MOLECULAR BASIS OF DELAYED CELL CYCLE PROGRESSION IN HYPOXIC CARDIAC FIBROBLASTS:

A NOVEL MECHANISM OF G1-S REGULATION INVOLVING p38 MAPK

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DOCTOR OF PHILOSOPHY

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CERTIFICATE

I, Malini S Pillai, hereby certify that I had personally carried out the work depicted in the thesis entitled **"Molecular basis of delayed cell cycle progression in hypoxic cardiac fibroblasts: A novel mechanism of G1-S regulation involving p38 MAPK"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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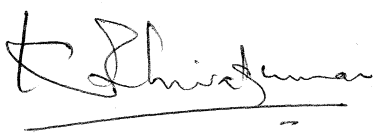
This is to certify that **Malini S Pillai**, in the Division of Cellular & Molecular Cardiology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The thesis entitled **"Molecular basis of delayed cell cycle progression in hypoxic cardiac fibroblasts: A novel mechanism of G1-S regulation involving p38 MAPK"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Clearance was obtained from the Institutional Animal Ethics Committee for carrying out the study.



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SYNOPSIS

Cardiac fibroblasts, a major intra-cardiac source of extra-cellular matrix proteins, matrix metalloproteinases and several growth factors and cytokines, are importantly involved in myocardial repair following injury. Unlike cardiomyocytes that have limited proliferative potential in the adult heart, cardiac fibroblasts retain their replicative capacity throughout adult life. In response to myocardial injury, normally quiescent cardiac fibroblasts undergo phenotypic transformation into activated myofibroblasts that proliferate and produce matrix components to replace the damaged myocytes and facilitate healing. The intrinsic ability of these cells to enter into and exit from the cell cycle in response to extracellular signals can significantly impact myocardial remodeling associated with hypertension, myocardial infarction, and myocardial reperfusion injury. Surprisingly, there is very little information on the regulation of the cardiac fibroblast cell cycle by a multitude of factors in the diseased myocardium that can potentially act as positive and negative modulators of fibroblast proliferation.

Hypoxia is a major constituent of several disease states of the myocardium. Although underlying tissue hypoxia, as in myocardial ischemia, may modify the functional response of cardiac fibroblasts, with significant implications in myocardial response to injury and disease progression, its effects on cell cycle regulatory elements in cardiac fibroblasts have not been addressed.

In this regard, a critical event in the eukaryotic cell cycle is its transition through the G1-S checkpoint, which is driven by specific cyclin-cyclin-dependent kinase (CDK) complexes that phosphorylate the retinoblastoma (Rb) protein, facilitating S phase entry. Inhibition of CDK activity by cyclin-dependent kinase inhibitors (CDKIs)

such as p27 promotes Rb hypophosphorylation and cell cycle arrest. In turn, p27 is degraded by the ubiquitin pathway involving the ubiquitin ligase SCF^{Skp2}. Apart from these molecules that are integral to the cell cycle machinery, the mitogen-activated protein kinases (MAPKs) have been linked to cell proliferation in different cell types.

Against this backdrop, the present study sought to understand the mechanisms involved in the hypoxic regulation of the cell cycle in adult rat cardiac fibroblasts.

Methods:

Isolation and culture of adult rat cardiac fibroblasts:

Cardiac fibroblasts were isolated from the ventricular tissue of young adult Sprague Dawley (2-3 months) rats by a series of enzymatic digestions using collagenase, trypsin, DNase and pancreatin. Hypoxic (pO2 ~0.75 mmHg/5% CO2) and normoxic (pO2 ~150 mmHg/5% CO2) conditions were generated using the BBL-GasPak system and GasPak envelops. The pO2 of the hypoxic and normoxic media was ~23 and ~113 mmHg, respectively. Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% fetal calf serum were used in all the experiments.

Measurement of DNA synthesis, flow cytometry, western blot and Taqman quantitative real-time PCR analyses were performed following standard protocols. Statistical significance was assessed using Student's t-test. $p \leq 0.05$ was considered significant.

Major Findings:

1. Hypoxia delays G1-S transition in cardiac fibroblasts:

Previous studies in this laboratory had shown that hypoxia significantly decreases basal and serum (10%)-induced DNA synthesis and increases the population doubling time from 27.7 hours in normoxic cultures to 54.14 hours, which is reversed upon re-oxygenation, suggesting that hypoxia causes reversible inhibition of cardiac fibroblast proliferation. In the present study, the hypoxia-sensitive cell cycle checkpoint was identified by analysing the time-dependence of the inhibitory effect of hypoxia on DNA synthesis, and flow cytometry. Exposure of cells to hypoxia 15 hours after serum addition had no effect on cell proliferation, implying that hypoxia exerted its action in the early phase of the cell cycle. Further, flow cytometric analysis of cultures subjected to 42 hours of hypoxia confirmed a modest G0/G1 block, suggesting that G_1/S checkpoint in the cardiac fibroblast cell cycle is oxygen-sensitive.

2. Molecular mechanisms involved in hypoxic regulation of G1-S transition:

a) Effect of hypoxia on cell cycle regulatory proteins:

Hypoxia attenuated the expression of cyclin D1 and A (but not cyclin E). Importantly, hypoxia induced the expression of p27 (but not p21) and hypophosphorylation of pRb, consistent with decreased G1-S progression. Interestingly, real-time PCR analysis showed a 2-fold increase in p27 mRNA under hypoxia, showing that p27 is transcriptionally up-regulated in hypoxic cells. Since Skp2-mediated degradation is a major mechanism of p27 regulation, Skp2 expression in hypoxic cells was examined next. Skp2 protein levels were significantly lower and

inversely related to p27 expression in cells exposed to hypoxia, suggesting possible post-translational regulation of p27 as well under hypoxic conditions.

b) Identification of signaling pathways mediating the hypoxia effect:

p44/42 MAPK as positive modulator of cardiac fibroblast proliferation:

Activation of p44/42 MAPK observed in normoxic and cycling cardiac fibroblasts was significantly inhibited in cells exposed to 12 hours of hypoxia. Inhibition of p44/42 MAPK activity using the pharmacological inhibitor, PD 098059 (10μM), attenuated DNA synthesis under normoxic conditions. Thus, p44/42 MAPK was found to act as a positive modulator of cardiac fibroblast proliferation. Its inactivation under hypoxia can therefore contribute to the hypoxia-induced decrease in cell proliferation.

p38 MAPK as key determinant of hypoxia-induced G0/G1 block:

Significant activation of p38 MAPK was noted in cells exposed to 6 or 12 hours of hypoxia, suggesting a role for p38 MAPK in the hypoxic inhibition of cardiac fibroblast proliferation. Inhibition of p38 MAPK using SB203580 (10μM) attenuated the effects of hypoxia on cyclin D1, but cyclin A levels remained unaffected. Further, significant reduction in p27 protein expression in p38 MAPK-inhibited hypoxic cells correlated well with the reversal of hypoxia-induced reduction in DNA synthesis and the G0/G1 block observed in hypoxic cells. p38 MAPK inhibition, however, failed to abolish the hypoxia-induced increase in p27 mRNA, showing that the transcriptional up-regulation of p27 in hypoxic cells is not mediated by p38 MAPK. Since Skp2 levels were significantly lower and inversely related to p27 expression in hypoxic cells in which p38 MAPK was active, possible regulation of

Skp2 by p38 MAPK was examined in these cells. Skp2 levels were restored in p38 MAPK-inhibited hypoxic cells, indicating that p38 MAPK may negatively regulate Skp2 expression in cardiac fibroblasts, which in turn would impact p27 expression post-translationally in these cells. Together, the findings suggest a novel mechanism of G0/G1 block in hypoxic cardiac fibroblasts involving hypoxic induction of p27 via direct transcriptional regulation, independent of p38 MAPK, and post-translational regulation via p38 MAPK-dependent suppression of its degradation by Skp2.

Significance of the findings:

The three principal findings of this study and their implications are:

1) Hypoxia retards G1/S transition in cardiac fibroblasts:

Fibroblast hyperplasia is central to the role played by these cells in the regulation of multiple aspects of cardiac function in the context of myocardial injury. Hypoxic inhibition of cardiac fibroblast proliferation can potentially impact the response of these cells to injury in a setting of myocardial ischemia.

2. p38 MAPK acts as a key regulator of the cardiac fibroblast cell cycle under hypoxic stress:

p38 MAPK has for long been implicated in environmental stress responses, in the regulation of inflammation and in apoptosis but, more recently, its involvement in the regulation of cell proliferation is increasingly recognized. This study identifies a role for p38 MAPK in regulating the G1-S cell cycle checkpoint in response to hypoxic stress. It is important to recognize these less appreciated functions of p38

MAPK because these kinases can be potential therapeutic targets in treating diseases involving cardiac fibroblasts.

3. Skp2 as a potential downstream target of p38 MAPK

The finding that p38 MAPK regulates skp2 is new and is significant since Skp2-mediated degradation of p27 is an important regulator of G1-S transition. It suggests a novel mechanism of G1-S regulation, which, to the best of our knowledge, has not hitherto been reported for any cell type.

A serendipitous finding:

During the course of the study, attempts were made to understand the role of tyrosine kinases in regulating cardiac fibroblast proliferation using genistein, the best-known inhibitor of tyrosine kinases. Genistein is commonly used to assess the role of tyrosine kinase in cell proliferation by the standard technique of [^{3}H]-thymidine incorporation. The involvement of tyrosine kinase in the stimulatory action of any mitogen on DNA synthesis is reported if such stimulation is blocked by genisten. In this study, genistein was found to cause nearly total inhibition of [^{3}H]-thymidine incorporation into DNA in cardiac fibroblasts stimulated with 10% serum. Subsequent experiments, however, led to the serendipitous finding that the dramatic decrease in [^{3}H]-thymidine incorporation in response to genistein does not reflect inhibition of DNA synthesis or cell proliferation but is due to reduced intracellular levels of [^{3}H]-thymidine, possibly due to inhibition of nucleoside uptake by these cells. The effect was irreversible and was demonstrable in pulmonary fibroblasts as well. The findings suggest that nucleoside uptake mechanisms might be a novel target of genistein action in cardiac fibroblasts, which points to serious limitations in

using genistein to assess the role of tyrosine kinase in cell proliferation by the standard technique of [^{3}H]-thymidine incorporation.

I have published an article in Molecular and Cellular Biochemistry (2009). Two more have been communicated.

I. INTRODUCTION

The vertebrate myocardium is a highly organized structure composed of the parenchyma and stroma. The cardiac parenchyma consists of terminally differentiated cardiomyocytes that account for about one-third of the myocardial cell population and are the fundamental contractile cells in the heart. The stromal compartment, or the interstitium, is a dynamic metabolic entity composed of a scaffold of structural proteins and non-myocytes, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and fibroblasts (Laurent, 1987; Weber *et al.*, 1995). While the interstitial protein scaffold provides a structural and mechanical continuum for the functioning of the heart as a syncytium, the cellular components of the stroma interact in a dynamic fashion to maintain the electrical, chemical, and biomechanical responses of the organ in diverse pathophysiological conditions. Maintenance of the quantitative relationship between the parenchymal and stromal compartments is vital for the structural and functional integrity of the myocardium.

Cardiac fibroblasts represent the majority of cardiac interstitial cells (>90%) and about two-thirds of the total myocardial cell population. They are primarily responsible for the homeostatic maintenance of the extracellular matrix (ECM). Cardiac fibroblasts are the only source of fibrillar type I and type III collagens in the heart, and are a major source of various extracellular matrix proteins including collagens IV, V, VI, elastin, fibronectin, matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). In a setting of myocardial injury, cardiac fibroblasts play a cardinal role in the wound healing response. Further, they synthesise and secrete an array of bioactive molecules including growth factors, cytokines and immunomodulatory factors that exert autocrine/paracrine effects affecting cardiac cell function (Brown *et al.*, 2005).

I.1. Identification of the problem

I.1.1. Cardiac fibroblast proliferation

Unlike cardiomyocytes that have limited proliferative potential in the adult heart, cardiac fibroblasts are undifferentiated cells that remain quiescent in the normal heart but retain the capacity to proliferate throughout adult life and do so in response to several mitogenic stimuli (Koudssi *et al.*, 1998; Palmer *et al.*, 1995). Whereas myocyte hypertrophy may be physiological, fibroblast hyperplasia is invariably a hallmark of cardiac pathology. In the diseased myocardium, the functional effects of cardiac fibroblasts are mediated through phenotypic transformation of normally quiescent cells into active myofibroblasts that express smooth muscle α-actin and exhibit increased proliferative, migratory and secretory properties. Prolonged activation of cardiac fibroblasts, defined by increased proliferation and ECM secretion, is a major component of the structural and functional remodeling of the heart in conditions such as hypertensive heart disease and myocardial infarction. Over-expression of the fibroproliferative response leads to cardiac fibrosis, a condition characterized by excess ECM deposition and a stiff myocardium. The impaired compliance of the fibrotic heart ultimately compromises contractile performance (Burlew & Weber, 2002). Surprisingly, however, although the proliferative response of cardiac fibroblasts is central to their role in pathological states of the myocardium, little information is available on the fundamental mechanisms that regulate their entry into and exit from the cell cycle.

Cardiac fibroblast proliferation is modulated by a multitude of factors prevalent in pathophysiological states of the myocardium, including proinflammatory cytokines,

vasoactive peptides, hormones, mechanical stretch and changes in oxygen availability. These factors can act as positive and negative modulators of fibroblast proliferation and the balance between these positive and negative influences would determine the net functional response of these cells in a given situation, with profound effects on the structural and functional integrity of the myocardium. It is therefore of obvious interest to ascertain how diverse factors prevalent in the diseased myocardium would modulate cardiac fibroblast proliferation.

I.1.2. Hypoxia

Hypoxia is a major factor associated with several disease states of the myocardium including myocardial ischemia and infarction (Sabbah *et al.*, 2005). It results from the disproportion between oxygen supply and demand, and affects cardiac viability and function. Hypoxia induces a wide range of profound physiological and pathophysiological responses at both the systemic and cellular levels by affecting multiple aspects of cell function including proliferation (Douglas *et al.*, 2005; Douglas *et al.*, 2003), energy metabolism (Hochachka *et al.*, 1996; Webster, 2003), expression of various proteins such as cytoskeletal proteins, ECM proteins (Ben-Yosef *et al.*, 2002; Chen *et al.*, 2005), proteins associated with cell survival (Boutilier, 2001) and factors modulating growth and inflammation (Giaccia *et al.*, 2004; Michiels, 2004; Sarkar *et al.*, 2003; Wenger, 2002). Cellular responses to hypoxia vary depending on the degree of hypoxia, the extent of cell differentiation and the tissue or organ of origin (Gardner *et al.*, 2003). It is reported to exert both pro- and anti-mitogenic effects on cells. For example, hypoxia inhibits cell cycle progression in MEFs (Gardner *et al.*, 2001; Green *et al.*, 2001) but induces

proliferation in pulmonary artery adventitial fibroblasts (Das *et al.*, 2001, Das *et al.*, 2002, Short *et al.*, 2008), pulmonary artery smooth muscle cells (SMCs) (Yu *et al.*, 2006) and airway SMCs (Cogo *et al.*, 2003). Further, in contrast to the enhanced proliferation observed in hypoxic porcine aortic ECs (Schafer *et al.*, 2003), proliferation in bovine aortic and bovine pulmonary artery ECs is inhibited by hypoxia (Tucci *et al.*, 1997).

Although underlying tissue hypoxia, as in myocardial ischemia, may modify the functional response of cardiac fibroblasts, with significant implications in myocardial response to injury and disease progression, its effects on cell cycle regulatory elements in cardiac fibroblasts have not been addressed.

I.1.3. The Cell Cycle

The cell cycle is a highly ordered process by which cells duplicate their genome and transmit identical copies of the initial genetic material to daughter cells. In mammalian cells, it proceeds through four different phases: G1, S, G2 and M. The G1 phase that stands for "GAP-1" mediates cell growth and prepares the cell for DNA synthesis in the subsequent phase. The S-phase stands for "Synthesis" and is the stage in which DNA synthesis occurs. The G2 phase is "GAP-2" and is needed for cell growth and preparation for mitosis. The last phase M stands for "Mitosis", the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. The cell cycle events are organised such that late events occur only upon completion of early events. To monitor this dependency, cells are equipped with "checkpoints" set at various stages of the cell cycle that verify whether the processes at each phase of the cell cycle have

been accurately completed before progression into the next phase. Multiple checkpoints have been identified that include: G1/S (G1) checkpoint, intra-S phase checkpoint, G2/M checkpoint and Metaphase checkpoint.

G1/S checkpoint is a critical event in the eukaryotic cell cycle where the key decision of whether the cell should divide, delay division, or enter a resting stage is made. The basic cell cycle machinery that drives the cell cycle consists of a binary system of cell regulators, the cyclins and cyclin-dependent kinases (CDKs). Cyclins form active complexes with CDKs, and the periodic activation of different cyclin-CDK complexes through different phases ensures cell cycle progression. Unlike the expression of CDKs that is more or less constitutive, the expression of cyclins is "cyclic" and responsive to proliferative signals. In fact, fluctuations in cyclin levels represent the primary mechanism by which CDK activity is regulated. The cyclin-CDK complexes that orchestrate the advance of the cell through the G1-S checkpoint include the cyclin D-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2 complexes. Phosphorylation of nuclear control protein, Retinoblastoma (Rb), by cyclin D-CDK4/6 and cyclin E-CDK2 complexes results in the release of the E2F transcription factor leading to the transcription of genes required for the completion of the G1/S phase transition and S phase entry. In this regard, an important mechanism regulating cyclin/CDK activity is the interaction with inhibitory proteins (CDKIs) such as p27 and p21 that bind to and inhibit the catalytic activity of cyclin-CDK complexes, promoting Rb hypophosphorylation and cell cycle delay or arrest (Morgan *et al.*, 1995; Morgan *et al.*, 1997; Sherr & Roberts, 1999). Regulation of these negatively acting cell cycle effectors is important for orderly cell cycle progression. The abundance of CDKIs is regulated by their rate of degradation by the

proteolysis apparatus in a phosphorylation-dependent manner (Pagano *et al.*, 1995). The degradation of p27 requires its phosphorylation on Thr-187 and is mediated by Skp2, an F-box protein that associates with Skp1, Cul1, and Roc1/Rbx1 to form the SCF^{Skp2} ubiquitin ligase complex (Carrano *et al.*, 1999; Tsvetkov *et al.*, 1999). Skp2-mediated degradation of p27 is an important regulator of G1-S transition (Sutterlüty *et al.*, 1999). While its role in regulation of cell proliferation in various cell types (Wu *et al.*, 2009) has been well documented, its regulatory role in cardiac fibroblast proliferation has not been investigated.

I.1.4. Role of MAPKs in cell proliferation

Mitogen-activated protein kinase (MAPK) signal transduction pathways, involving a series of protein kinase cascades, relay, amplify and integrate signals from a diverse range of stimuli and elicit an appropriate physiological response including cellular proliferation, differentiation, development, inflammatory responses and apoptosis in mammalian cells. Multiple studies have shown that the MAPK pathways play a critical role in the regulation of cell proliferation in different cell types (Marc & Jonathan, 2000). Three MAPK families have been clearly characterized: namely, classical MAPK (also known as ERK1/2 or p44/42 MAPK), C-Jun N-terminal kinase/ stress-activated protein kinase (JNK/SAPK) and p38 kinase. Of the three MAPK sub-families, the ERK1/2 cascade is the best characterised MAPK signaling pathway that is activated by mitogens and is a key transducer of proliferation signals (Gao *et al.*, 2004). In contrast, p38 MAPKs and JNKs are stress kinases that are activated in response to a variety of stress stimuli including pro-inflammatory cytokines, heat and osmotic shock, UV radiation and hypoxia (Derijard *et al.*, 1994;

Raingeaud *et al.*, 1995). While the signaling pathways downstream of JNK activation appear to be non-mitogenic (Orsini *et al.*, 1999), the p38 MAPK pathway is lately reported to regulate cell proliferation depending on the cell type and the stress stimulus but the underlying mechanisms remain largely unknown (Thornton *et al.*, 2009).

I.2. Broad objective of the study

Against this backdrop, this study aimed at delineating the molecular mechanisms involved in the hypoxic regulation of the cardiac fibroblast cell cycle, focusing on the role of p38 MAPK.

The specific objectives of the study included:

- Identification of the hypoxia-sensitive cell cycle checkpoint
- Determination of the expression levels of cell cycle regulatory proteins, cyclins and CDKIs, and the phosphorylation status of Rb in hypoxic cardiac fibroblasts
- Elucidation of the role of p38 MAPK in the hypoxic regulation of the cardiac fibroblast cell cycle

I.3. Major Findings

The principal findings of this study can be summarized as follows:

I.3.1. Hypoxia retards G1/S transition in cardiac fibroblasts

I.3.2. Hypoxia differentially regulates the expression of cell cycle regulatory proteins. It reduces the expression of G1 regulators, cyclin D and cyclin A, but cyclin

E is unaffected. Importantly, hypoxia induces both protein and mRNA expression of p27, but not of p21, leading to hypophosphorylation of Rb.

I.3.3. p44/42 MAPK is a positive modulator of cardiac fibroblast proliferation

I.3.4. p38 MAPK plays a key role in the hypoxic regulation of the cardiac fibroblast cell cycle:

Several lines of evidence point to a significant role for p38 MAPK in the hypoxic inhibition of cardiac fibroblast proliferation. These include: (i) marked increase in p38 MAPK activity in hypoxic cardiac fibroblasts (ii) attenuation of the negative effect of hypoxia on cell cycle progression upon p38 MAPK inhibition, and (iii) differential regulation of cyclin D, p27 and Skp2 by p38 MAPK under hypoxia.

The findings show that p38 MAPK inhibition fails to abolish the hypoxia-induced increase in p27 mRNA, suggesting that the transcriptional up-regulation of p27 in hypoxic cells is not mediated by p38 MAPK. However, the negative regulation of Skp2 by p38 MAPK, as evident from the inverse relationship between p38 MAPK activity and Skp2 expression, would impact p27 protein expression post-translationally in these cells.

I.4. Brief overview of main chapters in the thesis:

I.4.1. Literature Review

This chapter summarizes the major developments in the field of cardiac fibroblast biology. Further, a critical review of the molecular mechanisms involved in the regulation of cell proliferation, focusing on the role of different cell cycle proteins and their interactions with various signal transduction pathways that mediate cell

proliferation, is presented. An attempt is made to identify the gaps in the literature on cardiac fibroblast proliferation so as to place in perspective the issues addressed in the present study.

I.4.2. Study design

Specifically, it dwells upon the experimental model and approaches employed in the fulfilment of the objectives. It lists the reagents, chemicals, and equipments used in the study, with the names of manufacturers and suppliers. The statistical methods used to evaluate the data have been indicated.

I.4.3. Results

This section presents the findings of the study and their statistical evaluation.

I.4.4. Discussion

This section discusses the observations and stresses their significance. What is new is highlighted so as to give an idea as to how the outcome of this study pushes the field forward.

I.4.5. Summary and conclusions

This section summarizes the major findings and their significance.

I.4.6. List of references

All publications cited in the thesis are presented in this section in the prescribed format.

I.4.7. Appendix

This chapter is devoted to a serendipitous observation during the course of the study. Briefly, attempts were made to understand the role of tyrosine kinases in regulating cardiac fibroblast proliferation using genistein, the best-known inhibitor of tyrosine kinases. Genistein is commonly used to assess the role of tyrosine kinase in cell proliferation by the standard technique of [^{3}H]-thymidine incorporation. In this study, genistein was found to cause nearly total inhibition of [^{3}H]-thymidine incorporation into DNA in cardiac fibroblasts stimulated with 10% serum. Subsequent experiments, however, led to the serendipitous finding that the dramatic decrease in [^{3}H]-thymidine incorporation in response to genistein does not reflect inhibition of cell proliferation but is due to reduced intracellular levels of [^{3}H]-thymidine, possibly due to inhibition of nucleoside uptake by these cells. The findings suggested that nucleoside uptake mechanisms might be a novel target of genistein action in cardiac fibroblasts, which points to serious limitations in using genistein to assess the role of tyrosine kinase in cell proliferation by the standard technique of [^{3}H]-thymidine incorporation.

I.4.8. List of publications

II. LITERATURE REVIEW

The myocardium consists of two inter-dependent compartments: the parenchyma, composed of highly differentiated cardiac myocytes, and the stroma comprising less differentiated cells embedded in an ECM. Myocytes, though fewer in number, account for the bulk volume of the myocardium. They are the functional contractile units of the myocardium that confer the pump function. Being terminally differentiated, myocytes of the postnatal heart have limited capacity to proliferate but grow by an increase in cell mass in response to mitogenic stimuli, a process termed 'hypertrophy'. Myocyte hypertrophy can be physiological, resulting from normal growth and exercise, or pathological as in chronic hypertension, valvular disease and ischemia/infarction (Weber *et al.*, 1995).

The stromal component of the heart, representing the cardiac interstitium, is a complex network of interstitial cells or the non-myocytes including resident fibroblasts, capillary ECs, monocytic cells and vascular cells from coronary arteries and veins as well as the acellular compartment composed of the structural proteins of the ECM (Weber *et al.*, 1995). The non-myocytes constitute more than two-thirds of the myocardial cell population of which cardiac fibroblasts represent a majority. While myocytes respond to pathophysiological stimuli by hypertrophic growth, fibroblasts increase in number (hyperplasia) and synthesize more ECM proteins, leading to structural remodeling of the interstitium (Campbell *et al.*, 1995). Fibroblast hyperplasia is almost always associated with pathologic conditions of the heart (Brown *et al.*, 2005).

II.1. Cardiac Fibroblasts

Cardiac fibroblasts are the most abundant cell type in the heart that account for about 90% of the non-myocytes. They play a pivotal role in regulating normal myocardial function, and in myocardial remodelling associated with hypertension, cardiac hypertrophy, myocardial infarction and heart failure (Brown *et al.*, 2005; Brilla & Maisch, 1994).

II.1.1. Origin of cardiac fibroblasts

Fibroblasts are traditionally defined as cells of mesenchymal origin that produce interstitial collagen. Cardiac fibroblasts are derived from mesenchymal cells originating in the proepicardial organ (Moorman & Christoeffels, 2003; Norris *et al.*, 2008). These cells migrate over the surface of the heart to form the epicardium that gives rise to epicardial-derived cells (EPDCs) (Manner *et al.*, 2001). In vivo, EPDCs are the major source of cardiac fibroblasts that progressively differentiate into these cells within the wall of the heart, a process regulated by a number of growth factors including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Kalluri & Neilson, 2003; Wessels & Perez-Pomares, 2004). In addition to EPDC-derived cardiac fibroblasts, other sources of fibroblasts have been demonstrated. Studies have shown that in the neonatal and adult heart, fibroblasts can arise from endogenous cell populations by epithelial-mesenchymal transition (Potts & Runyan, 1989) and from bone marrow-derived cells such as the mesangioblasts (Visconti *et al.*, 2006). Recent evidence has also shown post-natal recruitment of circulating fibroblast progenitor cells into the ventricular myocardium (Visconti & Markwald, 2006).

II.1.2. Structural organisation of cardiac fibroblasts in the heart

It has been stated that the adult heart consists of 30% myocytes and 70% nonmyocytes, of which the majority are cardiac fibroblasts (Nag, 1980). Fibroblasts form a complex 3-D network within the connective tissue matrix that they occupy. Fibroblasts within the endomysial collagen network then surround the groups of myocytes in a lamellar function (Camelliti *et al.*, 2005). Cardiac fibroblasts interact with the extracellular matrix through integrins and Discoidin Domain Receptor 2 (DDR2), a collagen receptor that is expressed specifically in cardiac fibroblasts (Goldsmith *et al.*, 2004). The intercellular connections of fibroblasts appear to be through two different types of cell-cell molecules, connexins and cadherins. The connexin that connects fibroblasts to myocytes (heterotypic) is Cx43, and the connexin that connects fibroblasts to other fibroblasts (homotypic) is Cx45 (Banerjee *et al.*, 2006; Camelliti *et al.*, 2005). Cadherin-11 appears to be a specific fibroblast cadherin and has been associated with VEGF-D signaling (Orlandini M & Oliviero, 2001). This in vivo arrangement of fibroblasts within the collagen network can allow the fibroblasts to contract the endomysial collagen, exerting force on the myocytes. Changes in fibroblast contractility have been suggested to impact myocardial relaxation. Such an organization facilitates a role for fibroblasts in maintaining the structural integrity of the heart through cell–cell and cell–ECM interactions, as well as through proliferation and ECM degradation and synthesis (Camelliti *et al.*, 2005).

