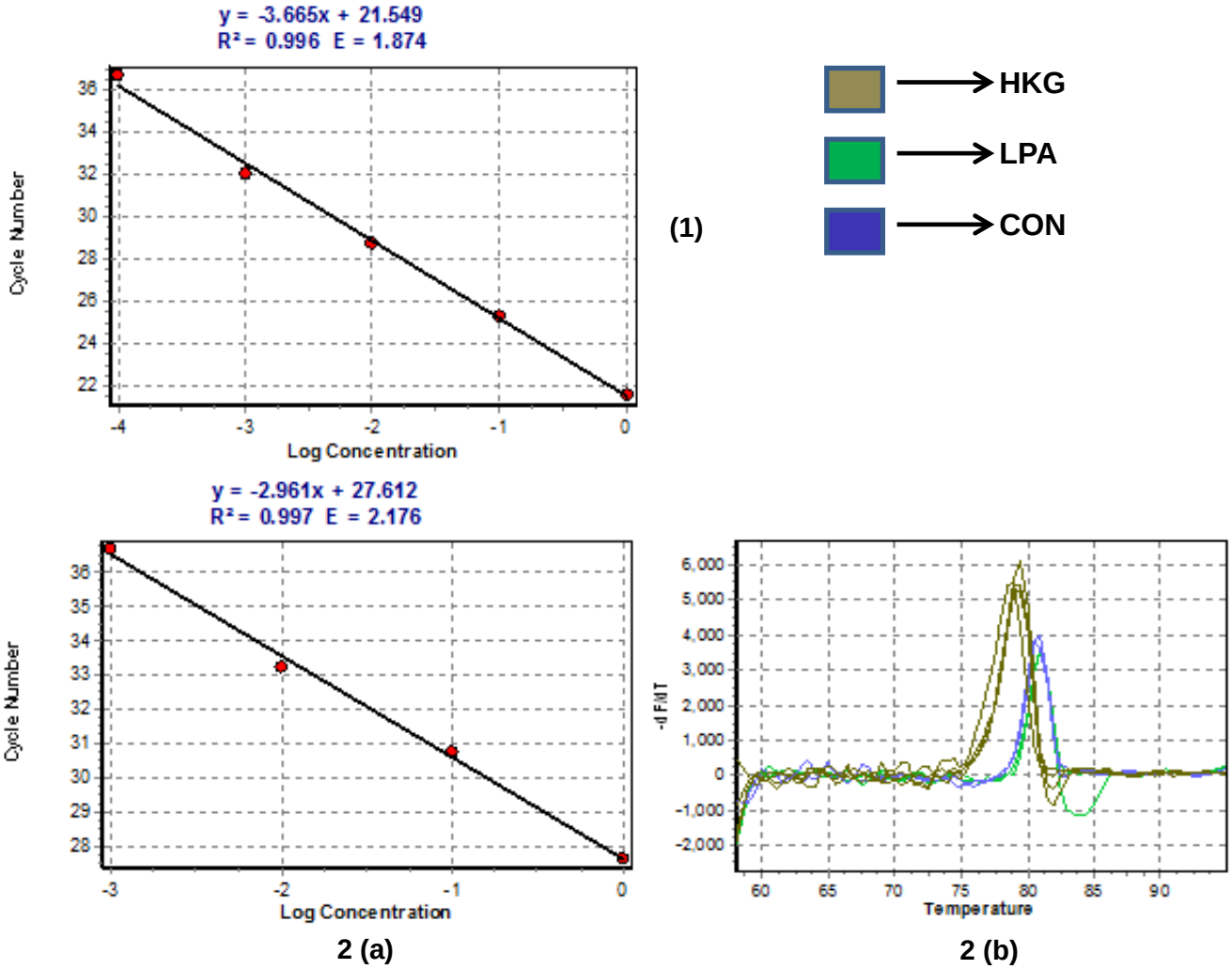


Figure 18. Standard and dissociation curves for both CANX and LPA receptor 1 gene in P19 cells.

Total RNA isolated from cells was DNase treated, reversed transcribed using specific primers and subjected to PCR analysis on a Quantica Thermal Cycler as described in the methods. The panels shows: Panel 1 = cDNA standard curve for CANX; Panel 2a = cDNA standard curve and 2b = dissociation curve for LPAR1. The data is representative of three independent studies with two replicates in each.

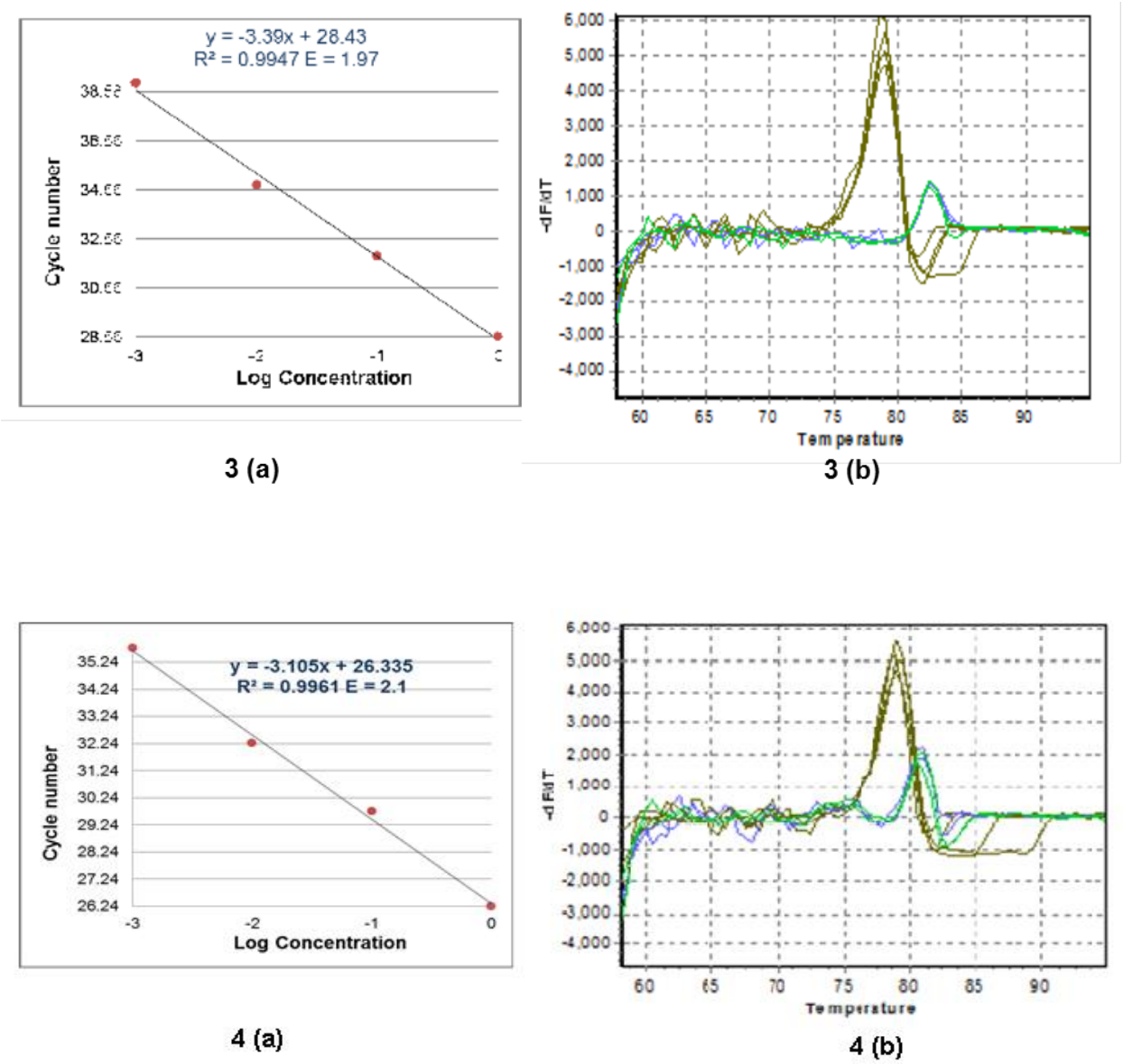
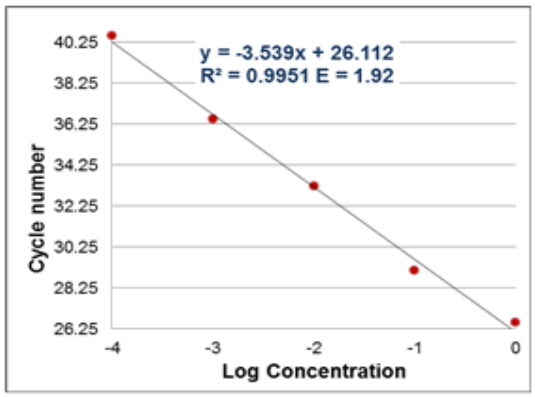
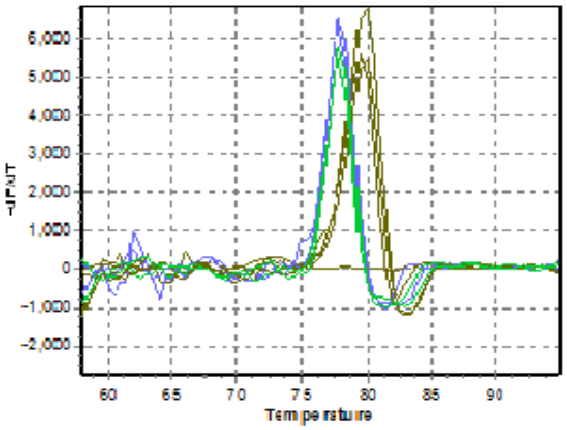


Figure 19. Standard and dissociation curves for LPA receptor 2 and 3 genes in P19 cells.

Total RNA isolated from cells was DNase treated, reversed transcribed using specific primers and subjected to PCR analysis on a Quantica Thermal Cycler as described in the methods. The panels shows: Panel 3a = cDNA standard curve and 3b = dissociation curve for LPAR2; Panel 4(a) = cDNA standard curve and 4(b) = dissociation curve for LPAR3. The data is representative of three independent studies with two replicates in each.



5 (a)



5 (b)

Figure 20. Standard and dissociation curves for LPA receptor 4 gene in P19 cells.

Total RNA isolated from cells was DNase treated, reversed transcribed using specific primers and subjected to PCR analysis on a Quantica Thermal Cycler as described in the methods. The panels show: Panel 5a = cDNA standard curve and 5b = dissociation curve for LPAR4. The data is representative of three independent studies with two replicates in each.

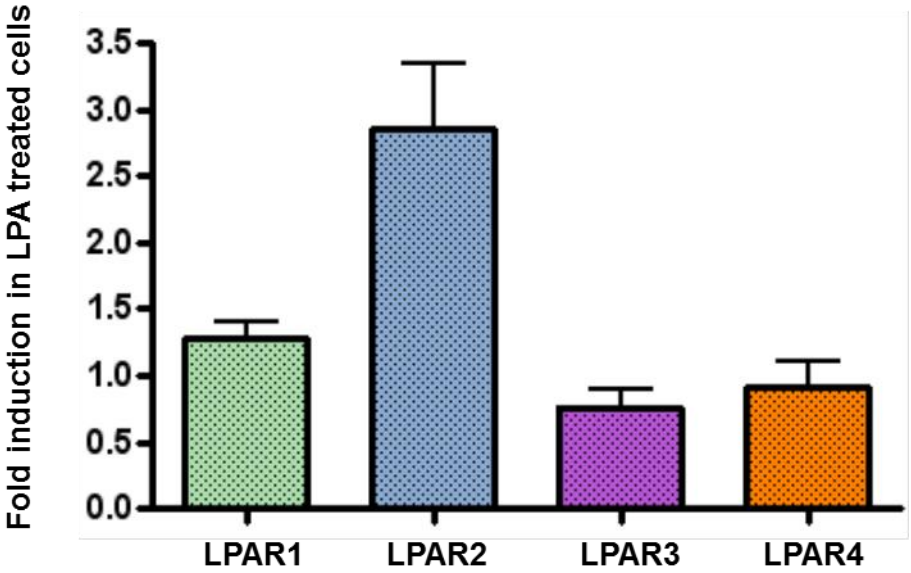


Figure 21. Fold change in expression of LPA receptors in LPA-induced P19 cells.

P19 cells were either incubated with culture medium alone or treated with 5 μM LPA during the EB formation stage. Cells were then incubated for 4 days before plating in cell culture 6-well dishes. Total RNA isolated after 6 days was DNase treated, reversed transcribed using LPAR1, LPAR2, LPAR3 and LPAR4 specific primers respectively and subjected to PCR analysis using a Quantica Thermal Cycler as described in the Methods (section 2.10). The graph shows fold change in LPA receptors mRNA levels on day 6 in LPA (5μM) treated cells normalised against CANX. The data is normalised against the housekeeping gene and the fold increase expressed against levels detected in non-LPA treated respective controls. The data represent the mean ± S.E.M. from three independent experiments with two replicates in each.

3.1.1.8 Identification of LPA receptors that mediate the differentiation of P19 stem cells into cardiomyocytes

i. The role of LPAR1/3

To investigate whether LPA-induced differentiation is mediated through LPA1/3 receptors, the LPA1/3 receptors antagonist Ki16425 (IC_{50}, LPA1=0.34μM, LPA2=6.5μM, LPA3=0.93μM) was used. The effect of Ki16425 on the cell viability was initially examined using the MTT assay as described in the methods. Ki16425 had no toxic effects on P19 cells except when the very high concentration of 50μM was used (Figure 22). Cells were next treated with Ki16425 (1, 10 and 50μM) for 60 min before the addition of LPA (5μM) and lysates generated on day 6 after plating for western blotting. As shown in Figure 23, Ki16425 inhibited LPA-induced differentiation of P19 cells in a concentration-dependent manner, completely abolishing MLC-1v expression at 1μM and above. This suggests that LPA-induced cardiac differentiation is mediated, at least in part, through LPA1/3 receptors.

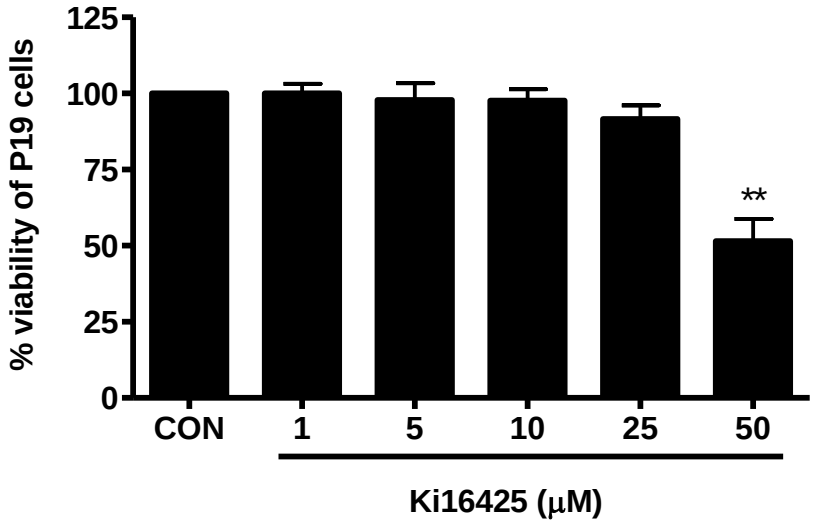


Figure 22. Viability of P19 cells in the presence of various concentrations LPA1/3 inhibitor Ki16425.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of Ki16425 indicated on the graph for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. **denotes $p<0.01$ when compared to the untreated control samples.

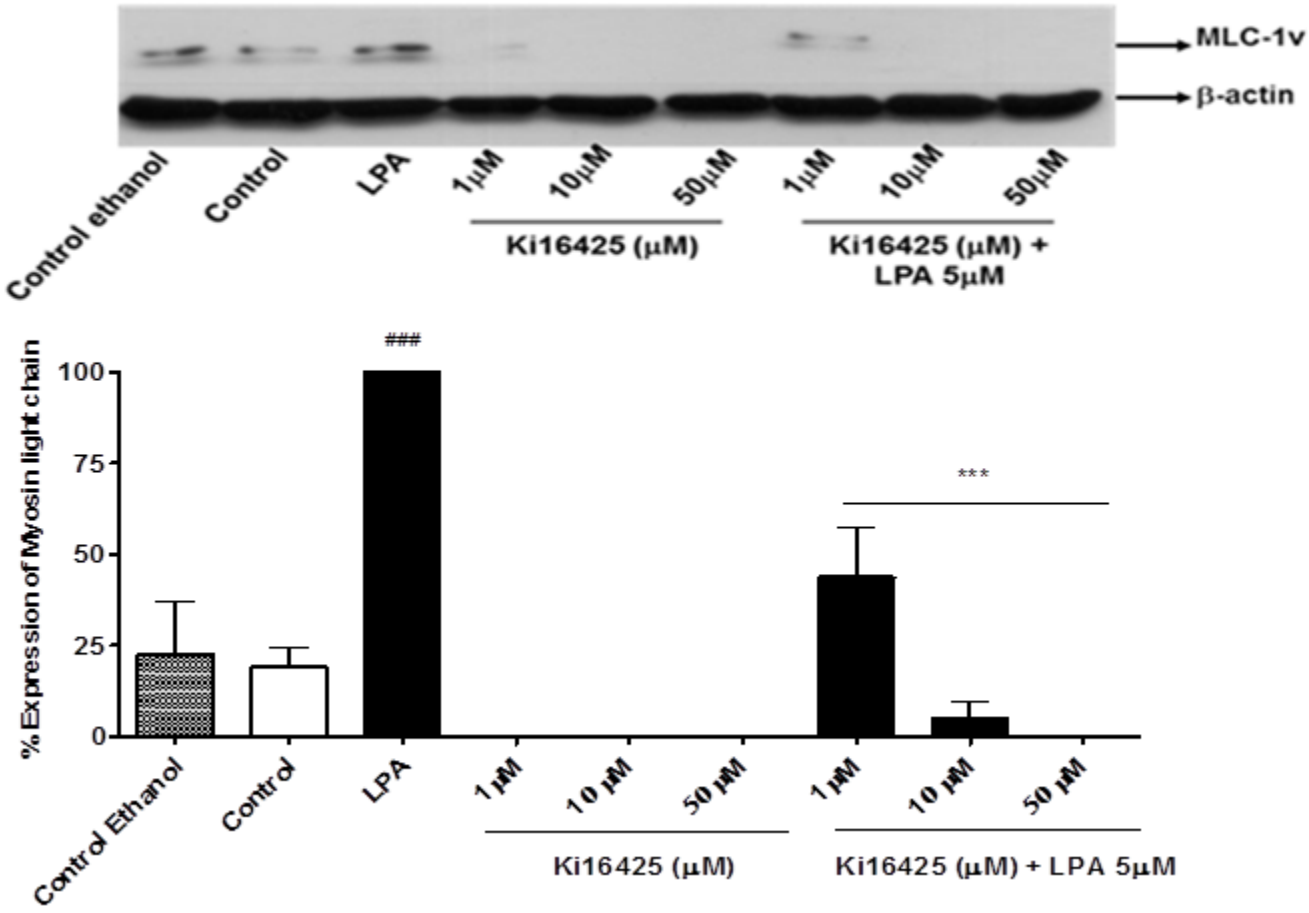


Figure 23. Effects of Ki16425 on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), control (0.1 % ethanol), Ki16425 at the concentrations shown, LPA (5μM) or LPA (5μM) and Ki16425 during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole Cell lysates were generated after 6 days of plating, and subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***$p<0.01$, when compared to the LPA treated sample and $^{###}p<0.01$ when compared to the untreated sample.

ii. Role of LPAR4

To investigate whether LPA-induced differentiation is mediated through the LPA4 receptor, the non-selective LPA4 receptor antagonist suramin was used (IC_{50} for purinergic receptors, 43 μM). The effect of suramin on cell viability was initially determined using the MTT assay as described in the methods. The results obtained revealed that suramin had no toxic effects on P19 cells except when a very high concentration of 1 and 5 mg/ml was used (Figure 24). Cells were next treated with suramin (1,5 and 50 μg/ml) for 60 min before the addition of LPA (5μM) during the EB forming stage and lysates were generated 6 days after plating the EBs. Changes in expression of MLC-1v were determined by western blotting.

As shown in Figure 25 suramin inhibited LPA-induced cardiac differentiation in a concentration-dependent manner suggesting that LPA may act, at least in part, through LPA4 receptor. At the highest concentration of 50μg/ml, suramin completely abolished the induction of differentiation to the basal level. Interestingly, suramin itself could induce the differentiation of P19 cells into cardiomyocytes in the absence of LPA at the very low concentration of 1μg/ml (Figure 25).

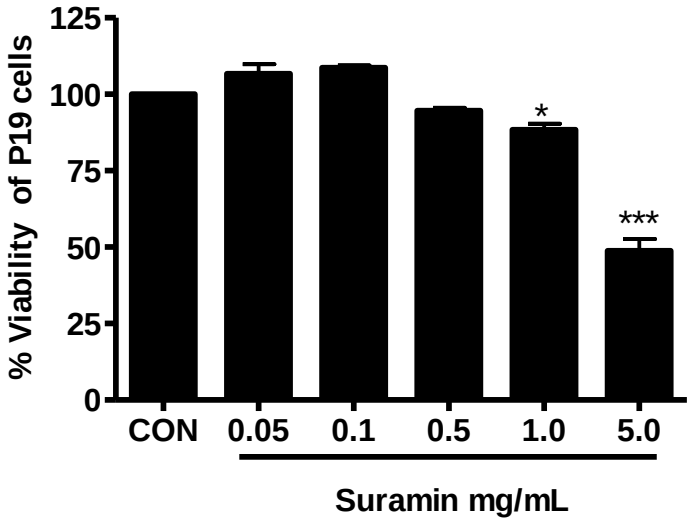


Figure 24. Viability of P19 cells in the presence of suramin.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of suramin indicated on the graph for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. *$p<0.05$, *** $p<0.001$ when compared to the untreated control samples.

