SIGNAL TRANSDUCTION MECHANISMS FOR STEM CELL DIFFERENTIATION INTO CARDIOMYOCYTES

By

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ABSTRACT

Cardiovascular diseases are among the leading causes of death worldwide and particularly in the developed World. The search for new therapeutic approaches for improving the functions of the damaged heart is therefore a critical endeavour. Myocardial infarction, which can lead to heart failure, is associated with irreversible loss of functional cardiomyocytes. The loss of cardiomyocytes poses a major difficulty for treating the damaged heart since terminally differentiated cardiomyocytes have very limited regeneration potential. Currently, the only effective treatment for severe heart failure is heart transplantation but this option is limited by the acute shortage of donor hearts. The high incidence of heart diseases and the scarcity donor hearts underline the urgent need to find alternative therapeutic approaches for treating cardiovascular diseases.

Pluripotent embryonic stem (ES) cells can differentiate into functional cardiomyocytes. Therefore the engraftment of ES cell-derived functional cardiomyocytes or cardiac progenitor cells into the damaged heart to regenerate healthy myocardial tissues may be used to treat damaged hearts. Stem cell-based therapy therefore holds a great potential as a very attractive alternative to heart transplant for treating heart failure and other cardiovascular diseases. A major obstacle to the realisation of stem cell-based therapy is the lack of donor cells and this in turn is due to the fact that, currently, the molecular mechanisms or the regulatory signal transduction mechanisms that are responsible for mediating ES cell differentiation into cardiomyocytes are not well

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understood. Overcoming this huge scientific challenge is absolutely necessary before the use of stem cell-derived cardiomyocytes to treat the damaged heart can become a reality.

Therefore the aim of this thesis was to investigate the signal transduction pathways that are involved in the differentiation of stem cells into cardiomyocytes. The first objective was the establishment and use of cardiomyocyte differentiation models using H9c2 cells and P19 stem cells to accomplish the specific objectives of the thesis. The specific objectives of the thesis were, the investigation of the roles of (i) nitric oxide (ii) protein kinase C (PKC), (iii) p38 mitogen-activated protein kinase (p38 MAPK) (vi) phosphoinositide 3-kinase (PI3K) and (vi) nuclear factor-kappa B (NF-kB) signalling pathways in the differentiation of stem cells to cardiomyocytes and, more importantly, to identify where possible any points of convergence and potential cross-talk between pathways that may be critical for differentiation to occur.

P19 cells were routinely cultured in alpha minimal essential medium (α-MEM) supplemented with 100 units/ml penicillin /100 µg/ml streptomycin and 10% foetal bovine serum (FBS). P19 cell differentiation was initiated by culturing the cells in microbiological plates in medium containing 0.8 % DMSO to form embryoid bodies (EB). This was followed by transfer of EBs to cell culture grade dishes after four days.
H9c2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS. Differentiation was initiated by incubating the cells in medium containing 1% FBS.

In both models, when drugs were employed, they were added to cells for one hour prior to initiating differentiation. Cell monolayers were monitored daily over a period of 12 or 14 days. H9c2 cells were monitored for morphological changes and P19 cells were monitored for beating cardiomyocytes. Lysates were generated in parallel for western blot analysis of changes in cardiac myosin heavy chain (MHC), ventricular myosin chain light chain 1(MLC-1v) or troponin I (cTnI) using specific monoclonal antibodies.

H9c2 cells cultured in 1% serum underwent differentiation as shown by the time-dependent formation of myotubes, accompanied by a parallel increase in expression of both MHC and MLC-1v. These changes were however not apparent until 4 to 6 days after growth arrest and increased with time, reaching a peak at day 12 to 14. P19 stem cells cultured in DMSO containing medium differentiated as shown by the time-dependent appearance of beating cardiomyocytes and this was accompanied by the expression of cTnI.

The differentiation of both P19 stem cells and H9c2 into cardiomyocytes was blocked by the PI3K inhibitor LY294002, PKC inhibitor BIM-I and the p38 MAPK inhibitor SB2035800. However when LY294002, BIM-I or SB2035800 were added after the initiation of DMSO-induced P19 stem cell differentiation, each inhibitor failed to block the cell differentiation into beating cardiomyocytes. The NF-kB activation inhibitor, CAPE, blocked H9c2 cell differentiation into cardiomyocytes. Fast nitric oxide releasing donors (SIN-1 and NOC-5) markedly delayed the onset of differentiation of H9c2 cells into cardiomyocytes while slow nitric oxide releasing donors (SNAP and NOC-18) were less effective in delaying the onset of differentiation or long term differentiation of H9c2

cells into cardiomyocytes. Akt (protein kinase B) is the key downstream target of PI3K. Our cross-talk data also showed that PKC inhibition and p38 MAPK inhibition respectively enhanced and reduced the activation of Akt, as determined by the phosphorylation of Akt at serine residue 473.

In conclusion, PKC, PI3K, p38 MAPK and NF-kB are relevant for the differentiation of stem cells into cardiomyocytes. Our data also show that the PKC, PI3K and p38 MAPK signalling pathways are activated as very early events during the differentiation of stem cells into cardiomyocytes. Our data also suggest that PKC may negatively regulate Akt activation while p38 MAPK inhibition inhibits Akt activation. Our fast NO releasing donor data suggest that nitric oxide may negatively regulate H9c2 cell differentiation.

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ABBREVIATIONS

AKT	Protein kinase B
ASK	apoptosis stimulating kinase
APS	Ammonium persulfate
ATF2	Activating Transcription factor 2
ATRA	All-*trans*retinoic acid
BCA	Bicinchoninic acid
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
BSA	Bovine serum albumin
CO$_2$	Carbon dioxide
CRD	Cysteine-rich domain
DAG	1, 2-diacyglycerol
DEA/NO	2-(N, N-diethylamino)-diazenolate-2-oxide
DLK	Dual leucine zipper bearing kinase.
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EB	Embryoid body
ECL	Enhanced chemiluminescence
ELK-1	E-26-like protein 1
ERK	Extracellular signal-regulated kinase

ES cell	Embryonic Stem Cell
FBS	Foetal Bovine Serum
Grb2	Growth factor receptor-bound protein 2
Gab1	Grb2-associated binding protein 1
FOG	Friend Of GATA
ICM	Inner cell mass
IP3	Inositol (1,4,5)tris-phosphate
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
LIF	Leukaemia Inhibitory Factor
LIFRβ	Leukaemia Inhibitory Factor Receptor
MADS	An acronym for MCM1, Agamous, Deficiens SRF
MAPK	Mitogen activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MAPKKKK	MAP kinase kinase kinase kinase
MEK	Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase
MCM1	Minichromosomal Maintenance protein 1
MSC	Mesenchymal stem cells
MEF	Mouse Embryonic Fibroblast
MEF2	Myocyte Enhancer Factor 2
MEF2A	Myocyte Enhancer Factor 2 A

MEF2C   Myocyte Enhancer Factor 2 C

MEF2D   Myocyte Enhancer Factor 2 D

MEKK 1/4   Mitogen ERK kinase kinase, 1/4

MLK3   Mixed lineage kinase 3

MNK1   MAPK signal-activating kinase 1

MSK   MAPK/SAPK-activated kinase

MTT   (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyl-tetrazolium bromide

Nanog   Tir *nan Og*, a mythical celtic land of the ever young

NF-AT   Nuclear Factor of Activated T cells

NF-kB   Nuclear Factor -kappa B

NO   Nitric oxide

NOC-18   2, 2′-(Hydroxynitrosohydrazino)bis-ethanamine

NOC-5   3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine

p38 MAPK   p38 Mitogen activated protein kinase

PMA   Phorbol 12-myristate-13-acetate

PAPA/NO   (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate

PBS   Phosphate buffered saline

PDK1   3-Phosphoinositide-Dependent Protein Kinase 1

PDK2   3-Phosphoinositide-Dependent Protein Kinase 2

PI3K   Phosphatidylinositol-3 Phosphate Kinase

PIP2   Phosphatidylinositol (1, 4) bis-phosphate

PKB   Protein Kinase B

PKC	Protein Kinase C
PVDF	Polyvinylidene fluoride
RPM	Revolutions per minutes
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH-2	Src Homology Domain 2
SHP2	Sh-2-Containing Tyrosine Phosphatase
SIN-1	3-Morpholinosydnonimine
SMAD	Similar To Mothers Against Decapentaplegic
SNAP	(±)-S-Nitroso-N-acetylpenicillamine
SOCS	Suppressors Of Cytokine Signalling
SOS	Son Of Sevenless
SSEA	Stage specific embryonic antigen
STAT	Signal Transducers And Activators Of Transcription
TAK	transforming growth factor-β-activated kinase
TRA-1-60	
TRA-1-81	Battle of *Tra*falgar (i.e. named after the Battle of *Tra*falgar)
TBS	Tris buffered saline
TEMED	N,N,N′,N′-tetramethylethylenediamine

CHAPTER 1.0

INTRODUCTION

1   INTRODUCTION

1.1 Definition of stem cells

A stem cell is an unspecialised or undifferentiated cell from the embryo (i.e. embryonic stem cell) or from adult or foetus tissue (i.e. adult stem cell). An embryonic stem cell has two important fundamental properties: unlimited ability to divide and generate new stem cells (i.e. self-renewal) without going into senescence and under appropriate physiological or experimental conditions, can specialise or differentiate into other cell types (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998). Adult stem cells, like ES cells, are capable of self-renewal and can differentiate into various cell types. However, unlike ES cells, adult stem cells (at most) are only multipotent (i.e. are not pluripotent) and also have limited self-renewal ability. Self-renewal and pluripotency (multipotency for adult stem cells) allow stem cells to serve as building blocks in developmental processes in human and animals. In the adult human or animal, stem cells also maintain tissue homeostasis by providing a continuous supply of new cell types to replace highly differentiated cells that have become damaged or simply turn over as part of the normal physiology of organs or tissues regeneration. Embryonic stem cells are the only non-transformed (i.e. stable diploid karyotype) mammalian cells that can be continuously propagated *in vitro*.

1.2 Potential benefits of stem cell research

In the future, as our understanding of the essential properties and behaviour of stem cells increase, it may be possible to use them for cell-based therapies to treat human diseases (e.g. diabetes and heart diseases), to have more understanding of human development and developmental-related diseases (such as birth defects), the aging process and aging-related diseases (e.g. Parkinson's disease). Stem cell research will also allow for the use of stem cell-derived specialised cell types for the screening of new drugs and other chemical compounds. Stem cell research therefore has huge scientific and medical importance.

1.3 Classification of stem cells

On the basis of their origin, human and animal stem cells can be classified into two categories: somatic or adult stem cells and embryonic stem cells. Three types of mammalian embryonic stem cells have been derived: embryonic stem (ES) cells (Evans and Kaufman, 1981; Nagy *et al.*, 1990; Brook and Gardner, 1997; Thomson *et al.*, 1998), embryonic carcinoma (EC) Cells (Martin, 1981; McBurney and Rogers, 1982) and embryonic germ (EG) cells (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2003; Liu *et al.*, 2004) (Figure 1.10) On the basis of their developmental or differentiation potential ES cells can be further subdivided into the following types: totipotent, pluripotent, multipotent and unipotent stem cells. Totipotent stem cells (i.e. the zygote) can differentiate into all cell types of body. The three types of embryonic stem

cells (ES, EC and EG) originate from the zygote (Figure 1.10). Pluripotent stem cells (i.e. cells of the inner cell mass at the blastocyst stage) can differentiate into any cell type of the three primary germ layers i.e. endoderm (e.g. pancreatic cells, thyroid cells and lung alveolar cells), mesoderm (e.g. cardiac muscle cells, skeletal muscle cells, kidney tubule cells) and the ectoderm (e.g. epidermal tissues, neuron and pigment cells). Multipotent stem cells (e.g. haematopoietic cell) can differentiate into several but not all cell types. Unipotent stem cells (e.g. spermatogenic stem cells) give rise to only one cell type.

1.3.1 Embryonic stem (ES) cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of embryos at the blastocyst stage (3 to 5-day old embryo) (Evans and Kaufman, 1981; Martin, 1981; Doetschman *et al.*, 1985; Nagy *et al.*, 1990; Brook and Gardner, 1997; Thomson *et al.*, 1998) (Figure 1.10). When returned to the embryonic environment by transfer into a host blastocyst or aggregation with blastomere-stage embryos, cultivated ES cells reincorporate into normal embryonic development and have the full potential to develop into all lineages of the embryo proper (Bradley *et al.*, 1984; Nagy *et al.*, 1990).

The blastocyst includes three structures: the trophoblast, blastocoel and inner cell mass (ICM). The trophoblast is the layer of cells that surrounds the blastocyst. The blastocoel is the hollow cavity inside the blastocyst. The inner cell mass is a group of cells (approximately 30 cells) at one end of the

blastocoels (Figure 1.10). The ICM can be grown on mitotically arrested (mitomycin C treated or gamma irradiated) adherent mouse embryonic fibroblasts (MEF) or feeder layer as it is referred to. Both mitomycin-C and gamma irradiation induce DNA damage and cause cell cycle. Mitomycin-C binds to DNA and causes DNA cross-linking (Rink *et al.*, 1996; Volpato *et al.*, 2005; Nieto *et al.*, 2007). This interferes with DNA replication, RNA transcription and protein synthesis. Gamma irradiation treatment induces DNA damage irradiation (Bishay *et al.*, 2000; Ibuki *et al.*, 2003). Therefore the treatment of cells with either mitomycin C or gamma arrests the cell growth or proliferation. The feeder layer cells provide a sticky surface for the ES cells and also release nutrients into the culture medium. In developing tissues, a stem cell from the blastocyst is capable of giving rise to the multiple specialised cell types. ES cells have been differentiated into various cell types including cardiomyocytes (Doetschman *et al.*, 1985; Kehat *et al.*, 2001), skeletal and smooth muscle cells (Rohwedel *et al.*, 1994; Drab *et al.*, 1997), neuronal cells (Bain *et al.*, 1995; Bruneau *et al.*, 1999; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001), β islet pancreatic cells (Assady *et al.*, 2001), dipocytes (Dani *et al.*, 1997), endothelial cells (Levenberg *et al.*, 2002) and several other tissue types.

The other types of embryonic stem cells are embryonic (embryonal) carcinoma cells and embryonic germ (EG) cells. Embryonal carcinoma (EC), like ES, are fully pluripotent cells derived from malignant teratocarcinomas rather than, directly from , the ICM (Martin, 1981; McBurney and Rogers, 1982) (Figure 1.10). Embryonic germ (EG) cells are also pluripotent and are

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derived from primordial germ cells of foetal gonadal or genital ridges (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2003; Liu *et al.*, 2004) (Figure 1.10).

Mouse EG cells, like the ES cells, are cultured on MEF or in the presence of leukaemia inhibitory factor (LIF). Leukaemia inhibitory factor is a differentiation inhibitor that keeps stem cells in the undifferentiated state. In contrast to both ES and EG cells that require culture on embryonic fibroblast feeder layer cells or in the presence of LIF, EC cells (e.g. P19 cell line) are embryonic fibroblast feeder-cell independent (Bradley *et al.*, 1984).
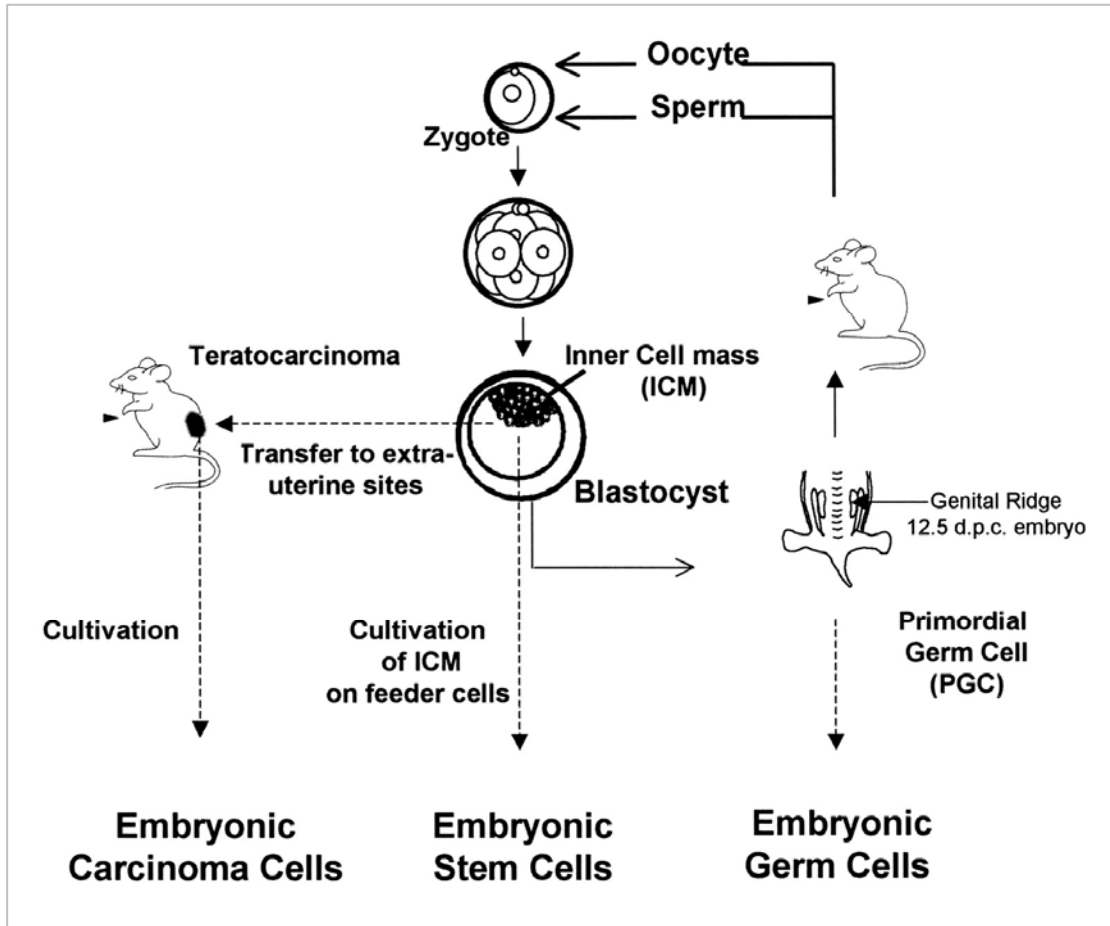
Figure 1.10: Pluripotent embryonic cell lines from mammals: Embryonic carcinoma (EC) cells, embryonic stem (ES) cells and embryonic germ (EG) cells. The ES cells are derived from the ICM and epiblast. EC cells are derived from germ cell tumours or teratocarcinomas. EG cell lines are derived from primordial germ cells of gondal or genital ridge of embryos. Taken from Boheler *et al.*, 2002.

1.3.2 Adult Stem Cells

Adult stem cells are undifferentiated cells found among differentiated cells in tissues. They can be isolated from foetal (after gastrulation) or adult tissues. Amniotic fluid stem cells are adult stem cells from foetal tissues (Perin *et al.*, 2008). Unlike ES cells which are pluripotent, adult stem cells are only multipotent and are generally believed to be programmed to differentiate into specialised cell types of the tissues or organs in which they reside. The primary role of adult stem cells in a living organism is to maintain tissue homeostasis and repair. They maintain homeostasis by generating new cells to replace terminally differentiated cells that are no longer functional. Adult stem cells have been isolated from a wide range of tissues or organs including the heart (Laugwitz *et al.*, 2005; Moretti *et al.*, 2007), bone marrow (Pittenger *et al.*, 1999; Reyes *et al.*, 2001), brain (Clarke *et al.*, 2000), liver (Yang *et al.*, 2002), skin (Toma *et al.*, 2001), adipose (Zuk *et al.*, 2002), skeletal muscle (Jackson *et al.*, 1999) and blood (Zhao *et al.*, 2003).

1.4 Stem cell niche

The decision between stem cell self-renewal and differentiation is thought to be a highly regulated process under the control of molecular and cellular factors. This *in vivo* regulatory microenvironment is known as a stem cell niche. Stem cell number, cell division, self-renewal and differentiation are likely to be controlled by both extrinsic factors and intrinsic factors. Extrinsic factors, including Wnt (Lam *et al.*, 2006; Aicher *et al.*, 2008; Fleming *et al.*, 2008), BMP (Kosinski *et al.*, 2007), Notch (Alexson *et al.*, 2006; Song *et al.*,

2007) and intrinsic factors, including upstream controllers such as the *unpaired* (*upd*) gene (Kiger *et al.*, 2001; Tulina and Matunis, 2001; Boyle *et al.*, 2007) and transcription factors, such as Early Growth Response *1(EGR1)* (Min *et al.*, 2008), Pharynx and Intestine in Excess (PIE-1) (Mello *et al.*, 1996; Seydoux *et al.*, 1996) play roles in stem cell niche. Stem cell niches have been proposed to exist in several adult tissues, including the haematopoietic system (Calvi *et al.*, 2003; Zhang *et al.*, 2003b; Arai *et al.*, 2004), neural tissues (Shen *et al.*, 2004) and in epithelial tissues including the gut epithelium (He *et al.*, 2004) and the skin (Tumbar *et al.*, 2004). *In vivo*, the stem cell niche favours the maintenance of the stem cells in the undifferentiated state. The ultimate decision of a stem cell to remain in the undifferentiated state or to differentiate into specific cell types may depend upon the expression of several regulatory molecules, including transcription factors within the niche. The expression of these regulatory molecules may be induced either by intrinsic or extrinsic events or by both. A clear understanding of how the decision between stem cell self-renewal and differentiation is made will make it easier to culture adult stem cells in the differentiated state and also to direct their differentiation into specific cell types using the appropriate conditions. This clear understanding may be part of the first steps in the direction toward the realisation of the huge therapeutic potentials of adult stem cells.

1.5 Bone marrow stem cells

The bone marrow (BM) is a good source of adult stem cells. The bone marrow contains at least two kinds of stem cells: haematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). The HSC give rise to all the blood cells: red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, macrophages, and platelets (Spangrude *et al.*, 1988; Okada *et al.*, 1991; Bhatia *et al.*, 1997; Yang *et al.*, 2005). Mesenchymal stem cells have the potential to differentiate into multiple lineages but they are rare (i.e. 1 in 10,000 of nucleated BM cells). Mesenchymal stem cells express several markers but none of the markers expressed are specific to their identification. The identification of MSC is therefore based on a composite marker expression and a lack or absence of haematopoietic stem cell marker expression such as CD45 (Lu *et al.*, 2005; Polisetty *et al.*, 2008), CD34 (Conrad *et al.*, 2002; Polisetty *et al.*, 2008), CD14 (Lu *et al.*, 2005) or CD11 (Polisetty *et al.*, 2008). Mesenchymal stem cells also do not express some co-stimulatory molecules such as CD80 (Sun *et al.*, 2003; Lu *et al.*, 2005), CD86 (Lu *et al.*, 2005; Liu *et al.*, 2006) or CD40 (Liu *et al.*, 2006). Human MSC can however express CD105 (Barry *et al.*, 1999; Conrad *et al.*, 2002; Roura *et al.*, 2006; Odabas *et al.*, 2008), CD73 (Barry *et al.*, 2001; Trivedi and Hematti, 2007; Odabas *et al.*, 2008), CD44 (Oswald *et al.*, 2004; Yanada *et al.*, 2006; Zhu *et al.*, 2006), CD90 (Oswald *et al.*, 2004; Wiesmann *et al.*, 2006) and some adhesion molecules (e.g. CD106 (Polisetty *et al.*, 2008), CD166 and CD29 (Oswald *et al.*, 2004; Polisetty *et al.*, 2008)).

Mesenchymal stem cells have the potential to contribute to the regeneration of various mesenchymal tissues including bone (osteocytes), cartilage cells (chondrocytes) (Worster *et al.*, 2001; Bai *et al.*, 2004), ligament, tendon, fat (adipocytes) (Scavo *et al.*, 2004) and smooth muscles (Jeon *et al.*, 2006; Wang *et al.*, 2006). In addition to their differentiation into mesenchymal tissues, recent research suggests that MSC can also differentiate into cardiomyocytes (Hakuno *et al.*, 2002; Li *et al.*, 2006; Wang *et al.*, 2006; Tomita *et al.*, 2007; Muscari *et al.*, 2008) and this can be achieved experimentally using 5-azacytidine (Fukuda, 2001; Xu *et al.*, 2004b; Antonitsis *et al.*, 2007). 5-azacytidine is a nucleoside analog of cytidine. Carbon 5 on the pyrimidine ring of 5-azacytidine is replaced by a nitrogen atom. Therefore when incorporated into DNA, 5-azacytidine cannot be methylated thus leaving the new synthesized genomic DNA largely in the unmethylated state (Jones and Taylor, 1980; Taylor and Jones, 1982). DNA methylation is one of the key mechanisms of gene regulation. The incorporation of 5-azacytidine into DNA can activate gene expression and also alter the differentiation of some eukaryotic cells (Jones and Taylor, 1980; Jones *et al.*, 1983). The successful cardiac differentiation of the MSC was noted by expression of mRNA for Nkx2.5, GATA-4, TEF-1, and MEF2C (Myocyte Enhancer Factor 2 C) before and further expression of MEF2A and MEF2D after 5-azacytidine treatment. Cardiac proteins including atrial natriuretic peptide (ANP) were also detected (Fukuda, 2001) . The analysis of their contractile protein, such as myosin and α-actin, indicated that their phenotype was similar to foetal ventricular cardiomyocytes (Fukuda, 2001).

The differentiation of MSC into cardiomyocytes as determined by the expression of α-cardiac actin, β-myosin heavy chain and cardiac troponin-T by human MSC has also been demonstrated (Xu *et al.,* 2004b; Antonitsis *et al.,* 2007). An important observation in these studies is that both 5-azacytidine-treated cells and non-treated cells expressed cardiac specific genes (Fukuda, 2001; Antonitsis *et al.,* 2007). It was further shown that cardiac specific genes were not expressed in undifferentiated MSC that were not being expanded in culture (Antonitsis *et al.,* 2007). This suggests that while MSC can differentiate into cardiomyocytes, they do not do so randomly or spontaneously but only in the presence inducing factors (e.g. 5-azacytidine or conditions such as being expanded in culture). While the physiological stimulus that drives MSC differentiation into cardiomyocytes is not yet known, these results raise a possibility that MSC (or cardiomyocytes derived from bone marrow sources) can potentially be valuable for the treatment of myocardial infarction. A better understanding of the MSC differentiation into cardiomyocytes may allow the use of MSC-derived cardiomyocytes to replace cardiomyocytes in the damaged heart.

1.6 Potential advantages and disadvantages of ES and adult stem cells

1.6.1 Embryonic stem cells

Embryonic stem cells are obtained from embryos and can be expanded in culture for a very long time. Pluripotent ES cells can undergo flexible differentiation into many different cell types. However, the pluripotent nature of

ES cells means that they are difficult to differentiate into a uniform or homogeneous population of one cell type. Due to their pluripotent capacity, undifferentiated ES cells transplanted into target tissues may form teratomas. Apart from the risks of tumour formation, ES cell-derived target cell types may also cause an immune reaction in the recipient, thus, causing the rejection of transplanted cells. Apart from the above scientific issues with ES cells that have to be resolved, there is also an ethical dimension to human ES cell research and potential future use. Currently available stem cell technologies, for the derivation of pluripotent human ES cells, require the "destruction" of human embryos at the blastocyst stage (Thomson *et al.*, 1998). Therefore for some people, ES cell research and potential use may pose ethical problems. However the creation of induced pluripotent stem cells (iPS) from adult human cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007) may offer a viable alternative to the human embryo as a source for pluripotent stem cells. Octamer-binding protein (Oct-3/4), a transcription and pluripotency marker, is expressed in embryonal carcinoma cells, embryonic stem cells and embryonic germ cells (Thomson *et al.*, 1998; Hansis *et al.*, 2000; Reubinoff *et al.*, 2000). Human amniotic fluid stem cells have also been shown to express Oct-3/4. Human amniotic fluid stem cells can be obtained without the destruction of human embryos (Prusa *et al.*, 2003; De Coppi *et al.*, 2007; Perin *et al.*, 2008; Zheng *et al.*, 2008b). In the future iPS and adult stem cells, like amniotic fluid stem cells with higher pluripotency, may offer viable alternatives to the human embryo as a source for pluripotent stem cells.

1.6.2 Adult stem cells

Adult stem cells are rare in mature tissues and methods for harvesting pure adult stem cells and growing them culture are difficult. Therefore adult stem cells unlike ES are relatively more difficult to obtain in large numbers.

For research purposes, the need for a pure population of adult stem cells is a critical consideration because the reliability of experimental results from adult stem studies may be compromised by the presence of other cell type in the cell culture or stem cell preparation from adult tissues. The presence of non-stem cell populations in isolated adult stem cell population can lead to the wrong interpretation of results, especially if the contaminating cell type has or express the phenotypes of the final or target cell type for the differentiation studies.

Adult stem cells exist *in vivo* and this means that they must be able to self-renew in order to be able to participate in the maintenance of long term tissue homeostasis in human and animals, but isolated adult stem cells cannot undergo long-term self-renewal *in vitro*. The renewal capacity of adult stem is therefore less than that of ES cells hence adult stem cells may not live as long as ES cells in culture. For future stem cell therapies, the rarity of adult stem cells and their low self-renewal capacity are very important considerations, since a large number of cells may be needed on a routine basis for patient treatment.

Adult stem cells are programmed to differentiate into cell types of the tissues in which they are found. However emerging evidence suggest that under appropriate conditions, animal and human adult stem cells can differentiate into cell types of tissues other than that of their origin. Human MSC have been differentiated into cardiomyocytes (Grauss *et al.*, 2007)*,* chondrocytes (Bai *et al.*, 2004), adipocytes (Scavo *et al.*, 2004) and smooth muscle (Jeon *et al.*, 2006). Mouse MSC have been differentiated into cardiomyocytes (Kudo *et al.*, 2003) and neurons (Tropel *et al.*, 2006). Rat MSC have been differentiated into neurons (Lou, et al. 2003) and smooth muscle (Wang *et al.*, 2006) and horse MSC have been differentiated into chondrocytes (Worster *et al.,* 2001). However, generally, adult stem cells have less differentiation potentials then ES cells and may, therefore, be more difficult to differentiate into cell of other tissue other than the tissue of origin. A key potential advantage of using adult stem cells is that if the patient's own cells are expanded in culture and then re-introduced into the patient, the risk of immune rejection of the transplanted cells may be avoided. In terms of ethical considerations, adult stem cell research and their therapeutic use may be more acceptable than ES cells, since adult stem cells can be isolated without any potential harm to the donors.

1.7 Embryonic stem cell culture models

Embryonic stem cells are cultured at low density on a mitotically inactivated embryonic fibroblast feeder layer or in the presence of LIF or both. Under these culture conditions, the stem cells are prevented from undergoing

undirected spontaneous differentiation processes thus maintaining their stem cell pluripotency (Evans and Kaufman, 1981; Martin, 1981; Smith *et al.*, 1988; Williams *et al.*, 1988; Niwa *et al.*, 1998)**.**

A huge amount of what we know about stem cell and stem differentiation comes from studies using mouse ES cell models. Experience with human ES cells is relatively new and limited, but comparison between human and mouse models using the limited data from human have revealed important differences between the two models. These differences include the cell culture requirements to keep the stem cells in the undifferentiated state, morphology, growth kinetics and antigenic phenotypes or stem cell specific molecular markers.

1.8 Differences between human and mouse embryonic stem cells

1.8.1 Differences in leukaemia inhibitory factor requirement

The leukaemia inhibitory factor (LIF) (fully discussed below under LIF signalling) alone is sufficient, even in the absence of feeder layer cells, to maintain mouse stem cell cultures in the undifferentiated state. However LIF alone is not sufficient to prevent the differentiation of human (and other primates) ES cells (Schuringa *et al.*, 2002). This means that human ES cells, unlike mouse ES cells, are maintained in an undifferentiated state through a signalling pathway(s) that may be independent of or in addition to LIF signalling**.**

1.8.2 Differences in cell morphologies and growth kinetics

Mouse ES cells grow in tightly attached rounded masses while human ES cells form relatively flat compact colonies with high nuclear to cytoplasm ratio and prominent nucleoli (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Park *et al.*, 2004). Human colonies can be easily dissociated into single cells with conventional mechanical and enzymatic techniques. The population doubling time of human ES cells of approximately 36 hours (Hovatta *et al.*, 2003; Park *et al.*, 2004) is significantly longer than that of mouse ES cells (approximately 10-16 hours) (Viswanathan *et al.*, 2002; Berrill *et al.*, 2004; Fok and Zandstra, 2005; Greenlee *et al.*, 2005).

1.8.3 Differences in stem cell specific markers

In addition to morphological differences, human and mouse pluripotent stem cells differ in their expression of a number of antigenic phenotypes or stem cell specific molecular markers. Common stem cell markers that are used in characterisation of ES cells include the glycolipid surface Stage Specific Embryonic Antigens (SSEA-1, SSEA-3, SSEA-4) and the keratan sulfate-related antigens (TRA-1-60 and TRA-1-81) (Solter and Knowles, 1978; Gooi *et al.*, 1981; Ozawa *et al.*, 1985; Krupnick *et al.*, 1994; Draper *et al.*, 2002). The TRA antigens (or antibodies) were named after the Battle of *Tra*falgar. Undifferentiated primate (human and nonhuman primates) ES cells express the specific cell surface markers: SSEA-3, SSEA-4, TRA-l-60 and TRA-1-81 (Draper *et al.*, 2002; Henderson *et al.*, 2002). They also express alkaline phosphatase. However differentiated primate ES cells strongly expressed

SSEA-I (Krupnick *et al.*, 1994; Thomson *et al.*, 1995)**.** Differences between mouse and human is shown by the fact that mouse inner cell mass cells, undifferentiated ES cells and EC cells express SSEA-1 but do not express SSEA-3, SSEA-4, TRA-1-60, or TRA-1-81, in contrast to   undifferentiated primate ES cells. SSEA-1 appears during the late cleavage stages of mouse embryogenesis. SSEA-1 is strongly expressed by undifferentiated mouse ES cells (Solter and Knowles, 1978; Gooi *et al.*, 1981; Ozawa *et al.*, 1985; Krupnick *et al.*, 1994). Differentiated murine ES cells are characterised by the loss of SSEA-1 expression (Cui *et al.*, 2004). The loss of SSEA-1 expression may be accompanied in some instances by the appearance of SSEA-3 and SSEA-4. In contrast to undifferentiated mouse ES cells, undifferentiated human ES cells are characterised by the expression of SSEA3, SSEA4, TRA-1-60, and TRA-1-81, and by the lack of SSEA1 (Draper *et al.*, 2002; Henderson *et al.*, 2002). The differentiation of human ES and EC cells is characterised by down-regulation of SSEA-3 and SSEA-4 (Draper *et al.*, 2002). In contrast to the differential expression of the above stem cell markers, both mouse and human embryonic stem cell express octamer-binding transcription factor-3/4 (Oct-3/4) (Thomson *et al.*, 1998; Hansis *et al.*, 2000; Reubinoff *et al.*, 2000), Nanog (see section 1.10.3)   (Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Hart *et al.*, 2004; Sato *et al.*, 2004) and have high telomerase activity (Thomson *et al.*, 1998; Amit *et al.*, 2000; Armstrong *et al.*, 2000; Yang *et al.*, 2008). Stem cell markers are proteins found in stem cells (and rarely in other cells) and as such they have specific physiological functions. For example, Oct-3/4 is a transcription factor that is expressed in totipotent and pluripotent stem cells of the pregastrulation embryo and is

required to sustain stem cell self-renewal and pluripotency (Rosner *et al.*, 1990; Palmieri *et al.*, 1994; Nichols, 1998; Niwa *et al.*, 2000). While the physiological functions of many stem cell markers are not currently known, stem cell markers are important research tools for the identification of undifferentiated stem cells and the characterization of differentiated cell types.

1.9 Stem cell self-renewal and pluripotent state

While research effort has been directed toward clearly understanding the mechanisms that are involved in stem cell differentiation into various cell types, it is also equally important to understand the regulatory mechanisms that are responsible for ES cell self-renewal and pluripotency. A clear understanding of the key self-renewal/pluripotency regulatory mechanisms can lead to better differentiation protocols and this may lead to the realisation of ES cells full therapeutic potentials. While intracellular signalling events that are involved in the maintenance of ES cell self-renewal and pluripotency are complex and not yet fully understood, research data point to the involvement of various extrinsic factors (e.g. LIF, BMP, Wnt) and key ES cell specific factors (e.g. Nanog, Oct-4, Sox2).

1.9.1 Leukaemia inhibitory factor signalling and stem cell self-renewal

In   cell culture medium, pluripotent ES cells undergo spontaneous differentiation to generate the three germ layers: endoderm, mesoderm and ectoderm leading to a mixed population of cell lineages and cell types (Amit

et al., 2000; Schuldiner *et al.*, 2000). The presence of leukaemia inhibitory factor (LIF) or closely related cytokines is required in culture, in order to prevent this spontaneous differentiation (Smith and Hooper, 1987; Smith *et al.*, 1988; Williams *et al.*, 1988; Niwa *et al.*, 1998). LIF, a multifunctional cytokine of the interleukin-6 (IL-6) cytokine family, sustains ES cell self-renewal and pluripotency by signalling mechanisms that are not yet fully understood.

Leukaemia inhibitory factor acts on a cell surface receptor complex that is composed of two chains [(i.e. an 80-kDa LIF-binding α-chain and a 130-kDa signal transducer β-chain (gp130)].The gp130 chain is a common receptor subunit that is shared by receptors for other cytokines of the IL-6 family, including IL-6, IL-11, cardiotrophin-1, oncostatin M, and ciliary neurotrophic factor (Hibi *et al.*, 1990; Gearing *et al.*, 1991; Gearing and Bruce, 1992; Ip *et al.*, 1992; Davis *et al.*, 1993; Murakami *et al.*, 1993). Interleukin-6 can substitute for LIF in mouse ES cells. Mouse ES cells can be maintained in the undifferentiated state using a combination of interleukin-6 and soluble interleukin-6 receptor (IL-6/sIL-6R) (Nichols *et al.*, 1994; Yoshida *et al.*, 1994; Yoshida *et al.*, 1996)

The binding of LIF induces the dimerisation of the receptor chains to form a high affinity heterodimer complex (Davis *et al.*, 1993; Murakami *et al.*, 1993). The high affinity receptor complex formation leads to the recruitment and activation of cytosolic tyrosine kinases of the Janus kinase (JAK) family (i.e. JAK-1, JAK-2, JAK-3 and Tyk2). The activated JAK cross phosphorylate themselves (Narazaki *et al.*, 1994; Stahl *et al.*, 1994) and then phosphorylate

the tyrosine residues on the intracellular domain of gp130 (Stahl *et al.*, 1994; Nakamura *et al.*, 1998). This phosphorylation by JAKs creates binding or docking sites (i.e. the phosphorylated tyrosine residues) on the receptor for the binding of proteins containing the Src-homology 2 (SH2) domain. The creation of docking sites on the gp130 receptor, is followed by the activation of two major signalling cascades: JAK-STAT which promotes ES cell self-renewal (Boeuf *et al.,* 1997; Niwa *et al.*, 1998; Matsuda *et al.*, 1999; Raz *et al.*, 1999) and SHP2-Ras/Raf/ERK/MAPK which promotes ES cell differentiation (Burdon *et al.*, 1999). This means that the balance between the JAK-STAT cascade and the SHP-2-ERK cascade may be crucial in determining whether ES cells differentiate or remain the differentiate state.

1.9.2 Leukaemia inhibitory factor signalling: STAT cascade

Docking sites on gp130 are required for LIF/STAT signalling to maintain ES cell self-renewal (Niwa *et al.*, 1998). The STAT (signal transducer and activator of transcription) protein family include STAT1, 2, 3, 4, 5A, 5B and 6. Non-phosphorylated latent STAT proteins are activated to become active transcription factors by phosphorylation. STAT3 is the major or critical mediator of LIF signalling that maintain ES cells in the differentiated state. Over-expression of STAT3F (dominant negative mutant form of STAT3 with tyrosine 705 changed to phenylalanine) in ES cells growing in the presence of LIF can cause loss of pluripotency and enhanced cell differentiation (Boeuf *et al.*, 1997; Niwa *et al.*, 1998). STAT3 activation is required and sufficient to maintain mouse ES cells in the undifferentiated state (Matsuda *et*

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al., 1999; Raz *et al.*, 1999). It has been shown that the inhibition of STAT3 activation blocked ES cell self-renewal and promoted differentiation (Niwa *et al.*, 1998).

When STAT3 docks on the cytosolic domain of the receptor via SH-2-phosphotyrosine interactions, it (i.e. STAT3) is phosphorylated by receptor-bound JAKs (or other nonreceptor tyrosine kinases). STAT3 phosphorylation at Tyr705 is required for dimerisation and nuclear translocation because over-expression of STAT3F has been shown to block the activation of endogenous STAT3 in myeloid leukemia MI cells (Minami *et al.*, 1996; Nakajima *et al.*, 1996). The activated STAT factors dissociate from the receptor complex, undergo homodimerisation or heterodimerisation and then translocate into the nucleus where they bind to elements in the promoters of STAT responsive or target genes to control their transcription (Figure 1.11)

1.9.3 Leukaemia inhibitory factor signalling: ERK/MAPK cascade

The SHP-2-ERK cascade is activated by docking of growth factor receptor-bound protein 2 (Grb2) on phosphorylated SHP2 on the activated gp130 receptor. Grb2 and the son of sevenless (SOS) form a complex. The complex formation leads to the activation of Ras which in turn activates Raf. Many other pathways also use Ras signalling to achieve various outputs or functions. It is not yet fully understood how specificity is generated. Specificity of Ras action may be determined by cell type or the presence (or absence) of

specific signal-activated and tissue-restricted signalling molecules (e.g. transcription factors).

Ras-activated Raf activates mitogen-activated protein kinase kinase (MEK) which in turn finally activates ERK (Figure 1.11). Activated SHP-2-ERK cascade antagonises the STAT3 cascade to promote stem cell differentiation. ERK1 and ERK2 play a role in mediating mitogenic responses of cells to growth factors (Pages *et al.*, 1993; Cowley *et al.*, 1994; Weber *et al.*, 1997). SHP-2-ERK activation is also not unique to controlling the balance between ES cell renewal and differentiation. However, SHP-2-ERK activation has been linked to the regulation of mouse ES cell self-renewal. SHP-2-ERK signalling can repress the expression of Nanog in mouse ES cells (Hamazaki *et al.*, 2006). A dominant-negative mutant of Ras has been found to suppress the expression of GATA-4, GATA-6 and other markers of extraembryonic endoderm differentiation. It has also been reported that a constitutively active Ras promoted ES cell differentiation and this promoted ES cell differentiation was inhibited by the application of a MEK inhibitor (U0126) (Yoshida-Koide *et al.*, 2004). Similarly it has been shown that the inhibition of ERK signalling promotes self-renewal of mouse ES cells (Burdon *et al.*, 1999).
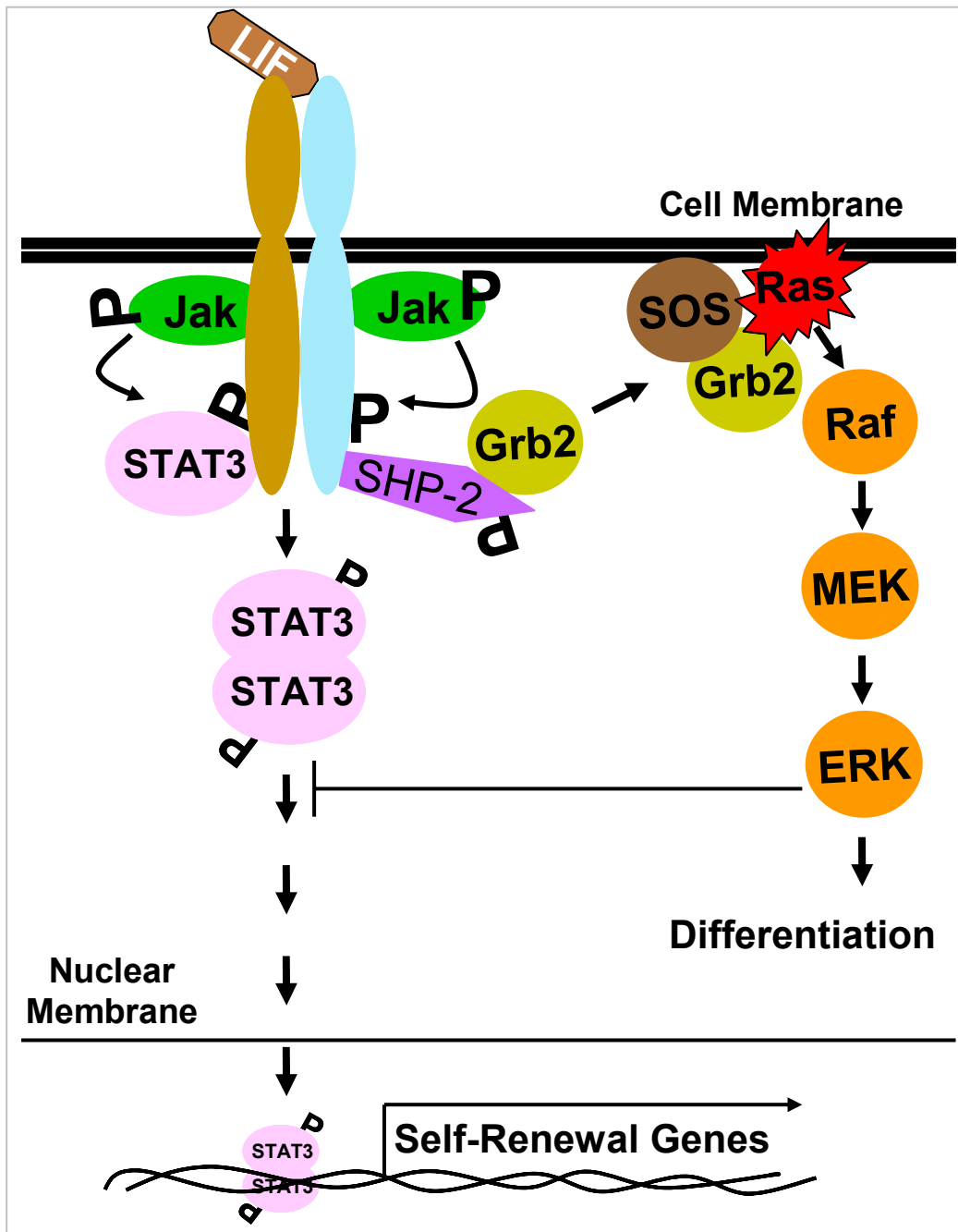
Figure 1.11: LIF signalling and stem cell self-renewal. This diagram shows a simplified view of LIF-induced signalling events. LIF binding to the receptor leads to recruitment of JAK and the activation of STAT. STAT dimerise, translocate to the nucleus and switch on the expressions of cytokine (e.g., LIF) target genes. In the case of LIF binding to the receptor, in stem cells, genes that keep the stem cells in the undifferentiated state are activated. Abbreviations: JAK (Janus kinase); MEK (MAPK kinase); P (Phosphate group); SOS (Son of sevenless) and STAT (Signal transducers and activators of transcription); Sh-2-Containing Tyrosine Phosphatase (SHP2),

1.9.3.1 Negative regulation of LIF signalling by SHP-2 and SOCS

The gp130-dependent signalling pathway is regulated by action of Src-homology tyrosine phosphatases 2 (SHP-2) and suppressor of cytokine signalling (SOCS) proteins (Figure 1.12). SHP-2 recruited to the phosphorylated gp130 receptor, in addition to their activation of ERK cascade, also negatively regulate gp130-induced STAT3 activation by dephosphorylation of phosphotyrosines (Anhuf *et al.*, 2000; Ohtani *et al.*, 2000). In SHP2 signal–deficient mice, gp130-induced STAT3 activation showed prolonged gp130-induced STAT3 activation (Ohtani *et al.*, 2000). Suppression of SHP-2 signalling promotes mouse ES cell self-renewal (Burdon *et al.*, 1999).

The eight SOCS proteins (SOCS1-SOSC7 and CIS) are characterised by a conserved C-terminal SOCS box, a central SH2 domain and a variable N-terminal (Hilton *et al.*, 1998). It has been shown that over-expression of SOCS3 can completely suppress LIF-induced signalling events (Yasukawa *et al.*, 2001). Over-expression of SOCS3 in a trophoblast stem cell line (Rcho-1) also suppressed the cell differentiation while SOCS3-deficient Rcho-1 cells were found to rapidly differentiate thus demonstrating that SOCS3 negatively regulates the ES cell differentiation (Takahashi *et al.*, 2003).

The mechanism by which SOCS inhibit gp130 receptor signalling is not yet entirely clear. SOCS proteins can be recruited to specific phosphotyrosine residues in activated gp130 receptor or can bind to receptor bound-JAKs and inhibit LIF signalling. For example it has been shown that SOCS1 can inhibit

the activities of JAK1, JAK2 and JAK3 by direct interaction (Endo *et al.*, 1997; Nicholson *et al.*, 1999). In contrast SOCS3 inhibitory activity may depend on its direct interaction with the phosphorylated gp130 receptor (Nicholson *et al.*, 2000). The gp130 receptor activation is known to induce rapid and transient up-regulation of the expression of SOCS proteins. The SOCS proteins then inhibit the signalling pathways that initially led to their production in a negative feedback loop (Krebs and Hilton, 2001).

The importance of the negative regulatory role of the SOCS proteins may be the reason why LIF can keep mouse ES cells but not human (including other primates) ES cells in the undifferentiated state. It has been shown that human but not mouse ES cells are rich in SOCS proteins (e.g. SOCS-1) (Schuringa *et al.*, 2002; Sato *et al.*, 2003).The negative regulation by SOCS proteins and SHP-2 ensure that LIF/STAT signalling has the appropriate magnitude and the durations.
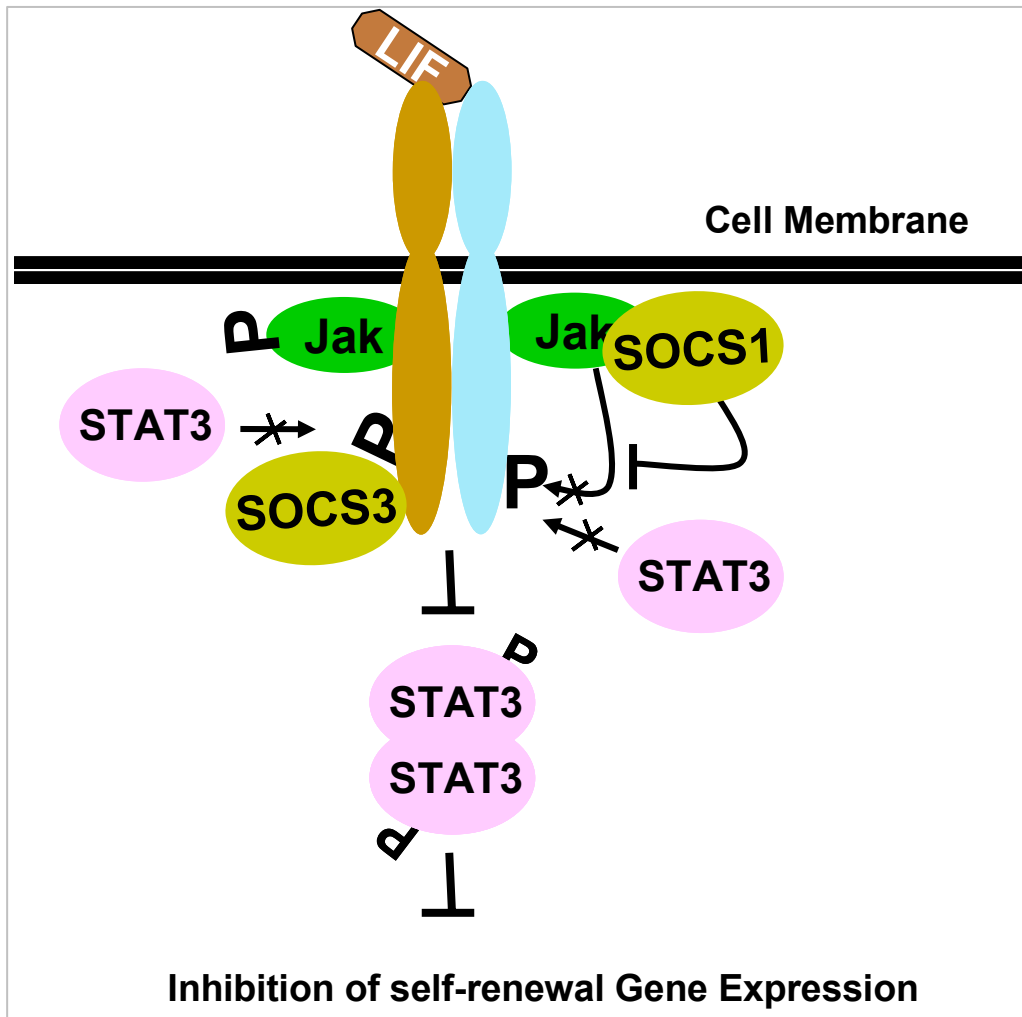
Figure 1.12: Negative regulation of LIF signalling by socs proteins.
Cytokine binding to the receptor activates the JAK-STAT pathway, leading to
the induction of SOCS protein. The induced SOCS proteins act in a negative
feedback loop to inhibit LIF signalling either by interacting with JAK or with the
phosphorylated gp130 receptor. Redrawn from (Krebs and Hilton, 2001) with
modifications. Abbreviations: JAK (Janus kinase); STAT (Signal transducers
and activators of transcription); SOCS (suppressor of cytokine signalling)

1.10 Regulation of stem cell self-renewal and pluripotency

1.10.1 LIF signalling and pluripotent transcription factors

LIF signalling post STAT3 is poorly understood. Effectors downstream of STAT3 are poorly understood. Whether LIF signalling has any effect on key transcription factors that are responsible for ES cell self-renewal and pluripotency, is not yet clearly established. Evidence of direct connection between LIF/STAT signalling (i.e. post STAT3 activation) and ES cell self-renewal associated transcription factors (such as Nanog, Sox2 and Oct-4) is still missing. Information linking LIF/STAT signalling to these transcription factors can lead to the better understanding of LIF effects on ES cells and may also lead to the development of efficient stem cell culture and differentiation protocols.

LIF/STAT3 signalling has been given a prominent role in the maintenance of ES self-renewal and pluripotency in many studies (Niwa *et al.*, 1998). However LIF signalling pathway is not essential for the maintenance of ICM and human ES cell self-renewal and pluripotency. For example Nanog is capable of maintaining ES cell self-renewal independently of LIF/STAT3 signalling (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). LIF is also not specific to ES cells because it can act on a wide range of cell types. LIF can mediate growth arrest and apoptosis in myeloid leukemia MI cells (Minami *et al.*, 1996). Moreover LIF also does not exert a consistent effect on all cells. LIF can inhibit differentiation (i.e. promotes cell self-renewal) or LIF can induce differentiation (i.e. inhibit cell self-renewal). Both of these two mutually

exclusive processes (differentiation and self-renewal) require the activation of LIF signalling so the presence (or absence) of a set of genes (which may or may not be unique to the target cell type) with which LIF can interact, may ultimately determine whether LIF will promote self-renewal or induce of differentiation. In fact, ES cell pluripotency is thought to be maintained by a few key transcription factors including Oct-4, Nanog, Sox2 and other. These factors bind to regulatory sequences in the promoters of self-renewal and pluripotency specific genes and control their expression, thus, regulating ES cells self-renewal and pluripotency.

1.10.2 Octamer-binding protein -3/4

Octamer-binding protein -3/4 (Oct-3/4) belongs to POU (PIT/OCT/UNC) family of transcription factors. It binds to octameric sequence AGTCAAAT in target genes. Oct-4 is expressed in totipotent and pluripotent stem cells of the pregastrulation embryo and the germ cell lineage (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Yeom *et al.*, 1996; Nichols, 1998). When the embryo differentiates into the endoderm and mesoderm, Oct-4 expression is down-regulated (Rosner *et al.*, 1990). Oct-4 is expressed in the ICM but not in the trophectoderm (Palmieri *et al.*, 1994). Oct4-deficient embryos develop normally to the blastocyst stage, but their ICM cells are not pluripotent (Nichols, 1998). The trophectoderm (TE) is the first differentiated cell lineage or tissue of the mammalian blastocyst. The TE surrounds the ICM. Forced repression of Oct-4 has been shown to induce the loss of pluripotency in ES cells causing their differentiation into the TE lineage (Niwa *et al.*, 2000). The

above study results strong evidence in support of the requirement of Oct-4 for ES cell pluripotency.

The precise levels of Oct-4 are critical for proper maintenance of ES self-renewal and pluripotency. Repression of Oct-4 (~50% of normal level) induces differentiation toward the trophectoderm formation (i.e. differentiation) while over-expression of Oct-4 (~150% of normal level) induces differentiation mainly toward extraembryonic endoderm (Niwa *et al.*, 2000). It is generally accepted that Oct-4 expression is unique to ES cells. However this view is been increasingly challenged by several studies that reported the expression of Oct-4 in the MSC of human (D'Ippolito *et al.*, 2004; Izadpanah *et al.*, 2006), rhesus monkey (Izadpanah *et al.*, 2006) and mouse (Pallante *et al.*, 2007). MSC "pluripotency" was demonstrated by the expression of Oct-4 and also by MSC ability to differentiate into beating cardiomyocytes and also expressed cardiac specific protein cardiac genes including Nkx2.5, cTnT, α-cardiac actin, MLC-2v, Cx43, and Cx40 (Pallante *et al.*, 2007). These results suggest that Oct-4 critical roles in pluripotency may not be unique for ES cells but may also apply to adult stem cells.

1.10.3 Nanog

Nanog was named after Tir nan Og, a mythical celtic land of the ever young, by Chamber *et al.*, 2003, in recognition of its ability to maintain ES cell self-renewal and Pluripotent state. Nanog is both necessary and sufficient for self-renewal and pluripotency of mouse epiblast and ES cells (Chambers *et al.*,

2003; Mitsui *et al.*, 2003). It has been found that Nanog-deficient ICM can not generate epiblast and also that nanog-deficient ES cells can not maintain pluripotency (Mitsui *et al.*, 2003). Over-expression of Nanog is sufficient to maintain ES cell self-renewal independently of LIF signalling (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). These studies demonstrate that Nanog is required for the maintenance of pluripotency in both ICM and ES cells.

1.10.4 Sox2

Sox2, like Oct4, is expressed in the ICM, epiblast, and germ cells (Avilion *et al.*, 2003). Sox2 and Oct-4 synergistically bind in the enhancers of several genes (Yuan *et al.*, 1995; Ambrosetti *et al.*, 1997; Okuda *et al.*, 1998; Nishimoto *et al.*, 1999; Ambrosetti *et al.*, 2000; Boyer *et al.*, 2005; Tan *et al.*, 2007). Octamer-Sox enhancers can regulate the expression of pluripotent stem cell-specific genes, including Nanog, Oct-4 and Sox2 itself. It has been shown that forced expression of Oct-4 can rescue the pluripotency of Sox2-null ES cells (Masui *et al.*, 2007). These results suggest that Sox2 is dispensable for the activation of Oct–Sox enhancers but is essential for maintaining the correct levels of Oct-4 in order to stabilise ES cells in a proper pluripotent state (Masui *et al.*, 2007)

1.10.5 Cooperative Interaction of Oct-4, Sox2 and Nanog

The cooperative interaction of Oct-4, Sox2 and Nanog is required in early development and also for the maintenance of the pluripotent and

undifferentiated states. Cooperation of Oct-4 and Sox2 has been detected in the enhancers of several genes including fibroblast growth factor (fgf)-4 (Yuan *et al.*, 1995; Ambrosetti *et al.*, 1997; Ambrosetti *et al.*, 2000), undifferentiated embryonic cell transcription factor 1 (UTF1)(Nishimoto *et al.*, 1999; Boyer *et al.*, 2005), Fbox15 (Tokuzawa *et al.*, 2003) and Nanog (Kuroda *et al.*, 2005; Rodda *et al.*, 2005).

UTF1 is expressed in human and mouse ES cells and the ICM (Okuda *et al.*, 1998; Boyer *et al.*, 2005; Tan *et al.*, 2007) but its transcription stops at the onset of differentiation (Okuda *et al.*, 1998). Similarly the expressions of Oct-4, Sox-2 and UTF1 are down-regulated following the induction of differentiation in ES cells (Nishimoto *et al.*, 1999; Boyer *et al.*, 2005).. The simultaneous down-regulation of Oct-4, Sox-2 and UTF1 suggest that UTF1 expression may be controlled by synergistic binding of Oct-4 and Sox-2 to an Oct–Sox regulatory sequence in UTF1 (Nishimoto *et al.*, 1999; Boyer *et al.*, 2005). Like UTF1, fgf-4 gene expressions in the ICM and in EC cells requires the cooperative activity of Sox-2 and Oct-4 (Yuan *et al.*, 1995; Ambrosetti *et al.*, 1997; Ambrosetti *et al.*, 2000). The cooperative activity of Sox-2 and Oct-4 may also be required for the   transcription of Nanog. It has been shown that specific knockdown of Oct4 and Sox2 mRNA by RNA interference inhibited the expression of Nanog (Rodda *et al.*, 2005).

The above data suggest that the combinatorial regulatory actions of Oct-4 and Sox2 may be a general mechanism by which Sox-2 and Oct-4 can control the expression of several genes, especially those that are involved in the

maintenance of ES cell self-renewal and pluripotent state. Oct4, Sox2, Nanog and possibly other factors may be part of a very complex auto-regulatory network that is required to balance ES cell decisions between self-renewal and differentiation.

1.11 Embryonic stem cell differentiation protocols

1.11.1 Embryoid body formation

The most common method used for *in vitro* differentiation is to culture ES cells in non-treated dishes (e.g. Petri dishes) and in absence of a feeder layer or LIF. Standard non-treated culture plates (e.g. microbiological plates) are usually sufficient for this purpose. ES cells that are cultured under these conditions form multicellular aggregates called embryoid bodies (EBs) and spontaneously and irreversibly differentiate into cell types of the three embryonic germ layers (ectoderm, mesoderm and endoderm) (Evans and Kaufman, 1981; Martin, 1981; Doetschman *et al.*, 1985; Smith *et al.*, 1988; Williams *et al.*, 1988; Niwa *et al.*, 1998). The three-dimensional structure of EBs is thought to initiate signalling that induces the differentiation of ES cells into cell types of embryonic germ layers (Keller, 1995; Itskovitz-Eldor *et al.*, 2000). The differentiation of ES cells into specific subsets of cells may be induced by treatment with specific factors (e.g. growth factors).

1.11.2 Formation of EB by the Hanging Drops Method

Different protocols have been used in mouse ES cell culture for initiating the formation of EBs. One of the widely used methods is the "hanging drops" technique. In this technique, a definite number of mouse ES cells (usually 400) are cultivated in hanging drops (droplet of medium on the inside of a culture dish for a short period (e.g. 2 days). The EBs are then collected and further cultured for an additional period (e.g. 4-5 days) in suspension and then plated on gelatin-coated culture dishes for further differentiation. A simpler method than the "hanging drop method" is the liquid suspension culture. In the method, a definite number of stem cells are seeded in bacterial-grade dishes in an appropriate growth medium and left to form EBs. After the EB formation, they are then transferred to cell culture-grade dishes.

1.11.3 Embryonic stem cell differentiation into cardiomyocytes

The fundamental question of whether stem cells can actually differentiate into functional cardiomyocytes has been scientifically and convincingly answered. Under the appropriate experimental conditions, mouse and human ES cells can be differentiated into cardiomyocytes (Wobus *et al.*, 1991; Xu *et al.*, 1998; Kehat *et al.*, 2001; He *et al.*, 2003; Mummery *et al.*, 2003). The differentiation of ES cells into cardiomyocytes can be monitored by expression of several cardiac specific structural proteins including tropomyosin (Moore *et al.*, 2004), α-actinin (Denning *et al.*, 2006), cardiac troponin I (cTnI) (Kehat *et al.*, 2001), cardiac troponin T (cTnT) (Kehat *et al.*, 2001), atrial natriuretic peptide (ANP) (Denning *et al.,* 2006), cardiac myosin

light chain isoforms (MLC-2v and MLC-2a) (Kehat *et al.*, 2001; Moore *et al.*, 2004), MLC-1v & MLC-1a and cardiac myosin heavy chain isoforms (α-MHC and β-MHC) (Kehat *et al.*, 2001; Denning *et al.*, 2006; Anderson *et al.*, 2007). ES cell differentiation into cardiomyocytes can be also be tracked by the expression of several cardiac specific transcription factors including Nkx2.5, GATA-4 (Kehat *et al.*, 2001; Nakamura *et al.*, 2003; Anderson *et al.*, 2007), and myocyte enhancer factor (MEF2C) (Denning *et al.*, 2006; Anderson *et al.*, 2007).

CARDIAC TRANSCRIPTION FACTORS AND STEM CELL DIFFERENTIATION INTO CARDIOMYOCYTES

1.12 Cardiac transcription factors and stem cell differentiation into cardiomyocytes

Cardiac development requires the interactions of many cardiac transcription factors. Cardiac transcription factors regulate cardiac specification and differentiation of cardiac cell types by controlling the expression of various cardiac genes that code for structural proteins or regulatory proteins. From Drosophila to man, cardiac specification and differentiation is guided by a complex transcriptional regulatory network that include the GATA family of transcription factors, cardiac-specific homeobox transcription factor (Csx/Nkx2.5), MEF2 transcription factors and the T-box transcription factors. The roles of GATA and the Nkx2.5 transcription factors are discussed below as examples of the complex roles of transcription factors in cardiomyocyte differentiation

1.13 GATA TRANSCRIPTION FACTORS

GATA transcription factors belong to a family of proteins that contain either one or two highly conserved zinc finger DNA-binding domains. The family name "GATA" is based on the a recognition of the consensus DNA sequence (T/A)GATA(A/G) (Orkin, 1992; Lowry and Atchley, 2000). Within the promoters and enhancers of target genes, the GATA family members bind this consensus sequence with high efficiency and specificity (Orkin, 1992). The zinc finger motif in the GATA DNA binding domain is in the form $Cys-X_2-Cys-X_{17-20}-Cys-X_2-Cys$ followed by a basic region (Patient and McGhee, 2002). In animals, where GATA factors have been shown to play critical roles in development, differentiation and control of cell proliferation, the GATA DNA binding domain adopts the form $Cys-X_2-Cys-X_{17}-Cys-X_2-Cys$ (Patient and McGhee, 2002). The C-terminal zinc finger and the adjacent basic domain are required for DNA consensus sequence recognition and binding to the DNA (Tsai *et al.*, 1989; Lee *et al.*, 1991; Morrisey *et al.*, 1996) (Figure 1.13).

Six members of the GATA family of transcription factors (GATA-1 to -6) have been identified in vertebrates and a wide range of other eukaryotic organisms including plants (Reyes *et al.*, 2004), insects, and echinoderms (Lowry and Atchley, 2000) and fungi (Maxon and Herskowitz, 2001). Based on sequence homology and expression pattern, GATA transcription factors have been divided into two subgroups. The first subgroup of GATA proteins (GATA-1, -2 and -3) plays important roles mainly in haematopoiesis. GATA-2 is indispensable for development of definitive haematopoiesis (Mouthon *et al.*, 1993; Labbaye *et al.*, 1995; Orlic *et al.*, 1995; Tsai and Orkin, 1997;

Shimizu and Yamamoto, 2005). The second subgroup includes GATA-4, -5
and -6. These are found predominantly in the heart, digestive system and the
extraembryonic endoderm. GATA-4, -5 and -6 bind to similar consensus
sequences in cardiac genes (Charron *et al.*, 1999). There are confirmed
phosphorylation sites in GATA -4 (but not in GATA -5 or -6) (Grepin *et al.*,
1994; Ip *et al.*, 1994; Jiang and Evans, 1996) (Figure 5).
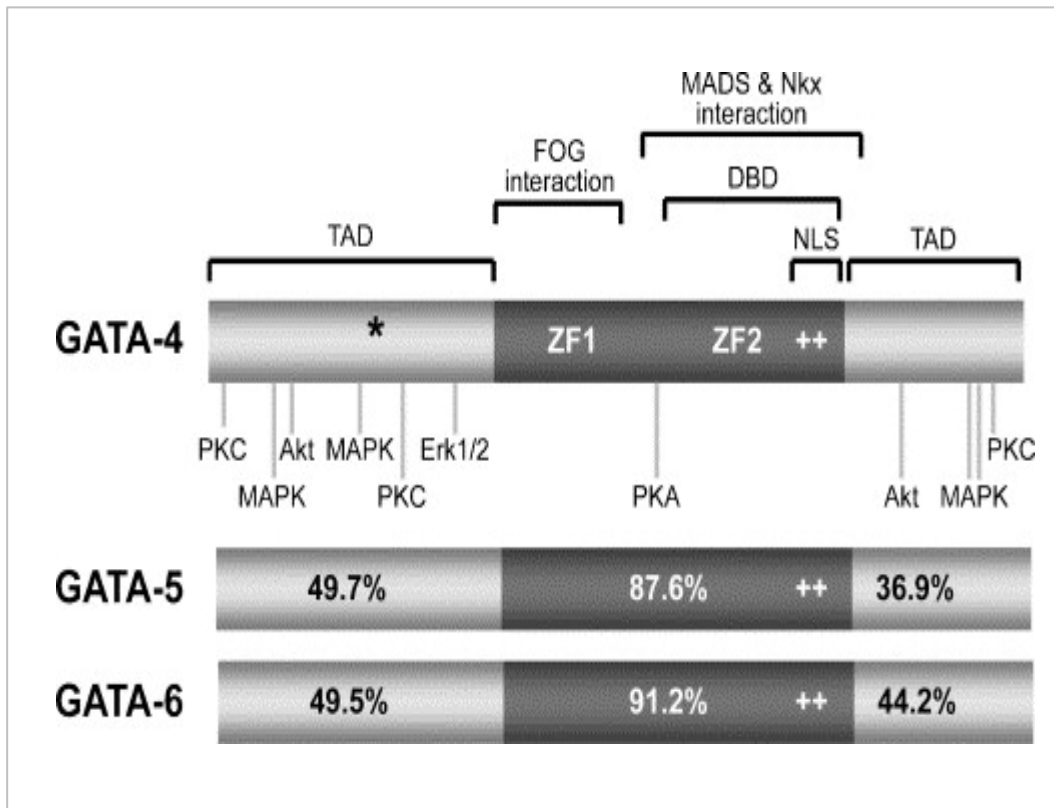
Figure 1.13: Structural similarity among the GATA transcription factors. Domains and the putative phosphorylation sites with their respective kinases are shown. The * denotes a confirmed MAPK phosphorylation site. The percentage shown represents the degree of homology for each protein in comparison to GATA-4. ZF = zinc finger; TAD = transactivation domain; DBD =DNA binding domain; NLS = nuclear localisation signal, FOG = Friend of GATA) and MADS is an acronym that refers to the genes in which the sequence element was first identified: <u>M</u>CM1 (minichromosomal maintenance protein 1) in *Saccharomyces* cerevisiae, <u>A</u>gamous from Arabidopsis thaliana, <u>D</u>eficiens from the snapdragon *Antirrhinum* majus and <u>S</u>RF from human. Taken from Temsah *et a*l. (Temsah *et al.*, 2005)

1.13.1 Roles of GATA transcription factors in cardiogenesis

In the developing embryo, the expression of GATA-4, -5 and -6 is detected and persists later in the developing heart. GATA-6 gene expression is restricted to the precardiac mesoderm, the embryonic heart tube and the primitive gut (Morrisey *et al.*, 1996). GATA-4 and GATA-6 transcript localisation overlap throughout the developing cardiogenic region (Jiang and Evans, 1996). GATA-5 is also expressed in cardiac crescent prior to formation of the primordial heart tube. After the tube heart, the expression of GATA-5 is then seen in both endocardium and myocardium (Laverriere *et al.*, 1994). GATA-4 is a potent transcriptional regulator of the promoters of several cardiac specific genes including ANP, BNP (Grepin *et al.*, 1994; Charron *et al.*, 1999; McBride and Nemer, 2001), α-MHC, cTnI (Molkentin *et al.*, 1997; Murphy *et al.*, 1997; Di Lisi *et al.*, 1998; Charron *et al.*, 1999), and cTnC (Ip *et al.*, 1994)and MLC (McGrew *et al.*, 1996). All the above are strong evidence to suggest that these transcription factors play key roles in cardiogenesis.

1.13.2 Role of GATA-4 in cardiogenesis

Homozygous GATA4 null (GATA-4$^{-/-}$) mice arrested and died between 7.0 and 10.5 days post coitum (dpc) because of severe developmental abnormalities including ventral foregut closure and cardiac bifida (Kuo *et al.*, 1997; Molkentin *et al.,* 1997; Xin *et al.,* 2006). The GATA-4$^{-/-}$ embryos showed severe defects in the "rostral-to-caudal" and "lateral-to-ventral folding" (Kuo *et al.*, 1997). The embryos of these GATA-4$^{-/-}$ mice developed splanchnic mesoderm that differentiated into primitive cardiomyocytes that expressed

contractile proteins (Kuo *et al.,* 1997; Molkentin *et al.*, 1997). However the GATA-4 null embryos lacked primitive heart tubes because the procardiomyocytes failed to migrate to the ventral midline to form a linear heart. The GATA-4$^{-/-}$ cardiomyocytes and endocardial cells were still able to populate the hearts of GATA-4$^{-/-}$-C57BL/6 chimeric mice(Kuo *et al.*, 1997). The two bilaterally symmetric promyocardial primordia in the mutants did not migrate ventrally but remained lateral and formed two independent heart tubes containing differentiated cardiomyocytes (Molkentin *et al.*, 1997).These results suggest a role for GATA-4 in cardiac morphogenesis but not in cardiomyocyte lineage specification.

Small interfering RNAs (siRNA) that specifically target GATA-4 in cardiac mesodermal cells suppressed the expression of N-cadherin mRNA   without affecting the expression of the other cardiac marker mRNAs however it caused cardia bifida in chick embryos (Zhang *et al.*, 2003a). The selective suppression of N-cadherin, one of the genes essential for the single heart formation, with the lack of effect on cardiac specific markers by GATA-4 siRNA also suggests that GATA-4 may not be essential for cardiomyocyte commitment during cardiogenesis.

Similarly, the inhibition of GATA-4 using GATA-4 antisense transcripts blocked P19 stem cell cardiac differentiation at the premyocardial (cardioblast) stage but brachyury T was also found to be expressed. Brachyury T   is a T-box transcription factor (Kispert *et al.*, 1995) and is necessary for the establishment of the mesoderm in vertebrates (Schulte-

Merker *et al.*, 1992). Mesodermal lineage gives rise to cardiomyocytes. The expression of brachyury T suggests that mesodermal lineage commitment occur in the absence of GATA-4 (Grepin *et al.*, 1997) (Grepin *et al.*, 1997). The expression of brachyury T in absence of GATA-4 is further strong supporting evidence that GATA-4 is not essential for the initiation of cardiac lineage commitment.

Zebrafish embryos depleted of GATA-5 and GATA-6 have no heart. In contrast, embryos depleted of GATA-4 in the presence of either functional GATA-5 or GATA-6 develop defective heart tubes (Holtzinger and Evans, 2007) rather than total blockage of cardiomyocyte specification. These results also support an essential role for GATA-4 in cardiac morphogenesis but not in cardiomyocyte lineage specification.

All the above data when considered together, suggest that GATA-4 may be required for the events that control normal cardiac morphogenesis after the early stages of cardiomyocyte commitment but may not be essential for cardiomyocyte commitment during the early stages of cardiogenesis. The lack of normal cardiac morphogenesis in the absence of functional GATA-4 but in the presence of intact GATA-5 and GATA-6 also suggest that there may not be functional redundancy between GATA-4 and GATA-5 or between GATA-4 and GATA-6.

1.13.3 Role of GATA-5 and GATA-6 in cardiogenesis

GATA-6 is expressed at the blastocyst stage in part of the inner cell mass and in the trophectoderm (Koutsourakis *et al.*, 1999). This suggests that GATA-6 may have a role to play in the very early events of heart development. Inactivation of GATA-6 gene in mice is lethal at gastrulation, with differentiation defects in the ectoderm and part of the visceral endoderm (Koutsourakis *et al.*, 1999). GATA-6 is expressed in cardiac mesoderm before gastrulation (Peterkin *et al.*, 2003). In both *Xenopus* and zebrafish embryos, the injection of anti-sense oligonucleotides to specifically block the translation of GATA-6 protein severely reduced the expression of cardiac proteins in embryos (Peterkin *et al.*, 2003).

The fact that zebrafish embryos lacking GATA-5 and GATA-6 did not develop hearts (i.e. lack of cardiomyocyte specification) (Holtzinger and Evans, 2007) but those lacking only GATA-4 in the presence of a functional GATA-5 or GATA-4 can developed defective tubular hearts (i.e. cardiomyocyte specification) suggest that GATA-5 or GATA-6 may be essential and also perform redundant functions during cardiomyocyte specification. Similarly, the ablation of both GATA-5 and GATA-6 resulted in complete lack of the expression of Nkx2.5, vMHC and MLC-2 in zebrafish embryos (Peterkin *et al.*, 2007), thus also suggesting important roles for either GATA-5 or GATA-6 or both. Embryos null for both GATA-4 and GATA-6 ($GATA4^{-/-}GATA6^{-/-}$) completely lack hearts (Zhao *et al.*, 2008).These studies point to non-essential roles for GATA-4 but possible essential roles for GATA-5 or GATA-6. It is beginning to emerge that, in contrast to the more prominent roles that

are attributed to GATA-4 in literature, GATA-5 or GATA-6 rather than GATA-4 may have essential roles to play in the induction of cardiomyocyte differentiation.

