

**THE ROLE OF FOLLISTATIN-LIKE 3 (FSTL3) IN
CARDIAC HYPERTROPHY AND REMODELLING**

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ABSTRACT

Follistatins are extracellular inhibitors of TGF- β family ligands like activin A, myostatin and bone morphogenetic proteins. Follistatin-like 3 (Fstl3) is a potent inhibitor of activin signalling and antagonises the cardioprotective role of activin A in the heart. The expression of Fstl3 is elevated in patients with heart failure and upregulated by hypertrophic stimuli in cardiomyocytes. However its role in cardiac remodelling is largely unknown. The aim of this thesis was to analyse the function of Fstl3 in the myocardium in response to hypertrophic stimuli using both *in vivo* and *in vitro* approaches.

To explore the role of Fstl3 in cardiac hypertrophy, cardiac-specific *Fstl3* knock-out mice (*Fstl3* KO) were subjected to pressure overload induced by trans-aortic constriction. They showed attenuated cardiac hypertrophy and improved cardiac function compared to wild type littermate control mice. Knock-out of *Fstl3* specifically from cardiomyocytes was sufficient to reduce the total expression of *Fstl3* in the heart following pressure overload, implying that cardiomyocytes are the major source of Fstl3 in the heart after TAC. *Fstl3* KO mice also showed reduced expression of typical hypertrophic markers including ANP, BNP, α -skeletal actin and β -MHC. Similarly, treatment of neonatal rat cardiomyocytes with Fstl3 resulted in hypertrophy as measured by increased cell size and protein synthesis. This was also accompanied by activation of p38 and JNK signalling pathways. Microarray analysis of ventricular samples from these mice indicated reduced expression of genes involved in protein binding and extracellular matrix. Verification by quantitative real-time RT-PCR revealed reduced expression of collagens and TGF- β 1 in the myocardium, indicating reduced fibrosis. This was supported by histological analysis showing reduced interstitial fibrosis.

As an *in vitro* model of pressure overload, cardiomyocytes were subjected to mechanical stretch, which elevated the expression of Fstl3. In order to explore the role of cardiomyocyte-derived Fstl3 in modulating fibroblast function, cardiac fibroblasts were treated with the conditioned medium from stretched cardiomyocytes and collagen synthesis was measured. Fstl3 was found to be necessary for conditioned medium to induce an increase in collagen synthesis in fibroblasts. Fstl3 was also shown to induce low levels of cell death in cardiac fibroblasts. In order to identify stretched

cardiomyocyte derived factors necessary for Fstl3 action on fibroblast collagen synthesis, a yeast two hybrid analysis was undertaken. Results indicated that Fstl3 may act, at least in part, through binding to proteins of the extracellular matrix as well as pro-fibrotic factors, including connective tissue growth factor (CTGF). While CTGF did not affect fibroblast collagen synthesis in the presence of Fstl3, it inhibited Fstl3 induced cell death, indicating a protective role. In summary, data presented in this thesis demonstrates that Fstl3 is upregulated after cardiac injury and functions by activating the pro-hypertrophic and pro-fibrotic responses in the myocardium, making it an attractive therapeutic target for intervention of cardiac pathologies.

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DECLARATION

The work presented in this thesis was conducted by the author and any contributions from other members are appropriately referenced.

Specifically for the data presented in Chapter 3, experimental protocols including RNA extraction from human cardiac tissues and quantitative real-time RT-PCR were carried out by Dr. Leanne Felkin. Microarray analysis was performed by Dr. Enrique Lara-Pezzi and immunohistochemical studies on human cardiac tissues were carried out by Dr. Padmini Sarathchandra. *In vivo* procedures of myocardial infarction and ischemia-reperfusion on mice as well as analysis of *Fstl3* and *Activin βA* mRNA expression were carried out by Dr. Yuichi Oshima and Kyriakos Papanicolaou.

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1. Trans-aortic constriction on 10 mice by Dr. Shimano
2. ELISA for activin A from mouse serum samples by Dr. Shimano

Additional contributions from other members are referenced in Chapter 2 in the respective methods sections.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	4
DECLARATION	6
TABLE OF CONTENTS	7
LIST OF TABLES	11
LIST OF ABBREVIATIONS	112
CHAPTER 1: INTRODUCTION	13
1.1 EPIDEMIOLOGY OF CARDIOVASCULAR DISEASES	13
1.2 CARDIAC HYPERTROPHY	13
Figure 1.1: Classification of left ventricle geometry in pathological cardiac hypertrophy.....	15
1.3 INITIATION OF PATHOLOGICAL CARDIAC HYPERTROPHY	16
1.3.1 Mechanosensors in the heart.....	16
1.3.2 Neurohumoral stimuli.....	20
Figure 1.2: G protein mediated intracellular signalling pathways in cardiac hypertrophy.....	21
1.4 INTRACELLULAR SIGNALLING CASCADES	24
Figure 1.3: Major intracellular signalling cascades activated by hypertrophic stimuli.	24
1.4.1 MAPK signalling pathway.....	25
1.4.2 Calcineurin-NFAT signalling axis.....	27
1.4.3 PI3K - Akt/PKB pathway	28
1.5 MARKERS OF CARDIAC HYPERTROPHY AND HEART FAILURE	29
1.6 METABOLIC REMODELLING IN THE HYPERTROPHIED HEART.....	30
1.7 DECOMPENSATION AND PROGRESSION TO HEART FAILURE (HF).....	32
1.7.1 Alterations in excitation-contraction coupling in cardiomyocytes	32
1.7.2 Angiogenesis mismatch.....	33
1.7.3 Cardiac fibrosis and extracellular matrix remodelling.....	34
1.7.4 Cell death.....	36
1.8 TGF- β SUPERFAMILY.....	37
1.8.1 Ligands and Receptors.....	37
Figure 1.4: TGF- β family ligands, receptors and intracellular Smad proteins.....	38
1.8.2 Intracellular signalling mechanisms.....	38
Figure 1.5: Schematic of TGF- β family signal transduction by Smad proteins.....	39
1.8.3 TGF- β family ligands in cardiac disease.....	40
1.9 FOLLISTATIN-LIKE 3 (FSTL3).....	41
1.9.1 Domain organisation of FSTL3	41
Figure 1.6: Sequence alignment of FST and FSTL3.....	42
Figure 1.7: Domain sequence comparison of FST and FSTL3.....	43
1.9.2 Expression pattern of FSTL3	43
Figure 1.8: Evolutionary conservation of FSTL3 sequence.....	44
Figure 1.9: Expression pattern of Follistatin and FSTL3 in human tissues.	45
1.9.3 Regulation of Fstl3 expression	45
Figure 1.10: Transcriptional response sites in the Fstl3 promoter.	46
1.9.4: FSTL3 interaction with Activins	46
Figure 1.11: The x-ray crystallographic structure of activin A-FSTL3 complex.....	48
1.9.5 Protein binding partners of FSTL3.....	49
1.9.6: Expression of FSTL3 in various diseases	50
1.10 HYPOTHESIS AND AIMS.....	51
CHAPTER 2: MATERIALS & METHODS	52
2.1 CLONING OF FSTL3 IN pCMS-EGFP	52
Table 2.1: Fstl3 construct	52
2.1.1 Vector and Insert preparation	52
Table 2.2: Restriction digestion of Fstl3 cDNA vector and target vector.....	52
2.1.2 Agarose gel electrophoresis	52
2.1.3 DNA extraction and purification from agarose gels.....	53
2.1.4 Ligation of vector-insert.....	54

Table 2.3: Ligation reaction setup.....	54
2.1.5 Transformation of competent <i>E.coli</i> cells and colony selection	54
2.1.6 Map of pCMS-EGFP-Fstl3.....	55
Figure 2.1: Schematic of pCMS-EGFP-Fstl3.....	55
2.2 PLASMID DNA EXTRACTION	56
2.2.1 Plasmid DNA isolation from bacterial cells: Miniprep.....	56
2.2.2 Plasmid DNA isolation from yeast cells	57
2.2.3 Plasmid DNA isolation from bacterial cells: Maxiprep.....	57
2.2.4 DNA quantification using spectrophotometry.....	58
2.3 NEONATAL RAT VENTRICULAR CARDIOMYOCYTES AND FIBROBLASTS ISOLATION AND CULTURE	59
2.4 RNA EXTRACTION FROM CELLS.....	61
2.5 RNA EXTRACTION FROM MOUSE CARDIAC TISSUE.....	62
2.5.1 Sample disruption and homogenization	62
2.5.2 RNA isolation	63
2.5.3 RNA quantification	64
2.6 MICROARRAY ANALYSIS	64
2.7 cDNA SYNTHESIS.....	65
Table 2.4: cDNA synthesis reaction setup	65
Table 2.5: cDNA synthesis reaction conditions	66
2.8 QUANTITATIVE REAL-TIME RT-PCR.....	66
Table 2.6: Real-time PCR reaction setup	67
Table 2.7: Real-time PCR cycling conditions.....	68
2.9 PROTEIN SAMPLE PREPARATION FROM CELLS AND TISSUES	68
2.10 PROTEIN QUANTIFICATION.....	69
Table 2.8: Protein standards	69
Figure 2.2: Representative standard curve for protein quantification.	70
2.11 ONE-DIMENSIONAL GEL ELECTROPHORESIS OF PROTEINS.....	70
Table 2.9: Resolving gel formulation.....	71
Table 2.10: Stacking gel formulation.....	71
2.12 ELECTROBLOTTING OF PROTEINS	72
Figure 2.3: Assembly of protein transfer apparatus in trans-blot cassette.....	72
2.13 WESTERN BLOT	73
2.14 CO-IMMUNOPRECIPITATION (CO-IP) OF PROTEINS	74
Figure 2.4: Schematic of co-immunoprecipitation technique.	75
2.15 CARDIOMYOCYTE CELL SURFACE AREA MEASUREMENT	76
2.16 MEASUREMENT OF CARDIOMYOCYTE PROTEIN SYNTHESIS.....	77
2.17 MECHANICAL STRETCH OF NEONATAL CARDIOMYOCYTES AND FIBROBLASTS	78
Figure 2.5: Schematic of BioFlex [®] plates and application of equibiaxial strain.....	78
2.18 MEASUREMENT OF COLLAGEN USING SIRCOL COLLAGEN ASSAY.....	79
Figure 2.6: Standard curve for collagen measurement using Sircol assay.	80
2.19 MEASUREMENT OF CELL DEATH - LDH ASSAY.....	81
2.20 LUCIFERASE ASSAY	81
2.21 CARDIAC-SPECIFIC <i>FSTL3</i> KO MICE	82
2.22 TRANS-AORTIC CONSTRICTION (AORTIC BANDING)	82
Figure 2.7: Schematic of trans-aortic constriction (TAC).	83
2.23 ECHOCARDIOGRAPHY.....	83
2.24 TISSUE SAMPLING.....	84
2.25 STAINING OF PARAFFIN SECTIONS WITH PICROSIRIUS RED.....	84
Table 2.11: Deparaffinization and rehydration of paraffin sections	85
Table 2.12: Dehydration and clearing.....	85
2.26 STAINING OF PARAFFIN SECTIONS WITH WHEAT-GERM AGGLUTININ (WGA)	86
Table 2.13: Immunofluorescence staining.....	86
Figure 2.8: Negative control for α -sarcomeric actin antibody.....	87
2.27 ELISA FOR ACTIVIN A.....	87
2.28 YEAST TWO-HYBRID ANALYSIS.....	88
2.28.1 Cloning of <i>Fstl3</i> in pBD-Gal4-Cam: Bait plasmid construction.....	88
Figure 2.9: Schematic representation of bait plasmid encoding the Gal4-BD with <i>Fstl3</i>	89
2.28.2 Transformation of yeast with bait plasmid.....	89
2.28.3 Checking leakiness of reporter gene <i>HIS3</i>	90

2.28.4 Transformation of yeast with target DNA library	90
Figure 2.10: Schematic representation of the target plasmid construct.	90
2.28.5 Screening of positive colonies.....	91
Figure 2.11: Flow diagram of yeast two-hybrid analysis of protein-protein interactions.....	92
CHAPTER 3: FSTL3 IN HUMAN HEART FAILURE	93
3.1 INTRODUCTION.....	93
3.2 PATIENT CHARACTERISTICS AND MYOCARDIAL SAMPLES	94
3.3 EXPRESSION OF FSTL3 IS ELEVATED IN HEART FAILURE	94
Figure 3.1: Expression of TGF- β family ligands and their inhibitors in heart failure.	95
Figure 3.2: Expression of FSTL3 in heart failure and recovery.....	96
Figure 3.3: Tissue distribution of FSTL3 in heart failure patients.	97
3.4 MICROARRAY ANALYSIS OF FSTL3 IN HEART FAILURE	97
Figure 3.4: Gene ontology analysis of FSTL3-correlating genes in heart failure.....	98
3.5 EXPRESSION OF FSTL3 IN MOUSE MODELS OF CARDIAC INJURY	98
Figure 3.5: Fstl3 mRNA expression is elevated in different models of cardiac injury.	99
Figure 3.6: Elevated Activin β A mRNA expression in different models of cardiac injury.	100
3.6 REGULATION OF FSTL3 EXPRESSION BY HYPERTROPHIC STIMULI IN THE HEART	100
Figure 3.7: Expression of Fstl3 mRNA is elevated by TGF- β 1 in cardiomyocytes.....	101
3.7 DISCUSSION	101
CHAPTER 4: CARDIAC HYPERTROPHY IN FSTL3 KO MICE	104
4.1 INTRODUCTION.....	104
4.2 BASELINE CHARACTERISTICS OF CARDIAC-SPECIFIC FSTL3 KO MICE	104
Figure 4.1: Similar baseline cardiac size and function of wild type and cardiac-specific Fstl3 KO mice.....	105
4.3 ANALYSIS OF FSTL3 EXPRESSION POST-INJURY IN CARDIOMYOCYTE-SPECIFIC FSTL3 KO MICE	105
Figure 4.2: Normalised expression of Fstl3 in KO mice after injury.	106
Figure 4.3: Myocardial expression of Activin A in WT and Fstl3 KO mice is unchanged.	107
4.4 ANALYSIS OF CARDIAC HYPERTROPHY IN FSTL3 KO MICE AFTER TAC.....	107
Figure 4.4: Reduced HW/BW and LW/BW ratio in Fstl3 KO mice after TAC.....	108
Figure 4.5: Reduced cardiomyocyte cross-sectional area in Fstl3 KO mice after TAC.	109
4.5 EXPRESSION OF MARKERS OF CARDIAC HYPERTROPHY AFTER TAC	110
Figure 4.6: Reduced expression of hypertrophic markers in Fstl3 KO mice after banding.	110
4.6 CARDIAC FUNCTIONAL STUDIES ON FSTL3 KO MICE AFTER TAC	111
Figure 4.7: Improved cardiac function in Fstl3 KO mice after TAC.	111
4.7 METABOLIC CHARACTERISTICS OF FSTL3 KO MICE	112
Figure 4.8: Normalised expression of fatty acid oxidation markers in Fstl3 KO mice after TAC.	113
4.8 DISCUSSION	113
CHAPTER 5: FSTL3 AS A PRO-HYPERTROPHIC FACTOR IN THE MYOCARDIUM	118
5.1 INTRODUCTION.....	118
5.2 EFFECT OF FSTL3 ON CARDIOMYOCYTE HYPERTROPHY.....	118
Figure 5.1: Fstl3 induces increased protein synthesis in cardiomyocytes.....	119
Figure 5.2: Fstl3 is sufficient to increase cardiomyocyte surface area.....	120
5.3 EFFECT OF FSTL3 ON CARDIOMYOCYTE SIGNALLING PATHWAYS.....	120
Figure 5.3: Fstl3 may activate two MAPK pathways in cardiomyocytes.	121
5.4 DISCUSSION	122
CHAPTER 6: CARDIAC REMODELLING IN FSTL3 KO MICE	125
6.1 INTRODUCTION.....	125
6.2 MICROARRAY ANALYSIS OF FSTL3 KO MICE	125
Table 6.1 (A) KO TAC < WT TAC Representative Genes	127
Table 6.1 (B) KO TAC < WT TAC Gene Ontology (GO)	128
6.3 ANALYSIS OF CARDIAC FIBROSIS IN FSTL3 KO MICE IN RESPONSE TO TAC.....	129
Figure 6.1: Reduced cardiac fibrosis in Fstl3 KO mice after TAC.	129
Figure 6.2: Reduced expression of fibrosis markers in Fstl3 KO mice.	130
Figure 6.3: Reduced expression of pro-fibrotic cytokines in Fstl3 KO mice.	131
6.4 EXPRESSION OF MATRIX METALLOPROTEINASES AND THEIR INHIBITORS IN FSTL3 KO MICE	131

Figure 6.4: Reduced expression of markers of extracellular matrix remodelling in <i>Fstl3</i> KO hearts.	132
6.5 INFLAMMATORY RESPONSE IN <i>FSTL3</i> KO MICE AFTER TAC	133
Figure 6.5: Deranged expression of inflammatory cytokines in <i>Fstl3</i> KO mice.	133
6.6 DISCUSSION	133
CHAPTER 7: ROLE OF FSTL3 IN INDUCTION OF CARDIAC FIBROSIS	138
7.1 INTRODUCTION	138
7.2 EFFECT OF RECOMBINANT FSTL3 ON CARDIAC FIBROBLASTS	138
Figure 7.1: <i>Fstl3</i> is not sufficient to increase collagen synthesis in fibroblasts.	139
7.2.1 Effect of <i>Fstl3</i> on fibroblast death and proliferation	139
Figure 7.2: <i>Fstl3</i> induces a dose dependent increase in LDH release from fibroblasts.	140
Figure 7.3: <i>Fstl3</i> does not affect fibroblast proliferation.	141
7.3 EFFECT OF MECHANICAL STRETCH AND FSTL3 ON FIBROBLASTS	141
Figure 7.4: Mechanical stretch of fibroblasts does not induce <i>Fstl3</i> mRNA expression.	142
Figure 7.5: <i>Fstl3</i> induces collagen synthesis in stretched fibroblasts.	143
7.3.1 Effect of <i>Fstl3</i> on Smad activation in fibroblasts	143
Figure 7.6: <i>Fstl3</i> activates the Smad pathway in cardiac fibroblasts.	144
7.4 IN VITRO MODEL OF TAC: MECHANICAL STRETCH OF CARDIOMYOCYTES	144
Figure 7.7: Mechanical stretch of cardiomyocytes induces <i>Fstl3</i> mRNA synthesis.	145
7.4.1 Effect of stretched cardiomyocytes on fibroblasts	145
Figure 7.8: <i>Fstl3</i> is necessary for stretch-mediated paracrine induction of fibroblast collagen synthesis.	146
7.5 DISCUSSION	146
CHAPTER 8: YEAST TWO-HYBRID ANALYSIS FOR FSTL3 INTERACTION PARTNERS	150
8.1 INTRODUCTION	150
8.2 CHECKING FOR ACTIVATION OF REPORTER GENE BY FSTL3	150
Figure 8.1: The <i>Fstl3</i> bait protein does not auto-activate the reporter gene <i>HIS3</i> .	151
8.3 IDENTIFICATION OF POTENTIAL BINDING PARTNERS OF FSTL3	151
Table 8.1 Probable binding partners of <i>Fstl3</i> .	153
8.4 ANALYSIS OF FSTL3 INTERACTION WITH CTGF	154
Figure 8.2: Anti-CTGF antibody immunoprecipitates CTGF from fibroblast cell lysate.	155
Figure 8.3: Co-immunoprecipitation of CTGF and <i>Fstl3</i> from fibroblast cell lysate.	156
8.5 FUNCTIONAL SIGNIFICANCE OF FSTL3 INTERACTION WITH CTGF	156
Figure 8.4: <i>Fstl3</i> and CTGF together are not sufficient to enhance collagen synthesis in fibroblasts.	157
Figure 8.5 CTGF inhibits <i>Fstl3</i> -induced LDH release in cardiac fibroblasts.	158
8.6 DISCUSSION	158
CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS	161
9.1 GENERAL DISCUSSION AND CONCLUDING REMARKS	161
9.2 LIMITATIONS AND FUTURE WORK	166
9.3 SUMMARY	167
Figure 9.1: Schematic of <i>Fstl3</i> action in the heart.	168
REFERENCES	169
APPENDIX I: TAQMAN® GENE EXPRESSION ASSAYS	198
APPENDIX II: ANTIBODIES USED FOR WESTERN BLOT ANALYSIS	199
APPENDIX III: YEAST TWO-HYBRID REAGENTS	199
APPENDIX IV: MICROARRAY ANALYSIS	202

LIST OF TABLES

Table 2.1: Fstl3 construct	52
Table 2.2: Restriction digestion of <i>Fstl3</i> cDNA vector and target vector	52
Table 2.3: Ligation reaction setup	54
Table 2.4: cDNA synthesis reaction setup	65
Table 2.5: cDNA synthesis reaction conditions	66
Table 2.6: Real-time PCR reaction setup	67
Table 2.7: Real-time PCR cycling conditions	68
Table 2.8: Protein standards	69
Table 2.9: Resolving gel formulation	71
Table 2.10: Stacking gel formulation	71
Table 2.11: Deparaffinization and rehydration of paraffin sections.....	85
Table 2.12: Dehydration and clearing	85
Table 2.13: Immunofluorescence staining	86
Table 6.1 (A) KO TAC < WT TAC Representative Genes.....	127
Table 6.1 (B) KO TAC < WT TAC Gene Ontology (GO)	128
Table 8.1 Probable binding partners of Fstl3	153

LIST OF ABBREVIATIONS

<i>Abbreviation</i>	<i>Name</i>	<i>Abbreviation</i>	<i>Name</i>
ACE	angiotensin converting enzyme	IP	immunoprecipitation
ADAM	a disintegrin and metalloproteinase	JNK	Jun N terminal protein kinase
ALK	activin receptor like kinases	KO	knock-out
Ang-II	angiotensin II	LDH	lactate dehydrogenase
ANP	atrial natriuretic peptide	LVAD	left ventricular assist devices
BMP	bone morphogenetic proteins	MAPK	mitogen activated protein kinases
BSA	bovine serum albumin	MCAD	medium chain acyl-coA dehydrogenase
Cn	Calcineurin	mCPT1 or CPT1 β	muscle type carnitine palmitoyltransferase I
CT-1	cardiotrophin 1	MMP	matrix metalloproteinases
CTGF	connective tissue growth factor	NFAT	nuclear factor of activated T cells
DMSO	dimethyl sulphoxide	NRVF	neonata rat ventricular fibroblasts
DNA	deoxyribonucleic acid	NRVM	neonatal rat ventricular myocytes
ECC	excitation-contraction coupling	PAGE	polyacrylamide gel electrophoresis
ECM	extracellular matrix	PCR	polymerase chain reaction
EF	ejection fraction	PE	phenylephrine
EGF	epidermal growth factor	PGC-1	PPAR gamma coactivator
ELISA	enzyme-linked immunosorbent assay	PI3K	phosphoinositide 3 kinase
ERK	extracellular regulated kinase	PLC	phospholipase C
ET-1	endothelin 1	PPAR	peroxisome proliferator-activated receptor
FAK	focal adhesion kinase	RNA	ribonucleic acid
FAO	fatty acid oxidation	RT	room temperature
FRET	fluorescence resonance energy transfer	RyR	ryanodine receptors
FS	fractional shortening	SDS	sodium dodecyl sulphate
Fst	folliculin	SERCA	sarcoplasmic/endoplasmic reticulum Ca ²⁺ ATPase
Fstl3	folliculin-like 3	SKA	skeletal α actin
GFP	green fluorescent protein	TAC	trans-aortic constriction
GO	gene ontology	TGF- β	transforming growth factor β
GPCR	G protein coupled receptors	TIMP	tissue inhibitor of metalloproteinases
Hif-1	hypoxia-inducible factor 1	VEGF	vascular endothelial growth factor
IGF	insulin-like growth factor	WGA	wheat-germ agglutinin
IL	Interleukin	WT	wild type
		β -MHC	β myosin heavy chain

CHAPTER 1: Introduction

1.1 Epidemiology of cardiovascular diseases

Cardiovascular diseases (CVD) encompass a number of pathologies such as coronary heart disease (CHD) including angina and myocardial infarction, stroke, cardiomyopathies, rheumatic and inflammatory heart diseases, hypertension, etc. CVDs are the most common cause of death with an estimated 17.5 million deaths per year globally [1]. In Europe, CVDs account for about 4.3 million deaths per year, representing about 48% of all deaths [2]. In the UK alone, about 35% of the deaths each year are accounted for by CVD, wherein about half of these (48%) are due to CHD and 28% due to stroke [3]. Currently, 23% of all disability-adjusted life years (DALYs) are lost to CVD in Europe [2]. Thus it is not surprising that the overall costs of CVD in the EU are about 192 million € per year with about 57% of these due to direct healthcare costs [4].

1.2 Cardiac hypertrophy

In most cases, cardiovascular diseases eventually lead to heart failure where the ability of the heart to adequately pump blood in response to systemic demands is compromised. However, initial compensation of the heart to increased systemic demands is provided in the form of cardiac hypertrophy, where individual cardiomyocytes grow in length and/or width in order to increase cardiac pump function and reduce ventricular wall tension [5-7]. Although cardiac hypertrophy is most commonly observed in cases where the underlying CVD leads to hemodynamic overload, it is also seen as an adaptive phase following myocardial infarction or due to genetic disposition [8-12]. Cardiac hypertrophy can be broadly classified into physiological and pathological hypertrophy. Physiological cardiac hypertrophy is commonly seen in response to exercise training or during pregnancy, where the increased workload is matched by preserved contractility [13]. In contrast, hypertrophic growth due to arterial hypertension, aortic stenosis or regurgitation develops initially as a compensatory mechanism gradually leading to contractile dysfunction and is called as pathological hypertrophy [6, 14]. These definitions are not taken absolutely, as the

mechanisms that drive cardiac growth responses are often common in physiological and pathological hypertrophy.

Both physiological and pathological hypertrophy can be sub-classified into concentric and eccentric hypertrophy. Concentric hypertrophy develops in response to pathological stimuli such as hypertension or aortic stenosis in order to normalize the increased wall stress. This results in thickening of the ventricular walls due to parallel addition of sarcomeres and also results in smaller ventricular chamber dimensions [15, 16]. Concentric hypertrophy is also seen as an adaptation to isometric or static physical exercises such as weight lifting [13]. On the other hand, eccentric hypertrophy develops in response to increased diastolic wall stress and is characterized by chamber dilation and wall thinning due to addition of sarcomeres in series [15, 17]. It develops in response to isotonic exercise training as well as pathological stimuli such as aortic or mitral regurgitation resulting in volume overload [18, 19]. Hypertrophy following myocardial infarction shows the characteristics of both concentric and eccentric hypertrophy due to a combination of pressure and volume overload [10, 20]. These patterns of ventricular geometry are summarized below in Figure 1.1 based on the left ventricular (LV) mass and relative wall thickness defined as the ratio of LV wall thickness to diastolic chamber diameter. Besides concentric and eccentric hypertrophy, a third type of LV geometry is also observed *i.e.* concentric remodelling, characterized by normal LV mass but increased relative wall thickness, and is often seen in chronic hypertension [21].

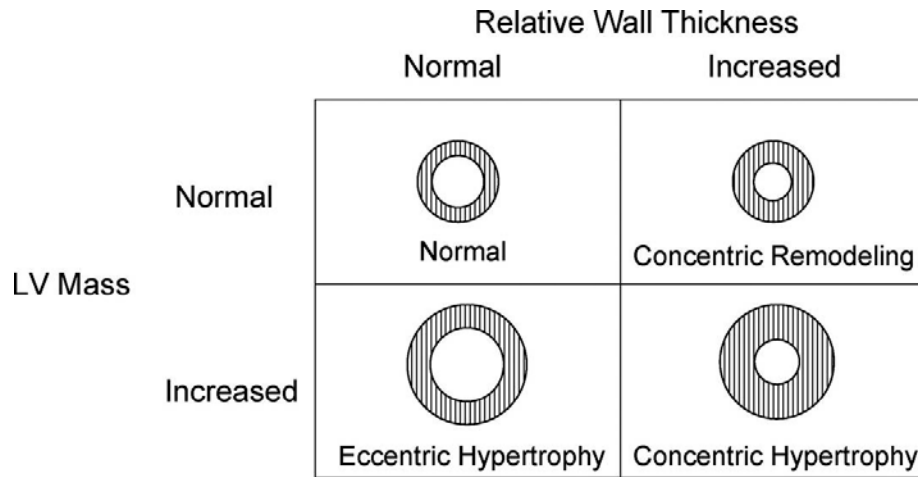


Figure 1.1: Classification of left ventricle geometry in pathological cardiac hypertrophy. Relative wall thickness is the ratio of LV mass to diastolic diameter. The striped area represents LV wall thickness and the area of the inner circle represents LV volume. Concentric hypertrophy is present when LV mass increases and is accompanied by increased relative wall thickness. In eccentric hypertrophy, LV mass increases while relative wall thickness is maintained. Concentric remodelling is characterized by increased relative wall thickness with normal LV mass. Source: Drazner, M. H. Circulation 2011;123:327-334 [22].

At the cellular level, a combination of hypertrophic stimuli, both mechanical and neurohumoral, initiate the cardiac growth response in the absence of hyperplasia. These have been extensively studied using neonatal cardiomyocytes as model systems [23, 24]. The cardiomyocyte growth response is characterized by the activation of immediate early genes such as *c-jun*, *c-fos*, *jun B* and *egr* family. Following this, the ‘fetal gene program’ is activated, wherein elevated expression of genes normally involved in developmental processes is observed. These include atrial natriuretic peptide/factor (ANP or ANF), α - skeletal actin and isoform switch from α – myosin heavy chain (α -MHC) to β – myosin heavy chain (β -MHC or Myh7) [24-26]. This is accompanied by an increase in protein synthesis and cell size as well as sarcomeric organization, thus initiating cardiomyocyte hypertrophy [24, 27-30].

1.3 Initiation of pathological cardiac hypertrophy

Cardiac hypertrophy is initiated by various biomechanical and neurohumoral stress stimuli coming from the underlying CVD. These lead to changes in the intracellular signalling cascades resulting in increased protein synthesis/stability thereby leading to increase in the size of individual cardiac myocytes. It is possible that mechanical stress is the primary stimulus for cardiac hypertrophy as blockade of adrenoreceptors (humoral stimuli) or sympathectomy (neural stimuli) does not affect development of cardiac hypertrophy in response to hemodynamic overload [31].

1.3.1 Mechanosensors in the heart

Increased hemodynamic load is sensed by specific classes of mechanosensors including integrins, cytoskeleton and sarcolemmal proteins as well as guanine nucleotide binding proteins (G proteins). Integrins are a family of cell-surface receptors that link the extracellular matrix to the cytoskeleton and intracellular signalling proteins like focal adhesion kinase (FAK). Mechanical stretch of cardiac fibroblasts was shown to activate the extracellular regulated kinase (ERK) and Jun N-terminal protein kinase (JNK) pathways in an integrin-dependent manner [32], thus proving the ability of integrins to act as mechanotransducers. Integrins can also influence the hypertrophic response of cardiomyocytes, as overexpression of $\beta 1$ integrin in cardiomyocytes was shown to increase protein synthesis and atrial natriuretic peptide (ANP) expression [33]. Moreover, integrins may be directly involved in activating signalling cascades by recruiting non-receptor tyrosine kinases such as FAK and Src, cytoskeletal proteins (α -actinin) and signal transducing molecules (Grb2, Sos, Ras, Raf, PLC γ , ERKs, and SAPKs) as part of the focal adhesion complexes (FACs) [34, 35]. In fact integrins can activate FAK through Src [36] which can further activate the ERK pathway through Grb2-Sos-Ras-Raf or through PLC γ [35, 37]. These signalling molecules can activate various downstream cascades such as those involving p21^{ras}, mitogen-activated protein kinases (MAPK), protein kinase C and p70^{S6K} [38, 39], leading to increased protein synthesis and activation of the hypertrophic response. Moreover, FAK has been shown to mediate sarcomeric organization as well as activation of hypertrophic genes like ANP, in co-ordination with Src substrate protein p130Cas [40]. Other ECM proteins in contact with integrins such as collagens, laminin and fibronectin may also be involved

in triggering signals. For example, mechanical stretch was shown to induce the activation of JNK1 in cardiac fibroblasts cultured on fibronectin, laminin or vitronectin whereas activation of ERK2 could only be observed if fibroblasts were cultured on fibronectin [32].

Cytoplasmic proteins like melusin and MLP have also been implicated as sensors of mechanical stretch in cardiomyocytes. Melusin is an integrin interacting protein and is essential for attenuation of cardiac hypertrophy in response to hemodynamic load but not to humoral stimuli, via the phosphorylation (inactivation) of glycogen synthase kinase - 3β (GSK- 3β) [41]. The muscle LIM domain protein (MLP) is anchored to the Z-disc and may act as an internal stretch-sensor as mutations in MLP have been associated with dilated cardiomyopathy and heart failure [42]. Mechanical stretch can also lead to the expression and release of various growth factors and hormones that can directly activate the cardiac growth response [43]. These include endothelin 1 (ET-1) [44], angiotensin II (Ang-II) [45], transforming growth factor β (TGF- β) [46, 47], insulin-like growth factor 1 (IGF-1) [46], etc. Thus, integrins can establish a well-coordinated cardiac growth response to stretch by integrating the various signalling pathways activated by ECM and growth factors.

Sarcolemmal proteins that can act as mechanosensors in the heart include enzymes such as phospholipases C, D and A_2 (PLC, PLD, PLA_2) and protein kinase C (PKC), stretch activated ion channels and ion exchange channels like the Na^+/H^+ exchanger (NHE). Phospholipases are enzymes that catalytically breakdown plasma membrane phospholipids in a Ca^{2+} dependent manner to generate various lipid-derived second messengers [48]. In cardiomyocytes, mechanical stretch is shown to activate PLC within 1 min and also PLD and PLA_2 . Activated phospholipases can hydrolyse phospholipids to release second messengers like inositol-1,4,5-trisphosphate (IP_3), diacyl glycerol (DAG), arachidonic acids, and phosphatidic acid [49]. IP_3 causes the release of Ca^{2+} from intracellular Ca^{2+} stores, which can then activate some isoforms of PKC (α , β , γ) as well as other signalling intermediates like calmodulin and calcineurin. PKC (α , β , γ , δ , ϵ , η , θ , μ) can also be activated by DAG [50], and is involved in the expression of stretch-induced immediate early genes like c-fos and early growth response 1 (Egr-1) [49, 51].

Stretch-activated channels (SAC) and ion exchange channels allow the passage of free ions like Na^+ , K^+ and Ca^{2+} into the cell cytoplasm. Opening of SAC leads to an influx of Ca^{2+} ions into the cells which is known to cause Ca^{2+} induced Ca^{2+} release [52]. At resting membrane potentials, this can lead to depolarisation of the membrane thereby increasing its excitability. Whole cell stretch experiments have shown that stretch affects a variety of ion channels including K^+ ATP channel [53], L-type Ca^{2+} channel [54] and Na^+/K^+ pump current [55]. In guinea pig ventricular myocytes, it has been reported that whole-cell uniaxial stretch causes a time-independent current, most likely through a Ca^{2+} -activated non-selective cation channel or the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [56]. However, it is yet unclear if SAC can function as the initial mechanosensors for stretch-induced cardiac hypertrophy as they may be activated secondarily by Ca^{2+} release or fatty acids [56, 57].

The Na^+/H^+ exchanger (NHE) is located in the sarcolemma and regulates the exchange of Na^+ (influx) for H^+ (efflux), thereby increasing intracellular pH [58]. In cultured cardiomyocytes, specific inhibition of NHE was shown to attenuate stretch-mediated MAP kinase activation as well as protein synthesis independently of Ang II and ET-1. Similarly, acid loading of cardiomyocytes was also found to reduce MAP kinase activation [59]. Enhanced activity of NHE and the subsequent increase in intracellular Na^+ can activate the $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) channel and thereby cause an increase in intracellular Ca^{2+} [60], which is known to activate Ca^{2+} /calmodulin-dependent kinase II (CaMKII) and calcineurin. To this effect, it was reported that ANP through its receptor activates guanylyl-cyclase A (GC-A) to moderate the hypertrophic response to pressure overload by inhibiting excessive activation of NHE and the subsequent increase in intracellular Ca^{2+} and activity of CaMKII and Akt/ protein kinase B (PKB) [61]. Thus, NHE may play an important role in converting mechanical stress into intracellular biochemical signals.

Guanine nucleotide binding proteins (G proteins) couple cell surface receptors to intracellular signalling intermediates by switching between an inactive guanosine diphosphate (GDP) bound state and an active guanosine triphosphate (GTP) bound state [62]. G proteins exist in two forms – the ‘small G proteins’ and the ‘heterotrimeric G proteins’. The small G proteins are single polypeptides consisting of five families – Rho, Ras, ARFs, Rab, Ran [63]. Ras can promote activation of Raf-1, phosphoinositide

3-kinase (PI3K) [64], small GTPase Ral proteins and GTPase activating proteins (GAP) p120GAP and p190GAP, thereby leading to activation of Rho [65]. In addition, Ras can also activate all three MAPK pathways – ERK1/2 [66], JNK and p38 [67]. Ras, when expressed in a constitutively active form in mice, was sufficient to induce cardiac hypertrophy and diastolic dysfunction [68]. Thus, activation of Ras can contribute significantly to multiple MAPK signalling responses in the heart, as shown by the phenotype of Ras overexpressing transgenic mice [69]. The Rho family is involved in organisation of the actin cytoskeleton and the formation of focal adhesion complexes by regulation of integrin clustering [38, 70]. Also, some members of the Rho family (Rac and cdc42) have been shown to activate the Jun N-terminal protein kinase (JNK) and p38 MAP kinase pathways [71]. RhoA can also activate Rho kinase (ROCK) which leads to sarcomeric organisation by activation of myosin light chain kinase (MLCK) [72]. Phenylephrine (PE) and endothelin-1 (ET-1) mediated cardiomyocyte hypertrophy can also be blocked using either dominant negative mutant of RhoA or ROCK inhibitor [73, 74]. However, overexpression of RhoA in mice was not sufficient to cause ventricular hypertrophy. It resulted in conduction abnormalities and atrial fibrillation leading to ventricular dilation and heart failure [75]. Similarly, overexpression of the Rab family member Rab1a in transgenic mice was found to be sufficient for cardiac hypertrophy leading to dilation and heart failure through activation of PKC [76]. Thus, small G proteins are important mediators of cardiac hypertrophy and act by converting membrane signals to intracellular biochemical signals in the heart.

Heterotrimeric G proteins consist of G_α and $G_{\beta\gamma}$ subunits and transduce stimulatory or inhibitory signals originating from cell surface receptors – G protein coupled receptors (GPCR). Activation of G proteins results in exchange of GDP for GTP on the G_α subunit and dissociation of G_α from the $G_{\beta\gamma}$ subunits, which can then activate downstream signalling effectors – typically adenylyl cyclase, phospholipase C, MAPK, PI3K, *etc.* [77, 78]. G proteins, specifically the G_{α_q} and $G_{\alpha_{i1}}$ subunits in the adult rat cardiac fibroblasts have been shown to be activated within 1 min of mechanical stretch and this response was modulated by the amount and duration of the strain applied [79]. Moreover, transient overexpression of constitutively active G_{α_q} in the heart of mice was found to be sufficient to induce cardiac hypertrophy leading to heart failure through calcineurin-dependent and independent mechanisms [80]. Also, specific inhibition of G_{α_q} signalling by overexpression of a carboxyl terminal G_{α_q} peptide transgenically in

mice was shown to attenuate the cardiac hypertrophy response to pressure overload [81]. These studies indicate a crucial role of G proteins in the mechanotransduction of stretch signals in the heart leading to cardiac hypertrophy and heart failure.

1.3.2 Neurohumoral stimuli

Neurohumoral stimuli that initiate hypertrophic signalling include endocrine hormones and growth factors such as catecholamines (noradrenaline and adrenaline), endothelin-1, angiotensin-II, insulin-like growth factor (IGF-1), *etc.* These ligands are sensed by cardiomyocytes through various receptors such as GPCRs, receptors with intracellular tyrosine kinase domains (RTK), serine/threonine kinase domains and gp-130 linked receptors. Activation of receptors in turn initiates various signalling cascades that are central in controlling the cardiac growth and remodelling response.

G protein coupled receptors (GPCRs) are a family of seven transmembrane domain containing receptors that couple to intracellular heterotrimeric G proteins and mediate stimulatory or inhibitory signals from the cell surface. They exist primarily as three functional classes of receptors depending on the class of G_α protein that they couple to. These are the β -adrenergic receptors that mainly couple to G_{as} , the muscarinic cholinergic receptors coupled to G_{ai} and the α -adrenergic receptors, endothelin receptor and angiotensin receptors that are coupled to G_{aq} proteins. Examples of GPCR agonists include β -adrenergic agonists adrenaline, noradrenaline and isoproterenol, cholinergic receptor agonist acetylcholine and α -adrenergic agonists adrenaline, noradrenaline, endothelin-I (ET-1) and angiotensin-II (ang-II). When an agonist binds the extracellular or transmembrane domain of the receptor, it results in the catalysis of GDP to GTP exchange on the G_α subunit and its dissociation from the $G_{\beta\gamma}$ subunits. These G protein subunits then transmit the intracellular signals via activation of effector proteins - G_{as} and G_{ai} activate adenylyl cyclase (AC) while G_{aq} activates phospholipase C [82]. This signal is further propagated by production of second messengers like cyclic AMP (cAMP), IP₃ and DAG. Adenylyl cyclase activity leads to the accumulation of cAMP and consequent activation of protein kinase A (PKA), which can phosphorylate several proteins involved in cardiomyocyte contraction such as the L-type calcium channel, ryanodine receptors and phospholamban, thereby augmenting cardiac contractility. Phospholipase C hydrolyses PIP₂ to IP₃ and DAG, which in turn activates protein

kinase C – an important mediator of GPCR mediated cardiac hypertrophy [83]. In addition, free $G_{\beta\gamma}$ subunits directly enhance MAPK signalling, PI3-K and Ras activity [78, 84]. Figure 1.2 shows a schematic of various GPCRs signalling through their respective G proteins.

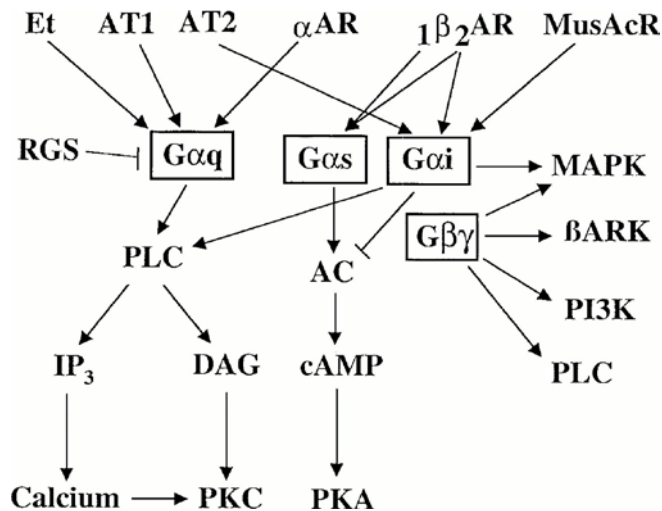


Figure 1.2: G protein mediated intracellular signalling pathways in cardiac hypertrophy. Various G protein coupled receptors activate different classes of G proteins such as $G_{\alpha q}$, $G_{\alpha s}$ and $G_{\alpha i}$ to initiate intracellular signalling pathways mainly activating phospholipase C (PLC) and adenylyl cyclase (AC). Source: Molkenstein JD, Dorn GW. Annual Review of Physiology 2001; 63: 391-426 [85]

G proteins and GPCRs have been extensively studied and implicated in the development of cardiac hypertrophy. Since $G_{\alpha i}$ mediated inhibition of AC can attenuate contractility, its expression has been hypothesized and proven to be elevated in the pathogenesis of cardiac hypertrophy and heart failure in patients as well as animal models [86-88]. Thus, upregulation of $G_{\alpha i}$ and the subsequent reduction in AC activity may be an important step in the transition from compensated hypertrophy to decompensated heart failure. The role of $G_{\alpha s}$ in heart failure is controversial as AC activity has been shown to be unaffected by transgenic overexpression of $G_{\alpha s}$ in the heart [89]. Although agonist induced contractility was elevated in these mice, it did not affect basal contractility but increased cellular necrosis, cardiac fibrosis and hypertrophy [90]. In contrast, $G_{\alpha q}$ has been shown to play a significant role in cardiac disease as demonstrated by its direct and necessary action on cardiac hypertrophy [81,

91, 92]. This is further exemplified by the clinical success of angiotensin receptor blockers in prevention or regression of cardiac hypertrophy [93, 94]. G-protein mediated signalling by agonist-activated GPCRs can be inhibited by desensitization of the receptor through phosphorylation by GPCR kinases (GRKs), leading to receptor internalisation and its uncoupling from the G proteins [95]. In addition, regulator of G protein signalling (RGS) proteins, which activate GTPase and induce GTP hydrolysis, have also been shown to inhibit GPCR signalling and subsequent cardiac hypertrophy [96, 97]. Thus GPCR mediated signalling functions to transmit the cardiomyocyte response to various hypertrophic stimuli.

Receptors with intrinsic tyrosine kinase activity (RTK) have been implicated to play a role in both physiological and pathological response of the heart. Examples of RTK involved in hypertrophy regulation include insulin-like growth factors (IGF-I and II) receptor and epidermal growth factor (EGF) receptor amongst others [98]. IGF receptor binding leads to activation of the receptor tyrosine kinase, which phosphorylates insulin receptor substrates (IRS-1 and 2). This subsequently leads to activation of phosphoinositide-3 kinase (PI3-K) which further activates Akt (or protein kinase B) and p70/85 S6K. Other signalling intermediates are also activated by IGF receptor, including Ras and PLC which can later activate MAPK proteins as well as protein kinase C (PKC) (Reviewed in [99]). The role of IGFs in regulation of cardiac hypertrophy is controversial. *In vitro* studies on cardiomyocytes have shown hypertrophy in response to IGF-1 stimulation [100]. Transgenic overexpression of IGF-1 has been reported to increase cardiomyocyte numbers and therefore cardiac mass without causing cardiomyocyte hypertrophy [101]. In contrast, local expression of IGF-1 in the heart was shown to induce cardiac hypertrophy and age dependent systolic dysfunction [102]. Due to the pleiotropic nature of IGFs, a cardioprotective role has also been documented [103-107]. Another RTK implicated in cardiac hypertrophy is the EGF receptor, which can be activated by EGF, heparin binding EGF (HB-EGF) and TGF- α amongst others [108]. Furthermore, transactivation of EGF receptor has been shown to be necessary for hypertrophic signalling following angiotensin II type I (AT1) receptor activation [109, 110]. It has since been suggested that GPCRs such as AT1 receptor, which lack intrinsic kinase activity may use RTK to transduce hypertrophic signals in the myocardium [111, 112]. Activation of EGF receptor by HB-EGF shedding, which can be induced by a disintegrin and metalloproteinase – 12

(ADAM12), has also been shown to induce cardiac hypertrophy [113, 114]. Thus, receptor tyrosine kinases play a central role in the development of cardiac hypertrophy.

The best studied receptors with intrinsic serine/threonine kinase activity involved in cardiac pathologies are the TGF- β superfamily receptors and will be discussed in Section 1.8.1. Another example of receptors involved in sensing neurohumoral stimuli in cardiac hypertrophy is the gp130 linked receptors which are activated in response to IL-6 family cytokines including cardiotrophin-1 (CT-1), leukemia inhibitor factor (LIF) and IL-6 [115]. Signalling pathways activated by gp130 linked receptors include the janus kinase (Jak)-signal transducer and activator of transcription (Stat) pathway and the MAPK pathway. CT-1 induced cardiac hypertrophy is transduced predominantly through the MEK5-ERK5 signalling axis while LIF induced hypertrophy is transduced through Ras-ERK1/2 signalling [116-118]. Moreover, continuous activation of gp130 pathway by constitutive overexpression of both IL-6 and IL-6 receptor was shown to cause cardiac hypertrophy in transgenic mice [119]. It is interesting to note that in contrast with adrenergic receptor stimulation, gp130 mediated hypertrophy (CT-1 stimulation) is mainly manifested by increase in cell length of individual cardiomyocytes by addition of sarcomeres in series [120]. Furthermore, interdependence of angiotensin II mediated signalling and IL-6/gp130 pathway has recently been recognized. In this respect, IL-6 signalling was shown to be necessary for angiotensin II mediated hypertension and cardiac hypertrophy via gp130 linked receptor [121]. Similarly, CT-1 induced cardiac hypertrophy was shown to be more pronounced in hypertensive hearts with increased angiotensinogen [122]. In addition to regulation of cardiac hypertrophy, gp130 signalling also promotes cardiomyocyte survival and may thus be crucial to regulating the progression to heart failure [123, 124].

These various receptor mediated pathways activated by both mechanical and neurohumoral stimuli converge on specific intracellular signal-transduction circuits that mediate the hypertrophic response of cardiomyocytes. These signalling pathways further coordinate the hypertrophic growth regulating nuclear gene expression. Subsequently, this leads to augmented rates of protein translation and reduced rates of protein degradation in the cytoplasm.

1.4 Intracellular signalling cascades

Most hypertrophic stimuli transduce their effects via major intracellular signalling cascades such as protein kinase C (PKC), Calcineurin – nuclear factor of activated T cells (NFAT), mitogen activated protein kinase (MAPK) and Akt/PKB pathways. A schematic of activation of these pathways is shown in Figure 1.3.

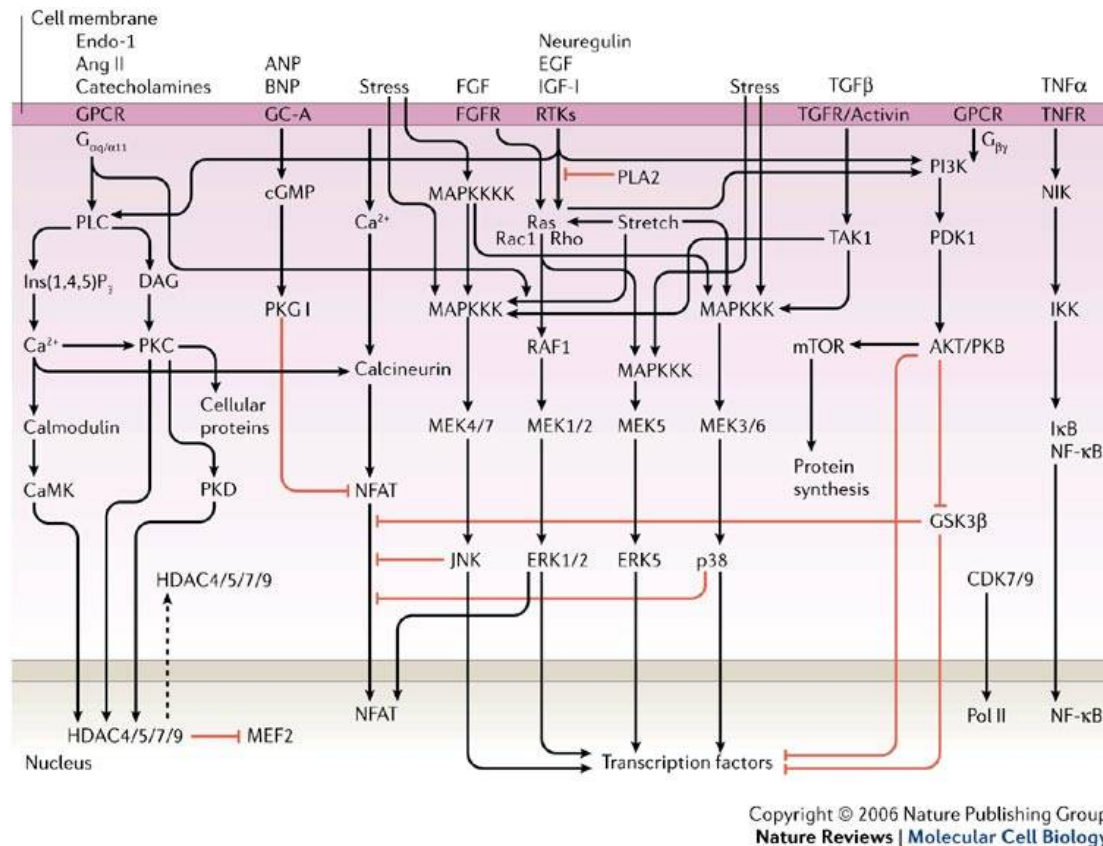


Figure 1.3: Major intracellular signalling cascades activated by hypertrophic stimuli. Mechanical and neurohumoral stimuli are sensed by receptors in the sarcolemmal membrane of cardiomyocytes (top purple panel). These then activate intracellular protein signalling pathways, eventually culminating in signal transduction to the nucleus (lower brown panel). Major pathways include PKC, Calcineurin-NFAT, MAPK such as JNK, ERK and p38 and Akt/PKB. Source: Heineke *et al.* *Nature Reviews Molecular Cell Biology* 2006; 7: 589–600 [125]

1.4.1 MAPK signalling pathway

MAPK pathways are organized as a three member protein kinase cascade. In the core module, MAPKs are phosphorylated by MAPK kinases (MKK), which are themselves activated via phosphorylation by serine/threonine kinases MKKKs. MAPK proteins include the JNK, ERK and p38 family members while MKK are MEK family proteins. Low molecular weight G proteins as well as other protein kinases form the MKKK family. MAPK pathways are activated in cardiomyocytes in response to a number of stimuli such as mechanical loading, GPCR agonists, RTK agonists like IGF-1, EGF, serine/threonine kinase receptor activation by TGF- β family ligands as well as IL-6 family ligands mediated gp130 linked receptor activation (reviewed in [126]). MAPK proteins p38, JNK and ERK have been implicated in cardiomyocyte hypertrophy. Cardiomyocytes expressing constitutively active MKK3 or MKK6, which specifically activate p38 MAPK, showed characteristic hypertrophic responses including sarcomeric organization and expression of skeletal α -actin and ANF. MKK3 also increased apoptosis upon overexpression, coordinating with p38 α but not p38 β , which has been suggested to regulate hypertrophy rather than apoptosis [30, 127]. The *in vivo* data concerning the role of p38 in cardiac pathologies is however conflicting with the *in vitro* data. Transgenic overexpression of activated MKK3 and MKK6 in cardiomyocytes was shown to cause age-dependent fibrosis and heart failure, with no evidence of cardiac hypertrophy [128]. Moreover, cardiac specific dominant negative p38 α and p38 β transgenic mice displayed either similar or enhanced cardiac hypertrophy compared to wild type mice following pressure overload [129]. Similarly, dominant negative expression of MKK3, MKK6 or p38 α in the heart of transgenic mice resulted in an enhanced hypertrophic response to pressure overload, in addition to exhibiting age dependent cardiac hypertrophy via activation of NFAT pathway [130]. Thus the p38 pathway may serve a protective function against cardiac hypertrophy but may also promote cardiac remodelling and heart failure progression. Similarly, while JNK expression in cardiomyocytes has been shown to induce hypertrophic responses, it may function as an inhibitor of *in vivo* cardiac hypertrophy in mice. Expression of constitutively active MKK7 in cardiomyocytes was shown to specifically activate JNK signalling and hypertrophic responses [131]. However, transgenic overexpression of MKK7 in the heart resulted in age dependent cardiomyopathy in the absence of cardiac hypertrophy [132]. Loss of function analysis using dnMKK4 demonstrated reduced

activation of JNK in response to ET-1 stimulation or *in vivo* pressure overload, thereby reducing hypertrophy [133, 134]. In contrast, both cardiac specific dnJNK1/2 transgenic mice and JNK1 and JNK2 null mice showed enhanced cardiac hypertrophy with age or in response to pressure overload, via activation of calcineurin-NFAT signalling pathway [135]. Thus JNK signalling may serve to inhibit cardiac growth responses.