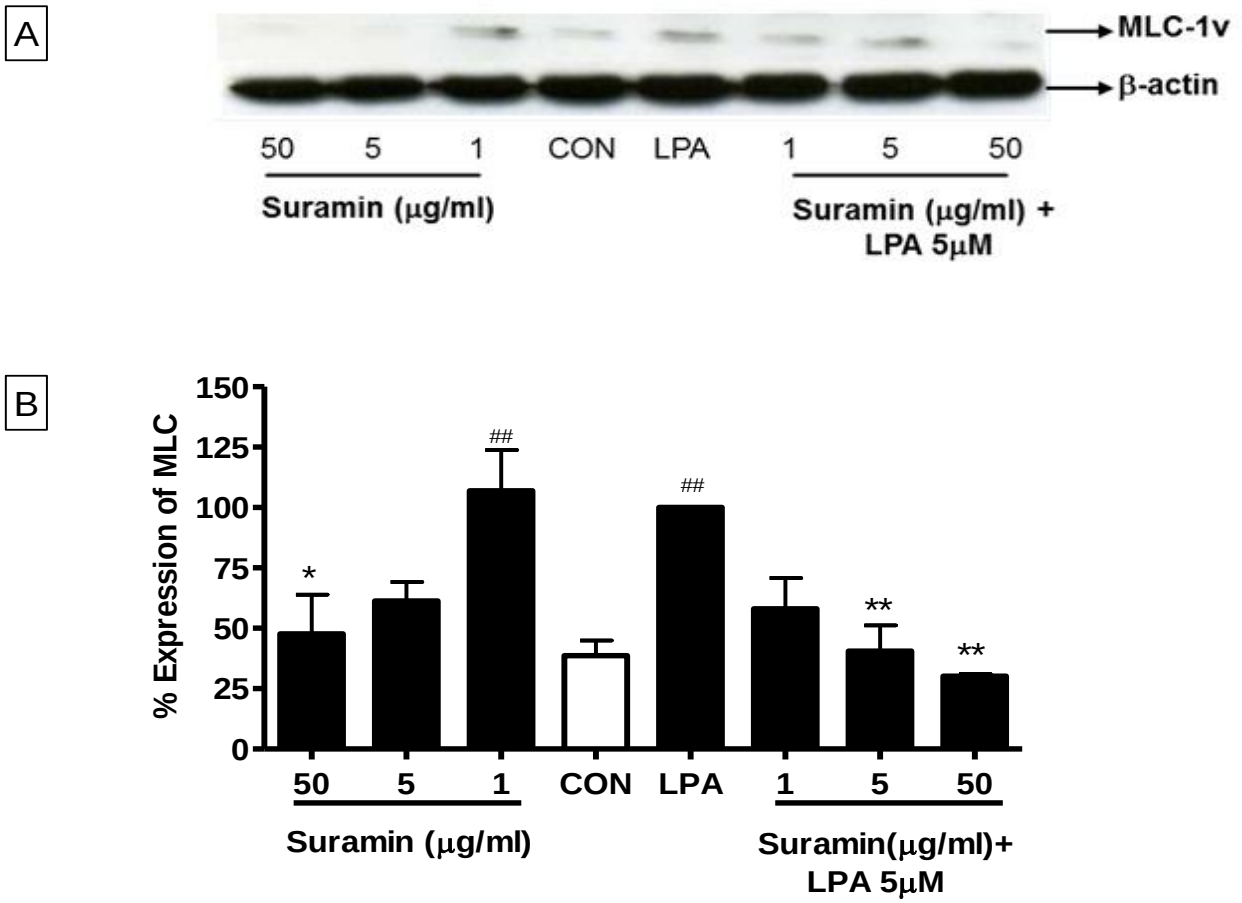


Figure 25. Effects of suramin on MLC-1v expression in controls and in LPA-induced cardiac differentiation of P19 cells.

P19 cells were either incubated with culture medium alone (control), increasing concentrations of suramin, LPA (5μM) or suramin with LPA (5μM) during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole Cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data is transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). The data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. **p<0.01, *p<0.05 when compared to the LPA treated controls and $^{\#\#}$p<0.01 when compared to the untreated sample.

Chapter 3.II

Role of G proteins and signalling molecules in LPA-induced P19 stem cells differentiation

The research carried out for this section was aimed at determining which signalling mechanisms may mediate the effects of LPA in our studies. Studies were therefore carried out investigating the role of $G\alpha_i$ and specific signalling pathways including PI3K, Akt, PKC, ROCK, p38 MAPK and p42/44 MAPK which have been implicated not only in the induction stem cell differentiation but also linked to LPA receptor signalling in different biological systems. The results obtained are discussed below.

3.2.1 Role of $G\alpha_i$ in LPA-induced differentiation of P19 stem cells into cardiomyocytes

To examine the involvement of $G\alpha_i$ in the actions of LPA, pertussis toxin (PTX), a potent inhibitor of this G-protein was used (IC_{50} for PTX on ADP –ribosylation of $G\alpha_i$, 150 +/- 40pg/ml). The effect of PTX on cell viability was initially determined using the MTT assay as already described in the methods. P19 cells were treated with various concentrations of PTX (20,100, 200 and 1000 ng/ml) for 24h prior to the cytotoxicity assay being carried out. As shown in Figure 26 PTX did not cause any statistically significant change in cell viability. Cells were next treated with a range of concentrations of PTX (100, 200 ng/ml) for 24 hours before the addition of LPA (5μM) during the EB forming phase. Lysates were generated 6 days after plating the EB into tissue culture plates and used in western blotting to detect changes in expression of MLC-1v. In these experiments PTX at both concentrations of 100 and 200 ng/ml failed to inhibit the expression of MLC-1v confirming that the $G\alpha_i$ may not be involved in the differentiation process and that LPA may not signal through this molecule in inducing differentiation of P19 cells into cardiomyocytes (Figure 27).

Although these findings and conclusions are supported by the data, it is possible that the experiments may possibly be underpowered. Each study was however carried out in three independent experiments and although the validity of the statistical tests for underpowered studies may be limited, there is confidence that the trends obtained are indeed reproducible and consistent with both our previous work and with those of many other studies. Thus, the conclusions reached from the findings in this thesis would still be considered valid and indicative of the critical cellular events that regulate the induction of differentiation of P19 cells by LPA.

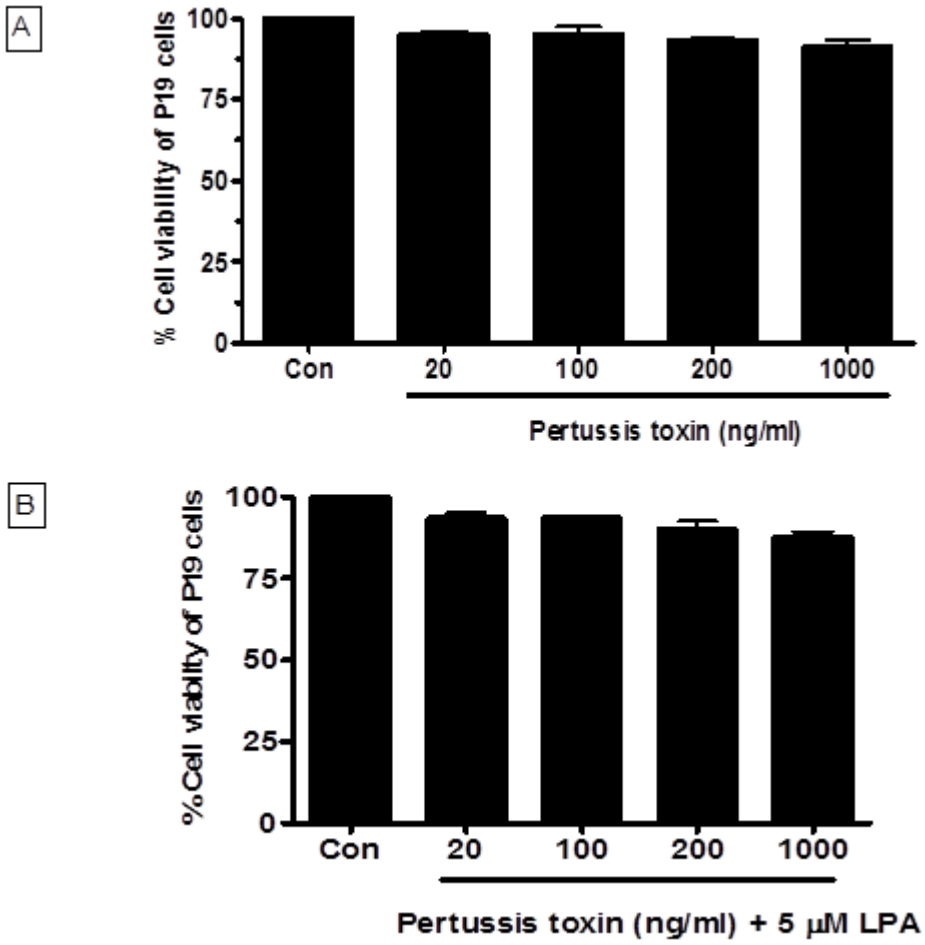


Figure 26. Viability of P19 cells in the presence of various concentrations Pertussis toxin in the absence and presence of LPA

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of PTX in the absence and presence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of PTX alone, (**B**) MTT assay for increasing concentrations of PTX along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test but there was no statistical significance between test conditions.

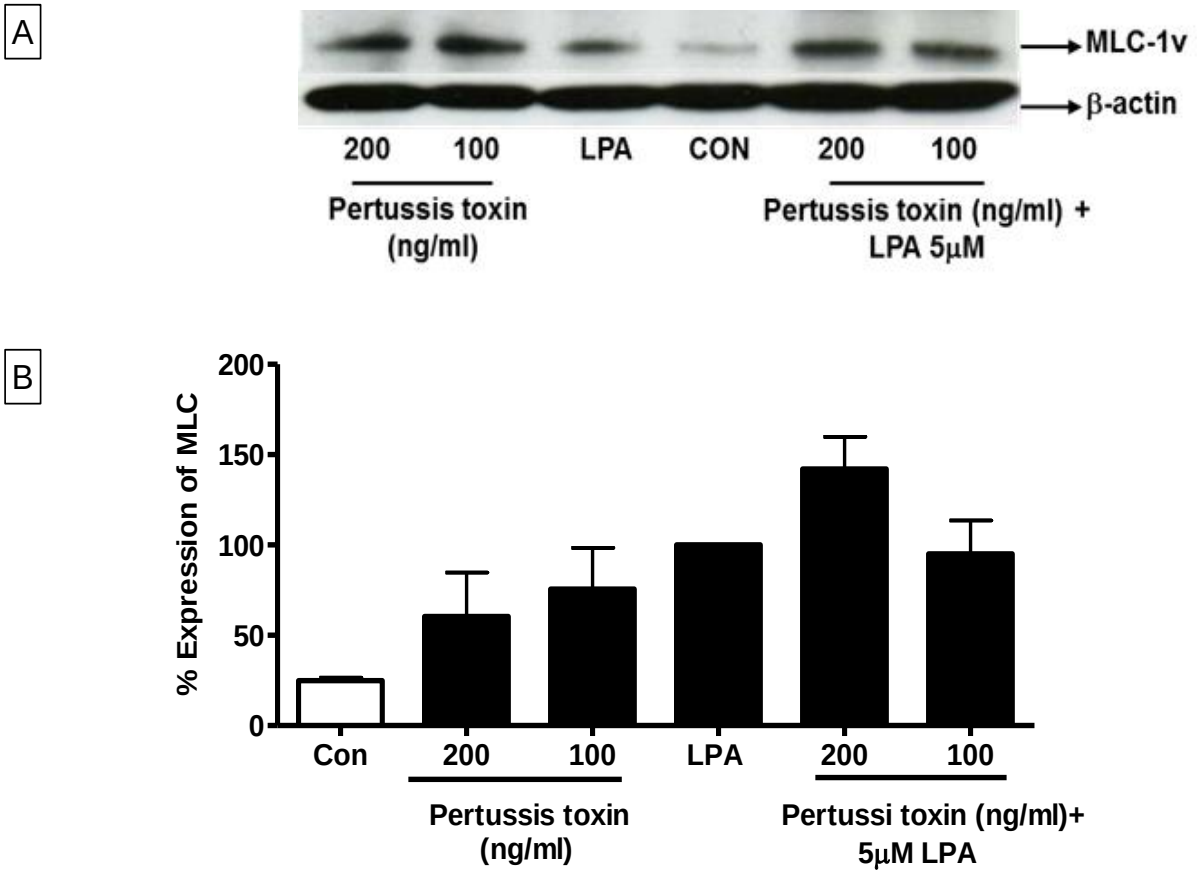


Figure 27. Effects of PTX on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), various concentrations of PTX alone, LPA (5μM) alone, or LPA and PTX that was added 24 hours before LPA during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data.

3.2.2 Role of PI3K in LPA-induced differentiation of P19 stem cells into cardiomyocytes

In these studies, cells were treated with the potent PI3K inhibitor LY294002 (IC_{50} = 1.4 μM) for 24 hours in the absence and presence of LPA (5μM) to initially determine any potential cytotoxic actions of the compound. LY294002 had no toxic effects on P19 cells except at the high concentrations of 10μM and 20μM (Figure 28). In parallel studies, treatment of cells with LY294002 at 1, 2, 5, 10 and 20μM for 60 minutes before the addition of LPA (5μM) caused a concentration-dependent inhibition of MLC-1v expression (Figure 29). The inhibition was significant with 5μM LY294002, which was found not to be cytotoxic to the cells. These findings strongly suggest an inhibition of the differentiation of the P19 cells into cardiomyocytes and more importantly implicates the PI3K pathways as being critical for this process.

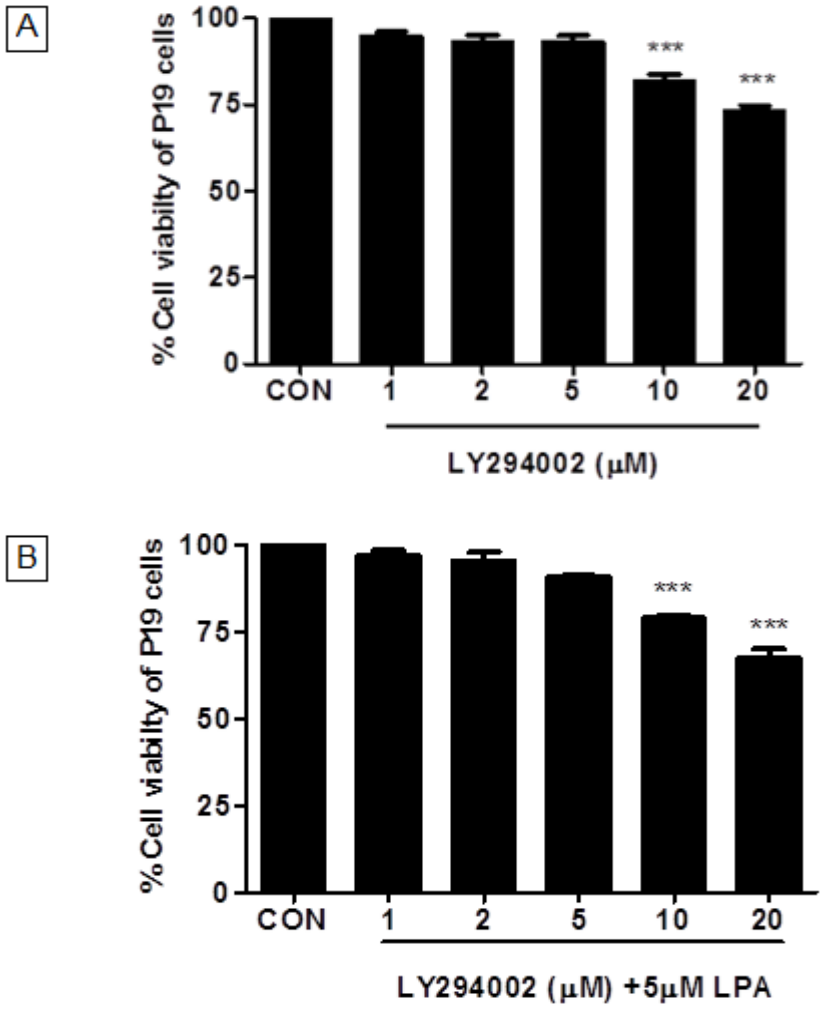


Figure 28. Viability of P19 cells in the presence of various concentrations LY294002.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of LY294002 in the absence and presence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of LY294002 inhibitor alone, (**B**) MTT assay for increasing concentrations of LY294002 inhibitor along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. ***$p<0.001$, when compared to the controls.

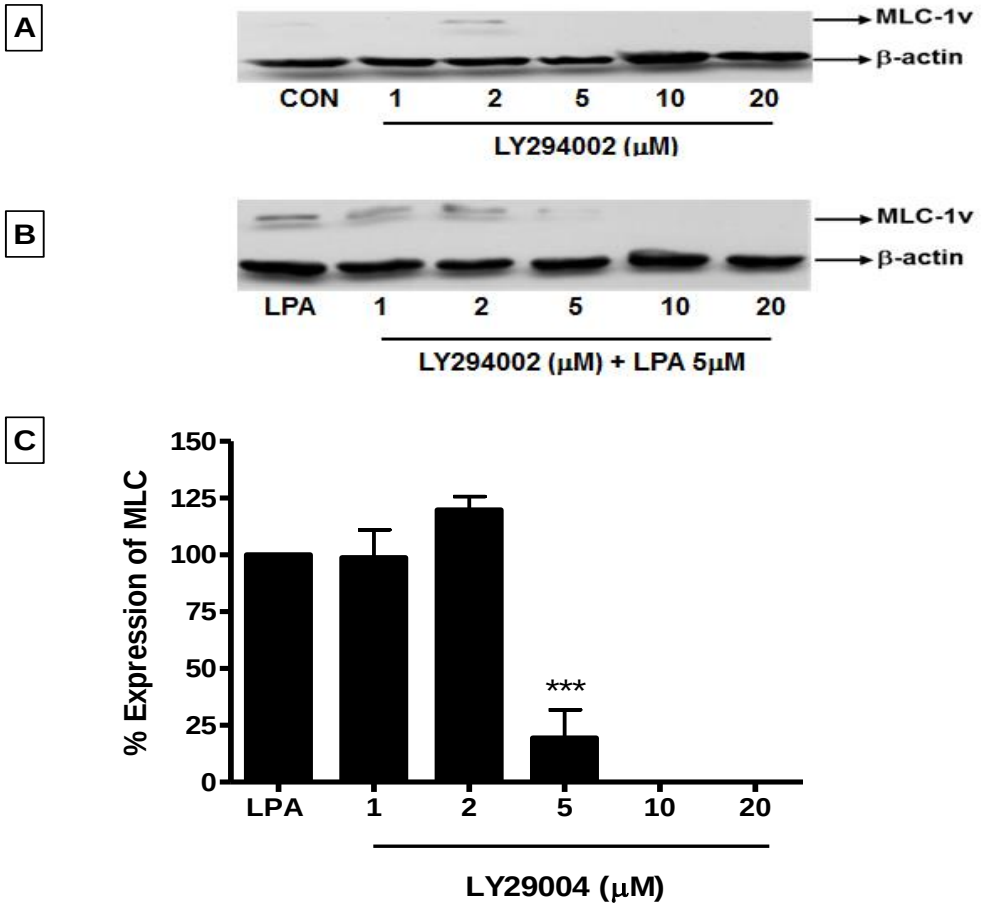


Figure 29. Effects of LY294002 on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), various concentrations of LY294002 alone, LPA (5μM) alone, or LPA and LY294002 during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A** and **B**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **C**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***$p<0.001$, when compared to the LPA treated sample.

3.2.3 Role of Akt/Protein Kinase B (PKB) in LPA-induced differentiation of P19 stem cells into cardiomyocytes

The role of Akt in LPA-induced differentiation of P19 cellls to cardiomyocytes was next investigated. In these studies, cells were treated with the potent Akt inhibitor, Akt inhibitor XIII (IC_{50} = 560 nM, 390 nM and 7.8 μM for Akt1, Ak2, and Akt3, respectively) for 24 hours in the absence and presence of LPA (5μM) to initially determine any potential cytotoxic actions of the compound. Akt inhibitor XIII had no toxic effects on P19 cells even at 30μM (Figure 30). In parallel studies, treatment of cells with Akt inhibitor XIII at 0.1, 0.5 and 1.0μM for 60 min before the addition of LPA (5μM) caused a concentration-dependent inhibition of MLC-1v expression (Figure 31) when lysates were generated 6 days after plating the EBs in tissue culture grade 6-well plates. The inhibition was significant and virtually complete with 1μM Akt inhibitor XIII that was found not to be cytotoxic to the cells. Akt inhibitor XIII alone appeared to increase MLC-1V in a concentration dependent manner but these effects, although higher than basal, were not statistically different to controls. These findings strongly suggesting an inhibition of the differentiation of the P19 cells into cardiomyocytes and more importantly implicates the Akt pathways as being critical for this process.