II.1.3. Functions of cardiac fibroblast in the myocardium

Cardiac fibroblasts play an essential role in myocardial function and pathophysiology, contributing to structural, biochemical, mechanical and electrical

properties of the healthy and diseased myocardium (Camelliti *et al.*, 2005; Porter *et al.*, 2009; Souders *et al.*, 2009). The following sections will address relevant aspects of their function.

II.1.3.1. Cardiac fibroblasts as key regulators of ECM turnover

Cardiac fibroblasts are recognized as the cell type primarily responsible for homeostatic maintenance of ECM in the normal heart. Cardiac ECM is a highly differentiated structure. The major constituents include interstitial fibrillar collagens [type I (~80%) and type III collagens (~10%)] along with smaller amounts of other collagens (IV, V, VI), elastin, proteoglycans, glycoproteins, cytokines, growth factors, and proteases (Corda *et al.*, 2000; Bowers *et al.*, 2009). ECM forms an organized network that surrounds and interconnects cellular structures. Under normal physiological conditions, it provides a scaffold for the myoycte and non-myocyte populations of cells, distributes mechanical forces throughout the myocardium, transmits mechanical signals to individual cells via cell surface ECM receptors (mechanotransduction) and participates in fluid movement in the extracellular environment.

ECM homeostasis requires equilibrium between its synthesis and degradation. Cardiac fibroblasts (CF) synthesize and secrete several components of ECM, predominantly fibrillar collagens type I and III, the major collagens in the heart (Bowers *et al.*, 2009). Cardiac fibroblasts are also the primary source of regulators of collagen biosynthesis such as TGF-β (Porter *et al.*, 2009). Transforming growth factor-β (TGF-β) is a fibrogenic growth factor that enhances matrix deposition by increasing the synthesis of matrix components such as collagen and fibronectin and

promotes the expression of inhibitors of extracellular matrix proteases down regulating interstitial collagenases that function to degrade matrix components (Massague, 1990; Slack *et al.*, 1993).

ECM degradation is regulated by two key enzymes: MMPs and TIMPs. MMPs constitute a family of proteolytic enzymes including collgenases, gelatinases, stromelysins, and membrane type MMPs that collectively have the capacity to degrade all the components of ECM. TIMPs, on the other hand, are locally synthesised secretory proteins principally involved in inhibiting MMPs. The MMP/TIMP system is a key contributor to ECM turnover and its dysregulation affects ECM remodeling and function. Cardiac fibroblasts from a variety of species have been shown to express several MMPs including collagenases (MMP-1, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3), and membrane type MMPs (predominantly MT1-MMP), and TIMPs, predominantly TIMP-1 and TIMP-2 (Weber et l, 1995; Tyagi *et al.*, 1996; Eghbali, 1992). MMP/TIMP expression by cardiac fibroblasts can be modulated by several chemical, physical or environmental stimuli affecting ECM homeostasis.

II.1.3.2. Cardiac fibroblasts serve as an intra-cardiac source of autocrine and paracrine factors

A major function of cardiac fibroblasts is to produce and secrete growth factors (VEGF, bFGF, IGF-1), cytokines (IL-1β, IL-6, TNF-α, TGF-β), and other signaling molecules (Ang II, ET-1) (Baudino *et al.*, 2006; Porter *et al.*, 2009; Souders *et al.*, 2009). These factors act both synergistically and antagonistically to affect cardiac cell functions by autocrine and paracrine mechanisms. Cardiac fibroblasts also serve

important roles as intermediate sensors and amplifiers of signals from myocytes and immune cells in the normal and injured myocardium through the production of the autocrine and paracrine mediators. The specific signaling factors that are secreted by cardiac fibroblasts depend largely on the stimuli such as chemical factors, such as pro- or anti-inflammatory cytokines and growth factors, electrical signals, hypoxia, or mechanical stretch. For example, mechanical stretch induced TNF-α, TGF-β, ET-1 and Angiotensinogen gene expression in rat cardiac fibroblasts (Yokoyama *et al.*, 1999; Pikkarainen *et al.*, 2006). Hypoxia was found to stimulate cardiac fibroblasts to secrete cytokines and growth factors such as TNF-α and VEGF (Shivakumar *et al.*, 2008). Reoxygenation of hypoxic cardiac fibroblasts leads to induction of TGF-β and BNP synthesis in neonatal rat cardiac fibroblasts (Grobe *et al.*, 2007; Makino *et al.*, 2006).

II.1.3.3. Cardiac fibroblasts – role in myocardial wound healing and repair

Cardiac fibroblasts participate as key cells in the wound healing response (van den Borne *et al.*, 2010). Wound healing entails a cascade of events involving several different cell types, including inflammatory cells, endothelial cells and fibroblasts. Although initiation of the wound healing response is dominated by cells such as macrophages derived from the circulation, cardiac fibroblasts play a significant role in regulating the subsequent phases (Cleutjens *et al.*, 1995 b). In acute injury, the death of cardiomyocytes invokes the recruitment of inflammatory cells, which remove the necrotic cell debris by phagocytosis. Granulation tissue begins to form that consists of inflammatory cells, newly formed blood vessels (to restore the blood supply) and fibroblasts that deposit collagen. Eventually, this granulation tissue

matures into a scar that has been perceived as a passive replacement for the lost cardiomyocytes with little biological activity, devoid of inflammatory cells but rich in ECM and cardiac fibroblasts. Fibroblasts are activated to differentiate into myofibroblasts, so-called as this cell type expresses the pro-contractile protein α-smooth muscle actin, which converge on the damaged region, proliferate and accelerate the synthesis of various ECM proteins, such as collagen, fibronectin and laminin to replace the necrotic cardiomyocytes as scar (Brown *et al.*, 2005; Cleutjens *et al.*, 1999; Sun & Weber, 2000). Myofibroblasts, therefore, maintain the structural integrity of healing scars preventing infarct expansion and ventricular dilatation. The inevitable proliferation and production of collagen by myofibroblasts in the remote area, however, can contribute to adverse ventricular remodeling and unfavourable outcomes.

II. 1.4. Cardiac fibroblasts in disease

Prolonged activation of cardiac fibroblasts, defined by increased proliferation and subsequent ECM secretion, is a direct consequence of several pathological conditions such as hypertension, myocardial infarction, cardiac hypertrophy and heart failure and leads to cardiac fibrosis, a condition characterized by excess accumulation of ECM proteins, especially fibrillar collagens (Cleutjens *et al.*, 1995a; Cleutjens *et al.*, 1995b). This fibrosis may be reparative, replacing areas of myocyte loss with structural scar supporting ventricular morphology, or reactive, involving diffuse increases in ECM deposition at sites unrelated to focal injury affecting the amount of collagen, the collagen phenotype, and the degree of collagen cross linking.

Perivascular fibrosis surrounding coronary arterioles is also noted. Differences in the characteristics of fibrosis depend on the etiology of the disease.

Fibrosis has important functional consequences in the heart. First, increased ECM content results in exaggerated mechanical stiffness and contributes to diastolic dysfunction. Progressive increases in fibrosis can cause systolic dysfunction and left ventricular hypertrophy (LVH). Second, increased collagen content disrupts electrotonic connectivity between cardiac myocytes and provides an electrical substrate for re-entrant arrhythmogenesis. Third, perivascular fibrosis surrounding intracoronary arterioles impairs myocyte oxygen availability, reduces coronary reserve, and exacerbates myocyte ischemia (Brown *et al.*, 2005).

The importance of fibrosis as a determinant of myocardial performance and disease outcome is increasingly appreciated. In mild hypertension, reactive fibrosis with net increase in collagen accumulation in endomysium and perimysium and myocyte hypertrophy was reported, but the overall structure of the ECM was preserved. With increasing LVH, greatly expanded and disorganized ECM deposition was observed, reflecting areas of myocyte death and replacement fibrosis interspersed with extreme myocyte hypertrophy (Rossi, 1998). The causes of cardiac fibrosis in hypertensive heart disease have been attributed to a combination of hemodynamic (pressure overload) and humoral factors (AngII, ET-1, TGFβ) (Kenchaiah & Pfeffer, 2004).

Dilated cardiomyopathy (DCM) is characterized by myocyte loss, hypertrophy of residual myocytes and abnormalities of cytoskeleton. DCM involves changes in collagenous framework. Weber *et al.* show that there is reduction in number of fibrillar collagen tethers in DCM and loss of these tethers is important in

pathogenesis of myocardial dilation allowing for myocyte slippage (Weber, 1989). Studies by Paul *et al.* reveal that TIMP-3 deficiency leads to DCM (Fedak *et al.*, 2004). Further, significant associations of increased collagen deposition and elevated collagen I: collagen III composition has been observed. These data suggest a definite association of DCM with fibrosis.

Fibrosis exerts adverse impact on cardiac electrical properties in addition to effects on the mechanical properties of the myocardium. A specific relationship between fibrosis and arrhythmogenesis exists that depends on the cardiomyopathy and the structural details of myocardial remodelling (Eckardt *et al.*, 2000). Myocyte loss owing to apoptotic or necrotic mechanisms is followed by replacement fibrosis, resulting in electrical isolation of myocytes, and introduction of alternate conductance pathways for reentrant arrhythmias. These conductance disturbances may be exacerbated by perivascular or reactive fibrosis, which impairs myocyte oxygen availability. Arrhythmogenic right ventricular dysplasia (ARVD) is an extreme example of a fibrotic cardiomyopathy and its consequences (Tabib *et al.*, 2003). Arrhythmogenesis in ARVD commonly appears to be associated with re-entrant pathways arising from replacement fibrosis at sites of myocyte loss.

Fibroblast proliferation and tissue remodeling are cardinal features of many of these cardiac pathologies. This necessitates an examination of the broader issue of cell proliferation in higher eukaryotes.

II.2. The Mammalian Cell Cycle

Cell cycle is a vital process that forms the basis of growth and development in living organisms. The cell-division cycle may be defined as a sequential series of events

that take place in a cell leading to its division and duplication. Each cell cycle has four co-ordinate processes: cell growth, DNA replication, distribution of diploid chromosome to daughter cells and cell division.

In cycling or proliferating cells, the chromosomal cycle can be broadly classified into two brief periods. The first part of the cell cycle and generally by far the longest is termed Interphase that involves accumulation of nutrients needed for duplicating its DNA and other cellular components including cytoplasmic organelles, membrane, structural proteins and RNAs. It is further divided into G_1 (Gap 1) phase, S (Synthesis) phase, and G_2 (Gap 2) phase. The second major phase of the cell cycle is the M phase that typically comprises two major events: nuclear division (mitosis) and cell division (cytokinesis) to produce daughter cells that are genetically identical to each other and to the parent cell.

Duration of cell-division cycle varies in different organisms. Cell division in E.coli takes about 20 minutes, while in yeast it takes 90-120 minutes and in mammalian cells about 24 hours. In the typical dividing eukaryotic cell, G1 phase lasts approximately 12 hours, S phase 6 to 8 hours, G2 phase 3 to 6 hours, and mitosis about 30 min, although the exact length of each phase varies with cell type and growth conditions (Murray & Hunt, 1993). The events associated with different phases of the cell cycle are as follows:

G1 (Gap 1) phase – G1 is the gap phase during which cells prepare for the process of DNA replication. In this phase, the cells are not metabolically quiescent. The DNA is compactly folded as chromatin and the genes and proteins necessary for DNA synthesis and replication are activated. The proteins that help the cells progress

through the S phase are synthesized and accumulated. Compared to DNA replication and mitosis, which follow canonical steps that vary little from cell to cell, the steps controlling entry and progression through G1 are largely dependent on cell type and context. It is during this phase that diverse metabolic, stress and environmental cues are integrated and interpreted and the cell makes the decision to proceed, pause, or exit the cell cycle. To this end, during G1, the cell makes further decisions regarding whether to self-renew, differentiate or die.

S (Synthesis) phase - S phase is defined as the stage in which DNA synthesis occurs. The double helical DNA is replicated into two daughter DNA molecules. Histones and various proteins responsible for the activity of chromosomes bind rapidly to the newly formed DNA molecules.

G2 (Gap 2) phase - G2 is the second gap phase during which the cell prepares for the process of division. This phase is marked by proof reading of replicated DNA and repairing any mismatches and/or mispairing in the newly synthesized DNA.

M (Mitosis or Maturation) phase – Mitosis consists of karyokinesis and cytokinesis. The prominent stages include prophase, prometaphase metaphase, anaphase and telophase. Mitosis involves chromatin condensation, nuclear envelope breakdown, chromatid separation and cytokinesis, resulting in the formation of two daughter cells genetically identical to the parent cell.

In addition to G1, S, G2, and M, the term **G0** is used to describe cells that have exited the cell cycle and become quiescent.

G_0 (Gap 0) phase – Depending on environmental and developmental signals, cells in G_1 may temporarily or permanently leave the cell cycle and enter a quiescent or

arrested phase known as **G_0**. This phase may extend from about 8 hours to years, during which the cells are not committed to progress into the G_1 phase of the cell cycle. G_0 phase cells show G_1- phase DNA content but are metabolically quiescent and exhibit low rates of RNA and protein synthesis and low enzymatic activity. Apparently, G_0 represents a stage when there is active repression of genes needed for mitosis, resulting in the exit of cells from the cell cycle and entry into a state of differentiation or quiescence.

II.2.1. Regulation of the cell cycle

The progression through different phases of the cell cycle is regulated by a binary system of cell regulators consisting of a family of regulatory subunits called the cyclins, which bind to (and help to activate) the catalytic subunits, the cyclin-dependent protein kinases (CDKs). The cyclin-CDK complexes integrate information flow from outside the cell to trigger the transition through subsequent phases of the cell cycle in response to mitogenic signals (Epstein, 2003; Israels & Israels, 2001).

Cyclins- Cyclins were first identified in marine invertebrates as proteins whose accumulation and degradation oscillated during the cell cycle (Rosenthal *et al.*, 1980). As the name suggests, the level of cyclins independently increase or decrease within the phases of the cycle in response to the proliferative signals. Cyclins undergo sequential binding to the CDKs throughout the cell cycle to form active complexes that phosphorylate nuclear control proteins that have specific functions during different stages of the cell cycle. It is the periodicity of the cyclins, mediated by their synthesis and subsequent proteolytic degradation that result in sequential

activation and inactivation of CDKs, ensuring well-delineated transitions between cell cycle stages and providing the primary means of cell cycle regulation.

Cyclins are grouped into classes that relate to the phase of the cell cycle they regulate. Thus, depending on cell cycle phase-specificity, cyclins are classified into:

- ✓ G1 cyclins - cyclin D1(D2, D3)
- ✓ G1/S cyclins - cyclin E
- ✓ S-phase (or late G1/S) cyclins - cyclin A (also termed late G_1/S cyclin due to its presence at later stages of G_1/S transition)
- ✓ Mitotic cyclins - cyclins A & B

The D-type cyclins are the first cyclins to be induced as G0 cells are stimulated to enter the cell cycle (Sherr, 1994). In cycling or proliferating cells, cyclin D expression is increased during the mid-G_1 phase and persists till the onset of the S phase. Unlike many other cyclins, D-type cyclins do not oscillate throughout the cell cycle. Cyclin D has a relatively short half-life (~20 min) and rapidly disappears with the removal of mitogenic stimuli or the addition of anti-proliferative agents. Cyclin E is the next cyclin to be induced during the progression of cells through G1. Cyclin E expression is essential for the transition of cells through G_1/S checkpoint of the cell cycle and its levels are increased towards the later stages of G_1, persisting through the G_1/S checkpoint and at the initiation of the S phase. D and E cyclins are, therefore, sometimes referred to as 'Start' cyclins since they govern the entry into, progression through and the exit from the G_1 phase in response to external mitogenic

cues (Sherr & Roberts, 2004). Cyclin A-associated kinase activity is required for entry into S phase, completion of S phase, and entry into M phase (Girard *et al.*, 1991). Cyclin A colocalizes with sites of DNA replication, suggesting that cyclin A may actively participate in DNA synthesis or perhaps play a role in preventing excess DNA replication. Mitosis is regulated by specific CDK (CDK1) in association with cyclins A, B1, and B2 (Arellano & Moreno, 1997). The proteins these cyclin-CDK1 complexes phosphorylate include cytoskeleton proteins such as lamins, histone H1, and possibly components of the mitotic spindle. The degradation of cyclins A and B is necessary for cells to exit mitosis.

CDKs- CDKs are a group of serine/threonine kinases that form active heterodimeric complexes by binding to their regulatory subunits, cyclins. Unlike cyclins, CDKs are expressed constitutively and are activated in a cell cycle phase-dependent manner. Different members of the CDK family, in association with different cyclins, turn key switches throughout the cell cycle. The cell cycle can therefore be viewed as a recurring CDK cycle in which specific patterns of cyclin-CDK complexes enable the transition of cells through the phases and checkpoints of the cell cycle. The specific cyclin-CDK combinations for the different phases of cell cycle are as follows:

- ✓ D-type cyclins (G1 cyclins) - bind CDK4/ 6
- ✓ cyclin E & A (G1/S cyclins)- bind CDK2
- ✓ cyclin A and B (mitotic cyclins) - bind CDK1

Several CDKs work cooperatively to drive cells through different phases of the cell cycle. Mitogenic signals act by means of CDK4/6, inducing transcription of cyclins and other components resulting in CDK2 activation and entry into S phase. CDK4

and CDK6 that form active complexes with the D type of cyclins (cyclins D1, D2, and D3) are involved in early G_1 phase, whereas CDK2 is sequentially activated by the E type cyclins, cyclins E1 and E2, during G_1/S transition stage and is required to complete G_1 phase and initiate S phase. CDK2-cyclin A complex appears during late S phase and plays a role in progression of DNA replication. CDK2 activation results in the recruitment of DNA helicases, primases, and polymerases by pre-replicative complexes (PRCs), causing unwinding of the double helix and DNA replication. CDK activity is essential for the unwinding step and several components of the PRC become phosphorylated in the process. The newly replicated origins cannot reassemble new PRCs until CDK activity once again drops at the end of mitosis. The prototypic CDK, CDK1 associates with cyclins A and B and acts at the G2/M interface. The progressive accumulation of cyclins A and B during the cell cycle and their abrupt degradation at the onset of anaphase, mediates entry and exit from mitosis, respectively. The drop in CDK1 activity at the end of M phase allows replication origins to be reloaded with PRC (Kelly & Brown, 2000; King *et al.*, 1994; Sherr & Roberts, 2004).

Many levels of regulation impinge upon CDKs to impose tight control over cell-cycle progression. The enzymatic activity of a CDK is regulated at three levels: i) cyclin association, ii) subunit phosphorylation, and iii) association with cyclin-dependent kinase inhibitors (CDKIs).

i) Regulation of CDK activity by cyclin levels:

Regulation of CDK activity during cell cycle progression involves regulation of the timing of cyclin protein accumulation and degradation. Once the CDKs have

completed their role, cyclins undergo a rapid programmed proteolysis via ubiquitin-mediated delivery to the proteasome complex.

ii) Regulation of CDK activity by positive and negative phosphorylation of CDKs:

In all eukaryotes studied, CDKs are regulated by phosphorylation and dephosphorylation of critical residues. Important targets of this regulation are the neighbouring threonine and tyrosine in the ATP-binding loop (GXGTYG). Once complexed with their cyclin subunit, CDK4, CDK2 and CDK1 (Cdc2) must be phosphorylated on a regulatory threonine residue (Thr-172, Thr-160 and Thr-161 in humans, respectively) to become active. This activating phosphorylation is accomplished by the CDK-activating kinase (CAK), which is composed of CDK7, cyclin H, and a RING-finger protein MAT1 (Nigg, 1996; Draetta, 1997). However, before the phosphorylation of CDKs occurs, an inhibiting kinase phosphorylates CDKs at two other locations that block the active site. For example, the inhibitory phosphorylation of CDK1 molecules on Thr-14 and Tyr-15 amino acid residues in late S phase and G_2 is accomplished by dual-specificity kinases including Wee1, Mik1, and Myt1 (Booher *et al.*, 1997; Lundgren *et al.*, 1991). Thr-14 and Tyr-15 are positioned within the Cdc2 ATP-binding cleft and phosphorylations of these residues are thought to inhibit kinase activity by disrupting the orientation of ATP molecules bound in this cleft. These inhibitory phosphorylations appear to be an important mechanism employed by cells to prevent premature activation of cyclin B/Cdc2 complexes before entry into mitosis (Obaya & Sedivy, 2002). This inhibition can be relieved by the removal of inhibitory phosphate by the action of duel-specificity phosphatase, Cdc25C. The extremely rapid activation of cyclin B/Cdc2 at the G_2/M

border is thought to be brought about by an autocatalytic positive feedback loop involving cyclin B/Cdc2 and Cdc25C. This occurs when Cdc25C binds to cyclin B/Cdc2, dephosphorylating Cdc2 and activating the protein kinase complex. Cyclin B/Cdc2 in turn phosphorylates Cdc25C, which increases its phosphatase activity, resulting in the activation of more cyclin B/Cdc2 complexes, and in turn resulting in a rapid activation of both the Cdc25C phosphatase and cyclin B/Cdc2 (Obaya & Sedivy, 2002). Support for this model comes from the observations that hyperphosphorylation of Cdc25C correlates with increased phosphatase activity. (Hoffmann *et al.*, 1993).

ii) Regulation of CDK activity by (CKIs) :

Association with small inhibitory proteins is a universal mechanism of CDK regulation (Sherr & Roberts, 1999). Two major families of CDKIs exist: 1) The INK4 family, 2) Cip/Kip family. Members of the Cip/Kip family of proteins are universal CDK inhibitors that bind to all known cyclin-CDK complexes and inhibit their activation and kinase activity, whereas the INK4 inhibitors bind to the cyclin D kinases (CDK4 and CDK6) and inhibit their association with the cognate cyclins. These two families of CDKIs play distinctly different roles in the regulation of the cell cycle.

INK4 family of CDKIs:

The INK4 family members which include $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ are distinguished by the presence of an ankyrin-like repeat domain, which binds to CDK4 and CDK6 at the expense of cyclin D. The site recognized on CDK4 overlaps with the region required for cyclin binding, and INK4 CDKIs have been shown to

block the formation of cyclin D/CDK4 complexes. The primary function of INK4 prototype, $p16^{INK4A}$, is to downregulate CDK4 and CDK6 after progression to S-phase, or to prevent accidental activation of cyclin-CDK complexes and maintain states of quiescence and differentiation. Although $p16^{INK4A}$ can be considered an intrinsic cell-cycle regulator, $p15^{INK4B}$ appears to function as an inducible CDK inhibitor that responds to extracellular signals. The functions of $p18^{INK4C}$ and $p19^{INK4D}$ are, however, less well understood. Expression of $p19^{INK4D}$ is induced as cells enter S phase, suggesting that it may also function to downregulate cyclin D-CDK activity after Rb phosphorylation. The exact role of INK4 proteins and whether they exhibit any functional overlap at all remains unknown (Ca′ nepa *et al.*, 2007; Sherr & Roberts, 1999).

Cip/Kip family of CDKIs:

The Cip/Kip family of CDKIs are more broadly acting inhibitors whose actions affect the activities of cyclin D-, E-, and A-dependent kinases. Cip/Kip members include $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, all of which contain characteristic motifs within their amino-terminal moieties that enable them to bind both to cyclin and CDK subunits (Sherr & Roberts, 1999; Denicourt & Dowdy, 2004).

$p21^{Cip1}$:

$p21^{WAF1/Cip1}$ was the first CDKI identified. Induction of p21 inhibits cell cycle progression in two ways: (*a*) It functions as a regulator of cell cycle progression at G1 by inhibiting the activity of a broad range of cyclin/CDK complexes, with a preference for those containing CDK2 (Harper *et al.*, 1995), and (*b*) It plays a regulatory role in S phase DNA replication through its interaction with Proliferating

cell nuclear antigen (PCNA), an elongation factor for DNA polymerase δ and a component of the DNA repair machinery (Waga *et al.*, 1994). The functions of p21 in regulating DNA repair through its interactions with PCNA and associated proteins, however, remain controversial. The CDK-inhibitory domain resides in the amino-terminus of p21, while PCNA binds to part of C-terminal domain. p21 expression is low in G1 and increases as the cell-cycle progresses such that the proper growth stimulus must be present during G1 in order to induce a parallel increase in cyclin D-CDK complexes that can overcome the inhibitory effect of p21. The expression of p21 is tightly controlled by the tumor suppressor protein, p53, through which it mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli including DNA damage (El-Deiry *et al.*, 1993; El-Deiry *et al.*, 1994; Dulic *et al.*, 1994). Under stress, the p53 protein is stabilized and activated as a transcription factor. The p21 gene promoter contains a p53-binding site that allows p53 to transcriptionally activate the p21 gene. In addition to its role as a proliferation inhibitor, it has been shown to play a role in cellular senescence (Noda *et al.*, 1994, Stein *et al.*, 1999). This protein has also been reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation (Gartel & Tyner, 2002).

$p57^{Kip2}$:

$p57^{kip2}$ contains four structurally distinct domains: 1) an amino-terminal CDK-inhibitory domain, 2) a proline-rich domain, 3) an acidic domain, and 4) a carboxy-terminal domain that shares sequence similarity to $p27^{Kip1}$. Like p21, p57 has been

shown to interact with PCNA through the PCNA-binding domain within its C-terminus, and this function is important in the ability of p57 to suppress cellular transformation. Disruption of either CDK/cyclin or PCNA binding partially reduced p57's ability to suppress transformation in primary cells, while loss of both inhibitory functions completely eliminated p57's suppressive activity. Thus, control of the cell cycle and suppression of cell transformation by p57 require both CDK and PCNA inhibitory activity, and disruption of either or both functions may lead to uncontrolled cell growth. contains that, when separated from its N-terminal CDK-cyclin binding domain, can prevent DNA replication in vitro and S phase entry in vivo (Watanabe *et al.*, 1998). In contrast to the ubiquitous expression of p21 and p27, p57 displays a tissue-specific expression pattern and appears highest in well-differentiated cells, suggesting a specialized role in coordinating cell-cycle arrest with differentiation (Lee *et al.*, 1995). Mutations in this gene are implicated in sporadic cancers and Beckwith-Wiedemann syndrome, suggesting that this gene is a tumor suppressor candidate (Matsuoka *et al.*, 1995).

p27 ^{kip1}:

p27 functions as an integral brake of the cell cycle (Sherr & Roberts, 1999). It was originally identified as an inhibitor of cyclin-CDK complexes in Mv1Lu mink-lung epithelial cells arrested by TGF-β (Polyak *et al.*, 1994). In contrast to p21, the levels of p27 are highest during G0 and early G1 phases and decline upon mitogenic stimulation as cells progress from G1 to S phase. p27 primarily acts as a negative regulator of G_1 progression by binding to and inhibiting the activity of cyclin E or cyclin A: CDK2 complexes (Sherr & Roberts,1999; Denicourt & Dowdy, 2004).

Recent studies, however, attribute a disparate role for p27 as a positive regulator of cell cycle progression by promoting the assembly and activity of cyclin D–CDK4/6 complexes (Cheng *et al.*, 1999).

Interaction between p27 and cyclin E-CDK2 complex:

The interaction between p27 and cyclin E-CDK2 is quite complex. It has been suggested that p27 might potentially inhibit cyclin-CDK activity in a variety of ways: (1) By potentially obstructing substrate access to the complex by physically occupying the substrate binding domain on the cyclin subunit and (2) By preventing activating phosphorylation of the cyclin-CDK complex by the CDK Activating Kinase (CAK), which physically alters the conformation of the CDK subunit. (3) CDK inhibitory model: This is the most widely accepted mechanism, where p27 functions both as a substrate and an inhibitor of the kinase complex (Sheaff *et al.*, 1997; Nho & Sheaff, 2003). It involves blocking the catalytic active site of the CDK, preventing ATP access. p27 structure includes an N-terminus inhibitory domain and the C-terminus domain containing the cyclin E-CDK2 T187 phosphorylation site. Two distinct cyclin E-CDK2-p27 complexes are formed: one in which the C-terminus of p27 interacts with cyclin E-CDK2 to form a loosely bound complex (the enzyme: substrate interaction); and another in which the N-terminus of p27 interacts with cyclin E-CDK2 to form a tightly bound thermodynamically favoured complex (the enzyme: inhibitor interaction). The mechanism of inhibition of cyclin E-CDK2 complex by p27 involves a p27-induced conformational change in the CDK2 catalytic cleft, as well as p27 intercalation into the ATP binding site of CDK2 (Nho & Sheaff, 2003). ATP concentration, therefore, is an important determinant in the

conversion of the loosely bound complex to the tightly bound state. Higher ATP concentration promotes enzyme: substrate interaction and p27 phosphorylation. ATP can bind the loose p27-cyclin E-CDK2 complex (or it can bind cyclin E-CDK2 first), resulting in p27 phosphorylation. At this point phosphorylated p27 can either be released (restarting the cycle) or converted to the tightly bound state (Cheng *et al.*, 1999).