1.13.4 Cooperative Interaction between GATA-4 and GATA-6

No single GATA transcription factor has been found to be both necessary and sufficient for cardiogenesis or cardiomyocyte specification. This along with their co-expression and localisation, developmental regulation during cardiogenesis and their structural similarities had led to the suggestion of possible functional redundancy among cardiogenic GATA factors. However instead of a possible redundancy of GATA-4 and GATA-6 function, several studies (as discussed above) point to a possible cooperative interaction between GATA-4 and GATA-6. GATA-4 and -6 have similar potency in activating cardiac genes including ANP, BNP (Charron *et al.*, 1999) and troponin C (Morrisey *et al.*, 1996). Furthermore the down-regulation of either GATA-4 or GATA-6 proteins in primary cardiomyocytes resulted in reduced mRNA expression of several cardiac genes including BNP, α-MHC, β-MHC and cTnI (Charron *et al.*, 1999). Mice that are heterozygous for either a GATA4 or GATA6 null allele are normal but compound heterozygosity of GATA4 and GATA6 is lethal by E13.5 (Xin *et al.*, 2006). These results suggest a possible cooperative interaction between GATA-4 and GATA-6 the regulation of cardiac specific gene expression.

1.13.5 Partners or Targets for GATA-4

GATA-4 interacts with Nkx2.5, a key cardiac transcription factor. ANP gene is a direct downstream target for Nkx2.5 and DNA binding sites for this transcription factor are found in the ANP promoter (Durocher *et al.*, 1996). GATA-4 physically associates with Nkx2.5 to cooperatively activate the transcription of cardiac-restricted genes including ANP (Durocher *et al.*, 1996; Durocher *et al.*, 1997; Durocher and Nemer, 1998; Lee *et al.*, 1998; Sepulveda *et al.*, 1998). BMP activation of cardiac-specific proteins appears to be mediated by the transcription factors GATA-4 and Nkx-2.5. Application of BMP-2 *in vivo* induced ectopic expression of Nkx2.5 and GATA-4, 5 and 6 (Merika and Orkin, 1993; Zhu *et al.*, 1997; Svensson *et al.*, 1999).

More supporting evidence for crosstalk between GATA-4 and Nkx2.5 comes from experimental data which showed that simultaneous over-expression of Nkx2.5 and GATA-4 in P19Cl6[noggin] cells induced cardiac differentiation while differentiation was not observed when either Nkx2.5 or GATA-4 alone was over-expressed in P19Cl6noggin cells (Monzen *et al.*, 1999). P19Cl6noggin over-express the noggin gene. The noggin gene codes for a family of secreted proteins that bind to BMP and inhibit their interaction with their receptor thus blocking their signalling roles (Zimmerman *et al.*, 1996).

Besides Nkx2.5, GATA-4 also associates with other transcription factors including NFAT(Nuclear Factor of Activated T cells) (Suzuki *et al.*, 1999; Diedrichs *et al.*, 2004; Jiang *et al.*, 2007), SRF (Belaguli *et al.*, 2000; Moore *et al.*, 2001), p300 (Slepak *et al.*, 2001; Yanazume *et al.*, 2003), MEF2 (Morin *et*

al., 2000) and Friend of GATA-2 (FOG-2) (Svensson *et al.*, 1999). GATA-4 association with these other transcription factors that are targets of several signalling pathways underlies the complex mechanisms by which GATA proteins can regulate many important processes during cardiogenesis.

1.13.6 GATA-4 and Upstream Protein Kinases

Phosphorylation is a key regulatory mechanism in many biological systems. The location of numerous phosphorylation sites in GATA-4 protein (Figure 1.13) suggests that it is a direct downstream target for several kinases, including the MAP Kinases ERK (Liang *et al.*, 2001). It has been shown that RhoA potentiates the transcriptional activity of GATA-4 via a p38 MAPK-dependent pathway that phosphorylates GATA-4 activation domains. It was also shown that GATA binding sites mediate RhoA activation of target cardiac promoters (Charron *et al.*, 2001). Over-expression of TAK1 [a mitogen-activated protein kinase kinase kinase (MAPKKK)] can restore the ability of P19CL6noggin cells to differentiate into cardiomyocytes (Monzen *et al.*, 1999). The over-expression of the dominant negative form of TAK1 in parental P19CL6 cells blocked cardiomyocyte differentiation. While the over-expression of Nkx2.5 or GATA-4 alone can not restore the ability of P19CL6noggin cells to differentiate into cardiomyocytes, the over-expression of both GATA-4 and Nkx2.5 can rescue P19CL6noggin ability to differentiate into cardiomyocytes (Monzen *et al.*, 1999). These results suggest that TAK1, Nkx-2.5 and GATA-4 may cooperatively interact in BMP induced cardiogenic signalling.

1.13.7 Summary for GATA transcription factors

GATA transcription factors are key regulator molecules in animal development. GATA-1, 2 and 3 are relevant in the haematopoietic system while GATA-4, 5 and 6 have roles to play in cardiogenesis. The promoters of many cardiac specific proteins including ANF, BNF, cTnl, α-MHC, MLC contain consensus sequence for GATA-4 in their genes. GATA-4 also interacts with the specific cardiac transaction factor Nkx2.5 and other transcription factors including p300, FOG-2 and NFAT. Great prominence is attributed to the role of GATA-4 in cardiomyocyte commitment but emerging evidence suggests that GATA-4 may not be essential for cardiomyocyte commitment. GATA-4 may play an important role in the events that control normal cardiac morphogenesis after the early stages of cardiomyocyte commitment or cardiogenesis. Increasingly important roles for GATA-5 or GATA-6 or both are also emerging. Finally, all the research data or evidence about the roles of GATA transcription factors in cardiogenesis in vertebrates (e.g. mouse, *Xenopus* and Zebrafish Chicken) strongly supports the existence of critical roles for GATA-4, 5 and 6 during normal cardiogenesis.

1.14 CARDIAC HOMEOBOX TRANSCRIPTION FACTOR

The discovery of the tinman gene in Drosophila contributed enormously to our understanding of some of the molecular events that are involved in the vertebrate heart development. Tinman expression is essential for the specification of cardiac cells and the formation of Drosophila heart (Kim and Nirenberg, 1989; Azpiazu and Frasch, 1993; Bodmer, 1993; Zaffran and Frasch, 2005).

Csx/Nkx2.5 [i.e. Cardiac-Specific homeobox (Csx) or the fifth vertebrate gene identified in the NK2 homeobox gene family (Nkx2.5)] is a member of the NK homeobox gene family that is conserved in evolution. The gene products act as DNA-binding transcriptional activators (Kim and Nirenberg, 1989). Each NK2 protein consists of a highly conserved structure that is composed of an N-terminal TN domain, the homeodomain and the NK-2-specific domain (NK2-SD). Four NK genes (i.e. NK1, NK2, NK3, and NK4) were originally identified in Drosophila. The equivalent of Drosophila tinman gene in mammal is Csx/Nkx2.5 (i.e. Nkx2.5) (Komuro and Izumo, 1993; Lints et al., 1993). Nkx2.5 is a universal marker for early heart development and its homeodomain has a helix-turn-helix motif that binds to DNA sequences. The specific consensus DNA sequence is 5′ T(C/T) AAGTG 3′. Nkx2.5 is expressed in a wide range of species including XNkx2.3 and XNkx2.9 in Xenopus (Tonissen et al., 1994; Evans et al., 1995; Cleaver et al., 1996; Newman and Krieg, 1998), cNkx2.3 and cNkx2.8 in chicken (Buchberger et al., 1996; Boettger et al., 1997; Brand et al., 1997; Reecy et al., 1997), nkx2.3 and nkx2.7 in zebrafish (Lee et al., 1996) and Nkx2.6 in mouse (Biben and

Harvey, 1997; Nikolova *et al.*, 1997). Nkx2.5 is expressed in the human heart (Kasahara *et al.*, 2000; Inga *et al.*, 2005; Zheng *et al.*, 2008a). The loss of function of human NKX2.5 (due to mutations) is associated with congenital heart diseases (Kasahara *et al.*, 2000; Inga *et al.*, 2005).

1.14.1 Role of the transcription factor Nkx2.5 in cardiogenesis

Nkx2.5 is one of the earliest markers of heart field mesoderm in Drosophila and vertebrates. In the mouse, the expression of Nkx2.5 starts early (about 7.5 dpc) in the precardiac mesoderm. This early expression of Nkx2.5 in the heart progenitor cells in the first heart field (FHF) and the second hearts field (SHF) continues at a high level in the heart through adulthood (Komuro and Izumo, 1993; Lints *et al.*, 1993; Lee *et al.*, 2004). The specification of cardiogenic cells and the development of the Drosophila dorsal vessel (the insect equivalent of the mammalian heart) requires tinman (Kim and Nirenberg, 1989; Azpiazu and Frasch, 1993; Bodmer, 1993). Drosophila with mutated tinman are unable to express myosin and the precardiac markers, zfh-1 and eve in the heart (Azpiazu and Frasch, 1993; Bodmer, 1993). Wild-type tinman expression can partially rescue the mutant phenotype, but widespread expression of tinman does not result in ectopic expression of cardiac markers (Bodmer, 1993). This suggests that tinman alone is not sufficient to initiate the regulatory pathway leading to heart formation. Tinman may require the cooperation of additional regulatory proteins. Indeed as already mentioned above Nkx2.5 needs cooperative interaction with other factors such as GATA-4. Cell culture studies suggest that the Nkx2.5 protein

may act together with other transcription factors to regulate the expression of cardiac genes. For example, the ability of Nkx2.5 to activate transcription from the cardiac α-actin promoter is strongly increased by the presence of the MADS domain protein, serum response factor (SRF) (Chen and Schwartz, 1996). Even higher levels of transcriptional activation are achieved from this promoter when Nkx2.5, SRF, and the zinc finger protein GATA-4 are all present in the same cell (Sepulveda *et al.*, 1998).

The over-expression of either XNkx2.5 or XNkx2.3 in *Xenopus* (Cleaver *et al.*, 1996) or zebrafish (Chen and Fishman, 1996) embryos caused enlarged hearts which were perfectly normal in all other features. The enlarged heart was due to the thickening of the myocardium caused by an increase in the overall number of myocardial cells (hyperplasia) (Cleaver *et al.*, 1996). On the other hand the expression of inhibitory or dominant negative mutants of either XNkx2.3 or XNkx2.5 in the *Xenopus* embryos resulted in complete lack of cardiac gene expression and complete absence of heart formation (Fu *et al.*, 1998; Grow and Krieg, 1998). This inhibition can be rescued by expression of wild-type Nkx2.5 (i.e. tinman) sequences (Grow and Krieg, 1998) thus underlining the importance of the role of Nkx2.5 in cardiogenesis. The gain-of-function results by Nkx2.5 over-expression and the inhibitory effects of dominant negative Nkx2.5 suggest that Nkx2.5 has an important role in cardiogenesis.

Nkx2.5 knockout mice die *in utero* shortly after looping morphogenesis. Heart tube formation was normal in mutant embryos, but looping morphogenesis, a

critical determinant of heart formation was not initiated at the linear heart tube stage (8.25-8.5 days p.c.) (Lyons *et al.*, 1995). It has also been found that hearts of homozygous null embryos underwent looping but showed arrest of cardiac development and poor development of blood vessels (Tanaka *et al.*, 1999). Commitment to the cardiac muscle lineage was not compromised (Lyons *et al.*, 1995). The ventricular specific marker, MLC-2v was not expressed in mutant hearts or in mutant ES cell-derived cardiomyocytes, but β-MHC, another ventricular specific marker was expressed in the mutant mouse hearts. Cardiac development is multi-step process. The arrest of cardiac development in the Nkx2.5 knockout mice suggests that Nkx2.5 may be required for the expression of several essential factors in the early stages of the developing heart. However the expression of β-MHC in the mutant hearts indicated that not all ventricular-specific genes depend on Nkx2.5 for their expression (Lyons *et al.*, 1995). But the data however demonstrate that Nkx2.5 is required for cardiogenesis. In addition, many studies have shown that the lack of Nkx2.5 compromised or reduces the expression of several cardiac specific genes including the transcription factors eHAND (Biben *et al.*, 1997), Msx2, MEF2C, N-Myc (Tanaka *et al.*, 1999), CARP (Zou *et al.*, 1997) and Irx4 (Bruneau *et al.*, 2000) and many other cardiac genes including ANF, BNP, MLC-2v (Lyons *et al.*, 1995; Tanaka *et al.*, 1999). Cardiac transcription factors are critical players in cardiogenesis and the fact that many may be under the control of Nkx2.5 is an indication of the importance of Nkx2.5 in this process.

1.14.2 Nkx2.5 role in stem cell differentiation into cardiomyocytes

P19 stem cells differentiate into cardiomyocytes in 1% DMSO (McBurney *et al.*, 1982; Habara-Ohkubo, 1996; Angello *et al.*, 2006). The expression of a dominant-negative mutant of Nkx2.5 blocked this differentiation (Jamali *et al.*, 2001). The inability of the cells to form cardiomyocytes was accompanied by the loss of endogenous expression of cardiac genes including GATA-4 and MEF2C. This indicated that the function of Nkx2.5, as well as being critical for cardiogenesis *in vivo*, is also critical for stem cell differentiation into cardiomyocytes.

1.14.3 Summary for the Roles of the Homeobox Nkx2.5

Research data from Drosophila, mouse, *Xenopus* and zebrafish support important regulatory roles for Nkx2.5 in cardiogenesis and the functions of the normal heart. Nkx2.5 interacts with other transcription factors including GATA transcription factors (as discussed earlier) and T-box factors (discussed below) and serum response factor. Nkx2.5 is also involved in the roles of cardiogenic extracellular signalling proteins including BMPs and Wnts (discussed below). Results from both loss and gain of function experiments have established Nkx2.5 as a very important cardiac transcription factor and support a transcriptional regulatory role of Nkx2.5 over a cascade of other cardiac transcription factors and cardiac specific genes.

1.15 T-BOX TRANSCRIPTION FACTORS

The T-box proteins are mainly of importance during the specification of the chamber myocardium after the linear tube heart has been formed. Their roles come into play after cardiac lineage commitment. They are therefore discussed very briefly in this introduction.

The T-box proteins belong to a family of transcription factors that are essential for early cardiac lineage determination, chamber specification and valvuloseptal development. This protein family is characterised by the presence of a highly conserved DNA-binding domain (i.e. the T-box). Seventeen T-box proteins been described in vertebrates (Naiche *et al.*, 2005). Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20 are expressed in vertebrate embryonic hearts and play specific roles in the heart development.

1.15.1 Role of T-box transcription factors in cardiogenesis

The T-box proteins are conserved regulators of heart development from Drosophila to human and other vertebrates. Drosophila Dorsocross complex (Doc), the three linked T-box genes (equivalent to mammalian Tbx4/Tbx5/Tbx6) are critical for Drosophila cardiac specification. Doc is required for the Drosophila cardiac specification (Reim and Frasch, 2005). Drosophila T-box genes midline and H15 pannier (i.e. equivalent to mammalian Tbx20) participate in cardiac specification in determining cardioblast differentiation, polarity and patterning (Miskolczi-McCallum *et al.*, 2005; Reim *et al.*, 2005). Midline represses Doc in a subset of cardioblasts,

and this is crucial for establishing the morphological and functional characteristics of the distinct classes of muscle cells, one functioning in the propulsion of haemolymph and another acting as valves (Reim *et al.*, 2005).

1.15.2 Mechanisms of action of T-Box transcription factors

T-box proteins perform their functions by combinatorial and cooperative interaction with other transcription factors. Tbx1 and Tbx2 (Bruneau *et al.*, 2001; Habets *et al.*, 2002; Xu *et al.*, 2004a) and Tbx5 (Hiroi *et al.*, 2001) can cooperatively interact with Nkx2.5. Tbx5 can also cooperatively interact with GATA-4 (Garg *et al.*, 2003). In the developing hearts, Tbx20 directly interacts with Nkx2.5, GATA-4 and GATA-5 in regulation of gene expression in the developing heart (Stennard *et al.*, 2003). Drosophila Doc, like the mammalian T-box proteins, also functions in association with two Drosophila transcription factors, tinman (i.e. equivalent to mammalian Nkx2.5) and pannier (i.e. equivalent to mammalian GATA-4), during cardiac specification (Reim and Frasch, 2005).

1.15.3 Roles of Tbx2 and Tbx5 in heart chamber development

Mouse Tbx5 is expressed in the cardiac crescent, indicating its involvement in the earliest stages of cardiac induction, then in a graded fashion along the heart tube with the highest levels in the sinoatrial region (Bruneau *et al.*, 1999). In Tbx5 null embryos, the left ventricular and sinoatrial regions are severely hypoplastic   (Bruneau *et al.*, 2001) . However the formation of the

linear heart tube suggests that Tbx5 may be essential for regulation of chamber-specific gene expression and morphogenesis of the early heart but is not essential for cardiac lineage specification.

Tbx2 functions as a transcriptional repressor in non-chamber myocardium zones (i.e. outflow tract, inflow tract and AVC) of the tube heart in order to restrict chamber myocardium formation. Tbx2 cooperatively interacts with Nkx2.5. Their combinatorial interaction represses the expression of chamber myocardium specific genes in non-chamber myocardium zones (i.e. outflow tract, inflow tract and AVC) of the tube heart. ANF gene (first marker for chamber formation) is specifically repressed by Tbx2/Nkx2.5 interaction (Charron *et al.*, 1992; Habets *et al.*, 2002; Harrelson *et al.*, 2004; Cai *et al.*, 2005).

In contrast to Tbx2, Tbx5 combinatorial interaction with Nkx2.5 activates the expression of chamber myocardium specific genes in chamber myocardium zones (i.e. left ventricle and left atrium) of the tube heart (Bruneau *et al.*, 2001; Habets *et al.*, 2002). ANF gene is specifically induced by Tbx5/Nkx2.5 interaction in chamber myocardium forming zones (left ventricle and left atrium) of the tube heart (Bruneau *et al.*, 2001). The opposing actions of Tbx2 and Tbx5 are required at various cardiac developmental stages for the correct formation of the heart.

EXTRACELLULAR SIGNALLING PROTEINS AND STEM DIFFERENTIATION INTO CARDIOMYOCYTES

1.16 ROLES OF EXTRACELLULAR SIGNALLING PROTEINS IN STEM DIFFERENTIATION INTO CARDIOMYOCYTES

Extracellular signalling factors are central for stem cell differentiation or for remaining in the undifferentiated state. Stem cell cardiomyocyte lineage commitment involves complex networks of extracellular signalling molecules that activate various signalling pathways. These activated signal transduction pathways in turn exert complex positive or negative controls on this process.

Well known peptide growth factors or extracellular signalling molecules that have been identified as essential regulators during the development of vertebrate heart are also involved in stem cell differentiation into cardiomyocytes. These include the bone morphogenetic proteins (BMP) (Zhang and Bradley, 1996; Schlange *et al.*, 2000), sonic hedgehog (SHH) (Yamagishi *et al.*, 2003; Hu *et al.*, 2004), fibroblast growth factor (FGF) (Kelly *et al.*, 2001; Vitelli *et al.*, 2002), Wnt (Ku and Melton, 1993; Schneider and Mercola, 2001; Garriock *et al.*, 2005) and Notch proteins (Loomes *et al.*, 2002; Timmerman *et al.*, 2004). The role of BMP and Wnts in cardiogenesis and stem cell differentiation into cardiomyocytes are discussed as examples of the essential roles of these extracellular signalling factors in cardiomyocyte lineage commitment

1.17 BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily of structurally related secreted proteins that are expressed in lateral endoderm and ectoderm and play central roles in the induction of heart formation in vertebrate embryos. The other members of this superfamily are TGF-β, activins, nodal, anti-Mullerian hormone (AMH) and myostatins. BMPs were originally identified as proteins that induce ectopic bone and cartilage formation *in vivo* (Wozney *et al.*, 1988). BMP and its family members play crucial roles during embryonic development, cell division or proliferation, differentiation, organisation migration, adhesion, extracellular matrix production and apoptosis.

BMPs exert their biological effects by binding to heteromeric complexes of two types of receptors (types I and II receptors) with intrinsic serine/threonine kinase activity and activation of specific downstream intracellular effectors known as Smad proteins (Wrana *et al.*, 1994). Smads relay the signal from the cell membrane to the nucleus, where they affect the transcription of target genes.

BMP type II and I receptors are cell surface receptors. BMP type II receptors are single transmembrane proteins with glycosylated cysteine-rich extracellular ligand binding domains and cytoplasmic domains with intrinsic serine/threonine kinase activity(Lin *et al.*, 1992). Type I receptors share a glycine/serine residue-rich stretch (GS-domain) in the juxtamembrane region, which is essential for type I receptor activation (Wrana *et al.*, 1994). BMP bind

weakly to type I or type II receptors alone. BMPs bind to heteromeric type I/type II receptor complex with high affinity (ten Dijke *et al.*, 1994; Liu *et al.*, 1995; Rosenzweig *et al.*, 1995). BMP signalling occurs via at least two pathways: MAPK and Smad pathways.

1.17.1 BMP receptor Intracellular signalling and Smad proteins

The Smad (Similar To Mothers Against Decapentaplegic) transcription factors are the major intracellular signalling molecules or effectors of BMP-induced signalling pathway (Canalis *et al* 2003; Shi and Massague 2003; Kawabata *et al* 1998). BMP, TGF-β and other family members induce their membrane receptors to directly activate the Smads which then form transcriptional complexes to control the expression of target genes. Eight different Smad proteins (Smads 1 through 8) have been identified in mammals. Five of the mammalian Smads (Smad 1, 2, 3, 5 and 8) act as substrates for the TGF-β receptor family. These five Smads are known as receptor-regulated Smads (R-Smads). R-Smads 1, 5 and 8 serve mainly as specific substrates for activated BMP and Muellerian type I receptors while R-Smads 2 and 3 serves as specific substrates for activated TGF-β, activin, and Nodal type I receptors. Smad 4 serves as a type I receptors common mediator or common partner Smad (Co-Smad) for all R-Smads. R-Smads activation by BMP receptors leads to their heterodimerization with the Co-Smad (i.e. Smad 4). Smad 6 and 7 are inhibitory Smads (I-Smad) that serve as decoys interfering with Smad–receptor or Smad–Smad interactions. In addition to this intracellular regulation by I-Smads, BMP signally is also regulated at the receptor level by

extracellular proteins (e.g. Noggin) that directly bind BMP molecules and inhibit their interaction with the BMP receptors.

Upon BMP-induced heteromeric complex formation, the constitutively active serine/threonine kinase of type II receptor phosphorylates type I receptor in its intracellular domain. Phosphorylation activate the kinase activity of type I receptor. The activated type I receptor phosphorylates and activates target proteins of the R-Smads (Wrana *et al.,* 1994; Chen and Weinberg, 1995). The phosphorylation of R-Smad causes a conformational change, which promotes heterodimerization with Smad4. The R-Smad/Smad4 complex translocates to the nucleus. Within the nucleus R-Smad/Smad4 bind to specific sequences in the promoters of BMP target genes and control their expression in a cell-type specific manner (Figure 1.14).
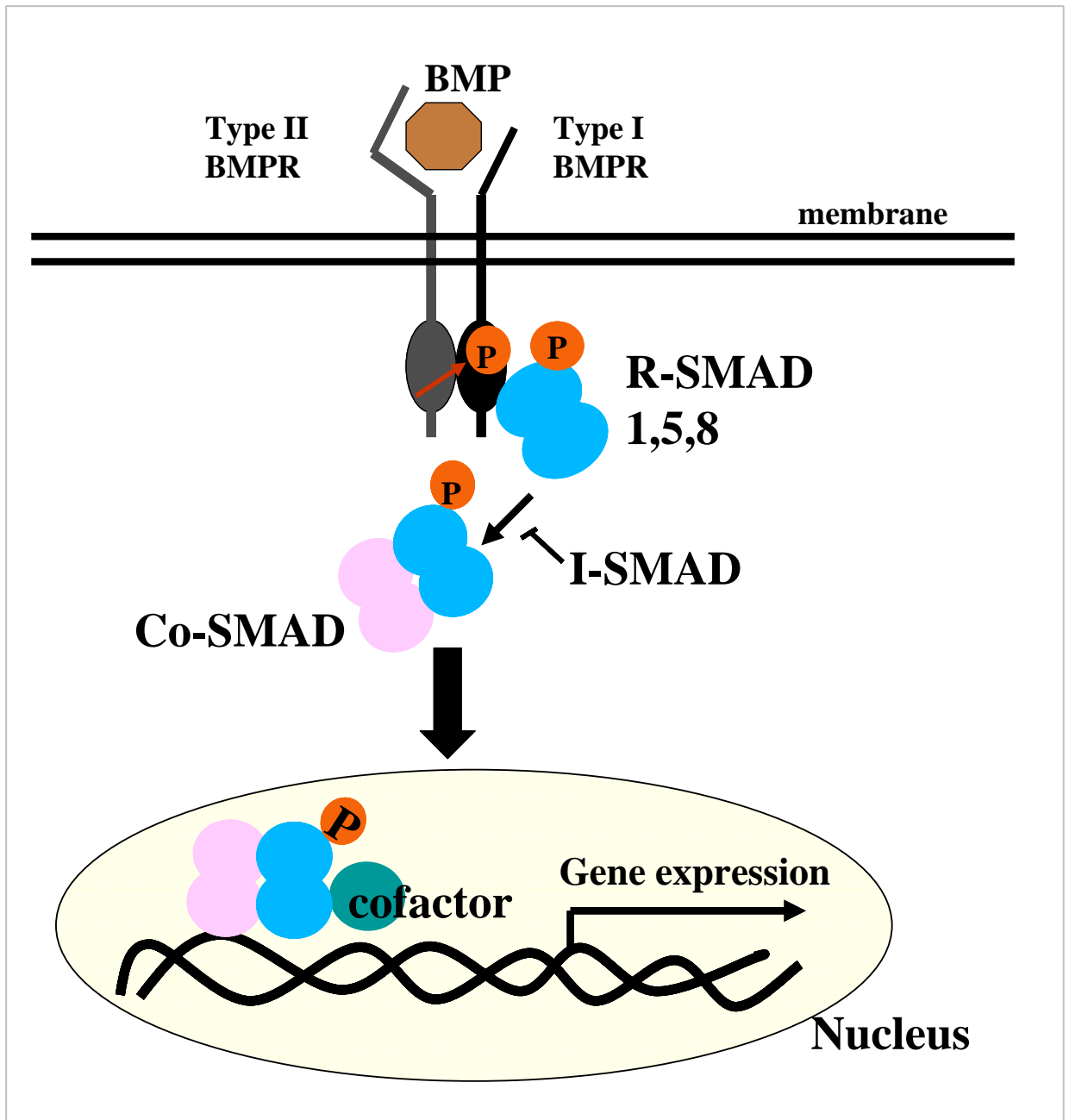
Figure 1.14: BMP signalling pathway. BMP bind and form a heteromeric complex with type I and type II BMP receptors. The type II receptor phosphorylates the type I receptor. This leads to the phosphorylation of receptor-regulated smads (SMAD1,5 or 8). The phosphorylated SMAD forms a complex with the common mediator or common partner Smad (SMAD4). This complex is then and is transported into the nucleus leading to the activation of target genes. The intracellular inhibitor of BMP signal transduction is SMAD6. BMP= bone morphogenetic protein, R-Smads= receptor-regulated Smads, Co-SMAD = common mediator or common partner Smad, I-SMAD = inhibitory Smads and P= phosphate group.

1.17.2 Role of BMPs in stem cell differentiation into cardiomyocytes

As already stated, BMPs are expressed in lateral endoderm and ectoderm of vertebrate embryos. Stimulatory factors from the anterior endoderm in combination with BMP signals in the anterior lateral mesoderm promote heart formation (Schultheiss *et al.*, 1997; Andrée *et al.*, 1998; Schlange *et al.*, 2000; Tzahor and Lassar, 2001). It has been shown that BMP-2 soaked beads can induce the   ectopic expression of Nkx2.5 and GATA-4 in chick embryos (Schultheiss *et al.*, 1997). BMP-2 can   induce the expression of cardiac specific proteins in chick embryos and the addition of Noggin to explants resulted in loss of the expression of Nkx2.5, GATA-4, eHAND, MEF2A and MHC expression (Schlange *et al.*, 2000). This suggests that BMP-2 is upstream of Nkx2.5 and GATA-4, and as already mentioned, eHAND, MEF2A and MHC are under the control of GATA-4 and Nkx2.5.

DMSO induces the cardiac differentiation of P19CL6 (a derivative of P19 stem cells). Over-expression of noggin (a natural inhibitor of BMP) in P19CL6 cells (P19CL6 [noggin] cells) prevented DMSO-induced differentiation of P19 cells into cardiomyocytes (Monzen *et al.*, 1999). Spontaneously beating cells did not result and cardiac transcription factor and contractile proteins were not present in P19Cl6[noggin] cells. This cardiac differentiation arrest could be rescued by over-expression of BMP-2 or by directly adding BMP proteins to the culture medium (Monzen *et al.*, 1999). The data provide strong evidence for the involvement of BMPs in stem cell differentiation into cardiomyocytes.

1.17.3 Role of BMP and MAPK signalling in cardiomyocyte differentiation

In addition to the Smad pathways discussed above, BMP signalling also occurs via MAPK signalling pathways. The MAPK TGF-β-activated kinase 1 (TAK1) is expressed in P19CL6 cells and therefore could be involved in the DMSO-induced differentiation of these cells into cardiomyocytes. Over-expression of dominant negative TAK1 led to an inhibition P19CL6 cell differentiation into cardiomyocytes while over-expression of wild-type TAK1 or constitutively active TAK1 rescued cardiac differentiation in P19CL6[noggin] cells (Monzen *et al.*, 1999). The above data suggests that BMP-induce activation MAPK signalling can lead to cardiac lineage specification in ES cells.

1.17.4 Role of BMP and Smad Signalling in Cardiomyocyte Differentiation

P19CL6noggin cells, which constitutively over-express the BMP antagonist noggin, do not differentiate into cardiomyocytes when treated with DMSO. Over-expression of both Smad1 and Smad4 has been found to be sufficient to rescue cardiac differentiation in DMSO-treated P19Cl6[noggin] cells (Monzen *et al.*, 2001). The same studies demonstrated that over-expression of the inhibitory Smad6 completely blocked differentiation of P19CL6 (Monzen *et al.*, 2001), thus suggesting that like the BMP-mediated activation of the MAPK signalling pathway, the BMP-activated Smad pathway is also necessary for ES cell differentiation into cardiomyocytes

1.17.5 Crosstalk between Smad and MAPK signalling

Experimental evidence suggests that ATF-2 (activating transcription factor 2) may be a common target for both Smad and TAK1 pathways in TGF-β signalling (Makino *et al.*, 1999). ATF-2 activity is enhanced after phosphorylation by stress-activated protein kinases such as c-Jun N-terminal kinase and p38. ATF-2 has also been found to be bound by Smad3 and Smad4 upon TGF-β stimulation (Makino *et al.*, 1999). It has also been found that the over-expression of dominant negative forms of ATF-2 inhibited the P19CL6 cell differentiation into cardiomyocytes, while wild type ATF-2 enhanced the ability of Smad 1&4 and TAK1 to rescue cardiac differentiation in P19CL6noggin cells (Monzen *et al.*, 2001).

ATF-2 was also found to stimulate β-MHC promoter activity in a synergistic manner with Smad1&4 and TAK1 and promoted terminal cardiomyocyte differentiation of P19CL6noggin, whereas over-expression of the dominant negative form of ATF-2 reduced the promoter activities of several cardiac-specific genes including GATA-4, MEF2C, MLC2v β-MHC, ANP and BNP and inhibited differentiation of P19CL6 (Monzen *et al.*, 2001). These results suggest that Smads, TAK1, and their common target ATF-2 cooperatively play a critical role during the differentiation of stem cells into cardiomyocytes.

1.17.6 Summary of bone morphogenetic proteins

BMP have important roles in both *in vivo* cardiogenesis and the differentiation of stem cells into cardiomyocytes. BMP are expressed very early in the endoderm and ectoderm and play central roles in the induction of heart

formation in vertebrate embryos. In addition to the Smad pathways BMP signalling also occurs via MAPK signalling pathways. This is very important since phosphorylation is a key post-transcriptional mode of gene control. The MAPK signalling when activated could control many other targets including transcription factors and other kinases.

1.18 Wnt Proteins

Wnt proteins belong to a family of cysteine-rich and lipid-modified signalling proteins (Willert *et al.*, 2003). The Wnt signalling pathways are highly regulated and play major roles in embryological development, tissue morphogenesis, homeostasis and regeneration, cell proliferation (van de Wetering *et al.*, 2002) and differentiation, cell fate decisions, apoptosis, axial polarity and axonal guidance.

Wnt signalling cascade is initiated by Wnt binding to the cysteine-rich, extra-cellular domain of a frizzled (Fz) receptor. For the effective Wnt binding, in addition to the Fz receptor, the presence of single-pass transmembrane molecule of the low density lipoprotein receptor-related proteins (LRP) (also known as the arrow protein in Drosophila) (Wehrli *et al.*, 2000) or LRP5/6 (i.e. vertebrate equivalent of Drosophila arrow protein) (Pinson *et al.*, 2000; Tamai *et al.*, 2000) is required. The LRPs are co-receptors to the Fz receptors. The Fz receptors, like the LRPs, are also transmembrane proteins with a long N-terminal extension called a cysteine-rich domain (CRD). Wnt proteins bind directly to the CRD region (Bhanot *et al.*, 1996; Hsieh *et al.*, 1999; Dann *et al.*, 2001).

1.18.1 Wnt Signalling pathways

Wnt binding to Fz receptors can result in the activation of one of three different Wnt regulated pathways: Wnt/β-catenin (canonical signalling

pathway) and two non-canonical Wnt signalling pathways (i.e. the Wnt/planar cell polarity (Wnt/PCP) and Wnt/calcium (Wnt/Ca^{2+}) pathway)

1.18.1.1 Wnt canonical (Wnt/β-catenin) pathway

In the absence of Wnt/receptor interactions, the level of β-catenin is kept low by degradation induced by β-catenin association with a large multiprotein β-catenin destruction complex that includes two scaffolding proteins (i.e. axin and adenomatous polyposis coli (APC)), two serine/threonine kinases β (i.e. glycogen synthase k inase 3β (GSK3β) and casein kinase 1 (CK1) (Giles *et al.*, 2003; Logan and   Nusse, 2004). In this complex, β-catenin is phosphorylated by CK1 and GSK3β. This phosphorylation triggers the ubiquitination of β-catenin resulting in its subsequent degradation in the proteosome.

Wnt binding to Fz and LRP receptor complex induces the phosphorylation of LRP (Tamai *et al.*, 2004; Davidson *et al.*, 2005). A cytoplasmic protein known as Dishevelled (Dsh/Dv1) is also phosphorylated and activated (Figure 1.15). The activation of Dsh leads to the recruitment of Axin to the membrane, where it interacts with the cytoslic tail of LRP. Axin recruitment to the membrane from the multiprotein β-catenin destruction complex (i.e. Axin/APC/CK1/GSK3β) leads to the subsequent disruption or inhibition of the destruction complex. GSK3β is therefore not able to phosphorylate β-catenin. The net effect of GSK3β inhibition is the stabilisation and the accumulation of the non-phosphorylated form of β-catenin in   the cytosol.   The non-

phosphorylated cytosolic β-catenin then translocates into the nucleus, where it competes with members of the groucho family of co-factors for interactions with the T-cell factor/lymphoid-enhancing factor 1 (TCF/LEF) family of transcription factors (Molenaar *et al.*, 1996; van de Wetering *et al.*, 1997; Cavallo *et al.*, 1998; Eastman and Grosschedl, 1999) . Once an active (LEF/TCF)-β-catenin complex is formed the transcription of Wnt target genes is activated (Yamaguchi *et al.*, 1999; Ai *et al.*, 2000). TCF/LEFs bind to a conserved DNA sequence known as the Wnt-response element (WRE) (i.e. C/T-C-T-T-T-G-A/T-A/T) via their HMG domain (van de Wetering *et al.*, 1997). In the absence of Wnt, TCF form a complex with Groucho. This complex acts as transcriptional repressor of Wnt responsive genes (Cavallo *et al.*, 1998; Eastman and Grosschedl, 1999).
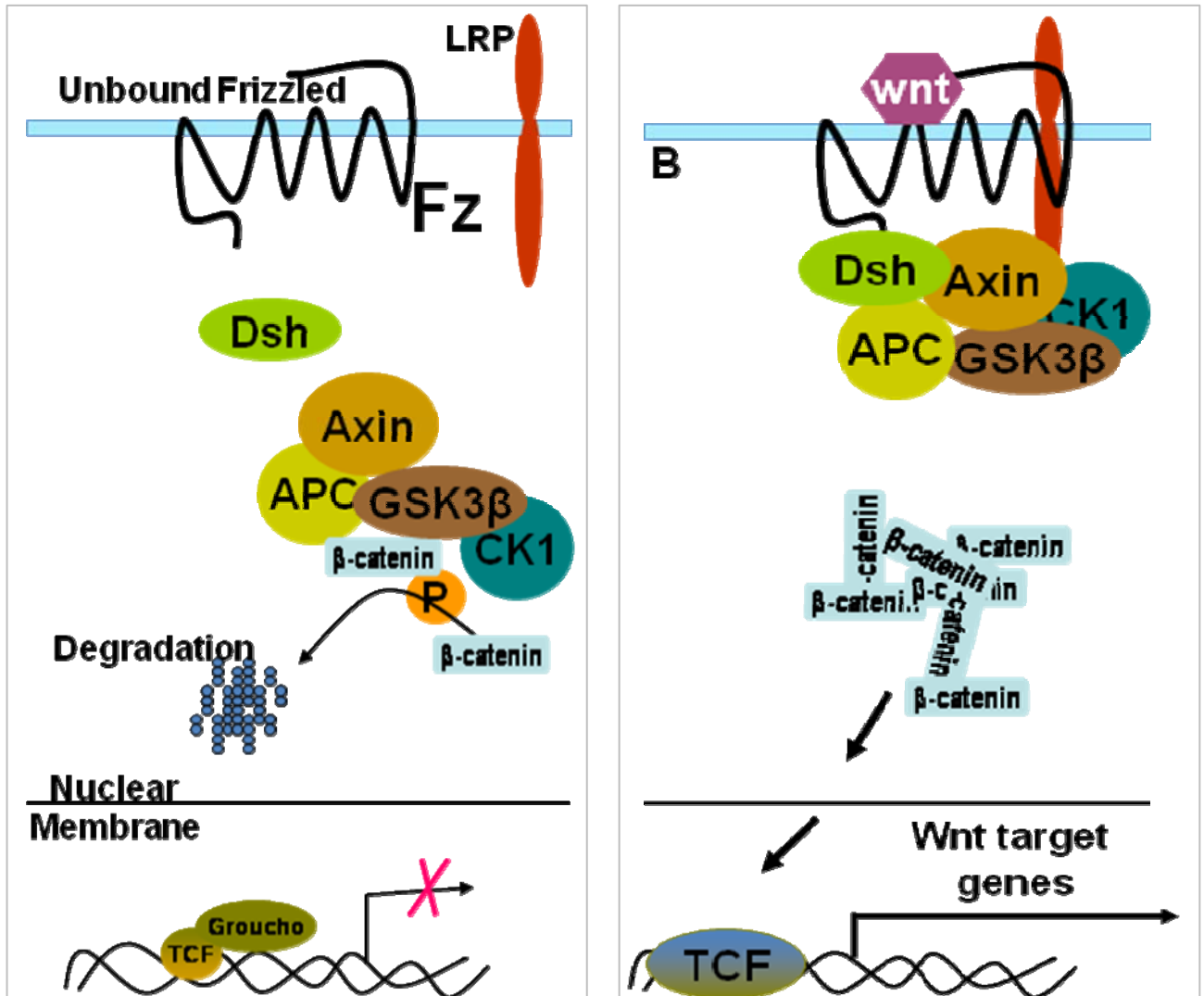
Figure 1.15: Activation of canonical (Wnt/ β-catenin) pathway. **(A)** In the absence of Wnt β-catenin associates with the Axin-APC- GSK3β complex. β-catenin is phosphorylated !and then targeted for degradation. At the same time, Wnt target genes are repressed by the association of TCF with Groucho**. (B)** Wnt binding to the Frizzled and LRP receptors induces phosphorylation of LRP and recruitment of Axin. Dsh is also phosphorylated, and the Axin·APC·GSK-3 complex is inhibited, leading to accumulation of cytosolic β-catenin and its subsequent translocation into the nucleus. In the nucleus β-catenin replaces Groucho from the T-cell factor/lymphoid-enhancing factor 1 (TCF/LEF) and then activates target genes.

1.18.1.2 Non-canonical Wnt/Ca2+ signalling pathway

In addition to the canonical pathway described above, a non-canonical pathway known as the Wnt/Ca^{2+} signalling pathway can also be activated by binding of Wnt proteins to Fz receptors. However unlike canonical pathway, the downstream pathways activated by this binding do not involve GSK-3β or β-catenin. The activation of the Wnt/Ca2+ signalling pathway is thought to involved that activation of G-proteins and phospholipase C (PLC) leading to the release of intracellular Ca^{2+} ions, The increased intracellular calcium levels in turn activate key enzymes such protein kinase C (PKC) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Sheldahl *et al.*, 1999; Pandur *et al.*, 2002; Sheldahl *et al.*, 2003) (Figure 1.16). It has been shown in zebrafish blastulae, that the over-expression of Wnt5a or rat Fz2 (i.e. Frizzled receptor) stimulates the frequency of calcium fluxes in the enveloping layer (EVL) cells (Slusarski *et al.*, 1997). Similarly in *Xenopus* embryos, over-expression of Wnt5a or Wnt1 activated calcium-sensitive kinase protein kinase C (Sheldahl *et al.*, 1999) and calcium/calmodulin-dependent kinase II (CamKII) (Kuhl *et al.*, 2000). In contrast blocking the over-expressed Wnt signalling using antibodies to Frizzled-5 (wnt5a the receptor) was found to inhibit PKC activity (Weeraratna *et al.*, 2002).
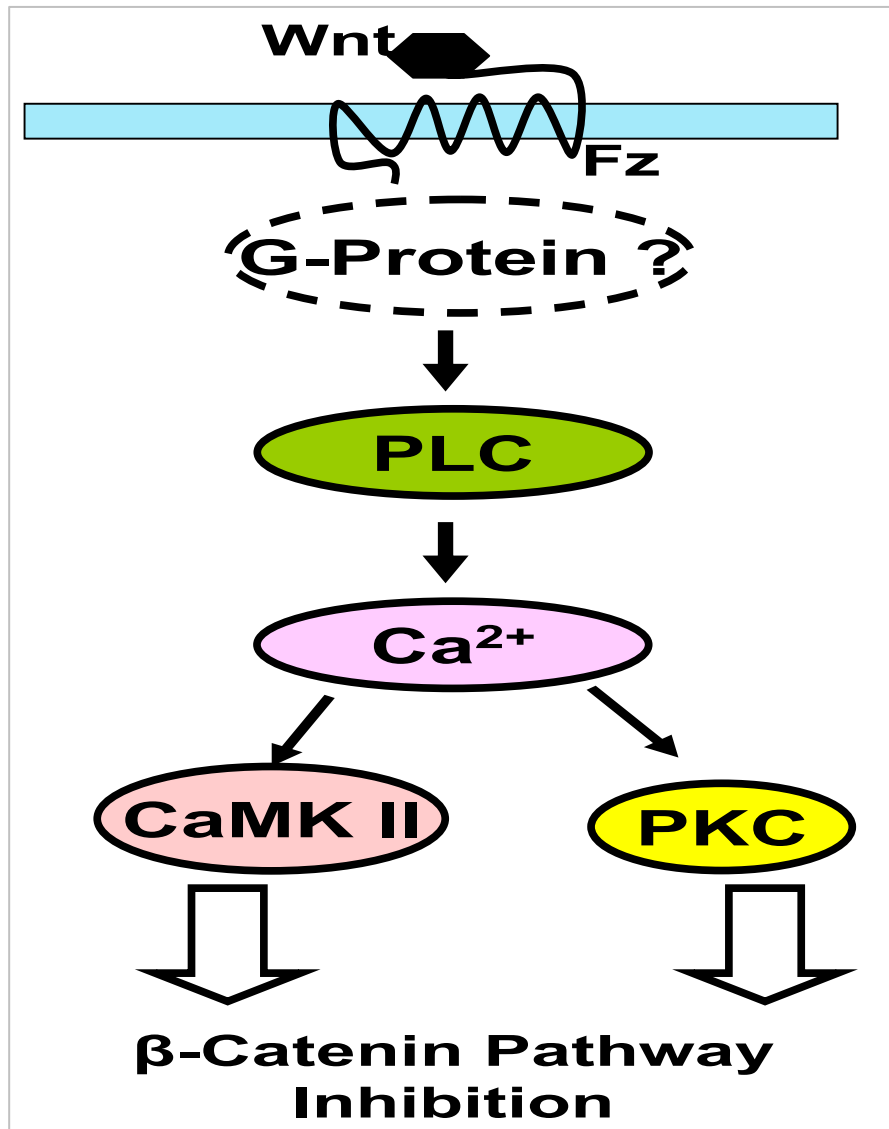
Figure 1.16: Activation of Wnt/ca^{2+} non-canonical pathway. Wnt binding to the receptor stimulates G-proteins which in turn activate phospholipase C. This leads to the release of calcium ions and the activation of protein kinase C and Ca2+/calmodulin-dependent protein kinase II (CaMKII). Signalling events downstream from this stage may lead to the inhibition of the canonical wnt signalling pathway.

1.18.1.3 Non-canonical Wnt/PCP signalling pathway

The second non-canonical signalling is the Wnt/PCP pathway. The Wnt/PCP signalling pathway regulates the generation of planar cell polarity (PCP). Like in the other Wnt pathways, in this non-canonical pathway, Wnt binding to the Fz receptor activates Dsh but in this case, Dsh activation does not lead to either a stabilisation of β-catenin or to an influx or release of intracellular calcium ions. Instead Dsh is localised to the cell membrane where it mediates the activation of small GTPases (RhoA and Rac) which then activate Rho kinase and c-Jun N-terminal kinase (JNK) (Endo *et al.*, 2008) (Figure 1.17).
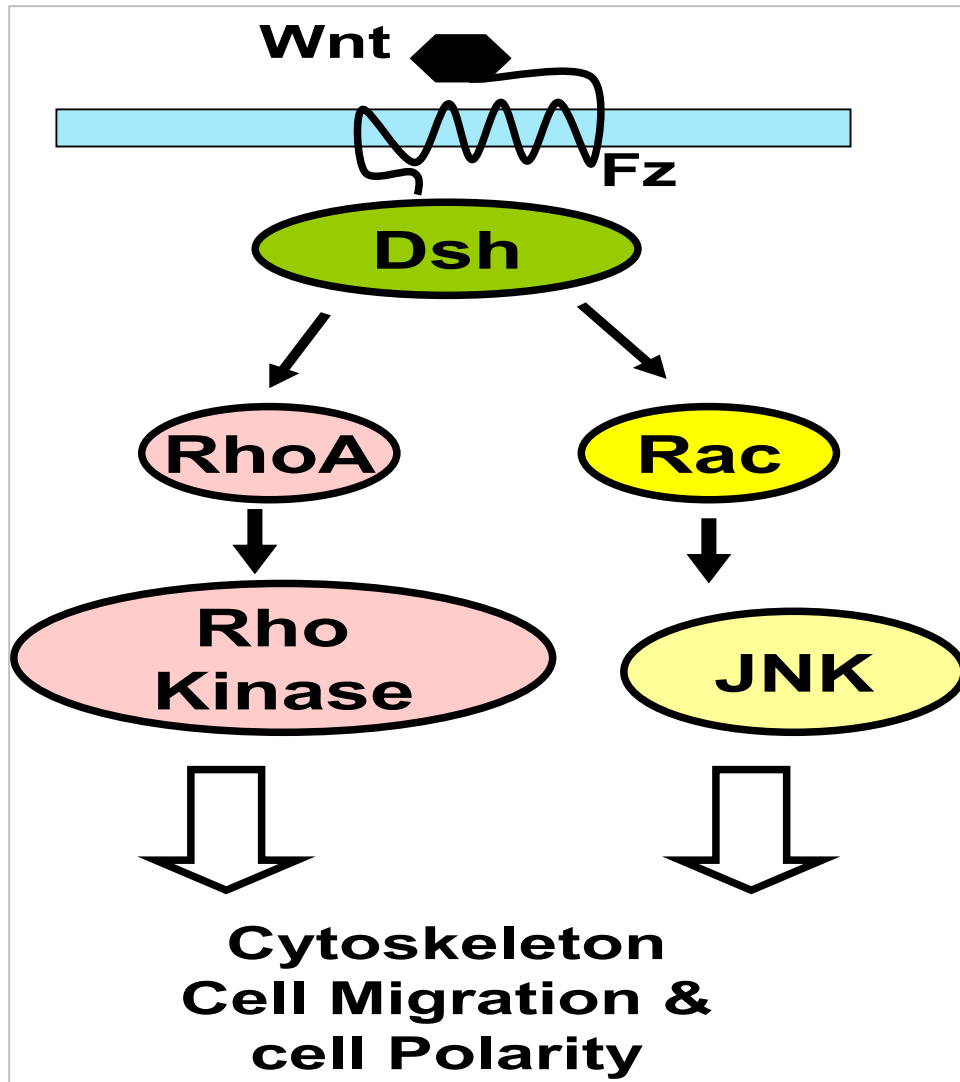
Figure 1.17: Activation of Wnt/PCP non-canonical pathway. Wnt binding to the receptor stimulates Dsh protein which in turn RhoA and Rac. RhoA and Rac respective activation of Rho kinase and JNK is thought to control cell migration and polarity and the cytoskeleton.

1.18.2 Classes of Wnt proteins

Based on the preference or ability to signal through the canonical or non-canonical pathway, Wnt proteins are divided into two groups: Wnt1 group (e.g. Wnt1, Wnt3a, and Wnt8) and wnt5a group (e.g. Wnt4, Wnt5a, and Wnt11). Wnt1 group are thought to signal exclusively via the canonical Wnt/β-catenin pathway while the Wnt5a group normally signal through the non-canonical Wnt signalling (Tada and Smith, 2000; Pandur *et al.*, 2002; Maurus *et al.*, 2005). The signals generated by the Wnt5a group can suppress β-catenin-mediated signalling (Torres *et al.*, 1996; Topol *et al.*, 2003; Westfall *et al.*, 2003; Maye *et al.*, 2004). This suggests that wnt5a group of Wnt proteins function as negative regulators of Wnt1 class proteins.

1.18.3 Wnt inhibitors

1.18.3.1 Endogenous Wnt inhibitors: secreted frizzled-related proteins

Secreted frizzled-related proteins (sFRP) are distinct but related to the Fz cell membrane receptor proteins (Rattner *et al.*, 1997). The Wnt binding domain is conserved among sFRP and Fz proteins. The sFRP exert their biological activity acting as competitive inhibitor for Wnts binding to their cell membrane receptors (Bafico *et al.*, 1999; Golan *et al.*, 2004; Roman-Roman *et al.*, 2004).

1.18.3.2 Endogenous Wnt inhibitors: Dickkopf (Dkk) proteins

Dickkopf (Dkk) like the sFRP also   inhibit the canonical Wnt signalling (Glinka *et al.,* 1998). However, unlike the sFRP which bind to Fz receptors, the Dkk exert their inhibitory activities on   canonical Wnt by competitively binding to the extracellular domains of the co-receptors (LRP5 and LRP6) (Mao *et al.,* 2001; Semenov *et al.,* 2001).   Beside their competitive inhibition of the canonical pathway at the LRP5/6 receptor level, Dkk signalling may also   regulate   the activity of canonical Wnt proteins   (Bafico *et al.,* 2001; Davidson *et al.,* 2002; Mao *et al.,* 2002).

1.18.4 Frizzled receptor family in the mesoderm

In addition to the enormous number of Wnt ligands, many different Wnt receptors of the Frizzled family have been detected in the mesoderm of the heart-forming fields, cardiac neural crest cells or the adult heart, including Fz-4 (DeRossi *et al.,* 2000), Fz-7 and Fz-8 (Wheeler and Hoppler, 1999; Saitoh *et al.,* 2001), Fz-10a and -10b (Moriwaki *et al.,* 2000), Fz-1 and Fz-2 (van Gijn *et al.,* 2001) and Fz-9 (Sagara *et al.,* 1998; Wang *et al.,* 1999). The sFRP-1 is expressed in the heart and in the visceral yolk sac during mouse development, and that sFRP-1 and mWnt-8 display overlapping expression patterns during heart morphogenesis (Jaspard *et al.,* 2000). The extensive expression of different Wnt proteins and receptors in the developing hearts is strong indication that Wnt proteins have a role to play in the different stages of cardiogenesis.

1.18.5 Roles of Wnt roles in cardiogenesis

Vertebrate heart development starts with specification of myocardial precursor cells within the mesodermal germ layer of the early embryo. Several Wnt ligands and Frizzled receptors have been shown to be expressed in a special and temporal manner regulated during early heart development. Wnt-11 expression is found in the heart-forming fields in chicken, mouse and *Xenopus* (Ku and Melton, 1993). It has also been shown that Wnt11-R, a protein closely related to mammalian Wnt11, is required for heart morphogenesis in *Xenopus* (Garriock *et al.*, 2005). It has also be shown that Wnt (Wnt-11) gene is expressed by newly gastrulated mesoderm cells within chicken embryos in a pattern which suggests that it may be involved in formation of the cardiogenic fields (Eisenberg *et al.*, 1997).   In mouse embryos, by 7.5-8.5 dpc , the expression of Wnt2 in the early heart field overlaps with that of Wnt-11 before it becomes restricted to the pericardium (Monkley *et al.*, 1996).

1.18.6 Roles of the canonical Wnt/β-catenin signalling in ES cell differentiation into cardiomyocyte

The current model of the role of Wnt in development of the heart is that inhibition of canonical Wnt/β-catenin signalling pathway is essential for the induction cardiac lineage commitment (Marvin *et al.*, 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001; Pandur *et al.*, 2002). However this model is not universally supported by published data. Data from some studies suggest that canonical Wnt/β-catenin signalling inhibits cardiogenesis while

data from other studies support positive regulation of ES cell differentiation into cardiomyocytes by the Wnt/β-catenin signalling (Nakamura *et al.*, 2003; Naito *et al.*, 2006; Kwon *et al.*, 2007; Liu *et al.*, 2007).

It has been shown in *Xenopus* laevis that the administration of Dkk-1 and Crescent (Wnt inhibitors) and also the ectopic expression of GSK-3β can initiate cardiogenesis in ventral marginal zone mesoderm (Schneider and Mercola, 2001). Similarly in chick embryos Crescent and Dkk-1 were found to induce heart muscle formation in the posterior lateral plate mesoderm (Marvin *et al.*, 2001). These studies suggest that the Wnt/β-catenin signalling negatively regulates cardiogenesis and the activation of pathways that inhibit Wnt/β-catenin signalling is required for the induction of cardiogenesis.

In contrast to the inhibitory roles suggested by the above studies, data from other studies suggest that Wnt/β-catenin signalling positively regulates ES cell cardiac differentiation during the early stages. Using mouse embryos, it was found that Wnt8a was also specifically expressed in heart precursors at E8.5 and also expressed initially throughout the developing heart tube (Kwon *et al.*, 2007). It was also found that the expression of the early cardiac genes Nkx2.5 and Tbx5 in EBs was up-regulated by Wnt3a and down-regulated by Dkk-1.The addition of Wnt3a to EBs before the induction of early cardiac genes increased the number of beating EBs to more than 50% while the addition of Fz8/Fc or Dkk-1 and Fz8/Fc, at this stage resulted in a complete absence of beating EBs (Naito *et al.*, 2006; Kwon *et al.*, 2007). The second heart (SHF) field marker, Islet1 was similarly affected by Wnt3a and Dkk-1

(Kwon *et al.*, 2007). Constitutively active GSK-3β (i.e. degradation of β-catenin) also completely blocked Tbx5 (a mesoderm marker) and GATA4 (Nakamura *et al.*, 2003; Kwon *et al.*, 2007) and also inhibited the expression of BMP2, BMP4, and FGF8 (Nakamura *et al.*, 2003). In contrast, LiCl (a GSK-3β inhibitor) significantly increased the expression of Nkx2.5, GATA4, Tbx5, BMP2, and BMP4 (Nakamura *et al.*, 2003). These results strongly suggest a positive rather than a negative regulation of the stem cell differentiation into cardiomyocytes.

In addition to functional data mentioned above, the wide expression of canonical Wnts in the developing heart may suggest positive roles for canonical Wnts during the myocardial fate specification. For example canonical Wnts including Wnt2 (Monkley *et al.*, 1996; Karasawa *et al.*, 2002), Wnt9a (Karasawa *et al.*, 2002; Person *et al.*, 2005) and Wnt6 (Rodriguez-Niedenfuhr *et al.*, 2003) are also expressed in the developing heart.

In addition to a positive role for Wnt/β-catenin signalling during ES cell cardiac differentiation, it was shown that this pathway exhibited biphasic effects. While Wnt/β-catenin signalling positively regulated ES cell differentiation into cardiomyocytes during the early phases, it negatively regulated ES cell cardiac differentiation during the late stages of cardiomyocyte differentiation (Nakamura *et al.*, 2003; Naito *et al.*, 2006; Kwon *et al.*, 2007). In support of a negative role of Wnt/β-catenin signalling during the late stage cardiomyocyte differentiation, it was shown that the treatment of EBs with Dkk-1 and Fz8/Fc, by day 5 (i.e. late cardiomyocyte differentiation

stage in those experiments) increased the number of cardiomyocytes (Naito *et al.*, 2006; Kwon *et al.*, 2007) thus suggesting a negative role of Wnt at this stage.

Support of a positive role for Wnt during the early phase of cardiogenesis comes from the high expression of Wnt3 and Wnt8a in the pre-streak and early gastrula-stage embryo (Kemp *et al.,* 2005). In ES cells, Wnt3 and Wnt8a are transiently induced between day 2 and 3 of differentiation. It was also shown that Dkk-1 addition at day 0, 1 or 1.5, completely inhibited the generation of cells expressing Flk1 (a lateral mesoderm early marker). Lineage commitment was reduced by 80%. In contrast Dkk1 added at day 2 and later had negligible effects (Lindsley *et al.,* 2006).

All the above data when considered together, suggest that Wnt/β-catenin signalling is required for commitment of mesodermal cells into cardiomyocytes during the early phase of the of ES differentiation into cardiomyocytes. The down-regulation of of cardiac gene expression by Dkk-1, Fz8/Fc and constitutively active GSK-3β suggest that Wnt/β-catenin signalling played positive roles during the early phases of ES cell cardiac differentiation. The inhibition of the expression of Nkx2.5, GATA-4, Tbx5, BMP2, and BMP4 by constitutively active GSK-3β and the induction of these genes by LiCl (a GSK-3β inhibitor) also suggest that Wnt/β-catenin signalling is required for commitment of mesodermal cells into a cardiomyocyte lineage and the differentiation of the committed cells into cardiomyocytes. The inhibition of the

expression of BMP2, BMP4, and FGF8 also suggest that Wnt pathway is upstream of the BMP and FGF signalling pathways.

1.18.7 Summary of Wnt Proteins

There is extensive expression of different Wnt proteins and their receptors in the developing hearts. Wnt and their receptors are expressed in the heart forming fields, cardiac crest and also in the adult heart. Wnt signalling is very complex. Some Wnts activate the canonical pathway, which signals through stabilisation of β-catenin. Wnts in Wnt1 group signal mainly through β-catenin. Some Wnts in wnt5a group signal through Wnt/PCP pathway leading to the regulation of cell migration and polarity and the cytoskeleton. Other Wnts in wnt5a group signal through activation of phospholipase C leading to of Ca^{2+} ion release, the activation of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and this has been suggested to cause to the inhibition of the canonical Wnt/β-catenin signalling.

While data from previous studies suggest that the Wnt/β-catenin signalling inhibit cardiogenesis, more recent data suggest that the canonical Wnt/β-catenin signalling play positive role during the early phases of cell lineage specification *in vivo* and in ES cell cardiac differentiation. The fact that data from some reports support a positive role for the Wnt/β-catenin signalling in the commitment of mesodermal cells into a cardiomyocyte lineage and the differentiation of the committed cells into cardiomyocytes, while data from other reports suggest a negative role, may be a reflection of the existence

large number of Wnts and also our lack of clear understanding of the roles of

these proteins in cardiomyocyte differentiation.

1.19 Overview of extracellular signalling molecules and transcription factors in cardiogenesis and stem cell differentiation into cardiomyocytes

There is no doubt that stem cells offer many important potential benefits for treating many diseases including cardiovascular diseases. The key problem is how to derive a particular cell type from stem cells. There are data on the process of stem cell differentiation into cardiomyocytes. Many signal molecules and signal transduction pathways have been implicated in stem cell differentiation but there is no complete account of the mechanisms that keep stem cells in the undifferentiated state. Equally, there is no comprehensive account of the mechanisms that can drive stem cell differentiation into a particular cell type.

Investigations of the molecular mechanisms of many transcription factors including Nkx2.5, and GATA transcription factors have helped clarify understanding of this area but these studies are still at an early stage and exact details of their modes of action are not fully understood. As it has been seen in other systems, it is more likely that it is the actual interactions (or combinatorial actions) rather than the role of each factor alone that may hold the keys to our understanding of their roles in cardiogenesis.

At the receptor level many molecules including Wnt, BMP have been shown to play important roles in stem cell and stem cell differentiation (Figure 1.18). Signalling downstream of various classical signalling pathways   including

PI3K, PKC, MAPK and others have been implicated to have roles to play in stem cell differentiation into cardiomyocytes but the exact molecular mechanisms by which these may mediate the stem cell cardiomyocyte lineage commitment and differentiation is currently not fully understood.

Extracellular molecules are released by cells or chemical compounds are added to ES cells

Wnts, BMP, Activin, FgF, TgF-β, SHH, Notch, NO, DMSO, 5-Azacytidine & others

Pluripotent embryonic stem cells expressing pluripotent markers

Oct-4   SOX2   NANOG

Extracellular molecules bind to their membrane receptors

Signalling events and Molecules e.g. kinases second messengers , etc

PKC, p38 MAPK, MAPK ERK, TAK1, PI3K and others

Cardiac transcription factors interact to activate cardiac specific genes. This leads to cardiogenesis

Nkx2.5   GATA4   GATA4   GATA6

Stem cell Cardiac Differentiation

MLC-2v, MLC-1v, TnI α-MHC, β-MHC, MLC-2a, ANP & Other

Figure 1.18: Extracellular signalling molecules and transcription factor position in cardiogenesis. In response external conditions or factors signal molecules including, BMP, wnts and other molecules are released. A range of specific signalling pathways are activated. Downstream proteins including kinases are activated. Cardiac specific transcription factors are expressed and these factors then initiate cardiac differentiation or cardiac lineage commitment. BMP= bone morphogenetic proteins, Oct-4= Octamer-binding protein-4, SHH= sonic hedgehog, FgF= fibroblast growth factor, TGF-β = transforming growth factor-β and DMSO= dimethylsulfoxide

MECHANISMS OF HEART DEVELOPMENT

1.20 Mechanisms of heart development

A clear understanding of the basic molecular mechanisms of heart development and the signalling pathways of several transcription factors that promote cardiac differentiation is relevant for stem cell differentiation into cardiomyocytes. Identification of soluble growth factors, transcription factors and signalling cascades capable of priming cardiogenesis is a crucial issue for the *in vitro* development of cardiomyocytes from ES cells. Many key transcription factors including, GATA factor, Nkx2.5 and T-box factors are expressed in the developing heart. In addition to these key transcription factors, many extracellular molecules including BMP and Wnt proteins that are upstream of these transcriptions factors are also expressed in the developing heart.

Early development of the heart is composed of two major stages: cardiac myocyte specification/differentiation and heart morphogenesis (i.e. the organisation of tissue into the specific anatomical structure). A great deal of research information about heart morphogenesis has been collected using *in vivo* and *in vitro* genetic manipulation, biochemical, and structural analyses. On the other hand there is less research information about the factors or mechanisms that regulate the cardiomyocyte differentiation.

The vertebrate heart formation involves cell commitment to cardiac lineage, proliferation, differentiation, migration and integrated multicellular morphogenesis. This complex process is regulated by various signalling molecules, including transcription factors. Vertebrate heart formation is highly

susceptible to errors that can result to congenital heart diseases (CHD). Congenital heart diseases with incidence of nearly 1% of live birth, are the most common forms of genetic diseases (Hoffman and Kaplan, 2002; Garg, 2006; Thom *et al.*, 2006).

1.20.1 Origin of cardiac progenitor cells

1.20.1.1 First heart field (FHF)

The heart is the first embryonic organ to become functional in the developing embryos. Heart formation is a process that is based on a series of progressive lineage restrictions. The whole heart is likely to be derived from a single mesodermal cardiac progenitor cell (Meilhac *et al.*, 2004).

However, an early lineage restriction that occurs prior to heart formation creates two profoundly distinct pools of cardiac progenitor cells. The myocytes of vertebrate heart are derived from (at least) these progenitor cell populations. The cardiogenic committed progenitor cells found in two bilaterally symmetrical regions of anterior lateral mesoderm constitute the first lineage known as the first heart field (FHF) (Buckingham *et al.*, 2005). The FHF forms the cardiac crescent (Meilhac *et al.*, 2004). The FHF cells begin differentiation at the crescent stage at approximately embryonic day 7.5 to 8.0 (E7.5-8.0) in the mouse embryo (corresponds to week 2-3 of human gestation). The two lateral halves of this cell population migrate, fuse at the anterior midline undergo early differentiation at the cardiac crescent stage.

These cell then form a simple linear heart tube   (Franco *et al.*, 1998;
Buckingham *et al.*, 2005).

From the very beginning this tubular heart exhibits slow autonomous
peristaltic contractions (Garg, 2006; Srivastava, 2006) and consists of an
interior layer of endocardial cells and an exterior layer of myocardial cells. The
linear heart tube consists of cardiac progenitors for parts of the ventricles
(Buckingham *et al.*, 2005). The other parts or compartments are formed by
the addition of cells to its cranial and caudal poles (De La Cruz *et al.*, 1977;
Noden, 1991; Redkar *et al.*, 2001). Subsequently, the linear heart tube
undergoes rightward looping and is remodelled into a mature four-chambered
heart i.e. ventricular and atrial chambers with inflow and outflow regions and
valves to control the flow of blood.   In mammals and also in birds, the four-
chambered heart is separated into two ventricles and two atria by a septum
that separates oxygenated blood from deoxygenated blood.

1.20.1.2 Second heart field (SHF)

A progenitor cell lineage occupies a position caudally and medially to the first
lineage at the cardiac crescent stage. This is the second heart field (SHF).
The SHF is a population of undifferentiated multipotent cardiac progenitor
cells that contribute to the heart tube growth at both inflow and outflow poles.
A small number of myocytes in left ventricle (LV) and atria are derived from
SHF. However The SHF provides the majority of myocytes in the right

ventricle (RV) and outflow tract (OFT) (Kelly *et al.*, 2001; Mjaatvedt *et al.*, 2001; Cai *et al.*, 2003).

The FHF and SHF comprises the ventral and dorsal-medial aspect of the cardiogenic plate respectively (Cai *et al.*, 2003). The FHF differentiates at the cardiac crescent stage while the SHF differentiates relatively late as the second lineage migrates in to join the already differentiated cells of the first lineage. Both lineages appear to be regulated by complex positive and negative signalling networks involving members of the bone morphogenetic protein (BMP), sonic hedgehog (SHH), fibroblast growth factor (FGF), Wnt, and Notch proteins.

1.20.2 Parallel between heart formation and ES cell differentiation into cardiomyocytes

Cardiogenesis is very complex process that involves many signalling molecules and signal transduction pathways. This process is regulated by various extracellular signalling molecules and transcription factors. A clear understanding of the basic molecular mechanisms of heart development is essential for the selective differentiation of ES cells to generate pure cardiac cell lineages. Some examples of the parallel between ES differentiation into cardiomyocytes and the vertebrate heart formation mechanisms are discussed to highlight the similarities between *in vivo* and *in vitro* cardiomyogenesis.

Mesodermal lineage in the FHF gives rise to cardiomyocytes. P19 stem cells differentiating into cardiomyocytes express brachyury T (mesodermal lineage commitment marker) (Grepin *et al.,* 1997). Brachyury T is a T-box transcription factor (Kispert *et al.,* 1995) and is necessary for the establishment of the mesoderm in vertebrates (Schulte-Merker *et al.,* 1992). ES cells undergoing cardiac differentiation brachyury T and cardiac specific transcription factors and structural proteins including Nkx2.5, GATA-4, MLC-2a MLC-2v, ANF, MHC and troponin T (Schwanke *et al.,* 2006). This suggests that vertebrate stem cells in culture follow the same cardiac differentiation programme as *in vivo*.

Nkx2.5 is one of the early markers during stem cell differentiation into cardiomyocytes (Nakamura *et al.,* 2003; Naito *et al.,* 2006; Kwon *et al.,* 2007). Nkx2.5 is also one of the earliest markers of heart field mesoderm. In the mouse, the expression of Nkx2.5 starts early in heart progenitor cells in the FHF and SHF continues at a high level in the heart through adulthood (Komuro and Izumo, 1993; Lints *et al.,* 1993; Lee *et al.,* 2004).

In *Xenopus* embryos, the expression of inhibitory or dominant negative mutants forms of Nkx2.5 (Nkx2.3 or XNkx2.5) resulted to complete lack of cardiac gene expression and complete absence of heart formation (Fu *et al.,* 1998; Grow and Krieg, 1998). Similarly in P19 stem cells the expression of a dominant-negative mutant of Nkx2.5 blocked the cell differentiation into cardiomyocytes (Jamali *et al.,* 2001).

GATA-6 is expressed in cardiac mesoderm before gastrulation (Peterkin *et al.*, 2003). In both *Xenopus* and zebrafish embryos, the inhibition of the GATA-6 mRNA translation into protein by anti-sense oligonucleotides led to markedly reduced levels of cardiac proteins in the embryos (Peterkin *et al.*, 2003). Zebrafish embryos depleted of both GATA-5 and GATA-6 can not develop hearts (Holtzinger and Evans, 2007). Mouse embryos null for both GATA-4 and GATA-6 had no hearts (Zhao *et al.*, 2008). Similarly, the inhibition of GATA-4 using GATA-4 antisense transcripts blocked P19 stem cell cardiac differentiation at the premyocardial (cardioblast) (Kispert *et al.*, 1995).

BMPs are expressed in lateral endoderm and ectoderm of vertebrate embryos. BMP signals in the anterior lateral mesoderm promote heart formation (Schultheiss *et al.*, 1997; Andrée *et al.*, 1998; Schlange *et al.*, 2000; Tzahor and Lassar, 2001). It has been shown that BMP soaked beads can induce the ectopic expression of Nkx2.5 and GATA-4 in chick embryos (Schultheiss *et al.*, 1997). Over-expression of noggin (a natural inhibitor of BMP) in P19 stem cells prevent their differentiation into cardiomyocytes while the over-expression of BMP-2 or direct addition of BMP proteins to the culture medium rescued the cell cardiac differentiation (Monzen *et al.*, 1999).

Wnt proteins, Wnts receptors, Wnt receptor antagonists and Wnt antagonists are important for the signalling events that control the development of the heart. These proteins have been detected in the mesoderm of the heart-

forming fields in vertebrates (DeRossi *et al.*, 2000) (Wang *et al.*, 1999; Wheeler and Hoppler, 1999; van Gijn *et al.*, 2001).

In ES cells differentiating into cardiomyocytes it was found that the addition of Wnt3a to EBs increased th expression of cardiac genes and differentiation into beating cardiomyocytes while the addition of Fz8/Fc or Dkk-1 and Fz8/Fc (wnt antagonists) resulted in a complete absence of beating EBs (Naito *et al.*, 2006; Kwon *et al.*, 2007).The SHF marker, Islet1 was similarly affected by Wnt3a and Dkk-1 (Kwon *et al.*, 2007).

1.20.3 Summary of mechanisms of heart development

In summary the few selected examples discussed above, show the remarkable similarities between heart formation in the vertebrates and ES cell differentiation. This similarities show the importance or the relevance of understanding the basic mechanisms of heart development to the differentiation of ES cells into cardiomyocytes.

STEM CELL RESEARCH AND CARDIOVASCULAR DISEASES

1.21 Stem cell research and cardiovascular diseases

Cardiovascular diseases (e.g. heart failure) are among the leading causes of mortality and morbidity worldwide (Murray and Lopez, 1997) and particularly in the developed world (Rosamond *et al.*, 1998). The high incidence of cardiovascular diseases is a combination of non-modifiable (non-preventable) and modifiable (preventable) risk factors. The non-modifiable risk factors include increasing age (Jousilahti *et al.*, 1999; Asia_Pacific_Cohort_Studies_Collaboration, 2006), gender (being male) (Jousilahti *et al.*, 1999; Lloyd-Jones *et al.*, 1999) and heredity. The modifiable risk factors include tobacco smoke (Kawachi *et al.*, 1993; Raftopoulos *et al.*, 1999; Van Berkel *et al.*, 1999; Kaprio *et al.*, 2000), high blood total cholesterol and LDL (Manolio *et al.*, 1992; Stamler *et al.*, 1993; Woodward *et al.*, 2008), high systolic blood pressure (Rutan *et al.*, 1989; Navas-Nacher *et al.*, 2001; Sundaram *et al.*, 2005; Prugger *et al.*, 2008) and physical inactivity (Kaprio *et al.*, 2000; Kruger *et al.*, 2003; Sundaram *et al.*, 2005; Jenum *et al.*, 2006), obesity (Hubert *et al.*, 1983; Li *et al.*, 2002; Sundaram *et al.*, 2005) and diabetes mellitus (Sundaram *et al.*, 2005; Prugger *et al.*, 2008). The high incidence of cardiovascular diseases makes the search for new therapeutic approaches for improving the functions of the damaged heart is a critical endeavour. Myocardial infarction is associated with irreversible loss of cardiomyocytes. Cardiomyocytes undergo apoptosis during the acute and chronic phases of myocardial infarction. This has been shown in human (Itoh *et al.*, 1995; Saraste *et al.*, 1997; Baldi *et al.*, 2002; Akasaka *et al.*, 2006), rat (Fliss and Gattinger, 1996; Palojoki *et al.*, 2001; Chandrashekhar, 2005; Louhelainen *et al.*, 2007) and the mouse (Bialik *et al.*, 1997; Engel *et al.*,

2004). Studies have shown that the inhibition of cardiomyocyte apoptosis can slow down post-infarction cardiac remodelling and also improve cardiac function in the mouse (Diwan, 2007), rat (Chandrashekhar, 2005; Chen *et al.*, 2007) and rabbit (Qin *et al.*, 2007).

Myocardial infarction can lead to heart failure. The loss of cardiomyocytes poses a major difficulty or challenge in the treatment of the damaged heart since cardiomyocytes have very limited regeneration potential. This means that, currently, the only effective treatment for severe heart failure is heart transplantation (Anyanwu *et al.*, 2002; Hertz *et al.*, 2002). The worldwide heart transplant data from the International Society for Heart and Lung Transplantation (ISHLT) shows that transplant half-life (i.e. the time at which 50% of those transplanted remain alive) for heart transplant patients is 10 years (Taylor *et al.*, 2007). This represents a good extension to life, especially for elderly patients (e.g. those aged 60 years and above).

Despite the good survival rates of heart transplant patients, heart transplantation is not a viable treatment option and has not delivered or met the high expectation that it generated among patients with heart diseases, when it was first introduced. This is due to the increasing number of patients on the heart transplant waiting list as the result of the continuous decrease in the availability of hearts for transplant. Heart transplantation as a treatment for heart failure is limited by the acute shortage of donor hearts (Anyanwu *et al.*, 2002). The ISHLT data showed that the number of annual reported heart transplants since 1982 peaked at 4428 in 1994 (Trulock *et al.*, 2003; Taylor *et*

al., 2007). This peak was then followed by a gradual but steady decline as the results of decreased number of heart transplant procedures in many countries (Taylor *et al.*, 2006; Taylor *et al.*, 2007). The latest annual figure for heart transplants worldwide stands at 3095 in 2005 (Taylor *et al.*, 2007). The worldwide number of patients on the waiting list far exceeds the number of hearts available for transplant. In the United Kingdom alone, there were 379 patients on the cardiothoracic waiting list in 2006-2007 while in the same period only 292 cardiothoracic transplants were carried out (UK Transplant, 2007). The high incidence of heart diseases and the acute scarcity of donor hearts underline the urgent need to find alternative therapeutic approaches to heart transplantation.

Stem cell research may lead to a viable alternative to heart transplant for the treatment of heart disease. Several animal and human studies have demonstrated that transplantation of cardiomyocytes (or cardiac progenitor cells) may be an alternative treatment to whole heart transplant for patients with severe heart failure. ES cells therefore represent a potential alternative source of functionally intact cardiomyocytes for the treatment of cardiovascular diseases. Research evidence on the regenerative potential of stem cell-derived cardiomyocytes and progenitor cells for the prevention or treatment of heart failure has created intense interests in stem cell-based therapy for heart failure. In a rat model of myocardial infarction (created by the ligation of the left anterior descending coronary artery) it has been shown that implantation of cardiac progenitor cells rescued scarred infarcted myocardium and improved cardiac function in rats (Bonaros *et al.*, 2006;

Gonzalez *et al.*, 2008; Rota *et al.*, 2008). Similarly, in a mouse myocardial infarction (also created by the ligation of the left anterior descending coronary artery), transplantation of human ES cell-derived cardiomyocytes improved left ventricular function (Cao *et al.*, 2008). The above data show that differentiation of stem cells into functional cardiomyocytes or cardiac progenitor cells follows by their engraftment into the damaged heart to regenerate healthy myocardial tissues to replace the damaged tissues may be used to slow the deterioration of the acutely or chronically damaged heart after myocardial infraction. This means that stem cell-based therapy holds a great potential as a very attractive alternative to heart transplant for the treating heart failure and other cardiovascular diseases.