In contrast to the roles of p38 and JNK, ERK signalling has been established to play a central role in mediating cardiac hypertrophy. ERK proteins are activated in response to GPCR stimulation by angiotensin II, endothelin-1 and adrenergic agonists, RTK activation by IGF-1 as well as mechanical loading (reviewed in [136]). Cardiac specific overexpression of activated MEK1 was shown to specifically induce constitutive ERK1/2 activation and was sufficient for the development of age dependent concentric hypertrophy without decompensation over time [137]. In contrast, simple ERK2 overexpression in the heart did not induce cardiac hypertrophy but showed synergistic hypertrophic responses when crossed with MEK1 transgenic mice [138]. Thus activation of MEK1-ERK1/2 pathway in the heart may be sufficient to induce cardiac hypertrophy. Knock-out of dual specificity phosphatase-6 (*Dusp6*), a specific regulator of ERK1/2 dephosphorylation, showed increased basal but not agonist induced activity of ERK1/2 in the heart amongst other tissues. These mice exhibited increased cardiac size due to cardiomyocyte hyperplasia and resistance to pressure overload induced hypertrophy [139]. Taken together, these results suggest that the phenotype of MEK1 transgenic mice may simply be a result of non-physiological constitutive activation of ERK1/2 in contrast with the physiological increase in ERK1/2 activity which did not induce hypertrophy [140]. Moreover ERK1/2 are not necessary for the development of cardiac hypertrophy in response to physiological or pathological stimuli, as demonstrated with *Erk1*^{-/-} and *Erk2*^{+/-} mice [141]. However, these mice exhibited increased cardiomyocyte apoptosis and progression to heart failure following chronic pressure overload. Recently, ERK5 has been identified as an atypical MAPK protein activated in the stressed heart and has been demonstrated to play a critical role in pressure overload induced cardiac hypertrophy. *Erk5* knock-out in the heart was shown to reduce hypertrophy following pressure overload, accompanied by increased apoptosis and expression of p53 and Bad proteins [142]. Thus, ERK signalling has been increasingly implicated to play an anti-apoptotic role in the myocardium [137, 141-143].

1.4.2 Calcineurin-NFAT signalling axis

Calcineurin (Cn) is a Ca^{2+} dependent heterodimeric protein with serine/threonine phosphatase activity. It consists of two subunits, the catalytic subunit CnA and the regulatory subunit CnB. Calcineurin is activated by binding of the Ca^{2+} dependent adaptor protein calmodulin upon its saturation with Ca^{2+} . Activated calcineurin dephosphorylates transcription factors of the NFAT family, which translocate to the nucleus and activate target genes involved in hypertrophy [144]. CnA catalytic subunit is encoded by three genes *i.e.* CnA α , CnA β and CnA γ , while the regulatory subunit CnB is encoded by two genes, CnB1 and CnB2. Out of these only CnA α , CnA β and CnB1 expression is detected in the heart [145]. Four out of five NFAT transcription factors have been shown to be regulated by calcineurin, NFATc1 to NFATc4 [145]. Numerous studies have shown that calcineurin signalling is sufficient to induce cardiac hypertrophy. Overexpression of activated calcineurin catalytic subunit in the heart of transgenic mice was shown to induce extensive cardiac hypertrophy as well as interstitial fibrosis. Moreover, cardiac expression of constitutively active NFATc3, the molecular target of calcineurin, was sufficient to replicate this phenotype [146]. Similarly, inhibition of calcineurin-NFAT signalling axis in the heart by genetic means in *CnA β ^{-/-}*, *Nfatc3^{-/-}* and *Nfatc2^{-/-}* mice or by transgenic overexpression of myocyte-enriched calcineurin-interacting protein (MCIP1), an endogenous inhibitor of CnA, has demonstrated the necessary role of this pathway in cardiac hypertrophy development in response to neurohumoral agonist infusion or mechanical loading [145, 147-149]. Moreover, as mentioned above, the calcineurin-NFAT pathway is also used by MAPK proteins to induce hypertrophic responses. It is interesting to note that inhibition of calcineurin signalling by overexpression of MCIP1 reduced cardiac hypertrophy while preserving ventricular systolic function [147]. In contrast, MCIP2 overexpression in the heart also reduced cardiac hypertrophy in response to pressure overload, but resulted in diastolic dysfunction and cardiac stiffness [148]. Similarly, in the mouse model of dilated cardiomyopathy from muscle lim protein (*Mlp*) knock-out, inhibition of calcineurin function by knock-out of *CnA β* , calcineurin was suggested to function in a cardioprotective manner by reducing cardiac remodelling [149]. Thus the calcineurin-NFAT signalling axis is a key regulator of cardiac hypertrophy and remodelling.

1.4.3 PI3K - Akt/PKB pathway

Phosphoinositide 3-kinase (PI3K) can be activated by stimulation of RTK or GPCRs with their agonists [150-152]. Upon activation, PI3K subunit I α (called p110 α) phosphorylates phosphatidylinositol 4, 5-bisphosphate, which leads to recruitment of 3-phosphoinositide-dependent kinase-1 (PDK1) and Akt/PKB to the membrane. PDK1 then activates Akt by phosphorylation of Thr308 residues. Further phosphorylation of Akt on Ser473 by the mTORC2 complex can activate Akt or define its substrate specificity for phosphorylation of glycogen synthase kinase 3 (GSK-3 α or GSK-3 β , inactivated by phosphorylation) and Forkhead transcription factors (Foxo) amongst others [152, 153]. The PI3K-Akt pathway has been implicated in regulation of basal cardiac growth and physiological hypertrophy. Expression of constitutive active PI3K lead to increased heart size while that of dnPI3K or *Akt1*^{-/-} lead to smaller sized hearts in transgenic and knock-out mice, with neither model showing signs of fibrosis and cardiac dysfunction [154, 155]. Moreover, transgenic mice with expression of dominant negative p110 α in the heart demonstrated a necessary role for PI3K in development of physiological hypertrophy in response to exercise training. However, pathological hypertrophy was observed following pressure overload which lead to cardiac dysfunction and heart failure, suggesting a protective action of PI3K [156]. Analysis of transgenic mice with constitutively active Akt1 in the myocardium demonstrated cardiac hypertrophy with preserved or enhanced systolic function and protection from ischemic injury and cell death [157-159]. In contrast, overexpression of Akt3 in the heart resulted in compensated hypertrophy and cardioprotection in the short term, which progressed to reduced contractility and loss of cardioprotection in an age dependent manner [160]. Similarly, constitutive active Akt1 expression in the myocardium was shown to induce adaptive cardiac hypertrophy with preserved cardiac function in the short term. Sustained activation of Akt1 in these mice lead to cardiac dilation and heart failure attributed to insufficient angiogenic responses [161]. Loss of function study on *Akt1*^{-/-} hearts showed exacerbated cardiac hypertrophy and dysfunction following pressure overload [155]. An important target of Akt mediated phosphorylation and inactivation is GSK-3 β , which is known to inhibit cardiac hypertrophic transcriptional regulators such as GATA-4, NFAT and β -catenin [162-164]. Transgenic mice overexpressing active GSK-3 β were shown to inhibit cardiac hypertrophy in response to a number of stimuli including β -adrenergic stimulation, calcineurin activation as well as

pressure overload [165, 166]. Thus PI3K-Akt signalling represents an important molecular switch which may regulate both physiological and pathological hypertrophic responses of the heart.

In summary, signalling pathways MAPK, PI3K and TGF- β may play an important role in progression of cardiac hypertrophy. They also variably regulate physiological vs. pathological hypertrophy and may thus be involved in regulating their development. Whilst the p38 and JNK pathways may play a protective role against cardiac hypertrophy, they may also regulate progression to heart failure. Similarly, the PI3K-Akt pathway may also play a protective role against cardiac hypertrophy, but may also regulate decompensation and progression to heart failure due to its role in angiogenesis. The calcineurin-NFAT pathway however, seems to play a central role in the development of cardiac hypertrophy. Similarly, the TGF- β pathway may be central to regulation of both hypertrophy and fibrosis in the myocardium. These signalling cascades are also multi-functional and are involved in regulating a number of cellular processes such as proliferation, differentiation, protein synthesis, cell death, *etc.* thereby making therapeutic targeting ineffective by itself. Owing to the complexity of these signalling cascades and their extensive cross-regulation by each other, essential downstream targets of these pathways need to be identified in order to delineate their exact function whilst avoiding activation of other pathways.

1.5 Markers of cardiac hypertrophy and heart failure

Initiation of the cardiac hypertrophic program is marked by re-expression of the ‘fetal gene program’ wherein genes normally expressed in the developing heart and repressed in the adult myocardium are reactivated during conditions of stress. These include natriuretic peptide genes (ANF, BNP), skeletal α -actin (SKA) as well as isoform switch from α -myosin heavy chain (α -MHC) to β -MHC. Reactivation of the fetal gene program permits a compensatory response of the stressed myocardium to increase protein synthesis and cell size as well as activation of appropriate pathways to maintain cardiac function while meeting the increased contractile or cardiac output demands. However, fetal gene program activation is mostly seen with pathological hypertrophy and not with physiological hypertrophy [25, 156, 167-170]. This indicates that physiological hypertrophy induces variable gene expression responses to compensate increases in

systemic demands while preventing decompensation. Also, in this respect, the fetal gene program induction is probably insufficient to maintain cardiac function and eventually leads to ventricular dysfunction. Both ANF and BNP are recognised as markers of pathological hypertrophy and their expression levels have useful prognostic rationale towards disease severity [171]. Elevated ANF and BNP levels play a compensatory role against cardiac overload through their natriuretic, diuretic and vasodilatory actions which serve to reduce both preload and afterload [172]. Under conditions of cardiac stress, contractile proteins also undergo an isoform switch from the fast α -MHC to slow β -MHC, thereby altering the α -MHC/ β -MHC ratio [173], which can dramatically affect cardiac contractility and output. In fact, constitutive overexpression of β -MHC in mouse hearts was found to be sufficient to reduce systolic function [174]. Moreover, treatment of patients with dilated cardiomyopathy with β -blocker therapy lead to recovery accompanied by increased α -MHC and reduced β -MHC expression [175]. Similarly, increased expression of α -skeletal actin (SKA) has been reported following pressure overload at the compensated hypertrophic phase where cardiac contractility is maintained in response to increased pressure [176]. This has been shown to generate enough force of contraction despite the mechanical load and without affecting cell shortening. However, cardiomyocytes are unable to maintain the increased levels of SKA in chronic pressure overload and this may be crucial in the transition from compensated hypertrophy to decompensated heart failure [177]. Thus, increased α -skeletal actin expression serves as a marker for cardiac hypertrophy.

1.6 Metabolic remodelling in the hypertrophied heart

Reactivation of the fetal gene programme in cardiac hypertrophy and heart failure is accompanied by a switch to fetal myocardial energy substrate preference - increased glucose utilisation and reduced fatty acid oxidation (FAO) [178-180]. This switch enables the hypertrophied heart to meet the enhanced energy requirements under cardiac stress while optimising oxygen consumption and maintaining cardiac contractility [181]. Reduced fatty acid oxidation and increased glucose utilisation is a characteristic of pathological cardiac hypertrophy [178, 182] and is necessary for immediate cell survival. Glucose oxidation requires less oxygen than fatty acid oxidation (FAO), although it generates less energy than FAO, and is thus important in conditions of cardiac stress to provide sufficient energy in an oxygen-efficient manner. This also

increases the glycogen stores in the heart, thereby promoting cell survival and providing sufficient energy during conditions of decreased substrate availability [183-185].

Nuclear receptor peroxisome proliferator-activated receptor α (PPAR α) is a ligand-activated transcription factor that is involved in modulating the expression of genes involved in fatty acid uptake and oxidation. These include muscle-type carnitine palmitoyltransferase I (mCPT1 or CPT1 β) and medium-chain acyl-coA dehydrogenase (MCAD) [186-188]. PPAR gamma coactivator-1 (PGC-1 α) is involved in mitochondrial biogenesis and functions as a coactivator of PPAR α , cooperatively inducing the expression of PPAR α target genes involved in FAO [189]. PPAR α expression is normally enriched in tissues where oxidative energy demands are high, like heart and liver, and mitochondrial FAO is the primary source of energy [190]. CPT1 β plays a crucial role in importing long-chain acyl-CoA into the mitochondria while MCAD catalyses the first step in the mitochondrial β -oxidation of fatty acids. The expression of PPAR α and its target genes CPT1 β and MCAD is reduced in response to hypertrophic stimuli such as pressure overload [191-193].

Although compensatory at first, increased glycolysis may contribute to cardiac remodelling and pathological hypertrophy, eventually leading to heart failure due to diminished energy production compared to FAO as well as accumulation of intracellular lipids and metabolic intermediates [191, 194]. Altered metabolic homeostasis in the heart has itself been suggested to be a primary cause for the development of pathological hypertrophy and heart failure. This is further supported by the observation that treatment of patients with heart failure using β -adrenergic receptor blockers resulted in functional improvement accompanied by increased FAO [195, 196]. Knock-out of PPAR α was shown to reduce the expression of its target genes in the heart while leading to age dependent cardiac fibrosis [197]. In contrast, cardiac specific overexpression of PPAR α induced cardiac dysfunction exhibiting a diabetic cardiomyopathy phenotype [198]. Similarly, downregulation of PPAR α expression in the hypertrophied heart was shown to be necessary for preserving cardiac contractility, as reactivation was shown to induce cardiac dysfunction [199]. PGC-1 α has emerged as a key regulator of metabolic responses owing to its control of the expression of FAO enzymes. Overexpression of PGC-1 α in the heart was shown to cause dilated cardiomyopathy [200]. In contrast, loss of PGC-1 α resulted in age dependent cardiac dysfunction and remodelling [201, 202].

Mutations in MCAD have been associated with hypertrophic cardiomyopathy and sudden death [203]. Similarly, inhibition of fatty acid import into the mitochondria resulted in cardiomyocyte hypertrophy [204, 205], suggesting that any alteration in the myocardial energy substrate may be sufficient to induce cardiac hypertrophy. Thus the PPAR-PGC system is crucial in controlling the myocardial metabolic phenotype and any alterations are likely to cause maladaptive cardiac pathologies.

1.7 Decompensation and progression to heart failure (HF)

The state of ‘compensated hypertrophy’ in the long run progresses to decompensation and is associated with an increased risk of sudden death and/or development of heart failure, independent of the underlying cause of myocardial hypertrophy [206]. Similarly, inhibition of cardiac hypertrophy using drugs such as angiotensin-converting enzyme (ACE) inhibitors was shown to reduce the risk of death and progression to heart failure in high risk patients [207]. These observations raise the question if stress-induced adaptive hypertrophy is beneficial initially and detrimental if prolonged. Molecular and cellular changes during cardiac hypertrophy progression eventually lead to alterations in excitation-contraction coupling in myocytes, fibrosis and ECM remodelling, angiogenesis mismatch and cell death by apoptosis and necrosis, resulting in chamber dilation, cardiac dysfunction and eventually heart failure. A detailed understanding of the mechanisms of heart failure progression will help identifying therapeutic targets to prevent or reverse pathological remodelling. The following sections briefly summarize these potential mechanisms of heart failure progression.

1.7.1 Alterations in excitation-contraction coupling in cardiomyocytes

Excitation-contraction coupling (ECC) is responsible for the generation of contractile force of cardiomyocytes via calcium-induced calcium release followed by activation of ryanodine receptors (RyR) which further cause Ca^{2+} entry from the sarcoplasmic reticulum. The increase in Ca^{2+} concentration leads to conformational changes in the myofilaments, thus causing contraction. Relaxation is initiated by the removal of Ca^{2+} from the cytosol via the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (reviewed in [208]). During normal function, the amount of Ca^{2+} entering the cell during contraction is exactly matched by

the amount of Ca^{2+} leaving it. A number of alterations have been described during cardiac hypertrophy which alter the calcium homeostasis and may play a role in progression to heart failure.

Reduced SR Ca^{2+} release, reduced Ca^{2+} rate as well as increased diastolic Ca^{2+} levels have been observed in heart failure [209]. These are affected by reduced expression and/or activity of RyR and SERCA2a, which are both prominent in heart failure [210, 211]. SERCA2a gene therapy has been shown to restore contractile function in heart failure in animal models [212-215] and is currently being tested in a clinical trial for patients with heart failure [216]. Dyssynchrony at the organ as well as the cellular level has been suggested as one of the mechanisms for contractile dysfunction leading to heart failure. At the cellular level, Ca^{2+} release from the SR and contraction are dyssynchronized, possibly due to alterations in t-tubule geometry and expression and function of RyRs and L-type Ca^{2+} channels in the sarcolemma (reviewed in [217]). A role for PKC α has been demonstrated in the regulation of Ca^{2+} handling in heart failure. Elevated expression and activity of PKC α has been noted in heart failure in different models [218-220]. Moreover, mice lacking PKC α showed cardiac hypercontractility which was cardioprotective against pressure overload induced heart failure or cardiac dilation in a genetic mouse model. Similarly, overexpression of PKC α resulted in hypocontractility, hypertrophy and cardiac dysfunction, possibly via activation of protein phosphatase inhibitor -1 (PP I-1) which indirectly affects phospholamban and therefore SERCA [221]. Inhibition of PKC α by pharmacological or genetic means was also shown to protect against heart failure while improving contractility [222, 223]. The calcium sensor S100A1 protein, which is downregulated in heart failure, can interact with SERCA/phospholamban and acts as a regulator of RyR and calcium homeostasis [224]. Mice lacking S100A1 show increased cardiac dysfunction induced by ischemia or pressure overload, while those overexpressing S100A1 are hypercontractile and maintain cardiac function following myocardial infarction [225, 226]. Thus alterations in regulators of excitation-contraction coupling contribute to impaired cardiac function in progression of heart failure.

1.7.2 Angiogenesis mismatch

Cardiac hypertrophy is accompanied by an increased demand for myocardial oxygen and the adaptive phase of hypertrophy usually shows increased capillary density. However, sustained hypertrophy and the ensuing molecular changes lead to a mismatch between the required capillary density and cardiac growth. This may result in hypoxia or micro-ischemic areas that lead to further damage [227]. Angiogenesis mismatch has been suggested as one of the major mechanisms for transition to heart failure. Following severe pressure overload in the heart, the compensatory phase of hypertrophy showed increased capillary density, which progressively reduced in the pathological state, and could be reversed by the induction of angiogenic signalling [228]. Similarly, the central role of vascular endothelial growth factor (VEGF) was demonstrated by its inhibition using soluble VEGF receptors, which lead to reduced capillary density following pressure overload and faster transition to chamber dilation and heart failure. Thus VEGF was shown to be essential for the vascular network growth during adaptive hypertrophic responses [229]. In this respect, Akt signalling is implicated in the angiogenic responses in the stressed heart. Acute Akt activation in the heart was shown to cause reversible compensatory hypertrophy without affecting the myocardial capillary density. However, prolonged Akt activation lead to chamber dilation and heart failure, accompanied by reduced capillary density. Interestingly, inhibition of angiogenesis in physiological hypertrophy state by blocking VEGF action also led to cardiac dysfunction and failure [161]. At the molecular level, hypoxia-inducible factor-1 α (Hif-1 α) is known to regulate the expression of pro-angiogenic factors VEGF and angiopoietin-2. In a model of pressure overload, initial adaptive hypertrophy was associated with an increase in Hif-1 α and its target angiogenic proteins. Transition from hypertrophy to heart failure was dependent on inhibition of angiogenesis and increased p53 expression, which inhibited Hif-1 α and the angiogenic response [228]. Thus, reduced angiogenesis plays an important role in the transition to heart failure.

1.7.3 Cardiac fibrosis and extracellular matrix remodelling

Cardiac structure is maintained by the extracellular matrix (ECM), which provides support for maintaining proper contractile function of the heart. The ECM is composed of fibrillar collagens (predominantly type I and type III), basement membrane components like fibronectin and laminin, proteoglycans and glucosaminoglycans [230]. Adaptation to myocardial stress induces a number of cellular and molecular alterations

which lead to changes in the ECM composition and the collagen network as well as the cardiac structure, together called remodelling. During conditions of chronic hypertension and pressure overload, cardiac hypertrophy gradually decompensates and reactive interstitial fibrosis is observed, which increases ventricular stiffness and progressively affects contractility and diastolic chamber filling capacity [231-233]. The ECM structure is maintained by a balance between the synthesis and degradation of matrix components, which is regulated by matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Increased collagen deposition takes place as a result of increased collagen synthesis and reduced collagen degradation, which is commonly observed in pathological remodelling [234, 235]. Progression to heart failure is often associated with increased matrix degradation due to altered activities of MMPs and TIMPs, thereby causing chamber dilation and contractile dysfunction [236-238]. This was also demonstrated in a mouse model of mutated collagen type I, which made it resistant to degradation by MMPs and lead to age dependent cardiac fibrosis and contractile dysfunction [239]. In fact, inhibition of MMP activity in a number of animal models of heart failure has demonstrated beneficial effects on cardiac remodelling and function [240-242]. Similarly, gene targeted MMP2 and MMP9 mice exhibited reduced cardiac rupture, ventricular remodelling and dilation following myocardial infarction [243, 244]. However, MMPs are also known to regulate angiogenesis and MMP2 in particular promotes VEGF expression during compensatory hypertrophy, while MMP9 is anti-angiogenic [245]. In this respect, hydrogen sulphide therapy of pressure overloaded hearts was shown to inhibit the transition to heart failure by inducing MMP2 while inhibiting MMP9 in the heart [246].

TGF- β is an important mediator of cardiac fibrosis and is induced in response to a number of stress stimuli including angiotensin II [247, 248]. The hypertrophic and fibrotic functions of angiotensin II in the heart have been shown to be mediated by TGF- β [249-251]. TGF- β increases ECM synthesis by cardiac fibroblasts and also induces cardiomyocyte hypertrophic responses, as demonstrated by mice overexpressing TGF- β 1 [252]. It has also been shown to inhibit MMPs expression and activity and induce TIMP expression, further contributing to remodelling [253-255]. TGF- β 1 signal transduction takes place via Smad-dependent and independent pathways in cardiomyocytes and fibroblasts and is extensively discussed below (section 1.8.2). While inhibition of TGF- β signalling using soluble TGF- β receptor was shown to be

beneficial initially following infarction, it lead to progressive dysfunction characterized by chamber dilation [255]. In contrast, in a pressure overload model, inhibition of TGF- β by neutralizing antibody therapy was shown to have beneficial effects by attenuation of fibrosis and diastolic dysfunction [256]. This may be due to the pleiotropic nature of this cytokine. Thus, experimentation with its downstream signalling components may be useful in delineating the exact physiological effects of each of them. In this respect, knock-out of Smad3 in the myocardium resulted in reduced fibrosis and improved cardiac function in mouse models of angiotensin II infused hypertension, pressure overload and myocardial infarction [257-259]. It is interesting to note that in the pressure overload model, mice lacking Smad3 developed increased cardiac hypertrophy but showed reduced fibrosis and better survival rates, indicating compensated hypertrophy in the absence of Smad3 [257]. In contrast, knock-out of co-activator Smad4 from the myocardium resulted in age dependent cardiac hypertrophy, fibrosis and reduced contractility, while causing spontaneous death in some mice between the age of 5-12 months [260]. Thus, the TGF- β -Smad pathway plays a significant role in cardiac remodelling and progression to heart failure.

1.7.4 Cell death

Cardiomyocyte cell death occurs during pathological remodelling and has been implicated in the transition to chamber dilation and heart failure [261]. Cardiomyocyte loss due to apoptosis, necrosis as well as autophagy is extensively being studied in attempts to identify new targets for therapeutic intervention. Cardiomyocyte apoptosis has been observed in animal models of heart failure as well as in patients with heart failure [262-264]. Apoptosis signal-regulating kinase (ASK)1 is an upstream MAPKKK protein that regulates cell death via effector proteins of the MAPK family [265]. Although its role in cardiac hypertrophy is contested [266-268], it has been shown to play an important role in myocardial remodelling following injury. Cardiac specific induction of ASK1 expression was shown to increase apoptosis and fibrosis in response to pressure overload and ischemic injury, further progressing to cardiac decompensation [269]. Similarly, knock-out of *Ask1* resulted in reduced apoptotic cell death and ventricular remodelling following ischemic injury, pressure overload or angiotensin II infusion [266, 267, 270]. Bcl family proteins like Bnip3 and Nix have also been

implicated in modulating cell death in cardiac remodelling. Cardiac Nix deletion was shown to attenuate cardiomyocyte death, ventricular remodelling and cardiac dysfunction following pressure overload, while Nix overexpression resulted in variable age dependent apoptotic cardiomyopathy [271, 272]. Similarly, knock-out of *Bnip3* resulted in reduced post-infarction ventricular remodelling without affecting infarct size and preserved cardiac function associated with reduced apoptosis [273]. Another key regulator of apoptosis is the tumor suppressor gene p53, whose expression is elevated during the transition phase to heart failure. In addition to inhibiting angiogenesis, p53 also induces apoptosis and knock-out of *p53* was shown to inhibit heart failure progression and improved cardiac function in the myotrophin transgenic mouse model [274]. Thus, cardiomyocyte apoptosis inhibition is a clinically relevant therapeutic option which is being explored.

1.8 TGF- β superfamily

TGF- β superfamily is involved in regulating a numerous cellular processes such as proliferation, differentiation, cell adhesion, growth, development, apoptosis, migration, immune responses, *etc.* and any alterations in their function can result in serious pathologies such as cancer, wound-healing defects and fibrosis (reviewed in [275, 276]).

1.8.1 Ligands and Receptors

The ligands of the TGF- β superfamily are synthesized as dimeric pre-proteins and are characterized as having a conserved ‘cysteine knot’ structure formed by three disulphide bonds between six conserved cysteine residues. The ligands can be subdivided into TGF- β isoforms, activin, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and nodal. TGF- β exists in three isoforms - TGF- β 1, TGF- β 2 and TGF- β 3; while four activin β monomers exist and form homo or heterodimers like activin A (β_A - β_A) and activin AB (β_A - β_B). A total of 10 BMPs and 11 GDFs have been identified until now. All TGF- β superfamily members bind to dimeric transmembrane serine/threonine kinase receptors of two functional subclasses – type II and type I (also called activin receptor like kinases ALKs). Ligands of the TGF- β /activin subclass bind the type II dimeric receptors which induces recruitment and phosphorylation of type I receptor dimeric complex and initiates the conserved

intracellular signalling pathway via Smad proteins (reviewed in [277]). Figure 1.4 below shows the different TGF- β superfamily ligands and their receptors.

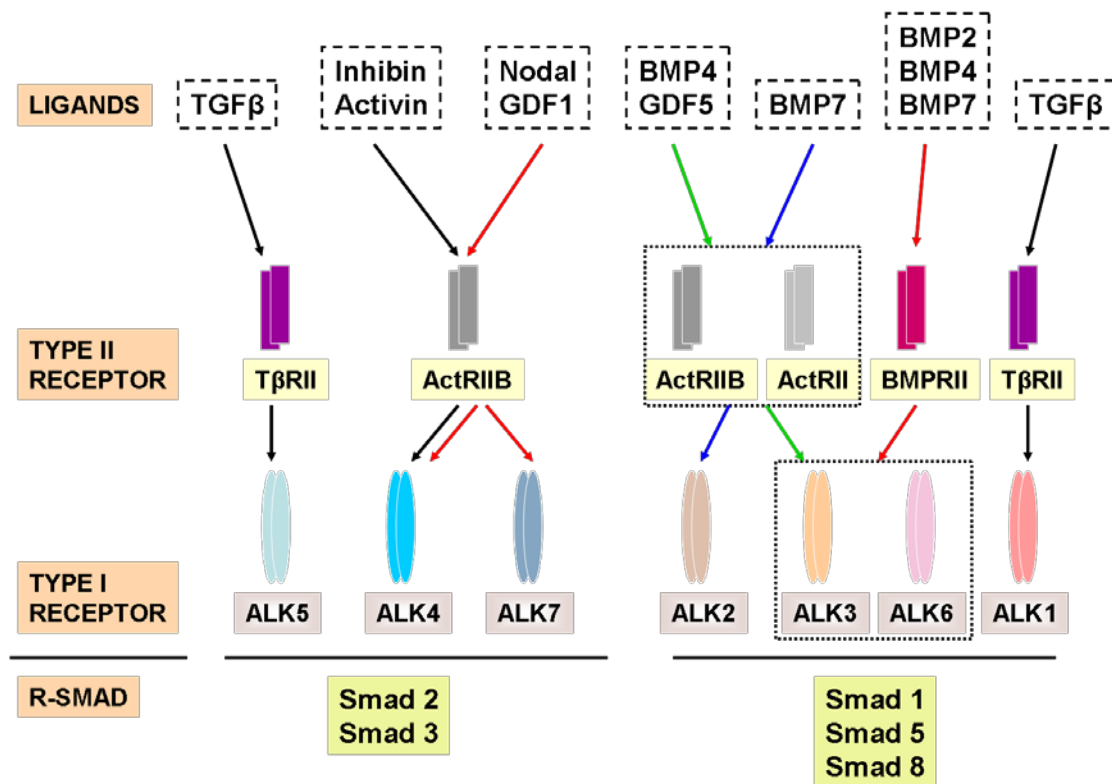


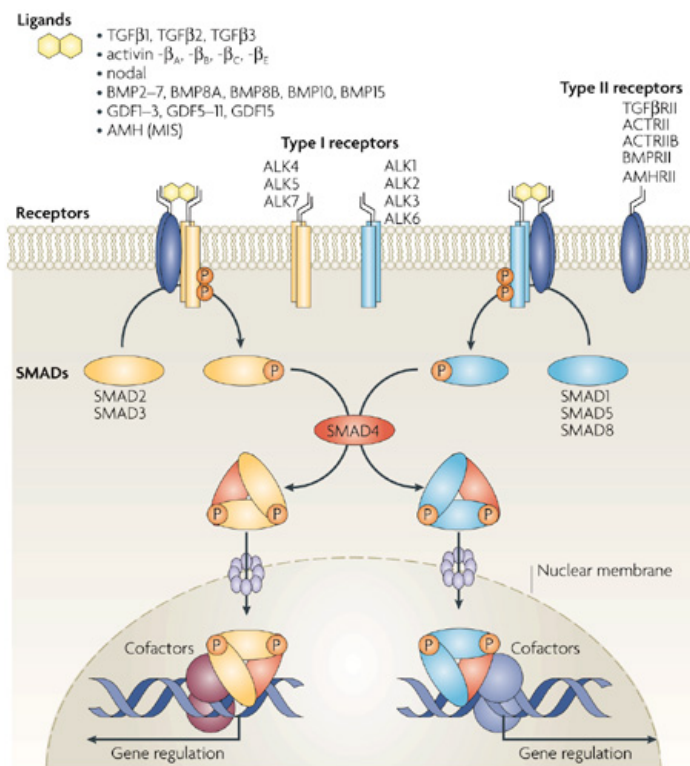
Figure 1.4: TGF- β family ligands, receptors and intracellular Smad proteins.

Ligands of the TGF- β superfamily like TGF- β , Activin, Nodal, BMPs and GDFs bind to different combinations of type II and type I receptors and initiate intracellular signalling via various R-Smad proteins. Smad2 and Smad3 are preferentially phosphorylated by ALK4, 5, 7 while Smad1, Smad5 and Smad8 are phosphorylated by ALK1, 2, 3, 6.

1.8.2 Intracellular signalling mechanisms

Signal transduction by the TGF- β family ligands is mediated mainly by intracellular Smad proteins. Some Smad-independent pathways have also been identified in specific tissues. Smad proteins can be subdivided into three functional classes: Receptor activated Smad (R-Smad), common mediator Smad (co-Smad) and inhibitory Smad (I-Smad). Type II receptor binding by TGF- β /activin subclass ligands and subsequent type I receptor activation leads to the recruitment and phosphorylation of the R-Smad by the type I receptor. R-Smads include Smad2 and Smad3 which are activated by TGF- β /activin ligands via type I receptors ALK4, ALK5 and ALK7; as well as Smad1,

Smad5 and Smad8 activated mainly by BMPs and GDFs via ALK1, 2, 3 and 6. Phosphorylation of R-Smad induces its binding to Smad4 as well as homomerization with another R-Smad. This complex then translocates to the nucleus where it can function as a transcriptional regulator of the target genes (reviewed in [277, 278]). Figure 1.5 below shows a schematic of the intracellular signal transduction via Smad proteins. Inhibition of the Smad pathway is mediated by inhibitory Smad6 and Smad7 by a number of potential mechanisms including competitive binding to Type I receptors, receptor inactivation by dephosphorylation or degradation as well as transcriptional inhibition [279-282]. While ligands of the TGF- β family are numerous and multiple type I and type II receptor combinations exist, their signalling mechanisms converge on a limited number of intracellular Smad proteins. Thus, signalling specificity is generated via regulation of signal type, duration and magnitude as well as inhibitory pathways (reviewed in [283]).



Nature Reviews | Molecular Cell Biology

Figure 1.5: Schematic of TGF- β family signal transduction by Smad proteins. Ligands of the TGF- β superfamily bind type II receptors which recruit and phosphorylate type I receptors. This leads to phosphorylation of R-Smad which then forms a complex with another R-Smad and Smad4 common mediator. This complex

enters the nucleus and induces transcription control of target genes. Source: Schmierer B and Hill CS; Nature Reviews Molecular Cell Biology 2007; 8: 970-982 [283]

In addition to signalling via Smad pathway, TGF- β ligands can also activate a number of different signalling pathways including MAPK via activation of TGF- β activated kinase 1 (TAK1), Ras and RhoA (reviewed in [278]).

1.8.3 TGF- β family ligands in cardiac disease

TGF- β family members have been widely implicated in the pathogenesis of heart failure. TGF- β 1 is a profibrotic cytokine and stimulates the production of extracellular matrix proteins in many tissues, thereby promoting fibrosis [284-287]. In both hypertrophic and dilated cardiomyopathies, TGF- β 1 is found to be elevated at the mRNA and protein levels [288, 289]. Angiotensin II is known to increase the expression of TGF- β 1 in the myocardium and TGF- β 1 is the primary mediator of Angiotensin II induced cardiac fibrosis [290]. Thus the beneficial effects of ACE inhibitors are partially dependent on their ability to reduce TGF- β 1 expression [250, 291]. Similarly, direct blocking of TGF- β 1 action in rats subjected to pressure-overload induced cardiac hypertrophy was found to reduce cardiac fibrosis and diastolic dysfunction without affecting cardiac hypertrophy [256]. Regulation of fibrosis by TGF- β was discussed in section 1.7.3.

Activins are homodimeric cytokines of the TGF- β superfamily and are known to modulate diverse pathophysiological processes including inflammation and wound healing, cell proliferation, differentiation, metabolism and fibrosis (reviewed in [292]). Activins function by binding to a complex of two serine/threonine kinase receptors (Type I and Type II) which then activate pathway-restricted Smad proteins and propagate the signal to the nucleus (reviewed in [293]). In patients with heart failure caused by coronary artery disease or dilated cardiomyopathy, elevated serum levels of Activin A were detected as well as increased expression of Activin β_A subunit was found in the T cells. Similarly, increased expression of Activin A was observed in the myocardium of animals subjected to coronary artery ligation, pressure-overload induced cardiac hypertrophy as well as ischemia/reperfusion injury [294]. *In vitro* studies on cardiomyocytes treated with Activin A showed increased expression of markers of

cardiac remodelling and hypertrophy including ANP, BNP, TGF- β 1, MCP-1, MMP-9 and TIMP-1 [295]. Activin A was also found to inhibit the increase in cardiomyocyte length and sarcomeric organisation induced by leukocyte inhibitory factor (LIF) [296]. Thus, activin functions to regulate both cardiac hypertrophy and remodelling and may present an important therapeutic target.

1.9 Follistatin-like 3 (FSTL3)

The follistatin family of proteins, whose founding member is follistatin (FST), are potent inhibitors of activin signalling. Follistatin-like 3, also known as follistatin-related gene (FLRG) or follistatin-related protein (FSRP) is a member of the follistatin-module family of proteins with characteristic cysteine-rich follistatin domains (FSD). Both follistatin and follistatin-like 3 (FSTL3) can bind activin with a high affinity and neutralise its action by preventing its binding to the activin receptors [297]. FSTL3 was identified as the target of a chromosomal translocation t(11;19)(q13;p13) observed in B-cell chronic lymphocytic leukemia, wherein the *CCND1* gene was found to be juxtaposed to the coding region of FSTL3 resulting in an aberrant transcript [298].

1.9.1 Domain organisation of FSTL3

FSTL3 contains a signal peptide, N-terminal domain, followed by two FSD and a C-terminal domain enriched in acidic residues. The overall structure of FSTL3 is similar to FST, which contains three FSD, and they share 45% primary sequence identity with FSTL3 [299]. Figure 1.6 shows the alignment of FST and FSTL3 proteins indicating high sequence homology. Domain sequence arrangement of FST and FSTL3 is shown in Figure 1.7. Conserved cysteine residues in a characteristic arrangement within the follistatin domains are highlighted 'C' and the heparin-binding sequence in FST, which is absent in FSTL3 is shown. The lack of heparin-binding sequence in FSTL3 makes it the dominantly available protein in the serum compared to FST [300, 301].

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CLUSTAL W (1.83) multiple sequence alignment

hFST      MVRARHQP-----GGLCLLLLLLCQFMEDRSAQAGNCWLFQAKNGRCQVLYKTELSKEE 54
hFSTL3    MRPGAPGPLWPLPWGALAWAVGFVSSMSGNPPAPGGVCWLQQGQEAATCSLVLTQDVTAE 60
          * . * * . * . : : : : . . . . * . * * * : * : : . * : : : *

hFST      CCSTGRLLSTWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNK 114
hFSTL3    CCASGNIDTAWSNLTHPGNKIN-LLGFLG-LVHCLPCKDSCDGV ECGPGKACRMLG-GRP 117
          * * : * . . * : : : . . . : * * : * * : * : * : * * * * * : *

hFST      RCVCAPDCSNITWKGPVCGLDGKTYRNECALRKARCKEQPELEVQYQGRCKKTCRDVFCP 174
hFSTL3    RCECAPDCSGLPARLQVCGSDGATYRDECELRAARCRGHPDL SVMYRGRCRKSCHEHVVC 177
          * * * * * . : : * * * * * * : * * * : * : * * : * : * * *

hFST      GSSTCVVDQTNNAYCVTCN-RICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGRSIGL 233
hFSTL3    RPQSCVVDQTGSAHCVVCRAAPCPVPSSPGQELCGNNNVTYISSCHMRQATCFLGRSIGV 237
          . : * * * * . * : * * . * * * * : * * * : * : * : * * * :

hFST      AYESGCIKAKSCEDIQCTGGKKCLWDFKVGGRGCSLCDELCPDSKSDEPVCASDNATYAS 293
hFSTL3    RHAGSCAGTP-----EEPPGGESAE-----EEENFV- 263
          : * . * : * * * * * * * * * * : : . .

hFST      ECAMKEAACSSGVLLVVKHSGSCN 317
hFSTL3    -----

```

Figure 1.6: Sequence alignment of FST and FSTL3. Protein sequences of human FST and FSTL3 were obtained from Ensemble database and aligned using Clustal W 1.83 software. Aligned sequence is shown with amino acid position on the right. Key: * - identical residue; : - conserved substitution; . - non-conserved substitution; **Red** – small hydrophobic, aromatic residues; **Blue** – acidic residues; **Magenta** – basic residues; **Green** – hydroxyl, amine, basic residues.

SIGNAL DOMAIN				
hFS315	-29	MVRARHQPGGLCLLLLLLCQFMEDRSAQA		-1
hFSRP	-35	MRPGAPGPLWPLPWGALAWAVGFVSS-MGSGNPAPG		-1
N-TERMINAL DOMAIN				
hFS315	1	GN C WLRQAKNGR C QVLYKTELSKEE CC STGRLSTS W TEEDVNDNTLFWKMFNGGAPN CIP C K	63	
hFSRP	1	GV C WLQQGQEAT C SLVLQTDVTRAE CC ASGNIDTA W SNLTHPGNKIN-LLGFLGLVH- CLP C K	62	
FOLLISTATIN DOMAIN 1 <i>heparin binding motif</i>				
hFS315	64	ET C ENVDCGPGKK C RMNKKNKPR C VCAPD C SNITWKG PV C GLDGKTYRNE C ALLKAR C KEQPELEVQYQGR C K	136	
hFSRP	63	DS C DGVE C GPGKA C RMLGGR-PR C E C APD C SGLPARLQ V C GSDGATYRDE C ELRAAR C RGHPDLSVMYRGR C R	134	
FOLLISTATIN DOMAIN 2				
hFS315	137	KT C RDV F C PGSST C VVDQTN NAY C VT C NRI- C PEPASSEQY L C GNDGVTYSSA C HLRKAT CL LGRSIGLAYEG K C I	211	
hFSRP	135	KS C EHVV C PRPQS C VVDQTGSAH C VV C RAAP C PVPSSPGQEL C GNNNVTYISS C HMRQAT C FLGRSIGVRHAGS C A	210	
FOLLISTATIN DOMAIN 3				
hFS315	212	KAKS C EDIQ C TGGKK C LWDFKVGRGR C SL C DEL C PD SKS DEPV C ASDNATYASE C AMKEAA C SSGVLL EV KHSGS C N	288	
C-TERMINAL DOMAIN				
hFS315	289	SISEDTEEEEEDEDQDYSPFISSILEW	315	
hFSRP	211	GTPEEPPGGESAEEEEENFV	229	

Figure 1.7: Domain sequence comparison of FST and FSTL3. The domain sequences of FST (FS315) and FSTL3 (FSRP) are aligned and conserved cysteine residues ‘C’ are indicated. Tryptophan residues at positions 4 and 36, which are responsible for the activin-binding capacity of FST are shown as ‘W’. Source: Tortoriello DV, Sidis Y, et.al; Endocrinology 2001;142(8):3426-34 [302]

1.9.2 Expression pattern of FSTL3

FSTL3 is evolutionarily conserved and encodes a secreted glycoprotein. However, there have been some reports of nuclear localisation of FSTL3 in cell lines like HeLa (human cervical carcinoma) and HEK293 (human embryonic kidney) [302]. Figure 1.8 shows the sequence alignment of FSTL3 across various species, indicating evolutionary sequence conservation. Both FST and FSTL3 are ubiquitously expressed in a number of tissues and cell types, but differ in their expression pattern. Whereas highest expression of FSTL3 in adult tissues is found in the placenta, testis, heart and pancreas, in the fetal tissues FSTL3 is abundantly expressed in the lung, spleen, thymus, liver and heart [302]. At the same time, highest expression of FST is found in the ovary, pituitary, testis, kidney, fetal heart and fetal liver (Figure 1.9). During mouse heart development, Fstl3 expression is found in the aorta and pulmonary artery smooth muscle, ventricles as well as valve leaflets of the mitral and tricuspid valves [303].

CLUSTAL W (1.83) multiple sequence alignment

```

Human      MRPGAPGPLWPLPWGALAWAVGVFSS--MGSGNPAPGGVCWLQQGQEATCSLVLTQTDVTRA 59
Chimpanzee MRPGAPGPLWPLPWGALAWAVGVFSS--MGSGNPAPGGVCWLQQGQEATCSLVLTQTDVTRA 59
Rat        MRPGA---LWPLLWGALVWAVGSGVAVMGSGDSVPGGVCWLQQGQEATCSLVLTQTVSRE 57
Mouse      MRSQA---LWPLLWGALVWTVGSGVAVMGSEDSVPGGVCWLQQGQEATCSLVLTQTVSRE 57
Opossum    -----PGGICWLRQGRDAQCSLILRTGVSW 26
Chicken    ---SQRQSVWGLRAAGMVPWGLMGALCAGRGVLEGGICWLQQGQEAKCTMILKTGVW 57
Xenopus    -----MLQFLLPLLLCTVTRGHPIPGGDTLQSGGTCWLLQGTSTCSRMLLSSVTWD 53
Zebrafish   -----MNVLAVLHCVILISVCEILGEHRTSAGMCWLQQGQPEHRCDMVLMRGVSRE 50
                                     . * * * * * : * : * * :

Human      ECCASGNIDTAWSNLTHPGNKINLLGFLGLVHCLPCKDS--CDGVECGPGKACRMLGGRP 117
Chimpanzee ECCASGNIDTAWSNLTHPGNKINLLGFLGLVHCLPCKDS--CDGVECGPGKACRMLGGRP 117
Rat        ECCASGNINTAWSNFTHPGNKISLLGFLGLVHCLPCKDS--CDGVECGPGKACRMLGGRP 115
Mouse      ECCASGNINTAWSNFTHPGNKISLLGFLGLVHCLPCKDS--CDGVECGPGKACRMLGGRP 115
Opossum    ECCSAGRVDTAWSNYTHQGNKISLLGFLGLVPCHPCKET--CEGVDCGPGKVCKMHGGRP 84
Chicken    ECCANGNVDAWSNYTPGNKISLLGFLGLVTCHPCKES--CEGVVCGPDKVCKMKHGRP 115
Xenopus    ECCKDGHIDTAWSNYTEPMNKISLLGFLGLVSCQPCRDS--CEGVQCPGKTCFLKDGPR 111
Zebrafish   ECCAAGRLDTAWSNASLPINEVSLGFLGIVSCKPKCDTENC EGVNCGLGKVCVRMKSGRP 110
***  * : : : * * * : * : : * * * : * : * * : * : * : * * : * * : * *

Human      RCECAPDCSGLPARLQVCGSDGATYRDECELR AARCRGHPDL SVMYRGRCRKSC EHV VCP 177
Chimpanzee RCECAPDCSGLPARLQVCGSDGATYRDECELR AARCRGHPDL SVMYRGRCRKSC EHV VCP 177
Rat        HCECVSNCEGVPA GFQVCGSDGATYRDECELR TARCRGHPDL RVMYRGRCQKSCAQV VCP 175
Mouse      HCECVPNCEGLPA GFQVCGSDGATYRDECELR TARCRGHPDL RVMYRGRCQKSCAQV VCP 175
Opossum    HCACAPDCSGLSMQLQVCGSDGVTYRDECELR TI KCQGH PDLGV MYQHCKKSCSLV VCP 144
Chicken    QCACAPDCSSLPRLQVCGSDGYTYRDECDLLTAKCRDHPDLEVMYQ GKCKKSCSSV VCP 175
Xenopus    QCECTPDSCGLEADV P VCGSDGHTYQDECELRVTKCRGHPDLEVMY YGKCKKSCSNV VCP 171
Zebrafish   QCVCS PDCSNISTKHAVCGSDGNSYKDECALLMARCKGHPDLEIMYQGECKKSCSNV VCP 170
: * * : : * : * * * : * : * * * : * : * * : * : * : * * : * *

Human      RPQSCVVDQTGSAHCVVCRAAPCPVPSSPGQELCGNNNVTYISSCHMRQATCFLGRSIGV 237
Chimpanzee RPQSCVV--QTGSAHCVVCRAAPCPVPSSPGQELCGNNNVTYISSCHMRQATCFLGRSIGV 236
Rat        RPQSCLVDQTGSAHCVVCRAAPCPVP PNPQELCGNNNVTYISSCHLRQATCFLGRSIGV 235
Mouse      RPQSCLVDQTGSAHCVVCRAAPCPVP SNPGQELCGNNNVTYISSCHLRQATCFLGRSIGV 235
Opossum    GTHTCVVDQTGSAHCVICRSAPCPVPSSPNQQLCGNNNITYASSCHLRQATCFLGRSIGV 204
Chicken    GTHTCVVDQTGSAHCVMCRTAPCPEPTSLDHS LCGNNNVTYPSACHLRRATCFLGRSIGV 235
Xenopus    GTHSCVVDQTGSAHCVVCRTAPCPQPLAFGNMLCGNNNVTYPSACHLRRATCYLGK SIGV 231
Zebrafish   GTHTCVTDQTNSAHCVMCR TAPCPMFMITEQTICGNDNITYQSACHLRRATCFLGRSIGV 230
: : * : * * : * * * : * * * : : * * : * * : * * : * * : * *

Human      RHAGSCAGTPEEPGGESAEEEEENFV- 263
Chimpanzee RHAGSCAGTPKEPPDGESAEEEEENFV- 262
Rat        RHPGICTGGPKVP-----AEEEEENFV- 256
Mouse      RHPGICTGGPKVP-----AEEEEENFV- 256
Opossum    RHSGSCTGSTTFP--GLLAEVEDNSV- 228
Chicken    RHYGSCSG----- 243
Xenopus    RHTGSCADIPEFS--MDLDDSVKNYI- 255
Zebrafish   RHYGHCRSN-----EASEENSLF 248
** * * .

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Figure 1.8: Evolutionary conservation of FSTL3 sequence. Protein sequences of FSTL3 from various species were obtained from the Ensemble database and aligned using Clustal W 1.83 software. Aligned sequence is shown with amino acid position on the right. Key: * - identical residue; : - conserved substitution; . - non-conserved substitution.

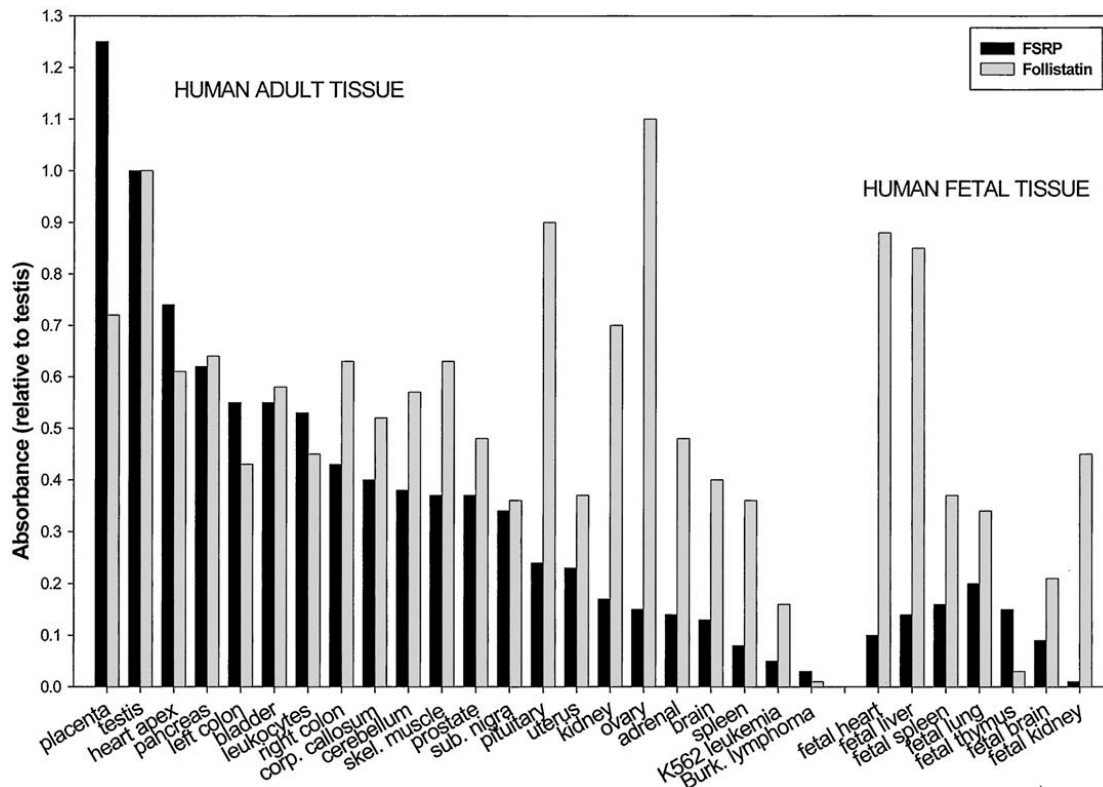


Figure 1.9: Expression pattern of Follistatin and FSTL3 in human tissues. mRNA expression of FST and FSTL3 (FSRP) was assessed using multiple tissue array from a number of adult and fetal human tissues. Source: Tortoriello DV, Sidis Y, et.al; Endocrinology 2001;142(8):3426-34 [302]

1.9.3 Regulation of Fstl3 expression

Owing to its ubiquitous expression in many tissues and cell types, the expression of Fstl3 is tightly regulated by a number of cytokines and growth factors. Using Fstl3 promoter-reporter constructs, the proximal -550 base pair region of Fstl3 promoter was identified to be sufficient for basal promoter activity in HeLa and HEK293 cells [304]. This region is highly GC rich and contains five Sp1/Ap2 consensus sites as well as a number of Smad binding elements. The Sp1 sites are essential for basal transcriptional activation as their deletion results in complete abrogation of Fstl3 promoter activity in HepG2 cells (hepatocarcinoma) [305]. Both TGF- β and activin A stimulate the expression of Fstl3 via Smad proteins [305, 306]. In case of activin, this functions as a negative feedback loop as activin stimulated Fstl3 can further inhibit activin signalling [306]. Also, the Sp1 sites are essential for optimal response of Fstl3 to TGF- β stimulation [305]. The expression of Fstl3 is also regulated by tumor necrosis factor α

(TNF α) in HepG2 cells and this response is potentiated by TGF- β through Smad proteins. Four TNF α responsive elements are present in the Fstl3 promoter, which bind to nuclear factor kappa-B (NF- κ B) transcription factor and regulate the expression of Fstl3 [307]. A schematic of the various transcription response elements in the Fstl3 promoter is shown in Figure 1.10, wherein the proximal -2000 base pairs of the Fstl3 promoter are represented.

