

**REGULATION OF CARDIAC REMODELLING BY ADIPONECTIN IN
RESPONSE TO PRESSURE OVERLOAD AND UNLOADING:
A FOCUS ON THE EXTRACELLULAR MATRIX**

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ABSTRACT

Cardiac remodelling, the reorganization of the heart which occurs in response to factors impacting its function, includes remodelling of the extracellular matrix (ECM) and hypertrophic cardiomyocyte growth. Pressure overload (PO) induced remodelling of the ECM is initially considered a compensatory mechanism to maintain myocardial integrity, but is also considered a progressive, negative event increasing myocardial stiffness. Adiponectin, an adipokine inversely correlated with type 2 diabetes and obesity, plays an important role in the adaptive response of the heart in various cardiomyopathies, however, adiponectin signalling leading to ECM regulation remains unclear. The studies presented here investigate the role of adiponectin in regulating cardiac remodelling with a particular focus on the ECM from a physiological and mechanistic perspective.

Studies using wild-type (WT) and adiponectin deficient (AdKO) mice showed that PO induced left ventricular (LV) cardiac remodelling is delayed by adiponectin deficiency. The appearance of thick collagen fibres and activation of pro-fibrotic genes (MMPs and TIMPs) is delayed in AdKO mice subjected to PO when compared to WT mice. Cardiac hypertrophy and dysfunction, measured by echocardiography, is similarly delayed in AdKO mice. Furthermore, MEF2 activation determined using MEF2-lacZ reporter mice, is decreased in AdKO mice compared to WT mice following PO.

Studies in primary neonatal cardiac fibroblasts identified the APPL1-AMPK signalling axis as the mediator of adiponectin stimulated ECM remodelling through membrane localization of APPL1 and subsequent phosphorylation of AMPK, leading to MT1-MMP re-localization, MMP2 activation, and fibroblast migration. Also, adiponectin pre-treatment inhibited angiotensin II induced fibroblast to myofibroblast differentiation. Furthermore, in primary neonatal cardiomyocytes we identify the hypertrophic regulators Myocyte Enhancing Factor-2 (MEF2) and Atrial Natriuretic Factor (ANF) as downstream targets of adiponectin signalling.

Lastly, using an in vivo model of reverse remodeling, we show that myocardial strain and cardiac hypertrophy are regressed following LV unloading. However, regression of cardiac fibrosis was incomplete leading to persistent small fibre fibrosis.

Together these studies establish adiponectin as an important regulator of cardiac remodelling via the APPL1-AMPK signalling axis and MEF2 activation. Furthermore, we show that adiponectin deficiency confers protection against PO induced remodelling.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ACC	Acetyl-CoA carboxylase
ACE inhibitors	Angiotensin converting enzyme inhibitors
ACM	Adult cardiomyocytes
AdipoR1	Adiponectin Receptor 1
AdipoR2	Adiponectin Receptor 2
AdKO	Adiponectin knockout mouse
AdWT	Wild-type mouse
AGE	Advanced glycation end products
AMPK	AMP –activated protein kinase
ANF	Atrial natriuretic factor
AngII	Angiotensin II
ANP	Atrial natriuretic peptide
APPL1	Adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif
ARB	Angiotensin II receptor antagonists
ATP	Adenosine tri-phosphate
BMI	Body mass index
BNP	Brain natriuretic peptide
CaMKII	Ca ²⁺ /calmodulin–dependent kinase

CD36	Fatty acid translocase
CF	Cardiac fibroblasts
CH	Concentric hypertrophy
CHF	Congestive heart failure
CNP	C-type natriuretic peptide
CO	Cardiac output
Col I	Collagen type I
Col III	Collagen type III
Col IV	Collagen type IV
Col V	Collagen type V
Col VI	Collagen type VI
CPT1	Carnitine palmitoyltransferase-1
CVD	Cardiovascular Disease
DBCM	Diabetic Cardiomyopathy
db/db mice	Leptin receptor deficient mice
DO	Diastolic overload
DsbA-L	Disulfide bond A oxidoreductase-like protein
ECM	Extracellular matrix
EDD	End diastolic diameter
EDV	End diastolic volume
EF	Ejection fraction

EH	Eccentric hypertrophy
EMT	Epithelial-to-mesenchymal cell transition
EndMT	Endothelial-to-mesenchymal cell transition
ERK	Extracellular Receptor Tyrosine Signalling Kinase
Erp44	Endoplasmic reticulum protein 44
ESD	End systolic diameter
ESV	End systolic volume
ET-1	Endothelin-1
FA	Fatty Acids
FABP3	Fatty acid binding protein 3
fAd	Full length isoforms of adiponectin
FoxO	FoxO (forkhead box proteins O)
FTMI	Fibrosis tissue mass index
gAd	Globular domain of adiponectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
GPCR	G-protein coupled receptors
HDAC	Histone deacetylase
HF	Heart failure
HG	Hyperglycaemia
HI	Hyperinsulinemia

HMW	High molecular weight isoform of adiponectin
IL-1 β , IL-6	Interlukin - 1 β , Interlukin-6
IRS-1	Insulin Receptor Substrate – 1
JNK	c-Jun N-terminal kinases
LMW	Low molecular weight isoform of adiponectin
LV	Left ventricle
LVAD	Left ventricular assist device
LVH	Left ventricular hypertrophy
LVPWd	LV posterior wall dimension in diastole
MEF2	Myocyte enhancing factor 2
MEK	MAPK/ERK kinase
MMP	Matrix metalloproteinase
MMW	Medium molecular weight isoform of adiponectin
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane bound type – 1 MMP
mTOR	Mamalian target of rapamycin
myoCF	Myofibroblasts
NFAT	Calcineurin-nuclear factor of activated T-cells
NFF	Natriuretic factor family
NF- κ B	Nuclear factor- κ B
NIDMM	Non-Insulin Dependent Diabetes Mellitus

p38-MAPK	p38 mitogen-activated protein kinase
PARP-1	poly (ADP-ribose) polymerase-1
PDI	Protein disulfide bond isomerise
PDK	Pyruvate dehydrogenate kinase
PI3K	Phosphoinositide 3-kinase
PKB	Protein Kinase B
PO	Pressure overload
POH	Pressure overload induced hypertrophy
PPAR α	Peroxisome Proliferator Activated Receptor – α
PPAR γ	Peroxisome Proliferator Activated Receptor – γ
PTB	Phosphotyrosine binding domain
RAAS	Renin Angiotensin Aldosterone System
ROS	Reactive Oxygen Species
SK α -actin	Skeletal muscle α -actin
SO	Systolic overload
SREBP	Sterol regulatory element binding protein
STZ	Streptozotocin
SV	Stroke volume
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGF β	Transforming growth factor β

TIMP	Tissue inhibitors of matrix metalloproteinase
TNF α	Tumour necrosis factor – α
TZD	Thiazolidinedione
VO	Volume overload
VOH	Volume overload induced hypertrophy
α -MHC	α -myosin heavy chain
β -MHC	β -myosin heavy chain

CHAPTER 1: REVIEW OF LITERATURE

Excerpts from this chapter are taken from Dadson K, Liu Y, and Sweeney G. *Adiponectin action: a combination of endocrine and autocrine/paracrine effects*. Front Endocrinol (Lausanne), 2011. **2**: p.62.

1.1 Diabetes and Cardiovascular Disease

1.1.1 Prevalence and Impact of Diabetes in Canada

Diabetes is a major chronic disease in both Canada and the United States, as 2.4 million Canadians [1] and 25 million Americans [2] currently live with the disease, accounting for 6.8% of the Canadian, and 8% of the American population in 2013. Alarmingly, approximately 5.0 million Canadians and nearly 70 million Americans are currently classified as pre-diabetic (having a >30% chance of developing diabetes over their lifetime), and this number is commonly predicted to rise significantly. The impact of the rise of diabetes on our health care system is significant. The most recent available data estimates the total cost of diabetes in the year 2000 was \$2.5 billion dollars [1]. When including costs associated with long-term complications (cardiovascular disease, kidney and eye disease, neurological disorders), it is estimated that the cost of diabetes increases 3.6 fold. Indeed, in addition to the increased prevalence of diabetes with age, the treatment and management of diabetic co-morbidities such as cardiovascular disease (CVD) represents the major leading expenditure for diabetic care.

1.1.2 Types of Diabetes and the Metabolic Syndrome

The group of metabolic pathologies termed *diabetes* encompasses a family of diseases of which *diabetes mellitus* is the most common and widely studied sub-group. Diabetes mellitus is characterized by high circulating levels of blood glucose leading

to polydipsia (increased thirst), polyuria (excessive urination) and glucosuria (glucose excretion through the urine), while by contrast *diabetes insipidus* caused by kidney dysfunction or a deficiency in anti-diuretic hormone resulting in excessive urination without glucosuria or insulin resistance [3]. Diabetes mellitus is divided into 4 subgroups: Type 1 Diabetes, also termed juvenile diabetes or insulin-dependent diabetes mellitus; Type 2 Diabetes, also referred to as adult-onset diabetes, or non-insulin dependent diabetes mellitus (NIDDM); gestational diabetes, a variant which occurs during pregnancy, and may precede the development of NIDMM; other rare forms of hyperglycemia due to genetic defects in insulin secretion, treatment with high doses of glucocorticoids, or disorders such as cystic fibrosis.