1.21.1 Stem cell therapy for infarcted hearts

One of the key issues still to be resolved is whether undifferentiated ES cells transplanted into normal or infarcted heart can be guided by the local environment in the heart to differentiate and contribute to cardiac functions. It has been shown that undifferentiated hES are not directed to specifically differentiate into cardiomyocytes in normal or infarcted heart of the nude mouse (Leor *et al.*, 2007). Undifferentiated ES cells transplanted into normal or infarcted heart undergo non-specific lineage differentiation (Leor *et al.*, 2007; Nussbaum *et al.*, 2007). In contrast to the above other groups have shown that transplantation of undifferentiated ES cells into infracted myocardium can induce the cardiac differentiation of the transplanted ES and improved cardiac functions. This has been shown in the rat (Behfar *et al.*,

2002; Hodgson *et al.*, 2004) and in the mouse (Nelson *et al.*, 2006; Singla *et al.*, 2006). Being pluripotent, ES cells may be difficult to differentiate into cardiac specific cells, if undifferentiated ES cells are directly transplanted into infarcted hearts**.**

As already mentioned *in vitro* differentiation of ES cells into functional cardiomyocytes has been achieved. Under appropriate experimental conditions, ES cells can faithfully follow the same course of cardiomyocyte differentiation as *in vivo* (Wobus *et al.*, 1991; Xu *et al.*, 1998; Kehat *et al.*, 2001; He *et al.*, 2003; Mummery *et al.*, 2003) There is also extensive evidence in the scientific literature that adult stem cells (e.g. MSC) can differentiate into cardiomyocytes (Makino *et al.,* 1999; Fukuda, 2001; Toma *et al.*, 2002; Xu *et al.*, 2004b; Antonitsis *et al.*, 2007; Bartunek *et al.*, 2007). These adult stem cell-derived cardiomyocytes, like ES cell-derived cardiomyocytes, express cardiac specific transcriptions factors (including GATA-4, and Nkx2.5, TEF1, MEF2C, MEF2A and MEF2D) during the course of their differentiation (Makino *et al.*, 1999; Fukuda, 2001). These cardiomyocytes also express β-MHC, desmin, and cardiac α-actin and cardiac troponin T (Xu *et al.*, 2004b).

Beyond the fundamental question of whether of ES cells can differentiate into functional cardiomyocytes, the next question is: can ES cell-derived cardiomyocytes successfully engraft into adult animal hearts and improve post MI heart? Functional cardiomyocytes have been derived from cultures of human H1, H7, H9, and H14 embryonic stem cells (He *et al.*, 2003). These ES cells were originally derived from human blastocysts (Thomson *et al.*,

1998; Lavon and Benvenisty, 2003). Cardiomyocytes derived from these ES cells displayed expected cardiomyocyte morphology such Z bands and intercalated disks and they express numerous cardiac myocyte specific proteins including α-cardiac actin, atrial myosin light chain, ventricular myosin light chain, α-myosin heavy chain, atrial natriuretic peptide, and cardiac troponin T and I (He *et al.*, 2003). They also showed rhythmic contractions and long duration of action potentials (He *et al.*, 2003). Interestingly, similar results have been report with cardiomyocytes derived from MSC (Makino *et al.*, 1999; Fukuda, 2001; Toma *et al.*, 2002; Antonitsis *et al.*, 2007).

Despite the close similarities between stem cell-derived cardiomyocytes and normal cardiomyocytes, the long-term effects of cardiac cell transplantation on cardiac functions are unknown at the moment. However several animal studies demonstrate that successful engraftment of stem-cell derived cardiomyocytes into the adult heart is possible. ES-derived cardiomyocytes can successfully engraft into adult animal hearts and improve post myocardial infarction heart This has been shown in pig complete atrioventricular block model [created by ablating the His bundle (the major electrical conduction pathway, connecting the atria with the ventricles)] (Kehat *et al.*, 2004) and rat myocardial infarction model (created by the ligation of the left anterior descending coronary artery) (Etzion *et al.*, 2001; Behfar *et al.*, 2002; Muller-Ehmsen *et al.*, 2002; Laflamme *et al.*, 2005; Caspi *et al.*, 2007b; Leor *et al.*, 2007). In the rat model of myocardial infarction, cardiomyocytes differentiated from human ES cells showed *in vivo* functional integration in the animal infarcted hearts (Laflamme *et al.*, 2007). The transplanted cardiomyocytes thickened the wall of the left

ventricle and enhanced ejection fraction and reduced paradoxical systolic bulging of the infarct in post myocardial infarction rats (Müller-Ehmsen *et al.*, 2002; Caspi *et al.*, 2007a). Interestingly results similar to those reported with ES cells have also been obtained using MSC. It has been shown, in the mouse (Kudo *et al.*, 2003; Grauss *et al.*, 2007) and the rat (Hahn *et al.*, 2008; Pasha *et al.*, 2008) models of myocardial infarction (both created by the ligation of the left anterior descending coronary artery), that transplantation of MSC led to reduction in infarct size, improved LV function and survival. The rat myocardial infarction model also showed that the transplanted MSC promoted neovascularisation and myogenesis (Pasha *et al.*, 2008) and a regain of 80−90% of the lost myocardial volume and completely normalised systolic and diastolic cardiac functions (Mangi *et al.*, 2003).

Like the need for prior differentiation of ES cells into cardiomyocytes before transplantation into infarcted hearts, MSC may need specific pre-treatment before transplantation. For example it was also shown that mice transplanted with MSC that were pre-treated with growth factors (i.e. BMP-2, FGF-2, IGF-1) before transplantation had fewer apoptotic cells in infarcted hearts, smaller infarct size and better cardiac function than mice transplanted with untreated MSC (Hahn *et al.*, 2008). Almost identical results were obtained with dogs that were treated MSC that were induce to differentiate by BMP-2, FGF-2 and IGF-1) (Bartunek *et al.*, 2007). The treated MSC expressed muscle-specific (MEF2) and cardiac-specific (GATA-4 and Nkx-2.5) transcription factors (Bartunek *et al.*, 2007), a strong evidence for their differentiation along the cardiac lineage. Myocardial injection of these cardiomyocytes differentiated

from autologous MSC into dogs with chronic myocardial infarction resulted in cardiac regeneration of the chronically infarcted myocardium (Bartunek *et al.*, 2007).

The role of cardiac endothelial cells in the use of stem cells to treat an infarcted heart is also important. It has also been demonstrated that transplantation of human MSC (Grauss *et al.*, 2007) or mouse MSC (Kudo *et al.*, 2003) into ischemic hearts of mice caused the transplanted MSC to differentiate into myocytes and endothelial cells and contribute to reduced infarct size, reduced fibrosis, significant preservation of LV ejection fraction, increased vascularity of the infarct scar and a marked reduction in the thinning of the infarcted wall in the mice (Kudo *et al.*, 2003; Grauss *et al.*, 2007). While it was not determined whether the cardiac improvements reported in these studies were the results of cell fusion (Terada *et al.*, 2002) or MSC actually differentiating into cardiac cells, these results suggest that both cardiomyocytes and cardiac endothelial cells are important for cardiac regeneration in infarcted hearts.

Collectively, the all the above studies with both ES cell-derived and adult stem cell-derived cardiomyocytes have demonstrated that stem cell based therapy can be used to improve the function of the damaged heart and also improve survival after myocardial injury. They also show that stem cell therapy may become an alternative therapeutic approach to the current use of human heart transplants for the treatment of heart failure in patients. However there are still

many critical hurdles that must be overcome before the therapeutic potential of stem cells can be realised.

1.21.2 Challenges facing stem cell-based therapy

The potentials and challenges of stem therapy for treating human cardiovascular diseases are presented below. The challenges are by no means limited to cardiovascular diseases but are equally the concerns of stem cell-based therapies that may be targeting other human diseases. Progress has been made in the identification, isolation characterization, and maintenance of stem cells in culture (Evans and Kaufman, 1981; Martin, 1981; Shamblott *et al.,* 1998; Thomson *et al.,* 1998) but the specific differentiation of ES cell into cardiomyocytes (or any other particular cell type)   remains a problem.

1.21.2.1 Challenge of cardiac-specific differentiation of stem cells

Cardiac-specific differentiation of stem cells or the selection of lineages consisting of pure embryonic cardiomyocytes is essential and represents a scientific challenge. The search for growth factors and signalling molecules that are involved in cardiac-specific differentiation is therefore an active area of research. There is need for the design and establishment of suitable and reliable differentiation techniques that can overcome the problem of inefficient and also spontaneous stem cell differentiation. The homogeneous differentiation of stem cells into cardiomyocytes with sufficient yield is a critical requirement for the clinical use of stem cells in cardiovascular medicine. A

serious limiting factor of stem cell therapy for the treatment of cardiovascular diseases is the current difficulty in generation sufficient number of stem-cell derived cardiac cells. Across many laboratories, differentiation methods are not only inefficient but sometime difficult to reproduce. The problems of variable reproducibility may be due to the variations in the conditions under which ES cells are routinely maintained and cultured or could be due to variation in differentiation conditions or both. There is a need for common stem cell culture conditions for the maintenance and for differentiation. This will not only simplify maintenance of multiple lines in single research but may help to improve the poor reproducibility of specific differentiation protocols among various research teams. Using two independently derived human embryonic stem cell lines (BG01 and HUES-7), it has been demonstrated in principle that it is possible to develop protocols that can be applied to multiple stem cell lines (Denning *et al.*, 2006). Reproducible differentiation methods at the research levels may eventually develop into technologies that lead to generation of a large number of homogeneous stem-cell derived cell types. Such future technologies may in turn lead to easy availability of needed stem-cell-derived cell types, which can be applied in sufficient doses in order to achieve the maximum desired therapeutic benefits.

1.21.2.2 Challenge of post-transplantation tumour formation

Indefinite self-renewal capacity and pluripotency are the key attractive features of stem cells. However these features also carry the potential risks for tumour formation after transplants. Undifferentiated or partially

differentiated ES cells can subsequently form post-transplantation teratomas (i.e. germline tumours consisting of various cell types). Therefore the development of robust and reproducible methods to derive a homogeneous population of cardiomyocytes from ES cells is essential for therapeutic application of ES cells. A homogeneous cell type not only ensures that a sufficient numbers of the desired cell type for a maximum therapeutic benefit are applied but most importantly it ensures the removal of unwanted cell types that could lead to harmful side effects.

Tumorigenesis following transplantation of undifferentiated ES cells or cell types differentiated from ES has been described in many reports (Thomson *et al.*, 1998; Swijnenburg *et al.*, 2005; Caspi *et al.*, 2007a; Nussbaum *et al.*, 2007). A test of five different human embryonic cell lines found that all produced teratomas after injection into severe combined immunodeficient (SCID)-beige mice (Thomson *et al.*, 1998). Mostly important was the finding that each injected mouse formed teratomas that included cell types of the three germ layers - i.e. gut epithelium (endoderm), cartilage, striated muscle, bone, and smooth muscle and (mesoderm); and neural epithelium, embryonic ganglia and stratified squamous epithelium (ectoderm) (Thomson *et al.*, 1998). The generation of all cell types from the three germ layers is an indication that uncontrolled differentiation occurred. Efforts to remove undifferentiated ES cell or enrich differentiated cell types is therefore an active area of research. It is also possible, in principle, to deplete undifferentiated cells from a heterogeneous cell population using molecular methods. A heterogeneous cell population (obtained from the differentiation of stem cells)

can be treated with a vector that puts an effector gene under control of a gene element that allows the specific and higher expression of transgene in the undifferentiated subpopulation. The transgene product can then be used to separate the undifferentiated cell population from the differentiated cells using specific antibodies. This has the potential to produce a cell population that is relatively enriched for differentiated cell types. For example, it has been shown that cardiomyocytes differentiated from ES cells that were transfected with a fusion gene consisting of alpha-cardiac myosin heavy chain promoter driving the aminoglycoside phosphotransferase (neomycin resistance gene) led to higher harvest of cardiomyocytes with undetectable levels of Oct-4 (Zandstra *et al.*, 2003). The undetectable level of Oct-4 suggests that undifferentiated ES cells were depleted from the ES cell-derived cardiomyocyte population. Similarly, it has been shown that, when a construct of murine α-MHC promoter driving the neomycin-resistance gene, was introduced into hES3 cells, a differentiated cell population with more than 99% cardiomyocytes resulted, after cardiac differentiation that was followed by G418 selection (Xu *et al.*, 2008). These studies demonstrated not only that ES cell-derived cardiomyocytes can be enriched but also that the elimination of undifferentiated cells is possible. Another potential procedure to remove undifferentiated ES cells is by selection against undifferentiated ES cells using monoclonal antibodies against well-characterised human ES cell surface antigens (e.g.SSEA-4) (Shibata *et al.*, 2006). Flow-cytometric cell sorting (i.e. fluorescence-activated cell sorting; FACS) and monoclonal antibodies can also be used to enrich differentiated cell types from

undifferentiated stem using antibodies against the specific marker of the differentiated cell types (Fukuda *et al.*, 2006).

1.21.2.3 Summary of Stem cell research and cardiovascular diseases

The search for new therapeutic approaches for improving the function of the damaged heart is urgent. Cardiomyocytes have very limited regeneration potential so their loss, one of the underlying causes of myocardial infarction and eventual heart failure, is a major problem for treating heart failure. Currently, heart transplantation is the only cure for heart failure but this is not a viable treatment option due to the acute shortage of donor hearts.

There is sufficient evidence to conclude that the therapeutic application of stem cell-derived cardiomyocytes can be a realised. Various animal models have shown that stem cell-derived cardiomyocytes can successfully engraft into adult hearts and can also improve the function of infarcted hearts. However, realisation of the full potential of stem cells for the treatment of various heart diseases will be possible only after the resolution of key issues including the limited availability of homogenous stem-cell derived cardiomyocytes, potential immune rejection, post transplant teratoma formation and others. While there have been advances in the differentiation of stem cells into cardiomyocytes and better understanding of potential post transplantation risks (e.g. teratoma formation), a fully understanding about the above problems and how to avoid them is needed. The various reports of post-transplantation tumour formation in various models of stem cell-based

cell therapy and the various attempts to resolve this issue is a reflection of the seriousness of all the critical hurdles that must be overcome before stem cell therapy can become a clinical reality.

1.22 Aim of the PhD project

As already discussed above, ES cell-derived cardiomyocytes are potential or promising alternative to heart transplant in the treatment of heart diseases for cardiac repair. The realisation of stem cell therapy is been currently hampered by the critical lack of reliable, selective and readily reproducible differentiation methods. This difficulty is in turn due to the lack of a clear understanding of mechanisms of signal transduction pathways that mediate stem cell differentiation into cardiomyocytes. Without a clear understanding of the mechanism of stem cell cardiac lineage commitment and differentiation into cardiomyocytes, it would be difficult to obtain homogenous and sufficient number of cardiomyocytes for use in stem cell base therapy. The specific, efficient and selective differentiation of stem cells into cardiomyocytes is absolutely necessary for realisation of stem cell therapy for treating the damaged heart and this remains a tough scientific challenge.

The aim of this PhD thesis is therefore to investigate the cell signalling pathways that are responsible for mediating the differentiation of stem cells into cardiomyocytes. This PhD project, besides being a scientific challenge for the reason stated above, it has another challenging dimension, in that, it is the first of its kind in the host laboratory. The project is the first attempt by our

laboratory to direct our strong   background in cell signalling research to the studies of stem cell differentiation into cardiomyocytes.

The project specific aims/objectives are as follows:

1. The first objective is to establish cardiomyocyte differentiation models using H9c2 and P19 stem cells and the use of these models to accomplish the other specific objectives, which are, to:

2. Investigate the role of nitric oxide (NO) in the differentiation of stem cells into cardiomyocytes

3. Investigate the role of phosphoinositide 3-kinase (PI3K) signalling pathway in the differentiation of stem cells into cardiomyocytes

4. Investigate the role of protein kinase C (PKC) signalling pathway in the differentiation of stem cells into cardiomyocytes

5. Investigate the role of nuclear factor-kappa B (NF-kB) signalling pathway in the differentiation of stem cells into cardiomyocytes

6. Investigate the role of p38 mitogen-activated protein kinase (p38 MAPK) signalling pathway in the differentiation of stem cells   into cardiomyocytes

Possible alternative research approaches, to the above that we took, include investigating the roles of traditional molecules (e.g. BMP, TGF-β, Fgf, Wnt and growth factors) that have been implicated in stem cell differentiation into cardiomyocytes The signal transduction pathways that we investigated may be downstream of these molecules. Therefore an understanding of the roles of these downstream signal transduction pathways, in the differentiation of stem cells into cardiomyocytes, may give us a better insights or understanding of the roles of these traditional molecules mentioned above. This project is using two relevant cardiomyocyte differentiation models (H9c2 cell and P19

stem cell models) to investigate the roles of the signalling pathways (listed above) in stem cell differentiation into cardiomyocytes.

CHAPTER 2.0

MATERIALS AND METHODS

2 Materials and Methods

2.1 Resuscitation and culture of H9c2 cells from frozen stock

The H9c2 (2-1) line (H9c2 cells), at passage 8, was purchased from the European Collection of Cell Cultures [ECACC (No: 88092904), Salisbury, Wiltshire, UK]. On day 1, a 1ml vial of frozen H9c2 stem cells was quickly thawed in a water bath at $37^{o}C$ (Katayama *et al.*, 1997). The cells were then resuspended in 9ml of fresh complete culture medium (i.e. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100units/ml penicillin/100µg/ml streptomycin) in a 15ml centrifuge tube. All the components of the full medium were purchased from Invitrogen, UK. The tube was spun in a centrifuge at 1000 RPM at $4^{o}C$ for 5 minutes. The medium was then aspirated and the pellet was kept. Next, 5 ml of new fresh complete medium was added to the tube and the clumped cells were dissociated by trituration (i.e. pipetting up and down). The dissociated cells were transferred into a new T25 (25 cm^2 of cell growth area) culture flask in a total volume of 5ml of fresh culture medium and cultured in a cell culture incubator at $37^{o}C$, 95% air and 5% CO_2.

On day 2, the cells in the T25 flask were observed under a microscope to ensure that they were growing and also healthy. The spent medium was removed at day 3 and replaced with fresh complete growth medium. The cells were subsequently trypsinised and subcultured on day 4, as described below (Section 2.4), when they were approximately 60-70 % confluent. The cell

suspension was then placed in a T75 (75 cm^2 of cell growth area) flask and placed back into the incubator at 37°C, 95% air and 5% CO_2. The cells were left to become 60-70 % confluent and then brought into suspension again. The cell suspension was split into five parts (1:5 splitting). One part (1ml) of the cell suspension was subcultured in a new T75 flask. The cells from this flask were used to generate more cells for routine cell culture (i.e. cell generation for experiments and storage). The rest of the cell suspension (i.e. 4 parts or 4ml) were frozen and stored in liquid nitrogen for future use as described below (Section 2.5).

2.2 Resuscitation and culture of P19 stem cell from frozen stock

P19 embryonal carcinoma stem cell (P19 stem cells) (passage 18) were purchased from the European Collection of Cell Cultures [ECACC (No: 95102107), Salisbury, Wiltshire, UK]. The resuscitation and culture of P19 stem cell from frozen stock was carried out as already described above for H9c2 cells (Section 2.1). The only modification made to the above was that the frozen vial of P19 stem cells was directly cultured in a T75 flask (i.e. without the initial culture in T25 as was the case with H9c2 cells). P19 stem cells do not need the initial culture in a small growth area (i.e. T25 flask) as they have high growth rates. The frozen vial of P19 stem cells was resuspended in fresh complete culture medium (i.e. alpha Minimum Essential Medium (α-MEM) supplemented with 10% FBS and 100units/ml penicillin/100µg/ml streptomycin) in a 15 ml centrifuge tube. All the

components for the full medium were purchased from Invitrogen, UK. The cells were processed exactly as detailed for H9c2 cells.

2.3 Routine cell culture

2.3.1 Routine cell culture of H9c2 cells

H9c2 cells were routinely cultured in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin). The cells were maintained in a tissue culture incubator at 37°C, 95% air and 5% CO_2. Cells were routinely subcultured when 60-70% confluence. This precaution was to prevent the loss of differentiation potential should the cells reach confluency.

2.3.2 Routine cell culture of P19 stem cells

P19 stem cells were routinely cultured in full culture medium (i.e. α-MEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin). The cells were maintained in a tissue culture incubator at 37°C, 95% air and 5% CO_2. The cells were routinely subcultured when 60-70% confluent. This precaution of not allowing the cells to become confluent was to prevent their spontaneous or non-directed differentiation.

2.4 Subculture of cells

P19 stem cells or H9c2 cells were subcultured when 60-70 % confluent. This was carried out by removing the culture medium from the flask, washing cells 3 times with warm (37^oC) phosphate buffered saline (PBS) (Invitrogen, UK) and then adding 3ml of warm (37^oC) 0.05% trypsin/EDTA solution (Invitrogen, UK) cells in T75 flasks (i.e. 120µl trypsin/cm^2). Flasks were then placed in an incubator at 37^oC, 95% air and 5% CO_2 for 3-5 minutes. The trypsin was subsequently inactivated when all the cells had detached by adding 7ml of full growth medium. The serum in the full growth medium contains inhibitors that inhibit or neutralise the trpypsin. If the cells were not required for studies, the cell suspension was placed in a T75 flask and cultured to 60-70% confluent. If required, the cells were used for experiments.

2.5 Cryopreservation of cells

Excess cells or cells not required for immediate experimentation, were routinely cryopreserved and stored for later use. To freeze the cells, they were brought into suspension as before. The cell suspension was transferred into a 15ml centrifuge tube and spun at 1000 RPM for 5 minutes at 4^oC. The medium was then aspirated and the pellet was kept. The H9c2 cell pellet was re-suspended in freezing medium (i.e. 90% FBS and 10% dimethylsulfoxide (DMSO) (Invitrogen, UK)). The P19 stem cell pellet was re-suspended in freezing medium (i.e. 90% FBS and 10% DMSO or 10% glycerol (Invitrogen, UK)). The cell suspension was gently but thoroughly triturated to break apart

any cell clumps. The cell suspension was then aliquoted in 1ml volumes in cryovials and placed in a Thermo Scientific NALGENE® Mr. Frosty (i.e. a controlled rate freezing container) (Fisher Scientific, UK) for freezing at -80°C overnight before transferring into liquid nitrogen for long term storage. Normally one T75 flask of cells was frozen into two cryovials, each containing 1ml of resuspended cells (i.e. splitting ratio of 1:2)

2.6 Quantification of cells

In order to plate cells at the required densities, the total number of cells from each flask was determined using the Trypan Blue exclusion assay. The flask of cells was first trypsinised as described (Section 2.4). The cell suspension was transferred into a 15ml centrifuge tube and spun at 1000 RPM for 5 minutes at 4°C. The medium was then aspirated and the pellet was kept. The pellet resuspension was carried out in a small known volume (usually, 1-2 ml) of complete cell culture medium for each T75 flask of cells. Then 100µl of the cell suspension was mixed with an equal volume of 0.4% w/v) Trypan blue in an eppendorff tube. A moistened cover-slip was slipped over the chamber of a clean Neubauer haemocytometer. A 10µl aliquot of the cell/Trypan blue suspension was then added to each chamber on the haemocytometer and viewed under a light microscope using a 10X magnification on the eyepiece lens and a 20X objective lens.

Cells were counted in the two chambers of haemocytometer. All the cells in the 1mm center square (E) and four 1mm corner squares (A, B, C and D)

were counted in the first chamber (Figure 2.10). Separate counts of viable (seen as translucent with no blue staining) and non-viable (stained blue) cells were recorded. This procedure was repeated for the second chamber. In total, the cells in ten squares (five from each chamber) were counted.
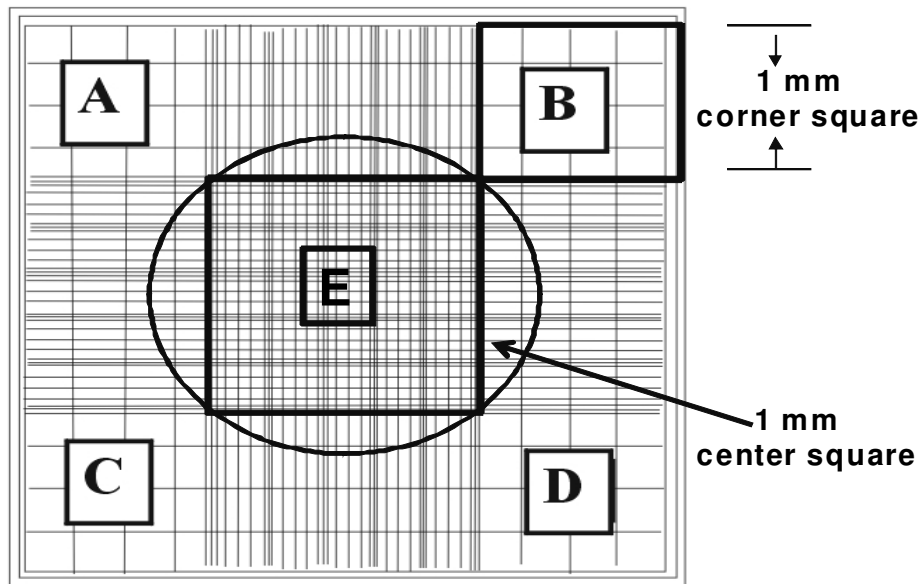
Figure 2.10: A schematic diagram (cartoon) of a haemocytometer a. Diagram shows a chamber of a haemocytometer and the areas (A, B, C, D and E) from which the cells were counted.

The cell concentration in the cell suspension was determined as follows:

Concentration of viable cells (cells/ml) = **A x C x D**

Where:

A is the mean number of viable cells counted i.e. $\dfrac{\text{Total number of viable cells counted}}{\text{Number of squares used}}$

C is the dilution factor and

D is the correction factor ($\times 10^4$)

Total number of viable cells per flask =

$$\text{concentration of viable cells} \times \text{volume of cell suspension}$$

2.7 Cell viability (MTT) assay

In order to eliminate any non selective cytotoxic actions of the drug used, cell viability was determined after each treatment by monitoring the cellular metabolism of (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyl-tetrazolium bromide (MTT) to formazan. This is a widely used cytotoxicity assay in which the water soluble tetrazolium salt is converted into an insoluble purple compound, formazan, by cleavage of the tetrazolium ring by mitochondrial dehydrogenases in viable cells (Mosmann, 1983). This assay is therefore largely a measure of mitochondrial activity. The MTT can be used to determine cytotoxicity of compounds. The water insoluble formazan dissolves in acidic isopropanol. The absorbance of formazan in the solution is proportional to the concentration of MTT metabolised and is believed to be a good indicator of cell viability. Toxic concentrations of a compound would lead to a reduction in the mitochondrial dehydrogenase ability to metabolise (MTT) to formazan. Toxic concentrations of a compound would therefore produce a reduced absorbance in the MTT assay. In contrast to the MTT assay, the Trypan Blue exclusion assay used to determine the number of cells for experiments, is based on the ability of viable (live) cells to exclude Trypan Blue and appear translucent (with no blue staining) under the microscope. Dead (non-viable) cells, on the other hand, cannot exclude Trypan Blue and are therefore stained blue, as the result of Trypan blue uptake, and appear blue under the microscope.

H9c2 cells (2×10^3 cells/cm^2) were seeded in p60 dishes or 6-well plates in full culture medium and allowed to grow to 60-70% confluence. The full growth medium was removed and the cells were washed twice with warm (37°C) PBS. The differentiation medium (i.e. DMEM supplemented with 1% FBS and 100units/ml penicillin/100µg/ml streptomycin) containing the appropriate concentration of the selected drug was added to the cells. The cells were incubated at 37°C, 95% air and 5% CO_2 for defined time periods. Control cells were incubated in the differentiation medium without the selected drug.

At the end of the incubation period, 200µl of MTT (Sigma, UK) stock (5mg/ml, made in PBS) was added to 2ml of culture medium in each p60 dish, giving a final concentration of 0.5mg/ml. The cells were incubated at 37°C, 95% air and 5% CO_2 for one hour. At the end of the incubation period, the cells were removed from the incubator and the culture medium was removed. The cells were incubated for 10 minutes with 1ml of acidic isopropanol (0.04M HCl in absolute isopropanol) to dissolve the formazan crystals. The resulting formazan solution was thoroughly vortexed to dissolve precipitates and the absorbance was measured at 540 nm using a microplate reader (Labsystems Multiskan Ascent, UK).

The cell viability in the control cells (untreated cells) was considered as 100%. The drug concentration-dependent (or time-dependent) viability in the treated cells was calculated a percentage of control viability. The cell viability at each drug concentration (or time point) was calculated as a percentage of control viability at that drug concentration (or time point). The viability values

were calculated using the formula shown below and reported as percentage of control cells.

$$\% \, \text{Cell Viability} = \frac{A_{540} \, \text{Drug Treated Cells}}{A_{540} \, \text{Control Cells}} \, \text{x} \, 100\%$$

2.8 Differentiation of H9c2 cells into cardiomyocytes

2.8.1 Initiation of H9c2 Cell differentiation into cardiomyocytes

H9c2 cells (2×10^3 cells/cm^2) (passage number not more than 25) were seeded in p60 dishes or 6-well plates and allowed to become 60-70% confluent in full culture medium. The monolayers of cells were washed twice with warm (37°C) PBS and re-incubated in differentiation medium (DMEM supplemented with 1% FBS, 100units/ml penicillin/100µg/ml streptomycin) to initiate differentiation (Kageyama *et al.*, 2002; Pagano *et al.*, 2004; Hunter *et al.*, 2007). The total proteins in a parallel dish were extracted and used as the zero time point when analysing changes in expression of targeted cell differentiation markers. Other dishes were incubated in the differentiation medium for defined time periods. Changes in cell morphology were recorded for up to 12 or 14 days by taking photos every two days, using a standard microscope and a digital eyepiece. Cell lysates were generated in parallel at the end of each time point and stored at -20°C until analysed by western blotting, using specific monoclonal antibodies against cardiac myosin heavy chain (MHC), cardiac troponin I (cTnI) or cardiac ventricular myosin light chain (MLC-1v), as described below (Section 2.14)

2.8.2 Effects of drugs (signalling pathway inhibitors) on the differentiation of H9c2 cells into cardiomyocytes

To determine whether the activation of a particular signalling pathway is required during the differentiation of H9c2 cells into cardiomyocytes, a drug (i.e. selective and specific inhibitor of that signalling pathway) was used to block the pathway. The effects of the pathway inhibition were then observed or measured. For the determination of the effects of drugs on the differentiation of H9c2 cells into cardiomyocytes, both the designated control cells and the cells to be treated with the selected drug (i.e. drug treated cells) were separately seeded in p60 dishes or 6-well plates at a density of $2x10^3$ cells/cm^2. The cells were placed in the incubator at 37^oC, 95 % air and 5% CO_2 and allowed to become 60-70% confluent in full culture medium in the absence of the selected drug. The culture medium was removed and the monolayers of cells were washed twice with warm (37^oC) PBS. The control cells were re-incubated in fresh full growth medium. The drug treated cells were re-incubated in the fresh full growth medium supplemented with the appropriate concentration of the selected drug. After one hour of incubation the culture medium was removed from both the control and the treated cells. The monolayers of cells were washed twice with warm (37^oC) PBS. The control cells were re-incubated in fresh differentiation medium (i.e. DMEM supplemented with 1% FBS, 100 units/ml penicillin/100 µg/ml streptomycin) to initiate differentiation. The fresh differentiation medium added to the control H9c2 cells did not contain any drug. The drug treated cells were re-incubated in the fresh differentiation medium supplemented with the appropriate concentration of the selected drug. The total proteins in a parallel dish were

extracted and used as the zero time point when analysing changes in expression of targeted cell differentiation markers. Other dishes were incubated in the differentiation medium in the presence or absence of selected drug as appropriate for defined time periods. Changes in cell morphology were recorded for up to 12 or 14 days by taking photos every two days, using a standard microscope and a digital eyepiece. Cell lysates were generated in parallel at the end of each time point and stored at -20°C until analysed by western blotting, using specific monoclonal antibodies against cardiac myosin heavy chain (MHC) or ventricular cardiac myosin light chain 1(MLC-1v) as described below (Section 2.14).

2.9 Differentiation of P19 Stem cells into beating cardiomyocytes

2.9.1 Initiation of P19 cell differentiation into cardiomyocytes

To initiate differentiation, P19 stem cells (1x10^6 cells) (passage not more than 30) were seeded in P100 Petri (i.e. microbiological) dishes in the differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 µg/ml streptomycin and 0.8% DMSO). P19 cells grown in Petri dishes (i.e. non-adherent conditions) do not attach but instead form cell aggregates known as embryoid bodies (EB) which subsequently differentiate into cardiomyocytes (McBurney *et al.*, 1982; Habara-Ohkubo, 1996; Brewer *et al.*, 2005). Embryoid bodies were allowed to form over a period of 4 days before transferring these into cell culture grade p60 dishes containing 4ml of full growth medium without DMSO. The day on which the embryoid bodies

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were transferred to cell culture grade dishes was labelled or designated as day 0. According to this labelling system, day 2 and day 4, for example, refer to the sixth and eighth day respectively since the P19 stem cell differentiation experiment was started with the setup of embryoid bodies. The growth medium was changed every other day and the cell monolayers were monitored daily for up to 12 or 14 days for the appearance of beating cardiomyocytes. Photographs were taken and where beating cardiomyocytes were present, videos were also recorded. Cell lysates were generated in parallel for western blot analysis using specific monoclonal antibody against cardiac troponin I (TnI) as described below (Section 2.14).

2.9.2 Effects of drugs (signalling pathway inhibitors) on P19 stem cell differentiation into cardiomyocytes

To determine whether the activation of a particular signalling pathway is required during the differentiation of P19 stem cells into cardiomyocytes, a selective and specific inhibitor of that signalling pathway was used to block the pathway. The effects of the pathway inhibition were then observed or measured. For the determination of the effects of drugs on the differentiation of P19 stem cells into cardiomyocytes, both control and drug-treated P19 stem cells (1×10^6 cells) were separately seeded in p100 Petri dishes. Control P19 stem cells were seeded in differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100units/ml penicillin/100μg/ml streptomycin and 0.8% DMSO). The drug-treated cells were initially seeded in culture medium as above but in the absence DMSO. The cells were placed in the incubator at 37^oC, 95% air and 5% CO_2. After one hour of incubation, the cell

suspensions in the Petri dishes of the drug-treated cells were supplemented with 0.8% DMSO and the cells were returned to the incubator. The control cells were left as before.

Four days after DMSO treatment, the EBs were transferred to cell culture grade p60 dishes or 6-well plates in full growth medium (i.e. without DMSO). Hereafter, the growth medium was changed every other day. The cell monolayers were monitored daily for up to 12 or 14 days for the appearance of beating cardiomyocytes. Cell photographs were taken and where beating cardiomyocytes were present, videos were also recorded. Cell lysates were generated in parallel for western blot analysis using appropriate selective antibodies as described below (Section 2.14).

2.9.3 Effects of delayed inhibition of signalling pathways on P19 stem cell differentiation into beating cardiomyocytes

The activation of a signalling pathway may be required only at a particular stage of a biological process. To determine the stage at which a particular signalling pathway is activated or required during the differentiation of P19 stem cells into cardiomyocytes, a selective and specific inhibitor of that signalling pathway was separately added to one batch of cells during DMSO-induced differentiation at the EB formation stage. The inhibitor was also separately added to another batch of cells four days after DMSO-initiated differentiation (i.e. inhibitor was only added after the formation of EB in DMSO). EB formation can be carried out over several days but a four day period is commonly used. We therefore decided to test the effects of the

addition of inhibitor after four days of DMSO-induced differentiation of P19 stem cells.

The control P19 cells were treated with differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100units/ml penicillin/100μg/ml streptomycin and 0.8% DMSO) containing an appropriate concentration of the selected inhibitor on the day the EBs were set up in Petri dishes. Control cells were exposed to the inhibitor only during the EB formation stage (i.e. no inhibitor exposure after the EB formation stage).

The delay-inhibition P19 cells were not initially treated with the inhibitor on the day the EBs were setup and were also not treated with the inhibitor throughout the EB formation stage, in Petri dishes. The delay-inhibition P19 cells were initially treated with differentiation medium (as above) in the absence of the selected inhibitor on the day the EBs were set up in Petri dishes. The delay-inhibition P19 stem cells were only treated with the differentiation medium containing the inhibitor from the day the EBs were transferred from Petri dishes to cell culture dishes (i.e. only after the four-day EB formation stage).

For differentiation experiments, both control P19 cells and delay-inhibition P19 cells (1×10^6 cells) were separately seeded in p100 Petri dishes. Control P19 cells were seeded in DMSO-free medium containing the appropriate concentration of the selected inhibitor. The delay-inhibition P19 cells were in seeded in differentiation medium (i.e. α-MEM supplemented with 10% FBS,

100units/ml penicillin/100μg/ml streptomycin and 0.8% DMSO) in the absence of the selected inhibitor. All the cells were placed in the incubator at 37°C, 95 % air and 5% CO_2. After one hour of incubation, the cell suspensions in the Petri dishes of the control P19 cells were supplemented with 0.8% DMSO (i.e. became differentiation medium plus drug) and the cells were returned to the incubator. The delay-inhibition P19 cells were left as initially set (i.e. differentiation medium only).

Four days after DMSO treatment, the EB of the control P19 cells were transferred to cell culture grade p60 dishes or 6-well plates in full growth medium (i.e. without DMSO). For the EB of the delay-inhibition P19 cells, after EB transfer to cell culture grade p60 dishes or 6-well plates, the differentiation medium was supplemented with the appropriate concentration of the selected inhibitor (i.e. became differentiation medium plus drug) for upto day 12.

The monolayers of both groups of cells were monitored daily for up to 12 days for the appearance of beating cardiomyocytes. Cell photos were taken and where beating cardiomyocytes were present, videos were also recorded. Cell lysates were generated in parallel for western blot analysis using a specific monoclonal antibody against cardiac troponin I as described below (Section 2.14).

2.10 Cross-talks between PI3K signalling pathway and other signalling pathways (PKC and p38 MAPK)

2.10.1 Time course of Akt phosphorylation at serine 473 residue in H9c2 cells differentiating into cardiomyocytes

To examine possible crosstalk(s) between the PI3K signalling pathway and the other signalling pathways (PKC and p38 MAPK), cross talk studies were carried out. For determination of the time course of Akt phosphorylation at serine 473 residue, H9c2 cells were seeded in p60 dishes at a density of 2×10^3 cells/cm^2 in full growth medium (i.e. DMEM supplemented with 10% FBS and 100units/ml penicillin/100μg/ml streptomycin). The cells were placed in the incubator at 37°C, 95 % air and 5% CO_2 and allowed to become 60-70% confluent. The culture medium was removed and the monolayers of cells were washed twice with warm (37°C) PBS. All the cells were incubated in fresh serum-free culture medium (i.e. DMEM supplemented with 100units/ml penicillin/100μg/ml streptomycin but no FBS) for 24 hours. After this period, the serum-free culture medium was removed and the monolayers of cells were washed twice with warm (37°C) PBS. Control cells were re-incubated in fresh serum-free medium and *1% FBS-treated H9c2 cells* (i.e. cells treated with low serum or e differentiation medium) were re-incubated in fresh differentiation medium (i.e. DMEM supplemented with 1% FBS and 100units/ml penicillin/100μg/ml streptomycin). All the cells were placed in the incubator at 37°C, 95 % air and 5% CO_2 and left for 0, 5, 10, 20, 30 minutes, 1, 2, 4, 6, 48 and 72 hours. Cell lysates were generated at the end of each time point and stored at -20°C until analysed by western blotting, using a

specific monoclonal antibody against phospho-Akt (Ser473) (Cell Signaling Technology via New England Biolabs, UK) as described below (Section 2.14).

2.10.2 Effects of drugs (signalling pathway inhibitors) on Akt phosphorylation at serine 473 residue in H9c2 cells differentiating into cardiomyocytes

To examine the effects of drugs (i.e. selective and specific inhibitors of signalling pathways) on Akt phosphorylation at serine 473 residue, H9c2 cells were seeded in p60 dishes at a density of $2x10^3$ cells/cm^2 in full growth medium (i.e. DMEM supplemented with 10% FBS and 100units/ml penicillin/100µg/ml streptomycin). The cells were placed in the incubator at 37^oC, 95 % air and 5% CO_2 and allowed to become 60-70% confluent. The culture medium was removed and the monolayers of cells were washed twice with warm (37^oC) PBS. The cells were incubated in fresh serum-free culture medium (i.e. DMEM supplemented with 100units/ml penicillin/100µg/ml streptomycin but no FBS) for 24 hours. After this period, the serum-free culture medium was removed from the dishes and the monolayers of cells were washed twice with warm (37^oC) PBS and the following cell treatment were carried out. Three experimental groups were then designated according how each particular group of cells was treated.

Control H9c2 cells: Control H9c2 cells (labelled as control) were treated with only serum-free medium throughout the Akt phosphorylation procedure (including the initial 24 hour treatment with serum-free medium). These cells

were not treated with any inhibitor or drug and were also not exposed to 1% FBS medium.

1 % FBS-treated H9c2 cells: 1 % FBS-treated H9c2 cells (labelled as 1% FBS) were treated only with differentiation medium (i.e. DMEM supplemented with 1% FBS and 100units/ml penicillin/100μg/ml streptomycin) (i.e. after the initial 24 hour treatment with serum-free medium). These cells were also not treated with any inhibitor or drug.

Inhibitor-treated H9c2 cells were labelled with the name of the inhibitor. After the initial 24 hour treatment with serum-free medium, the Inhibitor-treated H9c2 cells were first treated in the appropriate concentration of the selected drug in fresh serum-free medium. After one hour incubation at $37^{o}C$, 95 % air and 5% CO_2, the serum-free medium, containing the drug, was removed and the differentiation medium (i.e. DMEM supplemented with only with 1% FBS and 100units/ml penicillin/100μg/ml streptomycin) containing a fresh preparation the appropriate concentration of the selected drug was added to the cells.

After the appropriate treatment, cells were placed in the incubator at $37^{o}C$, 95 % air and 5% CO_2 and left for 15 minute incubation. After this period, cell lysates were generated and stored at $-20^{o}C$ until analysed by western blotting, using specific monoclonal antibodies against Akt phosphorylated at serine 473 residue (phospho-Akt-Ser473) as described below (Section 2.14).

2.11 Total protein extraction for western blotting

When required, lysates were generated from cells using a lysis buffer consisting of 20mM Tris-HCl, pH7.4, 1% SDS and 150mM NaCl. Cells were washed twice with ice-cold PBS and up to 500µl of hot (heated to 95°C) lysis buffer was added to cells in wells or dishes. The plates/dishes were immediately placed on ice and then 2.5µl of 200mM PMSF (i.e. 1mM PMSF final concentration) was added to cells in the dish. The cells were scraped off the dishes. The dishes with the scraped cells were left on ice for 15 minutes. During this time the cell lyates were regularly agitated by gentle trituration to obtain a homogenous lysate. The cell lysate or extract was transferred into a microcentrifuge tube and heated at 95°C for 5 minutes. The sample was then sonicated at 5-10 second interval for 1 minute. The homogenate or clear cell extract was transferred into a microcentrifuge tube and centrifuged at 1400 RPM at 4°C for 30 minutes to pellet insoluble materials. The pellet was discarded. The supernatant was transferred to a new microcentrifuge tube. The total proteins in the lysates were determined (see Section 2.12). The cell lysates or protein samples were prepared for loading onto gels as described below (section 2.13.1). Western blotting was usually carried out immediately or samples were stored at -20°C for later western blot analysis (Section 2.14).

2.12 Total protein determination in cell lysates using the BCA assay

The Pierce bicinchoninic acid (BCA) protein assay (Perbio Science, UK Ltd) was used to determine the total protein concentration in the cell lysates. This is a detergent-compatible formulation for the colorimetric detection and quantitation of total proteins. This method is a combination of the reduction of cupric ions [Cu (II)] to cuprous ions [Cu (I)] by proteins in an alkaline medium (i.e. biuret reaction) and a selective and sensitive colorimetric detection of the cuprous ions. The purple-colour reaction product of BCA assay is formed by the chelating of one Cu (I) ion by two molecules of BCA (Smith *et al.*, 1985; Wiechelman *et al.*, 1988). The purple complex formed has a maximum absorbance at 562nm. The absorbance is directly proportional to protein concentration (Smith *et al.*, 1985).

The colour produced from this reaction is stable and increases in a proportional manner over a broad range of increasing protein concentrations (Smith *et al.*, 1985). This colour complex exhibits a strong absorbance at 562 nm. The BCA method is not a true end-point method (i.e. colour development does not stop at the time of absorbance measurement). However, following incubation, the rate of continued colour development is sufficiently insignificant to allow large numbers of samples to be assayed together.

2.12.1 Preparation of bovine serum albumin (BSA) standards for the BCA assay

The microplate version of the BCA assay was carried out on the cell lysates using bovine serum albumin (BSA) (Sigma, UK) standard concentrations (0.0 to 0.5µg/µl). The microplate version of the BCA is linear between 0.02 to 2µg/µl of BSA or proteins. The BSA standard concentrations were prepared as shown in Table 1 from a 1mg/ml stock solution of BSA. All dilutions were carried out in the same lysis buffer used to lyse cells.

Table 1: Preparation of Diluted Albumin (BSA) Standards

Volume (µl) of 1mg/ml solution	Volume (µl) of lysis buffer	Final BSA concentration (µg/µl)
0	1000	0
25	975	0.025
50	950	0.050
100	900	0.100
200	800	0.200
300	700	0.300
400	600	0.400
500	500	0.500

2.12.2 Procedure of the BCA assay

Pierce BCA assay reagent A contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide. Reagent B contains 4 % (w/v) Copper (II) sulfate pentahydrate (CuSO4 • 5H$_2$O). A 1:50 dilution of reagent B in reagent A was prepared. A 10 µl aliquot of either standards or cell lysate was added in triplicate to a 96-well plate. To each well containing standards or cell samples, 100µl of the BCA reagent was added.

The lid or cover was put on the plate. The plate was incubated for 45-60 minutes at room temperature and the absorbance measured at 620nm on a multiscan plate reader (Labsystems Multiskan Ascent, UK). The average absorbance measured for the blanks (i.e. lysate buffer alone) was subtracted from the absorbance measurements of all other values. A protein standard curve was generated by plotting the average corrected absorbance (i.e. measured absorbance – blank absorbance) for each BSA standard against its concentration in µg/µl, using Microsoft Excel 2003 software. The equation for the best fit of standard curve was used to determine the protein concentration of each unknown sample. Alternative to the standard curve generation, using Microsoft Excel, the linear regression routine in GraphPad Prism software (version 4) was used to work out the protein concentration in lysates. In Microsoft Excel and GraphPad Prism software, only standard curves with r^2 value (a measure of linearity of BSA standard concentrations) of 97% or better were used. A dilution factor of 11 was applied to the calculations to determine the protein concentration in the cell lysates. A representative BCA protein standard curve is shown in Figure 2.11.
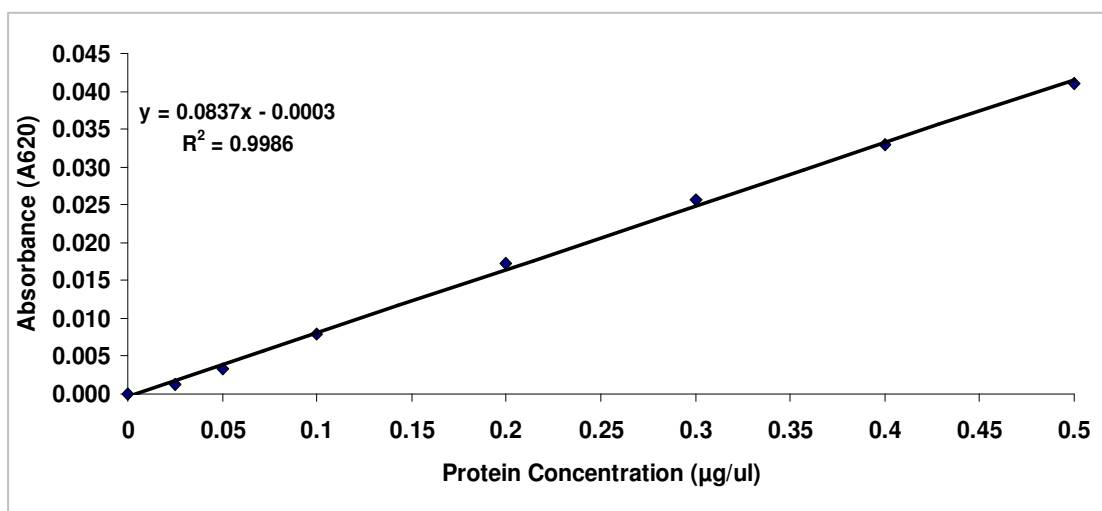
Figure 2.11: A Representative BCA Standard Curve. The protein standard curve was set up as described above. The average absorbance measurement of the blank was subtracted from the absorbance values of each standard used. The mean of triplicate readings was then taken and plotted against the BSA concentration used. This standard curve is representative of several BCA assays carried out during the course of the studies.

Using the equation generated from the standard curve (Figure 2.11), for a sample with an absorbance of 0.039 at 620nm; the corresponding protein concentration was calculated as follows:

$$y = 0.0837x - 0.0003$$
$$x = \frac{y + 0.0003}{0.0837} = \frac{0.039 + 0.0003}{0.0837} = 0.4735 \ \mu g/\mu l$$

Applying a dilution factor of 11 (10µl of sample in 110µl total volume) gave:

$$\text{Protein Concentration} = 0.4735 \times 11 = 5.21 \ \mu g/\mu l$$

Volume of sample containing the selected amount of total protein was calculated and this was used to load gels for western blotting as required.

2.13 Western blot analysis

The Western blotting procedure carried out consisted of the following steps: cell lysate preparation, separation or resolution of the proteins in the lysate by gel electrophoresis, transfer of the resolved proteins from the gel to a membrane, immunoblotting (i.e. incubation antibody of the transferred proteins on the membrane with antibodies), detection of the proteins bands that have been recognised or bound by the antibodies on x-ray films and analysis of the bands captured on the films.

2.13.1 Sample preparation for western blot analysis

A volume of protein extract containing an appropriate amount (e.g. 40, 60 or 80μg) of total proteins was added to an equal volume of 2X concentrated sample buffer containing Tris-HCl, pH6.8 (120mM), SDS (4%), glycerol (10%), β-mercaptoethanol (2%) and bromophenol blue 0.006%).

2.13.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

A resolving and stacking gel were prepared on a Bio-Rad Mini-PROTEAN II casting stand (Bio-Rad, UK). For the casting of 5ml of an 8.0% resolving gel (enough for one mini blot), the following components were added in the order listed: double distilled water (2.34ml), 30% acrylamide/bisacrylamide 37.5:1 (1.30ml), 1.5M Tris-HCl, pH8.8 (1.25ml), 10% sodium dodecyl sulphate (SDS) (0.050ml), 10% ammonium persulfate (APS) (0.025ml) and N,N,N′,N′-

tetramethylethylenediamine (TEMED) (0.005ml). For a 10% resolving gel, the water and acrylamide/bisacrylamide volumes were adjusted to 2.0ml and 1.67ml respectively and everything else stayed the same. Similarly for a 12% resolving gel, the water and acrylamide/bisacrylamide volumes were adjusted to 2.0ml and 1.67ml respectively and everything else stayed the same.

After polymerisation of gels was complete, 5% stacking gel was prepared by adding the following components: water (2.84ml), 30% acrylamide/bisacrylamide 37.5:1 (0.83ml), 0.5M Tris-HCl pH6.8 (1.25ml), 10% SDS (0.050ml), 10% APS (0.025ml) and TEMED (0.005ml). The stacking gel was layered on top of the resolving gel and allowed to polymerise.

The gel was transferred to a Bio-Rad Mini-PROTEAN II (Bio-Rad, UK) electrophoresis tank. A 1x electrophoresis tank buffer was prepared from a 10X stock consisting of: 30.28 g/L of Tris base (0.25M, pH 8.3), 144.0 g/L of glycine (1.92M), 100ml of 10% SDS (1%). Enough 1x diluted electrophoresis buffer to cover the top of the gel, was added to the gel tank. The prepared lysates along with a biotinylated protein ladder (Cell Signaling Technology via NEB, UK) were heated at 95°C for 5 minutes prior to loading onto the gel. The gel was run at a constant voltage of 100V for 40-60 minutes or until the bromophenol blue front had migrated to the bottom of the gel.

2.13.3 Transfer of proteins from gel to PVDF Membrane

The semi-dry transfer with a single buffer system was used to transfer the protein proteins from the gel to a 0.45µm pore size polyvinylidene fluoride (PVDF) membrane (Amersham, UK). Following SDS-PAGE, a 10X transfer buffer (1litre) was prepared using the following: 58.2 g/L of Tris base (0.48M, pH 8.3), 29.3 g/L of glycine (0.39M), 3.75ml of 10% SDS (0.038%). This was then stored at 4^0C and diluted 1:10 in double distilled water to give a 1x working solution containing 20% methanol. The PVDF membrane was cut to the same size as the gel, soaked in methanol for 1 minute, rinsed in excess distilled water for 1 minute and then equilibrated in the transfer buffer for at least 5 minutes.

Six filter papers, each cut to the same size as the gel, were soaked in the diluted transfer buffer. When available, two pieces of extra thick blot papers (Bio-Rad, UK) (each used in place of three filter papers) were used. Three of the six buffer-soaked filters were placed on the electroblotter (semi-dry transfer systems) (Bio-Rad, UK). The PVDF membrane was then placed on top of the stacked soaked filter papers on the electroblotter. A reference of the protein side on the PVDF membrane was made by cutting a small notch in one corner of the membrane. The gel was removed from the gel cast and placed on top of the PVDF membrane (Figure 2.12). The other three buffer-soaked filter papers were positioned over the gel and air bubbles between the gel and the filter papers were removed by gently rolling a clean 25ml pipette over the top filter or blot paper. A constant current of 0.8mA/cm^2 was applied to the gel and the protein transfer was carried out for 2-3 hours.
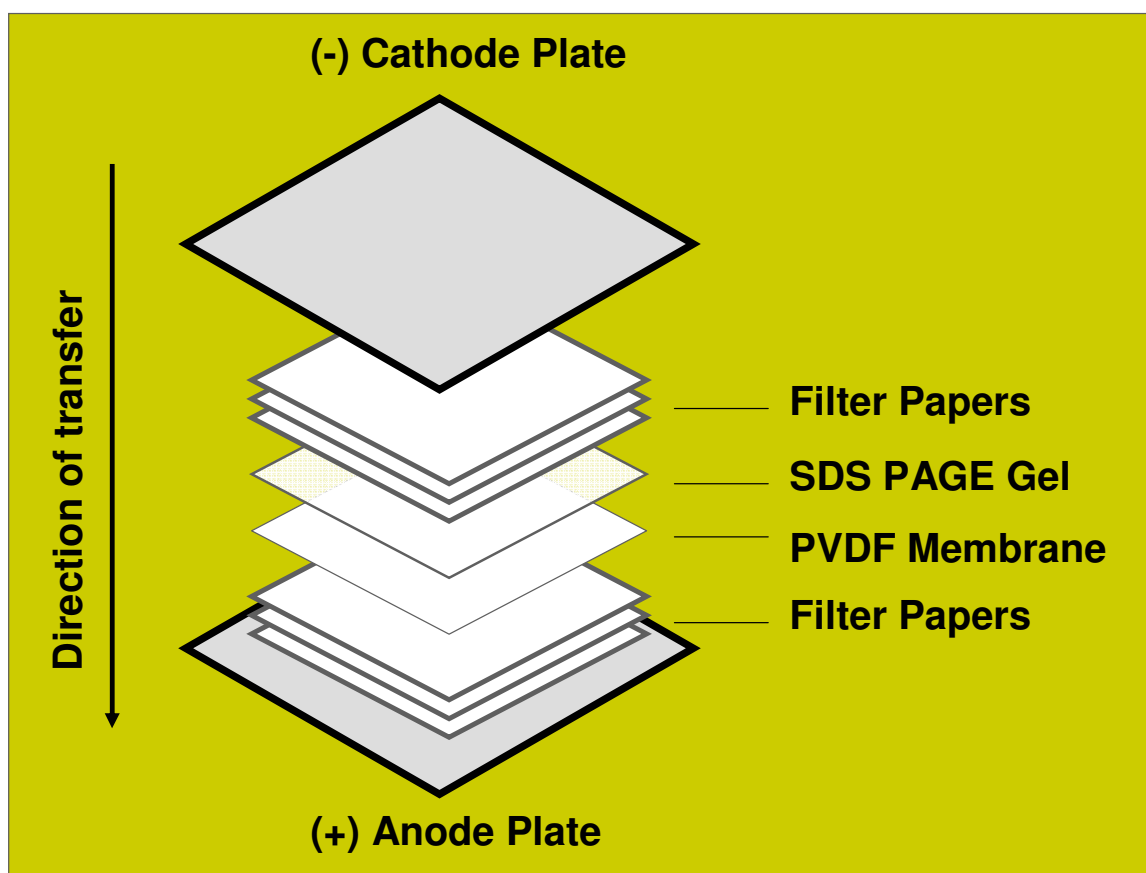
Figure 2.12: Semi-dry transfer of protein from gel to PVDF. This schematic diagram illustrates the arrangement of components in the semi-dry transfer assembly.

2.13.4 Blocking of the membrane

The membranes were blocked to prevent non-specific binding of antibodies to charged and other molecules on the membrane, thus preventing or reducing background noise. One litre of 10X Tris-buffered saline (TBS) solution containing 24.2g/L of Tris base (20mM; pH 7.5), 87.7g/L of NaCl (150mM) was prepared and normally stored at 4°C. The 10x TBS was used to prepare a 100ml of blocking buffer as follows: 10ml of 10x TBS, 5g (5% w/v) dry virtually fat-free milk (Marvel Brand) and 100µl (0.1% v/v) of Tween 20 (Sigma, UK) in double distilled water. The membrane was incubated in the blocking buffer for 1 hour at room temperature or overnight at 4°C.

2.13.5 Western blot antibody staining

After blocking, the membrane was incubated, using gentle agitation on a shaker, with the primary antibody in a sealed plastic bag. The incubation was carried out at room temperature for one hour. The primary antibody used to probe the blots was mouse monoclonal antibody to cardiac myosin heavy chain (1:2500) or mouse monoclonal antibody to cardiac troponin I (1:2500) or mouse monoclonal antibody to cardiac ventricular myosin light 1 (MLC-1v) (1:2500) or mouse monoclonal antibody to troponin I (1:1000) in blocking buffer. These primary antibodies were purchased from Abcam (Cambridge, UK). For the lysates generated from cross-talk studies, the primary antibody used in the blocking buffer to probe the blot, was the mouse monoclonal antibody to phospho-Akt (Ser473) (i.e. Akt phosphorylated at serine 473) (1:1000) (Cell Signaling Technology via New England Biolabs (NEB), UK).

After incubation with the primary antibody, the blot was washed three times (each wash lasting for 5- 10 minutes on a shaker) in wash buffer made up with 1x TBS containing 0.1% Tween 20. The incubation reactions and post incubation washes as described for the primary antibodies were repeated using goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000) (Cell Signaling Technology via NEB, UK) as the secondary antibody. An anti-biotin antibody (1:2000) (Cell Signaling Technology via NEB, UK) also conjugated to horseradish peroxidase was added in order to detect the protein ladder. Anti-β-actin antibody (1:10000) (Sigma, UK) conjugated to horseradish peroxidase was added in order to detect β-actin which was used as a loading control.

2.13.6 Detection of Protein Bands Using ECL Reagents

Detection or visualization of targeted protein bands (i.e. bound antibodies) was carried out in a dark room using enhanced chemiluminescence (ECL). The ECL reagent was prepared following the manufacturer's instructions by mixing equal volumes of reagent 1 and 2 supplied in the ECL Western Blotting Detection kit (Amersham, UK). The solution was immediately added to the membrane and incubated for 1 minute at room temperature. Excess detection reagent was drained from the membrane using a tissue paper. The blot was covered with cling film, ensuring there were no trapped air bubbles on the membrane. A sheet of autoradiography film, Hyperfilm ECL (Amersham, UK) was placed on top of the wrapped membrane in a film cassette and exposed for 5 minutes. The membrane was then developed,

fixed, rinsed under running tap water and then dried in air at room temperature.

2.13.7 Quantification of protein bands

The developed protein bands on the film were scanned using Epson (Perfection 2480 Photo) Scanner and the intensities of the protein bands on the scanned image were measured using densitometery software, Syngene Gene Tools (version 3.00).

2.14 Data Analysis

Data obtained from at least 3 independent experiments, (performed in single or duplicates, where possible), were used for statistical analysis. The one-way analysis of variance (ANOVA) test, followed by post-hoc Bonferroni's multiple comparison test or Dunnett's multiple comparison test, was carried out using GraphPad Prism version 4. A difference between the treatments was considered significant when $P< 0.05$. Data presented are means $\pm$ SD from at least 3 independent experiments.

CHAPTER 3.0

ESTABLISHMENT OF CARDIAC DIFFERENTIATION MODELS

3.1 INTRODUCTION

In order to be able to conduct the studies described in this thesis, it was essential to develop an *in vitro* model of cardiomyocyte differentiation. This was achieved by exploiting the ability of H9c2 cells to differentiate into cardiomyocytes when cultured in growth medium containing a reduced concentration of serum (Hescheler *et al.*, 1991) and also by using the mouse pluripotent P19 embryonal carcinoma stem cell line which can be maintained and differentiated into cardiomyocytes under standard cell culture conditions (e.g. 10% FBS and without the need for LIF or feeder cells) (McBurney *et al.*, 1982; Wobus *et al.*, 1991; Angello *et al.*, 2006).

3.1.1 H9c2 cell line model of cardiomyocyte differentiation

The H9c2 cell line is a well established cardiomyocyte differentiation model. These cells were established from embryonic rat ventricles by Kimes and Brandt (1976) and are now widely used by researchers as an *in vitro* model for generating cardiomyocytes (Kim *et al.*, 1999; Menard *et al.*, 1999; Hong *et al.*, 2001; Kageyama *et al.*, 2002; Giusti *et al.*, 2004; Pagano *et al.*, 2004).

In addition to differentiation studies, H9c2 cells have been used as a simple and relevant model to study various mechanisms in cardiomyocytes including apoptosis (Ekhterae *et al.*, 1999; Chae *et al.*, 2001; Hong *et al.*, 2001; Kageyama *et al.*, 2002; Bonavita *et al.*, 2003; Kim *et al.*, 2005; Pesant *et al.*, 2006; Qin *et al.*, 2006), glucose transport (Armoni *et al.*, 2005), opioid-induced

acute cardioprotection (Gross *et al.*, 2006) and reactive oxygen species (ROS)-induced cardiotoxicity in cardiomyocytes (Salvatorelli *et al.*, 2006).

The differentiation of these cells into cardiomyocytes is a highly ordered and regulated multi-step process that is initiated in response to a number of environmental signals including exposure to low serum content (Tamai *et al.*, 2000). The differentiation is initiated by mononucleated cell alignment, elongation and fusion leading to the formation of multinucleated myotubes. This process is accompanied by an increase in the expression of muscle specific proteins (Ludolph and Konieczny, 1995). Manipulations such the effect of drugs (e.g. inhibitors of signalling pathways) can be easily monitored during the cardiac differentiation of these cells. The H9c2 cell line is therefore a good model for investigating the signalling events that control cardiomyocyte differentiation.

3.1.2 P19 cell line model of cardiomyocyte differentiation

The P19 embryonal carcinoma stem cell line (P19 stem cells) is pluripotent and has been used as an *in vitro* model of stem cell differentiation into cardiomyocytes (Wobus *et al.*, 1991; Angello *et al.*, 2006). The cardiomyocyte differentiation of P19 stem cells is initiated in the presence of DMSO (McBurney *et al.*, 1982). The cardiac specific transcription factors, GATA-4 and Nkx2-5, are expressed in during the differentiation of P19 cells into cardiomyocytes (Skerjanc *et al.*, 1998).

Using these two cell types, we have developed systems which generate cardiomyocytes and thus provide models which we could exploit to investigate the mechanisms that regulate the differentiation of stem cells into cardiomyocytes

3.2 MATERIALS AND METHODS

3.2.1 Culture and differentiation of H9c2 Cells

The routine culture of H9c2 cells was carried out as described in section 2.3.1 (Chapter 2). H9c2 cells were trypsinised and the appropriate cell density for the differentiation experiments was calculated as described in section 2.6 (Chapter 2). H9c2 cells (2×10^3 cells/cm^2) were seeded in p60 dishes or 6-well plates in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin), placed in a tissue culture incubator at 37°C, 95% air and 5% CO_2 and allowed to become 60-70% confluent. The differentiation of H9c2 cells into cardiomyocytes was carried out in 1% serum as described in section 2.8.1 (Chapter 2).

3.2.2 Culture and differentiation of P19 stem cells

The routine culture of P19 stem cells was carried out as described in section 2.3.2 (Chapter 2). To initiate differentiation, P19 stem cells (1×10^6 cells) were seeded in P100 Petri (i.e. microbiological) dishes in the differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 µg/ml streptomycin and 0.8% DMSO) and the differentiation of P19 stem cells into beating cardiomyocytes was carried out as described in section 2.9.1 (Chapter 2).

3.2.3 Western blot analysis

In order to perform the western blot analysis, the following procedures were carried out as described in Chapter 2 under the sections indicated in parenthesis: cell lysate generation (section 2.11), preparation of BSA standards (section 2.12.1) and BCA assay (section 2.12.2). The western blotting was carried out as described in section 2.13.

3.2.4 Data analysis

Data obtained from independent experiments were used for statistical analysis as described in section 2.14 (Chapter 2).

3.3 RESULTS

3.3.1 Establishment and characterization of H9c2 Cell differentiation model

Freshly cultured undifferentiated H9c2 cells in full growth medium containing 10 % FBS initially were rounded but subsequently attached and grew as a monolayer. These undifferentiated H9c2 cells grew as single cells with clearly defined boundaries and looked almost spindly-shaped in morphology (Figure 3.10). This morphology is maintained throughout culture if the cells are not induced to undergo differentiation. The cells became highly confluent but failed to initiate cell alignment, elongation and fusion to form myotubes (Figure 3.11). In agreement with the lack of morphological changes in H9c2 cultured in full growth medium cardiac myosin heavy chain (alpha or beta isoform) (α/β-MHC) was not expressed as determined by western blotting (Figure 3.12).
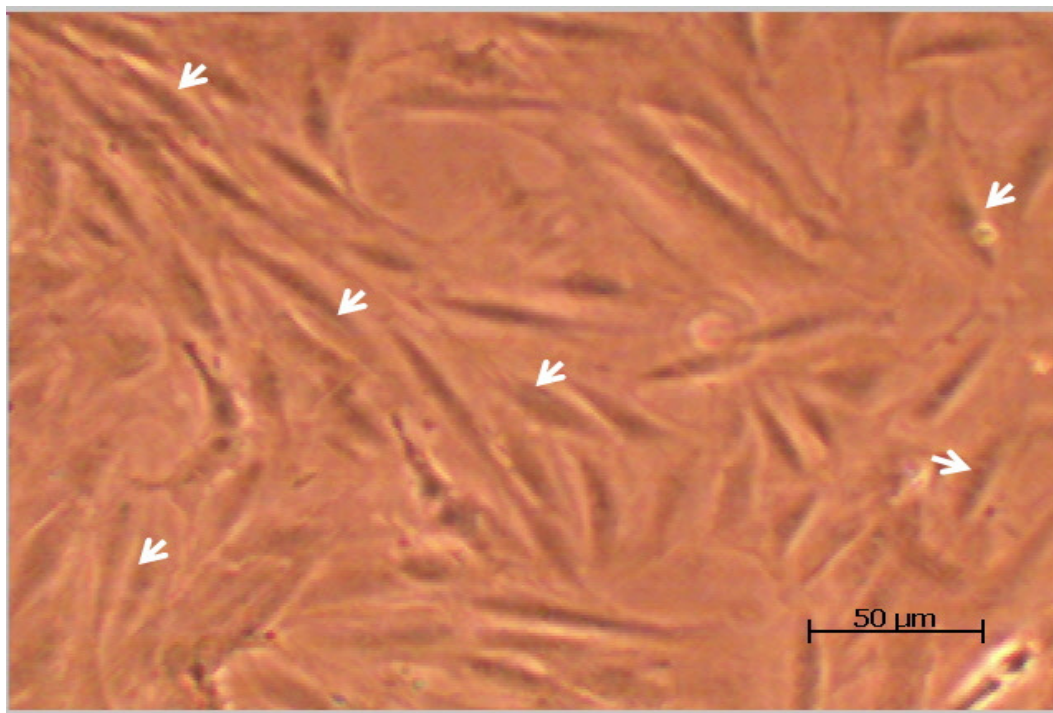
Figure 3.10: Morphology of H9c2 cells (undifferentiated) in routine culture.
H9c2 cells cultured in full growth medium consisting of DMEM supplemented with 10% FBS and 100 units/ml penicillin/ 100 µg/ml streptomycin grew as single elongated cells (white arrows). The photograph at day 4 was taken under 200X magnification and is representative of several microscope fields from several cultured flasks.

Day 0	Day 2	Day 4	Day 6

Day 8	Day 10	Day 12	Day 14
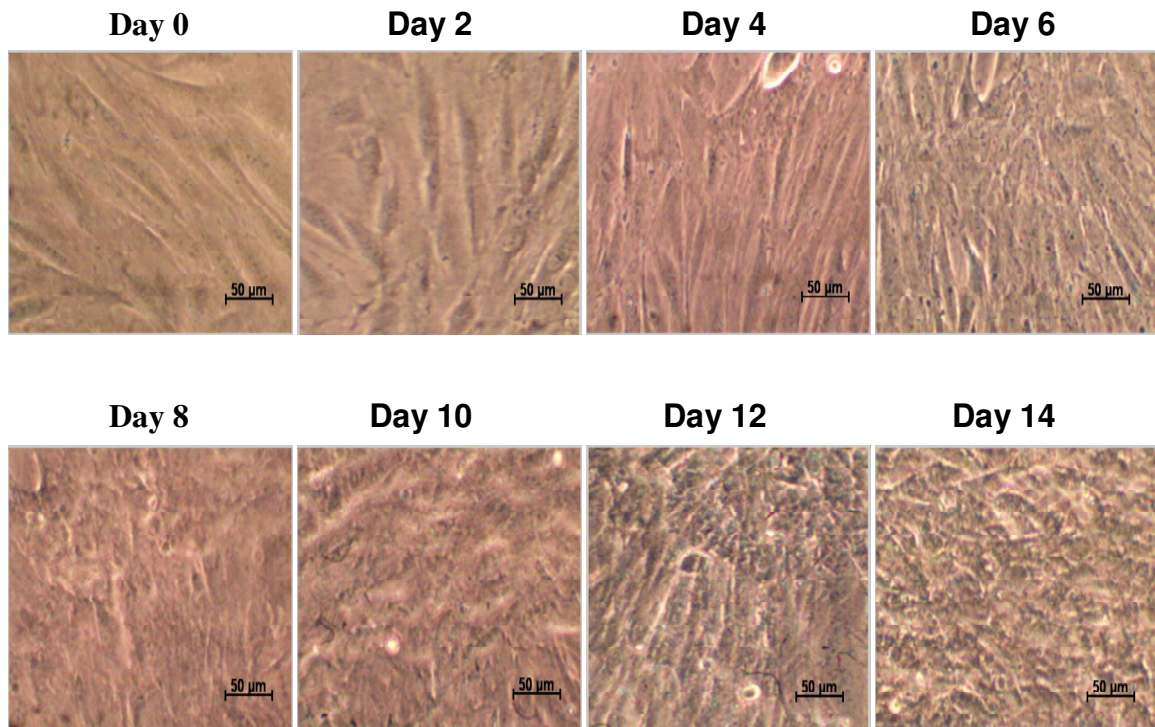
Figure 3.11: Morphology of undifferentiated H9c2 cells in full culture medium
H9c2 cells cultured in full growth medium consisting of DMEM supplemented with 10% FBS and 100 units/ml penicillin/100 µg/ml streptomycin grew as single elongated cells becoming very tightly packed and undefined after 10-14 days in culture. The photographs (200X) are representative microscope fields for the indicated days.
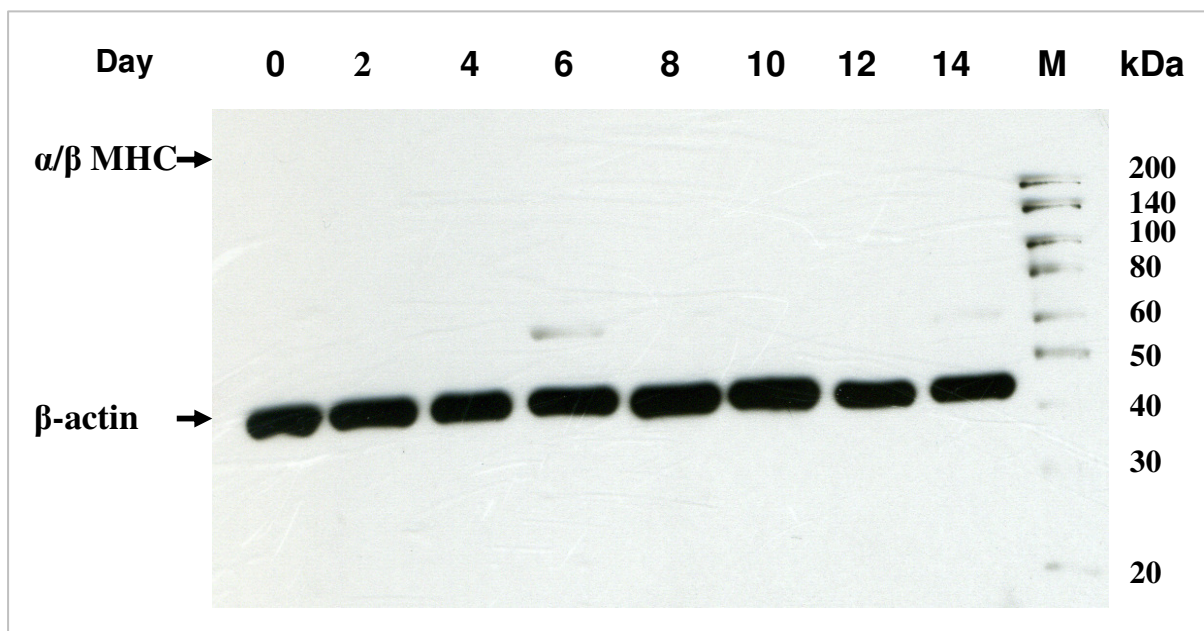
Figure 3.12: Expression of myosin heavy chain in undifferentiated H9c2 cells. In agreement with the lack of morphological changes in H9c2 cultured in full growth medium (i.e. DMEM supplemented with 10% FBS and 100 units/ml penicillin/100 µg/ml streptomycin), cardiac myosin heavy chain (MHC) was not expressed as determined by western blotting using a monoclonal antibody specific for the α and β isoforms of the protein. A blot of lysates from H9c2 cells cultured in full medium is shown and is representative of three independent experiments.

3.3.2 Differentiation of H9c2 cells into cardiomyocytes

In contrast to the growth pattern of H9c2 cells observed in the full growth medium, when these cells were cultured in 1% serum, they initially grew as flat, elongated mononucleated myoblasts similar to the morphology seen in the 10% FBS culture medium. However after four days in low serum culture, neighbouring cells began to fuse with complete differentiation becoming evident at day 12 to day 14. The differentiated cells acquired a myotube-like morphology (Figure 3.13). Spontaneous contraction, a distinguishing feature of cardiomyocytes, was however not observed in these cells.