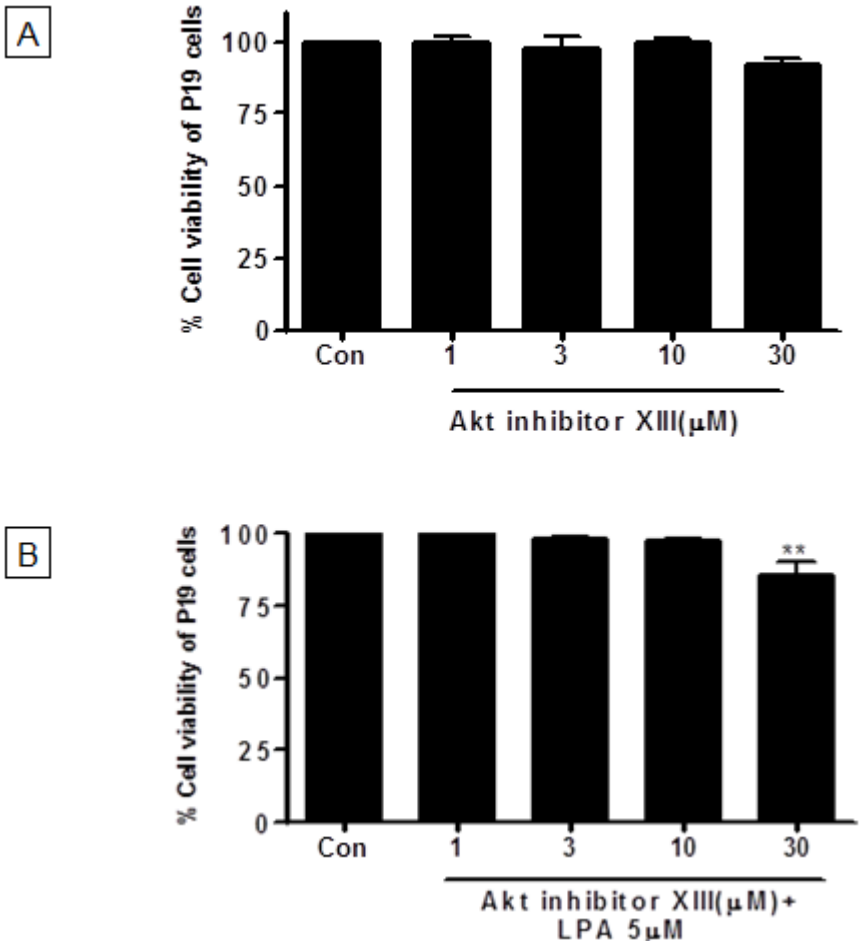


Figure 30. Viability of P19 cells in the presence of various concentrations Akt inhibitor XIII.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of Akt inhibitor XIII in the absence and presence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of Akt inhibitor XIII alone, (**B**) MTT assay for increasing concentrations of Akt inhibitor XIII along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. **p<0.01, when compared to the controls.

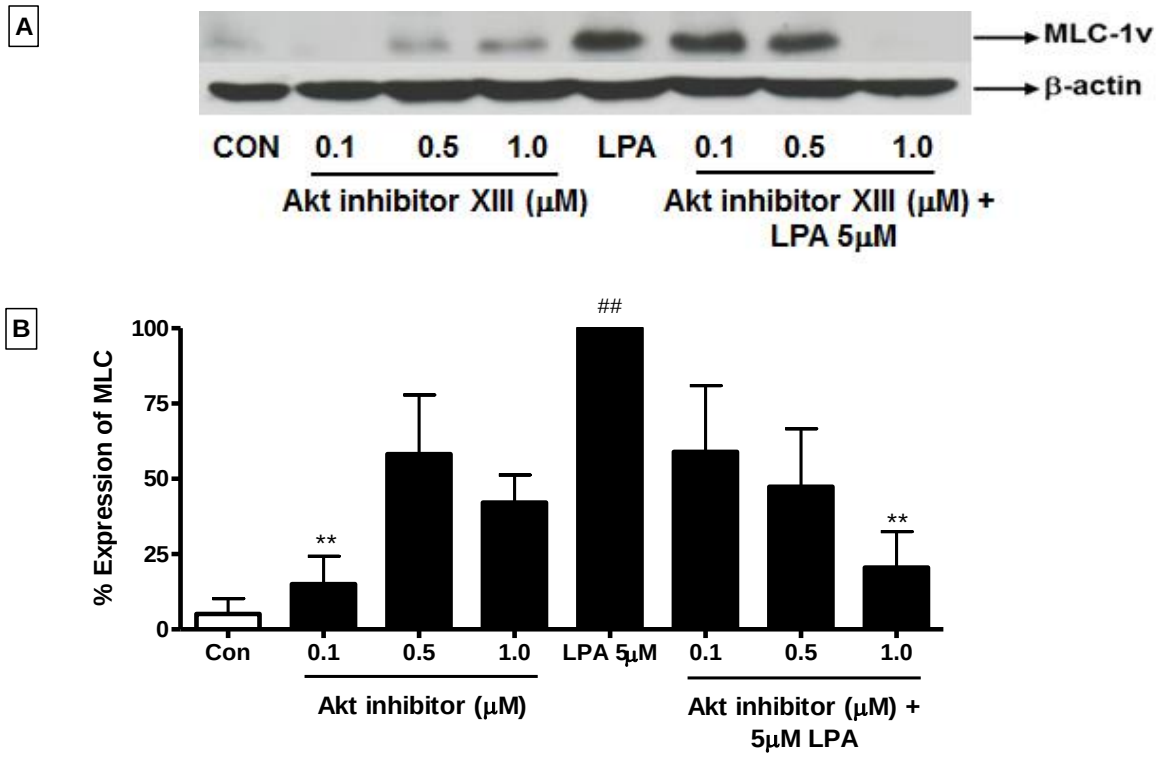


Figure 31. Effects of Akt inhibitor XIII on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), or ranges of concentrations of Akt inhibitor XIII alone, LPA (5μM) or LPA (5μM) and Akt inhibitor XIII during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. $^{**}p<0.01$, when compared to the LPA treated sample and $^{\#\#}p<001$, when compared to the untreated control.

3.2.4 Role of PKC in LPA-induced differentiation of P19 stem cells into cardiomyocytes

In these studies, cells were treated with the potent PKC inhibitor, bisindolylmaleimide I (BIM-I) (IC_{50} = 10 nM) for 24 hours in the absence and presence of LPA (5μM) to initially determine any potential cytotoxic actions of the compound. BIM-I is a potent competitive inhibitor which competes for the ATP binding site in the PKC protein. Bisindolylmaleimide I showed only marginal cytotoxic effects at 10 and 50 μM on P19 cells (Figure 32). However, the morphology of the EBs with 10 and 50μM concentrations of BIM-I alone and in presence of LPA 5μM showed no differences to the EBs formed in the presence of lower concentrations of these compounds. In parallel studies, treatment of cells with BIM-I at 0.1, 0.5, 1, 5 and 10μM for 60 min before the addition of LPA (5μM) and initiation of EB formation caused a concentration- dependent inhibition of MLC-1v expression (Figure 33). The inhibition was however significant with 5 and 10 μM BIM-I.

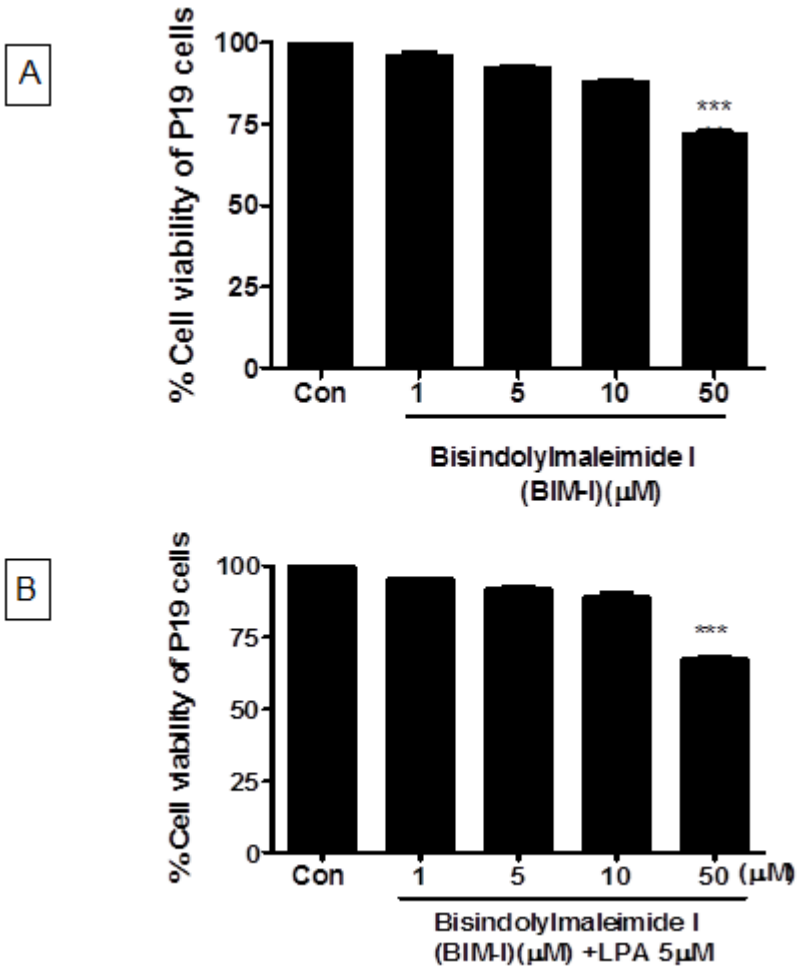


Figure 32. Viability of P19 cells in the presence of various concentrations bisindolylmaleimide I (BIM-I)

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of BIM-I in the absence and presence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of BIM-I inhibitor alone, (**B**) MTT assay for increasing concentrations of BIM-I inhibitor along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. $^{***}p<0.001$, when compared to the controls.

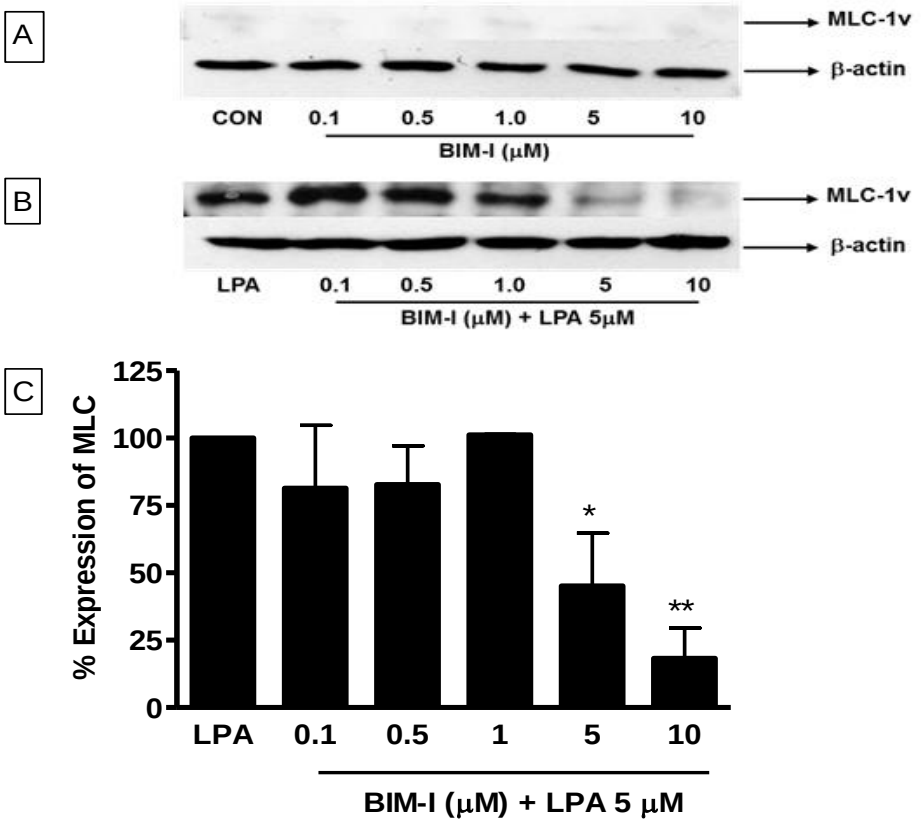


Figure 33. Effects of bisindolylmaleimide I (BIM-I) on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), or ranges of concentrations of BIM-I alone, LPA (5μM) alone, or LPA (5μM) with BIM-1 during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A** and **B**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **C**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. *$p<0.05$ and **$p<0.01$, when compared to the LPA treated sample.

3.2.5 Role of p38 MAPK in LPA-induced differentiation of P19 stem cells into cardiomyocytes

In these studies, cells were treated with the specific competitive inhibitor of p38 MAP kinase, SB203580 [4-(3-Iodophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole] (IC_{50} = 34 nM) for 24 hours in the absence and presence of LPA (5μM) to initially determine any potential cytotoxic actions of the compound (Figure 34.a). This was further analysed by Repeated Measured Anova and showed that there was significant difference between the means (P value, 0.0054) but no significant effect between the pairings (P value, 0.1970) (Figure 34.b).

SB203580 is a potent competitive inhibitor which competes for the ATP binding site on the p38 MAPK protein (Cuenda *et al.*,1995). Cells were next treated with SB203580 (1and 10μM) for 60 min before the addition of LPA (5μM) during the EB forming phase. Lysates were generated on day 6 and used in western blotting to detect changes in expression of MLC-1v. In these experiments, SB203580 at both concentrations of 1 μM and 10 μM failed to inhibit the expression of MLC-1v. In fact, SB203580 appear to increase MLC-1v expression albeit marginally at 10 μM. The data with this inhibitor suggests that LPA may not signal through P38 in inducing differentiation of P19 cells into cardiomyocytes (Figure 35). Whether SB203580 has, other effects that may enhance MLC-1v expression have not been investigated.

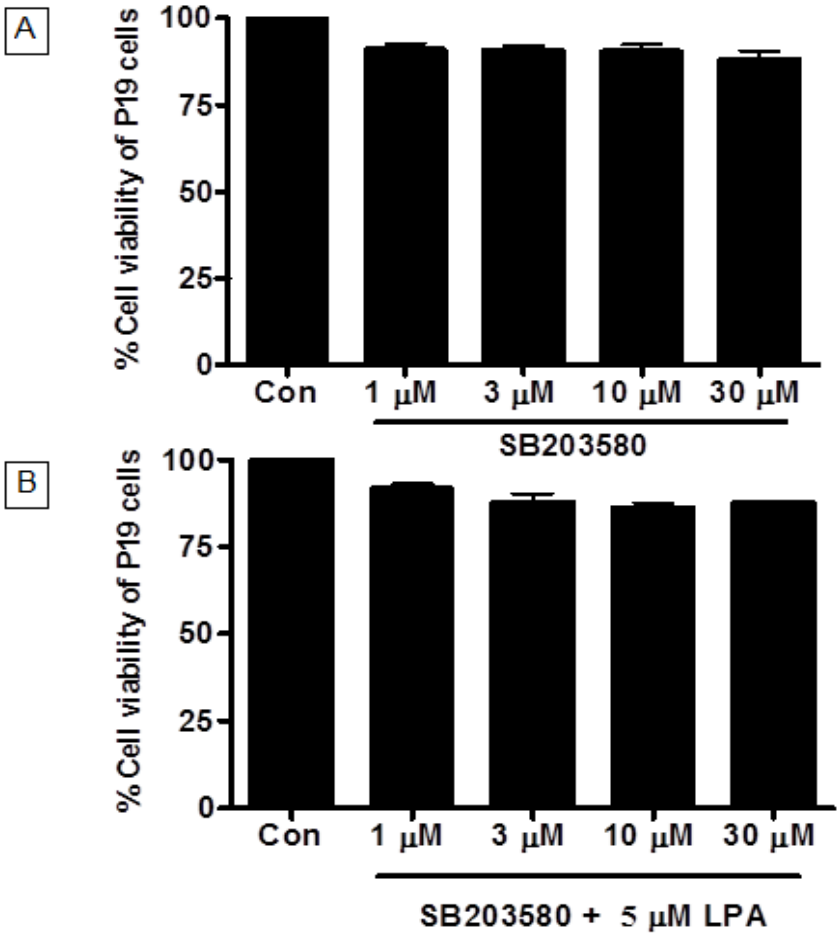


Figure 34. a. Viability of P19 cells in the presence of various concentrations of SB203580.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of SB203580 in the presence and absence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of SB203580 inhibitor alone, (**B**) MTT assay for increasing concentrations of SB203580 inhibitor along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

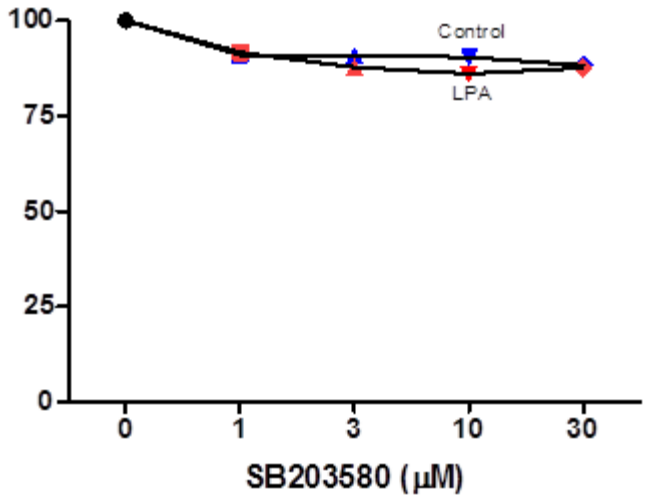


Figure 34. b. Viability of P19 cells in the presence of various concentrations of SB203580.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of SB203580 in the presence and absence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using Repeated Measured ANOVA.

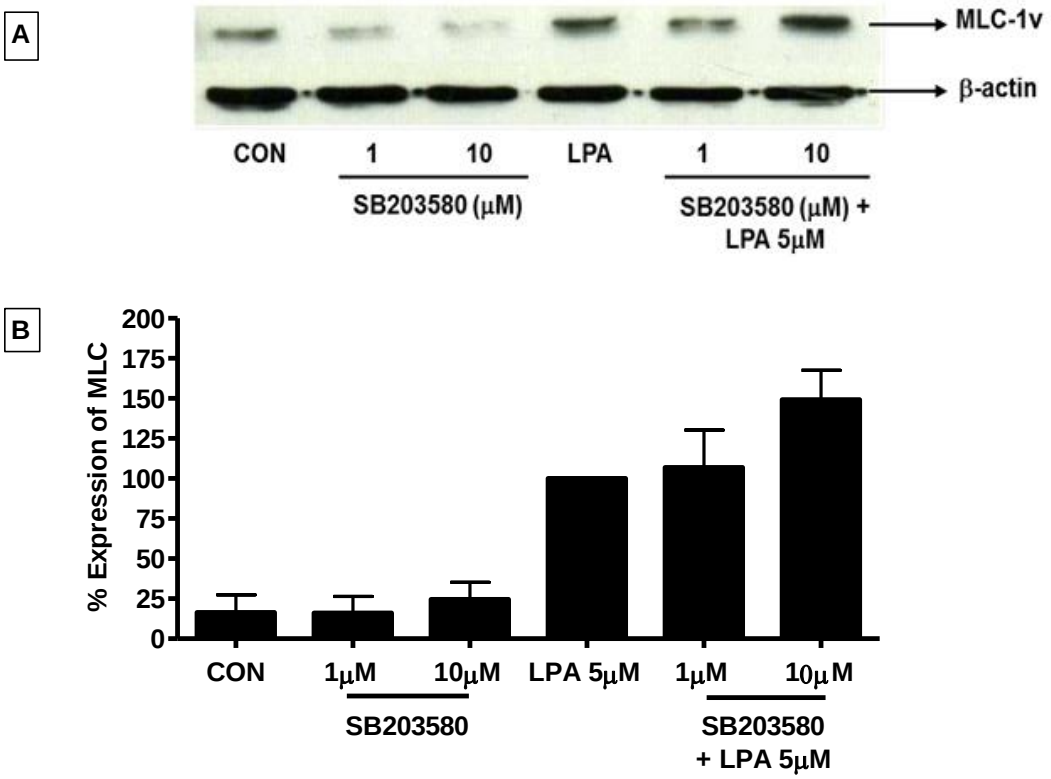


Figure 35. Effects of SB203580 on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), a range of concentrations of SB203580 alone, LPA (5μM), or LPA (5μM) and SB203580 during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data.

3.2.6 Role of ERK1/2 in LPA-induced differentiation of P19 stem cells into cardiomyocytes

The other kinase signalling pathway investigated was that involving ERK1/2 (p44/p42 MAPK). In these studies, cells were treated with the potent ERK1/2 (p44/p42 MAPK) inhibitor, PD98059 (Alessi *et al.*, 1995) (IC_{50} = 2μM) for 24 hours in the absence and presence of LPA (5μM) to initially determine any potential cytotoxic actions of the compound. PD98059 had no toxic effects on P19 cells even at 50μM (Figure 36). In parallel studies, treatment of cells with PD98059 caused a concentration-dependent inhibition of MLC-1v expression (Figure 37). The inhibition was significant with concentrations of 1μM and above. These findings implicate the ERK1/2 (p44/p42 MAPK) pathways as being critical for the LPA-induced differentiation in P19 stem cells.