Type 2 diabetes (T2D) is the most common form of the disease, responsible for approximately 90% of the diabetic population worldwide, largely due to the obesity epidemic. The number of obese individuals around the world is growing at a staggering rate [4-7], and the population of Canada is not exempt: by 2007 it was estimated that 32% of Canadian adults were considered overweight, with 16% considered obese having a BMI ≥ 30 [8]. With the number of obese individuals on the rise globally, an alarming number of clinical studies have suggested a causal relationship between obesity and cardiovascular disease [9-12] in both men and women [13]. Given the close connection between obesity, insulin resistance, and heart function [14, 15] the term 'The Metabolic Syndrome' (and the related cardiometabolic syndrome), was coined to define the constellation of risk factors which greatly increase

the risk of T2D and CVD (table 1). The link between T2D and CVD, outlined by the now foundational Framingham study, is well accepted: diabetic patients have a 2 to 4 fold higher chance of developing heart disease, while nearly 70% of diabetes related deaths are due to CVD. As such, diagnosis of individuals with the metabolic syndrome accounts for approximately 15% of Canadians [1], and as many as 23% of Americans [16]. First described by Rubler et al in 1972, myocardial dysfunction in patients with diabetes (in the absence of coronary artery disease, hypertension, or valvular heart disease) has been termed *diabetic cardiomyopathy* (DCM) and has gained attention as a major non-ischemic heart disease [17].

Table 1.1: Parameters of the metabolic syndrome.	
The metabolic syndrome may be diagnosed when 3 or more of the following are present.	
Adapted from [1]	
Elevated Waist Circumference	≥102 cm in males; ≥88 cm in females
Reduced high-density lipoprotein (HDL) cholesterol	<1.0 mmol/L in males; <1.3 mmol/L in females
Elevated triglycerides	≥1.7 mmol/L
Elevated blood pressure	systolic ≥130 mmHg and/or diastolic ≥85 mmHg
Elevated fasting plasma glucose	≥5.6 mmol/L

1.1.3 Diabetic Cardiomyopathy

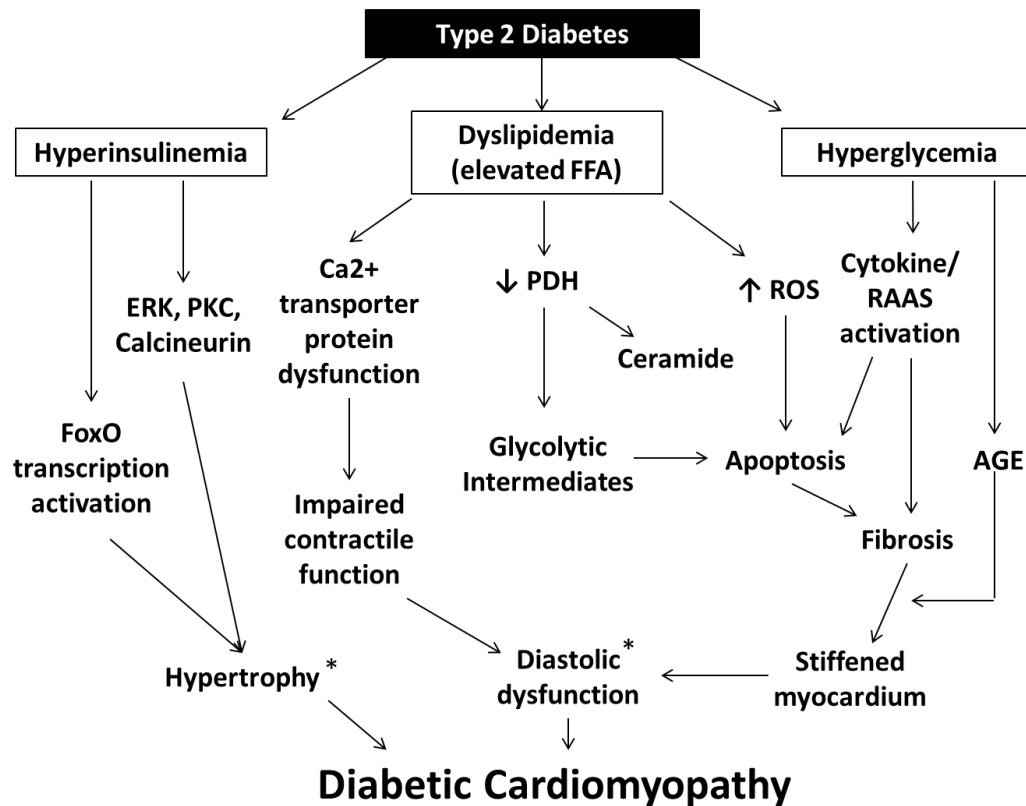
The hyperglycaemic (HG), hyperinsulinemic (HI), and dyslipidemic environment in T2D has direct and severe effects on cardiac function and structure (figure 1.1). The association between HI and cardiac hypertrophy was the subject of early clinical studies [18, 19] and is now well established, perhaps due to the cross-talk between insulin signalling and growth. Indeed, insulin signalling has been

implicated in both physiological (PI3K/PKB/Akt/mTOR) and pathological (ERK, PKC, calcineurin) hypertrophy [20, 21], as well as activation of the FoxO (forkhead box proteins O) family of transcription factors. In fact, FoxO proteins are now established as critical regulators of insulin sensitivity, glucose metabolism, and cardiac hypertrophy [22], where high fat diet induced hyperinsulinemia and cardiac hypertrophy are associated with chronic FoxO activation leading to heart failure [23]. Increased circulating levels of fatty acids (FA) is a persistent finding in T2DM and DBCM patients, and is associated with impaired cardiomyocyte metabolism as myocyte FA uptake and oxidation fail to counter balance increased adipocyte lipolysis and hepatocyte lipid synthesis, leading to oxidative stress [15]. Stressed oxidative capacity and lipid accumulation lead to ceramide production and ROS generation in parallel with IRS-1 (insulin receptor substrate) serine phosphorylation, resulting in insulin resistance [24]. The normal heart derives 70% of its performance energy from the oxidation of fatty acids. Hemodynamic stress is known to induce a switch in energy substrate to glucose [25], although hyperglycaemia is less efficiently handled by mitochondria in the heart, resulting in higher ROS production, activation of poly (ADP-ribose) polymerase-1 (PARP-1), and the apoptosis cascade [24]. PARP-1 activity itself suppresses GAPDH activity, leading to multiple pathways resulting in cellular damage: advanced glycation end product (AGE) accumulation, the polyol pathway, protein kinase C activation, and overactivity of the hexosamine pathway [26]. AGE accumulation in the myocardium is closely associated with collagen cross-linking, and HG induced oxidative stress also leads to increased collagen deposition [27,

28], myocardial fibrosis [29], and diastolic stiffness through ERK1/2 signalling [30, 31].

Angiotensin II, a strong marker of cardiac dysfunction and member of the renin-angiotensin-aldosterone system (RAAS) signalling pathway, is closely associated with many features of diabetic cardiomyopathy [15]. Angiotensin receptor density is increased in the diabetic heart [32] and was shown to induce insulin resistance, and stimulate the switch in cardiac substrate utilization through increased PDK expression [33]. Importantly, activation of the local RAAS in DBCM may induce oxidative damage, cardiomyocyte apoptosis, and cardiac fibrosis [34]. However, cardiomyocyte derived IGF-I can downregulate local AngII expression and cardiomyocyte apoptosis [15]. Therapeutically, AngII stimulated cardiac hypertrophy and fibrosis is attenuated by pioglitazone, a PPAR γ targeting anti-diabetic therapy that has been extensively used in DBCM patients [35].

The toxic diabetic milieu of dyslipidemia, HG, and HI leading to cardiac hypertrophy, metabolic disturbance, apoptosis, and cardiac fibrosis, present clinically as cardiac dysfunction, and ultimately lead to heart failure. Within this complex environment, the dysregulation of numerous circulating factors such as the cardio-important adipokine adiponectin plays an important role in the etymology of DBCM.



* = clinical diagnostic of DBCM

Figure 1.1: Progression of hyperinsulinemia, dyslipidemia, and hyperglycemia leading to diabetic cardiomyopathy.

Adapted from [36]. Cardiac fibrosis and hypertrophy are central features of the diabetic heart. Diastolic dysfunction may be an important early screening metric for DBCM [37].