The morphological changes seen in H9c2 cells undergoing differentiation into cardiomyocytes were accompanied by parallel increases in the expression of cardiac specific markers, including myosin heavy chain (MHC) (Figure 3.14) and ventricular myosin light chain (MLC-1v) (Figure 3.15). The expression of MHC was not detected in differentiated cells between days 0 to 2 in cells induced to differentiate. Myosin heavy chain expression was however evident at day 4 and continued to increase up to day 10, reaching a peak from day 12 to day 14. By comparison, MLC-1v expression, although absent in lysates from cells at day 0, was quite evident from day 2, reaching a peak between day 10 to 12.
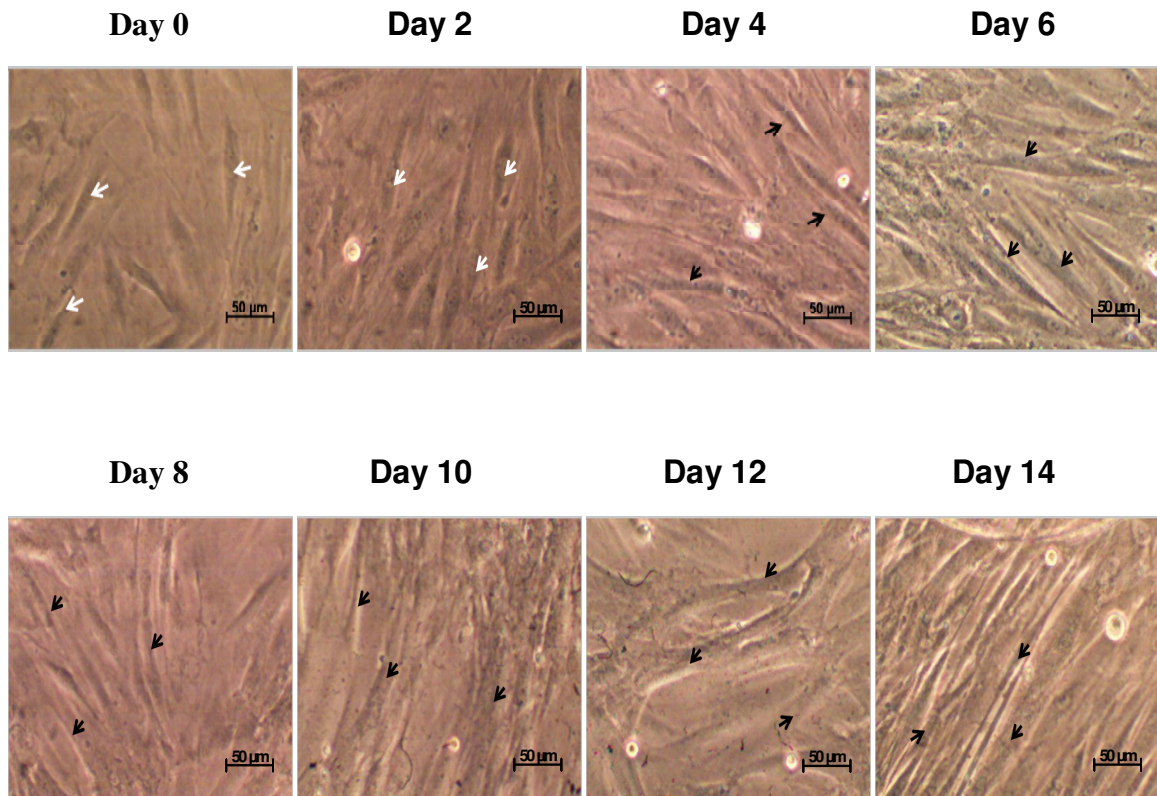
Figure 3.13: Morphology of differentiated H9c2 cells in culture. H9c2 cells cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100 units/ml penicillin/100 µg/ml streptomycin grew initially grew as single elongated cells (white arrows on day 0 and day 2) which by day 4 began to fuse, forming myotubes that were more evident at days 12 to 14 (black arrows on day 4 to 14). The photographs were taken under 200X magnification are representative microscope fields for the indicated days.
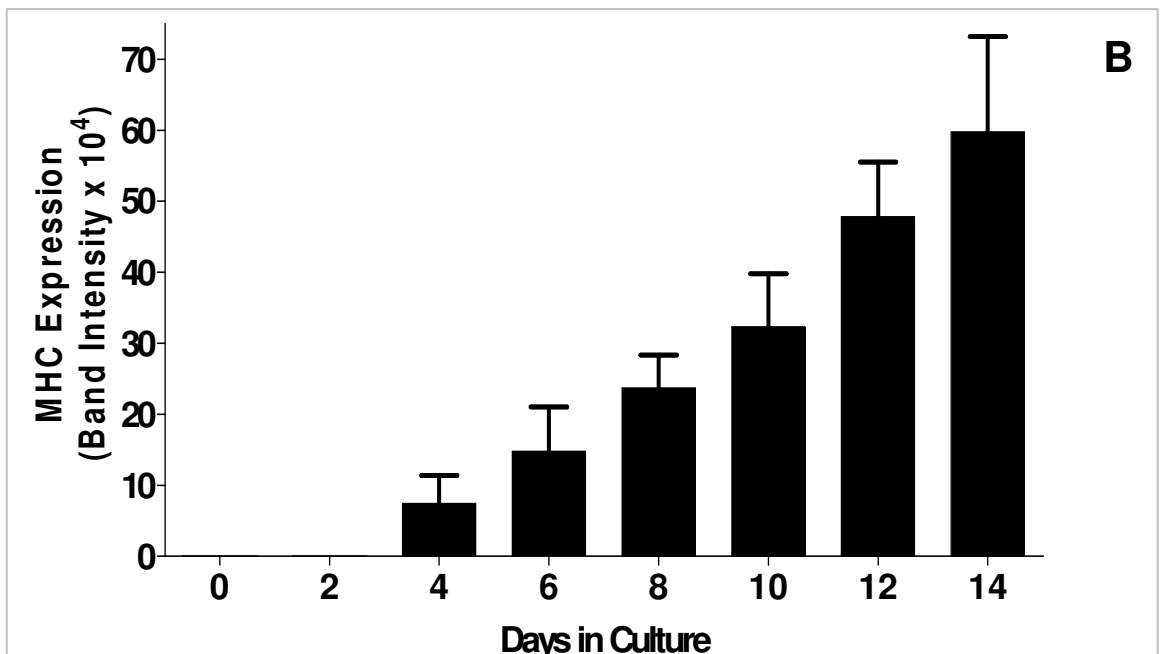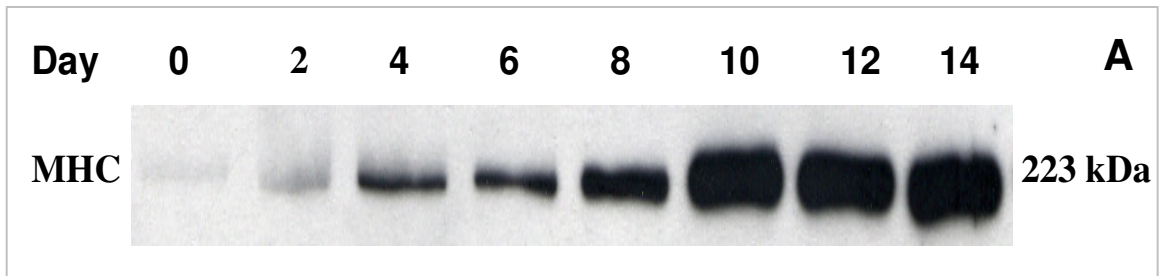
Figure 3.14: Changes in the expression of myosin heavy chain in differentiated H9c2 cells. H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100 units/ml penicillin/100 µg/ml streptomycin. Cell lysates were prepared at the end of each time point specified on the graph and subjected to western blotting using a specific monoclonal anti-MHC antibody. A blot of lysates from cells cultured in is shown (Panel A). The bar graph is the densitometric data (mean ± SD) from three independent experiments (Panel B).
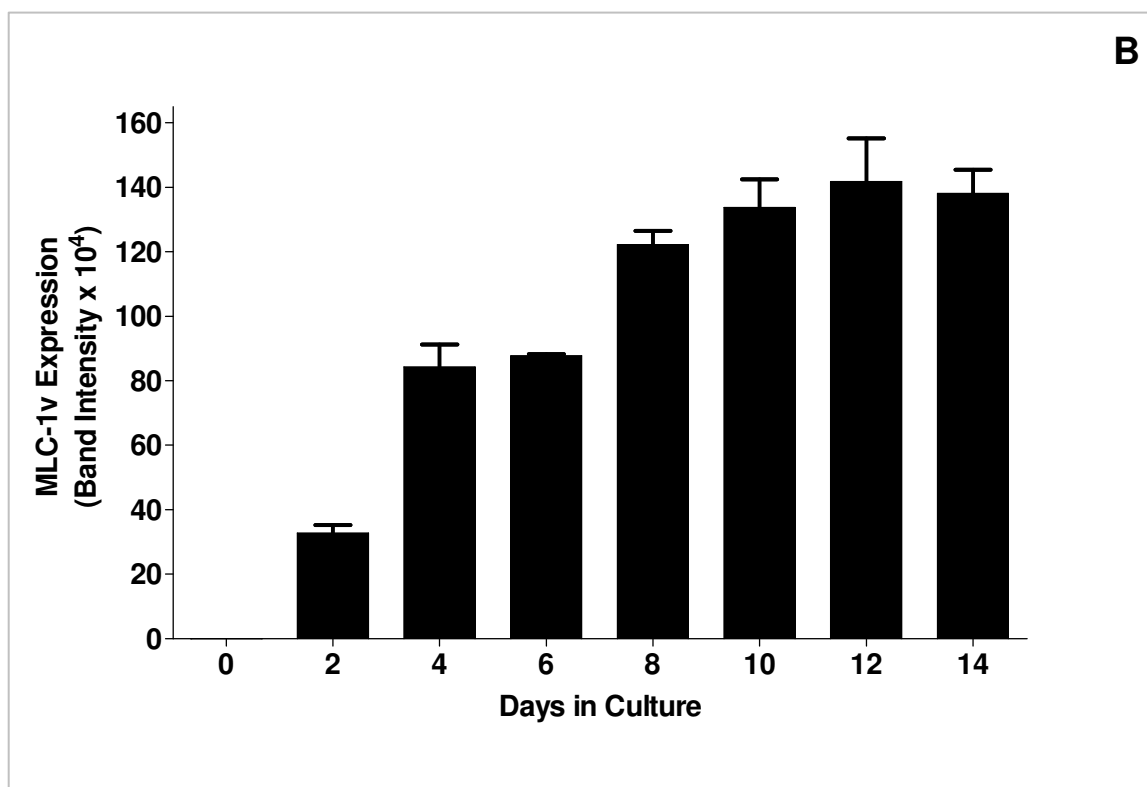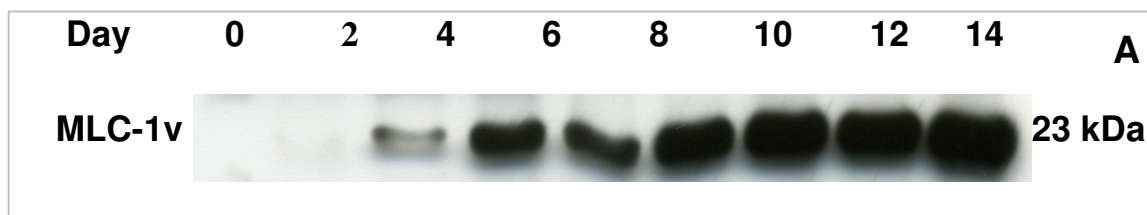
Figure 3.15: Changes in the expression of ventricular myosin light chain in differentiated H9c2 cells. H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% foetal bovine serum (FBS) and 100 units/ml penicillin/100 µg/ml streptomycin. Cell lysates were prepared at the end of each time point specified on the graph and subjected to western blotting using a specific monoclonal anti-MLC-1v antibody. A blot of lysates from cells cultured in is shown (Panel A). The bar graph is the densitometric data (mean ± SD) from three independent experiments (Panel B).

3.3.3 Establishment and characterization of P19 stem cell differentiation model

P19 embryonal carcinoma stem cells cultured in normal cell culture grade dishes in complete culture medium did not form embryoid bodies and did not differentiate into cardiomyocytes. Instead, they grew as a single cell monolayer but in clusters (Figure 3.16). In contrast, P19 cells cultured in microbiological Petri dishes in complete cell culture medium resulted in the cells aggregating and growing in suspension as embryoid bodies (EBs). This occurred within a few hours with the aggregates growing in size over time. The sizes and shapes of the formed EBs were however highly variable. The EBs increased in size over time (Figure 3.17).

Adherent P19 Stem Cells
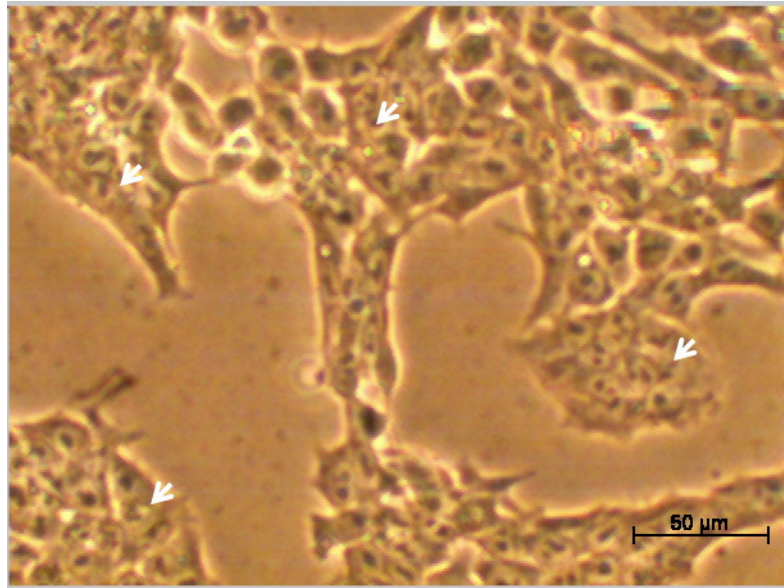
Figure 3.16: Normal growth pattern of P19 embryonal carcinoma stem cells in culture. P19 cells were seeded into standard cell culture dishes and allowed to grow in complete culture medium (α-MEM supplemented with 10%. The cells grew as clusters (white arrows) in a monolayer FBS and 100 units/ml penicillin/100 µg/ml streptomycin) did not form embryoid bodies. The photograph was taken under 200X magnification and is representative of several microscope fields from more than three cultured flasks.

P19 stem cell Embryoid Bodies at Day 1
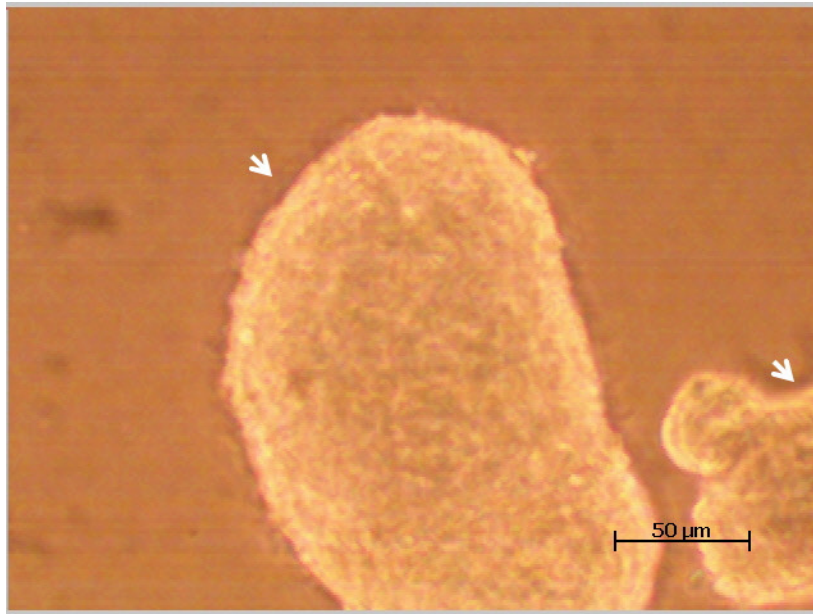
Figure 3.17: Formation of embryoid bodies from P19 embryonal carcinoma stem cells in culture. P19 cells were seeded into Petri dishes and allowed to grow in complete culture medium (α-MEM supplemented with 10% FBS and 100 units/ml penicillin/100 μg/ml streptomycin) and 0.8% DMSO to form embryoid bodies (white arrows). The photograph of the developing embryoid body at 24 hour was taken under 200X magnification and it is representative of several microscope fields from three independent experiments.

3.3.4 Differentiation of P19 stem cells into beating cardiomyocytes

The transfer of EB from Petri dishes to standard tissue culture dishes resulted in cell adhesion. The cell growth pattern changed from the three dimensional EB to a two dimensional monolayer (Figure 3.18). Twenty fours later, EB adhesion was followed by cell migration and proliferation. A rapid cell growth was observed in these early stages with monolayers becoming confluent by day 4. The first beating clusters of cardiomyocytes were routinely detected by day 6 or 7. When present, the size and number of beating clusters of cardiomyocytes usually increased up to day 14. There was a high degree of variability both in term of the clusters of beating cells and in the frequency at which they appeared to beat. Sometimes, isolated clusters of beating cells within monolayers (day 6 and day 14) were seen. At other times, uniform beating monolayers (day 8, day 10 and day 12) were seen. Some colonies demonstrated a much more rhythmic and rapid rate of contraction (day 8, day 10 and day 12) while others showed an intermediate or much slower contraction rates or uncoordinated contractions (day 6, and day 14). The slow or uncoordinated contractions appeared to occur more in isolated clusters of cells within monolayers while the much more rhythmic and rapid rate of contraction was evident in uniform monolayers.
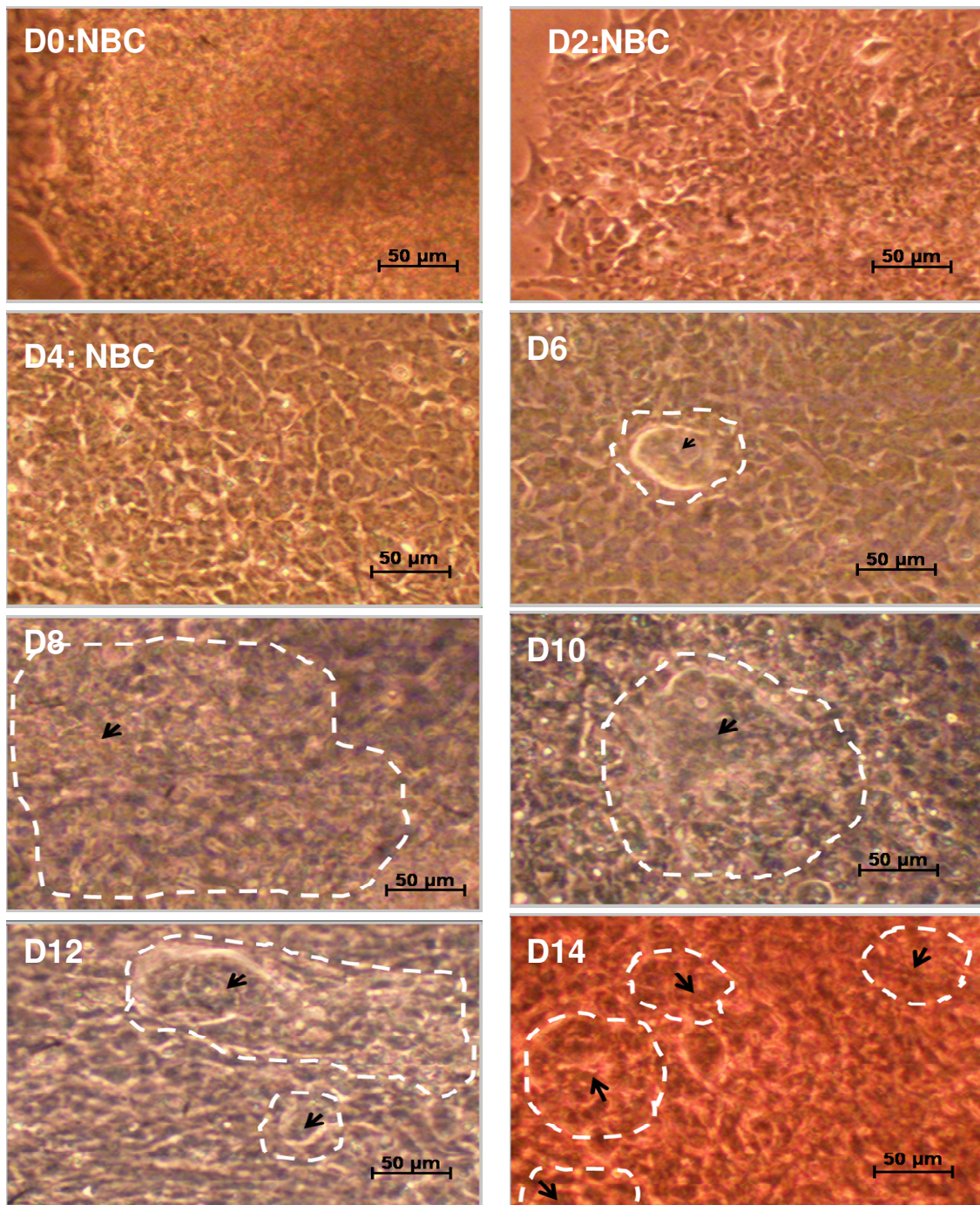
Figure 3.18: DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes. P19 stem cell EBs were formed in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 µg/ml streptomycin and 0.8% DMSO for four days and then transferred into standard cell culture dishes in DMSO-free medium. DMSO caused the differentiation of P19 stem cells into beating cardiomyocytes. The beating areas of cardiomyocytes are indicated by the dotted white circles. The beating focus of each beating area is shown by a black arrow. Photographs and movie clips of beating cells were taken under 200X magnification on the days indicated and are representative of microscope fields (for their respective days) from at least three independent differentiation experiments. NBC = no beating cardiomyocytes.

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3.4 DISCUSSION

This project is the first of its kind in the laboratory in which it was carried out. The host laboratory has strong background in the research field of cell signalling in cardiovascular biology but had carried out no research work on stem cell differentiation at the beginning of this project. This project is therefore a new direction in the research activity in our laboratory.

As discussed in the background to this project, cardiomyocyte lineage specification during cardiogenesis is a complex event. Therefore in the design of a study to explore cardiomyocyte differentiation *in vitro* the choice of a model has to be given careful consideration. Thus the first challenge was to find a model to investigate the cell signalling events that mediates cardiomyocyte differentiation. It was decided to use a model that is not only relevant but also simple so that it can be easily manipulated to address key questions about signalling events during cardiomyocyte differentiation. The P19 stem cell and H9c2 cell models meet this condition. Therefore given that this is the first stem cell project in our laboratory, the P19 stem and H9c2 cell lines were   attractive models to seek key answers or cues to signalling pathway that mediate cardiac differentiation.

The H9c2 cell line was chosen due to its relevance in cardiac regeneration, simplicity of use as well as the fact that this cell line has been extensively used by other researchers as a cardiomyocyte differentiation model   and also for the investigation of   other molecular or biological process in cardiomyocytes (Kim *et al.*, 1999; Menard *et al.*, 1999; Hong *et al.*, 2001;

Kageyama *et al.*, 2002; Giusti *et al.*, 2004; Pagano *et al.*, 2004). In addition to the differentiation studies, H9c2 cells have been used as a simple and relevant model to study various mechanisms in cardiac muscle cells including apoptosis (Ekhterae *et al.*, 1999; Chae *et al.*, 2001; Hong *et al.*, 2001; Kageyama *et al.*, 2002; Bonavita *et al.*, 2003; Pesant *et al.*, 2006; Qin *et al.*, 2006), glucose transport (Armoni *et al.*, 2005), opioid-induced acute cardioprotection (Gross *et al.*, 2006) and reactive oxygen species (ROS) cardiotoxicity (Salvatorelli *et al.*, 2006). Differentiated H9c2 cells preserve the receptor systems, signalling pathways and cardiac proteins (e.g. cardiac L-type Ca2+ channels) found in primary cardiomyocytes (Hescheler *et al.*, 1991; Sipido and Marban, 1991). The H9c2 cell line is therefore an appropriate model for our studies aimed at investigating the signalling mechanisms that regulate differentiation into cardiomyocytes.

Freshly cultured H9c2 cells in a suspension of complete growth medium looked rounded. Once attached, the undifferentiated cells grew as spindle-shaped mononucleated cells and did not express cardiac specific markers. The cells maintained this morphology throughout their period in culture (14 days). On the other hand, differentiated cells looked elongated and often fuse to form multinucleated myotubes. The expression of both MHC and MLC-1v was also evident in the cells differentiated in culture medium containing low serum (1% FBS).

The mechanism by which low serum induces the differentiation of H9c2 cells into cardiomyocytes is not clear; however there are possible and/or plausible

explanations. A critical event in the activation of the canonical Wnt/β-catenin pathway is level of cytosolic β-catenin. The phosphorylation of β-catenin by Glycogen synthase kinase 3β (GSK-3β) mediates the ubiquitination of β-catenin resulting in the subsequent degradation of β-catenin by proteosomes (Giles *et al.*, 2003; Logan and Nusse, 2004). While phosphorylated β-catenin is targeted for degradation, the non-phosphorylated form of β-catenin is degradation-resistant. A decreased in phosphorylation of β-catenin causes an increase in the levels of β-catenin in the cytoplasm and this leads to its subsequent translocation into the nucleus. In the nucleus, β-catenin, in a complex with LEF/TCF, activates the transcription of Wnt target genes (Yamaguchi *et al.*, 1999; Ai *et al.*, 2000).

Lithium (a GSK-3β inhibitor) treatment mimics the activation of the canonical Wnt/β-catenin signalling pathway by inducing the stabilization and nuclear translocation of β-catenin via inhibition of β-catenin phosphorylation by GSK-3β (Klein and Melton, 1996; Hedgepeth *et al.*, 1997; Rao *et al.*, 2005). Our data showed that H9c2 cells in 10% serum do not differentiate into cardiomyocytes but do so in low serum medium. It has also been shown that the treatment of H9c2 cells with lithium can cause H9c2 cell to differentiate into cardiomyocytes (Kashour *et al.*, 2003). This suggests that lithium treatment mimics low serum condition and also that low serum, like lithium, may activate some elements of the canonical Wnt/β-catenin signalling pathway which may play roles in the initiation of H9c2 cell differentiation into cardiomyocytes. Whether low serum, like lithium, can induce the activation of the canonical Wnt/β-catenin signaling pathway warrants further investigation.

H9c2 cells generally grow very slowly. The splitting ratio or cell density is therefore a critical consideration and once determined for optimal growth, the ratio should be adhered to in order to avoid inconsistent growth rates which, from our observations, can influence differentiation. Another factor worth mentioning is the fact that when brought from long term storage (in liquid nitrogen) the cells should first be cultured at high density in a smaller culture flask than that in which they were originally frozen from. This was found to be critical in helping the cells to regain their growth potential after a long stay in the liquid nitrogen. Where this is not carried out, the cells will either not recover from the freezing or may show sluggish growth and may never fully recover in future passages. Routine splitting ratios of 1:3-1:5 of 60-70% confluent H9c2 were found to be appropriate for maintaining the cells in a viable healthy state.

While H9c2 cells are easy to manipulate and differentiate into cardiomyocytes under standard laboratory conditions, the myoblast population in the cell line can easily be lost if proper care is not taken during routine cell culture. Therefore when H9c2 cells are used for cardiac differentiation experiments, the prevention of the depletion of the myoblast population is essential. Prolonged or continual culture of H9c2 cells without subculturing, during routine cell culture, will lead to a rapid depletion of the myoblast population in the cell line. This was avoided in our studies by making sure that at all times while in culture the cells were not allowed to become more than 60-70 % confluent. If the cells became fully confluent for any reason they were

discarded and a new batch was resuscitated from the liquid nitrogen cell stock.

The H9c2 cardiac differentiation model, like most models, is not a perfect substitute for the *in vivo* processes. Despite the many attractive features of working with these cells, one of the serious limitations of the H9c2 model for cardiac differentiation is that the cell line was derived from an embryonic heart. This means the cardiac lineage commitment may have already taken place. However cardiac specific genes such as those which code for cardiac specific myosin light chain and ventricular myosin light chain-1 are not constitutively expressed in undifferentiated H9c2 cells but are strongly expressed when the cells are induced to differentiate into cardiomyocytes. This makes these cells relevant for exploring the signalling transduction events that drives their differentiation into cardiomyocytes.

The use of P19 stem cell line as a model for stem cell differentiation into cardiomyocytes was also developed in parallel with the H9c2 cell model. The P19 stem cell line, like H9c2 cells, is relatively simple and can be easily manipulated in experiments. With proper care for the cells during routine cell culture and also during differentiation experiments, P19 stem cell differentiation into cardiomyocytes can be highly regulated and reproducible. An essential element in ensuring differentiation of ES cells is the formation of three-dimensional (3D) aggregates known as embryoid bodies (EB). P19 cells when cultured in the suspension form EB. The EB consists of ectodermal, mesodermal and endodermal cell types and the 3D environment in the EB helps in the recapitulation of the early stages of embryonic development thus

initiating the cell differentiation into cell types of all the three germ layers including cardiomyocytes (Wobus *et al.*, 1991; Maltsev *et al.*, 1993; Denning *et al.*, 2006), endothelial cells (Zhang *et al.*, 2005), neuronal cells (Bain *et al.*, 1995), adipocytes (Dani *et al.*, 1997) and blood cell precursors (Schmitt *et al.*, 1991). In P19 stem cells, EB formation in the presence of DMSO induces the cells to specifically differentiate into cardiomyocytes.

Between 6 to 8 days after EB transfer into cell culture grade dishes, beating cardiomyocytes were regularly observed in DMSO-induced P19 stem cells. While beating cardiomyocytes can be readily obtained, the population is highly heterogeneous. The beating rates from one cluster to another varied considerably even within the same culture dish. Where the main research focus is the unravelling of the signalling pathways that are mediating the differentiation of P19 stem cells into cardiomyocytes, this lack of homogenous P19 stem-derived beating cardiomyocytes may not be a major problem.

P19 stem cells have several good features. The cells are pluripotent and can be maintained continuously in serum-supplemented growth medium without the need for LIF or mitotically inactive feeder cells to keep them in the undifferentiated state. The fact that these cells can be maintained under standard cell culture conditions means that key experiments can be performed at relatively very low cost in comparison to the same experiments being performed using other ES cells that may need special conditions such as knockout serum, LIF or feeder cells. Another strong point for the use of P19 stem cells is that the cell line is a well-established model of

cardiomyocyte differentiation (Wobus *et al.*, 1994; Zhang *et al* 2005; Angello *et al.*, 2006). Moreover P19 cells can be induced to differentiate into cardiomyocytes fairly readily following exposure to DMSO (McBurney *et al.*, 1982; Monzen *et al.*, 1999; Naito *et al.*, 2003; Brown *et al.*, 2004; Brewer *et al.*, 2005).

In culture medium containing DMSO, P19 cells differentiating into cardiomyocytes recapitulate the principal biological features or stages of embryonic cardiogenesis. The expression of cardiac transcription factors including Nkx-2.5 (Skerjanc *et al.*, 1998; Monzen *et al.*, 1999; Wen *et al.*, 2007) GATA-4 (Monzen *et al.*, 1999; Uchida *et al.*, 2007; Wen *et al.*, 2007) and MEF2C (Skerjanc *et al.*, 1998; Wen *et al.*, 2007) have been well documented in P19 stem cells differentiating into cardiomyocytes. Cardiac specific proteins, including MHC (Habara-Ohkubo, 1996) and MLC (Habara-Ohkubo, 1996; Moore *et al.*, 2004), are expressed by cardiomyocytes derived from P19 stem cells. This means that P19 stem cells differentiating into cardiomyocytes follow the same mechanisms as normal embryonic stem cells.

It is not entirely clear how DMSO induces P19 stem cells to differentiate along the cardiac lineage. DMSO actions are not specific for P19 stem cells and neither are its actions limited to induction of cell differentiation. Beside the induction of cell differentiation, many other biological effects including membrane transport, vasodilation, muscle relaxation and antagonism of

platelet aggregation, have been attributed to DMSO (Jacob and Herschler, 1986).

Some available evidence suggests a possible role of DMSO in the Wnt/β-catenin signalling pathway for the commitment of P19 stem cell to differentiate into cardiomyocytes. Evidence for a regulatory role of DMSO on canonical Wnt/β-catenin signalling was shown by the ability of DMSO to induce a decrease in levels of phosphorylated β-catenin and the elevation of the non-phosphorylated form of β-catenin in the cytoplasm (Nakamura *et al.*, 2003). It was also shown that the levels of Wnt3a and Wnt8a were up-regulated by DMSO before the expression of cardiac specific transcription factors such as Nkx2.5, GATA-4 and Tbx5 (Nakamura *et al.*, 2003). Wnt3a and Wnt8a are believed to signal exclusively via the canonical Wnt/β-catenin pathway (Tada and Smith, 2000; Pandur *et al.*, 2002; Maurus *et al.*, 2005). The DMSO induced elevation of cytoplasmic β-catenin levels was suppressed by Frizzled-8/Fc (Fz-8/Fc) (Nakamura *et al.*, 2003), a protein that belongs to the secreted frizzled-related proteins. Secreted frizzled-related proteins are *in vivo* Wnt antagonists (Rattner *et al.*, 1997; Bafico *et al.*, 1999; Pera and De Robertis, 2000; Shibata *et al.*, 2000; Marvin *et al.*, 2001). The treatment of P19 stem cells with Fz-8/Fc also inhibited the DMSO-induced expression of cardiac transcription factors, cardiogenic growth factors (i.e BMP2, BMP4 and FGF8) and cardiac α-MHC (Nakamura *et al.*, 2003). This is a strong evidence that DMSO activates or modulates the the Wnt/β-catenin signalling pathway.

Glycogen synthase kinase 3β, as mentioned above, is responsible for keeping the canonical Wnt/β-catenin pathway in the "off" or inactive state through the phosphorylation of β-catenin canonical wnt/β-catenin signalling. Constitutively active GSK-3β blocks DMSO-induced differentiation of P19 stem cells while lithium and Wnt3A-conditioned medium up-regulated early cardiac markers and the proportion of differentiated P19 stem cells in the presence of DMSO (Nakamura *et al.*, 2003). The inhibition of DMSO-induced cardiac differentiation of P19 stem cells by constitutively active GSK-3β also suggests that DMSO may act through the Wnt/β-catenin signaling pathway. All the above data when taken together form a strong evidence to suggest that Wnt/β-catenin signalling is activated during the initiation of cardiac differentiation of P19 stem cell and that DMSO mode of actions may be the activation or modulation of the canonical wnt/β-catenin signalling pathway.

DMSO is not an endogenous signalling molecule but a clear understanding of The mode of action of DMSO in the induction of P19 stem cell differentiation into cardiomyocytes may increase our understanding of stem cell differentiation into cardiomyocytes. For example the suppression of DMSO-induced cardiac specific transcription factors (such as Nkx2.5 and GATA-4) in P19 cells by Wnt antagonists and GSK-3β inhibitors (Nakamura *et al.*, 2003) is also seen in other stem cell cardiac differentiation models (e.g. AB2.2 ES cells) where DMSO is not the cardiac inducing agent (Liu *et al.*, 2007). Thus a clearer understanding of DMSO roles in P19 stem cell differentiation into cardiomyocytes may lead to improved protocols for the differentiation of stem cells into cardiomyocytes.

There are several potential cardiac markers for monitoring the differentiation of stem cells into cardiomyocytes. These include Nkx2.5, GATA-4 (Kehat *et al.*, 2001; Nakamura *et al.*, 2003; Anderson *et al.*, 2007), myocyte enhancer factor (MEF2C) (Denning *et al.*, 2006; Anderson *et al.*, 2007), tropomyosin (Moore *et al.*, 2004), α-actinin (Denning *et al.*, 2006), cardiac troponin I (cTnI) (Kehat *et al.*, 2001), cardiac troponin T (cTnT) (Kehat *et al.*, 2001), atrial natriuretic peptide (ANP) (Denning *et al.*, 2006), cardiac myosin light chain isoforms (MLC-2v and MLC-2a) (Kehat *et al.*, 2001; Moore *et al.*, 2004), MLC-1v & MLC-1a and cardiac myosin heavy chain isoforms (α-MHC and β-MHC) (Kehat *et al.*, 2001; Denning *et al.*, 2006; Anderson *et al.*, 2007). For this project we monitored cardiac differentiation by the expression of cardiac α-MHC/β-MHC, MLC-1v (in H9c2 cells)   and cTnI (in P19stem cells). Ventricular myosin light chain 1 (MLC-1v) along with MLC-2v is present in both ventricles of the heart (Chuva de Sousa Lopes *et al.*, 2006).

3.5 Summary

Freshly cultured undifferentiated H9c2 cells in full growth medium containing 10 % FBS attached and grew as a monolayer with spindly-shaped morphology. In agreement with the lack of morphological changes, undifferentiated cells did not expressed cardiac myosin heavy chain as determined by western blotting. In contrast to their growth pattern in full medium, when H9c2 cells were cultured in 1% serum, the cells became aligned, elongated, fused and formed myotubes. The morphological changes seen in H9c2 cells undergoing differentiation into cardiomyocytes were accompanied by parallel increases in

the expression of cardiac specific markers, including cardiac myosin heavy chain (MHC) and cardiac ventricular myosin light chain (MLC-1v).

P19 embryonal carcinoma stem cells cultured in normal cell culture grade dishes in complete culture medium (with or without 0.8% DMSO) did not form embryoid bodies (EB) but grew as clusters, in a single cell monolayer, which did not differentiate into cardiomyocytes. In contrast, P19 cells cultured in microbiological Petri dishes in complete culture medium, supplemented with 0.8% DMSO, formed EBs. The transfer of EB from Petri dishes to standard tissue culture dishes resulted in cell adhesion, the formation of a two-dimensional monolayer, cell migration and proliferation. The first beating clusters of cardiomyocytes were routinely detected by day 6 or 7. The size and number of beating clusters of cardiomyocytes usually increased up to day 14. There was a high degree of variability both in term of the clusters of beating cells and in the frequency at which they appeared to beat.

In conclusion, models of cardiomyocyte differentiation using P19 cells and H9c2 cells were successfully established in this project. These models were subsequently used in further studies exploring the mechanisms that may regulate stem cell differentiation into cardiomyocytes. Both P19 stem cells and H9c2 cells are relevant models for cardiomyocyte differentiation studies.

CHAPTER 4.0

ROLE OF NITRIC OXIDE IN CARDIOMYOCYTE

DIFFERENTIATION

4.1 INTRODUCTION

4.1.1 Physiological functions of nitric oxide

Nitric oxide (NO) is a gas that plays many important roles as signalling molecule in the cardiovascular system. A key feature of this molecule is its high but time limited reactivity. The roles of nitric oxide in the cardiovascular system include the maintenance of vascular integrity through the inhibition of platelet aggregation (Freedman *et al.*, 1999), leukocyte–endothelium adhesion (Kubes *et al.*, 1991; Lefer *et al.*, 1999) and the regulation of cardiac smooth muscle contraction (Skarsgard *et al.*, 2000) to cause vasodilatation. NO relaxes the smooth muscles to cause vasodilation. Nitric oxide also functions as neurotransmitter in the nervous system.

4.1.2 Biosynthesis of nitric oxide

The generation of nitric oxide is controlled by nitric oxide synthases (NOS) of which three isoforms exist: cytokine-inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). These enzymes display tissue-specific expression and their activation is also related to the cell status and specific stages during development. Neuronal NOS is predominantly expressed in certain neurons and in skeletal muscle, eNOS is predominantly expressed in endothelial cells and iNOS is expressed by macrophages and monocytes. Monocytes are phagocytes circulating in the blood. When a monocyte leaves the blood and enters into tissues, it undergoes a series of cellular changes to become a macrophage.

4.1.3 Molecular or physiological targets of nitric oxide

Nitric oxide mediates its second messenger or signalling roles through soluble guanylate cyclase (sGC), its main physiological target (Gold *et al.*, 1990; Trigo-Rocha *et al.*, 1993; Phung *et al.*, 1999). Nitric oxide activates guanylate cyclase by binding to the heme group. The activation of sGC by NO results in increased levels of cGMP. Nitric oxide, through cGMP, regulates various downstream protein kinases (e.g. cGMP-dependent kinase) (Archer *et al.*, 1994; Pineda *et al.*, 1996), ion channels (i.e. cGMP-dependent ion channels) (D'Ascenzo *et al.*, 2002) and cGMP-dependent phosphodiesterases (Wexler *et al.*, 1998; Zima *et al.*, 2000). In the vasculature NO relaxation of vascular smooth muscle is also mediated through cGMP. The role of nitric oxide as a neurotransmitter in the nervous system is also mediated by increase in cGMP level.

In contrast to the beneficial cGMP-dependent signalling roles of NO, cGMP-independent actions may be toxic to the cells and tissues. For example, NO reactions with superoxide anion to form peroxynitrite anion(Brealey *et al.*, 2002), the inhibition of mitochondrial complexes I and IV (Takehara *et al.*, 1995; Okada *et al.*, 1996; Brealey *et al.*, 2002) and the activation of poly-ADP ribose polymerase (PARP) (Endres *et al.*, 1998) leading to depletion of NAD+ (a key glycolytic substrate) and the eventual depletion of cellular energy stores are all general mechanisms that underlie some of the toxic effects of NO.

4.1.4 Nitric oxide and stem cell differentiation

Some available data suggest that NO may have positive roles during stem cell commitment to cardiac lineage. On the hand, there are other reports with data to suggest that NO may negatively regulate stem cell differentiation into cardiomyocytes. Therefore currently the exact role of NO during stem cell commitment to cardiac lineage is not clear.

Reports that NO may be involved in stem differentiation into cardiomyocytes have been published (Kanno *et al.*, 2004). NO treatment of ES D3 cells using NO donors and also iNOS transfected in the ES cell were found to increase the number and the size of beating foci in EB outgrowths (Kanno *et al.*, 2004). It has also been demonstrated that incubation of EBs with the NOS inhibitor N (G)-methyl-L-arginine acetate (L-NMMA) markedly attenuates their differentiation into cardiomyocytes with the inhibition being reversed by the application of the NO-donor spermine-NONOate (Bloch *et al.*, 1999). Taken together, these findings indicate a positive role for NO in the differentiation of stem cells into cardiomyocytes. In addition to stem cells, NO may also regulate differentiation of numerous other cell types. For example, NO has been reported to promote the differentiation of mouse bone marrow clonal stromal cells (ST2 cells) to osteoblast-like cells (Hikiji *et al.*, 1997; Koyama *et al.*, 2000), human neuroblastoma cells (Obregon *et al.*, 1997; Ghigo *et al.*, 1998), human trophoblast (Lyall *et al.*, 1998; Sanyal *et al.*, 2000), as well as regulate differentiation of NG108-15 (Cote *et al.*, 1998) and PC12 cells (Phung *et al.*, 1999; Nakagawa *et al.*, 2000) into neurones.

The role of NOS and NO in heart muscle during embryonic development has been extensively investigated. On day 7.5, no NOS isoform was detected in mouse and rat embryos (Bloch *et al.*, 1999). However from E9.5 both rat and mouse embryos displayed prominent expression of iNOS and eNOS, which correlated with high expression of soluble guanylate cyclase (sGC) and also with elevated levels of cyclic guanosine monophosphate (cGMP) (Bloch *et al.*, 1999).

It was found that after E14.5, the expression of both iNOS and eNOS was down-regulated and also shortly after birth, eNOS expression was detectable while that of iNOS was barely detectable (Bloch *et al.*, 1999). In ES D3 cells, the NOS expression pattern in seen *in vivo* was also seen in these cells (Bloch *et al.*, 1999). Similarly, the expression of eNOS and nNOS but not iNOS in undifferentiated ES cells was also reported in another study (Krumenacker *et al.*, 2006). The expression of all three NOS isoforms were detected at different phases after the initiation of differentiation of ES cells into cardiomyocytes (Krumenacker *et al.*, 2006). In these studies, nNOS expression decreased within one day while iNOS mRNA became detectable after several days, and eNOS increased after 7-10 days (Krumenacker *et al.*, 2006). The differential expression of the various NOS isoforms suggest that NO may be involved in early stages of cardiogenesis and also that there may be change in NO requirement as differentiation progresses.

Similarly it was shown that oxytocin-induced differentiation of P19 stem cells into cardiomyocytes was inhibited by the NOS inhibitor N (G)-nitro-L- arginine

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methyl ester (L-NAME). The NO donor SNAP was able to reverse L-NAME mediated inhibition of P19 cell differentiation into cardiomyocytes (Danalache *et al.*, 2007). These studies clearly indicate a role for NO and the NOS enzymes in stem cell differentiation but what is evident is that this may be a complex process. This complexity is highlighted by the fact that suppression of NOS activity by L-NAME has also been shown to increase the number of stem and progenitor cells in the bone marrow of irradiated mice (Michurina *et al.*, 2004), indicating a negative regulatory role for NO in the generation of, at least, bone marrow derived stem and progenitor cells. The role of NO therefore clearly warrants further investigation, especially in determining the precise role of the different NOS isoforms in the differentiation process and indeed the levels and profile of NO release that may be required for promoting or regulating differentiation.

The aim of these studies was therefore to confirm the role of NO in the generation of stem cell derived cardiomyocytes and to further investigate the potential mechanisms that may mediate the process. To achieve these aims NO donors with varying profiles of NO release were used in these investigations. The following nitric oxide donors were used:

NOC-5 *(3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine)* is a stable NO donor that can spontaneously release two equivalents of nitric oxide in solution under physiological conditions without any cofactor. NOC-5 is fast releasing NO donor with a half-life of 93 minutes. *SIN-1 (i.e. 3-Morpholinosydnonimine)* is also a fast releasing NO donor with a half-life of 40 minutes (Lomonosova *et al.*, 1998). It spontaneously decomposes to yield

nitric oxide and superoxide anion radicals. NOC-18 *(i.e. 2, 2'-(Hydroxynitrosohydrazino)bis-ethanamine)* is a slow releasing NO donor half-life with a half-life of 20 hours (Shami *et al*., 1998). SNAP (i.e. *(±)-S-Nitroso-N-acetylpenicillamine)* is also a slow releasing NO donor with a half-life of 37 hours (Ferrero *et al*., 1999).

In further studies examining the role of NO in cardiac differentiation, guanosine 8-Bromo-,3′,5′-cyclic monophosphate (8-Bromo-cGMP) was also used to determine whether NO may act through the cGMP pathway as indicated indirectly in other studies. 8-Bromo-cGMP is a cell-permeable cGMP analog that referentially activates protein kinase G. It is more resistant than cGMP to cleavage by phosphodiesterases.

The rationale for taking this approach is because NO generally acts by elevating cGMP levels within vascular cells in exerting its actions. Thus if NO was activating the guanylate cyclase pathway and enhancing cGMP levels in H9c2 cells then we would anticipate that treatment of cells with 8-Bromo-cGMP would mimic the effects seen with the NO donors. These studies were carried out using the H9c2 cells initially and it was hoped that the studies will be extended to the P19 stem cell line. The latter was however not used because of the negative data obtained with the H9c2 cells and time constraints.

4.2 MATERIALS AND METHODS

4.2.1 Culture and differentiation of H9c2 Cells

The routine culture of H9c2 cells was carried out as described in section 2.3.1 (Chapter 2). H9c2 cells were trypsinised and the appropriate cell density for the differentiation experiments was calculated as described in section 2.6 (Chapter 2). H9c2 cells ($2x10^3$ cells/cm^2) were seeded in p60 dishes or 6-well plates in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin), placed in a tissue culture incubator at 37°C, 95% air and 5% CO_2 and allowed to become 60-70% confluent. The differentiation of H9c2 cells into cardiomyocytes was carried out in 1% serum as described in section 2.8.1 (Chapter 2). The effects of the nitric oxide donors (NOC-5, NOC-18, SIN-1 or SNAP, each at a concentration of 100µM) on the differentiation of H9c2 cells into cardiomyocytes were investigated as described in section 2.8.2 (Chapter 2). The role of 8-Bromo-cGMP (100µM) on the differentiation of H9c2 cells into cardiomyocytes was also investigated as described in section 2.8.2 (Chapter 2). The concentrations of NOC-5, NOC-18, SIN-1, SNAP and 8-Bromo-cGMP, used in experiments, were prepared by diluting the stock concentrations. The preparation of stock concentration, for each of the above compounds, was carried out as explained (Appendix III).

4.2.2 Cell viability assay

To examine the effects of various NO donors on H9c2 cell viability, the MTT assay was carried out as described in section 2.7 (Chapter 2).

4.2.3 Western blot analysis

In order to perform the western blot analysis, the following procedures were carried out as described in the Chapter 2 under the sections indicated in parenthesis: cell lysate generation (section 2.11), preparation of BSA standards (section 2.12.1) and BCA assay (section 2.12.2). The western blotting was carried out as described in section 2.13.

4.2.4 Data analysis

Data obtained from independent experiments were used for statistical analysis as described in section 2.14 (Chapter 2).

4.3 RESULTS

4.3.1 Effects of the NO donor NOC-5 on H9c2 cell differentiation into cardiomyocytes

Differentiation of H9c2 cells into cardiomyocytes, as determined by elongation and cell fusion, was significantly delayed in cells treated with NOC-5 when compared to controls. By day 4, differentiation was visible in control H9c2 cells. In contrast, NOC-5 treated cells maintained their normal single cell and spindle shaped morphology. However, by day 8, both controls and NOC-5 treated cells appear differentiated and this was clearly evident at day 12 (Figure 4.10). Thus although NOC-5 did not inhibit the long term differentiation of H9c2 cells into cardiomyocytes, it appeared to delay the onset of the process.

In parallel with the morphological changes, exposure to NOC-5 also caused a significant reduction in the expression of MLC-1v in treated cells. This was more evident at day 4 where MLC-1v expression was less than 50% of the levels seen in the control cells ($p < 0.01$) (Figure 4.11). Although MLC-1v levels increased at day 8 ($p > 0.05$) and at day 12 ($p > 0.05$), the levels detected were still only about 80% of those detected in control cells.
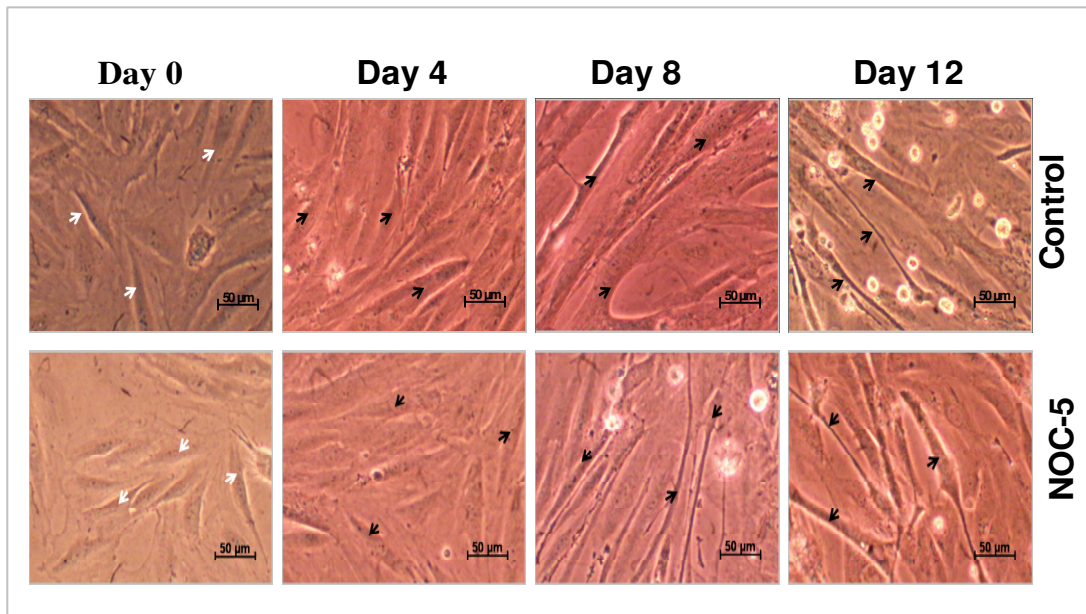
Figure 4.10: Effects of the NO donor NOC-5 on H9c2 cell differentiation into cardiomyocytes: morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100 units/ml penicillin/100 μg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 100μM of the nitric oxide donor, NOC-5. Cell alignment, elongation and myotube formation in H9c2 cells treated with 100μM of NOC-5 was slow in onset when compared to the control cells. While the control cells showed visible differentiation by day 4 (shown by black arrows), NOC-5 treated cells appeared to maintain their normal single cell and spindle shaped morphology. Morphologically, both control and NOC-5 treated H9c2 cells appeared to show marked differentiation by day 12 (shown by black arrows). Both control cells and NOC-5-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three differentiation experiments.
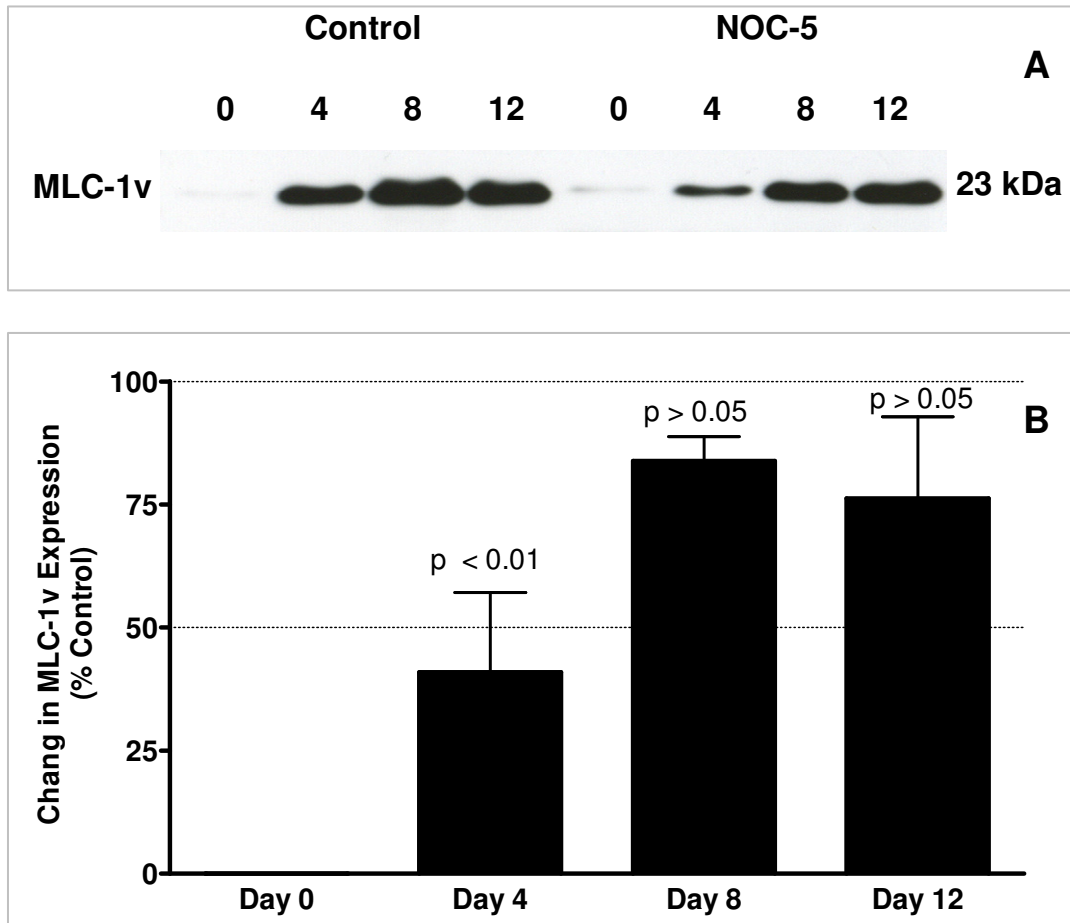
Figure 4.11: Effects of the NO donor NOC-5 on H9c2 cell differentiation into cardiomyocytes: cardiac ventricular myosin light chain 1 expression. Western blot analysis of MLC-1v revealed that expression of this protein was suppressed by NOC-5 in comparison to the control expression. The blot is representative of at least three differentiation experiments (panel A). The densitometric data (mean ± SD), from three independent experiments, show the time dependent changes in MLC-1v expression in NOC-5 treated H9c2 cells (panel B).

4.3.2 Effects of the NO donor SIN-1 on H9c2 cell differentiation into cardiomyocytes

SIN-1, like NOC-5, inhibited the early phase (day 4) of H9c2 cell differentiation into cardiomyocytes but NOC-5 appeared to have more effect at this stage. Cell elongation and cell fusion was slow in the H9c2 cells that were treated with SIN-1 in comparison to the control cells. By day 4, the usual differentiation was visible in the control H9c2 cells but not in the SIN-1 treated H9c2 cells. SIN-1 treated cells maintained their normal single cell and spindle shaped morphology (Figure 4.12). H9c2 cells in both control medium and SIN-1 supplemented medium showed differentiation at day 8 and day 12. Differentiation in both control and SIN-1 treated H9c2 cells was extensive by day 12.

The expression of MLC-1v in SIN-1 treated H9c2 cells was reduced at day 4, 8 and 12 in comparison to control cells ($p > 0.05$). MLC-1v levels in SIN-1 treated were just a little above 50% the control levels by day 4 ($p > 0.05$) (Figure 4.13). By day 8 MLC-1v levels in treated cells had recovered to more than 75% of control levels ($p > 0.05$). But by day 12, MLC-1v levels had only recovered to a little over 90% of MLC-1v levels in the control cells. MLC-1v in levels SIN-1 treated H9c2 cells never reached the levels seen in the control cells ($p > 0.05$). MLC-1v levels in both control and SIN-1 treated H9c2 cells were in agreement with the differentiation or morphological changes observed in these cells (Figure 4.13).
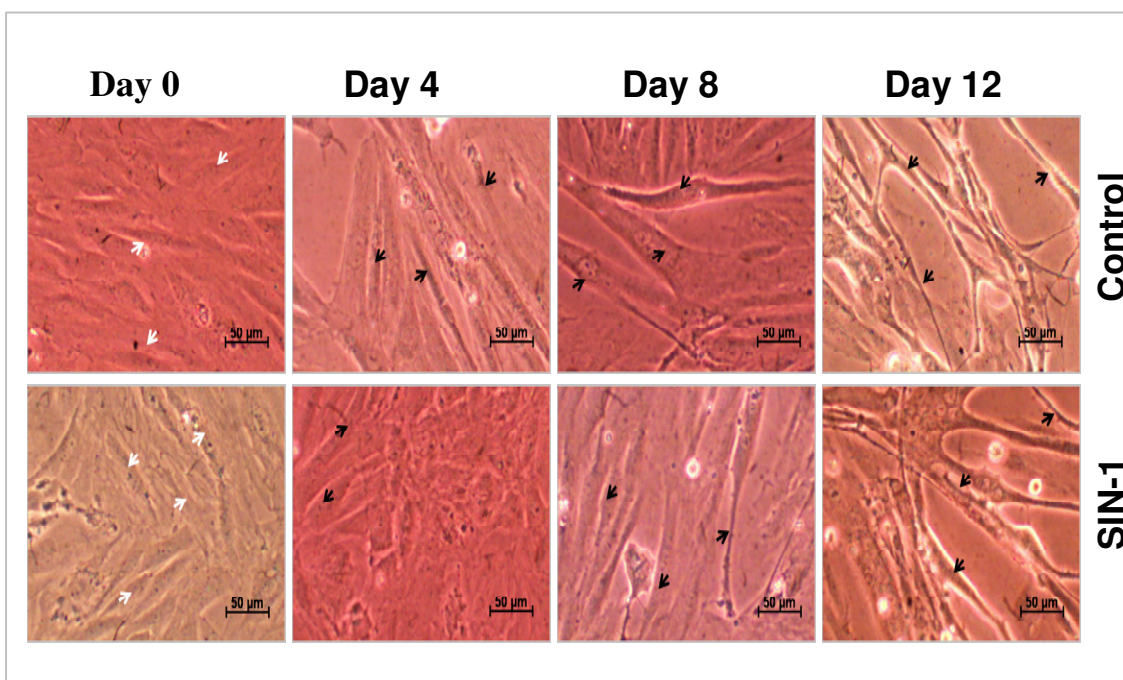
Figure 4.12: Effects of the NO donor SIN-1 on H9c2 cell Differentiation into Cardiomyocytes: Morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% 1% FBS and 100 units/ml penicillin/100 µg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 100µM of the NO donor, SIN-1. Cell alignment, elongation and myotube formation in H9c2 cells treated with 100µM of the SIN-1 was slow in onset when compared to the control cells. Control cells showed visible differentiation by day 4, but NOC-5 treated cells appeared to maintain their normal single cell and spindle shaped morphology (shown by black arrows). Morphologically, both control and SIN-1 treated H9c2 cells appeared to show marked differentiation by day 12. Both control cells and SIN-1-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three differentiation experiments.
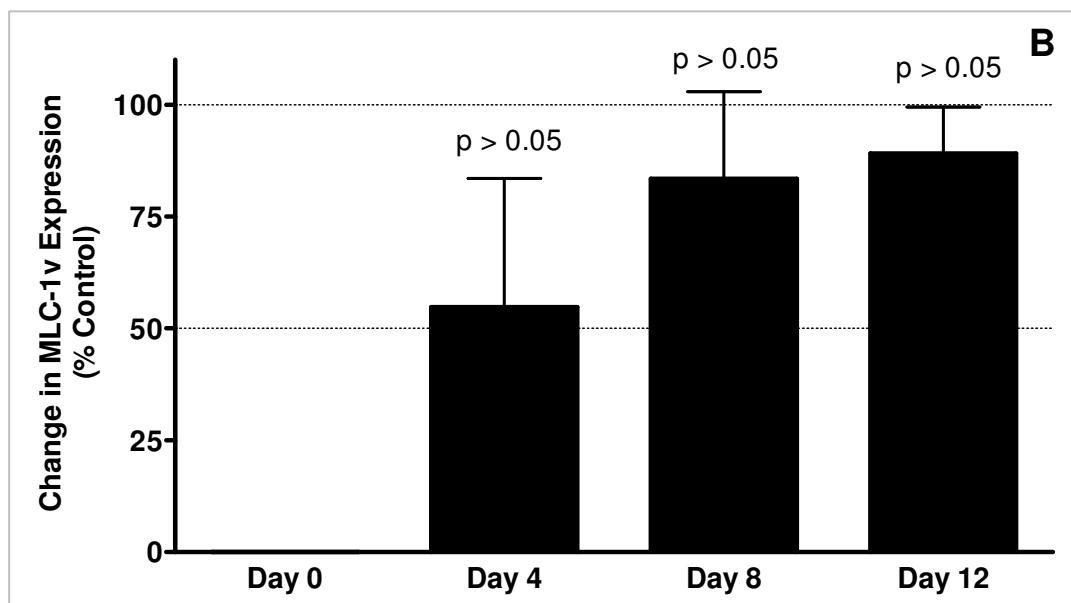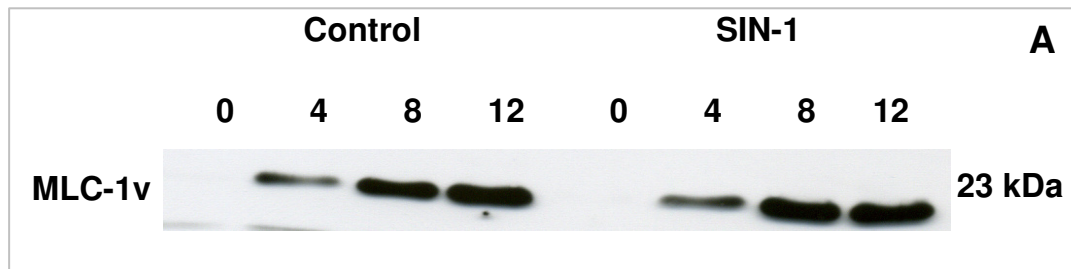
Figure 4.13: Effects of the NO donor SIN-1 on H9c2 cell Differentiation into Cardiomyocytes: cardiac ventricular myosin light chain 1 expression. Western blot analysis of MLC-1v revealed that expression of this protein was slightly suppressed by SIN-1 in comparison to the control expression. The blot is representative of at least three independent differentiation experiments (panel A). The densitometric data (mean ± SD), from three independent experiments, show the time dependent changes in MLC-1v expression in SIN-1 treated H9c2 cells (panel B).

4.3.3 Effects of the NO donor NOC-18 on H9c2 cell differentiation into cardiomyocytes

Treatment of H9c2 cells with NOC-18 delayed their differentiation into cardiomyocytes as determined by the lack of differentiation associated morphological changes. By day 4, the usual differentiation was visible in the control H9c2 cells but not in the NOC-18 treated H9c2 cells. Like the SIN-1 treated cells, cell elongation and fusion was slow in the H9c2 cells that were treated with NOC-18 in comparison to the control cells. NOC-18 treated cells maintained their normal single cell and spindle shaped morphology (Figure 4.14). Both control and NOC-18 treated cells showed increased differentiation from day 8 to day 12. Differentiation or morphological changes in both control and NOC-18 treated H9c2 cells were extensive by day 12.

By day 4, MLC-1v levels in NOC-18 treated H9c2 cells were lower than the levels determined in the control cells ($p > 0.05$). The delay in MLC-1v expression, although not statistically significant, was in agreement with the lack morphological changes seen in these cells. By day 8, MLC-1v levels in NOC-18 cells had recovered to more than 95% of control levels $p > 0.05$)(Figure 4.15). By day 12, MLC-1v   levels in NOC-18 treated slightly fell to about 80% of control levels $p > 0.05$). The levels of MLC-1v determined in the NOC-18 treated H9c2 cells never reached the levels seen in the control cells. In both control cells and NOC-18 treated H9c2 cells the levels of MLC-1v determined were in agreement with the differentiation or morphological changes observed in these cells.
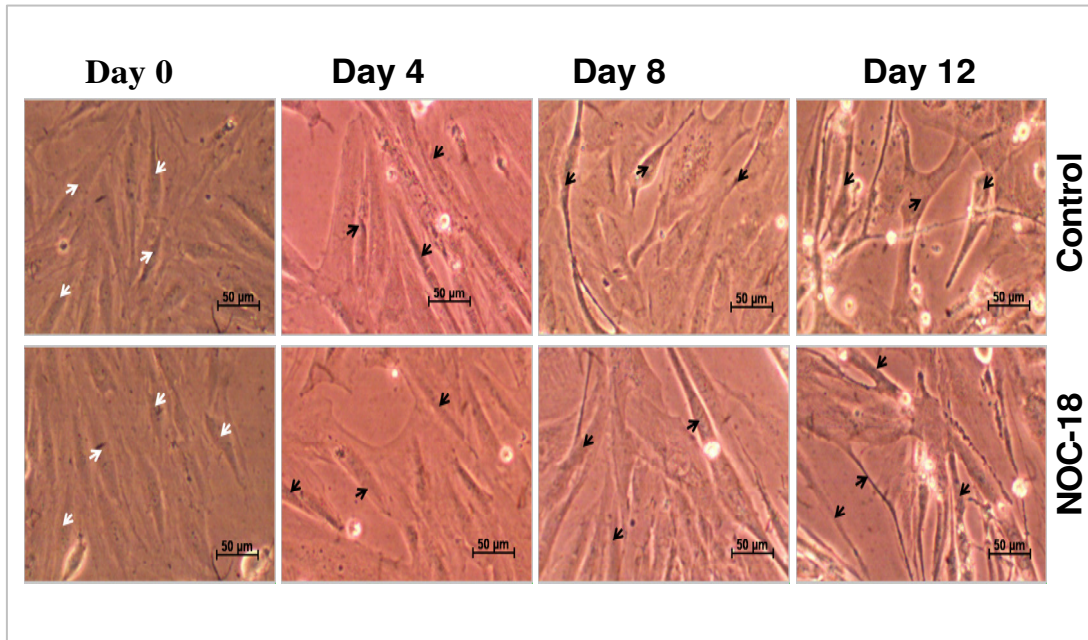
Figure 4.14: Effects of the NO donor NOC-18 on H9c2 cell Differentiation into Cardiomyocytes: Morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% 1% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 100µM of the nitric oxide donor, NOC-18. Differentiation (i.e. cell alignment, elongation and myotube formation) in H9c2 cells treated with NOC-18 was slow in onset when compared to the control cells. By day 4, differentiation (shown by black arrows) was visible in the control cells but not in NOC-18 treated cells which appear to maintain their normal single cell and spindle shaped morphology (shown by black arrows). Morphologically, both control and NOC-18 treated H9c2 cells appeared to show marked differentiation by day 12. Both control cells and NOC-18-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three differentiation experiments.
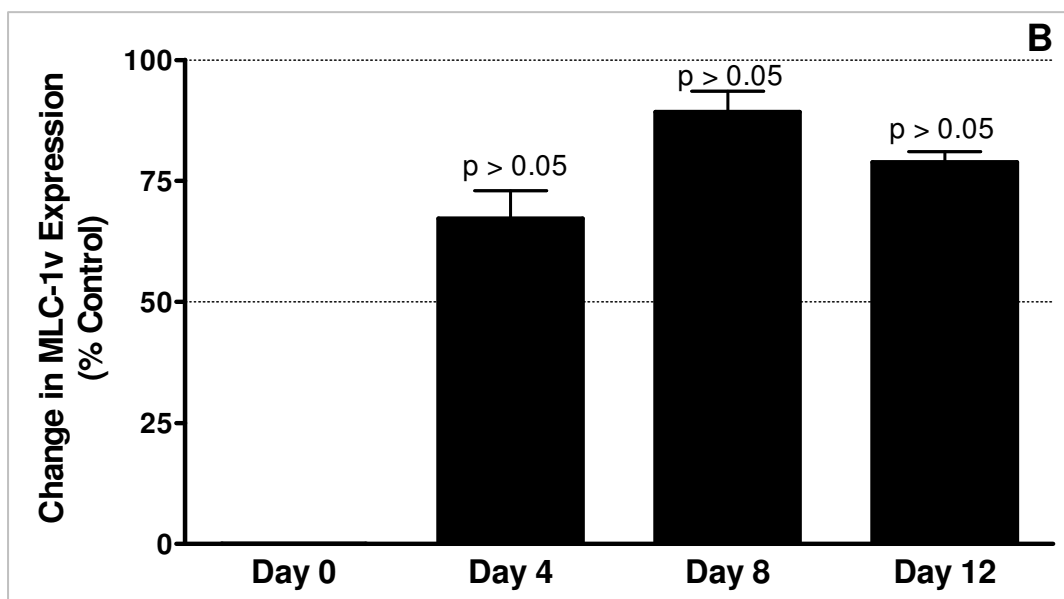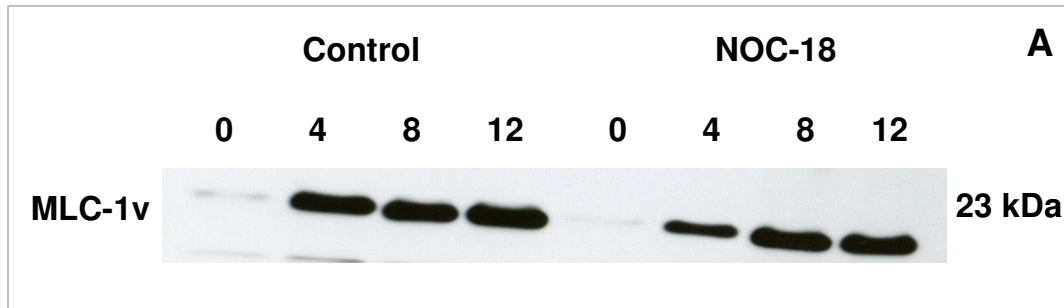
Figure 4.15: Effects of the NO donor NOC-18 on H9c2 cell Differentiation into Cardiomyocytes: cardiac ventricular myosin light chain expression. Western blot analysis of MLC-1v revealed that expression of this protein was slightly suppressed by NOC-18 in comparison to the control expression. The blot is representative of at least three differentiation experiments (panel A). The densitometric data (mean ± SD), from three independent experiments, show the time dependent changes in MLC-1v expression in NOC-18 treated H9c2 cells (panel B).

4.3.4 Effects of the NO donor SNAP on H9c2 cell differentiation into cardiomyocytes

H9c2 cells treated with SNAP appeared to have differentiated to the same extent as the control cells. By day 4, the usual differentiation was visible in both control and SNAP treated H9c2 cells. The degree of cell elongation and fusion in both H9c2 and SNAP treated H9c2 cells were very similar. Morphological changes in both control and SNAP treated H9c2 cells increased from day 4 and by day 12, these changes were extensive as usual (Figure 4.16).

In agreement with the morphological seen in the control cells and SNAP treated cells, MLC-1v levels in SNAP treated H9c2 cells were almost equal to those seen in the control cells. SNAP treatment slightly reduced the expression of MLC-1v ($p > 0.05$) (Figure 4.17). By day 8, SNAP treatment increased the expression of MLC-1v by more than 100% of control levels (Figure 4.17). However the SNAP mediated increase in MLC-1v levels was not statistically significant ($p > 0.05$). By day 12, both control and SNAP treated cells had similar levels of MLC-1v ($p > 0.05$). MLC-1v levels in both control and SNAP-treated H9c2 cells were in agreement with the differentiation or morphological changes observed in these cells.
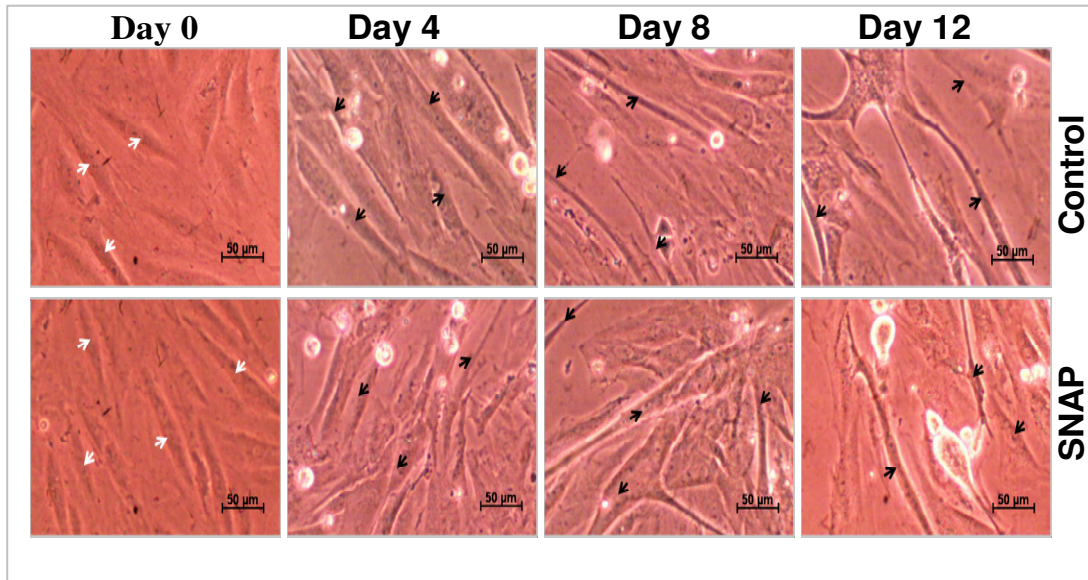
Figure 4.16: Effects of the NO donor SNAP on H9c2 cell Differentiation into Cardiomyocytes: Morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% 1% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 100µM of the nitric oxide donor, SNAP. The differentiation (i.e. cell alignment, elongation and myotube formation) (shown by black arrows) of H9c2 cells treated with SNAP was almost as much as the control cells. The degrees of cell elongation and fusion in both H9c2 and SNAP treated H9c2 cells were very similar. Morphological changes in both control and SNAP treated H9c2 cells increased from day 4 and by day 12, these changes were extensive as usual. Both control cells and SNAP-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three independent experiments.
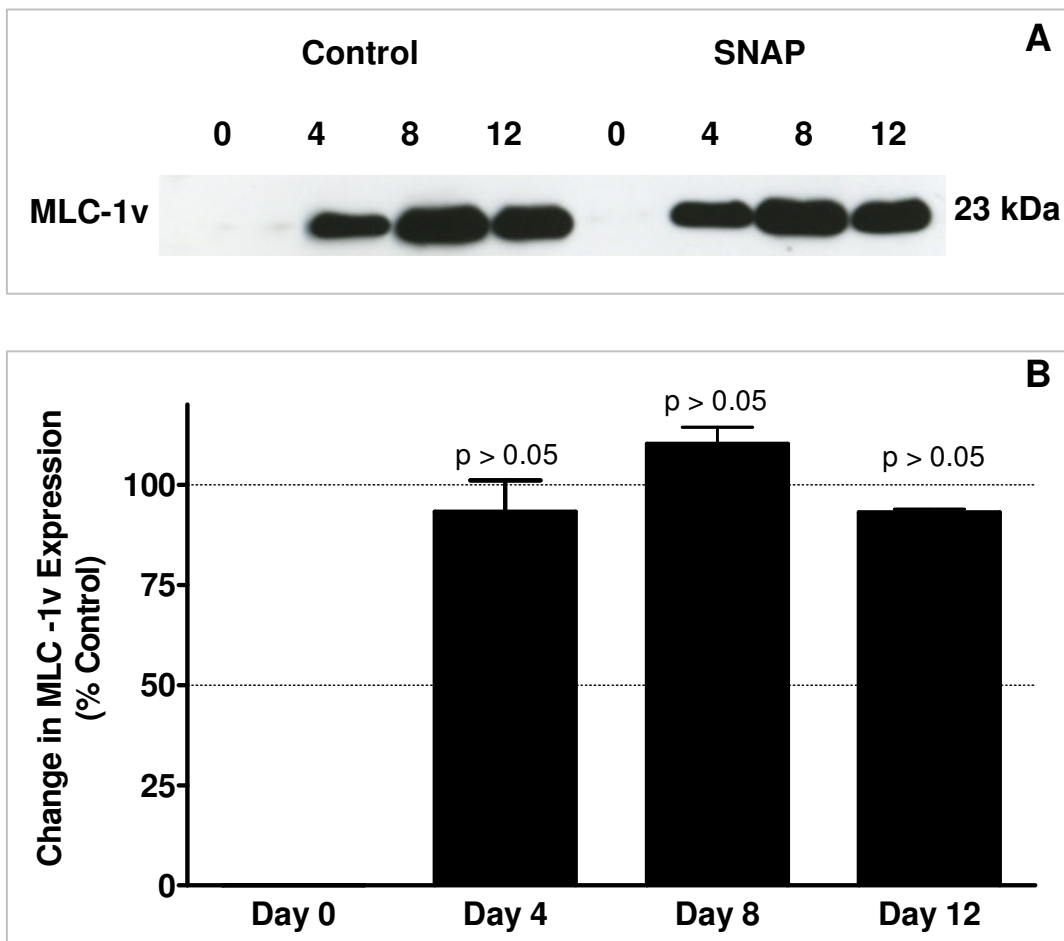
Figure 4.17: Effects of the NO donor SNAP on H9c2 cell Differentiation into Cardiomyocytes: cardiac ventricular myosin light chain 1 expression. MLC-1v levels in control and SNAP treated H9c2 cells were almost equal. SNAP treatment did not inhibit the expression of MLC-1v in H9c2 cells differentiating into cardiomyocytes. SNAP treatment increased MLC-1v levels in comparison to the control cells. The blot is representative of at least three independent experiments (panel A). The densitometric data (mean ± SD), from three independent experiments, show the time dependent changes in MLC-1v expression in SNAP treated H9c2 cells (panel B).

