The role of ms1 in cardiac physiology
and disease

Thesis submitted for the degree of
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by

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The role of ms1 in cardiac physiology and disease

Abstract

Left ventricular hypertrophy (LVH) is an adaptive response to increased workload, stress or injury. Initially this adaptation is beneficial but sustained hypertrophy is a predisposing risk factor for cardiac morbidity and death. Many signalling pathways regulate hypertrophy but the molecular controllers that sense pressure overload and initiate hypertrophy are unclear.

Previous work in the group identified a novel gene, designated myocyte stress 1 (ms1), which is up-regulated within 1 hour in the left ventricle following aortic banding in the rat, suggesting a possible role for ms1 in the initial signalling of the hypertrophic response. ms1 is also expressed during cardiac development and is transiently up-regulated during ischaemia-reperfusion in vitro. This suggests that ms1 may play a more widespread role in cardiac physiology.

The aim of the work in this thesis was to better understand the role of ms1 in cardiac physiology and disease through a combination of in vitro and in vivo approaches. It was demonstrated that transient over-expression of a c-Myc-ms1 fusion protein into a heart-derived rat cell line, H9c2, colocalised with actin and altered expression of known hypertrophic and cardioprotective target genes of the myocardin-related transcription factor (MRTF)/serum response factor (SRF) transcriptional pathway. The size of cells over-expressing ms1 significantly increased by 47% compared to untransfected cells and over-expression of ms1 markedly inhibited staurosporine-induced apoptosis by 88%. A Cre/loxP system based construct was developed to assess the in vivo effects of increased ms1 expression and was confirmed to work in a cell-based system. However, two independent attempts to make a transgenic mouse over-expressing ms1 were unsuccessful despite successful integration of the transgene.

Overall the findings suggest that ms1 induces a hypertrophic response and provides cardioprotection via a MRTF-SRF signalling mechanism. The findings provide for the first time direct evidence of the involvement of ms1 in hypertrophy and cardioprotection.
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ABLIM actin-binding LIM
α-MHC α-myosin heavy chain
ANP atrial natriuretic peptide
AMPK adenosine monophosphate-activated protein kinase
AR adrenergic receptor
ARC apoptosis repressor with caspase recruitment domain
AT angiotensin II receptor
A-V atrioventricular
β-MHC β-myosin heavy chain
BNP brain natriuretic peptide
BSA bovine serum albumin
cDNA complementary deoxyribonucleic acid
CMV cytomegalo virus
Ct threshold cycle
dH₂O distilled water
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DNase I deoxyribonuclease I
dNTP deoxyribonucleotide 5' triphosphate
DTT dithiothreitol
E embryonic day
ECL enhanced chemiluminescence
EDTA Ethylenediaminetetraacetic acid
EGF epidermal growth factor
EGFP enhanced green fluorescent protein
ERKs extracellular regulated kinases
ES embryonic stem
Et endothelin receptor
FISH fluorescence in situ hybridisation
FITC fluorescein isothiocyanate
Fra-1 fos-related antigen-1
GFP green fluorescent protein
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<td>glycogen synthase-3β</td>
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<td>histone acetyltransferases</td>
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<td>hours post fertilisation</td>
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<td>insulin-like growth factor 1</td>
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<td>IL-6</td>
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<td>IMS</td>
<td>industrial methylated spirit</td>
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<td>IRES</td>
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<td>C-Jun N-terminal kinases</td>
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<td>lysophosphatidic acid</td>
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<td>left ventricle</td>
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<td>MLC2v</td>
<td>myosin light chain 2 ventricular</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MRTFs</td>
<td>myocardin-related transcription factors</td>
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<td>myocyte stress 1</td>
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<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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<td>poly(ADP-ribose) polymerase</td>
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<td>serum response factor</td>
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<td>STARS</td>
<td>striated muscle activator of rho signalling</td>
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<td>TSS</td>
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<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling</td>
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Chapter 1

Introduction

1.1 Development of the vertebrate heart

The first organ to form in the embryo is the heart (Olson, 2004; Olson and Schneider, 2003). In response to an axial signalling system, differentiated cardiac myocytes become organised into a linear heart tube that undergoes rightward looping and forms asymmetry across the left-right axis of the embryo (Kelly and Buckingham, 2002; Olson, 2004). Growth of the looped heart tube and septation forms the multichambered heart (Olson, 2004). This is shown in figure 1.1. In order for blood to be delivered to and from the lungs and body, the right (pulmonary) and left (systemic) ventricular and atrial chambers of the heart are separated by an interventricular and interatrial septum. The sinoatrial node (pacemaker) controls each heart beat and a synchronised round of cardiac contraction and relaxation. The cardiac conduction system and direct cell-cell coupling of cardiac myocytes propagates electrical impulses through the heart (Olson, 2004).

Figure 1.1. Heart development (Olson, 2004).

Progenitors of the cardiogenic mesoderm form the linear heart tube and ultimately the four chambers of the heart. The cardiac conduction system components within the multichambered heart are shown. A-V, atrioventricular; LA, Left atrium; LV, Left ventricle; RA, Right atrium; RV, Right ventricle; S-A, sinoatrial.
1.2 Cardiac hypertrophy

1.2.1 Definition

Growth of the heart during embryogenesis occurs primarily through proliferation of cardiac myocytes (hyperplasia). However, after birth, the general consensus is that the cardiac myocytes withdraw from the cell cycle and subsequent growth of the heart occurs predominantly through hypertrophy (increase in cell size) rather than myocyte hyperplasia (increase in cell number). In broad terms there are two forms of cardiac hypertrophy, physiological and pathological (Olson and Schneider, 2003).

1.2.2 Physiological hypertrophy

In physiological hypertrophy growth of the heart occurs in the following 3 situations: during normal postnatal development, in pregnancy and in response to exercise (Chien and Olson, 2002; Lorell and Carabello, 2000), see figure 1.2. In humans the heart grows in proportion to the growth of the body where the left ventricle weight in grams is 3 to 4 times the body weight in kilograms. The 10-fold increase in left ventricle mass that occurs from childhood to adulthood is essential and not harmful. The requirement for an increased stroke volume and cardiac output during pregnancy is accompanied by a considerable increase in left ventricle mass, which regresses over months in the postpartum period (Lorell and Carabello, 2000). Concentric hypertrophy (section 1.3.1) occurs in the trained athlete who specialises in sports such as weight lifting and wrestling. However, eccentric hypertrophy (section 1.3.1) is seen in sports such as long-distance running and cycling (Lorell and Carabello, 2000).

1.2.3 Pathological hypertrophy

Pathological hypertrophy occurs in response to stress signals, which include hypertension, pressure or volume overload, endocrine disorders, myocardial infarction, and contractile dysfunction from inherited mutations in sarcomeric or cytoskeletal proteins (Olson and Schneider, 2003). This is shown in figure 1.2. In pressure overload hypertrophy such as aortic stenosis or hypertension, concentric hypertrophy (section 1.3.1) occurs which increases in wall thickness (Lorell and Carabello, 2000). In valvular disease and mitral regurgitation eccentric hypertrophy (section 1.3.1) occurs (Lorell and Carabello, 2000; Sugden and Clerk, 1998).
Physiological hypertrophy occurs in response to growth signals such as exercise and post-natal growth. Stress signals that induce pathological hypertrophy include hypertension and myocardial infarction (MI). Little is known whether each form of hypertrophy derives from unique signals or whether overstimulation evokes a pathological response.

1.3 Importance of pathological hypertrophy

One of the most important contributions to heart disease leading to heart failure and cardiac death is cardiac hypertrophy. Hypertrophy of the left or right ventricles is an adaptation of the heart in response to variety of biochemical, hemodynamic and hormonal stimuli (Pokharel et al., 2003). The primary inducing stimulus for left ventricular hypertrophy (LVH) is an increase in hemodynamic load (pressure or volume overload) and the heart adapts to accommodate this extra workload by increasing muscle mass. This adaptation of the heart is initially beneficial but in the long term LVH is an important independent risk factor for cardiovascular morbidity and mortality (Hunter and Chien, 1999; Pokharel et al., 2003). Pathological hypertrophy may be associated with an absence of symptoms for many years before the development of heart failure and sudden death (Lorell and Carabello, 2000). Thus it is important to recognise molecular mechanisms involved in the hypertrophic development of
hypertrophy and transformation to heart failure as well as to identify commonalities and differences in the signalling system that promote pathological versus physiological hypertrophy (Frey et al., 2004).

1.3.1 Classification of LVH

Two forms of LVH have been characterised, concentric and eccentric hypertrophy (reviewed by Lorell and Carabello (2000)). In concentric hypertrophy that develops from a sustained increase in pressure overload, there is an increase in myocyte cross-sectional area relative to cell length, with parallel deposition of sarcomeres that results in an increased ventricular wall thickness (increase in the ratio of wall thickness to chamber dimension). In eccentric hypertrophy which develops from sustained volume overload, chamber volume increases with a decrease in the ratio of wall thickness to chamber dimension. It is associated with an increase in myocyte length relative to cross-sectional area, with deposition of sarcomeres in series.

1.3.2 Diagnosis of LVH

The absence of symptoms associated with the development of LVH, means that detection is reliant on non-invasive techniques. Echocardiography provides an accurate non-invasive means of estimation of LV mass that is more sensitive and specific than previous routine methods of the electrocardiogram for detecting LVH (Reichek and Devereux, 1981). The detection of LVH requires adjustments for sex, height and body mass. The echocardiographic criteria for LVH was identified from analysis of healthy men and women within the large original cohort and offspring subjects (n = 6148) of the Framingham Heart Study (Levy et al., 1987). The normal mean values and normal upper limit criteria for LVH based on mean plus 2 standard deviation for LV mass, LV mass corrected for body surface area and LV mass corrected for height are, respectively, 294 g, 150 g/m² and 164 g/m in men and 198 g, 120 g/m² and 121 g/m in women (Levy et al., 1987).

Analysis from the Framingham Heart Study demonstrated that echocardiographic LVH identifies subjects with an increased risk for cardiovascular disease. Subjects with LVH are older, more obese, have higher blood pressures and are more likely to have pre-existing coronary disease and depressed LV systolic function (ejection fraction) (Levy et al., 1992). Increased LV mass determined echocardiographically also predicts an increased risk of cardiovascular morbidity and mortality, even after adjustment for other
major risk factors such as age, blood pressure, pulse pressure, treatment for hypertension, smoking, diabetes, obesity, cholesterol profile and electrocardiographic evidence for LVH. In addition, Haider et al. (1998) reported increased LV mass and hypertrophy are associated with increased risk for sudden death after accounting for known risk factors. Collectively these findings suggest that echocardiographically detected LVH is of prognostic value for cardiovascular morbidity and mortality.

1.4 Molecular changes in LVH

The cellular hallmark of hypertrophy is an increase in size of the cardiac myocyte, enhanced protein synthesis and a re-organisation of contractile elements from a series to a parallel arrangement. These changes in cellular phenotype are usually accompanied by complex changes in gene reprogramming. These changes include an induction of transcription factor-coding early genes such as the proto-oncogenes c-myc, c-jun and c-fos. This is followed by re-expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) that are only otherwise expressed during foetal development. Skeletal muscle α-actin and β-myosin heavy chain (β-MHC) expression are also up-regulated. Constitutively expressed contractile protein genes (cardiac muscle α-actin and myosin light chain 2 ventricular (MLC2v)) are up-regulated in the longer term (Chien et al., 1991; Finn et al., 1999; Sugden and Clerk, 1998). In addition, there is a modification of intracellular ion homeostasis, for example, down regulation of sarcoplasmic reticulum calcium ATPase with variable up-regulation of the Na⁺/Ca²⁺ exchanger, and down regulation of parasympathetic and sympathetic receptors (for example down regulation of [beta]1-adrenergic receptors and M2 muscarinic receptors) (Lorell and Carabello, 2000).

The molecular mechanisms that lead to cardiac hypertrophy are not fully understood. Much effort has concentrated on identifying the signals that mediate the pathways that are associated with hypertrophy and subsequently heart failure. A number of signalling pathways involving downstream molecules (figure 1.3) have been implicated such as G proteins, protein kinase C, small G proteins, members of the mitogen-activated protein kinase (MAPK) family and calcineurin. Transcription factors such as GATA 4 and myocyte enhancer factor 2C (MEF2C) have also been implicated (Molkentin and Dorn II, 2001). No single pathway regulates cardiac hypertrophy independently, but instead each pathway works together to regulate cardiac hypertrophy (Frey and Olson, 2003), see figure 1.3.
The main hypertrophic signalling pathways include small GTP-binding proteins and sarcomeric signalling, calcineurin-nuclear factor of activated T cells (NFAT) signalling, mitogen-activated protein kinase (MAPK) pathways and phosphoinositide 3-OH kinase (PI3K)/Akt/glycogen synthase-3 (GSK-3)-dependent signalling. Hypertrophic pathways ultimately converge on common downstream targets such as the transcription factors serum response factor (SRF), myocyte enhancer factor 2 (MEF2), NFAT and GATA 4.
1.5 **Signalling pathways**

Several signalling pathways have been implicated in cardiac hypertrophy where the G protein-coupled receptors (GPCRs), small G proteins, MAPKs, Akt, calcineurin and the transcription factors SRF, GATA 4 and MEF2C are considered the most important molecules (figure 1.3).

1.5.1 *G protein-coupled receptors*

Three functional classes of G protein-coupled receptors correspond to the three major classes of G proteins within the cardiovascular system. All G proteins consist of separate Gα and Gβγ subunits and are classified according to their α subunits into subfamilies, for example Gαs, Gαi and Gαq. β-adrenergic receptors couple primarily to Gαs and mediate heart rate and myocardial contractility in response to epinephrine and norepinephrine stimulation. Cholinergic receptors couple to Gαi, which are activated by acetylcholine. Angiotensin II, endothelin, and α-adrenergic receptors are the third class of receptors that couple to Gαq/α11 and have been studied and implicated in cardiac hypertrophy (reviewed by Molkentin and Dorn (2001)). For example, over-expression of Gαq/α11 in transgenic models resulted in cardiac hypertrophy (Molkentin and Dorn II, 2001). Cardiac-specific ablation of Gα11/αq in an adult animal model of aortic banding resulted in a lack of cardiac hypertrophy. Also over-expression of a dominant-negative mutant of Gαq in hearts of transgenic mice blunts pressure overload hypertrophy (Frey *et al.*, 2004). These studies demonstrate that Gαq/α11 are both essential for mediating cardiac hypertrophy.

1.5.2 *Small G proteins*

Small G proteins play an important role in sarcomeric and cytoskeletal organisation, hallmark features of the hypertrophic phenotype (Frey *et al.*, 2004). They have been characterised into five subfamilies (Ras, Rho, ADP ribosylation factors, Rab and Ran), each consisting of multiple members. Ras and Rho subfamilies have been mainly studied in the heart (Clerk and Sugden, 2000).

Ras induced a significant increase in cardiac mass when a constitutively active mutant was over-expressed in transgenic mouse hearts. This Ras mutant expressed in neonatal rat cardiac myocytes also resulted in hypertrophic gene expression (Frey *et al.*, 2004).
The Rho family of small G proteins, consists of RhoA, Rac, and cdc42 subfamilies (Frey et al., 2004). Transfection of cardiac myocytes with Rac increased ANF and BNP expression and promotes morphological changes associated with myocyte hypertrophy (Clerk and Sugden, 2000). Constitutive activation of Rac in cardiac myocytes in vitro and in vivo leads to hypertrophy linked with alterations in focal adhesions (Frey et al., 2004).

Rho plays an important role in a variety of cytoskeletal-dependent cell functions that include actin polymerisation, f-actin bundling, myosin-based contractility and focal adhesion formation (Yanazume et al., 2002). Activated Rho induced myofibrillar organisation and stimulated the expression of ANP in cardiac myocytes (Hoshijima et al., 1998). RhoA activates several protein kinases, specifically Rho-associated kinase (ROCK). The Rho/ROCK pathway has been linked to GATA 4 transcriptional activity during hypertrophy in neonatal rat cardiac myocytes (Yanazume et al., 2002). There is evidence that Rho activates transcription via serum response factor (SRF) (Hill et al., 1995). SRF regulates serum-inducible genes and genes expressed in skeletal, smooth and cardiac muscle such as ANP and α-skeletal actin. SRF is a MADS (Mcm 1 and Arg80 in yeast, Agamous and Deficiens in plants, and SRF in animals) box transcription factor and regulates these genes by binding to a DNA sequence (CC[A/T]6GG) known as the CArG box elements in their promoter/enhancer sequences (Frey and Olson, 2003; Sotiropoulos et al., 1999). Current evidence suggest that Rho activates transcription via SRF through a mechanism mediated by changes in actin treadmilling (Hill et al., 1995; Sotiropoulos et al., 1999). These results implicate Rho, Rac and Ras in cardiac hypertrophy; however, the precise signalling pathways are still unclear (Clerk and Sugden, 2000).

1.5.3 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) have been implicated as mediators of the hypertrophic response. MAPKs are classified into three major phosphorylation cascades, the extracellular regulated kinases (ERKs), C-Jun N-terminal kinases (JNKs) and the p38MAPKs (see figure 1.4). The latter two are termed the stress-activated MAPKs. ERKs are activated by classical hypertrophic agonists such as phenylephrine and endothelin-1, whereas the stress-activated MAPKs are activated more strongly by cell stresses such as ischaemia or cytotoxic agents (Finn et al., 1999; Wang et al., 1998b). MAPKs have been well established as transducers of growth and stress.
responses in many cell types. However, considerable controversy exists concerning their importance as mediators of cardiomyocyte hypertrophy. A number of studies have demonstrated roles for ERKs, p38MAPKs or JNKs as hypertrophic mediators but an almost equal number of studies have not found MAPKs involved in mediating hypertrophy (Bueno et al., 2001). The evidence for whether MAPKs play a role in mediating cardiac hypertrophy is discussed in the following sections.

Figure 1.4. MAPK signalling pathways (Liang and Molkentin, 2003).

G protein-coupled receptors (endothelin receptor (Et), angiotensin II receptor (AT), adrenergic receptor (AR) and receptor tyrosine kinases (RTKs) initiate signalling through G proteins that promote small G proteins. These upstream events promote activation of MAPK kinase kinases (MAPKKKs), which in turn regulate the MAPK kinases (MAPKKs), resulting in activation of JNKs, ERKs and p38 MAPKs and phosphorylation of transcription factors within the nucleus.
1.5.3.1 ERKs

Evidence that hypertrophic agonists activate ERKs initially implicated the ERK cascade in the hypertrophic response (Sugden and Clerk, 1998). There are at least five different ERK proteins that have been identified in mammalian cells named ERK1 to 5. The first MAPKs to be identified and the most highly studied and abundantly expressed are ERK1 and ERK2 (also known as p44 and p42 respectively). These are directly regulated by two MAPK kinases (MAPKKs), MEK1 and MEK2 (also referred to as MKK1 and MKK2). Upstream of the MAPKKs, multiple MAPK kinase kinases (MAPKKKs) exist that directly sense stress stimulation or are directly regulated by effectors such as the low molecular weight G proteins (Ras, Rac, Rho, Cdc42 etc). Both ERK1 and ERK2 function in this protein kinase cascade that plays a role in the regulation of cell growth and differentiation (Bueno and Molkentin, 2002).

Agonist stimulation or cell stretching in cultured cardiac myocytes activated ERK1/2 (Bueno and Molkentin, 2002) and there was a low magnitude of ERK activation that was maximal within the first 30 minutes after pressure overload in a rat model of pressure-overload hypertrophy by aortic banding, suggesting a very early and transient response (Fischer et al., 2001). Transfection of constitutively active MEK1 (upstream activator of ERK1/2) increased ANP activity in cultured cardiomyocytes, whereas a dominant-negative MEK1 construct inhibited its activity (Gillespie-Brown et al., 1995). MEK1-ERK1/2 was also found to be required for sarcomeric organisation induced by hypertrophic agonists and MEK1 transgenic mice demonstrated concentric hypertrophy and partially resistant to ischemia/reperfusion-induced apoptosis. Collectively the MEK1-ERK1/2 pathway could induce a hypertrophic response in vitro and in vivo (Bueno and Molkentin, 2002). ERK1/2 signalling has also been associated with phosphorylation and activation of the cardiac-enriched transcription factor GATA 4. GATA 4 is a critical regulator of most cardiac-expressed structural genes and hypertrophy responsive genes (Bueno and Molkentin, 2002). ERKs along with the JNKs and p38MAPKs can phosphorylate and activate the ternary complex factors Elk1 and SAP1a, which act at the serum response element in conjunction with SRF to up-regulate c-fos expression (early hallmark of the hypertrophic response) (Sugden and Clerk, 1998).

All these observations have implicated ERKs as regulators of cardiac hypertrophy but there are studies that do not support this. For example, stimulated ERK activation was
completely normalised or desensitised within 1-2 hours despite the continued presence of the agonists (Liang and Molkentin, 2003). However, other studies have observed transient ERK activation during an acute or initiation phase of long term stimulation that otherwise produces hypertrophic growth (Hoshijima and Chien, 2002). A number of negative regulators of cardiac signalling have been described. One is of a negative feedback system for the cardiac MAPK signalling cascade in which forced expression of MAPK phosphatase-1 (MKP-1) negatively regulates the cardiac hypertrophic response by down regulating ERK1/2 (Bueno et al., 2001). Other studies have also found ERK1/2 to be down regulated through MKP-1 and attenuate or decrease hypertrophy (Andersson et al., 1998; Black et al., 2002; Bueno et al., 2001; Takahashi et al., 2003). Recently ERK deactivation has also been found via adenosine monophosphate-activated protein kinase (AMPK). AMPK is a stress activated protein kinase that is involved in regulating energy and metabolic homeostasis. AMPK is increase during acute and chronic stresses such as hypoxia, ischaemia and cardiac hypertrophy (Shibata et al., 2004). Researchers investigated the role of adiponectin (circulating adipose-derived cytokine that is down regulated in cardiovascular disease) in regulating cardiac hypertrophy. They found that in cultures of cardiac myocytes, adiponectin activated AMPK and inhibited agonist stimulated hypertrophy and ERK activation. Also the effects were reversed when a dominant-negative form of AMPK was used (Shibata et al., 2004).

Finally studies have shown that ERK could be cardiomyocyte protective as inhibition of ERK caused increased hydrogen peroxide-induced apoptosis (Turner et al., 1998) and increased daunomycin-induced apoptosis (Bueno and Molkentin, 2002) in cardiomyocytes. Also ERK activation attenuated the amount of apoptosis subsequent to reperfusion injury (Bueno and Molkentin, 2002).

1.5.3.2 p38MAPKs

p38MAPKs are activated by a wide range of stress stimuli including chemical stress, physical stress, osmolar stress, radiation stress and G protein-coupled receptor activation. Four separate p38MAPK isoforms have been described including p38α, p38β, p38γ and p38δ. p38α and p38β are expressed in the human heart and thought to be the most important isoforms whereas p38γ and p38δ are undetectable in the heart (Molkentin and Dorn II, 2001). The upstream activators of p38MAPK are MEK3 and MEK6 (also referred to as MKK3 and MKK6) but less is known of the MAPKKK
factors which lie upstream of MEK3 and MEK6 (Molkentin and Dorn II, 2001; Sugden and Clerk, 1998). p38MAPK can phosphorylate and activate the transcription factors ATF-2 and MEF2C. MEF2C may regulate expression of c-jun (an early response to hypertrophic stimulation or cell stress) and other genes (Sugden and Clerk, 1998). p38MAPK can also phosphorylate and activate other protein kinases which in turn phosphorylate the small heat shock protein 25/27 which may increase its cytoprotective ability and maybe important in cell survival (Sugden and Clerk, 1998). As observed for ERK, an increase in p38MAPK was found in a rat model of pressure overload hypertrophy by aortic banding. This was a very early and transient response as it reached maximal activation within the first 30 minutes after pressure overload (Fischer et al., 2001). Activated MEK3/6 over-expression induced hypertrophy and ANP expression in neonatal cardiomyocytes (Wang et al., 1998a; Zechner et al., 1997). Inhibitors of p38MAPK were found to block agonist-stimulated cardiomyocyte hypertrophy in culture (Zechner et al., 1997). In addition, the hypertrophic response in neonatal cardiomyocytes was blunted in response to adenovirus-mediated gene transfer of a dominant negative p38βMAPK (Wang et al., 1998b).

p38MAPK signalling has also been indicated to not function as a forward regulator of the hypertrophic response. For example as found for ERK activation, p38MAPK activation has only been transiently observed during an acute or initiation phase of long term stimulation that otherwise produces hypertrophic growth (Hoshijima and Chien, 2002). In contrast to the study discussed earlier where over-expression of MEK3/6 induced hypertrophy (Wang et al., 1998a; Zechner et al., 1997) another study found that over-expression of MEK3/6 in the heart did not induce hypertrophic growth (Liao et al., 2001). As observed for ERK, transgenic mice expressing MKP-1 in the heart caused reduced p38MAPK and attenuated cardiac hypertrophy in response to aortic banding (Bueno et al., 2001). Another study concluded that p38 MAPK plays an essential role in the cardiomyocyte survival pathway but not in cardiac hypertrophy in response to pressure overload (Nishida et al., 2004). p38MAPKs are known to regulate cell viability; however, it is still unknown whether p38MAPKs are cytoprotective or pro-apoptotic. The majority of evidence suggests that p38MAPK signalling serves a pro-apoptotic role in the heart (reviewed by Baines and Molkentin) (Baines and Molkentin, 2005).
1.5.3.3 JNKs

The JNKs which are also known as the stress-activated protein kinases (SAPKs) are involved in cellular responses to pathological stresses and cytotoxic agents (Sugden and Clerk, 1998). MEK4 and MEK7 (also known as MKK4 and MKK7) appear to be upstream activators of JNKs and in turn are activated by MEKK1 or MEKK2. The MAPKKKs upstream of MEKK1 and MEKK2 include other protein kinases such as PAKs, GCKs and MLKs and members of the low-molecular-weight G proteins (Ras) (Molkentin and Dorn II, 2001). Phosphorylation of the transcription factors c-jun and ATF-2 are mediated by JNKs and this has been demonstrated in ventricular myocytes in response to hypertrophic agonists or cellular stresses (Sugden and Clerk, 1998). JNKs have been found to be activated in myocytes by cellular stresses such as hyperosmotic shock and by hypertrophic interventions such as stretch and Angiotensin II (Sugden and Clerk, 1998). These findings suggest the role for JNKs in the hypertrophic response. Also a marked transient increase in JNK (also observed for p38MAPK) was observed in a rat model of pressure overload hypertrophy by aortic banding (Fischer et al., 2001). It has been suggested that the α1-adrenergic receptor mediates ANP gene expression through a Ras-MEKK-JNK pathway and that elevated JNK activity is important in the development of hypertrophy in vitro and in vivo (Ramirez et al., 1997). Over-expression of MEK1 or MEK7, upstream activators of the JNK pathway, induced transcriptional and morphological features of the hypertrophic response (Bogoyevitch et al., 1996; Wang et al., 1998b). Adenovirus-mediated gene transfer of a dominant-negative MEK4 factor attenuated agonist-induced cardiomyocyte hypertrophy in vitro and reduced hypertrophy in hearts of aortic-banded rats (Molkentin and Dorn II, 2001).

JNK signalling has also been suggested not to function as a positive regulator of hypertrophy just like ERK and p38MAPK. As observed for ERK and p38MAPK, a transient activation of JNK was found during the initial phase of a long-term stimulation that otherwise produces a hypertrophic growth response (Hoshijima and Chien, 2002) and transgenic mice over-expressing MKP-1 in the heart, had reduced JNK and attenuated cardiac hypertrophy in response to aortic banding (Bueno et al., 2001). In contrast to researchers showing that a dominant negative MEKK1 attenuated ANP activity (Ramirez et al., 1997), transfection of MEKK1 was reported to attenuate sarcomeric organisation, suggesting an antihypertrophic effect of the JNK signalling pathway (Thorburn et al., 1997). Also in contrast to over-expression of MEK7 inducing
features of hypertrophy (Wang et al., 1998b), over-expression of activated MEK7 in transgenic mice did not cause hypertrophy (Liang and Molkentin, 2003).

JNKs (along with p38MAPK signalling), have been shown to regulate cell viability through cytoprotective and proapoptotic effects, with the majority of evidence suggesting a pro-apoptotic regulatory role for JNK signalling in the heart (Baines and Molkentin, 2005).

However, that ERK, p38MAPK, and JNK may not positively regulate cardiac hypertrophy does not exclude their potential importance in modulating the hypertrophic response or the transition towards heart failure in concert with other signalling pathways (Molkentin, 2004).

1.5.4 Akt

Akt (also known as protein kinase B) regulates cellular processes associated with growth (e.g. protein synthesis), survival (e.g. apoptosis), and carbohydrate metabolism (e.g. glycogenesis and possibly glycolytic flux and glucose uptake). Akt is also important in insulin and insulin-like growth factor 1 (IGF1) signalling (Sugden, 2003). It is activated through the phosphoinositide 3-OH kinase (PI3K) pathway. PI3K is activated by receptor protein tyrosine kinases or by G protein-coupled receptors and catalyses the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (Ptd InsP$_3$). PDK1 (Ptd InsP$_3$-dependent protein kinase) is then activated which causes phosphorylation and activation of Akt (Clerk et al., 2003). Phosphorylation and activation of Akt has been observed at 15 minutes and 1 day after aortic banding in rabbits prior to morphological changes and downstream targets of Akt, glycogen synthase-3β (GSK-3β) and p70 ribosomal s6 kinase (p70$^{6k}$) was observed at 15 minutes after pressure overload (Miyamoto et al., 2004). Prolonged (6 weeks) activation of Akt in the adult mouse heart resulted in cardiac hypertrophy, severe defects in contractility and massive fibrosis (Schiekofer et al., 2006). However, there was no hypertrophy observed by another study expressing Akt in the mouse heart (Tsujita et al., 2006). In most systems, Akt activation occurs downstream of PI3K and the PI3K-Akt pathway has been implicated as regulating body/organ size, growth and apoptosis (Burgering and Coffer, 1995; Franke et al., 1995; Shioi et al., 2000). PI3K has emerged as being involved in membrane trafficking, cytoskeletal organisation, cell growth and apoptosis (Van Empel and De Windt, 2004).
Expression of constitutively active or dominant negative mutants of PI3K in the heart resulted in mice with larger or smaller hearts, respectively (Shioi et al., 2000). Transgenic over-expression of Akt caused cardiac hypertrophy in mice (Frey and Olson, 2003). In contrast, over-expression of GSK-3β (a downstream target of Akt) in transgenic mouse hearts inhibited hypertrophy by aortic banding and partially prevented cardiac hypertrophy caused by an activated form of calcineurin, suggesting an important role of GSK-3β in regulating cardiac hypertrophy (Antos et al., 2002).

Akt is also known to mediate cell survival by inhibiting apoptosis through phosphorylation of Bad and tethers Bad in the cytoplasm, thereby diminishing its pro-apoptotic ability (Sugden, 2003). Over-expression of PI3K or Akt provides protection against myocyte apoptosis induced by doxorubicin, hypoxia or serum deprivation in cultured cells. Over-expression of Akt also protected against myocyte apoptosis resulting from ischaemia-reperfusion in vivo (Clerk et al., 2003). The PI3K/Akt pathway has been shown to be required for protein synthesis in cardiac myocytes, both in the basal state or due to hypertrophic stimulation, but signalling through this pathway also has clear cytoprotective effects. Over-expression of the phosphatase PTEN, which dephosphorylates Ptd InsP_3 to inactivate the pathway, causes myocyte apoptosis. Thus, a basal PI3K/Akt signal may be required for cardiac myocyte protection (Clerk et al., 2003). Akt activation was also shown to promote cardiomyocyte viability in another study; however, its role in hypertrophy was not reported (Molkentin and Dorn II, 2001).