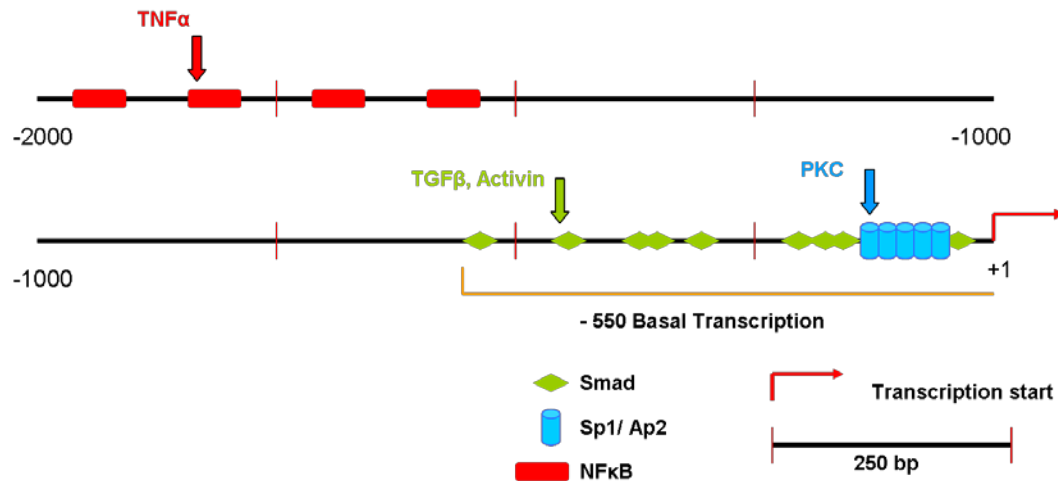


Figure 1.10: Transcriptional response sites in the Fstl3 promoter. The proximal -2000 base pairs of the promoter are represented in units of 250 base pairs. The transcription start site is indicated. Basal promoter activity is regulated by the proximal -550 base pairs of Fstl3 promoter containing five Sp1/Ap2 consensus sites which are necessary for basal promoter activity. This region also contains several smad binding elements which are activated by TGF- β and activin. Four TNF α response elements able to bind NF- κ B are indicated in red.

1.9.4: FSTL3 interaction with Activins

Both FST and FSTL3 can bind to and inhibit the activity of TGF- β family ligands like activin, myostatin and bone morphogenetic proteins (BMPs) [308]. FST and FSTL3 bind activin A with a high affinity ($K_d = 23.6 - 30.2\text{pM}$ and 39.3pM respectively) and near irreversibility [308] and inhibit its binding to the activin receptors [309, 310], thereby regulating activin bio-availability in different tissues. FSTL3 can also bind activin B, but with a lower affinity compared to activin A [297]. The C-terminal domain of FSTL3, including the second follistatin domain is principally involved in activin

binding and inhibition [310-312]. However, in the case of FST, the N-terminal domain has been implicated in activin-binding [313], in spite of contradictory reports regarding the necessity of the N-terminal domain in activin-binding [314]. Recently, the crystal structure of activin A - FSTL3 complex was solved and illustrated the intimate contact and necessity of the FSTL3 N-terminal domain in binding activin A with a high affinity [311]. As shown in Figure 1.11, two molecules of FSTL3 surround one molecule of homodimeric activin A (a) in a way that blocks both the type II and type I receptor interaction sites on activin A (c). The N-terminal domain (ND) of FSTL3 interacts with the activin dimer interface while the two follistatin domains cover the convex portion of activin A, thereby blocking the activin type II receptor interaction site. The FSTL3 ND interaction with activin A was found to be stronger than that for FST ND, the substitution of which resulted in 60% loss of activin-binding capacity. The interaction of activin A-FST is shown in Figure 1.11 (b), where the ND and FSD1 and 2 interact with activin in a similar manner as FSTL3. However, FSD3 of one FST interacts with the ND of the second FST, thereby completely blocking the type I receptor interaction site. In conclusion, both FST and FSTL3 completely block the activin receptor interaction sites on activin, albeit in a dissimilar manner. Furthermore, this differential binding translates to differential activin-inhibition capacities for FST and FSTL3. Thus, exogenous FST and FSTL3 are equivalent in inhibition of exogenously added activin. However, if either of these is endogenously produced, then FST has superior activin-inhibition capability, consistent with its superior cell-surface binding ability [308]. Thus, although FST and FSTL3 have strong overall structural and sequence similarity, their varied tissue distribution and activin neutralising capacities suggest that they might not be functional homologues.

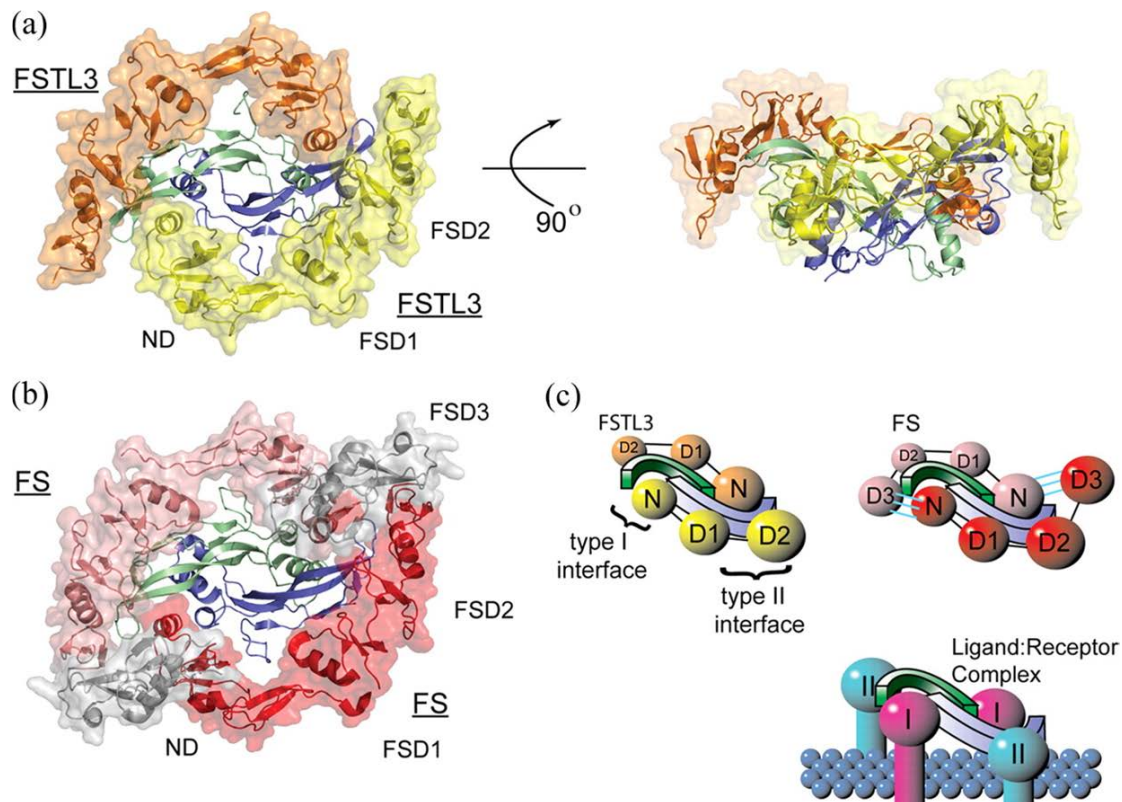


Figure 1.11: The x-ray crystallographic structure of activin A-FSTL3 complex. (Resolution 2.5 Å) Two molecules of FSTL3 (a) or FST (b) surround the central activin A dimer through interaction of N-terminal domain (ND) with the type I receptor interaction site and follistatin domains (FSD) with the type II receptor interaction site (c). Source: Stamler R et al. J. Biol. Chem. 2008; 283: 32831-32838 [311]

In vitro, Fstl3-activin binding has been shown to inhibit activin-dependent transcriptional activity in a dose-dependent manner [310]. However, unlike FST, it is unable to suppress activin-mediated secretion of follicle stimulating hormone (FSH) in pituitary cells [299]. In breast cancer cell lines, activin-mediated growth inhibition was abolished by expression of FSTL3, while silencing of FSTL3 resulted in tumor growth inhibition [315]. Transgenic over-expression of FSTL3 in gonads of mice resulted in gonadal developmental defects and reduced fertility – both thought to be arising from inhibition of activin in these tissues, which is critical for normal reproductive development and function [316]. On the other hand, whole-body knock-out of Fstl3 resulted in an extensive metabolic phenotype in the form of increased pancreatic islet numbers and size, β cell hyperplasia, increased insulin sensitivity and glucose tolerance – most likely mediated by increased activin bioactivity in these tissues [317]. More

recently, we have reported that activin-mediated protection against ischemic injury in the heart is abrogated by *Fstl3*, and knock-out of *Fstl3* in the heart resulted in smaller cardiac infarct size following ischemia-reperfusion injury [294]. Thus the activin A – *Fstl3* axis is important in determining normal function and homeostasis in a number of tissue systems.

1.9.5 Protein binding partners of FSTL3

Besides activin A and B, FSTL3 is known to bind to and inhibit other members of the TGF- β superfamily including myostatin and BMP-2. Myostatin is a muscle growth inhibitor and increased expression of myostatin results in severe cachexia and reduced adipose tissue and muscle mass [318, 319]. Circulating myostatin in the serum is found as a complex with FSTL3, which inhibits myostatin activity in reporter gene assays [300]. Thus FSTL3 may be involved in regulating the amount of free myostatin availability. Whole-body *Fstl3* KO mice show reduced visceral fat deposition, consistent with increased myostatin activity in these mice. These mice also show improved glucose tolerance and insulin sensitivity, which may be secondary to the altered fat deposition [317]. Thus the *Fstl3*-myostatin interaction may play an important role in regulating metabolic homeostasis. *Fstl3* can also bind BMP-2, albeit with a lower affinity than activin, and repress BMP-2 induced transcriptional activity [310]. Whether this interaction plays a significant physiological role is currently unknown.

Besides TGF- β superfamily ligands, other binding partners of *Fstl3* include fibronectin, AF-10 and a disintegrin and metalloproteinase – 12 (ADAM-12). Yeast two-hybrid analysis identified fibronectin as a binding partner for FSTL3 from a placental cDNA library. FSTL3 binding to fibronectin type I domains via follistatin domains was found to increase the hematopoietic cell adhesiveness to fibronectin and increased the adhesiveness of primary hematopoietic progenitor cells [320]. FSTL3 may thus be involved in regulation of haematopoiesis. FSTL3 was also shown to bind AF10 ([ALL1 (acute lymphoblastic leukaemia) fused gene from chromosome 10], enhance AF10 homo-oligomerisation and thus increase AF10 dependent transcription [321]. Similarly, ADAM-12 was identified as a binding protein for FSTL3 using yeast two-hybrid screening of human placental cDNA library. ADAM-12 interaction with FSTL3 inhibited osteoclast differentiation by reducing the number of osteoclasts as well as the

number of nuclei per osteoclast [322]. Whether this interaction exists in other tissues is unknown. Importantly, ADAM-12 is involved in ectodomain shedding of heparin-binding epidermal growth factor (HB-EGF), which then binds to the EGF receptor and induces cardiac hypertrophy. Inhibition of ADAM-12 mediated HB-EGF shedding using a specific inhibitor was shown to inhibit the development of cardiac hypertrophy in response to chronic pressure overload in mice [113].

1.9.6: Expression of FSTL3 in various diseases

Aberrant expression of FSTL3 is found in a number of pathologies. In women with pre-eclampsia, increased expression of FSTL3 is found in the placenta as well as serum [323]. Similarly, in women with intrauterine growth restriction, the expression of FSTL3 was highly elevated in the placenta compared to controls [324]. In placental trophoblast cells, the expression of FSTL3 is induced by hypoxia [325]. On the other hand, reduced levels of FSTL3 are found in women with ovarian endometriosis [326], as well as in patients with endometrial adenocarcinoma [327]. Recently, elevated expression of FSTL3 was reported in patients with heart failure compared to control subjects [328]. Similarly, increased expression of Fstl3 was reported in mouse hearts subjected to ischemic injury or pressure overload induced cardiac hypertrophy [294]. Interestingly, the expression of Fstl3 in the serum was shown to be elevated in response to heavy resistance training in humans [329]. A role for Fstl3 was found in modulating metabolic homeostasis in mice as deletion of Fstl3 resulted in an altered metabolic phenotype as well as fat deposition, as described above [317]. Interestingly, mRNA expression of Fstl3 is increased in the subcutaneous fat in obese mice (*ob/ob* mice) while the levels in visceral fat are reduced compared to wild type mice [330]. Thus, Fstl3 may play a significant role in both physiological as well as pathological responses of the body.

1.10 Hypothesis and Aims

Data from heart failure patients undergoing left ventricular assist device implantation and pharmacological treatment showed that the expression of follistatin family proteins was altered in the myocardium. Specifically, the expression of FSTL3 was elevated in heart failure and normalized following recovery. Chapter 3 presents the evidence for this observation. Microarray analysis on these samples indicated a potential role for FSTL3 in regulation of signal transduction and cell growth.

Hypothesis

Based on the above observations, a role for Fstl3 in regulating cardiac hypertrophy was hypothesized. In particular, it was hypothesized that elevated expression of Fstl3 is detrimental to cardiac function by regulating various signalling pathways involved in cardiac hypertrophy and/or remodelling.

Specific Aims

1. To analyze the expression of Fstl3 in animal models of cardiac stress and injury as well as in cardiomyocytes in response to hypertrophic stimuli.
2. To create a mouse model of cardiac-specific loss of function of Fstl3 in order to understand the role of Fstl3.
3. To examine the effects of Fstl3 absence on development of cardiac hypertrophy and remodelling in response to pressure overload in the heart.
4. To identify mechanism of action of Fstl3 in the stressed myocardium.

CHAPTER 2: Materials & Methods

2.1 Cloning of *Fstl3* in pCMS-EGFP

Mouse *Fstl3* cDNA clone was obtained from RZPD (Berlin, Germany) and used for cloning *Fstl3* in the following constructs:

Table 2.1: *Fstl3* construct

Name of construct	Vector	Insert	Promoter	Restriction sites
pCMS-EGFP - <i>Fstl3</i>	pCMS-EGFP (BD Biosciences Clontech, USA)	Mouse <i>Fstl3</i> (1718 bp)	CMV	EcoRI/XbaI

2.1.1 Vector and Insert preparation

For cloning of *Fstl3*, the cDNA containing vector and the target vector were digested with restriction enzymes EcoRI and XbaI (Roche Applied Sciences, UK - # 11175084001 and 10674257001 respectively) as described below:

Table 2.2: Restriction digestion of *Fstl3* cDNA vector and target vector

Insert			Vector		
Reagent	Amount	Final concentration	Reagent	Amount	Final concentration
SuRE/Cut Buffer H	2µl	1X	SuRE/Cut Buffer H	2µl	1X
<i>Fstl3</i> cDNA vector	1µl	1µg	Target vector*	1µl	1µg
EcoRI	1µl	1 unit	EcoRI	1µl	1 unit
XbaI	1µl	1 unit	XbaI	1µl	1 unit
MilliQ water	15µl		MilliQ water	15µl	
Total		20µl	Total		20µl
Incubation temperature		37°C	Incubation temperature		37°C
Incubation time		1 hour	Incubation time		1 hour

* Target vector = pcDNA 3.1 (+) or pCMS-EGFP

10X Sure/Cut buffer H: 500 mM Tris-Hcl, 100 mM MgCl₂, 1 M NaCl, pH 7.5

2.1.2 Agarose gel electrophoresis

Following restriction digestion, the insert and vector reactions were loaded on a 1% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich Co., UK - # E1510)

for visualization of DNA. The samples were diluted using 6X loading buffer (B7021S; New England Biolabs Inc.) and loaded in the wells of the gel along with 1 Kb plus DNA ladderTM (Invitrogen Ltd., UK - # 10787018). The samples were electrophoresed in 1X TAE buffer by applying a constant voltage of 100V. The gel was then visualized under UV light to detect the migration of DNA bands.

2.1.3 DNA extraction and purification from agarose gels

For extraction and purification of DNA samples from agarose gels, the QIAquick Gel Extraction kit (QIAGEN, UK - # 28706) was used. Following agarose gel electrophoresis, DNA fragment was excised from the gel by brief visualization under UV light in order to minimize DNA damage. The gel slice was transferred to a 1.5 ml microtube and weighed. Three volumes of buffer QG containing pH indicator dye were added to the tube *e.g.* 100 mg gel slice + 300 µl buffer QG. The microtube was incubated at 50°C for 10 minutes with intermittent shaking to help dissolve the agarose. After the gel slice had dissolved completely, the color of the mixture was checked to be yellow indicating optimal pH (<7.5) for DNA binding. One gel volume *e.g.* 100 µl of isopropanol was added to the mixture and mixed by inverting the tube 2-3 times. The mixture was then applied to the spin column contained in a 2 ml collection tube. The tube was centrifuged at 13,000 rpm for 1 minute at RT in order to enable binding of DNA contained in the sample to the silica membrane in the spin column. The flow through was discarded and 0.5 ml of buffer QG was added to the column and centrifuged as before in order to remove traces of agarose. Again, the flow through was discarded and 0.75 ml of ethanol containing wash buffer PE was added to the column and incubated at RT for 3 minutes. The column was centrifuged for 1 minute at 13,000 rpm to remove salts and other impurities. The flow through was discarded and the column was centrifuged again for 1 minute at 13,000 rpm to remove any residual ethanol from the membrane. The spin column was transferred to a fresh DNase-free 1.5 ml microtube. To elute the DNA, 30 µl of DNase-free water (pH 7.0 - 8.5) was added exactly at the centre of the column and allowed to incubate for 1 minute at RT. The column was then centrifuged for 1 minute at 13,000 rpm and DNA containing sample was quantified using spectrophotometry as described in section 2.2.4.

2.1.4 Ligation of vector-insert

Ligation of EcoRI/XbaI digested vector and insert was carried out using T4 DNA ligase (Invitrogen Ltd., UK - # 15224-090). T4 DNA ligase catalyses the formation of phosphodiester bonds between double stranded DNA molecules with 5' – phosphate and 3' – hydroxyl termini, thereby ligating DNA fragments. The following reaction was set up using a 3:1 insert : vector molar ratio and incubated at RT for 5 minutes.

Table 2.3: Ligation reaction setup

Reagent	Amount	Final concentration
5X ligase buffer	4µl	1X
Vector DNA	1µl	30fmol
Insert DNA	3µl	90fmol
T4 DNA Ligase	1µl	1 unit
MilliQ water	11µl	
Total		20µl
Incubation temperature		RT (24°C)
Incubation time		5 minutes

5X ligase buffer: 250 mM Tris-HCl (pH 7.6), 50 mM MgCl₂ , 5 mM ATP, 5 mM dithiothreitol (DTT), 25% (w/v) polyethylene glycol-8000

2.1.5 Transformation of competent E.coli cells and colony selection

The ligated constructs were transformed into One Shot[®] TOP10 Chemically Competent E.coli cells (Invitrogen Ltd., UK - # C4040-10) using manufacturer's guidelines. One vial per reaction containing 50 µl of One Shot[®] TOP10 cells was thawed on ice. To the cells, 5 µl of ligation product was added gently and incubated for 30 minutes on ice. Following incubation, heat-shock was given to the competent cells by placing them in a water bath equilibrated at 42°C for 30 seconds. The vial was removed from the water bath and briefly kept at room temperature. Pre-warmed 250 µl S.O.C medium was added aseptically to the vial. The cells were allowed to grow by placing them horizontally in a shaking incubator at 37°C for 1 hour at 225 rpm. The vial was centrifuged at 6000 rpm for 1 minute and 300 µl of the supernatant was discarded. The bacterial cell pellet was resuspended in the remaining volume of S.O.C medium and spread on a pre-warmed LB-agar plate containing 100 µg/ml ampicillin. The plate was inverted and placed overnight in an incubator at 37°C. Colonies growing on the

selective medium are properly ligated constructs capable of ampicillin resistance. The following day, 20 colonies were randomly picked from the plate, inoculated in 3 ml LB medium for plasmid DNA isolation using ‘miniprep’ protocol. DNA was sequenced to confirm proper ligation of the insert into the vector. Colonies containing required constructs were then inoculated in 200 ml LB medium for DNA isolation using ‘maxiprep’ protocol. DNA was stored at -20°C until required. ‘Glycerol stocks’ of constructs were made by adding 20-25% (v/v) glycerol to LB medium containing required clones grown overnight.

2.1.6 Map of pCMS-EGFP-FstI3

A schematic map of constructs resulting from cloning of mouse *FstI3* in the multiple cloning site (MCS) of pCMS-EGFP is shown in Figures 2.1

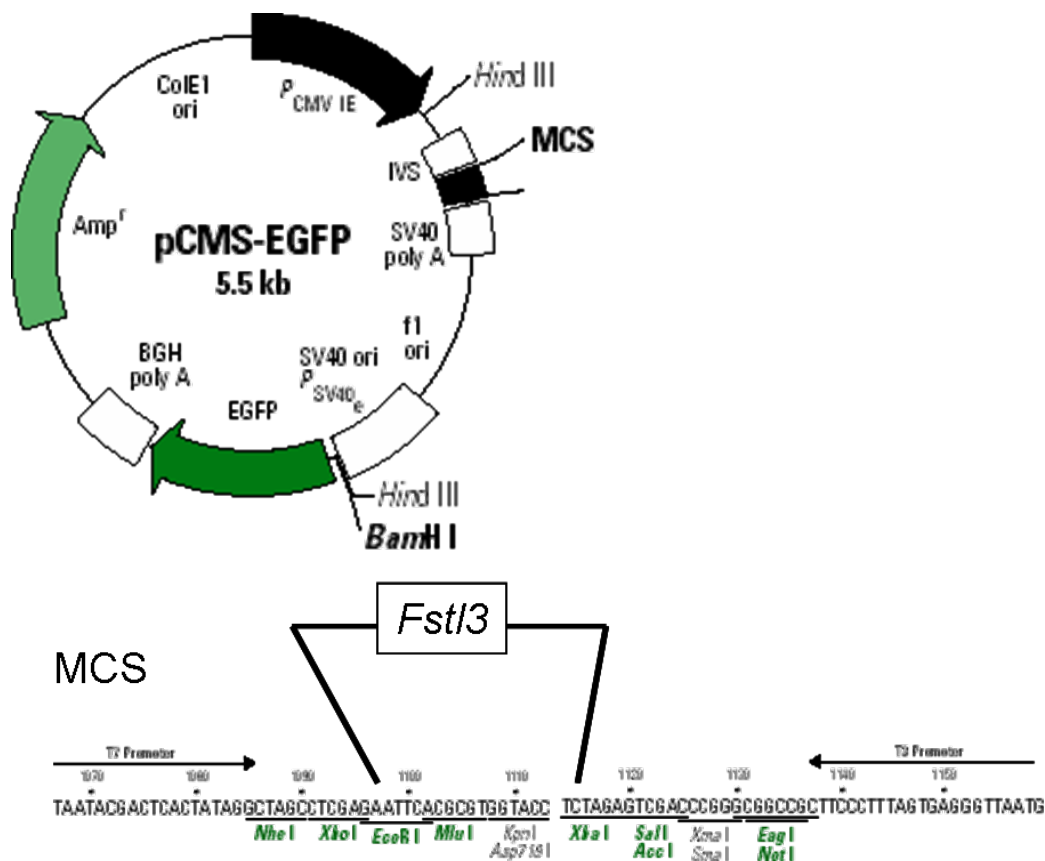


Figure 2.1: Schematic of pCMS-EGFP-FstI3. Adapted from BD Biosciences Clontech, USA.

2.2 Plasmid DNA extraction

2.2.1 Plasmid DNA isolation from bacterial cells: Miniprep

Plasmid DNA from bacterial cells (up to 20 µg) was isolated using the QIAprep spin miniprep kit (QIAGEN, UK - # 27106). Bacterial cultures (*E.coli* DH5α or TOP10) were inoculated in 3 ml Luria-Bertani (LB) medium containing selection antibiotics (e.g. Ampicillin 100 µg/ml) and grown for 12-18 hours at 37°C in a shaker at 250 rpm. Cells were harvested by centrifugation at 10,000 rpm for 2 minutes at RT and resuspended in 250 µl of buffer 'P1' containing 0.1 mg/ml RNase A and LyseBlue reagent. The cell suspension was vortexed in order to ensure complete mixing of LyseBlue with the cells, and was transferred into a 1.5 ml microtube. To this, 250 µl of SDS containing lysis buffer 'P2' was added and mixed by inverting the tube 5-6 times, resulting in a homogeneously blue colored suspension. SDS in the lysis buffer solubilises the protein and phospholipid components of plasma membrane, thereby leading to cell lysis and release of intracellular components. The lysis buffer also contains sodium hydroxide, which facilitates denaturing of chromosomal and plasmid DNA as well as intracellular proteins in the alkaline environment. Neutralisation buffer 'N3', 350 µl, was added to the lysate and mixed thoroughly by inversion, as indicated by the lysate turning homogeneously colorless due to SDS precipitation. The high-salt concentration in the neutralization buffer causes denatured proteins and chromosomal DNA to precipitate with the SDS, while ensuring that the smaller plasmid DNA renatures appropriately and remains solubilised. The samples were centrifuged at 13,000 rpm for 10 minutes at RT in a microcentrifuge, resulting in a compact white pellet of precipitated cell debris. The DNA containing supernatant was applied to a QIAprep spin column by decanting and centrifuged for 1 minute at 13,000 rpm to allow DNA adsorption onto the column. The flow-through was discarded and the spin column was washed using 0.5 ml buffer 'PB' by centrifugation for 1 minute at 13,000 rpm. The spin column was washed with 0.75 ml ethanol containing buffer 'PE' by centrifugation for 1 minute at 13,000 rpm and the flow-through was discarded, thereby removing contaminating salts from the DNA. The spin column was centrifuged again at 13,000 rpm for 1 minute in order to completely remove any traces of buffer 'PE', thus minimizing ethanol contamination of eluted DNA. The spin column was transferred into a fresh 1.5 ml collection tube and 50 µl of elution buffer 'EB' (10 mM Tris-Cl, pH 8.5)

was added directly on the centre of the silica gel membrane. The column was incubated for 2 minutes at RT and DNA was eluted by centrifugation at 13,000 rpm for 1 minute. DNA obtained was quantified using spectrophotometry and stored at -20°C until required.

2.2.2 Plasmid DNA isolation from yeast cells

For isolation of plasmid DNA from yeast cells, the QIAprep spin miniprep kit was used (QIAGEN, UK - # 27106) with some modifications. Single yeast colonies were inoculated in 5 ml YPAD medium and grown for 24 hours at 30°C in a shaker at 250 rpm. Cells were harvested by centrifugation at 10,000 rpm for 5 minutes at RT and resuspended in 250 µl buffer 'P1' containing 0.1 mg/ml RNase A. The cell suspension was transferred into a 1.5 ml microtube and 100µl of acid-washed glass beads (425-600 µm; Sigma-Aldrich Co., UK - # G8772) were added. Cell membranes were broken by vortexing for 5 minutes, after which the glass beads were allowed to settle at the bottom of the tube. The cell lysate was transferred into a fresh 1.5 ml microtube and 250 µl of lysis buffer 'P2' was added and mixed by inverting the tube. The miniprep protocol was followed from here onwards, as detailed in section 2.1.1.

2.2.3 Plasmid DNA isolation from bacterial cells: Maxiprep

When large quantities of plasmid DNA were required, a 'Maxiprep' protocol was followed to isolate up to 500 µg of DNA from bacterial cultures between 100-200 ml. The QIAfilter plasmid maxi kit was used (QIAGEN, UK - # 12263). Bacterial colonies were inoculated in 200 ml LB medium containing the appropriate selection antibiotic and grown overnight for 12-18 hours at 37°C in a shaker at 300 rpm. Bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4°C and the pellet was resuspended in 10 ml buffer 'P1' containing 0.1 mg/ml RNase A and LyseBlue. Following complete mixing, 10 ml of lysis buffer 'P2' was added and mixed thoroughly and incubated at RT for 5 minutes, turning the cell suspension uniformly blue. 10 ml of chilled neutralization buffer 'P3' was added to the lysate and mixed immediately by inversion. This results in precipitation of genomic DNA, proteins and cell debris together with potassium dodecyl sulphate (KDS), making the lysate less viscous and colorless while forming a white precipitate. The lysate was immediately poured into a

QIAfilter cartridge and incubated at RT for 10 minutes. This results in the precipitate floating in a layer above the clear lysate. During this incubation, a QIAGEN-Tip was equilibrated by adding 10 ml equilibration buffer 'QBT' and allowing it to flow through the column under gravity. Following the 10 minute incubation of the lysate, a plunger was inserted gently on top of the cartridge and the cell lysate was filtered directly into the column of the previously equilibrated QIAGEN-Tip. The plasmid DNA from the lysate was allowed to bind the resin by gravity flow until the column was empty. The QIAGEN-Tip was washed twice with 30 ml buffer 'QC', allowing the column to empty each time under gravity flow, thus removing all impurities in the sample. The QIAGEN-Tip was then transferred into a fresh 50 ml centrifuge tube and the plasmid DNA was eluted using 15 ml buffer 'QF', allowing the column to empty completely by gravity flow. To concentrate the DNA sample and remove salt contaminants, DNA was precipitated by addition of 0.7 volumes (10.5 ml) of isopropanol. The suspension was mixed properly and centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatant was carefully decanted and a clear, glassy pellet was observed at the bottom of the tube. The DNA pellet was resuspended in 300 µl TE buffer, transferred into a 1.5 ml microtube. The DNA was precipitated by adding 1/10th volume 3 M sodium acetate and 3 volumes 100% ethanol. The solution was mixed and centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was decanted and the pellet was washed once with 1 ml 70% ethanol and centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and the pellet was air-dried for 5-10 minutes. The purified plasmid DNA was resuspended in 300 µl TE buffer and quantified by spectrophotometry. Plasmid DNA samples were stored at -20°C until required.

2.2.4 DNA quantification using spectrophotometry

DNA concentration can be determined by spectrophotometry by measuring the absorbance at 260 nm (A_{260}). DNA samples are diluted in such a way as to get A_{260} values between 0.1 and 1. One unit absorbance at A_{260} corresponds to 50 µg plasmid DNA/ml. DNA samples were diluted 1:100 (v/v) in TE buffer, pH 8.0, transferred into cuvettes and absorbance was measured at 260 nm and 280 nm. The concentration of DNA in the sample was calculated using the formula: DNA concentration (µg/ml) = A_{260} X dilution factor X 50. The purity of the DNA samples was estimated by

calculating the $A_{260/280}$ ratio. For pure DNA samples, the value of the $A_{260/280}$ ratio is 1.8 (1.7 to 1.9 is acceptable).

2.3 Neonatal rat ventricular cardiomyocytes and fibroblasts isolation and culture

Neonatal rat ventricular cardiomyocytes (NRVM) were prepared essentially by the method described by Toraason M, *et.al.* [331], with some modifications. All the procedures were performed in Class II Biological Safety Cabinet (NUAIRE™) under sterile conditions. Dissection tools used for removing the hearts from neonatal rats were sterilized by autoclaving and disinfected in 70% ethanol after each use. 10-15 Sprague-Dawley rat pups (1-3 day old) were used per preparation. Rat pups were cleaned with 70% ethanol, sacrificed by cervical dislocation and decapitated. The chest cavity was opened and beating hearts were quickly excised using forceps and placed in a petri dish containing ice-cold Ca^{2+} and Mg^{2+} free Hank's buffered salt solution (CMF – HBSS; Sigma-Aldrich Co., UK - # H6648). Atria and surrounding connective tissue were removed and the ventricles were transferred to a tube containing ice-cold CMF-HBSS. The tissues were rinsed twice with CMF-HBSS in order to remove traces of blood and then transferred into a petri dish. Most of the CMF-HBSS was removed by aspiration, leaving about 1 ml in the dish. The ventricles were minced using sterile scalpels into small pieces about 1mm thick. To these, 8ml CMF-HBSS was added followed by 1 ml trypsin (Worthington Biochemical Corporation, USA - # LK003225) resuspended in CMF-HBSS, giving a final concentration of 50 μ g/ml trypsin. These were incubated overnight (14-16 hours) at 4°C. The following morning, 0.5 ml soybean trypsin inhibitor (Worthington Biochemical Corporation, USA - # LK003235) resuspended in CMF-HBSS, was added to the partially-digested tissues and incubated at 37°C in a 5% CO₂ (v/v) incubator for 20 minutes to fully inactivate Trypsin activity. Following this, the samples were transferred into a T75 tissue-culture grade flask (BD Biosciences, UK - # 353136) and 2.5 ml Collagenase (Worthington Biochemical Corporation, USA - # LK003245), resuspended in Leibovitz L-15 serum-free medium (Sigma-Aldrich Co., UK - # L1518) was added, equivalent to 750 units collagenase. The tissues were then fully digested by placing the flask vertically in a shaking water bath at 37°C for 30-45 minutes. Meanwhile, 20 ml serum-free L-15 medium was equilibrated by placing it in a T25 flask (BD Biosciences, UK - # 353108) with a vented cap, at 37°C in a 5% CO₂ incubator. After 30-45 minutes of collagenase digestion, the cells were dislodged by

tritulating the suspension 10 times using a 10 ml serological pipette. Cells were washed once with equilibrated L-15 medium and filtered through a 0.70 μm cell strainer (BD Biosciences, UK - # 352350) in L-15 medium. Cells were collected by centrifugation at 100 g (800 rpm) for 5 minutes. The cells were resuspended gently in 25 ml 'complete medium' - 4500 mg/L glucose containing Dulbecco's Modified Eagle Medium [DMEM] with 200 mM L-glutamine (Sigma-Aldrich Co., UK - # D6429), 10% [v/v] fetal calf serum (Sigma-Aldrich Co., UK - # F4135), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich Co., UK - # P4333). Cardiomyocytes were purified from the thus obtained mixture of cells by differential pre-plating. In this, the property of cardiac fibroblasts to adhere faster to tissue-culture plates was exploited to separate them from the cardiac myocytes. The cell mixture was sequentially pre-plated thrice in T75 flasks for 30, 30 and 20 minutes. The adherent fibroblasts were cultured further in 'complete medium' while the cardiomyocyte enriched medium after the third pre-plating was transferred to a separate tube. The cell density was measured after exclusion of dead cells with Trypan Blue (Sigma-Aldrich Co., UK - # T8154) and counting with the help of a Neubauer Hemocytometer. Cells were plated in type I fibronectin (Sigma-Aldrich Co., UK - # F1141) – coated ($1\ \mu\text{g}/\text{cm}^2$) 6 well tissue culture plates (BD Biosciences, UK - # 353934) at 1 million cells/well (area = $9.6\ \text{cm}^2$; cardiomyocyte density = $105,000/\text{cm}^2$), in a total of 2 ml medium per well. Cardiomyocytes obtained by this protocol routinely had a purity of 97-99%.

Cardiomyocytes were allowed to attach firmly for 3 days at which point, most cells were beating synchronously in culture. Cardiomyocytes were then serum-starved overnight – 'complete medium' changed to 'serum-free' medium – before starting experiments.

Cardiac fibroblasts isolated using the above protocol were allowed to multiply for 1 week, split 1:3 and cultured for another week (P1). Fibroblasts were then displaced by washing twice with 1X phosphate buffered saline (PBS), followed by addition of 1-2 ml trypsin-EDTA solution (Sigma-Aldrich Co., UK - # T3924) and incubation at 37°C in a 5% CO_2 incubator. Fibroblasts were passaged twice in this way to remove contaminating endothelial cells. For experimental setup, the cells were suspended in 'complete medium', counted using a hemocytometer and plated at 1 million cells/well (density = $105,000/\text{cm}^2$) of 6 well plates in a total of 2 ml medium/well. After allowing

them to attach for 24 hours, fibroblasts were serum-starved overnight before any experiments.

2.4 RNA extraction from cells

RNase-free eppendorfs and plastic-ware were used at all steps. All surfaces and pipettes were sprayed with RNaseZap[®] (Ambion, Inc. Applied Biosystems, UK - # AM9780), an RNase decontamination solution. Filtered, RNase-free pipette tips were used at every stage.

TRIzol[®] reagent (Invitrogen[™], UK - # 15596-018) was used to prepare RNA samples from cells. It is a ready-to-use mixture of acidic phenol, guanidine isothiocyanate and other proprietary components which allows simultaneous isolation of total cellular RNA, DNA and protein. When the cellular structure is broken down in the presence of TRIzol[®], the RNA and protein components differentially partition into the aqueous and organic phase of the solution respectively, while high molecular weight DNA will collect at the interphase [332]. This is mainly because of the acidic pH of the solution which ensures that RNA stays in the aqueous phase while DNA in the interphase. RNA is then precipitated from the aqueous phase.

Neonatal rat ventricular cardiomyocytes (NRVM) or neonatal rat cardiac fibroblasts were cultured in 6 well tissue culture plates (well diameter = 35 mm) at a density of 1 million/well. Cell culture medium was removed by aspiration and the cells were washed twice with 1X PBS. TRIzol[®], 1ml per well was added to preserve RNA integrity during cell lysis. The cells were scraped from the wells in the presence of TRIzol[®] and the lysates were transferred into 1.5 ml RNase-free eppendorf tubes and stored at -80°C if required. Frozen samples were incubated at room temperature (RT) for 5 minutes to permit complete dissociation of nucleoprotein complexes. 200 µl of chloroform per ml TRIzol[®] was added to each sample and mixed by vortexing for 15 seconds. The samples were then incubated at RT for 3 minutes followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. This results in separation of the phases into lower phenol/organic phase, an interphase and the aqueous upper phase containing RNA. The aqueous phase was gently aspirated by pipetting and transferred into a fresh 1.5ml microtube and 0.5ml isopropanol was added to the tube for precipitation of the RNA. The tubes were

vortexed briefly to mix the isopropanol and then incubated at RT for 10 minutes. Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C and the RNA pellet was visible at the bottom of the tube. The pellet was washed once with 1 ml of 75% ethanol followed by centrifugation at 8000 rpm for 5 minutes at 4°C. The RNA pellet was allowed to dry briefly and resuspended in 40 µl of RNase-free water. All RNA samples were stored at -80°C.

2.5 RNA extraction from mouse cardiac tissue

Myocardial samples from mouse ventricles were snap frozen in liquid nitrogen and stored at -80°C. RNase-free eppendorfs and plastic-ware were used at all steps. Mortar and pestle used for lysing frozen tissues were baked at 220°C for 4 hours to inactivate RNases. All surfaces and pipettes were sprayed with RNaseZap[®] (Ambion, Inc. Applied Biosystems, UK) to remove RNase. Filtered, RNase-free pipette tips were used at every stage. RNA was isolated by using the RNeasy Mini kit (Qiagen, UK - # 74106). The RNeasy kit is based upon a spin-column containing silica gel membrane fitting into a microtube. By altering the salt concentration in the column, RNA can be adsorbed onto or eluted from the membrane. The membrane enriches for longer RNA molecules by excluding RNA less than 200 bases. Elution is carried out in RNase-free water.

2.5.1 Sample disruption and homogenization

Sample disruption of frozen tissues was done using a mortar-pestle. Sample disruption helps to break up the extracellular matrix in the tissue samples and opens up the plasma membrane of cells and intracellular organelles in order to ensure complete RNA solubilisation. Frozen tissue samples (20-30 mg) were placed in liquid nitrogen (N₂) containing mortar and quickly crushed with the pestle. The frozen tissue powder was transferred to a 1.5 ml microtube and 500 µl lysis buffer (RLT) was added quickly while the sample was still frozen. This is a critical parameter in obtaining high quality RNA as thawing of frozen tissue must be minimized prior to disruption. The lysis buffer contains guanidine thiocyanate, a chaotropic agent which denatures proteins by disrupting hydrogen bonds and thereby their secondary structure. It also contains β-mercaptoethanol (1% v/v) which disrupts the tertiary and quaternary structure of proteins by breaking disulphide bonds. Together, these inhibit RNase activity and

promote cell lysis. The disrupted tissue in lysis buffer was kept on ice until homogenization.

Sample homogenization ensures reduced viscosity of disrupted samples by shearing genomic DNA and other high molecular weight cellular components. Complete sample homogenization was achieved by passing the disrupted sample through a QIAshredder (Qiagen, UK - # 79654). The QIAshredder is a microcentrifuge spin column containing a proprietary biopolymer shredding system. The disrupted samples were loaded on QIAshredder spin columns placed in a collection tube, centrifuged at 13,000 rpm for 2 minutes at room temperature and the homogenized lysate was collected.

2.5.2 RNA isolation

Homogenized lysates (500 µl) were diluted by adding 983 µl double-distilled water (ddH₂O) and 17 µl proteinase K (20 µg/µl concentration) was added to the lysate. They were incubated at 55°C for 10 minutes in a water bath. The tissue debris was pelleted by centrifugation at 13,000 rpm for 3 minutes at room temperature. The supernatant was transferred to a fresh 2 ml microtube and 0.5 volumes of 100% ethanol were added and mixed by pipetting. To adsorb RNA from the sample onto the membrane, 700 µl of sample was added to an RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 seconds at 13,000 rpm. The flow-through was discarded and the step repeated until the entire sample was passed through the column for adsorption. Wash buffer (RW1) was added to the column (700 µl) and incubated for 5 minutes at room temperature followed by centrifugation for 15 seconds at 13,000 rpm. The flow-through was discarded and 80 µl of DNase I mixture (concentration = 0.34 U/µl; Qiagen, UK - # 79254) was carefully added directly on top of the membrane. The column was left at room temperature for 15 minutes, followed by another wash with 700 µl of wash buffer RW1. The column was transferred to a new collection tube and washed with 500 µl ethanol-containing wash buffer RPE by centrifugation at 13,000 rpm for 15 seconds followed by another wash with RPE by centrifugation for 2 minutes. To ensure no carry-over of ethanol to the eluted sample, the collection tube was changed and the membrane dried by centrifugation for 1 minute at 13,000 rpm. The flow-through and collection tube were discarded. To elute the bound RNA sample from the column, the column was transferred to a fresh 1.5 ml microtube and 30 µl of RNase-free water was

pipetted on the membrane avoiding direct contact. These were left at room temperature for 10 minutes before centrifugation for 1 minute at 13,000 rpm. Another elution was carried out similarly by adding 20 µl RNase-free water to the column and centrifugation. The column was discarded and the RNA sample was quantified and stored at -80°C until required.

2.5.3 RNA quantification

RNA obtained from cells or tissues was quantified by spectrophotometry by measuring the absorbance at 260 nm ($A_{260\text{nm}}$). One unit absorbance at 260 nm is equivalent to 40 µg RNA per ml. Samples were diluted 1:12 in RNase-free water and absorbance was measured at 260 nm using BioPhotometer Plus (Eppendorf, UK). The RNA concentration was calculated as: $\text{RNA } (\mu\text{g /ml}) = A_{260\text{nm}} \times \text{dilution factor} \times 40$. For estimation of sample purity, the RNA samples were diluted 1:12 in TE buffer pH 8.0. $A_{260\text{nm}}$ and $A_{280\text{nm}}$ were measured and the ratio of $A_{260/280}$ was calculated. Pure RNA samples usually have an $A_{260/280}$ ratio of 2.0 – 2.1.

2.6 Microarray analysis

The GeneChip[®] Gene 1.0 ST Array System (Affymetrix, Santa Clara, CA) was selected to perform gene expression analysis on TAC and sham RNA samples from WT and *Fstl3* KO mice. The Mouse Gene 1.0 ST Array is designed to measure the gene expression of well-annotated genes using approximately 25 probes/ gene (a total of 770,317 distinct probes) designed across the full-length of 28,853 mouse genes. It uses a perfect match (PM)-only design where probes hybridize to the sense strands. Background is estimated by using approximately 17,000 generic background probes while standard poly-A controls and hybridization controls are represented on the arrays thus permitting troubleshooting throughout the experimental process. Gene-level analysis of multiple probes on different exons is summarized into a single expression value that represents all transcripts from the same gene. It thus provides a more complete and accurate picture of gene expression than 3' biased expression array designs. It also gives a more robust analysis of the cumulative transcription activities of the gene of interest. The design of the array was based on the February 2006 mouse genome sequence (UCSC mm8, NCBI build 36) with comprehensive coverage of

RefSeq, putative complete CDS GenBank transcripts, all Ensembl transcript classes and syntenically mapped full-length mRNAs and RefSeq NMs. The Mouse Gene 1.0 ST (Sense Target) Array has 100 percent coverage of NM sequences present in the April 3, 2007, RefSeq database.

Microarray data analysis was performed by Dr. Enrique Lara-Pezzi. Total RNA was hybridized on the Mouse Gene 1.0 ST Array chip in triplicate and data was analyzed using the GeneSpring GX software suite (Agilent Technologies, USA). After scanning, raw data in each chip was normalized to the 50th percentile and to the median of the chip. Samples were then further normalized on a gene per gene basis to the values obtained for sham wild type hearts and gene lists were filtered for raw expression > 50. Fold change lists comparing two conditions were generated using a cut-off value of 1.4-fold. For gene ontology (GO) analysis, the generated gene lists were compared to existing GO lists in GeneSpring. Only those GO lists showing a correlation *p*-value < 0.01 with the studied gene list are shown.

2.7 cDNA synthesis

For cDNA synthesis, 0.2 µg RNA samples were used. RNA samples were always thawed and kept on ice. TaqMan[®] reverse transcription kit (Applied Biosystems, UK - # N8080234) was used and a master mix of all the reagents was prepared on ice as detailed in Table 2.4. To every tube, 13.05 µl of the master mix was added, followed by a total of 200 ng RNA sample and RNase-free water to make up the total volume to 20 µl.

Table 2.4: cDNA synthesis reaction setup

Reagent	Final concentration	Amount (µl) for 20µl reaction
RT Buffer, 10X	1X	2
MgCl ₂ 25mM	5.5mM	4.4
dNTP mix (10mM)	500µM each dNTP	4
Random hexamers 50µM	2.5µM	1
RNase inhibitor	0.4U/µl	0.4
MultiScribe™ Reverse Transcriptase	1.25U/µl	1.25
RNase-free water	-	
RNA sample	200ng	6.95

cDNA was synthesized in a Techne PHC-3 thermal cycler (Helix technologies Ltd., UK). The cycling conditions used for cDNA synthesis are as follows:

Table 2.5: cDNA synthesis reaction conditions

Step	Temperature (°C)	Time (minutes)
Primer binding	25	10
Extension	48	30
Enzyme denaturation	95	5

cDNA samples were stored at -20°C until required.

2.8 Quantitative real-time RT-PCR

Quantitative real-time PCR (qRT-PCR) was performed using off-the-shelf TaqMan[®] Gene Expression assays (Applied Biosystems, UK). These assays consist of target-specific PCR primers flanking a target sequence-specific hybridization probe. The probe is fluorescently labeled with a reporter dye (FAM) on the 5' end and a quencher (NFQ – non-fluorescent quencher) combined with a MGB (minor groove binder) moiety on the 3' end, which stabilizes the hybridized probe and raises the melting temperature (T_m). When the probe is intact, either in the target-bound or unbound state, fluorescence from the reporter dye is transferred to the quencher dye by a phenomenon called Fluorescence Resonance Energy Transfer (FRET) and is measured. When the primers and probe are bound to the target sequence and the extension phase begins, DNA polymerase cleaves the bound probe resulting in separation of the reporter dye and quencher dye, preventing FRET. Fluorescence from the reporter dye now predominates and is proportional to the amount of PCR amplicon. This is measured at every PCR cycle and the cycle at which fluorescence of the reporter dye exceeds background threshold fluorescence is called the Cycle Threshold (C_t). This value is used to calculate the relative gene expression by the 'Comparative C_t method'. To enable this, amplification of an endogenous reference gene (18S rRNA) was carried out at the same time using primers and probe labeled with VIC reporter dye and TAMRA quencher dye. This allows measurement of C_t values for target gene and control in the same tube and the ΔC_t is calculated as the difference between C_t of target gene and C_t of endogenous reference genes. From this, the relative expression of the gene is calculated as:

Relative gene expression = $2^{-\Delta[\Delta Ct]}$; where $\Delta[\Delta Ct]$ is the difference between ΔCt of test sample and ΔCt of blank/calibrator sample. Thus the abundance of the target gene is calculated, normalized to an endogenous reference gene like 18S.

The qRT-PCR reaction was set-up as a 25 μ l reaction using 1 μ l cDNA prepared from 200 ng RNA. TaqMan[®] Gene Expression master mix and target gene assays as well as 18S rRNA assay were purchased from Applied Biosystems, UK. The details of all target gene assays are given in Appendix I. TaqMan[®] Gene Expression master mix is optimized for all TaqMan[®] Gene Expression assays and contains AmpliTaq Gold[®] DNA polymerase UP (ultra pure), Uracil-DNA Glycosylase (UDG), dNTPs containing dUTP, ROX[™] passive reference and buffer components proprietary to Applied Biosystems. ROX functions as an internal reference against which reporter dye signal is normalized during amplification. UDG prevents reamplification of carryover PCR products by removing uracil incorporated into PCR amplicons created with a master mix containing dUTP, thus minimizing false positives.

Table 2.6: Real-time PCR reaction setup

Reagent	Final concentration	Amount (μ l) for 25 μ l reaction
2X TaqMan [®] Gene Expression master mix	1X	12.5
20X 18S rRNA assay (VIC reporter)	1X	1.25
20X Target gene assay (FAM reporter)	1X (250nM probe, 900nM each primer)	1.25
cDNA sample	-	1
DNase/RNase-free water	-	9

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, UK). The cycling conditions are shown in Table 2.7. Data were collected and analyzed using Sequence Detection System software version 1.9.1 after setting the baseline and threshold for each reporter dye manually. Relative gene expression was calculated by the comparative Ct method.

Table 2.7: Real-time PCR cycling conditions

Step	Temperature (°C)	Time (minutes)	Purpose
1 st Hold	50	2	UDG incubation
2 nd Hold	95	10	Activates DNA polymerase
PCR: 40 cycles	95	15	Denaturation
	60	1	Annealing and extension*

* Primers anneal during the 60°C incubation while probes anneal as the temperature reduces from 95°C to 60°C

2.9 Protein sample preparation from cells and tissues

Cultured neonatal rat cardiac myocytes or fibroblasts were cultured as described above. Cells were serum-starved for 24 hours prior to all experiments. Cells were treated with inhibitors of signalling cascades for 1 hour prior to stimulation for specific time periods, as per experimental requirements. The culture medium was then aspirated and the cells were washed twice with cold 1X PBS. For cell lysis, 150 µl of cold 1X Ripa buffer (Cell Signaling Technology, UK - # 9806 : 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF; Roche Applied Science, UK - # 10837091001), 1X PhosSTOP Phosphatase Inhibitor Cocktail (Roche Applied Science, UK - # 04906837001), 1X Complete Protease Inhibitor Cocktail (Roche Applied Science, UK - # 04693132001) was added and the cells were scraped from the tissue culture plates. The cell lysate was incubated in ice for 20 minutes to permit complete lysis and was then centrifuged at 13,000 rpm for 2 minutes at 4°C to precipitate the cell debris. The resulting supernatant was used as protein extract and quantified as described below.

For isolating total cellular proteins from frozen mouse cardiac tissues, the tissue samples were disrupted (powdered) in liquid nitrogen using a mortar-pestle apparatus. This was then transferred to a 1.5 ml microtube and 100 µl of 1X Ripa buffer supplemented with 1 mM PMSF, 1X PhosSTOP Phosphatase Inhibitor Cocktail and 1X Complete Protease Inhibitor Cocktail was added. The lysate was then briefly sonicated for complete homogenisation and the resulting homogenate was incubated on ice for 20 minutes. The cell debris was removed by centrifugation at 13,000 rpm for 2 minutes at 4°C and the supernatant was used to quantify the amount of proteins in the sample.

Following protein quantification, the protein samples were diluted using 3X reducing loading buffer (Cell signaling technology, Inc., UK - # 7722: 187.5mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) bromophenol blue supplemented with 125 mM dithiothreitol (DTT)).