Interaction between p27 and cyclin D-CDK4 complex:

Unlike the other CDKIs, $p27^{Kip1}$ is constitutively expressed throughout the cell cycle, and the majority of p27 in the proliferating cell appears to be associated with cyclin D-CDK4 complexes, presenting a conundrum in defining the mechanism of a protein that is supposed to be an inhibitor. Recent studies show that p27 may be a required assembly factor for the cyclin D-CDK4 complex in early G1 that stabilizes it, without necessarily inhibiting cyclin D-associated kinase activity (Cheng *et al.*, LaBaer *et al.* 1997). In addition to contributing to D-type cyclin stabilization, the assembly of p27-cyclin D-CDK complexes would contribute to G1-to-S progression via at least two other mechanisms. First, cyclin D1, CDK4, and CDK6 lack nuclear localization signals. LaBaer *et al.* provided evidence that p27 could facilitate nuclear import of the assembled complexes (LaBaer *et al.* 1997). Second, a shift in the p27 binding equilibrium toward cyclin D-CDKs could potentiate cyclin E-CDK2 activation in late G1, favouring S phase progression (Sherr & Roberts, 1999). A possible molecular "switch" has been indentified that interconverts p27 from a cyclin D-CDK4 inhibitor to a non-inhibitor. p27 is preferentially phosphorylated at Tyrosine residues-Y88 and Y89 in the p27 CDK interaction domain, causing it to

bind cyclin D-CDK4 in a non-inhibitory mode (James *et al.*, 2007). Thus, p27-cyclin D-CDK4 complexes are catalytically active in proliferating cells where tyrosine kinases are active, but p27-cyclin D-CDK4 complexes are inactive in confluent cells where tyrosine kinase activity is reduced. Another possible mechanism that promotes the assembly of cyclin D-CDK4/6-p27 complex in early to mid G1 is the phosphorylation of p27 at Thr157 and Thr198 by AGC kinases through PI3K activation (Blain *et al.*, 2008).

Regulation of the activity of $p27^{kip1}$:

Although regulation of p27 is mostly post-translational, various mechanisms at the transcriptional and translational levels have been reported to regulate p27 cellular abundance and activity during different phases of the cell cycle.

1) Regulation by the proteolytic degradation of p27 :

One of the key mechanisms involved in the regulation of p27 abundance is proteolysis by the ubiquitin–proteasome pathway (Pagano *et al.*, 1995). p27 proteolysis rises dramatically during G1 phase progression, and the p27 half-life falls five- to eight-fold during G0 to S phase (Nakayama & Nakayama, 2006). Multiple mechanisms regulate ubiquitin-dependent p27 proteolysis. In early G1, mitogens seem to activate an export linked degradation mechanism that is followed in late G1 and S phases by a cyclin E–CDK2-dependent degradation of p27. One can broadly classify these into Thr187-phosphorylation dependent and independent p27 proteolytic mechanisms.

Thr187-phosphorylation-dependent p27 proteolysis:

A well conserved class of E3 ubiquitin–protein ligases, the SCF complex, formed by Skp1, Cul1, an F-box protein, the accessory protein CKS1B and the ring domain proteins, ROC1/Rbx1p/Hrt1p, has been identified as an upstream effector of p27 (Nakayama & Nakayama, 2006; Patton *et al.*, 1998). It was originally identified as a protein associated with the S-phase promoting kinase cyclin A-CDK2 (Zhang *et al.*, 1995). SCF (Skp1/Cul1/F-box protein) complexes are modular ubiquitin ligases whose specificity is determined by a substrate-binding F-box protein. Skp2 is an important F-box protein responsible for p27 recognition and subsequent degradation (Carrano *et al.*, 1999; Sutterlüty *et al.*, 1999). Substrate recognition by Skp2 depends on specific phosphorylation of p27 on Thr187 by the cyclin E-CDK2 complex and its interaction with the extreme COOH terminus of phosphorylated p27 (Tsvetkov *et al.*, 1999). Cyclin-CDK2-bound, threonine 187 (Thr187)-phosphorylated p27 binds to the phosphate-binding site of CKS1B, to SKP2 and to the CKS1-SKP2 interface. This association of Skp2 with p27 results in recruitment of the latter to the SCF core complex, thereby promoting its ubiquitination and degradation.

The expression of Skp2 correlates inversely with that of p27. In the normal cell cycle, levels of Skp2 are low in G0/G1 and rise in the S-phase (Zhang *et al.*, 1995). The amount of Skp2, therefore, varies in a cell-cycle dependent manner, such that it is detected first at the transition between G1 and S phases, accumulates during S and G2 phases, and then decreases in abundance as cells proceed through M phase. Both transcriptional and post-translational regulations are thought to underlie these changes. Skp2 is predominantly regulated post-translationally by APC/C (anaphase-

promoting complex/cyclosome), the other major cell cycle ubiquitin ligase (Bashir *et al.*, 2004). The abundance of Skp2 mRNA has also been demonstrated to be regulated by a transcription factor, GABP (Malek *et al.*., 2001). The stability and abundance of Skp2 is modulated by several signalling pathways including the Ras-Raf-MAPK pathway and PI3K pathway (Auld *et al.*, 2007; Tzu-Ping *et al.*, 2007).

Thr187-phosphorylation independent p27 proteolysis:

The activity of p27 is at least in part regulated by its sub-cellular compartmentalization. p27 is nuclear in G0 and early G1 and appears transiently in the cytoplasm at the G1/S transition in proliferating cells. It interacts with its targets (CDK2 complexes) in the nucleus and consequently inhibits G_1 progression. Subsequent degradation of p27 requires its cytoplasmic re-localization. Export of p27 in early G1, therefore, allows degradation of cytoplasmic p27 by a Skp2- and Thr187-independent pathway. In early G1, p27 phosphorylation at serine 10 (Ser10) by kinase-interacting stathmin (KIS) (Boehm *et al.*, 2002), or the minibrain-related kinase, MIRK (or dual tyrosine phosphorylation related kinase 1, DYRK1) (Deng *et al.*, 2004) increases p27 binding, through its nuclear export signal (NES), to CRM1 (or exportin 1) to promote nuclear export of p27 (Connor *et al.*, 2003; Ishida *et al.*, 2002; Rodier *et al.*, 2001). The export of p27 requires loss of cyclin-CDK2 binding, as CRM1 binds p27 in its CDK2 interaction site. Cytoplasmic p27 can be ubiquitylated by the ubiquitin ligase Kip1 ubiquitylation-promoting complex (KPC) (Nakayama & Nakayama, 2006). p27 has also been shown to physically interact with Jab1, a component of the COP9–signalosome complex, whose over expression

induces p27 translocation to the cytoplasm and promotes p27 degradation (Tomoda *et al.*, 1999).

Skp2-mediated p27 proteolysis can also occur in G1 independently of Thr187 phosphorylation, indicating that an additional Skp2-dependent ubiquitin ligase complex exists that may not require CKS1B for p27 recruitment. Recently a complex of Skp2, Cullin 4A (CUL4A), damaged-DNA binding protein 1 (DDB1) and the COP9 signalosome was shown to target p27 for proteolysis (Bondar *et al.*, 2006). That this complex regulates p27 proteolysis is supported by the observation that another COP9 signalosome subunit, COPS5, also binds to p27 independently of Thr187 and accelerates its proteolysis (Deng *et al.*, 2004; Malek *et al..*, 2001).

In quiescent G0 cells, another proteolytic mechanism regulates p27 stability. The degradation of p27 in G0 might involve cyclin-CDK binding or binding of another protein to its CDK-binding motif. Phosphorylation of threonine 198 (Thr198) of p27 by kinases, including AMP-activated protein kinase (AMPK), seems to stabilize p27 in G0 and thus Thr198 might play a part in this pathway (Kossatz, *et al.*, 2006).

2) Translational regulation of p27:

p27 mRNA translation is maximal in quiescence and early G1 and decreases after mitogen stimulation as cells progress toward S-phase. Cell cycle-dependent translation of p27 is mediated by the 5′ UTR of *CDKN1B*, which contain an upstream open-reading frame and an internal ribosome entry site (IRES). The IRES maintains *CDKN1B* translation during quiescence when most cellular cap-dependent translation is reduced (Hengst & Reed, 1996).

Several proteins regulate *CDKN1B* translation. The polypyrimidine tract-binding protein, PTB (also known as PTBP1), binds the *CDKN1B* IRES to increase IRES-dependent translation. Small interfering RNA-mediated PTB depletion shortens G1 phase and reduces p27 levels (Cho *et al.*, 2005). By contrast, Elav (*Drosophila* embryonic lethal, abnormal vision-like 1) family members, ELAVL1 (also known as Hu antigen R) and ELAVL4 (Hu antigen D), bind the *CDKN1B* IRES to inhibit translation (Kullmann *et al.*, 2002). Rho and CDK4 can both impair *CDKN1B* translation through its 3′ UTR (Gonzalez *et al.*, 2003; Vidal *et al.*, 2002).

Translation of *CDKN1B* can also be down regulated by microRNAs (miRNAs), which also act on the 3′ UTR (le Sage *et al.*, 2007). This highly conserved mechanism regulating p27 protein expression is found in flies and humans: in *Drosophila melanogaster*, inactivation of Dicer 1, the double-stranded RNase III isozyme that is essential for miRNA biogenesis, delayed the G1-S transition of germline stem cells through increased translation of the p27 homologue, dacapo. Using independent experimental approaches, down regulation of human p27 by miRNAs was recently demonstrated in an elegant screen for p27 regulatory miRNAs, in cells for which the cell cycle was inhibited by Dicer inactivation, and by a bioinformatics screen. Two polycistronic miRNAs sharing the same seed sequence, miR-221 and miR-222 (miR-221/222), bind to the *CDKN1B* 3′ UTR and inhibit its translation (le Sage *et al.*, 2007). Down regulation of high endogenous levels of miR-221/222 in different tumour cells increased p27 expression and inhibited tumour cell proliferation (le Sage *et al.*, 2007). Dead end 1 (DND1) prevents the access of miR221/222 to the p27 transcript (Kedde, M. *et al.*, 2007), and can protect the p27 transcript from miRNA-mediated down regulation.

3) Transcriptional regulation of p27:

Whereas cell cycle-dependent changes in p27 are largely post-transcriptionally regulated, several transcription factors act on the *CDKN1B* promoter. Forkhead box class O family (FoxO) proteins activate *CDKN1B* transcription in response to cytokines and nuclear Akt signalling (Dijkers *et al.*, 2000; Medema *et al.*, 2000). Besides regulating *CDKN1B* transcription, FOXO4 inhibits Akt to promote nuclear p27 localization and decreases expression of JAB1, a protein that mediates p27 degradation (Yang *et al.*, 2005). FOXM1, also increases p27 stability (Wang *et al.*, 2002). Thus, p27 regulation by FoxO proteins exceeds effects on transcription. The E2F1 transcription factor, which is itself activated by cyclin-CDK2, activates the *CDKN1B* promoter, leading to feedback inhibition of E2F1 action (Wang *et al.*, 2005). Several other transcription factors including SP1, NFYHES1 and AP-1 are involved in p27 regulation (Inoue *et al.*, 1999; Murata, K. *et al.*, 2005; Khattar & Kumar, 2010).

II.2.2. Molecular events during cell cycle progression

In early G1 phase, mitogen-induced signal transduction pathways promote the activation of cyclin D-CDK complexes at many levels, including gene transcription, cyclin D translation and stability, assembly of D cyclins with their CDK partners (CDK4/6), and import of holoenzyme into the nucleus, where they are phosphorylated by a CAK to be able to phosphorylate protein substrates. The most recognised function of cyclin D-CDK4/6 complex is the phosphorylation of Rb and Rb family members (p107 and p130). Rb, the first identified tumour suppressor protein, is recognised to be a central component of cell proliferation. Quiescent cells

are proposed to have a hypophosphorylated form of Rb that complexes with the E2F family of transcription factors, inhibiting the transcriptional activity of E2F, which is essential for overcoming the G_1/S transition point and progression to the S phase .The phosphorylation of Rb in mid-G1-phase by cyclin D-CDK4/6 leads to its dissociation from E2F, allowing this transcription factor to initiate the transcription of various genes. This phosphorylation–dephosphorylation cycle is believed to be one of the key cell-cycle control events in mammalian cells. E2F target genes that are upregulated as a result of Rb inactivation include genes required for the completion of the G1/S phase transition, as well as genes necessary for DNA replication. Among the E2F targets are genes encoding a second class of G1 cyclins, cyclin E and the associated kinase CDK2. E2F activation of cyclin E/CDK2 complex leads to further phosphorylation and inactivation of Rb, thus further enhancing E2F activity and increasing the accumulation of cyclin E/CDK2. This positive feedback loop, therefore, leads to continual inactivation of Rb, independent of the action of cyclin D/CDK4. Cyclin E-CDK2 complex not only perpetuates Rb phosphorylation, but also phosphorylates other substrates to promote continued cell cycle progression and passage through the restriction point. Cyclin A expression, which is induced subsequent to cyclin E and is required for transition through S phase, is also influenced by E2F. Cyclin A forms active complex with CDK2 that helps initiate DNA replication and progression through S phase. Whereas maximal periodic cyclin E-CDK2 activity is detected at G1/S, low levels of cyclin A/CDK2 activity is detected in late G1 phase, steadily increase as cells begin to replicate their DNA and donot decline until cyclin A is degraded in early mitosis, suggesting a role for this

complex in cell cycle progression at both G_1/S and G_2/M checkpoints. When cells complete the S phase and enter G_2, Mitosis Promoting Factor (MPF) is synthesized. Cyclin A/CDK2 activity is essential for the entry of cells into prophase and progression through mitosis and promotes the initiation of chromosome condensation, disassembly of nucleoli, and activation of MPF. The MPF is composed of cyclin B and CDK1 and this holoenzyme activates several proteins that are essential for mitosis. The resultant activities trigger nuclear envelope breakdown, spindle formation and chromatid segregation. On activation and translocation of MPF to the nucleus, cells enter metaphase and undergo changes characteristic of this phase. The progression of cycling cells from metaphase to anaphase requires the activation of Anaphase-promoting complex (APC/C), a multi-subunit ubiquitin ligase which mediates the changes that mark anaphase. The exit of cells from anaphase requires the degradation of cyclin B and inactivation of CDK1 by the proteolytic activity of APC/C. The last phase of mitosis, the telophase, is marked by events mediating cytokinesis leading to the separation of the two daughter cells, complete with a copy of the genetic material and cytoplasmic elements (Murray & Hunt, 1993; Sherr , 1994; Sherr & Roberts, 2004).

These cell cycle events are under the regulation of quality control mechanisms that ensure faulty cells correct their defects or self-destruct. Cell cycle checkpoints, which are the points of transition from one phase to the next, are control mechanisms that ensure the fidelity of cell division in eukaryotic cells.

Cell cycle checkpoints – Cell cycle checkpoints are the transition states within a cell cycle where the cells and the cell cycle events are monitored to ensure the

coordinated and error-proof completion of each stage of the cell cycle before the cell moves on to the next phase. At these points, the cells are screened for damage and, accordingly, the cell cycle regulatory proteins direct the cells towards repair or death, depending on the extent of damage. Thus, checkpoints represent a proof-reading mechanism for preventing the replication of damaged or un-repaired genome and, therefore, progression and propagation of mutated or damaged cells (Douglas & Haddad, 2003). The major cell cycle checkpoints are G_1/S transition, G_2/M transition, Spindle checkpoint and G_0/G_1 transition.

G_1/S transition – This checkpoint appearing at late G_1, is designated 'Start' or 'Restriction point' and is sensitive to the availability of extracellular mitogens. This transition ensures that the enzymes and other proteins required for the S phase have been synthesized and that the cell is ready to replicate DNA. Within this transition is the G_1 DNA damage checkpoint, which senses damage to the DNA, and activates pathways that delay cell cycle progression in G_1 before DNA synthesis (Epstein, 2003). Activation of the DNA damage checkpoint involves the PI3K homologs ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-related protein (ATR), which function at both G_1 and G_2. This checkpoint imposes a G_1/S block mediated by activation of proteins such as ATM, ATR, and p53. In the presence of genomic damage, p53 is activated that in turn up-regulates the expression of p21, a CDK inhibitor, to interrupt cycling by inhibiting Rb phosphorylation. This provides time for DNA repair. The progression of G_1-phase cells to the S-phase, in the absence of DNA damage, is a critical regulatory step in both normal and neoplastic cell growth

(Israels & Israels, 2001; Lundberg & Weinberg, 1999). Once a cell transits this checkpoint and enters the S phase, the progression through the rest of the cycle is not dependent on extracellular influences and hence the cell becomes committed to completing DNA synthesis. The progression from the S phase to G_2 lacks checkpoint control.

G2 /M transition - This checkpoint checks whether the DNA has been correctly and fully replicated and that the daughter chromosomes lack any replication error such as mispairing of nucleotides or incompletely synthesized DNA. This function of G_2/M is mediated by the presence of a DNA replication check point that imposes a cell cycle arrest or delay in cell cycle progression at G_2, before the cell enters the mitotic phase [51]. Unlike G_1 checkpoint, the G_2 checkpoint does not require p53 but functions by inhibiting the activation of CDK1, the protein kinase essential for the transition of cells at this point of cell cycle. The S and M phases of the cell cycle are tightly coupled and the G_2/M transition is important in preventing division of cells containing incompletely replicated DNA. This transition phase involves nuclear envelope dissolution and chromosome condensation.

Spindle checkpoint – This checkpoint ensures that a functional mitotic spindle is formed as a part of karyokinesis. This checkpoint also detects any failure of spindle fibre to attach to kinetochore and arrests the cells at metaphase (hence, also referred to as M checkpoint). Spindle checkpoint senses the improper alignment of the spindle itself and blocks cytokinesis and triggers apoptosis of the cell if the damage is irreparable. The critical transition from metaphase to anaphase and the separation

of sister chromatids is monitored by the spindle checkpoint gene products that include the Mad (mitotic arrest defective) proteins, Mad1-3p, the Bub (budding uninhibited by benomyl) proteins, Bub1-3p, and Mps1. To progress through this transition, cells must proteolytically degrade a number of proteins that are required earlier for entry into mitosis and this is accomplished by the activation of the proteasome, a component of the large multiprotein complex referred to as the anaphase-promoting complex or APC. Ubiquitin conjugation and proteolysis by APC results in the degradation of cyclin B proteins and the inactivation of MPF that is necessary for exit from mitosis as well as the degradation of proteins involved in sister chromatid cohesion such as Pds1p and proteins involved in cross-linking spindle microtubules such as A se1p (Shackelford *et al.*, 1999).

G_0/G_1 transition – A major determinant of cell growth is the efficiency of the cells to move from G_0 to the G_1 phase of the cell cycle across the G_0/G_1 transition. The transition involves the activation of quiescent G_0 cells with unduplicated DNA and the progression of the activated cells into G_1. This switch is mediated by the induction of immediate early gene products such as Fos and Myc in response to mitogenic stimuli. However, apart from triggering immediate early gene expression in cycling cells, mitogens also ensure that the cells traverse the entire duration of G_1 and the G_1/S transition (Israels & Israels, 2001). Most of the cell cycle checkpoints are linked to the apoptosis machinery, which enables the channeling of cells to apoptosis when cell or DNA damage, and replication error, is irreparable (Epstein, 2003; Kirshenbaum, 2001; Lundberg & Weinberg, 1999)

II.2.3. Role of MAPKs in cell proliferation

Mitogenic signals are relayed from the cytoplasm to the nucleus through the preferential activation of various intracellular signaling pathways. Work from many different groups using a variety of model systems has identified the MAPK cascade as a key mechanism by which such signals are transduced by the cell. In mammalian cells, three MAPK families have been clearly characterized: namely classical MAPK (also known as ERK 1/2 or p44/42 MAPK), C-Jun N-terminal kinase/ stress activated protein kinase (JNK/SAPK) and p38 MAPK. The core of this cascade consists of an evolutionarily conserved module of three sequentially activated protein kinases. Catalytic activation of MAPK requires phosphorylation on conserved tyrosine and threonine residues by a dual-specificity MAPK kinase (MAP2K/MAPKK/ MEK). The MAP2K is itself activated by phosphorylation on conserved serine and threonine residues by a serine/threonine MAP2K kinase (MAP3K/MAPKKK/ MEKK). In addition to phosphorylation of cytoplasmic targets, activation of MAP kinases promotes their nuclear translocation and subsequent modulation of transcription factors leading to stimulus-dependent alterations in gene expression. In mammalian cells the ability of extracellular signals to influence the cell cycle machinery is restricted to either G0 or G1 phases. After passage through the restriction point, cells are refractory to external cues and become committed to completing a full mitotic cycle. The effect of growth factor-dependent signal transduction pathways on the mammalian cell cycle consequently centres largely on the control of these kinase complexes at the G1/S transition and is one of the major functions of the MAPK pathway (Roovers & Assoian, 2000).

II.2.3.1. ERK pathway

Raf-MEK-ERK pathway represents one of the best characterised MAPK signaling pathways. Six different isoforms of ERK have been identified: ERK1–5 and ERK7/8, of which ERK1/2 (or p44/42 MAPK) is the best characterized member. It is predominantly involved in the control of cell proliferation, migration, cell division and differentiation (Cobb, 1999; Schaeffer & Weber, 1999). A number of observations have demonstrated the importance of this cascade in the regulation of cell cycle progression. In fact, the ERK family (p44/42 MAPK) is known to be an intracellular checkpoint for cellular mitogenesis. In cultured cell lines, mitogenic stimulation by growth factors correlated with stimulation of p44/42 MAP kinase, whereas inhibition of the ERK pathway by different means results in G1 arrest in a variety of cell types (Kerkhoff & Rapp, 1998; Squires *et al.*, 2002). In Chinese hamster lung fibroblasts and ovary cells a biphasic activation of MAPK at G1 was correlated with the ability to enter S phase (Tamemoto *et al.*, 1992).

Activated ERK1/2 translocates to the nucleus and phosphorylates transcription factors including the ternary complex factor (TCF) Elk-1, serum response factor accessory protein Sap-1a, Ets1, c-Myc, Tal , AP-1 etc. thereby positively regulating their activity (Murphy *et al.*, 2002).

Reported functions of ERK1/2 at different phases of the cell cycle are as follows:

1. **Preparation for the cell cycle:** ERK1/2 prepares the cell for the cell cycle by i) synthesis of ribosomal RNA, ii) contribution to chromatin remodeling, iii) increased pyrimidine nucleotide synthesis iv) increased protein synthesis , and v) participation in protein translation

2. **G_0-G_1 transition:** ERK1/2 are also implicated in the stabilization of c-Myc, which together with Max forms a heterodimeric transcription factor, one of the essential steps for cells to proceed from G_0 to late G_1.

3. **G_1-S transition:** Activation of this MAPK pathway promotes the expression of cyclin D1 that serves as a link between cell cycle progression and growth factor signalling. Activated Ras or MEK proteins were shown to induce the expression of reporter genes driven by the cyclin D1 promoter (Lavoie *et al.*, 1996). Besides regulation of the expression of cyclin D1, Raf-MEK-ERK cascade can also regulate the posttranslational regulation of the assembly of cyclin D-CDK4/6 complexes. ERK1/2 can also up-regulate cyclin D1 via increased stability of c-Myc transcription factor (Chambard *et al.*, 2007). These key events result in the activation of CDK4/6 which promotes cell cycle entry by phosphorylating Rb leading to the release of the transcription factor E2F. This in turn promotes the transcription of cyclins A and E resulting in the activation of CDK2. CDK2 activation is dependent on its localization in the nucleus. Blanchard *et al.* reported that nuclear translocation of CDK2 and the resulting G1/S transition of IL-2 dependent Kit 225 T cell is directly associated with the physical interaction of CDK2 with MAPK and dependent on MAPK activity (Blanchand *et al.*, 2000). Ras/Raf signaling is involved in the induction of c-myc expression. Co-expression of Ras with Myc allows the generation of cyclin E-dependent kinase activity and the induction of S phase (Leone *et al.*, 1997). Recent data show that high level of c-Myc protein prevents the association of p27 with cyclin E-CDK2 complexes driving p27 protein out of cyclin E-CDK2

complexes facilitating the phosphorylation of p27 and subsequent degradation. The ERKs can also directly phosphorylate p27 triggering its degradation by the ubiquitin proteasome pathway (Zhang *et al.*, 2002). It has also been shown to activate p21 (Balmanno & Cook, 1999). The key molecular events that contribute to the irreversible decision to enter S-phase at the restriction point can therefore all be modulated by ERK pathway activity: 1) the initiation of cyclin A and cyclin E mRNA synthesis as a consequence of cyclin D1-CDK4/6-mediated phosphorylation of Rb and liberation of E2F 2) the assembly of cyclin A- and cyclin E–CDK2 complexes by the up-regulation of the pool of CDKIs acting as assembly factors and 3) the release of catalytically active CDK2 complexes by the degradation of the inhibitory CDKI, p27 that is bound tightly to these complexes in late G1 4) ERK1/2 mediate inhibition of anti-proliferative gene transcription throughout the G_1 phase by an AP-1-dependent mechanism (Chambard *et al.*, 2007; Meloche and Pouyssegur, 2007).

4. **G_2-M transition:** A novel role for the ERK MAPK pathway in G2/M cell cycle control has recently been suggested. ERK1/2 activity is required during the early G_2 phase and DNA damage checkpoints during G_2 but is dispensable from this point on for the rest of mitosis (Shinohara *et al.*, 2006).

II.2.3.2. JNK pathway

Although significantly less well characterized, an involvement in cell cycle control of the SAPKs has recently emerged. JNK can bind the NH2-terminal activation domain of c-Jun and phosphorylate c-Jun on Ser-63 and Ser-73. Transactivation of c-

Jun leads to increased expression of genes with AP-1 sites in their promoters. Pedram *et al.* reported that through a novel ERK to JNK cross-activation and subsequent JNK action, the important events for VEGF-induced G1/S progression and cell proliferation are enhanced (Pedram *et al.*, 1998). The identified role of JNK and the importance of ERK/JNK cross-activation is specifically seen for the stimulation of important G1 cell cycle events that lead to progression to S phase (DNA synthesis) (Pedram *et al.*, 1998). JNK1 has been shown to bind to and phosphorylate the E2F1 transcription factor *in vitro* and consequently reduce its DNA binding affinity. Thus, JNK activation *in vivo* may have the consequence of disrupting E2F-containing transcriptional complexes and imparting a stress-induced G1 arrest subsequent to CDK-mediated phosphorylation of pRB. In addition, c-Jun has also recently been shown to promote proliferation by acting as a transcriptional repressor of p53 and consequently its target gene, the CDKI p21 (Zhang & Liu, 2002).

II.2.3.3. p38 MAPK pathway

The mammalian p38 MAPK families are activated by cellular stress including UV irradiation, heat shock, high osmotic stress, lipopolysaccharide, protein synthesis inhibitors, pro-inflammatory cytokines (such as IL-1 and TNF-α) and certain mitogens (Kacimi *et al.*, 2000; Raingeaud *et al.*, 1995). Four isoforms of p38 MAPK have been identified: p38α, p38β, p38γ and p38δ. While p38α and p38β are universally expressed, p38γ and p38 δ appear to have a more tissue specific expression pattern. p38γ is most abundant in skeletal muscle while p38δ is highly expressed in testes, pancreas, kidney and small intestine (Zarubin & Han, 2005). p38

MAPK appears to play a major role in apoptosis, differentiation, survival, development, inflammation and other Stress responses. Recent studies reveal a role for this MAPK in cell proliferation (Engel *et al.*, 2005; Khiema *et al.*, 2008; Thorton *et al.*, 2009).

p38 MAPK has been to shown to be a negative regulator of cell proliferation in different cell types in response to variety of stress stimuli. Its role in arresting the cell cycle possibly prevents inappropriate execution of key transitions in the presence of DNA damaging agents or other stressful stimuli. The p38 MAPK pathway is involved in the induction of the two major cell cycle checkpoints: G1/S and G2/M, although its role in the G2/M checkpoint is better established (Thorton *et al.*, 2009).