Taken together, these results suggest that the Akt pathway plays a dual role in producing the hypertrophic response of the heart and also in modulating cell survival (Van Empel and De Windt, 2004).

1.5.5 Calcineurin

Intense research has focused on the calcium-dependent signalling pathways implicated in cardiac hypertrophy (Olson, 2004) (figure 1.5). One such pathway involves calcineurin, a calcium calmodulin-dependent phosphatase. Calcineurin is activated by a rise of intracellular calcium and dephosphorylates a range of cellular substrates including the nuclear factor of activated T cells (NFAT) transcription factor, facilitating translocation of NFAT to the nucleus where it can mediate expression of pro-hypertrophic genes (Molkentin et al., 1998; Olson, 2004).
Figure 1.5. Calcium-dependent signalling pathways implicated in the hypertrophic growth of the adult heart (Olson, 2004).

The calcineurin phosphatase is activated by sustained elevation of intracellular calcium and dephosphorylates a range of cellular substrates including the nuclear factor of activated T cells (NFAT) transcription factor to induce hypertrophy, characterised by fetal gene activation and remodelling. The calcineurin pathway is integrated with other pathways such as the stress-responsive MAPKs.

Transgenic mice that over-express these components of the calcineurin signalling pathway developed hypertrophy that lead to heart failure and cyclosporine, an inhibitor of calcineurin, was found to suppress hypertrophy (Molkentin et al., 1998). However, calcineurin inhibitors failed to suppress experimental hypertrophy in several animal models (Ding et al., 1999; Zhang et al., 1999). In vitro, activated calcineurin expression in cardiomyocytes resulted in hypertrophy (De Windt et al., 2000) and a number of groups have reported elevated calcineurin activity in hypertrophic hearts induced by aortic banding in rats or mice (De Windt et al., 2001; Eto et al., 2000; Lim et al., 2000; Saito et al., 2003; Shimoyama et al., 1999; Zou et al., 2001). A significant increase in calcineurin activity was also observed in patients with cardiac hypertrophy (Ritter et al., 2002). There are a few studies that do not implicate that calcineurin is activated in the hypertrophic heart. For example one group found no change in calcineurin activity in response to pressure overload stimulation (Zhang et al., 1999).
and another group reported decreased calcineurin activity following pressure overload (Ding et al., 1999).

Calcineurin has also been shown to function in concert with members of the MAPK family. Mice expressing the activated calcineurin transgene resulted in JNK and ERK activation in the heart and isoproterenol stimulation of cardiac myocytes caused activation of ERK via a mechanism involving calcineurin activation (Molkentin, 2004). Inhibition of the MEK1-ERK1/2 signalling pathway in cultured cardiomyocytes attenuated hypertrophic growth directed by activated calcineurin (Sanna et al., 2005).

Recently it has been suggested that calcineurin plays a role in the modulation of cardiomyocyte apoptosis (De Windt et al., 2000; Molkentin et al., 1998) where calcineurin has been involved in both pro- and anti-apoptotic roles in cardiomyocytes (Bueno et al., 2004; De Windt et al., 2000; Kakita et al., 2001; Saito et al., 2000). These findings implicate calcineurin in both cardiac hypertrophy and apoptosis.

### 1.5.6 SRF

Expression of SRF in the adult mouse is high in skeletal and cardiac muscle, but low in the liver, lung and spleen tissues (Belaguli et al., 1997). During embryogenesis, SRF is expressed preferentially in differentiating cardiac and skeletal muscle cells (Zhang et al., 2001). Certain muscle-specific genes containing mutations in CArG boxes in their promoters lead to a loss of their expression in cardiac muscle cells (Zhang et al., 2001). Inactivation of SRF in cardiac/smooth muscle cells resulted in embryonic lethality at approximately embryonic day 11.5 (E11.5). SRF deficient mice display defects in sarcomeric assembly and absence of smooth muscle cell actin/intermediate filament bundles (Miano et al., 2004). In another study, Tamoxifen-inducible Cre recombinase was used to disrupt SRF in the hearts of mice. This caused impaired left ventricular function with reduced contractility, leading to dilated cardiomyopathy and subsequent death from heart failure (Parlakian et al., 2005). Taken together these studies indicate that SRF is involved in the organisation of cytoskeletal functions of cardiomyocytes and vascular smooth muscle cells.

The majority of SRF target genes identified in a genome-wide (in silico) screen were experimentally validated to be involved in cell growth, migration, cytoskeletal organisation, and myogenesis (Sun et al., 2006) suggesting a role for SRF in the regulation of genes responsible for cardiac structure and function.
SRF has also been implicated as a potential regulator of cardiac gene expression in response to hypertrophic signals. Transgenic mice that over-express SRF in the heart displayed massive cardiac hypertrophy and subsequently died within a few weeks after birth (Zhang et al., 2001). In hypertrophy transgenic hearts, SRF was found required for the induction of a range of genes such as ANP, c-fos, BNP, α-actin, and β-MHC (Nelson et al., 2005). Together these findings suggest a role for SRF in cardiac hypertrophy but the mechanisms that connect SRF to hypertrophic signalling remains unknown.

1.5.7 GATA4

The family of GATA transcription factors consists of six proteins (GATA 1-6) that regulate differentiation, growth and survival of a wide range of cell types (Pikkarainen et al., 2004). GATA 4 is one of the earliest transcription factors expressed in the developing mouse heart. GATA 4 mRNA is detected as early as at 7.0-7.5 days postcoitum in the precardiac mesoderm and is expressed during formation of the heart tube at 8.0 days postcoitum (Heikinheimo et al., 1994). GATA 4 mRNA is expressed in cardiac myocytes through to adulthood and in the mouse GATA 4 is expressed in the gut, gonads, liver, visceral endoderm and parietal endoderm (Arceci et al., 1993; Laverriere et al., 1994). After initial studies identified GATA 4 as a major GATA-binding factor in the heart, its role in development has emerged. Increased differentiation of beating hearts was observed in PI9 embryonal carcinoma cells when over-expressing GATA 4, while inhibition of GATA 4 prevents cardiomyocyte differentiation and triggers apoptosis (Pikkarainen et al., 2004). Inactivation of GATA 4 in transgenic mice causes death during embryonic development (Kuo et al., 1997; Molkentin et al., 1997).

GATA 4 is one of the targets of hypertrophic signals that mediate transcriptional activity of genes encoding α- and β-MHC, angiotensin II type 1 receptor, ANP, BNP, and endothelin-1 (Molkentin, 2000; Pikkarainen et al., 2004). GATA 4 also regulates cardiac transcription factors such as Nkx2.5, Hand2 and MEF2C (myocyte enhancer factor 2C) (Pikkarainen et al., 2004; Xin et al., 2006).

The DNA binding activity of GATA 4 was found to increase during the hypertrophic process (Yanazume et al., 2002). Binding is rapid in pressure-overloaded rat hearts and peaks at 30 minutes, which is similar to endothelin-1 stimulated neonatal rat cardiac
myocytes (Pikkarainen et al., 2004). Cardiac hypertrophy was observed when GATA 4 was over-expressed in cultured cardiomyocytes and transgenic mice (Liang et al., 2001) and features of cardiomyocyte hypertrophy induced by phenylephrine and endothelin-1 in culture were diminished when a dominant negative GATA 4 or antisense GATA 4 mRNA were used (Charron et al., 2001; Liang et al., 2001). Mice heterozygous for deletion of the second exon of GATA 4 (G4D) resulted in mild systolic and diastolic dysfunction with reduced heart weight and cardiomyocyte apoptosis. G4D mice developed overt heart failure and eccentric cardiac hypertrophy associated with significant increased fibrosis and cardiomyocyte apoptosis following pressure overload (Bisping et al., 2006). Another group (Oka et al., 2006) who used a Cre/loxP system to ablate GATA 4 in cardiomyocytes also observed cardiomyocyte apoptosis at baseline and after pressure overload. However, they found that hypertrophy was blunted after pressure overload. Taken together, these findings suggest that GATA 4 plays important roles in cardiac development, differentiation, growth and survival.

1.5.8 Members of the myocyte enhancer factor 2 (MEF2) family

The MEF2 family of transcription factors control cardiomyocyte differentiation, myofibrillogenesis and muscle metabolism (Olson and Schneider, 2003). Members of the MEF2 family like SRF contain a MADS domain; however, MEF2 and SRF bind only to their respective sites. MEF2 activates transcription by binding to the consensus sequence, called the MEF2-binding site CTA(A/T)4TAG, which is found in the promoter regions of numerous muscle specific genes (Black and Olson, 1998). In vertebrates there are four MEF2 genes, MEF2A, MEF2B, MEF2C, and MEF2D. Mice lacking in MEF2C die around E9.5 by improper development of the heart (Lin et al., 1997) and transgenic expression of a dominant-negative mutant of MEF2 in mouse hearts results in impaired growth of the myocardium (Kolodziejczyk et al., 1999).

The MEF2 transcription factor appears to be a critical target for hypertrophic signals (Chien and Olson, 2002). MEF2 activity is controlled by direct association with Class II histone deacetylases (HDACs). HDACs deacetylate nucleosomal histones, thus promoting chromatin condensation and transcriptional repression of MEF2 target genes. HDAC activity is opposed by histone acetyltransferases (HATs), which activate target genes by acetylating nucleosomal histones, thereby relaxing chromatin (Frey and Olson, 2003). It has been proposed that HDACs associate with MEF2 and repress hypertrophy and the fetal gene programme. Hypertrophic signals cause activation of a cardiac
HDAC kinase that phosphorylates the serine sites in HDACs. When phosphorylated, HDACs dissociate from MEF2 and are exported from the nucleus. Upon release of HDACs, MEF2 can associate with HATs and activate downstream target genes that govern a hypertrophic response (Zhang *et al.*, 2002). Therefore to inhibit the HDAC kinase could be therapeutically beneficial to prevent cardiac hypertrophy.

The various intracellular signalling pathways discussed in this section have been implicated as important pathways of the hypertrophic response but the key regulatory molecules involved and their interactions are still unknown. Until these findings are discovered, the molecular mechanisms of LVH remain unclear.

### 1.6 Myocyte stress 1 (mst1)

Previous work in the group applied a molecular indexing approach (Mahadeva *et al.*, 1998) to identify genes that are regulated during the early stages of pressure-induced LVH in a rat model of aortic banding. This approach for the isolation of differentially expressed genes involves digestion of cDNA pools from different samples with class II restriction endonucleases that generates fragments with non-identical cohesive ends. The ligation of adapters with perfectly complementary overhangs, partitions the cDNA fragments into non-overlapping subpopulations. These internal cDNA restriction fragments are then exponentially amplified by adapter primer PCR and visualised by non-denaturing polyacrylamide gel electrophoresis (Mahadeva *et al.*, 1998). Using the molecular indexing approach to identify genes that are differentially expressed in the early stages of aortic banding in the rat, a model of pressure-induced LVH, led to the identification and characterisation of a novel gene, myocyte stress 1 (mst1). mst1 was up-regulated within 1 hour in the left ventricle following aortic banding in the rat, with a peak expression before 1 day at 4 hours, well before any detectable increase in LV mass suggesting a possible role for mst1 in the initial signalling of the hypertrophic response (Mahadeva *et al.*, 2002). Interestingly mst1 is preferentially expressed in striated muscle as mst1 mRNA was found expressed at high levels in the adult heart and skeletal muscle of the rat. mst1 mRNA expression was observed in the rat left ventricle during embryogenesis and was found to be regulated post-natally through to adulthood. The rat mst1 gene is located on chromosome 7 and its full-length transcript size is approximately 1.3 kb. mst1 has a relatively simple genomic organisation of two exons and one intron. mst1 is a 375-amino acid protein and BLAST searches revealed that mst1 full-length cDNA and putative protein show no significant homology to any known
genes at date of submission. However, there is significant identity of specific regions that are well conserved between species implying evolutionary conservation and thus functional importance (Mahadeva et al., 2002).

At the same time the mouse homologue of ms1 termed STARS (striated muscle activator of Rho signalling) was identified in the early embryonic heart (E8.25) and shown to be a sarcomeric actin-binding protein which stimulates SRF-dependent transcription through a mechanism involving RhoA signalling and actin polymerisation (Arai et al., 2002). The molecular mechanism by which STARS activates SRF-dependent transcription was discovered a few years later and during the time of our own investigations into identifying the downstream targets of ms1. Kuwahara et al. (2005) have shown that STARS activates SRF-dependent transcription by inducing the nuclear accumulation of the SRF co-factors, myocardin-related transcription factors (MRTFs) MRTF-A and MRTF-B through a mechanism dependent on RhoA and actin polymerisation. MRTF-A (MAL/MKL-1) and MRTF-B (MKL-2) are expressed in a wide range of embryonic and adult tissues (Wang et al., 2002). MRTFs, like myocardin (belongs to a family of nuclear proteins that plays a role in cardiac gene expression and is a master regulator of smooth muscle development (Li et al., 2003)), enhance the transcriptional activity of SRF by forming a stable ternary complex on CArG boxes by associating with SRF and providing their powerful transcription activation domains (Wang et al., 2001b; Wang et al., 2002). MRTFs also convey stimulatory signals to SRF from Rho GTPase and the actin cytoskeleton by the use of their regulated translocation into the nucleus (Cen et al., 2003; Du et al., 2003; Miralles et al., 2003). Kuwahara et al. (2005) propose that STARS binds to actin and promotes actin polymerisation in the presence of Rho activity, which releases MRTFs from the inhibitory influence of G-actin, allowing the nuclear import of MRTFs and stimulation of SRF dependent gene activation (figure 1.6) (Kuwahara et al., 2005).
Figure 1.6. The proposed mechanism by which STARS activates SRF-dependent transcription via MRTFs and Rho-actin signalling (Kuwahara et al., 2005).

STARS stimulates SRF-dependent transcription by binding to G-Actin and promoting actin polymerisation in the presence of basal Rho activity, thereby allowing the nuclear accumulation of MRTFs and subsequent SRF-dependent gene activation. Actin itself is regulated via SRF thus generating a negative feedback loop.

More recently it has emerged that members of the actin-binding LIM (ABLIM) protein family interact with STARS, bind to actin and positively modulate STARS dependent activation of SRF transcriptional activity (figure 1.7) (Barrientos et al., 2007). The novel ABLIM proteins, ABLIM-2 and ABLIM-3 were identified to interact with STARS by performing a yeast-two hybrid screen of a human skeletal muscle cDNA library using STARS as bait and by co-immunoprecipitation. Another member of the ABLIM protein family, ABLIM-1 was also found to interact with STARS. ABLIM-1 is expressed in the retina, brain and muscle tissue and postulated to regulate actin dependent signalling. ABLIM-2 is highly expressed in skeletal muscle and not in the heart whereas ABLIM-3 is predominantly expressed in the heart. These ABLIM proteins bind to actin, are localised to the sarcomere in striated muscle and enhance STARS-dependent SRF-activation. Moreover, knockdown of ABLIM-1, -2 and -3 inhibited STARS-mediated SRF-activation, further supporting a role for ABLIM proteins to modulate actin-dependent SRF activity via binding of STARS (Barrientos et al., 2007).
Figure 1.7. ABLIM proteins associate with STARS and regulate STARS mediated activation of SRF transcription (Barrientos et al., 2007).

ABLIM proteins can augment STARS-dependent SRF-activation and are likely to serve as a scaffold to integrate signals from small G proteins via STARS towards the actin cytoskeleton.

As discussed earlier in section 1.5.2 Rho has been demonstrated to play a critical role in hypertrophy and Rho activates transcription via SRF, whereby the mechanism involves alterations in actin dynamics. Actin itself is regulated via SRF thus generating a negative feedback loop. Members of the ABLIM protein family are expressed in striated muscle tissue and localised to the sarcomere. ms1 is also a striated muscle specific protein and its mRNA increased markedly in the left ventricle of the rat from embryo to adulthood. ms1 expression was found to be up-regulated in a model of pressure-induced hypertrophy (Mahadeva et al., 2002) and as sacromeric and cytoskeletal organisation are hallmarks of the hypertrophic phenotype these findings, along with others (Arai et al., 2002; Barrientos et al., 2007; Kuwahara et al., 2005), suggest that the ABLIM proteins and the ms1-MRTF/SRF pathway may play an important role in the aetiology of certain heart disease.

In subsequent work our group has identified that ms1 is transiently up-regulated during simulated ischaemia and reperfusion in a heart-derived rat cell line, H9c2. Ischaemia alone had no effect on ms1 mRNA expression. However, ms1 mRNA is up-regulated following 1 hour to 2 hours of reperfusion and then returns to basal levels by 4 hours (Chong, unpublished results). Furthermore, the role of ms1 in cardiac development was...
investigated in the zebrafish. The earliest site of ms1 expression is the somites, where strong expression is noted by 16 somites approximately 17 hours post fertilisation (hpf). At 24 hpf ms1 expression is evident in the developing brain and at 48 hpf expression is detectable in both chambers of the heart. Following injection of the ms1 antisense morpholino, by 48-56 hpf, cardiac contractility was decreased, and the atrium was markedly enlarged; ventricular contraction was also impaired. These cardiac abnormalities resulted in significant pericardial oedema and decreased or absent circulation. There was also a variable degree of curvature and shortening of the longitudinal axis. These observations suggest an important role of ms1 in cardiac development and function (Mahadeva et al., 2007). These findings coupled with other findings of ms1’s rapid induction in LVH (Mahadeva et al., 2002), expression of ms1 during embryonic development (Arai et al., 2002; Mahadeva et al., 2002), its transient up-regulation during ischaemia-reperfusion and that ms1 is a striated muscle specific protein (Arai et al., 2002; Mahadeva et al., 2002) suggests that ms1 plays an important widespread role in cardiac development and physiology.

1.7 Aims

Multiple signalling pathways have been shown to regulate hypertrophy; however, the molecular controllers that sense pressure overload and initiate hypertrophy are unclear. The determinants of progression from cardiac hypertrophy to failure are not fully understood either and may be a consequence of alterations in specific signalling molecules and their downstream pathways. Cardiac hypertrophy is one of the most important causes of heart disease leading to heart failure, an increase risk of arrhythmia and sudden death; therefore, it is essential to identify the molecular events involved whose actions could be interrupted thereby halting or perhaps reversing clinical deterioration.

The novel gene ms1, may play a role in the initial signalling of the hypertrophic response and an important role in cardiac physiology and disease. This study proposes to further examine the function of ms1 in cardiac physiology and disease by using a combination of in vitro and in vivo approaches:

1. To identify putative target genes and downstream pathways of ms1 by over-expressing a c-Myc-ms1 fusion protein in vitro and examining altered mRNA and protein expression.
2. To examine the cellular consequences following over-expression of msl \textit{in vitro}.

3. To investigate the physiological and pathophysiological consequences of msl over-expression \textit{in vivo}. 
Chapter 2

Materials and Methods

In this chapter the materials and methods used in this thesis will be presented, starting with the materials and general methods of DNA, RNA and protein manipulation, followed by specific methods of cell culture, flow cytometry and genetic manipulation. Other specific methods are detailed in individual chapters where relevant.

2.1 Materials

2.1.1 Reagents

All chemicals were obtained from Sigma or Fisher unless otherwise stated. Antibiotics and X-Gal were purchased from Melford Laboratories. Plasmid preparation, DNA purification and RNeasy kits were purchased from Qiagen. TRIzol was purchased from Invitrogen and genomic DNA extraction kits (GenElute Mammalian Genomic DNA Miniprep) were bought from Sigma. M-PER Mammalian Protein Extraction reagent was bought from Pierce. DNA markers were purchased from Promega. Restriction enzymes, enzymes for reverse transcription (Superscript II enzyme) and synthetic oligonucleotides were obtained from Invitrogen. Enzymes for polymerase chain reaction (PCR) were purchased from Abgene, Qiagen (HotStarTaq DNA polymerase) or Roche (Expand High Fidelity PCR System). T4 ligase used for DNA ligations was bought from Promega. Positively charged nylon membranes used for Southern blot analysis were purchased from Amersham. $^{32}$P dCTP was obtained from MP Biomedicals. Radioactive labelling of DNA was performed using the RadPrime DNA Labeling System bought from Invitrogen. Cells were fixed and permeabilised prior to flow cytometry and microscopy using Leucoperm purchased from Serotec. Vybrant DyeCycle Violet Stain to stain DNA, Alexa fluor 350 phalloidin and Prolong Gold Antifade were purchased from Invitrogen. For sodium dodecyl sulphate (SDS) polyacrylamide protein gel electrophoresis 30% (w/v) acrylamide: 0.8% (w/v) bis acrylamide solution was bought from Flowgen. Protein markers (SeeBlue Plus2 pre-stained standard) were obtained from Invitrogen and nitrocellulose membranes (Protran) for immunoblots were purchased from Schleicher and Schuell Bioscience. Enhanced chemiluminescence (ECL) was performed using ECL Advanced Western blotting detection kit and the luminescent signal was detected on Hyperfilm ECL, both purchased from Amersham.
2.1.2 Antibodies

The α-tubulin (Tu-02) mouse monoclonal antibody and c-Myc (9E10) mouse monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. Phospho-Akt (Ser473) (193H12) rabbit monoclonal, phospho-p44/42 MAPK (Thr202/Tyr204) (197G2) rabbit monoclonal and Akt2 (5B5) rabbit monoclonal antibodies were all purchased from Cell Signalling Technology Inc. Anti c-Myc fluorescein isothiocyanate (FITC) antibody was bought from Serotec and rabbit GFP polyclonal antibody was purchased from Molecular Probes. Rabbit polyclonal antisera (C-48) raised against ms1 (immunising peptide was GRPKEGSKTAERAKRAEEHI) was produced at Pepceuticals Ltd. Biotinylated swine anti-rabbit antibody was obtained from DakoCytomation. Anti-rabbit and anti-mouse horseradish peroxidase (HRP) conjugates were purchased from Amersham.

2.1.3 Mammalian cell culture and reagents

The H9c2 cell line derived from the embryonic rat ventricle was obtained from the European Collection of Cell Cultures and NIH3T3 cells (murine embryonic fibroblasts) were purchased from the American Type Culture Collection.

Dulbecco’s Modified Eagle’s Medium, fetal bovine serum, penicillin and streptomycin were all purchased from Invitrogen. Trypsin-Ethylene diamine tetraacetic acid (EDTA) was bought from BioWhittaker and JetPEI cationic transfection reagent was purchased from Qbiogene.

2.1.4 Plasmids

pAlpha-MHCpromoter-ms1sense (mouse) was provided by H Mahadeva. pGEM-T Easy vector was purchased from Promega. The mammalian expression vector pcDNA3.1(+) was kindly given by Professor G Brooks. The transgenic vector PCCALL2-IRES-EGFP/anton (pi Z/EG) was kindly provided by C Lobe and the pCre-Pac vector was a generous gift from C Pritchard.

All vector maps are shown in appendix 1 (section 2.8) and shown in figure 2.1 is how these plasmids were experimentally used.
Figure 2.1. A diagram to show the plasmids involved to generate an expression vector to over-express msl \textit{in vitro} and a msl transgene for \textit{in vitro} and \textit{in vivo} msl over-expression.

2.2 DNA Manipulation

2.2.1 High Fidelity Polymerase Chain Reaction (PCR)

The full-length coding sequence of mouse msl was amplified using the Roche Expand High Fidelity PCR System (a mixture of Taq DNA polymerase plus another proof reading polymerase). 5 ng of a plasmid containing mouse msl termed pAlpha-MHCPromoter-mslsense (mouse) provided by H Mahadeva was PCR-amplified using forward

\[
\text{BgIII site}\quad \mid \quad \mid \quad \text{c-myc}\mid \quad \text{mouse msl}\mid (5'-\text{ccagatctatggagcagaagctcatcagcgaggaggacctggctccaggagaaagggaaagggag}-3')
\]

\[
\text{XhoI site}\quad \mid \quad \mid \quad \text{mouse msl}\mid \quad \text{mouse msl}\mid (5'-\text{cgccctcgagttactcaagzagataatcacaacatg}-3')\text{ primers.}
\]
Materials and Methods

Multiple reactions were performed in a master mix. A typical 50 μl reaction would contain the following:

- 5 ng Template DNA
- 2.5 μl 10 μM Forward Primer (final concentration of 0.5 μM)
- 2.5 μl 10 μM Reverse Primer (final concentration of 0.5 μM)
- 5 μl 10 × Expand High Fidelity PCR buffer with MgCl₂
- 5 μl 2 mM dNTP mix (final concentration of 0.2 mM)
- 3.5 U/μl Expand High Fidelity PCR System
- dH₂O to a final volume of 50 μl

Oil (2 drops) was then placed on the top of each reaction and the tubes were placed in a Techne Techgene thermal cycler. The thermocycling parameters were 1 cycle of 94°C, 2 minutes; 30 cycles of 94°C, 45 seconds; 59°C, 45 seconds; 72°C, 1 minute 10 seconds; a final extension step was performed at 72°C for 5 minutes. The PCR product was then ran alongside a 1 kb marker on a 1% (w/v) agarose gel as described in section 2.2.2. When the correct sized fragment for ms1 (approximately 1.3 kb) was obtained by electrophoresis the band was cut from the agarose gel and extracted and purified using the QIAquick Gel Extraction Kit (Qiagen) as stated by the manufacturer’s protocol.

2.2.2 Agarose gel electrophoresis

The appropriate volume of 6 × loading buffer (40% (v/v) glycerol, 60% (v/v) TE buffer (Tris Ethylene diamine tetraacetic acid (EDTA), 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and very small amount of bromophenol blue) was added to the sample. Samples were centrifuged for 1 minute at 13,000 rpm, loaded onto the appropriate percentage agarose gel in 1 × Tris-Acetate EDTA (TAE) buffer (40 mM Tris-HCl, 20 mM Glacial Acetic Acid, 0.1 mM EDTA, pH 8.0) and run alongside an appropriate sized marker (Promega). Ethidium bromide was present in the gel at a concentration of 0.035 μg/ml. The gel was electrophoresed in 1 × TAE buffer at a constant 80 V for 1 hour. The bands were visualised on ultraviolet light.

2.2.3 Extraction and purification of DNA from an agarose gel

When the correct sized fragment was obtained by electrophoresis the band was cut from the agarose gel and extracted and purified using the QIAquick Gel Extraction Kit
Materials and Methods

(Qiagen). The method was followed as stated in the QIAquick Spin Handbook (Qiagen). The DNA was stored at -20°C until required.

2.2.4 Quantification of DNA

The concentration of DNA was determined by diluting the DNA 1:50 with dH2O. The absorbance was read in a spectrophotometer at wavelengths of 260 nm and 280 nm. An $A_{260}$ of 1 was equivalent to 50 μg DNA/ml. The concentration of DNA in ng/μl was calculated as follows:

$$50 \mu g/ml \times A_{260} \times \text{dilution factor}$$

2.2.5 Ligation of DNA from PCR product or Plasmid into Vector

This method was based on the pGEM-T and pGEM-T Easy Vector Systems for cloning of PCR products (Promega). 100 ng of vector was placed into the ligation reaction with an appropriate amount of DNA (insert) calculated so there was $3 \times$ insert : $1 \times$ vector ratio as follows:

$$(\text{ng of vector} \times \text{kb size of insert}) \div \text{kb size of vector} \times \text{insert:vector ratio} = \text{ng of insert.}$$

The total reaction volume was 10 μl and to this 3 U of T4 ligase, T4 DNA ligation buffer (10 ×) and H2O (to make final volume) was added. A control was also performed where the insert was omitted; this indicated the presence of re-circularised vector plasmid. The reactions were incubated overnight at 4°C.

2.2.6 Transformation of bacteria with DNA from PCR product or Plasmid

Half the ligation reaction was added to 100 μl DH5α chemically competent cells (Invitrogen), mixed gently by tapping and incubated on ice for 30 minutes. The reaction was then heat shocked for exactly 45 seconds at 37°C using a water bath. Next the reaction was placed on ice for 2 minutes and then 400 μl pre-warmed SOC was added. The reaction was then incubated at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. Following the incubation, half the reaction was spread onto Luria Broth (LB) agar plates containing ampicillin (100 μg/ml) for selection of the transformed cells. Where possible, 40 μl X-Gal (20 mg/ml) was added to the LB agar plates containing the appropriate antibiotic to enable blue/white selection of transformants. The plates were inverted and incubated at 37°C overnight.
Materials and Methods

Single putative transformant colonies were isolated and grown in approximately 10 ml of LB (containing the appropriate antibiotic) at 37°C overnight at 225 rpm in a shaking incubator. 6 ml of these cultures were used to extract plasmid DNA using QIAprep Spin Miniprep kit (Qiagen) when DNA was needed to digest with appropriate restriction enzymes to allow identification of the plasmids containing the insert. The method for extracting DNA for this purpose was followed as stated in the QIAprep Spin Miniprep kit protocol. If a positive clone was obtained the remaining 4 ml culture was used to make a 20% (v/v) glycerol stock for long-term storage at -80°C and the rest was used to set up more culture (approximately 10 ml) to extract plasmid DNA using Qiagen-tip 20 Plasmid Mini Kit when DNA was to be cloned, sequenced or transfected. The method for extracting plasmid DNA for these purposes was followed as stated in the Qiagen-tip 20 Plasmid Mini Kit protocol. When DNA was used to be injected into mouse oocytes the remaining 4 ml culture was used to make a 20% (v/v) glycerol stock for long-term storage at -80°C and the rest was used to set up more culture (approximately 250 ml) to extract plasmid DNA using the Qiagen Endofree Maxi Kit by following the manufacturer’s instructions.

2.2.7 Restriction digest of DNA

To allow identification of the plasmid containing the PCR fragment (insert), the required amount of DNA was added with the restriction enzyme (or enzymes) (Invitrogen) of choice in a reaction volume containing the appropriate amount of reaction buffer (Invitrogen) at the optimum temperature for the enzyme(s) to work. This reaction was incubated overnight and the total reaction volume was then run out on an appropriate percentage agarose gel as described in section 2.2.2 to check that the enzyme had cut the DNA. If a restriction digest was performed to isolate a band of DNA from, for example a plasmid, then the digest reaction was incubated for a maximum of 2 hours, run out on an appropriate percentage agarose gel as described in section 2.2.2 and the correct sized band was cut from the agarose gel and extracted and purified as already described in section 2.2.3. All cloned PCR fragments were sequenced using the automated sequencing facility at the University of Leicester PNAACL.
2.3 RNA Manipulation

2.3.1 RNA extraction from tissue and cells

RNA was extracted from tissue using the RNeasy Maxi kit (Qiagen) by following the manufacturer’s protocol.

Cell culture media was removed and total RNA was extracted using TRIzol (Invitrogen) by following the manufacturer’s protocol.

Quantification of RNA was performed as described in section 2.2.4 except that an A_{260} of 1 was equivalent to 40 μg RNA/ml. The concentration of RNA in ng/μl was calculated as follows:

\[ 40 \, \text{μg/ml} \times A_{260} \times \text{dilution factor} \]

2.3.2 Deoxyribonuclease I (DNase I) treatment of RNA

1 μg total RNA was DNase I (Sigma) treated following the manufacturer’s protocol and then cDNA synthesis by Reverse Transcription (RT) was performed as stated below.