2.10 Protein quantification

Quantification of protein samples was performed using the *DC* protein assay (Bio-rad, USA - # 500-0112). This method is based on the Lowry assay with some modifications. The assay is compatible with a number of detergents commonly used in protein lysis buffers. Proteins in the sample are made detergent soluble followed by a reaction with alkaline copper tartrate solution. The resulting protein-copper complex is then able to reduce Folin reagent, consequently producing a characteristic blue color with maximum absorbance at 750 nm. The amount of colored product is proportional to the number of tyrosine and tryptophan amino acids in the protein sample. A standard assay using known amounts of bovine serum albumin (BSA) is performed at the same time and the amount of proteins in the test samples is quantified by matching the absorbance values of the test samples against the standard curve. BSA standards from 0 to 10 μ g were made in 1.5 ml microtubes as follows:

Table 2.8: Protein standards

Tube #	1	2	3	4	5	6	7	8	9
Protein amount (μ g)	0	1	2	3	4	5	6	8	10
Amount of 2mg/ml BSA (μ l)	0	5	10	15	20	25	30	40	15
Amount of milliQ water (μ l)	15	45	40	35	30	25	20	10	0

Test samples and lysis buffer were diluted 1:2 and 5 μ l of standards and test samples were added to the wells of a 96-well flat-bottom plate in duplicate. Reagent ‘A’ was prepared by adding 20 μ l of surfactant solution ‘S’ per 1ml of alkaline copper tartrate solution. To the standards and test samples, 25 μ l of reagent ‘A’ was added, followed by 200 μ l of Folin reagent. The resulting mix was incubated for 15 minutes at room temperature and the absorbance was read at 750 nm (A_{750nm}) in a microplate spectrophotometer: μ Quant (BIO-TEK Instruments, Inc.) using the software KC Junior v1.41.3. The values of A_{750nm} were plotted against the amount of proteins in the

standards and a standard curve was obtained using linear regression. The standard curve routinely had an 'R square' value of >0.99, confirming a good fit to linearity. The amounts of protein in the test sample were then obtained by matching their $A_{750\text{nm}}$ values against the standard curve. A representative standard curve obtained using BSA standards is shown in Figure 2.2.

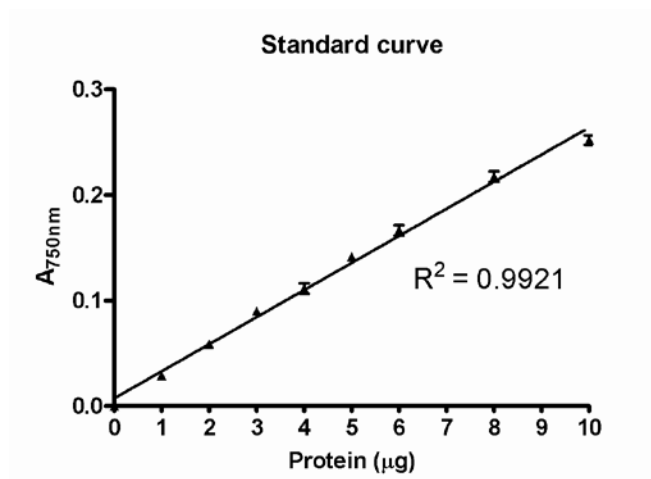


Figure 2.2: Representative standard curve for protein quantification. BSA standards with varying amount of protein were made and the $A_{750\text{nm}}$ was measured after protein quantification using DC protein assay. The assay was performed in duplicate and $A_{750\text{nm}}$ was plotted against the amount of protein. Linear regression analysis was performed and the R^2 value obtained is indicated.

2.11 One-dimensional Gel electrophoresis of proteins

Polyacrylamide Gel Electrophoresis (PAGE) is used to separate a complex mixture of proteins based on their size and charge. Gels are made by crosslinking acrylamide and bis-acrylamide to create specific gel pore sizes through which the protein mixture can migrate. A discontinuous buffer system is used for making separate stacking gel and resolving gel in order to stack all proteins into a single starting band and then finely resolve and separate them. In order to separate proteins based on their molecular weight, sodium dodecyl sulphate (SDS) is used in the gel system. Protein samples are denatured by heating them in the presence of SDS and a thiol reducing agent like β -mercaptoethanol, which reduces disulphide bonds in the proteins. This results in denatured proteins with a uniform charge-to-mass ratio proportional to their molecular weights, thus resulting in separation based on molecular weights.

The Mini-PROTEAN[®] system (Bio-Rad Laboratories, Inc.; USA) was used to prepare resolving gel according to the following table (2.9). The reagents were added in the order listed. 10% ammonium persulphate (APS; Bio-Rad # 161-0700) and N,N,N,N-Tetramethylethylenediamine (TEMED; Bio-Rad # 161-0801) catalyse the crosslinking and polymerisation of the acrylamide and bis-acrylamide (40% acrylamide-bis-acrylamide solution 29:1; Bio-Rad Laboratories, Inc. - # 161-0146). These were added immediately prior to pouring the gel in the casting plates. Lower Tris: 1.5 M Tris-Cl pH 8.8, 0.4% SDS. 12% resolving gel was made and poured into casting plates with 1mm spacer thickness. In order to prevent oxidation, the resolving gel was layered with 100% ethanol until polymerisation was complete.

Table 2.9: Resolving gel formulation

Reagents	Resolving gel					
	6%	7.5%	9%	12%	15%	20%
MilliQ water	1.75 ml	1.6 ml	1.4 ml	1 ml	0.6 ml	0 ml
Lower Tris (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Acrylamide: Bis-acrylamide solution (29 to 1) (40%)	0.75 ml	0.9 ml	1.1 ml	1.5 ml	1.9 ml	2.6 ml
10% APS	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl

After polymerization of the resolving gel, the ethanol layer was poured off. The stacking gel was made as detailed in Table 2.10 and poured over the resolving gel. Gel comb of 1 mm thickness containing 10 wells for loading samples was inserted. Once polymerization was complete, the comb was removed and the gel was attached to the buffer chamber. The wells were washed with SDS-electrophoresis buffer while the chamber was filled with the same buffer. Upper Tris: 0.5 M Tris-Cl pH 6.8, 0.4% SDS.

Table 2.10: Stacking gel formulation

Reagents	Stacking gel
MilliQ water	1.575 ml
Upper Tris (pH 6.8)	625 µl
Acrylamide: Bis-acrylamide solution (29 to 1) (40%)	225 µl
10% APS	40 µl
TEMED	4 µl

Equal amounts (30-60 μg) of denatured protein samples were loaded in the wells along with a molecular weight marker (Novex[®] Sharp pre-stained protein marker: Invitrogen[™], UK - # LC5800). SDS-electrophoresis buffer was poured in the outer chamber and the samples were electrophoresed at 120V until the bromophenol blue dye in the sample buffer reached the bottom of the gel. SDS-electrophoresis buffer 1 L: Tris base (3 g), Glycine (14.4 g), 0.1% SDS.

2.12 Electroblotting of proteins

Electroblotting of proteins is performed to transfer electrophoresed protein samples from polyacrylamide gels to membranes (nitrocellulose or polyvinylidene difluoride (PVDF)) prior to detection and analysis. Negatively charged proteins from the gel are transferred to membranes by applying a constant voltage through a buffer system, thereby facilitating electric transfer towards the anode. The transferred proteins are bound to the surface of the membrane, facilitating further immunodetection of specific proteins. The Mini Trans-Blot cell (Bio-Rad Laboratories, Inc.; USA) apparatus was used to assemble the transfer membrane, polyacrylamide gel and absorbent filter papers in Trans-blot cassettes. A 'wet transfer' system was used by soaking the gel, membrane and filter papers thoroughly in the transfer buffer. The assembly is summarized below in Figure 2.3.

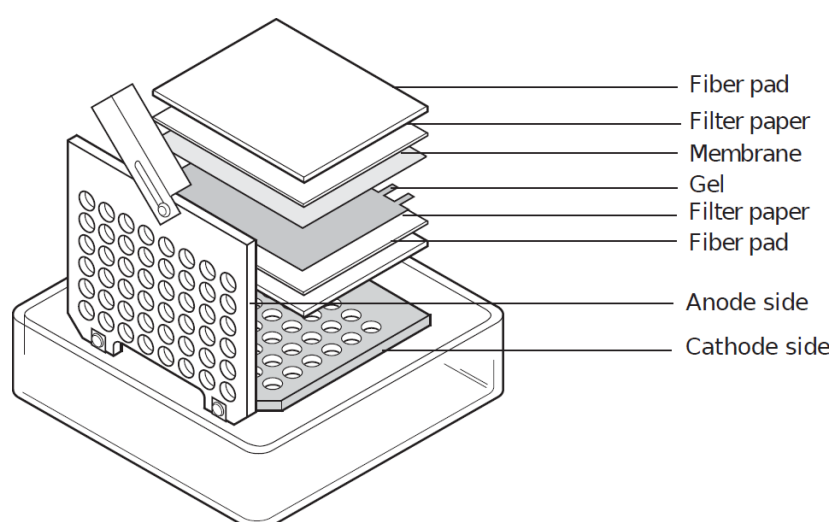


Figure 2.3: Assembly of protein transfer apparatus in trans-blot cassette. Adapted from Bio-Rad Laboratories Inc. USA (<http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1703930.pdf>)

In a tray containing transfer buffer, the transfer cassette was placed with the cathode side (dark) downwards. The fiber pads, filter papers and the nitrocellulose membrane were pre-wetted in transfer buffer for 10 minutes. One fiber pad was placed on the cathode side and any air bubbles in the pad were removed by rolling them out. A set of two filter papers (Whatman) were placed above the fiber pad and air bubbles removed. The polyacrylamide gel containing the resolved proteins was removed from the apparatus, the stacking gel was cut and the resolving gel was washed thoroughly in the transfer buffer. The gel was placed on the filter papers – protein side upwards – followed by nitrocellulose membrane (Bio-Rad Laboratories Inc. USA - # 162-0112). The air bubbles were removed and another set of filter papers and fiber pad were placed on top. The air bubbles were removed and the cassette was closed and locked tightly. The cassette was placed in the trans-blot core with the cathode side and anode side rightly orientated. This was then placed in the trans-blot cell and transfer buffer was filled up to the top. An ice pack was placed in the remaining space in order to avoid overheating due to Joule's heat produced during the electric transfer. The apparatus was connected to a Power-pack and transfer was carried out at 100V for 90 minutes at constant voltage. Transfer buffer 1 L: Tris base (3 g), Glycine (14.4 g), 20% Methanol.

2.13 Western blot

Following transfer of proteins to nitrocellulose membrane, the membrane was stained with Ponceau S – a reversible stain which allows further immunological analysis of protein samples – to ascertain uniform transfer of protein bands. Ponceau S binds to the positively charged amino groups of the proteins as well as non-covalently to non-polar regions. The membranes were immersed in Ponceau S staining solution (Sigma-Aldrich Co., UK – # P7170) for 5 minutes at room temperature. After staining, the membranes were quickly washed twice in 1X Tris-buffered sulphate (TBS) solution and transferred protein bands were observed. To destain, the membranes were immersed in TBS solution thrice for 5 minutes, while placing them on a rotary shaker. Ponceau S staining solution: 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid.

Following Ponceau S staining, membranes were used for specific immunological detection of proteins, also called 'Western Blot' analysis. The protein samples are bound to the surface of the nitrocellulose membrane and all other binding sites on the

membrane are blocked. The membrane is then probed for specific proteins using primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase (HRP). Chemiluminescent substrates are then added in order to visualize the position of the proteins by exposure to an autoradiography film. The following protocol was followed for western blot analysis of proteins. Membranes were blocked by immersing them in 5% (w/v) non-fat dry milk solution (Marvel) in TBS-Tween solution (TBS-T), for 2 hours at room temperature, placing them on a shaker. The blocking buffer was then removed and primary antibody solution (1:1000 v/v) in blocking buffer was added to the membranes. They were incubated overnight (12-18 hours) at 4°C with constant shaking to ensure uniform binding of primary antibody to the target proteins. The membranes were washed thrice in TBS-T solution for 5 minutes each. Secondary antibody directed against the primary antibody 'Fc' region and conjugated with HRP was used at a dilution of 1:2000 (v/v) in blocking buffer. Details of all antibodies used for western blotting are given in Appendix II. The membranes were incubated with the secondary antibody solution for 1 hour at room temperature with constant shaking. Following this, the membranes were washed thrice in TBS-T solution was 15 minutes each. During the last incubation, chemiluminescent substrate solution was prepared by mixing ECL Plus reagent A: reagent B, 40:1 (AmershamTM ECL PlusTM Detection Reagent kit; GE Healthcare, UK - # RPN 2132). After the last wash with TBS-T, chemiluminescent substrate solution was added evenly on the membrane (0.1 ml/cm² of the membrane) and incubated for 5 minutes at room temperature. The excess detection reagent was drained off and the membrane was enclosed in Saran wrap, making sure there were no air bubbles on the protein side. The membrane was then exposed to autoradiography film (AmershamTM Hyperfilm ECLTM, GE Healthcare, UK - # 28-9068-35) for various time periods until optimal protein bands were detected. Protein bands were detected by developing the film as follows: Immersion in developer solution for 5 minutes, water for 1 minute, fixer solution for 5 minutes (Kodak[®] GBX Developer and Fixer; Sigma-Aldrich Co., UK - # P7042, P7167).

2.14 Co-immunoprecipitation (Co-IP) of proteins

Co-Immunoprecipitation is a technique for analyzing protein-protein interactions. It is commonly used to verify protein interactions discovered through screening studies like yeast two-hybrid analysis. Immunoprecipitation is based on the principles of

precipitation of antibody-antigen complex by binding to protein A or protein G immobilized on agarose beads or other supports. The antibody is allowed to bind the target antigen in a mixture of proteins *e.g.* cell lysates. This antibody-antigen immune complex is then captured by binding of antibody Fc regions to protein A or protein G immobilized on a solid support. In co-IP, the antibody is used to precipitate not only the antigen, but also another protein that binds to the antigen. Thus the target antigen ‘co-precipitates’ another binding protein from the lysate and is thus immobilized on the protein A/protein G support. Any proteins not ‘precipitated’ from the mixture are washed away and the components of the bound immune complex are eluted from the support. They are then analyzed by SDS-PAGE and western blot for precise identification of the bound protein. A schematic of the steps involved in co-IP is shown in Figure 2.4.

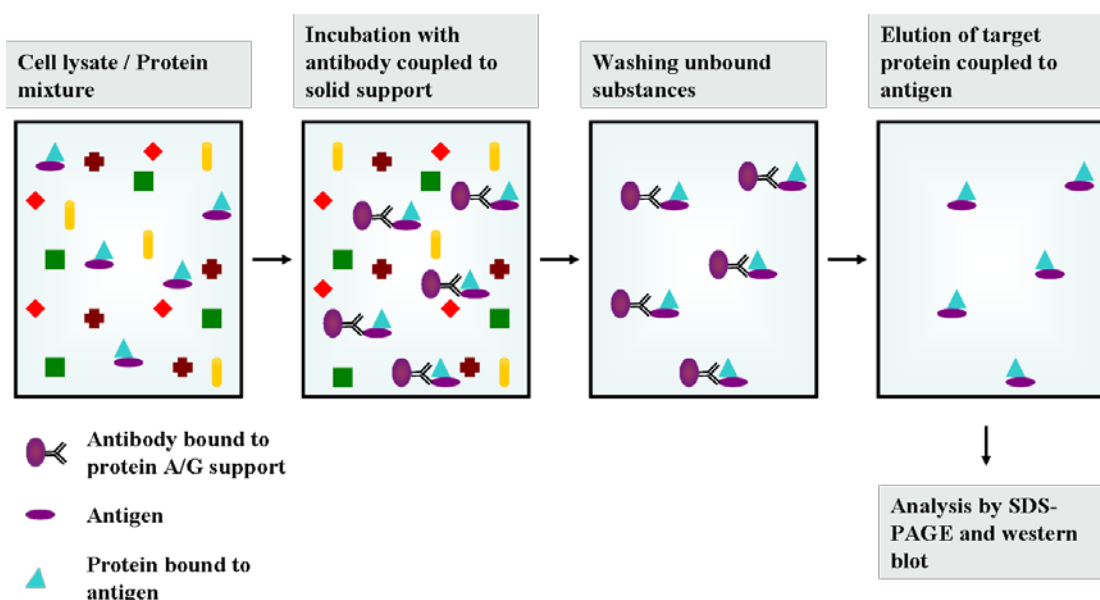


Figure 2.4: Schematic of co-immunoprecipitation technique. Target protein bound to an antigen (protein) is precipitated from a mixture of proteins by means of an antibody coupled to protein A or protein G immobilised on a solid support. The bound immune complex is then eluted and analysed by SDS-PAGE and western blot.

Co-IP was used to verify interaction between connective tissue growth factor (CTGF) and Fstl3. Neonatal rat ventricular fibroblasts (NRVF) cell lysate was used for co-IP. NRVFs were passaged twice, serum-starved for 24 hours and lysed in 1 ml cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM NaF, mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM PMSO and 1% Triton-

X100. The lysate was kept on ice for 15 minutes, following which it was pre-cleared using 50 µl protein A/G agarose beads (sc-2003 - Santacruz Biotechnology[®], USA) by rotation at 4°C for 1 hour. Following this, the tubes were centrifuged at 2000 rpm at 4°C for 5 minutes and the supernatant was transferred into new microtubes kept on ice. The lysate was incubated with 4 µg goat polyclonal anti-CTGF immunoprecipitation antibody (G14, sc-34772 - Santacruz Biotechnology[®], USA) or normal goat IgG (Santacruz Biotechnology[®], USA) as control. The lysates were incubated with the antibodies for 3 h at 4°C with rotation, following which 50 µl protein A/G agarose beads were added. The tubes were incubated overnight at 4°C with rotation to immunoprecipitate the complexes. The following day, tubes were centrifuged at 2000 rpm at 4°C for 5 minutes and the supernatant was carefully removed. The pellets were gently washed twice with cold 500 µl lysis buffer by centrifugation. The pellets were then resuspended in 100 µl 1X reducing loading buffer (Cell signaling technology, Inc., UK - # 7722) and boiled at 95°C for 5 minutes to release the bound proteins. The tubes were then kept on ice briefly and centrifuged at 2000 rpm at 4°C for 5 minutes. The supernatant was transferred into new microtubes and analysed by western blot. To check whether Fstl3 was co-immunoprecipitated, 1 µg/ml anti-Fstl3 antibody (MAB1255 – R&D systems UK) was used for detection during western blot.

2.15 Cardiomyocyte cell surface area measurement

To measure cell surface area, cardiomyocytes were grown in fibronectin-coated chamber slides (Lab-Tek[™] Permanox[™] 4 or 8 - well slides; Thermo Scientific Nunc, UK - # 177437, # 177445 respectively). The cells were cultured for 3 days, following which they were serum-starved for 24 hours prior to experiments. Cardiomyocytes were transfected with expression vector for mouse Fstl3 (pCMS-EGFP-Fstl3) or empty vector (pCMS-EGFP). Enhanced green fluorescent protein (EGFP) is expressed in this vector by use of a separate promoter (SV40) from the one used for cloning of Fstl3 (CMV). Thus, transfected cells can be detected by visualization of EGFP. Following transfection, the cells were cultured for 2 days. The culture medium was aspirated and the cells were washed twice with 1X PBS containing 1 mM MgCl₂ and 1 mM CaCl₂ to prevent their detachment. The cells were fixed by adding 4% paraformaldehyde (PFA) solution containing 1 mM MgCl₂ and 1 mM CaCl₂ for 15 minutes at room temperature. Following fixation, cell were stained with troponin-I antibody for detection of

cardiomyocytes and DAPI as a nuclear stain. The following protocol was used for immunofluorescent staining.

Fixed cells were washed thrice with 1X PBS solution for 2 minutes at RT. Cells were permeabilised by adding 0.1% (v/v) Triton X-100 in PBS for 10 minutes at RT. Cells were washed thrice with 1X PBS solution for 2 minutes at RT. To reduce non-specific binding, blocking was performed by adding 3% (w/v) BSA in PBS-T buffer for 30 minutes at room temperature. Following this, primary antibody solution – anti-troponin T (Sigma - # T6277; 1:200 v/v in blocking solution) was added and incubated for 1 hour at RT in a humidified chamber. The antibody solution was decanted and the cells were washed three times with 1X PBS-T for 5 minutes. The secondary antibody solution (anti-sheep IgG – Alexafluor[®] 594; Invitrogen, UK - # 11016) was prepared by diluting the antibody 1:1000 (v/v) in blocking solution and incubated with the cells for 1 hour at RT. The cells were washed thrice with DAPI (0.1 µg/ml) containing PBS solution for 5 minutes each. The chamber apparatus was removed from the slide and the slide was mounted using PermaFluor[™] aqueous mounting medium (Thermo Scientific, UK - # TA-030-FM). The slides were observed by fluorescent microscopy using Zeiss Axioscope microscope fitted with a Nikon digital camera (DXM1200F). Fluorescent micrographs were obtained using the software NIS Elements AR 3.0 (Nikon). Cardiomyocytes were identified by positive staining for troponin T and cell areas were measured by tracing around the cells using the same software. A total of 150-190 cell areas were measured per sample from 10 separate fields.

2.16 Measurement of cardiomyocyte protein synthesis

Protein synthesis in cardiomyocytes is used as a measure of hypertrophy. Cultured cardiomyocytes were treated in serum-free medium with different concentration of recombinant Fstl3 or 100 µM phenylephrine (PE) as a positive control. Protein synthesis was measured by calculating radioactive ³H-Leucine incorporation using scintillation counting, as described in [133] with some modifications. Scintillation counts were normalized to cell numbers reflected by total LDH release upon cell lysis, as described in section 2.19 below. All radioactive procedures were performed by Dr. Renee Germack. Briefly, the following protocol was followed:

Neonatal rat cardiomyocytes were cultured in 24-well tissue culture plates in 1 ml complete medium and serum-starved for 24 h prior to stimulation. Cells were stimulated with Fstl3 and/or PE in 500 μ l serum free medium for 24 h in the presence of 1 μ Ci [3 H]leucine/ml. Following this, cells were washed twice with 1 ml ice cold 1 X PBS and incubated with 500 μ l of 10% trichloroacetic acid (TCA) on ice for 1 h. Cells were washed twice with 1 ml ice cold 1 X PBS and 500 μ l of 1N NaOH was added for 30 minutes to dissolve precipitated proteins. The cells were then scraped and transferred to scintillation vials and radioactivity was calculated by liquid scintillation counting (TRI-CARB Liquid Scintillation Analyzer 1900CA, Parkard).

2.17 Mechanical stretch of neonatal cardiomyocytes and fibroblasts

Mechanical stretch was applied using Flexcell[®] FX-4000 Tension Plus system (Flexcell[®] International Corporation, USA). The system utilizes regulated vacuum pressure to deform flexible bottomed culture plates, thereby applying static or cyclic tensile strain to cultured cells at defined frequency, amplitude and waveforms. Cylindrical loading posts are used to apply equibiaxial strain while arcangle loading posts are used for uniaxial strain. The BioFlex[®] culture plates utilize a flexible silicone elastomer membrane covalently bonded to collagen I, collagen IV, laminin, etc. if required. A schematic diagram of the BioFlex[®] plate on the loading posts is shown in Figure 2.5. When controlled vacuum is applied, the flexible membrane is deformed and stretched and the cells grown in monolayer on the membrane are equally stretched.

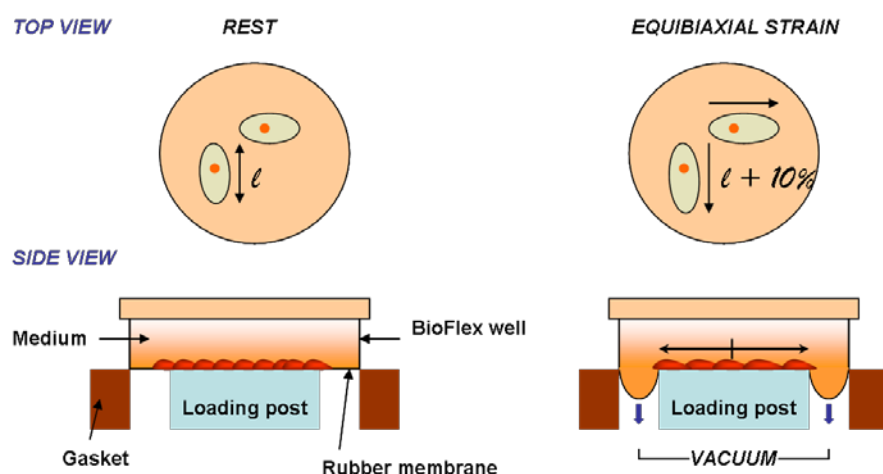


Figure 2.5: Schematic of BioFlex[®] plates and application of equibiaxial strain.

Neonatal rat ventricular cardiomyocytes were isolated as described in section 2.3 and plated on Bioflex[®] 6-well plates coated with collagen (Flexcell[®] International Corp. USA - # BF-3001C) and fibronectin I. Cells were plated at a density of 1×10^5 /cm² on the BioFlex[®] culture plates. Cardiomyocytes were cultured for 2 days in complete medium and serum-starved for 24 hours prior to experiments. Control cells were cultured similarly on 6-well tissue culture plates coated with collagen I and fibronectin I, and not subjected to stretch. The cells were treated as per experimental requirements in a total of 3 ml serum-free medium. The plates were loaded on 25 mm cylindrical loading posts and 10% equibiaxial stretch was applied at a frequency of 0.6Hz for 24 hours. The conditioned medium was collected and stored at -80°C until required. The cells were washed once with cold 1X PBS and RNA samples were obtained by addition of 1 ml TRIzol[®] per well, as described in section 2.4.

2.18 Measurement of collagen using Sircol collagen assay

Newly synthesized soluble collagen from supernatant of fibroblast cell cultures was assayed using Sircol collagen assay (Biocolor Ltd., UK). It is a colorimetric assay based on the binding of Sirius Red dye (in picric acid) to soluble collagen. A standard curve is obtained by using known quantities of collagen standards and the bound dye is quantified using absorbance at 555 nm. The following protocol was used:

Cardiac fibroblasts were cultured in 24-well tissue culture plates at 250,000 cells/well in 1 ml complete medium. Cells were serum-starved for 24 h and the medium was changed to conditioned medium from stretched cardiomyocytes. Conditioned medium from similarly treated but un-stretched cardiomyocytes was used as control. For some experiments, neutralizing antibody to Fstl3 was added to the medium (AF1255 - Goat polyclonal IgG, 7 µg/ml; R&D systems, UK). Normal goat IgG was used at the same concentration as control (sc-2028 - Santacruz Biotechnology[®] inc., USA). Fibroblasts were then cultured for 72 h following which collagen released into the supernatant was measured using Sircol assay. Supernatant from cultured fibroblasts (1 ml), culture medium (blank), and collagen standards dissolved in culture medium were transferred into low protein binding 1.5 ml microtubes. 200 µl of isolation and concentration reagent from the kit was added to each tube and mixed by inversion. The microtubes were incubated overnight in an ice-water mix at 4°C. The tubes were removed gently

without shaking their contents and centrifuged at 12,000 rpm for 10 minutes at 4°C. This results in a pellet of hydrated and concentrated collagen which is invisible. The supernatant from each tube was removed slowly by pipetting without disturbing the pellet. To these, 1 ml of Sircol dye reagent was added and mixed by gentle agitation followed by brief vortexing. The tubes were centrifuged at 12,000 rpm for 10 minutes and the pellet of collagen bound to the dye was visible. The supernatant was carefully drained by inverting the tubes. Ice cold acid-salt wash reagent (750 µl/tube) was gently layered on the pellet in order to remove unbound dye from the inside surface of the microtubes. The tubes were centrifuged at 12,000 rpm for 10 minutes, the supernatant was decanted and the tubes were briefly dried on paper towels. To these, 250 µl of alkali reagent was added and the tubes were vortexed to release the collagen bound dye. 200 µl from each tube was then transferred into individual wells of a 96-well plate and absorbance was measured at 555nm. Each assay was carried out in triplicates. A standard curve was plotted of A_{555nm} to collagen amounts from the standards after subtracting the blank values. Collagen from the test samples was calculated from the standard curve. Figure 2.6 below shows a representative standard curve.

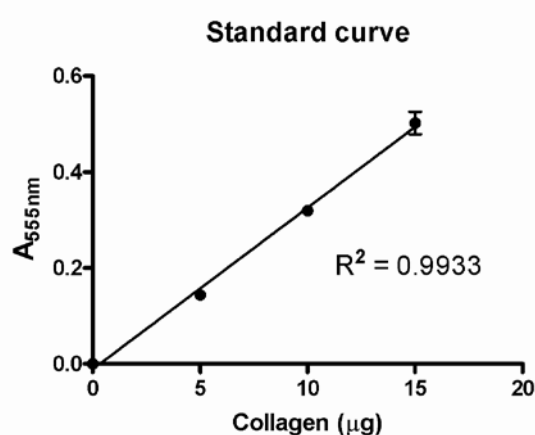


Figure 2.6: Standard curve for collagen measurement using Sircol assay. Collagen standards with varying amounts were made and the A_{555nm} was measured after quantification using Sircol collagen assay. The assay was performed in triplicate and A_{555nm} was plotted against the amount of collagen. Linear regression analysis was performed and the R^2 value obtained is indicated.

2.19 Measurement of cell death - LDH assay

Cell death in cultured cardiac fibroblasts was measured using quantification of lactate dehydrogenase (LDH), normally present in the cytoplasm, released into the supernatant. The CytoTox 96[®] non-radioactive cytotoxicity assay kit (Promega, UK) was used according to the manufacturer's guidelines. The assay is based on measurement of LDH using an enzymatic procedure which converts tetrazolium salt (INT) into a red coloured formazan product, proportional to the number of lysed cells. This is measured by calculating the absorbance at 490 nm ($A_{490\text{nm}}$). The following protocol was followed:

Cardiac fibroblasts were serum-starved for 24 h prior to stimulation with various concentrations of recombinant mouse Fstl3 (1255-F3-025/CF; R&D Systems, UK) or recombinant CTGF (5 µg/ml, 14-8503-80; eBioscience[®]) for 24 or 48 h in serum-free medium. Cells were cultured in 96-well plates at 50,000 cells/well in a total of 200 µl/well medium and all stimulations were carried out in triplicate. To measure LDH released into the supernatant, 50 µl medium from each well was transferred into a second 96-well plate and 50 µl of reconstituted substrate mix was added to each well. The plate was incubated for 30 minutes at RT and protected from light. Following this, 50 µl of stop solution was added to each well and any bubbles in the wells were popped using a syringe. Absorbance was measured at 490nm. Similarly, total cell numbers per plate were also calculated using an additional step in this procedure. For this purpose, cells were lysed using the lysis buffer provided in the kit by adding the 10X lysis solution to a final concentration of 1X and incubating the plate at 37°C for 1 hour. Following this, substrate solution was added to each well and the remaining protocol followed to measure $A_{490\text{nm}}$. Reaction blank and positive control were provided in the kit and used according to manufacturer's guidelines. Thus, for each sample, LDH released into the supernatant was normalized to total LDH released upon cell lysis and compared to control untreated cells.

2.20 Luciferase assay

Cardiac fibroblasts were cultured in 6 well plates at 1 million cells/well and transfected by lipofection using Lipofectamine[™] 2000 (# 11668-019; Invitrogen, UK) in serum-free medium without antibiotics. The DNA:Lipofectamine complexes were prepared

following the manufacturer's guidelines at a ratio of 1:3 (μg : μl) using 3 μg of SBE-luciferase plasmid (three tandem Smad binding elements) and 20 ng Renilla reporter plasmid (pRL-TK) for normalisation. These were added to the cells and incubated for 5-6 hours. After transfection, medium was changed to complete medium with antibiotics and 200 ng/ml recombinant Fstl3 was added. After 72 h, cells were washed twice with 1x PBS buffer and lysed in 100 μl 1X Passive Lysis Buffer (Promega, Madison, WI, USA) for 20 min on ice. The luciferase and renilla activities were measured in a Luminometer (TD-20/20-Turner designs) using a Dual Luciferase Kit (Promega).

2.21 Cardiac-specific *Fstl3* KO mice

All animal procedures including breeding were carried out at the Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts, USA in the laboratory of Professor Kenneth Walsh. Mice homozygous for a *Fstl3* allele containing two *loxP* sites flanking exons 3-5 (*Fstl3*^{flx/flx}) were backcrossed and maintained on a C57BL/6 background. These mice were crossed with α -myosin heavy chain (α -MHC)-Cre transgenic mice, also on a C57BL/6 background, which express Cre recombinase under the control of α -MHC promoter. Recombination by Cre leads to excision of *Fstl3* exons 3, 4 and 5. Thus, progeny containing *Fstl3*^{flx/flx} as well as α -MHC-Cre lack *Fstl3* expression in the post-natal cardiomyocytes, and are called cardiomyocyte-specific *Fstl3* KO mice. Only male mice were included in the study.

2.22 Trans-aortic constriction (Aortic Banding)

Trans-aortic constriction (TAC) or aortic banding is performed to induce left ventricular pressure overload in mice. It is a good *in vivo* model of outflow tract obstruction (*e.g.* aortic stenosis) and systemic hypertension leading to increased cardiac afterload and cardiac hypertrophy. TAC was performed in 6-8 week old *Fstl3* knock-out (KO) mice and wild type (WT) littermate controls. Prior to induction of anesthesia, all mice were given buprenorphine (0.25 mg/kg subcutaneously). General anesthesia was induced by inhalation of isoflurane (3-4% (v/v) in oxygen) and animals were restrained. The adequacy of anesthesia was ascertained by examination for loss of pedal and tail pinch reflexes. Anesthesia was maintained by 1-1.5% isoflurane inhalation throughout the procedure. The chest hair was removed by application of hair removal cream (Nair) and

the chest was disinfected with 70% ethanol followed by povidone iodine. Upper chest midline skin incision followed by median sternotomy was performed to open the chest cavity. The thymus was gently retracted until the transverse aorta was discernible. A loose suture (7-0 silk) was placed around the aorta and a 27 gauge blunted needle, between the right innominate artery and left common carotid artery (Figure 2.7). The suture was tightened to fully constrict the aorta and the needle was removed, thereby leading to partial constriction of the transverse aorta. The chest cavity, followed by the muscle and skin layer, was closed using 6-0 absorbable vicryl sutures. The chest was disinfected with povidone iodine and the animals were allowed to recover in a warm chamber. Analgesia (0.1 mg/kg buprenorphine) was given up to twice daily for 2-3 days following surgery. Some of the TAC procedures were performed by Dr. Shimano. 'Sham' operation was similarly performed with the exception that the suture around the aorta was not constricted.

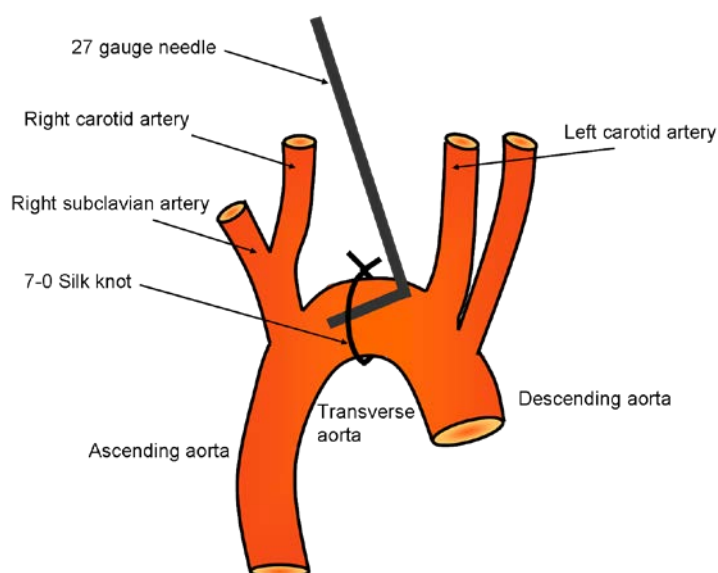


Figure 2.7: Schematic of trans-aortic constriction (TAC). The transverse aorta between the right subclavian artery and the left carotid artery is constricted by tying a suture around the aorta and a 27 gauge needle. The needle is then removed to leave the aorta partially constricted.

2.23 Echocardiography

Cardiac function was measured by trans-thoracic echocardiography before and 3 weeks after TAC procedure. An Acuson Sequoia C-256 machine with a 15-MHz probe was used on conscious but restrained mice. M-mode images were obtained at the papillary

muscle level of the cardiac chamber. The left ventricular (LV) chamber dimensions during systole and diastole were marked and the percent ejection fraction (% EF) and fractional shortening (% FS) were calculated. Three independent measurements were taken per mouse and average values were used.

2.24 Tissue sampling

Three weeks after TAC, mice were weighed (body weight – BW) and general anesthesia was induced as described in section 2.22. The abdominal cavity was opened by blunt dissection and the inferior vena cava was identified. Up to 900 µl blood was removed using a 22 gauge insulin syringe. The chest cavity was opened and the lung and heart were removed by dissection. The lung weight (LW) and heart weight (HW) were measured and the LW/BW and HW/BW ratios were calculated. The heart was cut transversely at the papillary muscle level. The upper part, including the atria was placed in a 1.5 ml microtube containing 10% formalin for fixation of the tissue. The LV was dissected out, divided into two pieces and snap-frozen in liquid nitrogen for obtaining protein and RNA samples respectively.

2.25 Staining of paraffin sections with Picrosirius red

Picrosirius red staining was performed essentially according to the method described in [333] with some modifications. Sirius red is an anionic dye which binds to the basic groups on collagen molecules through its sulphonyl groups. In the presence of picric acid (pH 2.0), the dye molecules bind collagen fibers in such a way as the axis of both the dye and collagen are aligned parallel, thereby increasing the birefringence of the collagen. Observation by polarized microscopy thus makes the picrosirius red staining highly specific to collagen fibers (types I, II and III). Increased birefringence enables very thin collagen fibrils to become visible as a source of light against a dark background, thereby increasing the sensitivity and resolution of the technique.

Picrosirius red staining was performed by Dr. Padmini Sarathchandra. Paraffin sections of mouse myocardium were cut at 5 µm thickness, deparaffinized and rehydrated by immersing them in the following solutions in the order and time specified in Table 2.11.

Table 2.11: Deparaffinization and rehydration of paraffin sections

Reagent	Time
Xylene	3 min
Xylene	3 min
Ethanol (100%)	3 min
Ethanol (100%)	3 min
Ethanol I (95%)	3 min
Ethanol (70%)	3 min
Running cold tap water	5 min

Following rehydration, the slides were immersed in Weigert's haematoxylin for 8 minutes to stain the nuclei (blue). The slides were then washed for 10 minutes under running tap water and blot dried. Picrosirius red solution (0.1% Sirius Red F3B in saturated aqueous picric acid solution; Sigma-Aldrich Co. Ltd., UK - # 365548 and P6744 respectively) was added on the tissue sections and incubated for 1 hour at RT. The slides were then washed twice with 0.5% glacial acetic acid and blotted dry. The sections were then dehydrated in ethanol and cleared in xylene by immersing them in the following solutions in the order and time specified in Table 2.12. The slides were then mounted using DPX mounting medium (Sigma-Aldrich Co. Ltd., UK - # 44581).

Table 2.12: Dehydration and clearing

Reagent	Time
Ethanol (70%)	3 min
Ethanol I (95%)	3 min
Ethanol (100%)	3 min
Ethanol (100%)	3 min
Xylene	3 min
Xylene	3 min

On the following day, the sections were viewed under a light microscope and fluorescent microscope. A total of 10 micrographs were captured per sample at 10X magnification and the fluorescence intensity and area were measured using the software NIS Elements AR 3.0 (Nikon). From this, the percent fibrotic area was calculated as the fraction of Sirius red stained area compared to the total measured area.

2.26 Staining of paraffin sections with wheat-germ agglutinin (WGA)

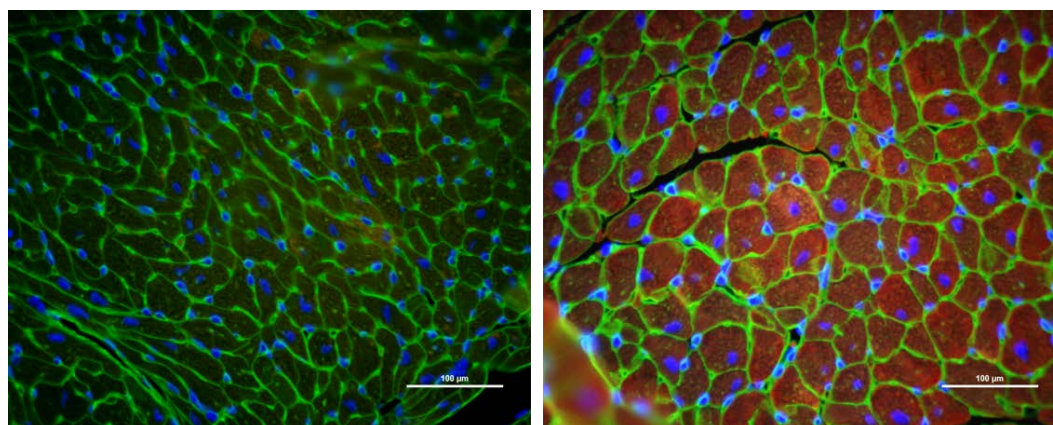
Wheat-germ agglutinin (WGA) is a lectin which selectively binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues [334]. WGA can be used to selectively label plasma membranes of eukaryotic cells. WGA conjugated to fluorescein (Excitation = 494 nm; Emission = 518 nm; Sigma-Aldrich Co., Ltd. - # L4895) was used at a concentration of 5 µg/ml in PBS for plasma membrane labelling. Cardiomyocytes were identified by α -sarcomeric actin staining and nuclei were labelled with DNA-binding fluorescent dye DAPI. The following protocol was followed.

Formalin fixed, paraffin embedded sections were cut at 5 µm thickness and allowed to dry for 24 hours. Deparaffinization and rehydration was performed as described in Table 2.11. During fixation, methylene bridges are formed which crosslink proteins and mask the antigenic sites. Formalin-fixed tissues therefore require an antigen retrieval step before immunohistochemical staining in order to break the methylene bridges and expose the antigenic sites. For antigen retrieval, the slides were immersed in 10 mM sodium citrate buffer (pH 6.0) and heated in a microwave for 5 minutes. The slides were then cooled down first by leaving in the citrate buffer for 10 minutes followed by running cold tap water in the container for 10 minutes. Following antigen retrieval, immunofluorescence staining was performed as detailed in Table 2.13 below.

Table 2.13: Immunofluorescence staining

Procedure	Reagent	Diluent	Condition	Time
Permeabilization	0.1% Triton-X	1X PBS	Humidified chamber	5 min
Blocking	3% BSA + 5% normal mouse serum	1X PBS + 0.1% Tween-20	Humidified chamber	45 min
Blot dry	-	-	-	-
Primary antibody	Mouse IgM Anti- α -sarcomeric actin (Sigma; Clone 5C5 - # A2172) 1:100 Wheat-germ agglutinin (Sigma - # L4895) 5µg/ml	1.5% BSA in 1X PBS + 0.1% Tween-20	Humidified chamber/ 4°C	Overnight
Wash	1X PBS	-	Gentle agitation	2 x 4 min
Secondary antibody	Goat anti-mouse IgM-AlexoFluor® 594 (Invitrogen - # A-21044) 1:1000	2% goat serum in 1X PBS	Humidified chamber	1 hour
Wash	1X PBS	-	Gentle agitation	4 min
Wash	DAPI (Sigma - # D9542) 100ng/ml	1X PBS	Humidified chamber	2 x 4 min
Mount	Permafluor (Thermo Scientific - # TA-030-FM)	-	-	-

The slides were observed by fluorescent microscopy using Zeiss Axioscope microscope fitted with a Nikon digital camera (DXM1200F). Fluorescent micrographs were obtained using the software NIS Elements AR 3.0 (Nikon). A total of 10 fluorescent micrographs per sample were obtained at 40X magnification. Cell areas were measured by tracing around the WGA-stained plasma membranes of cells using the same software. A total of 225 cell areas were measured per sample from 10 separate fields. Figure 2.8 below shows representative fluorescent micrographs for paraffin sections stained with WGA (green), α -sarcomeric actin (red) and DAPI (blue) along with the negative control stained in a similar fashion except for lack of anti- α -sarcomeric actin antibody.



Negative control

α – sarcomeric actin

Figure 2.8: Negative control for α -sarcomeric actin antibody. Sections of myocardium embedded in paraffin were stained with wheat-germ agglutinin, α -sarcomeric actin antibody and DAPI. For negative control, the primary α -sarcomeric actin antibody was omitted and the remaining protocol was followed as described in section 2.26. Scale = 100 μ m

2.27 ELISA for Activin A

Enzyme-linked immunosorbent assay (ELISA) was used for quantification of circulating Activin A in the serum of mice undergoing TAC. Activin A Quantikine ELISA kit (R&D Systems Inc., UK - # DAC00B) was used according to manufacturer's instructions. The assay was performed on all samples by Dr. Shimano.

2.28 Yeast two-hybrid analysis

A yeast two-hybrid assay was performed using the HybriZAP[®]-2.1 Two-Hybrid pre-digested vector kit (Stratagene) to identify *in vivo* protein interaction partners for Fstl3. Truncated Fstl3 was used as a bait to test interaction with target proteins expressed artificially in the yeast from a neonatal rat cardiomyocyte cDNA library. Fstl3 was cloned in frame with the yeast Gal4 DNA-binding domain (BD) while the library proteins were expressed as hybrid proteins with the Gal4 transcriptional activation domain (AD). Neither hybrid protein is capable of initiating activation of the reporter gene by itself. When a specific interaction takes place between the bait and target protein, the Gal4 BD and AD co-localise and activate expression of the reporter gene histidine (*HIS3*). Colonies which are positive for the reporter are selected and analysed for the sequence of the encoded target protein. Details of all reagents and culture media used are given in Appendix III. *Yeast host strain YRG-2*: YRG-2 is defective in uracil, histidine, adenine, lysine, tryptophan and leucine.

2.28.1 Cloning of Fstl3 in pBD-Gal4-Cam: Bait plasmid construction

A truncated form of Fstl3 lacking the signal peptide was cloned in frame with Gal4 BD in the vector pBD-Gal4 Cam (Figure 2.9) and verified by sequencing.

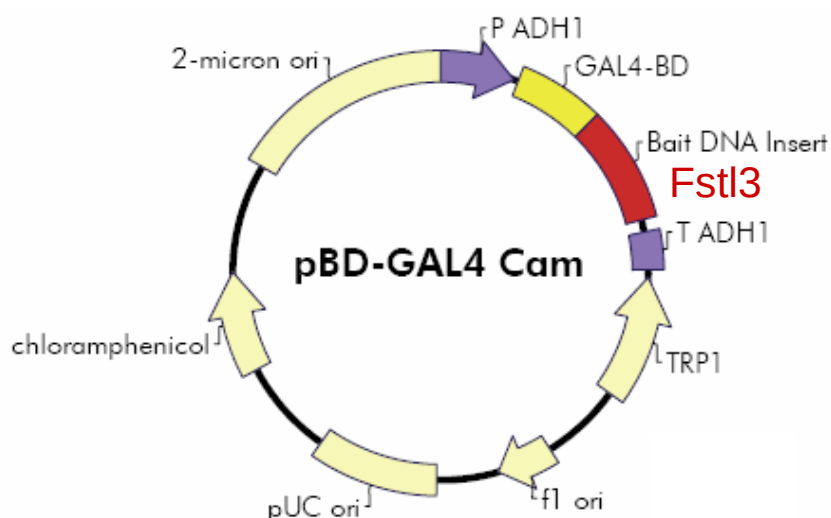


Figure 2.9: Schematic representation of bait plasmid encoding the Gal4-BD with Fstl3. The vector pBD-Gal4 Cam carries the Tryptophan synthesis gene *TRP1* as a selection marker. It encodes Fstl3 as fusion protein with Gal4-BD

2.28.2 Transformation of yeast with bait plasmid

The yeast strain YRG-2 is defective in tryptophan. Transformation of YRG-2 with the bait plasmid results in expression of the *TRP1* gene (tryptophan) and successful transformants are selected on Trp-deficient media. YPAD medium inoculated with a single colony of YRG-2 was grown overnight at 30°C in a shaking incubator. Yeast cells were obtained from 60 ml culture at an OD_{600nm} of 0.5 to 0.8 by centrifugation at 4000 rpm for 10 minutes and washed once with sterile water. The cells were resuspended in freshly prepared 1.5 ml TE-LiAc solution and 100 µg of denatured salmon sperm DNA was added with 100 ng of the bait plasmid and 600 µl of TE-LiAc-PEG solution to a 100 µl aliquot of the cell suspension. The mixture was incubated at 30°C for 30 minutes at 200 rpm and 70 µl dimethyl sulphoxide (DMSO, Sigma) was added. The cells were given a heat shock by incubation at 42°C for 15 minutes, harvested by centrifugation and resuspended in 0.5 ml TE buffer. A total of 150 µl of the sample was plated on selection medium lacking tryptophan (SD-Trp) and incubated at 30°C for 3-4 days. Four colonies were randomly selected and maintained on SD-Trp medium.

2.28.3 Checking leakiness of reporter gene *HIS3*

The use of certain proteins as baits, such as transcriptional activators, may result in activation of the reporter gene in the absence of the target plasmid construct. To check for auto-activation of the reporter gene *HIS3* before transformation with the target library, four YRG-2-Fstl3 colonies were inoculated in 1 ml of sterile water and 100 μ l of each sample was plated on SD-Trp and SD-Trp-His agar plates at dilutions of 1:0 (undiluted), 1:10 and 1:100. The plates were incubated at 30°C for 3-4 days and the colonies were counted. The YRG-2-Fstl3 samples that gave no colonies on SD-Trp-His medium were selected for further transformation as these colonies do not auto-activate the reporter gene.

2.28.4 Transformation of yeast with target DNA library

Transformation of YRG-2-Fstl3 with target DNA library results in expression of the Leucine synthesis gene *LEU2*. Successful transformants in which specific interaction takes place between the bait Fstl3 and target protein activate the reporter gene *HIS3*. These are selected on SD-His-Trp-Leu medium deficient in Histidine, Tryptophan and Leucine.

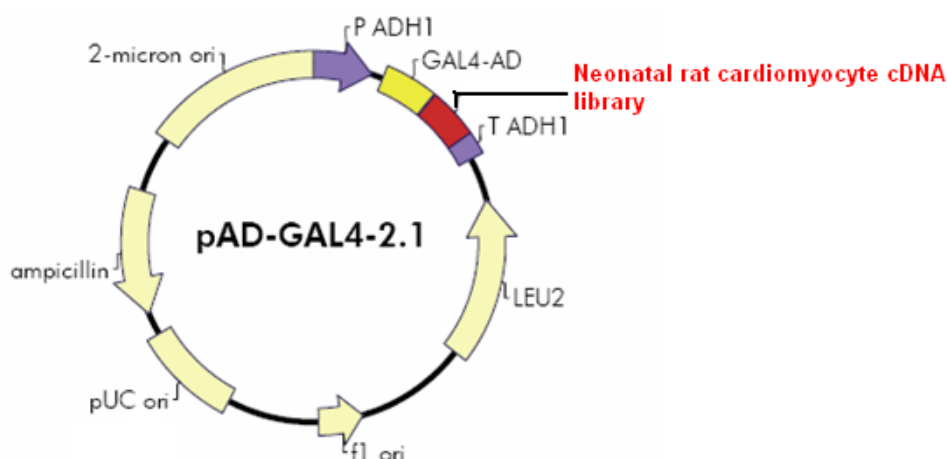


Figure 2.10: Schematic representation of the target plasmid construct. The target plasmid construct encodes the hybrid proteins from neonatal rat cardiomyocyte cDNA library linked to the Gal4-AD. The selection marker is the Leucine synthesis gene *LEU2*

YPAD medium inoculated with a single colony of YRG-2-FstI3 was grown overnight at 30°C in a shaking incubator at 250 rpm. Yeast cells were obtained from 500 ml of culture at an OD_{600nm} of 0.8 by centrifugation at 4000 rpm for 10 minutes and washed once with sterile water. The cells were resuspended in 5 ml of freshly prepared LiSORB solution and incubated at 30°C for 10 minutes. Cells were centrifuged again and the pellet was resuspended in LiSORB to a final volume of 1 ml. A total of 1.2 ml of denatured salmon sperm LiSORB solution (3.3 mg/ml) was added, followed by 9 ml of TE-LiAc-PEG solution. A total of 20 µg of target library was added and the mixture was incubated at 30°C for 30 minutes. Samples were given a heat-shock at 42°C for 8 minutes, followed by incubation on ice for 8 minutes and heat-shock at 42°C for 8 minutes. Cells were incubated in 10 ml SD-Trp-Leu medium at 30°C for 1 hour in a shaking incubator, centrifuged and the pellet was gently resuspended in 5 ml of SD-Trp-Leu medium. 250 µl aliquots were plated on 20 SD-His-Leu-Trp selection agar plates and incubated at 30°C for 4-5 days. The transformation rate was measured by plating 10 µl of transformation sample at dilutions of 1:0, 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000 on SD-Leu agar plates and the number of colonies were counted. Colonies that grew on SD-His-Leu-Trp selection plates were picked and streaked on SD-His-Leu-Trp plates to confirm positivity.

2.28.5 Screening of positive colonies

To obtain enough DNA from yeast colonies for sequencing and verification of interaction, the following steps were performed. Colonies from SD-His-Leu-Trp were grown overnight in YPAD medium and plasmid DNA was extracted using a standard miniprep protocol (QIAprep spin miniprep kit, Qiagen, UK – see section 2.2.2). This was then transformed into One Shot[®] TOP10 chemically competent *E. Coli* cells (Invitrogen) following manufacturers instructions by incubating the bacteria for 30 seconds at 42°C (Section 2.1.5). Transformed colonies were grown in LB medium and plasmid DNA was extracted using the QIAprep kit and sequenced using a Gal4-AD primer. The sequences obtained were analyzed using the nucleotide BLAST tool (Basic Local Alignment Search Tool, NCBI, USA). Positive interactions were validated by co-immunoprecipitation. Figure 2.11 summarises the important steps in a yeast two-hybrid analysis.