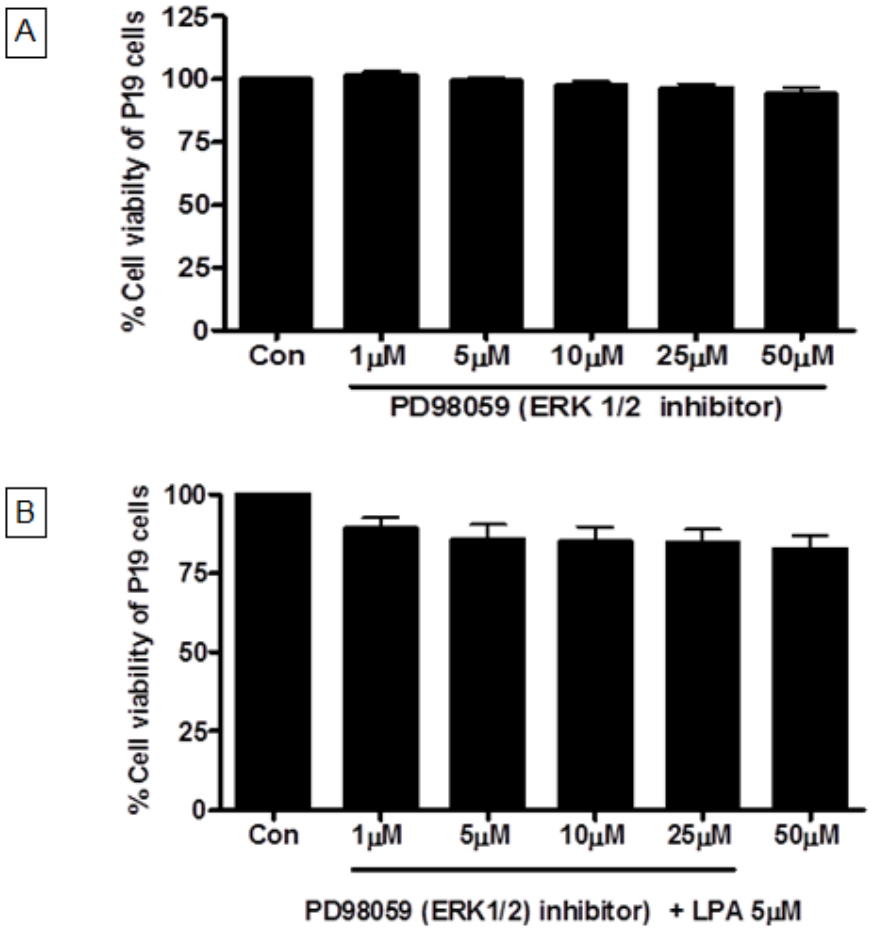


Figure 36. Viability of P19 cells in the presence of various concentrations PD98059.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of PD98059 in the presence and absence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of PD98059 inhibitor alone, (**B**) MTT assay for increasing concentrations of PD98059 inhibitor along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

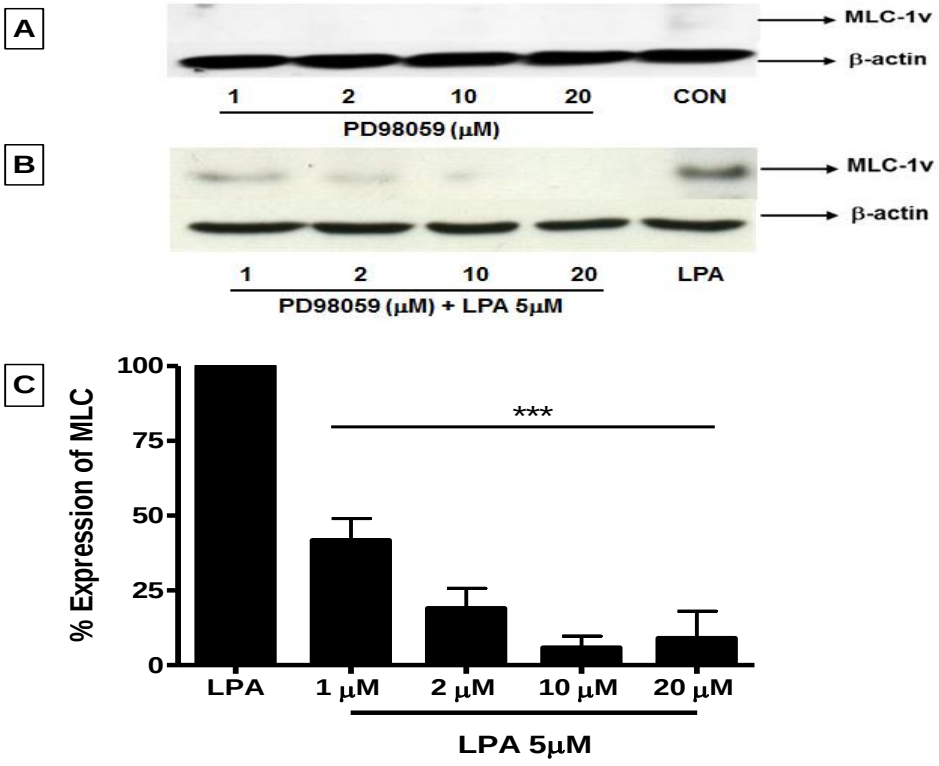


Figure 37. Effects of PD98059 on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), a ranges of concentrations of PD98059 alone, LPA (5μM), or LPA (5μM) with PD98059 during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A** and **B**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **C**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***$p<0.001$, when compared to the LPA treated sample.

3.2.7 Role of ROCK in LPA-induced differentiation of P19 stem cells into cardiomyocytes

In these studies, cells were treated for 24 hours, in the absence and presence of LPA (5μM), with Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride] (IC_{50} = 700nM) a potent Rho-associated protein serine/threonine kinase (ROCK) family of protein kinases inhibitor (Uehata *et al.*, 1997), to initially determine any potential cytotoxic actions of the compound (Figure 38). Once again these effects were marginal with the maximum decrease in MTT metabolism not being greater that 25%. However, these concentrations did not alter EB formation, which showed normal growth and development.

In parallel studies, treatment of cells with Y-27632 at 10μM for 60 min before the addition of LPA (5μM) caused a concentration- dependent inhibition of LPA-induced MLC-1v expression (Figure 39). These findings strongly suggest a role for the ROCK in the differentiation of the P19 cells into cardiomyocytes. Interestingly, the inhibitor on its own also appears to induce differentiation of P19 cells into cardiomyocytes (Figure 39) which contradicts the observations showing that it inhibits the effects induced by LPA. These observations are unclear at the moment and require further investigation.

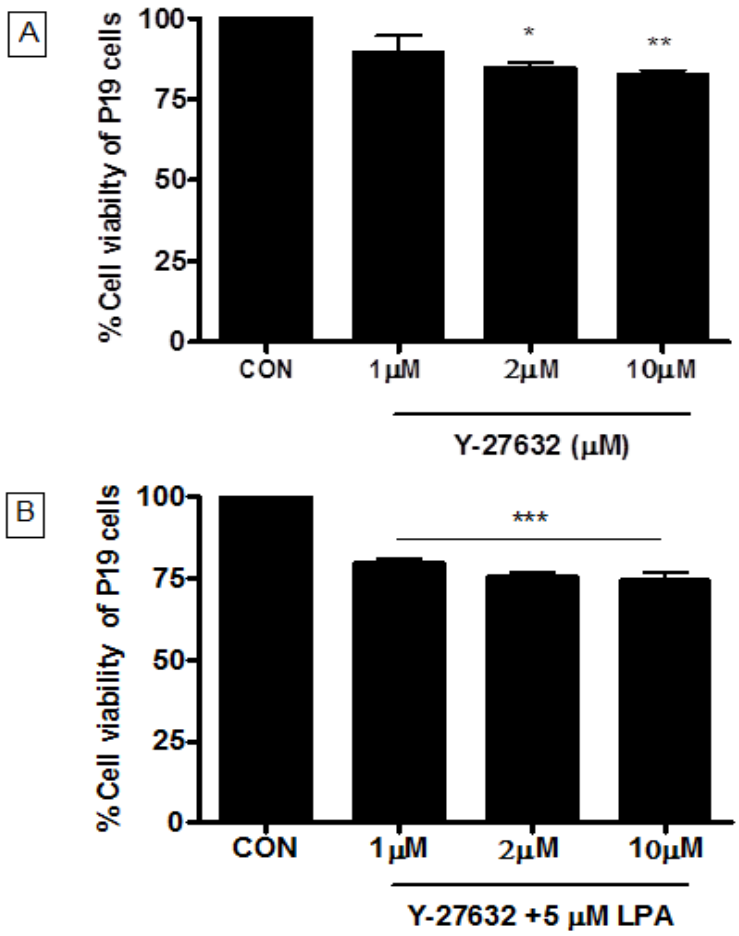


Figure 38. Viability of P19 cells in the presence of various concentrations Y-27632.

Confluent monolayer of P19 cells were plated in 96 well plates and treated with increasing concentrations of Y-27632 in the presence and absence of LPA for 24 h. Cells were then incubated with MTT for 4 h and the MTT assay carried out after treatment as described in the Methods (section 2.9). Panel (**A**) MTT assay for increasing concentrations of Y-27632 inhibitor alone, (**B**) MTT assay for increasing concentrations of Y-27632 inhibitor along with 5μM LPA. Data represent the mean ± S.E.M. from three independent experiments with five replicates in each. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ when compared to the controls.

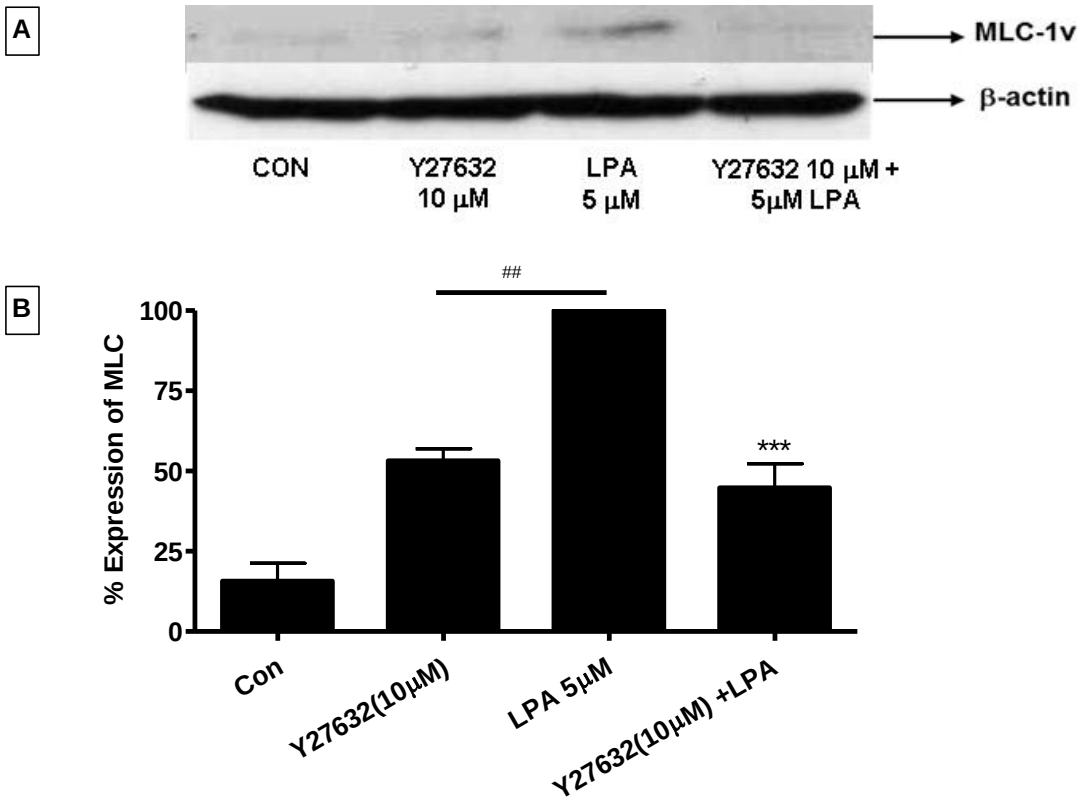


Figure 39. Effects of Y27632 on LPA-induced differentiation of P19 cells into cardiomyocytes.

P19 cells were either incubated with culture medium alone (control), Y27632 (10μM) alone, LPA (5μM), or LPA (5μM) with Y27632 (10μM) during the EB forming phase. Cells were then incubated for 4 days before plating in cell culture 6-well dishes as described in the Methods (section 2.5). Whole cell lysates were generated after 6 days of plating, and were subjected to western blot analysis for MLC-1v expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of MLC-1v protein (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***$p<0.001$, when compared to the LPA treated sample and $^{\#\#}p<0.01$ when compared to the untreated control.

Chapter 3.III

LPA-induced phosphorylation of different downstream signalling pathway

Phospho-Protein studies

The inhibitor studies reported in the previous chapter suggest that LPA may signal through selective protein kinase pathways. To confirm this, further experiments were carried out to establish whether the presence of LPA results in the phosphorylation of the targeted kinases. These studies were however limited to Akt, p38 MAPK and pERK1/2 because of time constraints and because these are amongst the kinases widely implicated to regulate stem cell differentiation.

3.3.1 Time dependent phosphorylation of Akt by LPA.

This experiment was conducted to verify the phosphorylation of Akt at 473 serine residue following the activation with LPA (5μM). In these studies, P19 cells were seeded into the 35mm petri dish at a density of 2 X 10^4 cells/petri dish in complete medium. When the cells were 60-70% confluent the spent media was removed and replaced with fresh medium containing 1% FBS for 24 hours. After a 24-hour period of culture in low serum, cells were treated with LPA (5 μM) for 0, 1, 3, 5, 10, 15, 30, 60 and 120 minutes. After each time points, lysates were generated with 1X lysate buffer containing phosphatase inhibitor and subjected to western blot analysis for phospho-Akt (Ser473). Figure 40 show that LPA induces Akt phosphorylation that was evident at 5 min and sustained for up to 1 hour after treatment.

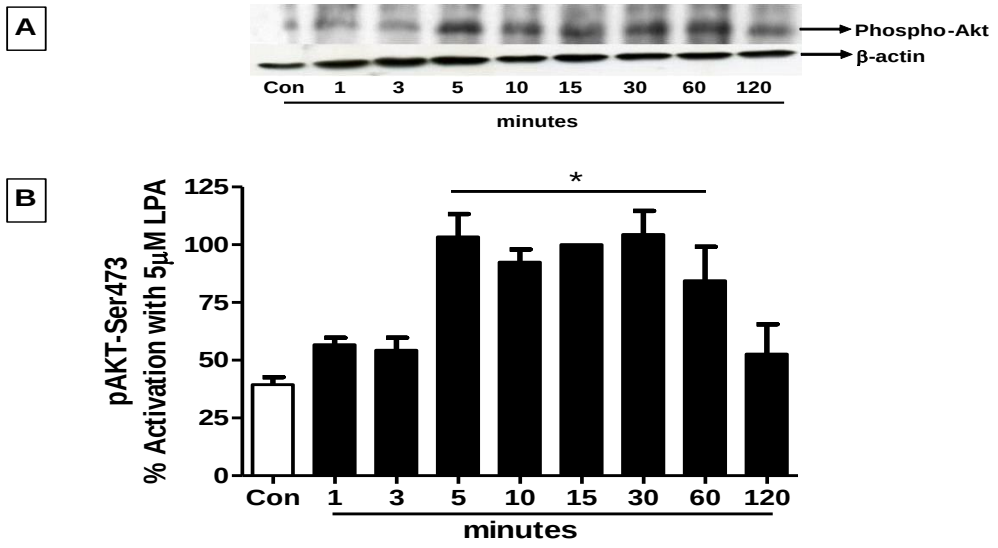


Figure 40. Time course of Akt phosphorylation induced by LPA.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated for specific time points with LPA (5μM); lysates generated and subjected to western blot analysis for phospho-Akt (Ser473) expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of phospho-Akt (Ser473) (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data.

*p<0.05when compared to the untreated control samples.

3.3.2 Time dependent phosphorylation of ERK1/2 by LPA.

This experiment was conducted to verify the phosphorylation of p-ERK1/2 (Thr202/Tyr204) residue following the activation with LPA (5μM). The experiments were set up as described previously and then activated with LPA (5μM) for 0, 1, 3, 5, 10, 15, 30, and 60 minutes. Lysates generated were subjected to western blotting for p-ERK1/2 (Thr202/Tyr204). Figure 41 shows that LPA induces ERK1/2 phosphorylation, which was evident at the very early time point of 3 min and rapidly declining from 5 minutes becoming barely detectable 10 minutes after LPA treatment.

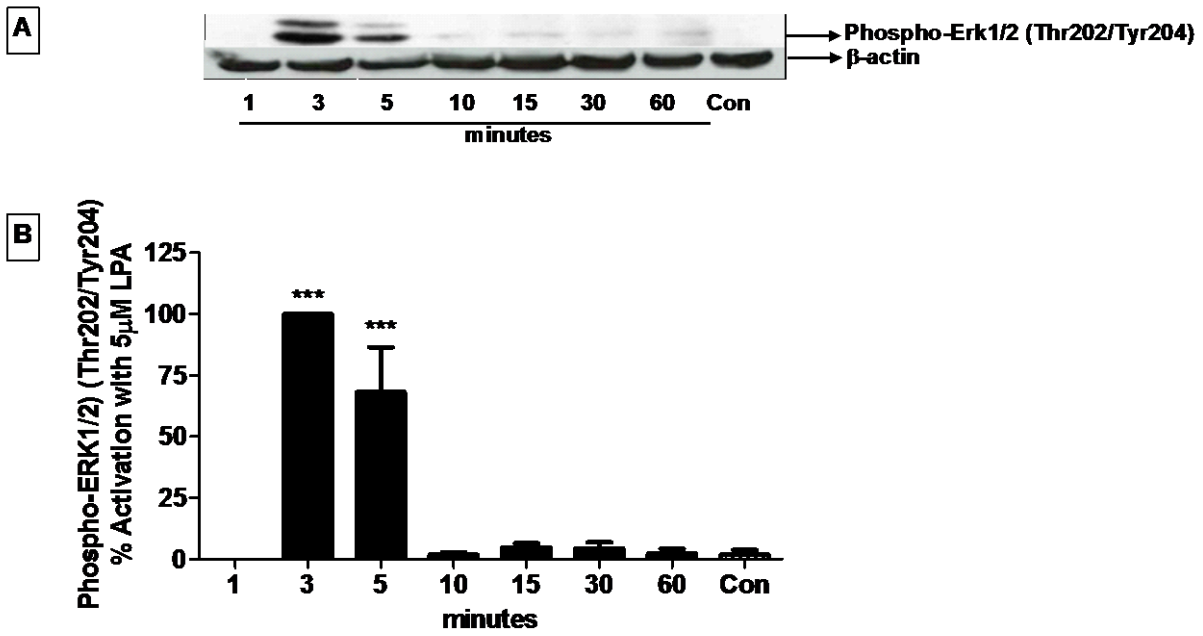


Figure 41. Time course of ERK1/2 phosphorylation induced by LPA.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated for specific time points with LPA (5μM), lysates were generated and subjected to western blot analysis for phosph-ERK1/2(Thr202/Tyr204) expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of phosph-ERK1/2(Thr202/Tyr204) (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***p<0.001, when compared to the untreated control samples.

3.3.3 Time dependent phosphorylation of p38 MAPK by LPA.

To establish whether LPA induced P38 phosphorylation, lysates were generated as described for the other studies following treatment with LPA (5μM) for 0, 1, 3, 5, 10, 15, 30, and 60 minutes. Western blot analysis was conducted for phospho-p38MAPK. Figure 42 show that LPA induces p38- MAPK phosphorylation, which was evident at 3 min and sustained for up to 30 minutes but declining 1 hour after LPA treatment.

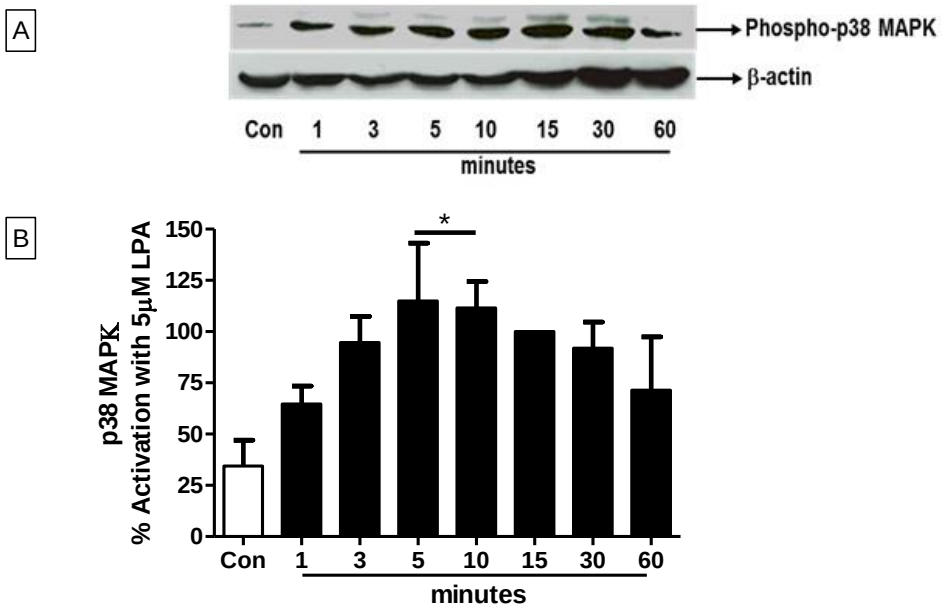


Figure 42. Time course of p38 MAPK phosphorylation induced by LPA.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated for specific time points with LPA (5μM), lysates were generated and subjected to western blot analysis for Phospho-p38 MAPK expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of Phospho-p38 MAPK (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. *$p<0.05$, when compared to the untreated control samples.

3.3.4 Effects of suramin and Ki16452 on LPA-induced Akt phosphorylation

To establish whether the effects of suramin (LPAR4 inhibitor) and Ki16425 (LPAR1/3 inhibitor) reported earlier are linked to the regulation of Akt phosphorylation, P19 cells were treated with 50μg/ml or 10 μM concentrations of these inhibitors respectively prior to activation with 5 μM LPA for 15 min. Lysates generated were analysed by western blotting for AKT phosphorylation. Figure 43 shows that LPA induced Akt phosphorylation was not inhibited by either suramin or Ki16425 suggesting that neither inhibitor regulated LPA-induced differentiation of P19 cells through suppression of Akt activation.