2.3.3 cDNA synthesis by Reverse Transcription (RT)

1 μl oligo dT (0.5 μg) and 1 μl of a 10 mM dNTP mix (final concentration of 0.5 mM each) was added to the DNase I treated RNA and placed in a Perkin Elmer Cetus DNA thermal cycler at 70°C for 10 minutes. The reaction was then placed on ice for 1 minute and briefly centrifuged (pulse spin) to remove condensation. To the reaction, 4 μl of 5 x First strand buffer (Invitrogen), 2 μl of 0.1 M DTT (Invitrogen, final concentration of 10 mM) and 1 μl Superscript II enzyme (Invitrogen, 200 U) was added giving a total reaction volume of 20 μl. Controls (minus RT) were also included that contained 1 μl dH₂O instead of the 1 μl Superscript II enzyme (200 U). The reaction was then placed back in the thermal cycler for 42°C, 55 minutes; 70°C, 10 minutes; and then back on ice.
2.3.4 Semi-quantitative RT-PCR

The cDNA synthesised by RT was diluted 1:5 with dH₂O prior to semi-quantitative RT-PCR. Multiple reactions were performed in a master mix. A typical 20 μl reaction would contain the following:

- 1 μl Template cDNA
- 1 μl 10 μM Forward Primer (final concentration of 0.5 μM)
- 1 μl 10 μM Reverse Primer (final concentration of 0.5 μM)
- 2 μl Abgene 10x buffer (final concentration in the reaction was 1×)
- 1.2 μl Abgene 25 mM MgCl₂ (final concentration of 1.5 mM)
- 2 μl 2 mM dNTP mix (final concentration of 0.2 mM)
- 0.3 μl Abgene Taq polymerase (final concentration of 1.25 U)
- dH₂O to a final volume of 20 μl

A no template control was also included which contained 1 μl dH₂O instead of 1 μl template DNA. Ribosomal Protein L32 (RPL32) was used as a loading control. Oil (2 drops) was then placed on the top of each reaction and the tubes were placed in a Techne Techgene thermal cycler. The genes, ms1, GATA 4, ARC, myocardin, SRF, BNP, calcineurin, MEF2C, cardiac α-actin, and the loading control RPL32 were PCR-amplified using the forward and reverse primers shown in table 2.1. The thermocycling parameters were 1 cycle of 94°C, 2 minutes; see table 2.1 for the number of cycles of 94°C, 45 seconds; 59°C, 45 seconds; 72°C, 45 seconds; a final extension step was performed at 72°C for 5 minutes.
### Materials and Methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>Cycle Number</th>
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</thead>
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<tr>
<td>msl Forward Primer</td>
<td>GTGACAGCATAGACACAGAGGACGTGCTGCCAACCTGCTT</td>
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</tr>
<tr>
<td>msl Reverse Primer</td>
<td>CACTGCTGCCAACCTGCTT</td>
<td></td>
</tr>
<tr>
<td>GATA-4 Forward Primer</td>
<td>CTGGGCCCTCTCATGATGAAAGCCTGGAATGCTGCACTGCTT</td>
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<tr>
<td>GATA-4 Reverse Primer</td>
<td>CTGAATGCTGTGGATGACCATGGA</td>
<td></td>
</tr>
<tr>
<td>ARC Forward Primer</td>
<td>CACTGGGCCCTGAGATGAAAGCCTGGAATGCTGCACTGCTT</td>
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</tr>
<tr>
<td>ARC Reverse Primer</td>
<td>CTGAATGCTGTGGATGACCATGGA</td>
<td></td>
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<tr>
<td>Myocardin Forward Primer</td>
<td>CCATCGCCTAACAACCATTACT</td>
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</tr>
<tr>
<td>Myocardin Reverse Primer</td>
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<td></td>
</tr>
<tr>
<td>SRF Forward Primer</td>
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<tr>
<td>SRF Reverse Primer</td>
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<td>BNP Reverse Primer</td>
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<td>Calcineurin Forward Primer</td>
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<tr>
<td>RPL32 Reverse Primer</td>
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</table>

Table 2.1. Primer Sequences and optimised cycle number for each gene.

Samples were electrophoresed as described earlier in section 2.2.2.

Gel images were documented directly onto a PC and relative mRNA expression was analysed by band quantification (using gene tools from Syngene software) and corrected for RPL32.

2.3.4.1 Statistical analysis

Statistical analysis was carried out using a Student’s t test. A P value < 0.05 was considered to be significant.

2.3.5 Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using either TaqMan® Gene Expression Assays (Applied Biosystems) and analysed using the comparative (ΔΔCt) method or using SYBR® Green with custom designed primers and analysed using the Pfaffl method.
2.3.5.1 Real-time quantitative RT-PCR using TaqMan® Gene Expression Assays and analysed using the comparative (ΔΔC_T) method

The cDNA synthesised by RT (as described in section 2.3.3) was diluted 1:5 with dH_2O prior to real-time RT-PCR. Multiple reactions were performed in a master mix. A typical 20 μl reaction would contain the following:

- 1-2 μl Template cDNA
- 1 μl 20 × Pre-formulated assay mix (Applied Biosystems) containing:
  - 2 unlabeled PCR primers (900 nM final concentration)
  - 1 FAM™ dye-labeled TaqMan® MGB probe (250 nM final concentration). Final concentration in the reaction was 1 ×
- 10 μl 2 x TaqMan® Universal PCR Master Mix, No AmpErase® UNG
  (Applied Biosystems, final concentration in the reaction was 1 ×)
- dH_2O to a final volume of 20 μl

Tata-binding protein (TBP) was used as a reference gene to normalise mRNA levels between different samples.

The reaction was carried out using the ABI Prism® 7900 HT sequence detection system. The thermal cycling conditions were set at 50°C for 2 minutes (hold step), 95°C for 10 minutes (hold step) and then the reaction ran for 40 cycles at 95°C for 15 seconds (denature) and 60°C for 1 minute (anneal/extend).

The data was analysed using the comparative (ΔΔC_T) method (Livak and Schmittgen, 2001) using the formula:

F{}^{\text{old induction}} = 2^{\Delta \Delta C_T} \\
\Delta C_T = \text{mean } C_T^{(\text{gene of interest})} - \text{mean } C_T^{(\text{reference gene})} \\
\Delta \Delta C_T = \Delta C_T^{(\text{unknown})} - \Delta C_T^{(\text{calibrator})}

The calibrator sample (e.g. nontreated control sample) allows corrections to be made for inter-assay variation and represents 1 × expression of the gene of interest.

The formula is based on the assumption that the efficiency of the PCR for both the gene of interest and reference gene is identical with a doubling of product every cycle.
2.3.5.2 Real-time quantitative RT-PCR using SYBR® Green and analysed using the Pfaffl method

Relative mRNA quantification in real-time RT-PCR was analysed using the Pfaffl method (Pfaffl, 2001). The cDNA synthesised by RT (as described in section 2.3.3) was diluted 1:5 with dH2O prior to real-time RT-PCR. Multiple reactions were performed in a master mix. A typical 25 μl reaction would contain the following:

- 0.01-3 μl Template cDNA
- 0.5 μl 10 μM Forward Primer (final concentration of 0.2 μM)
- 0.5 μl 10 μM Reverse Primer (final concentration of 0.2 μM)
- 12.5 μl 2 × Power SYBR® Green PCR Master Mix, (Applied Biosystems, final concentration in the reaction was 1 ×)
- dH2O to a final volume of 25 μl

RPL32 was used as a reference gene to normalise mRNA levels between different samples.

The reaction was carried out using the ABI Prism® 7900 HT sequence detection system. The thermal cycling conditions were set at 95°C for 10 minutes (hold step) and then the reaction ran for 40 cycles at 95°C for 45 seconds, 59/62°C for 45 seconds, 72°C for 45 seconds and then a final step at 95°C for 15 seconds and 60°C for 1 minute. The primer sequences for the reference gene and genes of interest are given in table 2.2.
Table 2.2. Primer Sequences and annealing temperature for each gene.

A series of cDNA dilutions were first generated to obtain a standard curve for the reference gene and gene of interest by plotting log of input cDNA verse $C_T$. From this the efficiency ($E$) was calculated according to the equation:

$$ E = 10^{[-1/slope]} $$

The efficiency of the reference gene and gene of interest do not have to be equal, but are incorporated into the analysis, resulting in increased accuracy. Thus the relative expression ratio (fold change) is calculated as follows:

1. mean $C_T$ values are determined for calibrator (e.g. nontreated control sample) and samples for the reference gene and gene of interest

2. gene of interest $\Delta C_p = \text{mean gene of interest}.C_{T}\text{ (calibrator)} - \text{mean gene of interest}.C_{T}\text{ (sample)}$
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3. reference gene $\Delta C_p = \text{mean reference gene.}C_T \text{ (calibrator)} - \text{mean reference gene.}C_T \text{ (sample)}$

4. The values for efficiency of each gene is then raised to the power of the respective $\Delta C_p$, and the ratio of gene of interest:reference gene is calculated using the formula:

$$\text{Ratio (fold change)} = \frac{\text{Efficiency (gene of interest)}^{\Delta C_p \text{ (gene of interest)}}}{\text{Efficiency (reference gene)}^{\Delta C_p \text{ (reference gene)}}}$$

The formula makes the assumption that while the efficiencies of the reference gene and gene of interest may differ, the efficiency of amplification for each sample within a PCR reaction is the same.

2.3.5.3 Statistical analysis

Statistical analysis was carried out using a Student's $t$ test. A $P$ value $< 0.05$ was considered to be significant.

2.4 Protein Manipulation

2.4.1 Extraction of proteins from cultured mammalian cells

M-PER Mammalian Protein Extraction reagent (Pierce) was used to extract proteins from H9c2 cells following transfection as described following the manufacturers instructions. 1 $\times$ Complete mini protease inhibitor cocktail (Roche) was added to the reagent before proceeding with the cell lysis to inhibit proteases to ensure that the protein of interest remains intact.

2.4.2 Quantification of Protein

Protein was quantified using the Bio-Rad Protein Assay (Bio-Rad) as instructed by the manufacturer. The protein standard used was bovine serum albumin (BSA) at concentrations of 0.2 $\mu$g/$\mu$l, 0.4 $\mu$g/$\mu$l, 0.6 $\mu$g/$\mu$l, 0.8 $\mu$g/$\mu$l and 1.0 $\mu$g/$\mu$l. The total volume used for each standard and sample was 20 $\mu$l. The amount of diluted dye reagent added to each was 1 ml.

2.4.3 Western blotting

This method was based on a previous method described (Bollag et al., 1996; Sambrook et al., 1989). To 15 $\mu$g protein, a half volume of 3 $\times$ loading buffer (188 mM Tris-HCl
(pH 6.8), 6% (v/v) Sodium dodecyl sulfate (SDS), 30% (v/v) Glycerol, 15% (v/v) β-mercaptoethanol, 0.03% (w/v) bromophenol blue) was added. Samples were then boiled (100°C) for 5 minutes prior to loading onto an appropriate percentage SDS-Polyacrylamide gel. Samples were run alongside a SeeBlue Plus2 pre-stained standard (Invitrogen) in gel running buffer (25 mM Tris, 250 mM Glycine, 0.1% (v/v) SDS) at a constant current of 10 mA overnight.

When the SDS-polyacrylamide gel had run to completion the proteins were transferred from the gel to a Protran nitrocellulose transfer membrane (Schleicher and Schuell BioScience) at a constant current of 400 mA for 3 hours using a wet module system (Bio-rad trans-blot cell) containing transfer buffer (48 mM Tris, 39 mM Glycine, 0.0375% (v/v) SDS, 20% Methanol) that was surrounded by ice. Membranes were stored dry until ready to process further.

Ponceau S solution (enough to just cover the membrane, Sigma) was used to stain the membrane to check there was protein on the membrane. This solution was washed off prior to blocking the membrane with three 5 minute washes in TBS-T pH 7.6 (20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween-20). The membrane was then blocked for 1 hour at room temperature with shaking in TBS-MT (TBS-T, 5% (w/v) skimmed milk powder (Somerfield)). Next the membrane was incubated with the primary antibody diluted in TBS-MT at 4°C overnight with shaking. After this three 5 minute washes in TBS-T were carried out at room temperature with shaking prior to incubation with the secondary antibody (carrying a horseradish peroxidase (HRP) conjugate, Amersham) for 1 hour at 4°C with shaking. A further three 5 minute washes in TBS-T at room temperature with shaking were carried out and then enhanced chemiluminescence (ECL) was performed using an ECL Advanced Western blotting detection kit (Amersham) as stated by the manufacturer’s instructions. The luminescent signal created was detected on Hyperfilm ECL (Amersham).

Developed films were scanned into a PC and relative protein expression was analysed by band quantification (using gene tools from Syngene software) and corrected for α-tubulin.

The primary antibodies used were the loading control α-tubulin (Tu-02) mouse monoclonal (1:1000, Santa Cruz Biotechnology Inc.), ms1 (rabbit C-48 polyclonal, 1:250, Pepceuticals Ltd), c-Myc (9E10) mouse monoclonal (1:200, Santa Cruz
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Biotechnology Inc.), phospho-Akt (Ser473) (193H12) rabbit monoclonal (1:1000, Cell Signalling Technology Inc.), phospho-p44/42 MAPK (Thr202/Tyr204) (197G2) rabbit monoclonal (1:1000, Cell Signalling Technology Inc.) and Akt2 (5B5) rabbit monoclonal (1:1000, Cell Signalling Technology Inc.).

2.4.3.1 Statistical analysis

Statistical analysis was carried out using a Student’s t test. A P value < 0.05 was considered to be significant.

2.4.4 Immunofluorescence

Approximately 40,000 H9c2 cells/well of a 6 well plate were plated out onto ethanol treated coverslips. Cells were left for 48 hours to attach onto the coverslips and then transfected as described in section 2.5.2. Cells were left for 24 or 72 hours and then fixed and permeabilised using a Leucoperm kit (Serotec) by following the manufacturer’s protocol. During permeabilisation mouse anti c-Myc fluorescein isothiocyanate (FITC) (Serotec) was added at a 1:10 dilution in phosphate buffered saline (PBS) and left for 30 minutes at room temperature in the dark. After this three 5 minute washes in PBS at room temperature were carried out and then Alexa fluor 350 phalloidin (Invitrogen) was added at a 1:40 dilution in 1% BSA (in PBS) and left for 20 minutes at room temperature in the dark. Following this three 5 minute washes in PBS at room temperature were carried out. Coverslips were mounted onto slides using Prolong Gold Antifade (Invitrogen). Images were captured using a Nikon Eclipse TE2000-E microscope and processed using Volocity 4 software (Improvision).

2.4.5 LacZ expression in mammalian cells by histochemical staining

NIH3T3 cells were left untransfected or transfected as described in section 2.5.2 with 1 μg of the empty vector (pi Z/EG) or the ms1 transgene (pi Z/EG-ms1). Staining of transiently transfected cells began following 48 hours of transfection using the BetaBlue Staining Kit (Novagen) by following the manufacturer’s instructions. Images were viewed using an Olympus CKX41 microscope and photographed using a Nikon MXA 5400 digital camera.

2.4.6 GFP Visualisation in mammalian cells

NIH3T3 cells were left untransfected, transfected as described in section 2.5.2 with 1 μg of the empty vector (pi Z/EG) or the ms1 transgene (pi Z/EG-ms1) or the pCre-Pac...
vector (Taniguchi et al., 1998) (appendix 1) or co-transfected with 1 μg of the empty vector (pi Z/EG) and 1 μg of the pCre-Pac vector or with 1 μg of the msl transgene (pi Z/EG-msl) and 1 μg of the pCre-Pac vector. GFP fluorescence was observed following 48 hours of transfection via a FITC filter, using a Zeiss Axiovert 135 microscope. Images were captured using a C14 digital camera.

2.5 **Cell Culture**

2.5.1 **Cell Maintenance**

The H9c2 cell line derived from the embryonic rat ventricle was obtained from the European Collection of Cell Cultures and NIH3T3 cells (murine embryonic fibroblasts) were purchased from the American Type Culture Collection and cultured in Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen). Cells were grown and passaged regularly in a tissue culture 80 cm² filter cap flask (Nunc) in an atmosphere of 5% CO₂/95% humidified air at 37°C.

2.5.2 **Transfection**

Approximately 40,000 H9c2 cells/well or 20,000 NIH3T3 cells/well of a 6 well plate were plated out so that cells were about 50% confluent 48 hours later for transfection. 1 μg DNA of pcDNA3.1(+) containing c-Myc tagged msl (pcDNA3.1(+) + msl) or the empty expression vector control (pcDNA3.1(+)) was transfected using the JetPEI Cationic Transfection Reagent (Qbiogene) as stated by the manufacturer’s protocol.

2.6 **Flow cytometric analysis**

2.6.1 **Cell size**

H9c2 cells were left untransfected or transfected as stated in section 2.5.2 except that approximately 100,000 cells/25 cm² filter cap flask were plated to obtain cells that were about 50% confluent 48 hours later for transfection using 3 μg DNA. Cells were left for 24 or 72 hours following transfection and then cells were washed once with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA (BioWhittaker)), incubated with trypsin-EDTA for a few minutes and subjected to gentle agitation to detach cells from the flask. 2.5 ml Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) was then added and the cells were collected into a 20 ml sterilin. Cells
were then centrifuged at 1200 rpm for 5 minutes at room temperature. Following centrifugation the supernatant was removed, cells were washed with PBS and then centrifuged at 1200 rpm for 5 minutes at room temperature. The supernatant was removed and cells were re-suspended in 1 ml PBSA (PBS containing 0.03% (w/v) Sodium Azide and 0.2% (w/v) BSA). Viable cells were counted using 0.4% Trypan blue solution (Sigma) and a haemocytometer chamber where 10 μl of cells were added to 10 μl of Tryphan blue, this was mixed and 10 μl of this cell suspension was added to the haemocytometer. Cells that were live appeared translucent and dead cells appeared blue. Cells were counted in the four 0.1 mm³ corner squares of the haemocytometer, averaged, multiplied by 2 and then multiplied by 10⁴ to give the number of cells per ml. Cells were adjusted to have at least 1 × 10⁵ cells in 100 μl PBSA.

Next cells were fixed and permeabilised using a Leucoperm kit (Serotec) by following the manufacturer's protocol. During permeabilisation mouse anti c-Myc fluorescein isothiocyanate (FITC) antibody (Serotec) was added at a 1:10 dilution in PBS and left for 30 minutes at room temperature in the dark.

Cells were analysed using a Beckman Coulter Epics XL-MCL flow cytometer and System II software. Cells were passed through a laser beam where the emission wavelength is 488 nm and analysed for size (forward scatter) and granularity (side scatter). The c-Myc FITC fluorescence of individual cells was measured and the data were registered on a logarithmic scale. This gave two cell populations comprising of cells that were and were not green. The total number of cells, the number of cells that were not green and the number of cells that were green were counted and presented as percentages. From these two populations cell size (forward scatter) of individual cells was measured and the data were registered on a linear scale and presented as the median thus allowing the median cell size of untransfected cells (not green) versus msi transfected cells (green) to be compared.

2.6.1.1 Statistical analysis

Statistical analysis was carried out using a Student's t test. A P value < 0.05 was considered to be significant.

2.6.2 Detection of DNA fragmentation (apoptosis)

The assay was based on a method previously described using propidium iodide (Nicoletti et al., 1991). Cells were transfected as described in section 2.6.1 and
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following transfection for 24 hours, cells were treated with 5 nM staurosporine (Sigma) for 24 hours and then collected, fixed and permeabilised as described in section 2.6.1 except that Hanks' balanced salt solution (HBSS (Invitrogen)) was used instead of PBS. Following permeabilisation and addition of the mouse anti c-Myc FITC antibody, cells were washed in HBSS and centrifuged at 1200 rpm for 5 minutes at room temperature. The supernatant was then removed and cells were re-suspended in 900 µl HBSS. To a 900 µl cell sample 100 µl RNase (1 mg/ml, Sigma) and 1 µl Vybrant DyeCycle Violet Stain (Invitrogen) was added. The sample was gently mixed and incubated in the dark at 37°C for 30 minutes.

Cells were measured using a DakoCytomation CyAn ADP flow cytometer and analysed using Summit v4.3 software. Cells were analysed as described in section 2.6.1 except that the violet fluorescence of individual cells was also measured in addition to the c-Myc FITC fluorescence of individual cells. The violet fluorescence was collected due to violet staining of DNA content and the data were registered on a linear scale. Cell cycle analysis based on measurements on DNA content generates a clear distribution where normal populations of growing cells have either one set of paired chromosomes per cell (G_0/G_1 phase of cell cycle), an intermediate amount of DNA, DNA synthesis (S phase), or two sets of paired chromosomes per cell, prior to cell division (G_2/M phase). During apoptosis the DNA of the cell fragments and following fixation and permeabilisation, the low molecular weight DNA inside the cytoplasm of apoptotic cells elutes during the wash procedure. Therefore, following staining with the violet dye, cells with lower DNA content contain less DNA stained and thus cells with lower DNA staining than that of G_1 cells show a sub-G_1 peak and are considered apoptotic (Nicoletti et al., 1991; Telford et al., 1991; Telford et al., 1992). The percentage of apoptotic cells was determined on the basis of the number in the sub-G_1 peak in the DNA violet fluorescence histogram thus allowing the percentage of apoptotic untransfected cells (not green) versus the percentage of apoptotic msl transfected cells (green) to be compared.

2.6.2.1 Statistical analysis

Statistical analysis was carried out using a Student's t test. A P value < 0.05 was considered to be significant.
2.7 Genetic Manipulation

2.7.1 Pronuclear microinjection

Prior to pronuclear microinjection the msl transgene was linearised at the SfiI and Scal sites by restriction digest to remove the plasmid back bone. The linearised plasmid DNA (msl transgene) was electroeluted, precipitated and purified through 2 clean up columns, QIAquick Nucleotide Removal Column (Qiagen) and Bio-Spin P-30 Column (Bio-Rad). The DNA was finally eluted in Injection Buffer (5 mM Tris-HCl pH 7.4, 0.1 mM EDTA) made with embryo grade solutions. These procedures were performed by S Munson at the Embryonic Stem Cell Facility, University of Leicester.

The msl transgene was then injected into the pronucleus of day 1 post-fertilisation oocytes by a continuous flow method of injection using the Eppendorff Femtojet system until pronuclear swelling was observed. Surviving oocytes were reimplanted into the oviducts of pseudopregnant females. This was performed by J Brown at the University of Leicester transgenic unit.

2.7.2 Animal husbandry

Mice were housed in internationally ventilated cages (Allentown). Bedding was aspen and sizzle nest and environmental enrichment products were mouse corner houses and fun tunnels. Water and diets were given ad lib. Diets were supplied by Special Diets Services. Mice were routinely maintained on Rat and Mouse No. 1 Maintenance (RM1) which contains 0.25% sodium, corresponding to approximately 0.7% sodium chloride. Lactating females were maintained on a higher protein diet (RM3).

2.7.3 Generation and identification of msl transgenic mice by PCR and Southern blotting

2.7.3.1 PCR analysis

For PCR analysis, at 4-6 weeks (following weaning) genomic DNA was extracted from an ear punch of the mouse (or yolk sac when staining embryos for lacZ) using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma) as stated by the manufacturer’s protocol. The mice were screened to assess inheritance of the msl transgene by PCR using lacZ and GFP vector specific primers. Interleukin 2 (IL-2) was used as an internal control.
The PCR reaction using the lacZ primers would contain the following:

- 2 μl Template DNA
- 2 μl 10 μM Forward Primer (final concentration of 1 μM)
- 2 μl 10 μM Reverse Primer (final concentration of 1 μM)
- 2 μl Qiagen 10 x buffer including 15mM MgCl₂ (final concentration in the reaction was 1 x buffer including 1.5mM MgCl₂)
- 2 μl 2 mM dNTP mix (final concentration of 0.2 mM)
- 0.1 μl 5 U/μl Qiagen HotStarTaq DNA Polymerase (final concentration of 0.5 U)

dH₂O to a final volume of 20 μl

Oil (2 drops) was then placed on the top of each reaction and the tubes were placed in a Techne Techgene thermal cycler. The thermocycling parameters were 1 cycle of 96°C, 15 minutes; 35 cycles of 96°C, 30 seconds; 59°C, 1 minute; 72°C, 1 minute; a final extension step was performed at 72°C for 5 minutes.

The PCR reaction using the GFP primers would contain the following:

- 2 μl Template DNA
- 1 μl 10 μM Forward Primer (final concentration of 0.5 μM)
- 1 μl 10 μM Reverse Primer (final concentration of 0.5 μM)
- 2 μl Qiagen 10 x buffer including 15mM MgCl₂ (final concentration in the reaction was 1 x buffer including 1.5mM MgCl₂)
- 2 μl 2 mM dNTP mix (final concentration of 0.2 mM)
- 0.2 μl 5 U/μl Qiagen HotStarTaq DNA Polymerase (final concentration of 1 U)
- 1 μl Dimethyl sulfoxide (DMSO) (final concentration of 5%)

dH₂O to a final volume of 20 μl

Oil (2 drops) was then placed on the top of each reaction and the tubes were placed in a Techne Techgene thermal cycler. The thermocycling parameters were 1 cycle of 96°C, 15 minutes; 35 cycles of 96°C, 45 seconds; 57°C, 1 minute; 72°C, 1 minute; a final extension step was performed at 72°C for 5 minutes.
The PCR reaction using the IL-2 primers contained the following:

- 0.5 µl Template cDNA
- 1 µl 10 µM Forward Primer (final concentration of 0.5 µM)
- 1 µl 10 µM Reverse Primer (final concentration of 0.5 µM)
- 2 µl Abgene 10 x buffer (final concentration in the reaction was 1 x)
- 1.2 µl Abgene 25 mM MgCl₂ (final concentration of 1.5 mM)
- 2 µl 2 mM dNTP mix (final concentration of 0.2 mM)
- 0.3 µl Abgene Taq polymerase (final concentration of 1.25 U)
- dH₂O to a final volume of 20 µl

Oil (2 drops) was then placed on the top of each reaction and the tubes were placed in a Techne Techgene thermal cycler. The thermocycling parameters were 1 cycle of 94°C, 1.5 minutes; 35 cycles of 94°C, 30 seconds; 60°C, 1 minute; 72°C, 1 minute; a final extension step was performed at 72°C for 2 minutes.

A no template control was included with all PCR reactions which contained 1 µl dH₂O instead of 1 µl template DNA. DNA from a mouse known to inherit the transgene was included as a positive control and DNA from a mouse known not to carry the transgene was used as a negative control.

All samples were electrophoresed as described earlier in section 2.2.2.

The primer sequences used are listed below in table 2.3.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacZ Forward Primer</td>
<td>ACTCTGGCTCACAGTACGCGT</td>
</tr>
<tr>
<td>LacZ Reverse Primer</td>
<td>CAGCGTTCGACCCAGGCCT</td>
</tr>
<tr>
<td>GFP Forward Primer</td>
<td>CCTACGGCAAGCTGACCCTGA</td>
</tr>
<tr>
<td>GFP Reverse Primer</td>
<td>ACGCCTGGCCGTCCTGATG</td>
</tr>
<tr>
<td>IL-2 Forward Primer</td>
<td>CTAGGCCACAGAATTGAAAGATCT</td>
</tr>
<tr>
<td>IL-2 Reverse Primer</td>
<td>GTAGGTGGAAATTCTAGCATCATCC</td>
</tr>
</tbody>
</table>

**Table 2.3. Primer Sequences.**

2.7.3.2 Southern blot analysis

For Southern blot analysis (Southern, 1975), 10 µg genomic DNA was extracted from mouse liver tissue using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma)
as stated by the manufacturer's protocol. Genomic DNA (10 μg) was digested with SstI or EcoRV and separated on a 0.6% agarose gel in 1 × TAE (Invitrogen) at 100 V for 30 minutes, 30 V overnight. After electrophoresis, agarose gels were depurinated for 10 minutes at room temperature with gentle agitation in 0.25 M HCl, denatured for 30 minutes at room temperature with gentle agitation in denaturisation solution (0.5 M NaOH, 1.5 M NaCl) and neutralised for 30 minutes at room temperature with gentle agitation in neutralisation solution (0.5 M Tris-HCl, 3 M NaCl, pH 7.5). DNA was then transferred from the agarose gel onto Hybond N membrane (Amersham) in 10 × Sodium saturated citrate (SSC), 1.5 M NaCl, 0.15 M Sodium citrate, pH 7 for approximately 24 hours. Membranes were then cross linked for 1 minute in a UV cross linker (120,000 microjoules/cm²) and rinsed briefly in 2 × SSC.

The 32P-labelled probe was a 655 bp NotI-BamHI fragment generated by restriction digest (section 2.2.7) that includes the rabbit β-globin polyadenylation sequence of the pi Z/EG vector. 20 ng of probe DNA was labelled with approximately 50 μCi [α32P]dCTP using the RadPrime DNA Labeling System (Invitrogen) following the manufacturer's instructions. Unincorporated nucleotides were removed using the Qiagen Nucleotide removal kit as stated by the manufacturer's protocol. Hybridisation was carried out in a Hybaid oven using Hybaid bottles and wire meshes. Membranes were placed onto the wire meshes (DNA facing upwards) and rolled up and placed into the Hybaid bottles. Membranes were pre-hybridised for 1 hour at 42°C in hybridisation buffer (0.5% (w/v) milk powder, 60 mg/ml Polyethylene glycol (PEG), 1 × SSPE (0.15 M NaCl, 0.2 mM Na2PO4, 1 mM EDTA, pH 7.4), 1% SDS) with gentle rotation and then the labelled probe was added to fresh hybridisation buffer, added to the membrane and left rotating overnight at 42°C.

After hybridisation the membranes were washed in 5 x SSC at 42°C for 10 minutes. The Geiger counter was used to assess if further washes were required to remove excess radioactivity. If further washes were require to remove excess radioactivity the membranes were washed in 3 x SSC at 42°C for 10 minutes.

The membrane was then left exposed to film (Kodak) overnight at -80°C and then developed.
2.7.4 

*ml* breeding colony

Positive founder mice that were identified to have inherited the *ml* transgene (*ml* transgenic) by PCR and Southern blotting were then bred with C57BL6 mice to establish a breeding colony. Lines that showed successful germ-line transmission of the *ml* transgene were chosen to cross further to create more breeding lines so enough mice were available for subsequent experiments. Positive offspring were identified using PCR analysis as discussed above.

2.7.5 

LacZ expression in embryos/tissues by histochemical staining and semi-quantitative RT-PCR

Embryos at E10.5 were rinsed in PBS and then fixed for 8 minutes in lacZ fix (2% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in PBS). Following fix, embryos were washed three times for 5 minutes in lacZ wash buffer (0.1% (v/v) sodium deoxycholate, 0.02% (v/v) nonidet-P40, 2 mM MgCl2 in 100 mM sodium phosphate, pH 7.3). Staining was carried out in 1 mg/ml X-gal, 6 mM potassium ferrocyanide, 5 mM potassium ferricyanide in lacZ wash buffer at 37°C overnight with protection from light. After completion of staining, embryos were washed twice for 5 minutes in PBS, fixed in lacZ fix for 15 minutes and stored in 70% ethanol at 4°C.