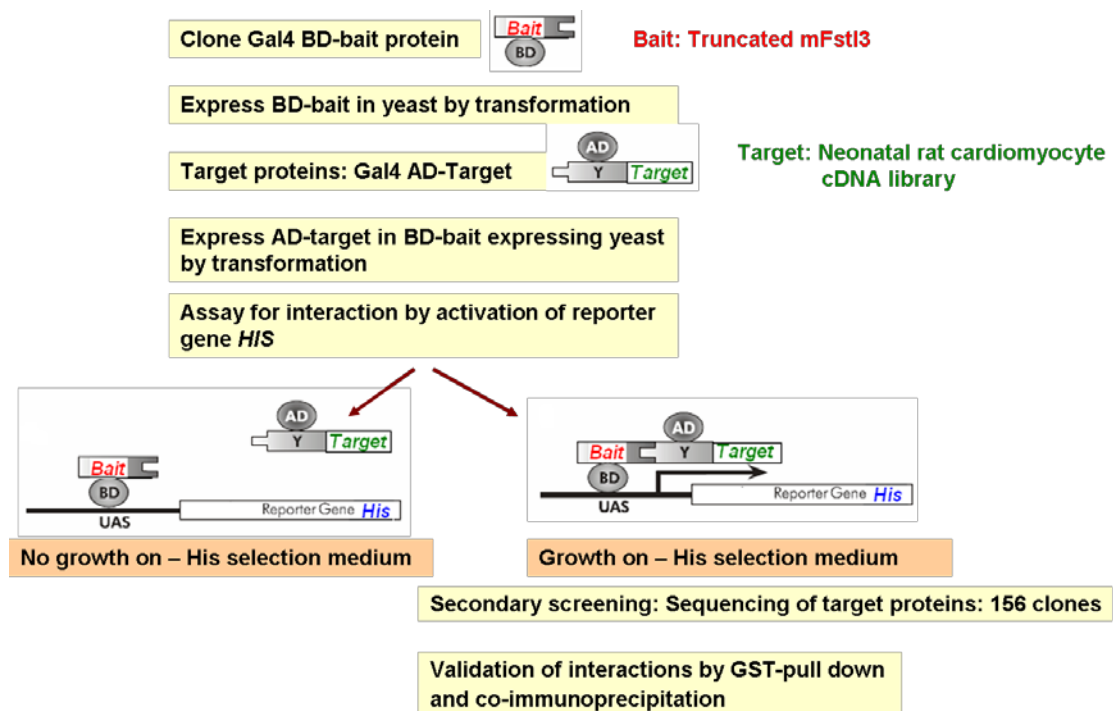


Figure 2.11: Flow diagram of yeast two-hybrid analysis of protein-protein interactions.

CHAPTER 3: FSTL3 in human heart failure

3.1 Introduction

The progression from underlying cardiac pathologies to heart failure is often characterised by cardiomyocyte hypertrophy, extracellular matrix remodelling and chamber dilatation resulting in compromised cardiac function. However, recent advances in the field have shown that in end-stage heart failure patients, mechanical unloading of the heart using left ventricular assist devices (LVAD) can reverse the progression to heart failure in some cases. This led to the notion that LVAD can also be used as a ‘bridge-to-recovery’ in addition to being used as a ‘bridge-to-transplantation’. Research at our institute has shown that a combination therapy using LVAD and a specific pharmacological regimen is highly effective in patients with non-ischemic cardiomyopathies and about two thirds of them showed sufficient recovery that allowed explantation of the LVAD, avoiding the need for heart transplantation [335]. Mechanical unloading of the heart combined with pharmacological therapy is aimed at maximising reverse remodelling of the heart, which leads to improved contractile function [336, 337]. Following this, induction of physiological hypertrophy by administration of the β_2 adrenergic receptor agonist Clenbuterol has shown beneficial effects towards recovery [338]. In a rat model of unloaded failing hearts, treatment with Clenbuterol was found to enhance physiological hypertrophy, cardiomyocyte contractility and normalised Ca^{2+} handling [339]. However, the molecular mechanisms underlying reverse remodelling and recovery are not fully understood.

TGF- β family members have been widely implicated in the pathogenesis of heart failure. Activins are homodimeric cytokines of the TGF- β superfamily and are known to modulate diverse pathophysiological processes including inflammation and wound healing, cell proliferation, differentiation, metabolism, fibrosis and cardiac remodelling (reviewed in [292, 295, 340]). Activin signalling is inhibited by the follistatin family of proteins. Both Follistatin (FST) and Follistatin-like 3 (FSTL3) can bind activin with a high affinity and neutralise its action by preventing its binding to the activin receptors [297]. On the other hand, both activin and TGF- β are known to transcriptionally regulate the expression of FST and FSTL3 through Smad proteins [305, 306]. Whereas a role for activin in heart disease has been proposed, the presence and possible function of

follistatins in the heart remains obscure. In this context, we hypothesized that the Activin-Follistatin interplay may play a role in the pathogenesis of heart failure. This chapter presents evidence for the importance of follistatins in heart failure based on their altered expression in both human and experimental models.

3.2 Patient characteristics and myocardial samples

The study conforms to the principles outlined in the Declaration of Helsinki. Myocardial biopsies were obtained from patients after informed consent and with the approval of the Royal Brompton and Harefield Research Ethics Committee. Patients with end-stage heart failure with dilated cardiomyopathies and without any histological evidence of myocarditis were included. Left ventricular biopsies were obtained at the time of LVAD implant (n = 27: 25 male, 2 female), and after recovery at the time of LVAD explant (n = 13) and at a period of 1 year follow-up (n = 9). Control samples were obtained from biopsies from healthy donor hearts presenting good hemodynamic function (n = 9) [328]. Mean age of recovery patients was 37.3 years with mean duration of disease being 47.3 months. Prior to LVAD implant, patients were treated with inotropes in addition to standard heart failure drugs. Following LVAD implantation, patients were treated with a pharmacological regimen which included β -blockers, ACE inhibitors, angiotensin II receptor antagonists and spironolactone followed by clenbuterol to induce physiological hypertrophy [335, 341].

3.3 Expression of FSTL3 is elevated in heart failure

Follistatin family proteins FST and FSTL3 have high sequence homology and both are involved in binding activin with a high affinity, inhibiting its function [297, 299]. The mRNA expression of FST and FSTL3 in myocardial samples from healthy controls and patients with heart failure was examined using qRT-PCR [328]. As shown in Figure 3.1, the expression of both FST (A) and Activin β_A subunit INHBA (B) mRNAs remained unchanged in end-stage heart failure patients and control samples. Similarly, the expression of Inhibin α subunit INHA also remained unchanged (C). However, FSTL3 mRNA expression was elevated in heart failure patients (D). The expression of FSTL3 was also examined in a cohort of patients undergoing combination therapy. As shown in Figure 3.2, FSTL3 mRNA expression was elevated at the time of LVAD implant.

However, following recovery and LVAD explant, FSTL3 expression was reduced and comparable to control levels after a period of 1 year following explant.

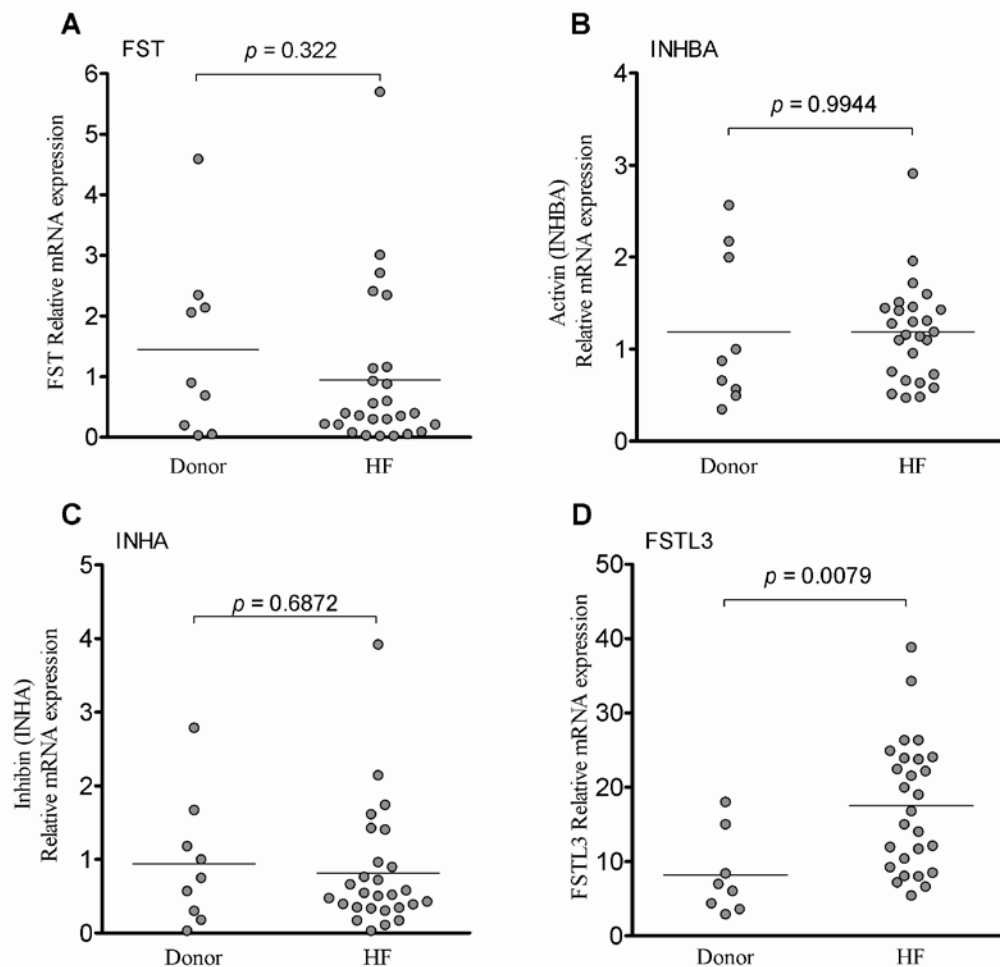


Figure 3.1: Expression of TGF- β family ligands and their inhibitors in heart failure. mRNA expression of FST (A), INHBA (B), INHA (C) and FSTL3 (D) in left ventricular samples from patients with heart failure (HF; n = 27) and healthy controls (Donor; n = 8-9) was analysed using quantitative real-time RT-PCR. Results are expressed in relative units normalised to endogenous 18S rRNA expression. Unpaired t-tests were used to calculate the p values, which are indicated in the figure.

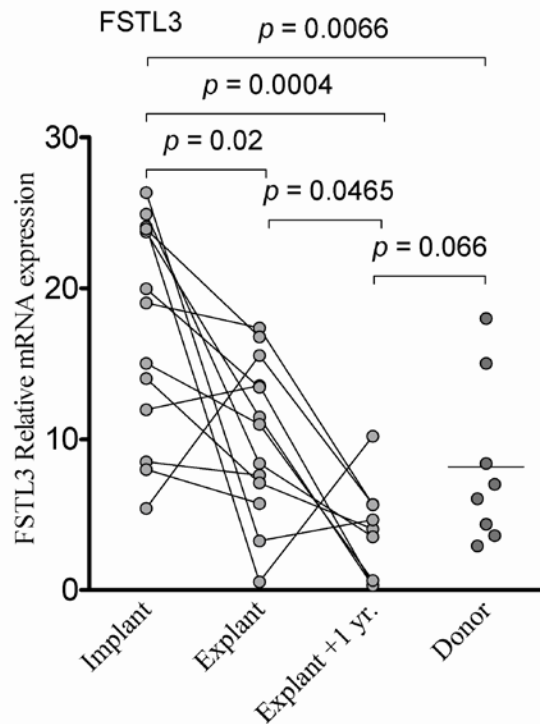


Figure 3.2: Expression of FSTL3 in heart failure and recovery. Expression of FSTL3 mRNA was examined using quantitative real-time RT-PCR in heart failure patients undergoing LVAD implant and combination therapy (Implant; $n = 13$), after recovery during LVAD explant (Explant; $n = 13$) and after a period of 1 year follow-up (Explant + 1 yr; $n = 9$). Results are expressed in relative units normalised to endogenous 18S rRNA expression. Paired t-tests were used to calculate the p value of FSTL3 expression at different time points while unpaired t-test was used for comparison with donor samples.

In order to examine the tissue distribution of FSTL3, immunohistochemical analysis was performed on myocardial sections from heart failure patients and controls with a specific antibody against FSTL3 [328]. As shown in Figure 3.3, sections from control samples showed low expression of FSTL3. However in patients with deteriorating heart failure, higher expression of FSTL3 was detected in cardiac myocytes and vascular endothelial cells while no staining was observed in either the fibroblasts surrounding the minor vessels or interstitial cells. FSTL3 was also found to be expressed in the nuclei of some cardiac myocytes (arrowheads).

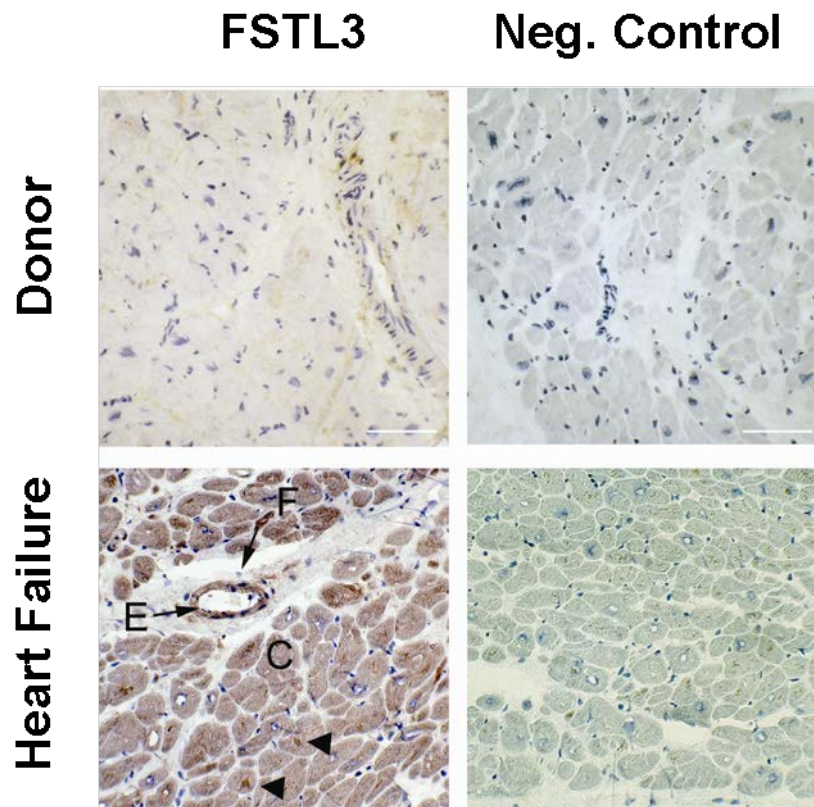


Figure 3.3: Tissue distribution of FSTL3 in heart failure patients. Sections of myocardium from control (Donor) and heart failure patients were stained using a specific antibody against FSTL3. Negative control represents staining in the absence of primary antibody. FSTL3 was found to be localised in cardiac myocytes (C) and endothelial cells (E), while fibroblasts (F) showed no expression of FSTL3. Some cardiac myocytes also showed nuclear expression of FSTL3 (arrowheads). Bar = 100 μ m.

3.4 Microarray analysis of FSTL3 in heart failure

As shown above, FSTL3 expression in patients with heart failure was found to be elevated and localised in cardiomyocytes and endothelial cells. In order to gain some initial understanding of the function of FSTL3 in the pathogenesis of heart failure, microarray analysis was performed on paired samples from patients taken at the time of LVAD implant and explant. Following this, lists of genes were compiled whose expression correlated with that of FSTL3 in the different samples and compared to existing gene ontology (GO) lists as detailed in [328]. As shown in Figure 3.4, FSTL3 showed good correlation with genes involved in transcriptional regulation, signal

transduction and protein phosphorylation, being associated mostly with the nuclear compartment.

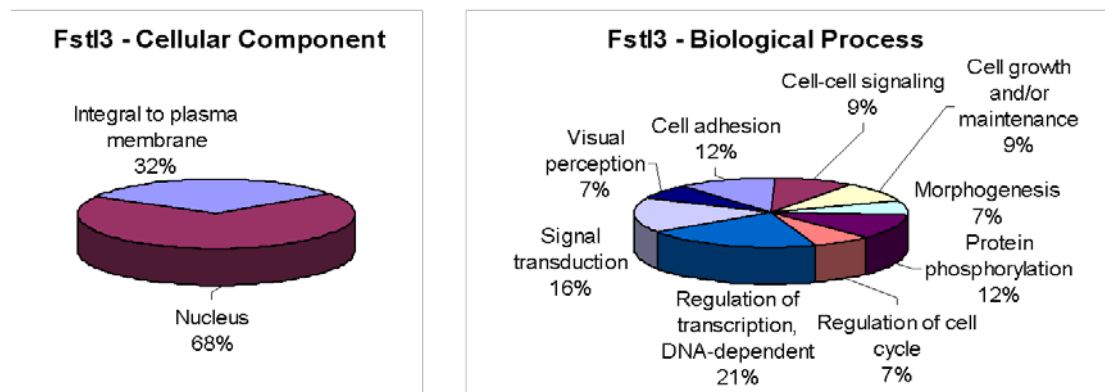


Figure 3.4: Gene ontology analysis of FSTL3-correlating genes in heart failure. Cardiac samples from heart failure patients were collected at the time of LVAD implant and explant and total RNA was analyzed using oligonucleotide microarrays. A list of genes correlating with FSTL3 in the different samples was compared with pre-existing gene ontology lists for cellular compartment and biological process. Lists with >3 genes common to the FSTL3 list and $p < 0.005$ were considered. The percentage of common genes in a list compared to the total number of common genes in all the lists is shown ($n = 16$).

3.5 Expression of *Fstl3* in mouse models of cardiac injury

Based on the observation of increased FSTL3 mRNA expression in human heart failure, *Fstl3* expression was examined in small animal models of cardiac injury. These included left anterior coronary artery ligation as a model of myocardial infarction (MI), ischemia-reperfusion (IR) injury and trans-aortic constriction (TAC) as a model of pressure-overload induced cardiac hypertrophy in mice. The mRNA expression of *Fstl3* in the myocardium was elevated in all models of cardiac injury compared to ‘sham’ operated mice [294], as shown in Figure 3.5. In the myocardium of infarcted mice (A), *Fstl3* mRNA was elevated in both the remote and infarct region three days after infarct. Similarly, in ischemia-reperfusion injury (B), *Fstl3* mRNA expression was elevated after both 12 h and 24 h of reperfusion. In pressure-overload induced cardiac hypertrophy, a prominent model of aortic stenosis, *Fstl3* mRNA expression was also elevated three weeks after banding (C).

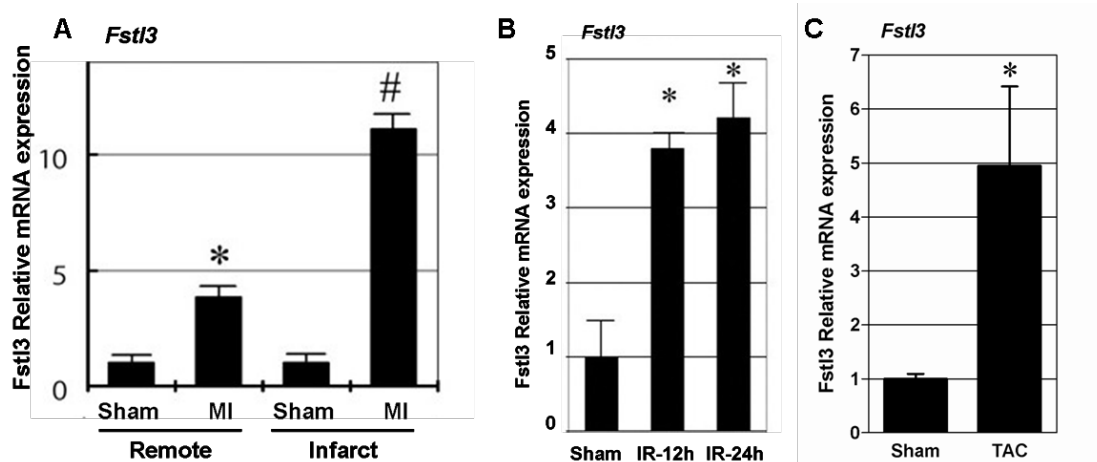


Figure 3.5: *Fstl3* mRNA expression is elevated in different models of cardiac injury. Expression of *Fstl3* mRNA was examined by quantitative real-time RT-PCR in cardiac samples from mice subjected to myocardial infarction (remote and infarct zone) at three days post-infarct (A), ischemia-reperfusion at 12 h and 24 h reperfusion (B) and trans-aortic banding at three weeks after banding (C), as well as ‘sham’ operated mice at respective time-points. Data are presented as relative expression of *Fstl3* as mean $\pm$ SEM and normalised to endogenous GAPDH (A, B) or 18S rRNA (C) expression. Unpaired t-tests were used to check statistical significance. * $p < 0.05$, # $p < 0.01$ compared to sham. $n = 3-6$.

Moreover, the mRNA expression of *Activin* β_A subunit was also tested in the same models and found to be elevated following injury. As shown in Figure 3.6, the expression of *Activin* β_A mRNA was elevated in both the remote and infarct zone at three days following myocardial infarction (A). Similarly, *Activin* β_A mRNA was also elevated after 12 h and normalised after 24 h following ischemia-reperfusion injury (B). In the cardiac hypertrophy model, *Activin* β_A mRNA expression was elevated three weeks after aortic banding (C).

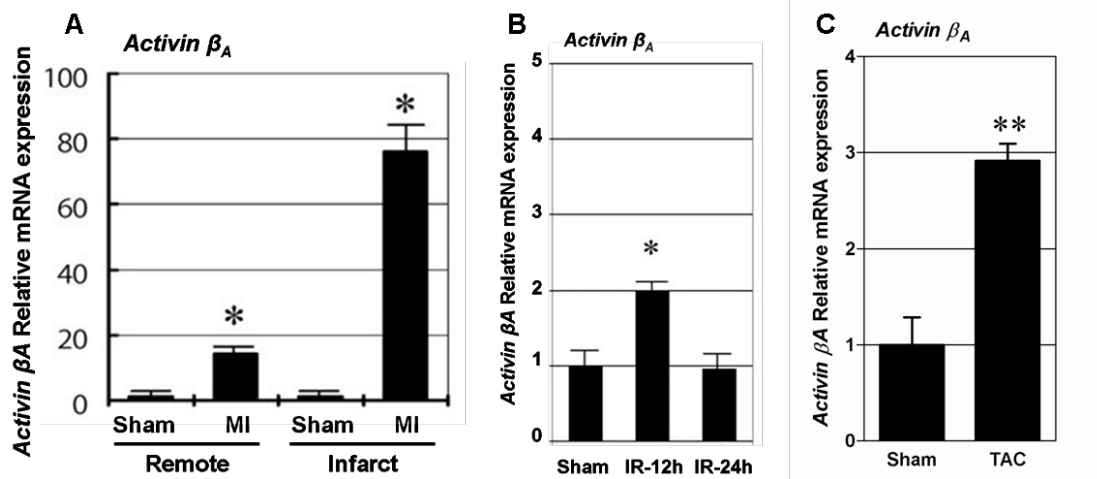


Figure 3.6: Elevated Activin β_A mRNA expression in different models of cardiac injury. Expression of *Activin β_A* mRNA was examined by quantitative real-time RT-PCR in cardiac samples from mice subjected to myocardial infarction (remote and infarct zone) at three days post-infarct (A), ischemia-reperfusion at 12 h and 24 h reperfusion (B) and trans-aortic banding at three weeks after banding (C), as well as ‘sham’ operated mice at respective time-points. Data are presented as relative expression of *Activin β_A* , expressed as mean $\pm$ SEM and normalised to endogenous GAPDH (A, B) or 18S rRNA (C) expression. Unpaired t-tests were used to check statistical significance. * $p < 0.05$, ** $p < 0.005$ compared to sham. $n = 3-6$.

3.6 Regulation of *Fstl3* expression by hypertrophic stimuli in the heart

Expression profile of FSTL3 from both heart failure patients as well as animal models of cardiac injury suggested that its expression may be regulated by hypertrophic stimuli in the heart. In order to test this hypothesis, expression of *Fstl3* mRNA was examined in neonatal rat cardiomyocytes in response to various pro-hypertrophic stimuli. As shown in Figure 3.7, the expression of *Fstl3* mRNA was elevated in response to TGF- β_1 , a hypertrophic [14, 15] and pro-fibrotic cytokine (A). Stimulation with phenylephrine (PE), isoproterenol, angiotensin II (AngII), cardiotrophin-I (CT-1) and endothelin-I (ET-1) did not have any effect on the expression of *Fstl3* mRNA in cardiomyocytes. However, stimulation of cardiomyocytes with TGF- β_1 led to a time-dependent increase in *Fstl3* mRNA expression (B).

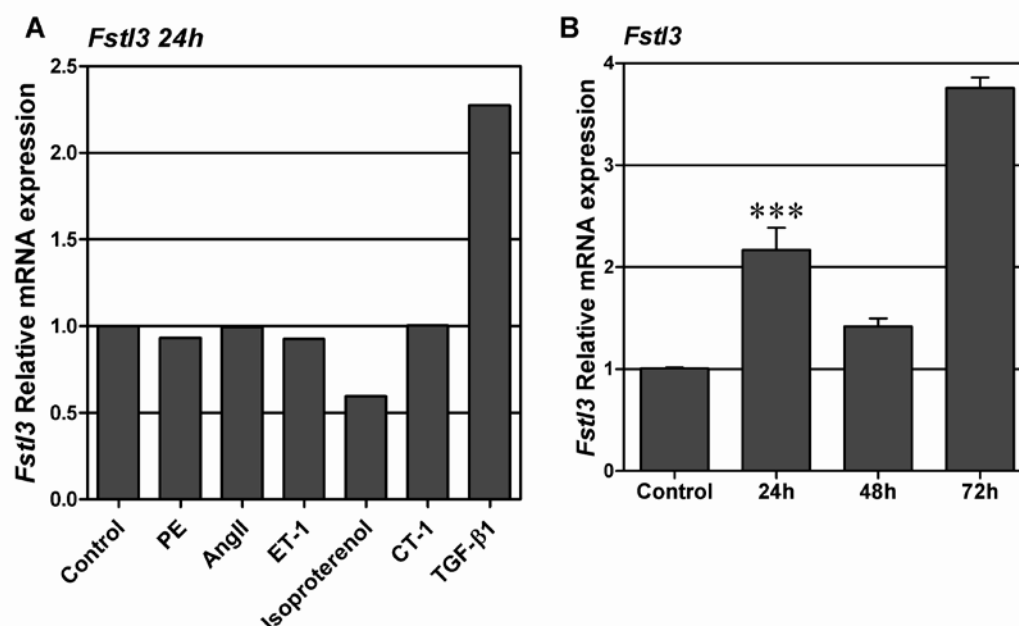


Figure 3.7: Expression of *Fstl3* mRNA is elevated by TGF-β1 in cardiomyocytes. Neonatal rat ventricular cardiomyocytes were cultured on fibronectin coated plates, serum-starved for 24h and treated with 100μM phenylephrine, 10μM angiotensin II, 1μM endothelin-I, 10μM isoproterenol, 1nM cardiotrophin-I or 10ng/ml TGF-β1 for different time periods as indicated. Expression of *Fstl3* mRNA was examined by quantitative real-time RT-PCR and normalised to endogenous 18S rRNA. (A) Expression of *Fstl3* mRNA at 24h post stimulation. n = 2. (B) Time course of *Fstl3* mRNA expression in cardiomyocytes stimulated with TGF-β1. One-way ANOVA was used to calculate the *p* value compared to control untreated samples. *** *p* < 0.001, n = 3-4.

3.7 Discussion

The activin - follistatin system is a complex regulatory network controlling the growth and development of many tissues. Follistatins are negative regulators of activin signalling and have been shown to be involved in tissue regeneration after injury in both the kidney and liver [16, 17], making them potential therapeutic targets. Follistatin was also shown to increase skeletal muscle mass and strength in dystrophic mice [342]. Follistatin-like 3 is a potent inhibitor of activin signalling with highest expression found in the placenta, testes, heart and pancreas [302]. Aberrant expression of FSTL3 has been reported in ovarian endometriosis [20], intrauterine growth restriction [324] and pre-

eclampsia [323]. In the failing heart, the expression of FSTL3 was highly elevated while that of FST remained unchanged. The expression of FSTL3 in heart failure samples was also found to correlate positively with α -skeletal actin and BNP, both markers of disease severity [328]. In heart failure recovery patients, the expression of FSTL3 after recovery was comparable to control samples, suggesting that FSTL3 may be used as a marker of heart failure. Immunostaining identified FSTL3 to be mainly expressed in the cardiomyocytes and endothelium in failing hearts and also in the nuclei of cardiomyocytes, consistent with previous reports of nuclear localisation of Fstl3 [302]. This is also consistent with the microarray analysis of FSTL3 expression where correlation was found to be highest with genes in the nuclear compartment and FSTL3 was associated with genes involved in transcription regulation and cell signalling.

Interestingly, the expression of *Fstl3* was found to be elevated in response to TGF- β 1 in cardiac myocytes. This is consistent with previous findings where TGF- β 1 was found to increase the expression of Fstl3 in bone marrow stromal cells [343] and HepG2 cells via Smad proteins [305]. However, FSTL3 is known to bind activin and myostatin [300], both TGF- β superfamily ligands and may be involved in restricting the freely available amounts of these proteins. Indeed, a whole body *Fstl3* knock-out mouse model was found to have altered visceral fat deposition attributed to increased myostatin action. These mice also had enhanced insulin sensitivity and glucose tolerance resulting from increased activin bioactivity in the pancreas [317]. Myostatin is known to regulate cardiac growth response and myostatin null mice show increased cardiomyocyte growth in response to hypertrophic stimuli [344]. Similarly, cardiac-specific over-expression of myostatin was found to have a negative impact on heart and skeletal muscle weights [345]. In the failing heart, increased expression of FSTL3 may negatively regulate myostatin and activin signalling. As shown above, in patients with heart failure, the expression of activin was found to be unchanged compared to normal control patients. This is in contrast to the elevated level of serum activin reported in a cohort of patients with heart failure caused by coronary artery disease or dilated cardiomyopathy [7]. However, the myocardial expression of activin was not examined in the study. The differences observed may also be due to the underlying nature of the disease *i.e.* dilated cardiomyopathy against coronary artery disease in which expression may be modulated by ischemia. As shown above, in different animal models of cardiac stress, the expression of both activin A and *Fstl3* was elevated immediately after injury. However,

it is possible that activin mRNA levels are reduced as the cardiac injury progresses to heart failure, as seen in end-stage heart failure patients. Recently it was reported that in the myocardial ischemia model, *Fstl3* functions to counteract the activin A-mediated cardioprotection against ischemic injury [294]. Similarly, in patients with angina, activin A was shown to have an anti-inflammatory effect, and dysregulated expression of activin and its receptors was suggested to be involved in the pathogenesis of coronary syndromes [346]. In this context, regulation of activin and related TGF- β family ligands by FSTL3 may have an impact on disease outcome and this signalling axis represents a new therapeutic target for cardiac diseases.

CHAPTER 4: Cardiac hypertrophy in *Fstl3* KO mice

4.1 Introduction

Systemic hypertension and outflow tract obstruction often result in pressure-overload in the heart, leading to increased aortic pressure and afterload. This greatly increases the ventricular wall stress, gradually leading to compensatory cardiac hypertrophy, which develops in order to preserve cardiac function under pressure-overload [6]. However, sustained pressure overload in the myocardium progressively leads to ventricular dilation [7] as cardiac hypertrophy by itself cannot reduce the afterload mismatch and cardiac contractility and output is adversely affected [347]. This is accompanied by various molecular, cellular and neurohormonal changes, together known as cardiac remodelling. Evidence presented in Chapter 3 illustrates the expression pattern of *Fstl3* in response to cardiac stress. Considering the increased expression of *FSTL3* in end-stage heart failure patients as well as in mouse models of cardiac hypertrophy and ischemic injury, we hypothesised that knock-out (KO) of *Fstl3* in the heart may be protective against cardiac hypertrophy. To test this hypothesis, trans-aortic constriction (TAC) or aortic banding was chosen as a model of pressure overload leading to cardiac hypertrophy.

In order to examine the *in vivo* role of *Fstl3*, cardiomyocyte-specific *Fstl3* KO mice were created by crossing floxed (LoxP - exon 3, 4, 5 – LoxP) *Fstl3* mice with α -MHC *Cre* mice (section 2.21). They were subjected to TAC or ‘sham’ surgery and analysed three weeks later. Both physiological and functional characteristics of the myocardium were analysed. Molecular markers of cardiac hypertrophy and heart failure were also measured. Data presented in this chapter will answer the question whether *Fstl3* is necessary for the development of cardiac hypertrophy.

4.2 Baseline characteristics of cardiac-specific *Fstl3* KO mice

In order to determine whether knock-out of *Fstl3* specifically in cardiomyocytes has an effect on cardiac structure or function, gravimetric analysis and echocardiography was performed on 6-8 week old male *Fstl3* KO mice and WT littermate controls. As shown in Figure 4.1, the body weight (A) or heart weight/body weight ratio (B) did not differ

between the KO and WT mice. Echocardiography revealed no differences in the % Fractional Shortening (C) or % Ejection Fraction (D) in the KO and WT mice. Thus, knock-out of *Fstl3* from cardiomyocytes did not affect the structure or function of the uninjured myocardium.

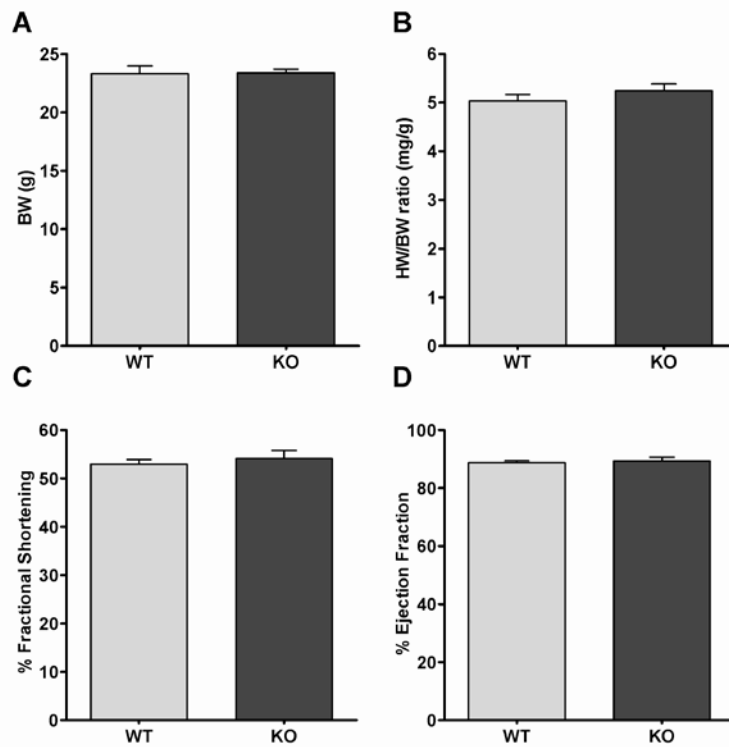


Figure 4.1: Similar baseline cardiac size and function of wild type and cardiac-specific *Fstl3* KO mice. (A) Body weight (BW) and (B) heart weight/body weight ratio (HW/BW) of 6-8 week old *Fstl3* KO mice and WT littermate controls was measured. Echocardiography was performed using an Acuson Sequoia C-256 machine with a 15-MHz probe on conscious mice and % Fractional Shortening (C) and % Ejection Fraction (D) were calculated from M-mode echocardiograms. Data are presented as mean ± SEM. n = 4-8.

4.3 Analysis of *Fstl3* expression post-injury in cardiomyocyte-specific *Fstl3* KO mice

In patients with heart failure, FSTL3 expression was mainly localised in the cardiomyocytes and endothelium (Figure 3.3). As shown in section 3.5, the expression of *Fstl3* was found to be highly elevated after cardiac injury and it was unclear if the expression was increased in any particular cell type in the myocardium. As such, knock-

out of *Fstl3* from cardiomyocytes may be functionally compensated for after injury by increased expression in any other cells such as endothelial cells or fibroblasts, making the cardiomyocyte-specific KO model ineffective. We tested this possibility by examining the expression of *Fstl3* mRNA in both KO and wild-type (WT) littermate control mice after ‘sham’ operation or TAC. As shown in Figure 4.2, the expression of *Fstl3* was elevated in WT mice after TAC compared to sham. However, in the cardiomyocyte-specific KO mice, *Fstl3* expression remained unchanged after TAC. The difference in the KO group between ‘sham’ and TAC was not statistically significant. Thus, cardiomyocytes are the main source of increased *Fstl3* in the myocardium post injury.

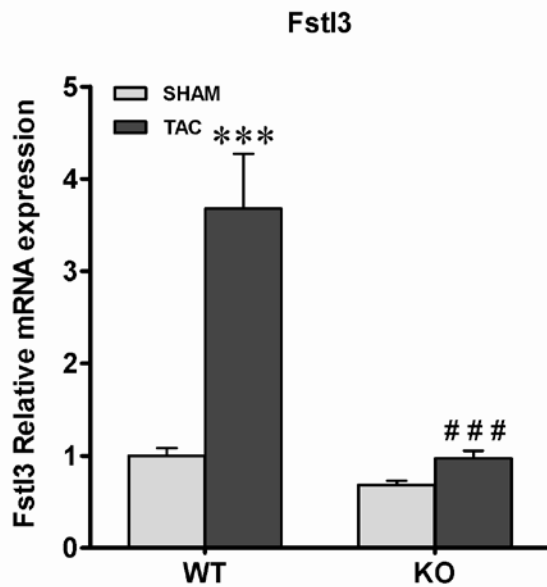


Figure 4.2: Normalised expression of *Fstl3* in KO mice after injury. Cardiomyocyte-specific *Fstl3* KO mice and WT littermate controls were subjected to trans-aortic constriction or ‘sham’ surgery to simulate pressure-overload induced cardiac hypertrophy. Three weeks later, myocardial expression of *Fstl3* was measured by quantitative real-time PCR. Two-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. Data are presented as mean ± SEM. *** $p < 0.001$ compared to WT SHAM. ### $p < 0.001$ compared to WT TAC. $n = 3-6$.

In order to determine if loss of *Fstl3*, an extracellular inhibitor of Activin A, had any effect on the expression of Activin in the heart, quantitative real-time RT-PCR was carried out on myocardial samples from WT and *Fstl3* KO mice. As shown in Figure 4.3 (A), the mRNA expression of Activin β_A increased in response to TAC. However,

no differences were observed between WT and *Fstl3* KO mice. Similarly, the serum levels of Activin A were also elevated following TAC and no differences were observed between the WT and *Fstl3* KO mice, either under ‘sham’ or TAC conditions (panel B).

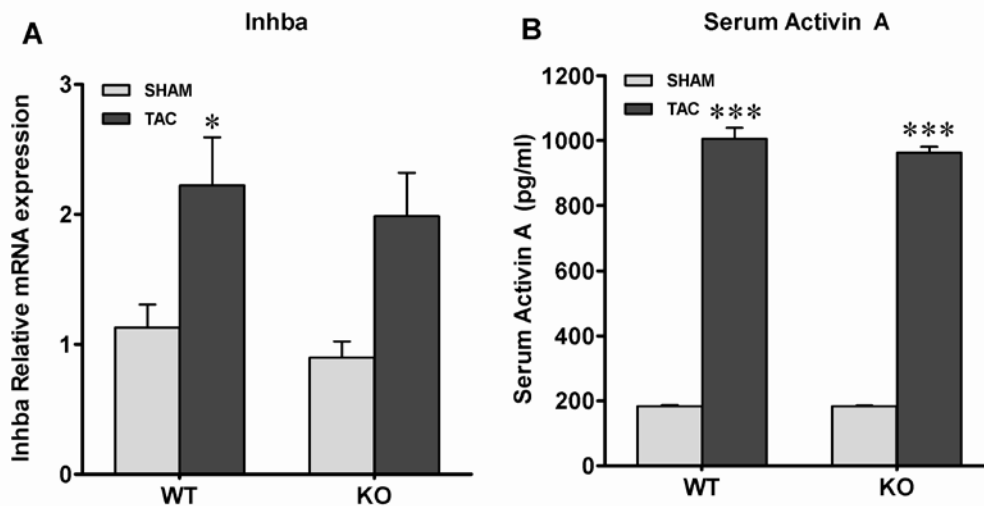


Figure 4.3: Myocardial expression of Activin A in WT and *Fstl3* KO mice is unchanged. (A) Three weeks after TAC or ‘sham’, the expression of Activin A (Inhibin β_A chains) was analysed by qRT-PCR. Data are presented as relative expression (mean $\pm$ SEM) of Activin A normalised to endogenous 18S rRNA. (B) Serum levels of Activin A were measured in WT and *Fstl3* KO mice 3 weeks after ‘sham’ or TAC using ELISA. Two-way ANOVA followed by Bonferroni post-test was used to calculate statistical significance. Data are presented as mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$ compared to WT SHAM. $n = 3-6$.

4.4 Analysis of cardiac hypertrophy in *Fstl3* KO mice after TAC

In order to examine the effect of *Fstl3* deficiency on the development of cardiac hypertrophy, KO and WT mice were subjected to trans-aortic constriction – a common model of pressure-overload induced cardiac hypertrophy (Section 2.22). Three weeks after ‘sham’ or ‘TAC’, mice were sacrificed and their hearts were analysed. As shown in Figure 4.4 (A), heart weight/body weight (HW/BW) ratio was increased on average from 5.1 to 9.3 after TAC in WT mice thereby confirming cardiac hypertrophy. Although KO mice developed some degree of hypertrophy, their average HW/BW ratio (6.8) was significantly lower than that of WT mice after TAC, suggesting that *Fstl3* is necessary for the full development of cardiac hypertrophy. To confirm these findings,

we determined the lung weight/body weight ratio (LW/BW) as a measure of pulmonary oedema – a marker of heart failure. As shown in Figure 4.4 (B), KO mice showed reduced LW/BW ratio compared to WT mice after TAC, although the values did not reach statistical significance.

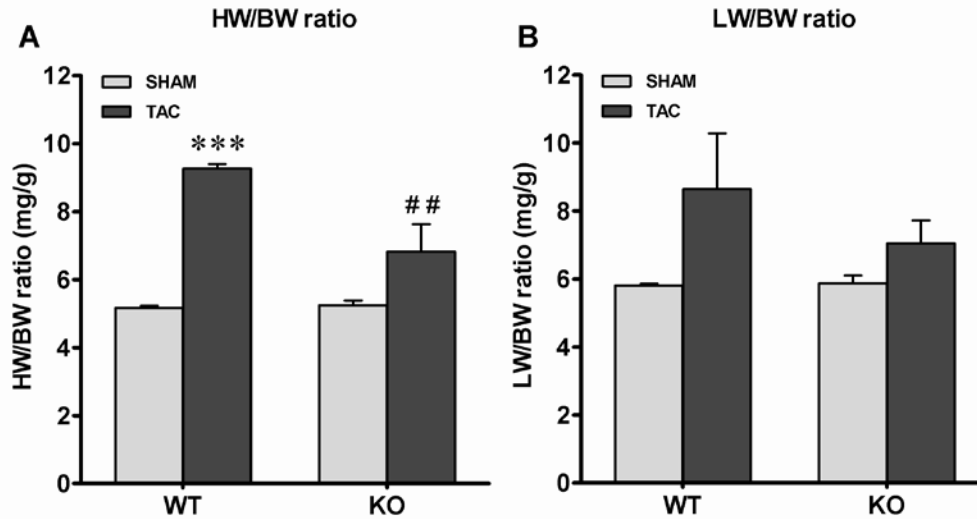


Figure 4.4: Reduced HW/BW and LW/BW ratio in *Fstl3* KO mice after TAC. *Fstl3* KO mice and WT littermate controls were subjected to TAC or ‘sham’ and HW/BW and LW/BW ratios were measured after 3 weeks. Two-way ANOVA followed by Bonferroni post-test was used to calculate the *p* values. Data are presented as mean $\pm$ SEM. *** *p* < 0.001 compared to WT SHAM; ## *p* < 0.01 compared to WT TAC. *n* = 3.

To confirm these results at a histological level, sections of myocardium from three WT and *Fstl3* KO mice per group, subjected to TAC or ‘sham’ were stained with α -sarcomeric actin and wheat-germ agglutinin (WGA) to determine the cell surface area. As shown in Figure 4.5 (B), average cardiomyocyte area increased in response to TAC in WT mice from 300 μm^2 to 495 μm^2 . This increase was significantly smaller in *Fstl3* KO mice, which only reached an average of 422 μm^2 after TAC. Taken together, these results confirm that the loss of *Fstl3* results in attenuated cardiac hypertrophy after TAC.

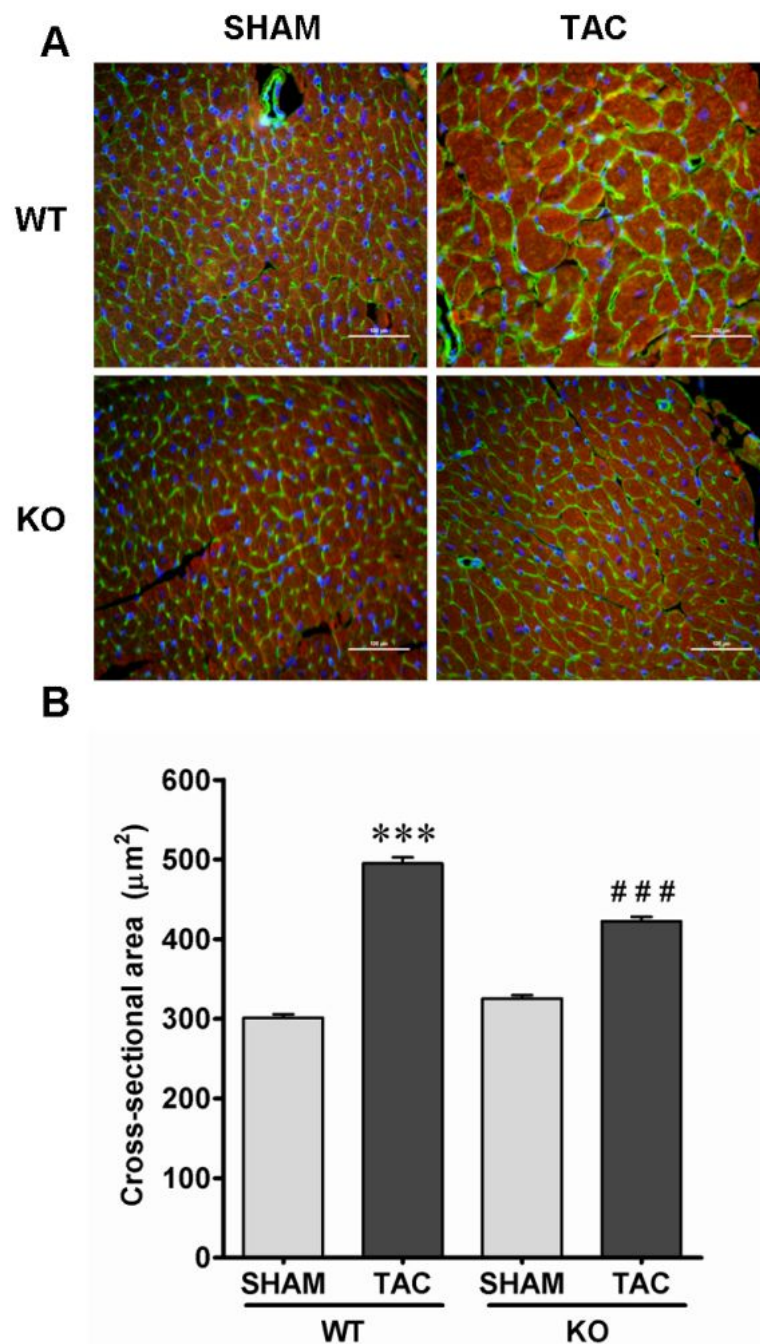


Figure 4.5: Reduced cardiomyocyte cross-sectional area in *Fstl3* KO mice after TAC. (A) Representative fluorescent micrographs of myocardial sections from WT and *Fstl3* KO mice are shown. Sections of myocardium were stained for α -sarcomeric actin (red), DAPI (blue) and wheat-germ agglutinin (green). Bar = 100 μm . (B) A total of 225 cross-sectional areas (CSA) per sample were measured from fluorescent micrographs using NISelements software. Quantification of CSA is shown with mean $\pm$ SEM. Two-way ANOVA followed by Bonferroni post-test was used to calculate statistical significance. Data are presented as mean $\pm$ SEM. *** $p < 0.001$ vs. WT SHAM; ### $p < 0.001$ vs. WT TAC. $n = 3$.

4.5 Expression of markers of cardiac hypertrophy after TAC

Re-expression of the fetal gene programme is a hallmark of cardiac hypertrophy [25, 348]. To determine whether *Fstl3* was necessary for the onset of this programme, the mRNA expression of fetal-specific genes β -myosin heavy chain (β -MHC or *Myh7*), α -skeletal actin (*Acta1*), ANP and BNP was examined in WT and *Fstl3* KO mice 3 weeks after aortic banding. As shown in Figure 4.6, aortic banding resulted in increased myocardial mRNA expression of ANP, BNP, *Acta1* and β -MHC in WT mice. However, the expression of these markers was strongly attenuated in *Fstl3* KO mice after banding, to levels comparable to sham operated mice, thereby indicating the necessity of *Fstl3* for induction of the fetal gene programme. The expression of BNP and β -MHC remained significantly elevated in the KO mice following TAC as compared to ‘sham’.

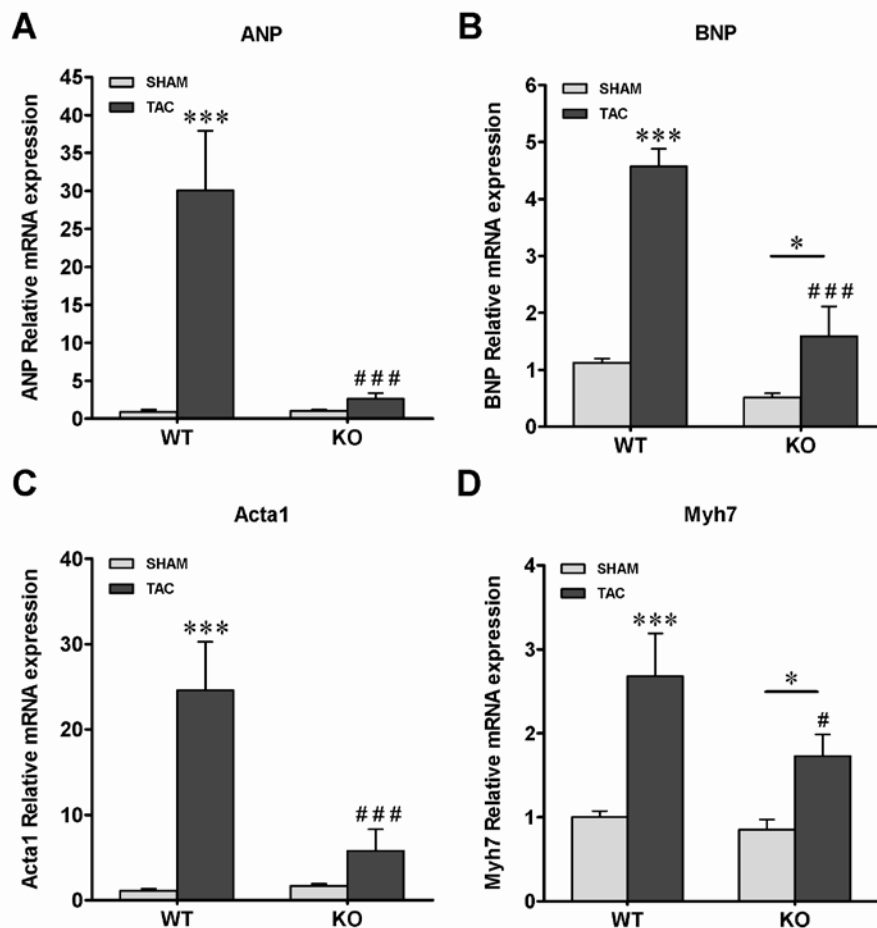


Figure 4.6: Reduced expression of hypertrophic markers in *Fstl3* KO mice after banding. *Fstl3* KO mice and WT littermate controls were subjected to trans-aortic constriction or ‘sham’ surgery. Three weeks later, mice were sacrificed and myocardial expression of ANP, BNP, *Acta1* and *Myh7* was analyzed by qRT-PCR and normalised

to endogenous 18S rRNA. Two-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. KO SHAM, *** $p < 0.001$ vs. WT SHAM, # $p < 0.05$, # # # $p < 0.001$ vs. WT TAC. n = 3-6.

4.6 Cardiac functional studies on *Fstl3* KO mice after TAC

To assess cardiac function after pressure-overload induced hypertrophy, WT and *Fstl3* KO mice were subjected to echocardiography (section 2.23). M-mode images were taken and percent fractional shortening and ejection fraction were calculated. As shown in Figure 4.7 (A), aortic banding resulted in a reduction of fractional shortening from 55% to 32%, indicating decreased cardiac contractility. Similarly, ejection fraction was also lowered from 90% to 67% in WT mice (panel B). In contrast, no significant reduction of fractional shortening or ejection fraction was observed in *Fstl3* KO mice after TAC compared to ‘sham’- operated mice. Compared to WT, KO mice showed significantly improved cardiac function following TAC.