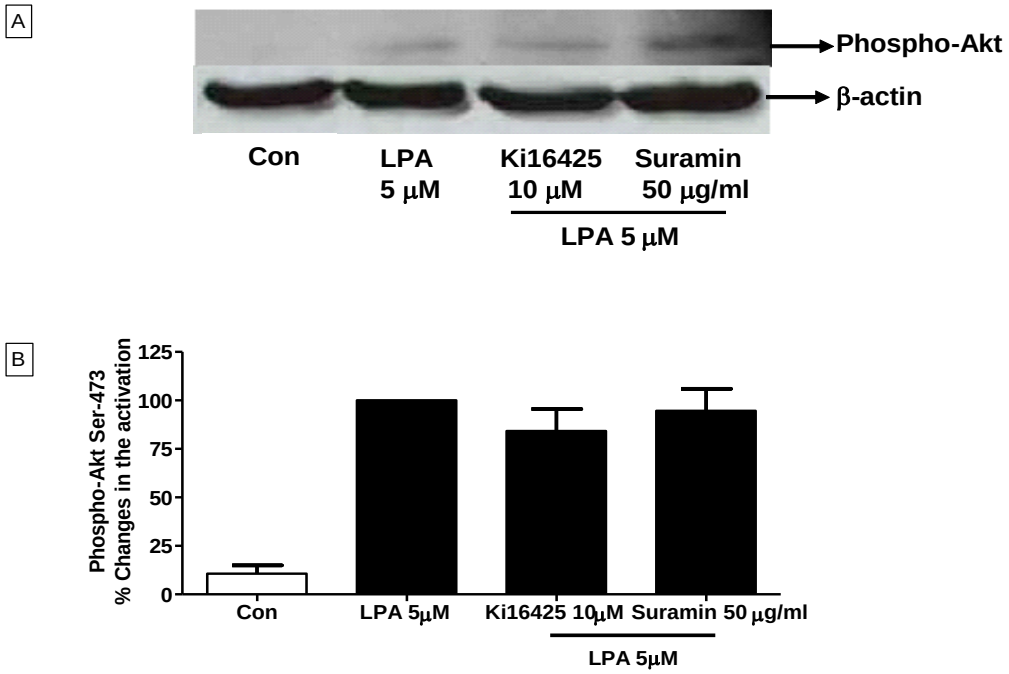


Figure 43. Effects of Suramin and Ki16425 on LPA-induced Akt-phosphorylation.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated with or without LPA (5μM) in the absence and presence of Ki16425 or suramin. The latter were added 60 minutes before the addition of LPA for a further 15 minutes; lysates were generated, and were subjected to western blot analysis for phospho-Akt (Ser473) expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of Phospho-p38 MAPK (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data but showed no statistical difference between treatments.

3.3.5 Effects of Suramin and Ki16452 on LPA induced phosphorylation of ERK1/2 (p42/44 MAPK)

In addition to investigating Akt, parallel experiments were also carried out to establish whether the effects of suramin (LPAR4 inhibitor) and Ki16425 (LPAR1/3 inhibitor) reported earlier are linked to the regulation of ERK1/2 (Thr202/Tyr204) phosphorylation. P19 cells were therefore treated with either 50μg/ml suramin or 10 μM Ki16425 prior to activation with 5 μM LPA for 3 minutes. Lysates generated were analysed by western blotting for phospho-ERK1/2 (Thr202/Tyr204). Figure 44 shows that LPA induced ERK1/2 phosphorylation was completely abolished by both receptor antagonists indicating that, unlike Akt, phosphorylation of ERK1/2 by LPA may be mediated via the activation of LPAR1/3 and potentially LPAR4.

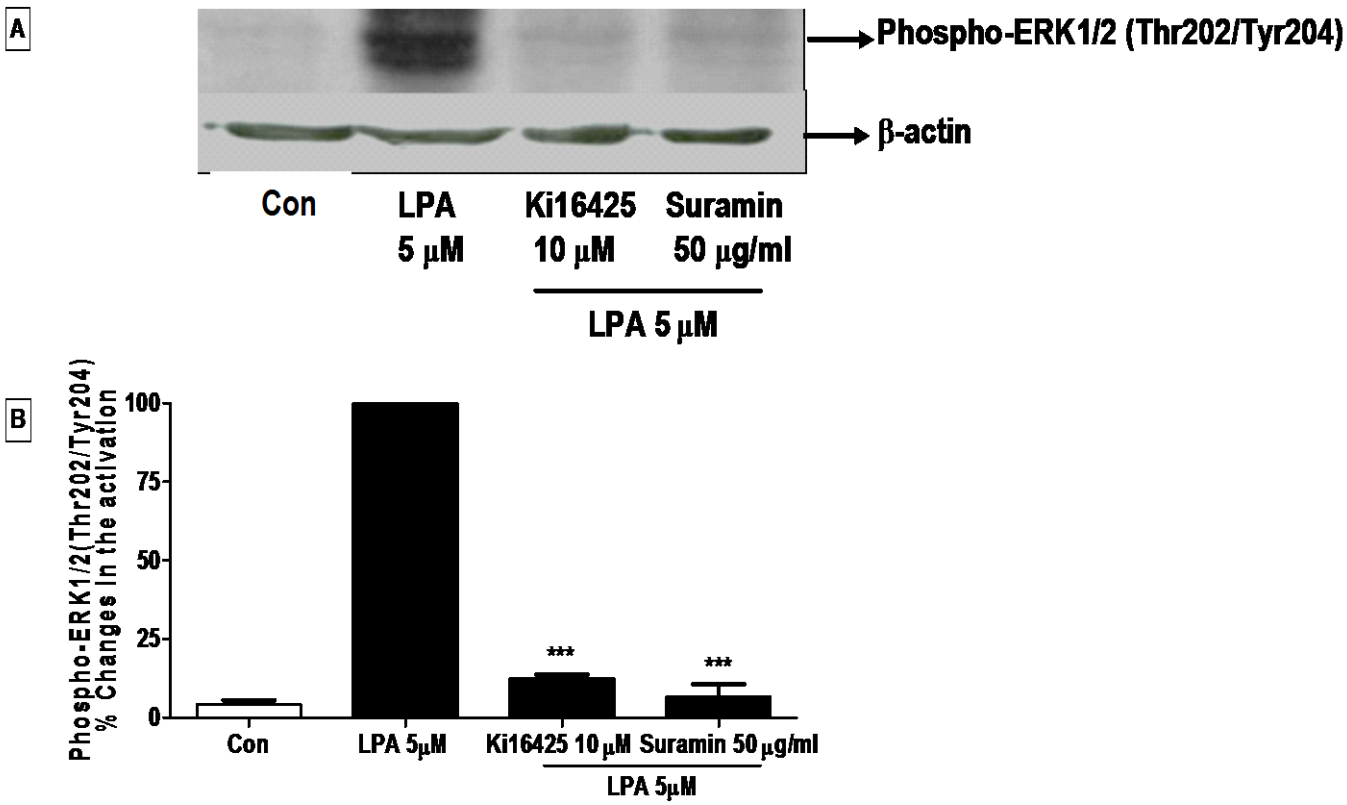


Figure 44. Effects of Suramin and Ki16425 on of ERK1/2 (Thr202/Tyr204) - phosphorylation in the differentiation of P19 cells into cardiomyocytes.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated with or without LPA (5μM) in the absence and presence of Ki16425 or suramin. The latter were added 60 minutes before the addition of LPA for a further 3 minutes; lysates were generated, and were subjected to western blot analysis for Phospho- ERK1/2 (Thr202/Tyr204) expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of Phospho- ERK1/2 (Thr202/Tyr204) (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***p<0.001 when compared to the LPA treated samples.

3.3.6 Effects of Suramin and Ki16452 on LPA-induced phosphorylation of p38 MAPK

Following on from the studies above, experiments were also carried out to establish whether the effects of suramin (LPAR4 inhibitor) and Ki16425 (LPAR1/3 inhibitor) reported earlier are linked to the regulation of p38 MAPK phosphorylation. P19 cells were therefore treated with 50μg/ml suramin or 10 μM Ki16425 prior to activation with 5 μM LPA for 10 minutes. Lysates generated were analysed by western blotting for phospho-p38 MAPK. Figure 45 shows that LPA induced p38 MAPK phosphorylation was also inhibited by suramin and by Ki16425, with both compounds abolishing expression of the phosphorylated protein. Interestingly, inhibition of P38 with SB203580 did not suppress differentiation and even appeared to enhance the latter.

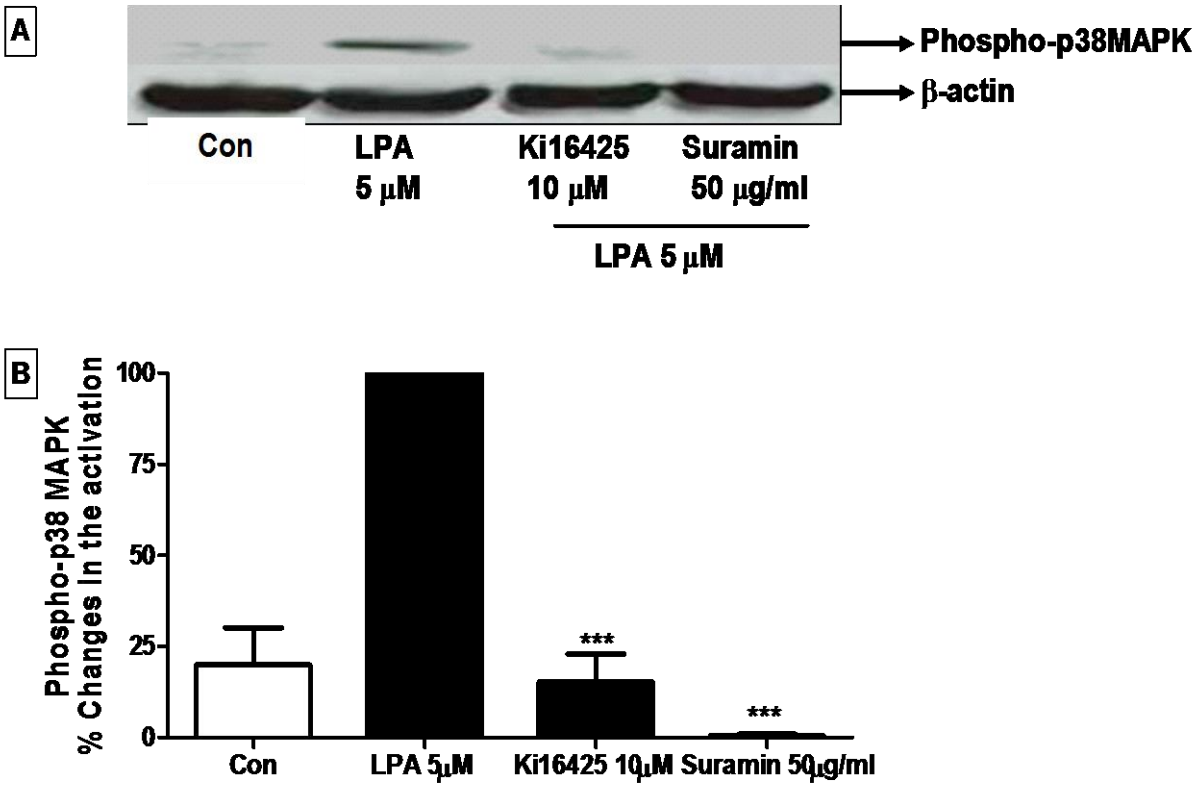


Figure 45. Effects of Suramin and Ki16425 on p38 MAPK-phosphorylation in the differentiation of P19 cells into cardiomyocytes.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated with or without LPA (5μM) in the absence and presence of Ki16425 or suramin. The latter were added 60 minutes before the addition of LPA for a further 10 minutes; lysates were generated, and were subjected to western blot analysis for Phospho-p38 MAPK expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of Phospho-p38 MAPK (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. ***$p<0.001$ when compared to the LPA treated samples.

3.3.7 Crosstalk signalling study

It is widely accepted that there may be crosstalk between cell signalling molecules, with the activation of one pathway resulting in the subsequent regulation of another. Thus, because of the diverse range of signalling proteins identified above as potentially being associated with the effects of LPA, additional studies were carried out to establish whether there was indeed crosstalk between the kinase pathways identified.

3.3.7.1 Crosstalk between other kinases and the Akt pathway

In these studies, experiments were conducted to examine the effects of LY294002 (5μM), BIM-I (10μM), PD98059 (5μM), ROCK (10μM), Akt inhibitor XIII (1μM), and SB203580 (10μM) on Akt phosphorylation by LPA (5μM). After a 24-hour period of culture in low serum, cells were incubated in medium containing 1% FBS with appropriate concentrations of the selected kinase inhibitors for 60 minutes. These were then challenged with 5 μM LPA for 15 minutes. Lysates were generated and subjected to western blotting for AKT phosphorylation using specific antibody. Figure 46 shows that LPA induced Akt phosphorylation at 15 minutes; however, none of the kinase inhibitors used significantly altered LPA-induced phosphorylation of AKT at ser-473.

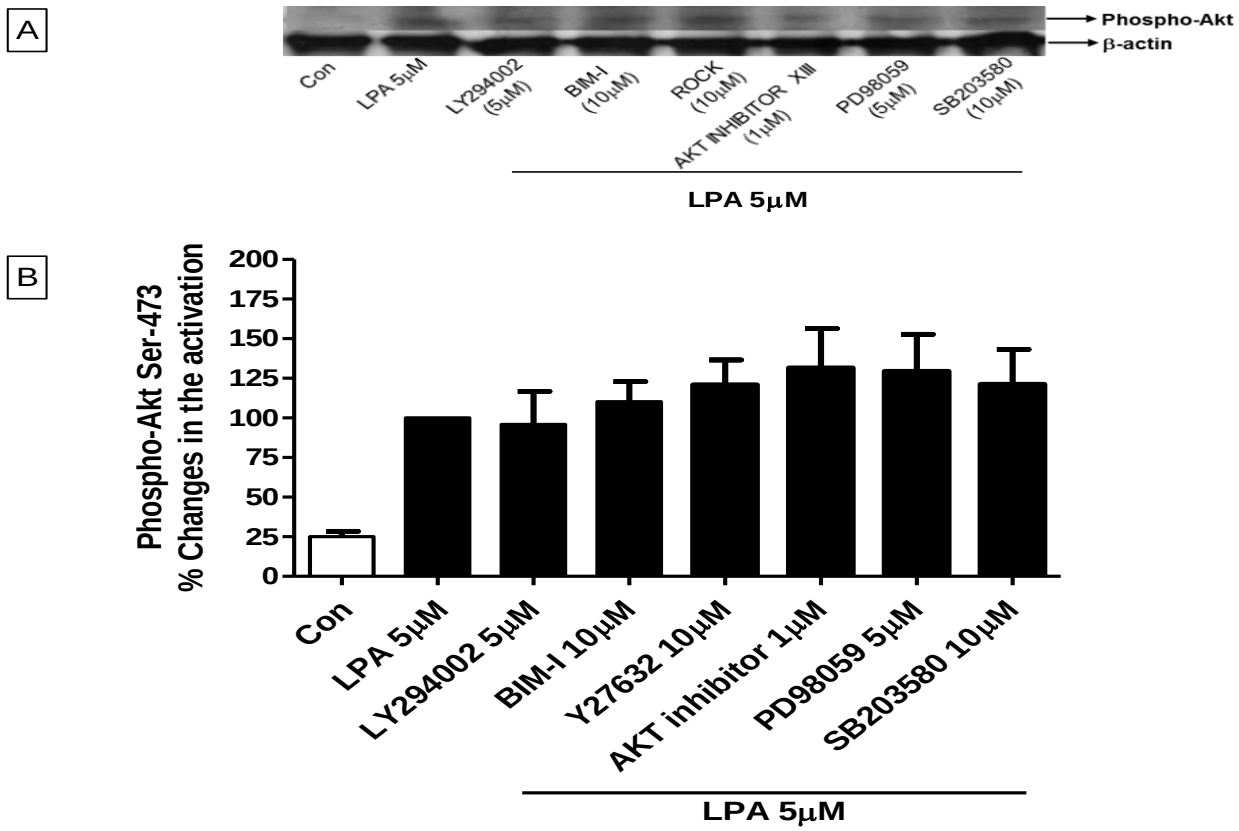


Figure 46. Regulation of LPA induced Akt-phosphorylation at Serine 473 residue by various kinase inhibitors

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated with or without LPA (5μM) in the absence and presence of LY294002 (5μM), BIM-I (10μM), PD98059 (5μM), ROCK (10μM), Akt inhibitor XIII (1μM), and SB203580 (10μM). The latter were added 60 minutes before the addition of LPA for a further 15 minutes; lysates were generated, and were subjected to western blot analysis for phospho-Akt (Ser473) expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of phospho-Akt (Ser473) (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data.

3.3.7.2 Crosstalk between other kinases and the ERK1/2 pathway

In these studies, experiments were conducted to examine the effects of LY294002 (5μM), BIM-I (10μM), PD98059 (5μM), ROCK (10μM), Akt inhibitor XIII (1μM), and SB203580 (10μM) on the activation of ERK1/2 (Thr202/Tyr204) phosphorylation. The experiments were setup as described previously and subsequently incubated with 5 μM LPA for 3 minutes. Lysates were generated and western blotting conducted for ERK1/2 (Thr202/Tyr204 phosphorylation using specific antibody. Figure 47 show that LPA induced ERK 1/2 phosphorylation was significantly inhibited by LY294002, BIM, PD98059, Y27632 and Akt inhibitor XIII, which completely abolish the activation of ERK1/2 by inhibiting its phosphorylation at Thr202/Tyr204. Interestingly, SB203580 failed to inhibit the activation of ERK1/2.

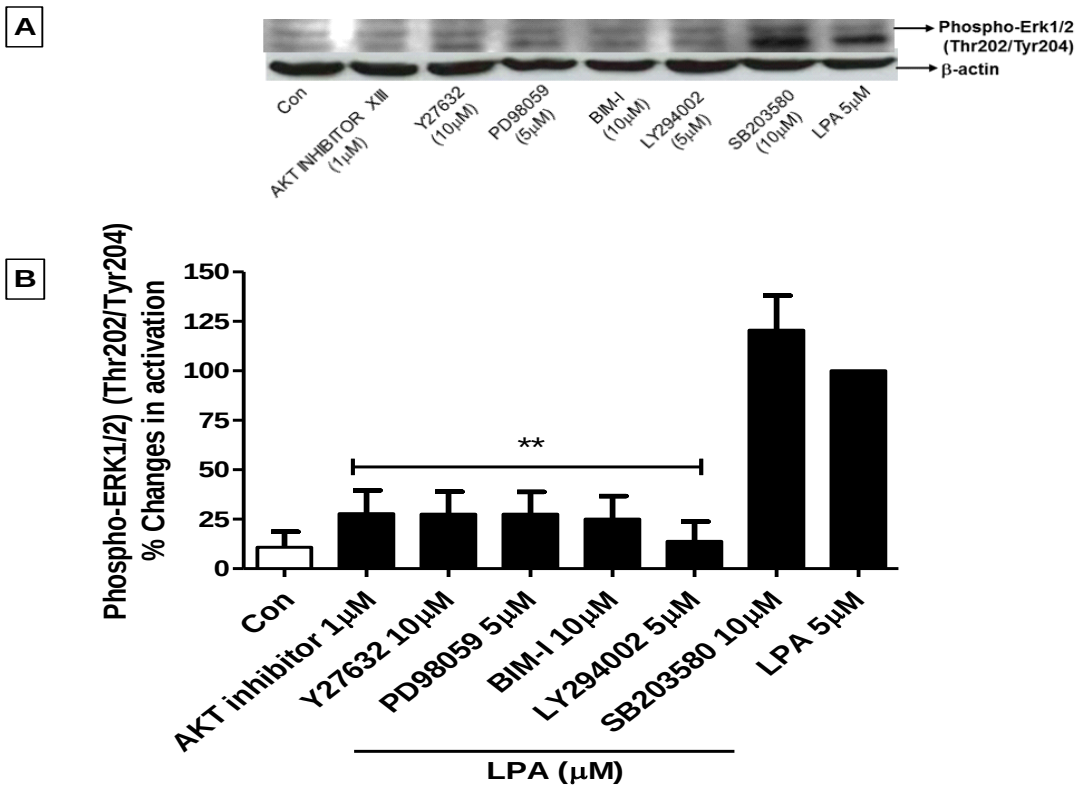


Figure 47. Regulation of LPA induced ERK1/2 (Thr202/Tyr204)-phosphorylation by various kinase inhibitors.

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated with or without LPA (5μM) in the absence and presence of LY294002 (5μM), BIM-I (10μM), PD98059 (5μM), ROCK (10μM), Akt inhibitor XIII (1μM), and SB203580 (10μM). The latter were added 60 minutes before the addition of LPA for a further 3 minutes; lysates were generated, and were subjected to western blot analysis for Phospho- ERK1/2 (Thr202/Tyr204) expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of Phospho- ERK1/2 (Thr202/Tyr204) (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. **$p<0.01$ when compared to the 5μM LPA samples.

3.3.7.3 Crosstalk between other kinases and the P38 pathway

In parallel to those above, experiments were also conducted to examine the effects of of LY294002 (5μM), BIM-I (10μM), PD98059 (5μM), ROCK (10μM), Akt inhibitor XIII (1μM), and SB203580 (10μM) on the activation of p38 MAPK through phosphorylation. The experiments were setup as described previously and subsequently incubated with 5 μM LPA for 10 minutes. Figure 48 shows that LPA induced p38 MAPK phosphorylation was inhibited by LY294002 and Y27632 but not by SB203580, Akt inhibitor XIII or PD98059. The inhibition cause by BIM although pronounced, was not statistically significant when compared to LPA 5μM.