*ml* transgenic mice (F2) at approximately 8 weeks of age from each line were visualised for lacZ expression in tissue slices of the liver, kidney and heart compared to a littermate wild type control by using the BetaBlue Staining Kit (Novagen). For those embryos and tissues expressing high levels of lacZ, a cytoplasmic blue colour appeared which determined expression of the *ml* transgene. Embryos and tissues were viewed using a LEICA M216F dissection microscope and photographed using a Nikon DXM1200 digital camera. *ml* transgenic mice from each line were also analysed for lacZ mRNA expression in the kidney of F2 animals at approximately 8 weeks of age compared to a littermate wild type control by semi-quantitative RT-PCR using the lacZ PCR conditions as stated in section 2.7.3. RPL32 was used as an internal control. Primers used were RPL32 forward (5’GTGAAGCCCAAGATCGTC-3’) and RPL32 reverse (5’CATCAGCAGCACCTCCAGC-3’). PCR conditions used are as stated in section 2.3.4.
2.7.6  *MLC2v Cre breeding colony*

The MLC2v Cre mouse breeding colony was already established at the University of Leicester transgenic unit and was initially obtained from K R Chien (Chen *et al.*, 1998b). Mice at generation 18 were bred with C57BL6 mice to carry on the breeding line and create our own breeding colony to use to cross with the msl transgenic line.

2.7.7  *Identification of MLC2v Cre positive mice*

DNA was extracted from an ear punch of the mouse at 4-6 weeks following weaning using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma) as stated by the manufacturer’s protocol. Cre positive mice were identified by PCR. Primers used were Cre forward (5'GTTCGCAAGAACATGATGGACA-3') and Cre reverse (5'CTAGAGCCTGTTTTGCACGTTC-3') (Chen *et al.*, 1998b).

The PCR reaction contained the following:

- 2 μl Template DNA
- 1 μl 10 μM Forward Primer (final concentration of 1 μM)
- 1 μl 10 μM Reverse Primer (final concentration of 1 μM)
- 2 μl Qiagen 10 × buffer including 15mM MgCl₂ (final concentration in the reaction was 1 × buffer including 1.5mM MgCl₂)
- 2 μl 2 mM dNTP mix (final concentration of 0.2 mM)
- 0.1 μl 5 Uμl Qiagen HotStarTaq DNA Polymerase (final concentration of 0.5 U)
- dH₂O to a final volume of 20 μl

A no template control was included with all PCR reactions which contained 1 μl dH₂O instead of 1 μl template DNA. DNA from a mouse known to carry the Cre locus was included as a positive control and DNA from a mouse known not to carry the Cre locus was used as a negative control.

Oil (2 drops) was then placed on the top of each reaction and the tubes were placed in a Techne Techgene thermal cycler. The thermocycling parameters were 1 cycle of 95°C, 15 minutes; 35 cycles of 94°C, 30 seconds; 66°C, 1 minute; 72°C, 1 minute; a final extension step was performed at 72°C for 10 minutes.
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IL-2 was used as an internal control and the primer sequences and thermocycling parameters for IL-2 are stated in section 2.7.3.1.

All samples were electrophoresed as described earlier in section 2.2.2.

2.7.8 Identification of double transgenic mice carrying the msl transgene and Cre locus

The two lines of transgenic mice (msl and MLC2v Cre) were mated to generate selective expression of msl in the heart. At 4-6 weeks following weaning DNA was extracted from an ear punch of the mouse using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma) by following the manufacturer’s instructions. Mice carrying the msl transgene and the Cre locus were identified by PCR using GFP primers (section 2.7.3.1) and Cre primers (section 2.7.7) respectively.

2.7.9 Cardiac over-expression of msl detected by semi-quantitative RT-PCR and GFP Immunostaining

Positive founder mice that were identified to have inherited the msl transgene and Cre locus (double transgenics) by PCR were then analysed by semi-quantitative RT-PCR for msl over-expression (section 2.3.4) and GFP expression (section 2.7.3.1) in the heart compared to littermate wild type and msl transgenic (transgenic mouse that carries the msl transgene) controls. RPL32 was used as an internal control. Primers used were RPL32 forward (5’GTGAAGCCCAAGATCGTC-3’) and RPL32 reverse (5’CATCAGCAGCACCTCCAGC-3’). PCR conditions used are as stated in section 2.3.4.

GFP Immunostaining was performed on hearts from mice that were identified to have inherited the msl transgene and Cre locus (double transgenics) compared to a transgenic mouse that carries the msl transgene (msl transgenic) that acts as a control. Hearts were rinsed in PBS and fixed in 4% (w/v) paraformaldehyde at room temperature with shaking overnight and then stored in 70% ethanol at 4°C until ready to process in paraffin wax.

Paraffin wax processing was performed by J Edwards, MRC Toxicology Unit, University of Leicester where the tissue was processed through a series of steps to dehydrate, clear and to wax impregnate the tissue. This was carried out using a Shandon Citadel 2000 where the tissue was in 70% Industrial Methylated Spirit (IMS) for 2 hours, 90% IMS for 2 hours, 100% IMS for 2 hours, 100% IMS for 2 hours, 100% IMS
for 2 hours, 100% IMS for 1 hour, 100% IMS for 1 hour, chloroform for 2 hours, chloroform for 2 hours, chloroform for 2 hours, wax for 2 hours and wax for 2 hours. Next the tissue was embedded in paraffin wax, cooled and stored at 4°C until ready to section.

Sections were cut at 5 microns using the LEICA RM 2135 microtome. Paraffin sections were then stained for GFP which included an extra section per sample to act as a 2°ry antibody only control (not incubated with the 1°ry antibody) and a section known to stain positive for GFP to act as a positive control. The paraffin wax was removed by submerging the sections twice in xylene for 10 minutes and then by removing the xylene by placing the sections twice in 100% ethanol for 10 minutes. Endogenous peroxidase was blocked by placing the sections in 0.18% (v/v) hydrogen peroxide in methanol for 10 minutes and then placed into dH2O. Next antigen retrieval was carried out by placing the sections into pre-heated 0.01 M citrate buffer pH 6 in a pressure cooker and boiling for 15 minutes using a microwave. After the antigen retrieval sections were placed into dH2O.

Sections were next incubated in 100 µl swine blocking serum (1:25, DakoCytomation) in PBS for 1 hour at room temperature in a humidifying chamber. The blocking serum was then removed from each section (except for the 2°ry antibody only control) and the sections were incubated in 100 µl of 1:200 1°ry antibody (rabbit GFP polyclonal antibody, Molecular Probes) in swine blocking serum at 4°C overnight in a humidifying chamber. Next the 1°ry antibody was removed from the sections and after this three 10 minute washes in PBS were carried out. The sections were then incubated in 100 µl of 1:500 biotinylated 2°ry swine anti-rabbit antibody (DakoCytomation) in PBS for 1 hour at room temperature in a humidifying chamber. Next the 2°ry antibody was removed from the sections and after this three 10 minute washes in PBS were carried out. The sections were then incubated in 100 µl of 1:500 streptavidin-HRP (DakoCytomation) in PBS for 45 minutes at room temperature in a humidifying chamber. Following this the strepavidin-HRP was removed from the sections and after this three 10 minute washes in PBS were performed. Next PBS was removed from the sections and the sections were incubated in DAB peroxidase substrate (Vector Laboratories) for 2 minutes at room temperature and then the sections were place in dH2O to wash. The sections were then dehydrated by placing the sections in 90% ethanol for 10 minutes, 100% ethanol
for 10 minutes and then sections were cleared in xylene and mounted using distrene-tri
cresyl phosphate in xylene (DPX-type mountant).

For each sample a section was also stained for Haematoxylin and Eosin to show
staining of nucleic acids and basic components in the cytoplasm. This was carried out
by J Edwards, MRC Toxicology Unit, University of Leicester using a slide staining
machine (Shandon Varistain 24-4) where the sections were incubated in xylene for 2
minutes, xylene for 3 minutes, IMS for 1 minute, IMS for 1 minute, 70% IMS for 1
minute, dH₂O for 1 minute, haematoxylin for 15 minutes, dH₂O for 1 minute, 1% acid
alcohol for 25 seconds, dH₂O for 6 minutes, dH₂O for 1 minute, dH₂O for 2 minutes,
1% aqueous eosin for 3 minutes, dH₂O for 2 minutes, 70% IMS for 1 minute, IMS for 1
minute, IMS for 1 minute, IMS for 1 minute, IMS for 1 minute, xylene for 2 minutes
and xylene for 5 minutes.

Sections were then mounted in DPX-type mountant. All sections were viewed using an
Olympus CKX41 microscope and photographed using a Nikon MXA 5400 digital
camera.

2.8 Appendix 1

![Figure 2.2. The main features of the pGEM-T Easy vector (Promega) used for the
TA cloning of PCR amplification products.](image-url)
Materials and Methods

Figure 2.3. The main features of the expression vector pcDNA3.1(+) (Invitrogen) used to over-express msl.

Figure 2.4. The pi Z/EG transgenic vector.
Figure 2.5. The pCre-Pac vector.
Chapter 3

Construction of an expression vector to transiently over-express a c-Myc-msl fusion protein in vitro

3.1 Introduction

To further explore the role of msl in cardiac physiology and disease, in particular whether msl leads to hypertrophy and has a cardioprotective role in vitro, an expression vector to transiently over-express a c-Myc-msl fusion protein was first generated. The expression vector used was the mammalian expression vector pcDNA3.1(+) that was kindly given by Professor G Brooks and is widely used for over-expression studies (Ekhterae et al., 1999; Turner et al., 1998). This vector is shown in appendix 1 of chapter 2. To generate an msl expression construct, the full-length coding sequence of mouse msl was amplified by PCR and cloned into the mammalian expression vector pcDNA3.1(+). A c-Myc tag is situated in the N-terminus before the coding sequence of msl and this modification has been previously used by others (Arai et al., 2002; Barrientos et al., 2007; Kuwahara et al., 2005). The c-Myc tag allows msl expression to be detected by western blotting and immunocytochemical approaches using antibodies for the c-Myc tag.

The msl expression construct was transiently transfected in vitro, in a heart-derived rat cell line, H9c2 and msl mRNA and msl protein over-expression was examined by semi-quantitative RT-PCR and western blotting, respectively, to confirm that the msl expression construct generated worked and cells over-expressed a c-Myc-msl fusion protein. Transient msl over-expression in cells was also analysed by immunofluorescence using a fluorescent labelled antibody for the c-Myc tag.

The heart-derived rat cell line, H9c2, was chosen as an in vitro model system as this cell line has been widely used by others to investigate hypertrophy (Brostrom et al., 2000; Huang et al., 2004; Hwang et al., 2006; Laufs et al., 2002; Liu et al., 2008) or cell death (Bonavita et al., 2003; Chen et al., 2000; Ekhterae et al., 1999; Gustafsson et al., 2004; Han et al., 2004; Pesant et al., 2006; Tanaka et al., 2003; Turner et al., 1998). The H9c2 cell line was established by the researchers, Kimes and Brandt (Kimes and Brandt, 1976) and is an immortalised cell line capable of proliferation and differentiation. Another in vitro model system would be the use of neonatal or adult mammalian cardiomyocytes in primary culture. Neonatal cardiac muscle cells can
divide in cell culture for a short period of time, but they ultimately withdraw permanently from the cell cycle, and adult cardiomyocytes can not divide and eventually die. Cardiomyocytes have been derived from embryonic stem cells (Doetschman et al., 1985), P19 cells (Edwards et al., 1983), and bone marrow stromal cells (Makino et al., 1999) to establish cardiac muscle cell lines; however, these cells differentiated into different cell types in culture and therefore were not a homogenous cell population. Other cell lines such as the H9c2 cell line derived from rat embryonic heart tissue, has the ability to retain the properties of muscle cells. The H9c2 cells are strictly mononucleated myoblasts that resemble both skeletal and cardiac muscle myoblasts. At the myoblast stage, H9c2 cells express both myosin light chain 2 atrial (MLC2a) and MLC2v cardiac markers that are also expressed in newborn rat cardiac myocytes in primary culture (Menard et al., 1999; Rybkin et al., 2003). Upon reaching confluence they begin to form multinucleated tubular structures. Also the H9c2 cells adopted features of skeletal muscle because the cells expressed nicotinic receptors and synthesised a muscle-specific creatine phosphokinase isoenzyme when the mononucleated myoblasts fused. The lengths, diameter and arrangement into sarcomeres observed in myotubes of H9c2 have also been observed in the developing skeletal and heart muscle and the ability to generate action potentials and to contract in response to electrical stimulation is more evidence to suggest that H9c2 resemble skeletal and heart muscle cells and can serve as a model system for a variety of investigations (Kimes and Brandt, 1976).

The aim of the work presented in this chapter was to construct an expression vector to transiently over-express a c-Myc-ms1 fusion protein in vitro and to confirm ms1 over-expression by semi-quantitative RT-PCR, western blotting and immunofluorescence microscopy.

3.2 Results

3.2.1 Construction of an expression vector to transiently over-express a c-Myc-ms1 fusion protein

To generate a ms1 expression construct, firstly the full-length coding sequence of mouse ms1 was amplified by PCR from a plasmid termed pAlpha-MHCpromoter-ms1sense (mouse) that was previously generated by H Mahadeva where restriction sites (BglII and XhoI) were included in the primers to allow cloning into the mammalian
Construction of a msl expression vector pcDNA3.1(+). A c-Myc tag comprising of 30 nucleotide sequences corresponding to the 10 amino acid sequence, EQKLISEEDL was added to the N-terminus of the msl coding sequence by oligonucleotide-mediated mutagenesis with PCR where the c-Myc tag codon sequences were incorporated into the primers. This c-Myc tag was added to allow msl expression to be detected by using antibodies for the c-Myc tag. The amplified full-length msl coding sequence (figure 3.1) was then cloned into the pGEM-T Easy vector because this is one of the most convenient systems for cloning of PCR products.

![Figure 3.1. The full-length msl coding sequence amplified by PCR.](image)

Single putative transformant colonies were isolated, extracted for DNA and restriction digest confirmed that the pGEM-T Easy vector contained msl by producing the correct sized fragment for msl (approximately 1.3 kb) and correct sized band for the pGEM-T Easy vector (approximately 3 kb) which is shown in figure 3.2. Sequencing confirmed msl to be cloned into the pGEM-T Easy vector.

![Figure 3.2. Restriction digest analysis of pGEM-T Easy + msl.](image)
Construction of a msl expression vector

The mammalian expression vector pcDNA3.1(+) and the pGEM-T Easy vector were both cut by the same restriction enzymes, EcoRI and XhoI. The full-length msl fragment (approximately 1.3 kb) was released from the pGEM-T Easy vector (approximately 3 kb) following the digest. The msl fragment and the pcDNA3.1(+) expression vector (approximately 5.4 kb) were agarose gel extracted and checked for DNA purity on an agarose gel before ligation of msl into pcDNA3.1(+) vector. The purified msl fragment was then cloned into the gel extracted and purified mammalian expression vector pcDNA3.1(+).

Restriction digest confirmed that the pcDNA3.1(+) vector contained msl by producing the correct approximate 1.3 kb fragment for msl and approximately 5.4 kb for pcDNA3.1(+) which is shown in figure 3.3. Sequencing was also performed to verify that msl was cloned into the pcDNA3.1(+) vector.

![Figure 3.3. Restriction digest analysis of pcDNA3.1(+) + msl.](image)

3.2.2 Transient transfection for 24 and 72 hours leads to over-expression of msl

H9c2 cells were transfected with the msl expression construct [pcDNA3.1(+) containing c-Myc tagged msl (pcDNA3.1(+) + msl)] or the empty expression vector control (pcDNA3.1(+)) for either 24 or 72 hours. The msl expression construct was transfected in H9c2 cells for 72 hours to allow sufficient time for cells to over-express msl. Semi-quantitative RT-PCR analysis confirmed msl mRNA over-expression compared to empty expression vector control (figure 3.4). Similar findings of msl mRNA over-expression were observed following transient transfection for 24 and 72 hours.
hours, where an example of this at 24 hours is shown in figure 3.4. Low ms1 expression was observed in the empty expression vector control samples (can not see this clearly on the image in figure 3.4) and ms1 was clearly over-expressed when compared to the empty expression vector control. RNA was DNase treated prior to cDNA synthesis and the reverse transcription enzyme was excluded during cDNA synthesis in order to separate ms1 mRNA from ms1 plasmid DNA. As shown in figure 3.4, there was no ms1 expression detected when the RNA was DNase treated and the enzyme was excluded. This suggests that DNase treatment of the RNA removed plasmid DNA contamination and confirms that the ms1 expression detected was ms1 mRNA over-expression and not from the ms1 expression plasmid. There was no RPL32 (internal control) expression when the reverse transcription enzyme was excluded, suggesting that genomic DNA contamination was not present in the RNA.

![Figure 3.4. ms1 mRNA over-expression in H9c2 cells.](image)

Semi-quantitative RT-PCR analysis of ms1 mRNA. RPL32 was used as an internal control for inaccuracies in initial RNA levels. C refers to the empty vector control (pcDNA3.1(+)) and M refers to the ms1 expression vector (pcDNA3.1(+) + ms1), n = 3. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given. - DNase indicates that the RNA was not DNase treated prior to reverse transcription (RT), + DNase indicates that the RNA was DNase treated prior to RT, + RT indicates that the RT enzyme was present during cDNA synthesis and - RT indicates that the RT enzyme was omitted.
Construction of a ms1 expression vector

Western blot analysis using the ms1 and c-Myc antibodies confirmed ms1 protein overexpression compared to empty expression vector control following transient transfection for 24 hours (figure 3.5 (a) and 3.6 (a)) and 72 hours (figure 3.5 (b) and 3.6 (b)). The ms1 protein (approximately 45 kDa) appeared as a doublet on the blot consisting of two separate bands referred to as the top and bottom band of the doublet. When this doublet was detected using the ms1 antibody (figure 3.5 (a) and (b)), ms1 protein expression was observed in the empty vector controls (endogenous ms1 in H9c2 cells) for the top band of the doublet but not for the bottom band of the doublet and for the ms1 overexpression samples for the top and bottom bands of the doublet. This finding was apparent following transient transfection for both 24 and 72 hours.

Figure 3.5. ms1 protein over-expression in H9c2 cells.

Western blot analysis confirms ms1 protein over-expression following (a) 24 hours and (b) 72 hours of transient transfection. α-tubulin was used as an internal control for inaccuracies in initial protein levels, C refers to the empty expression vector control (pcDNA3.1(+)) and M refers to the ms1 expression vector (pcDNA3.1(+) + ms1), n = 3. S refers to the SeeBlue Plus2 pre-stained standard and the molecular weights (kDa) are given to the right. TB refers to the top band of the doublet and BB refers to the bottom band of the doublet.
When the c-Myc antibody was used to confirm msl over-expression, c-Myc protein expression was not found in the empty vector controls but was for the msl over-expression samples for the top and bottom bands of the doublet indicating msl over-expression (figure 3.6). There was a faint band observed in the empty vector expression control samples from a 72 hour transfection that could be a non-specific band rather than the top band of the c-Myc protein doublet.

**Figure 3.6. msl protein over-expression confirmed by the use of a c-Myc antibody.**

Western blot analysis of c-Myc protein in H9c2 cells transiently transfected with an msl expression vector (M) or empty vector control (C), n = 3 for (a) 24 hours or (b) 72 hours. α-tubulin was used as an internal control for inaccuracies in initial protein levels. S refers to the SeeBlue Plus2 pre-stained standard and the molecular weights (kDa) are given to the right. NS refers to non-specific bands, TB refers to the top band of the doublet and BB refers to the bottom band of the doublet.

To confirm the msl protein doublet, the msl expression construct was transfected into another cell line, NIH3T3, for 72 hours. msl is expressed at much lower levels in NIH3T3 cells (murine embryonic fibroblasts) than H9c2 cells (Ounzain, personal
Construction of a ms1 expression vector

msl over-expression in NIH3T3 cells was detected by western blotting using both the msl and c-Myc antibodies (figure 3.7) and the msl protein appeared as a doublet on the blot as previously observed in H9c2 cells (figure 3.5 and 3.6). msl protein expression was not clearly identified in the empty vector controls but was for the msl over-expression samples for the top and bottom bands of the doublet when the msl antibody was used indicating msl over-expression (figure 3.7 (a)). There was a band observed in the empty expression control samples detected by the msl antibody and was thought not to be the top band of the msl protein doublet but a non-specific protein in between the top and bottom bands of the doublet. However, NIH3T3 cells express very low levels of msl and therefore the possibility of this being endogenous msl cannot be ruled out.

msl over-expression was detected using the c-Myc antibody and c-Myc protein expression was not found in the empty vector controls but was for the msl over-expression samples for the top and bottom bands of the doublet confirming msl over-expression (figure 3.7 (b)).
Figure 3.7. ms1 protein over-expression in NIH3T3 cells.

Western blot analysis using (a) ms1 and (b) c-Myc antibodies confirms ms1 protein over-expression in NIH3T3 cells. α-tubulin was used as an internal control for inaccuracies in initial protein levels. C refers to the empty expression vector control (pcDNA3.1(+) ) and M refers to the ms1 expression vector (pcDNA3.1(+) + ms1), n = 3. S refers to the SeeBlue Plus2 pre-stained standard and the molecular weights (kDa) are given to the right. NS indicates a non-specific band, TB refers to the top band of the doublet and BB refers to the bottom band of the doublet.

The empty expression vector protein samples and the ms1 over-expression protein samples from the 72 hour transfection in NIH3T3 cells were analysed again by western blotting but immunoblotted for just the secondary antibodies used to detect ms1 and c-Myc primary antibodies. This was done to confirm that the ms1 protein doublet was real and that one of the doublet bands was not due to the secondary antibody. Western blot analysis using just the secondary anti-rabbit HRP antibody (detects the ms1 primary antibody) confirmed that the ms1 protein doublet was real as no protein bands appeared that resembled the ms1 protein doublet (figure 3.8 (a)). The same result was
also observed by western blot analysis when the secondary anti-mouse HRP antibody that detects the c-Myc antibody was used alone (figure 3.8 (b)).

Figure 3.8. msl over-expression protein doublet is not due to the secondary antibody.

Western blot analysis using (a) secondary anti-rabbit HRP antibody and (b) secondary anti-mouse HRP antibody confirms that the msl protein doublet was present. α-tubulin was used as an internal control for inaccuracies in initial protein levels. C refers to the empty expression vector control (pcDNA3.1(+)) and M refers to the msl expression vector (pcDNA3.1(+) + msl), n = 3. S refers to the SeeBlue Plus2 pre-stained standard and the molecular weights (kDa) are given.
Immunofluorescence microscopy using a c-Myc FITC antibody confirmed that H9c2 cells over-expressed c-Myc-msl and hence were successfully transfected with the msl expression vector (figure 3.9). Transient transfection (24 and 72 hours) of the msl expression vector gave rise to a mixed population of untransfected cells and cells over-expressing msl. An example of this following transfection for 24 hours is shown in figure 3.9). To determine if msl colocalises with actin in H9c2 cells, H9c2 cells were transfected with the msl expression vector or the empty expression vector control and co-stained with c-Myc FITC antibody and Alexa fluor 350 phalloidin to detect msl and actin, respectively. Actin filaments were observed in all cells transfected with or without the msl expression construct and cells over-expressing msl colocalised with actin (figure 3.9).

Figure 3.9. Transient transfection leads to msl protein over-expression that colocalises with actin in H9c2 cells.

Immunofluorescence microscopy of msl protein in H9c2 cells transiently transfected with an msl expression vector (M) or empty vector control (C), n = 3. Cells were co-stained with c-Myc FITC antibody and Alexa fluor 350 phalloidin to detect msl and actin, respectively. Merge image shows msl colocalisation with actin in msl transfected cells. Bar = 20 μm. Magnification 40x.
Construction of a ms1 expression vector

3.3 Discussion

In order to explore the role of ms1 in cardiac hypertrophy and cell survival in vitro an expression vector to transiently over-express a c-Myc-ms1 fusion protein was first constructed. Semi-quantitative RT-PCR was used to confirm ms1 mRNA over-expression and western blotting and immunofluorescence microscopy were used to confirm ms1 protein over-expression.

3.3.1 Successful cloning of ms1 into the pcDNA3.1(+) vector to construct a ms1 expression vector

The full-length coding sequence of mouse ms1 was amplified by PCR (figure 3.1), and then cloned into the pGEM-T Easy vector (figure 3.2). ms1 was then moved from the pGEM-T Easy vector into the mammalian expression vector pcDNA3.1(+). Restriction digest confirmed that the pcDNA3.1(+) vector contained ms1 by producing the correct sized fragment for ms1 (approximately 1.3 kb) and correct sized band for the pcDNA3.1(+) vector (approximately 5.4 kb) which is shown in figure 3.3. In addition, ms1 was verified by sequencing to be cloned in frame into the pcDNA3.1(+) vector.

The ms1 expression vector was transiently transfected into a heart-derived rat cell line, H9c2, for 24 and 72 hours where 72 hours was used to allow enough time for the cells to over-express ms1. To confirm that the ms1 expression construct was transfected into cells and cells over-expressed ms1, ms1 mRNA was analysed by semi-quantitative RT-PCR and ms1 protein was analysed by western blotting and immunofluorescence microscopy.

3.3.2 ms1 mRNA and protein over-expression in vitro confirms successful transient transfection of the ms1 expression vector for 24 and 72 hours

Semi-quantitative RT-PCR analysis confirmed transient (24 and 72 hours) ms1 mRNA over-expression compared to empty expression vector control (figure 3.4). ms1 protein over-expression was confirmed by western blotting using the ms1 and c-Myc antibodies (figure 3.5 and 3.6). When the ms1 antibody was used to detect ms1 protein, ms1 protein appeared on the western blot as a doublet (figure 3.5). This was observed following transient transfection for 24 and 72 hours. There was much more ms1 protein detected in the ms1 over-expression samples compared to the empty vector control samples for the bottom band of the doublet. This suggests that the top band of the protein doublet could be non-specific and the actual ms1 protein was the bottom band of
the doublet. However, H9c2 cells express ms1 and there was no endogenous ms1 protein (bottom band of the doublet) detected in cells transfected with the empty vector control. To verify this, the c-Myc antibody was used to detect c-Myc protein and hence confirm ms1 over-expression as the c-Myc antibody detects the c-Myc tag situated in the N-terminus before the coding sequence of ms1. The c-Myc protein also appeared on the western blot as a doublet and ms1 was over-expressed compared to empty vector control for both bands of the doublet following transfection for 24 and 72 hours (figure 3.6). A faint protein band was observed in each of the empty expression vector control samples from a 72 hour transfection that were thought to be non-specific bands. However, this band could be a non-specific top band of the c-Myc protein and again raised the possibility that the bottom band was the real c-Myc-ms1 protein. This suggestion was an unlikely explanation because these non-specific bands were not observed following transfection for 24 hours and when the ms1 antibody was used there was no basal ms1 protein expression (bottom band of the doublet) detected and endogenous ms1 should have been detected as H9c2 cells express ms1.

To further investigate this protein doublet, the ms1 expression vector was transiently transfected in another cell line, NIH3T3. ms1 and c-Myc protein again appeared on the western blot as a doublet in cells transfected with the ms1 expression construct and ms1 protein (detected by both ms1 and c-Myc antibodies) was over-expressed compared to empty vector control for both bands of the doublet (figure 3.7). Western blot analysis using the ms1 antibody detected a protein band in cells transfected with empty vector control. This could be a non-specific band in between the top and bottom bands of the ms1 doublet rather than the top band of the ms1 protein doublet. It is feasible that this was endogenous ms1 (top band of the doublet) as NIH3T3 cells express low levels of ms1 and therefore this possibility cannot be disregarded. When the c-Myc antibody was used no protein bands were detected in cells transfected with empty vector control and the c-Myc protein doublet was observed in cells transfected with ms1, verifying the ms1 protein doublet was real.

One of the ms1 protein doublet bands could be due to the secondary antibodies used to detect ms1 and c-Myc primary antibodies. Figure 3.8 shows that when secondary antibodies were used alone, no protein bands were present on the western blot that could be the ms1 or c-Myc protein doublet. These findings suggest that the protein doublet appears to be real. Endogenous ms1 protein appeared as a single protein band on the
blot when cells were transfected with the empty vector control and ms1 protein was observed as a doublet consisting of two protein bands referred to the top and bottom bands of the doublet in cells over-expressing ms1.

Immunofluorescence microscopy using a c-Myc FITC antibody further confirmed that transient transfection for 24 and 72 hours of the ms1 expression vector leads to ms1 protein over-expression and phalloidin staining showed that ms1 associates with the actin cytoskeleton in transfected H9c2 cells (figure 3.9). This finding further strengthens the role of ms1 as an actin binding protein.

3.3.3 Reasons why the over-expressed ms1 protein appears as a doublet when analysed by western blotting

The top band of the protein doublet could be endogenous ms1 protein as it was present when cells were transfected with the empty vector control. ms1 protein appears to be mainly in the bottom band of the doublet when cells were transfected with the ms1 expression vector but there was some ms1 protein in the top band of the doublet confirmed by the additional use of a c-Myc antibody, suggesting there was endogenous and over-expressed ms1 protein in the top band. Therefore it appears that some of the over-expressed ms1 protein runs the same as endogenous ms1 protein but most runs lower. This is probably due the c-Myc tag possibly affecting the structure causing the over-expressed ms1 protein to run lower. The much higher levels of ms1 protein when over-expressed could be another reason why most of the over-expressed ms1 protein runs lower than the endogenous ms1 protein. Another explanation is due to secondary structure or post-translational modification of the protein where there is covalent modification that changes the properties of a protein. Examples of post-translational modifications include phosphorylation, glycosylation, acetylation and methylation (Mann and Jensen, 2003). A urea gel that allows much higher denaturation may resolve the doublet to a single band and explain any possible secondary structure. To evaluate the possibility of a post-translational modification for example phosphorylation, treatment with an enzyme such as a phosphatase could be performed.

Although there were two ms1 protein bands they were both definitely ms1 and secondary antibody only controls confirmed this. Immunofluorescence microscopy further confirmed that ms1 protein was over-expressed.
In summary, the main aim was to generate an expression vector to transiently over-express a c-Myc-ms1 fusion protein \textit{in vitro}. It was demonstrated in H9c2 cells that transient transfection of the ms1 expression vector for 24 hours and 72 hours resulted in ms1 mRNA and protein over-expression confirmed by semi-quantitative RT-PCR and western blotting, respectively. ms1 protein over-expression was further confirmed by immunofluorescence microscopy and ms1 binds to actin filaments in transfected H9c2 cells further demonstrating that ms1 associates with the actin cytoskeleton. The ms1 expression vector generated leads to ms1 over-expression when transfected into cells and thus this construct can be used \textit{in vitro} to investigate whether ms1 over-expression affects genes involved in hypertrophy and cell survival; therefore, allowing identification of target genes and downstream pathways of ms1 and to examine whether ms1 over-expression increases cell size and can protect against apoptotic cell death to determine if ms1 leads to cardiac hypertrophy and has an anti-apoptotic function.
Chapter 4

Effects of ms1 over-expression in vitro

4.1 Introduction

To identify putative target genes and downstream pathways of ms1, in particular ones involved in hypertrophy and cell survival, changes in mRNA and protein expression for a range of genes were examined in H9c2 cells over-expressing ms1. A range of genes were investigated that have been associated with cardiac hypertrophy such as the contractile gene cardiac α-actin, the transcription factor SRF and other known markers of hypertrophy such as BNP, calcineurin, GATA 4, myocardin and MEF2C. Protein kinases that are implicated as mediators of the hypertrophic response and involved in the cell survival pathway such as Akt and ERK1/2 were also examined. In addition, the apoptotic repressor ARC (apoptosis repressor with caspase recruitment domain) was investigated to determine if over-expression of ms1 altered ARC expression.

ms1 has been shown to activate SRF-dependent transcription via a Rho-MRTF pathway (Kuwahara et al., 2005). Microarray experiments using a cell line that expresses dominant negative MRTF-A identified twenty eight genes that were MRTF-dependent and five of these are known SRF target genes (Selvaraj and Prywes, 2004). It is possible that these twenty eight MRTF-dependent genes are putative targets of ms1 and therefore altered mRNA expression of six out of the twenty eight MRTF-dependent genes were analysed in ms1 over-expressing cells. The six genes chosen were adrenomedullin, interleukin-6 (IL-6), coagulation factor III (tissue factor), leukemia inhibitory factor (LIF) and two known SRF target genes, jun-B and fos-related antigen-1 (fra-1). These genes were examined because they have been associated with cardiac diseases such as cardiac hypertrophy, ischaemia/reperfusion injury and heart failure (discussed in section 4.3.3).