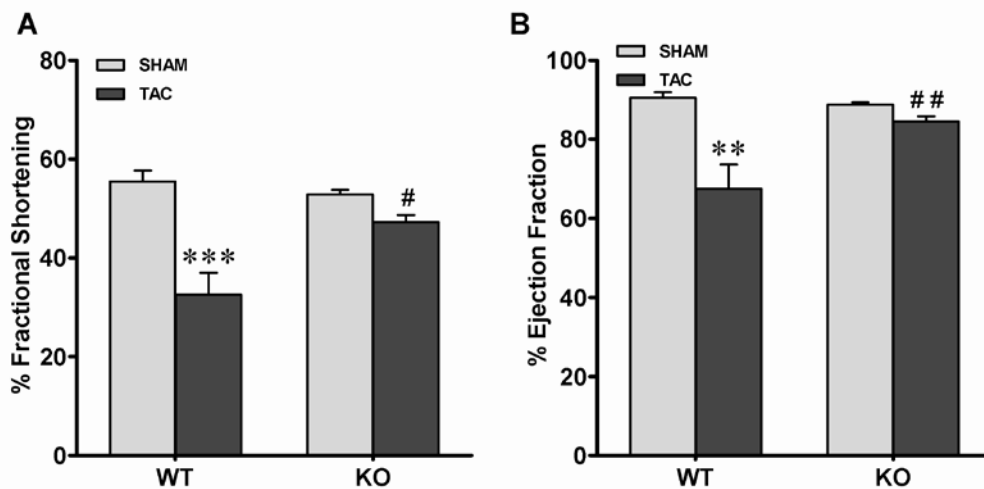


Figure 4.7: Improved cardiac function in *Fstl3* KO mice after TAC. *Fstl3* KO mice and WT littermate controls were subjected to trans-aortic constriction or ‘sham’ surgery and echocardiography was performed three weeks later. Percent fractional shortening and ejection fraction were calculated using measurements taken in M-mode. Two-way ANOVA was used to calculate the p values followed by Bonferonni post-tests to compare groups. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ vs. WT SHAM. # $p < 0.05$, # # $p < 0.01$ vs. WT TAC. n = 3.

4.7 Metabolic characteristics of *Fstl3* KO mice

Reactivation of the fetal gene programme in cardiac hypertrophy and heart failure is accompanied by a switch to fetal myocardial energy substrate preference - increased glucose utilisation and reduced fatty acid oxidation (FAO) [178-180]. Reduced hypertrophy and improved cardiac contractility in *Fstl3* KO mice following pressure-overload could be, at least in part, explained by improved metabolic responses. In order to test this hypothesis, mRNA expression of genes involved in regulating fatty acid uptake and oxidation was examined. These included nuclear receptor PPAR α , its target genes MCAD and mCPT1 (CPT1 β), as well as PPAR α coactivator PGC-1 α . As shown in Figure 4.8, the mRNA expression of PPAR α did not differ between WT and KO mice under 'sham' operated conditions (A). As the myocardium switches to increased glycolysis after pressure overload, the expression of PPAR α is downregulated significantly in WT mice. However, in the *Fstl3* KO mice, PPAR α expression after TAC was found to be comparable to 'sham' levels, indicating sustained fatty acid oxidation. Similarly, the expression of PPAR α target genes CPT1 β and MCAD and the PPAR α co-activator PGC-1 α in the myocardium were reduced in WT mice after TAC while remaining unchanged in KO mice (B-D). Overall, these results suggest that in *Fstl3* KO mice, fatty acid oxidation remains the dominant source of energy after TAC and the myocardium does not revert to the fetal metabolic gene program.

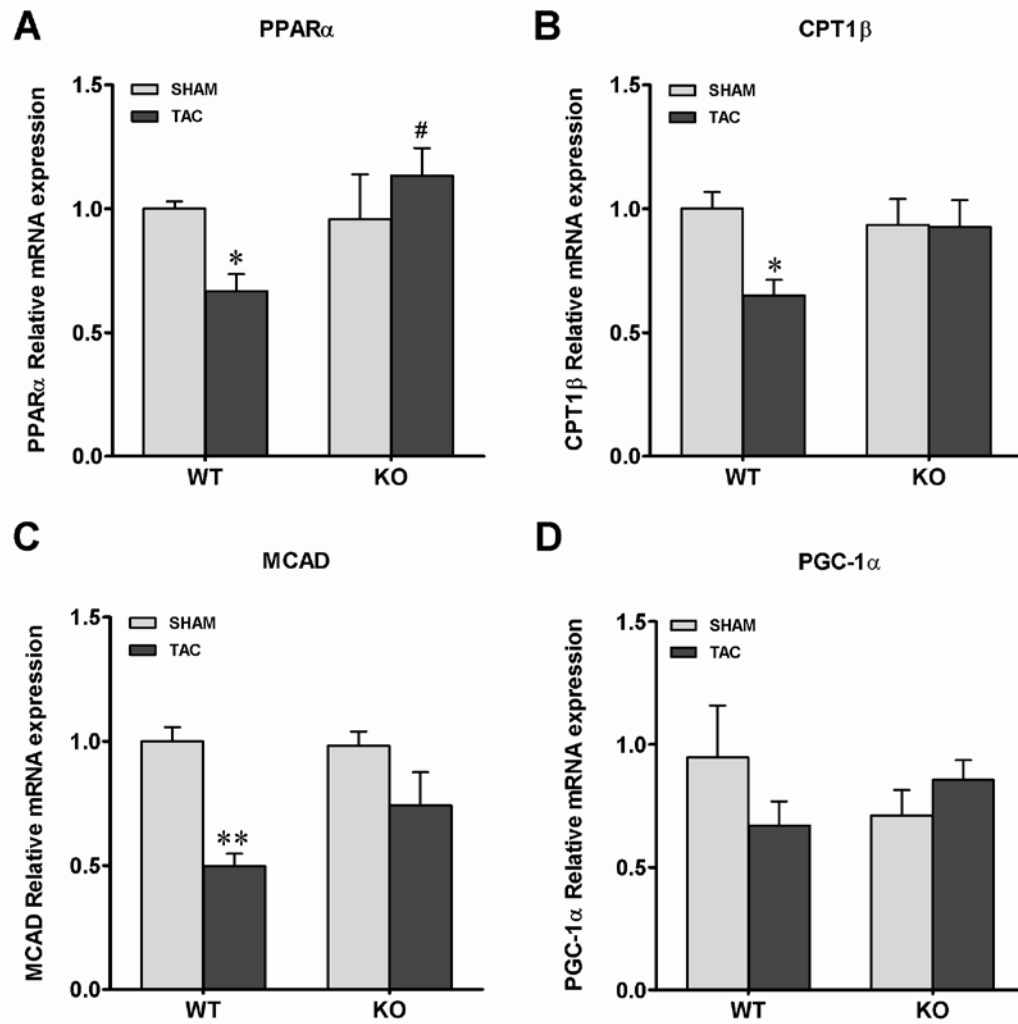


Figure 4.8: Normalised expression of fatty acid oxidation markers in *Fstl3* KO mice after TAC. *Fstl3* KO mice and WT littermate controls were subjected to trans-aortic constriction or ‘sham’ surgery. Expression of PPAR α , CPT1 β , MCAD and PGC-1 α was analyzed by quantitative real-time RT-PCR and normalised to endogenous 18S rRNA. Two-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. Data are presented as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ vs. WT SHAM; # $p < 0.05$ compared to WT TAC. n = 3-6.

4.8 Discussion

This chapter examined the effect of *Fstl3* ablation from cardiomyocytes on the cardiac structure and function following pressure-overload. The results presented here demonstrate the necessity of *Fstl3* for the full extent of hypertrophic responses of the injured heart leading to heart failure.

We and others have previously reported that the expression of Fstl3 is elevated in response to myocardial ischemia, pressure-overload induced hypertrophy, and heavy resistance training as well as in patients with heart failure [294, 328, 329]. However, the cellular source of this increased myocardial Fstl3 expression was not known. *Fstl3* KO in cardiomyocytes eliminated the increase in Fstl3 levels in the myocardium after aortic banding, thereby indicating that cardiomyocytes are the primary source of elevated Fstl3 levels after injury. This is in contrast with the observations in heart failure patients where Fstl3 was also detected in the vascular endothelial cells. Interestingly, 2 month old cardiac-specific *Fstl3* KO mice had normal cardiac function in the absence of injury, indicating that cardiomyocyte-derived Fstl3 does not play a significant role in adult cardiac homeostasis. This is in apparent disagreement with the findings of the study where whole-body *Fstl3* KO mice were reported to have mild cardiac hypertension by 9 months of age, possibly leading to the observed cardiac hypertrophy [317]. These differences may be attributed to the global effects of *Fstl3* knock-out from the body, which resulted in altered metabolic homeostasis in these mice due to increased bio-availability of Activin and Myostatin, as well as the differences in the age at which the mice were examined in the respective studies.

The balance of TGF- β family ligands and their inhibitors is important in determining the bio-availability of these cytokines in different tissues. Indeed, previous studies have shown that Activin A plays a cardioprotective role against ischemic injury and over-expression of Fstl3, an extracellular Activin A inhibitor, resulted in loss of activin-mediated cardioprotection [294]. In this study, it was observed that cardiac-specific *Fstl3* KO did not affect either the mRNA or protein levels of Activin A with or without injury, suggesting an activin-independent role for Fstl3 in the pressure-overloaded heart.

In the absence of Fstl3 from cardiomyocytes, mice displayed attenuated hypertrophy demonstrated by reduced heart weight/body weight ratio and cardiomyocyte surface area. They also showed improved cardiac contractility and reduced lung weight/body weight ratio indicating reduced pulmonary congestion. Thus, Fstl3 deficiency in cardiomyocytes was protective against pressure-overload induced cardiac hypertrophy and failure. Consistent with this observation, it was previously shown that cardiac-specific *Fstl3* KO mice display reduced infarct size upon ischemia/reperfusion injury

[294]. Thus *Fstl3* is necessary for the development of heart failure in response to different stimuli such as ischemia and pressure overload. Combined with the observation of increased *Fstl3* in patients with deteriorating heart failure and normalisation of its expression following recovery, it follows that *Fstl3* is an important mediator of the pathological responses to a number of underlying cardiovascular conditions progressing to heart failure.

At the molecular level, cardiac dysfunction following pressure-overload induced hypertrophy is known to be triggered by the activation of pathological signalling pathways and is characterised by reactivation of the fetal gene program [24]. In particular, both ANF and BNP are recognised as markers of pathological hypertrophy and their expression levels have useful prognostic rationale towards disease severity [171]. Reduced expression of ANF and BNP in *Fstl3* KO mice suggests that these mice do not develop heart failure following pressure overload. Under conditions of cardiac stress, contractile proteins also undergo an isoform switch from the fast α -MHC to slow β -MHC, thereby altering the α -MHC/ β -MHC ratio [26], which can dramatically affect cardiac contractility and output. In fact, constitutive over-expression of β -MHC in mouse hearts was found to be sufficient to reduce systolic function [174]. Similarly, increased expression of α -skeletal actin has been reported following pressure overload at the compensated hypertrophic phase where cardiac contractility is maintained in response to increased pressure [176]. This has been shown to generate enough force of contraction despite the mechanical load and without affecting cell shortening. However, cardiomyocytes are unable to maintain the increased levels of α -skeletal actin in chronic pressure overload and this may be crucial in the transition from compensated hypertrophy to decompensated heart failure [177]. Thus, increased α -skeletal actin expression serves as a marker for decompensation and progressing to heart failure. In this regard, *Fstl3* KO mice displayed attenuated expression of α -skeletal actin and β -MHC, further strengthening the observation of improved contractility seen by echocardiography and consistent with resistance shown by these mice against progression to heart failure.

Improved cardiac function after TAC may also be seen as a result of improved metabolism which is necessary to support the maintenance of cardiac contractility. PPAR α expression is necessary for both basal and inducible expression of

mitochondrial and peroxisomal FAO enzymes [194, 349]. In response to pressure overload, the expression of PPAR α and its coactivator PGC-1 α is reduced ([191] and Fig. 4.7.1). Besides regulating metabolic processes, PPAR α has also been implicated in modulating cardiac hypertrophy directly. PPAR α agonists like fenofibrate have been shown to attenuate cardiomyocyte hypertrophy by inhibiting endothelin I-induced Akt and GSK3 β phosphorylation [350]. Similarly, PPAR α KO mice show augmented cardiac hypertrophy in response to pressure overload [351], further validating the anti-hypertrophic role of PPAR α in the heart. Preserved expression of PPAR α , PGC-1 α and PPAR α target genes CPT1 β and MCAD was observed in *Fstl3* KO mice following TAC, suggesting that they are protected from progression to pathological hypertrophy and heart failure and that FAO remains the major source of energy. These results suggest that preserved energy status in *Fstl3* KO mice may be an important contributing factor towards improved cardiac function. However, more sophisticated metabolic characterisation of *Fstl3* KO mice is needed to understand the exact role played by it in alteration of energy consumption.

In summary, results presented in this chapter have identified an essential role for *Fstl3* in the development of hypertrophy and progression to heart failure. *Fstl3* is also necessary for the initiation of the fetal gene program and change in cytoskeletal contractile proteins. In the absence of *Fstl3*, myocardial energy metabolism was also maintained in response to pressure overload, further diminishing the hypertrophic responses. Accordingly, *Fstl3* may be an important player in delineating the compensated vs. decompensated stage of heart failure. It is also noteworthy that lack of *Fstl3* did not affect the myocardial or systemic levels of activin A, thereby making it an attractive candidate for therapeutic intervention.

Thus, *Fstl3* action on cardiac myocytes may be necessary for induction of the hypertrophic program at the molecular level, thereby making it an essential factor in the progression of hypertrophy. *Fstl3* may act directly on cardiomyocytes to induce the fetal gene program and the resulting cellular changes affecting cardiac hypertrophy and contractility. Similarly, *Fstl3* may also be involved during the compensatory phase in the regulation of cardiac metabolism. Thus, loss of *Fstl3* from cardiomyocytes may be beneficial in terms of reducing the continued expression of fetal genes following the compensatory phase. This may prevent progression of cardiac hypertrophy to heart

failure whilst maintaining a compensated state. It may also provide the necessary energy support in order to maintain cardiac contractility, thereby resulting in improved cardiac function. Thus loss of Fstl3 from the myocardium may be beneficial by preventing pathological hypertrophy in response to pressure overload.

CHAPTER 5: Fstl3 as a pro-hypertrophic factor in the myocardium

5.1 Introduction

Microarray analysis of heart failure patients (Section 3.4) suggested a correlation of FSTL3 with genes involved in cellular signalling and the nuclear compartment. Similarly, data presented in Chapter 4 substantiate the vital role of Fstl3 in the mouse heart for the development of hypertrophy in response to pressure overload. While it is clear that cardiomyocytes are the prominent source of increased Fstl3 due to cardiac injury, it remains unclear whether this has any effect on the cardiomyocytes themselves. Therefore, the aim of this chapter is to investigate whether Fstl3 promoted hypertrophic signalling in cardiomyocytes. In order to test this, neonatal rat ventricular cardiomyocytes (NRVM) were chosen as a model system as they display many characteristics of the *in vivo* heart in response to hypertrophic stimuli [24].

5.2 Effect of Fstl3 on cardiomyocyte hypertrophy

Hypertrophy of cardiomyocytes is often measured by increase in cell size and protein synthesis. To test the effect of Fstl3 on NRVM growth, cardiomyocytes were stimulated with recombinant Fstl3 and/or the α 1-adrenergic receptor agonist phenylephrine (PE) as a positive control and protein synthesis was measured by radioactive [³H] Leucine incorporation normalised to total cell number calculated by LDH released upon cell lysis. As shown in Figure 5.1, treatment with Fstl3 for 24 h increased protein synthesis by about 10% compared to control untreated cells. However, the difference did not reach statistical significance. Treatment with PE led to a significant increase in protein synthesis (20%). Interestingly, treatment with Fstl3 and PE together augmented the hypertrophic response and increased protein synthesis compared to cells treated with PE alone.

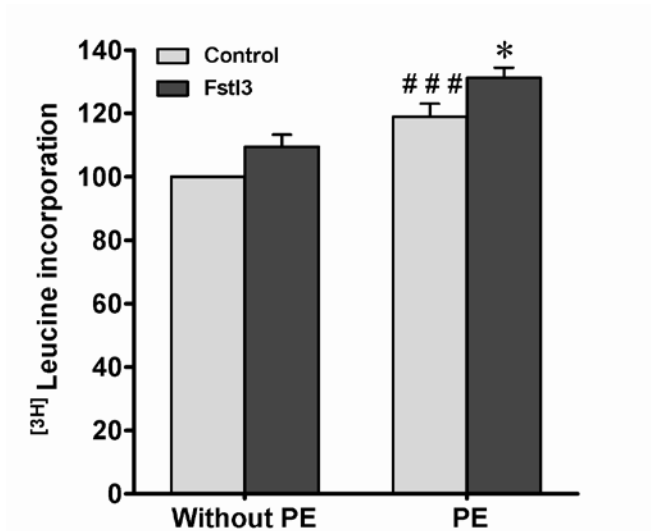


Figure 5.1: Fstl3 induces increased protein synthesis in cardiomyocytes. Neonatal rat cardiomyocytes were treated with 200 ng/ml Fstl3 and/or 100 μ M phenylephrine (PE) for 24 h and [3 H] Leucine incorporation was measured by scintillation counting normalised to total cell number. Data are presented as mean percent $\pm$ SEM of control untreated cells. Two-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. * $p < 0.05$ for control vs. Fstl3 in the presence of PE. # # # $p < 0.001$ for control vs. PE. n = 3-6.

To test whether cardiomyocyte overexpression of Fstl3 may have a similar effect on cell growth, NRVMs were transiently transfected with an expression vector for Fstl3 (also expressing GFP) or control vector (GFP alone), and stimulated with PE. Hypertrophy was measured by immunostaining and further calculation of the cell surface area of untransfected cells identified by absence of GFP. Transfected cells were excluded from the analysis as the aim was to examine the effect of secreted Fstl3 on neighbouring cells. Representative fluorescent micrographs are shown in Figure 5.2, panel A. Quantification of surface areas is shown in panel B. In the presence of Fstl3, cardiomyocyte surface area increased from an average 570 μm^2 to 820 μm^2 , an increase of 44%. PE increased the average surface area to 1100 μm^2 while the combination of PE and Fstl3 increased it to more than 1200 μm^2 . This cumulative effect of Fstl3 and PE reflects an additive response of their hypertrophic signalling pathways.

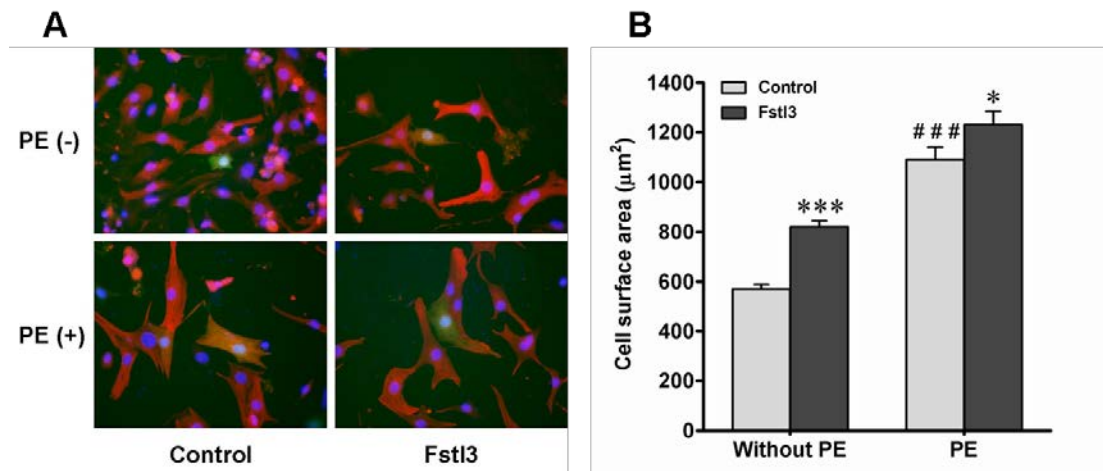


Figure 5.2: Fstl3 is sufficient to increase cardiomyocyte surface area. Neonatal rat cardiomyocytes were transfected with an Fstl3 expression vector (also expressing GFP) or a control vector and treated with 100 μ M phenylephrine for 48 h. Cells were stained with cardiac troponin I (red) and DAPI (blue). (A) Representative fluorescent micrographs of cardiomyocytes. (B) Quantification of cell surface areas (60-150 cells per sample) presented as mean $\pm$ SEM. Two-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. * $p < 0.05$, *** $p < 0.001$ for control vs. Fstl3 in the presence and absence of PE respectively. ### $p < 0.001$ for PE compared to control. $n = 3$ independent experiments.

5.3 Effect of Fstl3 on cardiomyocyte signalling pathways

In order to explore the molecular mechanisms underlying Fstl3-induced hypertrophy and its modulation by activin A, NRVMs were treated for 15 minutes with 200 ng/ml recombinant Fstl3 and/or recombinant activin A and protein samples were analysed by western blot. Phosphorylation of MAPK pathways was examined, as they are widely implicated in hypertrophic signalling in cardiomyocytes [85, 352]. Activation of the Smad pathway was also examined as a major pathway mediating signals from the TGF- β family members [283]. As shown in Figure 5.3 (A, B and C), a trend was observed for Fstl3 mediated increase in the phosphorylation of MAPK p38, JNK1 and JNK2 compared to control untreated cells. Activin A also had a similar effect on phosphorylation of these proteins. However, the differences did not reach statistical significance. When Fstl3 and activin A were added together, phosphorylation of p38, JNK1 and JNK2 was unchanged. This may be due to the binding of Fstl3 to activin A in the medium which would limit the availability of each of these proteins to activate

downstream signalling cascades. Fstl3 did not have any effect on ERK phosphorylation (panel D).

Activin A triggered activation of the Smad signalling pathway in cardiomyocytes. This effect was blocked by Fstl3, which had a variable effect on Smad2 phosphorylation on its own. Neither activin A nor Fstl3 showed any effect on Smad3 phosphorylation. In summary, Fstl3 may activate the MAPK p38 and JNK pathways and diminish the effect of activin A on these targets and Smad2.

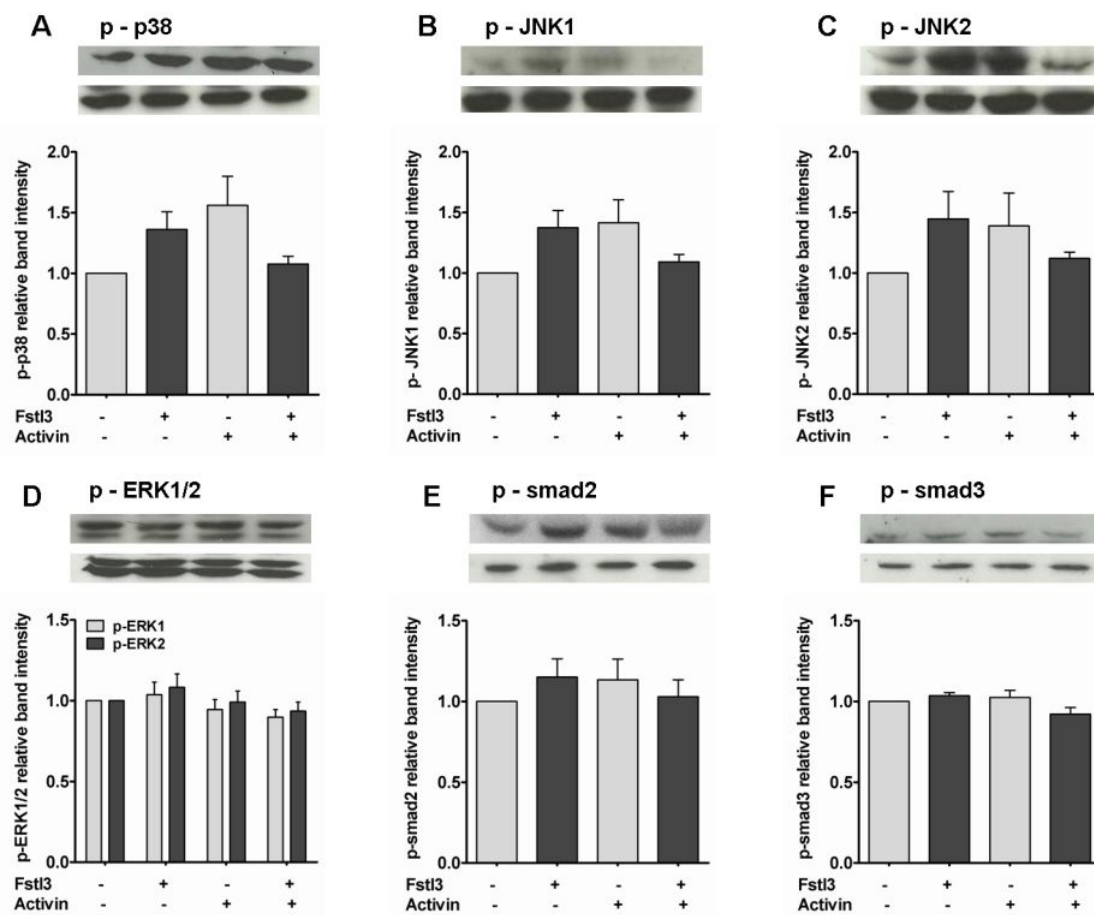


Figure 5.3: Fstl3 may activate two MAPK pathways in cardiomyocytes. Neonatal rat cardiomyocytes were treated with 200 ng/ml recombinant Fstl3 and/or 100 ng/ml activin A for 15 minutes and analysed by western blot. Representative blots are shown for phosphorylated p38, JNK1, JNK2, ERK1/2, Smad2 and Smad3. Phosphorylated proteins were normalised to total proteins respectively (lower blots). Quantification was performed using ImageJ software. Relative band intensity compared to control untreated samples is represented as mean $\pm$ SEM. n = 3-5.

5.4 Discussion

In vitro experiments on cardiomyocytes are often carried out to elucidate the specific mechanism of action of various molecules. This chapter presented evidence for the prohypertrophic effect of Fstl3 on cardiomyocytes and its activation of two MAPK pathways.

Phenylephrine, an α 1-adrenergic receptor agonist, is commonly used to stimulate cardiomyocyte hypertrophy [24]. It functions by activating the p38 and JNK pathways, amongst others, and was used as a positive control for measuring the cardiomyocyte hypertrophic response [69, 353]. Fstl3 increased hypertrophy of cardiomyocytes as measured by both elevated protein synthesis and surface area, although the prohypertrophic effect of Fstl3 was more evident on cell surface area than on protein synthesis. The difference in the effect of Fstl3 observed between the two methods may be explained by different concentration of Fstl3, possible post-translational modifications of the protein or the time after which hypertrophy was measured. In the experiments where Fstl3 expression vector was transfected in cardiomyocytes, cells were given 48 h to produce and secrete Fstl3, after which hypertrophy was measured by calculation of their surface area. It should be noted that this effect was caused by secreted Fstl3 from cardiomyocytes, as transfected cells (which also expressed GFP) were not included in the analysis. Interestingly, the hypertrophic effect of Fstl3 was augmented in the presence of PE and cardiomyocytes treated with both stimuli showed increased growth compared to PE or Fstl3 alone. Considering that stimulation of cardiomyocytes with PE did not increase the expression of Fstl3 (Chapter 3), these results suggest an additive effect of Fstl3 and PE on hypertrophic signalling pathways. Further investigation will be needed to determine whether both stimuli act through common or independent pathways.

A mechanistic insight into the effect of Fstl3 was obtained by stimulating cardiomyocytes and measuring the activation of different signalling pathways. Fstl3 was found to variably increase the phosphorylation of stress-activated MAPK proteins p38 and JNK1 and JNK2 – known mediators of cardiomyocyte hypertrophy in response to neuroendocrine stimuli and catecholamines [24, 85, 354, 355]. However, Fstl3 did not activate the ERK1/2 pathway. This pattern of activation of MAPK pathways is

interesting as only the ERK pathway has been established to play a causal role in cardiac hypertrophy *in vivo* [137]. Both p38 and JNK pathways are known to be involved in cardiomyocyte hypertrophy *in vitro* [30, 356]. However, they have been shown not to promote or play a role in cardiac hypertrophy *in vivo*, although they may be responsible for mediating a dilated cardiomyopathy phenotype [128, 132, 357-359]. Thus, possible activation of p38 and JNK pathways by Fstl3 is consistent with promoting cardiomyocyte hypertrophy *in vitro*. However, this may not necessarily be reflected in an *in vivo* mouse heart scenario.

The variable activation of MAPK proteins by Fstl3 following 15 minute stimulation of cardiomyocytes argues for a receptor-mediated action. However, to this day, a receptor for Fstl3 has not been identified. It is interesting to note that Fstl3, whose expression is elevated in response to TGF- β 1 in cardiomyocytes (Chapter 3), may stimulate cardiomyocyte hypertrophy *in vitro* through activation of p38 and JNK1/2 – the same proteins implicated in TGF- β 1 mediated cardiac hypertrophy via TAK-1 (TGF- β activated kinase) [251, 360, 361]. Thus Fstl3 may function to augment TGF- β -triggered signalling in cardiomyocytes or behave as a downstream effector through which TGF- β 1 mediates its hypertrophic action.

Interestingly, activin A was also found to increase phosphorylation of MAPK proteins p38 and JNK1/2. Activin-mediated phosphorylation of p38 has previously been described in other cells such as pancreatic cells, pituitary cells and breast cancer cells [362-364]. Furthermore, p38 activation was shown to be necessary for activin-mediated inhibition of growth of breast cancer cells [363]. Activation of these pathways was, however, diminished when activin A was combined with Fstl3, thereby ascertaining the inhibitory effect of either Fstl3 or activin A on each other. Previous studies have shown that Fstl3 inhibits activin A signalling by binding to it and preventing its binding to the activin receptors [309-311]. Similarly, in the heart, Fstl3 was shown to antagonise activin-mediated protection from cell death following ischemic injury [294]. However, the inhibitory effect of activin A on Fstl3 has not been described previously and could be an important therapeutic mechanism for inhibition of Fstl3 action in the myocardium. This apparent activation of MAPK signalling pathways by activin A and Fstl3 but inhibition in the presence of both may be attributed to either ligand sequestering, resulting in limited receptor binding and activation, or competitive binding to cell

surface receptors by Fstl3 and activin A. A similar response has been noted in previous studies where sFRP1, an extracellular inhibitor of Wnt signalling, was found to either inhibit or potentiate the response of Wingless (a *Drosophila* Wnt homologue) in a concentration and heparin dependent manner [365]. Similarly, Dkk2 was also found to inhibit or activate the Wnt/Lrp6 pathway depending on the presence of Kremen2, an additional Dkk receptor [366]. Accordingly, the interaction and downstream signalling of Fstl3 and activin A may be more complex than previously thought and one or more co-receptors or soluble factors might be involved in mediating the Fstl3 response in cardiomyocytes. This could add a further level of regulation on Fstl3/Activin A action in the injured myocardium where their expression levels are elevated (Chapter 3) but cardiomyocyte response to these could thus be limited.

Fstl3 diminished the effect of activin A on Smad2 phosphorylation, as reported previously [294]. The inhibitory effect of Fstl3 on Activin function may work as a negative feedback loop as activin A is known to regulate the expression of Fstl3 via Smad proteins in many tissues [306]. Interestingly, Fstl3-mediated activation of Smad pathway was sometimes observed although this could not be conclusively established. This is in contrast to a recent study where cardiac-specific Fstl3 knock-out mice were shown to have increased phosphorylation of Smad2 following pressure-overload in the heart, while no changes were observed in the expression of Smad1/5, Smad4 and Smad6 [367]. However, the expression of Smad7 – reported to be downregulated in the hypertrophied heart [368, 369]– was not examined. Smad7 is a direct inhibitor of Smad2 signalling [370] and thus the observed increase in Smad2 phosphorylation following TAC might be explained by Smad7 downregulation. Fstl3 may also be directly involved in regulating Smad7 expression, although further experiments are needed to test this hypothesis.

CHAPTER 6: Cardiac remodelling in *Fstl3* KO mice

6.1 Introduction

Chronic pressure overload and the resulting cardiac hypertrophy trigger disruption of the underlying cardiac structure, leading to a number of pathophysiological changes. These include cardiomyocyte death and changes in extracellular matrix (ECM) turnover and composition, and gradually lead to myocardial remodelling and heart failure [232, 262, 371, 372]. It has been recognised that the ECM plays a dynamic role in the adaptation of the heart to stress and regulates the process of remodelling [373-376]. In the ECM, fibrillar collagens I and III form the structural network necessary for maintaining cardiac structural integrity and function [377, 378]. Other components include the basement membrane, proteoglycans and glucosaminoglycans, as well as soluble proteins such as TGF- β [230]. The ECM structure is maintained by a balance between the synthesis and degradation of its constituents. Regulators of ECM turnover include a family of well conserved matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), the dynamic equilibrium between which controls the abundance of collagens, fibronectin and other ECM constituents [379-381].

This chapter examines the phenotype of *Fstl3* KO mice with respect to cardiac fibrosis in response to pressure overload. Microarray analysis comparing gene expression in the myocardium of *Fstl3* KO mice vs. WT mice in response to TAC is analyzed. Moreover, expression of various ECM components (collagens, fibronectin) as well as regulators and mediators of fibrosis is examined. Similarly, the expression of MMPs and TIMPs is also analyzed. Data presented in this chapter will answer the question whether knock-out of *Fstl3* from cardiomyocytes is sufficient to reduce myocardial fibrosis and remodelling in response to pressure overload.

6.2 Microarray analysis of *Fstl3* KO mice

In order to gain insights into the molecular mechanisms underlying the enhanced cardiac function following TAC in *Fstl3* KO mice, global transcription profile of the heart was analyzed using oligonucleotide microarrays, as described in section 2.6. Tables 6.1 A and B show the list of genes with reduced expression in the KO compared

to WT hearts three weeks after TAC (KO TAC < WT TAC) and a gene ontology (GO) analysis of this gene list respectively. Genes and GO for the following conditions are listed in Appendix IV:

1. WT TAC > WT SHAM
2. WT TAC < WT SHAM
3. KO TAC > KO SHAM
4. KO TAC < KO SHAM
5. KO SHAM > WT SHAM
6. KO SHAM < WT SHAM
7. KO TAC > WT TAC

Table 6.1 (A) KO TAC < WT TAC Representative Genes

Myocardial biopsies were taken from *Fstl3* KO and WT mice, three weeks after TAC and total RNA was hybridized on Mouse Gene 1.0 ST Array chips in triplicate and analyzed using GeneSpring X software suite. After scanning, raw data in each chip was normalized to the 50th percentile and to the median of the chip. Samples were further normalized on a gene per gene basis to the values obtained for sham operated WT hearts and gene lists were filtered for raw expression > 50. Fold change lists comparing two conditions were generated using a cut-off value of 1.4-fold.

<i>Affymetrix ID</i>	<i>Fold Reduction</i>	<i>Gene symbol</i>	<i>Description</i>
10398075	3.8514607	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N
10572897	3.2625666	Hmox1	heme oxygenase (decycling) 1
10458894	3.1543813	Lox	lysyl oxidase
10523717	2.8577793	Spp1	secreted phosphoprotein 1
10358476	2.8218665	Prg4	proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)
10492021	2.7843952	Postn	periostin, osteoblast specific factor
10462442	2.7407575	Il33	interleukin 33
10416181	2.6805735	Stc1	stanniocalcin 1
10598976	2.636716	Timp1	tissue inhibitor of metalloproteinase 1
10481627	2.4407265	Lcn2	lipocalin 2
10574023	2.3944807	Mt2	metallothionein 2
10355403	2.3532803	Fn1	fibronectin 1
10449452	2.1876445	Fkbp5	FK506 binding protein 5
10401527	2.1676607	Ltbp2	latent transforming growth factor beta binding protein 2
10513739	2.1075757	Tnc	tenascin C
10419934	2.0670037	Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta
10364494	2.0377984	Fstl3	folliculin-like 3
10440522	1.9075327	Adams1	a disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 1
10439009	1.8705704	Apod	apolipoprotein D
10376778	1.8641889	Mfap4	microfibrillar-associated protein 4
10557326	1.8601933	Il4ra	interleukin 4 receptor, alpha
10380419	1.8282303	Col1a1	collagen, type I, alpha 1
10440091	1.827271	Col8a1	collagen, type VIII, alpha 1
10424140	1.8161368	Col14a1	collagen, type XIV, alpha 1
10384223	1.7659196	Igfbp3	insulin-like growth factor binding protein 3
10435641	1.7086023	Fstl1	folliculin-like 1
10558961	1.7052909	Tspan4	tetraspanin 4
10536220	1.6890194	Col1a2	collagen, type I, alpha 2
10506714	1.6626495	Lrp8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
10360920	1.6470218	Tgfb2	transforming growth factor, beta 2

Table 6.1 (B) KO TAC < WT TAC Gene Ontology (GO)

Myocardial biopsies were taken from *Fstl3* KO and WT mice, three weeks after TAC and total RNA was hybridized on Mouse Gene 1.0 ST Array chips in triplicate and analyzed using GeneSpring X software suite. Gene lists were generated as above and compared to existing gene ontology lists in GeneSpring. Only those GO lists showing a correlation p -value < 0.01 with the studied gene list are shown.

GO ID	GO Term	p	n	q
GO:0005488	binding	2.22E-09	107	55.73
GO:0005576	extracellular region	1.08E-40	102	53.13
GO:0005515	protein binding	1.73E-07	86	44.79
GO:0044421	extracellular region part	1.47E-28	59	30.73
GO:0009987	cellular process	4.73E-03	51	26.56
GO:0031012	extracellular matrix	1.63E-23	37	19.27
GO:0050896	response to stimulus	1.97E-05	37	19.27
GO:0005578	proteinaceous extracellular matrix	2.19E-22	35	18.23
GO:0007155	cell adhesion	2.17E-07	28	14.58
GO:0022610	biological adhesion	2.17E-07	28	14.58
GO:0016043	cellular component organization and biogenesis	3.56E-03	25	13.02
GO:0002376	immune system process	1.94E-09	24	12.50
GO:0032502	developmental process	1.39E-06	24	12.50
GO:0007275	multicellular organismal development	6.54E-05	24	12.50
GO:0005615	extracellular space	2.75E-06	23	11.98
GO:0006955	immune response	4.81E-08	22	11.46
GO:0006950	response to stress	4.73E-03	18	9.38
GO:0009605	response to external stimulus	1.68E-07	17	8.85
GO:0006952	defense response	9.07E-06	15	7.81
GO:0001871	pattern binding	3.75E-10	14	7.29

legend: p - p value; n - number of counts in selection; q - percent counts in selection

As shown in Table 6.1 (A), of the total 28,856 genes analysed, 338 genes showed reduced expression (≤ 1.4 -fold) in *Fstl3* KO hearts compared to WT hearts after TAC. These included fibronectin-1, lysyl oxidase, collagens and other ECM-related genes. *Fstl3* was also included in this list, serving as a positive control. Gene ontology analysis of this list indicated that those genes downregulated in *Fstl3* KO hearts are mainly involved in protein binding and extracellular region/matrix, although *Fstl3* KO also resulted in decreased expression of genes related to the response to external stimuli and immune processes (Table 6.1 B). It is interesting to note that between 30 – 50% of

genes whose expression was reduced in KO mice belonged to categories related to the extracellular matrix, rather than growth or cytoskeleton categories. This suggests that the main pathological role of *Fstl3* in the heart may not be the promotion of hypertrophy but the regulation of fibrosis.

6.3 Analysis of cardiac fibrosis in *Fstl3* KO mice in response to TAC

Microarray analysis of WT and *Fstl3* KO mice after TAC indicated reduced expression of genes involved in cardiac fibrosis in the knockout animals. In order to confirm the observation *in vivo*, myocardial sections from WT and KO mice were stained with Sirius red, a collagen-binding dye. As shown in Figure 6.1 (A), Sirius red staining showed extensive interstitial and perivascular fibrosis (Inset) in WT mice after aortic constriction. However, this was attenuated in *Fstl3* KO mice. Percent fibrotic area was calculated as the ratio between collagen area and the total ventricular area in each section and quantified in panel B. In *Fstl3* KO mice the fractional fibrotic area is reduced as compared to WT mice after TAC, suggesting a role for *Fstl3* in modulation of cardiac fibrosis.

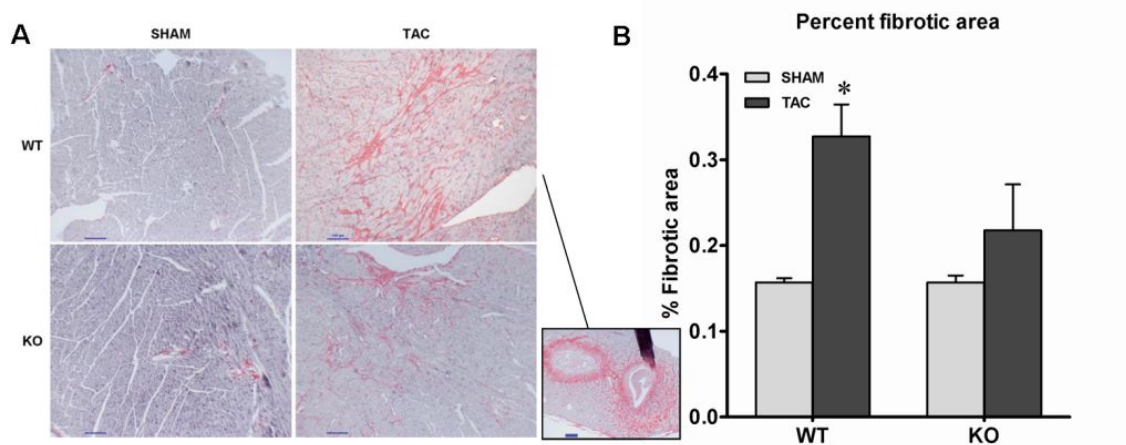


Figure 6.1: Reduced cardiac fibrosis in *Fstl3* KO mice after TAC. Cardiac fibrosis resulting from pressure overload was quantified by staining myocardial sections from WT and *Fstl3* KO mice with Sirius red. (A) Representative photomicrographs of Sirius red stained sections are shown; Inset: Representative perivascular fibrosis; bar = 100 μ m. (B) Percent fibrotic area was calculated from 15 Sirius red stained sections per sample, as the ratio of collagen deposition area to total ventricular area in the respective sections and expressed as a percentage. Two-way ANOVA followed by Bonferroni post-test was used to calculate statistical significance. * $p < 0.05$ compared to WT SHAM. $n = 3$.

Furthermore, collagen mRNA expression was measured using quantitative real-time RT-PCR. As shown in Figure 6.2, collagen I $\alpha 1$ (Col1a1) and collagen III $\alpha 1$ (Col3a1) mRNA was strongly induced after TAC in WT mice (A, B). Similarly, the mRNA expressions of lysyl oxidase (Lox), an enzyme cross-linking collagen fibres into mature collagen (C) [382], and fibronectin-1, which is crucial for collagen deposition (D) [383], were also elevated in the WT hearts. In contrast, *Fstl3* KO mice showed reduced expression of these markers following TAC, compared to WT, indicating reduced collagen deposition and fibrosis and further validating the microarray results.

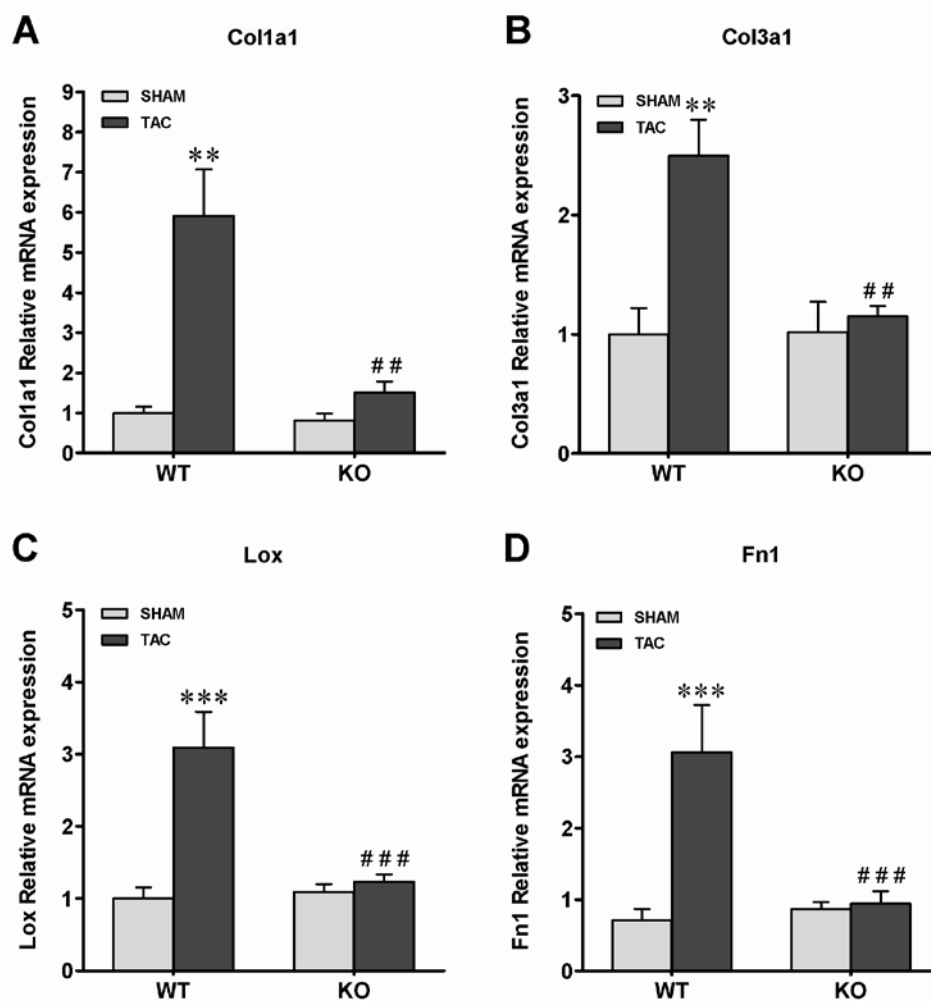


Figure 6.2: Reduced expression of fibrosis markers in *Fstl3* KO mice. Following TAC or 'sham', quantitative real-time RT-PCR was used to measure the myocardial mRNA expression of Col1a1 (A), Col3a1 (B), Lox (C) and fibronectin-1 (D), normalised to endogenous 18S rRNA. Two-way ANOVA followed by Bonferroni post-

tests were used to calculate statistical significance. ** $p < 0.01$, *** $p < 0.001$ vs. WT SHAM. ### $p < 0.001$ vs. WT TAC. $n = 3-6$ per group.

In the pressure-overloaded heart, the fibrotic response is initiated by various cytokine signalling pathways such as TGF- β . Therefore, the mRNA expression of typical mediators of fibrosis including TGF- β 1 and connective tissue growth factor (CTGF), one of its downstream mediators, was examined. As shown in Figure 6.3, the mRNA expression of both TGF- β 1 and CTGF was elevated after TAC in WT mice compared to ‘sham’ operated hearts. However, their expression remained unchanged in *Fstl3* KO hearts after TAC.

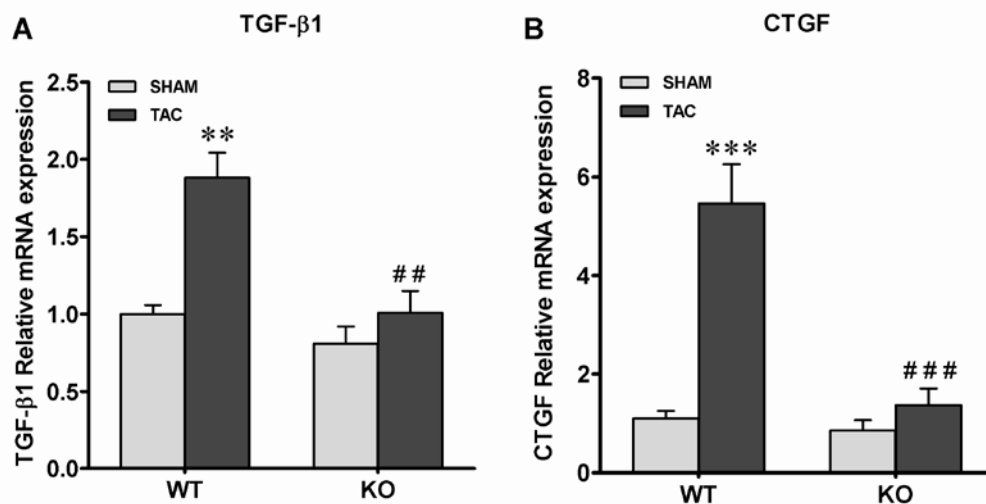


Figure 6.3: Reduced expression of pro-fibrotic cytokines in *Fstl3* KO mice. mRNA expression of TGF- β 1 (A) and CTGF (B) was analysed in myocardial samples from WT and *Fstl3* KO mice following TAC using qRT-PCR normalised to endogenous 18S rRNA. Two-way ANOVA followed by Bonferroni post-tests were used to calculate statistical significance. ** $p < 0.01$, *** $p < 0.001$ vs. WT SHAM. ## $p < 0.01$, ### $p < 0.001$ vs. WT TAC. $n = 3-6$ per group.

6.4 Expression of Matrix Metalloproteinases and their inhibitors in *Fstl3* KO mice

Pressure-overload in the heart often leads to myocardial remodelling and progression to heart failure. Extracellular matrix components like matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an active role in this process [379, 384-386]. In order to check if *Fstl3* was involved in the regulation of these molecules, the expression

of typical markers of myocardial remodelling was examined using qRT-PCR. As shown in Figure 6.4, the expression of gelatinases MMP2 and MMP9 increased in WT hearts in response to pressure overload. In contrast, *Fstl3* KO hearts showed attenuated expression of both MMP2 and MMP9 (Panels A, B). mRNA expression of ADAM12 – a disintegrin and metalloprotease – followed a similar pattern and showed attenuated expression in *Fstl3* KO hearts compared to WT hearts after TAC (Panel C), although it remained significantly elevated compared to ‘sham’ hearts. Furthermore, the expression of tissue inhibitor of MMPs 1, Timp1, was elevated in WT mice and remained unchanged in *Fstl3* KO hearts following TAC (Panel D). These results suggest an attenuation of the process of extracellular matrix remodelling in *Fstl3* KO mice.

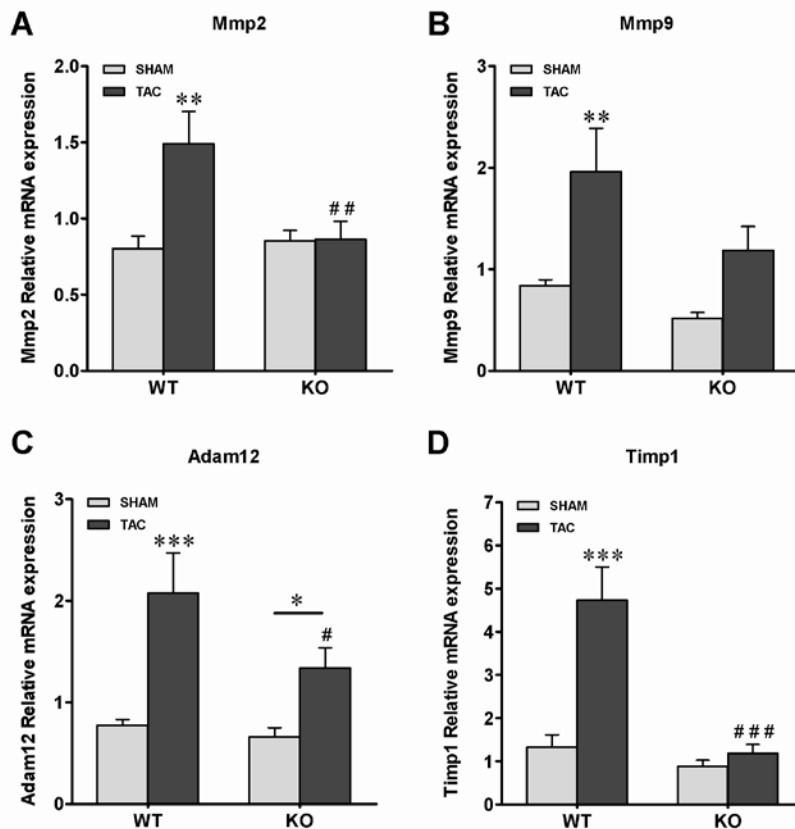


Figure 6.4: Reduced expression of markers of extracellular matrix remodelling in *Fstl3* KO hearts. Ventricular samples from WT and *Fstl3* KO hearts were analysed by qRT-PCR 3 weeks after ‘sham’ or TAC. Myocardial mRNA expression of Mmp2, Mmp9, Adam12 and Timp1 normalised to endogenous 18S rRNA is shown. Two-way ANOVA followed by Bonferroni post-tests were used to calculate statistical significance. * $p < 0.05$ vs. KO SHAM, ** $p < 0.01$, *** $p < 0.001$ vs. WT SHAM, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. WT TAC. $n = 3-6$ per group.

6.5 Inflammatory response in *Fstl3* KO mice after TAC

Myocardial remodelling and fibrosis is often initiated and accompanied by inflammation [387]. To investigate whether cardiomyocyte-secreted *Fstl3* was modulating the immune response, mRNA expression of pro-inflammatory cytokines IL-6 and IL-1 β was examined in the *Fstl3* KO mice after TAC. As shown in Figure 6.5 (A), the expression of IL-6 was elevated in response to TAC in WT mice but remained unchanged in *Fstl3* KO mice. Interestingly, *Fstl3* KO hearts seemed to have elevated expression of IL-1 β under ‘sham’ condition compared to WT mice, further remaining unchanged in response to TAC (Panel B). However, this could not be conclusively established as there was high variation among samples.