1.2 Adiponectin

1.2.1 Regulation of expression and post translational modification

Amongst the numerous adipose derived factors (adipokines), adiponectin has gained considerable interest with respect to DBCM due to its anti-diabetic and cardioprotective effects [38]. The concentration of adiponectin in circulation is relatively high in normal individuals (~2 to 17 ug/ml) [39-41], and has shown an

inverse relationship with the progression of obesity, type 2 diabetes, and the metabolic syndrome [42-44]. First identified by four independent groups [45-48], the adiponectin monomer is the 30 kDa protein product of the *APM1* gene mapped to human chromosome 3q27, a diabetes-susceptible locus [49], under transcriptional regulation by peroxisome proliferator activated receptor γ (PPAR γ), CCAAT/enhancer binding protein (C/EBP), sterol regulatory element-binding protein 1c (SREBP), E47, and Id3 protein, of which PPAR γ is the best characterized.

PPARs are ligand-activated transcription factors belonging to the nuclear family of receptors [50]. PPAR γ activity significantly increases adiponectin levels in circulation, while PPAR γ deletion reduces circulating adiponectin levels [51, 52], ablating adiponectin's anti-diabetic and cardioprotective effects [53, 54]. Accordingly, the thiazolidinedione (TZD) class of drugs (e.g. rosiglitazone and pioglitazone) are commonly prescribed treatments for T2D as a means to enhance insulin sensitivity and lower blood glucose levels and they work at least in part via increasing adiponectin levels through PPAR γ activation [55-57].

C/EBP α belongs to the basic lucine zipper family which plays a role in adipogenesis, and has been shown to regulate adiponectin expression either alone or in conjunction with PPAR γ [58]. SREBP overexpression upregulates adiponectin, but little is known of its role in DBCM, although SREBP-1c may play a role in adipogenesis and the cardiac parasympathetic response [59].

The downregulation of adiponectin in obesity, T2D, and cardiovascular disease is largely attributed to the increased inflammatory state and corresponding upregulation of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF α) although the transcription factors CREB and NFAT have been also identified as repressors of adiponectin gene expression [60]. cAMP response element binding protein, CREB, which contributes to hyperglycaemia and insulin resistance through the regulation of glucose homeostasis, inhibits adiponectin transcription through upregulation of ATF3, a transcriptional repressor. Adipose tissue specific overexpression of a dominant-negative CREB increases adiponectin levels independent of resistin, TNF α , and IL-1 β [60, 61].

NFAT (nuclear factor of activated T-cells) are a family of transcription factor proteins found in adipose tissue that are upregulated in obesity and T2D. Deletion or overexpression of the NFAT binding site in the mouse adiponectin promoter has an inverse effect on promoter activity [62].

TNF α , an acute-phase pro-inflammatory cytokine responsible for the regulation of immune cell function, is commonly cited as a down regulator of adiponectin expression. TNF α suppresses gene expression of numerous upstream adiponectin expression activators including PPAR γ , C/EBP, and SREBP [60], as well as inhibiting SP-1 promoter binding [63], thus decreasing adiponectin expression. TNF α may also acutely suppress adiponectin expression through PPAR γ

phosphorylation, leading to decreased PPAR γ binding activity, via JNK/PKC signalling [60].

The physiological effects of adiponectin may also be regulated post-translationally. Structurally, the highly conserved 244-amino-acid adiponectin monomer belongs to a soluble collagen defense superfamily, including collagens type VII and X, and complement factor C1q [46], and is composed of a signal peptide, a variable N-terminal domain, a collagen-like domain and a globular domain at the C-terminus [64]. The adiponectin protein must undergo post-translational modification before secretion: four conserved proline residues are hydroxylated and five lysine residues are hydroxylated then glycosylated before further processing to form adiponectin multimers [65]. Adiponectin oligomerization is now considered a critical step in the adiponectin production pathway, resulting in three adiponectin isoforms each of which exert independent responses in various target tissues: Trimer (low-molecular-weight form: LMW; ~90 kDa), hexamer (medium-molecular-weight form: MMW; ~180 kDa), and oligomer (high-molecular-weight form: HMW; > 250 kDa) [66-68]. These are collectively referred to as full-length adiponectin (fAd). The assembly of MMW and HMW adiponectin requires the formation of a cystine-cystine disulfide bond in the highly variable N-terminal region [68]. Adiponectin multimerization is regulated by the ER chaperone protein Ero1-La, protein disulfide bond isomerase (PDI) [69], and disulfide bond A oxidoreductase – like protein (DsbA-L) [70-73]. Expression of another chaperone protein Erp44, responsible for thiol-mediated

intracellular retention of adiponectin, has shown an inverse relationship with HMW secretion [74, 75]. In addition to directly stimulating adiponectin expression, PPAR γ regulation of the adiponectin oligomer ratio may be due to selective upregulation of Ero1-La and downregulation Erp44 [69, 71, 76].

Recent studies have proposed the importance of assessing high-to-low molecular weight isoform ratios when correlating adiponectin levels with clinical parameters assessing the metabolic syndrome [77, 78]. Accordingly, adipocyte conditioned media from STZ-induced diabetic rats mediates different metabolic effects in cardiac and skeletal muscle, and this correlates with changes in concentrations of oligomeric adiponectin forms, when compared with wild-type controls [79]. Furthermore, fAd may be cleaved by leukocyte elastase to liberate the globular C-terminal fragment (gAd) [68, 80] and exert direct cellular effects [81, 82].

1.2.2 Local production of adiponectin

Evidence of local expression has expanded the view of adiponectin from its classic role as an adipokine with wide ranging endocrine effects to a new concept in which the secretion of adiponectin from target tissues may off-set impaired bioavailability of circulating adiponectin. This may be especially important in tissues susceptible to pro-fibrotic processes in disease states such as the heart.

Adiponectin expression in the heart was first confirmed in isolated cardiomyocytes [83], and was subsequently found to be upregulated in the post-

ischemic murine heart [84], to attenuate cardiomyocyte hypertrophy *in vitro* [85], and to play a role in a porcine model of dilated cardiomyopathy [86]. Accordingly, the adiponectin transcription factor PPAR γ has been shown to inhibit cardiac hypertrophy and attenuate AngII-induced cardiac fibrosis [35, 87]. The clinical relevance of heart derived adiponectin has yet to be fully elucidated but the local adiponectin system was found to be dysregulated in patients with dilated cardiomyopathy [88].

Skeletal muscle has been shown to produce adiponectin with the same relative multimeric composition to adipose derived adiponectin [55, 89]. Rosiglitazone, another TZD and PPAR γ agonist, increases adiponectin mRNA transcription, as well as translation of intracellular and secreted protein from L6 skeletal muscle cells, which was shown to exert functional metabolic effects including enhanced insulin-stimulated Akt phosphorylation and glucose uptake [55]. Accordingly, PPAR γ -mediated skeletal muscle adiponectin production mediates autocrine effects to improve insulin sensitivity and could protect against high-fat diet-induced insulin resistance *in vivo* [85]. It has been suggested that skeletal muscle adiponectin content increases in response to certain inflammatory conditions and obesity in an attempt at providing local anti-inflammatory and antioxidative protection [90-92]. Indeed the globular form of adiponectin mediates potent metabolic effects in skeletal muscle and it is conceivable that elevated local amounts of gAd are produced in inflamed tissue by elastase enzyme derived from infiltrating inflammatory cells. Finally, autocrine

effects of gAd have recently been identified in the regulation of skeletal muscle cell differentiation [93, 94].

Adiponectin was also shown to be produced by the liver in response to carbon tetrachloride induced hepatofibrosis [95], in osteoblasts [96, 97], in the pulmonary vascular endothelium [98, 99], and in the pituitary [100, 101].

1.2.3 Adiponectin signalling

Intracellular adiponectin signalling is mediated through the binding of adiponectin with the transmembrane adiponectin receptor isoforms AdipoR1 and AdipoR2 [102] and also T-cadherin [103] which has been shown to mediate adiponectin stimulated cardioprotective effects [104] (figure 1.2). AdipoR1 and AdipoR2 have seven transmembrane domains with an intracellular N-terminus and an extracellular C-terminus, and are thought to function in a tyrosine phosphorylation independent manner [54], while less is known about the signalling mechanisms mediated by T-cadherin. AdipoR1 has highest binding affinity for gAd and is the major form found in cardiomyocytes and skeletal muscle [82] whereas AdipoR2 is most highly expressed in liver [105].