Altered mRNA expression of these six MRTF-dependent genes following ms1 over-expression may further support a role for ms1 in cardiac hypertrophy and cardioprotection and would suggest that ms1 regulates MRTF-dependent genes possibly via the Rho-MRTF pathway.
4.2 Results

4.2.1 Altered gene expression following 24 hours of ms1 over-expression

Gene expression changes following 24 hours of ms1 over-expression was examined by semi-quantitative RT-PCR (figure 4.1). RPL32 was used as an internal control. There was no significant change in the contractile gene cardiac α-actin, and markers for hypertrophy such as BNP, calcineurin and MEF2C following ms1 over-expression. Other markers for hypertrophy such as the transcription factors GATA 4 and myocardin were up-regulated approximately 1.4 fold in cells over-expressing ms1 but this fold change was not found to be significant for GATA 4 or myocardin. The transcription factor, SRF, and the apoptotic repressor, ARC, were increased approximately 2 fold in ms1 over-expressing cells. The fold change was significant for SRF ($P < 0.05$) and the approximate 2 fold change in ARC expression was borderline significant ($P = 0.082$).

When ARC mRNA was examined in the same cells over-expressing ms1 by real-time quantitative RT-PCR (figure 4.2) using the comparative ($\Delta\Delta C_T$) method, ARC expression was again increased approximately 2 fold and this fold change was borderline significant ($P = 0.048$).
Figure 4.1. Gene expression changes in cells over-expressing ms1 for 24 hours.

(a) Semi-quantitative RT-PCR analysis of altered transcript levels in H9c2 cells transiently transfected with an ms1 expression vector (M) or empty vector control (C), n = 3. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given. (b) Transcript levels for each gene in (a) were measured by semi-quantitative RT-PCR. Each gene was normalised to an internal control, RPL32, and the fold change in abundance is presented relative to empty vector control. Bars show mean ± SEM from 3 separate experiments. *P < 0.05 versus empty vector control.
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![Graph showing ARC expression](image)

**Figure 4.2. Increased ARC expression in cells over-expressing ms1 for 24 hours confirmed by real-time quantitative RT-PCR.**

Real-time quantitative RT-PCR analysis of ARC mRNA expression. ARC mRNA was normalised to TBP mRNA and the fold change in ARC expression was calculated relative to control (pcDNA3.1(+)). The results are the mean ± SEM from three separate experiments. Statistical significance by a Student’s *t* test: *P* < 0.05.

### 4.2.2 Altered protein expression following 24 hours of ms1 over-expression

Akt and ERK1/2 that have been implicated in cardiac hypertrophy and cell survival were examined for altered protein expression in cells over-expressing ms1 for 24 hours. Akt and ERK1/2 are activated when phosphorylated and therefore phosphorylated states of Akt and ERK1/2 were examined. In addition, total Akt2 protein expression was analysed following ms1 over-expression and α-tubulin was used as an internal control. As shown in figure 4.3 there was no significant change in total Akt2 and phosphorylated Akt following ms1 over-expression. An approximate 2 fold decrease in phosphorylated ERK1/2 protein was observed, albeit not statistically significant.
Figure 4.3. Changes in protein expression of kinases in H9c2 cells over-expressing ms1 for 24 hours.

(a) Altered protein levels in H9c2 cells transiently transfected with an ms1 expression vector (M) or empty vector control (C), n = 3, analysed by Western blotting. S refers to the SeeBlue Plus2 pre-stained standard and the molecular weights (kDa) are given. (b) Densitometric analysis for each protein kinase expression presented in (a). The percentage of protein expression is presented relative to empty vector control (pcDNA3.1(+)) after standardisation to α-tubulin as an internal control. Bars show mean ± SEM from 3 separate experiments. *P < 0.05 versus empty vector control.
4.2.3 Altered gene expression following 72 hours of ms1 over-expression

The majority of genes examined following 24 hours of ms1 over-expression (figure 4.1) were unaltered in expression and therefore the same genes were examined in cells over-expressing ms1 for a longer time of 72 hours by semi-quantitative RT-PCR. Again RPL32 was used as an internal control and by taking this into account there were approximately 2 to 4 fold changes found for a range of genes known to be involved in hypertrophy and cell survival that are shown in figure 4.4. The contractile gene cardiac α-actin was observed to increase by approximately 2 fold ($P < 0.01$) in cells over-expressing ms1. Markers for hypertrophy such as the fetal gene BNP and the transcription factor myocardin were up-regulated by approximately 4 fold ($P < 0.01$) and approximately 3 fold ($P < 0.05$), respectively, in cells over-expressing ms1. Other hypertrophic markers such as calcineurin, MEF2C and GATA 4 were increased approximately 2 fold ($P < 0.05$). There was no significant change in SRF expression following ms1 over-expression and the apoptotic repressor ARC was up-regulated approximately 4 fold ($P < 0.05$).
Figure 4.4. Over-expression of msl for 72 hours alters gene expression in H9c2 cells.

(a) Semi-quantitative RT-PCR analysis of altered mRNA in H9c2 cells transiently transfected with an msl expression vector (M) or empty vector control (C), n = 3. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given. (b) Transcript levels for each gene in (a) were measured by semi-quantitative RT-PCR. Each gene was normalised to an internal control, RPL32, and the fold change in abundance is presented relative to empty vector control. Bars show mean ± SEM from 3 separate experiments. *P < 0.05, **P < 0.01 versus empty vector control.
4.2.4 Altered protein expression following 72 hours of ms1 over-expression

There was no altered protein expression for total Akt2, phosphorylated Akt and phosphorylated ERK1/2 in cells over-expressing ms1 for 24 hours (figure 4.3) when analysed by western blotting. When these protein kinases were examined following 72 hours of ms1 over-expression there was no significant change in total Akt2 protein expression; however, phosphorylated Akt was down-regulated approximately 4 fold ($P < 0.05$) in ms1 over-expressing cells. Phosphorylated ERK1 and 2 were also down-regulated following ms1 over-expression by approximately 5 fold ($P < 0.05$) and approximately 4 fold ($P < 0.05$), respectively, see figure 4.5.
Figure 4.5. Over-expression of ms1 for 72 hours alters protein expression of kinases in H9c2 cells.

(a) Western blot analysis of altered protein levels in H9c2 cells transiently transfected with an ms1 expression vector (M) or empty vector control (C), n = 3. S refers to the SeeBlue Plus2 pre-stained standard and the molecular weights (kDa) are given. (b) Densitometric analysis for each protein kinase expression presented in (a). The percentage of protein expression is presented relative to empty vector control (pcDNA3.1(+)) after standardisation to α-tubulin as an internal control. Bars show mean ± SEM from 3 separate experiments. *P < 0.05 versus empty vector control.
4.2.5 Altered gene expression of MRTF-dependent genes in H9c2 cells over-expressing ms1

To determine whether MRTF-dependent genes are targets of ms1, changes in expression of six known MRTF-dependent genes, adrenomedullin, IL-6, tissue factor, LIF, jun-B and fra-1 following 24 hours of ms1 over-expression were examined by real-time quantitative RT-PCR using the Pfaffl method (Pfaffl, 2001) as explained in section 2.3.5.2 of the materials and methods chapter. Over-expression of ms1 significantly induced all the six known MRTF-dependent genes (figure 4.7). The cytokines LIF and IL-6 were up-regulated by approximately 2.5 fold ($P < 0.01$) and 6 fold ($P < 0.01$), respectively, in cells over-expressing ms1. An induction of approximately 1.5 fold was observed for tissue factor ($P < 0.01$) and the vasodilating peptide adrenomedullin ($P < 0.05$). The proto-oncogene jun-B and the transcription factor fra-1 were increased approximately 2 fold following ms1 over-expression. There was strong evidence that this fold change was significant for jun-B ($P < 0.01$) and fra-1 ($P < 0.01$).

MRTF-dependent genes were altered in expression following ms1 over-expression for 24 hours and therefore were not examined in cells over-expressing ms1 for 72 hours.

![Figure 4.7. Up-regulation of MRTF-dependent genes following ms1 over-expression.](image)

Over-expression of ms1 alters transcript levels in H9c2 cells. Transcript levels for each gene were quantified by real-time quantitative RT-PCR. Each gene was normalised to an internal control, RPL32 and the fold change in abundance is presented relative to empty vector control. Bars show mean ± SEM from 6 separate experiments. $*P < 0.05$, $**P < 0.01$ versus empty vector control.
4.2.6 Promoter analysis of ms1 and ms1 target genes for SRF binding sites

SRF regulates serum-inducible genes and genes expressed in skeletal, smooth and cardiac muscle by binding to CArG box elements (SRF binding sites) in their promoter sequences (Frey and Olson, 2003; Sotiropoulos et al., 1999). ms1 is able to stimulate SRF-dependent transcription by inducing the nuclear accumulation of MRTFs where they associate with SRF to activate transcription of genes containing SRF binding sites (Kuwahara et al., 2005). Therefore the promoters of putative target genes of ms1 were analysed for SRF binding sites to investigate whether ms1 alters gene expression of SRF target genes which would further support that activation of SRF target genes are mediated by ms1 via the Rho-MRTF pathway.

The ms1 promoter and the promoters of all genes examined in sections 4.2.1, 4.2.3 and 4.2.5 that are possible downstream target genes of ms1 were analysed for CArG box elements (SRF binding sites) using the programme DCODE.org Comparative Genomics Center (www.dcode.org). First the ECR browser in DCODE.org was used to extract the promoter sequences (-4000) and align human, mouse and rat sequences. rVista 2.0 was then used to identify conserved SRF binding sites. Exons and Introns were also analysed for SRF binding sites for certain genes known to have sites in these areas. SRF, cardiac α-actin, BNP, adrenomedullin, myocardin, jun-B and fra-1 are known to contain SRF binding sites (Adiseshaiah et al., 2005; Belaguli et al., 1997; Miano, 2003; Minty and Kedes, 1986; Miwa and Kedes, 1987; Nelson et al., 2005; Selvaraj and Prywes, 2004; Spencer and Misra, 1996; Sun et al., 2006; Wang et al., 2001b) but were analysed using DCODE.org to confirm that the database identified SRF binding sites.

Using published data and DCODE.org (ECR browser and rVista 2.0), conserved SRF binding sites were found in ms1 and nine out of the fourteen probable ms1 target genes (table 4.1) which are cardiac α-actin, SRF, BNP, myocardin, MEF2C, adrenomedullin, jun-B, fra-1, and LIF. Calcineurin (mouse) contains a possible SRF binding site but this sequence was not conserved in rat and human. A possible SRF binding site was identified in GATA 4, ARC and IL-6 that was conserved in mouse and rat but not in human. Tissue factor was not found to contain any likely SRF binding sites.
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Table 4.1. Summary of msl and msl possible target genes containing CArG sequences in the mouse genome.

<sup>a</sup>CArG boxes conserved in sequence between mouse, rat and human. Bold and italicised bases represent non-conserved nucleotides (1 or 2 bp tolerable mismatch), e.g. a mouse A might be a T in human or rat. <sup>b</sup>CArG boxes conserved in sequence between mouse and rat but not in human. <sup>c</sup>The position of each CArG element is relative to the transcriptional start site (TSS) according to Ensembl (www.ensembl.org/index.html).

Cardiac α-actin, SRF, BNP, myocardin, adrenomedullin, jun-B and fra-1 have been reported to contain SRF binding sites (Adiseshaiah et al., 2005; Belaguli et al., 1997; Miano, 2003; Minty and Kedes, 1986; Miwa and Kedes, 1987; Nelson et al., 2005; Selvaraj and Prywes, 2004; Spencer and Misra, 1996; Sun et al., 2006; Wang et al., 2001b) with the same sequences shown in table 4.1 which confirms the use of DCODE.org as a tool to search for SRF (transcription factor) binding sites. Out of these seven genes containing SRF binding sites, cardiac α-actin, BNP, adrenomedullin, jun-B
hours. There was a significant induction of BNP (approximately 4 fold) and myocardin (approximately 3 fold) when over-expressing ms1. However, SRF transcript levels were not significantly up-regulated following ms1 over-expression for 72 hours. At 24 hours only SRF was significantly up-regulated and this was by approximately 2 fold when compared to empty vector control. These findings suggest that ms1 appears to affect genes involved in cardiac hypertrophy and implicates these genes as putative targets of ms1 and downstream pathways of ms1 that collectively may be involved in the development of hypertrophy.

Expression of hypertrophic genes was up-regulated following ms1 over-expression for 72 hours and not following 24 hours. Induction of SRF mRNA was only observed in cells over-expressing ms1 for 24 hours and not for 72 hours. A possible explanation for this could be time-dependency where SRF was initially up-regulated and then the other hypertrophic genes were up-regulated. The cells were confluent by 72 hours in culture and H9c2 cells when confluent can differentiate into tubular structures (Kimes and Brandt, 1976). Many of these genes implicated in the hypertrophic response also regulate cell differentiation for example myocardin (Du et al., 2003; Wang et al., 2003), MEF2C (Gauthier-Rouviere et al., 1996) and GATA 4 (Pikkarainen et al., 2004) thus ms1 also appears to affect transcription factors that regulate cardiomyocyte differentiation, suggesting that ms1 may play a role in differentiation. ms1 mRNA was found up-regulated when H9c2 cells differentiate (Ounzain, personal communication) thus further supporting a role for ms1 in cell differentiation.

ms1 via the Rho-MRTF pathway can stimulate SRF activity by binding to actin, promoting actin polymerisation in the presence of Rho activity which releases MRTFs from the inhibitory influence of G-actin, allowing the nuclear import of MRTFs and stimulation of SRF dependant gene activation (Kuwahara et al., 2005). It is therefore feasible that the identified downstream target genes of ms1 were up-regulated following ms1 over-expression via the MRTF-SRF signalling pathway as many of theses genes could be SRF target genes. This will be discussed further in section 4.3.4.

4.3.2 ms1 appears to affect genes involved in cell survival

To establish if ms1 affects genes involved in cell survival pathways, transcript levels of the apoptotic repressor, ARC was examined by semi-quantitative RT-PCR and real-time quantitative RT-PCR following ms1 over-expression for 24 hours and 72 hours. ARC
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was investigated because like ms1, ARC was found expressed in the heart and skeletal muscle (Koseki et al., 1998). ARC was found to inhibit apoptosis by interacting with caspase 2 and 8 (Koseki et al., 1998), in contrast other studies have shown that inhibition of apoptosis via ARC does not necessarily operate through the caspases. For instance, hypoxia-induced apoptosis was suppressed by ARC inhibition of cytochrome C release from the mitochondria in a caspase-independent manner (Ekhterae et al., 1999). In addition, ARC over-expression inhibited hydrogen peroxide-induced cell death in H9c2 cells mediated totally or part through the blockade of hypoxia-induced cytochrome C release from the mitochondria (Neuss et al., 2001).

ARC has been reported to protect against ischaemia-reperfusion injury by reducing creatine kinase release and infarct size (Gustafsson et al., 2002). ARC and a mutant form of ARC (in the CARD domain) were then investigated in ischaemia-reperfusion injury and hydrogen peroxide-induced cell death and their effect on Bax (a pro-apoptotic Bcl-2 family protein that translocates to the mitochondria in response to death stimuli, causing cytochrome release from the mitochondria). It was found that the mutant form of ARC did not reduce creatine kinase release and infarct size after ischaemia-reperfusion or resulted in protection against hydrogen peroxide-mediated cell death in H9c2 cells. ARC co-immunoprecipitated with Bax and prevented Bax activation and cytochrome release in hydrogen peroxide treated cells but the mutant form of ARC did not. These results demonstrated that the CARD domain is important in mediating the protective effects of ARC and that ARC interacts with Bax which, prevents its activation and release of cytochrome C from the mitochondria (Gustafsson et al., 2004).

These results taken together with other results (Ekhterae et al., 1999; Neuss et al., 2001) suggest that ARC prevents cell death by interacting with multiple pathways (Gustafsson et al., 2004). More recently it was demonstrated that ARC protein levels were down regulated in human failing heart and ARC provided cardioprotection in response to pressure overload and ischaemia (Donath et al., 2006).

ARC expression was significantly up-regulated approximately 4 fold when ms1 was over-expressed for 72 hours and increased approximately 2 fold following ms1 over-expression for 24 hours. Therefore the anti-apoptotic factor ARC appears to be a target gene of ms1 and hence ms1 may play a role in cell survival via ARC. ARC expression like ms1 was found increased in differentiated cells (Hunter et al., 2007) and ARC
expression was significantly up-regulated in cells over-expressing msl for 72 hours where the cells were confluent and can start to differentiate further strengthening the role of msl in differentiation.

Subsequent work further investigated the role of msl in cell survival by examining the changes in protein expression of kinases involved in the cell survival pathway such as Akt and ERK1/2. Total Akt2, phosphorylated Akt and phosphorylated ERK1/2 were not significantly altered in their protein expression following msl over-expression for 24 hours. Again total Akt2 protein expression was unaffected in cells over-expressing msl for 72 hours; however, phosphorylated Akt and phosphorylated ERK1/2 were significantly down-regulated. Inhibition of ERK1/2 and Akt caused increased apoptosis (Bueno and Molkentin, 2002; Clerk et al., 2003; Turner et al., 1998; Yasuoka et al., 2004) and it was found that over-expressing msl significantly down-regulated ERK1/2 and Akt but no apoptosis (DNA fragmentation) was observed when analysed by flow cytometry as shown in figure 5.4 and 5.11 of chapter 5. Also msl over-expression caused an induction of the apoptosis repressor ARC; therefore, it is plausible that msl over-expression may provide a protective response against apoptosis and promote cell survival via deactivation of ERK1/2, Akt and activation of ARC.

Calcineurin-mediated NFAT activation has also been suggested to be critical in preventing cardiomyocyte apoptosis (Frey et al., 2004). Recent evidence has also linked a deactivation of Akt with elevated calcium in H9c2 cells and the inactivation of Akt was well correlated with the susceptibility to apoptosis (Yasuoka et al., 2004). The phosphatase, calcineurin is activated by a rise in intracellular calcium (Olson, 2004) and calcineurin also appears to be involved in apoptosis (Klumpp and Krieglstein, 2002). Thus, it is possible that over-expression of msl may also have an anti-apoptotic response via the deactivation of Akt in response to elevated calcium and therefore possible activation of calcineurin.

Taken together all these findings suggest that msl appears to affect genes involved in cell survival and may prevent apoptosis by interacting with multiple cell survival pathways.

It is important to note that numerous studies have implicated ERK1/2 and Akt in producing the hypertrophic response. Phosphorylated ERK1/2 and Akt were significantly down-regulated when msl was over-expressed and down-regulation of
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these kinases has been observed to decrease hypertrophy (Andersson et al., 1998; Black et al., 2002; Bueno et al., 2001; Frey and Olson, 2003; Hardt and Sadoshima, 2004; Shibata et al., 2004; Shioi et al., 2000; Takahashi et al., 2003) suggesting that over-expressing ms1 may produce an anti-hypertrophic response via ERK1/2 and Akt deactivation.

SRF regulates immediate early and muscle specific gene expression by binding to the CArG box elements in their promoter/enhancer sequences and this is thought to occur via different signalling pathways that involve MAPK signalling and actin dynamics (figure 4.8).

Figure 4.8. The possible mechanism to regulate the transcriptional activity of SRF through two parallel signalling pathways (Cen et al., 2004).

SRF bound to CArG boxes can be activated by either the ternary complex factor (TCF) family or MRTFs (MKL1). TCF is activated by MAPK phosphorylation. MRTFs are sequestered in the cytoplasm by binding to G-actin and are activated when extracellular signals stimulate actin polymerisation via Rho signalling causing MRTF translocation to the nucleus. Additional abbreviations: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; EGF, epidermal growth factor; LPA, lysophosphatidic acid.

Activation of MAPK pathways leads to phosphorylation of the ternary complex factor (TCF) family which associate with SRF on target genes that contain TCF binding sites (GGAA/T) adjacent to the CArG box (Janknecht et al., 1993). The binding of TCF and myocardin/MRTFs to SRF is in fact mutually exclusive (Wang et al., 2004). The B box of Elk-1 (one member of the TCF family) competes for MRTF binding to SRF, suggesting that they bind to the same region on SRF (Miralles et al., 2003). Signalling
by the Rho family of small GTPases stimulates SRF activity via actin polymerisation, independently of MAPK activation and TCFs (Sotiropoulos et al., 1999). MRTFs are sequestered in the cytoplasm by binding to G-actin monomers via their N-terminal RPEL motif. Extracellular signals stimulate actin polymerisation via Rho signalling and this causes MRTF translocation to the nucleus resulting in activation of SRF (Miralles et al., 2003).

In section 4.2.3 it was demonstrated that ms1 over-expression for 72 hours up-regulated expression of many known markers of hypertrophy and in section 4.3.1 it was suggested that ms1 may regulate expression of genes involved in hypertrophy via the MRTF-SRF pathway. If this MRTF-SRF pathway was involved the other signalling pathway, the MAP (ERK1/2) kinase-TCF pathway that stimulates SRF, probably would not be involved as competition has been shown between TCF and MRTF binding to SRF (Miralles et al., 2003). This could explain why ERK1/2 were down-regulated following ms1 over-expression and hence members of the TCF family were possibly not phosphorylated and associated with SRF to activate SRF dependent gene transcription. Instead SRF could have been stimulated via ms1-MRTF and therefore the finding that ERK1/2 protein levels were reduced when ms1 was over-expressed strengthens the possibility that ms1 may regulate expression of genes involved in hypertrophy via the MRTF-SRF pathway.

4.3.3 ms1 appears to affect MRTF-dependent genes associated with cardiac hypertrophy and cell survival

Selvaraj and Prywes used a cell line expressing dominant negative MRTF-A (can block activation by all of the members of the MRTF family) and identified many MRTF-dependent genes where a subset of these genes are known SRF targets (Selvaraj and Prywes, 2004). It has been recently demonstrated that ms1 via the Rho-MRTF pathway can activate SRF-dependent transcription (Kuwahara et al., 2005) and therefore it was speculated that the MRTF-dependent genes whether SRF target genes or not were putative targets of ms1. In order to identify ms1 target genes that are specifically dependent on the MRTF pathway altered mRNA expression of MRTF-dependent genes published by Selvaraj and Prywes (2004) were analysed in ms1 over-expressing cells. Adrenomedullin, IL-6, tissue factor, LIF and two known SRF target genes jun-B and fra-1 were examined because they have been associated with cardiac hypertrophy and cell survival. For example numerous studies have shown that the cytokines LIF and
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IL-6 can induce cardiac hypertrophy via glycoprotein 130 (gp130) signalling (Casey et al., 2005; Fredj et al., 2005; Hirota et al., 1995; Matsui et al., 1996; Sano et al., 2000) that involves activation of PI3K-Akt-p70s6k pathway (Hiraoka et al., 2003), Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Ancey et al., 2002; Kodama et al., 1997; Kunisada et al., 1996; Kunisada et al., 1998) and MAPK pathways (Ancey et al., 2002; Kunisada et al., 1998). LIF and IL-6 have also been shown to be cardioprotective (Matsushita et al., 2005; Smart et al., 2006; Yasukawa et al., 2001).

Elevated levels of tissue factor, the protease receptor initiating the coagulation cascade, was observed in hearts of rabbits subjected to myocardial ischaemia/reperfusion injury (Chong et al., 2003; Erlich et al., 2000; Golino et al., 1996). Administration of an inhibitory anti-rabbit tissue factor monoclonal antibody reduced infarct size (Chong et al., 2003; Erlich et al., 2000; Golino et al., 1996) suggesting that myocardial ischaemia/reperfusion injury could be decreased using anti-tissue factor therapy. Elevated tissue factor levels have also been found in patients with ischaemic heart disease (Falciani et al., 1998). In addition, tissue factor has been suggested to be involved in the maintenance of cardiac muscle structure (Luther et al., 2000).

Considerable controversy exists in the role that adrenomedullin plays in cardiac hypertrophy. Studies have found that adrenomedullin levels did not increase following aortic banding (Kaiser et al., 1998) and phenylephrine or endothelin-mediated hypertrophy (Autelitano et al., 2001) with many studies suggesting that adrenomedullin has anti-hypertrophic effects (Autelitano et al., 2001; Niu et al., 2004; Wang et al., 2001a; Zhang et al., 2000). However, there are an equal number of studies reporting an increase in adrenomedullin. One group observed this following aortic banding (Morimoto et al., 1999; Nishikimi et al., 2003), another following chronic pressure overload in rats by administrating angiotensin II (Romppanen et al., 1997) and many studies observed increased ventricular adrenomedullin levels in several hypertensive models with cardiac hypertrophy (Ishiyama et al., 1997; Shimokubo et al., 1995; Shimokubo et al., 1996). However, ventricular adrenomedullin gene expression is consistently induced in the failing heart (Jougasaki et al., 1995; Jougasaki et al., 1996; Kaiser et al., 1998; Nishikimi et al., 1997; Oie et al., 2000) and adrenomedullin was shown to inhibit cardiac myocyte apoptosis following doxorubicin (Tokudome et al.,
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2002) or ischaemia/reperfusion (Okumura et al., 2003; Okumura et al., 2004; Yin et al., 2004).

The proto-oncogene jun-B which is a known SRF target gene is transiently up-regulated after cardiac hypertrophy stimuli (Parker and Schneider, 1991; Ricci et al., 2005; Rockman et al., 1991) and induced in cardiac myocytes exposed to hypoxia (Webster et al., 1993) and ischaemia/reperfusion (Brand et al., 1992). However, there is conflicting data regarding the SRF target gene fra-1 upon cardiac hypertrophy stimuli. One group demonstrated no induction of fra-1 following aortic banding (Rockman et al., 1991) and another study observed both up-regulation and down-regulation of fra-1 expression in cardiomyocytes in vitro depending on the stimulus (van Wamel et al., 2000). Fra-1 knockout mice subjected to aortic banding have a normal hypertrophic response whereas over-expression of fra-1 lead to premature heart failure (Ricci et al., 2005).

These MRTF-dependent genes were examined by real-time quantitative RT-PCR following 24 hours of ms1 over-expression and as shown in figure 4.7 were all significantly up-regulated in H9c2 cells over-expressing ms1 compared to cells expressing empty vector control. Tissue factor and adrenomedullin were up-regulated by approximately 1.5 fold. Jun-B and fra-1 were increased approximately 2 fold in cells over-expressing ms1 and LIF and IL-6 were up-regulated by approximately 2.5 fold and 6 fold, respectively, following ms1 over-expression. These findings demonstrate that ms1 again appears to affect genes involved in cardiac hypertrophy and cell survival and implicates these MRTF-dependent genes as target genes of ms1. It is feasible that ms1 regulates these genes via endogenous MRTFs possibly present in H9c2 cells. The MRTF-dependent genes jun-B and fra-1 are known SRF target genes and as a result further provides evidence that ms1 activates SRF-dependent transcription via the Rho-MRTF pathway.

4.3.4 ms1 appears to affect SRF target genes associated with cardiac hypertrophy and cell survival

The evidence so far suggests that ms1 affects many genes known to be involved in cardiac hypertrophy and cell survival and ms1 affects MRTF-dependent genes also known to be involved in hypertrophy and cell survival further supporting a role for ms1 in cardiac hypertrophy and cardioprotection. ms1 may regulate these genes via the MRTF-SRF pathway where ms1 induces the nuclear accumulation of MRTFs and they
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al., 2003; Selvaraj and Prywes, 2004) and as shown in figure 4.4 and 4.7 they were up-regulated following ms1 over-expression. Collectively, these findings suggest that ms1 may regulate cardiac α-actin, jun-B, fra-1 and adrenomedullin via the MRTF-SRF pathway.

One conserved CArG sequence was found in the ms1 target gene LIF. This SRF binding site has not been reported elsewhere and therefore was identified as a novel CArG sequence. However, LIF has not been experimentally validated as a SRF target gene and was therefore considered as a probable target of SRF. IL-6 contained two CArG sequences that were conserved in mouse and rat but not in human. LIF and IL-6 were experimentally validated MRTF-dependent genes (Selvaraj and Prywes, 2004) and hence ms1 may regulate LIF and IL-6 via MRTF that involves an unknown mechanism or via the MRTF-SRF pathway.

BNP and MEF2C both contained one conserved SRF binding site approximately -200 bp from the TSS (according to Ensembl) where the SRF binding site in BNP has been experimentally validated (Nelson et al., 2005). The SRF binding site in MEF2C was a novel finding as this CArG sequence has not been reported to date in the literature and will require experimental validation to confirm that it is a SRF binding site. BNP and MEF2C dependency on MRTFs have not been reported to date. Both BNP and MEF2C were increased in cells over-expressing ms1 (figure 4.4) and therefore ms1 could regulate both via SRF especially BNP as BNP is a known SRF target gene (Nelson et al., 2005). This mechanism may or may not involve MRTFs.

The possible ms1 target gene myocardin contained one conserved SRF binding site when analysed using DCODE.org and this site was observed previously (Miano, 2003; Wang et al., 2001b). This suggests that myocardin may be a target of SRF and hence regulated by the ms1-MRTF-SRF pathway. Myocardin like MRTFs strongly associates with SRF to enhance the expression of SRF dependent genes (Wang et al., 2001b). Therefore myocardin is an unlikely target of MRTFs but could be a target of ms1-SRF where MRTFs are not involved.

SRF contained four conserved SRF binding sites which have been identified by others (Selvaraj and Prywes, 2004; Sun et al., 2006) and it is possible that SRF may regulate its own expression (Spencer and Misra, 1999). SRF was identified as a MRTF-dependent gene (Selvaraj and Prywes, 2004) and was up-regulated following transient
(24 hours) msl over-expression, suggesting that msl via MRTF-SRF may regulate SRF. msl itself was found to contain a conserved SRF binding site approximately -300 bp relative to the TSS as shown in table 4.1 and it has been demonstrated by chromatin immunoprecipitation assays performed with formaldehyde cross-linked chromatin isolated from feline adult cardiomyocytes that SRF binds to this site in the msl promoter (Ounsain, personnel communication), raising the possibility that msl is a SRF target, thereby creating a feedback loop (figure 4.9).

Many of the putative downstream targets of msl contain SRF binding sites (SRF target genes), were identified as MRTF-dependent genes and are associated with cardiac hypertrophy and cell survival pathways. Therefore msl appears to affect genes involved in cardiac hypertrophy and cell survival suggesting that msl may play a role in the development of hypertrophy and may provide cardioprotection via a MRTF-SRF signalling pathway.