Role of p38 MAPK in the G2/M cell cycle checkpoint

Exposure to ultraviolet light (UV), γ-irradiation and chemotherapeutic drugs results in the generation of DNA double strand breaks (DSBs). In response to DSBs, p38 MAPK is activated and leads to the establishment of a G2/M cell cycle checkpoint (Bulavin *et al.*, 2002). p38 MAPK activation in response to DNA DSBs has been reported to be dependent on the activation of Ser/Thr protein kinases that serve as DNA damage sensors, specifically the ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia-mutated and Rad3-related (ATR) kinases (Reinhardt *et al.*, 2007). However, the specific pathways connecting DNA damage to p38 MAPK activation may vary by cell type, the specific DNA damage stimuli and the extent of DNA damage.

Two pathways have been described that explains the mechanism by which p38 MAPK contributes to the induction of the G2/M cell cycle checkpoint. p38 MAPK

phosphorylates and activates p53 and this leads to the induction of a p53-dependent G2/M checkpoint (Thorton *et al.*, 2009). Phosphorylation of p53 leads to dissociation of p53 from Murine double minute 2 (Mdm2), a protein that binds to the transactivation domain of p53 to promote its ubiquitination and degradation. Disruption of this interaction promotes the accumulation of p53. p38 MAPK also associates with rowth arrest and DNA damage inducible 45α (Gadd45α) proteins and promotes their association with p53, further increasing p53 stability. The activation and stabilization of p53 by p38 MAPK leads to the transcription of p53-target genes such as p21, Gadd45α, 14-3-3. These proteins enforce a G2/M checkpoint by either directly or indirectly inactivating cyclin B-CDK1 complex, the major engine driving the transition from G2 to M (Thorton *et al.*, 2009).

p38 MAPK can also induce a G2/M checkpoint through the phosphorylation and inhibition of the phosphatase Cdc25B (Bulavin *et al.*, 2001). Cdc25B dephosphorylates CDK1 and activates the cyclin B/CDK1 complex driving progression of the cell cycle. It has been proposed that phosphorylation of Cdc25B inhibits its activity by promoting its association with 14-3-3 proteins and sequestering it in the cytoplasm (Morris *et al.*, 2000). While it was initially reported that p38 MAPK could directly phosphorylate Cdc25B *in vitro*, more recent studies have shown that p38 MAPK promoted the phosphorylation of Cdc25B indirectly through the activation of MK2 one of the well known substrates of p38 MAPK (Lemaire *et al.*, 2006). Activation of MK2 was also shown to be required for the induction of a G2/M checkpoint in response to UV in these studies.

Role of p38 MAPK in the G1/S checkpoint

Although less established, p38 MAPK activation can also contribute to the induction of a G1/S checkpoint in response to different stimuli (Thorton *et al.*, 2009). Several mechanisms by which p38 MAPK mediates a G1/S checkpoint have been described. p38 MAPK can induce a G1/S checkpoint through the activation of p53. In addition to its role in controlling G2 to M progression, p53 also regulates G1 to S progression. p38 MAPK activation of p53 results in the accumulation of p21 (Kim *et al.*, 2002). While p21 promotes a G2/M checkpoint by inactivating CDK1, to establish a G1/S checkpoint p21 inactivates CDK2. Another key target of p38 MAPK in controlling G1/S checkpoint is the regulation of cyclin D1expression (Page *et al.*, 2001). p38 MAPK affects the levels of cyclin D1 by two independent mechanisms. p38 MAPK can negatively regulate cyclin D1 at the level of transcription by phosphorylating and stabilizing HMG-box protein 1 (HBP1), a transcriptional repressor that inhibits cyclin D1 gene expression (Yee *et al.*, 2004). p38 MAPK can also directly phosphorylate cyclin D1 resulting in cyclin D1 ubiquitination and proteosomal degradation (Thorton *et al.*, 2009).

Unlike other members of the Cdc25 family that only regulate the G2/M transition, Cdc25A is a CDK phosphatase that can regulate the G1/S transition as well. p38 MAPK can phosphorylate and promote the degradation of Cdc25A contributing to the establishment of a G1/S checkpoint (Goloudina *et al.*, 2003). p38 MAPK has been reported to directly phosphorylate and stabilize p21 in vivo and regulate the other CDKIs such as p16INK4 and p19ARF (Kim *et al.*, 2002). It can mediate a G1/S checkpoint by phosphorylating Rb, perhaps directly, upon Fas receptor

stimulation in a cyclin-CDK independent way (Wang *et al.*, 2000) resulting in a cell cycle arrest. Thus, p38 MAPK can regulate the induction of a G1/S checkpoint by multiple distinct mechanisms which is context-dependent.

II.3. Hypoxia

Molecular oxygen is essential for the normal growth and development of most multicellular organisms. Hypoxia is a state in which diffusive oxygen transport fails to meet the metabolic oxygen demand of the tissue. But mammalian cells have evolved intrinsic mechanisms for the maintenance of oxygen homeostasis at the tissue level, the failure of which can lead to cellular dysfunction and/or irreversible cell damage. The cells within a tissue are exposed to different concentrations of oxygen depending on the specific localization of the cells and the functional status of the tissue. Atmospheric air contains about 21% oxygen. However, arterial pO_2 is only about 14%. Heart cells receive an oxygen supply of <10% under conditions of systemic normoxia (Roy *et al.*, 2003). During mild hypoxia, myocardial pO_2 drops to ~1% to 3% or lower (Roy *et al.*, 2003). Hypoxia is encountered in a variety of conditions – both physiological and pathological. The developing foetus may be exposed to hypoxic conditions *in utero* (Xu *et al.*, 2006). Further, hypoxia is an extremely common physiological stress, found at high altitudes due to oxygen-thin atmosphere (Pierson, 2000). Hypoxia also manifests under clinical conditions such as wound healing, anaemia, myocardial infarction, ischemic heart disease, stroke, retinopathy, chronic obstructive pulmonary disease and cancer (Ostadal *et al.*, 1999). Hypoxic condition within the body activates complex adaptive physiological mechanisms within cells aimed at optimizing oxygen delivery to metabolizing

tissues. However, persistence of hypoxia leads to severe pathological consequences both at the cellular and molecular levels (Giaccia *et al.*, 2004; Hochachka *et al.*, 1996; Michiels, 2004; Sarkar *et al.*, 2003; Webster, 2003; Wenger, 2002).

II.3.1. Cellular response to hypoxia and its effect on cell proliferation

Cells can respond differently to wide ranges of oxygen through alterations in both their metabolic states and growth rates. Although the understanding of hypoxia-induced cellular metabolic changes is rapidly emerging, our understanding of the effects of oxygen deprivation on the control of the cell cycle is still rudimentary. In recent years, several lines of evidence have indicated that hypoxia can alter cell proliferation. It can exert both pro- and anti-proliferative effects on cells depending on the degree of hypoxia, the extent of cell differentiation and the tissue or organ of origin. Hypoxia is found to delay cell cycle progression in a variety of cell types including bovine aortic and pulmonary artery ECs (Tucci *et al.*, 1997), MEFs (Gardner *et al.*, 2001; Gardner *et al.*, 2003; Green *et al.*, 2001) and splenic B-lymphocytes (Goda *et al.*, 2003). This inhibition or delay in cell cycle progression contributes to the reduction in utilization of available residual energy (Douglas *et al.*, 2005; Goda *et al.*, 2003; Gardner *et al.*, 2003). The hypoxia-induced inhibition of proliferation in many cell types, including murine embryo fibroblasts, is reported to be associated with changes in the cell cycle regulatory apparatus. The cell cycle may halt completely and irreversibly in hypoxia-sensitive cells leading to death whereas in hypoxia-tolerant cells, the cell cycle arrest can be transient lasting only for the duration of the stress and is resumed when the stress is removed (Amellem *et al.*, 1991; Amellem *et al.*, 1996). In certain cell types, however, hypoxia causes

hyperplasia as demonstrated in the pulmonary vasculature. Hypoxia induces human pulmonary fibroblast and pulmonary smooth muscle cell proliferation (Das *et al.*, 2001; Das *et al.*, 2002), mediated by PDGF and PAF. Hypoxic-induction of pulmonary artery adventitial fibroblast proliferation is reported to involve multiple pathways including p44/42 MAPK, JNK, p38 MAPK, and PKC (Durmowicz & Stenmark, 1999; Gerasimovskaya *et al.*, 2005. Hypoxia also regulates proliferation of aortic (Schafer *et al.*, 2003) and pulmonary artery ECs ((Tucci *et al.*, 1997).

Several mechanisms have been shown that underlie the specific cellular response to hypoxia. Severe hypoxia or anoxia (0.01% oxygen) causes inactivation of enzymes responsible for nucleotide synthesis, ultimately inhibiting DNA replication (Tanaka *et al.*, 1994). In the moderately hypoxic microenvironment (0.1 to ~1% oxygen), various biological reactions show significant changes relative to normoxia. Numerous studies on moderate hypoxia have indicated that hypoxic cells can undergo cell cycle arrest at the G1/S interface without any alteration in their long-term viability. Hypoxia-induced G1 arrest is associated with a decreased activity of certain cyclin-CDK complexes, leading to hypophosphorylation of Rb and inhibition of cell cycle progression (Gardner *et al.*, 2001; Amellem *et al.*, 1998; Krtolica *et al.*, 1998). Studies have also demonstrated an increase in cyclin-dependent kinase inhibitors, such as p27, and a decrease in cyclin-CDK components, such as cyclin D, cyclin E, and CDK4 (Gardner *et al.*, 2001).

A family of transcription factors called the hypoxia-inducible factor 1 (HIF-1) is a major regulator of the hypoxic response (Carmeliet *et al.*, 1998; Guillemin & Krasnow, 1997; Minet E *et al.*, 1999; Wenger, 2002). HIF-1 is a phosphorylation-

dependent and redox-sensitive heterodimeric protein composed of two distinct basic-helix-loop-helix-PAS transcription factors, the variable subunit and the constitutively expressed HIF-1β subunit. HIF-1α is sensitive to decreased oxygen levels and is degraded rapidly by the ubiquitin proteasomal pathway under normoxic conditions. HIF-1α degradation is accomplished by a family of enzymes, the prolyl hydroxylases, also referred to as oxygen sensors (Wenger, 2002). Hypoxia results in an altered availability of HIF-1α to the von Hippel-Lindau protein and ubiquitination, ultimately blocking its degradation. Once activated by hypoxia, the HIF-1α subunit binds to the HIF-1β subunit to modulate the expression of genes involved in several physiological processes including angiogenesis, proliferation, cell survival and cell death, erythropoiesis, energy metabolism and oxygen chemoreception (Wenger, 2002).

Apart from HIF proteins, a variety of other transcription factors and signaling molecules are involved in eliciting the hypoxia-induced changes. p53 and NFκB are among the transcription factors activated by hypoxia (Michiels *et al.*, 2002; Schmid *et al.*, 2004; Seta & Millhorn, 2004). Hypoxia has been reported to increase levels of the tumor suppressor protein, p53, and its accumulation in the mitochondria (Sansome *et al.*, 2001). p53 is associated with the activation of several processes that in turn mediate the effects of hypoxia on cells. For example, exposure of neonatal rat cardiac myocytes to prolonged hypoxia results in substantial cell loss by apoptosis and is associated with increased expression of p53 and its trans-activating ability (Pagano *et al.*, 1995). Hypoxia activates NFκB by tyrosine phosphorylation, inactivation and dissociation of IκB, the inhibitory subunit that binds and inactivates NFκB (Sansome *et al.*, 2001). Transcription factors such as activating protein-1 (AP-

1), CREB, GATA-1, GATA-2 and forkhead protein are also reported to be regulated by hypoxic stress (Bandyopadhyay *et al.*, 1995; Seta & Millhorn, 2004). Many hypoxia-inducible genes have their promoter and/or enhancer elements in their regulatory regions as binding sites for one or more of these hypoxia-responsive transcription factors. For example, most of the hypoxia-sensitive genes encoding cytokines such as VEGF, bFGF, and TNF-α have NFκB binding sites in or near their promoters, and therefore are regulated by NFκB (Seta & Millhorn, 2004). Hypoxic induction of these transcription factors is followed by their translocation to the nucleus where they modulate the expression of a wide array of genes encoding proteins with varied cellular functions (Wenger, 2002).

Several signaling pathways, including cAMP-protein kinase A, Ca^{2+}-calmodulin, phosphatidylinositol 3-kinase/Akt and p44/42 MAPK, SAPK or p38 MAPK mediate the effects of hypoxia through activation/inactivation of hypoxia-sensitive factors/proteins culminating in a functional phenotype that is characteristic of the response to hypoxia (Kacimi *et al.*, 2000; Seta & Millhorn, 2004). A large body of evidence indicates that enhanced p38 MAPK signaling is associated with cardiac hypertrophy, the onset of heart failure, and ischemic/reperfusion injury, whereas inhibition of p38 MAPK improves the survival and functional performance of ischemia/ reperfusion-injured hearts or cardiac myocytes, suggesting that activation of p38 MAPK might participate in cardiac ischemic/hypoxic injury (Conrad *et al.*, 1999; Kacimi *et al.*, 2000; Ma *et al.*, 1999; Petrich & Wang, 2004; Sodhi *et al.*, 2000; Wenzel *et al.*, 2005). Hypoxia-sensitive signaling pathways and factors are reported to modify the expression of several genes controlling mitochondrial

respiration and metabolism, activities of mitochondrial enzymes such as cytochrome oxidase and MnSOD, intercellular interactions, cytoskeletal structure, membrane transport, antioxidant defense mechanisms and cell survival (Seta & Millhorn, 2004).

II.3.2. Hypoxia in the heart

A constant supply of oxygen is indispensable for cardiac viability and function. The role of oxygen and oxygen-associated processes in the heart is complex, and they can be either beneficial or contribute to cardiac dysfunction and death. The heart can be exposed to hypoxia in several circumstances including pulmonary disease, pulmonary embolism, aspiration, during cardiac arrest, and at high altitude. Myocardial hypoxia could be caused due to a reduction in coronary blood flow (Ischemic hypoxia) or due to drop in pO_2 in arterial blood with adequate perfusion (Systemic hypoxia). Unlike localised myocardial ischemia, systemic hypoxia will affect the heart via direct and reflex effects on other sites in the circulation.

There have been many studies on the effects of hypoxia on cardiac cells. The effects of hypoxia on myocytes are similar to those in other cells. Hypoxia causes inhibition of oxidative phosphorylation and a switch to anaerobic glycolysis, resulting in decreased levels of high-energy phosphates, increased lactic acid production, and lower intracellular pH. Significant loss of cardiomyocytes by apoptosis is a prominent and an important pathogenic feature during cardiac ischemia and infarction (Webster *et al.*, 1999). Cardiac myocytes are reported to exhibit compromised cell viability even in the presence of hypoxic fibroblast-derived factors (Shivakumar *et al.*, 2008). ECs also undergo apoptosis in response to hypoxia (Matsushita *et al.*, 2000).

II.3.2.1. Hypoxia and cardiac fibroblasts

The effects of hypoxia on cardiac fibroblasts, however, remain largely unclear although these cells are exposed to oxygen deficit in several pathological states of the heart. Hypoxia is reported to enhance collagen type I synthesis but decrease total protein synthesis in cardiac fibroblasts (Agocha *et al.*, 1997b). Hypoxia has also been shown to enhance matrix metalloproteinases-2 synthesis in neonatal rat cardiac fibroblasts and augment the stimulatory effects of ET-1, Ang II and IL-1β on matrix metalloproteinases-2 synthesis (Bergman *et al.*, 2003).

II.3.2.1.1. Hypoxia and cardiac fibroblast proliferation

Agocha et al. (1997) have reported that hypoxia inhibits DNA synthesis in human cardiac fibroblasts and modifies the response of these cells to several growth factors (Agocha *et al.*, 1997b). Two other reports from the same laboratory (Griffin *et al.*, 2000; Zhao & Eghbali-Webb, 2002) examined gender-specific effects of hypoxia on rat cardiac fibroblast DNA synthesis, and the expression of cyclin D1, cyclin B1, NFκB, p53, ERK1/2, Bcl2, HIF-1 and Jun kinase. However, in all these studies, the observed inhibition of DNA synthesis was not corroborated with changes in cell number or proliferation doubling time. Further, these experiments were performed under serum-free conditions on confluent (contact-inhibited) fibroblast cultures that had been synchronized by serum-deprivation; under these conditions, cell proliferation cannot be expected to occur.

It is important to note that cardiac fibroblasts are resistant to apoptosis under hypoxic conditions and that the pathways mediating the cell cycle and cell death are coordinately regulated (Kirshenbaum, 2001). Delineation of molecular mechanisms

underlying hypoxic regulation of cell cycle activity may, therefore, provide useful insights into the pathways that confer hypoxia-resistance upon cardiac fibroblasts in contrast to hypoxia-sensitive cells.

Understanding the cell cycle regulatory pathways in cardiac fibroblasts is important to develop therapies targeting disease processes that involve this major cell type of the heart. Barring a single report on G_1/S arrest of the cardiac fibroblast cell cycle by IL-1β, the cardiac fibroblast cell cycle is poorly investigated.

III. MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Fine chemicals

M199, FBS, BSA, collagenase type IA, trypsin, pancreatin, DNase I, HEPES, EDTA, DMSO, amphotericin B, glucose, calcium chloride, disodium hydrogen phosphate, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, genistein, tyrphostin AG490, PD98059, 3-(4-Nitrobenzyl)-6-thioinosine, RNase A, propidium iodide, Hoechst 33342, TRI-reagent, DEPC, DNase I (amplification grade), aprotinin, pepstatin A, SDS, trizma base, agarose, glycine, sodium acetate, acrylamide, bis-acrylamide, mercaptoethanol, TEMED, APS, Ponceau S, monoclonal anti-vimentin antibody, anti-human von Willebrand antibody, immunostaining kit for desmin, anti-β-actin primary antibody, anti-rabbit and anti-mouse secondary antibodies were purchased from Sigma-Aldrich, USA. Cyclin D, cyclin E, cyclin A, p27, p21, Rb and phospho-Rb primary antibodies were from Santa Cruz Biotechnology, USA. SB203580, the p38 MAPK inhibitor, and the fine chemicals for cDNA synthesis including RT buffer, RNase inhibitor, random primers, dNTPs and M-MLV Reverse Transcriptase were purchased from Promega Corporation, Madison, WI, USA. BCA protein assay kit and ECL kit were from Pierce, USA. Cell lysis buffer and primary antibodies for phospho-p38MAPK, p38MAPK, phospho-p42/44 MAPK, p42/44 MAPK were obtained from Cell Signaling Technology, USA. Taqman gene expression assay was procured from Applied Biosystems, USA. Nitrocellulose membrane was from Millipore, USA.

III.1.2. Routine Chemicals

POPOP, PPO, sodium hydroxide, toluene, TCA, chloroform, isopropanol, hydrochloric acid, ethanol, ether, glycerol and phenol red were purchased from SISCO Research Laboratories, India. Skim milk was obtained Himedia, Mumbai, India. Methanol was from Merck, India. Gentamicin and Benzyl Penicillin were from Cadila pharmaceuticals, India and Alembic Limited, India respectively.

III.1.3. Radiochemicals

[^{3}H]-thymidine (Specific radioactivity 17.2 mCi/mmol) was obtained from the Bhabha Atomic Research Centre, India. [^{3}H]-proline (Specific radioactivity – 28 Ci/mmol) was procured from Amersham Radiochemicals, UK.

III.1.4. Cell culture ware

35mm and 100mm cell culture-treated dishes, GasPak environment systems and the anaerobic/aerobic envelopes to generate hypoxic/normoxic conditions were from Becton Dickinson, USA. Cell culture filter ware was procured from Millipore, USA. Cell scrapers were from Nunc, USA.

III.1.5. Equipments used

ELISA reader (Bio-Tek instruments, USA), liquid scintillation counter (Wallac 1409), UV-visible spectrophotometer (Shimadzu, Japan), high speed refrigerated centrifuge (Hitachi, Japan), weighing balance (Sartorius, Germany), water bath (LKB, Sweden), ice-flaker (Hoshizaki, Japan), pH meter (Labindia, India), CO_2 incubator (Sanyo, Japan), phase-contrast microscope (Nikon, Japan), fluorescence microscope (Zeiss Axioskop 2 Plus), laminar flow hood (CLAS, India), magnetic

stirrer (Schott, Germany), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), electrophoresis unit (Biorad laboratories, USA), Mini Blot (Biorad laboratories, USA), Programmable Thermal Cycler (MJ Research Inc, USA), Syngene Bio Imaging (Ingenius, Canada), UV-Transilluminator (Bangalore Genei, India), BD FACSAria benchtop flow cytometer (Becton and Dickinson, USA) and 7500 Real-Time PCR System (Applied Biosystems, USA).

III.2. COMPOSITION OF MEDIA, REAGENTS AND BUFFERS

III.2.1. Acrylamide 30%

29% (w/v) acrylamide and 1% (w/v) N, N'-methylene bisacrylamide in deionized water

III.2.2. Agarose gel (1%) for electrophoresis of DNA or RNA samples

For DNA - 200mg agarose in 20ml of 0.5X TBE

For RNA - 200mg agarose in 20ml of 1X MOPS buffer

III.2.3. Blocking solution

5% (w/v) skim milk or BSA in TBST containing 0.1% Tween-20

III.2.4. Cardiac fibroblast growth medium (pH 7.4)

M199 with Earle's salts containing FBS (10%), benzyl penicillin (50U/ml) and gentamycin (0.04mg/ml)

III.2.5. Cell Lysis buffer for westerns (1X)

20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA ,1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin

III.2.6. DAB substrate solution

6mg DAB in 10ml Tris (pH 7.6) containing 10µl of 30% H_2O_2

III.2.7. DEPC-treated deionized water

1ml of DEPC in one litre of deionized water, stirred overnight at room temperature and autoclaved

III.2.8. Dissociation medium for fibroblast isolation

The medium consisted of sodium chloride (116.4 mM); HEPES (20 mM); sodium dihydrogen phosphate (1.15 mM); glucose (5.55 mM); potassium chloride (5.37 mM); magnesium sulfate (0.81 mM), BSA (1mg/ml) and $CaCl_2$ (1mM). pH was adjusted to 7.4. Deoxyribonuclease I (5.5µg/ml), Amphotericin B (10mg/l) and antibiotics (50U/ml penicillin and 0.04mg/ml gentamycin) were added to the medium under sterile conditions at the time of isolation. The dissociation medium had collagenase type IA (0.5mg/ml) and trypsin 1:250 (1mg/ml), pancreatin (0.020mg/ml).

III.2.9. DNA/RNA gel-loading dye

Bromophenol blue (0.25%); xylene cyanol FF (0.25%); EDTA (1mM); glycerol (50%) in DEPC-treated deionized water

III.2.10. Electrode buffer (pH 8.3) for SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

Tris base (25mM), glycine (192mM), SDS (0.1%) in deionized water

III.2.11. Ethidium bromide (Stock solution)

1mg in 1ml de-ionized water; 5µl of this stock solution was added to 20ml of 1% agarose gel for DNA/RNA electrophoresis

III.2.12. EDTA (0.5M, pH 8.0)

930mg EDTA in 5 ml DEPC-treated deionized water

III.2.13. Lysis buffer

0.1M sodium hydroxide containing 0.1% SDS

III.2.14. MOPS electrophoresis buffer [10X]

MOPS (0.2 M, pH 7.0), sodium acetate (3M, pH 5.0), EDTA (0.5M, pH 8.0) in DEPC-treated deionized water

1X MOPS buffer was prepared from this stock solution by 1:10 dilution.

III.2.15. Phosphate-buffered saline (PBS) (pH 7.4)

Sodium chloride (137mM), potassium chloride (2.7mM), disodium hydrogen phosphate (10.14mM), potassium dihydrogen phosphate (1.76mM)

III.2.16. Propidium iodide staining solution

PI (25µg/ml), RNase (40µg/ml), and nonidet P-40 (0.3%) in PBS

III.2.17.a. Resolving Gel for SDS – PAGE (8%)

2.7ml 30% acrylamide, 2.5ml 1.5M Tris (pH 8.8), 0.1ml 10% SDS, 0.1ml 10% ammonium persulfate and 6µl TEMED were added to 4.6ml of deionized water

III.2.17.b. Resolving Gel for SDS–PAGE (12%)

4ml 30% acrylamide, 2.5ml 1.5M Tris (pH 8.8), 0.1ml 10% SDS, 0.1ml 10% ammonium persulfate and 4µl TEMED were added to 3.3ml of deionized water

III.2.17.c. Resolving Gel for SDS – PAGE (15%)

5ml 30% acrylamide, 2.5ml 1.5M Tris (pH 8.8), 0.1ml 10% SDS, 0.1ml 10% APS and 4µl TEMED were added to 2.3ml of deionized water.

III.2.18. Scintillation cocktail

PPO (0.6%) and POPOP (0.02%) in toluene

III.2.19. SDS gel-loading buffer [1X]

SDS (2% w/v), bromophenol blue (0.03%), β-mercaptoethanol (0.3%), glycerol (10% v/v) in Tris buffer (0.067M, pH 6.8)

III.2.20. Serum-free medium

M199 containing antibiotics (50U/ml penicillin and 0.04mg/ml gentamycin)

III.2.21. Sodium acetate (3M, pH 5-6)

1.23 g sodium acetate in 5 ml DEPC-treated deionized water

III.2.22. Stacking gel buffer (pH 6.8)

30ml from resolving buffer was measured, adjusted pH to 6.8 using HCl and was made up to 45ml. Stored at room temperature.

III.2.23. Stacking gel for SDS – PAGE (5%)

0.33ml 30% acrylamide, 0.25ml 1M Tris (pH 6.8), 0.02ml 10% SDS, 0.02ml 10% ammonium persulfate and 2µl TEMED were added to 1.4ml of deionized water

III.2.24. Substrate solution for alkaline phosphatase

1.0mg/ml Fast Red TR (4-Chloro-2-methylbenzenediazonium), 0.4mg/ml Naphthol AS-MX (3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate), 0.15mg/ml levamisol prepared in 0.1M Tris buffer, pH 7.4.

III.2.25. Towbin's buffer (Transfer buffer)

3.027g Tris base, 14.4g glycine, 200ml methanol made up to 1L with deionized water

III.2.26. Tris borate EDTA buffer (TBE) (5X, pH 8.3)

54g Tris base; 27.5g boric acid; 20ml EDTA (0.5M, pH 8.0) made up to 1L with deionized water

III.2.27. Tris-buffered saline (10X, pH 7.6)

24.2g Tris base, 80g sodium chloride in 1L deionized water

III.2.28. Tris-buffered saline with Tween-20 (TBST) [1X]

1X TBS containing 0.1% Tween-20

III.2.29. Trypsin-EDTA solution

2.5mg/ml trypsin and 0.38mg/ml EDTA in PBS (pH 7.4)

III.3. ISOLATION, CULTURE AND CHARACTERIZATION OF CARDIAC FIBROBLASTS

III.3.1. Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague-Dawley rats following the method of Kumaran and Shivakumar (2002) with some modifications. Rats (2-3 month old) were anaesthetized with ether. Heart was excised and collected in PBS (Ref. III.2.15) containing antibiotics (50U/ml penicillin and 0.04mg/ml gentamycin) and amphotericin B (10mg/l). The atria were removed and the ventricular tissue was washed in PBS, minced into bits of approximately 1 mm^3 size and subjected to a series of digestions in dissociation medium (Ref. III.2.8) containing Collagenase type IA (0.5mg/ml), Trypsin 1:250 (1mg/ml) and Pancreatin (0.020mg/ml). Digestion was aided by gentle shaking of the flask containing tissue bits on an orbital shaker maintained at 37°C. The supernatants were centrifuged at 1500 rpm for 5 minutes. The cell pellets were pooled, re-suspended in M199 supplemented with 10% FBS, seeded in two 35 mm cell culture dishes and incubated in a humidified CO_2 incubator at 37°C in 95% air-5% CO_2 for 150 minutes. At the end of this period, the supernatant containing unattached cells and debris was discarded, the dishes with the adherent fibroblasts were rinsed 3-4 times and incubated with M199 containing 10% FBS. The pre-plating step ensured preferential attachment of cardiac fibroblasts. At 24 hours after isolation, the dishes were washed and incubated with M199 containing 10% FBS (Ref. III.2.4) and maintained in a CO_2 incubator at 37°C.

III.3.1.1. Sub-culture of cardiac fibroblasts

At confluence, the culture supernatant was removed, cells were washed with PBS (Ref. III.2.15) and trypsinized at 37^{0}C in trypsin-EDTA solution (Ref. III.2.29). Trypsinization was stopped by addition of M199 containing 10% FBS (Ref. III.2.4) and the detached cells were collected immediately by centrifugation at 1500 rpm for 5 minutes. The cell pellet was suspended in M199 containing 10% FBS (Ref. III.2.4) and seeded in fresh culture dishes at a split ratio of 1:3.