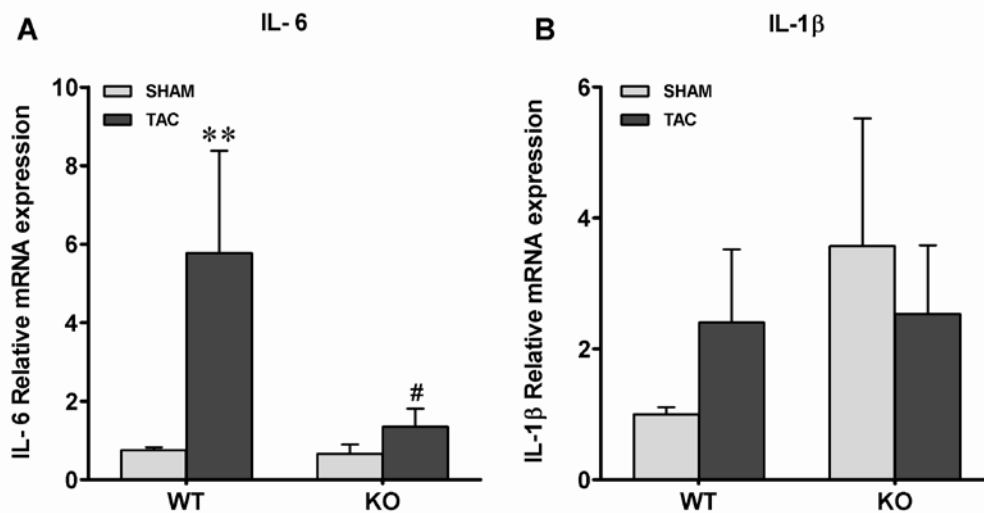


Figure 6.5: Deranged expression of inflammatory cytokines in *Fstl3* KO mice. Myocardial samples from WT and *Fstl3* KO mice were examined following TAC using qRT-PCR. Ventricular mRNA expression of IL-6 and IL-1 β is shown as mean $\pm$ SEM normalised to endogenous 18S rRNA. Two-way ANOVA followed by Bonferroni post-tests were used to calculate statistical significance. ** $p < 0.01$ vs. WT SHAM. # $p < 0.05$ vs. WT TAC. $n = 3-6$ per group.

6.6 Discussion

This Chapter examined the effect of *Fstl3* KO from cardiomyocytes on myocardial fibrosis in response to pressure overload. Microarray analysis on myocardial samples indicated reduced expression of genes related to the extracellular space/matrix in *Fstl3*

KO hearts. This was further confirmed by qRT-PCR which demonstrated reduced mRNA expression of collagens (type I and III), fibronectin and lysyl oxidase in the *Fstl3* KO hearts. It was accompanied by reduced expression of TGF- β 1 and CTGF – known mediators of cardiac fibrosis. Aberrant expression of ECM components and regulators has been consistently observed in patients with dilated cardiomyopathy and heart failure [384, 388, 389]. TGF- β can be considered as a master regulator of cardiac fibrosis and remodelling, acting as a major effector of Angiotensin II in the heart with important roles in cardiac hypertrophy, fibroblast homeostasis, ECM remodelling and inflammation [249, 250]. In this respect, TGF- β not only promotes increased collagen and fibronectin deposition, but also reduces ECM turnover by inhibition of MMPs and induction of TIMP activity [247, 248, 253-255]. Moreover, both TGF- β and Activin A, have been implicated in regulating Lox activity and thereby collagen cross-linking [390-392]. On the other hand, the role of CTGF as a mediator of cardiac fibrosis is controversial. *In vitro* experiments have demonstrated a role for CTGF in fibroblast proliferation and ECM synthesis [393, 394]. However, in overexpression studies, CTGF transgenic mice did not develop cardiac fibrosis. Nevertheless, they exhibited cardiac hypertrophy and an age-dependent progression to chamber dilation and heart failure [395]. CTGF expression can be used as a marker for cardiac fibrosis as its expression, mainly regulated by TGF- β , is elevated in heart failure patients [396] as well as in different models of fibrosis [397-403]. Thus, reduced expression of CTGF in *Fstl3* KO mice may in fact be considered beneficial against progression to heart failure. Interestingly, fibronectin has been shown to bind to Fstl3 leading to increased cell adhesion of hematopoietic stem cells [320]. However, whether this interaction also takes place in the myocardium and plays a role in collagen deposition or fibroblast proliferation is unknown. Thus, reduced expression of TGF- β and collagens in the hearts of *Fstl3* KO mice suggests a necessary role for Fstl3 in the development of cardiac fibrosis in response to pressure overload. Considering the stimulatory role of TGF- β on Fstl3 expression (Chapter 3, [305]), it remains unclear whether the observed reduced fibrosis is a direct response to loss of Fstl3 in the myocardium or as a secondary effect arising from reduced cardiac hypertrophy. If Fstl3 is involved in direct regulation of fibrosis in the presence/absence of cardiac disease, it would be interesting to delineate this pathway in future studies using an inducible knock-out of Fstl3 after TAC. Using this system, the exact role of Fstl3 – whether causative – may be clarified by removing Fstl3 after rather than before induction of hypertrophy. At this stage

however, it cannot be said confidently whether reduced fibrosis in *Fstl3* KO mice is secondary to reduced hypertrophy or a direct effect of lack of *Fstl3*. Considering the complexity and interconnectivity of cardiac remodelling processes, it is likely that fibrosis in the heart may be regulated to some extent by both *Fstl3* expression as well as the stage of disease progression.

ECM homeostasis is maintained by a balance in the synthesis and degradation of ECM components, controlled by the activities of proteinases and their inhibitors [404]. Myocardial collagens have low turnover and thus are mainly regulated by synthesis or degradation in response to stimuli. Increased MMP expression and activity in the myocardium plays a major role in chamber dilation and progression to heart failure, which can be inhibited by increased TIMP expression [240, 379, 384, 385, 405]. However, TIMPs are also known to regulate the activation of MMPs, *e.g.* TIMP2 and TIMP3 are known to regulate active MMP2 and MMP9 [406-408]. *Fstl3* KO mice showed attenuated expression of MMP2, MMP9 and Timp1 compared to WT mice after TAC, suggesting reduced cardiac remodelling. This does not necessarily indicate changes in the activity of these MMPs, as this was not examined. Both MMP2 and MMP9 have been implicated in induction of TGF- β expression, which subsequently inhibits their activity [409, 410]. The balance in the overall expression and activity of MMPs and TIMPs in *Fstl3* KO mice may be a reflection of the overall phenotype of reduced fibrosis during various stages of disease progression [379, 385, 404, 411, 412]. This may also be affected by the profile of inflammatory cytokines in the heart, which can directly affect MMP activity [413, 414]. Similarly, it cannot be ruled out that the observed reduction in fibrosis may simply be a secondary effect of reduced cardiac hypertrophy in response to pressure overload. It is interesting to note that PPAR α expression has been linked to reduced extracellular matrix remodelling, inflammation and oxidative stress in the heart [351, 415-417]. *Fstl3* KO mice show preserved expression of PPAR α in the heart following pressure overload (Chapter 5, Section 5.6.1), which may at least in part be responsible for reduced cardiac fibrosis in these mice.

Activin A has been demonstrated to play a pro-fibrotic role in various tissues leading to increased synthesis of collagen type I [418-420]. However, despite the elevated levels of Activin A in the heart and serum of *Fstl3* KO mice after TAC (Chapter 4), they

exhibited reduced fibrosis and collagen deposition, suggesting that the pro-fibrotic role of Activin A may be tissue-specific. Interestingly, Activin A is known to induce the expression of pro-MMPs 2, 3 and 9 as well as active MMP2 in endometrial stromal cells thereby promoting decidualization [421, 422]. Whether Activin A regulates ECM synthesis and MMP expression in cardiac fibroblasts remains unknown, but is unlikely based on the results presented here.

The expression of Adam12 was also reduced in *Fstl3* KO hearts following TAC. Interestingly, Adam12 has been suggested to play a causal role in cardiac hypertrophy by increasing HB-EGF shedding and subsequent activation of GPCR signalling [113]. Furthermore, an interaction between Fstl3 and Adam12 has been reported, and may be involved in Fstl3-mediated inhibition of osteoclast differentiation [322]. Although Adam12 binding to Fstl3 has not been tested in the myocardium, it might play a role in regulation of cardiac hypertrophy and remodelling.

Pro-inflammatory cytokines have been suggested to play a role in ECM remodelling in the heart, leading to chamber dilation and heart failure [423]. Knock-out of *Fstl3* in the myocardium reduced the expression of IL-6 following TAC. In contrast, the expression of IL-1 β seemed to be elevated in the KO hearts even under basal conditions, although samples showed high variation. In mice, Activin A was found to play a critical role in LPS induced inflammation as well as expression of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6. This action was found to be reversible upon administration of follistatin, an extracellular inhibitor, or activin receptor antagonism [424, 425]. In this respect, *Fstl3* KO mice showed elevated levels of Activin A in the serum, similar to WT mice in response to TAC (Chapter 4). Therefore, it is unlikely that the reduced expression of IL-6 in these mice is a direct consequence of the loss of Fstl3, and is probably secondary to reduced hypertrophy and remodelling.

Knock-out of Fstl3 from cardiomyocytes and the resulting reduced fibrosis following TAC implies that Fstl3 may act in a cell non-autonomous manner by acting on resident cardiac fibroblasts. Thus, Fstl3 produced in cardiomyocytes may be secreted and thus have an effect on other cell types such as fibroblasts, endothelial cells and smooth muscle cells. Fstl3 may thus induce fibrosis by affecting fibroblast proliferation or regulating matrix synthesis or degradation. Fstl3 may also have an effect on

myofibroblast differentiation in the heart. Considering that *Fstl3* expression was seen in the cardiac endothelial cells in heart failure patients (Chapter 3), *Fstl3* may play a role in endothelial-mesenchymal transition to induce myofibroblast differentiation, thereby resulting in perivascular fibrosis. However, myofibroblast differentiation was not examined in the *Fstl3* KO mice in this study.

In summary, results presented here suggest that *Fstl3* may play a role in the development of cardiac fibrosis in response to pressure overload.

CHAPTER 7: Role of Fstl3 in induction of cardiac fibrosis

7.1 Introduction

Fstl3 was demonstrated to be necessary for the induction of cardiac fibrosis in response to pressure overload, wherein reduced expression of collagens and other fibrosis modulators was observed in cardiomyocyte-specific *Fstl3* KO mice (Chapter 6). This Chapter further explores the role of Fstl3 as a paracrine mediator in the induction of fibrosis by using *in vitro* models. To simulate the conditions of *in vivo* pressure overload, an *in vitro* model was set up using mechanical stretch applied to cells in culture grown on flexible membranes (Section 2.17). Either recombinant Fstl3 or mechanical stretch induced Fstl3 from cardiomyocytes was used in these experiments. Col1a1 mRNA expression was measured as type I collagen has been reported to be the most abundant of newly synthesized collagens and is highly elevated in response to different types of mechanical stimuli on fibroblasts [426, 427].

7.2 Effect of recombinant Fstl3 on cardiac fibroblasts

Data presented in Chapter 6 indicated a necessary role for Fstl3 in development of cardiac fibrosis after TAC. However, it was unclear whether Fstl3 had a direct effect on fibroblasts. To examine this possibility *in vitro*, neonatal rat cardiac fibroblasts were treated with recombinant Fstl3 and collagen mRNA was measured by qRT-PCR. Newly synthesized acid-soluble total collagen released into the culture medium was also measured using the Sircol collagen assay [428, 429]. As shown in Figure 7.1, Fstl3 stimulation of fibroblasts did not increase Col1a1 mRNA or newly synthesized collagen release to the supernatant. However, it is possible that fibroblast stimulation was not established since a positive control for stimulation was not used.

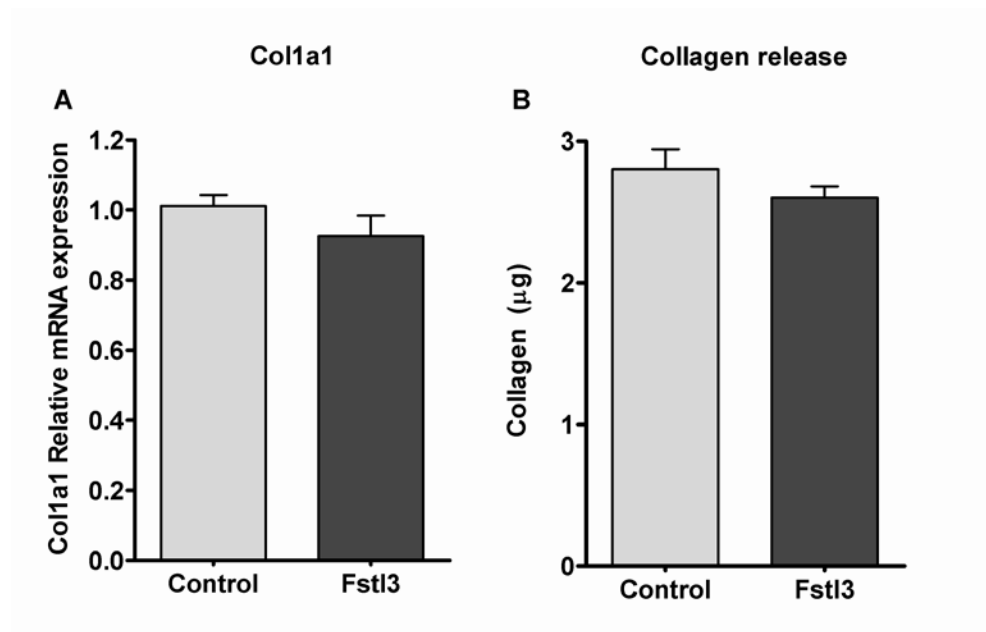


Figure 7.1: Fstl3 is not sufficient to increase collagen synthesis in fibroblasts. Neonatal rat cardiac fibroblasts were treated with 200 ng/ml recombinant Fstl3. (A) Fibroblasts were treated with Fstl3 for 24 h and Col1a1 mRNA expression was measured by qRT-PCR. (B) Fibroblasts were treated with Fstl3 for 72 h and collagen release was measured by colorimetric collagen assay. Data are presented as mean $\pm$ SEM. Unpaired t-test was used to calculate the *p* values. Mean differences did not reach statistical significance. *n* = 3.

7.2.1 Effect of Fstl3 on fibroblast death and proliferation

Cardiac remodelling is usually accompanied by fibroblast turnover. To test if Fstl3 had an effect on cell death or proliferation, cardiac fibroblasts were treated with recombinant Fstl3 at various concentrations and cell death was measured by LDH release into the supernatant normalized to total LDH released upon cell lysis. Figure 7.2 shows a dose response curve of fibroblast LDH release upon treatment with increasing concentrations of Fstl3 for 24 and 48 h. Fstl3 caused a dose and time-dependent increase in LDH release from fibroblasts, indicating cell death at higher concentrations.

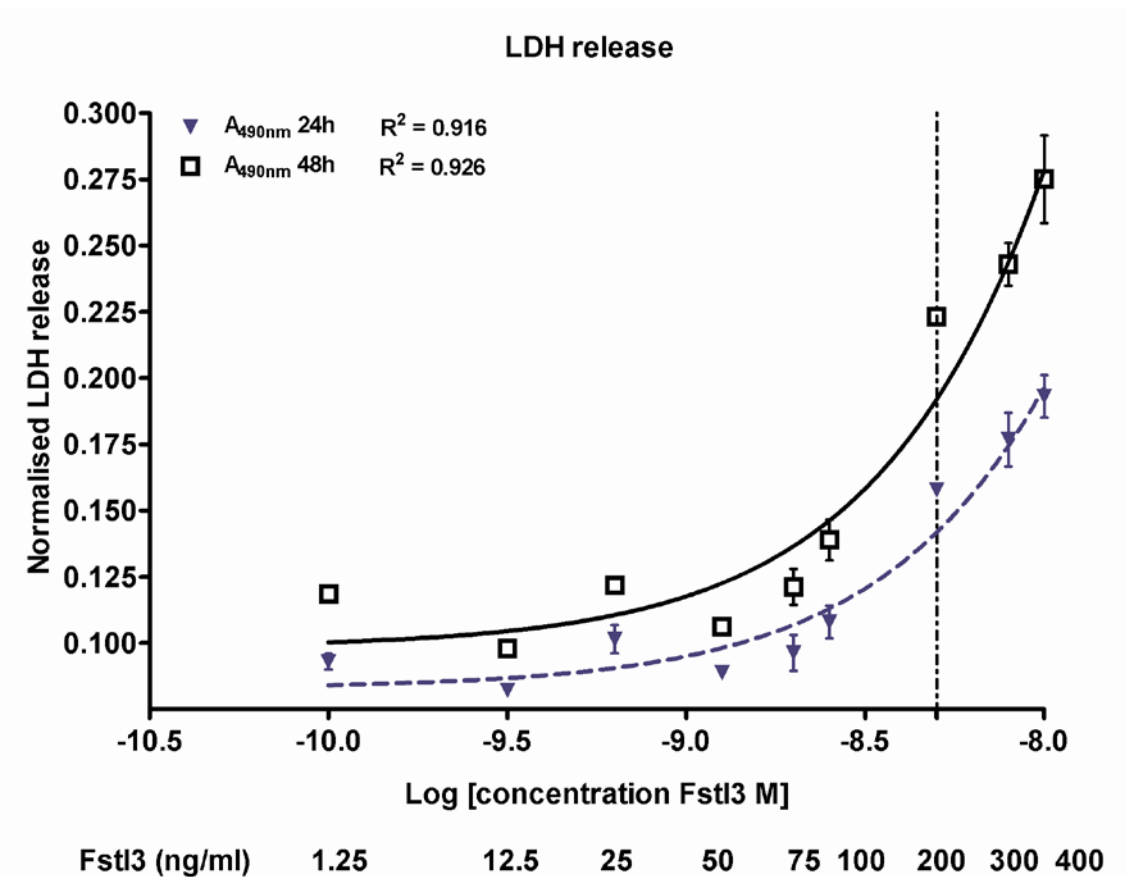


Figure 7.2: Fstl3 induces a dose dependent increase in LDH release from fibroblasts. Neonatal rat cardiac fibroblasts were treated with various concentrations of Fstl3 for 24 h and 48 h. LDH released in the supernatant was determined with a colorimetric assay by measuring absorbance at 490 nm and normalized to total LDH released after cell lysis. Data are expressed as mean $\pm$ SEM. Sigmoidal dose response curve was fit to the values. $n = 3$.

Fibroblast cell numbers depend on proliferation as well as cell death. Therefore, fibroblast cell proliferation in response to concentrations of Fstl3 that affected LDH release was examined using BrdU incorporation. As shown in Figure 7.3, Fstl3 treatment did not affect fibroblast proliferation at the concentrations examined. This also verified that the increased LDH release upon Fstl3 treatment was not due to increased cell proliferation.

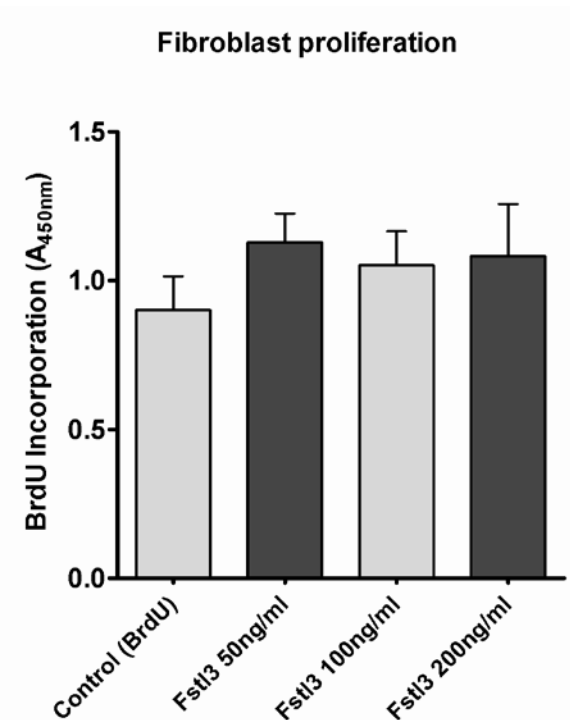


Figure 7.3: Fstl3 does not affect fibroblast proliferation. Neonatal rat cardiac fibroblasts were treated with Fstl3 at various concentrations and BrdU incorporation was measured over an 18 h period. Data are expressed as mean $\pm$ SEM. One-way ANOVA was used to calculate the *p* values and the difference between the means did not reach statistical significance. *n* = 3-4.

7.3 Effect of mechanical stretch and Fstl3 on fibroblasts

To test whether additional factors might be necessary for the action of Fstl3 on fibroblasts, an *in vitro* stimulus equivalent to pressure overload in the heart was set up using mechanical stretch applied to cultured cells. Fibroblasts were subjected to 15% equibiaxial stretch and expression of Fstl3 was measured. As shown in Figure 7.4, Fstl3 mRNA expression was not significantly changed upon mechanical stretch of fibroblasts.

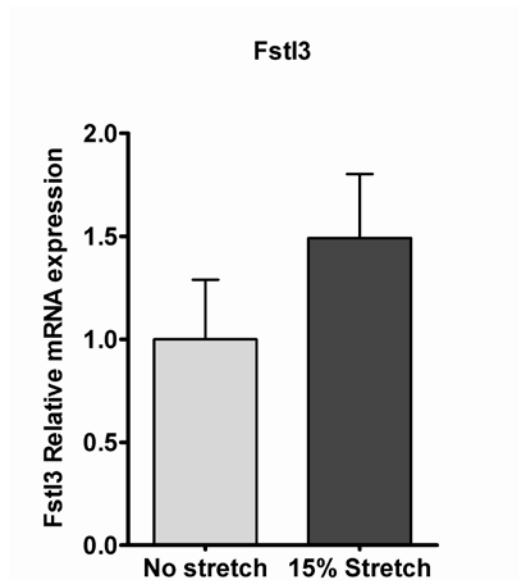


Figure 7.4: Mechanical stretch of fibroblasts does not induce Fstl3 mRNA expression. Neonatal rat cardiac fibroblasts were subjected to 15% equibiaxial stretch for 24 h and Fstl3 mRNA expression was measured using qRT-PCR and normalized to endogenous 18S rRNA. Data are expressed as mean $\pm$ SEM. Unpaired t-test was used to calculate the *p* value, which did not reach statistical significance. *n* = 3.

Next, the effect of combining Fstl3 and mechanical stretch on fibroblasts was analyzed. As shown in Figure 7.5, neither Fstl3 treatment nor 15% stretch was sufficient by itself to induce Col1a1 mRNA expression. However Col1a1 expression was elevated when both the stimuli were combined. This observation indicated that Fstl3 required additional factor(s) that were probably released upon mechanical stretch for induction of Col1a1 in fibroblasts.

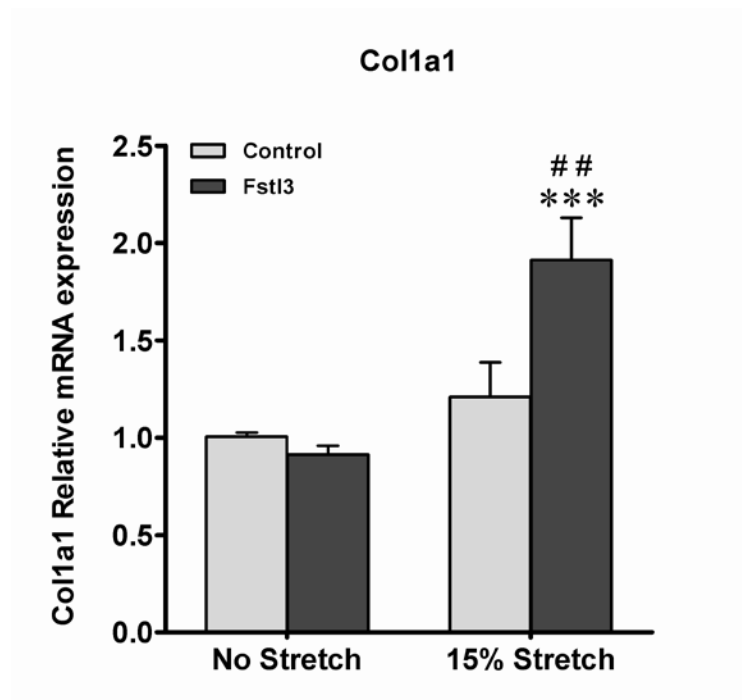


Figure 7.5: Fstl3 induces collagen synthesis in stretched fibroblasts. Neonatal rat cardiac fibroblasts were subjected to 15% equibiaxial stretch for 24 h and stimulated with 200 ng/ml recombinant Fstl3. Col1a1 mRNA expression was measured using qRT-PCR and normalized to endogenous 18S rRNA. Data are expressed as mean $\pm$ SEM. 2-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. *** $p < 0.001$ for stretch vs. no stretch with Fstl3 stimulation. ## $p < 0.01$ for Fstl3 vs. control at 15% stretch condition. $n = 3$.

7.3.1 Effect of Fstl3 on Smad activation in fibroblasts

The Smad pathway in fibroblasts has been shown to play an active role in fibroblast activation and collagen synthesis. To analyze whether Fstl3 was involved in activation of the Smad pathway in fibroblasts, phosphorylation of Smad2 was examined by western blot. As shown in Figure 7.6 (A), Fstl3 treatment increased Smad2 phosphorylation in fibroblast compared to control. Activation of Smad pathway usually leads to translocation of the Smad complex into the nucleus where they bind to Smad-responsive elements in the promoters of target genes causing their transcriptional activation. To confirm Smad activation, fibroblasts were transiently transfected with a luciferase-reporter construct with three tandem Smad-binding elements (SBE) and stimulated with recombinant Fstl3 for 72 h. As shown in Figure 7.6 (B), Fstl3

stimulation caused an increase in luciferase reporter signal, normalized to control renilla reporter. Thus, Fstl3 activated the Smad pathway in cardiac fibroblasts.

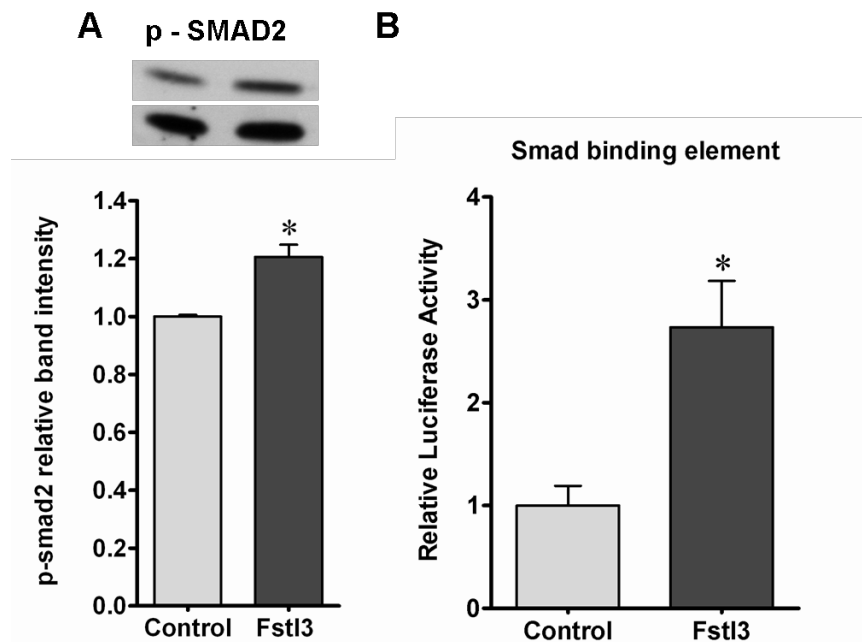


Figure 7.6: Fstl3 activates the Smad pathway in cardiac fibroblasts. (A) Neonatal cardiac fibroblasts were treated with 200 ng/ml Fstl3 for 15 min and phosphorylation of Smad2 was examined by western blot, quantified and normalized to total Smad2 (lower band) in the cells. Data are expressed as mean $\pm$ SEM. Unpaired t-test was used to calculate statistical significance. * $p < 0.05$, $n = 3$. (B) Time course showing phosphorylation of Smad2. (C) Fibroblasts were transfected with SBE-luciferase reporter construct or control renilla reporter construct and stimulated with 200 ng/ml Fstl3 for 72 h. Luciferase units normalized to renilla are shown as mean $\pm$ SEM. Unpaired t-test was used to calculate statistical significance. * $p < 0.05$, $n = 3$.

7.4 *In vitro* model of TAC: Mechanical stretch of cardiomyocytes

In vivo data from Chapter 4 demonstrated that the source of increased Fstl3 in the myocardium in response to TAC was cardiomyocytes. This implied that the effect on fibrosis could be mediated by paracrine action and/or cross-talk between cardiac myocytes and fibroblasts. Thus, an *in vitro* equivalent system was set up in which cardiomyocytes were subjected to mechanical stretch [430]. As shown in Figure 7.7 (A), mechanical stretch of cardiomyocytes increased mRNA expression of Fstl3, while the expression of Col1a1 was unaffected and that of Col3a1 was downregulated (Panels

B and C). However, it could not be established whether Fstl3 was released in the conditioned medium as an ELISA was not performed.

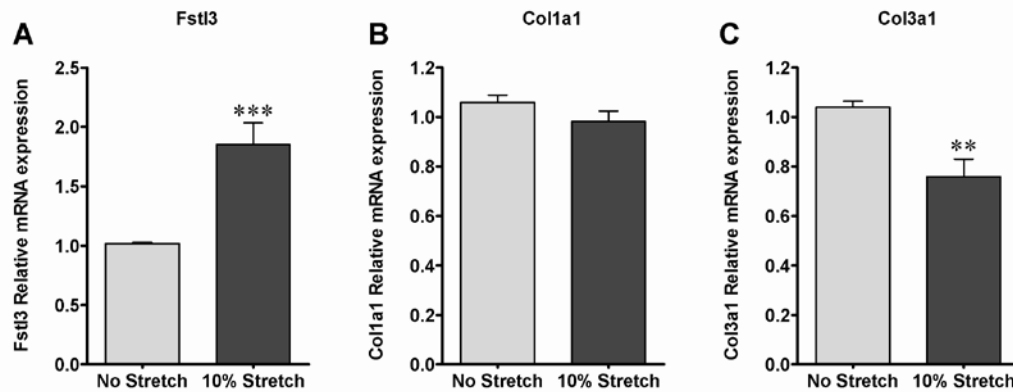


Figure 7.7: Mechanical stretch of cardiomyocytes induces Fstl3 mRNA synthesis.

Neonatal rat ventricular cardiomyocytes were subjected to 10% equibiaxial stretch for 24 h and mRNA expression of Fstl3 (A; $n = 6$), Col1a1 (B; $n = 7$) and Col3a1 (C; $n = 11$) was measured by qRT-PCR and normalized to endogenous 18S rRNA. Data are expressed as mean $\pm$ SEM. Unpaired t-tests were used to calculate the p values. ** $p < 0.01$, *** $p < 0.001$ compared to ‘no stretch’ control samples.

7.4.1 Effect of stretched cardiomyocytes on fibroblasts

To test whether stretched cardiomyocyte-derived Fstl3 affected collagen synthesis by fibroblasts, the conditioned medium from stretched cardiomyocytes was added to cultured fibroblasts and newly synthesized collagen secreted into the culture supernatant was measured using Sircol collagen assay. As shown in Figure 7.8 (A), conditioned medium from stretched cardiomyocytes caused an increase in collagen synthesis by cardiac fibroblasts. To determine whether Fstl3 was necessary for increased collagen production by fibroblasts in response to conditioned medium from stretched cardiomyocytes, a neutralizing antibody against Fstl3 or isotype-matched control IgG) was added to the cultured fibroblasts during stimulation with cardiomyocyte conditioned medium. As shown in Figure 7.8 (B), neither control nor neutralizing Fstl3 antibody had any effect on collagen synthesis by fibroblasts in the presence of conditioned medium from non-stretched cardiomyocytes. However, in the presence of stretched cardiomyocyte conditioned medium, neutralizing Fstl3 antibody reduced the collagen release from fibroblasts as compared to control antibody. Thus, Fstl3 was

found to be necessary for collagen synthesis by fibroblasts, albeit in the presence of unknown factors derived from stretched cardiomyocytes.

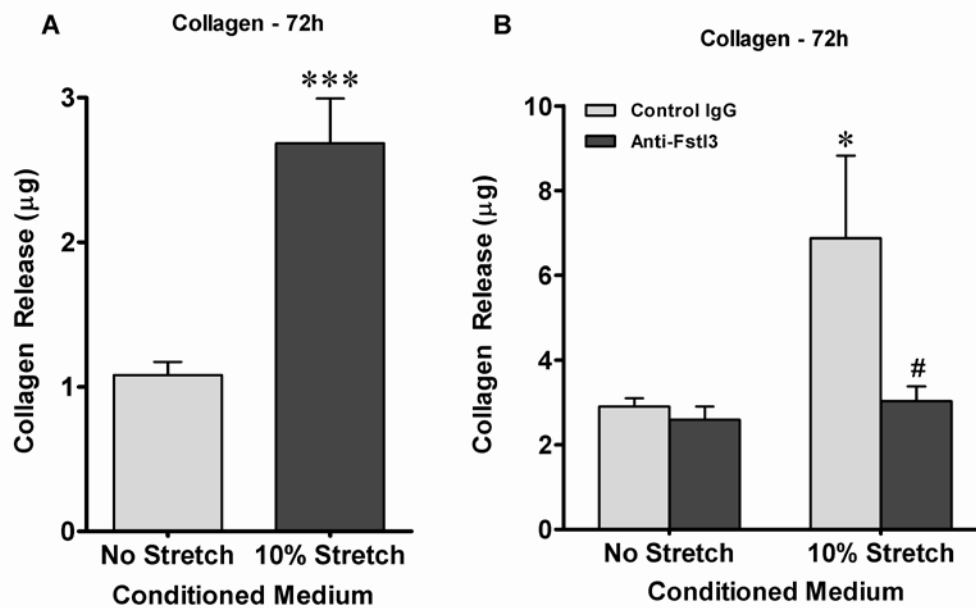


Figure 7.8: Fstl3 is necessary for stretch-mediated paracrine induction of fibroblast collagen synthesis. Conditioned medium from stretched cardiomyocytes was added to neonatal rat cardiac fibroblasts and newly formed collagen released to the medium was measured using a colorimetric collagen assay. (A) Addition of conditioned medium from stretched cardiomyocytes increases collagen synthesis in fibroblasts. Data are expressed as mean $\pm$ SEM. Unpaired t-test was used to calculate the p value. *** $p < 0.001$; $n = 7$. (B) Anti-Fstl3 neutralizing antibody or isotype matched control IgG were added to conditioned medium from stretched cardiomyocytes and collagen release from fibroblasts was measured. Data are expressed as mean $\pm$ SEM. 2-way ANOVA followed by Bonferonni post-test was used to calculate the statistical significance. * $p < 0.05$ for 10% stretch vs. no stretch; # $p < 0.05$ for anti-Fstl3 vs. control IgG at 10% stretch. $n = 4$.

7.5 Discussion

This Chapter presented evidence for a role of Fstl3 in collagen production by fibroblasts. Both recombinant Fstl3 and endogenously produced Fstl3 increased collagen synthesis in cardiac fibroblasts, albeit in the presence of unknown mechanical stretch-derived factors.

Stimulation of fibroblasts with recombinant Fstl3 did not affect collagen synthesis. However, a dose-dependent increase in cell death, independent of proliferation, was observed. In this respect, Fstl3 may be similar to other neurohumoral stimuli such as angiotensin II, which is known for its role in cardiac hypertrophy and remodelling, and also in regulation of cardiomyocyte and fibroblast cell death [431-435]. Cell death, both necrosis and apoptosis, has been shown to play an important role in the transition from compensated cardiac hypertrophy to dilation and heart failure [263, 436, 437]. Whether Fstl3 is sufficient for induction of cell death *in vivo* is still unknown. However, cardiomyocyte-specific *Fstl3* KO mice display increased expression of anti-apoptotic Bcl-2 in the heart as well as protection from ischemia-reperfusion induced cell death [294]. Thus, protection from cell death in *Fstl3* KO mice may represent an important mechanism by which progression to heart failure is arrested.

Interestingly, Fstl3 expression was not elevated in cardiac fibroblasts subjected to mechanical stretch. This confirmed the *in vivo* observation that cardiomyocytes are the major source of increased Fstl3 in the myocardium in response to stress (Chapter 4). Moreover, collagen expression remained unchanged in cardiac fibroblasts subjected to stretch. This may be due to the extent of stretch applied to the cells (cyclic 15% equibiaxial) or the duration of the stimulus [438]. This may also be affected by the growth substrate that the cells were cultured on and the type and maturity of fibroblasts [426]. However, increased collagen expression was observed in stretched fibroblasts stimulated with recombinant Fstl3. This indicated that Fstl3 required additional factors released/expressed upon mechanical loading to induce collagen expression in fibroblasts. Similar responses have been noted before in neonatal rat cardiac fibroblasts subjected to mechanical loading and stimulation with IGF-1, where increased expression of $\alpha 1$ collagen type I mRNA was observed [439]. Many humoral factors and cytokines including angiotensin II, endothelin-1, TNF- α and TGF- β have been known to be secreted by mechanically loaded fibroblasts and are crucial to collagen synthesis and ECM remodelling [440-443]. Although the identity of the factors cooperating with Fstl3 remains as yet unknown, it is possible that a cell surface receptor on fibroblasts may only be expressed or morphologically exposed upon mechanical stretch. This regulation has been shown for surface integrins on fibroblasts, whose expression is changed in

response to mechanical stimuli and they have been known to act as receptor complexes for mechanosensitive signal transduction [32, 444, 445].

The Smad pathway has been shown to play a major role in mediating the intracellular signaling triggered by pro-fibrotic factors like TGF- β [283]. Moreover, Fstl3 has been known to interact with members of the TGF- β superfamily like activin, myostatin and BMPs [308]. It was hypothesized that Fstl3 may exert its fibrotic effect by regulating the Smad pathway. Fstl3 induced phosphorylation of Smad2 in fibroblasts. Luciferase reporter linked to tandem Smad binding elements showed increased activity at 72 h post transfection, further indicating that the Smad pathway was activated by Fstl3. However, Fstl3 was not sufficient to induce collagen production in fibroblasts, suggesting that the Smad pathway was not fully activated or not for long enough. A time course study of activation of the different components of Smad pathway is necessary to fully delineate its role in Fstl3-induced collagen expression.

To simulate conditions of pressure overload in the heart, neonatal rat cardiomyocytes were subjected to mechanical loading [430]. This was found to increase the expression of Fstl3 from cardiomyocytes, in agreement with the observations made *in vivo* as mentioned before (Chapter 4), whereas it did not increase (type I or III) collagen α 1 mRNA expression. Conditioned medium from stretched cardiomyocytes increased collagen synthesis in fibroblasts in a Fstl3 sensitive manner. These results suggest that Fstl3 plays a crucial role in the paracrine activation of fibroblasts by cardiomyocytes, which would explain the *in vivo* observations of reduced fibrosis in *Fstl3* KO mice. Fstl3 can thus be considered as an important mediator of cardiomyocyte – fibroblast cross-talk that acts in an autocrine / paracrine manner on both cell types to regulate cardiac remodelling. Other factors have been similarly implicated to play a role in cardiomyocyte – fibroblast cross-talk and regulation of cardiac hypertrophy and remodelling. For example, TGF- β is secreted from mechanically stressed cardiac fibroblasts as well as cardiomyocytes and plays an important role in cardiac hypertrophy and remodelling [441, 443, 446]. Similarly, angiotensin II, endothelin-1, IGF-1 and TNF- α have also been implicated in mediating paracrine actions in the stressed myocardium [440, 442, 447]. Cross-talk between cardiomyocytes and non-cardiomyocytes in the heart has been shown to be necessary for the induction of hypertrophic markers ANF and BNP [448]. Similarly, angiotensin II induced

cardiotrophin-1, leukemia inhibitory factor (LIF) and IL-6 expression in fibroblasts was found to contribute to cardiomyocyte hypertrophy and also regulated fibroblast proliferation [449, 450]. Other factors implicated in fibroblast-cardiomyocyte communication include FGF2, IL-33, MMPs and TIMPs, connexin-43, *etc.* (reviewed in [451]). This model of cross-talk between the different myocardial cell types through paracrine factors or cell-surface and ECM proteins is probably the most comprehensive depiction of the *in vivo* complexity of responses in the stressed myocardium. Future studies aimed at understanding the function of specific proteins should take into consideration the possibility of their functional regulation by paracrine factors from different cell types.

CHAPTER 8: Yeast two-hybrid analysis for Fstl3 interaction partners

8.1 Introduction

Data presented in Chapter 7 demonstrated that cardiomyocyte-derived Fstl3 is necessary for the induction of collagen production by fibroblasts, thus playing a crucial role in cardiomyocyte – fibroblast cross-talk. Experiments on stretched fibroblasts as well as cardiomyocytes indicated that Fstl3 probably requires additional factors derived from mechanically stimulated cells to mediate its pro-fibrotic effects *in vitro*. On the basis of this observation, it was hypothesized that Fstl3 interaction with other myocardial proteins may be required for its function. To test this hypothesis, the first step was the identification of protein binding partners of Fstl3. The yeast two-hybrid system, also known as an ‘interaction trap’, is based on transcriptional activation of a reporter gene following interaction of the bait protein of interest with proteins expressed from a library. FSTL3 is believed to act, at least in part, through interaction with extracellular signalling components of the TGF- β - activin pathway such as myostatin, activin, BMPs, etc. However, there has been no systematic study on Fstl3 interacting factors, or of potential intracellular binding proteins in the heart. This chapter presents the setup and identification of Fstl3 interacting proteins from the myocardium using the yeast two-hybrid system and verification of their interaction in mammalian cells. Further, the functional consequences of the interaction are also examined.

8.2 Checking for activation of reporter gene by Fstl3

To identify protein interaction partners, Fstl3 was used as ‘bait’ in yeast YRG2 cells. Yeast cells were transformed and selection was carried out based on growth on media lacking tryptophan. The bait construct expressed truncated Fstl3 lacking the signal peptide as a chimeric protein linked to the Gal4 DNA binding domain (Gal4-DBD). To check if the bait protein activates the reporter gene *HIS3* (encoding histidine) on its own, four randomly selected YRG2-Fstl3 colonies were plated on SD-Trp and SD-Trp-His agar plates at dilutions of 1:0 (undiluted), 1:10 and 1:100. In this assay system, colonies that do not grow on medium lacking histidine (SD-Trp-His) do not activate the reporter gene and are considered ‘non-leaky’. As shown on Figure 8.1, YRG2-Fstl3 colonies show variable growth on SD-Trp medium depending on the plating dilution,

indicating successful transformation with the bait plasmid encoding Fstl3. However, when plated on SD-Trp-His selection medium, no growth was observed at any dilutions tested, demonstrating that Fstl3 does not activate the reporter gene *HIS3* on its own and thus cannot induce growth on medium lacking histidine.

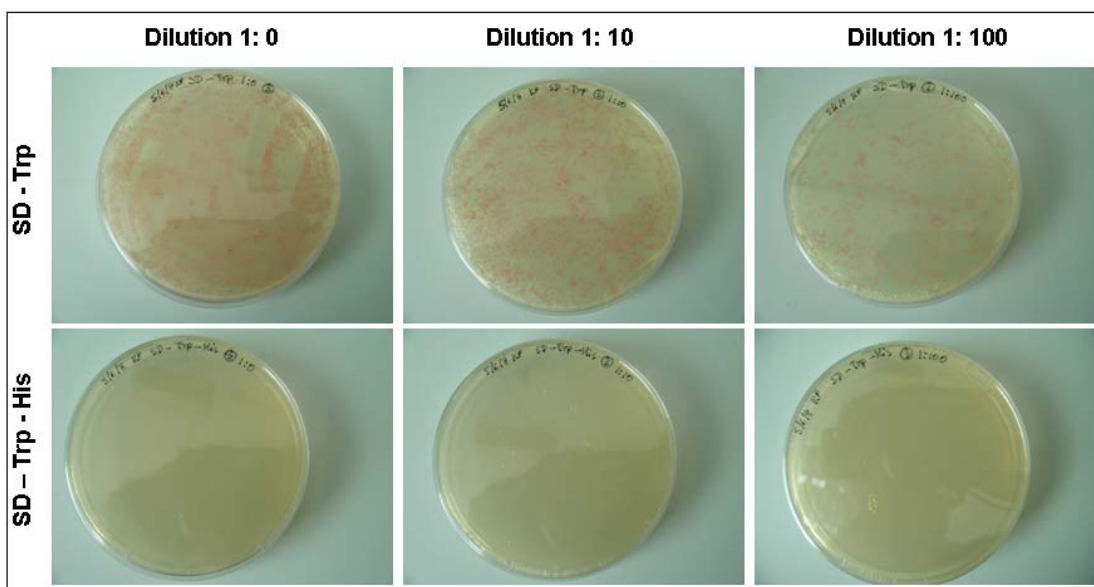


Figure 8.1: The Fstl3 bait protein does not auto-activate the reporter gene *HIS3*.

Yeast colonies expressing the Fstl3-Gal4 DBD chimera were plated on SD-Trp (top panel) and SD-Trp-His (bottom panel) selection medium to check for auto-activation of the reporter gene *HIS3*. The cells were plated at dilutions of 1:0, 1:10 and 1:100 and allowed to grow for 4 days. Representative images of colony growth at different dilutions are shown.

8.3 Identification of potential binding partners of Fstl3

Next, YRG2-Fstl3 was transformed with rat cardiomyocyte cDNA library linked to the transcription activation domain (AD) of Gal4 and plated on medium lacking tryptophan, leucine and histidine, thereby ensuring that the colonies that grow express both bait and target proteins and show interaction between the two by activation of reporter gene *HIS3*. The yield of transformation was measured to be > 1.15 million successful transformants. A total of 156 clones were selected based on activation of reporter gene and identified by sequencing. After eliminating probable false positives in the form of mitochondrial, ribosomal and cloning vector targets as well as out-of-frame sequences,

110 likely targets were identified. Table 8.1 lists the target proteins identified as well as the number of clones that encoded them.

As shown in Table 8.1, a number of targets identified were encoded in multiple individual clones, thereby indicating high likelihood of interaction with Fstl3. Interestingly, fibronectin 1 was encoded in 5 separate clones. This served as a positive control as interaction of FSTL3 with type I domains of fibronectin has been previously reported, although not in the heart [320]. A majority of 34 clones encoded syntenin, a cytoplasmic syndecan binding protein. The second most abundant transcript found was connective tissue growth factor (CTGF), a well-known fibrotic marker. A number of targets such as granulin, EFEMP2, fibronectin, laminins, fibulins or fibrillins are components of the extracellular matrix (ECM) and basement membranes. Thus it seems likely that Fstl3 may interact with members of the ECM to potentiate its downstream effects.

Table 8.1 Probable binding partners of Fstl3

<i>Target Protein</i>	<i>Number of hits</i>
Syntenin	34
Connective tissue growth factor (CTGF)	14
Granulin	12
EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2)	11
Fibronectin 1	5
Dickkopf homolog 3 (DKK3)	4
Laminin gamma 1	4
Latent transforming growth factor beta binding protein 2 (LTBP2)	4
Proprotein convertase subtilisin/kexin type 6	3
NUFIP2	2
Laminin beta 2	1
Sorting nexin 3 (SNX3)	1
Y box binding protein 1 (YBX1)	1
CD63 antigen	1
Fibulin 5	1
Fibulin 2	1
Cyclophilin D	1
Ribophorin 1	1
Sprouty homolog 2	1
ZNF 330	1
Rabin 3	1
Lactate dehydrogenase A	1
NDRG 4	1
Ceramide synthase 2	1
Fibrillin 1	1
HLA-B associated transcript 1	1
Thrombospondin 1	1
Total	110

8.4 Analysis of Fstl3 interaction with CTGF

From the collection of probable interaction partners of Fstl3 identified by yeast two-hybrid system, connective tissue growth factor (CTGF) seemed most promising due to its previously documented role in regulation of cardiac hypertrophy and fibrosis [395, 397, 452]. CTGF has been implicated to play a role in angiotensin II and TGF- β mediated fibrosis and is known to promote fibroblast proliferation [394, 453, 454]. *In vitro*, CTGF induces cardiomyocyte hypertrophy by activation of the Akt pathway [452]. Moreover CTGF is a secreted factor in contrast with syntenin, and was thus chosen for verification of its interaction with Fstl3.

To validate Fstl3 interaction with CTGF, co-immunoprecipitation (IP) was performed. Neonatal rat cardiac fibroblasts cell lysate was immunoprecipitated using anti-CTGF antibody immobilized on protein A/G beads and the bound proteins were analysed by SDS-PAGE followed by western blot using anti-CTGF (as control) or anti-Fstl3 antibody. As shown in Figure 8.2, anti-CTGF antibody successfully immunoprecipitated CTGF from fibroblast cell lysate, represented by the 50 kD band in the anti-CTGF lane, which is absent in the isotype-matched control IgG immunoprecipitation. As the western blot was run under reducing conditions, the 50 kD CTGF band would have been masked by the high-intensity IgG heavy chain band (from IP antibody) if conventional secondary antibody linked to HRP was used for detection. Therefore, protein G linked to HRP was used for detection of primary western blot antibodies as it only binds fully intact antibody and not the heavy and light immunoglobulin chains that have been separated by reducing PAGE. A fibroblast lysate was used as a positive control.

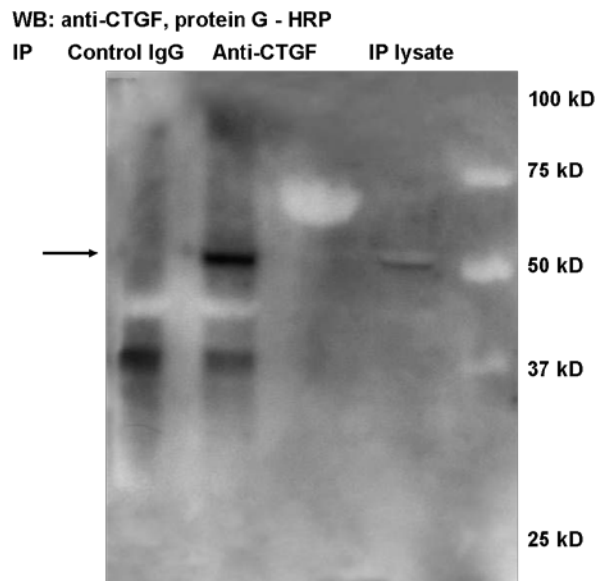


Figure 8.2: Anti-CTGF antibody immunoprecipitates CTGF from fibroblast cell lysate. CTGF was immunoprecipitated from fibroblast cell lysate using anti-CTGF antibody and the bound proteins detected by western blot using protein G - HRP for detection. Arrow indicates the CTGF band at 50 kD.

Once the CTGF antibody was successfully tested for IP of CTGF, co-immunoprecipitation of CTGF with Fstl3 was explored using the same anti-CTGF antibody (or control IgG) and analysed by western blot. Anti-Fstl3 antibody followed by HRP-linked secondary antibody was used for detection of Fstl3. Fstl3 band of about 72 kD was detected. This band was absent in the sample where isotype-matched control IgG was used for IP, thereby ruling out non-specific interaction of Fstl3 with either IgG fixed regions or the protein A/G beads. Recombinant Fstl3 was used as positive control for western blot. Thus, Fstl3 interacts with CTGF in cardiac fibroblast lysates.

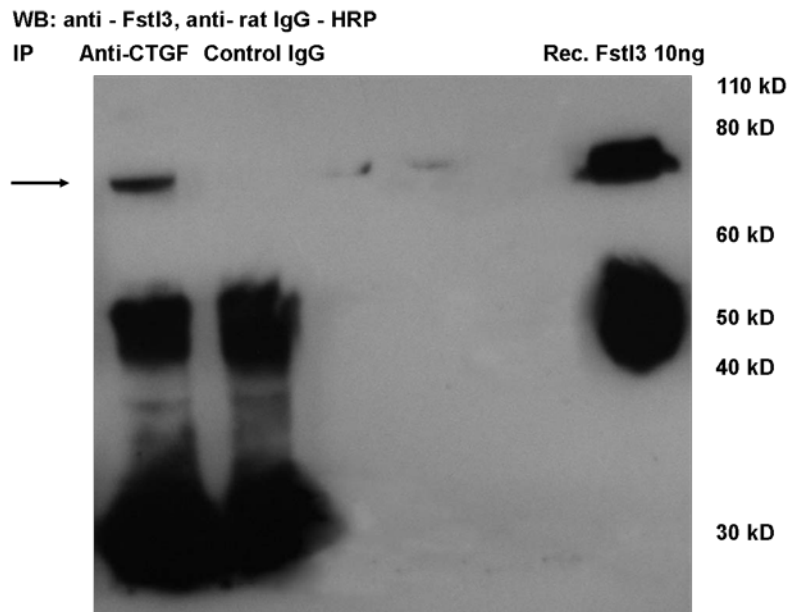


Figure 8.3: Co-immunoprecipitation of CTGF and Fstl3 from fibroblast cell lysate. CTGF was immunoprecipitated from fibroblast cell lysate using anti-CTGF antibody immobilized on protein A/G beads and the beads were washed several times to remove unbound molecules. The bound molecules were then eluted and analysed using western blot for detection of Fstl3. Recombinant Fstl3 was used as positive control. Arrow indicates an Fstl3 band at 72 kD in the anti-CTGF immunoprecipitated sample that is absent in the control IgG precipitated lane.