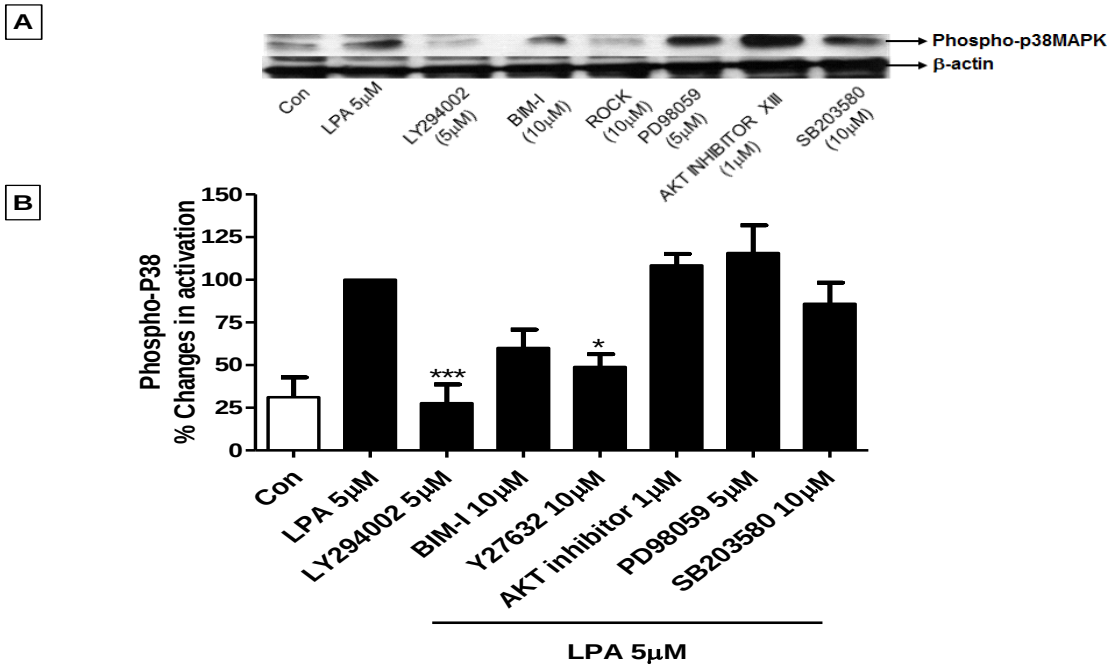


Figure 48. Regulation of LPA induced p38 MAPK- phosphorylation by various kinase inhibitors

Confluent monolayers of P19 cells were plated in 35mm petri dishes and cultured in medium consisting of α-MEM supplemented with 1% FBS, 100units/ml penicillin/100μg/ml streptomycin. Cells were incubated with or without LPA (5μM) in the absence and presence of LY294002 (5μM), BIM-I (10μM), PD98059 (5μM), ROCK (10μM), Akt inhibitor XIII (1μM), and SB203580 (10μM). The latter were added 60 minutes before the addition of LPA for a further 10 minutes as described in the Methods (section 2.6). Whole cell lysates were generated, and were subjected to western blot analysis for Phospho-p38 MAPK expression. The blot shown is representative of at least three independent experiments (Figure **A**). Respective band intensities were quantified by densitometric analysis and normalized to β-actin protein. The data was transformed and presented as percentage of relative intensity of the maximum expression of Phospho-p38 MAPK (Figure **B**). Data represent the mean ± S.E.M. from three independent experiments. Statistical differences between means were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test of the normalized data. *p<0.05, ***p<0.001 when compared to the 5μM LPA samples.

Key Findings

- Data confirms that LPA caused induction of differentiation of P19 stem cells into cardiomyocytes at physiological concentrations.
- P19 stem cells expressed LPA receptors 1 to 4.
- The LPA induced differentiation of P19 cells into cardiomyocytes through LPA receptors 1/3 and 4. LPAR2 was not investigated because of the lack of commercially available inhibitors.
- The induction of differentiation by LPA may be coupled to downstream signalling pathways, which include ROCK, PI3K, PKC and/or Akt and may converge on ERK1/2. Inhibition of any of these pathways has the potential to suppress differentiation.
- In contrast, signalling leading to P38 activation may potentially suppress the process but this needs further clarification.

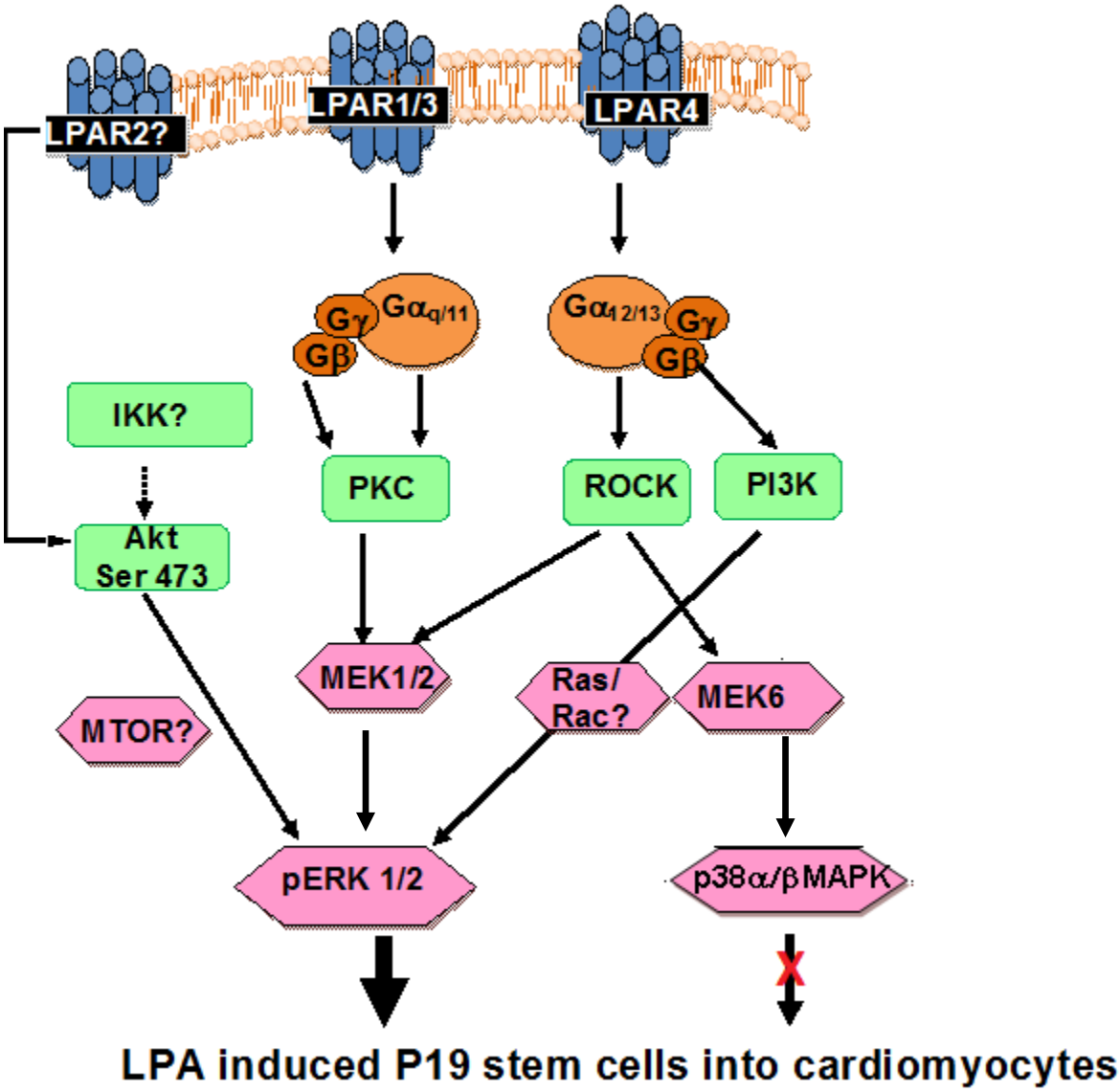


Figure 49. Regulation of P19 stem cells differentiation into cardiomyocytes.
LPA induces differentiation of P19 stem cells into cardiomyocytes potentially through ERK1/2. PI3K, ROCK and PKC are proposed as upstream pathways that activate MEK1/2 and stimulate ERK1/2. These PTX-insensitive pathways are $G\alpha q_{11}/G\alpha_{12/13}/G\beta\gamma$ coupled.

Chapter 4

Discussion

It is estimated that around two million people across the UK are affected with coronary heart disease and it still remains the major cause of death across the world. The only promising and effective treatment for this disease is heart transplantation. It is identified that stem cells could be effectively used in regenerative medicine and have the potential to regenerate infarcted myocardium, which could be an alternative treatment for coronary heart diseases.

Currently, stem cell differentiation can be induced experimentally using growth factors, hormones, solvents and chemicals such as dimethyl sulphoxide (Skerjanc, 1999) or 5-azacytidine (Rangappa *et al*., 2003). Despite the successes with these agents, most are not physiological and cannot be used in translational therapy because of the toxicity or unwanted effects associated with such chemicals. As a result there has been great interest and some progress in identifying more physiologically relevant molecules. In this regard, it has been reported that hormones like oxytocin and other molecules like retinoic acid induce differentiation of mouse embryonic stem cells into cardiomyocytes (Bugorsky *et al.*, 2009). In addition, Wnt and BMPs are active modulators of differentiation of human pluripotent stem cells into cardiomyocytes (Lian *et al*., 2012) and growth factors like fibroblast growth factors (FGF-2) stimulate the differentiation of neural stem cells (Hienola *et al*., 2004).

The studies reported in this thesis have extended current findings on physiological regulators of stem cell differentiation by focusing on identifying the role of LPA in the induction of differentiation of P19 cells into cardiomyocytes.

Lysophosphatidic acid is an interesting molecule because although it has been implicated in disease states, for instance contributing to endothelial permeability and stress fiber formation during early atherosclerotic lesion formation (Colangelo *et al*., 1998; Ross, 1993). it also elicits various biological functions such as triggering neuronal differentiation of cortical neuroblasts and induces proliferation (Fukushima *et al.*, 2007). In addition, LPA has also been reported to exert anti-apoptotic actions on mesenchymal stem cells, which may aid in facilitating their survival within the ischaemic myocardium (Karliner, 2004; Chen *et al.*, 2008). A closely related phospholipid, sphingosylphosphorylcholine (SPC) has also been reported to induce differentiation of mouse embryonic stem cells in to cardiomyocytes suggesting that biolipids such as LPA can potentially regulate stem cell differentiation *in vivo*. Few reports have also suggested a role for LPA in blood and lymphatic vessel formation during embryogenesis (Sumida *et al.,* 2010). It has also recently been shown to induce differentiation of rat embryonic stem cells but into neuroglial and cholinergic neurons (Cui *et al.*, 2006). However, there are no reports on the ability of LPA to generate cardiomyocytes from stem cells. This has therefore been the focus of this thesis that is aimed at investigating the effect of LPA on the ability of stem cells to differentiate into cardiomyocytes.

In our studies, we have used P19 stem cell line for conducting all of our experimental studies. P19 cells are relatively easy to culture and can be maintained in the undifferentiated state without the need for factors such as leukemia inhibitory factor

(LIF) or feeder layer, and provide a good model for differentiation of stem cells into cardiomyocytes. The protocols are also well established (Paquin *et al*., 2002; van der Heyden *et al*., 2003; Wobus *et al*., 1991) and have demonstrated the expression of cardiac transcription factors and specific cardiac protein in the differentiated P19 cells similar to other cardiac lineage-committed cells (Grepin *et al*., 1997; Maltsev *et al*., 1993; Monzen *et al*., 1999; Skerjanc, 1999). Moreover, they have also demonstrated that P19 cells undergo a similar process of the cardiac developmental stages during embryogenesis and express cardiac markers such as Nkx2.5, GATA-4, cardiac specific proteins like MHC, Troponin I and MLC (Habara-Ohkubo, 1996;Monzen *et al.*, 1999).

DMSO can induce P19 cells to differentiate into cardiomyocytes in part by the induction of the canonical Wnt/β-catenin signalling molecules (Nakamura *et al.*, 2003). Epigenetic studies have also shown the upregulation of DNA methyl transferase 3a and histone modification on exposure to DMSO (Iwatani *et al.*, 2006). Similarly, treatment of P19 cells with DMSO causes the expression of the alpha and beta-cardiac MHC along with sarcomere MHC and MLC (Habara-Ohkubo, 1996; Moore *et al*., 2004). These findings indicate that DMSO may be a good inducer of the differentiation of embryonic carcinoma cells into cardiomyocytes and has therefore been used in our studies as a positive control for the differentiation process.

The formation of 3-dimensional EBs is considered vital for the induction of differentiation of embryonic stem cells (Desbaillets *et al*., 2000; Grover *et al*., 1983).

Thus, in all our studies, P19 stem cells were initially plated into petri dishes during which they formed a 3-dimensional embryoid body with varying shapes and sizes, and with the cell mass formed expressing markers characteristic of the ectoderm, endoderm and mesoderm layers similar to that seen during the embryogenesis. On transferring these EBs to a tissue culture grade plate the adhered and formed monolayer of cells. Cells treated with 1% DMSO showed differentiation into cardiomyocytes from day 4 with beating clusters of cells from day 6-7 and sustained until day 12. The addition of LPA also induced differentiation of P19-derived EBs into cardiomyocytes. This effect was both time and concentration dependent and support studies with SPC, which have been reported to induce differentiation of mouse embryonic stem cells to cardiomyocytes.

The normal concentration of LPA in serum ranges between 1 and 5μM, but concentrations can reach up to 20μM when generated by activated platelets in the circulation (Baker *et al.*, 2002; Eichholtz *et al.*, 1993; Sano *et al*., 2002). In our studies, LPA showed a bell-shaped response with 5μM inducing optimum differentiation and this is well within the physiological range. As a result, all subsequent experiments were carried out using 5 μM LPA, which was also found not to exert any cytotoxic effects on cells. Interestingly at the higher concentrations where differentiation was suppressed (eg 25μM) LPA was shown to have marginal toxicity and this may account for the effects observed at these high concentrations. The concentration of 5μM is however consistent with previous studies which were carried out at a range of 1-10μM LPA (Fukushima *et al.*, 2007; Cui *et al.*, 2007).The differentiation of P19 cells

to cardiomyocytes induced by LPA was confirmed by the presence of beating clusters of cells and by western blotting probing for MLC-1v. Both these markers were evident on day 6 after plating EBs into tissue culture dishes and allowed to grow into monolayers. Interestingly, on day12, all the four concentrations (1, 5, 10 and 25μM) of LPA used could induce differentiation to the same extent and these were comparable to the induction of differentiation by 1% DMSO. In parallel to the above experiment, time course studies with 5μM LPA showed differentiation was time dependent with a significant increase in differentiation and sustained until day 14.

Further experiments were conducted to identify the LPA receptors that may be crucial for differentiation of P19 cells into cardiomyocytes. All the LPA receptors belong to the family of seven transmembrane spanning G protein-coupled receptors (GPCRs). LPA has been reported as a ligand for the ventricularzone-1 receptor identified and isolated from mouse complementary deoxyribonucleic acid (cDNA) (Hecht *et al.*, 1996). LPAR1-3 are members of the endothelial differentiation gene (Edg) family (Mutoh *et al.*, 2008; Virag *et al.*, 2003). These three members share 45-56% overall amino acid identity. Recently used nomenclature for LPA receptors are LPA1 (Edg-2), LPA2 (Edg-4), and LPA3 (Edg-7) and are in accordance with the guidelines of the International Union of Pharmacology (Contos *et al.*, 2002; Wang *et al.*, 2001). The newly identified receptors include LPA4/P2Y9/GPR23, LPA5/GPR92 (Lee *et al.*, 2007), and LPA6 (GPR87) (Ye, 2008). These receptors are also coupled to G-proteins such as $G\alpha_{12/13}$, $G\alpha_{i/o}$, $G\alpha_q$, and probably other G proteins except $G\alpha_s$ (Contos *et al.*, 2000). Moreover, all these receptors show distinct tissue distribution in the body. In relation to

stem cells, LPA receptors 1, 2 and 3 have been shown to be expressed in mouse embryonic stem cells, LPAR1 has also been shown to be present in mesenchymal stem cells and all 5 receptors are present in neuronal progenitor cells (Chen *et al.*, 2008; Dubin *et al*., 2010; Todorova *et al.*, 2009;).

Studies to determine the expression of LPA trans-membrane receptor expression profile in the P19 cells was carried out by reverse transcription and real time-PCR analysis of total mRNA from control non induced cells using the relative quantification method with cDNA standards (Pfaffl, 2001). This method is now routine and very sensitive as well as reproducible, permitting the quantification of transcripts with very high accuracy (Yuen *et al.*, 2002). A housekeeping gene (HKG) was used to standardise the data. Housekeeping genes are expressed in all cells and are required for the normal maintenance of cellular function both in normal and pathophysiological conditions. While a few genes like GAPDH, HSP90 and β-actin can remain unaltered other HKGs may be altered by various experimental condition. It is therefore important to discover authentic HKGs for analysis in a specific experimental condition to ensure that the its expression levels are not altered by the treatment. In our studies, the normalization was conducted using 8 HKGs, and Calnexin and tyrosine 3- monooxygenase/tryptophan 5-monooxygenase activation protein. Calnexin is an integral protein which acts as a chaperone and important for protein folding. YWHAZ is a protein which has been indicated to function in the regulation of insulin sensitivity. Calnexin was found to be the most stable under our experimental condition and therefore used as the housekeeping gene for normalizing changes in LPA receptor

expression.

The single product of the PCR amplicon that is crucial in these studies was confirmed using two principles. Firstly, the presence of a single peak in all PCR runs and secondly the separation of the amplicon based on amplicon size using a DNA ladder (50 base pairs). The agarose gel electrophoresis (2%) conducted with the DNA amplicons from the real time PCR showed the expression of LPA receptors 1, 2, 3, and 4 but not receptor 5. This confirms that P19 cells express LPA receptors 1-4 and not 5, hence these receptors could be involved in the differentiation to cardiomyocytes. This result is consistent with the previous report that has shown the involvement of LPA in the proliferation of P19 cells through cAMP response element binding protein, CREB (Kim *et al*., 2012). What is more interesting is the observation that when P19 cells were treated with LPA, upon differentiation, express higher levels of transcripts for the LPA receptors when compared to controls. Even more interesting is the observation that LPAR2 was increased by up to 3 fold under these conditions. This finding suggests that LPAR2 could be a potentially critical LPA receptor in P19 stem cell derived cardiomyocytes. This is the first report to show this profile of change in these receptors especially when stem cells are compared to the differentiated cells that have originated from them. The relevance of this finding however remains to be determined in full.

To identify which receptor may be associated with the induction of differentiation by LPA, studies were conducted using known pharmacological inhibitors of LPAR1/3 and LPAR4. To determine the role of LPAR1/3 the antagonist Ki16425 was used as this

has been reported to be selective for these receptors (Ohta *et al.*, 2003). It has a molecular formula of $C_{23}H_{23}ClN_2O_5S$ and molecular weight of 474.96 kDa. Studies have shown it to cause complete inhibition in Ca^{2+}rise observed during the LPA induced osteoblastic differentiation of human mesenchymal stem cells when used at a concentration of 10μM. It is also reported to inhibit neuronal differentiation of human embryonic stem cells at the same concentration (Dottori *et al.*, 2008). Thus, for our experiments Ki16425 was used at a range of concentrations of 1, 10 and 50 μM. The result demonstrated that Ki16425 concentration dependently inhibited the expression of MLC-1v with 10 μM virtually abolishing the latter. These results suggest that LPAR1 and/or 3 may be involved in the LPA mediated effects in inducing P19 cells differentiation into cardiomyocytes. We cannot however rule out that the effect may in fact only involve one or the other rather than both receptors but until selective inhibitors are available which can distinguish between LPAR1 and LPAR3 we cannot conclude which is the critical target for the actions of LPA. The implication of these receptors in the differentiation process however fits with at least one other study, which has shown that LPAR1/3 may be involved in neuronal differentiation and in the anti-apoptotic mechanisms associated with LPA (Chen *et al.*, 2008; Fukushima *et al.*, 2007).

P19 cells express LPAR1-4 and the other receptors not blocked by Ki16425 may in fact be critical for the differentiation process. To address this, a further study was carried out using the non-selective antagonist of LPAR4, suramin.This is a polysulphonated naphthylurea with a molecular mass of 1297.29 kDa, which shows

diverse biological effects and antagonises the P2Y receptors. LPAR4 shows a closer identity towards the P2Y receptor than the Edg family of receptors (20-24 %) (Yanagida *et al.*, 2007). Reported studies have revealed the inhibitory action brought about by suramin on LPA-induced effect is mediated through GPCR (van Corven *et al.*, 1992; van der Bend *et al.*, 1992). When used in our studies, experiments were conducted initially with 0.05, 0.1 and 0.5 mg/ml concentrations of suramin. All these three concentrations showed complete inhibition of the differentiation of stem cells induced by LPA. Further experiments were conducted with lower concentrations of 0.001 and 0.005 along with 0.05 mg/ml. A dose dependent response was observed and showed inhibitions back to control levels with 0.05 mg/ml with no significant inhibition at lower concentrations. This demonstrates the involvement of LPAR4 receptor in the LPA induced differentiation of P19 cells.