APPL1 (Adaptor protein containing PH domain, PTB domain and Leucine zipper motif) is an adaptor protein identified by Mao et al. [54] that interacts with the N-terminal intracellular domain of both AdipoR1 and AdipoR2 through its PTB domain. APPL1 is now regarded as a critical mediator of adiponectin signalling and

as a regulator of the crosstalk between the adiponectin and insulin signalling pathways [67]. APPL1 has been shown to interact with the p110 α catalytic and p85 regulatory subunits of PI3K [106], and Akt [107]. APPL1 was found to be a critical mediator of adiponectin stimulated cardiac fibroblast regulation of the ECM [108]. Indeed, adiponectin was found to stimulate APPL1 translocation towards to the cell membrane [108] where it can interact with AdipoRs to mediate downstream signalling and metabolic events [54]. Other more recently identified adaptor proteins which have been suggested to be involved in adiponectin's intracellular signal transduction include activated protein kinase C1 (RACK1; [109]), ER protein 46 (ERp46; [110]), and protein kinase CK2 β [111]. Among the adaptor proteins, only APPL1 associates with both AdipoR1 and AdipoR2 while RACK1, ERp46, and CK2 β bind to AdipoR1. It has now been shown that APPL1 plays an important role in mediating adiponectin's metabolic effects in liver, muscle, and endothelial cells [112] as well as its cardioprotective effects [113, 114]. RNAi-mediated RACK1 knockdown prevented adiponectin regulated glucose uptake in HepG2 cells [109]. Pharmacological inhibition of CK2 β attenuated adiponectin signalling in skeletal muscle cells [111]. Co-immunoprecipitation also confirmed the interaction between ERp46 and AdipoR1, but not AdipoR2, and interestingly the suppression of ERp46 expression resulted in increased cell surface AdipoR1 levels and enhanced adiponectin stimulated phosphorylation of AMP kinase (AMPK) but reduced phosphorylation of p38-mitogen-activated protein kinase (MAPK; [110]).

Although APPL1 shows nuclear localization following adiponectin treatment [67], APPL1 does not have a nuclear localization sequence (NLS). APPL1 localizes with early endosomes, and can undergo nucleo-cytoplasmic shuttling to regulate transcriptional pathways via HDAC interactions [115]. In fact, in addition to adiponectin signalling, APPL1 is currently known to interact with proteins involved in a variety of pathways including insulin, Wnt, and NF- κ B signalling [67, 115].

The ability of adiponectin to counteract deterioration in cardiac function is mediated by metabolic, anti-apoptotic, anti-fibrotic, and anti-hypertrophic effects [114, 116-120]. Adiponectin has been shown to regulate fatty acid β -oxidation in the heart. In cell based *in vitro* studies of isolated cardiomyocytes, adiponectin was shown to stimulate the phosphorylation of AMPK, IRS1, and Akt (T308 and S473) correlating with the regulation of glucose and fatty acid uptake and metabolism [121], and to target cofilin to mediate remodelling of the actin cytoskeleton leading to the translocation of lipoprotein lipase (LPL) to the cell surface [122]. Adiponectin was also shown to stimulate the phosphorylation of acetyl coenzyme A carboxylase (ACC), as well as to induce CPT-1 expression and activation through AMPK [123]. Recently [113], we demonstrated that adiponectin increases fatty acid uptake, CD36 translocation, and insulin-stimulated glucose transport as well as Akt phosphorylation in isolated adult cardiomyocytes, and enhances fatty acid oxidation in conjunction with AMPK and ACC phosphorylation in the isolated working heart. However, despite an increase in fatty acid oxidation and myocardial oxygen

consumption, adiponectin increased hydraulic work, and maintained cardiac efficiency [113]. Similarly, enhanced adiponectin sensitivity through transgenic overexpression of APPL1 was shown to protect mice fed a high-fat diet from cardiac dysfunction [124].

The phosphorylation of AMPK was shown to attenuate norepinephrine induced cardiomyocyte hypertrophy and ERK phosphorylation [116], angiotensin II induced NF- κ B activation and hypertrophy [125], and also shown to fully [118], or minimally [120] attenuate hypoxia–reoxygenation induced apoptosis. Adiponectin was shown to attenuate hypoxia–reoxygenation induced apoptosis in H9C2 cells through the AdipoR1/APPL1 signalling pathway [114]. Additionally, our recent study in isolated neonatal cardiac fibroblasts showed that adiponectin regulates remodelling of the ECM through activation of MMP2 and translocation of MT1-MMP via the APPL1-AMPK signalling axis [108].

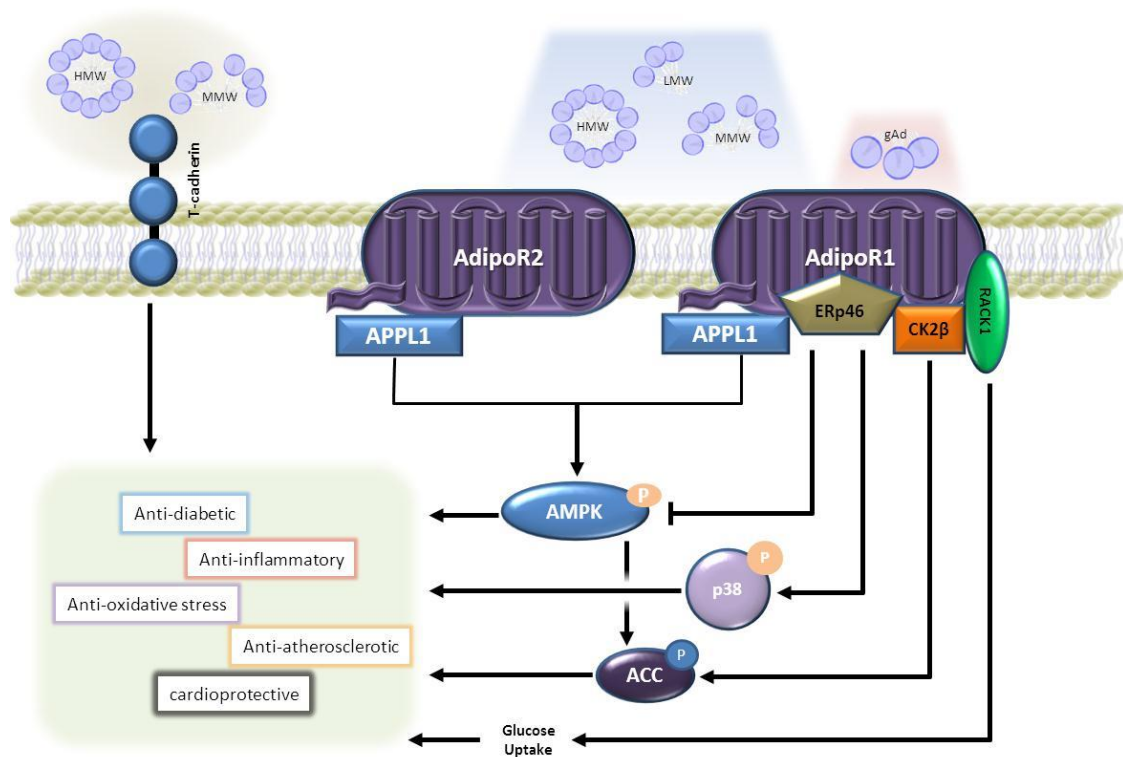


Figure 1.2: Adiponectin Signalling.

AdipoR1 and AdipoR2 are transmembrane adiponectin receptors that bind the adaptor protein APPL1 through the PTB domain and have been shown to mediate many of adiponectin's effects. T-cadherin lacks an intracellular domain but may be essential for adiponectin's effects in the heart.

1.3 Adiponectin and cardiovascular disease

With the growing appreciation of adipose tissue as a dynamic endocrine source of a comprehensive bioregulatory secretome (termed adipokines) [126, 127], and the close association between obesity, the metabolic syndrome, and CVD (section 1.1) there has been great interest in the direct myocardial effects of the adipose derived insulin-sensitizer adiponectin, which is now closely associated with CVD [38, 112,

128-133]. The *in vivo* study of adiponectin in the heart largely relies upon surgical intervention (coronary artery ligation: MI, transverse aortic banding: POH) or administration of inducers of cardiac dysfunction (e.g. Angiotensin II), in various mouse backgrounds. Sections 1.3.2 and 1.3.3. will discuss *in vivo* findings regarding adiponectin and CVD with specific emphasis on POH and AngII infusion as two well established *in vivo* models of non-ischemic cardiomyopathy.

1.3.1 Human studies

High expression from visceral adipose tissue was initially considered an important factor in the potential impact adiponectin might have in the pathogenesis of diseases associated with obesity such as CVD [134]. This was later supported with data proposing adiponectin as a regulator of endothelial adhesion molecules, while adiponectin protein was detected in ruptured vascular walls [135, 136]. Indeed, circulating levels of adiponectin showed an inverse relationship with coronary artery disease, acute myocardial infarction, and essential (idiopathic) hypertension, [137-140].