4.3.5 Effects of 8-Bromo-cGMP on H9c2 cell differentiation into cardiomyocytes

To further explore the actions of NO in regulating the differentiation of H9c2 cell into cardiomyocytes, we examined whether 8-Bromo-cGMP, an analogue of the second messenger cGMP would mimic the effects seen with the NO donors. As shown in Figure 4.18, H9c2 cells treated with 8-Bromo-cGMP appeared to have differentiated almost as much as the control cells. Similar to the NO donor treated cells, the degree of cell elongation and fusion in both controls and 8-Bromo-cGMP treated H9c2 cells appeared at day 8 and at day 12. In parallel, the expression of MLC-1v in 8-Bromo-cGMP-treated H9c2 cells was reduced at day 4 ($p > 0.05$) but indistinguishable from the levels detected in the control cells at day 8 ($p > 0.05$) and at day 12 ($p > 0.05$) (Figure 4.19). Thus, like the NO donors, 8-Bromo-cGMP regulated H9c2 cell differentiation into cardiomyocytes by delaying the induction of this process.

Day 0	Day 4	Day 8	Day 12	
				Control
				8-Bromo-cGMP
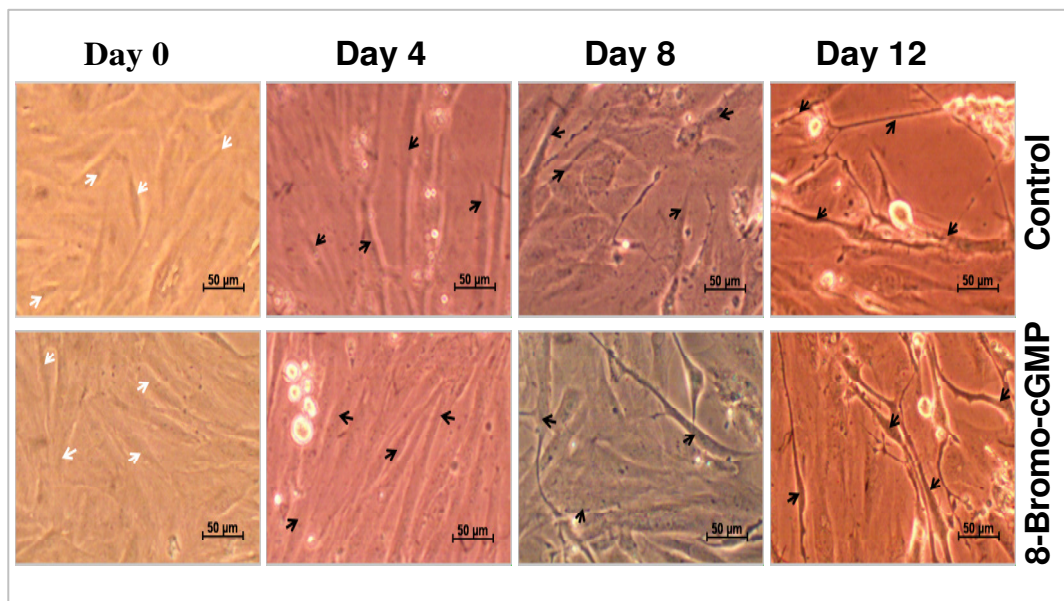
Figure 4.18: Effects of 8-Bromo-cGMP on H9c2 cell Differentiation into Cardiomyocytes: morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 100µM of 8-Bromo-cGMP. The differentiation (i.e. cell alignment, elongation and myotube formation) (shown by black arrows) of H9c2 cells treated with 8-Bromo-cGMP appeared to be almost as much as the control cells. The degrees of cell elongation and fusion in both H9c2 and 8-Bromo-cGMP treated H9c2 cells were very similar. Morphological changes in both control and 8-Bromo-cGMP treated H9c2 cells were extensive by day 12. Both control cells and SNAP-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three independent experiments.
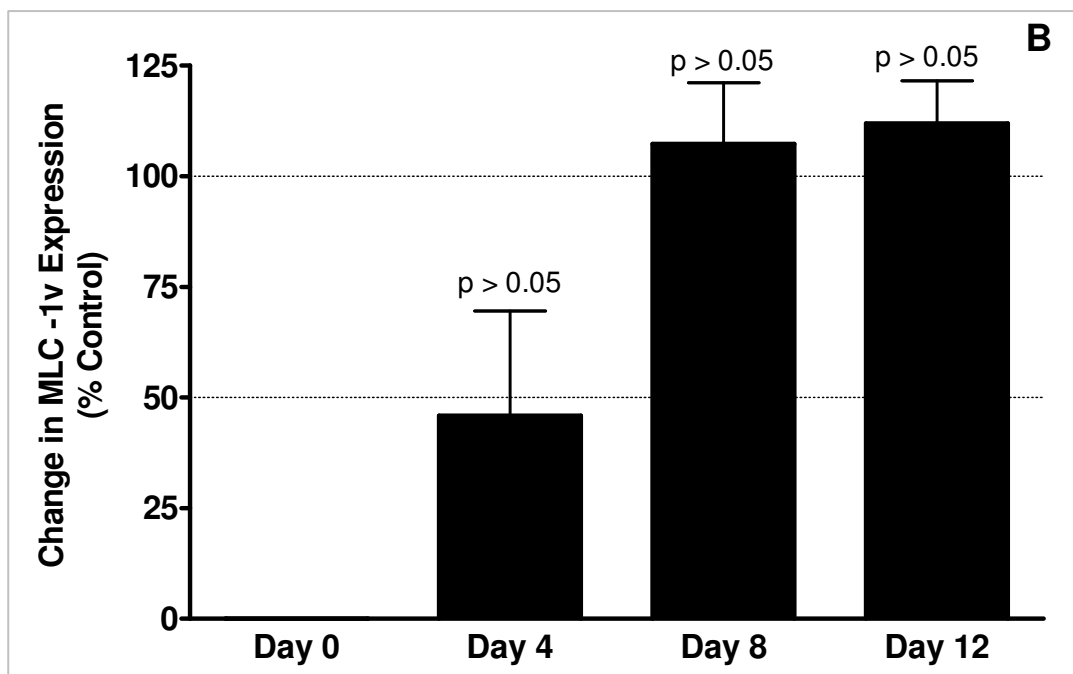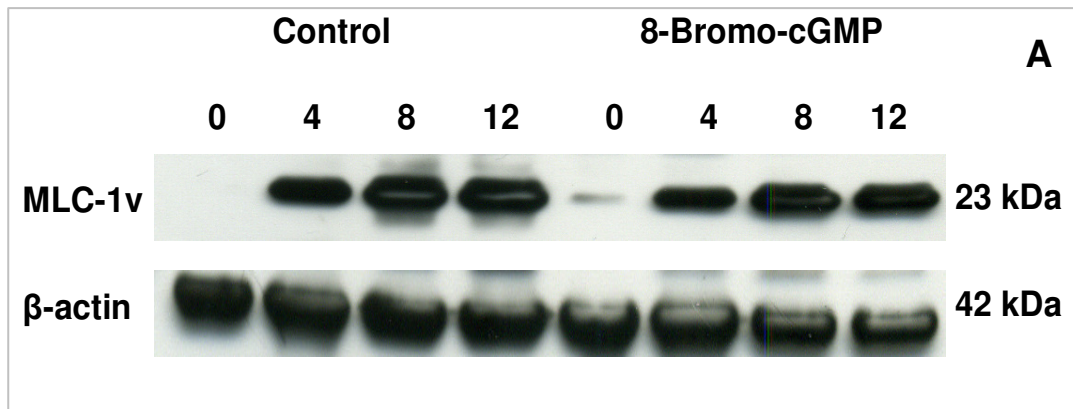
Figure 4.19: Effects of 8-Bromo-cGMP on H9c2 cell Differentiation into Cardiomyocytes: ventricular myosin light 1 (MLC-1v) expression. Western blot analysis of MLC-1v revealed that expression of this protein was initially delayed in 8-Bromo-cGMP treated H9c2 cells in comparison to the control expression (A). Thereafter MLC-1v levels increased to more than those seen in the control cells. The blot is representative of at least three independent experiments. The densitometric data (mean ± SD), from three independent experiments, show the time dependent changes in MLC-1v expression in 8-Bromo-cGMP treated H9c2 cells (B).

4.3.6 Viability of H9c2 cells in various concentration of NOC-5

There was no difference between the viability of control H9c2 cells and cells treated with NOC-5 at concentrations ranging from 3µM to 30µM (p>0.05) (Figure 4.20). There was however a significant difference between the viability of control H9c2 cells and those cell treated NOC-5 concentrations above 30µM (p<0.01). At these high concentrations, NOC-5 reduced cell viability to 66% at 100µM and 9.5 % at 1000µM. As a result 100µM was selected for investigating the effects of NOC-5 on H9c2 cell differentiation into cardiomyocytes.
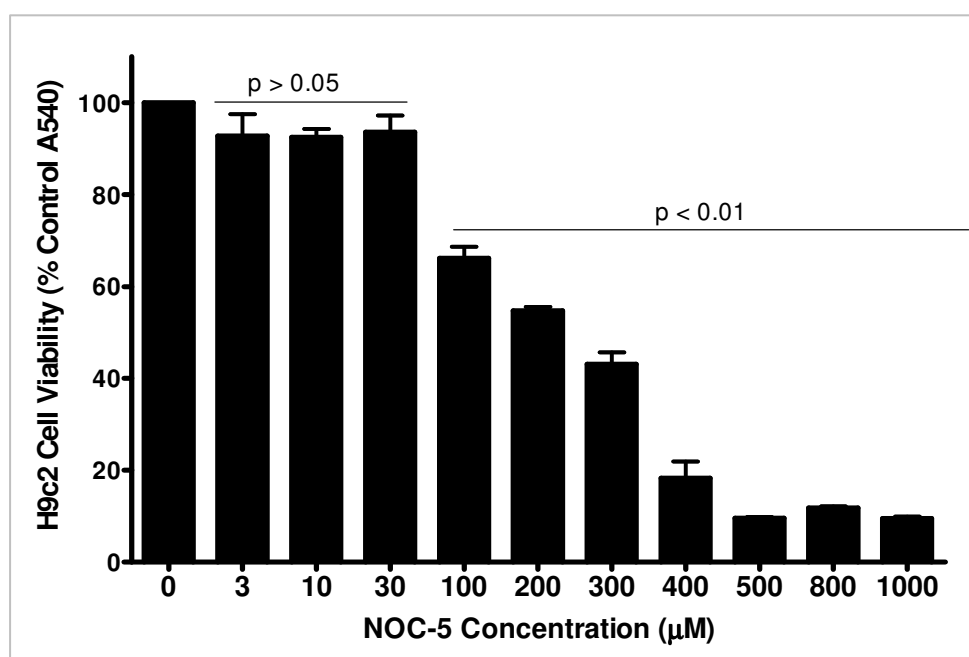
Figure 4.20: Viability of H9c2 cells in the presence of various concentration of NOC-5. H9c2 cells were seeded in 6-well plates and treated with NOC-5 as described in the methods section above   (Section 4.3.1). The MTT assay was carried out at the end of the treatment. The data were analysed by one-way analysis of variance (ANOVA) test. The control viability was compared to the viability at each concentration using post-hoc Dunnett's multiple comparison test. There was no significant difference in cell viability between the control and each NOC-5 concentrations from 3 to 30µM (p>0.05). There was a significant difference in cell viability between the control and each NOC-5 concentrations form 100 to 1000µM (p<0.01). The data presented in the graph are the mean ± SD from three independent experiments.

4.3.7 H9c2 Cell Viability in Various concentration of SIN-1

As seen with NOC-5, SIN-1 had no significant effect on cell viability at concentrations of 3µM to 30 (Figure 4.21; p>0.05). There was a significant difference between the viability of control H9c2 cells and the viability of the H9c2 cells that were treated with each SIN-1 concentration above 30µM (p<0.01). At 100uM SIN-1 reduced viability to 71.7% while the viability at with 1000µM SIN-1 was 10% that of control cells.
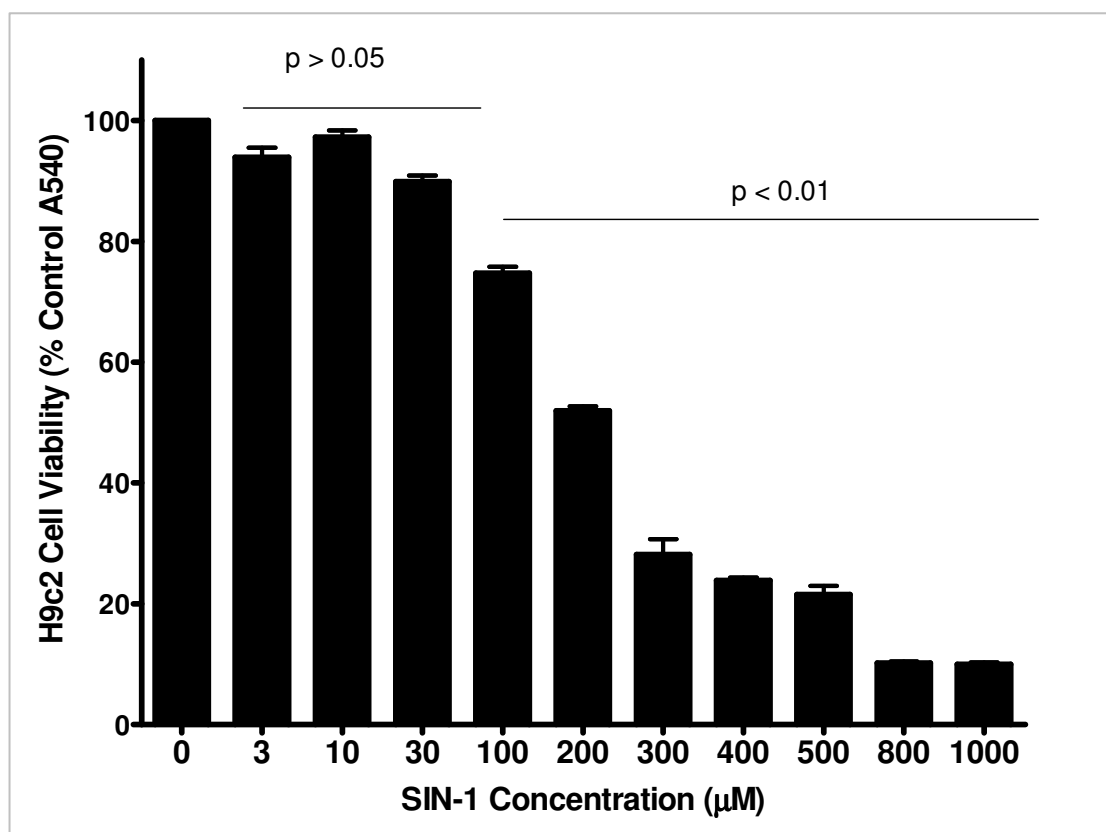
Figure 4.21: Viability of H9c2 cells in the presence of various concentration of SIN-1. H9c2 cells were seeded in 6-well plates and treated with SIN-1 as described in the methods section above (Section 4.3.1). The MTT assay was carried out at the end of the treatment. The data were analysed by one-way analysis of variance (ANOVA) test. The control viability was compared to the viability at each concentration using post-hoc Dunnett's multiple comparison test. There was no significant difference in cell viability between the control and each SIN-1 concentrations from 3 to 30µM (p>0.05). There was a significant difference in cell viability between the control and each SIN-1 concentrations from 100 to 1000µM (p<0.01). The data presented in the graph are the mean ± SD from three independent experiments.

4.3.8 H9c2 Cell Viability in Various concentration of NOC-18

Consistent with the other NO donors, NOC-18 did not cause any distinct cytotoxicity when used at concentrations of up 30µM (Figure 4.22; p>0.05). Concentrations of 100µM and above however caused marked cytotoxicity of the same magnitude seen with the other compounds. At 100µM NOC-5 the cell viability was reduced to 67.5% while the viability of H9c2 cells treated with 1000µM NOC-18 was only 9.8% of control cell viability.
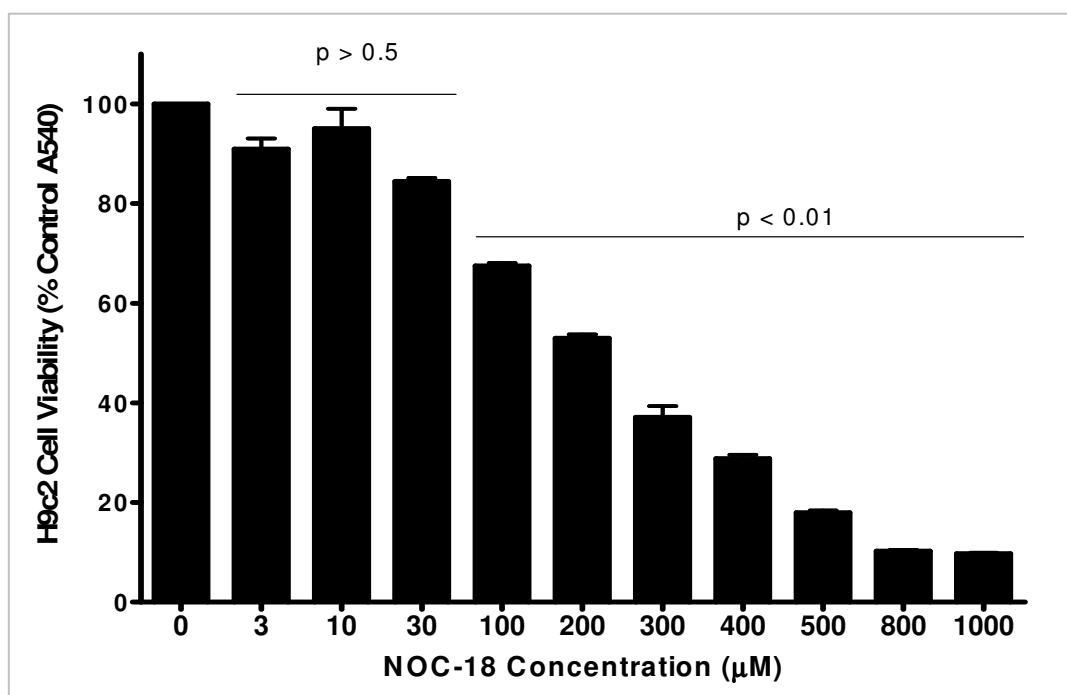
Figure 4.22: Viability of H9c2 cells in the presence of various concentration of NOC-18. H9c2 cells were seeded in 6-well plates and treated with NOC-18 as described in the methods section above (Section 4.3.1). The MTT assay was carried out at the end of the treatment. The data were analysed by one-way analysis of variance (ANOVA) test. The control viability was compared to the viability at each concentration using post-hoc Dunnett's multiple comparison test. There was no significant difference in cell viability between the control and each NOC-18 concentrations from 3 to 30µM (p>0.05). There was a significant difference in cell viability between the control and each NOC-18 concentrations from 100 to 1000µM (p<0.01). The data presented in the graph are the mean ± SD from three independent experiments.

4.3.9 H9c2 Cell Viability in Various concentration of SNAP

Of all the NO donors used, SNAP appears to be the least toxic and best tolerated since incubation of cells with this compound produced only marginal toxicity at concentrations of up to 200μM (Figure 4.23; $p>0.05$). SNAP at concentration at 300μM reduced viability to 63 % that of control H9c2 cells. SNAP concentration of 100μM, with cell viability of 92% was selected for investigating the effects of SNAP on H9c2 cell differentiation into cardiomyocytes.
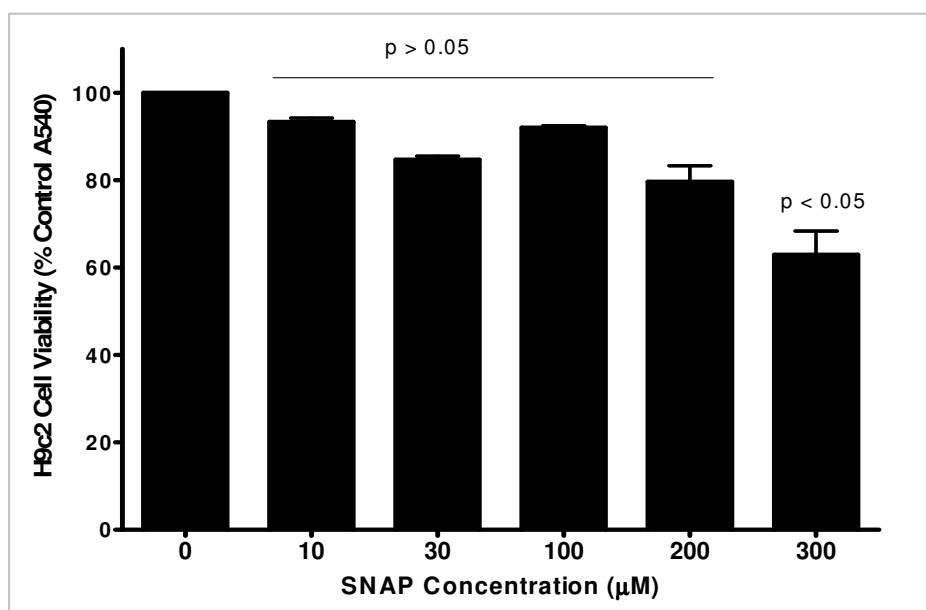
Figure 4.23: Viability of H9c2 cells in the presence of various concentration of SNAP. H9c2 cells were seeded in 6-well plates and treated with SNAP as described in the methods section above (Section 4.3.1). The MTT assay was carried out at the end of the treatment. The data were analysed by one-way analysis of variance (ANOVA) test. The control viability was compared to the viability at each concentration using post-hoc Dunnett's multiple comparison test. There was no significant difference in cell viability between the control and each SNAP concentrations from 10 to 200μM ($p>0.05$). There was a significant difference in cell viability between the control H9c2 cells an H9c2 cell treated with 300μM SNAP ($p<0.05$). The data presented in the graph are the mean ± SD from three independent experiments.

4.4 DISCUSSION

Nitric oxide has been suggested to play a role in stem cell differentiation into different lineages including cardiomyocytes. In this regard, NO has been reported to facilitate mouse embryonic stem cell differentiation into cardiomyocytes (Kanno *et al.*, 2004). In NO donor (SNAP, PAPA/NO and DEA/NO) treated ES D3 cells and also in ES cells transfected with iNOS, the number of EBs with beating foci and the size of beating foci in the EB outgrowths was increased. In support of the cardiac differentiation of these cells being mediated by NO, the number of EBs with beating foci and also the size of the foci were decreased by L-NAME (Kanno *et al.*, 2004). In similar study in ES D3 stem cells, it was shown that exposure of cells to arginine vasopressin (AVP) increased the number of beating embryoid bodies and also caused an increase in the expression of GATA-4. These AVP effects on the cells were also found to be antagonised by L-NAME (Gassanov *et al.*, 2007) thus again suggesting a positive role for nitric oxide in stem cell differentiation into cardiomyocytes. As already highlighted, expression of eNOS, iNOS and nNOS have also been found in undifferentiated ES cells (Bloch *et al.*, 1999; Krumenacker *et al.*, 2006) with the profile of expression changing during the differentiation process (Krumenacker *et al.*, 2006). The above mentioned data suggest a positive role of NO in the differentiation of stem cells into cardiomyocytes.

In addition to reported positive role of NO in the differentiation of stem cells into cardiomyocytes, NO has been reported to play positive role in the differentiation of other cell types, For example, over-expression of nNOS or

exogenous nitric oxide has also been shown to increase neuronal differentiation of neuroblastoma cells (Ciani *et al.*, 2004). A similar report has also shown that nNOS and nitric oxide are required for neuronal differentiation of PC12 cells. In the latter, transfection of PC12 cells with a nNOS dominant negative mutant block nerve cell growth factor-induced neuronal differentiation (Phung *et al.*, 1999), thus suggesting that nitric oxide has a positive role to play in PC12 cell neuronal differentiation. NO has also been shown to increased differentiation of cultured rat preadipocytes into adipocytes (Yan *et al.*, 2002).

In contrast to the positive roles of nitric oxide in cell differentiation, it has recently been reported that the differentiation of preadipocytes (3T3-L1 cells) into adipocytes is suppressed by NO (Kawachi *et al.*, 2007). In addition, inhibition of NO synthesis using L-NAME increased the number of stem and progenitor cells in the bone marrow of irradiated mice (Michurina *et al.*, 2004). Thus, it is evident that the actions of NO in regulating stem cell production and/or differentiation may be a complex process that requires further investigation. The conflicting reports about the role of NO in cell differentiation may reflect the variable nature of NO in cell differentiation or variation in experimental procedures used by different laboratories. Nitric oxide is a molecule with a transient half-life and a diverse array of biological functions in humans. Thus when examining the actions of NO, the experimental conditions may be critical in dictating the results obtained.

We investigated the role of NO in the differentiation of H9c2 cells into cardiomyocytes using both fast NO releasing donors, NOC-5 and SIN-1 with respective half-lives of 40 and 93 minutes and the slow NO releasing donors NOC-18 and SNAP with respective half-lives of 20 and 37 hours. Our data showed that NO donors with different profiles of NO release can have different effects on H9c2 cell differentiation into cardiomyocytes. Both SIN-1 and NOC-5 delayed the onset of differentiation of H9c2 cells into cardiomyocytes. However SNAP and NOC-18 were not as effective in delaying the onset of differentiation or long term differentiation of H9c2 cells into cardiomyocytes. By day 8, H9c2 cell differentiation, as determined by MLC-1v western blot, in the presence of SNAP or NOC-18 had recovered to levels that were almost the same as those seen in the control cells. In fact by day 8, the expression of MLC-1v in SNAP treated was greater than that seen in the control cells. The delayed effects of fast-releasing NO donors on the onset of H9c2 cell differentiation can be interpreted as evidence that NO (at least from that fast-releasing NO donors) negatively regulates H9c2 cell differentiation into cardiomyocytes. However it should be noted, that fast-releasing NO donors release large amounts of their NO content in a matter of minutes and this may be detrimental to cells independent of direct and specific negative effects on the signalling events mediating the H9c2 cells differentiation into cardiomyocytes.

NO produced in small or regulated amount (as produced by constitutively expressed nNOS and eNOS), is involved in cell signalling leading to the regulation of many downstream targets. Soluble guanylyl cyclase, an enzyme

that generates cGMP, is the main physiological target for NO (Gold *et al.*, 1990; Trigo-Rocha *et al.*, 1993; Phung *et al.*, 1999). Nitric oxide, by regulating the production of cGMP, also regulates various downstream protein kinases (e.g. cGMP-dependent kinase) (Archer *et al.*, 1994; Pineda *et al.*, 1996), ion channels (i.e. cGMP-dependent ion channels) (D'Ascenzo *et al.*, 2002) and cGMP-dependent phosphodiesterases (NO can cause stimulation or inhibition) (Wexler *et al.*, 1998; Zima *et al.*, 2000). However, excessive formation of NO may be detrimental to the cells and tissues. This is evident from several reports that implicate NO in the pathogenesis of many diseases including stroke (Archer *et al.*, 1994; Iadecola *et al.*, 1994; Ai *et al.*, 2000; Li and Forstermann, 2000) and sepsis (Brealey *et al.*, 2002). Excessive NO can disrupt cellular functions by binding to the heme group in cytochrome c oxidase and inhibit the enzyme leading to increased production of superoxide in the mitochondrial electron transport chain. The increased superoxide can further react with NO to yield peroxynitrite which can directly damage the enzymes in the mitochondrial electron transport chain (Brealey *et al.*, 2002). This can cause major disturbances in the cells ability to produce ATP that is required for the maintenance of many vital cellular functions. Peroxynitrite is also an unstable compound which can breakdown into hydroxyl ($OH^{.}$) and nitrite ($NO_2^{.}$) radicals. The hydroxyl radical causes the most damage to cells and tissues and may be responsible for many cellular damages that are attributed to excess production of NO. The nitrite radical can covalently modify proteins by nitration and this can also lead to detrimental effects on cells. Apoptosis is a vital biological process but abnormal apoptosis (i.e. too little or excessive apoptosis) as the results of breakdown of the balance between

anti-apoptotic and pro-apoptotic cellular signals, is detrimental to cells. Nitric oxide can mediate either apoptosis or cell survival. For example, it has been shown that over-expression of nNOS or the slow-releasing NO donor, DETA NONOate (i.e. NOC-18) protects human neuroblastoma SK-N-BE cells from serum withdrawal-induced apoptosis (Ciani *et al.*, 2002b). Normal levels of NO can prevent apoptosis by inhibiting the activities of caspases including caspase-3, caspase-8 and 9 (Kim *et al.*, 1997; Li *et al.*, 1999; Zhou *et al.*, 2005). Nitric oxide may also prevents apoptosis by regulating signalling from the fas receptor, another apoptotic mechanism (Hebestreit *et al.*, 1998). On the other hand higher levels of NO may overcome the cell protective mechanisms against oxidative stress leading to apoptosis or other cytotoxic effects. The expression of key pro-apoptotic proteins, such as bax and caspases have been shown to be repressed by endogenous NO while the inhibition of NO production induces the expression of these pro-apoptotic proteins (Thippeswamy *et al.*, 2001). Similarly the inhibition of NO production has also been shown to decrease the mRNA and protein levels of Bcl-2, an anti-apoptotic gene (Ciani *et al.*, 2002a). It has been shown that inhibition of NO production by L-NAME, resulted in progressive apoptosis in cochlear ganglion cells (CGC) cells while slow-releasing NO donors or a cGMP analogue rescued the L-NAME induces apoptosis (Ciani *et al.*, 2002a). From the above it can be appreciated that normal levels of NO can promote cell survival while excess NO can contribute to cell damage and death. Our MTT data, with all the NO donors used, showed that NO is toxic at high concentrations. Therefore, the balance between the transient but very high NO concentrations and the sustained production of NO at low concentrations

may determine whether NO plays a beneficial or detrimental role in a biological process. In cells undergoing differentiation, shifting this balance in favour of cell survival may play critical or positive roles, since cell differentiation is a very stressful process and this means that normal level of NO as generated by nNOS and eNOS or control released by slow-releasing NO donor may have positive effects on stem cell differentiation into cardiomyocytes. The fact that our data showed that fast NO releasing donors markedly delayed the onset of differentiation of H9c2 cells into cardiomyocytes while slow NO releasing donors were not as effective in delaying the onset of differentiation or long term differentiation of H9c2 cells into cardiomyocytes shows the need for a fine balance between the transient but very high NO concentrations and the sustained production of NO at low levels or concentrations.

To further explore the role of NO on the differentiation process, the effects of 8-Bromo-cGMP on H9c2 cell differentiation was investigated. As already mentioned, NO exerts most of its physiological actions via activation of soluble guanylate cyclase, which leads to an increase in intracellular concentration of the second messenger, cGMP (Gold *et al.*, 1990; Trigo-Rocha *et al.*, 1993; Phung *et al.*, 1999). The analog of cGMP, 8-Bromo-cGMP, is more resistant to phosphodiesterases than endogenous cGMP.

Our 8-Bromo-cGMP data are in agreement with the data obtained with some of the NO donors used in that treatment of cells with 8-Bromo-cGMP also delayed the onset of differentiation and reduced the expression of MLC-1v

significantly at the earlier stages of differentiation. However after prolonged incubations, MLC-1v expression was virtually indistinguishable in both controls and drug treated cells. What is not clear is whether this recovery is due to a loss in the actions of NO and/or 8-Bromo-cGMP over time. In our experiments, cells were exposed to the compounds for one hour prior to and then 48 hours after initiation of differentiation. After this period, cells were cultured in differentiation medium alone and it is possible that as a consequence of omitting the compounds from the incubation medium, their actions gradually diminished over time. What is clear however is the fact that NO (at least NO from fast-releasing NO donors) and indeed 8-Bromo-cGMP may inhibit rather than enhance the differentiation process, at least over the initial stages of the process. This is clearly contradictory to the other studies highlighted above which show a positive regulatory role of NO in the differentiation of stem cells not only into cardiomyocytes but also into other lineages. Our studies, as they stand, give little indication as to conflicting findings and further experiments are clearly needed especially in real stem cells or stem cell lines. These studies although planned were not carried out because of time constraints but are clearly needed. It would also be very useful in future studies to use NOS inhibitors and also combine NO donors with cGMP analogs (e.g. Br-cGMP) to complement the findings with the NO donors.

One possible but perhaps less convincing reason as to why the NO donors suppressed differentiation in our studies may be because of their ability to suppress cellular metabolic activity as indicated by the decreases in MTT

metabolism observed in the cytotoxicity assays carried out. Even if this did not result in cell death the reduction in metabolic activity within cells could regulate various cellular processes, including differentiation. The concentrations of the different compounds used were however well within the ranges used in several other studies. In fact NOC-5 has been used at concentrations ranging from 0.1-3000µM (Hotta *et al.*, 1999; Okuyama *et al.*, 2000; Kim *et al.*, 2005) while NOC-18 and SNAP have both been used in other studies at concentrations of up to 500µM (Palmer *et al.*, 2000; Sasaki *et al.*, 2000; Bal-Price and Brown, 2001; Niedbala *et al.*, 2002; Kim *et al.*, 2005). SIN-1 has been used at up to 2000µM (Mistry and Garland, 1998; Mathy-Hartert *et al.*, 2000; Eligini *et al.*, 2001; Kim *et al.*, 2005). These are all much higher than the 100µM used in our studies. The 100µM concentration was chosen because the cells appeared to recover, without adversely detrimental effects, after the NO donors compound were removed from the incubation medium. In the future research, dose-response studies to clearly establish effective and non-toxic concentrations of these compounds, would very useful

4.5 Summary

NOC-5 and SIN-1 appeared to delay the onset of H9c2 cell differentiation into cardiomyocytes, as determined by cell elongation and fusion to form myotubes. In parallel with the morphological changes, NOC-5 and SIN-1 also caused a reduction in the expression of MLC-1v in differentiating H9c2 cells. NOC-18 also delayed the onset of H9c2 cell differentiation into cardiomyocytes but to a lesser extent than NOC-5 and SIN-1. Differentiation

associated morphological changes in both control and SNAP-treated H9c2 cells were similar. In agreement with the morphological seen in the control cells and SNAP treated cells, MLC-1v levels in SNAP-treated and control H9c2 cells were almost equal. 8-Bromo-cGMP, an analogue of cGMP, appeared to delay the onset of H9c2 cell differentiation into cardiomyocytes. However, like NOC-5 and SIN-1, 8-Bromo-cGMP failed to block the long term differentiation of H9c2 cells into cardiomyocytes. In parallel, expression of MLC-1v was reduced at day 4 but thereafter MLC-1v expression in controls and 8-Bromo-cGMP-treated cells were indistinguishable.

In conclusion, our data suggest that generation of cardiomyocytes from H9c2 cells may be negatively regulated by NO and this may occur through generation of cGMP. Our data are however preliminary and further studies are needed to confirm these findings. Moreover, a detailed study of the full signalling pathway activated by NO may prove important in understanding how this molecule regulates differentiation.

CHAPTER 5.0

ROLE OF PROTEIN KINASE C IN CARDIOMYOCYTE

DIFFERENTIATION

5.1 INTRODUCTION

5.1.1 Structural features of protein kinase C isoforms

Protein kinases Cs (PKC) are members of the serine–threonine second messenger-dependent protein kinase family. They play pivotal roles in mediating cellular responses to extracellular stimuli that regulate cell proliferation, differentiation and apoptosis. PKC isoforms are single polypeptides with an N-terminal regulatory region and a C-terminal catalytic region. A key regulatory and structural feature of the PKC isoforms is the presence of four conserved domains (i.e. C1, C2, C3 and C4) (Coussens *et al.*, 1986) (Figure 5.10).

The C1 domain contains a cysteine-rich motif (Parker *et al.*, 1986) that forms the 1, 2-diacyglycerol (DAG) (endogenous ligand) binding site. Diacylglycerol and phorbol esters (PKC activators) are hydrophobic anchors that recruit PKC enzyme to cell membranes (Orr *et al.*, 1992; Mosior and Epand, 1993). Phorbol esters, which are non-hydrolysable analogues of DAG, also bind to the C1 domain (Johnson *et al.*, 2000). The C1 domain also contains the auto-inhibitory pseudosubstrate sequence upstream of the cysteine-rich motif (House and Kemp, 1987; House and Kemp, 1990). The C2 domain contains the recognition site for acidic lipids and in conventional PKC isoforms. It also contains the Ca^{2+} ion binding site. The C3 domains form the ATP binding site and the C4 domain form the substrate binding site.
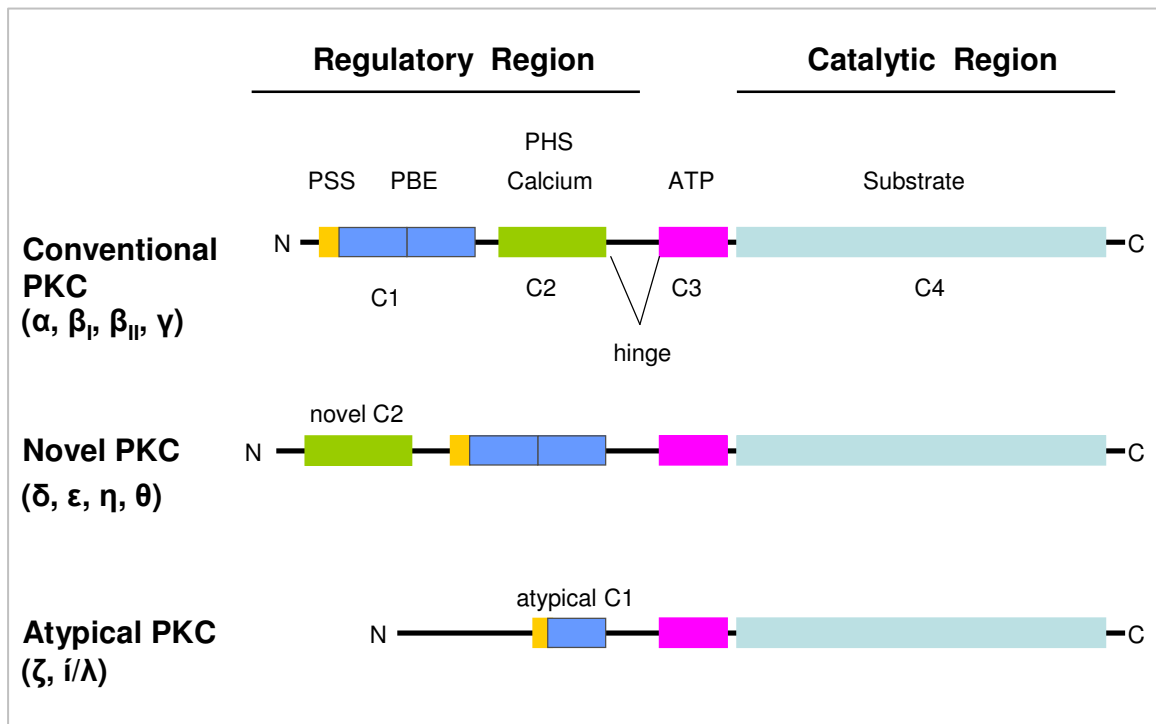
Figure 5.10: A schematic diagram showing the structure of conventional, novel and atypical PKCs. The pseudosubstrate domain (*gold*), C1 domain motifs (*blue*), C2 domain (green), C3 the ATP-binding lobe (*pink*) and C4 the substrate-binding lobe (gray) are shown. The C2 domain (i.e. novel C2) of novel nPKCs lacks Ca^{2+} binding capacity. The C1 (atypical C1) of atypical protein kinase Cs have only one Cys-rich motif which does not bind DAG or phorbol ester. PSS = pseudosubstrate, PHS = phosphatidyl-L-serine, PBE = phorbol esters and ATP= adenosine triphosphate

5.1.2 Generation of PKC second messengers

Stimulus (e.g. growth factor) mediated activation of membrane bound phospholipase C (PLC) plays a central role in the activation of both conventional PKCs and novel PKCs. The stimulation of many G-protein coupled receptors can lead to the activation of PLC. Activated PLC converts phosphatidylinositol (1, 4) bisphosphate (PIP2) into inositol (1, 4, 5) trisphosphate (IP3) and DAG.

5.1.3 Differential requirement of second messengers by PKC isoforms

All PKC isoforms via their C1 domains bind phosphatidyl-L-serine (phosphatidylserine) (Hurley *et al.*, 1997), a membrane acidic phospholipids that is located only on the cytoplasmic side of cell membranes. Phosphatidylserine binding is necessary for the high-affinity interaction of PKC with DAG containing membranes (Orr and Newton, 1994). The ten PKC isoforms are classified into three subgroups (conventional novel, and atypical PKCs) based on the composition of their regulatory domains moiety (Nishizuka, 1995; Musashi, 2000; Newton, 2003) (Figure 5.10). The particular configuration or domains in the regulatory region of each isoform determines the cofactor requirement of that isoform

5.1.3.1 Conventional PKC (cPKC) isoforms

The conventional PKC alpha, beta$_1$, beta$_2$ and gamma isoforms (α, β$_I$, β$_{II}$, γ) contain functional C1 and C2 domains (Figure 5.10). They therefore require DAG and Ca^{2+} ions for their full activities and functions.

5.1.3.2 Novel PKC (nPKC) isoforms

The novel PKC delta, epsilon, eta and theta isoforms (δ, ε, η, θ) contain a functional C1 domain and a non-ligand-binding (i.e. non-Ca^{2+}/membrane-binding) novel C2 domain (Figure 5.10). They are therefore Ca^{2+} independent but require DAG for their full activation and functions. The C1 domain of novel PKCs has an intrinsic affinity for DAG-containing membranes that is 2 fold higher than that of the C1 domain of conventional PKCs (Giorgione *et al.*, 2006). This allows the novel PKCs to respond to agonists that cause the production of DAG but not Ca^{2+} ion release. In contrast, cPKCs have to be targeted to membranes by their Ca^{2+}-binding C2 domain, in response to elevated of cytosolic Ca^{2+} ons, before they can to respond to DAG (Nalefski and Newton, 2001).

5.1.3.3 Atypical PKC (aPKC) isoforms

Atypical PKC zeta and tau/lamda isoforms (ζ, í/λ) contain a single non-DAG-binding ("atypical") C1 domain but no C2 domain (Figure 5.10). Therefore they do not respond to Ca^{2+} ion or DAG or phorbol esters.

5.1.4 PKC activation by phosphorylation,

All PKC isoyzmes have to be primed by a series of ordered phosphorylations before they can become structurally competent for catalysis. These kinases share three conserved phosphorylation motifs which must be primed by phosphorylations at critical residues or else kinases will remain catalytically inactive (Newton, 2003).

The processing of conventional PKC$_{\beta II}$ serves a model for the processing of PKC isoforms. The first phosphorylation of cPKC$_{\beta II}$ is catalyzed on a critical threonine residue (threonine 500), in the activation loop, by PDK-1 (i.e. the same kinase that activates Akt at threonine 308 in Akt activation loop). The importance of this phosphorylation event was demonstrated when it was found that mutation of the critical threonine residue to a neutral (i.e. non-phosphorylatable residue) inhibited PKC activity (Cazaubon *et al.*, 1994). PDK-1 is responsible for the phosphorylation-driven priming of all PKC isoforms. PDK-1 phosphorylates conventional PKCs (Dutil *et al.*, 1998), novel PKCs (Le Good *et al.*, 1998; Cenni *et al.*, 2002) and atypical PKCs (Chou *et al.*, 1998; Le Good *et al.*, 1998) at the critical threonine residues. In ES cells lacking PDK1 (PDK-1$^{-/-}$ cells), the intracellular levels of PKCα, PKCβI, PKCγ, PKCδ and PKCε, were markedly reduced (Balendran *et al.*, 2000). While PDK-1 activity directed at Akt is phosphoinositide-dependent (i.e. PIP3 must be bound to Akt PH domain), PDK-1-driven phosphorylation of PKCs is PIP3 independent (Sonnenburg *et al.*, 2001). This PDK-1-driven phosphorylation in the activation loop is followed by two further intramolecular autophosphorylations on two conserved residues; one on threonine 450 in the

turn motif in the C-terminal region and another on serine 660 in the hydrophobic motif (Newton, 2003). These phosphorylation events produce a mature (i.e. fully phosphorylated) PKC that is catalytically competent but not activated.

5.1.5 PKC activation by second messengers

The mature PKCs are localized to the cytosol and maintained in an inactive conformation by an auto-inhibitory pseudosubstrate sequence that blocks the active site. This auto-inhibition is relieved by allosteric interactions with second messengers. Diacylglycerol binds both cPKC and nPKC. Diacylglycerol binding recruits the enzymes to the membranes through their C1 and C2 domains. This induces a conformational change in the enzyme leading to the exposure of the auto-inhibitory pseudosubstrate domain and the removal of the pseudosubstrate from the substrate binding site thus allowing the substrate to bind and be phosphorylated (Orr *et al.*, 1992; Orr and Newton, 1994; Sakai *et al.*, 1997; Oancea and Meyer, 1998). In addition, IP3 mediates the release of Ca^{2+} ions from intracellular stores and the Ca^{2+} ions in turn potentiate the activation of cPKC. The activity of fully activated PKC activity is terminated by metabolism of DAG. As atypical PKCs do not respond to DAG or Ca^{2+}, the only clear regulation is their requirement for PDK-1 for the phosphorylation step that primes these isoforms (Chou *et al.*, 1998; Le Good *et al.*, 1998). These PKC isoforms have a pseudosubstrate sequence that allosterically regulates the enzyme. Their activation may also

depend on PI3K and increase in PIP3 (Standaert *et al.*, 2001) but the exact mechanism of this is not clear.

5.1.6 PKC isoforms and cell differentiation

PKC isoforms have been implicated in the differentiation of various cell types (Balazovich *et al.*, 1987; Melloni *et al.*, 1987; Kanakaraj *et al.*, 1998; Kim *et al.*, 2005; Marchisio *et al.*, 2005a). Differential regulation of PKC isoforms has been shown during the differentiation of various cell types. For example, PKC α, ζ, and δ are strongly and preferentially expressed and activated by phosphorylation during DMSO-induced erythroid differentiation of Friend erythroleukemia cells (Marchisio *et al.*, 2005a). Similarly it has also been shown that enhanced protein levels and activities of PKC βI and βII isoforms are required for the monocytic differentiation of HL-60 cells (Kim *et al.*, 2005). It has also been reported that the expressions of PKC α and ζ did not change significantly during the differentiation of immature mononuclear satellite cells to polynuclear myotubes (Boczan *et al.*, 2000). In contrast, the expression of PKC γ and η increased with differentiation while PKC θ showed high expression during the early phases of differentiation, but a decreased expression in the differentiated myotubes (Boczan *et al.*, 2000).

5.1.7 PKC isoforms and stem cell differentiation

Like other cell types, differential regulation of PKC isoforms has also been shown during the differentiation of stem cells. Studies have shown that the expression of specific PKC isoforms changed significantly as ES cells differentiate into cardiomyocytes. It has been shown that the differentiation of ES cells into beating cardiomyocytes requires the down-regulation of PKC β and ζ in conjunction with the up-regulation of PKC ε(Zhou *et al.*, 2003).

From the above, it can be seen that while PKC isoforms have been implicated in the differentiation of stem cells and other cell types, their exact roles are currently poorly understood. Therefore the aim of this study is to investigate the role of the PKC pathway in the cardiac differentiation of H9c2 cell and P19 stem cells. H9c2 and P19 stem cells are different cell types but both can differentiate in cardiomyocytes. Investigation of the role of PKC in the cardiac differentiation of these cells may yield information about the cell specific effects or possible cell-type independent effects of PKC during cardiac differentiation. To achieve the aim a PKC inhibitor, bisindolylmaleimide I (BIM-I) (Appendix VI (a)) was used for this study. BIM-I is a potent inhibitor for PKC (IC50=10nM) (Toullec *et al.*, 1991). It is highly selective for α (IC50=20nM), $β_I$ (IC50=17nM) $β_{II}$, (IC50=16nM), γ (IC50=20nM) (Toullec *et al.*, 1991). BIM-I also inhibit novel $PKC_δ$ (IC50=210nM), $PKC_ε$ (IC50=132nM) and atypical $PKC_ζ$ (IC50=5800nM)(Martiny-Baron *et al.*, 1993). BIM-I is a PKC competitive inhibitor and competes for the ATP-binding site of PKC (Toullec *et al.*, 1991).

5.2 MATERIALS AND METHODS

5.2.1 Culture and differentiation of H9c2 Cells

The routine culture of H9c2 cells was carried out as described in section 2.3.1 (Chapter 2). H9c2 cells were trypsinised and the appropriate cell density for the differentiation experiments was calculated as described in section 2.6 (Chapter 2). H9c2 cells (2×10^3 cells/cm^2) were seeded in p60 dishes or 6-well plates in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin), placed in a tissue culture incubator at 37°C, 95% air and 5% CO_2 and allowed to become 60-70% confluent. The differentiation of H9c2 cells into cardiomyocytes was carried out in 1% serum as described in section 2.8.1 (Chapter 2). The role of PKC signalling pathway in the differentiation of H9c2 cells into cardiomyocytes was investigated. The effects of the inhibition of the PKC signalling pathway were investigated using 10µM of BIM-I as described in section 2.8.2 (Chapter 2).

 A BIM-I stock (10 mM solution) was prepared by dissolving 1mg of BIM-I in 242µl of DMSO. The stock solution was distributed into 10 or 20µl aliquots and stored in the freezer at -20°C. The aliquots were protected from light during storage and use. The differentiation medium was used to dilute (1:1000) the frozen stock BIM-I solutions (10mM) to the BIM-I concentration (10µM) used in the differentiation experiments. The preparation of stock BIM-I concentration was carried out as explained in appendix VIII. More details about BIM-I are located in appendix VI (a).

5.2.2 Culture and differentiation of P19 stem cells

The routine culture of P19 stem cells was carried out as described in section 2.3.2 (Chapter 2). To initiate differentiation, P19 stem cells (1×10^6 cells) were seeded in P100 Petri (i.e. microbiological) dishes in the differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 μg/ml streptomycin and 0.8% DMSO) and the differentiation of P19 stem cells into beating cardiomyocytes carried out as described in section 2.9.1 (Chapter 2). The role of PKC signalling pathway in the DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes was investigated. The effect of the inhibition of PKC signalling pathway by 10μM of BIM-I on the DMSO-induced P19 stem cell differentiation into beating cardiomyocytes was investigated as described in section 2.9.2 (Chapter 2). Furthermore the effects of delayed inhibition of PKC on P19 stem cell differentiation into cardiomyocytes were also investigated as described in section 2.9.3 (Chapter 2).

5.2.3 Cell viability assay

To examine the effects of the PKC inhibition on H9c2 cell viability, the MTT assay was carried out as described in section 2.7 (Chapter 2).

5.2.4 Western blot analysis

In order to perform the western blot analysis, the following procedures were carried out as described in the Chapter 2 under the sections indicated in

parenthesis: cell lysate generation (section 2.11), preparation of BSA standards (section 2.12.1) and BCA assay (section 2.12.2). The western blotting was carried out as described in section 2.13.

5.2.5 Data analysis

Data obtained from independent experiments were used for statistical analysis as described in section 2.14 (Chapter 3).

5.3 RESULTS

5.3.1 Effects of protein kinase C inhibition on H9c2 cell differentiation into cardiomyocytes

The inhibition of the PKC signalling pathway, using BIM-I (10µM, caused marked inhibition of cell elongation, fusion and myotube formations that usually accompany H9c2 cell differentiation into cardiomyocytes. Control H9c2 cells cultured in the absence of BIM-I underwent differentiation and formed myotubes as seen previously. In contrast, cells exposed to BIM-I (10µM) maintained their undifferentiated single cell and spindle shaped morphology at day 4 and 8. By day 12 there were only marginal signs of cell elongation, fusion and myotube formation in the in BIM-I treated cells (Figure 5.11).

In agreement with the lack of morphological change observed in BIM-I treated H9c2 cells, the expression of MLC-1v protein in these cells was completely blocked up to day 4. Expression of MLC-1v in BIM-I treated cells was however detectable at day 8, increasing marginally by day 12. MLC-1v levels in BIM-I treated cells, by day 12, were just slightly above 50% of the levels seen in the control cells ($p < 0.001$) (Figure 5.12).
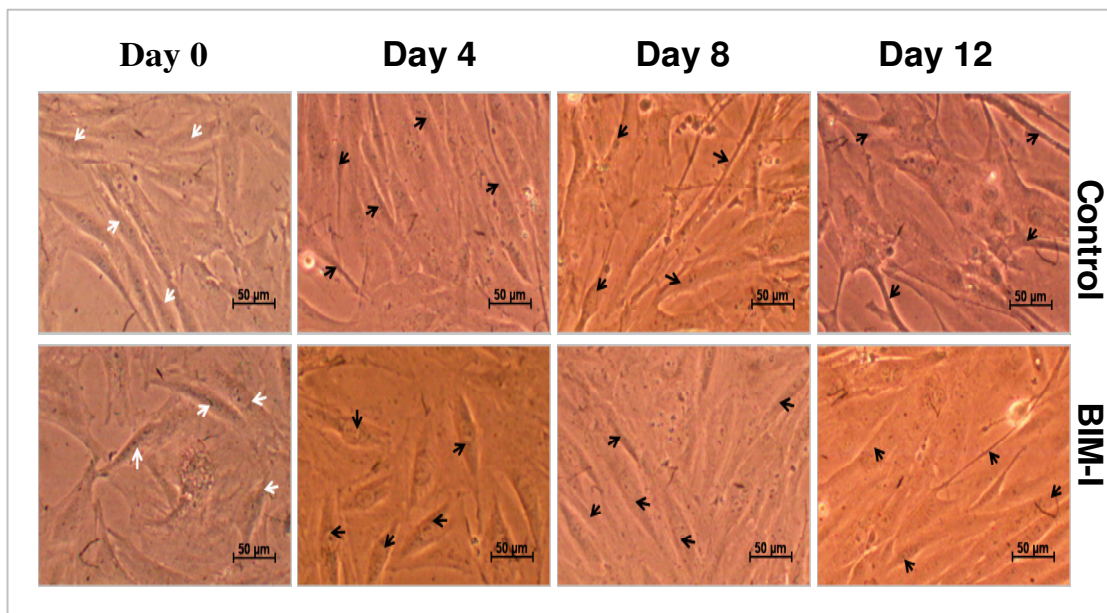
Figure 5.11: Effects of the protein kinase C inhibition on H9c2 cell differentiation into cardiomyocytes: morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100units/ml penicillin/100µg/ml streptomycin. The above differentiation medium was further supplemented with 10µM of BIM-I for the treated cells. BIM-I caused significant inhibition of H9c2 cell differentiation (i.e. cell alignment, elongation and myotube formation). Cells cultured in the absence of this compound followed the normal differentiation pattern (shown by black arrows in day 4 to day12 control cells). In contrast, cells exposed to BIM-I maintained their normal single cell and spindle shaped morphology (shown by black arrows in day 4 to day12 BIM-treated cells). Morphologically at day 4, 8 and 12 there were only marginal signs of differentiation in BIM-I treated H9c2 cells. Both control cells and BIM-I-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three independent differentiation experiments.
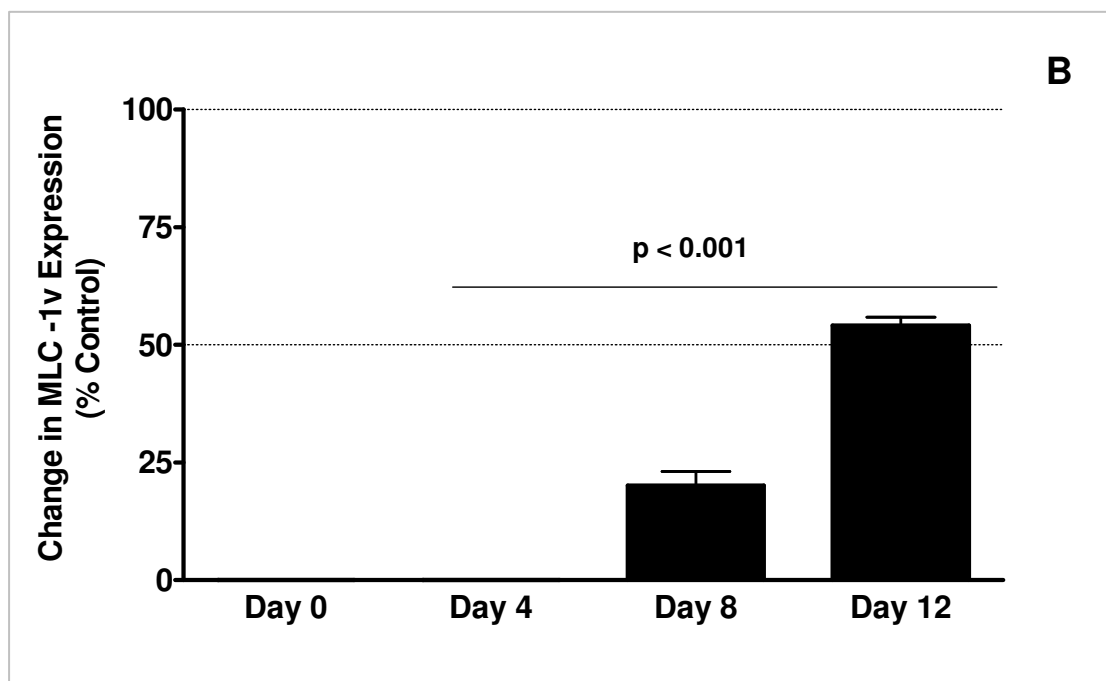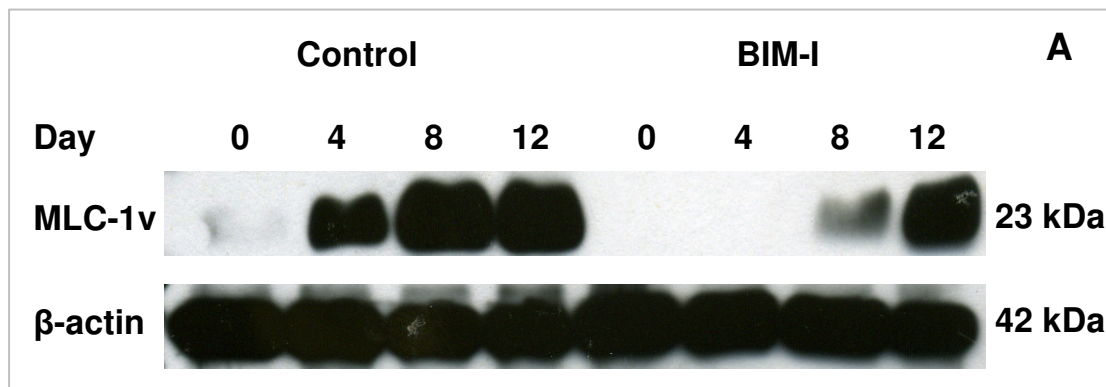
Figure 5.12: Effects of the protein kinase C inhibition on H9c2 cell differentiation into cardiomyocytes: cardiac ventricular myosin light 1(MLC-1v) expression. Western blot analysis of MLC-1v revealed that the expression of this protein in H9c2 cells was blocked by treating the cells with 10µM of BIM-I. . A a marginal expression started at day 8 and by day 12, MLC-1v levels in BIM-I treated cells were just slightly above 50% of the levels seen in the control cells. At day 4, day 8 and day 12, there was significant difference between control cells and BIM-treated cells ($p<0.001$). This is indicated by a horizontal line (drawn from day 4 to day 12) with the indicated p-value. The blot is representative of at least three differentiation experiments (panel A). The densitometric data (mean ± SD), from three independent differentiation experiments, show the time dependent changes in MLC-1v expression in BIM-I treated H9c2 cells (panel B).

5.3.2 Viability of H9c2 cells in 10µM of the protein kinase C inhibitor, BIM-I

The widely used concentration of 10µM for the PKC inhibitor BIM-I was selected for use in the experiments designed to investigate the effects of PKC inhibition on H9c2 differentiation into cardiomyocytes. BIM-I at the concentration of 10µM, like the other widely used concentrations of the other inhibitors used in this project, was not overtly toxic to the cells (Figure 5.13). The average viability was about 73% of the viability determined in the control cells. The control viability was significantly different than that in the treated cells at 6, 12, 24 and 48 hours ($p< 0.01$). At 48 hours (longest time that the cells were in the drug), the viability of treated cells was about 75% of the control viability and this was comparable or similar to the viability seen in the cells treated with other drugs.
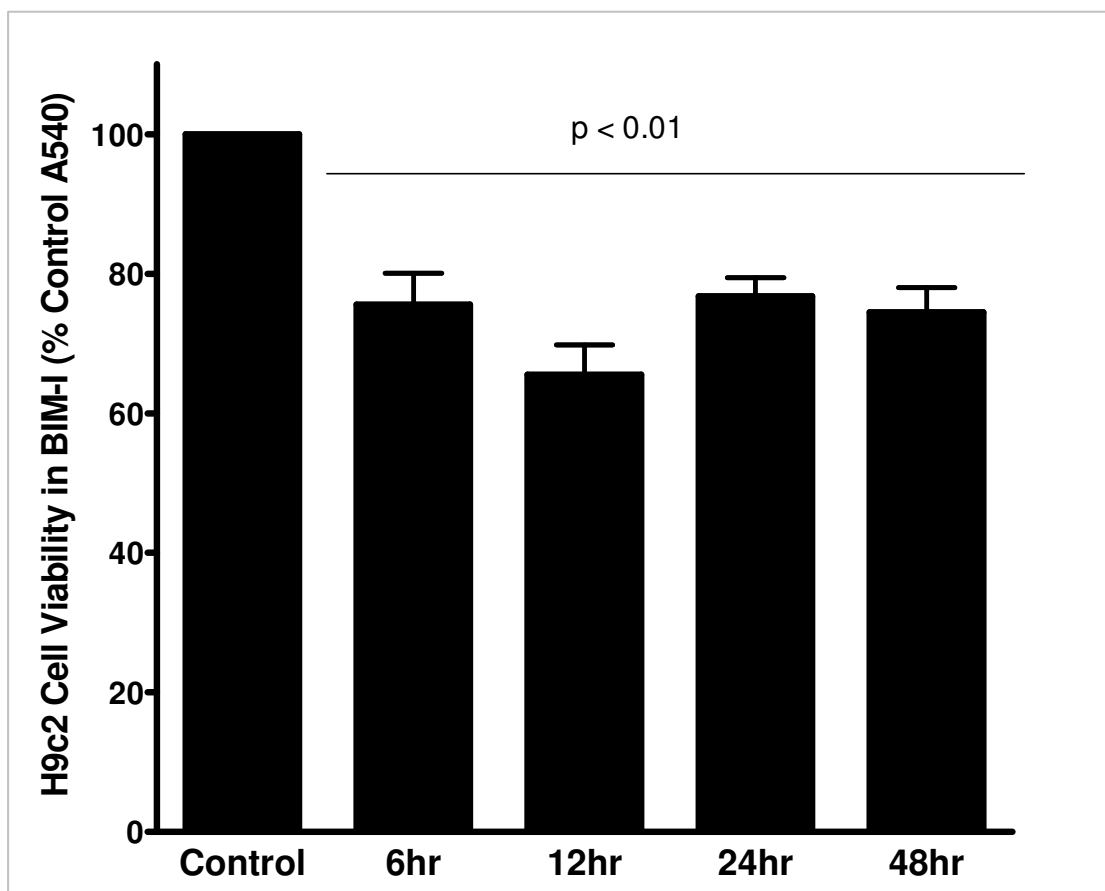
Figure 5.13: Viability of H9c2 Cells in the presence of BIM-I. H9c2 cells were seeded in 6-well plates and treated with 10µM BIM-I as described in the methods section above (section 5.3.1). The MTT assay was carried out at the end of each time point after the treatment. BIM-I at the concentration of 10µM was not toxic to the cells. The average viability was about 73% of the viability determined in the control cells. The control viability (control bar in the graph) was significantly different than the viability, calculated in BIM-I treated cells, at 6, 12, 24 and 48 hours (p< 0.01). At 48 hours (longest time that the cells were in the drug), the viability of treated cells was about 75% of the control viability and this was comparable or similar to the viability seen in the cells treated with other drugs. The MTT data presented in the graph are the mean ± SD from three independent experiments

5.3.3 Effects of the protein kinase C inhibition on P19 stem cell differentiation into beating cardiomyocytes

The control and BIM-treated P19 cell embryoid bodies followed their normal pattern of growth as already described in the establishment of the P19 cardiomyocyte differentiation model (Chapter 3). Upto day 5, beating cardiomyocytes were not usually observed in control or treated cells. Between day 6 and 7, P19 stem cells treated with DMSO in the absence of BIM-I usually began to show signs of beating cardiomyocytes (Figure 5.15). When present, the number of beating clusters of cardiomyocytes usually continued to increase up to day 14. In contrast, P19 stem cells treated with DMSO in the presence of 10μM BIM-I did not show any signs of beating cardiomyocytes during the same period. No beating cardiomyocytes were observed in any of the dishes containing BIM-I treated P19 stem cells over the whole course of the experiment (Figure 5.14).

P19 stem cell-derived cardiomyocytes were highly variable. Beating rates amongst the clusters of cardiomyocytes were highly variable. The patterns of cardiomyocyte beating clusters (including number and beating rates), from P19 stem cells treated with DMSO in the absence of BIM-I (i.e. no inhibition of PKC signalling pathway), were as already described in Chapter 3 (see Figure 5.15).

In agreement with the lack of appearance of beating cardiomyocytes in BIM-I treated P19 stem cells, the long term expression of cardiac restricted protein troponin I was inhibited at day 8 and day 12. (Figure 5.16)
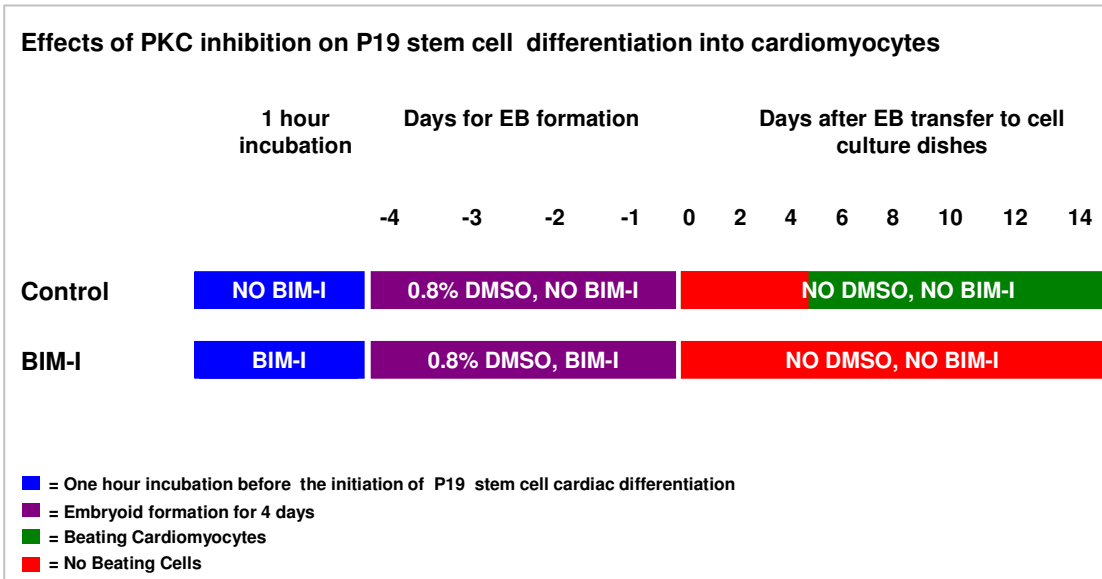
Figure 5.14: A schematic diagram of the effects of protein kinase C Inhibition on DMSO-induced differentiation of P19 stem cells into cardiomyocytes. PKC inhibition with BIM-I (10μM) blocked the differentiation of P19 stem cells into beating cardiomyocytes, when the inhibitors was added to the cells during the EB formation stage in the absence of BIM-I beating cardiomycytes were seen from day 6 to day 14 after the EB were transferred to cell culture dishes.
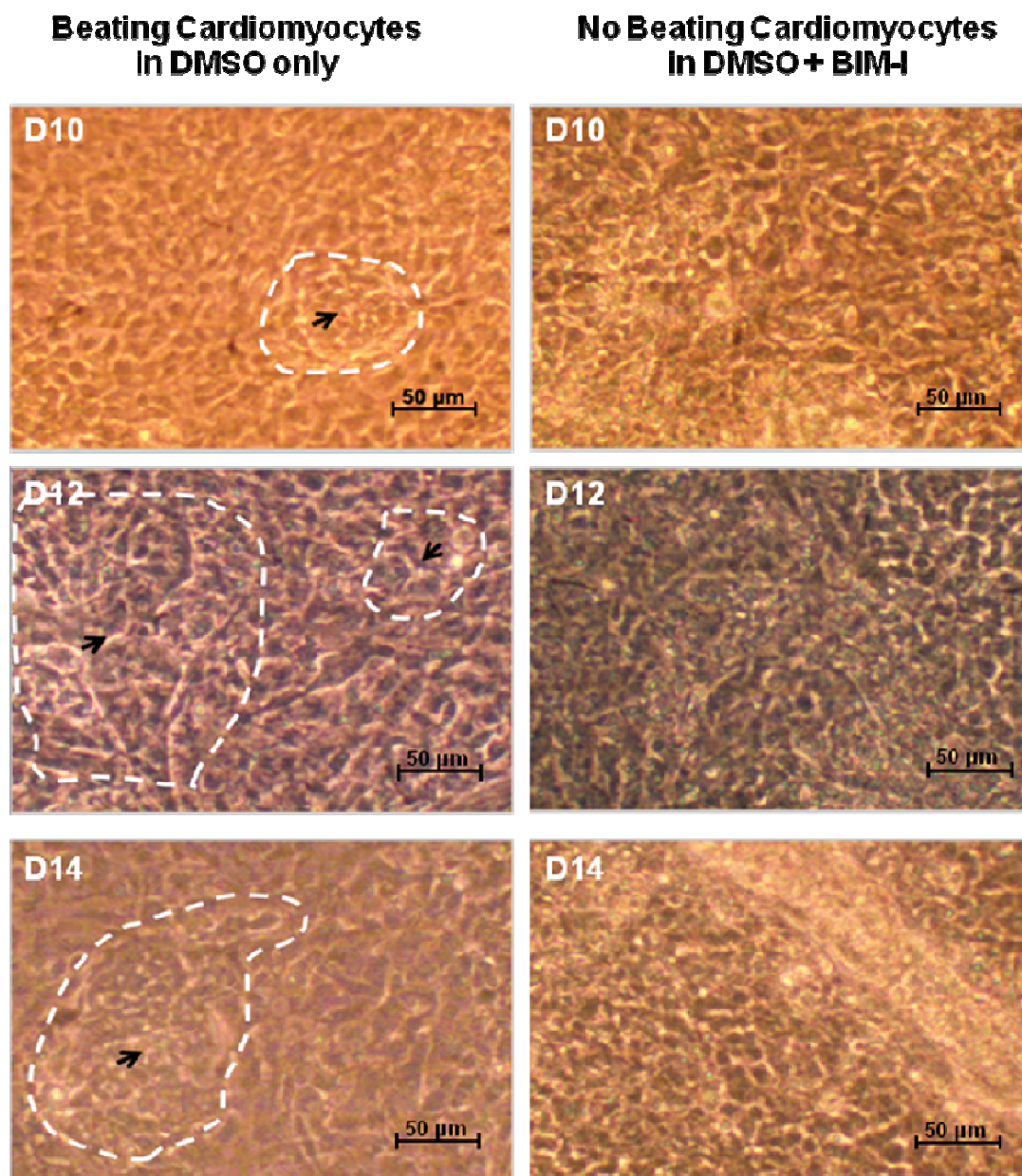
Figure 5.15: Effects of protein kinase C Inhibition on DMSO-induced differentiation of P19 stem cells into cardiomyocytes. Control P19 stem cell EBs were formed in differentiation medium consisting of α-MEM supplemented with 10% FBS and 100 units/ml penicillin/100 µg/ml streptomycin and 0.8% DMSO. The above differentiation medium was supplemented with 10µM of BIM-I for the treated P19 stem cells. DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes was inhibited by BIM-I. The areas of beating cardiomyocytes are shown within the closed figures drawn with white dotted lines. The beating focus of each beating area is shown by a black arrow. Photographs and movie clips of beating cells were taken under 200X magnification on the days indicated and are representative of microscope fields (for their respective days) from at least three independent differentiation experiments. D10 to D14 = day 10 to day 14.
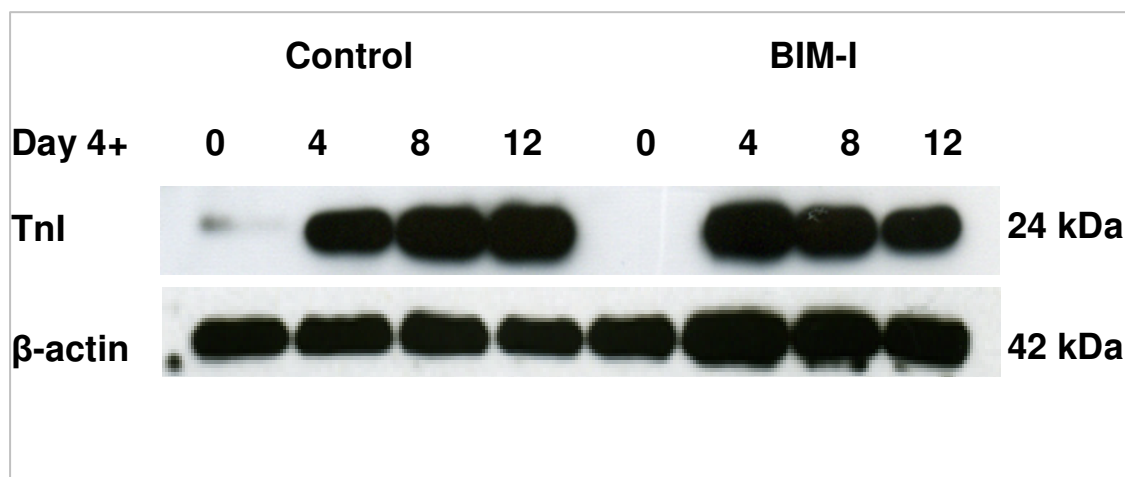
Figure 5.16: Effects of the protein kinase C inhibition on P19 stem cell differentiation into cardiomyocytes: Troponin I expression. Control P19 stem cells were cultured in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100 units/ml penicillin and 100µg/ml streptomycin as described in section 5.3.2. The above differentiation medium was further supplemented with 10µM of BIM-I for the treated cells. In BIM-treated P19 stem cell, the long term expression of cardiac restricted protein troponin I was inhibited at day 8 and day 12. While beating cardiomyocytes were observed in some dishes of the control cells, beating cardiomyocytes were not seen in any of the BIM-I treated P19 stem cells. The blot is representative of 3 differentiation experiments.

5.3.4 Effects of delayed inhibition of protein kinase C on P19 stem cell differentiation into beating cardiomyocytes

To determine the stage at which PKC may regulate the differentiation of P19 stem cells into cardiomyocytes, BIM-I was added to cell culture four days after exposure to DMSO (Figure 5.17). Control P19 stem cells were treated with DMSO and BIM-I at the EB formation stage and for comparison, parallel dishes of P19 cells (*delay-inhibition P19 cells*) were treated with DMSO only at EB formation stage and then only treated with BIM-I after the EB were transferred to culture dishes (section 5.3.2).

Twenty four hours after the setup of cells in Petri dishes, cell aggregates (EBs) were observed in the control cells and also in the *delay-inhibition P19 cells*. The EBs in both groups of cells underwent normal adhesion followed by proliferation and migration and both control and experimental cells were confluent by day 4. As already described, no beating cardiomyocytes were observed in both control and delay-treatment cells before day 6. By day 6 or 7, the first beating appeared in the *delay-inhibition P19 cells* (Figure 5.18). As expected, DMSO failed to induce P19 stem cell differentiation into beating cardiomyocytes, when 10µM of BIM-I was added at the EB formation stage.

However when DMSO was allowed to initiate P19 stem cell differentiation before the inhibition of the PKC signalling pathway (using 10µM of BIM-I), BIM-I failed to block the DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes (Figure 5.17). The patterns of beating cardiomyocyte clusters (including number and beating rates) from P19 stem cells, in which

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DMSO was allowed to initiate their differentiation before the inhibition of the

PKC signalling pathway, were as already described in Chapter 3 (see Figure

5.18).

Effects of delayed inhibition of PKC on P19 stem cell differentiation into cardiomyocytes

	1 hour incubation	Days for EB formation					Days after EB transfer to cell culture dishes							
		-4	-3	-2	-1	0	2	4	6	8	10	12	14	
Control	BIM-I	0.8% DMSO, BIM-I				NO DMSO, NO BIM-I								
BIM-I	NO BIM-I	0.8% DMSO, NO BIM-I				NO DMSO, BIM-I								

■ = one hour incubation before the initiation of P19 stem cell cardiac differentiation
■ = Embryoid formation for 4 days
■ = Beating Cardiomyocytes
■ = No Beating Cells

Figure 5.17: Effects of delayed inhibition of protein kinase C on P19 stem cell differentiation into beating cardiomyocytes. In P19 stem treated with BIM-I (10µM) four days after the initiation of DMSO-induced cardiac differentiation, BIM-I failed to block the cell differentiation into beating cardiomyocytes.