Interestingly, it was observed that suramin alone induced differentiation at 0.001mg/ml concentration. This is unexpected and requires further investigation. However in addition to inhibiting P2Y receptors, suramin has been reported to elicit many more biological roles like blocking the binding of various growth factors to their receptors (Hosang, 1985; Coffey *et al.*, 1987), inducing differentiation of mouse embryonic stem cells to sinus nodal like cells (Wiese *et al.,* 2011). The precise mechanism that underlies the direct induction by suramin of the differentiation of P19 cells into cardiomyocytes is currently unclear but its ability to induced differentiation of mouse embryonic stem cells to sinus nodal like cells is reported to occur through Ryanodine receptor. Whether this is the case in the P19 cells remains to be established.

Following the observations that LPA can induce the differentiation of p19 cells into cardiomyocytes and establishing that this effect is mediated through known LPA receptors, the next phase of the studies embarked on identifying the signalling mechanisms that may mediate the actions of LPA. Many reports have claimed that the LPA effects may be mediated through PTX sensitive and insensitive pathways which induce calcium mobilisation, gene transcription, MAPK stimulation, DNA synthesis, and cytoskeletal changes (Bandoh *et al.*, 1999; Fukushima *et al.*, 1998; Weiner *et al.*, 1999). For example, LPAR1 triggered survival of rat Schwann cells and proliferation of oligo-dendrocytes are mediated through PTX-sensitive Gi/o/PI3K/Akt signalling pathways (Weiner *et al*., 1999). Similarly, LPA triggered DNA synthesis has been shown to differ in its PTX-responsive effects in that there may be a PTX sensitive and a PTX insensitive component to the response (Tabuchi *et al*., 2000; Keller *et al*., 1997). In addition, LPA inhibiting hESCs differentiation to neuronal progenitor was also reported to occur through a PTX-insensitive pathway (Dottori *et al*., 2008). The divergence in the PTX sensitivity shown in the previous studies may be because of variations in the species, origin, developmental phases, and/or culture prerequisite. $G\alpha_i$ subunit is ADP-ribosylated by PTX there by leaving it in inactive state. This further inhibit the signalling events mediated through cAMP (Burns, 1988). To determine whether responses to LPA in our studies are PTX sensitive, experiments were conducted using PTX to inhibit LPA-induced differentiation of P19 cells. Our data revealed that PTX was without effect even though we used pharmacological concentrations, which have previously been used in other studies conducted in MSCs

and hESCs (Chen *et al*., 2008; Kim *et al*., 2005). This clearly reveals that the LPA induced differentiation to cardiomyocytes is not coupled to the PTX-sensitive $G\alpha_i$ signalling. Currently it is not clear whether the other G protein subunits including $G\alpha_q$, $G\alpha_{12/13}$ or $G\alpha_{\beta\gamma}$ are involved but it is likely that at least one of these will be part of the LPA receptor signalling process. Currently there are no commercially available inhibitors for these proteins and studies that have looked at their involvement have used a transfection approach which is currently beyond the scope of this thesis because of time constraints.

It is likely that any physiological actions are channelled through multiple signalling pathways. This is crucial and expected when a particular cell system shows multiple receptor expression similar to our study. To identify the upstream signalling, additional experiments were conducted focusing on kinase pathways including the mitogen activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), Akt/protein kinase B (PKB), protein kinase C (PKC), Rho- associated protein kinase (ROCK).

Initial experiments examined the effects of PI3K pathway in the regulation of differentiation of P19 cells with 5μM LPA. The role of PI3K pathway has been extensively studied in many cell systems and it has been reported to play a crucial role in many physiological functions (Bekhite *et al*., 2011; Okkenhaug *et al*., 2003). A previous study has reported the role of PI3K signalling in the induction of differentiation

of mESC into cardiomyocytes induced by Neuregulin-1β and this was shown to involve the activation of cAMP Response Element Binding Protein (CREB) (Okkenhaug *et al*., 2003). PI3K is also reported to be involved in LPA induced proliferation of rat chondrocytes and Schwann cells (Kim *et al*., 2005; Li *et al*., 2003). In addition, PI3K promotes the differentiation of cardiac derived stem cells into vascular endothelial cells mediated by Vascular Endothelial Growth Factors (VEGF) (Xiao *et al*., 2013). In studies like differentiation of hESC into endothelial cells are mediated through the PI3K signalling pathway (Merkely *et al*., 2014). Thus we conducted experiments using LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], a potent inhibitor of PI3K. This compound acts by binding at the ATP-binding site of the enzyme (Vlahos *et al.*, 1994) and is a specific inhibitor that does not show inhibition on the other Kinases like, Lipid Kinases, Serine/Threonine Kinases or Protein Tyrosine Kinases. In these studies, LY294002 was used at a range of concentrations from 1-20μM. This was based on published literature reporting the various concentration ranges of LY294002 showing inhibitory effects on various cellular systems (Pong *et al*., 1998; Watanabe *et al*., 2008; Xiao *et al*., 2013). The data obtained showed that LY294002 significantly inhibited the differentiation of P19 cells into cardiomyocytes, strongly implicating the PI3K signalling pathways as being critical for this process and was only cytotoxic at 20μM. These findings are consistent with other reports, which have indicated that treatment with LY294002 inhibited differentiation of ES to cardiomyocytes, and showed EBs to have reduced beating foci (Klinz *et al*., 1999).

The downstream target of PI3K signalling is Akt and, although this has not been

established yet in our studies, these two form a cascade that feeds into the nucleus to trigger differentiation. In this regard, it is worth noting that other reports have suggested effects of LPA may be mediated through PI3/Akt signalling. For instance, activation of the PI3K/Akt signalling pathway by LPA in Caco-2 colon cancer cells protects these cells from apoptosis (Rusovici *et al.*, 2007). Furthermore, the same pathway activated by LPA inhibits migration of MDA-MB-231 breast cancer cells (Du *et al.*, 2010). Thus, the PI3K/Akt cascade is central to most of the actions of LPA and it now remains to be show whether this is the case in its ability to induce the differentiation of P19 cells into cardiomyocytes.

Other than PI3K/Akt, LPA may mediate effects by the activation of downstream signalling pathways involving PI3K/Akt/PKB coupled to Wnt/GSK-3 (Fang *et al*., 2002). The latter is also critical for differentiation of stem cells into different lineages and it has been reported that Wnt/β-catenin signalling is crucial in DMSO induced differentiation of P19CL6 cells (Nakamura *et al.*, 2003). Thus a selective inhibitor for Akt, Akt inhibitor XIII isoenzyme-selective Akti-1/2 which has an IC50 for Akt1, Akt2, Akt3 are 58 nM, 210 nM, 2.12 μM respectively was used for our study. In these studies, Akt inhibitor XIII isoenzyme-selective Akti-1/2 at a low concentration of 1μM completely abolish expression of MLC-1v. Thus, the fact that the differentiation of P19 cells could be abolished in our studies with concentrations of the inhibitor as low as 1 μM suggested that Akt may be the most likely candidates that mediate the effects of LPA in our studies. Previous reports have indicated the involvement of Akt signalling in LPA and other phospholipid like sphingosine-1-phosphate mediated effects (Chen *et*

al., 2008).

The other signalling pathway investigated involves that mediated by PKC. Previous reports have suggested that a raised activity of PKC induces erythropoietin stimulated erythroid differentiation and PKC plays an important role in the self-renewal and lineage commitment of ESC (Myklebust *et al.*, 2000). Furthermore, DMSO triggered differentiation of erythroleukemia cells is associated with high expression and phosphorylation of PKC α, ε, ζ and δ (Marchisio *et al.*, 2005). All these findings suggest the importance of PKC and led us to investigate PKC in our studies. A range of concentrations of bisindolylmaleimide I (BIM-I) from 1-10μM were selected for the studies (Toullec *et al*., 1991). This compound is a potent competitive inhibitor that competes for the ATP binding site in the PKC protein. It was observed that BIM-1 at 10μM completely abolish the expression of MLC at a nontoxic concentration. The results are consistent with PKC also being critical for the differentiation of P19 cells into a cardiac lineage since the latter was significantly inhibited. PKC is stimulated by an increase in intracellular DAG or Ca^{2+}, which is triggered by phospholipases. Activated Wnt/Ca^{2+} increases intracellular calcium and activates PKC. In addition, Wnt/β-catenin signalling play a key role in DMSO induced cardiomyocytes formation from P19CL6 cells (Nakamura *et al*., 2003). Activated PKC further hampers GSK-3β there by leading to cytosolic accumulation of β -catenin. In the interim, lithium, which mimics Wnt/ β -catenin signalling, has been shown to activate the accumulation of β -catenin there by inhibiting the GSK-3 β phosphorylation (Hedgepeth *et al*., 1997; Klein

et al., 1996). Collectively from above studies we presume that LPA induced differentiation to cardiomyocytes is mediated through multi-signalling molecules which involves PI3K/Akt and PKC pathways. This is possibly due to the expression and activities of $G\alpha_q$ coupled receptors. However, the mechanism illustrated above has to be validated in order to confirm the role of wnt/catenin signalling.

In addition to the above signalling kinases, MAPKs have been reported to be involved in the differentiation of a variety of cells including stem cells (Gaur *et al.* 2010). However, previous reports have indicated that differentiation could be accelerated either by activation or inhibition of these MAPK families (Gaur *et al.* 2010; Li *et al.*, 2002).

Mitogen activated protein kinases are serine/threonine protein kinases which are involved in cellular processes including cell proliferation, cell survival, apoptosis, cellular differentiation process (Bokui *et al.*, 2008; Chen *et al.*, 2008; Zhang *et al.*, 2002). MAPKs are activated by sequential phosphorylation of MAP Kinase Kinase Kinase, MAP Kinase Kinase. MAPK signalling is involved in the activation of pathways by numerous stimuli such as stress, growth factors, cytokines (Moriuchi *et al.*, 2001; Niisato *et al.*, 2007; Park *et al.*, 2009). Inactivation of MAPK is achieved by the dephosphorylation by a MAPK phosphatase (MKPs) (Theodosiou *et al*., 2002). The MAPKs family include extracellular signal regulated kinases (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK). P38 MAPK is mainly present in cytoplasm and nucleus of a cell and is moved to the nucleus following activation (Raingeaud *et al.*, 1995). It plays a crucial role in normal chemotaxis, cell migration, apoptosis and differentiation (Fang

et al., 2000; Lee *et al.*, 2008). p38 MAPKs are activated by G protein activated MAPKKK which in turn activated MAPK through an intermediate MAPKK (MEK3/6). p38 exists in four isoforms namely alpha, beta, gamma and delta. It has been reported that MEK6 phosphorylates all isoforms of p38 MAPK, whereas alpha and beta isoforms of p38MAPK are phosphorylated by MEK3 (Enslen *et al.*, 2000) and this further phosphorylates numerous targets like transcription factors, phospholipase A2, and Tau protein (microtubule associated protein) (Kyriakis, 2000; Yang *et al.*, 1999).

ERK signalling is mainly involved in cellular responses like migration, proliferation, apoptosis and differentiation (Du *et al*., 2010*;* Liu *et al.*, 2009; Sarbassov *et al.*, 1997). Stimuli such as cytokines, stress, and growth factors activates the small GTP- binding protein called Ras, which can translocate and phosphorylate c-Raf proteins to the membrane (Beier *et al.*, 1999). The activated Raf phosphorylates ERK1/2 through MAPKK (MEK1 and 2). Further, this activates ERK 1/2 and subsequently activates kinases like ribosomal S6 kinases (RSKs), MAPK/SAPK-activated kinase (MSK) and MAPK signal-activating kinase 1 (MNK1). ERK1/2 also targets on transcription factors like c- Myc, STAT3, Elk-1, c-Fos.

The c-Jun N-terminal kinase/stress-activated protein kinases (JNK) are mainly involved in the cellular functions like proliferation, and apoptosis (Kostadinova *et al*., 2012; Xia *et al., 1995*). These signals are mediated by growth factors, cytokines and stress related responses (e.g. UV radiation and heat). These activates small GTP binding protein like Ras, Rac and also Cdc42 which then phosphorylates MAPKKKs, MAPKK

specifically MKK4 and MKK7. Further, it phosphorylates and activates JNK, which lead to the stimulation of downstream targets like c-Jun, HSF1, ELK1, p53, transcription factors including STAT3 and NF-kappa B.

It has been reported in various cell types that LPA mediated activities like migration, proliferation and differentiation (as in the case of VSMCs) are mediated through p38 and ERK1/2 (Gschwind *et al.*, 2002; Tangkijvanich *et al.*, 2003). LPA has also been involved in the activation of c-fos, which may be mediated by the ERK1/2 following mitogen, and stress activated kinases (MSKs) activation (Schuck *et al.*, 2003). Hence, our studies were extended to examine the involvement of MAPK specifically looking at the p38 and ERK pathways, which are both downstream to PI3K pathway.

To determine the role of ERK1/2, we used a potent inhibitor PD98059 and selective inhibitor of ERK1/2 that bind to the MEK1 (inactive form) and prevent the activation of ERK1/2. The results obtained showed that PD98059 completely abolishing MLC-1v expression induced by LPA at 5μM concentration. This observation supports reports that ERKs are a principal signalling pathway in the differentiation of stem cells to various lineages (Jaiswal *et al.*, 2000). ERK1/2 activation is crucial for the differentiation for different types of stem cells such as the stimulation of embryonic stem cells to neuronal cells (Li *et al* 2006), and also the differentiation of MSCs to chondrocytes (Lee *et al.*, 2004). ERK1/2 have also been shown to play a definite role in mesoderm development (Kunath *et al., 2007*). The activation of ERK signalling supports differentiation through the activin/smad 2, 3 by the inhibition of

GSK3β and further activates the targets of Wnt such as β-catenin (Singh *et al.* 2012). ERK1/2 signalling also plays an important role in other LPA mediated effects including migration of breast cancer cells, cell proliferation as in the case with human bladder smooth muscle cells (Dixon *et al.*, 1999; Stahle *et al.*, 2003). It has been reported that LPA protects MSCs against hypoxia or serum deprived induced apoptosis through ERK signalling (Chen *et al.*, 2008). Thus, our study showing the involvement of ERK1/2 signalling in the LPA induced differentiation of cardiomyocytes is in agreement with some of the findings above but provides the first report that this pathway mediates LPA-induced differentiation of P19 cells into cardiomyocytes.

In contrast to the ERKs, inhibition of the p38 MAPKs pathway using SB203580 failed to cause any statistically significant change in LPA-induced MLC-1v expression but appear to enhance the latter at 10 μM, a concentration that has been widely used in other studies (Barancik *et al.*, 2001; Kim *et al.*, 2009). This shows selectivity in the actions of the MAPKs and indicates that for P19 cells, differentiation into cardiomyocytes could be inhibited by blocking the ERKs but enhanced by inhibiting the p38 MAPK pathway.

P38 MAPK signalling has been reported to be involve in many biological activities mediated through LPA and related compound like sphingosine-1-phosphate (Li *et al.;* Zhou *et al.*, 2009). The role of the p38 MAPK in normal immune responses, chemotaxis, proliferation, apoptosis and differentiation has also been well documented

(Bokui *et al.*, 2008; Ohashi *et al.*, 2000; Zetser *et al.*, 1999; Zhang *et al.*, 2002). In addition, activation of p38 has been implicated in the induction of hESCs differentiation to cardiomyocytes (Matsuura *et al.*, 2009; Xu *et al.*, 2008) but at least one report has suggested that inhibition of p38 MAPK in the same cells stimulates their differentiation into cardiomyocytes (Gaur *et al.* 2010). This latter finding is consistent with our observations of enhanced MLC-1v expression following treatment of P19 cells with SB203580 even though this effect was not found to be statistically significant in our studies. SB203580 has been reported to inhibit specifically the α and β p38 MAPK isoforms. The fact that this compound enhanced MLC-1v expression, albeit marginally, suggest that in P19 cells, enhanced differentiation might be mediated through p38α and β. Further studies, for instance transfecting specific p38 isoforms into P19 cells or using specific inhibitors of β and γ that have recently been made commercially available may be worth doing. Additionally, n value of the experiments could be enhanced to show statistical significance of the trends currently observed.

Rho associated coiled-coil forming protein kinase (ROCK or ROK) belongs to the serine-threonine kinase family, and is composed of ROCK-1 and ROCK-2 in mammals and have an amino-terminal and significantly different carboxy-terminal domain (Nakagawa *et al.*, 1996; Riento *et al.*, 2003). RhoA GTPase which is the main upstream target of ROCK binds to the coiled-coil region of ROCK and stimulates the catalytic action (Bishop *et al.*, 2000). Stimulated ROCK is reported to be involved in actin myosin contraction and stress formation by increased myosin light chain phosphorylation, retraction of neurite and Schwann cells, cytokinesis, mitosis and

induced neurite formation on inhibition with Y-27632 (Hirose *et al.*, 1998). LPA has been reported to cause retraction of neurites and Shwann cells through LPA receptors (Fukushima *et al.*, 1998; Ishii *et al.*, 2000). Cell differentiation may also be regulated by ROCK which has been shown to participate in the maturation and differentiation of skeletal muscles (Pelosi *et al.*, 2007). There is however very little evidence showing the involvement of ROCK in the differentiation of stem cells. It has been reported that the ROCK pathway helps ES cells to remain as undifferentiated cells and inhibit their neural differentiation caused by high seeding density (Chang *et al.* 2010). Previous studies have indicated a role for ROCK in cell differentiation as mentioned in the introduction section. This pathway is also essential for myogenesis and differentiation of keratinocytes (Castellani *et al.*, 2006; McMullan *et al.*, 2003). In bone marrow-derived mesenchymal stem cells (BM-MSC) the ROCK pathway has been shown to induce differentiation and to potentiate the transdifferentiation of the cells (Pacary *et al.*, 2007). However, ROCK has been shown to be involved in the inhibitory action of LPA in the neuronal differentiation from neuronalprogenitor cells derived from hESC (Dottori *et al.*, 2008).

We examined the effect of ROCK in the LPA induced differentiation of P19 cells into cardiomyocytes using the ROCK specific inhibitor Y27632. Our data suggested that the pre-treatment of P19 cells with a ROCK inhibitor at the EB formation stage completely blocked the expression of MLC-1v and thus differentiation. This strongly suggests that activation of the ROCK pathway may also be crucial for LPA induced differentiation. These effects were seen with concentrations of the inhibitor which have been

widely used in other studies (Chang *et al.* 2010; Dottori *et al.*, 2008). Interestingly, in addition to the inhibitory action of Y27632 on the LPA induced differentiation, Y27632 (5μM and 10μM) by itself showed some induction of differentiation of P19 cells into cardiomyocytes. This is similar to observations in a recent report which demonstrated the differentiation of human adipose tissue-derived stem cells (hADSCs) could be induced in a dose dependent manner following treatment with Y27632.

Most of the biological effects are produced by means of crosstalk between various signalling cascades. Therefore, to identify the sequence of the signalling cascades, studies were conducted on the phosphorylation of selected proteins. The key pathways that participate in the differentiation of stem cells into cardiomyocytes are MAPKs and PI3K/Akt(PKB) pathways, however the sequence of these signalling mechanisms vary depending on various factors such as the type of cells and inducers used. Consequently, it is essential to identify the signalling cascade that facilitates LPA induced differentiation in P19 cells.

Akt activation is mediated by PDK1 and PDK2 successively phosphorylating at threonine 308 and serine 473 respectively (Alessi *et al*., 1996; Alessi *et al*., 1997). PI3K/Akt plays a key role in cellular proliferation and survival that are mediated through downstream effectors like Wnt signalling and nuclear factor Kappa-B (NF-κB). In addition, it is shown to inhibit Glycogen Synthase Kinase-3β (GSK-3β) a negative regulator of Wnt pathway. Akt plays multiple roles in maintaining the vital functions of cardiomyocytes. Over expression of Akt has also been shown to induce the expression

of vascular endothelial growth factor and angiopoitin-2 thus regulating myocardial capillary growth (Mora *et al*., 2003; Shiojima *et al*., 2005). Most importantly, during cardiomyocytes differentiation it stimulates cardiomyocytes cells, and cardiac progenitor cells proliferation/survival (Beltrami *et al*., 2003; Gude *et al*., 2006). Furthermore, Akt has been shown to play a crucial role in LPA mediated effects in cellular systems. The cell survival in ovarian cancer cells by LPA and anti-apoptosis mediated by C. difficile toxin via LPA are mediating through Akt activation. Thus to extend our studies on Akt, experiments were carried out to look at the phosphorylation of Akt at serine 473. The data obtained showed that Akt was phosphorylated by 5μM LPA, starting from 5 minutes. The peak of activation was found at a time point of 15 minutes, and was sustained until 30 minutes, declining thereafter. However, studies conducted to look at the involvement of the LPAR1/3 and LPAR4 receptors, with Ki16425 and suramin revealed no changes to the LPA induced activation of Akt at Ser-473. In addition, selective signalling molecule inhibitors of p38 MAPK (SB203580 10 μM), ERK1/2 (PD95080 5 μM), PI3K (LY294002 5 μM), PKC (BIM 10 μM), and ROCK (Y27632 10 μM) caused no significant change in the expression and activation of Akt at Ser-473 residue. These data suggest that the phosphorylation/activation of Akt at least on Ser-473 may not be directly regulated by the pathways highlighted above. However, Akt inhibitor XIII that selectively inhibits the activity of Akt1/Akt2 completely abolished the expression of MLC-1v strongly suggesting a role for Akt in the differentiation process.