While there is speculation that adiponectin may serve as an independent risk factor for the development of CVD [141-143], the high molecular weight isoform of adiponectin is now considered a more accurate independent predictor of heart failure outcome [144, 145]. Pioglitazone, a known PPAR γ agonist and insulin sensitizing therapeutic, increases circulating adiponectin levels, and was shown to decrease inflammation and atherogenesis independent of glycemic control in patients with

CVD, suggesting that adiponectin's influence was independent of its role as an insulin sensitizer [146, 147].

The volume of data examining the link between adiponectin and CVD is growing rapidly and there is increasing discord in the conclusions made in each study [132, 148]. For example, high circulating levels of adiponectin were associated with a low risk of MI in men aged 40 to 75 without signs of cardiovascular disease [149], and a 10 year follow up study in 70 year old men showed that serum adiponectin was associated with a lower risk of coronary heart disease, independent of BMI and glycemic control [150]. However, in the Jackson Study comprising a broad metabolic spectrum of the black community, adiponectin was found to correlate with LV mass in patients with hypertension and insulin resistance [151]. Accordingly, increased circulating adiponectin levels were associated with LV diastolic dysfunction in patients with hypertrophic cardiomyopathy [152], and was identified as a risk factor for coronary heart disease in elderly men [153]. Conversely, another study of the elderly population instead showed that higher adiponectin concentrations predicted reduced risk of non-fatal MI [154].

In an effort to clarify and summarize what is known about adiponectin in heart disease, two very thorough reviews of current clinical literature conclude there is no clear association between adiponectin and CVD [38, 133]. Clearly, the multifaceted etymology of cardiovascular disease and the complex regulation of adiponectin and its

isoforms in obesity, T2D and inflammatory conditions may be obscuring these results making definitive conclusions of observational data elusive.

1.3.2 Animal studies

Adiponectin knockout (AdKO) mice exhibit a normal cardiac phenotype when unstimulated: interstitial fibrosis, cardiomyocyte cross-sectional area, and echocardiographic assessment of cardiac function are similar between AdKO and wild-type (WT) mice. Transverse aortic banding (MTAB), a mouse surgical model commonly used to simulate clinical pressure overload of the left ventricle, is known to induce an increase in LV mass, increased fibrosis, and expanded myocyte cross-sectional area in WT mice [155]. Following MTAB surgery, AdKO mice exhibit signs of exacerbated LV hypertrophy, increased interstitial fibrosis, and increased LV posterior wall thickness, a common measure of hypertrophic cardiac remodelling, all of which are ameliorated upon adenoviral-adiponectin administration [116]. Accordingly, PPAR γ heterozygous, and db/db mice also show an exaggerated hypertrophic response to MTAB, suggesting that adiponectin deficiency, common in T2D and obesity, exacerbates PO induced cardiac dysfunction [116, 156]. While the exact mechanisms precipitating cardiac dysfunction in AdKO mice are not clear, impaired AMPK signalling, glucose metabolism and subsequent disruption of adiponectin mediated angiogenesis are noted in adiponectin deficient mice following MTAB [157, 158].

Adiponectin has also been shown to be protective against myocardial infarction [118]. Interestingly, despite a significant reduction in adiponectin levels (~50%) following MI [119] or MTAB [159], Adiponectin was shown to localize to the infarct scar both in wild-type mice and in patients following ischemic injury, through leakage from the vascular compartment [160]. Indeed, myocardial accumulation of adiponectin, through association with collagen [117], is a putative mechanism through which adiponectin could induce cardioprotective signalling in a hypoadiponectinemic environment. Accordingly, a local adiponectin system has been shown in patients with dilated cardiomyopathy which is regulated independently of the endocrine adiponectin system [88].

However, recent studies in adiponectin deficient mice using MTAB to induce pressure overload have yielded some conflicting data. The study published by Liao et al in 2005 clearly shows the predicted progressive decline in cardiac function following MTAB in WT mice, and the exacerbated decrease in ejection fraction (EF) and fractional shortening (FS) in AdKO mice beginning at 2 weeks following surgery [157]. Similarly, a study published comparing WT, AdKO, and T-cadherin KO mice, showed the decrease in FS and increase in heart weight-to-body weight ratio in both AdKO and T-cadherin KO mice compared to WT mice after 4 weeks of PO [104]. By contrast, two papers published in 2010 by the Stanley group show an increase in posterior wall thickness in AdKO mice in response to MTAB, but no change in other functional parameters (end diastolic volume, end systolic volume, ejection fraction) [161, 162]. A

follow-up study published by the same group in 2011 similarly failed to show an effect of MTAB on cardiac function in AdKO mice, and also showed that high-fat diet accelerated cardiac decline in WT but not AdKO mice [163]. They conclude that adiponectin does not confer cardiac protection in the overloaded myocardium, adiponectin deficiency may be protective against the development of cardiac dysfunction, and that adiponectin may play a permissive (detrimental) role in the progression of cardiac remodelling. Indeed, a finer analysis of the study published by the Walsh group in *nature medicine*, the first comprehensive comparison of WT and AdKO mice following MTAB, posits an alternative interpretation. After 1 week of PO, WT mice show a significant decrease in fractional shortening compared to AdKO mice despite indications of hypertrophic remodelling (increased heart weight-to-body weight ratio and LV posterior wall thickness) in both groups [116]. Decreased fractional shortening with increased LV posterior wall thickness is a negative indicator of cardiac function, which suggests that the WT mice from this study are in progressive heart failure while the AdKO mice remain in the compensated phase of cardiac remodelling [164, 165].

Currently, similar to clinical findings, the conclusions from detailed focused studies linking adiponectin action to cardiac dysfunction in mice are mixed. Certainly, compensatory upregulation of cardioprotective agents in whole body and germ line adiponectin knockout models need to be considered as possible confounding factors. While these considerations may lead to the discovery of new therapeutic targets, more

sophisticated approaches such as inducible or cardiac specific adiponectin deficient mouse models, as well as models targeting downstream adiponectin signalling (AdipoR1/2, APPL1/2), will help to further elucidate the role of adiponectin in heart disease.

1.3.3 Adiponectin and Angiotensin II

AngII, the pluripotent member of RAAS known to play a central role in the homeostatic regulation of the cardiovascular system [166], is known to exert hypertrophic and apoptotic effects on cardiomyocytes, and is a major therapeutic target [167]. RAAS is upregulated in obesity and CVD, and shows an inverse relationship with adiponectin [168]. Therapeutic inhibition of RAAS was shown to lead to increased adiponectin levels in circulation in patients with essential hypertension [169].

AngII infusion is a common *in vivo* methodology mimicking the pathology of dilated cardiomyopathy [170-173]. AngII potently targets cardiac fibroblasts to increase collagen production [174] leading to severe cardiac fibrosis and dysfunction. Owing to the hypoadiponectinemic-hypertensive environment in DBCM patients, numerous groups have studied AngII infusion in AdKO mice. AngII induces fibrosis (increased Col I and Col III), MMP2 and MMP9 activities, TGF β expression, and cardiac dysfunction, and these are consistently exacerbated by adiponectin deficiency [159, 175]. Administration of adiponectin or pioglitazone treatment attenuates these

effects [35, 116]. Accordingly, adiponectin cardioprotection is blocked in PPAR- α -KO mice treated with AngII [176].

There is growing evidence of systemic and intracellular negative cross-talk between AngII and adiponectin signalling (figure 1.3). Adiponectin inhibits AngII induced oxidative stress in renal tubular cells, vascular smooth muscle cells, and cardiomyocytes [177-179]. AngII was also shown to induce hypoadiponectinaemia [180, 181] and adiponectin resistance [182], while, interestingly, AngII type 1 receptor agonists have been shown to activate PPAR γ [183, 184].

PARP-1 has been shown to regulate adiponectin gene expression through poly(ADP-ribosyl)ation of PPAR γ . Knockdown of PARP-1 increases PPAR γ binding activity, and the expression of both adiponectin and adiponectin receptor 1 (AdipoR1) [185]. Importantly, PARP-1 activity is increased through various mechanisms in diabetes including hyperglycemia (section 1.1.3), and through AngII activity. Regulation of adiponectin expression by hyperglycemia [24] and Angiotensin II [186] through PARP-1 may be an important mechanism in DBCM.

The reciprocal relationship between AngII and adiponectin in the heart may be of great significance especially considering the discovery of the local RAAS, and the emerging recognition of a local adiponectin system within the heart.