No Beating Cardiomyocytes in DMSO + BIM-I

Beating Cardiomyocytes BIM-I after DMSO
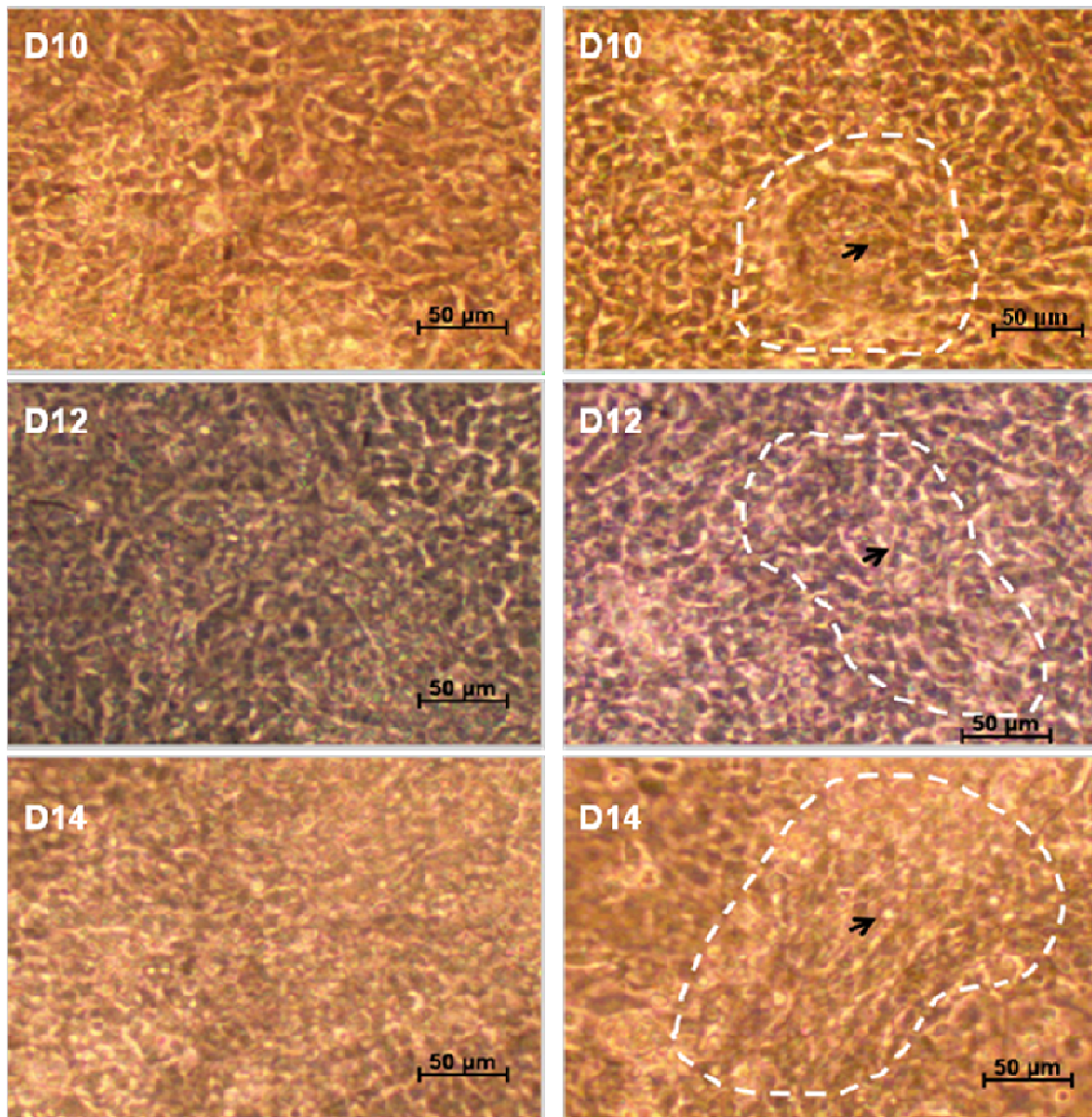
Figure 5.18: Effects of delayed inhibition of protein kinase C on P19 stem cell differentiation into beating cardiomyocytes. Control EBs were formed in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin, 0.8% DMSO and 10µM of BIM-I. For the delay inhibition of PKC, BIM-I was added four days after DMSO. Delayed addition of BIM-I has no effects on DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes. The beating areas of cardiomyocytes are indicated by the dotted white circles. The beating focus of each beating area is shown by a black arrow. Photographs and movie clips of beating cells were taken under 200X magnification on the days indicated and are representative of microscope fields (for their respective days) from at least three independent differentiation experiments. D10 to D14 = day 10 to day 14.

5.4 DISCUSSION

At both morphological and biochemical levels, the data showed that the inhibition of PKC by BIM-I markedly inhibited H9c2 cell differentiation into cardiomyocytes. Control cells cultured in the absence of BIM-I underwent the usual differentiation associated morphological changes (i.e. cell alignment, elongation and fusion to form myotues). In contrast BIM-treated H9c2 cells maintained their normal single cell and spindle shaped morphology with only marginal signs of morphological changes. This strongly suggests that the activation of PKC signalling may have key roles to play in the events that are responsible for causing these morphological changes during H9c2 cardiac differentiation. In agreement with the lack of morphological changes observed in BIM-I treated H9c2 cells, the BIM-I suppression of the expression of MLC-1v also suggest roles for PKC activation during H9c2 cell differentiation into cardiomyocytes.

In agreement with the PKC pathway having a role to play during H9c2 cell differentiation into beating cardiomyocytes, PKC inhibition also blocked the differentiation of P19 stem cells into cardiomyocytes. Control P19 stem cells differentiated into beating cardiomyocytes and expressed cardiac specific troponin I while BIM-I treated cells failed to differentiate into beating cardiomyocytes. Furthermore P19 stem cell cardiac differentiation as determined by the expression of cardiac specific troponin I was also inhibited. Our data are significant in that, it is suggestive of a need for PKC activation in both H9c2 and P19 stem cells. H9c2 and P19 stem cells are different cell types but both can differentiate into cardiomyocytes. The need for PKC

activation for the differentiation of both cell types (i.e. H9c2 and P19 cells) therefore suggests that the activation of PKC signalling pathway may be a fundamental or an essential signalling event during the differentiation of stem cells into cardiomyocytes.

The human embryonal carcinoma cell line NT2/D1 can differentiate into neuronal cells in the presence of retinoic acid. An increased PKC activity has been shown to accompany neuronal differentiation of these cells (McCarthy *et al.*, 1995). All-*trans*retinoic acid (ATRA) can induce the differentiation of the human promyelocytic leukaemia cell line (HL-60) into mature granulocyte-like cells. It has also been found that the activation of PKC is necessary for ATRA-induced differentiation of HL-60 cells (Kanakaraj *et al.*, 1998; Kim *et al.*, 2005).

PKC translocation from the cytoplasm to the plasma membrane is a central mechanism for the activation and activity of PKC isoforms, however selective nuclear accumulation of PKC ζ have been reported in HL-60 cells induced to differentiate along the granulocytic lineage by ATRA (Zauli *et al.*, 1996; Bertolaso *et al.*, 1998; Neri *et al.*, 1999). PKC ζ also accumulated in the nuclei of U937 cells (human promonocytic leukemia cells) that have undergone monocyte differentiation(Kiley and Parker, 1995). More importantly, it has been reported that PKC ζ nuclear accumulation, was blocked by the inhibition of PI3K using Wortmannin (Neri *et al.*, 1999).The inhibition of PKC ζ nuclear translocation, by wortmannin , has also been reported during ischemia in rat hearts (Mizukami *et al.*, 1997). Increased expression and phosphorylation of

PKC α, ε, ζ and δ has been shown following DMSO-induced differentiation in Friend erythroleukemia cells also suggest a possible role of this PKC in the differentiation of these cells (Marchisio *et al.*, 2005b). All the above results suggest that PKC may be involved in cell differentiation. These studies also suggest that the localisation of PKC isoforms to different subcellular compartments may be a mode of regulation or an indication of unique functions for individual PKC isoforms.

In agreement with suggestions from the previous studies that PKC isoforms have role to play in cell differentiation, our data suggest that PKC have may a role in the differentiation of both P19 stem cells and H9c2 cells. BIM-I blocked the differentiation of both cells into cardiomyocytes. While previous data suggest important roles for PKC during cell differentiation and our data specifically suggest a role for PKC in the differentiation of stem cells into cardiomyocytes, the exact mechanism by which PKC can mediate its positive effects on cell differentiation is not clearly defined. PKC isoforms are second messenger dependent kinases. PKC activation depends on either DAG or Ca^{2+} or both depending on the isoform. The increase in intracellular DAG or Ca^{2+} is mediated by phospholipase C (PLC). The elevation of intracellular Ca^{2+} ions is essential for the activation of conventional PKC isoforms. It is therefore very interesting to note that that some members of the Wnt-5a group that signal through the non-canonical Wnt/Ca^{2+} signalling pathway caused a PLC mediated increased in intracellular Ca^{2+} ion levels (Sheldahl *et al.*, 1999; Pandur *et al.*, 2002; Sheldahl *et al.*, 2003) that go on to activate PKC. It is/was generally thought that signalling events downstream of Wnts

belonging to Wnt-5a group, via the Wnt/Ca^{2+} signalling pathway, cause the inhibition   of the canonical Wnt/β-catenin signalling pathway (Torres *et al.*, 1996; Topol *et al.*, 2003; Westfall *et al.*, 2003; Maye *et al.*, 2004). However it is becoming clear that the assigned sole function of the non-canonical Wnt/Ca2+ signalling as an inhibitor of the canonical Wnt/β-catenin signalling pathway may not be   as simple as reported.

As discussed in Chapter 3, the available evidence suggests that DMSO-induced differentiation of P19 stem cells into cardiomyocytes may occur through the Wnt/β-catenin signalling pathway. Calcium ion release from the Wnt/Ca^{2+} signalling would be expected to activate PKCs. If the Wnt/Ca^{2+} signalling pathway function is the inhibition of the Wnt/β-catenin signalling pathway, then PKC (at least the conventional PKC isoforms) would be expected to play negative roles in the differentiation of P19 stem cell into cardiomyocytes. Clearly our data are in direct contrast to that expectation. Our data showed that inhibition of PKC led to the inhibition of the cardiac differentiation of P19 stem cells and H9c2 cells.

The activation of the Wnt/Ca^{2+} signalling pathway has been shown to cause calcium ion influx and the activation of PKC (Sheldahl *et al.*, 2003). Interestingly it has also been shown that the activation PKC inactivated GSK-3β and increased the accumulation of cytosolic β-catenin (Garrido *et al.*, 2002). In the same study, it was also found that the inhibition of Ca^{2+-}dependent PKC isoforms activated GSK-3β. Wnt-3a and lithium mimicked PKC activation (Garrido *et al.*, 2002; Sanai *et al.*, 2004). Clearly the

inactivation of GSK-3β by PKC and the accumulation of cytosolic β-catenin will enhance rather than inhibit the Wnt/β-catenin signalling pathway. Whether PKC in our experimental system can phosphorylate and inactivate GSK-3β remains to be examined. Whether PKC in our experimental system was activated by Ca^{2+} ions from the Wnt/Ca^{2+} signalling pathway also remains to be examined. It is possible that the PKC in our experimental system may be activated in a mechanism that is entirely independent of the Wnt/Ca^{2+} signalling pathway. However if PKC in our system was activated by Wnt/Ca^{2+} signalling pathway then our data suggest that Wnt/Ca2+ signalling pathway may also contribute to Wnt/β-catenin mediated differentiation of stem cells in cardiomyocytes in contrast to the inhibitory role assigned to this pathway during cardiogenesis.

5.5 Summary

The inhibition of the PKC signalling pathway, using BIM-I (10µM), caused marked inhibition of cell elongation, fusion and myotube formation that usually accompany H9c2 cell differentiation into cardiomyocytes. Control H9c2 cells cultured in the absence of BIM-I underwent differentiation and formed myotubes. In agreement with the lack of morphological change observed in BIM-I-treated H9c2 cells, the expression of MLC-1v protein in these cells was completely blocked up to day 4 and then significantly inhibited thereafter.

P19 stem cells cultured in 0.8% DMSO in the absence of BIM-I differentiated into beating cardiomyocytes. In contrast, P19 stem cells treated with 0.8%

DMSO in the presence BIM-I (10µM) did not differentiate into beating cardiomyocytes. However when DMSO was allowed to initiate P19 stem cell differentiation before the addition of BIM-I, the cells differentidate into beating cardiomyocytes (i.e. BIM-I failed to block their DMSO-induced cardiac differentiation). In agreement with the lack of appearance of beating cardiomyocytes in BIM-I treated P19 stem cells, the long term expression of cardiac restricted protein troponin I (TnI) was inhibited.

In conclusion, our data therefore suggest that the activation of PKC signalling pathway may be required for the differentiation of H9c2 cell and P19 stem cell differentiation into cardiomyocytes. The data also suggest that the activation of PKC signalling pathway may be required during the early stages of P19 stem cell differentiation into cardiomyocytes.

CHAPTER 6.0

ROLE OF p38 MAP KINASE IN H9C2 AND P19 STEM

CELL DIFFERENTIATION INTO CARDIOMYOCYTES

6.1 INTRODUCTION

6.1.1 Mitogen-activated protein kinase

The p38 kinases are serine/threonine kinases that belong to a very large protein kinase family known as the mitogen-activated protein kinases (MAPKs). The MAPKs control several cell functions including gene expression (Wu *et al.*, 2004; Lawrence *et al.*, 2007; Mochizuki *et al.*, 2007), differentiation (Zetser *et al.*, 1999; Jadlowiec *et al.*, 2004; Bokui *et al.*, 2008; Kook *et al.*, 2008), proliferation (Souza *et al.*, 2004; Erlich *et al.*, 2007; Jia *et al.*, 2008), migration (Goetze *et al.*, 1999; Rousseau *et al.*, 2006; Jia *et al.*, 2008) and cell survival or apoptosis (Chuang *et al.*, 2000; Pan *et al.*, 2002; Chen *et al.*, 2008; Cho *et al.*, 2008). They are activated by a range of extracellular stimuli (mitogens) including growth factors (Moriuchi *et al.*, 2001; McFarland and Pesall, 2008), hypotonic stress (Niisato *et al.*, 2007) and oxidative stress (Kurata, 2000; Gaitanaki *et al.*, 2003; Kim *et al.*, 2004; Frossi *et al.*, 2007). The effects of antioxidant on various MAPKs may depend on the MAPK and/or the activating stimulus. For example the antioxidants, ascorbic acid, catalase and superoxide dismutase, have been reported to suppress p38-MAPK signalling pathway in the perfused frog hearts (Gaitanaki *et al.*, 2006) . On the other hand, in cultured rat aortic smooth muscle cells, diphenyleneiodonium chloride (DPI), ascorbic acid, N-acetyl cysteine (NAC), diethyldithiocarbamic acid (DETC) and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox C) have no effects on angiotensin II-induced activation of ERK 1/2 activation (Kyaw *et al.*, 2001). In contrast, angiotensin II-induced activation of p38 MAPK was inhibited by DPI

and ascorbic acid, NAC (at high concentration) but not DETC or Trolox C (Kyaw *et al.*, 2001).

The MAPK signalling pathway architecture consists of an arrangement of three sequentially acting protein kinases (Figure 6.10). The pathway is activated by a small guanosine 5'-triphosphate (GTP)-binding protein (GTP) protein (of the Ras or Rho family) or by an adaptor protein. The adaptor protein transmits the signal directly to MAP kinase kinase kinase (MAPKKK) (i.e. the kinase at the top of this architecture). Alternatively, the adaptor protein can also transmit the signal through a mediator kinase (i.e. MAP kinase kinase kinase kinase (MAPKKKK)) which phosphorylates and activates MAPKKK. In the second step, the activated MAPKKK phosphorylate and activate MAP kinase kinase (MAPKK) which in turn activates MAPK. Activated MAPK then phosphorylate a wide range of substrates.

Specificity is achieved among this complex arrangement of competing MAPK pathways because the kinase activities at each sequential phosphorylation step are directed toward specific downstream kinases by specific protein-protein interactions. Specific scaffold proteins that interact with substrates and their corresponding kinases along with the presence of specific docking sites or sequences in the MAPKs ensure specific recognition and activation by appropriate upstream kinases (Bardwell *et al.*, 2003; Tatebayashi *et al.*, 2003; Sharma and Mondal, 2006). Termination of MAPK activation (i.e. inactivation) is regulated by dephosphorylation that is carried out by MAPK

phosphatases (MKPs) (Bardwell *et al.*, 2003; Noordman *et al.*, 2006).
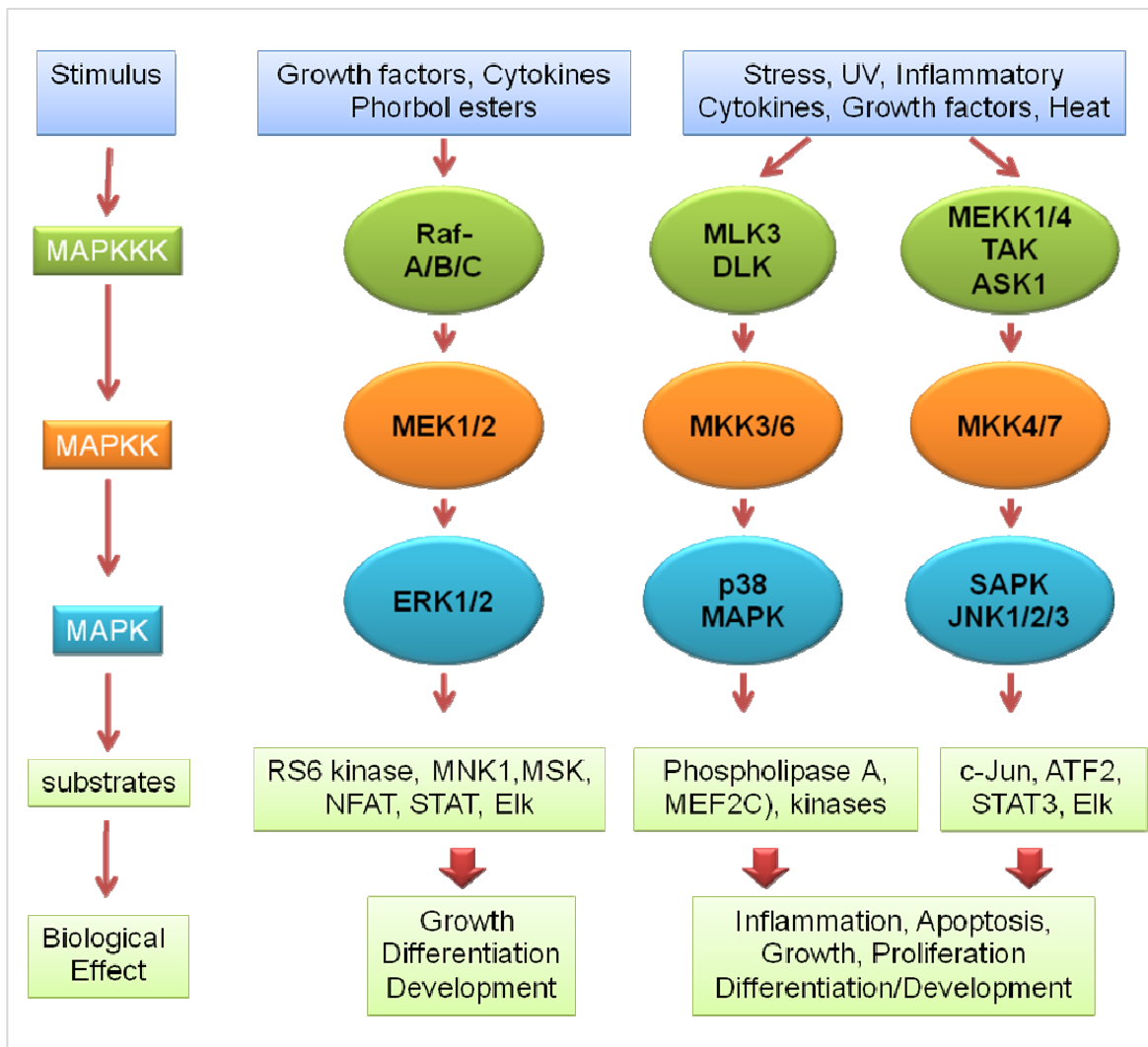
Figure 6.10: MAPK signalling pathway architecture. A schematic diagram showing the three sequential levels for the activation of downstream MAPKs by upstream MAPKs in the architecture of the MAPK signalling pathways. Some examples of targets for terminal kinases (i.e. p38, ERK and c-jun/SAPK) and downstream biological effects are shown in the diagram. NFAT = Nuclear Factor of Activated T cells, STAT= Signal Transducers And Activators Of Transcription, MEF2C = Myocyte Enhancer Factor 2 C, ATF2= Activating Transcription factor 2, MSK= MAPK/SAPK-activated kinase, MNK1= MAPK signal-activating kinase 1, Elk-1 = E26-like protein 1, Transforming growth factor-β-activated kinase (TAK), Mixed lineage kinase 3, MEKK1/4 = Mitogen ERK kinase kinase, 1/4, ASK= apoptosis stimulating kinase, DLK= dual leucine zipper bearing kinase, ERK= extracellular signal-regulated kinase JNK= c-Jun N-terminal kinase, SAPK= stress-activated protein kinase, MAPKKK=MAP kinase kinase kinase, MAPKK=MAP kinase kinase, MAPK = Mitogen activated protein kinase, MEK = Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase (MEK).

6.1.2 Mitogen-activated protein kinase family

The MAPK family consists of three subgroups: p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK).

6.1.2.1 P38 MAPK Pathway

Of the four p38 isoforms (p38α p38β, 38γ and p38δ), p38α is expressed in all tissues. The p38 MAPK pathway is activated by serum (Beier and LuValle, 1999), osmotic stress (Bell *et al.*, 2000; Volonte *et al.*, 2001), UV radiation (Bulavin *et al.*, 1999; Bodero *et al.*, 2003; Sethi and Sodhi, 2004) and heat shock (Dorion *et al.*, 2002; Zhou *et al.*, 2005; Venkatakrishnan *et al.*, 2006). The p38 MAPK pathway is also activated by proinflammatory cytokines (Nick *et al.*, 1999; Suzuki *et al.*, 2001). The p38 MAPK pathway is involved in apoptosis (Chuang *et al.*, 2000; Cho *et al.*, 2008), stress (Frossi *et al.*, 2007; Niisato *et al.*, 2007), proliferation (Souza *et al.*, 2004), cell differentiation (Morooka and Nishida, 1998; Cuenda and Cohen, 1999; Zetser *et al.*, 1999; Davidson and Morange, 2000; Kook *et al.*, 2008) and cell survival. In the p38 pathway, stimulus-activated G proteins activate MAPKKKs. The MAPKKKs in turn phosphorylate and activate the MAPKKs (e.g. MKK3 and MKK6). These MAPKK then activate p38. The substrates for activated p38 kinases include cytosolic phospholipase A2 (Kramer *et al.*, 1996; Hazan-Halevy and Levy, 2000; Degousee *et al.*, 2001; You *et al.*, 2005), the microtubule-associated protein Tau (Zhu *et al.*, 2000; Li *et al.*, 2003), transcription factors (MEF2C

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(Yang *et al.*, 1999; Zhao *et al.*, 1999; de Angelis *et al.*, 2005)) and other protein kinases.

6.1.2.2 Extracellular signal-regulated kinase (ERK)

The ERK are activated by a wide range of extracellular stimuli including growth factors (Bottazzi *et al.*, 1999; Ouwens *et al.*, 2002; Bobick *et al.*, 2007), serum (Frost *et al.*, 1994; Beier and LuValle, 1999), phorbol esters, cytokines and osmotic stress (Chiri *et al.*, 2004). These stimuli activate the small GTP-binding protein Ras. Activated Ras recruit Raf to the cell membrane where it becomes activated. The activated Raf (i.e. the MAPKKK) then phosphorylates and activates the MAPKK (MEK1 and 2) which in turn phosphorylate ERK1/2. The substrates for activated ERK1/2 include other downstream kinases such as ribosomal S6 kinases (RSKs) (Smith *et al.*, 1999), MAPK/SAPK-activated kinase (MSK) (Deak *et al.*, 1998) and MAPK signal-activating kinase 1 (MNK1) (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Activated ERK1/2 also phosphorylate transcription factors such as NF-AT (Ali *et al.*, 2000; Sanna *et al.*, 2005), Elk-1(Muller *et al.*, 1997; Vanhoutte *et al.*, 1999; Thiels *et al.*, 2002), c-Fos (Vanhoutte *et al.*, 1999; Monje *et al.*, 2003; Burch *et al.*, 2004; Pellegrino and Stork, 2006), c-Myc, (Pintus *et al.*, 2002; Ramljak *et al.*, 2003; Serra *et al.*, 2008), STAT3 (Kanai *et al.*, 2003) and apoptosis-related proteins (Jan *et al.*, 1999; Scheid *et al.*, 1999; Ellert-Miklaszewska *et al.*, 2005; Sawatzky *et al.*, 2006; Caraglia *et al.*, 2007). The ERK1/2 signaling pathway is involved in cell proliferation (Souza *et al.*, 2004), differentiation

(Kim *et al.*, 2005), transformation and apoptosis (Jan *et al.*, 1999; Chuang *et al.*, 2000; Ellert-Miklaszewska *et al.*, 2005; Caraglia *et al.*, 2007).

6.1.2.3 C-Jun N-terminal kinase/stress-activated protein kinase

The c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) pathway are activated by growth factors, cytokines and stress related responses (e.g. UV radiation and heat). JNK/SAPK signalling pathway is mainly involved in responses to stress. Like ERK, JNK/SAPK are also involved in cell proliferation (Sabapathy *et al.*, 2001; Zhang *et al.*, 2005; Ding *et al.*, 2007), differentiation (Cuenda and Cohen, 1999; Lemonnier *et al.*, 2004) and apoptosis (Chuang *et al.*, 2000; Cho *et al.*, 2008).

In the JNK/SAPK pathway, activated G proteins (Cdc42, Rac, or Ras) activate several MAPKKK (e.g. apoptosis stimulating kinase (ASK) and other molecules including transforming growth factor-β-activated kinase (TAK)). These MAPKKKs, in turn, activate the MAPKKs (e.g. MKK4 and MKK7). The activated MAPKKs in turn directly phosphorylate and activate JNK/SAPK. The substrate for activated JNK/SAPK includes several transcription factors such as c-jun (Hatzoglou *et al.*, 2000; Zhong *et al.*, 2007), ATF2 (Hayakawa *et al.*, 2003; Bailey and Europe-Finner, 2005), STAT3 (Turkson *et al.*, 1999) and Elk-1 (Hatzoglou *et al.*, 2000; Zhang *et al.*, 2007; Zhong *et al.*, 2007).

6.1.2.4 MAPK and stem cell differentiation

The MAPK have been implicated in the differentiation of various cells including the differentiation of stem cells into cardiomyocytes and other cell types (Zhang and Bradley, 1996; Schlange *et al.*, 2000). However the exact mechanism by which MAPK can regulate cell differentiation is not currently clear. There is evidence that GATA-4 actions may require the activation of MAPK (i.e. p38) dependent pathway (Charron *et al.*, 2001; Liang *et al.*, 2001). Given their implication in the differentiation of various cells and the lack of clear understanding of their role in differentiation, the MAPK are therefore good candidates for studies aimed at understanding the cell signalling pathways that are responsible for mediating stem cell differentiation into cardiomyocytes. P38 MAPK is downstream of PI3K and our data have already suggested that when PI3K is inhibited, the differentiation of H9c2 and P19 stem cell differentiation into cardiomyocytes, is also inhibited. We therefore extended our studies to determine whether the signalling events that control differentiation of these cells into cardiomyocytes are linked to the activation of the p38 MAPK.

The aim of this study was to investigate the role of the p38 MAPK pathway in the cardiac differentiation of H9c2 and P19 stem cells. Like p38 MAPK, ERK and JNK have also been implicated in differentiation of various cell types. We initially started by examining the role of p38 MAPK in the hope that the study could be extended to examination of the roles of ERK and JNK. The roles of JNK and ERK cardiac differentiation were not examined in this project due to time constraints and to other factors. The design of this study focused on the

use of a specific competitive inhibitor of p38 MAP kinase known as SB203580

(4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole)

(IC50=0.6µM) (Cuenda *et al.*, 1995). SB203580 (100µM) (i.e. ten times the

widely used concentration of 10µM), does not inhibit JNK/SAP kinase or p42

MAP kinase (Cuenda *et al.*, 1995). SB20380 competes for enzyme ATP

binding site of p38 MAPK (Cuenda *et al.*, 1995; Young *et al.*, 1997; Gum *et*

al., 1998).

6.2 MATERIALS AND METHODS

6.2.1 Culture and differentiation of H9c2 Cells

The routine culture of H9c2 cells was carried out as described in section 2.3.1 (Chapter 2). H9c2 cells were trypsinised and the appropriate cell density for the differentiation experiments was calculated as described in section 2.6 (Chapter 2). H9c2 cells ($2x10^3$ cells/cm^2) were seeded into p60 dishes or 6-well plates in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin), placed in a tissue culture incubator at 37°C, 95% air and 5% CO_2 and allowed to become 60-70% confluent. The differentiation of H9c2 cells into cardiomyocytes was carried out in 1% serum as described in section 2.8.1 (Chapter 2). Role of the p38 MAPK signalling pathway in the differentiation of H9c2 cells into cardiomyocytes was investigated using SB203580 (10µM) as described in section 2.8.2 (Chapter 2).

6.2.2 Culture and differentiation of P19 stem cells

The routine culture of P19 stem cells was carried out as described in section 2.3.2 (Chapter 2). To initiate differentiation, P19 stem cells ($1x10^6$ cells) were seeded in P100 Petri (i.e. microbiological) dishes in the differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 µg/ml streptomycin and 0.8% DMSO) and the differentiation of P19 stem cells into beating cardiomyocytes carried out as described in section 2.9.1 (Chapter 2). The role of p38 MAPK signalling pathway in the DMSO-induced differentiation

of P19 stem cells into beating cardiomyocytes was investigated using SB203580 (10µM) as described in section 2.9.2 (Chapter 2). An SB203580 stock (10 mM solution) was prepared by dissolving 1mg of SB203580 in 264.97µl of DMSO. The stock solution was distributed into 10 or 20µl aliquots and stored in the freezer at -20°C. The aliquots were protected from light during storage and use. The differentiation medium was used to dilute (1:1000) the frozen stock SB203580 solutions (10mM) to the SB203580 concentration (10µM) used in the differentiation experiments. The preparation of stock SB203580 concentration was carried out as explained in appendix VIII. More details about SB203580 are located in appendix VI (c). Furthermore the effects of delayed inhibition of p38 MAPK on P19 stem cell differentiation into cardiomyocytes were also investigated as described in section 2.9.3 (Chapter 2).

6.2.3 Cell viability assay

To examine the effects of the inhibition of p38 MAPK, with SB203580, on H9c2 cell viability, the MTT assay was carried out as described in section 2.7 (Chapter 2).

6.2.4 Western blot analysis

In order to perform the western blot analysis, the following procedures were carried out as described in the Chapter 2 under the sections indicated in parenthesis: cell lysate generation (section 2.11), preparation of BSA

standards (section 2.12.1) and BCA assay (section 2.12.2). The western

blotting was carried out as described in section 2.13.

6.2.5 Data analysis

Data obtained from three independent experiments were used for statistical

analysis as described in section 2.14 (Chapter 2).

6.3 RESULTS

6.3.1 Effects of the p38 MAPK inhibition on H9c2 cell differentiation into cardiomyocytes

As described in previous Chapters, morphological changes and marker expression were observed or determined during the course of the differentiation experiments. Morphologically, treatment of H9c2 cells with SB203580 (10µM) caused little or no inhibition of their differentiation into cardiomyocytes. In both control cell and cells treated with SB203580, cell elongation was visible from day 4. By day 8 to day 12, cell fusion and myotube formation were extensive and to the same degree in both groups (Figure 6.11).

In contrast to the apparent lack of inhibition of the usual morphological changes that accompanied H9c2 cell differentiation into cardiomyocytes, SB203580 treated cells decreased the expression of MLC-1v by day 4 (p<0.05) (Figure 6.12). However, MLC-1v I expression in SB203580 treated cell had reached the same level seen in the control cells by day 8 (p>0.05). By day 12, MLC-1v expression in SB203580 treated cells fell back to nearly the levels seen in these cells at day 4 (p<0.05) (Figure 6.12).
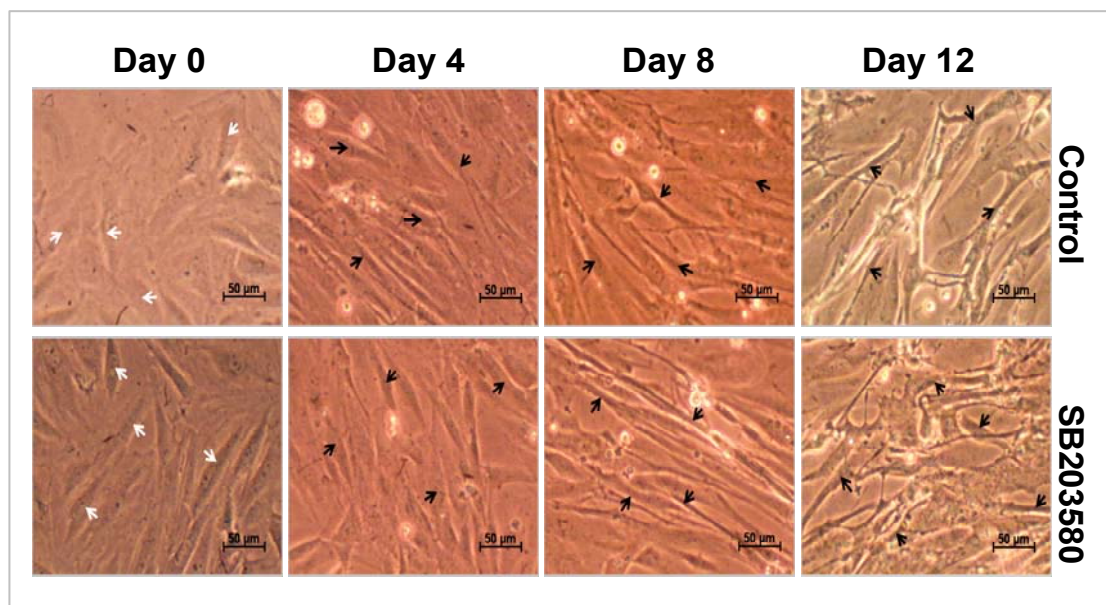
Figure 6.11: Effects of p38 MAPK inhibition on H9c2 cell differentiation into Cardiomyocytes: Morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 10µM of SB203580. Morphologically, SB203580 treatment of H9c2 cells shows very little signs of inhibition of their differentiation (i.e. cell alignment, elongation and myotube formation) into cardiomyocytes inhibited. Both control and SB203580 treated cells differentiated (shown by black arrows in day 4 to day12 control and SB20350-treated cells) to almost the same degree. By day 8 to day 12, cell fusion and myotube formation were extensive and to the same degree in both groups. Both control cells and SB20359-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three independent differentiation experiments.
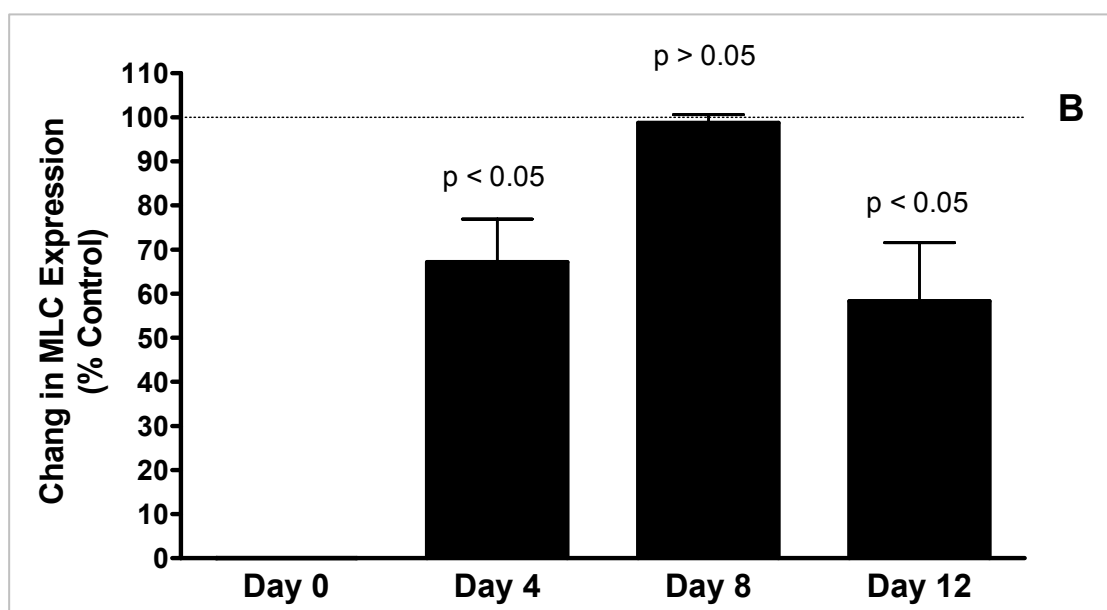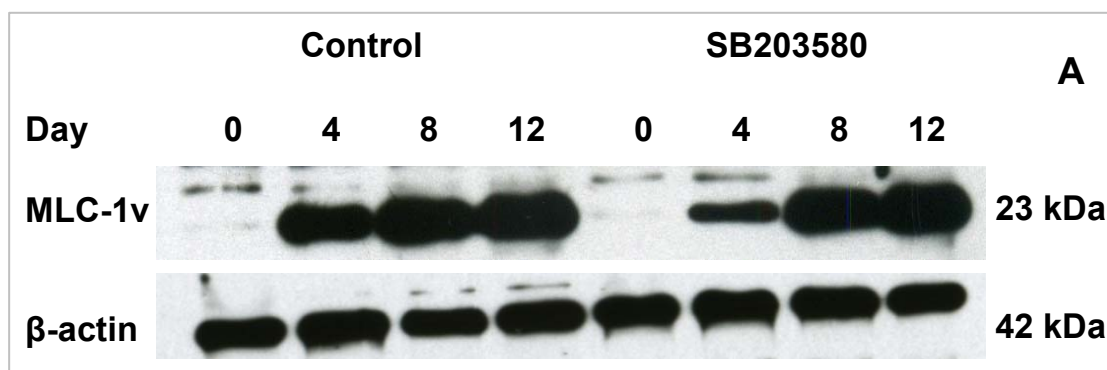
Figure 6.12: Effects of p38 MAPK inhibition on H9c2 cell differentiation into Cardiomyocytes: cardiac ventricular myosin light 1 expression. Western blot analysis of MLC-1v revealed that expression of this protein was initially delayed cells (by day 4, $p<0.05$) in comparison to the control expression (panel A). By day 8, MLC-1v expression in treated cell reached levels seen in control cells ($p>0.05$) but by day 12, MLC-1v level fell back to nearly the levels seen at day 4 ($p<0.05$). The blot is representative of at least three independent experiments. The densitometric data (mean ± SD), from three independent differentiation experiments, show the time dependent changes in MLC-1v expression in SB203580 treated H9c2 cells (panel B).

6.3.2 Viability of H9c2 cells in 10µM of the p38 MAPK inhibitor, SB203580

The widely reported concentration of 10µM for the p38 MPAK inhibitor SB203580 was selected for use in the experiments designed to investigate the effects of p38 MAPK inhibition on H9c2 differentiation into cardiomyocytes. The MTT cell viability test was carried out to determine if this concentration was toxic to the cells over the period that the cells were exposed to the drug. SB203580 at the concentration of 10µM was not toxic to the cells at any of the incubation time points. The average viability was about 89% of the viability determined in the control cells. There was a slight reduction in SB203580 treated cells at 12 and 24 hours ($p < 0.05$) but at 48 hours, the cell viability recovered to a value that was not significantly different from the control viability ($p > 0.05$) (Figure 6.12). At 48 hours the viability was about 91% of the viability determined in the control cells (Figure 6.13).
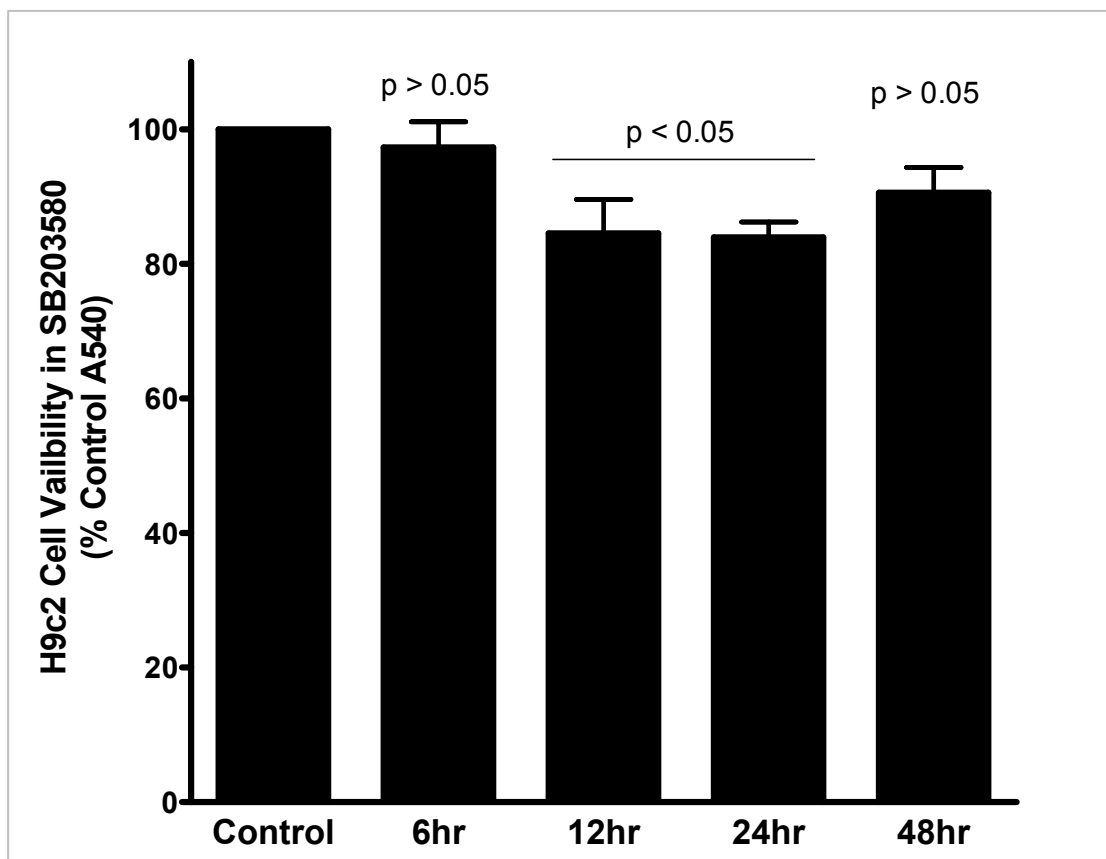
Figure 6.13: Viability of H9c2 Cells in the presence of the p38 MAPK inhibitor, SB203580. H9c2 cells were seeded in 6-well plates and treated with 10μM SB203580 as described in the methods section above (Section 6.3.3). The MTT assay was carried out at the end of the treatment. At 48 hours the viability of SB203580-treated cels was not significantly different from the control viability ($p > 0.05$). The data presented in the graph are the mean ± SD from three independent experiments.

6.3.3 Effects of p38 MAPK inhibition on P19 stem cell differentiation into cardiomyocytes

Given the lack of effects of the p38 MAPK inhibitor on H9c2 cell differentiation into cardiomyocytes, we next investigated how p38 MAPK inhibition may affect P19 cell differentiation into cardiomyocytes. In contrast to the lack of inhibition H9c2 cell differentiation into cardiomyocytes, SB203580 markedly inhibited P19 Stem cells differentiation into cardiomyocytes as determined by the expression of the cardiac restricted protein troponin (TnI). (Figure 6.16) Control P19 stem showed a small level of TnI expression at the time of EB transfer from Petri dishes to cell culture plates. In control P19 cells, there was a marked increase on day 4 and this was followed further increased on day 8. The levels of TnI in SB203580 treated P19 stem cells were markedly reduced at every time point of the differentiation experiment as determined by western blot for TnI. The inhibited or low levels of TnI detected in SB203580 treated cells are in agreement with the lack of appearance of beating cardiomyocyte clusters in these cells. While beating cardiomyocytes were observed in the control cells as usual, no beating cardiomyocytes were seen in the SB203580 treated P19 stem cells. As was observed in the cases of PKC inhibition (Chapter 5) and PI3K inhibition (Chapter 7), it was also observed that P19 stem treated with SB203580 four days after the initiation of DMSO-induced differentiation of P19 stem cells failed to block the cell differentiation into beating cardiomyocytes (Figure 6.15). However, SB203580 blocked P19 stem cell differentiation into beating cardiomyocytes, when added to the cells during the EB formation stage (Figure 6.14).
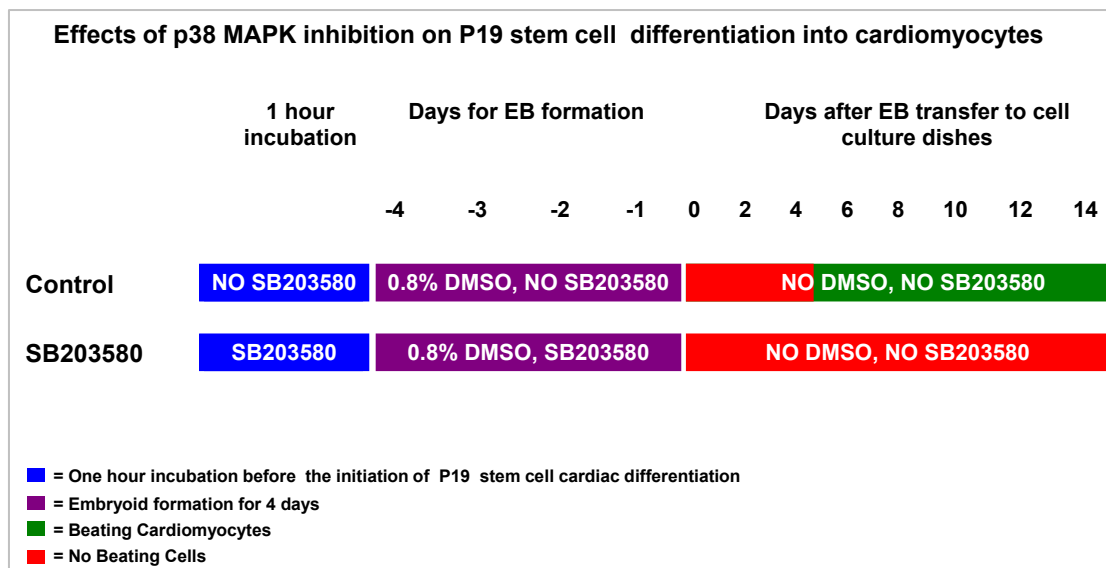
Figure 6.14; *A schematic diagram of the effects of p38 MAPK inhibition on P19 stem cell differentiation into cardiomyocytes* P38 MAPK inhibition with SB203580 (10µM) blocked the differentiation of P19 stem cells into beating cardiomyocytes, when the inhibitors was added to the cells during the EB formation stage in the absence of SB203580 beating cardiomycytes were seen from day 6 to day 14 after the EB were transferred to cell culture dishes.
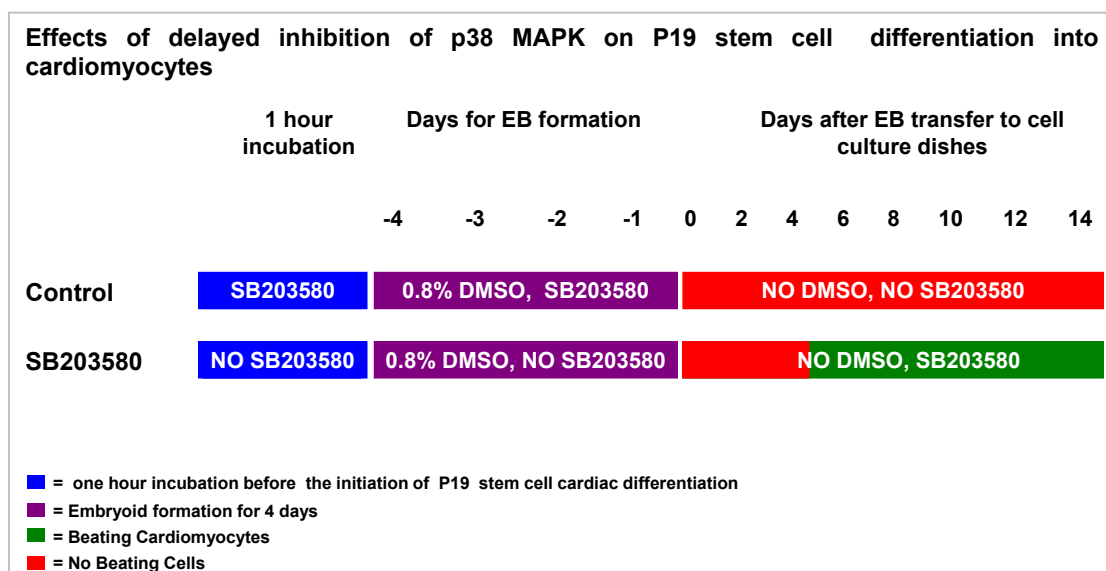
Figure 6.15; *A schematic diagram of the effects of delayed inhibition of p38 MAPK on P19 stem cell differentiation into cardiomyocytes.* In P19 stem treated with SB203580 (10µM) four days after the initiation of DMSO-induced cardiac differentiation, SB2093580 failed to block the cell differentiation into beating cardiomyocytes.
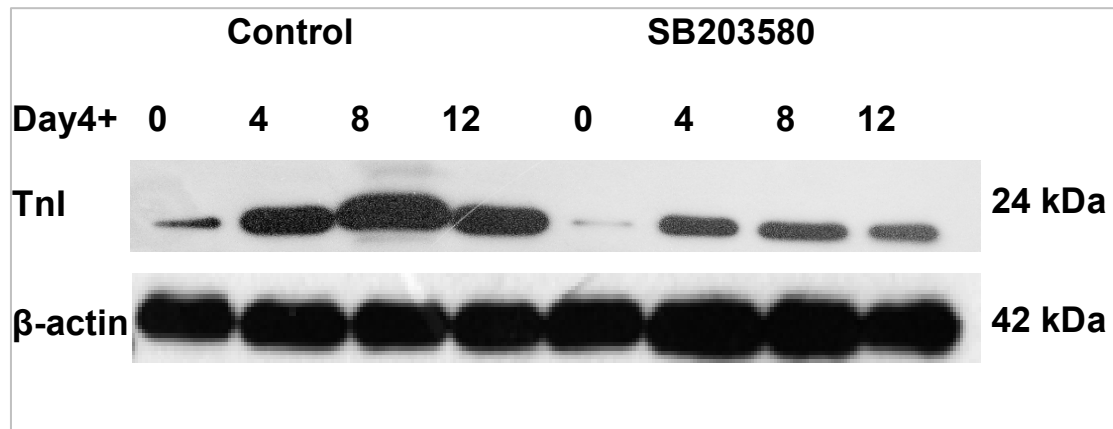
Figure 6.16: Effects of p38 MAPK inhibition on P19 stem cell differentiation into cardiomyocytes: troponin I expression. Control P19 stem cells were cultured in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100 units/ml penicillin and 100µg/ml streptomycin as described in section 6.3.2. The above differentiation medium was further supplemented with 10µM of SB203580 for the treated cells. SB203580 treatment markedly inhibited P19 Stem cells differentiation into cardiomyocytes as determined by the expression of cardiac restricted troponin I (TnI). While beating cardiomyocytes were observed in the some dishes of the control cells, beating cardiomyocytes were not seen in any of the SB203580 treated P19 stem cells. The blot is representative of three independent differentiation experiments.

6.4 DISCUSSION

The MAPKs are among the signalling pathways that have been well studied. The MAPKs are involved in the control of gene expression (Wu *et al.*, 2004; Lawrence *et al.*, 2007; Mochizuki *et al.*, 2007), proliferation (Souza *et al.*, 2004; Erlich *et al.*, 2007; Jia *et al.*, 2008), migration (Goetze *et al.*, 1999; Rousseau *et al.*, 2006; Jia *et al.*, 2008) and cell survival or apoptosis (Chuang *et al.*, 2000; Pan *et al.*, 2002; Chen *et al.*, 2008; Cho *et al.*, 2008).

The activation of the p38 MAPK signalling has been implicated in the differentiation of various cell types (Gallea *et al.*, 2001; Tuli *et al.*, 2003; Chang *et al.*, 2007). including stem cell differentiation into cardiomyocytes (Monzen *et al.*, 1999; Davidson and Morange, 2000; Aouadi *et al.*, 2006). The precise role of these kinases and how they relate to the complex network of signaling events that lead to differentiation is still not clearly understood.

One of the key MAPKs, the p38 MAPK, is downstream of PI3K (Salh *et al.*, 2002). However, the p38 MAPK pathways can also be activated through PI3K-independent mechanisms. As our data have already implicated PI3K in stem cell differentiation into cardiomyocyte (see Chapter 7) we therefore extended our studies to determine whether the signalling events that control H9c2 and/or P19 stem differentiation cells into cardiomyocytes are linked to the activation of the p38 MAPK. A specific inhibitor of the p38 MAPK signalling pathway, SB203580 (Cuenda *et al.*, 1995; Young *et al.*, 1997; Gum *et al.*, 1998) was used in this study. The 10µM of SB203580 used in this study has been reported in other studies (Nick *et al.*, 1999; Barancik *et al.*, 2001;

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Kwiecinska *et al.*, 2005). Our data also showed that this concentration is not toxic to H9c2 cells. The effect of SB203580 on stem cell differentiation into cardiomyocytes reported here may therefore be due to the specific pharmacological inhibition of the p38 MAPK signalling pathway by this compound.

Our data suggest that p38 MAPK signalling may have a role in the differentiation of P19 stem cells and H9c2 cells into cardiomyocytes. SB203580 (10µM) completely blocked the differentiation of P19 stem cells into beating cardiomyocytes. SB203580 also markedly reduced the expression of cardiac restricted troponin I in P19 stem cells. Similarly, during the differentiation of H9c2 cells into cardiomyocytes, SB203580 delayed the expression of MLC-1v. The expression of MLC-1v subsequently recovered as time progressed during the 14 day incubation period. Perhaps of interest is the fact that SB203580 did not significantly alter the morphological changes (i.e. cell elongation, cell fusion and formation of myotubes) that accompany the initiation of H9c2 differentiation into cardiomyocytes. This may however be due to the fact that the morphological changes generally become more apparent at the later stages of differentiation by which time the cells have started to recover from the inhibition caused by SB203580. Whether the lack of a significant effect, by SB203580, on the morphological changes that accompanied H9c2 differentiation into cardiomyocytes, is due to p38 MAPK having a lesser role in these changes (than in the regulation of MLC-1v expression) was not investigated. This may need further investigation in future studies. As our data stand, it would appear that the role of the p38 MAPK in

stem cell differentiation is more prominent in P19 when compared to the H9c2 cells. A role for the p38 MAPKs in stem cell differentiation indicated in our studies would be consistent with reports in other systems (Gallea *et al.*, 2001; Tuli *et al.*, 2003; Chang *et al.*, 2007); (Monzen *et al.*, 1999; Davidson and Morange, 2000; Aouadi *et al.*, 2006).

Our data have further suggested that the p38 MAPK mediated mechanisms or events that control P19 stem cells differentiation into cardiomyocytes are switched on very early following DMSO treatment. Since SB203580 (10µM) was able to block P19 stem differentiation into beating cardiomyocytes only when added at the time of initiation of P19 cell differentiation (i.e. at the EB formation stage). Addition of SB203580 to P19 cells at the EB formation stage completely blocked cell differentiation into beating cardiomyocytes. However when exposed to cells four day after DMSO treatment, SB203580 had no effect on the ability of the cells to differentiate into beating cardiomyocytes. This strongly suggests that activation of the p38 MAPK pathway occurs very early in the differentiation process. The data also suggest that the activation of p38 MAPK pathway may be transient since late application of SB2003580 failed to inhibit the differentiation process. Whether p38 activation is indeed transient or whether it is sustained but become irreverent after the initiation of the differentiation process remains to be determined.

Bone morphogenetic proteins are well known extracellular signalling molecules that are involved in stem cell differentiation into cardiomyocytes (Zhang and Bradley, 1996; Schultheiss *et al.*, 1997; Schlange *et al.*, 2000).

The core signalling mechanism of BMP is through the activation of SMAD proteins (Wrana *et al.*, 1994; Monzen *et al.*, 2001). However BMP signalling can also be channelled through MAPK via SMAD-independent mechanisms (Gallea *et al.*, 2001). Also GATA-4, a key cardiac transcription factor may be a direct downstream target of several kinases including the MAPK (Liang *et al.*, 2001) as there are many potential phosphorylation sites within this transcription factor. It has been reported that GATA-4, is a nuclear mediator of RhoA signalling. The convergence of RhoA on GATA signalling was suggested by the finding that RhoA potentiates the activity of GATA-4 via a p38 MAPK-dependent pathway that phosphorylates GATA-4 activation domains. It was also found that GATA binding sites mediate RhoA activation of target cardiac promoters. Thus, it is possible that p38 MAPK may regulate stem cell differentiation through its activation by upstream signaling events associated with the Rho pathway and subsequent downstream regulation of the transcriptional activities of GATA-4 (Charron *et al.*, 2001). Whether this is part of a sequence of events that also requires PKC and/or PI3K in our cell systems remain to be determined.

Besides showing that p38 MAPK is required for cardiomyocyte differentiation, the project has gone further to investigate if the p38 MAPK affects cardiac differentiation in conjunction with other leading pathways that were investigated in this project. This is explained in more detail in Chapter 7 where the 'cross-talk' studies are discussed.

6.5 Summary

The differentiation of H9c2 cells into cardiomyocytes was blocked by the inhibition of p38 MAPK signalling using SB203580 (10µM). However SB203580 had little or no effect on the differentiation associated morphological changes in H9c2 differentiating into cardiomyocytes. In both control cell and SB203580-treated cells, morphological changes were visible. In contrast to the apparent lack of inhibition of morphological changes in SB203580-treated H9c2 cells, the expression of MLC-1v was decreased in these cells.

In contrast H9c2 cells, SB203580 markedly inhibited the expression of cardiac restricted protein troponin (TnI) and also blocked the cell differentiation into beating cardiomyocytes. The small expression of TnI seen in control P19 stem cells, at the time of EB transfer to cell culture plates, increased upto day 8. In comparison to control cells, TnI levels in SB203580-treated P19 stem cells were reduced. The inhibited or low levels of TnI detected in SB203580-treated cells were in agreement with the lack of appearance of beating cardiomyocytes. It was also observed that SB203580 blocked P19 stem cell differentiation into beating cardiomyocytes, when added to the cells during the EB formation stage. In contrast, SB203580 failed to block the differentiation of P19 stem cells into beating cardiomyocytes, when the cells were treated, with this compound, four days after the initiation of DMSO-induced cardiac differentiation.

In conclusion, our data therefore suggest that the activation of p38 MAPK signalling pathway may be an essential signalling event during the differentiation of stem cells into cardiomyocytes. The data also suggest that the p38 MAPK exerts its effects at the early stages of P19 stem cell differentiation into cardiomyocytes.

CHAPTER 7.0

ROLE OF PHOSPHOINOSITIDE 3-KINASE IN H9c2

AND P19 STEM CELL DIFFERENTIATION INTO

CARDIOMYOCYTES

7.1 INTRODUCTION

7.1.1 Phosphoinositide 3-kinases

The phosphoinositide 3-kinase (PI3K) enzymes are present in all cell types and have been shown to be crucial regulators for many cell functions including cell cycle control (Rosenzweig *et al.*, 1997), proliferation (Erlich *et al.*, 2007; Nakao *et al.*, 2007; Ramos-Nino *et al.*, 2008; Rosseland *et al.*, 2008), survival (Li *et al.*, 2001; Hambardzumyan *et al.*, 2008; Lim *et al.*, 2008) and migration (Nakao *et al.*, 2007).

Class I PI3K (IA and IB) are the most extensively studied isoforms. Class 1A PI3Ks are heterodimers of regulatory (p85) and a catalytic (p110) subunits. Class II PI3Ks are monomeric proteins that lack adapter subunits. The preferential substrates of class II PI3Ks are phosphatidylinositol and phosphatidylinositol phosphate. Class III PI3Ks, like class I PI3Ks, are also heterodimeric enzymes consisting of regulatory and catalytic subunits.

The catalytic subunits of class I PI3K are activated by signals originating from upstream receptor tyrosine kinases (for Class IA PI3K) or G-protein-coupled receptors (for Class IB PI3K).

Phosphoinositide 3-kinase catalyses the transfer of the gamma-phosphate from ATP to the 3'-OH position of the inositol ring in the membrane bound phosphatidylinositol (4, 5)-bisphosphate (PIP_2) to phosphatidylinositol (3, 4,

5)-trisphosphate (PIP$_3$). The reverse reaction to convert PIP$_3$ back to PIP$_2$ is catalysed by PTEN (phosphatase and tensin homolog deleted on chromosome 10), a phosphatase (Maehama and Dixon, 1998).

Downstream events following the formation of PIP$_3$ lead to the activation of Akt or protein kinase B (PKB). Akt is a serine/threonine protein kinase (Jones *et al.*, 1991) and a critical component of the PI3K signalling. For example, PI3K transcriptional regulation of genes (e.g. induction of c-myc and Bcl-2) (Ahmed *et al.*, 1997) and stimulation of protein synthesis by controlling the translation initiation factor eIF-4E via phosphorylation of 4E-BP1 (Gingras *et al.*, 1998) are mediated via Akt. Akt-dependent regulation of growth factor and anti-apoptotic signals, by PI3K, have been shown in a variety of cell types including BAF/3 cells (Ahmed *et al.*, 1997), cerebellar neurons (Dudek *et al.*, 1997), H19-7 cells (Eves *et al.*, 1998) and human embryonic kidney 293-EBNA cells (Kauffmann-Zeh *et al.*, 1997).

The PIP$_3$ produced by the PI3K reaction activates the phosphatidylinositol-dependent protein kinases (PDK-1 and PDK-2) by recruiting them to the plasma membrane. PIP$_3$ also binds to the pleckstrin homology (PH) domain of Akt and anchors it to the plasma membrane (Klippel *et al.*, 1997) where Akt is activated. The activation of Akt is regulated through two distinct phosphorylation events which have been reported to occur in a sequential manner. The PDK1-mediated phosphorylation of Akt at threonine 308 (Thr308) in the domain activation loop precedes the PDK2-mediated phosphorylation at serine 473 (Ser473) (Alessi *et al.*, 1996; Alessi *et al.*,

1997a; Alessi *et al.*, 1997b). The phosphorylation of Akt at Thr308 by PDK-1 is believed to render the Ser473 site at Akt C-terminal tail accessible to PDK-2. Akt is fully activated only if it is phosphorylated at both Thr308 and ser473 sites (Alessi *et al.*, 1997a). Other downstream signalling molecules with PH domains that are target by PIP3 include protein kinase C and MAPK signalling pathway.

In addition to Akt activation, PDK1 is also a converging point for a number of different pathways including the phosphorylation conventional PKCs (Dutil *et al.*, 1998), novel PKCs (Le Good *et al.*, 1998; Cenni *et al.*, 2002) and atypical PKCs (Chou *et al.*, 1998; Le Good *et al.*, 1998; Dong *et al.*, 1999), p70 S6 kinase (Pullen *et al.*, 1998; Flynn *et al.*, 2000; Kuemmerle, 2003) and glucocorticoid-regulated kinase (SGK) (Kobayashi and Cohen, 1999; Kobayashi *et al.*, 1999).

PI3K signalling is terminated by the degradation of PIP3 by at least two different phosphatases known as SHIP (Src homology 2-containing inositol 5-phosphatase) and PTEN (tensin homolog deleted on chromosome 10 protein (PTEN) (Koyasu, 2003). PTEN and SHIP remove the 3-phosphate of PIP3 to re-generate $PI(4,5)P_2$. This means that PTEN activity blocks all the main downstream effects of PI3K. In contrast, SHIP removes the 5-phosphate from the inositol ring of PIP_3 to generate $PI(3,4)P_2$. This means that dephosphorylation of PIP3 by SHIP block some of the main downstream effects of PI3K.

7.1.2 PI3K and stem cell differentiation into cardiomyocytes

The PI3K pathway is one of the well studied signalling pathways whose signalling mechanisms are well established. PI3K signalling has been implicated in the differentiation of stem cells into cardiomyocytes (Klinz *et al.*, 1999; Sauer *et al.*, 2000; Naito *et al.*, 2003). Cardiogenesis or stem cell differentiation into cardiomyocytes is a very complex process. It is therefore very unlikely that this process will be controlled by a single signalling pathway. Interaction or cross-talk between pathways is more likely to be key mode of regulation of stem cells differentiation into cardiomyocytes. Our studies therefore take the approach of not only examining the role of PI3K but also investigated if there is any cross-talk between PI3K and PKC and also between PI3K and p38 MAPK during cardiac cardiomyocyte differentiation. Therefore the aim of this study is to investigate the role of the PI3K pathway and possible cross-talk between the PI3K and other well established pathways (i.e. PKC and p38 MAPK) that have been shown to have role to play in cardiac differentiation. To achieve the aims a specific PI3K inhibitor LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (IC_{50}=1.4µM) was used for this study (Vlahos *et al.*, 1994). LY294002 is cell-permeable, potent and specific PI3K inhibitor that acts on the enzyme ATP-binding site (Vlahos *et al.*, 1994). LY294002 (50µM) completely inhibited PI3K but failed to inhibit other kinases including serine/ threonine kinases (e.g. PKC, PKA, MAP kinase and S6 kinase), protein tyrosine kinases (e.g. EGF receptor tyrosine kinase and c-src kinase), lipid kinases (e.g. PI 4-kinase and diacylglycerol kinase) and rabbit kidney ATPase (Vlahos *et al.*, 1994).

7.2 MATERIALS AND METHODS

7.2.1 Culture and differentiation of H9c2 Cells

The routine culture of H9c2 cells was carried out as described in section 2.3.1 (Chapter 2). H9c2 cells were trypsinised and the appropriate cell density for the differentiation experiments was calculated as described in section 2.6 (Chapter 2). H9c2 cells (2×10^3 cells/cm^2) were seeded in p60 dishes or 6-well plates in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin), placed in a tissue culture incubator at 37°C, 95% air and 5% CO_2 and allowed to become 60-70% confluent. The differentiation of H9c2 cells into cardiomyocytes was carried out in 1% serum as described in section 2.8.1 (Chapter 2). The role of PI3K signalling pathway in the differentiation of H9c2 cells into cardiomyocytes was investigated. The effects of the inhibition of the PI3K signalling pathway were investigated using 20µM of LY294002 as described in section 2.8.2 (Chapter 2). A LY294002 stock (10mM solution) was prepared by dissolving 1mg of LY294002 in 325.3µl of DMSO. The stock solution was distributed into 10 or 20µl aliquots and stored in the freezer at -20°C. The aliquots were protected from light during storage and use. The differentiation medium was used to dilute (1:500) the frozen stock LY294002 solutions (10mM) to the LY294002 concentration (20µM) used in the differentiation experiments. The preparation of stock LY294002 concentration was carried out as explained in appendix VIII. More details about LY294002 are located in appendix VI (b).

7.2.2 Cross-talk studies

To examine possible PI3K/PKC and PI3K/p38 MAPK cross-talk, cross-talk studies were designed and carried out. In order to carried out the cross-talk investigation, the following procedures were carried out as described in the Chapter 2 under the sections indicated in parenthesis: time course of Akt phosphorylation at serine 473 (2.10.1) and the effects of drugs (inhibitors) on PI3K activation as determined by Akt phosphorylation at serine 473 (Section 2.10.2).

7.2.3 Culture and differentiation of P19 stem cells

The routine culture of P19 stem cells was carried out as described in section 2.3.2 (Chapter 2). To initiate differentiation, P19 stem cells (1×10^6 cells) were seeded in P100 Petri (i.e. microbiological) dishes in the differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 μg/ml streptomycin and 0.8% DMSO) and the differentiation of P19 stem cells into beating cardiomyocytes carried out as described in section 2.9.1 (Chapter 2). The role of PI3K signalling pathway in the DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes was investigated. The effect of the inhibition of PI3K signalling pathway by 20μM of LY294002 on the DMSO-induced P19 stem cell differentiation into beating cardiomyocytes was investigated as described in section 2.9.2 (Chapter 2). Furthermore the effects of delayed inhibition of PI3K on P19 stem cell differentiation into cardiomyocytes were also investigated as described in section 2.9.3 (Chapter 2).

7.2.4 Cell viability assay

To examine the effects of the PI3K inhibition on H9c2 cell viability, the MTT assay was carried out as described in section 2.7 (Chapter 2).

7.2.5 Western blot analysis

In order to perform the western blot analysis, the following procedures were carried out as described in Chapter 2 under the sections indicated in parenthesis: cell lysate generation (section 2.11), preparation of BSA standards (section 2.12.1) and BCA assay (section 2.12.2). The western blotting was carried out as described in section 2.13.

7.2.6 Data analysis

Data obtained from independent experiments were used for statistical analysis as described in section 2.14 (Chapter 2).

7.3 RESULTS

7.3.1 Effects of the PI3K Inhibition on H9c2 Cell Differentiation into Cardiomyocytes

The differentiation of H9c2 cells in culture medium supplemented with the PI3K inhibitor, LY294002, was completely blocked throughout the differentiation period. Control H9c2 cells differentiated into cardiomyocytes as determined by the usual morphological changes of cell elongation, fusion and myotube formation. In contrast LY294002 treated H9c2 cells maintained their normal single cell and spindle shaped morphology (Figure 7.10). By Day 4, the usual morphological changes that accompany H9c2 cell differentiation were visible in the control cells. In contrast, these changes only began at day 8 in LY294002 treated H9c2 cells and were never as extensive as those seen in the control cells by day 12.

In agreement with the lack of morphological changes observed in LY294002 treated H9c2 cells; the expression of MLC-1v protein was blocked at day 4 ($p<0.01$), day 8 ($p< 0.05$) and at day 12 ($p>0.05$) (Figure 7.11). The MLC-1v expression never reached the same levels as in control cells. MLC-1v levels in both control and LY294002 treated H9c2 cells were in agreement with the degree of differentiation or morphological changes observed in these cells. MLC-1v expression in LY294002 treated H9c2 cells was less than 25% of control by day 4 and the by day 8 the expression was still less than 50 % of the levels seen in the control cells.
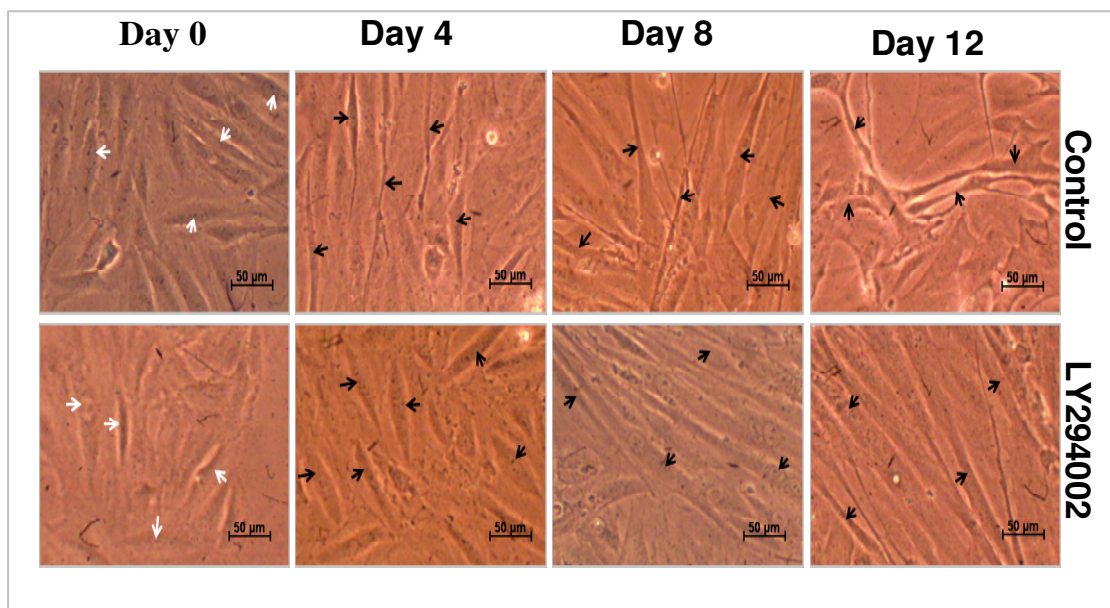
Figure 7.10: Effects of the PI3K inhibition on H9c2 cell differentiation into cardiomyocytes: morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100units/ml penicillin/100µg/ml streptomycin. The above differentiation medium was further supplemented with 20µM of LY294002 for the treated cells. Morphologically, LY294002 treatment significantly inhibited cell alignment, elongation and myotube formation. LY294002 treated H9c2 cells largely maintained their normal single cell and spindle shaped morphology which is in contrast to control H9c2 cells which showed the usual morphological changes. The photographs were taken under 200X magnification and are representative of at least 3 differentiation experiments.
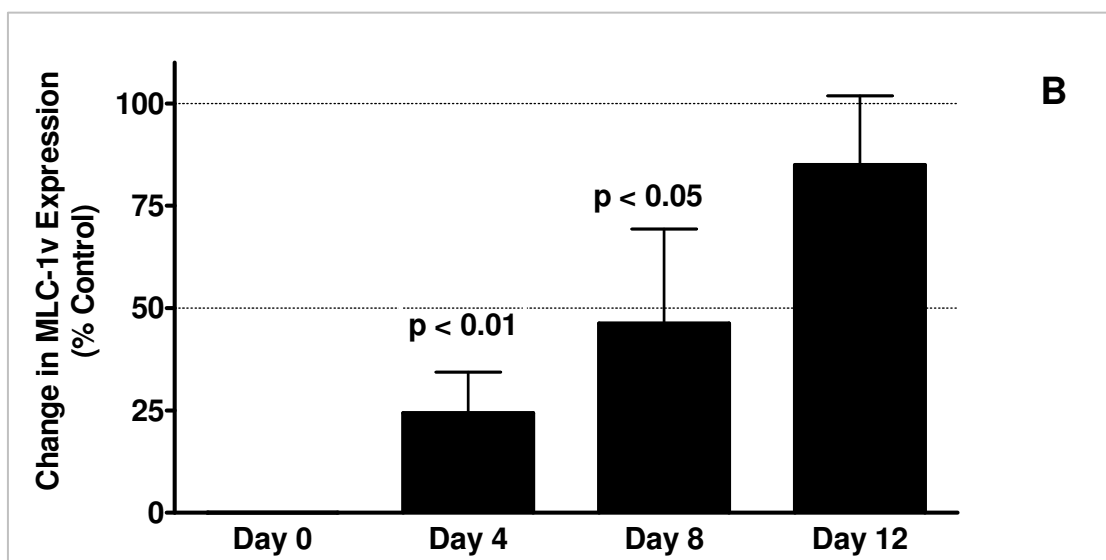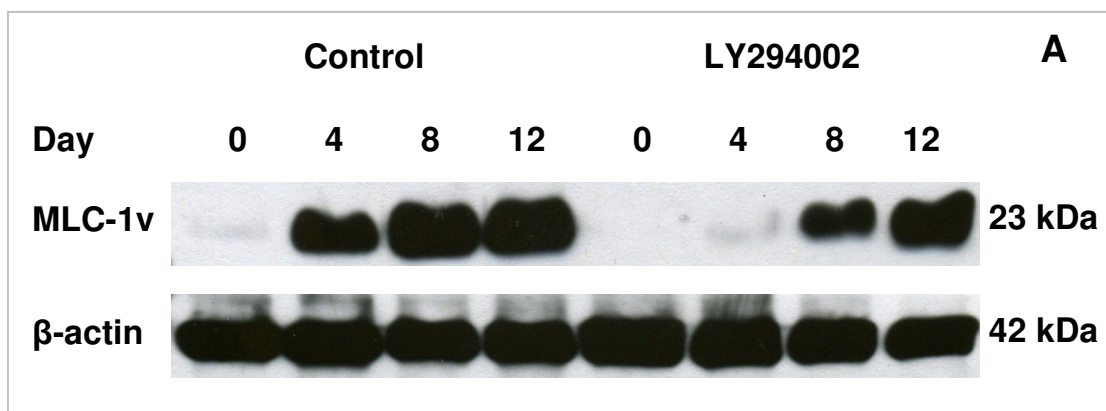
Figure 7.11: Effects of the PI3K inhibition on H9c2 cell differentiation into cardiomyocytes: cardiac ventricular myosin light 1(MLC-1v) expression. In agreement with the lack of morphological changes observed in LY294002 treated H9c2 cells, western blot analysis of MLC-1v revealed that LY294002 treatment significantly inhibited this protein expression up to day 8, in comparison to the control expression (A). Marginal expression was seen after day 8. The blot is representative of at least 3 differentiation experiments (panel A). The densitometric data (mean ± SD), from three independent differentiation experiments, show the time dependent changes in MLC-1v expression in LY294002 treated H9c2 cells (panel B)

7.3.2 Effects of LY294002 (20µM) on the viability of H9c2 cells

The widely reported concentration of 20µM for the PI3K inhibitor LY294002 was selected for use in the experiments designed to investigate the effects of PI3K inhibition on H9c2 differentiation into cardiomyocytes. The cell viability test (MTT assay) was carried out to determine if this concentration was toxic to the cells over the period that the cells were left in this drug. LY294002 at the concentration of 20µM was not toxic to the cells. There was no statistical difference between the viability of control cells and the cells cultured in LY294002 ($p > 0.05$) (Figure 7.12). The average viability was about 85% of the viability determined in the control cells. By 48 hours the viability was about 77% of the viability determined in the control cells.
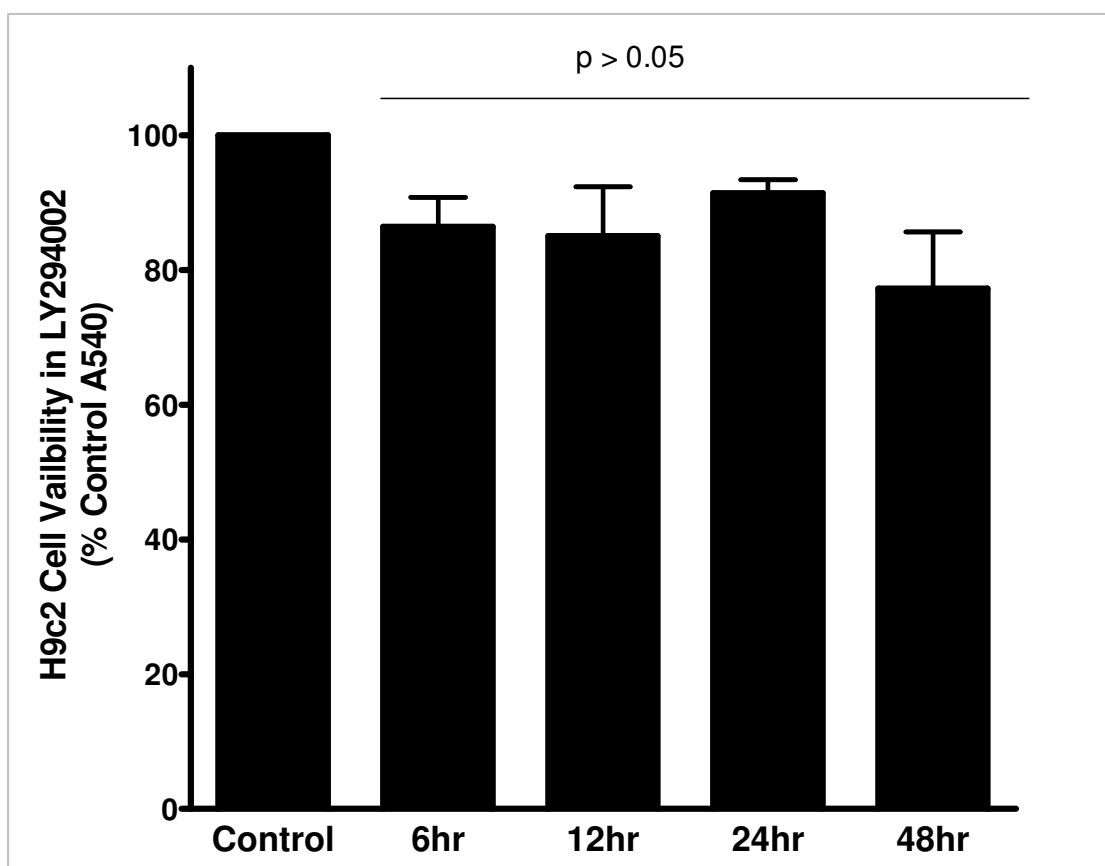
Figure 7.12: The viability of H9c2 cells in the presence of the PI3K inhibitor, LY294002. Control H9c2 cells were seeded in 6-well plates in differentiation medium consisting of DMEM supplemented with 1% FBS and 100units/ml penicillin/100µg/ml streptomycin. The above differentiation medium was further supplemented with 20µM of LY294002 for the treated cells as described in the method section above (Section 7.2.4). The MTT assay was carried out at the end of the treatment. Cell viability of LY29402 treated cells at each time point was calculated as a percentage of the cell viability in the control cells at that time point. There was no statistical difference between the viability of control H9c2 cells and LY294002 treated H9c2 cells (p > 0.05). The data presented in the graph are the mean ± SD from three independent experiments.