Reports have suggested induction of differentiation of H9C2 cells into cardiomyocytes

with lithium, an inducer of Wnt signalling and inhibitor of glycogen synthase kinase-3β. The above study has shown the differentiation was achieved through PI3K signalling however, was independent to Akt pathway (Kashour *et al*., 2003). Comparable to our studies, it might be that differentiation of P19 cells into cardiomyocytes is mediated through PI3K but in an Akt-Ser473 independent manner. The activation of Akt occurs via phosphoinositide-dependent kinase1 (PDK1) which phosphorylates T-308 and subsequently auto phosphorylates or by the action of PDK2 which phosphorylates Ser-473. In addition, Ser-473 phosphorylation could also be achieved through mammalian target of rapamycin complex 2 (mTORC2). LY294002, a selective inhibitor of PI3K, acts at the ATP binding site of the protein. Akt inhibitor XIII is a selective inhibitor of Akt which acts on plecktrin homology (PH) domain and inhibits activity of Akt1/Akt2. Our data from the inhibitory studies have showed that PI3K and Akt completely blocked the expression of the cardiac marker MLC-1v; however, neither PI3K nor Akt inhibitors could inhibit LPA induced activation of Akt at the Ser-473 residue. Thus, Akt and PI3K may be signalling independently in P19 cells with regards to LPA induced differentiation.

LPA has been shown to induce the proliferation of pre-adipocytes, which was mediated by dual pathways. The report indicated that the one mediated through GPCR was through PTX-insensitive mechanism in association with PI3K but Akt independent (Holmstrom *et al*., 2010). Interestingly, Akt activation at Thr-308 and Ser-473 additionally occurs by the signalling molecule IKBKE, known as IKKε (atypical IκB kinase ε) and IKKi. This report suggests that the phosphorylation via PDK1and PDK2 is

dependent on the pleckstrin homology (PH) domain, which normally occurs either through PI3K or by the mammalian target of rapamycin complex 2 (mTORC2) (Guo *et al*., 2011). In addition, there are similar reports that prove the activation of Akt through atypical IκB kinase ε and TANK-binding kinase 1 (IKKε/TBK1) (Xie *et al*., 2011). In both these scenario, it is observed that the activity of Akt is PI3K independent. Hence, in LPA induced differentiation of P19 cells, the activation of Akt may occur through a PI3K independent pathway.

PI3K/Akt signalling has many downstream binding effectors including Raf1, SMAD3 and reports suggest evidence of cross talk between the PI3K and MEK pathways (Conery *et al*., 2004; Remy *et al*., 2004; Reusch *et al*., 2001; Runyan *et al*., 2004). Experiments conducted with SB203580, a potent P38 inhibitor, did not however show any profound effect on LPA-induced differentiation of P19 cells, indicating that this kinase may either not be involved in mediating the effects of LPA or may not be fully activated by the latter. Thus to confirm whether P38 is in fact activated in these cells further studies were carried out examining the its phosphorylation in response to 5μM LPA. The results show clearly that p38 MAPK was activated in response to LPA and this was observed from 5 minutes, peaking at 10 minutes but declining after 30 minutes. Further to the above experiment, studies were conducted to look at the effect of inhibitors of LPA receptors and signalling molecules on the activation of p38 MAPK. Our data indicated that the p38 MAPK activation was completely abolished by LPA1/3 and LPA4 receptor antagonists, Ki16425 (10 μM) and suramin (0.05mg/ml).

Therefore, it proves the involvement of LPA1/3 and LPA4 receptors in the activation of p38 MAPK. In addition, LY294002 5 μM and Y27632 10 μM abolished the LPA induced activation of p38 MAPK while SB203580, BIM, Akt inhibitor XIII and PD95080 caused no significant changes to the phosphorylation of p38 MAPK. Hence, this suggests that the PI3K and ROCK signalling pathways through downstream targets MEK3/6 could be inducing the activation of p38 MAPK. This in turn may act to regulate MLC1v expression but only marginally.

ERKs are generally activated by ligands that bind to G protein coupled receptors, cytokines, growth factors, carcinogens. The two forms of ERKs, ERK1 and ERK2, are activated through Gα and Gβγ. The main effectors linked to these G proteins are PI3K and PKC. The activated PKC promote the activation of Ras, Raf and the MEK pathways (Ginnan *et al*., 2006; Osmond *et al*., 2005). MEK1 and 2 are phosphorylated by activated c-Ras and it in turn further phosphorylates and activates the downstream targets, ERK1 and ERK2 (Burgering *et al*.,1995). Studies conducted with 5μM LPA showed that it could phosphorylate and activate ERK1/2 at 3 minutes but declined after 5 minutes. This activation was completely abolished by LPAR1/3 and LPAR4 receptor antagonists, Ki16425 and suramin. This again demonstrates the involvement of LPAR1/3 and LPAR4 receptors in the activation of ERK1/2. In addition, all the signalling inhibitors mentioned above for PI3K, PKC, ROCK, Akt and ERK1/2 showed complete inhibition of activation of phospho-p42/42. SB203580 was without effect. Hence, it is very clear that ERK1/2 plays a central role in the differentiation process of P19 stem cells into cardiomyocytes induced by LPA and may be the point of

convergence of multiple signalling events originating from LPAR1/3 and 4 receptors. The only signalling event that could occur through $G\alpha_{12/13}$ is the Rho/ROCK pathway and our data has shown that ROCK completely abolished the activation of ERK1/2 induced by 5μM LPA. The implication of ROCK is consistent with a previous study showing that LPA mediated activation of Na/H exchangere3 through the LPA5 receptor was mediated via ROCK/MEK/ERK pathway in intestinal epithelial cells (Yoo *et al*., 2011). ROCK via $G\alpha_{12/13}$ has also been shown to abolish the activation of ERK. It is interesting to note that the ROCK pathway has been indicated to mediate cardiac myocyte hypertrophy mediated through the activation of p-ERK/GATA-4 (Yanazume *et al*., 2002). Similarly, in glioblastoma cell migration and proliferation, ROCK plays a crucial role signalling the events through ERK activation (Zohrabian *et al*., 2009).

PD98059 an ERK1/2 inhibitor abolished the activation of p-ERK induced by 5μM LPA. PD98059 is a selective inhibitor of p42/44 MAPK that bind to the MEK1 (inactive) and prevent the activation of ERK1/2. This could logically explain its inhibitory action on ERK1/2 phosphorylation. As already stated, inhibitors for PI3K, PKC, and Akt also blocked ERK1/2 activation suggesting cross talk between these pathways. It is likely however that signalling through PI3K and PKC may be distinct from Akt since inhibition of PI3K did not block Akt phosphorylation. A distinct role for Akt is consistent with a previous report in which glycogen-like peptide 2 (GLP2) stimulated the differentiation to neuronal cells via Akt/mTOR/ERK1/2 and was confirmed by the expression of vasoactive intestinal polypeptide (VIP) (de Heuvel *et al*., 2010). Similarly, LPA could induce differentiation of P19 cells into cardiomyocytes, through Akt/mTOR/ERK1/2

pathway. In contrast, PI3K/PKC could signal through Ras/B-Raf/ERK linked to Gβγ (Hawes *et al*., 1996).

In addition to the action of $G\alpha_{\beta\gamma}$, $G\alpha_q$ can stimulate PLC and PKC, which can further activate the MEK and ERK pathways. In nerve growth factor induced differentiation of PC12 cells, it was observed that this was mediated through the activation of atypical PKC through Src or Ras or PI3K mediated pathways. The activated PKC lead to the induction of IKK and resulted in regulating the activation of NF-κB. In addition, MEKs are downstream targets of PKC and in this study; the induction of differentiation was mediated through the involvement of MAPKs (Wooten *et al*., 2000).

The close links between PI3K and PKC are well demonstrated in vasculogenesis which is also dependent on the p110α and p1110δ subunits of PI3K that interacts with the downstream targets of PKC catalytic subunits, α/βII and PKCδ in mouse ES cells (Bekhite *et al*., 2011). Similarly, in IGF-I induced HL60 cells differentiation into macrophages the signalling events are reported to be through PI3K/PKCζ (Liu *et al*., 1998).

Chapter 5

Summary and conclusions

The objective of this thesis was to establish the effect of LPA in the differentiation of P19 stem cells into cardiomyocytes and to investigate the signalling mechanisms involved. The data obtained confirm that LPA can induce P19 stem cells to differentiate into cardiomyocytes and exerts this effect at known physiological concentrations, which can exist *in vivo.* In addition, the effects are linked to well characterised LPA receptors (LPAR1/3 and 4) which are in turn coupled to downstream signalling pathways of which those involving the ROCK, PI3K, PKC and/or Akt may be critical, and may converge on ERK1/2. Inhibition of any of these pathways has the potential to suppress differentiation. In contrast, signalling leading to P38 activation may potentially suppress the process but this needs further clarification. Cross inhibition studies revealed a complex network of cross talk between the pathways identified and this is summarised in Figure 49 below for clarification.

Future Work

The outcome from this thesis has generated further interesting areas that needs to be addressed and these are summarised below.

1. Our studies have confirmed the involvement of LPA1/3/4 receptors in the induction of differentiation with pharmacological inhibitors. However, we could not investigate the role of LPAR2 because of a lack of commercially available inhibitor for this receptor subtype. It is however important to establish this especially as PCR analysis of receptor expression profile showed that LPAR2 mRNA increased 2.5 fold when cells where treated with 5μM LPA. In the absence of pharmacological inhibitors, the studies could be performed using siRNA transfection to deplete LPAR2. .

2. Studies involving Akt seem to suggest that this pathway may be activated independently of PI3K. Thus, experiments should be carried out to determine the PI3K independent activation of Akt.

3. This thesis has uncovered the role of the MAPKs p38 MAPK and ERK but did not investigate JNK. The latter is a critical member of the MAPKs, which could regulate differentiation through AP1 signalling.

4. In addition to AP-1, further studies could be carried out to investigate the role of other transcription factors including those specific to a cardiac lineage commitment.

5. The data generated for the p38 MAPK suggest only a marginal role. This is an interesting observation which needs further investigation. In the first instance, it

would be important to confirm the current trends by increasing the n value to improve on statistical significance. Following this, it would be important to confirm whether LPA activates all different isoforms of P38 and whether these mediate different effects. This suggestion is based on the fact that SB203580 selectively blocks the α and β isoforms and we therefore currently have no data to suggest whether γ and δ isoforms play any role in mediating the effects of LPA or indeed whether the effects may be different to those seen with α and β. These studies could be carried out using transfection of cDNA to over express each isoform and determine whether this alters MLC-1v expression. Additionally siRNA could be exploited to deplete each isoform but prior to this it would be essential to demonstrate whether P19 cells express all 4 isoforms of P38 using western blotting.

6. Suramin, an LPAR4 non-selective antagonist at very low concentration showed induction of differentiation in the absence of LPA. This was also true for other inhibitors used even though they each blocked LPA induced differentiation. It would be worth investigating these unexpected effects and establish the cellular mechanisms associated with each drug.

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Appendix

Appendix 1. Cell Line

P19 Embryonal Carcinoma Stem Cell Line	
Cell Line Name	P19
Species	Mouse
Cell Line Description	The P19 cell line was derived from an embryonal carcinoma induced in a C3H/He strain mouse. The pluripotent P19 cells can be induced to differentiate into neuronal and glial cells in the presence of retinoic acid. Aggregates of P19 cells differentiate into cardiac and skeletal muscle in the presence of dimethyl sulfoxide (DMSO).
Tissue	Embry
Growth Mode	Adherent
Culture Medium	Alpha MEM + 2mM Glutamine + 10 % FBS
Subculture Routine	Split cells at 70-80% confluency using trypsin/EDTA.
Suppilier	European Collection of Cell Cultures (ECACC) http://www.hpacultures.org.uk/collections/ecacc.jsp
Catelogue Number	95102107

Appendix 2. Cell culture reagents

REAGENTS	DESCRIPTION	SUPPLIER	STORAGE
Minimal Essential Medium (MEM) Alpha Medium (α-MEM, no nucleosides	Contains non-essential amino acids, sodium pyruvate, lipoic acid, B_{12}, biotin, and ascorbic	Invitrogen, UK http://www.invitrogen.com (Product code:22561021)	Fridge 4°C
Foetal Bovine Serum	Heat inactivated	Invitrogen, UK http://www.invitrogen.com (Product code:10500064)	Stock: -20°C
Penicillin-Streptomycin (10,000 U/ml) Contains 10,000 units/ ml of penicillin and 10,000 µg/ ml of streptomycin.	Prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria.	Invitrogen, UK http://www.invitrogen.com (Product code: 15140122)	Stock: -20°C
0.5% Trypsin-EDTA (10X)-no phenol red	Contains 5.0 g/L of trypsin, 2.0 g/L of EDTA.4Na, 8.5 g/L of NaCl.	Invitrogen, UK http://www.invitrogen.com (Product code: 15400054)	Stock: -20°C Diluted aliquots of stock Solution: Fridge 4°C,

Appendix 3. Oleoyl-L-α-lysophosphatidic acid sodium salt

Synonym	1-Oleoyl-*sn*-glycerol 3-phosphate sodium salt, 3-*sn*-Lysophosphatidic acid, 1-oleoyl sodium salt, LPA sodium salt
Description	Endogenous agonist for LPA_1 and LPA_2 receptors. LPA does not induce angiogenesis, but has effects on endothelial cell physiology that are similar to those of sphingosine 1-phosphate. Induces cell migration of cancer and non-cancer cells.
Empirical Formula	$C_{21}H_{41}O_7P$
CAS Number	22556-62-3
Solubility	Solution has been achieved in 1% phosphate buffered saline (PBS), pH 7.2containing 0.1% Fatty acids free BSA
Storage	−20°C
Molecular Weight	436.5
Stock concentration	5mM
Supplier	Sigma (Sigma-Aldrich), UK http://www.sigmaaldrich.com
Catalogue number	L7260

Appendix 4. Monoclonal Anti-Cardiac Myosin Light Chain I Antibody

Antibody Name	Anti-Myosin light chain 3 antibody [MLM527]
Antibody Type	Primary antibodies
Description	Mouse monoclonal [MLM527] to Cardiac Myosin light chain
Immunogen	Human ventricle myosin light chain I
Specificity	Antibody reacts with ventricle myosin light chain I (MLC-1v)
Band size	Predicted band size : 22 kDa Observed band size : 25 kDa
Storage	Aliquot and store at -20°C or -80°
Dilution used	1:500 in blocking buffer
Secondary antibody Required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Abcam, UK http://www.abcam.com
Catalogue number	ab680

Appendix 5. Mouse Phospho-Akt (Ser473) (587F11) monoclonal antibody

Antibody Name	Mouse Phospho-Akt (Ser473) (587F11) monoclonal antibody
Antibody Type	Primary antibody
Description	Mouse monoclonal antibody to Akt phosphorylated at serine 473 (Ser473)
Immunogen	Synthetic phospho-peptide (KLH-coupled) corresponding to residues around Ser47 of mouse Akt.
Specificity	Phospho-Akt (Ser473) (587F11) Mouse mAb detects endogenous levels of Akt only when phosphorylated at serine 473. This antibody does not detect Akt phosphorylated at other sites or related kinases such as PKC and p70 S6 kinase.
Band size	60 kDa
Storage	Store at –20°C. Do not aliquot the antibody.
Dilution used	1:1000in blocking buffer
Secondary antibody Required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Cell Signallng Technology http://www.cellsignal.com
Catalogue number	4051

Appendix 6. Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb

Antibody Name	Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb
Antibody Type	Primary antibody
Description	Mouse monoclonal antibody to p38 MAP kinase (MAPK) phosphorylation at Thr180 and Tyr182.
Immunogen	Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues around Thr180/Tyr182 of human p38 MAPK.
Specificity	Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb detects p38 MAPK only when activated by dual phosphorylation at Thr180 and Tyr182. This antibody does not significantly cross-react with the corresponding phosphorylated forms of either p44/42 MAPK (Erk1/2) or SAPK/JNK. It does not detect nonphosphorylated p38 MAPK.
Band size	43 kDa
Storage	Store at –20°C. Do not aliquot the antibody.
Dilution used	1:2000in blocking buffer
Secondary antibody Required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Cell Signallng Technology http://www.cellsignal.com
Catalogue number	9216

Appendix 7. Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb

Antibody Name	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb
Antibody Type	Primary antibody
Description	Mouse monoclonal antibody to p38 MAP kinase (MAPK) phosphorylation at Thr180 and Tyr182.
Immunogen	Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAP kinase.
Specificity	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse mAb detects endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2), and singly phosphorylated at Tyr204. This antibody does not cross-react with the corresponding phosphorylated residues of either SAPK/JNK or p38 MAP kinase.
Band size	42, 44 kDa
Storage	Store at –20°C. Do not aliquot the antibody.
Dilution used	1:2000in blocking buffer
Secondary antibody required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Cell Signallng Technology http://www.cellsignal.com
Catalogue number	9106

Appendix 8. Monoclonal Anti-β-Actin antibody conjugated to horseradish peroxidise (HRP)

Antibody Name	Monoclonal Anti-β-Actin antibody conjugated to horseradish peroxidise (HRP)
Antibody Type	Primary antibody, isotype IgG1
Description	A purified fraction of mouse monoclonal anti-b-actin isolated from ascites fluid of the AC-15 and conjugated horseradish peroxidise (HRP)
Immunogen	slightly modified β-cytoplasmic actin N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys, conjugated to KLH.
Specificity	Antibody reacts specifically with β-actin found many tissues and species
Band size	42 kDa
Storage	Aliquot stored at -20°C or -80°C
Dilution used	1:10000 in blocking buffer
Secondary antibody Required	Not required
Supplier	Sigma (Sigma-Aldrich), UK http://www.sigmaaldrich.com
Catelogue numebr	A3854
Description	Suramin is a polysulfonated naphthylurea anticancer agent that inhibits tumor cell proliferation. It uncouples G-proteins from receptors. It is an broad spectrum antagonist at P2X and P2Y purinergic receptors.
Empirical Formula	C51H34N6Na6O23S6

Appendix 9. Suramin

Description	Suramin is a polysulfonated naphthylurea anticancer agent that inhibits tumor cell proliferation. It uncouples G-proteins from receptors. It is an broad spectrum antagonist at P2X and P2Y purinergic receptors.
Empirical Formula	$C_{51}H_{34}N_6Na_6O_{23}S_6$
CAS Number	129-46-4
Solubility	H_2O: >10 mg/ml
Storage	Store at room temperature protected from light.
Molecular Weight	1429.17
Stock concentration	0.5mg/ml
Supplier	Sigma (Sigma-Aldrich), UK http://www.sigmaaldrich.com
Catalogue number	S 2671

Appendix 10. SB203580

Synonym	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole
Description	A highly specific, cell-permeable inhibitor of p38 kinase (IC50 = 34 nM in vitro, 600 nM in cells).
Empirical Formula	C21H16N3OSF
CAS Number	152121-47-6
solubility	DMSO
Storage	Protect from light Solid: -20°C Stock solution Aliquots: -20°C
Molecular Weight	377.4
Stock concentration	10mM (1mg/265µl of DMSO)
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue number	559389

Appendix 11. LY294002

Synonym	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
Description	A phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor that acts on the ATP-binding site of the enzyme
Empirical Formula	C19H17NO3
CAS Number	154447-36-6
Solubility	DMSO
Storage	Protect from light Solid: -20°C Stock solution Aliquots: -20°C
Molecular Weight	307.4
Stock concentration	20mM
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue number	440202

Appendix 12. Bisindolylmaleimide I

Synonym	2-[1-(3-Dimethylaminopropyl)-1Hindol- 3-yl]-3-(1H-indol-3-yl)-maleimide
Description	A reversible protein kinase C (PKC) inhibitor that acts as a competitive inhibitor for the ATP-binding site
Empirical Formula	C25H24N4O2
CAS Number	133052-90-1
Solubility	DMSO
Storage	Protect from light Solid: -20°C Stock solution Aliquots: -20°C
Molecular Weight	412.5
Stock concentration	2.4mM
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue number	203290

Appendix 13.Y-27632

Synonym	Rho Kinase Inhibitor VI
Description	A highly potent, cell-permeable, selective inhibitor of Rho-associated protein kinase (Ki = 140 nM for p160 ROCK (ROCK-I). Also inhibits ROCK-II with almost equal potency. Inhibition is achieved by competing with ATP for binding to the catalytic site.
Empirical Formula	C14H21N3O · 2HCl · H2O
solubility	500 µg/296 µl solution of Y-27632 in H2O.
Storage	-20°C Protect from Light
Molecular Weight	338.3
Stock concentration	A 5 mM (500 µg/296 µl) solution of Y-27632 in H2O.
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue number	688001

Appendix 14. Pertussis Toxin

Description	Pertussis toxin uncouples G proteins from receptors by ADP ribosylating a cysteine residue near the carboxyl terminus of the α subunit.
Empirical Formula	C14H21N3O · 2HCl · H2O
solubility	50 micro gram/ 500 micro litre in sterile, distilled water
Storage	Store, as supplied, at 2°C to 8°C. Upon reconstitution, apportion into working aliquots and store at 2°C to 8°C. Suspensions are stable at 2°C to 8°C for up to six months. DO NOT FREEZE.
Molecular Weight	~117
Stock concentration	50 micro gram/ 500 micro litre in sterile, distilled water
Supplier	Invitrogen,UK https://www.lifetechnologies.com
Catalogue number	PHZ1174

Appendix 15. Akt inhibitor VIII isoenzyme-selective Akti-1/2

Synonym	1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one, Akti-1/2
Description	A cell-permeable and reversible quinoxaline compound that potently and selectively inhibits Akt1/Akt2 activity (IC50 = 58 nM, 210 nM, and 2.12 µM for Akt1, Akt2, and Akt3, respectively, in in vitro kinase assays). The inhibition appears to be pleckstrin homology (PH) domain-dependent.
Empirical Formula	$C_{34}H_{29}N_7O$
Solubility	A 10 mM (1 mg/181 µl) solution of Akt Inhibitor VIII
Storage	+2°C to +8°C
Molecular Weight	551.6
Stock concentration	10 mM
Supplier	EMD Millipore http://www.emdmillipore.com
Catalogue number	124018