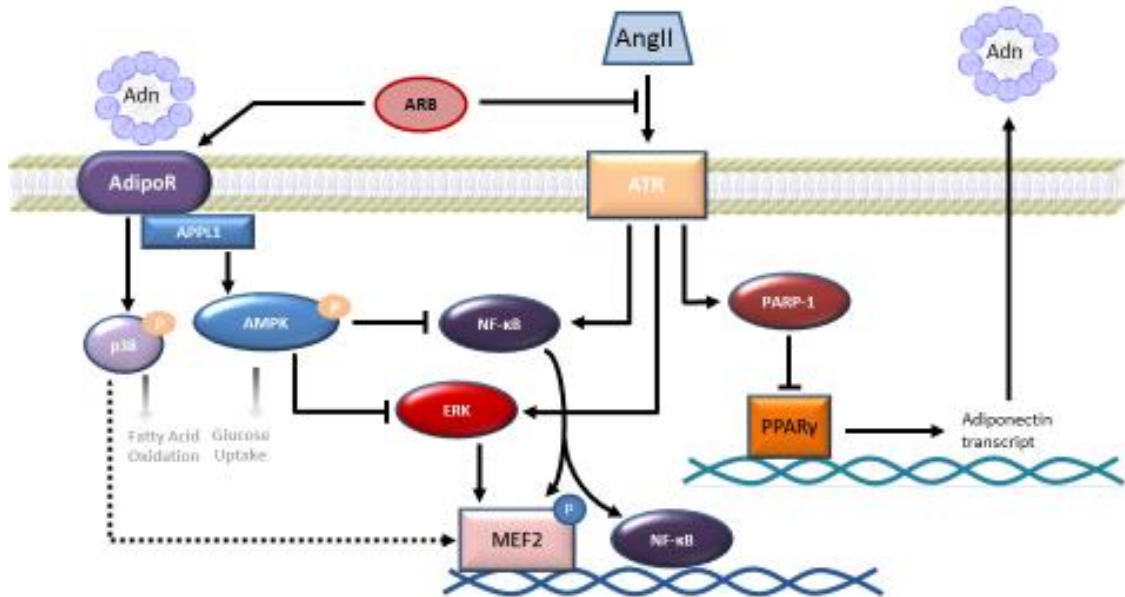


Figure 1.3: Inhibitory cross-talk between adiponectin and AngII signalling.

Adiponectin inhibits AngII induced hypertrophy via NF- κ B and ERK signalling. AngII attenuates adiponectin expression via poly(ADP-ribose)ation of PPAR γ via PARP-1 activation. Adiponectin upregulation of MEF2 activity may be through phosphorylation of p38 (dotted line).

1.4 Cardiac Remodelling in Cardiovascular Disease

Cardiac remodelling is the generalized term used to define reorganization of the heart in response to factors that impact the homeostasis of its function. Dynamic changes in the myocardium including gene expression, molecular, cellular and/or structural alterations are coordinated to adapt to various stressors (hemodynamic, pathological, idiopathic, etc...), or are dysregulated in the progression of heart disease leading to failure [14, 128, 187-192]. The heart is a heterogeneous organ composed of various tissues and cell types. Cardiomyocytes, providing contractile properties

within the heart, consume the majority of metabolic resources and account for approximately 70% of the myocardium by cell volume. Cardiac fibroblasts, primarily responsible for regulation of the extracellular matrix (ECM), are the most abundant cell type in the heart despite constituting a smaller volume than cardiomyocytes. Other cell types including vascular smooth muscle cells, endothelial cells and macrophages exert specialized and critical roles in the normal and remodelling heart.

Left ventricular (LV) structure and contractile function are coupled in the progression from a normal state to failure. LV structure is altered in non-ischemic heart disease in response to increased hemodynamic load as the heart adapts to maintain sufficient cardiac output to adequately supply blood to the body. The non-diseased heart adapts to subtle changes in hemodynamics (e.g. vasoconstriction) according to the Frank-Starling law: LV stroke volume increases in response to increased end diastolic volume (increased LV pre-load). The LV wall is stretched to accommodate the increased end diastolic volume, resulting in increased length of the myocyte sarcomere, the structure where in contraction-relaxation coupling is defined [193]. Small-angle X-ray diffraction studies of contracting ventricular preparations recently determined that systolic contractile force is increased when sarcomere stretch induces a reorientation that reduces the search volume required by the myosin head to find a stereo-specific actin binding site [194]. These subtle changes are clearly adept in providing acute adaptations to normal variation in vascular dynamics. However, the hemodynamic stresses in heart disease supercede compensation by the

Frank-Starling law. Instead, the myocardium reacts at the transcriptional level, leading to an expansion of LV mass and remodelling of the myocardium.

1.4.1 Mechanics of hypertrophy and clinical diagnosis

Increased LV mass, termed LV hypertrophy (LVH), is commonly considered a principle adaptive remodelling event, and is manifest in two distinct stress-dependent patterns: pressure overload hypertrophy (POH) and volume overload hypertrophy (VOH). An elegant catheterization experiment performed in 1975 in patients with LV POH, LV VOH, or no evidence of heart disease, for the first time demonstrated the differential LV wall stresses of pressure overload (PO) and volume overload (VO) during the contractile cycle [195]. It was theorized based on LaPlace's Law that distinct PO results in increased LV wall stress during contraction (systole) leading to concentric hypertrophy, whereas VO results in increased diastolic wall stress in the relaxed LV resulting in eccentric hypertrophy (figure 1.4). Structurally, concentric hypertrophy is characterized by the increase in LV wall thickness, reduction in LV volume, and increase in systolic contractile force as myofibrils are added in parallel to increase the coordinated stroke force. By contrast, in eccentric hypertrophy, LV wall thickness is decreased and volumetric capacity is increased as sarcomeres are added in series, reducing diastolic stress (figure 1.4) [196]. Many clinical studies in patients with PO (hypertension, ventricular outflow obstruction) and VO (aortic/mitral valve regurgitation) have supported these findings [197]. Echocardiography currently serves as the gold-standard by which to differentiate

these disparate geometric changes, and holds prognostic value in the prediction of patient outcome.

Table 1.2: Echocardiography measures of cardiac performance
Changes in measures of cardiac performance in pressure or volume overload. Cardiac function is decreased in the initial stages of pressure overload, but normalize following compensatory (comp) remodelling.

Functional Measure	Abbreviation	Δ in PO (initial)	Δ in PO (comp)	Δ in VO
Heart Rate	HR	-	-	-
End Systolic Diameter	ESD	↓	-	↑
End Diastolic Diameter	EDD	-	-	↑
End Systolic Volume	ESV	↓	-	↑
End Diastolic Volume	EDV	↓	-	↑
Stroke Volume	SV	↓	-	↑
Ejection Fraction	EF	↓	-	-
Fractional Shortening	FS	-	-	-
Cardiac Output	CO	↓	-	↑
Left Ventricular Mass	LV Mass	-	↑↑↑	↑
Posterior Wall Dimension in diastole	LVPWd	-	↑↑	↓

Ultrasound echocardiography is a critical tool used by researchers and clinicians to study the structure and function of the heart in real time. Typical measurements of LV function are listed in table 1.2 and the following progressions of VO and PO are depicted in figure 1.4. **Early VO**: the LV undergoes a passive expansion of EDD and EDV through the Frank Starling mechanism, resulting in initially high diastolic filling pressures and symptoms of heart failure. **Prolonged VO**: ESD and ESV increase (eccentric hypertrophy), resulting in an increase in SV, but normalized EF. These remodelling events lead to normal LV pressures, a relative decrease in wall stress, and the abolition of patient symptoms. The prognosis for patients with chronic

VO is favourable, with a 75% survival rate after 5 years, and 50% survival up to 10 years [198]. By contrast, in PO, initially compensated LV function decompensates over time leading to congestive heart failure (CHF). **Early PO:** Increasing LVPWd, a defining feature of compensatory concentric remodelling in response to PO, is accompanied by increased LV filling (diastolic) pressures. The LV can then exert greater contractile force to compensate for the increased wall stress. **Compensatory POH:** increased LVPWd and LV diastolic pressure lead to near-normal EDV, and normal indicators of cardiac function (EF, FS, and SV). In this phase, fibroblasts within the LV begin to expand the collagen ECM to provide support against the increased wall stress, and to provide an expanded contractile framework for the myocytes. **Prolonged/decompensated PO:** Excessive ECM expansion leads to LV stiffness which in turn begins to impair diastolic compliance (diastolic dysfunction), thereby reducing EDV and SV. To maintain SV, CO, and systolic pressure, diastolic pressures increase until pulmonary congestion occurs. Chronic PO leads to decompensation marked by reduced LV contractile function (due to apoptosis and necrosis), LV dilation, and severely impaired vascular dynamics leading to blood accumulation in peripheral tissues (congestive heart failure) [198].

Recently, an eight-year echocardiographic assessment study of LV geometry in 1020 hypertensive patients with either concentric or eccentric hypertrophy was published. Patients with concentric hypertrophy exhibited increased systolic and diastolic blood pressures, while patients with eccentric hypertrophy displayed

increased LV internal diameter. Furthermore, hearts with concentric and eccentric hypertrophy exhibited signs of cardiac dysfunction predicted by the remodelling patterns outlined above: hearts with eccentric hypertrophy showed decreased EF and FS, indices of systolic dysfunction, while hearts with concentric hypertrophy exhibited impaired LV isovolumetric relaxation time, and LV deceleration time, common features of LV fibrosis and diastolic dysfunction [199].