III.3.2. Characterization of cardiac fibroblasts in culture

Fibroblastic nature of the cells in culture was ascertained by morphology and immunocytochemistry.

III.3.2.1. Analysis of morphology

Sub-confluent and confluent cultures were examined under an inverted phase contrast microscope for morphologic characteristics.

III.3.2.2. Immunocytochemical analysis for vimentin, desmin and von Willebrand factor

Immunocytochemical staining was done as described by Eghbali *et al.* (1991) [55]. Cells from passage 2 or 3 grown to 60-70% confluence were washed with PBS (Ref.III.2.15) and fixed in 70% ice-cold methanol for 30 minutes. The fixed cells were treated with 3% hydrogen peroxide for 5 minutes, incubated for 10 minutes with 2% BSA in PBS and for 60 minutes with primary antibody diluted in PBS containing 1% BSA. The cells were then incubated for 30 minutes with diluted HRP- or ALP-conjugated secondary antibody. Following PBS wash, the cells were

incubated for up to 10 minutes with either the HRP-specific substrate reagent containing 3-amino, 9-ethyl-carbazole (AEC) or the ALP-specific substrate (Ref.III.2.24). After colour development, the cells were counterstained with hematoxylin, mounted in glycerol and observed under a microscope.

Monoclonal anti-vimentin, anti-mouse IgM and anti-mouse IgG antibodies were diluted 1:50; anti-human von Willebrand factor was diluted 1:800. Immunostaining for desmin was done using a commercially available kit.

III.3.3. Induction of hypoxia

The GasPak disposable hydrogen + CO_2 generator envelopes containing sodium borohydride and sodium bicarbonate + citric acid tablets and palladium pellets were used to generate hypoxic conditions. Upon addition of deionized water, the sodium borohydride tablet generates hydrogen which, in the presence of palladium catalyst, reduces the chamber oxygen to water, resulting in a chamber pO_2 of about < 0.1%, as indicated by anaerobic indicator strips. pO_2 of the medium was ~3%.

To generate normoxic conditions, GasPak disposable CO_2 generator envelopes containing sodium bicarbonate + citric acid tablet were used. The sodium bicarbonate + citric acid tablets in both normoxic and hypoxic systems release CO_2 upon addition of deionized water to provide a pCO_2 of about 5%, which was checked using a CO_2 indicator. The desired chamber oxygen and CO_2 levels were achieved within 50 minutes, at 37°C.

III.3.4. Assessment of cell viability by Hoechst 33342-PI staining

Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were exposed to hypoxia or normoxia for 20 hours and then incubated with Hoechst 33342 (10μM) at 37^{0}C for 10 minutes. Following incubation in the dark, propidium iodide (PI) was added (0.25μg/ml) and cultures were visualized under a fluorescence microscope (Zeiss Axioskop 2 Plus) at excitation wavelengths of 352 nm and 538 nm, respectively, for Hoechst 33342 and PI.

III.3.5. Cell cycle analysis by flow cytometry

Sub-confluent cultures of cardiac fibroblasts, synchronised for 24 hours, were exposed to 42 hours of hypoxia in M199 containing 10% FBS with or without SB203580 in DMSO (10 μM), with the control groups receiving the vehicle at an equivalent concentration. Cells were trypsinized with trypsin/EDTA (Ref III.2.29), washed with Ca^{2+} -/ Mg^{2+} -free PBS (Ref III.2.15) and fixed in 70% ethanol in PBS for 1 hour at 4^{o}C. The fixed cells were re-suspended in 0.25ml PBS (Ref III.2.15) and treated with 5μl of 10mg/ml RNase A for 10 min at 37^{o}C. Cellular DNA was stained with 10μg/ml propidium iodide (PI) and samples were filtered through a 70-mm nylon mesh to remove cell clumps.

Flow cytometric analysis was done using a BD FACSAria benchtop flow cytometer (Becton and Dickinson, USA). Single cell populations were gated using forward scatter, an indicator of cell size, versus side scatter, an indicator of cell granularity. The FL2 detector measures fluorescent light from PI, which emits a red color at 650-nm wavelength of the FACScan laser, and PI intensity is proportional to the DNA

content of the cell. The FL2-PI area versus width plots distinguished true cycling G2/M cells from doublets or aggregates of G0/G1 cells. Based on DNA content, the cells were sorted into G_0/G_1, S and G_2/M populations. At least 20,000 cells were collected per sample.

III.3.6. Measurement of DNA synthesis

DNA synthesis was measured in terms of [^{3}H]-thymidine incorporation into TCA-insoluble material, as described earlier [98], with some modifications. Sub-confluent cultures, synchronized by serum-deprivation for 24 hours, were exposed to hypoxic/normoxic treatments for the indicated durations in M199 containing 10% FBS and 1μCi/ml [^{3}H]-thymidine. The culture supernatant was discarded; cell layer was washed with PBS (Ref III.2.15), lysed in lysis buffer (Ref. III.2.13) and precipitated with 20% ice-cold TCA. The TCA precipitates were washed with 5% ice-cold TCA and 70% alcohol and air dried. Radioactivity associated with the TCA-insoluble fraction was determined using a liquid scintillation spectrometer.

III.3.7. Western blot analysis

Western blot analysis was carried out following the standard protocol. Briefly, sub-confluent cardiac fibroblast cultures in M199 containing 10% FBS (Ref. III.2.4) were exposed to hypoxic/ normoxic treatments for the indicated durations and lysed in cell lysis buffer (Ref.III.2.5). The lysates were centrifuged at 13,000 rpm for 30 minutes to remove cell debris, and the supernatant was aliquoted and stored at -80^0C until use. Protein was quantified using the BCA protein assay kit. The lysates were denatured by incubation with SDS-gel loading buffer (Ref.III.2.19) at 100°C for 10

minutes. 60μg of protein was electrophoretically fractionated on 8, 12 or 15% SDS-PAGE minigels (Ref.III.2.17) and electroblotted onto nitrocellulose membrane for 90 minutes at 100V. The membrane with the transferred proteins (ascertained by Ponceau S staining) was blocked for 1 hour with 5% skim milk or BSA (in case of phosphoproteins) (Ref. III.2.3) and incubated overnight at 4^0C with primary antibodies prepared at a dilution of 1:200 (anti-cyclin D1, anti-cyclin A, anti-cyclin E, anti-p27, anti-p21, anti-Rb, anti-phospho Rb, anti-Skp2) or 1:1000 (anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-p44/42 MAPK, anti-p44/42 MAPK) in 5% BSA in TBST (Ref. III.2.28). Unbound primary antibody was removed by washing (5x5' times) with TBST (Ref. III.2.28). Immunoblots were exposed for 1 hour to HRP-conjugated anti-mouse/anti-rabbit secondary antibody at 1:1000 dilution in 5% BSA containing TBST (Ref. III.2.28). The ECL detection system was used to detect the antigen-antibody complexes, with average exposure time of 5–60s. Duration of exposure of the membrane to X-ray film ranged from 30 seconds to 30 minutes. The membrane was then stripped overnight by washing with TBST (Ref. III.2.28) on a rocking platform, re-probed with anti-β actin antibody (1:1000) and was developed as described above. Protein expression was quantified by densitometric scanning (Syngene gel documentation system).

III.3.8. Isolation of total RNA

Glasswares used for RNA isolation were DEPC-treated and autoclaved. All reagents were prepared with DEPC-treated water (Ref. III.2.7).

Total RNA was isolated from sub-confluent cultures of cardiac fibroblasts exposed to hypoxia/normoxia for 12 hours in M199 containing 10% FBS (Ref. III.2.4) using

TRI reagent (4ml/100mm culture dish), according to the manufacturer's instructions. Following phase separation with chloroform, RNA was precipitated from the aqueous phase using isopropanol and was dissolved in DEPC-treated water (Ref. III.2.7). The yield and purity of the isolated RNA was determined spectrophotometrically by assessing A_{260} and $A_{260/280}$ respectively. The intactness was ascertained by 1% agarose gel (Ref. III.2.2) electrophoresis following standard protocol.

The isolated RNA samples were subjected to DNase I (amplification grade) treatment as per manufacturer's instructions to remove any genomic DNA contamination. This was further validated by PCR analysis of the DNase I treated-RNA using G3PDH primers.

III.3.9. cDNA synthesis

Following DNase I treatment, 2µg of total RNA was reverse transcribed to cDNA by the following procedure. The cDNA synthesizing mixture consisted of:

5X RT buffer	6.0 µl
dNTPs	2.5 µl
Random primers	3.0 µl
RNase inhibitor	0.5 µl
M-MLV RT	2.0 µl
Total RNA	10.0 µl
DEPC treated water	6.0 µl
	---
	30 µl

1µg total RNA, 10µl of DEPC-treated water (Ref. III.2.7) and 3.0µl random primers were initially mixed and heated at 70^0C for 5 min. The heated mixture was then snap-cooled on ice and mixed with the remaining constituents listed above. The reaction mixture was incubated at 37^0C for 60 minutes, heated at 90^0C for 5 minutes and then cooled on ice to inactivate M-MLV reverse transcriptase. The cDNA preparations were stored at -20^0C until use.

III.3.10. Real-time PCR analysis

Taqman quantitative real-time PCR analysis was carried out using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA) with specific, FAM-labelled probes. The oligonucleotide primers and probes for p27 mRNA (Assay ID, Rn00582195_m1) and 18S rRNA (4333760T) were from Applied Biosystems. PCR reactions were performed, as per the manufacturer's instructions. The following thermal cycling condition was used: 95°C for 10 min followed by denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min for each of 40 cycles. p27 expression was quantified using $\Delta\Delta Ct$ method with 18S rRNA as reference gene.

III.3.11. STATISTICAL ANALYSIS

Statistical significance was assessed using Student's t-test. $p \leq 0.05$ was considered significant. Values were expressed as Mean ± SD.

IV. RESULTS

IV.1. Characterization of adult rat cardiac fibroblasts

IV.1.1. Morphological analysis

Cardiac fibroblasts isolated from adult rat ventricular tissue were grown in culture as described under 'Methods'. Pre-plating for 150 minutes post-isolation ensured selective enrichment of cultures with fibroblasts, which constituted >99% of the cells in the cultures. Morphological analysis and immunocytochemical staining established the fibroblastic nature of the cells. At 150 minutes after isolation, the cells had a dense nest-like morphology (*Figure 1*) and the cells attained spindle-like appearance by 24 hours. At confluence, the cultures exhibited a monolayer rather than 'cobblestone' or 'hill and valley' pattern, ruling out contamination of the cultures by ECs and VSMCs, respectively (*Figure 2*). Cells at passages 2 or 3 were used for the experiments.

IV.1.2. Immunocytochemical staining

Cells were tested for immunoreactivity with antibodies against the cytoskeletal proteins, vimentin, desmin and the perinuclear Factor VIII-associated antigen. They were positive for vimentin (*Figure 3*) but negative for Factor VIII-related antigen (*Figure 4*) and desmin (*Figure 5*), confirming their fibroblastic nature and ruling out the presence of ECs and VSMCs in the cultures.

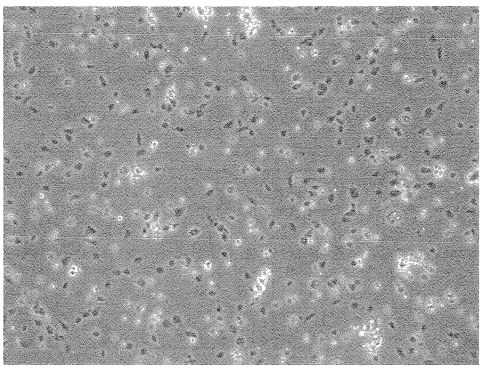


Figure 1: Phase contrast micrograph of adult rat cardiac fibroblasts at 150 minutes after isolation (100X)

Cardiac fibroblasts, isolated as described under 'Methods', were pre-plated for 150 minutes in M199 containing 10% FBS. At the end of this period, dishes enriched with the adherent fibroblasts were rinsed and incubated with M199 containing 10% FBS.

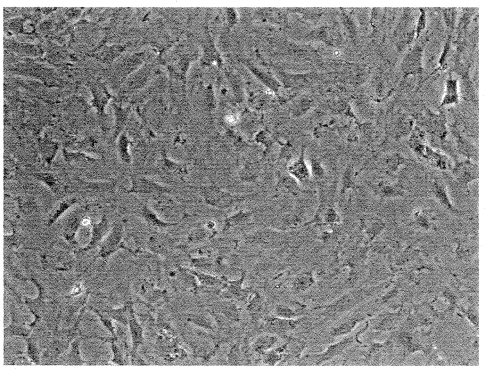
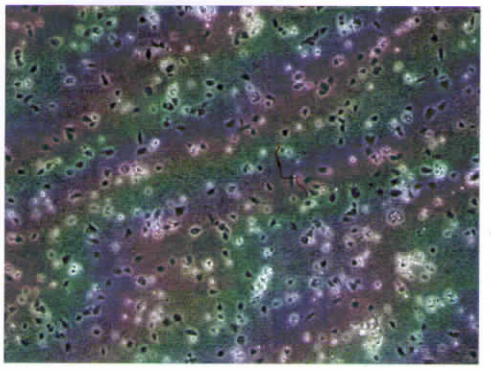


Figure 2: Phase contrast micrograph of adult rat cardiac fibroblasts at confluence (100X)

Cardiac fibroblasts, isolated as described under 'Methods' were grown to confluence in M199 containing 10% FBS to form a monolayer with spindle-shaped morphology



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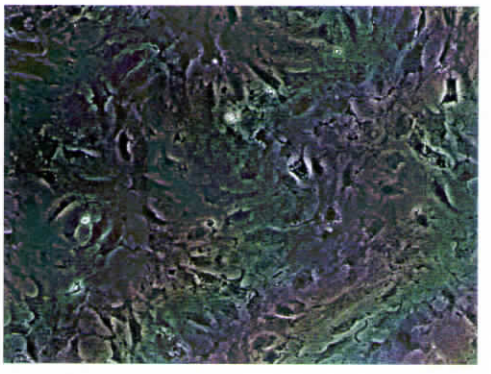

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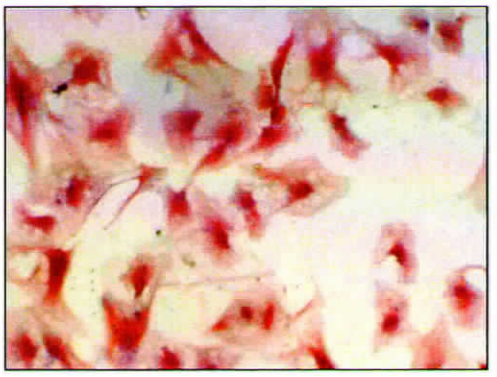


Figure 3: Photomicrograph of vimentin-positive adult rat cardiac fibroblasts (200X)

Sub-confluent cultures were methanol-fixed and incubated with anti-vimentin antibody, ALP-conjugated secondary antibody and ALP-specific chromogen. Nuclei were counter-stained using hematoxylin.

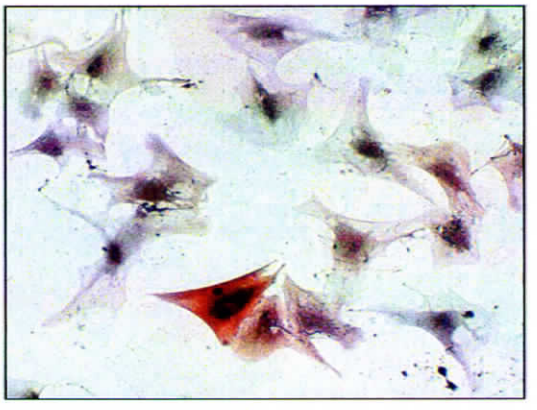


Figure 4: Photomicrograph of desmin-negative adult rat cardiac fibroblasts (200X)

Sub-confluent cultures were methanol-fixed and incubated with anti-desmin antibody, ALP-conjugated secondary antibody and ALP-specific chromogen. Nuclei were counter-stained using hematoxylin.

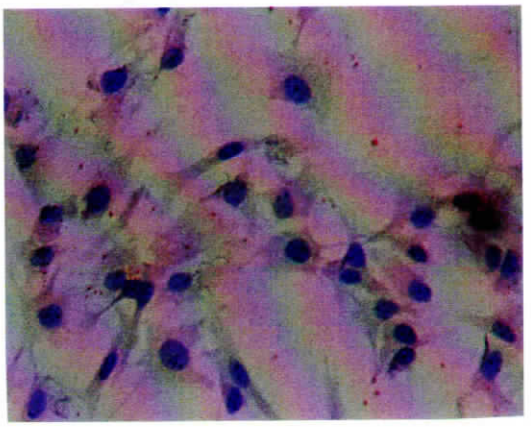


Figure 5: Photomicrograph of Factor VIII-negative cardiac fibroblasts (200X)

Sub-confluent cultures were methanol-fixed, and incubated with anti-Factor VIII antibody, ALP-conjugated secondary antibody and ALP-specific chromogen. Nuclei were counter-stained using hematoxylin.

IV.2. *In vitro* hypoxia model

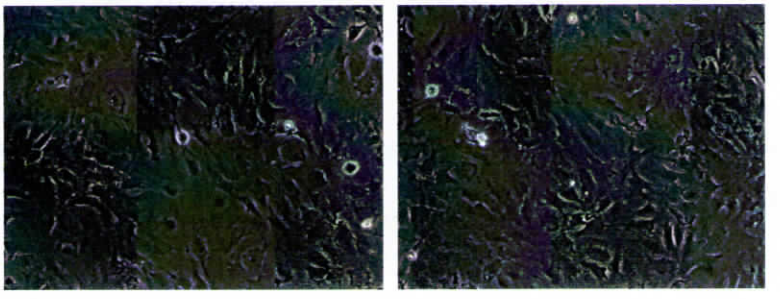
Hypoxic (pO2 ~0.75 mmHg) and normoxic (pO2 ~150 mmHg, "control") conditions were generated using the BBL-GasPak system and GasPak envelopes from Becton Dickinson, USA (Figure 6). The pO2 of the hypoxic and normoxic media was ~23 mmHg (3%) and ~113 mmHg (15%), respectively, and the pH was comparable at about 7.5. pCO2 in both normoxic and hypoxic systems was about 5%.

To test whether cellular viability is compromised under hypoxic conditions, sub-confluent cultures of cardiac fibroblasts were exposed to hypoxic or normoxic conditions in M199 containing 10% FBS and cell survival was evaluated by phase contrast microscopy and Hoechst/PI staining as described under 'Methods'. Consistent with earlier observations in this laboratory (Sapna, 2007), cardiac

fibroblasts exhibited normal morphology at 48 hours of hypoxia treatment (*Figure 7*) and were hardly positive for PI after 20 hours of exposure to hypoxia (*Figure 8*).

Figure 6 : In vitro cellular hypoxia system

GasPak anaerobic system containing (H2 + CO2) generator envelope was used to generate hypoxic conditions as described under 'Methods'.



Normoxia **Hypoxia**

Figure 7: Representative phase contrast micrograph of normoxic and hypoxic cardiac fibroblasts

Sub-confluent cultures of cardiac fibroblasts in M199 with 10% FBS were subjected to normoxic (N) or hypoxic (H) treatment for 48 hours. Hypoxia did not produce morphological changes in these cells.

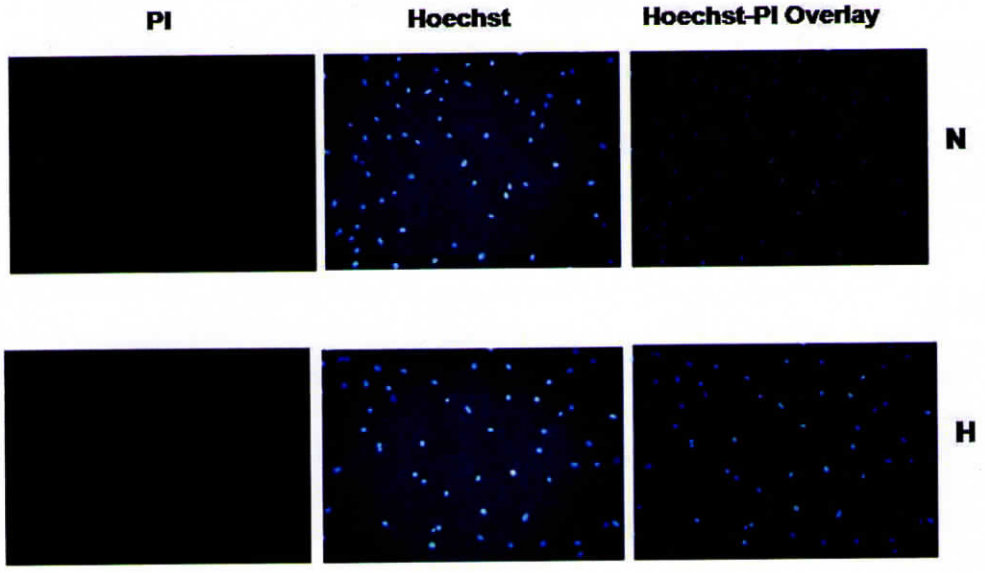


Figure 8: Representative fluorescent micrograph of Hoechst 33342-PI stained normoxic (N) and hypoxic (H) cardiac fibroblasts

Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were exposed to normoxic (N) or hypoxic (H) conditions for 20 hours and then stained with Hoechst 33342 and PI as described under 'Methods'. Very few cells took up PI, showing that hypoxia does not compromise cardiac fibroblast viability.

IV.3. Effect of hypoxia on cardiac fibroblast proliferation

IV.3.1. Hypoxia delays G1-S transition in cardiac fibroblasts

Previous studies in this laboratory had shown that hypoxia significantly decreases basal and serum (10%)-induced DNA synthesis and increases the population doubling time from 27.7 hours in normoxic cultures to 54.14 hours, which is reversed upon re-oxygenation, suggesting that hypoxia causes reversible inhibition of cardiac fibroblast proliferation (Sapna, 2007). The inhibition of proliferation by

hypoxia prompted investigations on the hypoxia-sensitive checkpoint(s) in the cardiac fibroblast cell cycle.

To broadly identify the hypoxia-sensitive checkpoint, the time-dependence of the inhibitory effect of hypoxia on DNA synthesis was first evaluated. Sub-confluent cultures of cardiac fibroblasts, synchronized by serum deprivation for 24 hours in M199, were exposed to normoxia or hypoxia with 1μCi/ml [^{3}H]-thymidine 5, 12 and 15 hours after addition of 10% FBS. At 24 hours after serum addition, 20% TCA-precipitable radioactivity was determined. Exposure of sub-confluent cultures of cardiac fibroblasts to hypoxia 15 hours after serum addition had no effect on cell proliferation (*Figure 9*), implying that hypoxia exerted its action in the early phase of the cell cycle. This was further confirmed by flow cytometry.

Flow cytometric analysis allows the measurement of DNA content on a per cell basis and is the technique of choice for the determination of cell cycle kinetics. The cell cycle phase distribution, analyzed by flow cytometry, showed that exposure of sub-confluent cultures of cardiac fibroblasts to 42 hours of hypoxia in M199 containing 10% FBS resulted in significant accumulation of cells at the G_0/G_1 phase, suggesting that G_1/S transition is oxygen-sensitive in hypoxic cardiac fibroblasts (*Figure 10*).

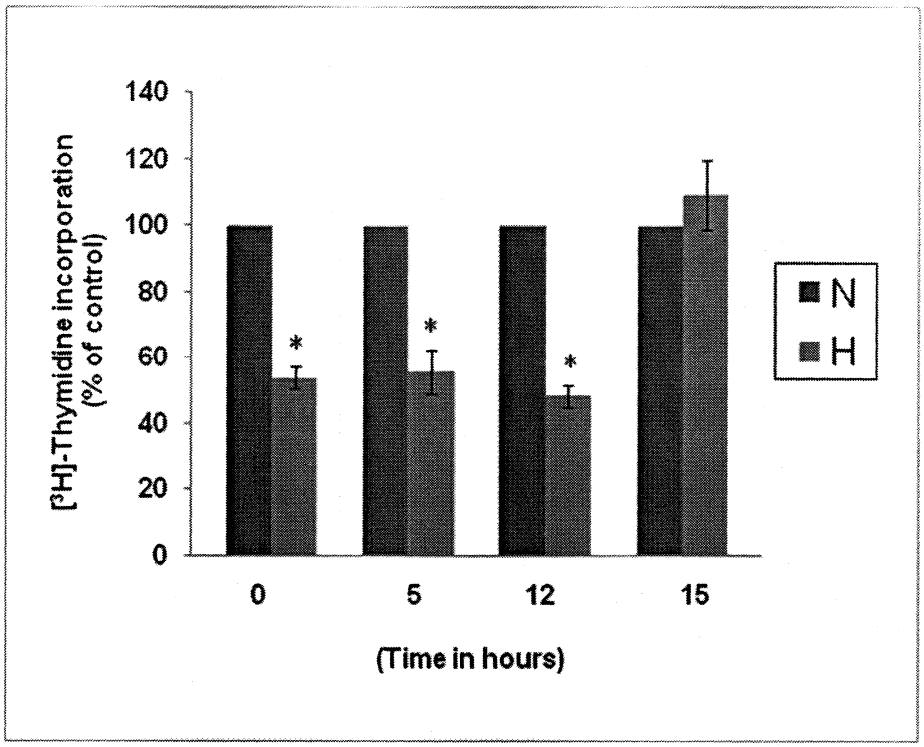
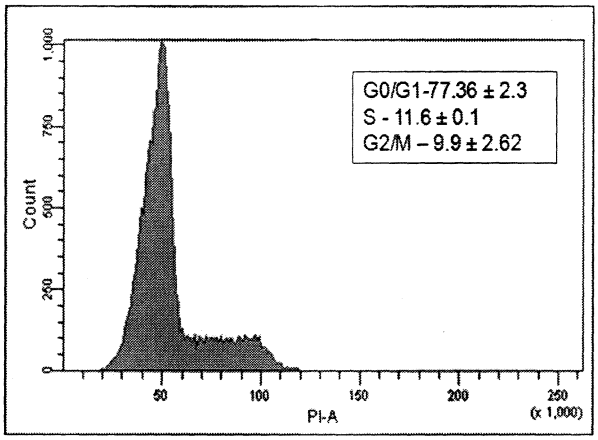


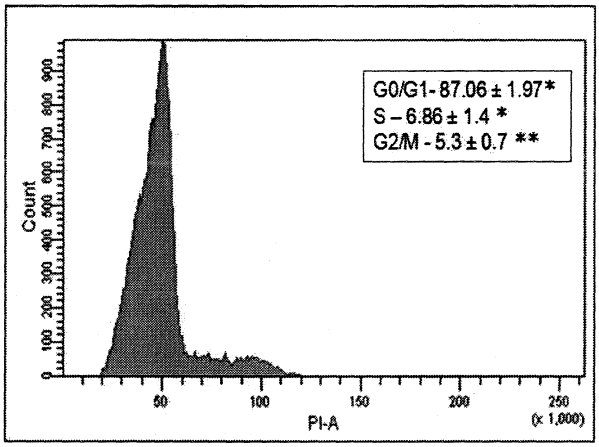
Figure 9: Time-dependence of the hypoxia effect on DNA synthesis

*Sub-confluent cultures of cardiac fibroblasts in M199 were synchronized by serum deprivation for 24 hours and then exposed to normoxia (N) or hypoxia (H) with 1μCi/ml [^{3}H]-thymidine 5, 12 and 15 hours after addition of 10% FBS. At 24 hours after serum addition, 20% TCA-precipitable radioactivity was determined. Values represent Mean ± SD of 5 determinations. H vs N, *p < 0.001.*

N



H



HSB

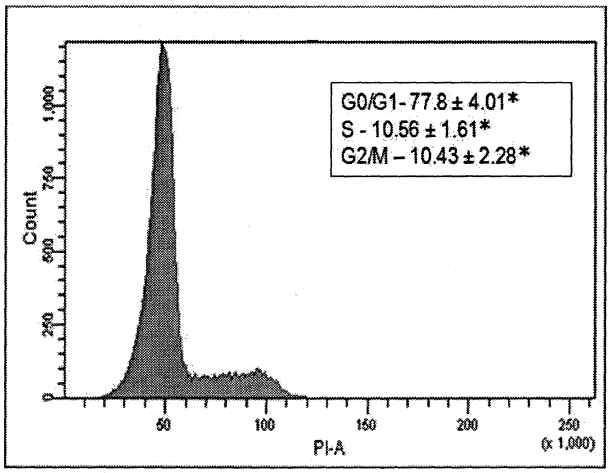


Figure 10: Hypoxia delays G1-S transition in cardiac fibroblasts

*Sub-confluent cultures of cardiac fibroblasts in M199 were synchronized by serum deprivation for 24 hours, exposed to 42 hours of hypoxia in M199 with 10% FBS and processed for flow cytometry as described under 'Methods'. Single cell populations were gated using forward scatter versus side scatter. The "gate" was created in the FL2-PI (I.e. propidium iodide intensity) versus cell width plot. Data were then converted into a standard histogram that could be analysed for the distribution of cells in each stage of the cell cycle. Representative flow cytometric profiles for normoxic (N), hypoxic (H) and p38 MAPK-inhibited hypoxic (HSB) cells are shown. Data in each panel, expressed as Mean ± SD, are the cumulative results of 3 independent determinations. H vs N, *p < 0.01, **p < 0.05; HSB vs H, *p < 0.05.*

IV.3.2. Molecular mechanisms involved in hypoxic regulation of G1-S transition

To elucidate the mechanisms underlying hypoxic regulation of the cardiac fibroblast cell cycle, subsequent experiments focused on:

(i) Expression of cell cycle regulatory proteins

(ii) Identification of signaling pathways mediating the anti-proliferative effect of hypoxia

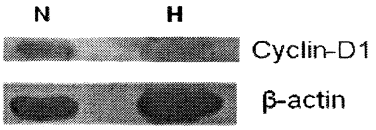
IV.3.2.1. Expression of cell cycle regulatory proteins

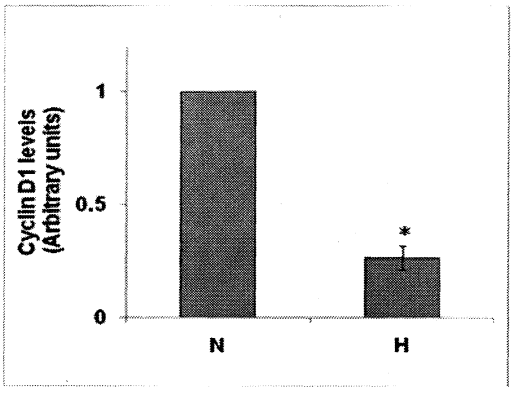
Mitogen-dependent progression through the first gap phase (G1) and initiation of DNA synthesis (S phase) are cooperatively regulated by certain classes of cyclin-CDK complexes including cyclin D1-CDK4 and cyclin E/A-CDK2 complexes, whose activities are in turn constrained by the CDKIs such as p27 and p21 (Morgan *et al.*, 1995; Morgan *et al.*, 1997; Sherr & Roberts, 1999). The present study sought to identify the components of the G1 checkpoint that respond to hypoxia and

contribute to delayed cell cycle progression in cardiac fibroblasts. The expression of G1/S regulators were analysed by western blotting using protein lysates prepared from sub-confluent cultures exposed to 18 hours of hypoxia or normoxia in M199 containing 10% FBS. Hypoxia reduced protein levels of cyclin D1 and cyclin A, but cyclin E was unaffected (*Figure 11*).