Figure 4.9. A proposed model to describe how msl may regulate MRTF-SRF target genes.

Putative target genes of msl may be regulated via the MRTF-SRF pathway. msl itself could be a SRF target gene, thus creating a feedback mechanism.
Chapter 5

Biological effects of msl over-expression in vitro

5.1 Introduction

In chapter 4 it was demonstrated that msl appears to affect genes involved in cardiac hypertrophy and affects genes known to protect against apoptotic cell death which possibly occurs via a MRTF-SRF signalling mechanism. These findings along with other findings of msl’s rapid induction in LVH and during ischaemia-reperfusion (discussed in chapter 1) suggest that msl may play a role in the development of hypertrophy and provide cardioprotection. Hence, the work in this chapter directly examined the role of msl in cardiac cellular hypertrophy and cardioprotection in vitro.

As explained in chapter 1 cardiac hypertrophy is usually accompanied by complex changes in gene reprogramming. In addition to these changes in the patterns of gene and protein expression, the defining characteristics of hypertrophy are an increase in cell size of cardiac myocytes, enhanced protein synthesis and increased myofibrillar assembly (Frey and Olson, 2003; Sugden and Clerk, 1998). An increase in cell size can be monitored by an enlargement in cell size where the cross-sectional area of the cell is measured (Hunter et al., 1995). Therefore, the first aim of this study was to investigate whether msl leads to hypertrophy by measuring an increase in the size of H9c2 cells following msl over-expression in vitro.

The second aim sought to determine whether msl can provide cardioprotection and protect H9c2 cells from apoptotic cell death. Apoptosis was a term first coined by Kerr et al. (1972) and proposed as a mechanism of controlled cell deletion, which occurs in two stages. The first is the formation of apoptotic bodies and the second is their phagocytosis and degradation by other cells. Apoptosis is defined as programmed cell death recognised by morphological and biochemical changes distinct from necrosis or accidental cell death (Arends and Wyllie, 1991). In apoptosis because the nucleus and cytoplasm shrink and often fragments, and the cells or fragments are phagocytosed, the contents of the cells do not leak into the extracellular space and therefore there is no inflammation. However, necrosis results from cell injury. The cells swell and lyse and the cell’s contents spill into the extracellular space, inducing an inflammatory response (Kerr et al., 1972; Raff, 1992). For cells to survive and not undergo apoptosis continuous signalling by cytokines, cell-cell contacts, cell-matrix interactions, growth
factors and hormones are required. Defects in cell death signalling, in membrane or cytoplasmic receptors or alterations in the genes that govern apoptosis have been implicated in the pathogenesis of cancer and autoimmune, neurodegenerative, and cardiovascular diseases (Thompson, 1995). In the cardiovascular system, apoptosis plays an essential role in homeostasis and development but also in the death of myocytes in animal models of myocardial ischaemia (Gottlieb et al., 1994), in models of pressure overload (Teiger et al., 1996), in aged spontaneously hypertensive rats (Li et al., 1997b), in humans with acute myocardial infarction (Saraste et al., 1997) and in congestive heart failure (Narula et al., 1996).

In summary, the aim of this chapter was to elucidate a role for ms1 in cardiac hypertrophy and cardioprotection by transiently over-expressing ms1 in vitro and examining cell size and the ability of ms1 to protect against apoptotic cell death.

5.2 Results

5.2.1 Cell size quantification of ms1 over-expressing cells by microscopy

The cell size of individual H9c2 cells over-expressing ms1 versus untransfected cells (ms1 untransfected cells following transfection of the ms1 expression vector), empty vector transfected cells and untreated cells were initially examined by immunofluorescence microscopy using the software Volocity 4 (Improvision) that allows the cell area of individual cells to be measured. H9c2 cells were left untreated or transfected with the ms1 expression vector or empty vector and co-stained with c-Myc FITC antibody and Alexafluor 350 phalloidin to detect ms1 and actin, respectively. The phalloidin antibody was used so that the whole area of the cell was visualised and hence measured. Transient transfection (72 hours) of the ms1 expression vector gave rise to a mixed population of untransfected cells and cells over-expressing ms1. The software identified c-Myc FITC labelled cells (ms1 transfected cells) that appeared green and calculated the area of each cell also marked by phalloidin (actin) staining versus untransfected cells (not green) marked by phalloidin staining. However, quantification of cell size using this method was problematic because the cells were confluent following 72 hours in culture after the transfection and therefore the software could not accurately measure the area of each individual cell as the cells were in contact with other cells or overlapping each other. Even following 24 hours of ms1 over-expression when the cells were less confluent the cell area was difficult to quantify accurately using
Biological effects of ms1 over-expression in vitro

the software because the cells still maintained cell to cell contact. Also the transfection efficiency was low which meant many cells must be counted to obtain accurate numbers and this would be very time consuming. Due to these reasons it was decided to use the flow cytometer which can measure the size of thousands of individual cells on the basis of light scatter parameters where the forward-angle light scatter relates to the cell size (Brown and Wittwer, 2000). Furthermore, the flow cytometer can rapidly analyse multiple characteristics of thousands of single cells, thus overcoming low transfection efficiencies and allowing different parameters to be measured.

5.2.2 Cell size quantification of ms1 over-expressing cells by flow cytometry

H9c2 cells were left untreated or transfected with the ms1 expression vector or the empty vector control for either 24 hours or 72 hours and then collected, fixed, permeabilised and stained with the c-Myc FITC antibody to detect ms1 over-expressing cells. Transient transfection of the ms1 expression vector will give rise to a percentage of cells transfected with the ms1 expression vector (ms1 transfected cells) and a percentage of cells will remain untransfected unlike stable transfections which is the formation of a stable cell line were all clonal descendants express the construct. This will also occur following transient transfection of the empty vector control which will give rise to a population of empty vector transfected cells and untransfected cells. The empty vector (pcDNA3.1(+)) does not contain a c-Myc tag or any other tag to distinguish empty vector transfected cells from untransfected cells and therefore was only included as a control to show there were no cells labelled with the c-Myc FITC antibody and hence over-expressing ms1. Using the flow cytometer the cells were analysed by forward scatter and side scatter parameters and displayed on a histogram as shown in figure 5.1 (a), (e) and (i). The whole cell population was gated (gate A in figure 5.1 (a), (e) and (i)) and the c-Myc FITC fluorescence of individual cells from this population was measured and registered on a logarithmic scale, see figure 5.1 (b), (f) and (j). The histogram displayed a population of cells that were not labelled with the c-Myc FITC antibody and a population of cells that were labelled.
Figure 5.1. Quantification of the size of cells over-expressing msl by flow cytometry.

H9c2 cells were left untreated or transiently transfected with the empty vector control (pcDNA3.1(+)) or the msl expression vector (pcDNA3.1(+) + msl), stained with c-Myc FITC antibody to detect msl over-expressing cells (gate C in (b), (f) and (j)) and analysed for cell size (gate F in (c), (g) and (k) and gate G in (d), (h) and (l)) by flow cytometry.
The untreated and empty vector control samples were first analysed because these cells were not over-expressing ms1 and hence not labelled with the c-Myc FITC antibody. The histogram displayed a single population of untreated cells not labelled with c-Myc FITC fluorescence which was gated in gate E as shown in figure 5.1 (b) and this confirmed there were no cells over-expressing ms1. As shown in figure 5.1 (f) a single population of cells were displayed on the histogram that contained empty vector transfected cells and untransfected cells which was gated in gate E. Empty vector transfected cells could not be gated separately from untransfected cells because there was no technique to identify empty vector transfected cells. There was no additional population of cells labelled with c-Myc FITC fluorescence which confirmed there were no cells over-expressing ms1. The ms1 expression vector sample was then analysed where within that population those cells that were not labelled with the c-Myc FITC antibody (untransfected cells) were gated in gate E (figure 5.1 (j)) and those cells that were labelled with the c-Myc FITC antibody (ms1 transfected cells) were gated in gate C (figure 5.1 (j)). The number of cells in gate C (cells over-expressing ms1) and the number of untransfected cells in gate E (figure 5.1 (j)) were counted and recorded as percentages thus allowing the transfection efficiency to be determined. From these two populations in gates C and E the cell size (forward scatter) of single cells was measured and displayed on a linear scale as shown in figure 5.1 (c), (d), (g), (h), (k) and (l). The data were recorded as the median thus allowing the median cell size of untransfected cells from gate G as shown in figure 5.1 (l) versus the median cell size of ms1 transfected cells from gate F (figure 5.1 (k)) to be compared.

5.2.3 Cell size analysis following 24 and 72 hours of ms1 over-expression

The cell size of single H9c2 cells following no treatment or transient transfection of the empty vector or the ms1 expression vector for 24 and 72 hours were examined by flow cytometry. As shown in figure 5.2 when cells were transfected with the ms1 expression vector, 13.0% ± 0.5% of cells were positive for c-Myc and hence were ms1 transfected cells that over-expressed ms1 compared to 86.9% ± 0.5% untransfected cells. This finding was similar at both 24 hour and 72 hour transfections. This low transfection efficiency of approximately 13% was observed previously in chapter 3 using immunofluorescence microscopy; however, because approximately 10,000 cells were analysed by flow cytometry, 13% of cells was equivalent to approximately 1300 cells quantified that over-expressed ms1.
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Figure 5.2. 13.0\% \pm 0.5\% of H9c2 cells over-expressed msl following transfection for 72 hours when analysed by flow cytometry.

H9c2 cells were transiently transfected with the msl expression vector (pcDNA3.1(+) + msl), stained with c-Myc FITC antibody and analysed using a flow cytometer for the percentage of cells over-expressing msl (msl transfected cells) detected by c-Myc FITC fluorescence. Bars show median $\pm$ SD, n = 4.

There was no change in the size of cells over-expressing msl (msl transfected cells) compared to untransfected cells, empty vector transfected and untransfected cells (following transfection of the empty vector, pcDNA3.1(+) or untreated cells following 24 hours (figure 5.3 (a)). Thus, over-expression of msl for 24 hours does not appear to alter cell size. However, there was a significant approximate 1.5 fold increase in cell size following 72 hours of msl over-expression (in msl transfected cells) compared to untransfected cells ($P < 0.01$), empty vector transfected and untransfected cells following transfection of the empty vector, pcDNA3.1(+) ($P < 0.01$) and untreated cells ($P < 0.01$) as shown in figure 5.3 (b). Thus, the size of H9c2 cells appears to increase following 72 hours of msl over-expression.
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Figure 5.3. Cell size following msl over-expression for 24 and 72 hours.

Flow cytometric analysis of the size of cells following no treatment (untreated) or transient transfection of the empty vector (pcDNA3.1(+) or the msl expression vector (pcDNA3.1(+) + msl) for (a) 24 hours and (b) 72 hours. The median size of msl transfected cells, untransfected cells, empty vector transfected and untransfected cells (pcDNA3.1(+) and untreated cells were quantified and the fold change in cell size was calculated relative to control (untransfected cells). Bars show mean ± SD from 4 separate experiments. *P < 0.01.
5.2.4 *Is the cell size increase following 72 hours of msl over-expression due to cell proliferation?*

H9c2 cells proliferate in culture and therefore the increase in cell size following 72 hours of msl over-expression could have been due to the cells increasing in size prior to cell division rather than hypertrophy of the cell (increase in cell size without associated increases in cell number). To investigate whether the size of cells over-expressing msl increased due to cell proliferation or hypertrophy, cell proliferation following 72 hours of msl over-expression was examined by flow cytometry.

The number of cells that are dividing in culture is known as cell proliferation and once the cell is instructed to divide, it enters the active phase of the cell cycle. The concept of the cell cycle and its subdivision into several phases was first introduced during the early 1950s (Hall and Levison, 1990; Howard and Pelc, 1951; Howard and Pelc, 1953). During the G₀/G₁ phase of cell cycle, the cell prepares to synthesise DNA. The cell undergoes DNA synthesis and replicates its genome during the S phase of the cell cycle. During the G₂ phase the cell prepares to undergo division and the cell undergoes division during the M phase and then re-enters G₀/G₁. DNA synthesis is often used as a marker of cell proliferation and can be measured in several ways including tritiated thymidine uptake and incorporation into DNA during the S phase (Kvaloy *et al.*, 1981; Kvaloy *et al.*, 1985) or by the incorporation of a thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Dean *et al.*, 1984; Dolbeare *et al.*, 1983; Gratzner, 1982). Both methods quantify the amount of labeled precursor incorporated into DNA which is directly proportional to the amount of cell division occurring in the culture. Flow cytometry using dyes that can bind to DNA such as propidium iodide can measure DNA content, thus, allowing the S phase fraction of the cell cycle to be determined (Braylan *et al.*, 1980; Lee *et al.*, 1992; Timm *et al.*, 1998). Cell proliferation can also be measured where molecules that regulate the cell cycle are measured either by their activity for example cdc2 kinases (Draetta *et al.*, 1988; Draetta and Beach, 1988) or by using antibodies that recognise proliferating cells such as ki67 (Gerdes *et al.*, 1983; Gerdes *et al.*, 1984; Verheijen *et al.*, 1989a; Verheijen *et al.*, 1989b). All these methods to assess cell proliferation have their advantages and disadvantages. It was initially decided to use flow cytometry using propidium iodide to measure the fraction of cells with S phase DNA content by DNA distribution analysis as a marker of proliferation in cells over-expressing msl versus untransfected cells. This method had advantages over
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other methods because thousands of cells can be analysed using the flow cytometer which was required because of the low transfection efficiency. Also propidium iodide can measure apoptosis (sub-G\textsubscript{1}) (Nicoletti \textit{et al.}, 1991; Telford \textit{et al.}, 1991; Telford \textit{et al.}, 1992) and all the cell cycle phases at the same time.

H9c2 cells were left untreated or transfected with the ms1 expression vector or empty vector control for 72 hours and then collected, fixed, permeabilised and stained with the c-Myc FITC antibody to detect ms1 over-expressing cells and propidium iodide to stain DNA content. When analysed by the flow cytometer, the cells did not take up propidium iodide. Cells are usually fixed in ice cold 70\% ethanol prior to the addition of propidium iodide and when this method was used, the cells did take up propidium iodide. However, in order to detect ms1 over-expressing cells using the c-Myc FITC antibody (that recognises the c-Myc tag on ms1), the cells were fixed and permeabilised prior to the addition of the antibody using a different method to the one used with propidium iodide, where the fixative was formaldehyde based. Although this method detected ms1 over-expressing cells it was not compatible with propidium iodide to stain DNA content. An alternative to propidium iodide is the Vybrant DyeCycle Violet Stain which stains DNA in the same way as propidium iodide. Vybrant DyeCycle Violet Stain can be used on unfixed or fixed cells and was compatible with the fixation and permeabilisation method used to detect ms1 over-expressing cells by the c-Myc FITC antibody. In brief, cells were transfected and following 72 hours the cells were collected, fixed, permeabilised and stained with the c-Myc FITC antibody to detect ms1 over-expressing cells and stained with Vybrant DyeCycle Violet Stain to detect DNA content. The Beckman Coulter Epics XL-MCL flow cytometer that was originally used to measure cell size does not have the correct fluorescence detector to identify the cells stained with the Vybrant DyeCycle Violet Stain and therefore cells were measured using a DakoCytomation CyAn ADP flow cytometer and analysed using Summit v4.3 software in subsequent experiments.

The cells were analysed as described previously in section 5.2.2 except that the whole cell population gated in R1 was also gated through another gate R2 where the R2 gate collected single cells stained with Vybrant DyeCycle Violet Stain (figure 5.4). The c-Myc FITC fluorescence of single cells from cells in gate R2 was measured and displayed on a logarithmic scale as shown in figure 5.4 (c), (g) and (k). Cells that were not over-expressing ms1 and hence not labelled with the c-Myc FITC antibody
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(untreated cells, empty vector transfected cells and untransfected cells following transfection of the empty vector and cells exposed to the transfection of the msl expression vector but remained untransfected) were gated in gate R8 (figure 5.4 (c), (g) and (k)). Cells over-expressing msl and therefore labelled with the c-Myc FITC antibody were gated in gate R9 (figure 5.4 (k)). The cell cycle distribution within these two populations in gates R8 and R9 was measured and displayed on a linear scale as shown in histograms (d), (h), (l) and (m) of figure 5.4. The percentages of cells in each cell cycle phase was recorded where the cells in gate R13/R16 represented the percentage of apoptotic cells (sub-G1), cells in gate R15/R14 represented cells in G0/G1 phase, cells in gate R19/17 represented cells in S phase (DNA synthesis) and the percentage of cells in gate R22/R23 represented cells in G2/M phase of the cell cycle. Cells that were not exposed to the transfection method (untreated cells) were included as a control to set the gates for each phase of the cell cycle (figure 5.4 (d)). Thus, the percentage of cells in each phase of the cell cycle in particular the S phase (marker of proliferating cells) was compared for those cells that over-expressed msl and those cells that did not.
Figure 5.4. Quantification of cell proliferation following msl over-expression.

Cells were left untreated or transfected with the empty vector control (pcDNA3.1(+)) or the msl expression vector (pcDNA3.1(+) + msl), stained with c-Myc FITC antibody to detect msl over-expressing cells (gate R9 in (e), (g), (k)) and Vybrant DyeCycle Violet Stain, to analyse S phase DNA content as a marker of proliferation ((d), (h), (l), (m)) by flow cytometry.
As shown in figure 5.5, after 72 hours of transfection there was no significant change in the number of cells over-expressing ms1 in S phase of the cell cycle (16.4% ± 4.0%) versus untransfected cells in S phase (15.8% ± 2.1%) or empty vector transfected and untransfected cells in S phase following transfection of the empty vector, pcDNA3.1(+) (13.7% ± 1.7%). However, there was a significant increase ($P < 0.05$) in the number of cells in S phase following transfection of the ms1 expression vector or empty vector control compared to untreated cells (9.3% ± 1.0%).

Figure 5.5. No change in the number of ms1 transfected cells in S phase versus untransfected cells in S phase following transfection of the ms1 expression vector for 72 hours.

Flow cytometric analysis of the percentage of cells in S phase following no treatment (untreated) or transient transfection of the empty vector (pcDNA3.1(+) or the ms1 expression vector (pcDNA3.1(+) + ms1) for 72 hours. Bars show mean ± SD from 4 separate experiments. *$P < 0.05$.

During the G2/M phase of the cell cycle the cell enters mitosis and divides into two similar daughter cells. Thus, the number of cells in the G2/M phase of the cell cycle that over-expressed ms1 was compared to the number of cells in the G2/M phase that did not over-express ms1. As shown in figure 5.6, after 72 hours of transfection there was no significant increase in the number of cells in the G2/M phase of the cell cycle that over-expressed ms1 (9.3% ± 2.1%) versus untransfected cells (7.6% ± 2.4%) or empty vector transfected and untransfected cells following transfection of the empty vector, pcDNA3.1(+) (8.6% ± 2.1%). However, the number of cells in S phase significantly
increased ($P < 0.05$) following transfection of the ms1 expression vector or empty vector control compared to untreated cells (3.5% ± 0.8%). Thus, it appears that the transfection itself of the empty vector or the ms1 expression vector increases the number of cells in S phase or G2/M phase but over-expression of ms1 for 72 hours does not appear to alter the number of cells in S phase or G2/M phase of the cell cycle compared to cells that did not over-express ms1 following transfection.

![Graph showing cell percentages in G2/M phase](image)

Figure 5.6. The number of ms1 transfected cells in G2/M phase did not increase compared to the number of untransfected cells in G2/M phase following transfection of the ms1 expression vector for 72 hours.

The number of H9c2 cells in G2/M phase following no treatment (untreated) or transient transfection of the empty vector (pcDNA3.1(+)) or the ms1 expression vector (pcDNA3.1(+) + ms1) for 72 hours was examined by flow cytometry. Bars show mean ± SD from 4 separate experiments. *$P < 0.05$.

In addition, cellular proliferation of cells that over-expressed ms1 compared to those that did not over-express ms1 was quantified by direct cell counting using a haemocytometer. There was no significant difference in the number of cells between the two transfected groups following 72 hours of transfection (figure 5.7). A significantly greater ($P < 0.01$) number of cells in the untreated group were observed probably due to the known cytotoxic effect of the transfection reagent. Therefore, over-expression of ms1 does not appear to increase cell proliferation.
Figure 5.7. Over-expression of ms1 for 72 hours did not increase the proliferation of H9c2 cells.

Viable cells were identified by trypan blue exclusion and counted using a haemocytometer following no treatment (untreated) or transient transfection of the empty vector (pcDNA3.1(+)) or the ms1 expression vector (pcDNA3.1(+) + ms1) for 72 hours. Bars show mean ± SD from 4 independent experiments. *P < 0.01.

5.2.5 Detection of apoptosis

As discussed earlier in section 5.1, apoptosis is a distinct form of cell death characterised by a sequence of morphological events that include cell shrinkage, cell shape change, condensation of the cytoplasm, nuclear envelope changes, nuclear fragmentation, formation of apoptotic bodies and eventual cell disintegration (Kerr et al., 1972; Wyllie, 1980). It was discovered that the percentage of cells over-expressing ms1 was low and hence quantification of apoptosis by microscopy would be rather cumbersome. To overcome this, the flow cytometer was used because of the advantage that it can analyse multiple characteristics of many thousands of single cells. As explained in section 5.2.4 it was found that the Vybrant DyeCycle Violet Stain could be used as an alternative to propidium iodide to stain DNA content of cells and therefore flow cytometry using this stain was the method chosen to measure apoptosis. Cell cycle analysis based on measurements on DNA content generates a clear distribution consisting of the G0/G1 phase of cell cycle, the S phase and the G2/M phase. During apoptosis the DNA of the cell fragments and following fixation and permeabilisation, the low molecular weight DNA inside the cytoplasm of apoptotic cells elutes during the
wash procedure. Therefore, following staining with the violet dye, cells with lower DNA content contain less DNA stained and thus cells with lower DNA staining than that of G1 cells show a sub-G1 peak and are considered apoptotic (Nicoletti et al., 1991; Telford et al., 1991; Telford et al., 1992). Other flow cytometric assays that measure apoptosis were considered such as Annexin V; however, could not be used because Annexin V can not be used on fixed samples and to detect the percentage of cells over-expressing ml using the c-Myc antibody, the cells had to be fixed and permeabilised prior to the addition of the antibody.

5.2.6 Analysis of apoptosis in H9c2 cells by DNA fragmentation (sub-G1)

5.2.6.1 Hydrogen peroxide (H2O2) treatment to induce apoptosis

Apoptosis is increased during oxidative stress and is involved in several cardiovascular diseases (Han et al., 2004). Hydrogen peroxide (H2O2) is a well known oxidant that has been used to induce apoptosis in H9c2 cells (Chen et al., 2000; Gupta et al., 2006; Gustafsson et al., 2004; Han et al., 2004; Hou and Hsu, 2005; Neuss et al., 2001; Pesant et al., 2006; Tanaka et al., 2003; Turner et al., 1998; Zhang and Herman, 2006) and was therefore used to induce apoptosis following transfection of the ml expression vector or empty vector control in H9c2 cells, to identify whether over-expression of ml can protect against H2O2 induced apoptosis.

Different concentrations of H2O2 (500 μM – 800 μM) was added to H9c2 cells for 24 hours to first determine the amount of H2O2 that caused H9c2 cells to undergo apoptosis. Cells were collected, fixed, permeabilised and stained with the c-Myc FITC antibody and Vybrant DyeCycle Violet Stain following 24 hours after H2O2 treatment. The cells were analysed using a DakoCytomation CyAn ADP flow cytometer as described previously in section 5.2.4 except that the gates R1, R2, R8 and R9 were extended to collect apoptotic cells, see figure 5.8 (a-c), (e-g) and (i-k).

The percentage of cells in each phase of the cell cycle was recorded where the cells in gate 13 (sub-G1 phase) represent the percentage of apoptotic cells (figure 5.8 (d), (h) and (l). The percentage of apoptotic H9c2 cells were quantified following treatment with various concentrations of H2O2 for 24 hours. As shown in figure 5.9 very few H9c2 cells were apoptotic following treatment with 500 μM (1.5% ± 0.2%) or 600 μM H2O2 (4.5% ± 0.6%) for 24 hours. With 700 μM H2O2 or more, cells appeared very apoptotic with condensed nuclei; however, cells would not pellet following centrifugation in order
to collect the cells probably because the cells were too fragmented and thus could not be analysed using the flow cytometer. Treatment of H9c2 cells with 500 µM or 600 µM H₂O₂ for 24 hours did not cause a sufficient amount of apoptosis and amounts above 600 µM H₂O₂ caused too much cell fragmentation where the cells could not be collected and therefore H₂O₂ was not used further to study apoptosis.
Figure 5.8. Quantification of H₂O₂ induced apoptosis in H9c2 cells by flow cytometry.

Cells were left untreated or treated with 500 µM H₂O₂ or 600 µM H₂O₂ for 24 hours, stained with c-Myc FITC antibody and Vybrant DyeCycle Violet Stain and analysed by flow cytometry for the percentage of apoptotic cells based on the sub-G₁ phase DNA content (gate R13 in (d), (h) and (l)).
Figure 5.9. Quantification of apoptosis following 24 hours of H$_2$O$_2$ treatment in H9c2 cells.

The percentage of apoptotic cells (sub-G$_1$ phase) following treatment with H$_2$O$_2$ for 24 hours was quantified using flow cytometry. Bars show mean ± SD from 3 independent experiments.

5.2.6.2 Staurosporine treatment to induce apoptosis

Another frequently used agent for the induction of apoptosis is staurosporine. Nearly all mammalian cells are able to become apoptotic when treated with staurosporine and it has been consistently shown that staurosporine-induced apoptosis involves cytochrome c release and results in activation of caspases that is prevented by over-expression of Bcl-2 (Bertrand et al., 1994; Chen et al., 2000; Gustafsson et al., 2004; Jacobsen et al., 1996; Jacobson et al., 1994; Neuss et al., 2001; Takahashi et al., 1997; Turner et al., 1998). Even though staurosporine is frequently used to induce apoptosis, the exact mechanism of staurosporine-induced apoptosis is unknown. Many important mechanisms involved in the apoptotic process have been demonstrated in staurosporine-induced apoptosis for example the activation of caspase 8 and Bid cleavage (Stepczynska et al., 2001) and the activation of the downstream effector caspase 3 (Stepczynska et al., 2001; Takahashi et al., 1997; Yue et al., 1998). Staurosporine was shown to induce apoptosis in cardiac myocytes (Yue et al., 1998) and in H9c2 cells (Ekhterae et al., 2003; Hou and Hsu, 2005; Lee et al., 2006; Mao et al., 2004)
demonstrating that staurosporine-induced apoptosis is a useful model to investigate apoptosis in mammalian cells and was therefore used as a substitute for H$_2$O$_2$.

H9c2 cells were treated with various concentrations of staurosporine (5 nM - 25 nM) for 24 hours and assayed as described above to quantify the percentage of cells that displayed apoptosis. As shown in figure 5.10 (a) the size of the sub-G$_1$ peak increased as the amount of staurosporine added to H9c2 cells increased and when the percentage of apoptotic cells under the sub-G$_1$ peak were quantified the amount of apoptotic cells increased with increasing concentrations of staurosporine. 28.5% ± 1.7% of cells were apoptotic following treatment with 5 nM staurosporine for 24 hours. With 15 nM staurosporine, 39.4% ± 1.1% of cells were apoptotic and just under half of the cells (44.5% ± 2.4%) displayed apoptosis following treatment with 25 nM staurosporine for 24 hours (figure 5.10 (b)). This demonstrated that flow cytometry using the Vybrant DyeCycle Violet Stain to detect DNA fragmentation (apoptotic cells present in the sub-G$_1$ phase) was a valid assay for detecting apoptosis in H9c2 cells when treated with various concentrations of staurosporine (5 nM - 25 nM) for 24 hours.
Biological effects of ms1 over-expression in vitro

Figure 5.10. Quantification of staurosporine induced apoptosis in H9c2 cells by flow cytometry.

H9c2 cells were left untreated or treated with staurosporine (5 nM - 25 nM) for 24 hours, stained with c-Myc FITC antibody and Vybrant DyeCycle Violet Stain to detect apoptotic cells (sub-G1 phase) by flow cytometry (a). (b) The percentage of apoptotic cells based on the sub-G1 phase DNA content (gate R13 in (a)) was quantified. The results are the mean ± SD from 3 independent experiments.
5.2.7 Analysis of staurosporine-induced apoptotic cell death following ms1 over-expression

The ability of ms1 to protect H9c2 cells against staurosporine-induced apoptotic cell death was examined by flow cytometry. Following transfection for 24 hours, cells were treated with 5 nM staurosporine for 24 hours. Transfection was performed for 24 hours because the majority of genes (in particular the MRTF-dependent genes) associated with cell survival pathways were up-regulated following ms1 over-expression for 24 hours. Although concentrations of 5 nM – 25 nM staurosporine all induced apoptosis (figure 5.10) in H9c2 cells, 5 nM staurosporine was the concentration used in further experiments as this concentration induced apoptosis that was consistently measured using flow cytometry mainly because the amount of apoptosis was substantial and hence the sub-G1 peak was not too large which enabled the amount of apoptosis in this peak to be quantified without difficulty. Following treatment with 5 nM staurosporine for 24 hours, cells were assayed as described in sections 5.2.4 and 5.2.6 where the percentage of cells over-expressing ms1 present in the sub-G1 phase (apoptotic cells) was compared against the percentage of apoptotic cells that did not over-express ms1.

As shown in figure 5.11, treatment with 5 nM staurosporine for 24 hours to untreated cells (unexposed to transfection) resulted in 26.8% ± 3.5% of cells being apoptotic versus 0.6% ± 0.1% of apoptotic cells following no staurosporine treatment (P < 0.01). Treatment with 5 nM staurosporine for 24 hours to cells following transfection with the empty vector resulted in 32.4% ± 3.8% apoptotic cells versus 2.1% ± 0.7% of apoptotic cells following no staurosporine treatment (P < 0.01). In untransfected H9c2 cells within the population of cells transfected with the ms1 expression vector, 1.6% ± 0.5% of cells underwent apoptosis under control conditions. Treatment with 5 nM staurosporine for 24 hours markedly increased the percentage of cells undergoing apoptosis to 29.0% ± 2.9% (P < 0.01). Less than 1% of ms1 transfected cells (0.7% ± 0.3%) underwent apoptosis following no treatment with staurosporine and this was significantly less than the amount of apoptotic untransfected cells (P < 0.05) and apoptotic cells following transfection with the empty vector (P < 0.05) when not treated with staurosporine. With 5 nM staurosporine for 24 hours, 3.5% ± 1.0% of ms1 transfected cells were apoptotic and this increase was significant (P < 0.05) compared to baseline ms1 transfected cells following no treatment. In contrast to the response of cells left untreated (not exposed to transfection), cells following transfection with the
Biological effects of msl over-expression \textit{in vitro}

empty vector and untransfected cells (within the population of cells transfected with the msl expression vector) to 5 nM staurosporine for 24 hours, H9c2 cells transfected with msl displayed very little apoptosis when exposed to the same conditions. Over-expression of msl significantly inhibited staurosporine-induced apoptosis compared with untreated cells (no transfection) ($P < 0.01$), cells following transfection with the empty vector ($P < 0.01$) and untransfected cells ($P < 0.01$). These results suggest that msl over-expression prevents staurosporine-induced apoptotic cell death.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.11.png}
\caption{msl inhibits apoptosis induced by staurosporine in H9c2 cells.}

Cells were left untreated or transfected (24 hours) with the empty vector (pcDNA3.1(+)) or msl expression vector (pcDNA3.1(+) + msl) and treated with or without 5 nM staurosporine for 24 hours, stained with c-Myc FITC antibody and Vybrant DyeCycle Violet Stain to detect the percentage of apoptotic cells (sub-G1 phase) by flow cytometry. The results are the mean ± SD from 4 independent experiments. $*P < 0.05$, $**P < 0.01$, $+P < 0.01$ versus untreated cells, empty vector cells and untransfected cells with staurosporine treatment, $#P < 0.05$ versus empty vector and untransfected cells without staurosporine treatment.