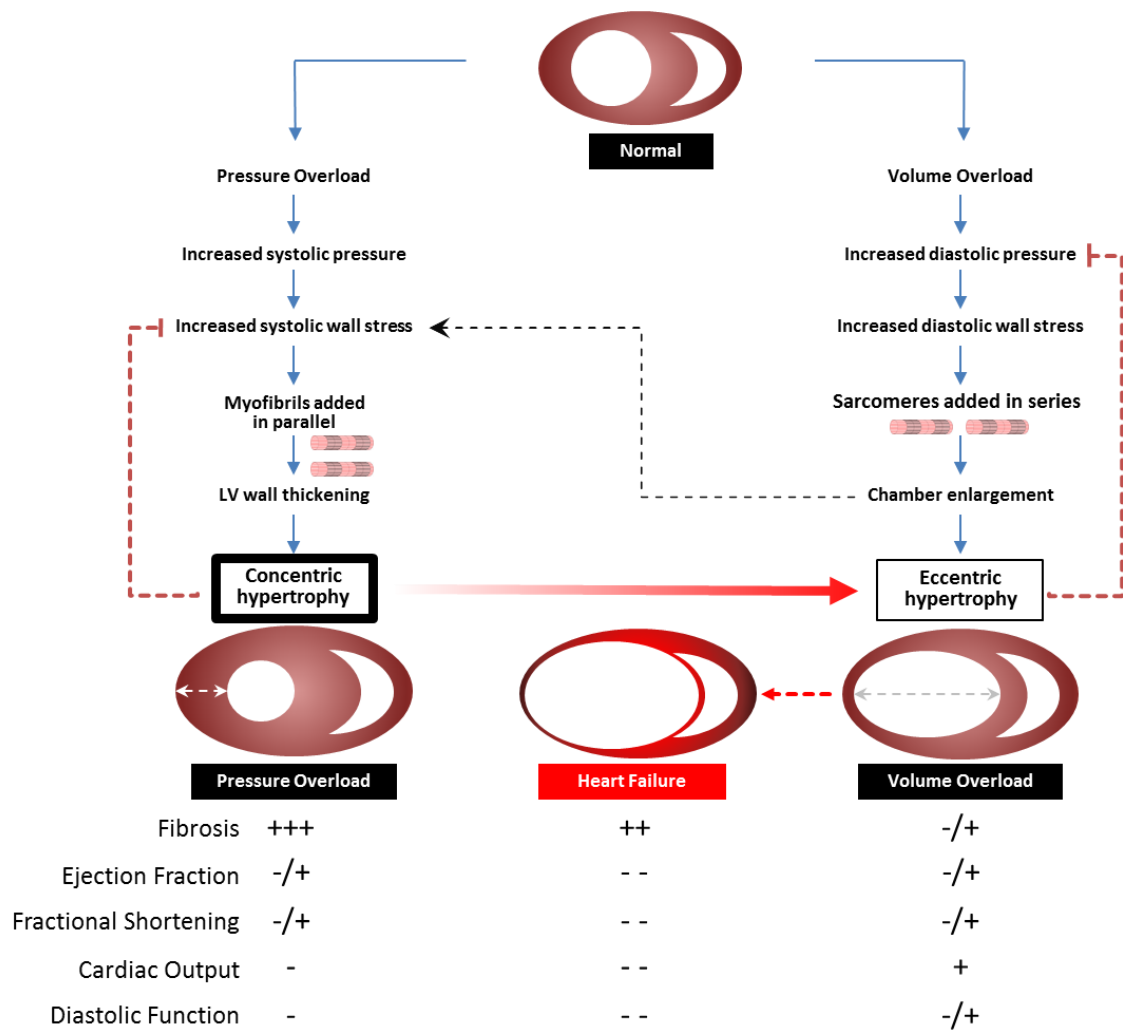


Figure 1.4: Schematic of the progression of POH and VOH leading to HF

The development of concentric hypertrophy in PO serves to decrease systolic wall stress, while increasing fibrosis decreases CO and induces diastolic dysfunction, leading to eccentric hypertrophy and failure. Adapted from [196, 200]

While LV fibrosis is largely considered absent or downregulated in VOH [201], increased LV fibrosis is a hallmark of POH that holds significant prognostic value. A study of patients with aortic valve stenosis (a clinical model of PO) demonstrated an

increased risk of mortality in the group with a higher fibrosis index as calculated through endomyocardial biopsy. Specifically, fibrosis tissue mass index was positively correlated with end systolic wall stress, and ESD and EDD, and negatively correlated with FS, EF, and LVPWd, indicating a negative correlation between myocardial fibrosis and cardiac function. Strikingly, patients diagnosed with severe fibrosis (high fibrosis tissue mass index) had the lowest survival rate (<50%) compared to the group with a relatively mild fibrosis mass tissue index (90%) after the 10 year follow-up [202].

The hypertrophic remodelling patterns seen in PO and VO seek to normalize cardiac function through the Law of LaPlace [$\sigma = (p * r)/2t$] which relates chamber wall stress (σ) to pressure within the cavity (p), chamber radius (r), and wall thickness (t). Accordingly, despite very different geometries and intraventricular pressures, VOH and POH result in initially normalized systolic stress patterns when compared to normal patients [195].

1.4.2 Hypertrophic signalling and gene regulation

In vivo studies have sought to delineate the distinct gene profiles induced in PO (aortic constriction) [203, 204] and VO (aortocaval fistula) [201, 205-207]. Direct temporal comparison of PO and VO in rats showed similar progressive changes to heart weight-to-body weight ratio and ANF expression (a feature of the fetal gene program) following PO and VO, whereas skeletal α -actin, β -MHC, and SERCA2 mRNA were increased following PO, and there was no detectable change in these

markers in VO compared to sham surgery. This suggests a difference in myocardial remodelling favouring increased contractility during PO in comparison to VO [208].

A recent study utilizing a novel balloon technique to impose cardiac phase-targeted LV load to the heart *ex vivo*, distinguished the molecular patterns elicited through the induction of systolic overload (SO; analogous to pressure overload) versus diastolic overload (DO; analogous to volume overload) [209]. Bioinformatics analysis revealed 140 and 77 distinct genes regulated in SO and DO respectively. Furthermore, SO activated proliferation and apoptosis gene profiles, and stimulated the activation of ERK, JNK, and CaMKII signalling. DO was associated with increased gene markers for fibrosis and connective tissue cells, as well as increased p38 signalling. ANP, a well-established marker of POH and VOH, was found in this study to be upregulated by only DO [209], although this may be due to the cyclical-temporal regulation of ANP in VOH [207].

Both POH and VOH are associated with activation of the fetal gene program, a protective reversion to the expression of genes involved in the growing heart, intended to slow the progression of heart disease [188, 210]. The fetal gene program is responsible for the change in expression of many genes (table 1.3); most notably: ATP-demanding α -myosin heavy chain (α -MHC) is replaced by the less demanding β -myosin heavy chain (β -MHC) to conserve energetics; the preferred energy substrate is switched from oxidation towards glycolysis; and activation of the natriuretic factor family (NFF) is increased [197].

However, there is evidence that the shift from α -MHC to β -MHC, as well as the reduced oxidative capacity, are maladaptive events leading to decompensated heart failure [211]. On the other hand, the NFF (ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; CNP: C-type peptide) exert well-characterized protective effects on the cardiovascular system: vasorelaxation, natriuresis (excretion of sodium in the urine leading to lower blood volume) and concomitant diuresis (increased excretion of urine), suppression of myocyte hypertrophy, and inhibition of the RAAS [212]. The NFF have also been shown to function through the cardiac fibroblasts to exert potent anti-fibrotic effects. BNP inhibited TGF β stimulated migration and collagen I expression from isolated human cardiac fibroblasts, and also inhibited fibroblast proliferation and conversion to the hyper-secretory myofibroblast cell type [213]. Accordingly, BNP knockout mice exhibit an increased basal fibrotic phenotype which presents as multi-focal fibrotic lesions in the LV that increase in number and size in response to PO [214]. Although more recently characterized with respect to heart failure [215], CNP also exerts antifibrotic and antiproliferative effects in the myocardium [216]. NFF transcription is regulated by numerous well characterized factors involved in cardiac hypertrophy such as GATA4/5/6, NF- κ B, and MEF2 (table 1.3).