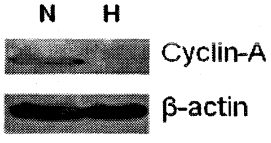
CDKIs such as p21 and p27 have been implicated in a variety of cell cycle arrest pathways induced by stress conditions, including hypoxia (Gardner *et al.*, 2001; Ji-Hong *et al.*, 2006). In this study, the effect of hypoxia on the expression of p21 and p27 was examined. Induction of p27 protein but not of p21 (*Figure 11*) was observed in hypoxic cardiac fibroblasts. Hypoxic induction of p27 in cardiac fibroblasts, noted here, is consistent with other reports that hypoxia up-regulates p27, which mediates hypoxia-induced cell cycle arrest (Gardner *et al.*, 2001).

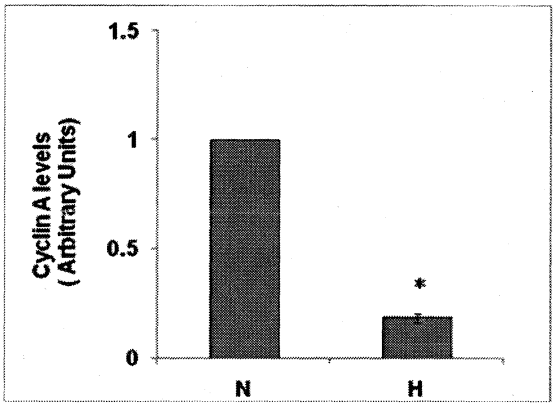
The best-characterized molecular event necessary for G_1/S phase transition is phosphorylation of the critical checkpoint control protein, Rb, by cyclin E-CDK2 complexes (Plana-Silva & Weinberg, 1997). An inhibition of CDK2 activity primarily mediated by p27 induction promotes Rb hypophosphorylation and subsequent cell cycle arrest. To determine whether the phosphorylation status of Rb correlated with proliferation inhibition, western blot analysis was performed using antibodies that detect both the hypophosphorylated and hyperphosphorylated forms of Rb. As shown in *Figure 12*, hypoxia decreased the phosphorylation state of Rb by about 35% in these cells.



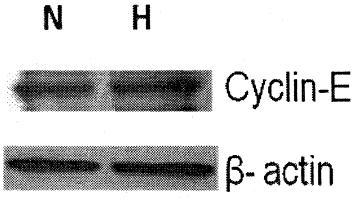


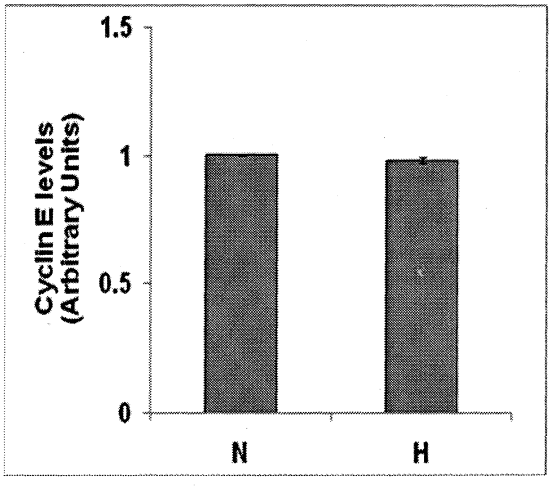
H vs N. *p < 0.005





H vs N. *p < 0.005





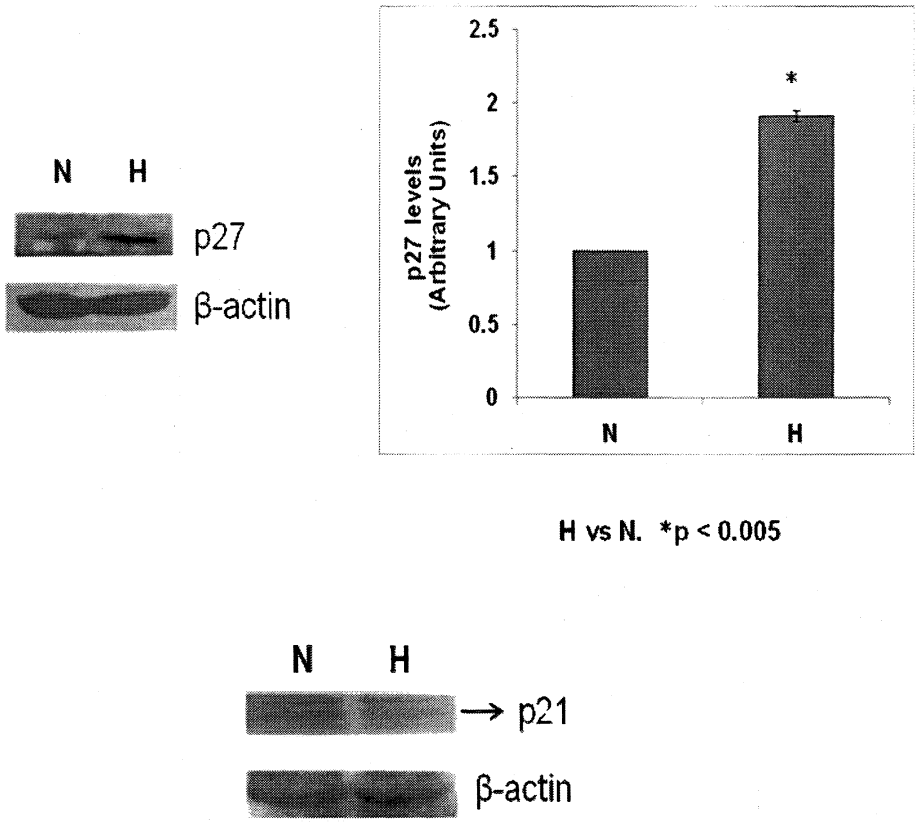


Figure 11: Effect of hypoxia on the expression of cell cycle regulatory proteins

Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were exposed to normoxia (N) or hypoxia (H) for 18 hours. Western blot analysis was performed as described under 'Methods'. Protein expression, quantified by densitometric scanning, was normalized to β-actin that served as the loading control. Values represent Mean ± SD of 3 independent experiments and are expressed in arbitrary units.

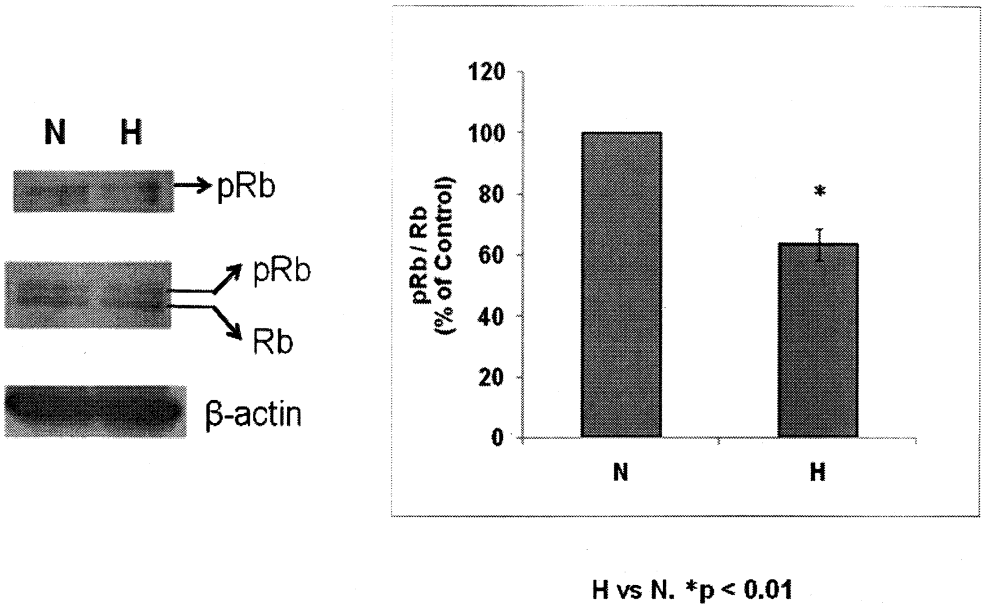


Figure 12: Hypoxia decreases Rb phosphorylation in cardiac fibroblasts

Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were exposed to normoxia (N) or hypoxia (H) for 18 hours. Western blot analysis was performed using both anti-phospho-Rb antibody that detects only the hyperphosphorylated form (pRb) and anti-Rb antibody that detects both the hyperphosphorylated and the hypophosphorylated (Rb) forms of Rb. The ratio of pRb to Rb was determined following densitometric analysis. β-actin served as the loading control. Values represent Mean ± SD of 3 independent experiments and are expressed as percent of control

IV.3.2.2. Identification of signaling pathways mediating the inhibitory effect of hypoxia on the cardiac fibroblast cell cycle

Hypoxia has been shown to regulate proliferation in several cell types through the activation/inactivation of different signaling pathways including tyrosine kinases, p44/42 MAPK and p38 MAPK (Conrad *et al.*, 1999; Das *et al.*, 2001). Further

experiments were, therefore, carried out to elucidate the role of these signaling pathways in the hypoxic inhibition of cardiac fibroblast proliferation.

Investigations aimed at determining the role of tyrosine kinases in cardiac fibroblast proliferation presented certain serendipitous findings that are discussed under "Appendix".

IV.3.2.2.1. Role of p44/42 MAPK in cardiac fibroblast proliferation

p44/42 MAPK is best known for its role in cell proliferation (Gao *et al.*, 2004). To determine the role of p44/42 MAPK in cardiac fibroblast proliferation, sub-confluent cultures, synchronized by serum deprivation for 24 hours in M199, were pre-incubated with the p44/42 MAPK inhibitor, PD 098059 (10μM), or 0.1% DMSO for one hour and exposed to normoxia for 24 hours in M199 containing 10% FBS in the presence of the inhibitor or vehicle and [^{3}H]-thymidine (1μCi/ml). Inhibition of p44/42 MAPK activity by PD 098059 (10μM), attenuated DNA synthesis under normoxic conditions (*Figure 13*) suggesting that p44/42 MAPK may act as a positive regulator of cardiac fibroblast proliferation.

To examine the possibility that p44/42 MAPK may be involved in mediating fibroblast responses to hypoxia, western blot analysis was performed to compare the activation (phosphorylation) status of p44/42 MAPK in cells under normoxic and hypoxic conditions. Sub-confluent cultures, serum deprived for 24 hours, were exposed to 12 hours of hypoxia/normoxia in M199 containing 10% FBS. Use of phospho-p44/42 MAPK-specific antibody revealed significant hypophosphorylation of p44/42 MAPK in hypoxic cells compared to normoxic, cycling cardiac fibroblasts

(*Figure 14*). The inactivation of p44/42 MAPK under hypoxia can, therefore, contribute to the hypoxia-induced decrease in cell proliferation.

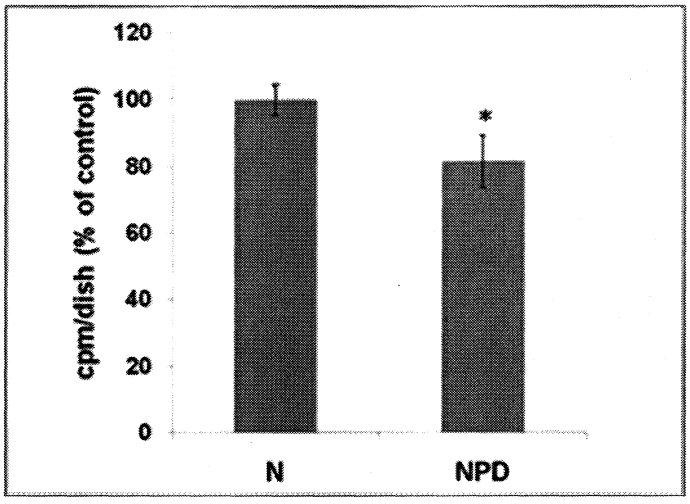


Figure 13: Effect of p44/42 MAPK inhibition on DNA synthesis

*Sub-confluent cultures of cardiac fibroblasts in M199 were synchronized by serum deprivation for 24 hours. Cell were then pre-incubated with PD 098059 (10μM) or 0.1% DMSO for one hour and exposed to normoxia for 24 hours in M199 containing 10% FBS in the presence of the inhibitor or vehicle and [^{3}H]-thymidine (1μCi/ml). DNA synthesis in normoxic (N), and p44/42 MAPK-inhibited normoxic (NPD) cells was measured as described under 'Methods'. Values represent Mean ± SD of 5 independent experiments. NPD vs. N, ** $p < 0.001$.*

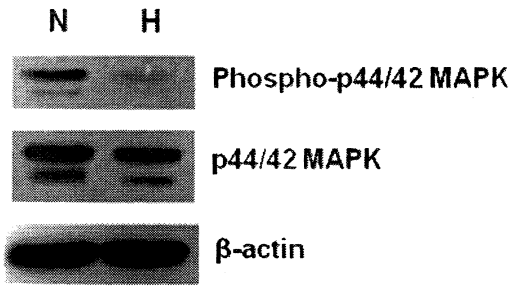


Figure 14: Activation status of p44/42 MAPK in hypoxic cardiac fibroblasts.

Sub-confluent cardiac fibroblast cultures, synchronised by serum deprivation for 24 hours, were exposed to hypoxia (H)/ normoxia (N) in M199 containing 10% FBS for 12 hours. Phosphorylation of p44/42 MAPK was assessed by western blot analysis using phospho-p44/42 MAPK-specific antibody as described under 'Methods'. The blots were stripped and re-probed with anti-total p44/42 MAPK and anti-β-actin antibodies to confirm equal loading. A representative profile from one of 3 experiments is shown.

IV.3.2.2.2. Role of p38 MAPK in hypoxic regulation of G1-S transition in cardiac fibroblasts

p38 MAPK has for long been implicated in environmental stress responses, in the regulation of inflammation and in apoptosis but, more recently, their involvement in the positive and negative regulation of cell proliferation is increasingly recognized (Engel *et al.*, 2005; Fernandes *et al.*, 2004). In the present study, p38 MAPK activity was examined, using phospho-p38 MAPK specific antibody, in synchronous sub-confluent cultures of cardiac fibroblasts exposed to 6 or 12 hours of hypoxia/normoxia in M199 containing 10% FBS. Significant activation of p38

MAPK, indicated by increased levels of the phosphorylated form of p38 MAPK, was observed in hypoxic cardiac fibroblasts (*Figure 15*).

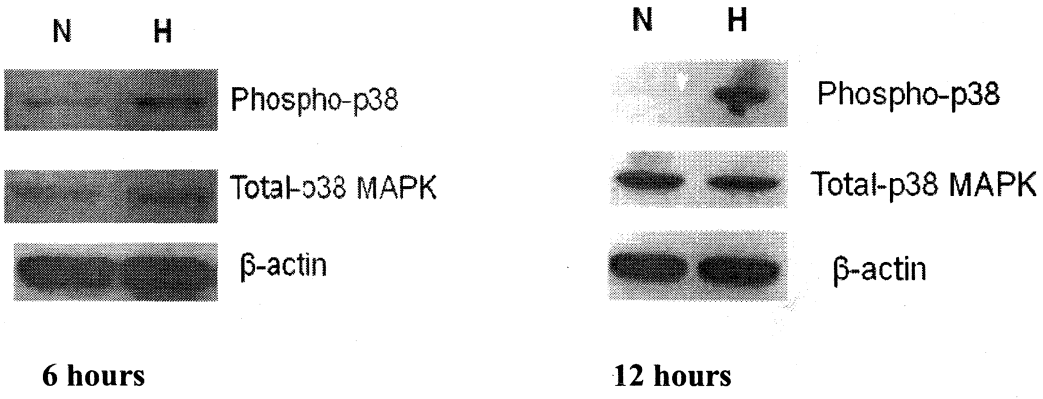


Figure 15: Activation status of p38 MAPK in hypoxic cardiac fibroblasts

Sub-confluent cardiac fibroblast cultures, synchronised by serum deprivation for 24 hours, were exposed to normoxia (N) or hypoxia (H) in M199 containing 10% FBS for 6 or 12 hours. Phosphorylation of p38 MAPK was assessed by western blot analysis using phospho-p38-specific antibody as described under 'Methods'. The blots were stripped and re-probed with anti-total p38 MAPK and anti-β-actin antibodies to confirm equal loading. A representative profile from one of 3 experiments is shown.

A series of p38 MAPK inhibition experiments using the pharmacological inhibitor, SB203580 (10μM in DMSO) was carried out to ascertain whether activated p38 MAPK contributed to delayed G1-S transition in hypoxic cardiac fibroblasts.

i) Effect of p38 MAPK inhibition on DNA synthesis:

Sub-confluent cultures, synchronized by serum deprivation for 24 hours in M199, were pre-incubated with the p38 MAPK inhibitor, SB203580 (10μM), or 0.1% DMSO for one hour and exposed to normoxia or hypoxia for 24 hours in M199

containing 10% FBS in the presence of the inhibitor or vehicle and [^{3}H]-thymidine (1μCi/ml). It was found that p38 MAPK inhibition does not affect DNA synthesis under normoxia but significantly attenuates the hypoxic inhibition of DNA synthesis (*Figure 16*).

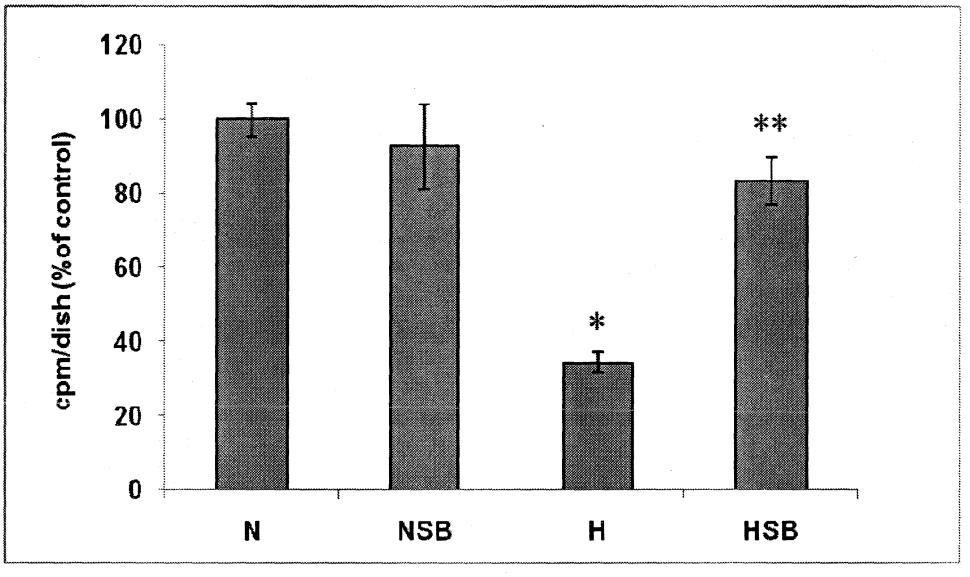


Figure 16: Effect of p38 MAPK inhibition on DNA synthesis in hypoxic cardiac fibroblasts

*Sub-confluent cultures of cardiac fibroblasts in M199 were synchronized by serum deprivation for 24 hours. Cell were then pre-incubated with SB203580 (10μM) or 0.1% DMSO for one hour and exposed for 24 hours to normoxia or hypoxia in M199 with 10% FBS in the presence of the inhibitor or vehicle and [^{3}H]-thymidine (1μCi/ml). DNA synthesis in normoxic (N), hypoxic (H) and p38 MAPK-inhibited normoxic (NSB) or hypoxic (HSB) cells was measured as described under 'Methods'. Values represent Mean ± SD of 5 independent experiments. H vs N, *p < 0.001; HSB vs H, **p < 0.001.*

ii) Effect of p38 MAPK inhibition on cell cycle phase distribution:

The site of action of p38 MAPK on cardiac fibroblast cell cycle progression under hypoxia was determined by flow cytometry. Sub-confluent cells, synchronized by serum deprivation for 24 hours in M199, were exposed to 42 hours of hypoxia in M199 containing 10% FBS and processed for flow cytometry. Consistent with the attenuation of hypoxia-induced reduction in DNA synthesis, p38 MAPK inhibition reversed the G0/G1 block observed in hypoxic cardiac fibroblasts (*Figure 10*), suggesting that p38 MAPK is a key determinant of hypoxia-induced G0/G1 block.

iii) Differential regulation of cell cycle regulatory protein expression by p38 MAPK:

To evaluate the effect of p38 MAPK inhibition on the expression of G1-S regulators under hypoxic conditions, sub-confluent cultures of cardiac fibroblasts were pre-incubated with SB203580 (10μM) or 0.1% DMSO for one hour and exposed to normoxia or hypoxia in M199 containing 10% FBS for 18 hours in the presence of the inhibitor or vehicle. Western blot analysis revealed that inhibition of p38 MAPK attenuated the effects of hypoxia on cyclin D1 (*Figure 17*), consistent with the reported regulation of cyclin D1 by p38 MAPK (Page *et al.*, 2001). Cyclin A levels, however, remained unaffected (*Figure 17*), demonstrating selective regulation of cell cycle regulatory molecules by p38 MAPK. Importantly, in p38 MAPK-inhibited hypoxic cells, significant reduction in p27 protein expression was observed (*Figure 17*). The decrease in p27 expression correlated well with the increase in DNA synthesis (*Figure 16*) and reversal of G0/G1 block (*Figure 10*) in p38 MAPK-inhibited hypoxic cells, suggesting that p38 MAPK-mediated p27 induction may retard G1-S transition in hypoxic cardiac fibroblasts.

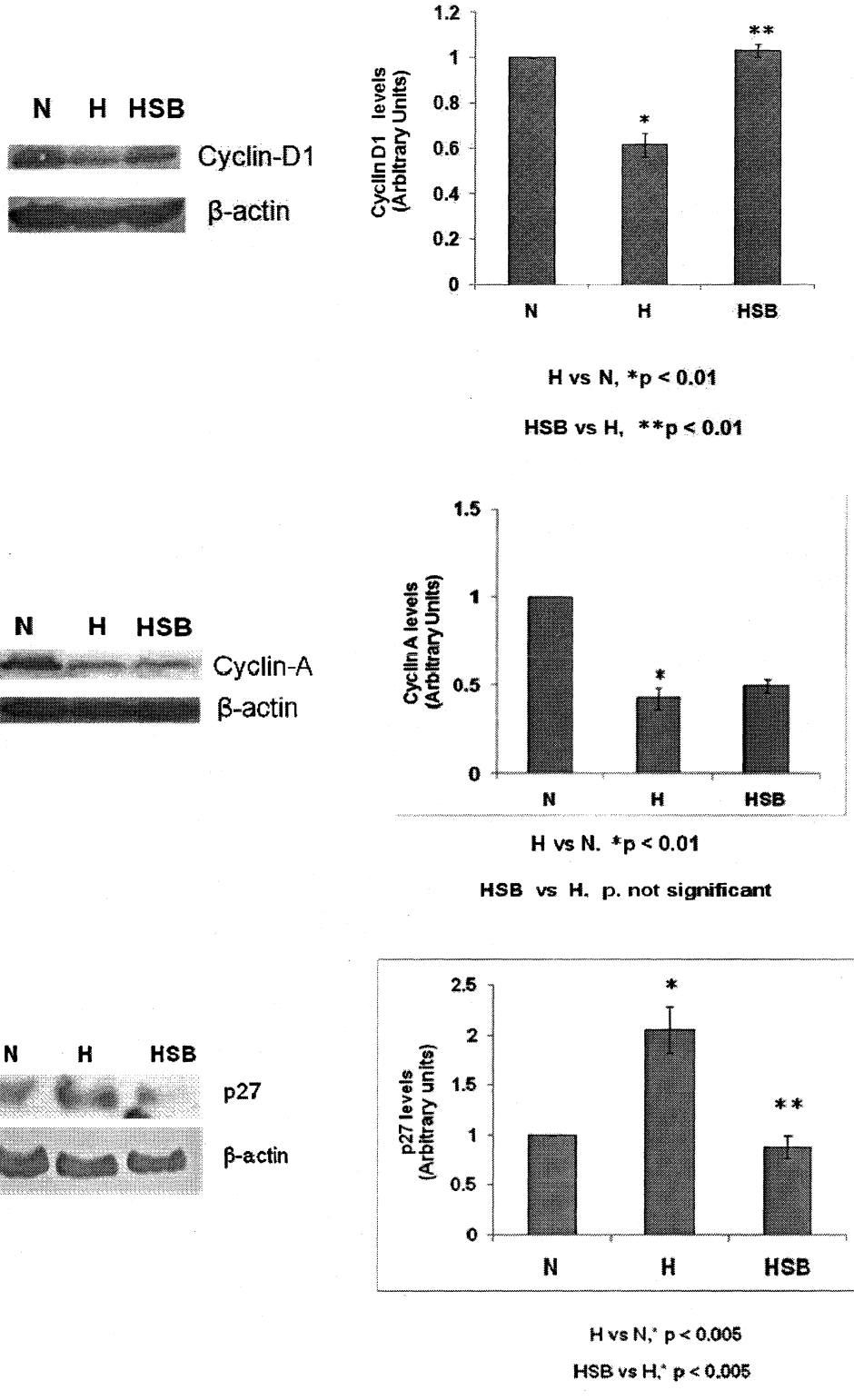


Figure 17: Differential regulation of cell cycle regulatory protein expression by p38 MAPK

Sub-confluent cultures of cardiac fibroblasts were pre-incubated with SB203580 (10μM) or 0.1% DMSO for one hour and exposed to normoxia or hypoxia in M199 containing 10% FBS for 18 hours in the presence of the inhibitor or vehicle. Western blot analysis was performed as described under 'Methods'. Protein expression in normoxic (N), hypoxic (H) and p38 MAPK-inhibited hypoxic (HSB) cells was quantified by densitometric scanning and normalized to β-actin that served as the loading control. Values represent Mean ± SD of 3 independent experiments and are expressed in arbitrary units.