During apoptosis the cell shrinks and this can be distinguished by flow cytometry on the basis of forward angle light scatter which relates to cell size because they bend less light into the forward scatter detector than a viable cell does. Thus, cells undergoing apoptosis should appear “smaller” than living cells in a flow cytometer. The median size of cells over-expressing msl compared to the median size of cells not over
expressing ms1 following treatment with 5 nM staurosporine for 24 hours was also measured.

As shown in figure 5.12, treatment with 5 nM staurosporine for 24 hours to untreated cells (unexposed to transfection) resulted in a significant reduction in cell size ($P < 0.01$). This finding was also observed when cells following transfection with the empty vector and untransfected cells (within the population of cells transfected with the ms1 expression vector) were treated with 5 nM staurosporine for 24 hours. However, the size of cells over-expressing ms1 following staurosporine treatment was similar to the size of cells that over-expressed ms1, the size of untransfected cells, the size of cells following transfection with the empty vector and the size of untreated cells with no staurosporine treatment. Cells over-expressing ms1 were approximately twice as large in cell size compared to untransfected cells ($P < 0.01$), cells following transfection with the empty vector ($P < 0.01$) and untreated cells (not exposed to transfection) ($P < 0.01$), subsequent to treatment with staurosporine. These findings suggest that the size of cells over-expressing ms1 remain unaltered following treatment with staurosporine.
Biological effects of msl over-expression in vitro

![Graph showing fold change in cell size](image)

Figure 5.12. msl prevents a reduction in the size of H9c2 cells induced by staurosporine.

The size of H9c2 cells was examined by flow cytometry following no treatment (untreated) or transient transfection of the empty vector (pcDNA3.1(+)) or the msl expression vector (pcDNA3.1(+)+msl) for 24 hours with or without 5 nM staurosporine for 24 hours. The median size of msl transfected cells, untransfected cells, empty vector transfected and untransfected cells (pcDNA3.1(+)) and untreated cells were quantified and the fold change in cell size was calculated relative to control (non treated untransfected cells). The results are the mean ± SD from 4 independent experiments. *P < 0.01.

5.3 Discussion

The main findings were:

1. msl increases the size of H9c2 cells without an increase in cell proliferation.
2. msl protects H9c2 cells from staurosporine-induced apoptosis.

5.3.1 msl increases the size of H9c2 cells without an increase in cell proliferation

In order to investigate the role of msl in cardiac hypertrophy, the size of cells over-expressing msl versus the size of cells that did not over-express msl were initially examined by immunofluorescence microscopy where the cell area of individual cells were measured. H9c2 cells were transfected with the msl expression vector and co-
stained with c-Myc FITC antibody and phalloidin to detect ms1 and actin, respectively. Phalloidin staining enabled the area of each cell to be visualised and hence measured. The transfection efficiency of H9c2 cells transfected with the ms1 expression vector for 24 hours or 72 hours was low and gave rise to a mixed population of untransfected cells and ms1 expressing cells. The low transfection efficiency meant quantification of cell size by microscopy would be time consuming. It was also discovered that H9c2 cells were confluent after 72 hours in culture and cells maintained cell to cell contact whether confluent or not, thus accurate measurement of cell size was impossible. To overcome these problems flow cytometry was used which allowed the cell size of many thousands of individual cells to be measured on the basis of light scatter parameters.

Following 24 hours or 72 hours of transfection with the ms1 expression vector, the transfection efficiency of H9c2 cells transfected with the ms1 expression vector and hence over-expressing ms1 was 13.0% ± 0.5% (figure 5.2) and equivalent to approximately a thousand cells that over-expressed ms1. Following 72 hours of transfection with the ms1 expression vector, the size of cells over-expressing ms1 were significantly increased by approximately 1.5 fold (figure 5.3 (b)) when compared to untransfected cells. However, there was no change in the size of cells over-expressing ms1 following a 24 hour transfection (figure 5.3 (a)) probably because 24 hours was too early to detect any changes in cell size.

The size of H9c2 cells increased following a longer time (72 hours) of ms1 over-expression and it was demonstrated in chapter 4 that expression of hypertrophic genes was up-regulated following ms1 over-expression for 72 hours and not following 24 hours. It was also demonstrated that ms1 appears to affect genes involved in cardiac hypertrophy that are MRTF-dependent genes and/or SRF target genes. It is therefore interesting to speculate that ms1 regulates genes associated with cardiac hypertrophy that collectively may promote an increase in cell size i.e. hypertrophy via the MRTF-SRF pathway.

H9c2 cells proliferate in culture and the increase in cell size could have been caused by cell proliferation (the number of dividing cells) rather than hypertrophy. It was investigated whether the increase in size of cells over-expressing ms1 was caused by cell proliferation by flow cytometry using a dye (Vybrant DyeCycle Violet Stain) that can bind to DNA, allowing the percentage of cells with S phase DNA content (DNA synthesis) by DNA distribution analysis to be measured as a marker of proliferation.
There was no change in the number of cells over-expressing ms1 in S phase (figure 5.5) or in G2/M phase (figure 5.6) of the cell cycle compared to the number of cells not over-expressing ms1 following transfection. In addition, there was no increase in the number of cells following ms1 over-expression when quantified by direct cell counting (figure 5.7). Therefore, ms1 does not appear to alter the rate of DNA synthesis and cell division, suggesting that ms1 does not stimulate proliferation. The increase in cell size following ms1 over-expression appears not to be due to the cell increasing in size as a result of the cell dividing but likely due to a hypertrophic increase in cell size which is supported by the finding that ms1 affects genes known to promote hypertrophy.

5.3.2 ms1 protects H9c2 cells from staurosporine-induced apoptosis

It was demonstrated that even with a low transfection efficiency of H9c2 cells with the ms1 expression vector, transient over-expression of ms1 protects H9c2 cells from staurosporine-induced apoptosis (figure 5.11). To confirm this finding, the size of cells over-expressing ms1 were investigated following staurosporine-induced apoptosis because during apoptosis the cell shrinks and appears smaller in size than viable cells when analysed using flow cytometry. The size of cells over-expressing ms1 were unaltered in size following staurosporine treatment; however, cells not over-expressing ms1 were much smaller in size (figure 5.12). Cells over-expressing ms1 were not smaller in size probably because these cells hardly underwent apoptosis compared to non ms1 over-expressing cells. These findings suggest that ms1 has a profound role in protection against staurosporine-induced apoptotic cell death.

Understanding the mechanism(s) through which staurosporine causes apoptosis could provide an insight into the mechanism of ms1’s protective effects. Unfortunately the exact mechanism by which staurosporine induces apoptosis is unknown. Caspase-3 activation (a critical effector caspase of the apoptotic process) has been observed in staurosporine-induced apoptosis in cardiomyocytes that may be a potential target in the treatment of heart diseases caused by excessive cell death (Yue et al., 1998). Given that ms1 is expressed in the heart, it is feasible that ms1 could protect cells from staurosporine-induced apoptosis by preventing caspase-3 activation. However, the experiments in this study were performed in the heart derived H9c2 cells; therefore, additional studies with cardiac myocytes, in culture and in vivo, will be necessary to establish how ms1 prevents staurosporine-induced apoptosis.
Biological effects of msl over-expression in vitro

The proapoptotic protein Bax of the Bcl-2 family plays an important role in apoptosis (Oltvai et al., 1993). Bax translocates from the cytosol to the mitochondria during apoptosis and is associated with the release of cytochrome c (Manon et al., 1997) and activation of caspases that lead to cell death (Li et al., 1997a). Bax translocation to the mitochondria and release of cytochrome c was observed in H9c2 cells upon induction of apoptosis by staurosporine (Hou and Hsu, 2005). Therefore it is possible that msl may protect H9c2 cells against apoptotic cell death by preventing Bax translocation to the mitochondria and subsequent cytochrome c release and activation of caspases. Further work would be required in order to determine which apoptotic pathway msl interferes with.

In chapter 4 it was shown that msl affects many genes known to be cardioprotective, for example, ARC, calcineurin, LIF, IL-6, tissue factor and adrenomedullin as well as Akt and ERK1/2. Thus msl could protect cells against staurosporine-induced apoptosis via one or a collection or all of these anti-apoptotic genes. Interestingly, ARC has been shown to inhibit staurosporine-induced apoptosis (Ekhterae et al., 2003) and ARC was observed to mediate its protective effects by interacting with Bax, preventing Bax activation and release of cytochrome c (Gustafsson et al., 2004); therefore it is reasonable to suggest that msl may prevent staurosporine-induced apoptosis via ARC. A combination of a variety of assays will be required to delineate which apoptotic pathway (extrinsic or intrinsic) and which signaling mechanism msl interacts with to inhibit apoptotic cell death.

5.3.3 Limitations of the study

There are a number of limitations that require consideration. Even though there was low transfection efficiency, msl was discovered to have an anti-apoptotic role. However, this is the first study to demonstrate msl's protective effects and requires confirmation using other apoptotic assays where many involve microscopy. Therefore it would be necessary to improve the transfection efficiency. It would also be an advantage to have increased transfection efficiency so that msl’s role in hypertrophy could be confirmed by additional assays including measuring cell size using microscopy.

The transfection efficiency can depend on many factors including the quality of plasmid DNA, optimised amounts of transfection reagent and DNA used, the expression vector.
Biological effects of ms1 over-expression in vitro

and the cell line. Plasmid DNA was extracted using a transfection-grade plasmid kit (Qiagen) and the purity of plasmid DNA extracted was determined using a 260 nm/280 nm ratio which gave a ratio of approximately 1.8 and therefore the quality of plasmid DNA did not cause low transfection efficiency. The transfection method was optimised and the amounts of transfection reagent (JetPEI) and plasmid DNA was at optimum to give the highest transfection reagent it could without toxicity. The low transfection efficiency could be due to the expression vector or the cell line used or a combination of both. Only one other group has used the JetPEI Cationic transfection reagent for transient transfection with H9c2 cells (Pesant et al., 2006); however, they did not use the same expression vector pcDNA3.1(+) and this was with the transfection reagent Lipofectamine (Miyazaki et al., 2006; Zhang and Herman, 2006). Lipofectamine could therefore be used to improve the efficiency of transient transfections. Other ways to improve the transfection efficiency would be to generate a stable cell line over-expressing ms1 and this has previously been successfully achieved using the expression vector pcDNA3.1(+) and the transfection reagent Lipofectamine in H9c2 cells (Ekhterae et al., 1999; Ekhterae et al., 2003; Kageyama et al., 2002; Turner et al., 1998), or to use adenovirus-mediated transduction.

A future consideration would be the use of cardiac myocytes as an alternative to the heart-derived H9c2 cells. Adult cardiac myocytes in culture cease to proliferate unlike H9c2 cells and therefore would be highly beneficial to use instead of H9c2 cells when examining cell size as a marker of hypertrophy. Neonatal cardiomyocytes have been experimentally used to assess hypertrophy by cell size (Hoshijima et al., 1998; Wang et al., 1998a) and successfully transfected with pcDNA3.1(+) DNA constructs (Charron et al., 2001; Liang et al., 2001; Oka et al., 2005; Yanazume et al., 2002).

Although ms1 appears to increase cell size and protect cells from apoptosis, additional controls are necessary to confirm this finding. ms1 increased cell size when compared to untransfected cells (figure 5.3 (b)) and inhibited staurosporine-induced apoptosis when compared to untransfected cells (figure 5.11) where untransfected cells were cells exposed to the transfection of the ms1 expression vector that did not take up the ms1 expression construct. However, it was not known whether ms1 itself increases cell size and prevents apoptosis or the transfection of the plasmid DNA increases cell size and prevents apoptosis. This is because ms1 transfected cells could not be accurately
Biological effects of ms1 over-expression \textit{in vitro} compared to empty vector transfected cells because when the empty vector was transfected the whole population was analysed and transfection of the empty vector would have resulted in a mixed population of cells that were untransfected and cells that were transfected with the empty vector. Cells transfected with the empty vector should have been analysed but were not because the empty vector (pcDNA3.1(+)) does not have a c-Myc tag or any other tag to distinguish empty vector transfected cells from untransfected cells. An empty vector expression construct engineered to contain a c-Myc tag is required to validate these findings.

In summary, the main aim was to investigate a role for ms1 in cardiac hypertrophy and cardioprotection by transiently over-expressing ms1 \textit{in vitro} and examining cell size and the ability of ms1 to protect against staurosporine-induced apoptotic cell death. ms1 appears to increase cell size and prevent staurosporine-induced apoptosis \textit{in vitro}, suggesting that ms1 may promote hypertrophy and have a cardioprotective role. It was demonstrated in chapter 4 that ms1 affects hypertrophic and cardioprotective genes possibly via a MRTF-SRF signalling pathway thereby further supporting a role for ms1 in cardiac hypertrophy and cardioprotection.
Chapter 6

Construction and initial analysis of a transgenic with cardiac specific over-expression of ms1

6.1 Introduction

In vitro, ms1 appears to affect genes associated with cardiac hypertrophy and increase cell size. In addition, ms1 was found to affect anti-apoptotic genes and appears to inhibit apoptotic cell death in vitro. However, it remains uncertain as to whether ms1 causes cardiac hypertrophy and can be cardioprotective in vivo. To further elucidate a role for ms1, the aim of the work in this chapter was to investigate the physiological and pathophysiological consequences of increased ms1 expression in vivo. This was carried out by generating a transgenic strain over-expressing ms1 selectively in the heart to study the effects of increased cardiac expression of ms1 on cardiac size and physiology, in particular whether it leads to LVH and provides cardioprotection in response to acute ischaemia.

A powerful approach in order to examine gene function in cardiac development, physiology and disease is the use of transgenic mice (Petrich et al., 2003; Sugden and Clerk, 1998). Genetic manipulation is a very useful approach to investigate the complex signalling pathways in cardiac development, function and pathology. However, conventional transgenic approaches using tissue specific promoters lack temporal specificity. Several problems can occur due to unregulated expression throughout cardiac development. One problem is where there is difficulty in establishing stable transgenic animal lines for functional studies when transgene expression leads to early lethality. Another problem is when developmental and pathological effects cannot be distinguished in the cardiac phenotype of adult animals leading to physiological studies being compromised (Petrich et al., 2003). To overcome this, controlled expression can be obtained using conditional transgenisis. This involves using the Cre/loxP system to modulate gene expression in a temporal and spatial manner (Lakso et al., 1992; Nagy, 2000; Orban et al., 1992). The Cre/loxP system involves Cre, a member of the α integrase family of site-specific recombinases. Cre recombinase of the P1 bacteriophage is a 38 kD protein that catalyses the recombination between two of its recognition sites, called loxP (locus of crossover) (Hamilton and Abremski, 1984). This is a 34 bp consensus sequence comprising two
13 bp palindromes separated by an asymmetric 8 bp core. The asymmetric core sequence defines an orientation to the \emph{loxP} site. A major advantage of this system is that no additional cellular factors are needed; the only requirements for DNA rearrangement are the integrase and the recombination sites. Recombination occurs within the spacer area of the \emph{loxP} sites when a single recombinase molecule binds to each palindromic half of a \emph{loxP} site, then the recombinase molecules form a tetramer, thus bringing the two \emph{loxP} sites together (Nagy, 2000; Ryding \emph{et al.}, 2001). \textit{In vitro} cell culture experiments in the late 80's and early 90's were the first to show that Cre recombinase would work in eukaryotic cells (Sauer and Henderson, 1988; Sauer and Henderson, 1989; Sauer and Henderson, 1990). It was then discovered that the Cre recombinase worked in the mouse as well when expressed from a transgene (Lakso \emph{et al.}, 1992; Orban \emph{et al.}, 1992).

The Cre recombinase system can be added to excise or invert \emph{loxP}-flanked DNA segments or create intermolecular recombination between different DNA molecules. Using the recombinase activity as a genetic activation or inactivation switch, conditional transgenesis or conditional knockouts become available (Nagy, 2000).

The mechanism for conditional transgene activation involves the promoter and the coding region for the gene of interest to be separated by a \emph{loxP}-flanked STOP region. This does not allow any transcription initiated from the promoter to read through and include the gene. Expression of the gene occurs when the region is removed by Cre-mediated excision (Nagy, 2000) (figure 6.1).
Construction and initial analysis of msl transgenics

Figure 6.1. The structure of a conditional transgene (Nagy, 2000).

The conditional transgene consists of a promoter and the coding region for the gene of interest to be separated by a loxP-flanked lacZ (STOP) region. This does not allow any transcription initiated from the promoter to read through and include the gene prior to Cre excision. The addition of Cre recombinase removes the lacZ region and expression of the gene occurs.

In order to generate a transgenic strain over-expressing msl in a cardiac-specific manner, a transgene was first constructed. The transgenic vector PCCALL2-IRES-EGFP/anton (pi Z/EG) was kindly provided by C Lobe (University of Toronto, Canada) and is based on the Z/EG vector (Novak et al., 2000) that has been previously used by other investigators (de Lange et al., 2003; Guo et al., 2002). The pi Z/EG vector is shown in appendix 1 of chapter 2 and was used because it allows controlled expression when using the Cre/loxP system that can be monitored in a double-reporter transgenic line. The advantage of using a pi Z/EG mouse line is that it provides a double-reporter system for Cre excision utilising lacZ and the enhanced green fluorescent protein (EGFP). Using this system it was discovered that lacZ was expressed throughout embryonic development and in adult stages and upon Cre excision, the lacZ gene was removed and the second reporter EGFP was activated (Lobe et al., 1999; Novak et al., 2000). Thus, this novel system is advantageous because Cre-mediated excision can be monitored in live samples and it can importantly show which tissues have not undergone Cre excision.

The first aim of this study was to use the pi Z/EG vector and generate a msl transgene that consists of the msl coding sequence driven by a pCAGGS promoter comprising the CMV (cytomegalovirus) enhancer and chicken β actin promoter. Following the
Construction and initial analysis of ms1 transgenics

Chicken β actin promoter is a loxP-flanked lacZ/neomycin-resistance fusion gene and three SV40 polyadenylation sequences. Thus read-through transcription will not occur due to the presence of a strong transcription STOP sequence (the polyadenylation sequences) at the end of the lacZ gene. Following the second loxP site and at the 3' side of the ms1 gene are an internal ribosome entry site (IRES), the coding sequence of the EGFP protein and a rabbit β globin polyadenylation sequence (Novak et al., 2000). The expression of Cre recombinase can remove the lacZ gene, thus, prior to Cre excision, the lacZ reporter and the neomycin resistance gene are expressed. After Cre excision the loxP-flanked lacZ/polyadenylation sequence is removed and the promoter is placed adjacent to the ms1 sequence and the EGFP reporter gene to direct ms1 and EGFP expression (Novak et al., 2000), see figure 6.2. This approach therefore allows conditional over-expression of ms1 and GFP positive cells allow identification of cells over-expressing ms1.

![Figure 6.2. The mechanism of the ms1 conditional transgene.](image)

The ms1 transgene consists of the ms1 and EGFP coding sequence driven by a pCAGGS promoter. Following the promoter is a loxP-flanked lacZ gene and three SV40 polyadenylation sequences. Thus read-through transcription will not occur due to the presence of a strong transcription STOP sequence (the polyadenylation sequences) at the end of the lacZ gene. Prior to Cre excision, only the lacZ reporter is expressed. The addition of Cre recombinase removes the lacZ gene, thus, ms1 and EGFP are expressed.

To confirm the ms1 transgene was functional, the transgene was transfected in vitro, in NIH3T3 cells to first check that lacZ was expressed and ms1/GFP was not over-expressed and then the ms1 transgene was co-transfected with a Cre recombinase expressing vector, pCre-Pac vector (Taniguchi et al., 1998) (appendix 1 of chapter 2) to
confirm that Cre recombinase removed lacZ and hence produced no lacZ expression but resulted in ms1/GFP over-expression.

The second aim of this study was to generate ms1 transgenic mice that showed successful germ-line transmission of the ms1 transgene identified using PCR, Southern blotting and lacZ mRNA and protein analysis. Once ms1 transgenic mice were generated they were crossed with mice expressing Cre recombinase to create cardiac specific over-expression of ms1 identified by ms1/GFP expression in the heart by semi-quantitative RT-PCR and immunostaining. To over-express ms1 specifically in the heart, the mice used to cross with ms1 transgenic mice were MLC2v Cre mice that were already established at the University of Leicester transgenic unit and initially obtained from K R Chien (Chen et al., 1998b). A knock-in of Cre recombinase into the MLC2v locus or the use of the α-myosin heavy chain (α-MHC) promoter are the most common models of cardiac-specific Cre transgene expression (Agah et al., 1997; Chen et al., 1998a). The α-MHC promoter drives expression in embryonic and adult atrial myocytes; however, expression in ventricular myocytes is observed mainly after birth (Palermo et al., 1996). In contrast, MLC2v is the earliest (E7.5-8.0) known ventricular-specific marker (O'Brien et al., 1993). In transgenic animals, the MLC2v promoter lacks uniform expression of the endogenous gene, displaying a strong bias to expression in the right ventricle (Ross et al., 1996). To overcome this transgenic artifact, Cre recombinase coding sequences were introduced into the chromosomal MLC2v gene by homologous recombinase. In this manner, Cre recombinase is expressed in a temporally and spatially restricted manner that corresponds to the endogenous MLC2v gene (Chen et al., 1998b). Mice that are heterozygous for this allele are normal, express normal levels of MLC2v protein, and have neither a molecular or physiological cardiac phenotype at base line or following hypertrophic stimuli (Chen et al., 1998b; Minamisawa et al., 1999). Thus the MLC2v Cre knock-in strategy is a valid approach for gene targeting and transgenesis to explore the molecular mechanisms of cardiac hypertrophy and heart development.

The endogenous MLC2v gene is expressed in ventricular cardiomyocytes and therefore so will Cre recombinase. When ms1 transgenic mice are crossed with MLC2v Cre mice, mice are generated that will over-express ms1/GFP in those cells that express Cre (ventricular cardiomyocytes).
In summary, the aim of the work presented in this chapter was to construct a ms1 transgene that was first confirmed to be functional by transiently over-expressing ms1/GFP \textit{in vitro} and then use the ms1 transgene to generate ms1 transgenic mice that over-express ms1 in a cardiac-specific manner when crossed with MLC2v Cre mice, in order to investigate whether increased cardiac expression of ms1 leads to LVH and provides cardioprotection.

\section*{6.2 Results}

\subsection*{6.2.1 Construction of the ms1 transgene}

The full-length mouse ms1 coding sequence was already cloned into the pGEM-T Easy vector as described in section 3.2.1 of chapter 3. The pGEM-T Easy vector was cut with the restriction enzymes \textit{BglII} and \textit{Xhol} and the full-length ms1 fragment (approximately 1.3 kb) was released. The transgenic vector pi Z/EG was also cut with the restriction enzymes \textit{BglII} and \textit{Xhol} and both the ms1 fragment and pi Z/EG vector (approximately 10.9 kb) were agarose gel extracted and ran on an agarose gel to check for DNA purity prior to ligation of ms1 into pi Z/EG.

ms1 was then cloned into the pi Z/EG vector and restriction digest confirmed that the pi Z/EG vector contained ms1 by producing the correct sized fragment for ms1 (approximately 1.3 kb) and the correct approximate 10.9 kb fragment for pi Z/EG which is shown in figure 6.3. ms1 was also confirmed by sequencing to be cloned into pi Z/EG.
Construction and initial analysis of msl transgenics

Figure 6.3. Agarose gel analysis following a restriction digest of the msl transgene using the enzymes BglII and Xhol confirmed that the msl transgene contained msl.

6.2.2 Transient transfection of the msl transgene in vitro leads to over-expression of msl

In order to determine that the constructed msl transgene was functional, NIH3T3 cells were left untransfected or transfected with various vectors for 48 hours as described in sections 2.4.5 and 2.4.6 of chapter 2 and analysed for lacZ and msl/GFP expression by semi-quantitative RT-PCR, histochemical staining and fluorescence microscopy. NIH3T3 cells were used because this cell line is one of the easiest to transfect and monitor expression from a transgene.

As shown in figure 6.4 lacZ mRNA expression was observed for cells transfected with the msl transgene or empty pi Z/EG vector control and for cells co-transfected with the msl transgene and pCre-Pac vector or the empty pi Z/EG vector control and the pCre-Pac vector compared to untransfected cells and pCre-Pac vector transfected cells.

The addition of Cre removes the lacZ gene; however, lacZ expression was still observed when cells were co-transfected with the msl transgene and pCre-Pac vector or the empty pi Z/EG vector control and the pCre-Pac vector. This was probably because co-transfection of the msl transgene/empty pi Z/EG vector and pCre-Pac vector would
have given a mixed population of untransfected cells, cells transfected with the ms1 transgene/empty pi Z/EG vector (expressed lacZ), cells transfected with the pCre-Pac vector (no lacZ expression) and cells transfected with the ms1 transgene/empty pi Z/EG vector and pCre-Pac vector (no lacZ expression). Hence, the lacZ expression observed would have come from the ms1 transgene/empty pi Z/EG vector transfected cells within the mixed transfected cell population.

The same finding was also observed using histochemical staining to detect lacZ expression, see figure 6.5. lacZ (β-galactosidase) activity was detected by the formation of visible, blue precipitates through the hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (MacGregor et al., 1987); therefore, those cells that appeared blue expressed lacZ and were transfected with the ms1 transgene or empty pi Z/EG vector. Thus, semi-quantitative RT-PCR and histochemical staining confirmed that the ms1 transgene expressed the first reporter lacZ prior to Cre excision.

ms1 mRNA over-expression was confirmed for cells co-transfected with the ms1 transgene and pCre-Pac vector (figure 6.4) by semi-quantitative RT-PCR. Therefore cells co-transfected with the ms1 transgene and pCre-Pac vector over-expressed ms1 (and hence GFP) due to expression of Cre recombinase from the pCre-Pac vector that removed the loxP-flanked lacZ/polyadenylation sequence resulting in ms1/GFP expression. However, ms1 over-expression was also observed for cells transfected with the ms1 transgene and there should only be ms1 over-expression when Cre recombinase was present. This expression was not endogenous ms1 because NIH3T3 cells express very low levels of ms1 and if detected it would also be observed for the other samples. Also there was no ms1 expression detected when the RNA extracted from cells transfected with the ms1 transgene was DNase treated and the reverse transcription enzyme was excluded (-RT) during cDNA synthesis (figure 6.4), suggesting that the ms1 expression observed was ms1 mRNA and not from the plasmid DNA. It was therefore possible that the ms1 transgene was leaky and the polyadenylation sequences at the end of the lacZ gene allowed read-through transcription.

To examine this further the second reporter GFP was analysed by fluorescence microscopy, see figure 6.5. GFP expression was only observed for cells co-transfected with the ms1 transgene and pCre-Pac vector or the empty pi Z/EG vector control and the pCre-Pac vector. Therefore cells co-transfected with the ms1 transgene and pCre-Pac vector appeared green and over-expressed ms1. As no GFP expression was
observed for cells transfected with the ms1 transgene but ms1 mRNA was detected this suggested that the ms1 transgene was possibly a little leaky and very low levels of ms1 transcript was detected because PCR is a highly sensitive technique.

Figure 6.4. Transient transfection of the ms1 transgene leads to lacZ expression prior to Cre excision and ms1 over-expression following Cre excision.

NIH3T3 cells were left untransfected (Un) or transfected with the constructs pCre-Pac (Cre), pi Z/EG (empty vector control), pi Z/EG-ms1 (ms1 transgene) or co-transfected with the pi Z/EG-ms1 (ms1 transgene) and pCre-Pac vector or the empty pi Z/EG vector control and the pCre-Pac vector for 48 hours, n = 2. LacZ and ms1 mRNA expression was determined by semi-quantitative RT-PCR. RPL32 was used as an internal control for inaccuracies in initial RNA levels. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given. + RT indicates that the RT enzyme was included during cDNA synthesis and –RT indicates that the RT enzyme was omitted.
Figure 6.5. Transient transfection of the msl transgene leads to msl over-expression detected by GFP staining.

Light microscopy and fluorescence microscopy of X-gal staining (lacZ) and GFP staining following transient (48 hours) transfection of NIH3T3 cells with the constructs pCre-Pac (Cre), pi Z/EG (empty vector control), pi Z/EG-msl (msl transgene) or cotransfection of pi Z/EG-msl (msl transgene) with the pCre-Pac vector or the empty pi Z/EG vector control with the pCre-Pac vector, n = 3. Bar = 5μm. Magnification 20x.
6.2.3 Generation of msl transgenic mice

The msl transgene (pi Z/EG-msl) was purified as described in section 2.7.1 of chapter 2) and linearised at the Sfil and ScaI sites (see appendix 1 of chapter 2) to remove the vector backbone by S Munson at the Embryonic Stem Cell Facility, University of Leicester. The linearised transgene was then injected into the pronucleus of day 1 post-fertilisation oocytes. This was performed by J Brown at the University of Leicester transgenic unit. A total of 434 oocytes were injected in batches with the msl transgene and just under half of them that survived were re-implanted into the oviducts of pseudopregnant females (table 6.1). 37 mice were born and screened to assess inheritance of the msl transgene by PCR using lacZ and GFP vector specific primers (figure 6.6) as described in section 2.7.3.1 of chapter 2. Out of 37 newborn mice, 5 carried the msl transgene (table 6.1).

<table>
<thead>
<tr>
<th>No. of oocytes injected</th>
<th>No. of oocytes transferred</th>
<th>No. Born (B6CBF1 Strain)</th>
<th>msl transgene positive</th>
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<tbody>
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<td>69</td>
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<tr>
<td><strong>Total</strong> 434</td>
<td>203</td>
<td><strong>37</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>

Table 6.1. The number of initial offspring carrying the msl transgene following microinjection of the transgene into the pronucleus of oocytes.
Figure 6.6. Inheritance of the msl transgene by PCR analysis

Founders were genotyped to identify inheritance of the msl transgene by PCR using lacZ and GFP vector specific primers. IL-2 was used as an internal control. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given.

The 5 mice found positive for carrying the msl transgene were bred with C57BL6 mice to establish breeding lines. In total 104 mice were born and 25 of these were found to be positive by PCR for carrying the msl transgene (table 6.2). Line 2 did not show successful germ-line transmission of the msl transgene but the other lines were found to show successful germ-line transmission. Positive founder mice that were identified to have inherited the msl transgene (msl transgenic) from lines 3, 4 and 5 were taken forward and bred further to create more breeding lines.
<table>
<thead>
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<th>Line</th>
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<th>msl transgene positive</th>
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<tr>
<td>Total</td>
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<td>25</td>
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</tbody>
</table>

Table 6.2. Successful germ-line transmission of the msl transgene.