7.3.3 Effects of PI3K inhibition on P19 stem cell differentiation into beating cardiomyocytes

To investigate the effects of PI3K inhibition on the differentiation of P19 stem cells into cardiomyocytes, LY294002 was added to cell culture of P19 stem cells during DMSO induced differentiation. Control P19 stem cells were treated with DMSO only while the differentiation medium of the LY294002-treated P19 cells was supplemented with 20µM of LY294002 at the EB formation stage. Control and LY2940002 treated EBs followed their normal pattern of growth as already described in the establishment of the P19 cardiomyocyte differentiation model (Chapter 3). The EBs seen on day 1 grew in size and number over time. Transfer of EBs from Petri dishes to standard tissue culture dishes resulted in cell adhesion and the subsequent growth pattern into a monolayer. This was then followed by cell migration and proliferation. By day four, both treated and control cells were confluent. By day 7, the first beating clusters of cardiomyocytes appeared in the DMSO treated but not in the cells exposed to LY294002 (Figure 7.14). No beating cardiomyocytes were observed in any of the dishes containing LY294002 treated P19 cells over the whole course of the experiment (Figure 7.13 & Figure 7.14). When present, the number of beating clusters of cardiomyocytes usually continues to increase up to day 14. The patterns of cardiomyocyte beating clusters (including number and beating rates) from P19 stem cells treated with DMSO in the absence of LY294002 (i.e. no inhibition of PI3K signalling pathway) were as already described in Chapter 3 (see Figure 7.14).

In agreement with the lack of appearance of beating cardiomyocytes LY294002 treated P19 stem cell, the expression of cardiac restricted protein troponin I was inhibited (Figure 7.15). Troponin levels in the LY294002 treated P19 stem cells were lower than those in the control cells at all time points between day 4 and day 12.

Effects of PI3K inhibition on P19 stem cell differentiation into cardiomyocytes

■ = One hour incubation before the initiation of P19 stem cell cardiac differentiation
■ = Embryoid formation for 4 days
■ = Beating Cardiomyocytes
■ = No Beating Cells
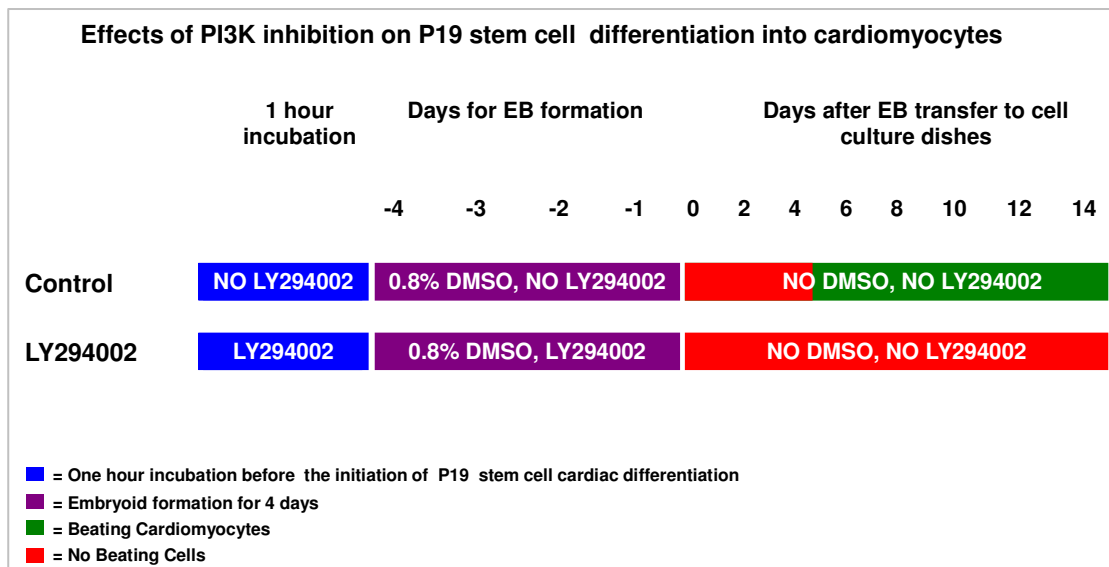
Figure 7.13: A schematic diagram of the effects of the PI3K inhibition on DMSO-induced differentiation of P19 stem cells into cardiomyocytes. PI3K inhibition with LY294002 (20µM) blocked the differentiation of P19 stem cells into beating cardiomyocytes, when the inhibitors was added to the cells during the EB formation stage in the absence of LY294002 beating cardiomycytes were seen from day 6 to day 14 after the EB were transferred to cell culture dishes.
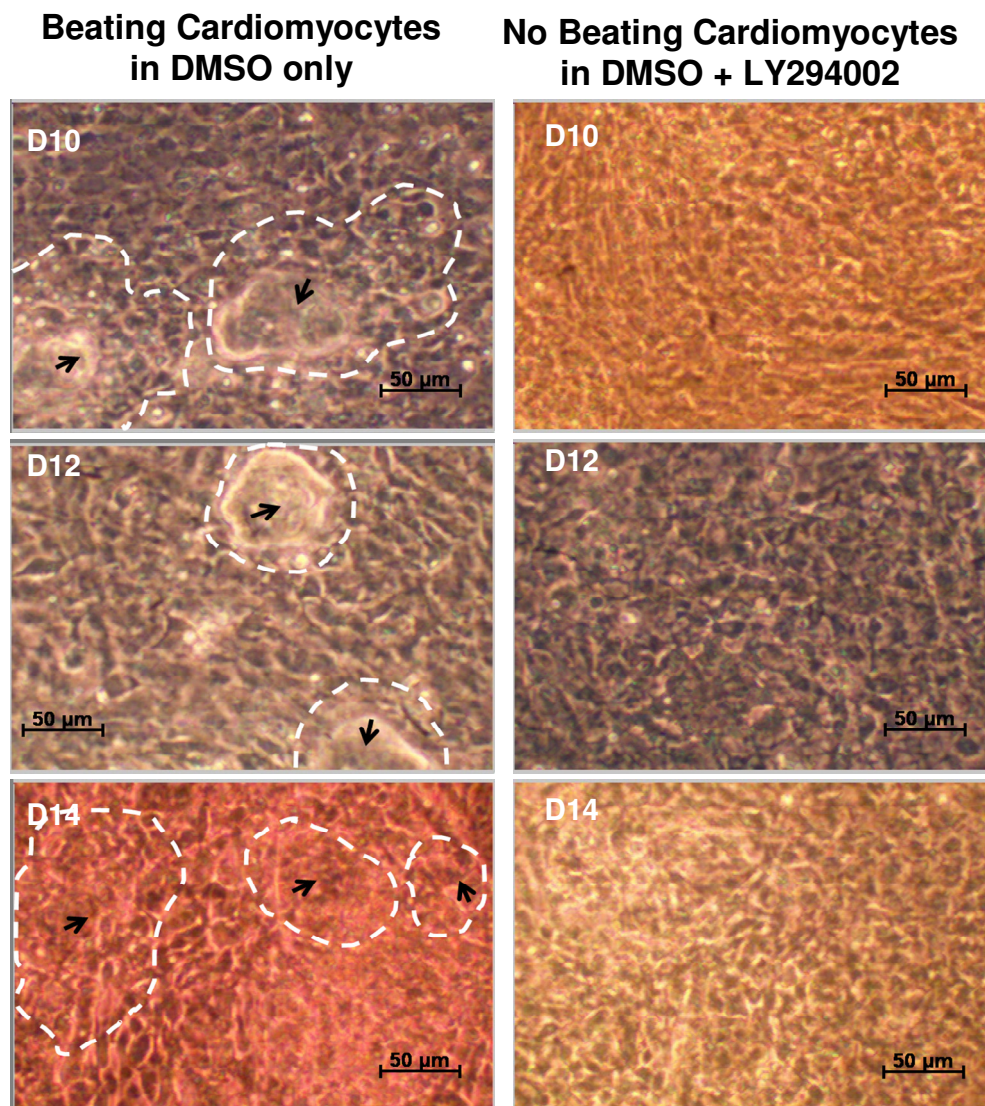
Figure 7.14: Effects of the PI3K inhibition on DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes. *Control P19 stem cell embryoid bodies were formed in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 μg/ml streptomycin and 0.8% DMSO. The above differentiation medium was further supplemented with 20μM of LY294002 for the treated P19 stem cells during the EB formation stage. DMSO caused the differentiation of P19 stem cells into beating cardiomyocytes. This DMSO-induced cardiac differentiation of the P19 stem cells was inhibited by LY294002. The areas of beating cardiomyocytes are shown within the closed figures drawn with white dotted lines. The beating focus of each beating area is shown by a black arrow. Photographs and movie clips of beating cells were taken under 200X magnification on the days indicated and are representative of microscope fields (for their respective days) from at least 3 differentiation experiments.*
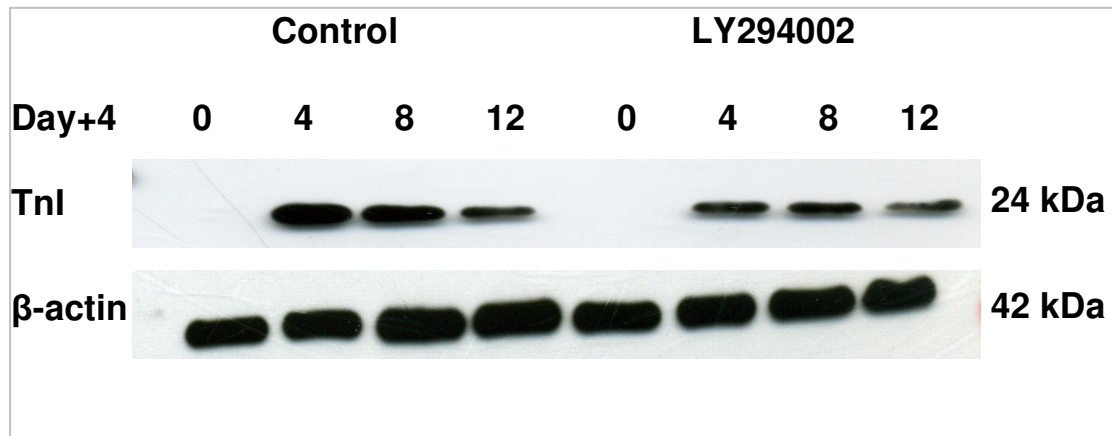
Figure 7.15: Effects of the PI3K inhibition on P19 stem cell differentiation into cardiomyocytes: cardiac troponin I expression. Control P19 stem cells were cultured in differentiation medium consisting of α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100μg/ml streptomycin. The above differentiation medium was further supplemented with 20μM of LY294002 for the treated cells. In agreement with the lack of appearance of beating cardiomyocytes in LY294002-treated P19 stem cell, the expression of the cardiac restricted protein, troponin I was inhibited. Troponin levels in the LY294002-treated P19 stem cells were lower than those in the control cells at all time points between day 4 and day 12. The blot is representative of three differentiation experiments.

7.3.4 Effects of delayed inhibition of PI3K   on P19 stem cell differentiation into beating Cardiomyocytes

To determine the stage at which PI3K may regulate the differentiation of P19 stem cells into cardiomyocytes, LY29402 was added to cell culture four days after exposure to DMSO. Control P19 stem cells were treated with DMSO and LY294002 at the EB formation stage and for comparison, parallel dishes of P19 cells (_delay-inhibition P19 cells_) were treated with DMSO only at EB formation stage and then only treated with LY294002 after the EB were transferred to culture dishes (see section 2.9.3). Four days later, after the transfer of EBs to cell normal culture dishes, the EBs in both groups of cells underwent normal adhesion, proliferation and migration were confluent by day 4.   No beating cardiomyocytes were observed in both control P19 cell and _delay-inhibition P19 cells_ before day 6. By day 6 or 7, the first beating cells appeared in the _delay-inhibition P19 cells_ (Figure 7.17). As expected, DMSO failed to induce P19 stem cell differentiation into beating cardiomyocytes, when LY294002 was added at the EB formation stage. However when DMSO was allowed to initiate P19 stem cell differentiation before 20µM of LY294002 was added to the cells, LY294002 failed to block the DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes (Figure 7.16 & Figure 7.17). The patterns of cardiomyocyte beating clusters from P19 cells, in which DMSO was allowed to initiate their differentiation before the inhibition of the PI3K signalling pathway, were as already described in Chapter 3 (see Figure 7.17).
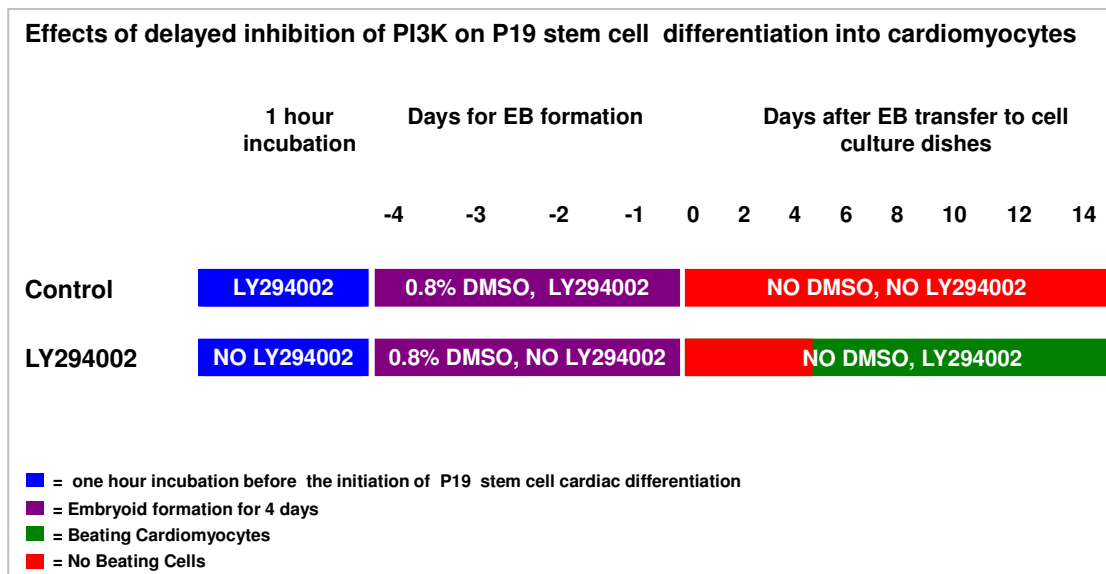
Figure 16: A schematic diagram of the effects of delayed inhibition of PI3K on P19 stem cell differentiation into beating Cardiomyocytes. In P19 stem treated with LY294002 (20µM) four days after the initiation of DMSO-induced cardiac differentiation, LY294002 failed to block the cell differentiation into beating cardiomyocytes

**No Beating Cardiomyocytes
in DMSO + LY294002**

**Beating Cardiomyocytes
LY294002 after DMSO**
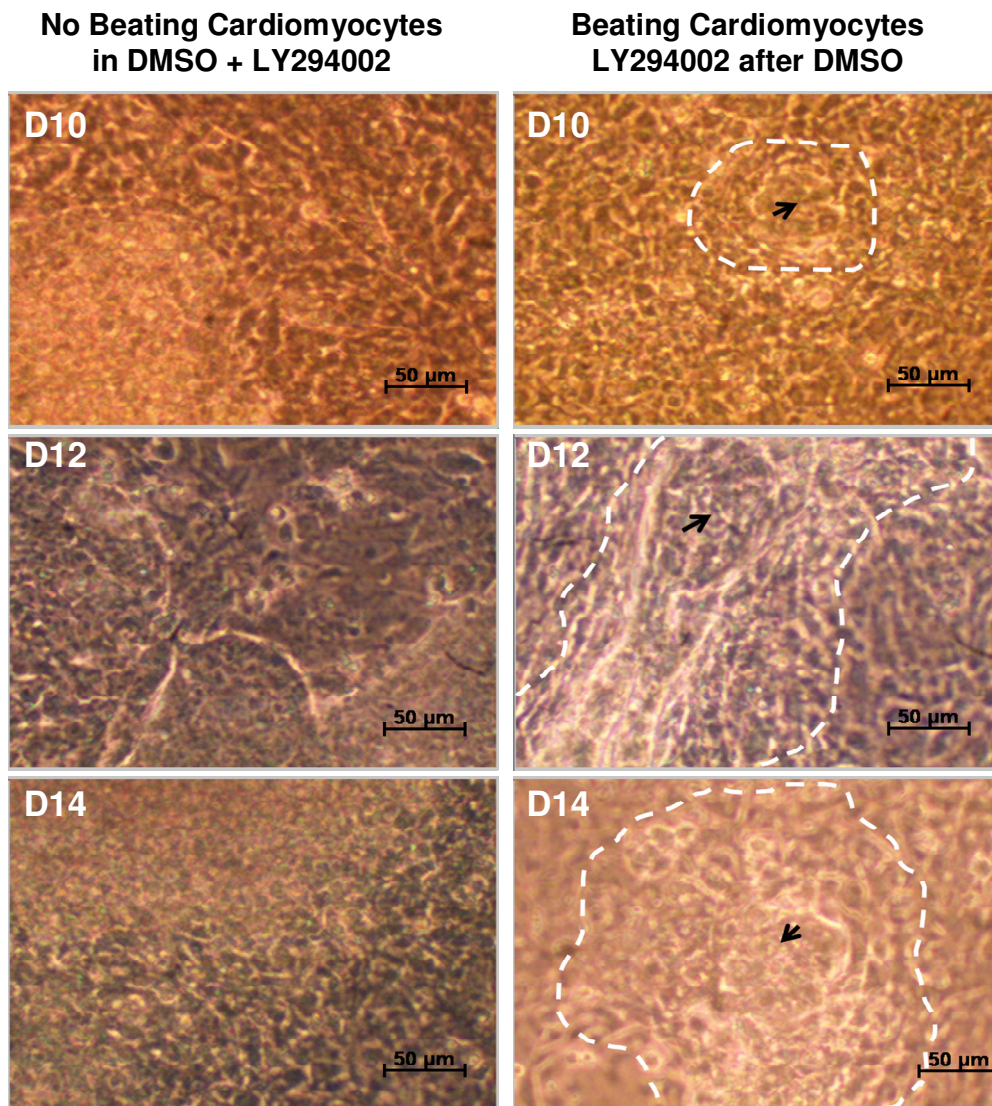
Figure 7.17: Effects of delayed inhibition of PI3K on P19 stem cell differentiation into beating cardiomyocytes. Control EBs were formed in differentiation medium (i.e. α-MEM supplemented with 10% FBS, 100 units/ml penicillin/100 µg/ml streptomycin, 0.8% DMSO and 20µM of LY294002). For the delay inhibition of PI3K, EBs were initially formed in the above differentiation medium but in the absence of LY294002. DMSO failed to induce P19 stem cell differentiation into beating cardiomyocytes when differentiation was initiated in the presence of LY294002. However when DMSO was allowed to initiate P19 stem cell differentiation before the addition of LY294002, the cell differentiated into beating cardiomyocytes. The areas of beating cardiomyocytes are shown within the closed figures drawn with white dotted lines. The beating focus of each beating area is shown by a black arrow. Photographs and movie clips of beating cells were taken under 200X magnification on the days indicated and are representative of microscope fields (for their respective days) from at least 3 differentiation experiments.

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7.4 CROSS-TALK STUDIES

7.4.1 Time course of Akt phosphorylation following the initiation of H9c2 cell differentiation into cardiomyocytes by 1% serum

Undifferentiating H9c2 cells maintained a baseline level of phosphorylated Akt

The initiation of H9c2 cell differentiation into cardiomyocytes induced a more

than 2-fold rapid increase in Akt phosphorylation at residue serine 473 within

5 minutes. The increased phosphorylated Akt levels were sustained for 6

hours before declining . The levels of phosphorylated Akt gradually fell back

to baseline levels after 24 hours and by 48-72 hours, phosphorylated Akt level

fell below the baseline values (Figure 7.18)
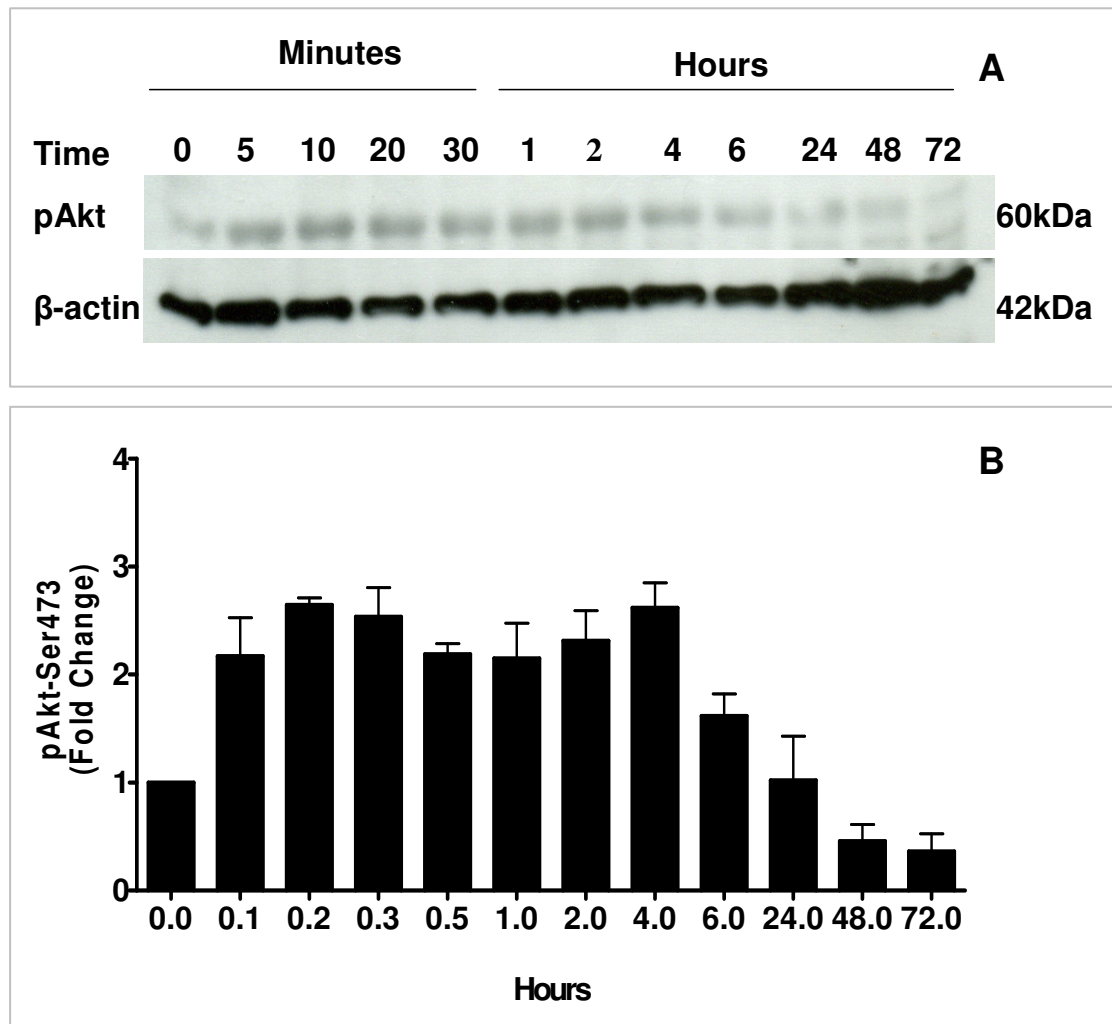
Figure 7.18: Time course of Akt phosphorylation following the initiation of H9c2 cell differentiation into cardiomyocytes by 1% serum. *Control* H9c2 cells were cultured in serum-free medium consisting of DMEM supplemented with 100units/ml penicillin/100µg/ml streptomycin in the absence of FBS. Serum-treated (1% FBS) control H9c2 cells were cultured in above supplemented with 1% FBS. A more than 2-fold increase in phosphorylated was induced by 1% FBS treatment and this was sustained for 6 hours before declining. Fold increase was calculated as the ratio of induced phosphorylated Akt level to baseline phosphorylated Akt. The blot is representative of at least 3 individual experiments (panel A). The densitometric data (mean ± SD), from three independent experiments, show the time dependent fold change in phosphorylated Akt in treated H9c2 cells (panel B).

7.4.2 Effects of PI3K inhibition on Akt phosphorylation at serine 473 in H9c2 cells differentiating into cardiomyocytes

To examine the effects of inhibiting PI3K signalling on the activation of this pathway, H9c2 cells *(LY294002-treated H9c2 cells)* were treated with 20µM of LY294002. For parallel comparison, cells exposed only to serum-free medium in the absence of LY294002 *(control H9c2 cells)* and another batch of cell exposed only to 1% FBS medium in the absence of LY294002 (*1 % FBS-treated H9c2 cells*) were included (see method section 2.10.2).

The initiation of H9c2 cell differentiation into cardiomyocytes, by 1% serum (i.e. 1% FBS), induced a marginal increase in Akt phosphorylation at serine 473 ($p > 0.05$) (Figure 7.20). This differentiation associated increase in Akt phosphorylation (in the 1 % FBS treated cells) was completely abolished by 20µM LY294002 ($p < 0.01$) (PI3K inhibition) (Figure 7.19). There was a significant fold reduction in Akt phosphorylation at serine 473, between control cells and 20µM of LY294002-treated cells ($p < 0.05$) (Figure 7.19).
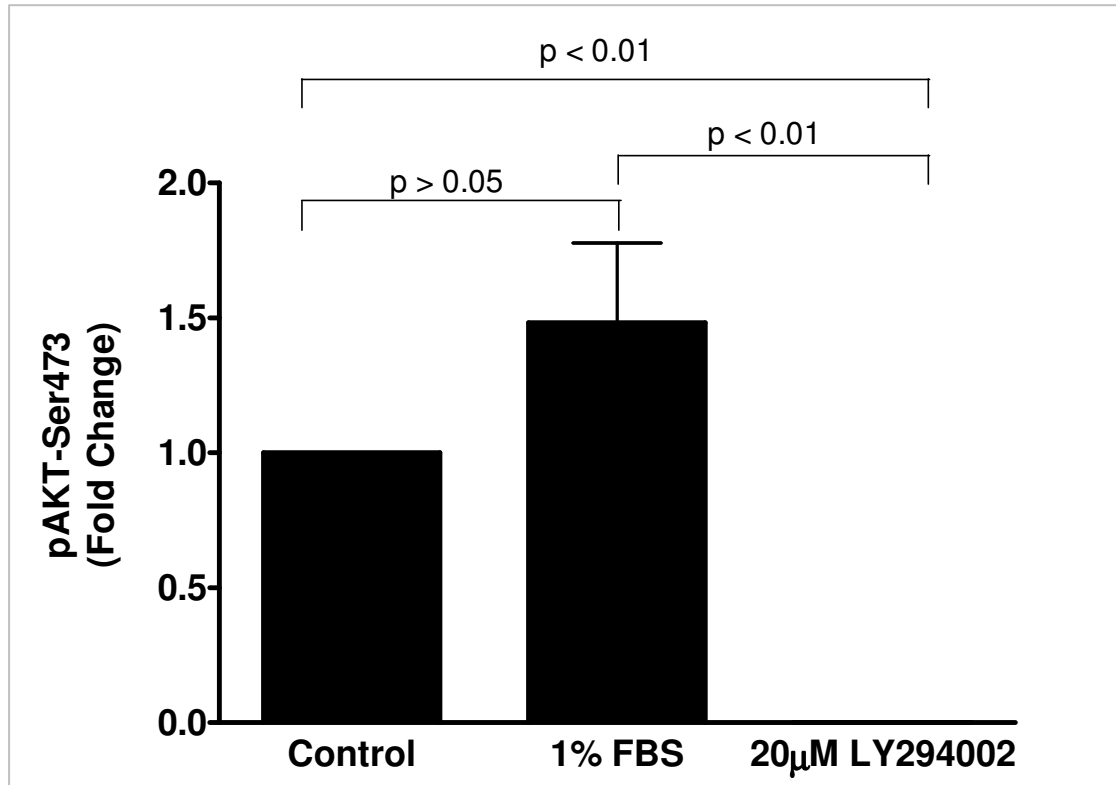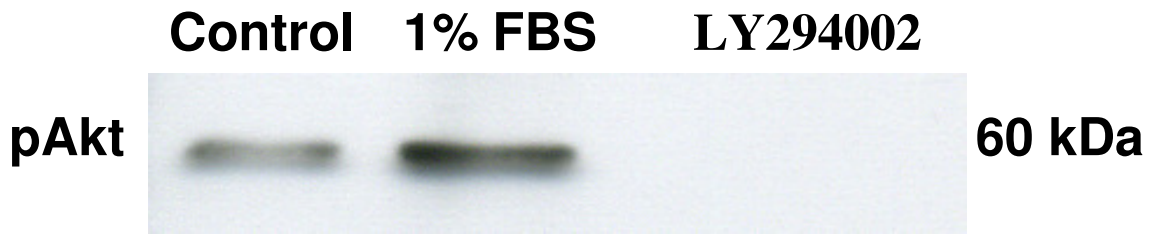
Figure 7.19: Effects of PI3K inhibitor LY29402 on Akt-ser473 phosphorylation in H9c2 cells differentiating into cardiomyocytes. Treatment of cells with 1% FBS induced a rapid increase in phosphorylated Akt levels. This increased Akt phosphorylation was completely abolished by 20μM of LY294002. There was no significant fold increase, in Akt phosphorylation at serine 473, between control cells and 1% FBS-treated cells (p>0.05). There was a significant fold reduction, in Akt phosphorylation at serine 473, between 1% FBS-treated cells and 20μM of LY294002-treated cells (p<0.01). There was also a significant fold reduction, in Akt phosphorylation at serine 473, between control cells and 20μM of LY294002-treated cells (p<0.01). Fold increase was calculated as the ratio of induced phosphorylated Akt level to baseline phosphorylated Akt. The comparisons between control vs 1% FBS (p>0.05), 1% FBS vs 20μM LY294002 (p<0.01) and control vs 20μM LY294002 (p<0.05) are indicated by lines connecting the respective pairs (i.e. treatments). The blot is representative of three independent experiments (panel A). The densitometric data (mean ± SD), from the three independent experiments, show the time dependent fold change in phosphorylated Akt in respective treatment shown on the graph (panel B).

7.4.3 Effects of protein kinase C inhibition on Akt phosphorylation at serine 473 in H9c2 cell differentiating into cardiomyocytes

To examine the effects of inhibiting PKC signalling on the activation of the PI3K signalling pathway, H9c2 cells *(BIM-I-treated H9c2 cells)* were treated with 10μM of BIM-I. For parallel comparison, cells exposed only to serum-free medium in the absence of BIM-I *(control H9c2 cells)* and another batch of cell exposed only to 1% FBS medium in the absence of BIM-I (*1 % FBS-treated H9c2 cells*) were included *(see method section 2.10.2).*

The initiation of H9c2 cell differentiation into cardiomyocytes, by 1% serum (i.e. 1% FBS), induced a marginal increase in Akt phosphorylation at serine 473 ($p > 0.05$) (Figure 7.20). This differentiation associated increase in Akt phosphorylation (in the 1 % FBS treated cells) was significantly enhanced by the 10μM BIM-I (i.e. PKC inhibition) ($p < 0.01$) There was a significant fold increase, in Akt phosphorylation at serine 473, between control cells and 10μM of BIM-I-treated cells ($p < 0.01$) (Figure 7.20).
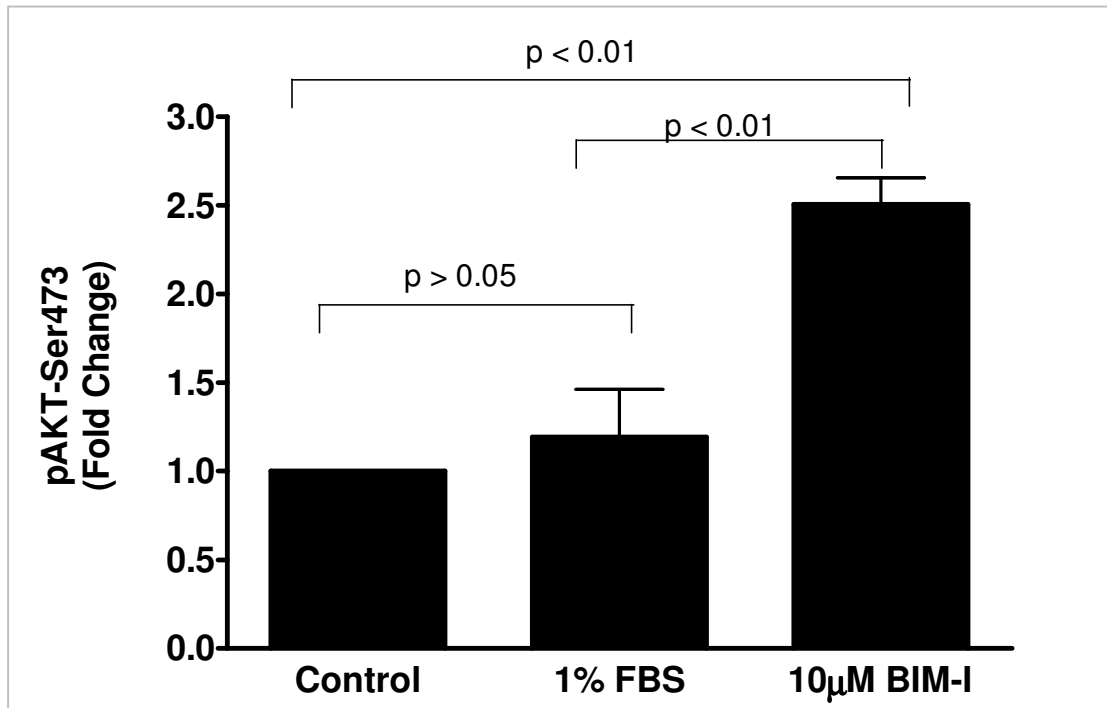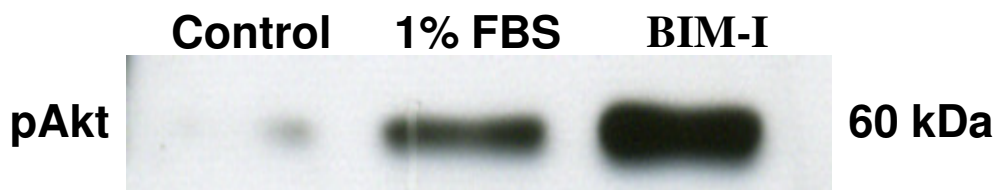
Figure 7.20: Effects of PKC inhibition on Akt phosphorylation at serine 473 residue in H9c2 cells differentiating into cardiomyocytes: PI3K and PKC cross-talk.. Treatment of cells with 1% FBS induced a rapid increase in phosphorylated Akt levels. This increased Akt phosphorylation was further enhanced by 10μM of BIM-I. There was no significant fold increase, in Akt phosphorylation at serine 473, between control cells and 1% FBS-treated cells ($p > 0.05$). There was a significant fold increase, in Akt phosphorylation at serine 473, between 1% FBS-treated cells and 10μM of BIM-I-treated cells ($p < 0.01$). There was a significant fold increase, in Akt phosphorylation at serine 473, between control cells and 10μM of BIM-I-treated cells ($p < 0.01$). Fold increase was calculated as the ratio of induced phosphorylated Akt level to baseline phosphorylated Akt. The comparisons between control vs 1% FBS ($p > 0.05$), 1% FBS vs 10μM BIM-I ($p < 0.01$) and control vs 10μM BIM-I ($p < 0.01$) are indicated by lines connecting the respective pairs (i.e. treatments). The blot is representative of three independent experiments (panel A). The densitometric data (mean ± SD), from the three independent experiments, show the time dependent fold change in phosphorylated Akt in respective treatment shown on the graph (panel B).

7.4.4 Effects of p38 MAPK inhibition on Akt phosphorylation at serine 473 in H9c2 cell differentiating into cardiomyocytes

To examine the effects of inhibiting PKC signalling on the activation of the PI3K signalling pathway, H9c2 cells _(SB203580-treated H9c2 cells)_ were treated with 10µM of SB203580. For parallel comparison, cells exposed only to serum-free medium in the absence of SB20580 _(control H9c2 cells)_ and another batch of cell exposed only to 1% FBS medium in the absence of SB203580 (_1 % FBS-treated H9c2 cells_) were included _(see method section 2.10.2)._

Initiation of H9c2 cell differentiation into cardiomyocytes, by 1% serum, led to a marginal increase in Akt phosphorylation at serine 473 ($p > 0.05$) (Figure 7.21).This differentiation associated increase in Akt phosphorylation (in the 1 % FBS treated cells) was significantly reduced by the 10µM SB203580 (i.e. p38 MAPK inhibition) ($p < 0.01$). There was also a significant fold reduction, in Akt phosphorylation at serine 473, between control cells and 10µM SB203580-treated cells ($p < 0.01$) (Figure 7.21).
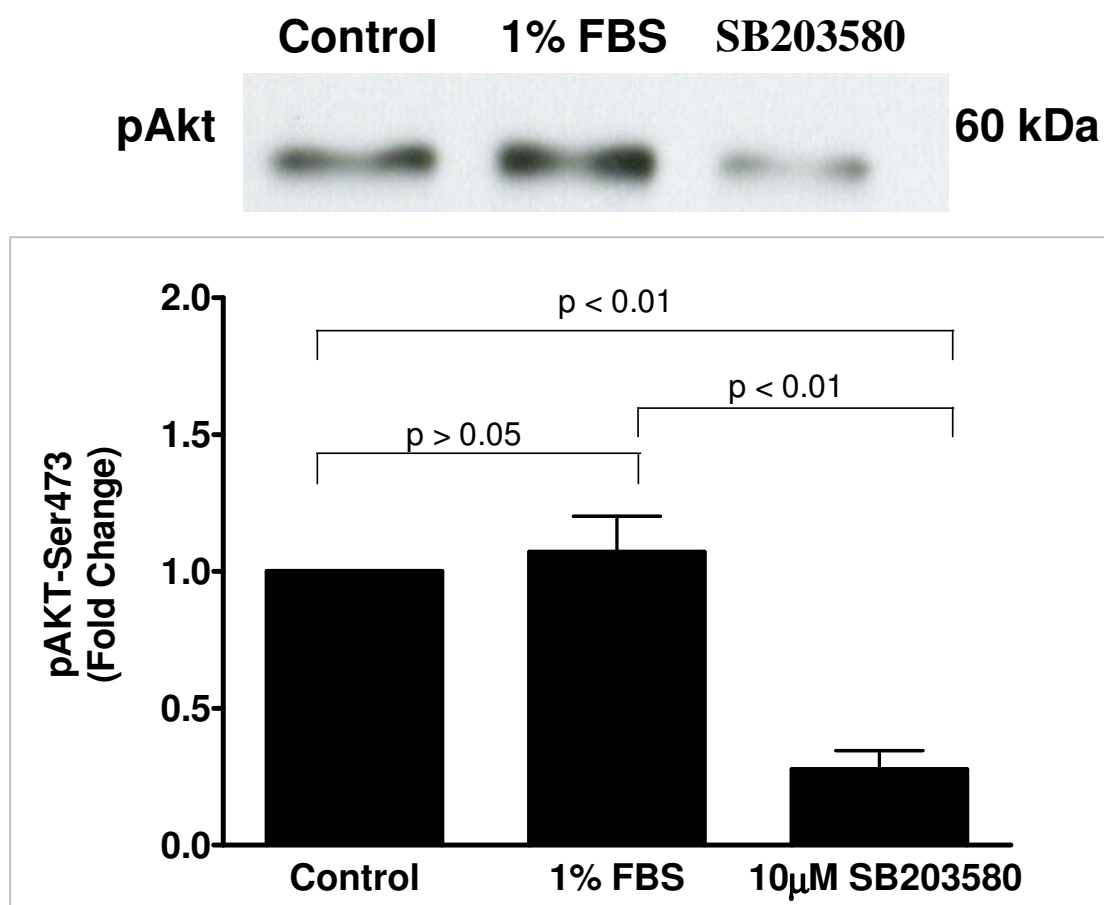
Figure 7.21: Effects of protein p38 MAPK inhibition on Akt phosphorylation at serine 473 residue in H9c2 cells differentiating into cardiomyocytes: PI3K and p38 MAPK cross-talk. Treatment of cells with 1% FBS induced a rapid increase in phosphorylated Akt levels. This increased Akt phosphorylation was significantly reduced by 10μM *SB203580*. There was no significant fold increase, in Akt phosphorylation at serine 473, between control cells and 1% FBS-treated cells ($p>0.05$). There was a significant fold reduction, in Akt phosphorylation at serine 473, between 1% FBS-treated cells and 10μM SB203580-treated cells ($p<0.01$). There was also a significant fold reduction, in Akt phosphorylation at serine 473, between control cells and 10μM SB203580-treated cells ($p<0.01$). Fold increase was calculated as the ratio of induced phosphorylated Akt level to baseline phosphorylated Akt. The comparisons between control vs 1% FBS ($p>0.05$), 1% FBS vs 10μM SB203580-treated cells ($p<0.01$) and control vs 10μM SB203580-treated cells ($p<0.01$) are indicated by lines connecting the respective pairs (i.e. treatments). The blot is representative of three independent experiments (panel A). The densitometric data (mean ± SD), from the three independent experiments, show the time dependent fold change in phosphorylated Akt in respective treatment shown on the graph (panel B).

7.5 DISCUSSION

Published data suggest important roles for PI3K signalling during the differentiation of ES cells into cardiomyocytes (Klinz *et al.*, 1999; Sauer *et al.*, 2000). Treatment of differentiating ES cell cultures with the PI3K inhibitor LY294002 was shown to reduce the number of α-actinin positive cardiomyocytes and the number of embryoid bodies containing beating foci (Klinz *et al.*, 1999).

Our data showed that the activation of the PI3K signalling pathway may be essential for differentiation of H9c2 cells into cardiomyocytes. Cell elongation and fusion forming myotubes, the main morphological changes or features associated with H9c2 cell differentiation into cardiomyocytes, were significantly inhibited by LY294002. The expression of cardiac specific ventricular myosin light chain 1 was also completely blocked in these cells. These results suggest that activation of the PI3K pathway may be essential for differentiation of H9c2 cells into cardiomyocytes.

Consistent with the H9c2 cell data, the inhibition of PI3K signalling also completely blocked the differentiation of P19 stem cells into beating cardiomyocytes and also inhibited the expression of cardiac restricted troponin I in these cells. Moreover, our data suggest that not only is activation of PI3K signalling critical for P19 stem cell differentiation into cardiomyocytes but that the early timing of this pathway may be critical. The inhibition of PI3K signalling, by LY294002 (20μM), four days after the initiation of DMSO induced differentiation failed to blocked P19 stem cell differentiation into

beating cardiomyocytes. These results suggest that activation of the PI3K pathway may be essential for differentiation of P19 stem cells into cardiomyocytes. The failure of LY294002 to block the differentiation of P19 stem cells into cardiomyocyte when added four days after DMSO also suggests that that PI3K signalling pathway is activated during the very early stage of P19 stem cell differentiation into cardiomyocytes. The data also suggest that the activation of PI3K pathway may be transient since late inhibition of PI3K by LY294002 failed to inhibit the differentiation process. Whether PI3K activation is indeed transient or whether it is sustained but become irreverent after the initiation of the differentiation process remains to be determined. Our data are in agreement with similar observations in which late LY294002 treatment failed to suppress the expression of Nkx-2.5 and GATA-4 in P19CL6 cells (a clonal derivative of P19 stem cells) (Naito *et al.*, 2003).

The 20μM concentration of LY294002 used in the present project was selected from published literature and appears to be a commonly used concentration for blocking PI3K activity (Qi *et al.*, 1999; Tamir and Bengal, 2000; Du *et al.*, 2001; Naito *et al.*, 2003; Fukuda *et al.*, 2005; Shaik *et al.*, 2008; Smyth *et al.*, 2008). LY294002 is not only potent but is also a specific inhibitor for blocking PI3K signalling (Vlahos *et al.*, 1994). LY294002, even at a higher concentration (50μM), specifically inhibited PI3K (Vlahos *et al.*, 1994). LY294002 (50μM) completely inhibited PI3K but failed to inhibit the activities of a wide range of other kinases including, PKC, PKA, MAP kinase, S6 kinase, EGF receptor tyrosine kinase, c-src kinase, PI 4-kinase and

diacylglycerol kinase) and ATPase (Vlahos *et al.*, 1994). Furthermore our cytotoxicity studies using MTT showed that LY294002, at 20uM concentration, had no cytotoxic effects. Thus, the inhibition of H9c2 and P19 stem cell differentiation into cardiomyocytes by LY294002 (20µM), as shown by our data, is due to LY294002 inhibition of the PI3K signalling pathway. Our data therefore suggest that the activation of the PI3K signalling is required for stem cell differentiation into cardiomyocytes. The data also suggest an early requirement for the activation of the PI3K signalling during the early stages of stem cell differentiation into cardiomyocytes.

Our data have also shed some light on whether activation of Akt by phosphorylation is required to drive cardiomyocyte differentiation. Akt is activated by sequential phosphorylation, first at threonine 308 by PDK1, and then, finally at serine 473 by PDK2 (Alessi *et al.*, 1996; Alessi *et al.*, 1997a; Alessi *et al.*, 1997b). Our data clearly showed that PI3K signalling is required for stem cell differentiation into cardiomyocytes. However the data obtained on Akt are less convincing. Indeed exposure of cells to LY294002 did result in inhibition of Akt phosphorylation at serine 473 which correlates well with the ability of the drug to modulate differentiation. However, our cross-talk studies using BIM-I, a specific and selective PKC inhibitor, to block the activation of the PKC signalling pathway, revealed an unexpected increase in Akt phosphorylation at serine 473, even though BIM-I blocked cardiac differentiation of both H9c2 cells and P19 stem cells. This would strongly suggest that phosphorylation of Akt in differentiating stem cells may have no direct relevance to the process itself.

The enhanced Akt activation (phosphorylation at serine 473) by PKC inhibition cannot be explained by non-specificity of the concentration (10µM) of BIM-I used in these experiments. It has been reported, in haematopoietic progenitor cells, that PKC inhibition, using another PKC inhibitor, Ro-31-8220 (bisindolylmaleimide IX) (5µM), enhanced Akt activation (increased phosphorylation at serine 473 and threonine 308), in response to c-Kit stimulation (Edling *et al.*, 2007). It has also been shown that Ro 31-8220 (3µM), bisindolylmaleimide VIII (3µM), and 30nM of LY 379196 (another bisindolylmaleimide) increase Akt phosphorylation at Serine 473 in A549 cells and HEK293 cells (Wen *et al.*, 2003). Increased Akt phosphorylation in HEK293 cells over-expressing Akt was diminished by phorbol 12-myristate-13-acetate (PMA) a potent PKC activator (Wen *et al.*, 2003). Our data therefore suggest that PI3K may be activating alternative downstream targets independent of Akt (Figure 7.22).

The question of whether Akt is required for stem cell differentiation into cardiomyocytes can be investigated by knocking out Akt, in stem cells. This could be done using pharmacological Akt specific inhibitors. Alternatively, Akt can be knocked out using molecular methods. For example, Akt siRNA can be used to block Akt mRNA translation into Akt protein. An Akt dominant negative stem cells, through the mutation of the critical amino acid residues (i.e. serine 473 or both threonine 308 and serine 473) can also be created. The mutant stem cells (either from Akt siRNA or Akt mutation) can then be induced to differentiate into cardiomyocytes. If Akt is not required, for stem cell differentiation into cardiomyocytes, then the mutant stem cells will retain

their ability to differentiate into cardiomyocytes. However, if Akt is required, for stem cell differentiation into cardiomyocytes, then the mutant stem cells will have reduced ability to differentiate into cardiomyocytes.

The Wnt/β-catenin signalling pathway has been reported to be involved in the cardiac lineage specification or differentiation. Wnt ligands and receptors are expressed in a spatially and temporally manner and regulated during early heart development. (Ku and Melton, 1993; Monkley et al., 1996; Eisenberg et al., 1997; Garriock et al., 2005). Moreover, the canonical Wnt/β-catenin signalling positively regulates ES cell differentiation. In the mouse, the expression of the early cardiac genes Nkx2.5 and Tbx5 (mesoderm marker) in EBs can be up-regulated by Wnt3a and down-regulated by Dkk-1(Wnt antagonist). The addition of Wnt3a to ES cells during the early phase of EB formation increased the number of beating EBs by more than 50% while the addition of Fz8/Fc (secreted or soluble Wnt antagonist) or Dkk-1 and Fz8/Fc at this stage leads to a complete absence of beating EBs (Naito et al., 2006; Kwon et al., 2007). Constitutively active GSK-3β (i.e. degradation of β-catenin) also completely blocked the expression of Tbx5 and GATA4 (Nakamura et al., 2003; Kwon et al., 2007). In contrast, lithium chloride (a GSK-3β inhibitor) significantly increased the expression of Nkx2.5, GATA4, Tbx5 (Nakamura et al., 2003). The down-regulation of Nkx2.5, GATA4 and Tbx5 expression by Dkk-1, Fz8/Fc and constitutively active GSK-3β and the induction of these genes by lithium suggests that Wnt/β-catenin signalling play positive roles in driving ES cell differentiation into cardiomyocytes.

Previous studies have suggested that PI3K signalling could involve activation of the Wnt/β-catenin pathway and this may be independent of Akt. Lithium treatment mimics Wnt/β-catenin signalling activation. Lithium induces the stabilisation and nuclear accumulation of β-catenin by inhibiting the phosphorylation of GSK-3β (Klein and Melton, 1996; Hedgepeth *et al.*, 1997; Rao *et al.*, 2005). Our data showed that H9c2 cells in 10% serum do not differentiate into cardiomyocytes. But it has been shown that treatment of H9c2 cells with lithium can overcome the inhibitory effects of serum and drive the cell differentiation into cardiomyocytes (Kashour *et al.*, 2003). LY294002 blocked lithium-induced differentiation of H9c2 cells (Kashour *et al.*, 2003) thus suggesting a link between the PI3K and the Wnt/β-catenin pathway. In contrast, it was also found that over-expression of Akt in H9c2 did not increase stabilisation or nuclear accumulation of β-catenin or increased H9c2 cell differentiation. Lithium also induced differentiation in H9c2 expressing dominant-negative Akt (Kashour *et al.*, 2003). These results suggest that PI3K signalling and Wnt/β-catenin signalling interacted in an Akt-independent manner or mechanism.

Our data suggest that PI3K regulates cardiomyocyte differentiation in an Akt-independent mechanism. Our data are in agreement with the previous data mentioned above. The new downstream target, independent of Akt, through which PI3K may cross-talk with Wnt/β-catenin signalling to regulate cardiomyocyte differentiation, is not known. Considering all of the above data, we are tempted to speculate that activation of PI3K in our cells models could drive differentiation mainly through activation of the Wnt/β-catenin signalling

pathway and not through phosphorylation of Akt. However further investigations (using Akt inhibitors, or Akt siRNA, or dominant negative Akt stem cells, along with studies to correlate the activation (or inhibition) of the Wnt/β-catenin signalling to stem cell cardiomyocyte differentiation) are required to confirm our data and determine the exact role of PI3K activation and Akt during the differentiation of stem cell into cardiomyocytes.

Further support of our suggestion that PI3K may regulate Wnt/β-catenin signalling through an Akt-independent mechanism comes from studies which showed that the activation of the Wnt/β-catenin signalling did not lead to GSK-3β phosphorylation (Ding *et al.*, 2000; McManus *et al.*, 2005). While Akt can inactivate GSK-3β by phosphorylation at serine 9 in response to the activation of other pathways, for example in response to insulin-like growth factor (IGF) and other factors (Desbois-Mouthon *et al.*, 2002), GSK-3β did not become phosphorylated at serine 9 in response to Wnt signalling (Ding *et al.*, 2000; McManus *et al.*, 2005).

As already discussed, GSK-3β inactivation is necessary for ES cell commitment to cardiac lineage differentiation. GSK-3β is constitutively active and downstream of Akt. Since Akt negatively regulates the activity of GSK-3β then activated Akt, following the activation of the PI3K signalling pathway, would be expected to inactivate GSK-3β and block its negative effects on cytoplasmic β-catenin levels thus leading to enhanced activation of the Wnt signalling and subsequent increase in ES cell differentiation into cardiomyocytes. However Akt-mediated inactivation of GSK-3β may not be a

relevant or a major event for the Wnt/β-catenin signalling pathway. In both wild type and knockin cells homozygous for both GSK-3β and GSK-3α, Wnt3a was found to inactivate GSK-3, stabilised β-catenin and stimulated Wnt-dependent gene transcription but failed to induce a detectable increase in the phosphorylation of GSK-3α (at seine 21) and GSK-3β (at serine 9) in the wild-type cells (McManus *et al.*, 2005). The above data show that Wnt-mediated inactivation of GSK-3β is not dependent on GSK-3β phosphorylation at serine 9. Thus the inactivation of GSK-3β by the Wnt signalling pathway may be achieved through a mechanism that is independent of Akt inactivation of GSK-3β. The above is in line with our suggestion the Akt phosphorylation may not be relevant for PI3K mediated regulatory effects on stem cell differentiation into cardiomyocytes.

PI3K inhibition in our experiments inhibited the differentiation of P19 stem cells in beating cardiomyocytes and the expression of troponin I. PI3K inhibition also inhibited the differentiation of H9c2 cells as determined by the lack of differentiation associated morphological changes and the inhibition of MLC-1v expression. While PI3K inhibition blocked the differentiation of both P19 stem cells and H9c2 cells into cardiomyocytes, our cross-talk data showed that PKC inhibition enhanced Akt activation (i.e. phosphorylation at serine 473). However as already shown (Chapter 5) PKC inhibition also blocked P19 stem cells and H9c2 cells into cardiomyocytes. Therefore our data suggest that PI3K positively regulates stem cell differentiation into cardiomyocytes not through Akt activation, but through a yet unknown signalling mechanism that is independent of Akt activation (Figure 7.22).

The data from the cross-talk studies suggested possible interactions between PI3K and PKC and also between PI3K and p38 MAPK signalling during cardiomyocyte differentiation. Our data suggest that activated PKC or p38 MAPK pathways may modulate the activation of the PI3K pathways as determined by Akt phosphorylation at serine 473. The inhibition of PKC pathway led to an elevation of Akt phosphorylation at serine 473, thus suggesting a possible negative role for PKC in the regulation of PI3K at the Akt level. In contrast the inhibition of p38 MAPK pathway led to a reduction in Akt phosphorylation at serine 473, thus suggesting a possible positive role for p38 MAPK in the regulation of PI3K also at the level of Akt. It not clear how PKC or p38 MAPK may regulate PI3K pathway activation by modulating Akt phosphorylation at serine 473.

The PI3K signalling controls many biological processes. It is therefore more likely that this pathway may be regulated by cross-talk with different signalling pathways. The fact that the PKC specific inhibitor BIM-I caused increased Akt phosphorylation suggests that PKC may play a negative role in the regulation of PI3K pathway. PKC is downstream of both PI3K and PDK1 (Chou *et al.*, 1998; Dutil *et al.*, 1998; Balendran *et al.*, 2000) therefore the effects of any cross-talk between PI3K and PKC on cardiac cardiomyocyte differentiation cannot be attributed to direct effect on either PI3K or PDK1.

Our data give ground for possible speculation about how BIM-I (i.e. PKC inhibition) might caused increase phosphorylation of Akt at serine 473. First,

PKC could inhibit Akt activation by targeting PDK2 (i.e. an elusive and currently unknown kinase that is thought to be responsible for Akt Akt phosphorylation at serine 473). If PKC does indeed control PDK2 as suggested by our data (i.e. increased Akt phosphorylation that accompanied the inhibition of PKC) then PKC may be a negative regulator of PDK2 since the inhibition of PKC by BIM-I appeared to "increase" the ability of "PDk2" to phosphorylate Akt-ser473.

The action of the lipid phosphatase, PTEN on PIP_3 limits the activation of Akt (Maehama and Dixon, 1998). However since PTEN does not directly dephosphorylate Akt, it is currently not known how Akt signalling is terminated once it is activated. While the dephosphorylation mechanism to directly inactivate Akt is yet to be found, evidence suggests that a phosphatase known as PHLPP (PH domain leucine-rich repeat protein phosphatase) selectively and specifically dephosphorylates Akt at serine 473 (Gao *et al.*, 2005). The second speculation therefore is that PKC may be an activator (i.e. positive regulator) of PHLPP "a putative phosphatase" that may be responsible for dephosphorylating AKt at serine 473 back to the baseline level since BIM-I would reduce the ability of PKC to activate that "putative phosphatase", thus limiting the ability of this "phosphatase to dephosphorylate phosphorylated Akt, hence the increased levels of phosphorylated Akt in presence of BIM-I.

If indeed as suggested by our data, PKC does negatively regulate PI3K via PDK2 or Akt-related "phosphatase" then the significance of such negative

regulation of Akt by PKC is worth exploring in order to get a better understanding of the interaction between PI3K and PKC signalling events. However according to our data, PKC regulation of PI3K either via PDK2 or Akt-related "phosphatase" may be of no or little relevance to the differentiation of stem cells into cardiomyocytes since our data also suggested that Akt activation is of no or little relevance in this process.

Our data also suggest a possible cross-talk between PI3K and p38 MAPK. PDK1 has been cloned and sequenced (Alessi *et al.*, 1997a) and it is well established that PDK1 phosphorylate Akt at threonine 308 (Alessi *et al.*, 1997a; Alessi *et al.*, 1997b). However the identity of the kinase that is responsible for Akt phosphorylation at serine 473 remains unknown. Our data showed reduced phosphorylation of Akt at serine 473 in the presence of SB203580 (10µM). We speculate that p38 MAPK may have an additional activity that can phosphorylate Akt at serine 473 in a phosphoinositide-dependent manner. Alternatively, p38 MAPK may be the unidentified PDK2. It is interesting to note that p38 MAPK is among a range of possible PDK2 candidates. Other candidate kinases include mitogen-activated protein (MAP) kinase-activated protein kinase-2 (MK2) (Alessi *et al.*, 1996), integrin-linked kinase (ILK) (Delcommenne *et al.*, 1998; Persad *et al.*, 2000), DNA-dependent protein kinase (DNK-PK), cPKC$_\alpha$ (Partovian and Simons, 2004) and cPKC$_\beta$ (Kawakami *et al.*, 2004). The question of whether p38 MAPK has a PI3K-dependent and phosphoinositide-dependent activity that can phosphorylate Akt at serine 473 or whether p38 MAPK and PDK2 are one and the same kinase remains. While Akt phosphorylation at serine 473 may not be

of major relevance to the differentiation of stem cells into cardiomyocytes since both PI3K and p38 MAPK are implicated in the differentiation of stem into cardiomyocytes, then the resolution of this question may help in advancing our understanding of the relationship between PI3K and p38 MAPK during cell differentiation and this needs further investigation.
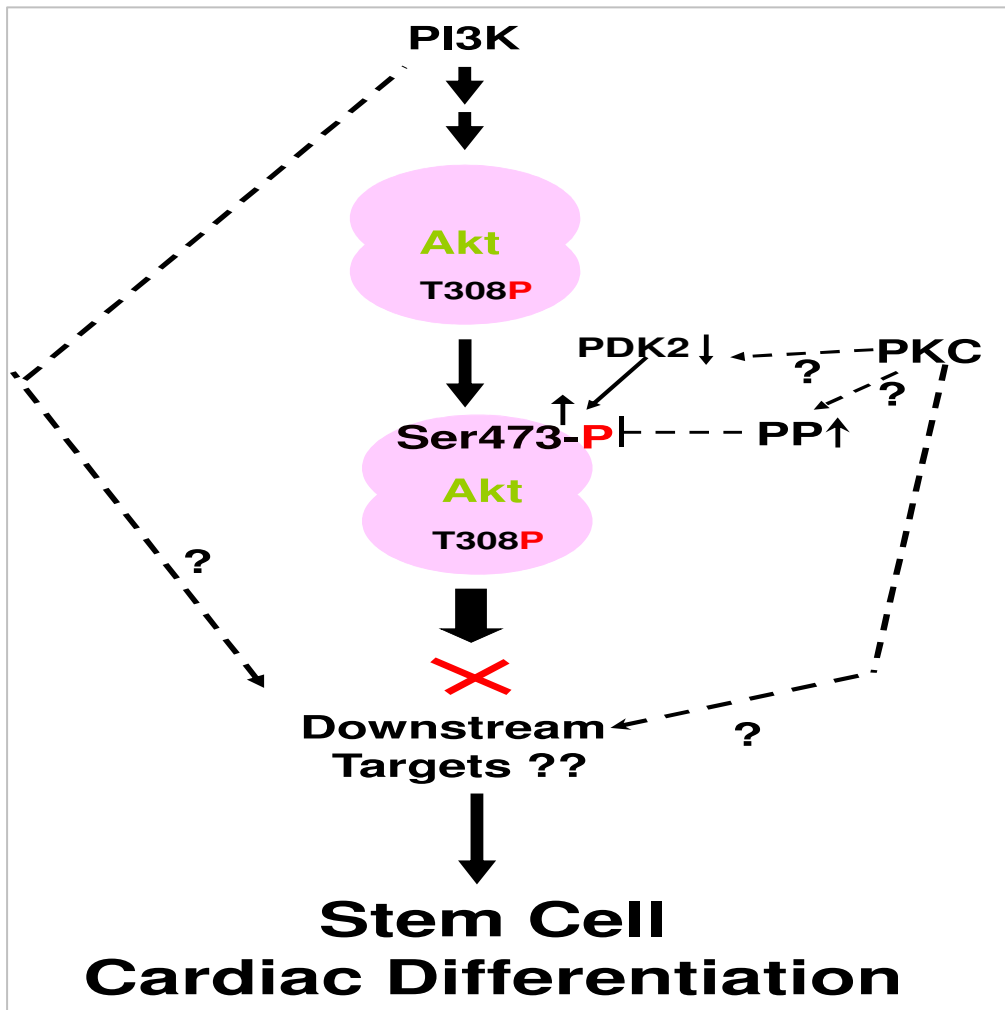
Figure 7.22: PI3K and PKC regulate stem cell differentiation into cardiomyocytes through possible Akt-independent mechanisms. PI3K activation causes Akt activation (phosphorylation at both threonine 308 and serine 473) but PI3K may regulate stem cell differentiation into cardiomyocytes through a possible Akt-independent mechanism. PKC may modulate the phosphorylated states of Akt, since PKC inhibition enhanced Akt phosphorylation at serine 473. PKC inhibition also blocked the differentiation of H9c2 cells and P19 stem cells into cardiomyocytes. Therefore PKC may regulate H9c2 cells and P19 stem cell differentiation into cardiomyocytes through possible Akt-independent mechanism(s). PKC may negatively regulate the activity of 3-Phosphoinositide-Dependent Protein Kinase 2 (PDK2) since the inhibition of PKC by BIM-I appeared to "increase" the ability of "PDK2" to phosphorylate Akt-ser473. Alternatively, PKC maybe an activator (i.e. positive regulator) of "a putative phosphatase" (PP) that may be responsible for dephosphorylating AKt at back to the baseline level. The possible Akt-independent mechanisms or downstream target(s), through which PI3K and PKC may regulate H9c2 cell and P19 stem cell differentiation into cardiomyocytes is/are currently unknown and warrants further investigation.

As stated above, the phosphorylation of Akt in differentiating stem cells may have no direct relevance to the cell differentiation into cardiomyocytes. Therefore, in addition to any possible modulation of Akt activity (as suggested by the inhibition of Akt activation by SB203580), the data also suggest that p38 MAPK may cause cardiac differentiation independent of Akt (Figure 7.23). Like in the case of PKC, the data also point to a possible target, downstream of/ independent of Akt, through which p38 MAPK might cause the differentiation of H9c2 cell and stem cell into cardiomyocytes. This possible key downstream target is currently unknown and warrants further investigation.
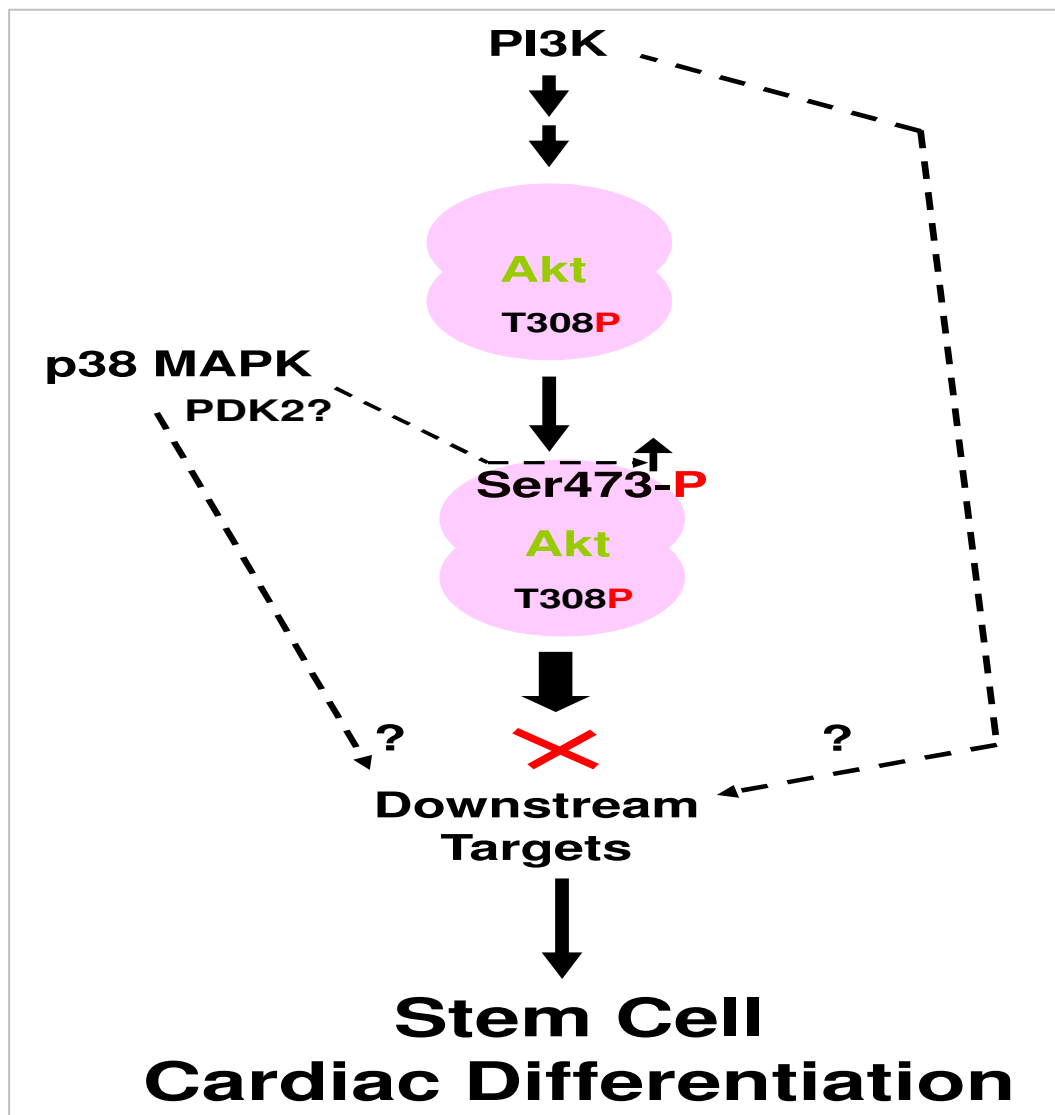
Figure 7.23: PI3K and p38 MAPK regulate stem cell differentiation into cardiomyocytes through possible Akt-independent mechanisms. PI3K activation causes the Akt activation (phosphorylation at both threonine 308 and serine 473) but PI3K may regulate stem cell differentiation into cardiomyocytes through a possible Akt-independent mechanism. P38 MAPK may modulate the phosphorylated states of Akt, since p38 MAPK inhibition inhibited Akt phosphorylation at serine 473. however, the inhibition of p38 MAPK also blocked the differentiation of H9c2 cells and P19 stem cells into cardiomyocytes. Therefore p38 MAPK may regulate H9c2 cells and P19 stem cell differentiation into cardiomyocytes through a possible Akt-independent mechanism(s). The possible Akt-independent mechanisms or downstream target, through which PI3K and p38 MAPK may regulate H9c2 cell and P19 stem cell differentiation into cardiomyocytes is/are currently unknown and warrants further investigation.

7.6 SUMMARY

The inhibition of PI3K using 20µM of LY294002 completely blocked the differentiation of H9c2 cells into cardiomyocytes (as as determined by the usual morphological changes of cell elongation, fusion and myotube formation. LY294002 treated H9c2 cells largely maintained their normal single cell and spindle shaped morphology which was in contrast to control H9c2 cells which showed the usual morphological changes. In agreement with the lack of morphological changes observed in LY294002 treated H9c2 cells, the expression of MLC-1v protein was significantly inhibited. Our data therefore suggest that activation of the PI3K signalling pathway is required for H9c2 cell differentiation into cardiomyocytes.

Treatment of H9c2 cells with 1% FBS induced a rapid increase in phosphorylation (at serine 473) but this differentiation associated increase in Akt phosphorylation was completely abolished by 20µM of LY294002. This differentiation associated increase in Akt phosphorylation was significantly enhanced by BIM-I (10µM) but significantly reduced by SB203580 (10µM). Our data therefore showed that PI3K is activated during 1% FBS-induced differentiation of H9c2 cells into cardiomyocytes and also that PKC and p38 MAPK may modulate Akt phosphorylation at serine 473.

P19 stem cultured in the presence of 0.8% DMSO but in the absence of LY294002 (absence of PI3K inhibition) differentiated into beating cardiomyocytes. However PI3K inhibition, with LY294002 (20µM), completely blocked the DMSO-induced differentiation of P19 stem cells into beating

cardiomyocytes. The expression of cardiac specific troponin I was also reduced in P19 stem cells treated with LY294002.

When DMSO was allowed to initiate P19 stem cell differentiation, for 4 days before the addition of LY294002 to the cells, LY294002 completely failed to block the cell differentiation into beating cardiomyocytes. Our data therefore suggest that activation of the PI3K signalling pathway is required for P19 stem cell differentiation into cardiomyocytes. In addition the data suggest that PI3K signalling is required during the early stages of P19 stem cell differentiation into cardiomyocytes.

In conclusion, the activation of the PI3K signalling pathway is required for P19 stem cells and H9c2 cell differentiation into cardiomyocytes. The activation of PI3K signalling is required during the early stages of stem cell differentiation into cardiomyocytes. Our data also suggest that PI3K positively regulates stem cell differentiation into cardiomyocytes not through Akt activation, but through a yet unknown signalling mechanism that is independent of Akt activation.

Our data also suggest that PKC may also be a negative regulator of Akt phosphorylation at serine 473 either via an unknown PDK2 or via positive regulation of a possible "phosphatase" that is responsible for the dephosphorylation of Akt at serine 473. It is also a suggestion from our data that p38 MAPK may have a PI3K-dependent and phosphoinositide-dependent

activity with the ability to phosphorylate Akt at serine 473 in or p38 MAPK and

PDK2 may be one and the same kinase.

CHAPTER 8.0

ROLE OF NF-kB IN H9C2 CELL DIFFERENTIATION

INTO CARDIOMYOCYTES

8.1 INTRODUCTION

NF-kB signalling pathway consists of a family of structurally related and evolutionarily conserved proteins known as NF-κB proteins. There are five mammalian NF-kB proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50/105) (i.e. p50 and its precursor p105), and NF-κB2 (p52/p100) (i.e. p52 and its precursor p100). These proteins can form homodimers and heterodimers. These dimers have different DNA site specificities and also different DNA binding affinities.

A common structural feature of NF-kB proteins is the presence of a highly conserved 300 amino acid domain at their N-terminals. This conserved sequence is known as the Rel homology domain (RHD). Rel homology domain is responsible for DNA binding, dimerisation, and association with inhibitory proteins (Coleman *et al.*, 1993; Toledano *et al.*, 1993). In unstimulated cells, NF-κB proteins exist in their transcriptionally inactive forms bound to proteins that inhibit their activation. These inhibitory proteins are known as inhibitors of NF-κB (IκB). Seven mammalian IκB proteins (IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p105 and p100) has been identified. This non-covalent interaction between the NF-kB dimers and the NF-kB inhibitors is thought to conceal the nuclear localisation signal (NLS) of NF-κB proteins thus preventing their translocation into the nucleus. Therefore inactive NF-kB proteins are retained in the cytoplasm. The NF-κB signalling can be activated either by the classical (canonical) pathway or by the alternative (noncanonical or novel) pathway.

In the classical pathway, IκB is phosphorylated (Li *et al.*, 1994; Naumann and Scheidereit, 1994; Traenckner *et al.*, 1995; Zandi *et al.*, 1998) by IκB kinase (IKK). This phosphorylation leads to polyubiquitination of IkB. This phosphorylation mediated polyubiquitination triggers IkB degradation by the ubiquitin-proteasome pathway. IkB degradation releases NF-κB of IkB inhibition and thereby activates NF-κB. The activated NF-κB then translocates into the nucleus (Naumann and Scheidereit, 1994). In the nucleus, NF-kB activate the expression of a various target genes including genes coding for other transcription factors, cytokines, growth factors and regulators of apoptosis.

NF-kB activation by the non-canonical pathway involves the activation of NF-kB inducing kinase (NIK) which in turn activates IKK (Tse *et al.*, 2007). The activated IKKα then phosphorylates p100 and this in turn undergoes proteasomal processing to generate p52. The generated p52 form a dimer with RelB (i.e. RelB/p52). The RelB/p52 complex then translocate to the nucleus to activate genes distinct from those regulated by the canonical pathway.

To ensure that the activation of NF-kB signalling is transient and self-limiting, a series of mechanisms including a negative feedback loop regulates NF-κB activation. The negative feedback loop involves the induction of IkB genes by the activated NF-kB. This leads to the synthesis of new IkBα proteins. The newly synthesised IkB then enter the nucleus, bind to activated NF-KB and return it to the cytoplasm in the inactive form

8.1.1 NF-κB signalling and stem cell differentiation

Transcription factors are among the key molecules for gene regulation at the level of transcription. Transcription factors are proteins that bind specific DNA or promoter sequences in promoters leading to the alteration of the transcription of downstream target genes under the promoter control. We have an understanding of the roles of cardiac transcription factors (e.g. GATA-4, Nkx.5) in cardiogenesis and ES cell differentiation into cardiomyocytes. However there is very little information on the role of other or non-cardiac transcription factors which may be upstream and may be activated much earlier than the cardiac transcription factors.