8.5 Functional significance of Fstl3 interaction with CTGF

CTGF is well known as a fibrosis marker and Fstl3 was shown to mediate fibroblast activation by stretched cardiomyocytes (Chapter 7). However, additional cardiomyocyte derived factors seemed to be necessary for the effect of Fstl3 on collagen synthesis. CTGF interaction with Fstl3 led to the hypothesis that CTGF could be one of the cardiomyocyte-derived factors through which Fstl3 exercised its pro-fibrotic role. To test this hypothesis, cardiac fibroblasts were treated with recombinant Fstl3 and/or CTGF for 72 h and newly synthesized collagen secreted into the culture supernatant was measured using Sircol collagen assay. Recombinant CTGF concentration of 500 ng/ml was chosen as it was shown to induce mRNA expression of ECM proteins including fibronectin, collagen I and III in H9c2 cardiomyocytes and dermal fibroblasts [455, 456]. As shown in Figure 8.4, neither Fstl3 not CTGF by itself was sufficient to induce collagen synthesis in fibroblasts. Similarly, when both Fstl3 and CTGF were present in

the medium, collagen synthesis remained unaffected. Thus, Fstl3 and CTGF were not sufficient to induce collagen synthesis in cardiac fibroblasts. However, the activity of recombinant CTGF was not tested.

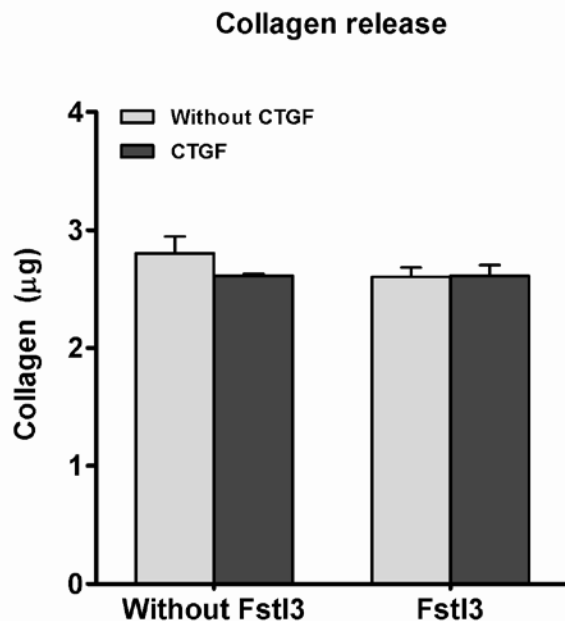


Figure 8.4: Fstl3 and CTGF together are not sufficient to enhance collagen synthesis in fibroblasts. Neonatal rat cardiac fibroblasts were treated with 200 ng/ml Fstl3 and/or 500 ng/ml CTGF for 72 h and collagen released into the culture supernatant was measured using Sircol assay, shown as mean $\pm$ SEM. n = 3.

The cardioprotective effects of CTGF on angiotensin induced cardiac dysfunction [395] suggested that CTGF might be involved in regulation of cell death. Moreover, Fstl3 was shown to induce cell death of fibroblasts (Chapter 7). Therefore, the combined effect of CTGF and Fstl3 was examined on LDH release by fibroblasts in order to determine whether their interaction affected cell death. Recombinant CTGF was used at a concentration of 5 μ g/ml as concentrations between 1 – 10 μ g/ml have previously been reported to inhibit cell death both *in vitro* and *in vivo* [457-459]. Cardiac fibroblasts were treated with increasing concentrations of Fstl3 and/or 5 μ g/ml CTGF for 24 h and LDH released into the culture supernatant was measured. Increasing concentrations of Fstl3 led to a dose-dependent increase in LDH release. However, in the presence of CTGF, LDH release was comparable to control cells. Thus, CTGF inhibited Fstl3-induced cell death in fibroblasts.

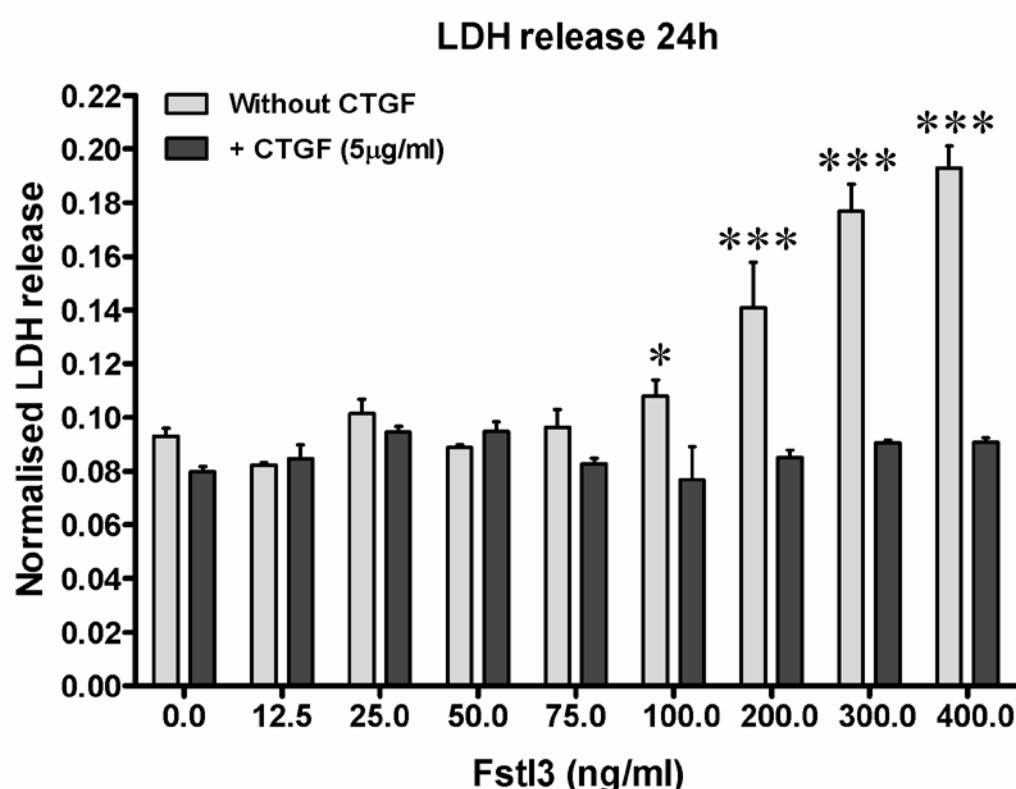


Figure 8.5 CTGF inhibits Fstl3-induced LDH release in cardiac fibroblasts. Fibroblasts were treated with increasing concentrations of recombinant Fstl3 and/or 5 µg/ml CTGF for 24 h. LDH released into the supernatant was determined with a colorimetric assay by measuring absorbance at 490 nm and normalised to total LDH released upon cell lysis. Data are presented as mean ± SEM. Two-way ANOVA followed by Bonferonni post-test was used to calculate statistical significance. * $p < 0.05$, *** $p < 0.001$ for CTGF vs. no CTGF at respective Fstl3 concentrations. $n = 3$.

8.6 Discussion

Data presented in this chapter identified a number of ECM and basement member constituent proteins as potential binding partners of Fstl3. Moreover, interaction of Fstl3 with CTGF was verified in cardiac fibroblasts using co-immunoprecipitation and CTGF was shown to inhibit Fstl3 mediated cell death in cardiac fibroblasts.

Yeast two-hybrid analysis proposed a number of targets to which Fstl3 may bind, most of these being members of the ECM involved in homeostasis, remodelling and structural integrity. This suggested that Fstl3 may act through these factors to mediate

its effect on cardiac hypertrophy and fibrosis. It is in agreement with the results of the microarray analysis of *Fstl3* KO hearts in which most proteins from the ECM were downregulated in response to pressure overload compared to WT mice (Chapter 6), which also suggested that *Fstl3* may primarily act by regulating ECM proteins. Interesting targets from the yeast two-hybrid analysis include syntenin (cytoplasmic), CTGF, fibronectin, LTBP2, fibrillin-1, fibulin-2 and 5, *etc.* Identification of fibronectin served as a good positive control for the procedure, as binding of *Fstl3* and fibronectin has been reported previously. Intriguingly, neither Activin A nor myostatin were identified from any of the colonies tested even though a strong interaction of both with *Fstl3* has been shown in previous studies. This could be simply because of the limited number of colonies tested or may be due to some physiological conditions that may be necessary for their interaction which were not fulfilled in the yeast two-hybrid system. A number of these proteins have been previously shown to be regulated by mechanical stretch in the heart. These include CTGF, fibronectin, thrombospondin-1, fibrillin-1 [460-464], thus making them attractive candidates for mediators of cardiomyocyte – fibroblast crosstalk alongside *Fstl3*.

Although the targets identified from the yeast two-hybrid screen seem promising, correct interpretation of this data requires consideration of its limitations which reduce its reliability [465]. For example, the interactions in the yeast cells are forced to take place in the nucleus due to fusion of bait and target proteins to nuclear localisation signals, which might not reflect the physiological conditions for proper folding of proteins or interaction amongst proteins. Similarly, fusion itself may result in misfolding of proteins and false positive targets. Moreover, some true interactions may be missed due to differences in expression of some target proteins in the yeast. Therefore, identified interaction proteins from the yeast must be verified in their native state in mammalian systems. In this regard, the interaction of CTGF with *Fstl3* was verified by immunoprecipitation and the functional effect of this interaction was explored.

Neither *Fstl3* nor CTGF were sufficient to induce fibroblast collagen synthesis and their interaction was not sufficient either to enhance collagen synthesis by fibroblasts. This suggested that CTGF may not be the stretched - cardiomyocyte derived factor, whose interaction with *Fstl3* may regulate collagen synthesis. It may also be possible that additional factors are required for the CTGF and *Fstl3* interaction to have a functional

significance in the regulation of fibrosis. Interaction of CTGF with Fstl3 was found to inhibit Fstl3 mediated fibroblast cell death. It should be noted that the role of CTGF in regulation of cell death is context-dependent as some reports have demonstrated increased cellular apoptosis upon treatment with CTGF [466-468]. Interestingly, a role for thrombospondin-1, also identified as a potential binding partner of Fstl3, has been reported in mechanosensitive initiation of fibroblast cell death [469]. However, in this study, the interaction of Fstl3 and thrombospondin-1 was not further verified. CTGF has been shown to play a role in fibrosis induction (*in vitro*) and is a recognised marker of *in vivo* fibrosis in multiple organs [398, 470]. However, transgenic mice with overexpression of CTGF in the heart developed age-dependent cardiac hypertrophy and heart failure albeit with no evidence of fibrosis. Moreover, CTGF transgenic mice displayed preserved cardiac function compared to littermate control mice upon treatment with angiotensin II, leading the authors to conclude that CTGF, by itself, is not profibrotic but is involved in development of cardiac hypertrophy and dysfunction [395]. Similarly, CTGF overexpression in the heart was shown to play a cardioprotective role against ischemia reperfusion injury via activation of Akt signalling [458]. Interestingly, Activin A has also been reported to play a cardioprotective role against ischemic injury and this effect was abolished by Fstl3 [294]. Thus Fstl3 may be involved in regulating the action of two different cardioprotective factors acting through diverse pathways and may thus contribute to cardiac dysfunction.

CHAPTER 9: General Discussion and Conclusions

9.1 General discussion and concluding remarks

Many aspects of the process of cardiac failure are still incompletely understood. A number of cardiac pathologies eventually progress to heart failure and surgical intervention is often needed to halt or slow down this process. This project was born out of an interesting clinical observation in end-stage heart failure patients undergoing implantation of left ventricular assist devices combined with pharmacological therapy. The expression of follistatin family genes was observed to be aberrant and FSTL3 expression in particular was elevated in myocardial samples from heart failure patients, returning to control levels following recovery [328]. At the time, Fstl3 was thought to mainly function as an extracellular inhibitor of activin and myostatin [315, 317, 471]. Fstl3 was peculiar in this respect as the expression of other TGF- β superfamily ligands, including *Activin* β_A remained unaltered in these patients. This observation was further replicated in mouse models of cardiac injury and suggested an activin independent role for the follistatin family proteins. The aim of this study was to explore the role of Fstl3 in cardiac hypertrophy.

The TGF- β superfamily regulates cardiac homeostasis as well as response to injury [287, 360, 472]. A complex interplay has been described between members of this family and their soluble inhibitors, in particular Fstl3. Both TGF- β and activin A can induce the expression of Fstl3 through Smad proteins [305, 306], while Fstl3 in turn binds to activin A with high affinity and inhibits its bioactivity by preventing the binding to its receptor [297]. The expression of activin A in the heart is upregulated in different cardiac injury models and has been demonstrated to play a cardioprotective role following ischemic injury [294]. In contrast, some studies have also noted a pro-fibrotic action of activin A [418, 420], although this hasn't been examined in the heart. On the other hand, cardiac specific *Fstl3* KO mice showed increased expression of activin A following pressure overload similar to that observed in WT mice. Due to the absence of Fstl3 inhibition of activin, the reduced cardiac hypertrophy in these mice might be a direct consequence of increased activin A function. Similar observations were reported in the whole body *Fstl3* KO mice, where the observed phenotype was attributed to increased tissue availability of activin A and myostatin, resulting in a

complex metabolic paradigm [317]. The expression of TGF- β in cardiac specific *Fstl3* KO mice was reduced following pressure overload, in contrast to activin A which remained elevated. TGF- β is known to induce cardiac hypertrophy and fibrosis and regulates the expression of other mediators of these responses in the myocardium [249, 252, 287, 473]. The pleiotropic effects of TGF- β in the myocardium are mediated by downstream effectors such as CTGF, whose expression is induced by TGF- β and is known to regulate cardiac hypertrophy and dysfunction [395, 474, 475]. Thus, the regulation of *Fstl3* expression by TGF- β in cardiomyocytes and its subsequent pro-hypertrophic and pro-fibrotic effects suggest that it may act as a downstream mediator of TGF- β action on cardiomyocytes and fibroblasts. The complex interplay between these proteins suggests that a balance in their temporal and spatial expression and activity may determine the course of disease progression in a context dependent manner.

Cardiac fibrosis is often seen as part of the remodelling response of the hypertrophied myocardium and thus many interventional studies have noted simultaneous reduction or increase in both cardiac hypertrophy and fibrosis. For example, inhibition of EGF receptor transactivation inhibited cardiac hypertrophy and fibrosis in response to pressure overload [20]. Similarly, cardiac specific knock-out of *Erk5* in mice resulted in reduced cardiac hypertrophy and fibrosis following pressure overload [142]. In overexpression studies, activated calcineurin subunit in the heart resulted in enhanced hypertrophy and fibrosis [146]. In contrast, a number of studies have also reported differential regulation of hypertrophy and fibrosis. The phenotype of mice with constitutive active PI3K or dominant negative PI3K in the heart showed increased or decreased cardiac size respectively without any effect on fibrosis in either models [154]. Cardiac specific *Fstl3* KO mice displayed reduced cardiac fibrosis and remodelling which may be a direct effect of reduced hypertrophy following pressure overload. However, microarray analysis in these mice suggested that *Fstl3* may function mainly by modulation of extracellular matrix components rather than hypertrophic mediators. Moreover, cardiomyocytes being the main source of increased *Fstl3* in the stressed heart suggested that *Fstl3* may function through autocrine and/or paracrine actions on other cell types in the myocardium. This was further supported by *in vitro* observations demonstrating a necessary role for cardiomyocyte-derived *Fstl3* in fibrosis development through paracrine actions.

Recently, a number of studies have focused on exploring the function of paracrine factors in mediation of cardiac hypertrophy and fibrosis. For example, cardiomyocyte-derived TGF- β has been recognized to act on fibroblasts in the presence of angiotensin II to enhance collagen production [476]. Furthermore, it was recently reported that paracrine action of TGF- β from cardiomyocytes is necessary for the protective effects of TGF- β type II receptor inhibition in the form of attenuated hypertrophy as well as fibrosis in response to pressure overload [477]. Similarly, IL-6 family cytokines have also been shown to play a paracrine role in fibrosis as well as cardiomyocyte hypertrophy. Pro-hypertrophic angiotensin II signalling induced IL-6, CT-1 and LIF expression in cardiac fibroblasts, which activated gp-130 signalling in cardiomyocytes to induce hypertrophic responses [449]. CT-1 stimulation was also sufficient to induce cardiac fibroblast growth and collagen synthesis and exhibited cooperation with endothelin-1/ET_A receptor signalling [478]. Moreover, stem cells expressing paracrine factors like IGF-1 and VEGF have been implicated in promoting cardiac repair following infarction, through activation of stromal cell-derived factor-1 α (SDF-1 α), which induced stem cell recruitment and enhanced angiogenesis [479, 480]. Reduced expression of TGF- β and IL-6 in *Fstl3* KO mice suggests that the phenotype observed in these mice may be a consequence of reduction in a number of paracrine regulators of cardiac remodelling in addition to *Fstl3*. Additionally, *Fstl3* may also act on endothelial and vascular smooth muscle cells in the myocardium, as has been previously reported for *Fstl1*, another member of the follistatin family proteins. *Fstl1* was demonstrated to be an Akt-inducible paracrine mediator of reduced apoptosis in the infarcted myocardium and also induced angiogenesis following ischemic injury via endothelial nitric oxide synthase signalling in skeletal muscle [481, 482]. Increased *Fstl3* expression was detected in the endothelium of failing hearts, and may have adverse effects in the response to injury, possibly by inducing perivascular fibrosis. In summary, *Fstl3* may now be considered as a novel cardiomyocyte-derived autocrine-paracrine mediator of cardiac hypertrophy and fibrosis.

The dependence of paracrine action of *Fstl3* on additional stretch-derived factors from the myocardium suggests a coordinated response of the heart undergoing remodelling. It was hoped that yeast two-hybrid analysis would identify potential partners essential for *Fstl3* action on fibroblasts. Although CTGF interaction with *Fstl3* was verified, it

did not alter collagen synthesis in fibroblasts. However, it was found to inhibit Fstl3 induced cell death and may thus be an important protective mechanism in the heart. Due to the modular structure of CTGF and its interaction with several extracellular signalling proteins as well as cell surface molecules, it is possible that Fstl3 may use CTGF to sequester itself to specific cell surface proteins, thereby acting in a context-specific manner. Cardioprotective role of CTGF was also recently demonstrated *in vivo* in a mouse model of ischemia-reperfusion injury where overexpression of CTGF in the heart was shown to reduce the scar size and protect the myocardium from ischemic injury [458]. Whether Fstl3-CTGF interaction plays a role in the cardioprotective actions of CTGF *in vivo* remains to be explored. In contrast, another study with cardiac restricted overexpression of CTGF reported age-dependent cardiac dysfunction but protection from pressure overload induced hypertrophy. Whether Fstl3 is required for the action of CTGF in development of heart failure could be analysed by crossing cardiac-specific CTGF transgenic mice with *Fstl3* KO mice.

It is interesting to note the dual effect of Fstl3 on cardiac fibroblasts – activation to induce collagen synthesis as well as induction of cell death. Such dual effects have been observed before for angiotensin II action on cardiac fibroblasts. While angiotensin II stimulation increases fibroblast collagen synthesis and expression of TGF- β [250, 483, 484], it has also been demonstrated to induce apoptotic cell death via PKC mediated mechanism [485]. Similarly, the dual effect of Fstl3 on fibroblasts may also function in coordination with disease progression, *i.e.* in the acute stage to induce collagen expression in fibroblasts in coordination with other factors, while in the chronic stage to induce cell death, thereby inducing remodelling and progression to heart failure. Interaction with CTGF may alter this balance. Other potential binding partners for Fstl3 included a number of ECM and basement membrane components, further suggesting Fstl3 action through manipulation of these factors. Precise identification of stretched cardiomyocyte derived factors coordinating Fstl3 effect on collagen synthesis in fibroblasts will require validation of the identified potential binding partners as well as sophisticated proteomic analysis including liquid chromatography - mass spectrometry analysis on conditioned medium from stretched cardiomyocytes.

Why cardiomyocytes would secrete a stress-activated factor like Fstl3 which would further exacerbate both hypertrophy and fibrosis is unknown. One explanation could be

that Fstl3 may act in response to mechanical loading to induce a compensatory hypertrophic state. However, in the chronically loaded myocardium, other factors would then be activated which in coordination with Fstl3 induce fibrosis and ECM remodeling. These may include Akt-1 induced factors as similar observations were made in cardiac specific inducible activation of Akt-1 where acute activation resulted in compensated and physiological hypertrophy with sustained cardiac function while chronic activation led to cardiac remodelling and contractile dysfunction due to angiogenic mismatch with cardiac growth [161]. Similarly, aberrant Smad signaling has also been suggested to play a role in cardiac decompensation and progression to heart failure. In this respect, lack of Smad3 in the heart was shown to induce compensated hypertrophy and resisted progression to heart failure in response to pressure overload or myocardial infarction [257-259]. Fstl3 may function in coordination with these factors and thus play an important role in cardiac remodelling. It would be interesting to explore this hypothesis using an inducible knock-out model of Fstl3 in the myocardium. Knock-out of Fstl3 following the acute responses to pressure overload may be more beneficial and prevent cardiac remodelling as opposed to knock-out of Fstl3 before or immediately following pressure overload, which would prevent hypertrophy.

A role for Fstl3 as a necessary mediator of cardiac hypertrophy *in vivo* is supported by similar observations *in vitro* in mediating phenylephrine induced cardiomyocyte hypertrophy [367]. In addition, cardiac specific *Fstl3* KO mice exhibited reduced scar formation following ischemic injury [11]. Thus Fstl3 may be an important mediator of the cardiac response to various forms of injury. Moreover, data presented in this thesis argue for a sufficient role of Fstl3 in induction of cardiomyocyte hypertrophy, possibly through activation of p38 and JNK pathways. Whether this response is directly mediated by receptor activation is currently unknown. The hypertrophic effect of TGF- β is also known to be transduced via activation of TAK which further activates p38 and JNK pathways in cardiomyocytes. Thus, induction of Fstl3 by TGF- β in cardiomyocytes may function to amplify the pro-hypertrophic action of TGF- β .

Fstl3 action on cardiomyocytes and fibroblasts and its role in their cross-talk represents an important target of therapeutic manipulation. In this regard, the action of Fstl3 from cardiomyocyte conditioned medium could be inhibited by the addition of a neutralising antibody. These have been recognized to be highly specific molecular tools to inhibit

the action of important disease regulators. For example, antibody therapy against both TGF- β 1 and TNF- α has been shown to effectively reduce cardiac fibrosis and progression to heart failure [256, 486]. However, inhibition of TGF- β during ischemic injury has also been reported to have detrimental effects associated with increased MMP activation and chamber dilation [487]. In this respect, Fstl3 inhibition by neutralising antibody may be used as an effective therapeutic strategy to minimize the harmful actions of Fstl3 on cardiomyocytes as well as fibroblasts and should be further explored using injection of Fstl3 neutralising antibody following cardiac injury.

9.2 Limitations and future work

An important limitation of the work presented here is the lack of sufficient numbers of *Fstl3* KO mice under study. This was mainly because of restricted availability of these mice in London due to their health status which made it imperative for all the mouse procedures to be performed at the Whittaker Cardiovascular Institute, Boston. Although post-operative mortality was only 10%, limited breeding regulations as well as available time introduced more restrictions in the number of KO mice available.

In vitro experiments were carried out on neonatal rat ventricular cardiomyocytes and fibroblasts as model systems. Although these primary cells are widely used for studying molecular mechanisms of hypertrophy and fibrosis, it is appreciated that the responses seen in these cells are not always reflective of the responses in adult cells. Moreover, the *in vivo* complexity of the tissue system with multiple intercellular and intracellular physical and chemical communication signals can not be fully simulated in the *in vitro* experimental systems. Similarly, cell culture systems are two dimensional while the *in vivo* tissue and organ systems are three dimensional. In addition, a number of systemic factors such as endocrine hormones or circulating cytokines and growth factors might in fact affect the phenotype of *Fstl3* KO mice following injury, which cannot be replicated in the *in vitro* mechanical stretch and cardiomyocyte – fibroblast cross-talk model.

Examination of the effects of Fstl3 on cardiomyocytes demonstrated increased cardiomyocyte hypertrophy and activation of the p38 and JNK signalling pathways. A detailed analysis of the molecular mechanism of Fstl3 mediated cardiomyocyte hypertrophy could not be performed mainly due to time limitations. In the future, it

would be interesting to assess whether Fstl3 action on cardiomyocytes is dependent on p38 and JNK pathways as well as the role of Smad pathway proteins in Fstl3 action in relation to activin A. Cardiac-specific *Fstl3* KO mice were reported to show increased phosphorylation of Smad2 in the heart following pressure overload, possibly explaining the observed reduction in cardiac hypertrophy [367]. It is possible that Fstl3 may directly regulate Smad7 expression and/or activation in the heart which might affect activation of its target protein Smad2 and thereby regulate the cardiomyocyte hypertrophic response. The role of Fstl3 as a cardiomyocyte – fibroblast cross-talk mediator was demonstrated in this study using a neutralising antibody against Fstl3. The exact molecular mechanism of mediating this paracrine effect should be explored to assess which signalling pathways are involved as well as the possibility of a cell surface receptor for Fstl3 through which it would transduce the pro-fibrotic signals. Similarly, the role of additional cardiomyocyte derived factors following mechanical loading and their relation with Fstl3 should also be examined. It would also be interesting to inspect whether Fstl3 has an effect on endothelial cell function or myofibroblast differentiation in the injured heart.

9.3 Summary

The expression of Fstl3 is elevated in the myocardium of heart failure patients as well as mouse models of cardiac injury, wherein cardiomyocytes were identified as the main source of increased Fstl3 following pressure overload. The role of Fstl3 in cardiac hypertrophy was tested using cardiac specific *Fstl3* KO mice subjected to pressure overload. These mice displayed attenuated cardiac hypertrophy and remodelling, resulting in improved cardiac function. *In vitro* analysis demonstrated that secreted Fstl3 from cardiomyocytes acts in an autocrine manner to induce hypertrophy of cardiomyocytes and activation of MAPK proteins p38 and JNK. Further, paracrine action of Fstl3 on fibroblasts was shown to increase collagen synthesis in coordination with unknown factors derived from mechanically loaded cells. Fstl3 also induced cell death in fibroblasts, which may contribute to heart failure progression. Furthermore, screening for cardiomyocyte derived protein binding partners of Fstl3 led to the identification of CTGF interaction with Fstl3, which inhibited Fstl3 induced cell death, but did not affect fibroblast collagen synthesis. Thus, Fstl3 has been identified as an

important paracrine mediator of the responses of injured myocardium. Figure 9.1 below shows a schematic of the proposed action of Fstl3 in the heart.

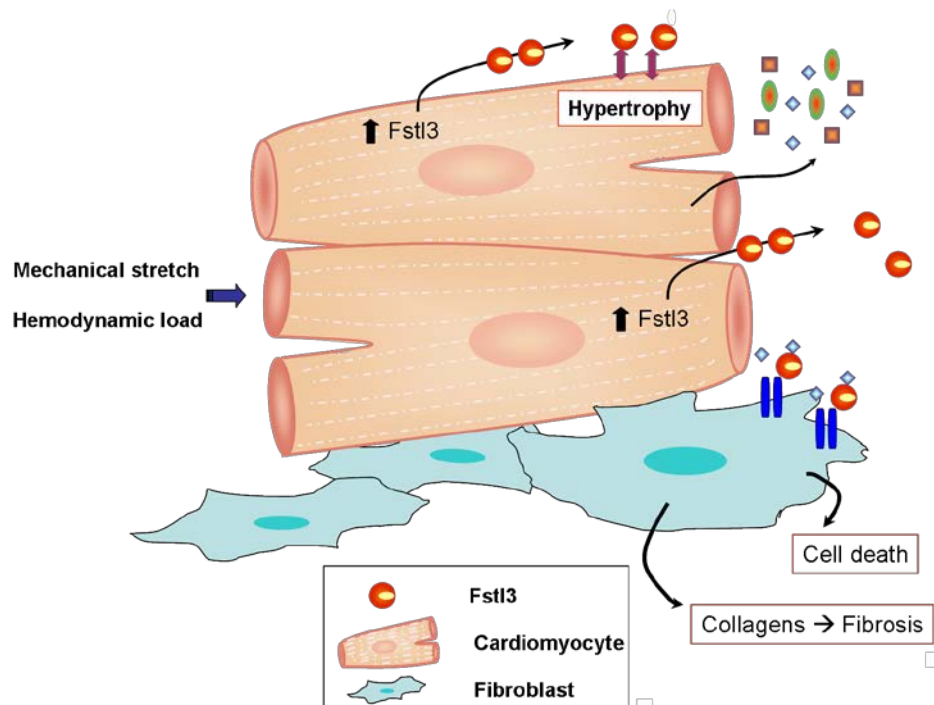


Figure 9.1: Schematic of Fstl3 action in the heart. Fstl3 is secreted from cardiomyocytes following mechanical loading and acts in an autocrine manner to induce cardiomyocyte hypertrophy. In coordination with unknown stretch-derived factors, Fstl3 acts in a paracrine manner on fibroblasts to induce collagen synthesis and cell death.

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Appendix I: TaqMan® Gene Expression assays

TaqMan® Gene Expression assay	Gene name	Assay ID
Human FST	Follistatin	Hs00246260_m1
18S rRNA	18S ribosomal RNA	4310893E
Human FSTL3 (FLRG)	Follistatin-like 3	Hs00610505_m1
Human INHA (Inhibin A)	Inhibin alpha	Hs0017410_m1
Human INHBA (Activin A)	Inhibin beta A	Hs00170103_m1
Mouse Acadm (MCAD)	Acyl-Coenzyme A dehydrogenase, medium chain	Mm00431611_m1
Mouse Acta1	Actin, alpha 1, skeletal muscle	Mm00808218_g1
Mouse Acta2 (α -SMA)	Actin, alpha 2, smooth muscle	Mm01546133_m1
Mouse Adam12	A disintegrin and metalloproteinase 12	Mm00475719_m1
Mouse Ccl2 (Mcp-1)	Chemokine (C-C motif) ligand 2	Mm00441242_m1
Mouse Ccl5 (Rantes)	Chemokine (C-C motif) ligand 5	Mm01302428_m1
Mouse CD68 (gp110)	CD68 antigen	Mm03047340_m1
Mouse Colla1	Collagen, type I, alpha 1	Mm00801666_g1
Mouse Col3a1	Collagen, type III, alpha 1	Mm01254476_m1
Mouse Cpt1b	Carnitine palmitoyltransferase 1b, muscle	Mm00487200_m1
Mouse Ctgf (Ccn2)	Connective tissue growth factor	Mm00515790_g1
Mouse Fn1	Fibronectin 1	Mm01256744_m1
Mouse Fstl3 (Flrg)	Follistatin-like 3	Mm00473194_m1
Mouse Igf-1	Insulin-like growth factor 1	Mm00439560_m1
Mouse IL-1b	Interleukin 1 beta	Mm01336189_m1
Mouse IL-6	Interleukin 6	Mm99999064_m1
Mouse Inhba (Activin A)	Inhibin beta A	Mm00434339_m1
Mouse Lox	Lysyl oxidase	Mm00495386_m1
Mouse Mmp2	Matrix metalloproteinase 2	Mm00439498_m1
Mouse Mmp9	Matrix metalloproteinase 9	Mm00442991_m1
Mouse Mt1	Metallothionein 1	Mm00496660_g1
Mouse Myh7 (β -MHC)	Myosin, heavy polypeptide 7, cardiac muscle, beta	Mm00600555_m1
Mouse Nppa (ANP)	Natriuretic peptide precursor type A	Mm01255747_g1
Mouse Ppara	Peroxisome proliferator activated receptor alpha	Mm00440939_m1
Mouse Ppargc1a (PGC1a)	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Mm01208835_m1
Mouse Rgs2	Regulator of G-protein signaling 2	Mm00501385_m1
Mouse Tgfb1	Transforming growth factor, beta 1	Mm01178820_m1
Mouse Timp1	Tissue inhibitor of metalloproteinase 1	Mm00441818_m1
Rat Colla1	Collagen, type I, alpha 1	Rn01463848_m1
Rat Ctgf	Connective tissue growth factor	Rn00573960_g1
Rat Fstl3	Follistatin-like 3	Rn00586203_m1

Appendix II: Antibodies used for western blot analysis

Target protein	Antibody	Dilution used	Catalog #	Company
Phospho - p38	12F8	1 : 1000	4631	Cell Signaling
Phospho - JNK/SAPK	G9	1 : 1000	9255	Cell Signaling
Phospho - Smad 2	138D4	1 : 1000	3108	Cell Signaling
Phospho - Smad 3	C25A9	1 : 1000	9520	Cell Signaling
Phospho - ERK 1/2	197G2	1 : 1000	4377	Cell Signaling
p38		1 : 1000	9212	Cell Signaling
JNK/SAPK		1 : 1000	9252	Cell Signaling
Smad 2/3		1 : 1000	3102	Cell Signaling
ERK1/2	137F5	1 : 1000	4695	Cell Signaling
Fstl3	210725	1 µg/ml	MAB1255	R&D Systems
CTGF	G-14	1 µg/ml	sc-34772	SantaCruz Biotechnology

Appendix III: Yeast two-hybrid reagents

1. Yeast culture media

YPAD medium (500ml)	25 g YPD media 10 mg Adenine sulphate (to give 0.02 %) 400 ml distilled water Autoclave 100 ml 10 % glucose
YPAD agar (500ml)	25 g YPD media 10 mg Adenine sulphate (to give 0.02 %) 17.5 g agar 400 ml distilled water Autoclave 100 ml 10 % glucose
SD Agar – Trp (500ml)	3.35 g yeast nitrogen base without amino acids 0.96 g yeast drop-out medium - Trp 17.5 g agar 400 ml distilled water Autoclave 100 ml 10 % glucose
SD Agar – Leu (500ml)	3.35 g yeast nitrogen base without amino acids 0.8 g yeast drop-out medium -Leu 17.5 g agar 400 ml distilled water Autoclave 100 ml 10 % glucose

SD Agar –His –Trp (1L)	6.7 g yeast nitrogen base without amino acids 20 mg L-uracil 20 mg L-adenine hemisulfate salt 30 mg L-lysine HCL 100 mg L-leucine 35 g agar 800 ml distilled water Autoclave 200 ml 10 % glucose
SD Media –His –Leu (1L):	6.7 g yeast nitrogen base without amino acids 20 mg L-uracil 20 mg L-adenine hemisulfate salt 30 mg L-lysine HCL 20 mg Histidine HCL monohydrate 800 ml distilled water Autoclave 200ml 10 % glucose
SD Media –Trp –Leu (1L)	6.7 g yeast nitrogen base without amino acids 1.54 g Yeast dropout media – Trp –Leu 800 ml distilled water Autoclave 200 ml 10% glucose
SD Agar –His –Leu -Trp (1L)	6.7 g yeast nitrogen base without amino acids 20 mg L-uracil 20 mg L-adenine hemisulfate salt 30 mg L-lysine HCL 35 g agar 800 ml distilled water Autoclave 200 ml 10 % glucose

2. Yeast solutions

10x Lithium acetate (LiAc or LiOAc)	1 M LiAc Adjust pH to 7.5 with dilute acetic acid Autoclave
10x TE	100 mM Tris-HCl, pH 7.5 10 mM EDTA, pH 8.0 Autoclave

50% w/v PEG 3350 solution	50 g PEG (molecular weight 3350) Distilled water up to 100 ml Autoclave
TE-LiAc solution (1x TE, 1x LiAc)	1 ml 10x TE 1 ml 10x LiAc 8 ml sterile water
TE-LiAc-PEG solution (1x TE, 1x LiAc, 40% w/v PEG 3350)	1 ml 10x TE 1 ml 10x LiAc 8 ml 50 % w/v PEG 3350
LiSORB (500ml)	100 mM LiOAc 10 mM Tris-Hcl (pH 8.0) 1 mM EDTA 1 M Sorbitol 500 ml distilled water Verify pH 8.0 Autoclave
10% Glucose (500 ml)	50 g glucose 500 ml autoclaved water

Appendix IV: Microarray Analysis

Table IV.1 (A) WT TAC > WT Sham: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Induction</i>	<i>Gene symbol</i>	<i>Description</i>
10398075	10.00	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N
10598976	7.67	Timp1	tissue inhibitor of metalloproteinase 1
10492021	7.58	Postn	periostin, osteoblast specific factor
10419934	6.17	Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta
10411082	5.30	Thbs4	thrombospondin 4
10458894	4.99	Lox	lysyl oxidase
10541496	4.83	Mfap5	microfibrillar associated protein 5
10481627	4.81	Lcn2	lipocalin 2
10582592	4.73	Acta1	actin, alpha 1, skeletal muscle
10440091	4.73	Col8a1	collagen, type VIII, alpha 1
10510265	4.57	Nppa	natriuretic peptide precursor type A
10401527	4.56	Ltbp2	latent transforming growth factor beta binding protein 2
10574023	4.36	Mt2	metallothionein 2
10417212	3.73	Itgb1	integrin, beta-like 1
10355403	3.51	Fn1	fibronectin 1
10416181	3.51	Stc1	stanniocalcin 1
10462442	3.41	Il33	interleukin 33
10435641	3.23	Fstl1	follicle-stimulating-like 1
10534667	3.21	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1
10484307	3.19	Frzb	frizzled-related protein
10513739	3.06	Tnc	tenascin C
10572897	3.03	Hmox1	heme oxygenase (decycling) 1
10478048	2.99	Lbp	lipopolysaccharide binding protein
10410931	2.91	Vcan	versican
10362201	2.89	Ctgf	connective tissue growth factor
10513208	2.86	Svep1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1
10360920	2.85	Tgfb2	transforming growth factor, beta 2
10380419	2.85	Col1a1	collagen, type I, alpha 1
10424140	2.80	Col14a1	collagen, type XIV, alpha 1
10595211	2.77	Col12a1	collagen, type XII, alpha 1
10570857	2.60	Sfrp1	secreted frizzled-related protein 1
10536220	2.53	Col1a2	collagen, type I, alpha 2
10586865	2.51	Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2
10540085	2.50	Fbln2	fibulin 2
10416215	2.43	Loxl2	lysyl oxidase-like 2
10375751	2.42	Adamts2	a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 2
10492798	2.37	Sfrp2	secreted frizzled-related protein 2
10506714	2.35	Lrp8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
10381962	2.35	Ace	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1

Table IV.1 (B) WT TAC > WT Sham: Gene Ontology

<i>GO ID</i>	<i>GO Term</i>	<i>p</i>	<i>n</i>	<i>q</i>
GO:0005488	binding	2.20E-13	337	58.10
GO:0018020	membrane	9.42E-07	295	50.86
GO:0005515	protein binding	5.54E-13	215	37.07
GO:0009987	cellular process	6.59E-04	187	32.24
GO:0005576	extracellular region	0.00E+00	184	31.72
GO:0043167	ion binding	2.54E-03	145	25.00
GO:0046872	metal ion binding	7.06E-03	143	24.66
GO:0005886	plasma membrane	4.82E-08	119	20.52
GO:0065007	biological regulation	7.54E-03	112	19.31
GO:0044421	extracellular region part	1.12E-37	107	18.45
GO:0032502	developmental process	2.39E-12	81	13.97
GO:0050896	response to stimulus	2.92E-12	81	13.97
GO:0007275	multicellular organismal development	3.56E-11	80	13.79
GO:0031012	extracellular matrix	5.60E-39	71	12.24
GO:0005578	proteinaceous extracellular matrix	4.57E-36	66	11.38
GO:0007155	cell adhesion	2.88E-13	61	10.52
GO:0022610	biological adhesion	2.88E-13	61	10.52
GO:0005509	calcium ion binding	2.97E-06	61	10.52
GO:0002376	immune system process	3.34E-14	53	9.14
GO:0048731	system development	3.54E-11	51	8.79

Table IV.2 (A) WT TAC < WT Sham: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Reduction</i>	<i>Gene symbol</i>	<i>Description</i>
10580635	7.04	Ces3	carboxylesterase 3
10454782	3.17	Egr1	early growth response 1
10441794	3.02	Mrgprh	MAS-related GPR, member H
10511363	2.95	Penk	preproenkephalin
10408543	2.83	Mylk4	myosin light chain kinase family, member 4
10428376	2.80	Angpt1	angiopoietin 1
10483000	2.76	Itgb6	integrin beta 6
10473444	2.68	Aplnr	apelin receptor
10556463	2.45	Arntl	aryl hydrocarbon receptor nuclear translocator-like
10442584	2.43	Rpl3l	ribosomal protein L3-like
10355893	2.38	Epha4	Eph receptor A4
10512145	2.35	Aqp7	aquaporin 7
10363224	2.30	Fabp7	fatty acid binding protein 7, brain
10602385	2.30	Pfkfb1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
10517655	2.29	Pla2g5	phospholipase A2, group V
10579958	2.24	Il15	interleukin 15
10492355	2.23	Mme	membrane metallo endopeptidase
10484190	2.16	Ttn	titin
10560481	2.15	Fosb	FBJ osteosarcoma oncogene B
10534085	2.15	Phkg1	phosphorylase kinase gamma 1
10472155	2.12	Kcnj3	potassium inwardly-rectifying channel, subfamily J, member 3
10409999	2.12	Fbp2	fructose biphosphatase 2
10462303	2.11	Kcnv2	potassium channel, subfamily V, member 2
10460556	2.08	Gal3st3	galactose-3-O-sulfotransferase 3
10374777	2.08	Efemp1	epidermal growth factor-containing fibulin-like extracellular matrix protein 1

Table IV.2 (B) WT TAC < WT Sham: Gene Ontology

<i>GO ID</i>	<i>GO Term</i>	<i>p</i>	<i>n</i>	<i>q</i>
GO:0005622	intracellular	8.16E-03	185	86.45
GO:0005737	cytoplasm	1.44E-04	167	78.04
GO:0044424	intracellular part	5.46E-03	167	78.04
GO:0043227	membrane-bounded organelle	4.70E-03	89	41.59
GO:0043231	intracellular membrane-bounded organelle	4.70E-03	89	41.59
GO:0044444	cytoplasmic part	1.93E-04	87	40.65
GO:0005739	mitochondrion	1.30E-12	81	37.85
GO:0008152	metabolic process	1.93E-04	44	20.56
GO:0003824	catalytic activity	3.46E-04	44	20.56
GO:0005743	mitochondrial inner membrane	9.02E-04	23	10.75
GO:0019866	organelle inner membrane	1.64E-03	23	10.75
GO:0031966	mitochondrial membrane	4.70E-03	23	10.75
GO:0044429	mitochondrial part	5.42E-03	23	10.75
GO:0005740	mitochondrial envelope	6.78E-03	23	10.75
GO:0005777	peroxisome	9.01E-05	14	6.54
GO:0042579	microbody	9.01E-05	14	6.54
GO:0006082	organic acid metabolic process	3.68E-06	10	4.67
GO:0006631	fatty acid metabolic process	3.68E-06	10	4.67
GO:0019752	carboxylic acid metabolic process	3.68E-06	10	4.67
GO:0032787	monocarboxylic acid metabolic process	3.68E-06	10	4.67

Table IV.3 (A) KO Sham > WT Sham: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Induction</i>	<i>Gene symbol</i>	<i>Description</i>
10373452	3.43	Gm129	predicted gene 129
10481627	2.99	Lcn2	lipocalin 2
10566254	2.75	Hbb-b1	hemoglobin, beta adult major chain
10553092	2.61	Dbp	D site albumin promoter binding protein
10523128	2.39	Ppbb	pro-platelet basic protein
10574023	2.38	Mt2	metallothionein 2
10538802	2.32	A930038C07Rik	RIKEN cDNA A930038C07 gene
10358389	2.09	Rgs2	regulator of G-protein signaling 2
10384458	2.03	Plek	pleckstrin
10534667	2.01	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1
10583100	1.99	Mmp8	matrix metalloproteinase 8
10496438	1.87	Adh1	alcohol dehydrogenase 1 (class I)
10347291	1.84	Cxcr2	chemokine (C-X-C motif) receptor 2
10479154	1.83	Tubb1	tubulin, beta 1
10416181	1.79	Stc1	stanniocalcin 1
10380887	1.78	Tcap	titin-cap
10545086	1.76	Snca	synuclein, alpha
10350516	1.70	Ptgs2	prostaglandin-endoperoxide synthase 2
10457168	1.66	Cd226	CD226 antigen
10558961	1.66	Tspan4	tetraspanin 4
10555378	1.65	Ucp3	uncoupling protein 3 (mitochondrial, proton carrier)
10487597	1.63	Il1b	interleukin 1 beta
10523156	1.61	Cxcl2	chemokine (C-X-C motif) ligand 2

Table IV.3 (B) KO Sham > WT Sham: Gene Ontology

<i>GO ID</i>	<i>GO Term</i>	<i>p</i>	<i>n</i>	<i>q</i>
GO:0005576	extracellular region	1.75E-05	31	65.96
GO:0048511	rhythmic process	2.41E-09	9	19.15
GO:0007623	circadian rhythm	3.61E-06	7	14.89
GO:0050896	response to stimulus	4.78E-06	5	10.64
GO:0005833	hemoglobin complex	1.67E-04	4	8.51
GO:0019825	oxygen binding	8.70E-04	4	8.51
GO:0005344	oxygen transporter activity	1.27E-03	4	8.51
GO:0015671	oxygen transport	1.27E-03	4	8.51
GO:0015669	gas transport	1.58E-03	4	8.51
GO:0006950	response to stress	2.89E-03	2	4.26
GO:0042221	response to chemical stimulus	6.92E-06	1	2.13

Table IV.4 (A) KO Sham < WT Sham: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Reduction</i>	<i>Gene symbol</i>	<i>Description</i>
10556463	3.22	Arntl	aryl hydrocarbon receptor nuclear translocator-like
10484190	2.24	Ttn	titin
10571599	2.19	Sorbs2	Sorbin and SH3 domain containing 2
10355998	1.83	Fam124b	family with sequence similarity 124, member B
10345675	1.83	Npas2	neuronal PAS domain protein 2
10540059	1.82	Slc41a3	solute carrier family 41, member 3
10363696	1.80	D630028G08Rik	RIKEN cDNA D630028G08 gene
10375402	1.75	Adam19	a disintegrin and metallopeptidase domain 19 (meltrin beta)
10473444	1.75	Aplnr	apelin receptor
10389581	1.59	Ypel2	yippee-like 2 (Drosophila)
10356084	1.57	Irs1	insulin receptor substrate 1
10363224	1.57	Fabp7	fatty acid binding protein 7, brain
10489053	1.56	4930518I15Rik	RIKEN cDNA 4930518I15 gene
10411459	1.54	Tmem171	transmembrane protein 171
10600604	1.53	Dmd	dystrophin, muscular dystrophy
10546450	1.52	Adamts9	a disintegrin-like and metallopeptidase (repolysin type) with thrombospondin type 1 motif, 9
10435075	1.50	Tfrc	transferrin receptor
10529264	1.46	Spon2	spondin 2, extracellular matrix protein
10497399	1.46	Pde7a	phosphodiesterase 7A
10510260	1.43	Nppb	natriuretic peptide precursor type B
10451198	1.43	Vegfa	vascular endothelial growth factor A
10485198	1.41	Tspan18	tetraspanin 18
10440993	1.41	Rcan1	regulator of calcineurin 1

Table IV.4 (B) KO Sham < WT Sham: Gene Ontology

<i>GO ID</i>	<i>GO Term</i>	<i>p</i>	<i>n</i>	<i>q</i>
GO:0048511	rhythmic process	8.13E-04	3	100.00

Table IV.5 (A) KO TAC > KO Sham: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Induction</i>	<i>Gene symbol</i>	<i>Description</i>
10510260	3.0078108	Nppb	natriuretic peptide precursor type B
10418934	2.865751	Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta
10582592	2.718127	Acta1	actin, alpha 1, skeletal muscle
10510265	2.5672863	Nppa	natriuretic peptide precursor type A
10411082	2.5555682	Thbs4	thrombospondin 4
10440091	2.5055985	Col8a1	collagen, type VIII, alpha 1
10492021	2.3362432	Postn	periostin, osteoblast specific factor
10541496	2.3086636	Mfap5	microfibrillar associated protein 5
10557300	2.2028258	Aqp8	aquaporin 8
10440993	2.0089958	Rcan1	regulator of calcineurin 1
10598976	1.9434552	Timp1	tissue inhibitor of metalloproteinase 1
10484307	1.9337001	Frzb	frizzled-related protein
10487040	1.9118756	Fbn1	fibrillin 1
10401527	1.8797361	Ltbp2	latent transforming growth factor beta binding protein 2
10371082	1.8671631	Itgb1bp3	integrin beta 1 binding protein 3
10435641	1.8326782	Fstl1	folliculin-like 1
10428370	1.780454	Abra	actin-binding Rho activating protein
10417212	1.7499126	Itgbl1	integrin, beta-like 1
10416215	1.7395872	Loxl2	lysyl oxidase-like 2
10504775	1.7298125	Col15a1	collagen, type XV, alpha 1
10362201	1.6256888	Ctgf	connective tissue growth factor
10360920	1.625665	Tgfb2	transforming growth factor, beta 2
10380419	1.6237258	Col1a1	collagen, type I, alpha 1

Table IV.5 (B) KO TAC > KO Sham: Gene Ontology

<i>GO ID</i>	<i>GO Term</i>	<i>p</i>	<i>n</i>	<i>q</i>
GO:0005488	binding	5.06E-06	60	73.17
GO:0005515	protein binding	5.66E-07	52	63.41
GO:0005576	extracellular region	3.77E-20	49	59.76
GO:0044421	extracellular region part	7.05E-19	31	37.80
GO:0031012	extracellular matrix	1.18E-26	31	37.80
GO:0005578	proteinaceous extracellular matrix	6.22E-25	29	35.37
GO:0032501	multicellular organismal process	9.77E-03	20	24.39
GO:0032502	developmental process	7.20E-06	20	24.39
GO:0007275	multicellular organismal development	2.19E-07	20	24.39
GO:0007155	cell adhesion	7.41E-10	19	23.17
GO:0022610	biological adhesion	7.41E-10	19	23.17
GO:0048856	anatomical structure development	1.23E-05	12	14.63
GO:0048731	system development	1.55E-05	12	14.63
GO:0048513	organ development	1.53E-05	12	14.63
GO:0005198	structural molecule activity	3.69E-05	12	14.63
GO:0044420	extracellular matrix part	1.48E-13	12	14.63
GO:0001944	vasculature development	6.48E-04	10	12.20
GO:0001568	blood vessel development	5.40E-04	10	12.20
GO:0043062	extracellular structure organization and biogenesis	8.25E-05	8	9.76
GO:0030198	extracellular matrix organization and biogenesis	6.76E-05	8	9.76

Table IV.6 (A) KO TAC < KO Sham: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Reduction</i>	<i>Gene symbol</i>	<i>Description</i>
10574434	2.58	Gm4638/Gm4671	predicted gene 4638/predicted gene 4671
10468487	2.58	LOC280487	pol polyprotein
10373452	2.51	Gm129	predicted gene 129
10358389	2.42	Rgs2	regulator of G-protein signaling 2
10580635	2.25	Ces3	carboxylesterase 3
10602385	2.21	Pfkfb1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
10555378	2.18	Ucp3	uncoupling protein 3 (mitochondrial, proton carrier)
10523128	2.16	Ppbp	pro-platelet basic protein
10427035	2.00	Nr4a1	nuclear receptor subfamily 4, group A, member 1
10428376	1.94	Angpt1	angiopoietin 1
10560481	1.94	Fosb	FBJ osteosarcoma oncogene B
10544932	1.92	Inmt	indolethylamine N-methyltransferase
10397346	1.90	Fos	FBJ osteosarcoma oncogene
10379524	1.89	Ccl11	chemokine (C-C motif) ligand 11
10377439	1.88	Per1	period homolog 1 (Drosophila)
10511363	1.85	Penk	preproenkephalin
10500272	1.85	Gm129	predicted gene 129
10480459	1.85	Hnmt	histamine N-methyltransferase
10565315	1.84	Fah	fumarylacetoacetate hydrolase
10553092	1.84	Dbp	D site albumin promoter binding protein
10428809	1.81	Klhl38	kelch-like 38 (Drosophila)
10374777	1.81	Efemp1	epidermal growth factor-containing fibulin-like extracellular matrix protein 1

Table IV.6 (B) KO TAC < KO Sham: Gene Ontology

<i>GO ID</i>	<i>GO Term</i>	<i>p</i>	<i>n</i>	<i>q</i>
GO:0004014	adenosylmethionine decarboxylase activity	7.04E-03	3	100.00

Table IV.7 (A) KO TAC > WT TAC: Representative Genes

<i>Affymetrix ID</i>	<i>Fold Induction</i>	<i>Gene symbol</i>	<i>Description</i>
10580635	3.37	Ces3	carboxylesterase 3
10473444	2.81	Aplnr	apelin receptor
10558694	2.41	Scgb1c1	secretoglobin, family 1C, member 1
10454782	2.31	Egr1	early growth response 1
10512145	2.07	Aqp7	aquaporin 7
10408543	1.97	Mylk4	myosin light chain kinase family, member 4
10483000	1.95	Itgb6	integrin beta 6
10366153	1.90	Rassf9	Ras association (RalGDS/AF-6) domain family (N-terminal) member 9
10511363	1.87	Penk	preproenkephalin
10517655	1.81	Pla2g5	phospholipase A2, group V
10491091	1.78	Tnfrsf10	tumor necrosis factor (ligand) superfamily, member 10
10442584	1.78	Rpl3l	ribosomal protein L3-like
10367591	1.78	Myct1	myc target 1
10494180	1.73	Tmod4	tropomodulin 4
10492002	1.73	Gm10727	predicted gene 10727
10358421	1.73	Rgs18	regulator of G-protein signaling 18
10530692	1.72	Kdr	kinase insert domain protein receptor
10356880	1.72	St8sia4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4
10374777	1.71	Efemp1	epidermal growth factor-containing fibulin-like extracellular matrix protein 1
10556463	1.71	Arntl	aryl hydrocarbon receptor nuclear translocator-like