IV.3.2.3. Regulation of p27 by p38 MAPK:

Generally, regulation of p27 occurs predominantly at the post-translational level (Pagano *et al.*, 1995), although increasing evidence supports p27 regulation at the transcriptional level (Dijkers *et al.*, 2000; Khattar & Kumar, 2010). Further experiments were carried out to assess whether p38 MAPK regulates p27 expression transcriptionally or post-translationally in cardiac fibroblasts.

IV.3.2.3.1. Transcriptional regulation of p27:

Sub-confluent cultures, pre-incubated with SB203580 (10μM) or DMSO for one hour, were exposed to hypoxia/normoxia in M199 containing 10% FBS for 12 hours in the presence of the inhibitor or vehicle and real-time PCR analysis was performed as described under 'Methods'. Briefly, total RNA was isolated and purity was ascertained by 1% agarose gel electrophoresis *(Figure 18).* To get rid of any genomic DNA contamination, total RNA was subjected to DNase I treatment as per manufacturer's instructions. PCR analysis of RNA samples subjected to DNase I treatment using G3PDH primers revealed no genomic DNA contamination after the

treatment *(Figure 19)*. 2μg of DNase I-treated RNA was reverse transcribed to cDNA with random hexamer primers and M-MLV reverse transcriptase. Taqman quantitative real-time polymerase chain reaction (RT-PCR) analysis was carried out using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA) with specific FAM-labelled probes under the following thermal cycling conditions: 95°C for 10 min followed by denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min for each of 40 cycles. 18S rRNA served as endogenous control. A 2-fold increase in p27 mRNA levels was observed in hypoxic cells *(Figure 20)*, indicating transcriptional control of p27 expression by hypoxia in these cells. The increase in p27 mRNA levels under hypoxia was, however, unaffected by concurrent treatment with SB203580 *(Figure 20)*, showing that transcriptional up-regulation of p27 in hypoxic cells is not mediated by p38 MAPK.

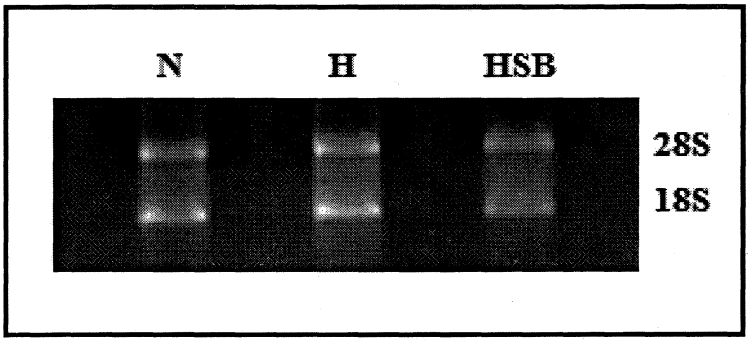


Figure 18: Agarose gel electrophoresis of RNA

RNA samples, isolated from normoxic (N), hypoxic (H) and p38 MAPK-inhibited hypoxic (HSB) cells as described under 'Methods', were subjected to 1% agarose gel electrophoresis. The presence of intact 28S and 18S bands was documented using Syngene Bio Imaging system.

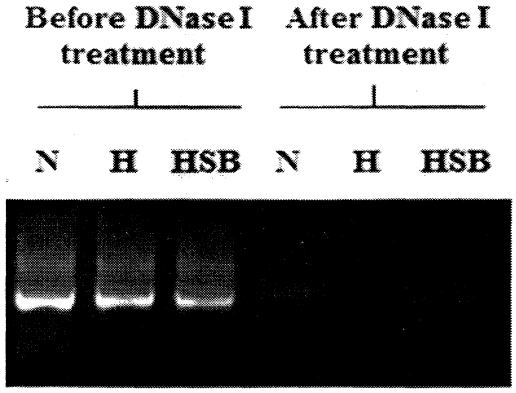


Figure 19: PCR analysis of RNA samples subjected to DNase I treatment

The isolated RNA samples from normoxic (N), hypoxic (H) and p38 MAPK-inhibited hypoxic (HSB) cells were subjected to DNase I treatment, as per manufacturer's instructions. This was validated by PCR analysis of the DNase I treated-RNA using G3PDH primers. PCR products were analyzed by 1% agarose gel electrophoresis. A representative profile shows no genomic DNA contamination after DNase I treatment.

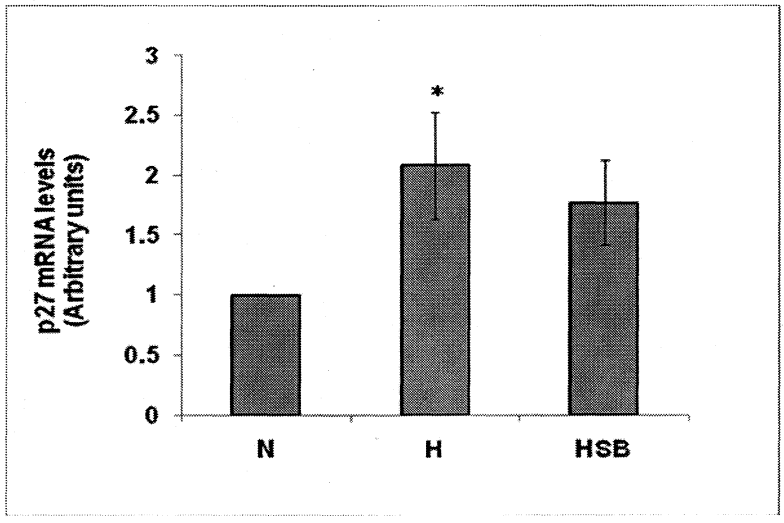


Figure 20: Real-time PCR analysis of p27 transcript levels in cardiac fibroblasts exposed to hypoxia in the presence or absence of SB203580

*Sub-confluent cultures of cardiac fibroblasts were pre-incubated with SB203580 (10μM) or DMSO for one hour and exposed to normoxia or hypoxia in M199 with 10% FBS for 12 hours in the presence of inhibitor or vehicle. p27 mRNA expression in normoxic (N), hypoxic (H) and p38 MAPK-inhibited hypoxic (HSB) cells was determined by Taqman quantitative real-time polymerase chain reaction analysis as described under 'Methods'. Values represent the Mean ± SD of 3 independent experiments and are expressed in arbitrary units. H vs N, *p = 0.01; HSB vs H, p, not significant.*

IV.3.2.3.2. Post-translational regulation of p27:

p27 expression is reported to be regulated predominantly by its rate of degradation (Pagano *et al.*, 1995). Skp2, an F-box protein of the SCF^{Skp2} ubiquitin ligase complex, specifically recognises p27 and targets it for proteasomal degradation (Carrano *et al.*, 1999; Tsvetkov *et al.*, 1999). Since Skp2-mediated degradation is a major mechanism of p27 regulation, the present study examined Skp2 expression in sub-confluent cultures of cardiac fibroblasts exposed to normoxia or hypoxia in M199 containing 10% FBS for 18 hours. Skp2 levels were significantly lower, and inversely related to p27 expression, in cells exposed to hypoxia *(Figure 21)*, suggesting additional post-translational regulation of p27 under hypoxic conditions.

Subsequent experiments examined the Skp2-dependent regulation of p27 by p38 MAPK. Sub-confluent cultures of cardiac fibroblasts, pre-incubated with SB203580 (10μM) or 0.1% DMSO for one hour, were exposed to normoxia or hypoxia in M199 containing 10% FBS for 18 hours in the presence of the inhibitor or vehicle. Western blot analysis using anti-Skp2 antibody revealed that low Skp2 levels in hypoxic cells

in which p38 MAPK was active were restored in p38 MAPK-inhibited hypoxic cells *(Figure 22)*, indicating that p38 MAPK may negatively regulate Skp2 expression in cardiac fibroblasts, which in turn would regulate p27 levels.

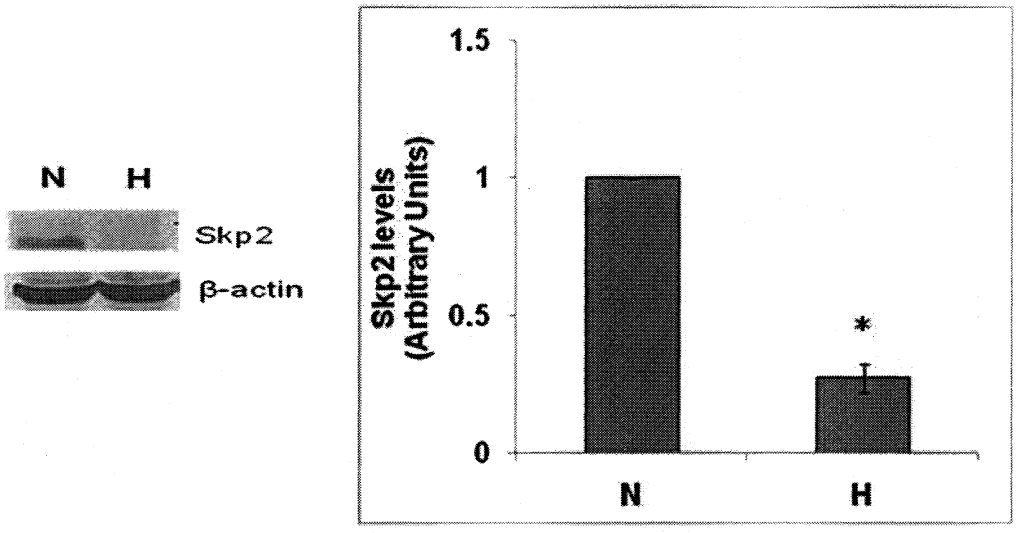


Figure 21: Western blot analysis of Skp2 expression in hypoxic cardiac fibroblasts

*Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were exposed to normoxia (N) or hypoxia (H) for 18 hours. Western blot analysis was performed as described under 'Methods'. Skp2 expression, quantified by densitometric scanning, was normalized to β-actin that served as the loading control. Values represent Mean ± SD of 3 independent experiments and are expressed in arbitrary units. H vs N, *p < 0.005.*

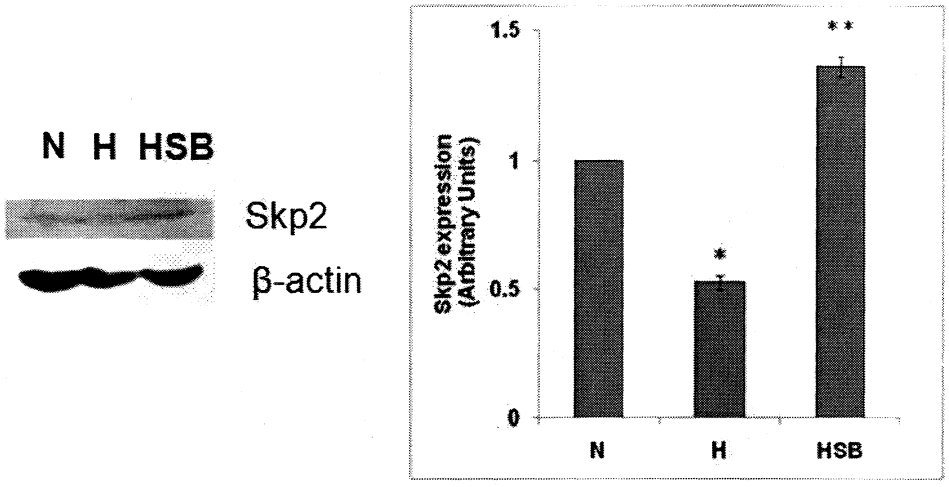


Figure 22: Skp2 is negatively regulated by p38 MAPK in hypoxic cardiac fibroblasts

*Sub-confluent cultures of cardiac fibroblasts were pre-incubated with SB203580 (10μM) or DMSO for one hour and exposed to normoxia or hypoxia in M199 with 10% FBS for 18 hours in the presence of inhibitor or vehicle. Western blot analysis was performed as described under 'Methods'. Skp2 expression in normoxic (N), hypoxic (H) and p38 MAPK-inhibited hypoxic (HSB) cells was quantified by densitometric scanning and normalized to β-actin that served as the loading control. Values represent Mean ± SD of 3 independent experiments and are expressed in arbitrary units. H vs N, *p < 0.0005; HSB vs H, *p < 0.0005.*

V. DISCUSSION

Hypoxia is reported to exert pro- and anti-mitogenic effects on cells, depending upon several factors such as the cell type, the extent of cell differentiation and the tissue or organ of origin. However, a common consequence of cellular hypoxia in non-transformed cells is cell cycle arrest at G1 (Gardner *et al.*, 2001). The present study sought to examine the mechanisms underlying hypoxic regulation of the cardiac fibroblast cell cycle.

Inherent difficulties associated with the evaluation of cardiac fibroblast responses to hypoxia *in vivo* include confounding influences from co-resident cells and the diverse array of growth factors/cytokines in the heart, and the inability to induce a uniform hypoxic insult across the tissue. It is pertinent to note that stable maintenance of partial blood flow reduction is difficult to achieve in *in vivo* experimental models so that a 1998 National Heart, Lung and Blood Institute Workshop identified the development of *in vitro* models of hypoxia as an area of priority (Kloner *et al.*, 1998). In the present study, we evaluated the effects of hypoxia on the cardiac fibroblast cell cycle using an *in vitro* cell culture model of hypoxia in the absence of other confounding influences obtained *in vivo*.

V.1. *In Vitro* Hypoxia Model

The *in vitro* hypoxia model used in the study provided a hypoxic atmosphere with a pO_2 of < 0.1% as against the normoxic pO_2 of ~20%. The pCO_2 in normoxic and hypoxic chambers was ~5%. The medium pO_2 was found to be ~3% in the hypoxic chambers and about 15% in the normoxic chambers (Sapna, 2007). It is important to note that although the atmospheric pO_2 is 20%, the arterial pO_2 is reported to be about 14% and, under conditions of normoxia, cardiac tissue pO_2 is <10% (Roy *et*

al., 2003). In the heart, a 30 to 60% decrease in oxygen levels resulting in a pO_2 of ~1 to 3% is considered moderate hypoxia (Roy *et al.*, 2003). Hence the hypoxic conditions (medium pO_2 ~3%) used in the present study may be considered moderate. Medium pH was comparable in both normoxic and hypoxic chambers and was about 7.5, ruling out a change in pH due to hypoxia.

Cardiac fibroblasts isolated from adult rat hearts were characterized and their fibroblastic nature was ascertained by morphology and immunocytochemical staining. The adherent cells had spindle morphology and formed a monolayer at confluence. Confluent cultures did not exhibit either 'cobblestone' or 'hill and valley' morphology, ruling out the presence of ECs and SMCs respectively, which was further confirmed by negative immunostaining for factor VIII-related antigen and desmin. The cells stained positive for the cytoskeletal protein, vimentin *(Figure 3)*. The *in vitro* cell culture model generated useful insights into the regulation of the cardiac fibroblast cell cycle under hypoxia.

V.2. Effect of hypoxia on cardiac fibroblast proliferation

V.2.1. Hypoxia delays G1-S transition in cardiac fibroblasts

Previous studies in this laboratory had shown that hypoxia causes reversible inhibition of cardiac fibroblast proliferation (Sapna, 2007). The oxygen sensitivity of the different phases of the cell cycle and the checkpoints differ in different cell types. Hypoxia may delay/arrest cell cycle progression at G_1/S (Gardner *et al.*, 2001), mid-S phase (Green *et al.*, 2001) or metaphase (Douglas *et al.*, 2005). In the present study, the evaluation of the time-dependence of the hypoxia effect on mitogen-stimulated cardiac fibroblast proliferation indicated that hypoxia exerts its action in

the early phases of the cell cycle since introduction of hypoxia 15 hours after addition of serum failed to inhibit proliferation (*Figure 9*). The cell cycle-specific site of action was further confirmed by flow cytometric analysis of hypoxic cultures (*Figure 10*) that showed conclusively that G1/S transition is oxygen-sensitive in cardiac fibroblasts. The finding is consistent with several studies that show that the transition at G_1/S is oxygen-sensitive in many cell types (Amellem *et al.*, 1991; Gardner *et al.*, 2001; Graeber *et al.*, 1994).

V.2.2. Molecular mechanisms involved in the hypoxic regulation of G1-S transition

Hypoxia may exert its inhibitory effect through the regulation of one or more of cell cycle regulatory proteins such as p53, Rb, cyclins, CDKs, and CDKIs (Amellem *et al.*, 1998; Gardner *et al.*, 2001; Goda *et al.*, 2003). The modulation of expression or activity of these proteins under hypoxic conditions has been found to be mediated by several pathways including p38 MAPK, p44/42 MAPK, Jun kinase, tyrosine kinase, PI3K/Akt and transcription factors such as HIF-1α (Conrad *et al.*, 1999; Goda *et al.*, 2003; Haddad *et al.*, 2007; Xiu-Mei *et al.*, 2009). Therefore, in looking for mechanisms underlying the hypoxic inhibition of cardiac fibroblast proliferation, this study focused on the effects of hypoxia on i) the expression of cell cycle regulatory proteins, and ii) the signaling pathways mediating the anti-proliferative effect of hypoxia.

V.2.2.1. Effect of hypoxia on the expression of cell cycle regulatory proteins

The delay in cell cycle progression in hypoxic cardiac fibroblasts was associated with hypoxia-mediated changes in G_1 regulators. Significant reductions in G1 regulators such as cyclin D and cyclin A (*Figure 11*) correlated well with the hypoxic inhibition of G1-S transition. Notably, cyclin E remained unaffected (*Figure 11*). This observation contrasts with the marked effect of hypoxia on cyclin E in transformed cells but is in line with the modest reduction in cyclin E noted in normal mouse embryo fibroblasts (Gardner *et al.*, 2001).

It is known that the Cip/Kip family of CDKIs play a critical role in regulating cell cycle progression under diverse stress conditions such as ionizing or UV radiation, TGF-β treatment, serum deprivation and hypoxia (Agocha *et al.*, 1997a; Gardner *et al.*, 2001). In this study, induction of p27 but not of p21 was observed in hypoxic cardiac fibroblasts (*Figure 11*). This is consistent with other reports that hypoxia up-regulates p27, which mediates hypoxia-induced arrest (Gardner *et al.*, 2001). In fact, a decrease in CDK2 activity primarily mediated by p27 is an important molecular mechanism implicated in hypoxia-associated G_1 arrest (Gardner *et al.*, 2001; Goda *et al.*, 2003). Forced expression of p27 is found to result in G_1 arrest in most cell types (Toyoshima & Hunter, 1994), while ablation of its synthesis delays withdrawal from the cell cycle. Studies by Gardner *et al.* (2001) suggest that p27 may be a major regulator of cell cycle progression in moderately hypoxic normal MEFs, as p27 null cells failed to arrest under these conditions and hypoxia-induced G_1 arrest in normal cells was overcome by decreasing p27 activity by antisense approaches. Additional evidence of the involvement of p27 is provided by Goda *et al.* (2003) who have

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demonstrated HIF-dependent increase in the expression of p27 during hypoxia-induced cell cycle arrest in MEFs and splenic B-lymphocytes. Further, p27 is reported to be constitutively expressed in post-mitotic cardiomyocytes (Sodhi *et al.*, 2000) and down-regulation of p27 activity in neonatal cardiomyocytes is associated with increase in proliferation (Tamamori-Adachi *et al.*, 2004). Growth arrest by contact inhibition and serum withdrawal is reported to be mediated at least in part by p27 (Polyak *et al.*, 1994).

Generally, the regulation of p27 expression is considered to be post-translational (Pagano *et al.*, 1995). But recent studies show evidence of its transcriptional regulation as well. The FOXO transcription factors have been shown to activate p27 transcription leading to cell cycle arrest (Dijkers *et al.*, 2000; Medema *et al.*, 2000). Other activators of the p27 promoter include Sp1, NF-Y, E2F1, and BRCA1 (Inoue *et al.*, 1999; Wang *et al.*, 2005; Williamson *et al.*, 2002). Recent studies by Khattar and Kumar (2010) report that mitogenic regulation of p27 is mediated by AP-1 transcription factors (Khattar & Kumar, 2010).

In the current study, a significant increase in p27 mRNA in hypoxic cardiac fibroblasts *(Figure 20)* suggested transcriptional control of its expression by hypoxia in these cells. Interestingly, p27 induction and down-regulation of Skp2 *(Figure 11 & Figure 21)*, an important F-box protein responsible for p27 recognition and subsequent degradation (Carrano *et al.*, 1999; Sutterlüty *et al.*, 1999), point to an inverse relationship between these two critical regulators of the G1 checkpoint in hypoxic cardiac fibroblasts. The association of Skp2 with p27 could result in the recruitment of the latter to the SCF^{Skp2} complex, a well conserved class of E3

ubiquitin–protein ligases that promote subsequent ubiquitination and degradation of p27. The findings presented here suggest that post-translational mechanisms mediated by the SCF^{skp2} complex may, additionally, play a role in p27 regulation in cardiac fibroblasts exposed to hypoxia.

Phosphorylation of Rb by cyclin-CDK complexes is a critical event in G1/S transition (Plana-Silva & Weinberg, 1997). Consistent with reports that hypoxia-induced arrest is associated with hypophosphorylation of Rb (Amellem *et al.*, 1998; Krtolica *et al.*, *1998)*, hypoxia decreased the phosphorylation state of Rb in cardiac fibroblasts (*Figure 12*). Interestingly, however, several studies also report the existence of an Rb-independent cell cycle arrest (Wirt *et al.*, 2010). Serum starvation of Rb null MEFs has been shown to induce G1 arrest (Herrera *et al.*, 1996). Similarly, over-expression of p27 induces G1 arrest in osteosarcoma cell lines with mutant Rb (Toyoshima & Hunter, 1994). In the present study, however, hypoxia appears to prevent the post-translational modification of Rb in response to mitogen by modulating the expression of the cyclins and the G1 CDKI, p27, leading to Rb-dependent cell cycle arrest.

These observations suggest that the cyclin-CDK complexes - cyclin D-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2 in early, mid and late G1 phases - are down-regulated under hypoxia. Reductions in cyclins D and A may regulate the activities of the cyclin D-CDK4/6 and cyclin A-CDK2 complexes whereas cyclin E-CDK2 activity may be inhibited by p27 without down-regulation of cyclin E expression. This in turn may impact Rb phosphorylation and prevent entry into S phase.

V.2.2.2. Signaling pathways mediating the anti-proliferative effect of hypoxia

Hypoxia activates multiple signaling pathways depending on the cell type and the stimulus (Seta *et al.*, 2004). Among hypoxia-activated pathways are the MAPK family of kinases that have a positive or negative influence on cell cycle progression (Das *et al.*, 2001; Kacimi *et al.*, 2000; Minet *et al.*, 2000; Schafer *et al.*, 2003).

V.2.2.2.1. Role of p44/42 MAPK in cardiac fibroblast proliferation

Of the three MAPK sub-families, the p44/42 MAPK cascade is activated by growth factors and is best known for its role in cell proliferation (Gao *et al.*, 2004). In the present study, activation of p44/42 MAPK was observed in normoxic cardiac fibroblasts, which was significantly inhibited in cells exposed to hypoxia (*Figure 14*). Further, inhibition of p44/42 MAPK using a pharmacological inhibitor decreased proliferation in normoxic cardiac fibroblasts *(Figure 13)*, suggesting that it may serve as a positive regulator of cardiac fibroblast proliferation. Given the positive influence of p44/42 MAPK on fibroblast proliferation, inactivation of p44/42 MAPK may contribute to the reduction in proliferation under hypoxic conditions. The finding is consistent with the growth-promoting role of the p44/42 MAPK pathway reported in mitogen-activated cells (Kortylewski *et al.*, 2001; Rivard *et al.*, 1999).

V.2.2.2.2. Role of p38 MAPK in cardiac fibroblast proliferation

p38 MAPK is activated in response to a variety of stress stimuli including heat and osmotic shock, UV radiation, pro-inflammatory cytokines and hypoxia (Raingeaud *et al.*, 1995; Kacimi *et al.*, 2000). Marked increase in p38 MAPK activity, as evident from the increased phosphorylation of p38 MAPK, was observed in cardiac

fibroblasts exposed to hypoxia (*Figure 15*). This is consistent with other studies reporting activation of p38 MAPK under hypoxia in various cell types (Kacimi *et al.*, 2000). Selective activation of p38γ and p38α isoforms of p38 family of MAPKs was observed in PC12 cell line (neural-like cell line) when exposed to moderate hypoxia (5%) (Conrad *et al.*, 1999). Hypoxia has also been shown to cause a substantial acute activation of p38 MAPK in mesangial cells (Sodhi *et al.*, 2000). Activation of the p38 MAPK family has been extensively studied in cardiac myocytes in relation to myocardial remodeling and failure, and has been shown to play an important role in myocyte hypertrophy, survival, and apoptosis (Ma *et al.*, 1999; Petrich & Wang, 2004; Wenzel *et al.*, 2005). However, there are few studies on the role of p38 MAPK signaling in relation to cardiac fibroblasts. p38 MAPK activation has been implicated in IL-1β-induced cardiac fibroblast migration (Mitchell *et al.*, 2007).

Although less established, p38 MAPK activation has been shown to regulate cellular growth in different ways depending on the cell type and the stimulus (Engel *et al.*, 2005; Khiema *et al.*, 2008; Thorton *et al.*, 2009). The p38 MAPK pathway is involved in the induction of two major cell cycle checkpoints, G1/S and G2/M, although its role in the G2/M checkpoint is better established (Thorton *et al.*, 2009). In the present study, several lines of evidence underscored the involvement of p38 MAPK in mediating delayed G1-S transition in hypoxic cardiac fibroblasts. Inhibition of p38 MAPK attenuated the hypoxia-induced reduction in DNA synthesis (*Figure 10)* and reversed the cell cycle phase distribution profile (*Figure 16*). p38 MAPK was also found to differentially regulate the cell cycle proteins, cyclin D1, cyclin A and p27, in hypoxic cardiac fibroblasts (*Figure 17*). Inhibition of p38 MAPK attenuated the effects of hypoxia on cyclin D1, but cyclin A levels remained

unaffected (*Figure 17*). Importantly, p38 MAPK inhibition was found to attenuate p27 expression in hypoxic cardiac fibroblasts suggesting that it may be a regulator of p27 expression in these cells (*Figure 17).* The findings are suggestive of a novel mechanism by which p38 MAPK may exert negative control over the cardiac fibroblast cell cycle. Notably, p38 MAPK inhibition failed to abolish the hypoxia-induced increase in p27 mRNA *(Figure 20)*, showing that the transcriptional up-regulation of p27 in hypoxic cells is not mediated by p38 MAPK. However, the negative regulation of Skp2 by p38 MAPK *(Figure 22)*, as evident from the inverse relationship between p38 MAPK activity and Skp2 expression, would potentially impact p27 protein expression post-translationally in these cells. Regulation of Skp2 through different pathways such as PI3K and p44/42 MAPK is reported (Auld *et al.*, 2007; Tzu-Ping *et al.*, 2007). The current findings identify Skp2 as a potential downstream target of p38 MAPK, suggesting a novel mechanism of G1-S regulation, which, to the best of our knowledge, has not hitherto been reported for any cell type.

The inhibitory role of p38 MAPK at the G1/S transition has been correlated with the down-regulation of cyclin D1 (Page *et al.*, 2001). p38 MAPK has also been reported to directly phosphorylate and stabilize p21 *in vivo* and regulate other CDKIs such as p16INK4 and p19ARF (Kim *et al.*, 2002; Thorton *et al.*, 2009).

To conclude, hypoxia acts as a negative regulator of G1-S transition in cardiac fibroblasts, marked by activation of p38 MAPK, induction of p27 and hypophosphorylation of Rb. One can propose a mechanism of G0/G1 block in hypoxic cardiac fibroblasts that involves p27 induction via direct transcriptional

of p38 MAPK-inhibited hypoxic cells with retrovirus expressing wild-type p27 or the T187V mutant that cannot be ubiquitinylated by SCF^{Skp2} for degradation would clarify whether inhibition of p27 degradation via p38 MAPK-dependent down-regulation of Skp2 is responsible for delayed cell cycle progression in hypoxic cardiac fibroblasts. Future investigations should uncover the molecular events that link p38 MAPK activation to Skp2 expression in the control of the cardiac fibroblast cell cycle under conditions of ambient stress. Further, the role of p44/42 MAPK as a positive regulator of cardiac fibroblast proliferation needs to be examined. Identification of the downstream targets of p44/42 MAPK may pave the way for therapeutic strategies to check excessive fibroplasia and adverse myocardial remodeling *post injury*.