If more information about transcription factors upstream of cardiac transcription factors become available then coupling that information with what we already known about cardiac transcription factors may tremendously increase our understanding of cardiogenesis and ES cell differentiation into cardiomyocytes. This may lead to better and more cardiac specific differentiation of ES into cardiomyocytes. This in turn will help in the realization of the therapeutic potential of stem cell-derived cardiomyocytes for the treatment of cardiovascular diseases.

We decided to investigate the role of NF-kB, which is downstream of PI3K. As already shown by our data, PI3K has role to play in the differentiation of stem cells into cardiomyocytes. The aim of this study is to investigate the role of NF-kB pathway in the cardiomyocyte differentiation. To examine the specific roles of NF-kB action during cardiomyocyte differentiation, we chose CAPE

(caffeic acid phenethyl ester), an inhibitor of NF-kB activation (Natarajan *et al.*, 1996) , in this study.

CAPE inhibits the activation of the classical NF-kB pathway by suppressing the degradation of IkB-α (Natarajan *et al.*, 1996; Abdel-Latif *et al.*, 2005; Song *et al.*, 2008). CAPE (IC_{50}= 2µM) is a potent NF-kB activation inhibitor (Reddy *et al.*, 2004).

8.2 MATERIALS AND METHODS

8.2.1 Culture and differentiation of H9c2 Cells

The routine culture of H9c2 cells was carried out as described in section 2.3.1 (Chapter 2). H9c2 cells were trypsinised and the appropriate cell density for the differentiation experiments was calculated as described in section 2.6 (Chapter 2). H9c2 cells ($2x10^3$ cells/cm^2) were seeded in p60 dishes or 6-well plates in full culture medium (i.e. DMEM supplemented with 10% FBS, 100units/ml penicillin/100µg/ml streptomycin), placed in a tissue culture incubator at 37^oC, 95% air and 5% CO_2 and allowed to become 60-70% confluent. The differentiation of H9c2 cells into cardiomyocytes was carried out in 1% serum as described in section 2.8.1 (Chapter 2). The role of NF-kB signalling pathway in the differentiation of H9c2 cells into cardiomyocytes was investigated. The effects of the inhibition of the activation of NF-kB signalling pathway were investigated using 30µM of CAPE as described in section 2.8.2 (Chapter 2). A CAPE stock (100mM solution) was prepared by dissolving 5mg of CAPE in 176µl of DMSO. The stock solution was distributed into 20 or 50µl aliquots and stored in the freezer at -20^oC. The aliquots were protected from light during storage and use. The differentiation medium was used to, first dilute the frozen stock CAPE solutions to 100µM (1:1000). The 100µM solution was then finally diluted (1:3.3) to the CAPE concentration (30µM) used in the differentiation experiments. The preparation of stock CAPE concentration was carried out as explained in appendix VIII. More details about CAPE are located in appendix VI (d).

8.2.2 Cell viability assay

To examine the effects of the NF-kB inhibition on H9c2 cell viability, the MTT assay was carried out as described in section 2.7 (Chapter 3).

8.2.3 Western blot analysis

In order to perform the western blot analysis, the following procedures were carried out as described in the Chapter 3 under the sections indicated in parenthesis: cell lysate generation (section 2.11), preparation of BSA standards (section 2.12.1) and BCA assay (section 2.12.2). The western blotting was carried out as described in section 2.13.

8.2.4 Data analysis

Data obtained from independent experiments were used for statistical analysis as described in section 2.14 (Chapter 3).

8.3 RESULTS

8.3.1 Effects of inhibition of NF-kB activation on H9c2 cell differentiation into cardiomyocytes

Consistent with previous data described above, culture of H9c2 cells in media with 1% serum resulted in a time dependent differentiation of the cells in to cardiomyocytes. More importantly, treatment with CAPE delayed the differentiation process with myotube formation becoming evident at day 8 to 12 (Figure 8.10). This effect is more evident when MLC-1v expression was monitored. As shown in Figure 8.11, levels of MLC-1v were significantly reduced at day 4 when compared to controls and even though levels recovered at day 8 and 12, expression was still marginally lower than in controls.
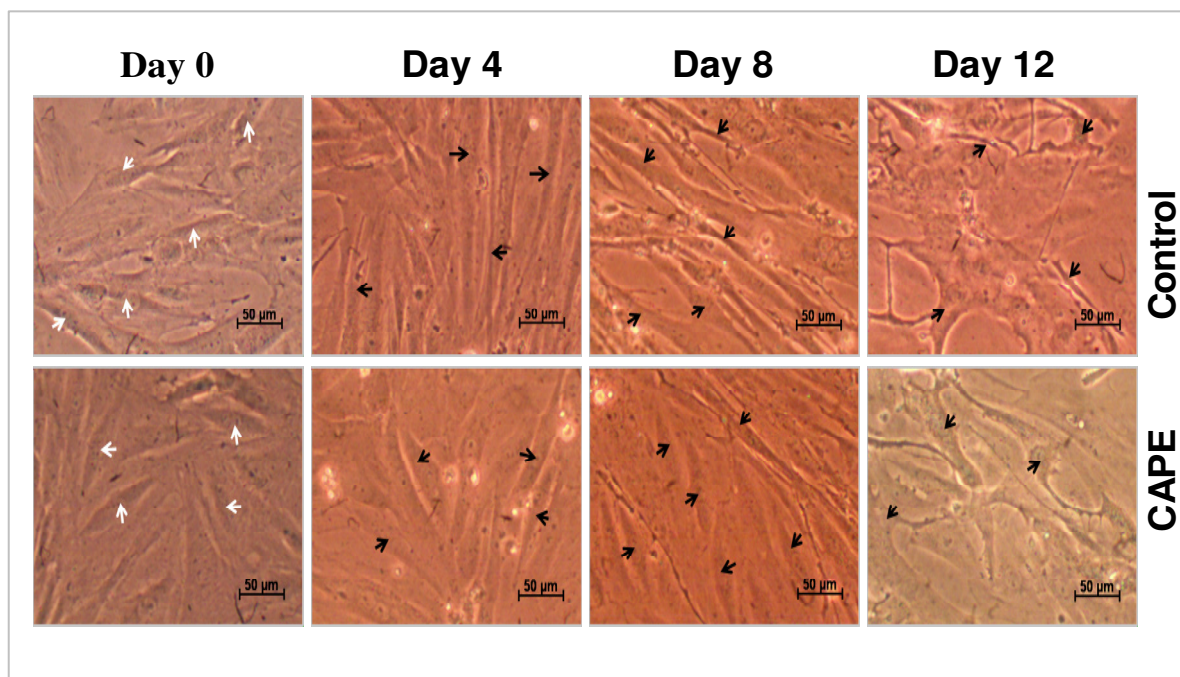
Figure 8.10: Effects of inhibition of NF-kB activation on H9c2 cell differentiation into cardiomyocytes: morphological changes. Control H9c2 cells were cultured in differentiation medium consisting of DMEM supplemented with 1% FBS and 100units/ml penicillin/100µg/ml streptomycin. The differentiation medium of treated cells was further supplemented with 30µM of CAPE. Incubation of H9c2 cells with CAPE, an inhibitor of the translocation of NF-kB into the nucleus, significantly inhibited H9c2 cell differentiation (i.e. cell alignment, elongation and myotube formation) into cardiomyocytes. CAPE treated H9c2 cells largely maintained their normal single cell and spindle shaped morphology (shown by black arrows in day 4 to day 12 NF-kB-treated cells). In contrast, control H9c2 cells showed the usual morphological changes (shown by black arrows in day 4 to day 12 control cells). Both control cells and NF-kB-treated cells showed the normal single cell morphology (shown by white arrows) at day 0. The photographs were taken under 200X magnification and are representative of at least three independent differentiation experiments.
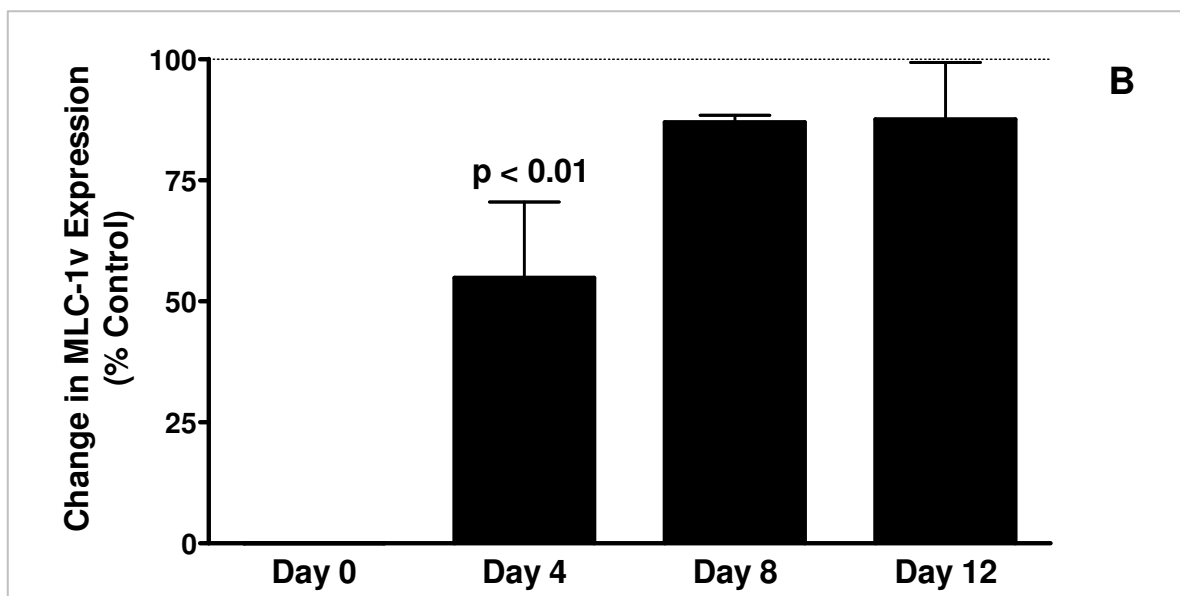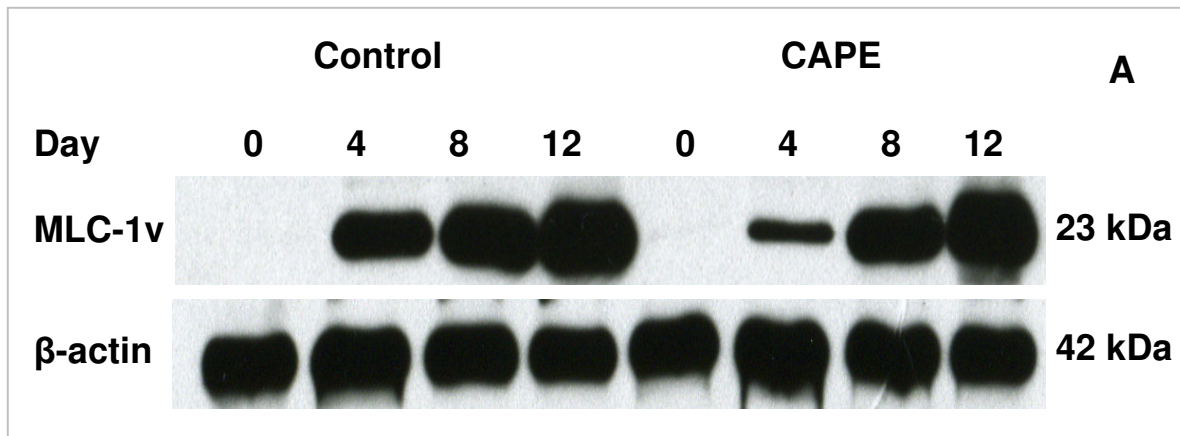
Figure 8.11: Effects of inhibition of NF-kB activation on H9c2 cell differentiation into cardiomyocytes: cardiac ventricular myosin light 1 expression. In agreement with the lack of morphological changes observed in CAPE treated H9c2 cells, western blot analysis of MLC-1v revealed that expression of this protein was also suppressed by CAPE in comparison to the control expression (panel A). The blot is representative of at least three differentiation experiments. The densitometric data (mean ± SD), from three independent experiments, show the time dependent changes in MLC-1v expression in CAPE treated H9c2 cells (panel B).

8.3.2 Viability of H9c2 cells in 30µM of NF-kB activation inhibitor CAPE

The 30µM of CAPE, used our experiments that were designed to investigate the effects of the inhibition of NF-kB activation on H9c2 differentiation into cardiomyocytes was not toxic to the cells. The average viability was about 89% of the viability determined in the control cells. Twenty four hours after the introduction of CAPE to the cells, there was no statistically significant difference between the viability calculated in control H9c2 cells and CAPE treated cells ($p > 0.05$) (Figure 8.12). By 48 hours, the maximum length of time for which the cells were exposed to CAPE, there was a slight reduction in viability of CAPE treated cells ($p < 0.05$). The viability of CAPE cells at this time was about 73% of the viability determined in the control cells.
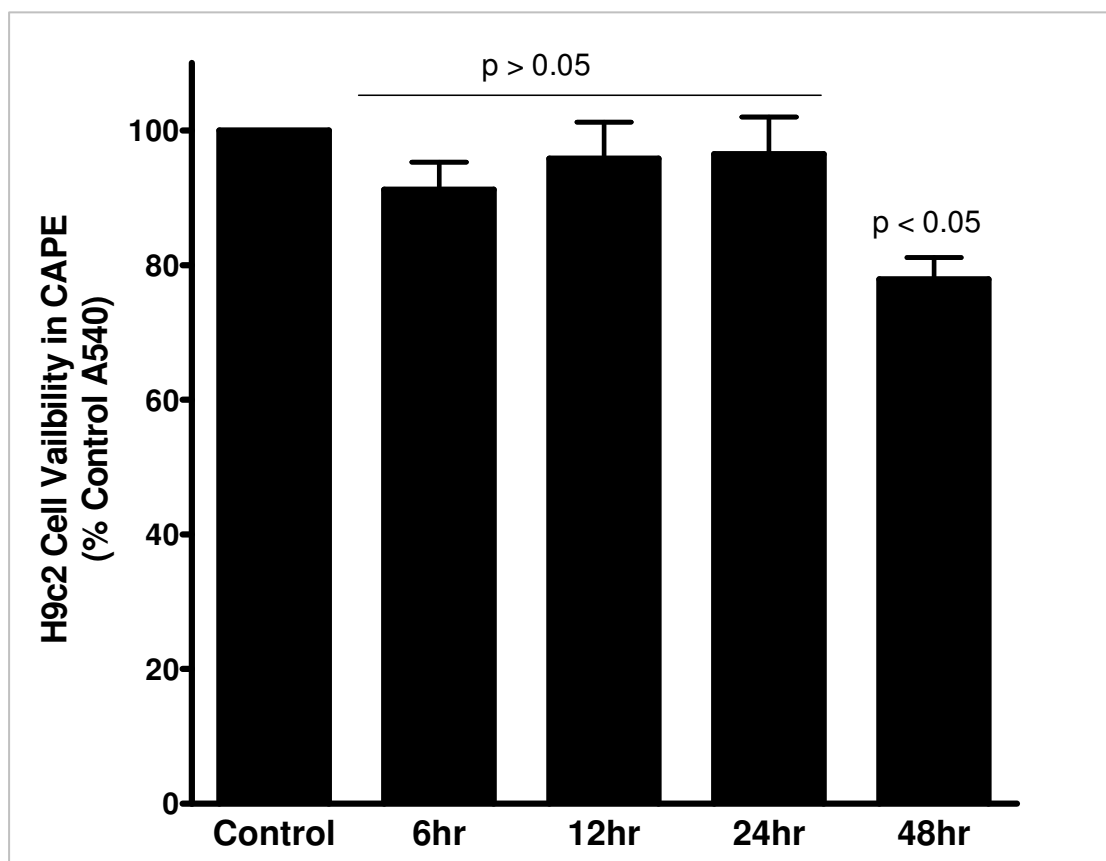
Figure 8.12: The viability of H9c2 cells in the presence of the NF-kB activation inhibitor, CAPE. H9c2 cells were seeded in 6-well plates in differentiation medium consisting of DMEM supplemented with 1% FBS and 100units/ml penicillin/100µg/ml streptomycin and 30µM of CAPE as described in the methods section above (Section 4.123). The MTT assay was carried out at the end of the treatment. Twenty four hours post CAPE, there was no difference in viability between control and CAPE treated cells ($p > 0.05$). A slight reduction in cell viability ($p < 0.05$) was seen at 48 hours. The data presented in the graph are the mean ± SD from three independent experiments.

8.4 DISCUSSION

There is a large amount of data on the role played by cardiac transcription factors in the differentiation process and this includes extended studies on the GATA factors (GATA 4, 5 and 6) (Laverriere *et al.*, 1994; Molkentin *et al.*, 1997; Murphy *et al.*, 1997; Di Lisi *et al.*, 1998; Charron *et al.*, 1999; McBride and Nemer, 2001), Nkx2.5 (Bodmer, 1993; Biben *et al.*, 1997; Tanaka *et al.*, 1999; Bruneau *et al.*, 2000; Jamali *et al.*, 2001; Lee *et al.*, 2004; Zaffran and Frasch, 2005) and T-box factors (Chapman *et al.*, 1996; Li *et al.*, 1997; Bruneau *et al.*, 1999; Bruneau *et al.*, 2001; Jerome and Papaioannou, 2001; Habets *et al.*, 2002; Christoffels *et al.*, 2004; Singh *et al.*, 2005).   However there is very little clear information on the role of NF-kB during the differentiation of stem cells into cardiomyocytes. NF-kB, a ubiquitous transcription factor, is present in most cells and   plays key roles in many cellular processes including cell proliferation and growth (Guttridge *et al.*, 1999; Hinz *et al.*, 1999; Preciado *et al.*, 2005), apoptosis (Wang *et al.*, 1998; Kaltschmidt *et al.*, 1999; Li *et al.*, 2004; Tse *et al.*, 2007) and in disease processes including inflammation (Tsao *et al.*, 1997; Miagkov *et al.*, 1998).

As far as we are aware, our data are the first to demonstrate a direct requirement of NF-kB activation for cardiac differentiation as demonstrated by the lack of differentiation of H9c2 cells into cardiomyocytes in the presence of the NF-kB inhibitor CAPE. CAPE is a potent and specific inhibitor of NF-kB activation (Natarajan *et al.*, 1996). CAPE was used at 30uM concentration and the MTT data showed that this compound was not markedly toxic at this concentration. Thus the effects of CAPE on the cell differentiation may be

mostly likely due to the inhibition of NF-kB signalling by blocking NF-kB translocation into the nucleus.

Our data showed that blocking NF-kB activation with CAPE delayed the onset of the differentiation of H9c2 cells into cardiomyocytes. Inhibition of H9c2 differentiation into cardiomyocytes as monitored by the morphological changes was evident upto day 8. While MLC-1v levels in CAPE treated cell recovered by day 8 and day 12 (the last day for this experiment), they were however never equal to the levels seen in the control cells. Our data therefore suggest that activation of NF-kB may support H9c2 cell differentiation into cardiomyocytes.

The suggestion by our data that NF-kB may have a role to play in H9c2 cell differentiation into cardiomyocytes is in agreement with recent emerging evidence that suggest positive roles for NF-kB in the differentiation of various other cells including osteoclast differentiation (Abu-Amer, 2001; Vaira *et al.*, 2008), the differentiation of HL-60 cells into monocytes (Tse *et al.*, 2007), neuronal differentiation of neuroblastoma cells (Feng and Porter, 1999), PC12 cells (Foehr *et al.*, 2000) and P19 stem cells (Liu *et al.*, 2004).

While our data and the reports mentioned above from other studies suggest a positive role for NF-kB in cell differentiation, other studies suggest that NF-kB may be a negative regulator of myogenesis due to its ability to promote cell proliferation or growth activity (Lehtinen *et al.*, 1996; Guttridge *et al.*, 1999; Hinz *et al.*, 1999; Guttridge *et al.*, 2000). NF-kB (p50 subunit and p65

subunit) activates the cyclin D1 promoter (Hinz *et al.*, 1999) and inhibits C2C12 myogenesis by means of its growth-promoting activity through a direct transcriptional regulation of cyclin D1 (Guttridge *et al.*, 1999). The inactivation of NF-kB caused a delayed and reduced expression of cyclin D1 in the G1 phase and also affected pRB phosphorylation and cell cycle progression from G1 to S-phase (Hinz *et al.*, 1999). Cyclins are key regulatory proteins that are involved in the regulation of cell cycle progression. They regulate the cell cycle progression by controlling the activities of cyclin-dependent protein kinases (CDK). Mutated IkB-α, with serine residues (ser32 and ser36) changed to alanines, is not subject to phosphorylation and subsequent degradation following an NF-kB-activating stimulus (Brockman *et al.*, 1995). C2C12 myoblasts containing this mutated IkB-α were found to proliferate slower than control cells and exit the cell cycle faster than control cells when myogenesis was induced   (Guttridge *et al.*, 1999).   Wild-type C2C12 myoblasts were also found to down-regulate the expression of NF-kB before the induction of myogenin and the initiation of myotube formation (Lehtinen *et al.*, 1996). In C2C12 myoblasts, the loss of p50 and p65 NF-kB DNA-binding activity, correlated with myogenic differentiation (Guttridge *et al.*, 1999). The above therefore suggest that NF-kB may negatively regulate myogenesis.

As cell proliferation and cell differentiation appear to be mutually exclusive processes, the growth promoting activity of NF-kB and also its ability to inhibit myogenic transcription factors (Guttridge *et al.*, 2000) may consistent with its role as a negative regulator of myogenesis. If NF-kB is fundamentally a negative regulator of cell differentiation then, CAPE would be expected to

improve H9c2 differentiation into cardiomyocytes. However according to our data CAPE instead blocked H9c2 cell differentiation into cardiomyocytes. Our data may appear to contradict the negative role suggested for NF-kB during cell differentiation, by the other studies (Lehtinen *et al.*, 1996; Guttridge *et al.*, 1999; Hinz *et al.*, 1999; Guttridge *et al.*, 2000). It should however be noted that an exit from the cell cycle is an essential condition for initiating differentiation. Therefore cells that have been induced to differentiate may transiently block NF-kB activation (i.e. transiently remove the growth promoting activity of NF-KB) in order to exit the cell cycle and initiate differentiation. However in the long term, after this transient inactivation of NF-kB, the restored NF-kB activity may be beneficial for the proliferation of the cells that have already differentiated or committed to differentiation. Nuclear factor-kappa B may indirectly promote H9c2 cell differentiation into cardiomyocytes by promoting the survival and proliferation of H9c2 cells that have already differentiated or committed to differentiation.

In an experimental condition, prolonged inhibition of NF-kB activation (for example in the presence of CAPE) may impair the ability of the cells, including those that have become committed to cardiomyocyte differentiation, to proliferate. In such experimental conditions, the inhibition of NF-kB activation may have a long term negative role on cell differentiation, due to its ability to impair cell proliferation, independent of any direct effect on the mechanisms mediating the differentiation. The grow-promoting activity of NF-kB, independent of any possible direct role on the induction of cell differentiation, is very important since cells undergoing differentiation are experiencing

tremendous stress in changing from cell type to another and need mechanisms that can enhance their survival. Thus the activation of NF-kB may be required during the differentiation of H9c2 cells into cardiomyocytes.

8.5 Summary

The time dependent differentiation of the H9c2 cells into cardiomyocytes in 1% FBS culture medium was significantly delayed by the inhibition of NF-kB signalling by CAPE (30µM). CAPE inhibition of H9c2 differentiation was more evident when MLC-1v expression was monitored as marker of H9c2 cell cardiac differentiation.

In conclusion, our data suggest that NF-kB activation may be required for H9c2 cell differentiation into cardiomyocytes. Whether NF-kB has a direct active role in inducing the differentiation of H9c2 cells into cardiomyocytes or that NF-kB promotes the survival of the cells that have already differentiated into cardiomyocytes or committed to cardiac differentiation, need further investigation.

CHAPTER 9.0

GENERAL DISCUSSION

9.1 Establishment of cardiac differentiation models

The aim of this thesis was to investigate the signal transduction pathways that that are involved in the differentiation of stem cells into cardiomyocytes. The first objective was to establish cardiomyocyte differentiation models using H9c2 and P19 stem cells and to use these models to accomplish the specific objectives of the thesis. The specific objectives were the investigation of the roles of (i) nitric oxide, (ii) phosphoinositide 3-kinase (PI3K), (iii) protein kinase C (PKC), (vi) NF-kB and (vii) p38 MAP kinase signalling pathways in the differentiation of stem cells cardiomyocytes. Stem cell research is a new area in our laboratory and the models needed for the studies carried out in this thesis had to be established and validated.

We have established the H9c2 cell line as a model of cardiomyocyte differentiation and used it for the investigation of the roles of various cell signalling pathways in the differentiation of stem cells into cardiomyocytes. The criteria used were relevance of the model to *in vivo* cardiomyocyte differentiation and simplicity in term of model manipulation. Both models met these conditions in term of being relevant and simple.

The H9c2 cell line was chosen because of its relevance to cardiac regeneration and simplicity of use. The H9c2 cell model has also extensively been used by other researchers not only as a cardiomyocyte differentiation model (Kim *et al.*, 1999; Menard *et al.*, 1999; Hong *et al.*, 2001; Kageyama *et al.*, 2002; Giusti *et al.*, 2004; Pagano *et al.*, 2004) but also in the investigation of   other biological processes in cardiomyocytes   (Ekhterae *et al.*, 1999;

Kageyama *et al.,* 2002; Armoni *et al.,* 2005; Gross *et al.,* 2006; Qin *et al.,* 2006; Salvatorelli *et al.,* 2006). The H9c2 cell line is therefore an appropriate model for our studies aimed at investigating the signalling mechanisms that regulate differentiation into cardiomyocytes.

Freshly cultured H9c2 cells in a suspension of complete medium were rounded. When undifferentiated H9c2 cells attach to culture dishes, they grow as spindle-shaped mononucleated cells and did not express cardiac specific markers. In agreement with the lack of morphological changes, undifferentiated cells did not expressed cardiac myosin heavy chain as determined by western blotting. However H9c2 cells cultured in low serum medium differentiate, become elongated and fuse to form multinucleated myotubes and express cardiac specific markers (cardiac myosin heavy chain (α/β-MHC) and cardiac ventricular myosin light chain (MLC-1v).

Despite the advantage of the simplicity of the H9c2 cardiac differentiation model, like most models, is not a perfect substitute for the *in vivo* processes. One of the limitations of the H9c2 model for cardiac differentiation is that the cell line was derived from an embryonic heart. This means that the cardiac lineage commitment may have already taken place. However cardiac specific genes such as that which code for cardiac specific myosin heavy chain and ventricular myosin light chain-1 are not constitutively expressed in undifferentiated H9c2 cells until the cells are induced to differentiate into cardiomyocytes. This makes these cells very relevant for exploring of the

signalling transduction events that drives their differentiation into cardiomyocytes.

The mouse P19 embryonal carcinoma cell line (P19 stem cells) was also developed as a model for stem cell differentiation into cardiomyocytes in parallel with the H9c2 cells. P19 embryonal carcinoma stem cells cultured in normal cell culture grade dishes in complete culture medium whether in the presence or absence of 0.8% DMSO did not form embryoid bodies (EB) but grew as clusters, in a single cell monolayers. These clusters did not differentiate into cardiomyocytes. In contrast, P19 cells cultured in microbiological Petri dishes in complete culture medium, supplemented with 0.8% DMSO, formed EBs. The transfer of EB from Petri dishes to standard tissue culture dishes resulted in cell adhesion, the formation of a two-dimensional monolayer, cell migration and proliferation. The first beating clusters of cardiomyocytes were routinely detected by day 6 or 7. The size and number of beating clusters of cardiomyocytes usually increased up to day 14. There was a high degree of variability both in term of the clusters of beating cells and in the frequency at which they appeared to beat.

The P19 stem cell model, like the H9c2 cell model is relatively simple and can be easily manipulated in experiments. P19 stem cells are pluripotent and can develop into cell types of the three germ layers. With proper care during their routine culture and also during experiments P19 stem cell differentiation into cardiomyocytes appears to be highly regulated and reproducible. Like other stem cell lines, P19 stem cells form embryoid bodies in suspension culture under non-adherent conditions. In the presence of DMSO, P19 stem cell

cardiomyocyte differentiation can be induced during the embryoid body formation.

The major advantage of the P19 cell models is that this pluripotent cell line can be maintained continuously in serum-supplemented cell growth medium without the need for LIF or feeder cells to keep them in the undifferentiated state. This allows stem cell research to be carried out at relatively very low cost in comparison to the use of other stem cells that may need special conditions such as knockout serum, LIF or feeder cells. Another strong point for the use of P19 stem cells is that the cell line is a well-established model of cardiomyocyte differentiation (McBurney *et al.*, 1982; Wobus *et al.*, 1991; Monzen *et al.*, 1999; Naito *et al.*, 2003; Brown *et al.*, 2004; Brewer *et al.*, 2005; Angello *et al.*, 2006). The ability of P19 stem cells to differentiate into cardiomyocytes has been clearly established by the expression of cardiac transcription factors including Nkx2.5 (Skerjanc *et al.*, 1998; Monzen *et al.*, 1999; Wen *et al.*, 2007) GATA-4 (Monzen *et al.*, 1999; Uchida *et al.*, 2007; Wen *et al.*, 2007), MEF2C (Skerjanc *et al.*, 1998; Wen *et al.*, 2007) and other cardiac specific proteins including cardiac troponin I (Choi *et al.*, 2004; Fathi *et al.*, 2008) during the cardiac differentiation of these cells.

In conclusion, models of cardiomyocyte differentiation using P19 cells and H9c2 cells were established. The established cardiac differentiation models were subsequently used in further studies exploring the mechanisms that may regulate stem cell differentiation into cardiomyocytes. New or better models may be used in future experiments. However these models, due to their

simplicity, relevance to cardiac differentiation and relative low cost may still be a good a starting point for researchers that are new to the study of signalling pathways in stem cell differentiation into cardiomyocytes.

9.2 Effects of nitric oxide donors on H9c2 differentiation into cardiomyocytes

We examined the role of nitric oxide (NO) in the differentiation of H9c2 cells into cardiomyocytes using fast NO releasing donors (NOC-5 and SIN-1) and slow NO releasing donors (NOC-18 and SNAP). SIN-1 and NOC-5, markedly delayed the onset of differentiation of H9c2 cells into cardiomyocytes while SNAP and NOC-18, were not as effective in delaying the onset of differentiation or long term differentiation of H9c2 cells into cardiomyocytes.

In parallel with the morphological changes, NOC-5 and SIN-1 also caused a reduction in the expression of MLC-1v in differentiating H9c2 cells. NOC-18 also delayed the onset of H9c2 cell differentiation into cardiomyocytes but to a lesser extent than NOC-5 and SIN-1. Differentiation associated morphological changes in both control and SNAP-treated H9c2 cells were similar. In agreement with the morphological seen in the control cells and SNAP treated cells, MLC-1v levels in SNAP-treated and control H9c2 cells were almost equal. 8-Bromo-cGMP, an analogue of cGMP, appeared to delay the onset of H9c2 cell differentiation into cardiomyocytes. However, like NOC-5 and SIN-1, 8-Bromo-cGMP failed to block the long term differentiation of H9c2 cells into cardiomyocytes. In parallel, expression of MLC-1v was reduced at day 4 but thereafter MLC-1v expression in controls and 8-Bromo-cGMP-treated cells were indistinguishable.

Fast NO releasing donors including NOC-5 ($t_{1/2}$ = 93 minutes) and SIN-1 ($t_{1/2}$ = 40 minutes) release a large part of their NO content in a matter of minutes.

In contrast, slow NO releasing donors including NOC-18 ($t_{1/2}$ = 20 hours) and SNAP ($t_{1/2}$ = 37 hours) release their NO content slowly over time in manner that may resemble the manner that NO is produced by constitutively expressed nNOS and eNOS. The differential affects of fast and slow releasing NO donors on the differentiation of H9c2 cells into cardiomyocytes may be due to their differences in the release of NO.

Excessive NO can disrupt cellular functions and also induce oxidative stress via events that lead to formation of superoxide, peroxynitrite, hydroxyl radical and nitrite radicals, all of which can cause damage to cells and tissues. Nitric oxide released by NOC-18 and SNAP   may protect the cells against apoptosis (Kim *et al.*, 1997; Hebestreit *et al.*, 1998) by the suppression of pro-apoptotic ghenes (Thippeswamy *et al.*, 2001) and induction of anti-apoptotic genes (Ciani *et al.*, 2002).   NOC-18 and SNAP may therefore promote cell survival and contribute to differentiation of stem cells into cardiomyocytes. In contrast, NOC-5 and SIN-1, by their excessive production of NO, may induce apoptosis and therefore have negative effects on stem cells into cardiomyocytes.

Our findings with 8-Bromo-cGMP is in agreement with the data obtained with some of the NO donors used in that treatment of cells with 8-Bromo-cGMP also delayed the onset of differentiation. The 8-Bromo-cGMP data also suggest role of endogenous NO in H9c2 cell differentiation. The fact that NO (at least NO from fast-releasing NO donors) and also 8-Bromo-cGMP inhibited rather than enhance the differentiation process, at least over the

initial stages of the process, is clearly contradictory to the other studies which show a positive regulatory role of NO in the differentiation of stem cells into cardiomyocytes and also into other lineages. Our studies, as they stand, give little indication as to conflicting findings and further experiments are clearly needed especially in real stem cells or stem cell lines. These studies although planned were not carried out because of time constraints but are clearly needed.

In conclusion, our data would suggest that generation of cardiomyocytes from H9c2 cells may be negatively regulated by endogenous NO and this may occur through generation of cGMP. Our data are however preliminary and further studies are needed to confirm these findings. Moreover, a detailed study of the full signalling pathway activated by NO may prove important in understanding how this molecule regulates differentiation.

9.3 Effects of PKC inhibition on H9c2 and P19 stem cell differentiation into cardiomyocytes

The inhibition of the PKC signalling pathway, using BIM-I (10µM), caused marked inhibition of differentiation associated morphological changes (i.e. cell elongation, fusion and myotube formation) that usually accompany H9c2 cell differentiation into cardiomyocytes. Control H9c2 cells cultured in the absence of BIM-I underwent differentiation and formed myotubes. In agreement with the lack of morphological change observed in BIM-I-treated H9c2 cells, the expression of a cardiac specific maker (MLC-1v protein) in these cells was completely blocked up to day 4 and then significantly inhibited thereafter. This suggests that the activation of PKC may have fundamental roles in the events that are responsible for H9c2 cell differentiation into cardiomyocytes.

Similarly PKC inhibition, with BIM-I (10µM), blocked the differentiation of P19 stem into beating cardiomyocytes. P19 stem cells cultured in 0.8% DMSO in the absence of BIM-I differentiated into beating cardiomyocytes. In contrast, P19 stem cells treated with 0.8% DMSO in the presence BIM-I (10µM) did not differentiate into beating cardiomyocytes. In agreement with the lack of appearance of beating cardiomyocytes in BIM-I treated P19 stem cells, the long term expression of cardiac restricted protein troponin I (TnI) was inhibited.

Our data also suggested that the PKC signalling events that are responsible for mediating P19 stem cell differentiation into cardiomyocytes are switched on very early following DMSO treatment since the PKC inhibitor, BIM-I was

only able to block P19 stem differentiation into beating cardiomyocytes only if added at the EB formation stage (i.e. at the time of initiating of P19 cell cardiac differentiation). BIM-I application to P19 stem cells during the four days of EB formation completely blocked cell differentiation into beating cardiomyocytes. However the treatment of P19 stem cells with BIM-I, four days after DMSO induced cardiac differentiation had started, had no effect on the ability of the cells to differentiate into beating cardiomyocytes.

The cross-talk data, discussed below, also showed that PKC may regulate the PI3K signalling pathway, another key signalling pathway that has been widely reported to be required for stem cell differentiation into cardiomyocytes and other cell types. This may however have no direct relevance to the differentiation process since PKC enhanced the phosphorylation of Akt, a protein that is thought to be central to many of PI3K biological effects, but blocked differentiation. This information suggests that while it is desirable to target and investigate the role of specific pathways individually, studies aimed at identifying points of convergence or divergence and indeed cross-talk between pathways are needed in order to get a clear and better understanding of the processes that regulate differentiation.

In conclusion, our data therefore suggest that the activation of PKC signalling pathway may be required for the differentiation of H9c2 cell and P19 stem cell differentiation into cardiomyocytes. The data also suggest that the activation of PKC signalling pathway may be required during the early stages of P19 stem cell differentiation into cardiomyocytes.

9.4 Effects of p38 MAPK inhibition on H9c2 and P19 stem cell differentiation into cardiomyocytes

The differentiation of H9c2 cells into cardiomyocytes was blocked by the inhibition of p38 MAPK signalling using SB203580 (10µM). However SB203580 had little or no effect on the differentiation associated morphological changes in H9c2 differentiating into cardiomyocytes. In both control cell and SB203580-treated cells, morphological changes were visible. In contrast to the apparent lack of inhibition of morphological changes in SB203580-treated H9c2 cells, the expression of MLC-1v was decreased in these cells. Our data therefore suggest that p38 MAPK signalling pathway may have a role in the differentiation of H9c2 cells into cardiomyocytes.

In contrast H9c2 cells, SB203580 markedly inhibited the expression of cardiac restricted protein troponin (TnI) and also blocked the cell differentiation into beating cardiomyocytes. The small expression of TnI seen in control P19 stem cells, at the time of EB transfer to cell culture plates, increased upto day 8. In comparison to control cells, TnI levels in SB203580-treated P19 stem cells were reduced. The inhibited or low levels of TnI detected in SB203580-treated cells were in agreement with the lack of appearance of beating cardiomyocytes. It was also observed that SB203580 blocked P19 stem cell differentiation into beating cardiomyocytes, when added to the cells during the EB formation stage. In contrast, SB203580 failed to block the differentiation of P19 stem cells into beating cardiomyocytes, when the cells were treated, with this compound, four days after the initiation of DMSO-induced cardiac differentiation. Our data therefore suggest that p38 MAPK signalling pathway signalling has a role in the differentiation of P19 stem cells into

cardiomyocytes and also that this signalling pathway is switched on during the early stages of P19 stem cell differentiation into cardiomyocytes.

While our data suggest that p38 MAPK signalling pathway may have roles to play in the differentiation of both H9c2 cells and P19 stem cells into cardiomyocytes, the data also showed that p38 MAPK signalling may have a more prominent role in the differentiation of P19 stem cells into cardiomyocytes than in the differentiation of H9c2 cells into cardiomyocytes. SB203580 ($10\mu M$) completely block the differentiation of P19 stem cells into beating cardiomyocytes and also markedly reduced the expression of troponin I. In contrast SB203580 failed to block the differentiation-induced morphological changes (i.e. cell elongation, cell fusion and formation of myotubes) in H9c2 cells but did delay the expression of MLC-1v during the differentiation of H9c2 cells into cardiomyocytes. The findings that SB203580 blocked both the differentiation of P19 stem cells into beating cardiomyocytes and the expression of troponin I (in P19 cells), while in H9c2 cells, only blocked the expression of MLC-1v but not   the differentiation-induced morphological changes of H9c2, may imply that p38 MAPK have a lesser role in these differentiation-induced morphological changes. This question of whether this is the case was not investigated and it may need further investigation in future studies. Nevertheless, our data suggest that p38 MAPK may be involved in the cascade that drives the differentiation of both cell types into cardiomyocytes. P19 stem cells are undifferentiated cells without any prior cardiac lineage specification. In contrast, H9c2 cells were derived from an embryonic rat heart.   The apparent lesser effect of SB203580 on the

differentiation of H9c2 cells into cardiomyocytes and the inability of SB203580 to block P19 cell differentiation into   cardiomyocytes, when added to P19 stem cells four after DMSO-induced differentiation, may imply that p38 MAPK activation may be more relevant at early stages of cardiac differentiation.

In conclusion, our data therefore suggest that the activation of p38 MAPK signalling pathway may be an essential signalling event during the differentiation of H9c2 cells and P19 stem cells into cardiomyocytes. The data also suggest that the p38 MAPK exerts its effects at the early stages of P19 stem cell differentiation into cardiomyocytes.

9.5 Effects of the PI3K Inhibition on P19 stem and H9c2 Cell differentiation into cardiomyocytes

Our data showed that the activation of the PI3K signalling pathway may be essential for differentiation of both H9c2 and P19 stem cells into cardiomyocytes. The differentiation of both P19 stem cells and H9c2 cells into cardiomyocytes were significantly inhibited by LY294002 (20µM) treatment of these cells.

The inhibition of PI3K, using LY294002 (20µM), completely blocked the differentiation of H9c2 cells into cardiomyocytes (as as determined by the usual morphological changes of cell elongation, fusion and myotube formation). LY294002 treated H9c2 cells largely maintained their normal single cell and spindle shaped morphology which was in contrast to control H9c2 cells which showed the usual morphological changes. In agreement with the lack of morphological changes observed in LY294002 treated H9c2 cells, the expression of cardiac specific ventricular myosin light chain 1 (MLC-1v protein) was significantly inhibited. Our data therefore suggest that activation of the PI3K signalling pathway is required for H9c2 cell differentiation into cardiomyocytes.

Treatment of H9c2 cells with 1% FBS induced a rapid increase in phosphorylation (at serine 473) but this differentiation associated increase in Akt phosphorylation was completely abolished by 20µM of LY294002. This differentiation associated increase in Akt phosphorylation was significantly enhanced by BIM-I (10µM) but significantly reduced by SB203580 (10µM).

Our data therefore show that PI3K is activated during 1% FBS-induced differentiation of H9c2 cells into cardiomyocytes and also that PKC and p38 MAPK may modulate Akt phosphorylation at serine 473.

P19 stem cultured in the presence of 0.8% DMSO but in the absence of LY294002 (absence of PI3K inhibition) differentiated into beating cardiomyocytes. However PI3K inhibition, with LY294002 (20μM), completely blocked the DMSO-induced differentiation of P19 stem cells into beating cardiomyocytes. The expression of cardiac specific troponin I was also reduced in P19 stem cells treated with LY294002.

When DMSO was allowed to initiate P19 stem cell differentiation, for four days before the addition of LY294002 to the cells, LY294002 completely failed to block the cell differentiation into beating cardiomyocytes. Our data therefore suggest that activation of the PI3K signalling pathway is required for P19 stem cell differentiation into cardiomyocytes. In addition, like the PKC and p38 MAPK data, these results suggest that PI3K signalling is required during the early stages of P19 stem cell differentiation into cardiomyocytes.

Akt activation by sequential phosphorylation is a critical event in the PI3K signalling and is generally thought to be a central event in most of the effects mediated by PI3K activation (Ahmed *et al.*, 1997; Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Eves *et al.*, 1998; Gingras *et al.*, 1998). Our data showed that while PI3K is required for stem cell differentiation into cardiomyocytes, Akt phosphorylation is not sufficient to induce cardiomyocyte

differentiation since enhanced Akt activation failed to induce H9c2 cell cardiac differentiation. Our cross-talk studies with PKC showed that PKC inhibition enhanced Akt phosphorylation at serine 473 (i.e. Akt activation). As already discussed (Chapter 7), the enhanced Akt activation by PKC inhibition cannot be explained by non-specificity BIM-I (10μM). Other studies using different PKC inhibitors including Ro-31-8220 (bisindolylmaleimide IX), bisindolylmaleimide VIII and LY 379196 (another bisindolylmaleimide) have shown that PKC inhibition increased Akt activation (Wen *et al.*, 2003; Edling *et al.*, 2007). Akt phosphorylation, in HEK293 cells over-expressing Akt (i.e. cells that show higher levels of Akt activation), was inhibited by phorbol 12-myristate-13-acetate (PMA) a potent PKC activator (Wen *et al.*, 2003).

The enhancement of Akt activation by PKC inhibition may therefore suggest that PKC modulates Akt activity. However, since PKC inhibition also inhibited the differentiation of both H9c2 cells and P19 stem cells into cardiomyocytes. These data suggest that the possible crucial role of PI3K in cardiac differentiation may be carried out via mechanism(s) that is/are independent of Akt activation since enhancement of Akt phosphorylation failed to enhance the differentiation of either P19 stem cell or H9c2 cells into cardiomyocytes. However, PI3K inhibition (i.e. prevention of Akt activation) by LY294002 also blocked H9c2 and P19 stem cell differentiation into cardiomyocytes. The data suggest that the differentiation of H9c2 and P19 stem may be dependent on PI3K signalling, but independent of Akt activation. The data further point to a possible target downstream of PI3K through which the effects of PI3K on cardiomyocyte differentiation may be channelled independently of Akt. This

possible key downstream target is currently unknown and warrants further investigation.

The fact that PKC inhibition enhanced the activation of Akt but at the same time also inhibited the cardiac differentiation of both H9c2 cells and P19 stem cells may suggest that, during cardiac differentiation, there is no cross-talk between PI3K and PKC, at least not at a level that involves Akt. The mechanism(s) by which BIM-I promotes Akt phosphorylation is not clear. The enhancement of Akt by the PKC specific inhibitor BIM-I suggests that PKC may play a negative role in the regulation of PI3K pathway. PKC acts downstream of both PI3K and PDK1 (Chou *et al.*, 1998; Dutil *et al.*, 1998; Balendran *et al.*, 2000). Therefore the potential negative regulation of the PI3K signalling pathway by PKC cannot be attributed to a direct effect on either PI3K or PDK1.

Our data give ground for possible speculation about how BIM-I (i.e. PKC inhibition) might have increased Akt phosphorylation at serine 473. PKC could inhibit Akt activation by targeting PDK2, an elusive and currently unknown kinase that is thought to be responsible for Akt phosphorylation at serine 473. Available evidence also suggests that a phosphatase known as PHLPP (PH domain leucine-rich repeat protein phosphatase) selectively and specifically dephosphorylates Akt at Ser473 (Gao *et al.*, 2005). Therefore another possible mechanism by which PKC inhibition might lead to increase Akt phosphorylation may be that PKC is an activator (i.e. positive regulator) of PHLPP "a putative phosphatase" that may be responsible for

dephosphorylating AKt at serine 73 back to the baseline level, following a phosphorylation event. If this is the case then the ability of BIM-I to increase Akt phosphorylation could be explained by the fact that BIM-I reduces the ability of PKC to activate that "putative phosphatase", thus limiting the ability of this "phosphatase to dephosphorylate AKt-ser473, hence the increased AKt-ser473 levels in presence of BIM-I. However it is not known whether PKC can regulate PHPLP.

Our data suggest possible cross-talk between PI3K and p38 MAPK. While PDK1 has been cloned and sequenced (Alessi *et al.*, 1997a) and it is established that PDK1 phosphorylates Akt at threonine 308 (Alessi *et al.*, 1997a; Alessi *et al.*, 1997b), the identity of the kinase that is responsible for Akt phosphorylation at serine 473 remains unknown. Our data showed reduced phosphorylation of Akt at serine 473 when the p38 MAPK inhibitor SB203580 (10μM) was added to the cells. While there is growing list of possible PDK2 candidates including p38 MAPK, the identity of PDK2 remains elusive. The question of whether p38 MAPK has a PI3K-dependent and phosphoinositide-dependent activity that can phosphorylate Akt at serine 473 or whether p38 MAPK and PDK2 are one and the same kinase remains. This needs further investigation.

In conclusion, the activation of the PI3K signalling pathway is required for P19 stem cells and H9c2 cell differentiation into cardiomyocytes. The activation of PI3K signalling is required during the early stages of stem cell differentiation into cardiomyocytes. Our data also suggest that PI3K positively regulates

stem cell differentiation into cardiomyocytes not through Akt activation, but through a yet unknown signalling mechanism that is independent of Akt activation.

Our data suggest no possible cross-talk between PI3K and PKC, at least not at a level that involves Akt. The data also suggest that PKC may also be a negative regulator of Akt phosphorylation at serine 473 either via an unknown PDK2 or via positive regulation of a possible "phosphatase" that is responsible for the dephosphorylation of Akt at serine 473. It is also a suggestion from our data that there may be a possible cross-talk between PI3K and p38 MAPK and that p38 MAPK may also have a PI3K-dependent and phosphoinositide-dependent activity with the ability to phosphorylate Akt at Serine 473 or p38 MAPK and PDK2 may be one and the same kinase.
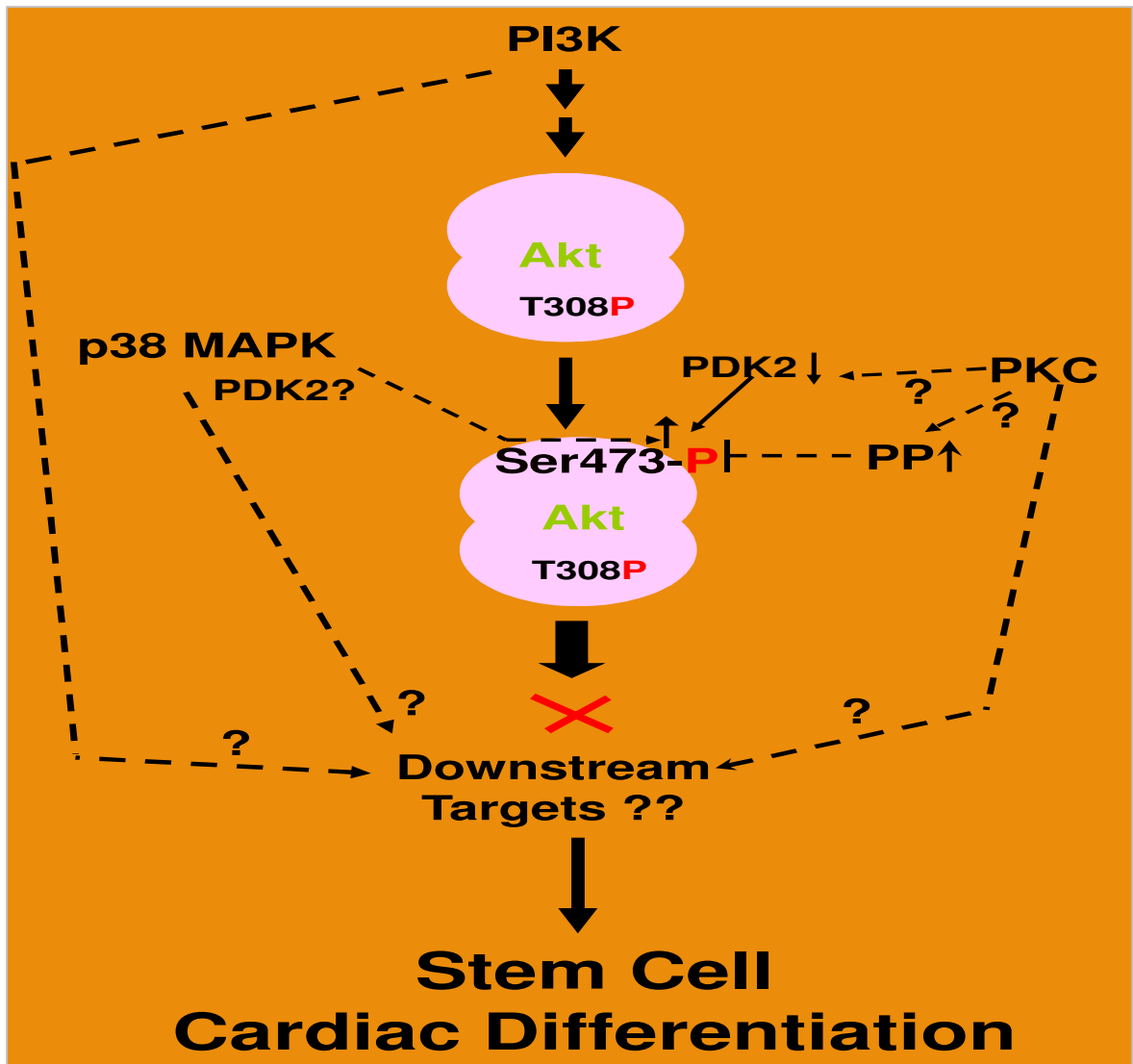
Figure 9.10: PI3K, PKC and p38 MAPK regulate stem cell differentiation into cardiomyocytes through possible Akt-independent mechanisms. PI3K activation causes the Akt activation (phosphorylation at both threonine 308 and serine 473) but PI3K may regulate stem cell differentiation into cardiomyocytes through a possible Akt-independent mechanism. Both PKC and P38 MAPK may modulate the phosphorylated states of Akt, However, the inhibition of PKC and p38 MAPK also blocked the differentiation of H9c2 cells and P19 stem cells into cardiomyocytes. Therefore PI3K, PKC, p38 MAPK may regulate H9c2 cells and P19 stem cell differentiation into cardiomyocytes through a possible Akt-independent mechanism(s). The possible Akt-independent mechanisms or downstream target, through which PI3K, PKC and p38 MAPK may regulate H9c2 cell and P19 stem cell differentiation into cardiomyocytes is/are currently unknown

9.6 Effects of the inhibition of the activation of NF-kB on H9c2 cell differentiation into cardiomyocytes

The differentiation of H9c2 cells into cardiomyocytes, in the presence of 1% FBS, was significantly delayed by the inhibition of NF-kB signalling by CAPE (30µM). CAPE inhibition of H9c2 differentiation was more evident when MLC-1v expression was monitored as marker of H9c2 cell cardiac differentiation.

There is a vast amount of data on the roles of cardiac transcription factors but very little information on other non cardiac transcription factors such as NF-κB on the differentiation of stem cells into cardiomyocytes. Our findings are the first to suggest a direct requirement for NF-kB activation for cardiac differentiation as shown by the lack of differentiation of H9c2 cells into cardiomyocytes in the presence of CAPE (30µM). Our data showed that blocking NF-kB activation using CAPE delayed the onset of the differentiation of H9c2 cells into cardiomyocytes.

NF-kB plays a major role in promoting cell survival. It promotes cell proliferation through a direct transcriptional regulation of cyclin D1 (Guttridge *et al.*, 1999). The inactivation of NF-B caused a delayed and reduced expression of cyclin D1 in the G1 phase and also affected pRB phosphorylation and cell cycle progression from G1 to S-phase (Hinz *et al.*, 1999).

As cell proliferation and cell differentiation appear to be mutually exclusive processes. NF-kB, due to its growth-promoting activity, may have negative

effect on cell differentiation (Lehtinen *et al.*, 1996). But in the long term, NF-kB may indirectly promote H9c2 cell differentiation into cardiomyocytes by promoting the survival of H9c2 cells that have already differentiated or become committed to differentiation into cardiomyocytes. This role of NF-kB is very important since cells undergoing differentiation are experiencing the stress of changing from one cell type to another and need mechanisms that can enhance their survival. Our data constitute important preliminary findings that warrant further investigations.

In conclusion, our data suggest that NF-kB activation may be required for H9c2 cell differentiation into cardiomyocytes. Whether NF-kB has a direct active role in inducing the differentiation of H9c2 cells into cardiomyocytes or that NF-kB promotes the survival of the cells that have already differentiated into cardiomyocytes or committed to cardiac differentiation, need further investigation.

9.7 CONCLUSION

H9c2 and P19 stem cell lines are relevant and complementary models for the investigation of the intracellular signalling mechanisms that mediate stem cell differentiation into cardiomyocytes. The PKC, PI3K, p38 MAPK and NF-kB signalling pathways are relevant and are involved in the differentiation of stem cells into cardiomyocytes as inhibition of these pathways was accompanied by inhibition of differentiation associated morphological changes (i.e. cell alignment, elongation and fusion to form myotubules in H9c2 cells), inhibition of functional or physiological changes (the appearance of beating cardiomyocytes in P19 stem cells) and inhibition of associated expression cardiac specific protein markers. The next logical stem step is to determine the exact role of these pathways in the initiation of stem cell cardiac differentiation. This would require the design of the experiments that will examine the direct effects of the inhibition or enhancement of these pathways on the expression of cardiac transcription factors (e.g. GATA-4, Nkx2.5). These further studies will establish whether they pathways are core key players or whether their role is the facilitation   of stem cardiac differentiation programme once it has been initiated.

Our nitric oxide data suggested that NO delayed the onset of differentiation. This is clearly contradictory to the other studies cited which show a positive regulatory role of NO in the differentiation of stem cells not only into cardiomyocytes but also into other lineages. Our NO studies, as they stand, give little indication as to the reason for these conflicting findings and further

experiments, including concentration-effects curves, are clearly needed

especially in real stem cells or stem cell lines.

FURTHER WORK

Further work

This thesis produced important new data which in turn give rise to new questions (which in a year or so won't seem to be important) that should be resolved in further work.

1. Our data suggest that the PI3K, PKC and p38MAPK pathways may be required for stem cell differentiation into cardiomyocytes. These pathways were implicated by monitoring terminal cardiac differentiation (i.e. beating cells or cardiac structural proteins). They question of whether these pathways have direct active roles in inducing the differentiation of stem cells into cardiomyocytes or whether they just facilitate the differentiation of stem into cardiomyocytes needs to be resolved. For each of the signalling pathways implicated a direct connection between their activation and the specific induction of stem cell cardiac lineage commitment needs to be established by monitoring the specific induction of the expression of early cardiac genes and cardiac transcription factors.

2. Our data suggest a role for NF-kB in the differentiation of H9c2 cells into cardiomyocytes. These are new data showing the involvement of this ubiquitous but non-cardiac transcription factor in cardiac differentiation. These data should be reproduced in stem cells or stem cell lines using the same criteria suggested for PI3K, PKC and p38 MAPK.

3. If the results from 1 or 2 are positive for any of pathways implicated (i.e. PI3K, PKC, p38MAPK or NF-kB) then to get better insight on the role of the implicated pathway, these studies should be extended to the examination of the connection of that pathway to major cardiac differentiation related pathways such as the BMP and Wnt signalling pathways.

4. For each of the pathways implicated, specific and selective activation should be carried out, in stem cells or stem cell lines, to complement the inhibition data obtained.

5. Our nitric oxide studies were carried out in H9c2 cells and failed to resolve previous conflicting findings. Further experiments should be carried out in stem cells or stem cell lines to resolve these conflicting results by monitoring the specific induction of the expression of early cardiac genes and cardiac transcription factors.

6. While our data suggest that Akt phosphorylation at serine 473 may not be of major relevance to the differentiation of stem cells into cardiomyocytes since PI3K, p38 MAPK and PKC are implicated in the differentiation of stem into cardiomyocytes then it may be worthwhile to use other p38 MAPK and PKC inhibitors (preferably more specific and selective)   to reproduce the inhibition of PI3K   (inhibition of Akt

phosphorylation at serine 473) by the inhibition of p38 MAPK and the activation of PI3K by the inhibition of PKC.

7. More specific and selective inhibitors for the various PKC isoforms should be examined in future studies in order to get a better insight of the role of individual PKC isoforms in the differentiation of stem cells into cardiomyocytes.

8. The concentrations of the various inhibitors, used in our studies, are in agreement with published concentrations. However in future studies, concentration-effect experiments should be carried out to examine the specificity of drugs used in our studies.

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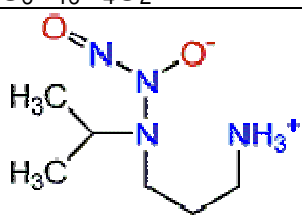
APPENDIX

APPENDIX I: CELL LINES

Appendix I (a):H9c2 (2-1) Cell Line

Cell Line Name	H9c2 (2-1)
Description	Rat BDIX heart myoblast . Cell form myotubes in 1% serum (FBS)
Species	Rat
Tissue	heart
Morphology	Myoblast
Growth Mode	Adherent
Culture Medium	DMEM + 2mM Glutamine + 10 % FBS
Subculture Routine	Split cells at 70-80% confluency using trypsin/EDTA. Important to passage at sub-confluence to avoid spontaneous cell fusion to form myotubes
Supplier	European Collection of Cell Cultures (ECACC) http://www.hpacultures.org.uk/collections/ecacc.jsp
Catalogue Number	88092904

Appendix I (b):P19 Embryonal Carcinoma Stem Cell Line

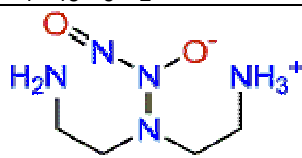|	
Cell Line Name	P19 embryonal carcinoma stem cells
Cell Line Description	The mouse teratocarcinoma pluripotent cell line was derived from an embryonal carcinoma induced in a C3H/He strain mouse. The P19 cells c differentiate into neuronal and glial cells in the presence of retinoic acid. The P19 cell Aggregates differentiate into cardiac and skeletal muscle in the presence of DMSO
Species	Mouse
Tissue	embryo
Growth Mode	Adherent
Culture Medium	Alpha MEM + 2mM Glutamine + 10 % FBS
Subculture Routine	Split cells at 70-80% confluency using trypsin/EDTA.
Karyotype:	Euploid
Supplier	European Collection of Cell Cultures (ECACC) http://www.hpacultures.org.uk/collections/ecacc.jsp
Catalogue No	95102107

APPENDIX II: CELL CULTURE REAGENTS

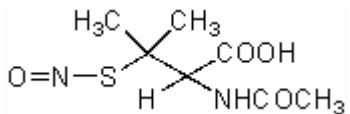| Reagents	Description	Storage	Supplier
Minimal Essential Medium (MEM) Alpha Medium (α-MEM) (Product code:22561021)	Without but Ribonucleosides and Deoxyribonucleosides but Contains Earle's Salts and L-glutamine	Fridge 4°C	Invitrogen, UK http://www.invitrogen.com
Dulbecco's Modified Eagle Medium (D-MEM) (1X) (Low Glucose) (Product code: 22320022)	Contains L-Glutamine, 1000 mg/L D-Glucose, Sodium Pyruvate, 25 mM HEPES.	Fridge 4°C,	Invitrogen, UK http://www.invitrogen.com
Foetal Bovine Serum (Product code: 10500064)	Heat inactivated Foetal Bovine Serum	Stock: -20°C,	Invitrogen, UK http://www.invitrogen.com
Penicillin-Streptomycin, liquid (10,000 units penicillin;10,000 µg streptomycin) (Product code: 15140122)	Contains 10,000 units of penicillin and 10,000 µg of streptomycin kills Gram-ve and +ve bacteria	Stock: -20°C,	Invitrogen, UK http://www.invitrogen.com
Trypsin, 0.5% (10x) with EDTA 4Na, (Product code: 15400054)	Contains 5.0 g/L of trypsin, 2.0 g/L of EDTA.4Na, 8.5 g/L of NaCl	Stock : -20°C, dilutedstock solution Aliquots: Fridge 4°C,	Invitrogen, UK http://www.invitrogen.com

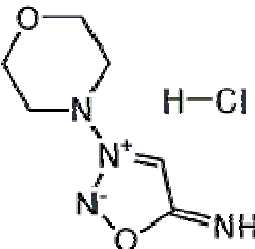# APPENDIX III: NITRIC OXIDE DONORS AND 8-BROMO-CGMP

Appendix III (a): NOC-5	
Alternate Names	3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine
Size:	10 mg
Description	A nitric oxide (NO) donor. Half-life ($t_{1/2}$) of NO release is 93 min (PBS, pH 7.4, 22°C).
CAS Number	146724-82-5
Molecular Weight	176.2
Molecular Formula	$C_6H_{16}N_4O_2$
Structure:	
Solubility	H_2O or NaOH (0.1 N, ≥ pH 10)
Stock Concentration Prepared	100mM (5mg/284µl of NaOH)
Storage	Protect from light Solid: -20°C Alkaline stock solution Aliquots: -20°C
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	487950

Appendix III (b): NOC-18

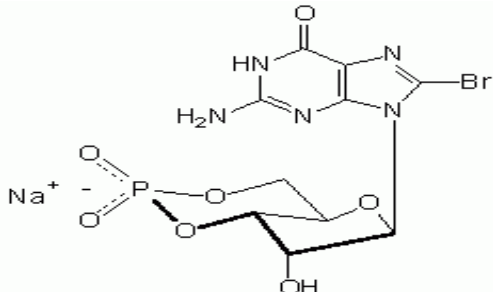|	
Alternate Names:	DETA NONOate; DETA/NO; 2,2′-(Hydroxynitrosohydrazino)*bis*-ethanamine
Description	A nitric oxide (NO) donor. Half-life for NO release ($t_{1/2}$ = 3400 min; PBS, pH 7.4, 22 °C)
CAS Number	146724-94-9
Molecular Weight	163.2
Molecular Formula	$C_4H_{13}N_5O_2$
Structure:	
Solubility	H_2O or NaOH
Stock Concentration Prepared	100mM (5mg/306µl of NaOH)
Storage	Protect from light Solid: -20 °C Stock solution Aliquots: -20 °C
Supplier	Calbiochem, http://www.merckbiosciences.co.uk
Catalogue Number	487957

Appendix III (c): SNAP

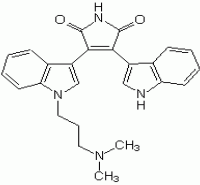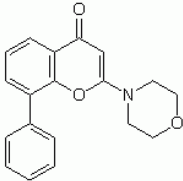|	
Alternate Names	(±)-S-Nitroso-N-acetylpenicillamine
Size:	20 mg
Description	A Nitric oxide donor with a half-life ($t_{1/2}$ = 10 h). SNAP begins to evolve nitric oxide immediately in aqueous buffers
CAS Number	79032-48-7
Molecular Weight	220.2
Molecular Formula	$C_7H_{12}N_2O_4S$
Structure	
Solubility	DMSO, Ethanol or Methanol
Stock Concentration Prepared	100mM (5mg/227µl of DMSO)
Storage	Protect from light store Solid: -20 °C Stock solution Aliquots: -20 °C
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	487910

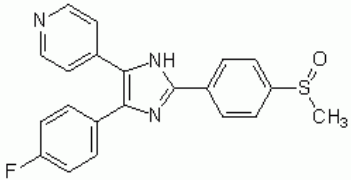| Appendix III (d) : SIN-1, Hydrochloride	
Alternate Names	3-Morpholinosydnonimine, HCl
Size:	20 mg
Description	Nitric oxide (NO) donor. Spontaneously decomposes to yield NO and superoxide anion radicals.
CAS Number	16142-27-1
Molecular Weight	206.7
Molecular Formula	$C_6H_{10}N_4O_2 \cdot HCl$
Structure	
Solubility	DMSO or Ethanol
Stock Concentration Prepared	100mM (5mg/242µl of DMSO)
Storage	Protect from light Solid: -20°C Stock solution Aliquots: -20°C
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	567028

Appendix III (e): Guanosine 3′,5′-cyclic Monophosphate	
Alternate Names	8-Bromo-cGMP; Br-cGMP, Na
Description	Cell-permeable cGMP analog
CAS Number	51116-01-9
Molecular Weight	446.1
Molecular Formula	$C_{10}H_{10}BrN_5O_7P \cdot Na$
Structure	
Solubility	H_2O
Stock Concentration Prepared	100mM (5mg/112µl of NaOH)
Storage	Solid: -20°C,stock solution Aliquots: -20°C
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	203820

APPENDIX VI: INHIBITORS

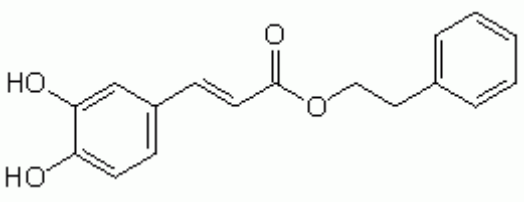| Appendix VI (a): Bisindolylmaleimide I	
Alternate Names	Gö 6850; GF109203X, **2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide**
Description	A reversible protein kinase C (PKC) inhibitor that acts as a competitive inhibitor for the ATP-binding site
CAS Number	133052-90-1
Molecular Weight	412.5
Molecular Formula	$C_{25}H_{24}N_4O_2$
Structure:	
Solubility	Anhydrous DMSO
Stock Concentration Prepared	10mM (1mg/242µl of DMSO)
Storage	Solid: -20°C, stock solution Aliquots: -20°C
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	203290

Appendix VI (b): LY294002	
Alternate Names	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
CAS Number	154447-36-6
Molecular Weight	307.4
Molecular Formula	$C_{19}H_{17}NO_3$
Structure:	
Solubility	DMSO
Stock Concentration Prepared	10mM (5mg/1627µl) of DMSO
Storage	Solid: -20°C, stock solution Aliquots: -20°C, Protect from light
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	440202

Appendix VI (c): SB203580

Alternate Names	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole
Description	A highly specific, cell-permeable inhibitor of p38 kinase (IC_{50} = 34 nM in vitro, 600 nM in cells).
CAS Number	152121-47-6
Molecular Weight	377.4
Molecular Formula	$C_{21}H_{16}N_3OSF$
Structure:	
Solubility	DMSO
Stock Concentration Prepared	10mM (1mg/265µl of DMSO)
Storage	Protect from light Solid: -20℃ Stock solution Aliquots: -20℃
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	559389

Appendix VI (d): CAPE

Alternate Names	Caffeic Acid Phenethyl Ester
Description	An active component of propolis from honeybee hive and a potent and specific inhibitor of NF-κB activation
CAS Number	104594-70-9
Molecular Weight	284.3
Molecular Formula	$C_{17}H_{16}O_4$
Structure	
Solubility	DMSO
Stock Concentration Prepared	100mM (5mg/176µl of DMSO)
Storage	Solid: -20℃,stock solution Aliquots: -20℃, Protect from light
Supplier	Calbiochem http://www.merckbiosciences.co.uk
Catalogue Number	211200

APPENDIX VII: ANTIBODIES

<table>
<tr><th colspan="2">Appendix VII (a): Monoclonal Anti-Cardiac Myosin Light Chain I Antibody (MLC-1v) Antibody</th></tr>
<tr><td>Antibody Name</td><td>Cardiac Myosin light chain I antibody [MLM527]</td></tr>
<tr><td>Antibody Type</td><td>Primary antibodies</td></tr>
<tr><td>Description</td><td>Mouse monoclonal [MLM527] to Cardiac Myosin light chain</td></tr>
<tr><td>Immunogen</td><td>Human ventricle myosin light chain I</td></tr>
<tr><td>Reacts with</td><td>Human, Mouse, Rat, Cow, Pig</td></tr>
<tr><td>Specificity</td><td>Antibody reacts with ventricle myosin light chain I (MLC-1v)</td></tr>
<tr><td>Application used</td><td>Western blotting (can also be used for ELISA, ICC/IF)</td></tr>
<tr><td>Band size</td><td>23 to 27kDa</td></tr>
<tr><td>Raised in</td><td>Mouse</td></tr>
<tr><td>Clonality</td><td>Monoclonal</td></tr>
<tr><td>Clone number</td><td>MLM527</td></tr>
<tr><td>Isotype</td><td>IgG2b</td></tr>
<tr><td>Purity</td><td>Protein A purified</td></tr>
<tr><td>Storage</td><td>Aliquot and store at -20°C or -80°</td></tr>
<tr><td>Concentration</td><td>1.0 mg/ml</td></tr>
<tr><td>Dilution used</td><td> 1:2500 in blocking buffer</td></tr>
<tr><td>Secondary antibody required</td><td>Anti-mouse antibody conjugated to horseradish peroxidase (HRP)</td></tr>
<tr><td>Supplier</td><td>Abcam, UK
http://www.abcam.com</td></tr>
<tr><td>Catalogue Number</td><td>ab680</td></tr>
</table>

Appendix VII (b): Monoclonal Anti-Cardiac Myosin Heavy Chain (α& β isoforms) Antibody

Antibody Name	Heavy Chain Cardiac Myosin antibody [3-48]
Antibody Type	Primary antibodies
Description	Mouse monoclonal [3-48] to heavy chain cardiac Myosin
Immunogen	Full length native protein (purified) (Human).
Reacts with	Human, Mouse, Rat, Cow, Dog, Pig, Rabbit
Specificity	Antibody reacts with alpha and beta isoform of myosin heavy chain
Application used	Western blotting (can also be used for ELISA, Flow Cyt, ICC/IF, IHC)
Band size	223 kDa
Raised in	Mouse
Clonality	Monoclonal
Clone number	3-48
Isotype	IgG1
Purity	IgG fraction
Storage	Aliquot and store at -20°C or -80°C
Concentration	1.0mg/ml
Dilution used	1:2500 in blocking buffer
Secondary antibody required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Abcam, UK http://www.abcam.com
Catalogue Number	ab15

Appendix VII (c): Monoclonal Anti-cardiac Troponin I Antibody

Antibody Name	Cardiac Troponin I antibody [284 (19C7)]
Antibody Type	Primary antibodies
Description	Mouse monoclonal [284 (19C7)] to cardiac Troponin
Immunogen	Native troponin complex
Reacts with	Human, Mouse, Rat, Cat, Cow, Dog, Fish, Goat, Monkey, Pig, Rabbit
Specificity	Reacts equally with free cardiac troponin I and cardiac troponin I forming complexes with other troponin components. No cross-reactivity with skeletal muscle troponin I.
Application used	Western blotting (can also be used for AP, ICC/IF, IHC-P, ELISA)
Band size	24 kDa
Raised in	Mouse
Clonality	Monoclonal
Clone number	284 (19C7)
Isotype	IgG2b
Purity	Protein A purified
Storage	Aliquot and store at -20°C or -80°C
Concentration	3.2 mg/ml
Dilution used	1:2500 in blocking buffer
Secondary antibody required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Abcam, UK http://www.abcam.com
Catalogue Number	ab19615

Appendix VII (d): Monoclonal Anti- phospho-Akt (Ser473) Antibody

Antibody Name	Mouse Phospho-Akt (Ser473) (587F11) monoclonal antibody
Antibody Type	Primary antibody
Description	A mouse monoclonal antibody to Akt phosphorylated at serine 473 (Ser473)
Immunogen	A synthetic phospho-peptide (KLH-coupled) corresponding to residues around Ser47 of mouse Akt.
Reacts with	Human, mouse Rat Hamster
Specificity	Antibody reacts with Akt only when phosphorylated at serine 473 and does not react with related kinases such as PKC and p70 S6 kinase.
Application used	Western blotting (can also be used IP)
Band size	60 kDa
Raised in	mouse
Clonality	monoclonal
Clone number	587F11
Isotype	IgG2b
Purity	Protein A purified
Storage	Whole tube (i.e. no *aliquot) o*f the antibody was stored at -20°C. Required portions of the antibody were taken from -20°C.
Concentration	1.0 mg/ml
Dilution used	1:1000
Secondary antibody required	Anti-mouse antibody conjugated to horseradish peroxidase (HRP)
Supplier	Cell Signallng Technology http://www.cellsignal.com
Catalogue Number	4051

Appendix VII (e): Monoclonal Anti-β-Actin antibody conjugated to horseradish peroxidise (HRP)	
Antibody Name	Monoclonal Anti-β-Actin-Peroxidase, Clone AC-15
Antibody Type	Primary antibody
Description	A Protein A purified fraction of mouse monoclonal anti-b-actin isolated from ascites fluid of the AC-15 and conjugated horseradish peroxidise (HRP)
Immunogen	A synthetic peptide corresponding to a slightly modified b-cytoplasmic actin N-terminal sequence: Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys conjugated to KLH
Reacts with	Human, mouse, rat, bovine, sheep, pig, rabbit, cat, dog, guinea pig, chicken, carp
Specificity	Antibody reacts specifically with β-actin found many tissues and species
Application used	Loading control for western blotting
Band size	42 kDa
Raised in	mouse
Clonality	Monoclonal
Clone number	AC-15
Isotype	IgG1
Purity	Protein A purified
Storage	Aliquot and store at -20°C or -80°C
Concentration	Conjugate concentration: 2.5–6 mg/mL Antibody Concentration: 2–4 mg/mL
Dilution used	1:25000
Secondary antibody required	None required
Supplier	Sigma (Sigma-Aldrich), UK http://www.sigmaaldrich.com
Catalogue Number	A3854

APPENDIX VIII: PREPARATION OF STOCK SOLUTIONS

Calculation of mole of a compound

Mole of compound

$$Mole = \frac{mass \; (g)}{mwt \; (molecular \; weight) \; g/mole}$$

Relationship between the mole, concentration and volume of a compound in solution

$$Concentration \; (mole/L(M)) = \frac{mole}{volume(L)} = \frac{mass/mwt}{Volume \; (L)}$$

$$\therefore Concentration \; (M) = \frac{mass}{mwt \; x \; volume(L)}$$

Calculation of required volume for dissolving a compound

The volume parameter from the above equation was made the subject and volume (μl) required to achieve a selected stock concentration of each compound was calculated.

$$Volume \; (L) = \frac{mass}{mwt \; x \; concentraion \; (M)}$$

$$Volume \; (\mu l) = \frac{mass \; (g)}{mwt \; x \; mole/L} = \frac{mass \; (g)}{mwt \; x \; mole/10^{-6}\mu l}$$

$$\therefore Volume \; (\mu l) = \frac{mass \; (g)}{mwt \; x \; mole/L} = \frac{mass \; (g)}{mwt \; x \; mole/\mu l} \; x \; 10^6$$

An Example Calculation

Example: Calculation of the volume of DMSO required for preparing the 10mM stock concentration of LY294002.

Mass of LY294002 used = 1mg = 1 x 10^{-3} g

Required Stock concentration = 10mM = 10 x 10^{-3} M = 10^{-2} M

Molecular weight of LY294002 = 307.4 g/mole

$$\therefore Volume\ (\mu l)\ = \frac{mass\ (g)}{mwt\ x\ {mole}/_L} = \frac{mass\ (g)}{mwt\ x\ {mole}/_{\mu l}}\ x\ 10^6$$

$$Volume\ (\mu L)\ = \frac{1\ x\ 10^{-3}g}{307.4\ {g}/_{mole}\ x10^{-2}\ {mole}/_{10^{-6}\mu L}}$$

$$Volume\ (\mu L)\ = \frac{1\ x\ 10^{-3}g}{307.4\ {g}/_{mole}\ x\ 10^{-2}\ {mole}/_{\mu L}}\ x\ 10^6$$

$$Volume\ (\mu L)\ of\ DMSO\ = 325.3\mu L$$

In a tissue culture hood (i.e. laminar flow cabinet), 325.3µL of DMSO was added to 1mg of LY294002. The LY294002 solid was through dissolved, aliquots of 10µL and 20 µL portions were made and stored at -20°C.