Two msl transgenic mice from each line were bred with C57BL6 mice to carry on the breeding line except for line 3 because there was only one founder that inherited the msl transgene and hence this one founder was bred to carry on line 3. Table 6.3 shows the total number of mice (F<sub>2</sub>) carrying the msl transgene out of the total number born for each line. The total number of mice found to carry the msl transgene for all three lines was much higher than expected. Approximately 80%, 70% and 90% of founders for lines 3, 4 and 5, respectively, inherited the msl transgene which is higher than the expected amount of 50% according to mendelian inheritance, thus, these genotyping results suggests multiple integrations of the msl transgene for all three lines.
<table>
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<th>No. -ve</th>
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Table 6.3. The total number of founders (F2) carrying the msl transgene (no. +ve) out of the total number born for each line.

6.2.4 msl transgene integration and copy number

In order to determine transgene integration and copy number, Southern blot analysis was performed on total liver DNA (10 μg) digested with SstI and hybridised with a 32P-labelled probe (655 bp NorI-BamHI fragment that includes the rabbit β-globin polyadenylation sequence of the pi Z/EG vector). The SstI restriction endonuclease recognition site only occurs once in the transgene, thus if the transgene inserted in a head-to-tail concatemer, cutting with SstI should result in one fragment the same size as the transgene (approximately 10.5 kb), and in the end fragments of the transgene array where they meet with genomic DNA that will be of unpredictable size (figure 6.7).
insertion is known as tandem integration. The intensity of the hybridisation signal corresponds to the copy number of the transgene in the insertion site. If the transgene has integrated more than once (multiple integration sites), a Southern blot will show multiple end fragments of unknown length corresponding to the number of integration events (figure 6.7). If it is single copy integration, a Southern blot should show a single fragment of unknown length (figure 6.7).

**Figure 6.7. Predicted possible integrations of the msl transgene following a digest with a restriction enzyme that cuts once in the transgene.**
Southern blot analysis of \textit{SstI} digested genomic DNA from mice (F$_2$) that were approximately 8 weeks old revealed a single fragment of approximately 10.5 kb (figure 6.8), suggesting tandem integration; however, there was no additional band (end fragment) present determined by the next \textit{SstI} restriction site in the mouse genome. This was observed for animals from all three lines. The intensity of the approximate 10.5 kb band was strong signifying multiple transgene copy numbers in the insertion site. Band intensity comparison suggests that the mouse from line 3 had more transgene copy numbers than the mouse from line 4 and line 5 and the mouse from line 4 had more copy numbers than the mouse from line 5.

![Figure 6.8. Southern blot analysis of \textit{SstI} digested genomic DNA isolated from mice (F$_2$) that was hybridised to radiolabeled probe.](image)

Total liver DNA (10 µg) was isolated from approximately 8 week old F$_2$ mice from all three lines, digested with \textit{SstI}, Southern blotted and hybridised with a $^{32}$P-labelled 655 bp \textit{NotI- BamHI} fragment that includes the rabbit $\beta$-globin polyadenylation sequence of the pi Z/EG vector. Southern blot analysis was performed on two animals identified to carry the msl transgene (+ve genotype) and two littermate animals identified not to inherit the msl transgene (-ve genotype) from each line. Shown is an example of a representative Southern blot.
To confirm this finding, total liver DNA (10 µg) was digested with a different restriction enzyme *EcoRV* and hybridised with a $^{32}$P-labelled 655 bp *NotI-BamHI* fragment that includes the rabbit β-globin polyadenylation sequence of the pi Z/EG vector. The *EcoRV* restriction cuts twice in the transgene, thus if the transgene inserted in a head-to-tail arrangement, cutting with *EcoRV* should result in two bands, one band of approximately 5.4 kb and a band of unknown length (figure 6.9).

![Diagram of genomic DNA digested with *EcoRV*](image)

**msl transgene**

**Mouse Genome**

- 10527 bp Transgene
- Hybridisation Probe
- *EcoRV*
- Restriction Enzyme

**Figure 6.9. Predicted tandem arrangement of the msl transgene following a digest with a restriction enzyme, *EcoRV* that cuts twice in the transgene.**

A single fragment of approximately 5.4 kb (figure 6.10), suggesting tandem integration of the msl transgene, was observed following Southern blot analysis of *EcoRV* digested genomic DNA from mice (F2) that were approximately 8 weeks old. This finding confirmed that the msl transgene integrated in a tandem arrangement; however, there was no additional band (end fragment) present for animals from all three lines as observed previously when genomic DNA from mice was digested with *SstI* (figure 6.8). As shown in figure 6.10 the intensity of the approximate 5.4 kb band was strong indicative of multiple transgene copy numbers in the insertion site. As seen before in figure 6.8, the band intensity suggests that the mouse from line 3 contained more transgene copy numbers than the mouse from line 4 and line 5 and the mouse from line 4 had more copy numbers than the mouse from line 5. This finding confirms that the msl transgene integrated in a tandem arrangement in all three lines.
Construction and initial analysis of ms1 transgenics

Figure 6.10. Southern blot analysis of total liver DNA digested with EcoRV and hybridised to radiolabeled probe confirmed tandem integration of the ms1 transgene.

Total liver DNA (10 µg) was isolated from approximately 8 week old F_2 mice from all three lines, digested with EcoRV, Southern blotted and hybridised with a 32P-labelled 655 bp NotI-BamHI fragment that includes the rabbit β-globin polyadenylation sequence of the pi Z/EG vector. Southern blot analysis was performed on two animals from each line. Shown is an example of a representative Southern blot.

6.2.5 Expression of the first reporter lacZ from the ms1 transgene

The ms1 transgene consists of a pCAGGS promoter comprising the CMV enhancer and chicken β actin promoter that drives lacZ expression ubiquitously prior to the addition of Cre recombinase. To determine that all three lines expressed lacZ and therefore the ms1 transgene, lacZ expression in the kidney was analysed by semi-quantitative RT-
PCR. As shown in figure 6.11 lacZ mRNA was not found in the kidneys of approximately 8 week old F₂ mice that were identified by genotyping to carry the msl transgene.

- [Figure 6.11. LacZ expression in the kidneys of F₂ mice.](image)

LacZ mRNA expression in the kidneys of approximately 8 week old F₂ mice (n = 3) from each line was analysed by semi-quantitative RT-PCR. RPL32 was used as an internal control for inaccuracies in initial RNA levels. N refers to the no template control. + RT indicates that the RT enzyme was included during cDNA synthesis and -RT indicates that the RT enzyme was omitted. Shown is an example of lacZ expression in the kidney from one animal identified to carry the msl transgene (+ve genotype) and a littermate animal identified not to inherit the msl transgene (-ve genotype) from each line. LacZ expression in the kidney from an animal known to express lacZ was included as a positive control.

The same finding was also observed using histochemical staining to detect lacZ expression, see figure 6.12. In addition, the heart and liver were also stained for lacZ expression and as observed in the kidney there was no visible blue staining present in the heart and liver and hence no lacZ expression. Kidney, heart and liver tissues from a mouse known to carry the pi Z/EG vector and express lacZ was provided by I. Shenje and included as a positive control. As shown in figures 6.11 and 6.12 these tissues expressed lacZ and therefore semi-quantitative RT-PCR and histochemical staining was performed correctly. These findings suggest that although the msl transgene was integrated it was not functional in mice that were approximately 8 weeks old from all three lines.
Figure 6.12. LacZ expression in tissues from three transgenic lines.

Histochemical staining using X-gal was performed to detect lacZ expression in tissues of ms1 transgenic mice (F2) at approximately 8 weeks of age from each line compared to a littermate wild type control, n = 2. A cytoplasmic blue colour appeared in tissues expressing high levels of lacZ, see +ve control tissues. Shown is an example of lacZ expression in the kidney, heart and liver from one animal identified to carry the ms1 transgene (+ve genotype) and a littermate animal identified not to inherit the ms1 transgene (-ve genotype) from each line. LacZ expression in the kidney, heart and liver from an animal known to express lacZ was included as a positive control. Magnification 1×.
It was possible that the msl1 transgene was not functional in mice at approximately 8 weeks of age because the msl1 transgene may have switched off in the adult. Therefore, embryos were analysed for lacZ expression to determine if the msl1 transgene was functional throughout embryonic development. Embryos at E10.5 from all three lines were genotyped to determine those that inherited the msl1 transgene and analysed for lacZ expression by histochemical staining, see figures 6.13 and 6.14 for an example from one transgenic line. Embryos from each line were identified to carry the msl1 transgene by genotyping. However, there was no visible blue staining present and hence no lacZ expression in embryos from all three lines. Therefore, lacZ was not expressed throughout embryonic development and in adult stages, confirming that the msl1 transgene integrated but was not functional.

![Genotyping of embryos](image)

**Figure 6.13. Genotyping of embryos to identify inheritance of the msl1 transgene.** Embryos (E10.5) from all three lines were genotyped to identify inheritance of the msl1 transgene by PCR using lacZ and GFP vector specific primers. IL-2 was used as an internal control. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given. Shown is genotyping analysis of embryos from one line.
Figure 6.14. LacZ expression in embryos (E10.5).
Embryos (E10.5) from each line were stained for lacZ expression. Shown is an example of lacZ staining from one embryo identified to carry the msl transgene (+ve genotype) and one embryo not to inherit the msl transgene (-ve genotype) from one line. Magnification 1.6x.

Even though msl transgenic mice did not express lacZ, two msl transgenic mice from each line were bred with C57BL6 mice to carry on the breeding lines and generate msl transgenic founders (table 6.4) to cross with MLC2v Cre mice as it was possible that the msl transgene might function when Cre was present. As observed previously, the total number of msl transgenic founders from all three lines was much higher than the expected amount of 50% according to mendelian inheritance (table 6.4).

<table>
<thead>
<tr>
<th>Line</th>
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<th>No. -ve</th>
</tr>
</thead>
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Table 6.4. The total number of founders (F3) carrying the msl transgene (no. +ve) out of the total number born for each line.
6.2.6 Generation of MLC2v Cre mice

The MLC2v Cre mouse breeding colony was already established at the University of Leicester transgenic unit and was initially obtained from K R Chien (Chen et al., 1998b). Mice at generation 18 were bred with C57BL6 mice to carry on the breeding line and create our own breeding colony to use to cross with the msl transgenic lines. Mice carrying the Cre locus (MLC2v Cre mice) were identified by PCR using Cre specific primers (figure 6.15)

**Figure 6.15. Identification of MLC2v Cre positive mice by PCR analysis.**
Founders were genotyped to identify mice carrying the Cre locus by PCR using Cre primers. IL-2 was used as an internal control. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given.

6.2.7 Generation of double transgenic mice carrying the msl transgene and Cre locus

MLC2v Cre mice were bred with two msl transgenic mice from each line to produce founders that carried both the msl transgene and the Cre locus (double transgenics) and therefore over-expressed msl specifically in the heart. As shown in figure 6.16 mice carrying the msl transgene and the Cre locus (double transgenics) were identified by PCR using GFP primers and Cre primers, respectively. The total number of double transgenics from all three lines was much higher than the expected amount of 1:4, see table 6.5. Approximately 72%, 36% and 44% of founders for lines AK1, AK2 and AK3, respectively, inherited the msl transgene and Cre locus.
Construction and initial analysis of msl transgenics

Figure 6.16. Identification of double transgenic mice carrying the msl transgene and Cre locus by PCR analysis.

Founders were genotyped to identify double transgenic mice carrying the msl transgene and Cre locus (+/+) , msl transgenic mice (+/-), MLC2v Cre mice (-/+ ) and wild type mice (-/- ) by PCR using GFP and Cre primers. IL-2 was used as an internal control. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given.

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Table 6.5. The total number of double transgenics (+/+), msl transgenics (+/-), MLC2v Cre mice (-/+ ) and wild type mice (-/- ) identified by genotyping the total number born for each line.
6.2.8 Cardiac over-expression of ms1

Cardiac specific over-expression of ms1 was analysed by ms1 and GFP expression in the hearts of double transgenic mice from all three lines by semi-quantitative RT-PCR and immunostaining. The hearts from double transgenics and ms1 transgenic controls were viewed for green fluorescence and there was green fluorescence present but the level of fluorescence looked similar in hearts from double transgenics and ms1 transgenic controls. Also it was hard to distinguish if the green fluorescence observed was real or not. Therefore, GFP immunostaining was performed as this technique is much more sensitive. As shown in figure 6.17, ms1 mRNA over-expression was not observed in the hearts of approximately 8 week old double transgenic mice from all three lines. ms1 transcript levels in the hearts of double transgenic mice from all three lines were similar in expression levels when compared to levels in the hearts from a littermate ms1 transgenic or wild type control. There was also no GFP mRNA in the hearts of double transgenics.
Construction and initial analysis of ms1 transgenics

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Figure 6.17. ms1/GFP expression in the hearts from three transgenic lines.

Semi-quantitative RT-PCR analysis of ms1 and GFP mRNA expression in the hearts of approximately 8 week old double transgenic mice (+/+), n=6 compared to a littermate ms1 transgenic (+/-), n=1 and a littermate wild type control (-/-), n=1 from each line. RPL32 was used as an internal control for inaccuracies in initial RNA levels. N refers to the no template control, L refers to the 100 bp DNA ladder and the band sizes (bp) are given. + RT indicates that the RT enzyme was included during cDNA synthesis and -RT indicates that the RT enzyme was omitted.
The same finding was also observed using immunostaining to detect GFP expression, see figure 6.18. cDNA was provided by C Prichard that has been used previously for semi-quantitative RT-PCR to detect GFP mRNA and was included as a positive control. A heart from a mouse known to carry the pi Z/EG vector and Cre locus and express GFP was provided by L Shenje and was included as a positive control for GFP immunostaining. GFP was expressed (figures 6.17 and 6.18) and therefore semi-quantitative RT-PCR and immunostaining was performed accurately.
Figure 6.18. Cardiac specific expression of GFP.

GFP expression in the hearts of approximately 8 week old double transgenics (+/+) compared to littermate ms1 transgenics (+/-), n=2, from each line was analysed by GFP immunostaining as described in materials and methods. Shown is an example of paraffin sections from +/- and +/- hearts from each line stained with rabbit GFP polyclonal antibody, 2°ry swine anti-rabbit antibody only or Haematoxylin and Eosin (H & E). Magnification 40x.
6.3 Discussion

In order to further explore the role of ms1 in cardiac hypertrophy and cardioprotection, an attempt was made to study the effect of increased expression of ms1 in vivo by over-expressing ms1 in a cardiac-specific manner in the mouse using a conditional ms1 transgene.

1. A ms1 transgene was constructed and transient transfection in vitro confirmed the transgene expressed lacZ mRNA and protein prior to Cre excision and ms1 mRNA and protein over-expression plus GFP expression following Cre-mediated deletion.

2. In vivo, the ms1 transgene integrated in a tandem arrangement; however, there was no lacZ expression in embryonic development or in the adult of ms1 transgenics prior to crossing with Cre recombinase expressing mice (MLC2v Cre).

3. When ms1 transgenic mice were bred with MLC2v Cre mice there was no cardiac over-expression of ms1 when ms1 and GFP expression was analysed in the hearts of double transgenics.

Thus, even though the ms1 transgene was functional in vitro, in vivo the ms1 transgene integrated into the mouse but did not over-express ms1 to allow investigation of increased levels of ms1 in the heart.

6.3.1 Cardiac-specific over-expression of ms1 was not observed in vivo following Cre-mediated excision

Even though the first reporter lacZ was not expressed from the ms1 transgene, the transgene was integrated and hence it was possible that Cre deletion may still switch on ms1 over-expression and the second reporter GFP. When ms1 transgenic mice carrying the ms1 transgene were crossed with MLC2v Cre mice carrying the Cre locus, the expected amount of 25% of the offspring carrying the ms1 transgene and Cre locus (double transgenics) in all three lines was not observed (table 6.5). 36% or more were identified as double transgenic mice and this finding possibly relates to the previous observation of a higher than expected number of ms1 transgenic offspring identified in the first instance. Although founders from each line were identified to carry both the ms1 transgene and Cre locus, there was no cardiac-specific over-expression of ms1 when analysed by semi-quantitative RT-PCR (figure 6.17) and the second reporter GFP.
was not expressed either when analysed by both semi-quantitative RT-PCR (figure 6.17) and immunostaining (figure 6.18).

It appears that although mice from all three lines carried the msl transgene, when they were crossed with MLC2v Cre mice that expressed Cre recombinase, msl was not over-expressed in the heart suggesting that Cre-mediated deletion did not switch on msl over-expression or the second reporter GFP probably because the msl transgene was not expressed in the first instance prior to Cre excision. The physiological and pathophysiological consequences of increased msl expression in vivo were therefore not investigated.

The finding that the msl transgene was integrated but was not expressed is difficult to interpret. It is not known how common this is as it has not been reported in the literature. It is possible that there was a structural problem with the transgene as there was no expression of the msl transgene in all three lines. However, the transgene was expressed in vitro and therefore this was an unlikely explanation.

Expression of a transgene can be affected by position effects (Hammes and Schedl, 2000). Position effects can reduce or abolish transgene expression and one way this can occur is due to insertion of the transgene into a transcriptionally inactive region of the genome (Hammes and Schedl, 2000). Therefore, it is feasible that there was no msl transgene expression because the construct inserted into an inactive region. In addition, multiple copies within a concatameric array can inactivate transgene expression (Garrick et al., 1998). Although poorly understood, it is believed to occur due to repeat-induced gene silencing where the repeat structure alters chromatin structure with the possibility that local heterochromatin formation may be responsible (Garrick et al., 1998; Henikoff, 1998).

Southern blot analysis showed that in all three lines the transgene was found to possibly integrate in a tandem arrangement. However, there was no additional band present on the blot indicative of the end fragment of the transgene array (figures 6.8 and 6.9). It was possible that the end fragment was not observed because if it was a single copy it would appear as a very faint band that may be difficult to detect in comparison to the very intense approximate 10.5 kb and 5.4 kb bands. Another possibility was that the end fragment was too large in size to transfer efficiently to the Southern membrane.
The most likely explanation was that the transgene integration was defective and hence the end fragment was not readily observed.

Fluorescence in situ hybridization (FISH) can be used to identify sites of transgene integration (Hammes and Schedl, 2000). FISH is mainly used on metaphase chromosomes, because at this stage integration sites on the chromosome are more easily determined. Gene (and transgene) expression occurs mainly during interphase and FISH can also be used to visualise transgenes and their transcripts during interphase from tissues (Santos et al., 2006). Due to time constraints transgene detection by FISH was not performed.

Recently a second attempt was made to make a transgenic mouse over-expressing msl using the same Cre/loxP system. This again was unsuccessful despite successful integration of the transgene.

Another approach to create transgenic mice is by transfection of the transgene into Embryonic Stem (ES) cells. The ES cells can be screened for lacZ expression by X-gal staining and those that express lacZ can then be analysed by Southern blotting to determine transgene integration. Those that have successfully integrated the transgene can undergo Cre deletion to monitor GFP expression. Candidate clones are injected back into a host blastocyst and re-implanted in a pseudopregnant mouse. This procedure is currently being performed as an alternative strategy to generate msl transgenic mice.

At the time of discovering that the transgenic mice did not over-express msl, Kuwahara et al. (2007) generated transgenic mice using the α-MHC promoter to over-express STARS in the heart. Increased expression of STARS in the heart did not cause hypertrophy or any histological abnormalities of the heart. However, when STARS transgenic mice were subjected to pressure overload there was an exaggerated deterioration in cardiac function. This was also observed when STARS transgenic mice were crossed with calcineurin transgenic mice known to display hypertrophy. Kuwahara et al. (2007) suggest that increased expression of STARS in response to hypertrophic stimuli facilitates the transition to cardiac dysfunction implicating STARS in the transition from cardiac hypertrophy to heart failure. Kuwahara et al. (2007) also discovered that STARS is a cardiac-specific and stress-inducible target of MEF2 and activator of SRF target genes, suggesting that STARS modulates adverse cardiac
remodelling by functioning as a key mediator of MEF2 signalling and SRF activity. The implications of these findings along with findings from chapters 4 and 5 will be discussed in more detail in chapter 7.
Chapter 7

General Discussion

The novel gene msl is rapidly up-regulated following pressure-induced LVH and during ischaemia-reperfusion, suggesting that msl may be involved in the development of hypertrophy and provide cardioprotection. The function of msl in cardiac physiology and disease, in particular whether msl leads to hypertrophy and has a cardioprotective role was investigated by examining the effects of msl over-expression in vitro and in vivo.

The main findings to emerge were as follows:

1. msl over-expression colocalised with actin in H9c2 cells.

2. Transient over-expression of msl in H9c2 cells altered gene expression of known hypertrophic and cardioprotective markers that are MRTF-SRF target genes.

3. Transient over-expression of msl increases the size of H9c2 cells and protects H9c2 cells from apoptotic cell death.

These findings suggest that msl may mediate a hypertrophic response and provide cardioprotection via a MRTF-SRF signalling mechanism where msl binds to actin, promotes actin polymerisation causing MRTF translocation to the nucleus resulting in activation of SRF dependent transcription of hypertrophic and cardioprotective genes.

7.1 Other evidence to support a role for msl in cardiac physiology and disease via a MRTF-SRF pathway

During this investigation our group investigated the effects of adding SRF mRNA with knockdown of msl in the zebrafish in collaboration with S Shaw (Cardiovascular Research Center, Massachusetts General Hospital, USA). Knockdown of msl in the zebrafish caused decreased cardiac contractility, an enlarged atrium and curvature and shortening of the longitudinal axis (figure 7.1). Injection of SRF mRNA remarkably reversed these cardiac abnormalities (unpublished results). SRF itself and many of the SRF target genes are involved in cardiac development, structure and function and msl via the MRTF-SRF pathway may regulate SRF target genes (chapter 4) that maintain cardiac function. It is therefore feasible that knockdown of msl caused a loss of SRF activity via MRTFs resulting in a loss of cardiac function. The addition of SRF
possibly restored SRF activity and transcription of muscle-specific genes to rescue the cardiac abnormalities. These findings support the concept of ms1 involved in a MRTF-SRF signalling pathway to regulate expression of genes that are important in cardiac physiology and disease.

Figure 7.1. Developmental phenotype of morpholino-induced knockdown of ms1. Lateral views of 56 hpf embryos. (a) Embryo injected with control morpholino (with 5 mismatches). The cardiac silhouette is demarcated by arrowheads. The heart tube is looped so that the ventricle (v) and atrium (a) are closely apposed (inset). Circulating red blood cells (rbc) are visible in a thin rim along the inferior aspect of the yolk and within the heart. (b) Embryo injected with ms1 morpholino. The heart tube is unlooped so that the ventricle (v) and atrium (a) are co-linear, with atrial dilation (inset). There is significant oedema (o) in the pericardium and over the yolk, with stasis of red blood cells (rbc) over the yolk. (c) Lateral view of entire 56 hpf embryo following injection of control, mismatch morpholino. (d) and (e) 56 hpf embryos showing representative phenotypes of ms1 morpholino injection.

In addition, Kuwahara et al. (2005) showed that knockdown of ms1 reduced SRF activity and it was demonstrated that cardiac-specific deletion of SRF in the embryonic heart results in cardiac defects (Miano et al., 2004) and deletion of SRF from the adult heart caused dilated cardiomyopathy (Parlakian et al., 2005). Therefore, loss of
function of both msl and SRF are detrimental to cardiac function probably because msl and SRF work together (msl-MRTF-SRF pathway).

In chapter 4 it was investigated whether msl over-expression altered a subset of known MRTF-dependent genes involved in hypertrophy and cell survival to strengthen a role for msl in cardiac hypertrophy and cardioprotection. Recently a similar approach was undertaken by Morita et al. (2007) who were interested to discover a direct role for the MRTF-SRF pathway in stress fiber and focal adhesion formation. It was discovered that the transcriptional regulation of a subset of cytoskeletal/focal adhesion genes caldesmon, tropomyosin, vinculin and zyxin identified by Selvaraj and Prywes (2004) as MRTF-dependent genes was mediated through the MRTF/SRF pathway (Morita et al., 2007). It was observed that msl colocalises with actin (chapter 3) and possibly regulates cardiac genes via the MRTF/SRF pathway (chapter 4); therefore, it is interesting to speculate that msl may play a role in the re-organisation of the actin cytoskeleton via regulation of the cytoskeletal/focal adhesion genes through a MRTF/SRF signalling mechanism. The re-organisation of the actin cytoskeleton is a characteristic of hypertrophy and if msl regulates the cytoskeletal/focal adhesion genes this would further support a role for msl in cardiac hypertrophy.

In chapter 5 it was demonstrated that over-expression of msl in vitro increases cell size and inhibits apoptosis. The cell size increase was not due to the cells increasing in size prior to cell division as there was no increase in the number of cells dividing following msl over-expression. The results imply that msl may regulate hypertrophic rather than general cellular growth. The finding that msl altered MRTF/SRF target genes involved in hypertrophy (chapter 4) strengthens a role for msl in hypertrophy via a MRTF-SRF signalling pathway. The involvement of msl in cardiac hypertrophy requires confirmation in vivo. The findings in vitro were achieved from a transient response of msl over-expression and the increase in levels of msl following pressure overload and ischaemia-reperfusion were transient. Hence, the increase in msl following pressure induced LVH and ischaemia-reperfusion could be a stress response and thus it is possible that in response to stress, msl is up-regulated, binds to actin and promotes actin polymerisation causing nuclear import of MRTFs and stimulation of SRF dependent gene transcription to mediate the hypertrophic response and provide cardioprotection as a compensatory response.
At the same time these observations were made, Kuwahara et al. (2007) discovered similar findings. STARS expression was up-regulated in mouse models of cardiac hypertrophy as initially observed by Mahadeva et al. (2002). STARS expression was also up-regulated in failing human hearts. The STARS promoter was found to be responsive to hypertrophic stress induced by crossing transgenic mice carrying the STARS promoter with calcineurin transgenic mice that display cardiac hypertrophy and induced by hypertrophic agonists in cardiomyocytes (Kuwahara et al., 2007). In addition, the STARS promoter was found to contain an essential MEF2-binding site and MEF2C was observed to control cardiac-specific and stress-inducible expression of STARS. The fetal cardiac genes BNP and cardiac α-actin were up-regulated following ms1 over-expression in vitro (chapter 4) and Kuwahara et al. (2007) also showed that these fetal cardiac genes that are known SRF target genes were up-regulated following STARS over-expression in vivo by generating transgenic mice. However, increased expression of STARS in the heart did not cause cardiac hypertrophy and when STARS transgenic mice were subjected to pressure overload or crossed with calcineurin transgenic mice, there was an exaggerated deterioration in cardiac function. Thus, it appears that ms1 is up-regulated in response to stress and sustained increase in ms1 results in the transition to heart failure (Kuwahara et al., 2007).

These observations along with findings from this work suggest that in response to stress stimuli, ms1 is up-regulated via MEF2 and ms1 binds to actin and sequesters actin monomers, thereby allowing MRTFs to translocate to the nucleus and activate SRF-dependent gene expression to mediate hypertrophy and provide cardioprotection initially as a compensatory response. Sustained increase in ms1 may result in an alteration of SRF target genes causing cardiac deterioration.

7.2 Future work

This study is the first to demonstrate that ms1 mediates hypertrophy and has an anti-apoptotic function and these novel findings require confirmation by the use of additional assays. Cell size and expression of known hypertrophic genes were used as a marker of hypertrophy. Hypertrophy is also characterised by an increase in protein synthesis and a re-organisation of the sarcomere, which could be examined following ms1 over-expression. In addition to analysing altered gene expression of known anti-apoptotic markers, the apoptotic assay employed was the use of a fluorescent dye to assess apoptosis on the basis of DNA fragmentation. Additional DNA fragmentation
assays include nucleosomal laddering of DNA fragments (Enari et al., 1998; Gottlieb et al., 1994; Wyllie, 1980) and the Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling (TUNEL) assay (Gavrieli et al., 1992). Other assays to measure apoptosis could be performed such as those to assess poly(ADP-ribose) polymerase (PARP) cleavage (Lazebnik et al., 1994), caspase activation (Bonavita et al., 2003) and cytochrome c release (Green and Leeuwenburgh, 2002; Liu et al., 1996; Reed, 1997). Morphological changes could be examined by the use of nuclear staining with the fluorescent dye bisbenzimide (Hoechst 33258) (Belloc et al., 1994) or the use of M30 cytodeath antibody to look for nuclear cytokeratin cleavage (Caulin et al., 1997; Leers et al., 1999; Schutte et al., 2004).

msl's hypertrophic and protective effects were demonstrated by over-expressing a c-Myc-msl fusion protein in vitro, another strategy to verify msl's role in hypertrophy and msl's anti-apoptotic function would be to inhibit expression of msl using RNA interefere (small interfering RNA (siRNA) of msl) in vitro, where knockdown of msl may not cause hypertrophy but cause apoptosis. The ability of msl to provide cardioprotection is currently being investigated in vivo by generating a transgenic mouse over-expressing msl specifically in the heart using an ES cell approach as discussed in chapter 6. This would also allow confirmation as to whether msl is involved in the development of cardiac hypertrophy.

To verify that msl regulates the hypertrophic and cardioprotective genes observed to increase following msl over-expression via the MRTF-SRF pathway, additional transfections to over-express msl with over-expression of MRTFs and SRF could be performed to examine if the same genes were up-regulated. Knockdown experiments using msl with and without knockdown of MRTFs/SRF could also be investigated to see if expression levels of hypertrophic and cardioprotective genes were reduced and hence indicate their regulation by msl via MRTF-SRF. To reveal the transcriptional regulation of hypertrophic and cardioprotective genes via msl-MRTF-SRF, luciferase reporter assays using the promoters of the genes involved in hypertrophic and cell survival pathways could be performed. Many of these genes have CArG boxes in their promoter regions (table 4.1 of chapter 4). Luciferase reporters containing the CArG boxes could be generated and co-transfected with the msl expression vector with and without MRTFs and SRF expression constructs to examine whether msl via MRTF-
SRF enhances promoter activities. In addition, the SRF binding sites may be mutated to see if promoter activities were abolished.

Once it is confirmed that msl is involved in the regulation of hypertrophic and anti-apoptotic genes, these genes could be over-expressed with msl to determine which genes with msl cause hypertrophy and which provide protection against apoptotic cell death. This would provide an important novel insight into the downstream pathways of msl and allow a more detailed hypertrophic and cell survival signalling pathway to emerge.

7.3 Concluding remarks

At the outset of this work the aims were to identify target genes and downstream pathways of msl by over-expressing msl and to examine the biological consequences of msl over-expression. Taken together, the findings presented suggest that msl induces a hypertrophic response and provides cardioprotection via a MRTF-SRF signalling pathway. It is important to note that many of the identified downstream target genes of msl implicated in hypertrophy and cardioprotection are also implicated in other important roles such as cardiac, skeletal and smooth muscle cell differentiation (SRF, GATA 4, MEF2C, myocardin and ARC) and cardiac development (SRF, GATA 4, MEF2C and myocardin). A role for msl in differentiation is supported by its developmental up-regulation (Mahadeva et al., 2002) and msl was shown to have an important role in cardiac development (Mahadeva et al., 2007). Thus, msl plays an important widespread role in cardiac development, differentiation, cardiac hypertrophy and cardioprotection and this may involve the MRTF-SRF signalling pathway.

Collectively, these findings may lead to further studies to explore the beneficial effects of the msl-MRTF-SRF pathway in cardiac development and function and as a therapeutic target for the treatment of cardiovascular disease.
References


References


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