VI. SUMMARY AND CONCLUSIONS

Fibroblast hyperplasia associated with increments in matrix components is a major component of wound healing in the injured heart. Optimal expression of the fibroproliferative response is a pre-requisite for the maintenance of the structural and functional integrity of the myocardium *post injury*. In this regard, diverse factors such as angiotensin II, TGF-β and pro inflammatory cytokines TNF-α, IL-1 and IL-6 prevalent in the injured/ diseased myocardium can act as positive and negative modulators of cardiac fibroblast proliferation. The balance between these positive and negative influences would determine the functional response of these cells in a given situation. Hypoxia is the most prevalent factor associated with cardiovascular diseases but its effects on the cardiac fibroblast cell cycle have not been investigated. Against this backdrop, the findings of the present study are as follows:

- Hypoxia retards G_1/S transition in cardiac fibroblasts
- Decrease in cyclin D1 and cyclin A expression was observed in hypoxic cells
- Hypoxia induces p27 but not p21
- Hypoxia promotes hypophosphorylation of Rb
- A 2-fold increase in p27 mRNA was observed in hypoxic cells
- p44/42 MAPK acts as a positive modulator of cardiac fibroblast proliferation

i. p44/42 MAPK activity increased in normoxic and cycling cardiac fibroblasts, which was significantly inhibited in hypoxic cells

ii. Inhibition of p44/42 MAPK activity attenuated DNA synthesis under normoxic conditions

- p38 MAPK appears to be a key determinant of delayed cell cycle progression under hypoxia:

i. p38 MAPK activity increases in hypoxic cells

ii. Inhibition of p38 MAPK reversed the hypoxia-induced reduction in DNA synthesis and the G0/G1 block in hypoxic cells

Underlying molecular mechanisms:

- ✓ Cyclin D1 levels were restored in p38 MAPK-inhibited hypoxic cells indicating that p38 MAPK may negatively regulate cyclin D1 expression
- ✓ Significant reduction in p27 expression in p38 MAPK-inhibited hypoxic cells suggests positive regulation of p27 expression by p38 MAPK in hypoxic cells
- ✓ The increase in p27 mRNA levels under hypoxia was unaffected upon p38 MAPK inhibition, suggesting that transcriptional regulation of p27 in hypoxic cells is independent of p38 MAPK
- ✓ p38 MAPK inhibition restored Skp2 levels in hypoxic cells, pointing to possible post-translational regulation of p27 by p38 MAPK via down-regulation of Skp2

To conclude, p38 MAPK is an important determinant of hypoxia-induced G0/G1 block in cardiac fibroblasts. p27 induction in hypoxic cardiac fibroblasts may involve direct transcriptional regulation, independent of p38 MAPK, and post-translational regulation via p38 MAPK-dependent suppression of its degradation by Skp2. The study identifies Skp2 as a potential downstream target of p38 MAPK, suggesting a

novel mechanism of G1-S regulation in cardiac fibroblasts exposed to stress conditions.

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APPENDIX

A serendipitous finding

In the course of investigations on the role of tyrosine kinase in the control of cardiac fibroblast proliferation, genistein, the best-known inhibitor of tyrosine kinases (Akiyama *et al.*, 1987; Yoon *et al.*, 1998), was found to cause total inhibition of [^{3}H]-thymidine incorporation into DNA in mitogen-stimulated cells, measured as TCA-insoluble radioactivity. Subsequent experiments, however, led to the serendipitous finding that the dramatic decrease in [^{3}H]-thymidine incorporation in response to genistein does not reflect inhibition of DNA synthesis or cell proliferation but is due to reduced intracellular pool of [^{3}H]-thymidine, measured as TCA-soluble radioactivity (Pillai & Shivakumar, 2009). The observation indicated possible inhibition of nucleoside uptake by genistein. This section discusses findings, which suggest that nucleoside uptake mechanisms might be a novel target of genistein action in cardiac fibroblasts. The data point to serious limitations in using genistein to assess the role of tyrosine kinase in cell proliferation by the standard technique of [^{3}H]-thymidine incorporation.

I. Methodology:

Measurement of [^{3}H]-thymidine uptake and incorporation into DNA:

Sub-confluent cultures of cardiac fibroblasts were synchronized by serum-deprivation for 24 hours and exposed to the indicated concentrations of genistein, Tyrphostin AG 490, or NBTI for 24 hours in Medium 199 containing 10% fetal bovine serum and 1 μCi/ml [^{3}H]-thymidine. At 24 hours, culture supernatant was discarded, cell layer was washed with PBS, lysed in lysis buffer (0.1 M NaOH containing 0.1% SDS) and precipitated with 20% ice-cold TCA. Radioactivity

associated with the acid-soluble and -insoluble fractions was determined using a liquid scintillation counter.

Measurement of [^{3}H]-proline incorporation and uptake:

Sub-confluent, synchronous cardiac fibroblast cultures were exposed to genistein at 100 μM (or DMSO) in DMEM with 2 μCi/ml [^{3}H]-proline for 12 hours. Culture supernatant was then discarded, cell layer was washed with PBS, lysed as described above and precipitated with 20% ice-cold TCA. Samples were processed for determination of acid-soluble and -insoluble radioactivity.

II. Results:

II.1. Genistein inhibits [^{3}H]-thymidine incorporation in cardiac fibroblasts:

i) Genistein was found to cause complete inhibition of [^{3}H]-thymidine incorporation into DNA in cardiac fibroblasts. A modest decrease in the incorporation of [^{3}H]-proline without any dramatic effect on proline uptake per se, excluded the possibility of non-specific effects of genistein on the plasma membrane (*Figure A-1*).

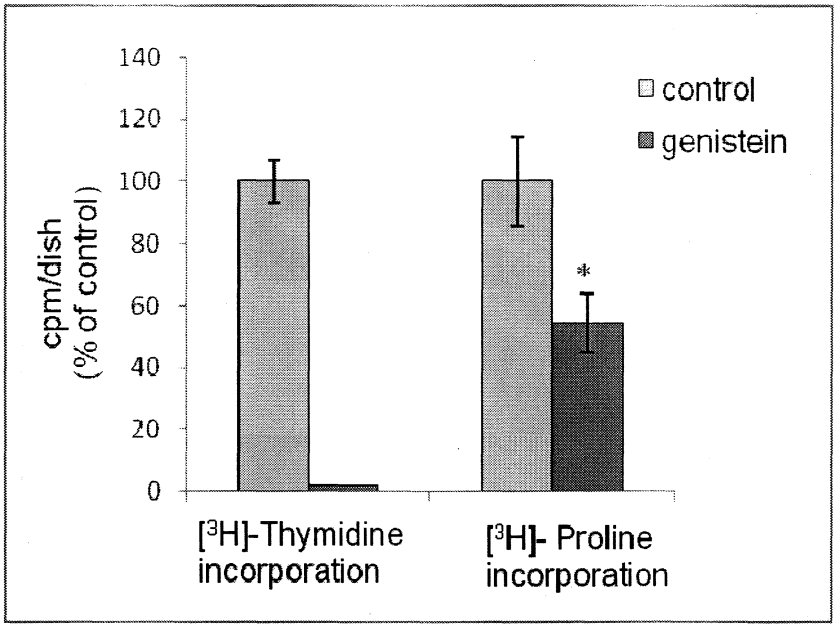

Figure A-1: Effect of genistein on [^{3}H]-thymidine and [^{3}H]-proline incorporation and cell proliferation in cardiac fibroblasts

*Sub-confluent, synchronous cultures of cardiac fibroblasts were incubated with genistein (100 μM) and [^{3}H]-thymidine (1μCi/ml) or [^{3}H]-proline (2μCi/ml) for 24 hours and 12 hours, respectively, and trichloroacetic acid-insoluble radioactivity was determined (n=4), Data are presented as Mean ± SD. Genistein vs. control, *p < 0.01.*

ii) The inhibitory effects of genistein on [^{3}H]-thymidine incorporation did not correlate with any change in cell number (*Figure A-2*), indicating that the genistein effect on [^{3}H]-thymidine incorporation was unrelated to cell proliferation. Further, loss of cell viability upon treatment with genistein was ruled out by Hoechst staining (*Figure A-3*).

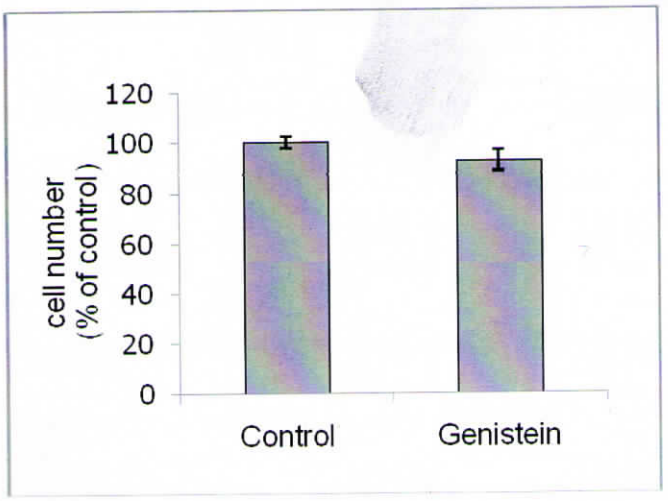

Figure A-2: Effect of genistein on cell number

Sub-confluent, synchronous cultures of cardiac fibroblasts were incubated with genistein (100 μM) for 24 hours and cell number was determined (n=3). Data are presented as Mean ± SD.

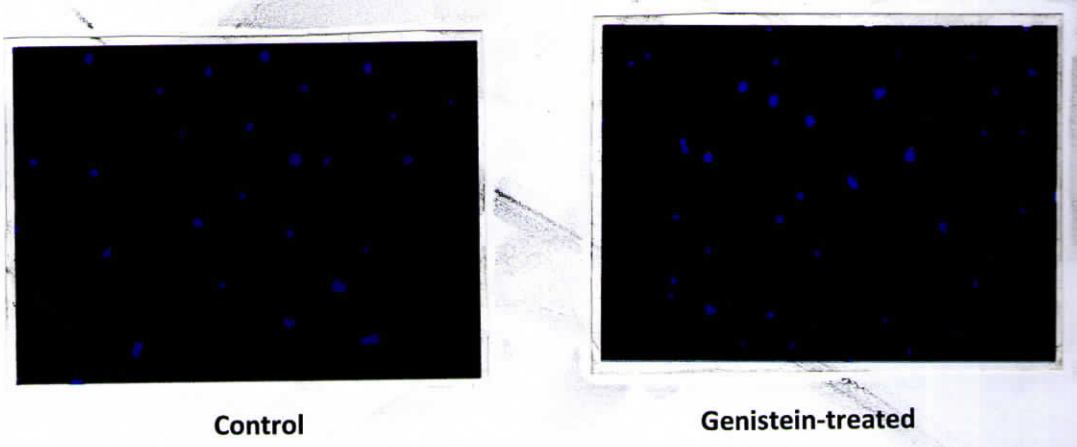

Figure A-3: Effect of genistein on cell viability

Sub-confluent, synchronous cultures of cardiac fibroblasts were incubated with genistein (100 μM) for 24 hours and cell viability was assessed using Hoechst 33324 staining as per manufacturer's protocol (n=3.)

II.2. Genistein inhibits [^{3}H]-thymidine uptake in cardiac fibroblasts:

Subsequent studies revealed that the decrease in [^{3}H]-thymidine incorporation was the result of reduced intracellular pool of [^{3}H]-thymidine, possibly due to inhibition of [^{3}H]-thymidine uptake.

i) The time-course of [^{3}H]-thymidine uptake (*Figure A-4*) showed near total inhibition of nucleoside uptake as early as 10 minutes after exposure to genistein whereas progressive increase in the intracellular levels of [^{3}H]-thymidine was evident in untreated cells.

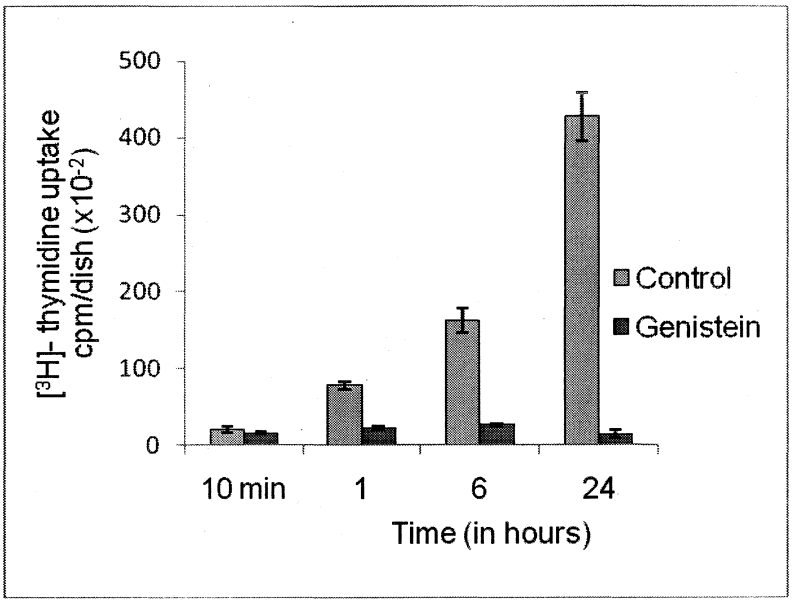

Figure A-4. Time- dependent effect of genistein on [^{3}H]-thymidine uptake in cardiac fibroblasts

*Sub-confluent cultures of cardiac fibroblasts were incubated with [^{3}H]-thymidine at 1μCi/ml and genistein (100 μM) for the indicated durations (n=3) and acid-soluble radioactivity was measured. Data are presented as Mean ± S.D. "n" denotes the number of determinations. Genistein vs. control, *p < 0.0001.*

ii) Furthermore, incubation of cells with genistein at concentrations ranging from *1*μM to 100μM induced a dose-dependent inhibition of [^{3}H]-thymidine uptake (*Figure A-5*). Thus, the apparent inhibition of incorporation of [^{3}H]-thymidine in cardiac fibroblasts by genistein was due to complete inhibition of nucleoside uptake as such. Moreover, either little or no apparent effect of other kinase inhibitors such as SB 203580 or PD 98059 on [^{3}H]-thymidine incorporation in these cells (*Figures 13 & 16*), stressing the specificity of the genistein effect.

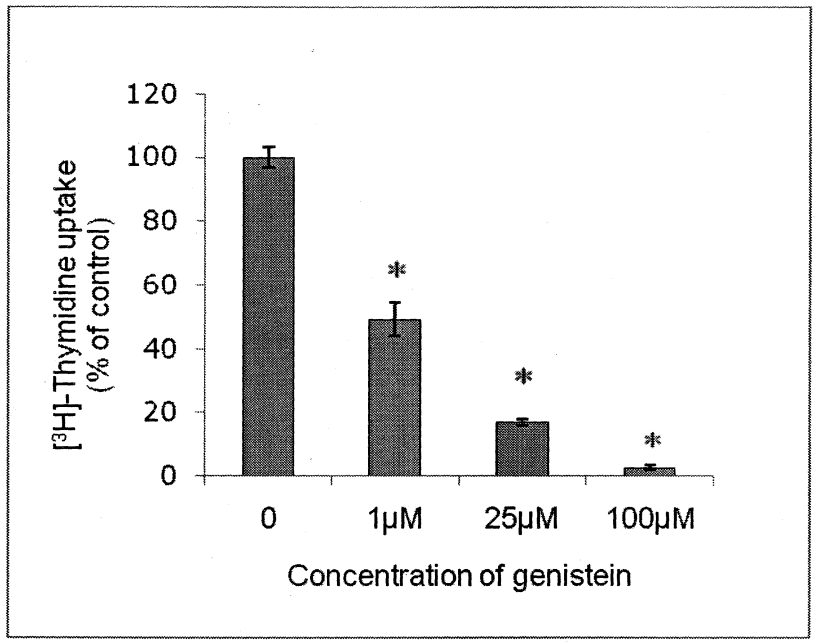

Figure A-5. Concentration-dependent effect of genistein on [^{3}H]-thymidine uptake in cardiac fibroblasts

*Sub-confluent cultures of cardiac fibroblasts were incubated with [^{3}H]-thymidine at 1μCi/ml and genistein (100μM) at the indicated concentrations for 24 hours (n=4), and acid-soluble radioactivity was measured. Data are presented as Mean ± S.D. "n" denotes the number of determinations. Genistein vs. control, *p < 0.0001.*

iii) The inhibitory effect of genistein on nucleoside uptake was irreversible since uptake of radiolabelled nucleoside remained inhibited when cultures were pre-treated for 24 hours with genistein followed by incubation with [^{3}H]-thymidine in the absence of genistein (*Figure A-6*).

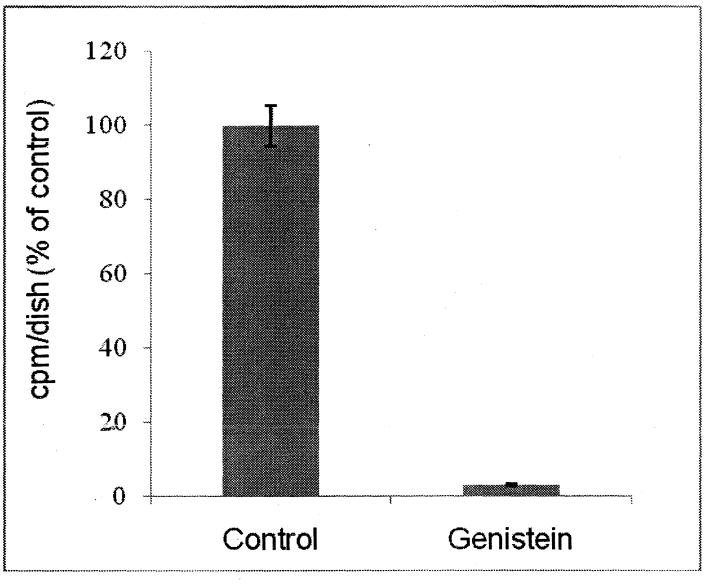

Figure A-6. Effect of genistein on [^{3}H]-thymidine uptake in cardiac fibroblasts is irreversible

*Sub-confluent cultures of cardiac fibroblasts were incubated with [^{3}H]-thymidine at 1μCi/ml and genistein (100 μM) for 24 hours. After 24 hours of incubation, fresh medium containing [^{3}H]-thymidine at 1μCi/ml, devoid of genistein, was added to the cells and was incubated for another 24 hours. Acid-soluble radioactivity was then measured. (n=3). Data are presented as Mean ± S.D. "n" denotes the number of determinations. Genistein vs. control, *p < 0.0001.*

iv) A structurally different but potent tyrosine kinase inhibitor, Tyrphostin AG 490, had no effect on cellular nucleoside uptake or incorporation into DNA, indicating that the genistein effect is independent of tyrosine kinase inhibition (*Figure A-7*).

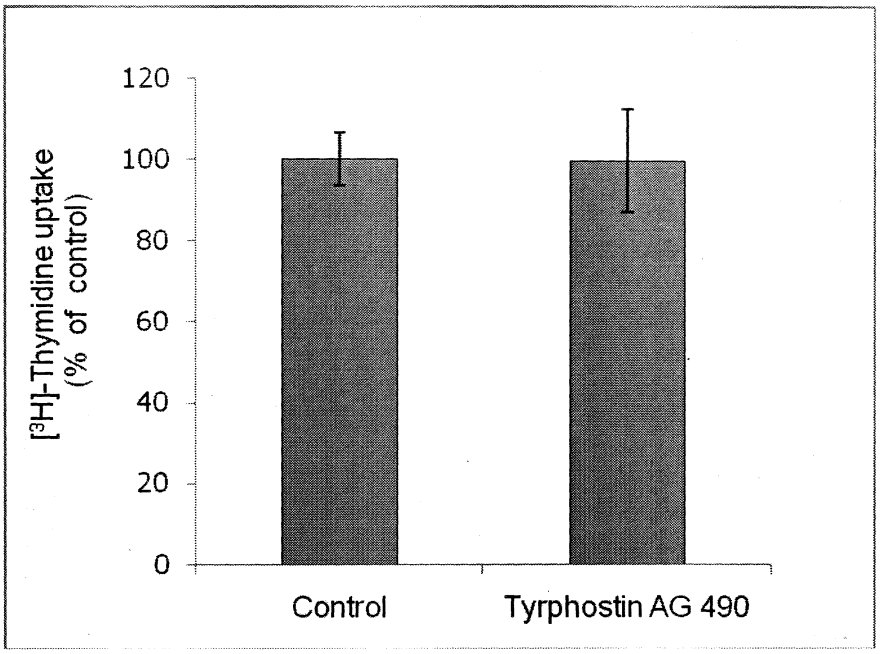

Figure A-7. Effect of Tyrphostin AG490 on [^{3}H]-thymidine uptake in cardiac fibroblasts

Sub-confluent, synchronous cultures of cardiac fibroblasts were incubated with 5μM Tyrphostin AG490 and [^{3}H]-thymidine at 1μCi/ml for 24 hours and acid-soluble radioactivity was measured (n=4). Data are presented as Mean ±S.D. "n" denotes the number of determinations. Tyrphostin had no significant effect on nucleoside uptake.

v) Genistein exerted a similar action on nucleoside uptake by pulmonary fibroblasts as well (*Figure A-8*), suggesting that the effect is not specific to cardiac fibroblasts.

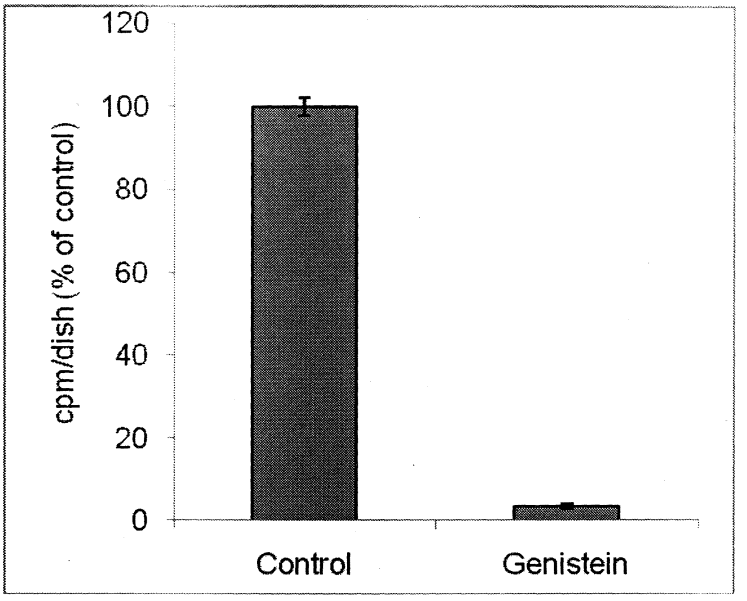

Figure A-8. Effect of genistein on [^{3}H]-thymidine uptake in pulmonary fibroblasts

*Sub-confluent cultures of pulmonary fibroblasts were incubated with [^{3}H]-thymidine at 1μCi/ml and genistein (100 μM) for the indicated durations (n=3) and acid-soluble radioactivity was measured. Data are presented as Mean ± S.D. "n" denotes the number of determinations. Genistein vs. control, *p < 0.0001.*

II.3. Genistein may exert its effect through nucleoside transporters

Nucleoside uptake in mammalian cells is generally believed to be through facilitated diffusion. Two classes of plasma membrane nucleoside transporters that facilitate the cellular import of nucleosides have been identified in mammalian cells, which include the sodium- dependent concentrative nucleoside transporters (CNTs) and the sodium-independent equilibrative nucleoside transporters (ENTs) (Cass *et al.*, 1999). ENTs are further defined by their differential sensitivity (es/ENT1) or insensitivity (ei/ENT2) to nitrobenzylthioionosine (NBTI). In this study, exposure of cardiac

fibroblasts to 10 μM NBTI, with [^{3}H] thymidine, produced a ~ 65% decrease in nucleoside uptake (*Figure A-9*), showing that the bulk of nucleoside uptake in these cells occurs via the NBTI-sensitive nucleoside transporter protein, ENT1. A wide variety of protein kinase inhibitors affect nucleoside uptake through selective inhibition of ENT1 nucleoside transporters. Therefore, total abolition of nucleoside uptake by genistein may involve inhibition of ENT1 nucleoside transporter and other routes of nucleoside uptake as well. Since the abolition of nucleoside entry into cardiac fibroblasts by genistein is nearly total, it appears that genistein may block all routes of nucleoside influx, including ENT1.

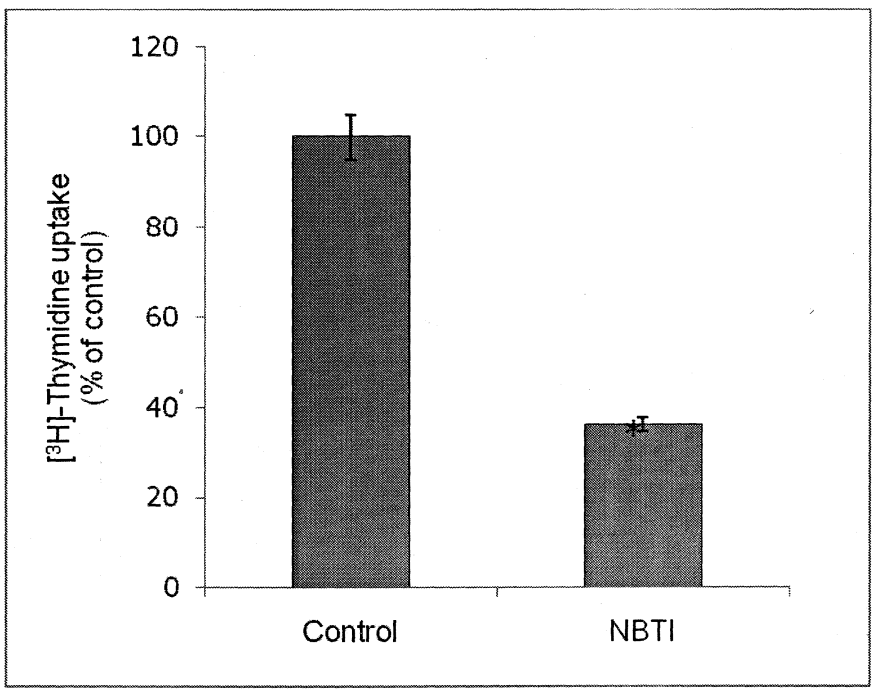

Figure A-9. Effect of NBTI on [^{3}H]-thymidine uptake in cardiac fibroblasts

The effect of NBTI on nucleoside uptake was determined by pre-incubating sub-confluent cardiac fibroblast cultures with 5μM NBTI for 1 hour, followed by 24 hour exposure to NBTI in the presence of 1μCi/ml [^{3}H]-thymidine (n=4). Data are

*presented as Mean ± SD. "n" denotes the number of determinations. NBTI vs. control, *p < 0.0001.*

In conclusion, inhibition of nucleoside uptake represents a novel target of genistein action in cardiac fibroblasts. Together, these observations indicate that the inhibition of [^{3}H]-thymidine incorporation into DNA by genistein, reported by many, may not necessarily reflect inhibition of DNA synthesis due to tyrosine kinase inhibition but may result from tyrosine kinase-independent abolition of radiolabeled nucleoside uptake by these cells. This limits the utility of genistein in assessing the role of tyrosine kinase in the regulation of cell proliferation in terms of [^{3}H]-thymidine incorporation. The study, therefore, stresses the need for caution while interpreting the data using this compound.

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LIST OF PUBLICATIONS

Articles published:

- ✓ **Malini S Pillai**, S Sapna, K Shivakumar (2011) p38 MARK Regulates G1-S Transition in Hypoxic Cardiac Fibroblasts. *The International Journal of Biochemistry and Cell Biology* 43: 919-927.
- ✓ **Malini S Pillai**, K Shivakumar (2009) Genistein Abolishes Nucleoside Uptake by Cardiac Fibroblasts. *Molecular and Cellular Biochemistry* 332: 121-125.
- ✓ M Sangeetha, **Malini S Pillai**, Linda Philip, Edward G Lakatta, K Shivakumar (2011) NF-κB Inhibition Comprises Cardiac Fibroblast Viability Under Hypoxia. *Experimental Cell Research* 317: 899-909.