Table 1.3: Transcriptional regulation of the fetal gene program

Gene	Transcriptional Regulation
ANP	MEF2, GATA4/5/6, SRF, Tbx5, Nkx2.5, ZPF260
BNP	NF-AT, NRSF, NF- κ B, SRF, TEF-1, XBP-1, GATA 4/5/6
β-MHC	Nkx2.5, MEF2C, GATA4/5/6
Sk-αActin	TEF, SRF, Sp1

The myocyte enhancing factor-2 family of transcription factors (MEF2A, B, C, D), are well established as mediators of tissue development [217], and are also known to play a role in cardiac hypertrophy [211, 218-220]. MEF2 DNA binding sequences have been identified in the promoter regions of α -MHC [221], SERCA2 [222], skeletal α -actin (Ska-actin) [223], and also dystrophin, desmin, and some myosin light-chain isoforms [219]. MEF2 activity is upregulated by both PO and VO [221], while transgenic overexpression of MEF2A and MEF2C increases NFF and SK α -actin expression, facilitates a switch towards β -MHC, and decreases ventricular performance, indices of dilated cardiomyopathy [218]. Transcriptional activity of MEF2 is regulated through MEF2-HDAC (repression) or MEF2-HAT (transcription) complex formation [211]. Phosphorylation of MEF2 by p38 or ERK also increases MEF2 transcriptional activity [224]. Although both p38 MAPK-MEF2 and ERK5-MEF2 signalling cascades have been implicated as pro-hypertrophic [219], ERK and p38 MAPK may induce distinct hypertrophic pathways through phosphorylation of different target sequences on MEF2, and also targeting of different members of the MEF2 family [224].

As detailed previously (chapter 1.1.3), insulin has been implicated in the pathogenesis of diabetic cardiomyopathy. However, streptozotocin induced diabetes (a common diabetic model lacking insulin) demonstrated the association of hypertrophic markers (AngII, MEF2, ANP and BNP) with high glucose and insulin deficiency [225, 226]. Indeed cardiac remodelling and LV hypertrophy can develop in the absence of hemodynamic stress, such as from metabolic or hormone disruption, genetic factors, or may be pathologically idiopathic. Detailed studies in cultured cardiomyocytes and animal models *in vivo* have allowed the characterization of many pro-hypertrophic pathways. The following is a brief summary of these complex signalling pathways, reviewed by [211, 219, 227]: Angiotensin II (AngII), Endothelin-1 (ET-1), and catecholamines bind G-protein coupled receptors (GPCR) to activate protein kinase C through the activation of phospholipase C (PLC), or Ca²⁺ influx, leading to HDAC translocation to the cytosol, and MEF2 transcription. HDAC translocation and activation of hypertrophy may alternatively be stimulated by the calcium-calmodulin-CaMK signalling pathway. Receptor tyrosine kinases mediate neuregulin, epidermal growth factor, and insulin-like growth factor-1 (IGF-1) signalling via the Rho/Rac1/Ras pathway, leading to MEK1/2-ERK1/2, and also MEK5-ERK5 activation of hypertrophy. TGFβ activates MEK3/6-p38 signalling and also MEK4/7-JNK signalling. Increased intracellular Ca²⁺ leads to calmodulin-dependent calcineurin activation and dephosphorylation of NFAT (calcineurin-nuclear factor of activated T-cells). NFAT then translocates to the nucleus (a process accelerated by ERK) where it upregulates hypertrophic genes (GAGA4, NFAT, AP1).

POH is associated with the significant increased expression of extracellular matrix constituents leading to interstitial fibrosis, impaired LV compliance, and diastolic dysfunction; whereas VOH is associated with a decreased expression of the ECM and overall dissolution of existing collagen by the matrix metalloproteinases (details of ECM regulation in the myocardium in various pathologies will be discussed in section 1.5). Expansion of the ECM in LV remodelling is a critical event in non-ischemic heart disease, and there is growing appreciation of the direct role the ECM plays in activating pro-hypertrophic signalling. ECM-integrin interaction may play a crucial role in transducing hemodynamic/mechanical load into biochemical events through 'outside-in' cascades: mechanical stress first exerts force upon the cardiomyocytes and the ECM which then stresses the focal adhesion complex and the integrins embedded in the cell membrane, thereby initiating intracellular signalling events such as activation of the pro-hypertrophic Ras-Raf-ERK1/2 pathway [190, 197]. The $\alpha 3\beta 1$ integrin heterodimer expressed in cardiomyocytes can bind numerous ECM ligands including collagen I, fibronectin, and laminin, and indeed the integrin subunits $\alpha 1$, $\alpha 5$, $\beta 1$ and $\beta 3$ are upregulated and/or activated by hypertrophy and PO [228-231]. VO however is associated with a progressive decrease in integrin expression [205]. Clearly, mechanisms mediating cardiac hypertrophy and cardiac fibrosis are intertwined in the overloaded myocardium. In the following section the effects of ventricular unloading on the remodelled/hypertrophic heart will be discussed.

1.4.3 Reverse remodelling

The remodelling events induced by PO and VO serve to offset systolic or diastolic wall stress, respectively, to preserve cardiac function (see section 1.4.1). However, numerous *in vivo* studies have demonstrated that when cardiac remodelling is avoided through genetic modification or pharmacological treatment, normal function is retained despite the induction of PO. Indeed, the Framingham Heart Study identified echocardiographic LVH as a significant risk factor for cardiovascular morbidity and death, even after normalization for other significant risk factors such as blood pressure, cigarette use, and cholesterol profile [232]. Accordingly, anti-hypertensive therapies (beta-blockers, ACE inhibitors and angiotensin II receptor antagonists (ARBs)) have been enormously successful in delaying the onset of maladaptive remodelling. Congestive heart failure (CHF) patients have benefitted from aggressive surgical techniques such as valve replacement and left ventricular assist device (LVAD) implantation, both of which directly alleviate LV PO [233, 234]. Indeed, accumulating clinical and experimental evidence has demonstrated restoration of cardiac function and regression of LV remodelling following LV unloading, a process now commonly termed reverse remodelling [235, 236].

Recent *in vivo* studies have begun to characterize the process of reverse remodelling in greater detail. LV unloading is achieved through removal of the constricting aortic band (debanding), and leads to reverse remodelling of the left ventricle, restoration of normal cardiac function [237-239], activation of a unique gene

expression profile [240], and normal pressure-volume relationships as long as debanding is performed before the heart enters decompensated failure [241]. Along with the regression of cardiomyocyte hypertrophy, debanding and the cessation of AngII infusion increased autophagic flux [242, 243].

However, less is known regarding changes to the ECM following unloading of the left ventricle. Increased fibrosis is universally regarded as a negative indicator of cardiac function and recent evidence using Doppler echocardiography and strain analysis has demonstrated the detection of early diastolic dysfunction in heart failure patients due to early upregulation of collagen in the myocardium. ECM expansion is typically viewed as a unidirectional progression [202, 244, 245]. In fact, evidence from recipients of valve replacements indicated the expansion of fibrosis despite LV unloading, leading to worsening diastolic indices of myocardial stiffness [246]. Indeed, debanding was also shown to differentially regulate the expression of collagen isoforms in *in vivo* [239, 247], and our studies (chapter 3) provide a clear structural picture of the 3-dimensional architecture of collagen networks in the debanded myocardium. We show that debanding results in the loss of the less compliant large collagen fibres, but also leads to an expansion of small fibre fibrosis which may have negative long-term implications for cardiac function.

1.5 Cardiac fibroblasts and the extracellular matrix regulation

1.5.1 Structure and function of the extracellular matrix

The cardiac extracellular matrix (ECM) is a highly organized interstitial network of structural proteins that forms a scaffold to perform functions such as tethering adjacent cardiac myocytes. It serves a vital role in the diseased myocardium, providing stress bearing support during overload, and reinforcing weak areas to prevent rupture in ischemic heart disease, but contributes to myocardial stiffness in later stages of heart failure. The cardiac ECM is separated into three layers. The outer layer, the *epimysium*, surrounds the myocardium and lies below the endothelial layer of the epi- and endo-cardium. The *perimysium* is a sheath of connective tissues consisting of tendon-like extensions of the epimysium. The perimysium aggregates myocytes into muscle fibres, bears shearing forces during contraction and relaxation, and minimizes the dissipation of force generated by cardiomyocytes. The *endomysium* surrounds and connects neighbouring myocytes and the intramuscular vasculature [248]. It is proposed that myocardial forces are transmitted through the endomysial ECM to the internal cytoskeleton of individual myocytes via ECM-integrin interactions at the cell surface [190, 228] (see section 1.4.3). All three layers of the ECM are composed mostly of collagen type I which has a tensile strength greater than that of steel [248], and allows for the mechanical co-ordination of forces generated by the myocytes. The ECM also contributes to LV expansion during diastole as coiled fibres release potential energy stored through compression in systole [249]. The

cardiac ECM is composed primarily of fibrillar collagens: collagen type I (~85%), type III (~10%), and type IV (~5%), although the endomysial and perimysial layers also have a percentage of elastin, allowing more flexibility during each cardiac cycle [250-252].

The ECM is not a static structure, rather it is a dynamic, complex environment containing bioactive matrix proteins, signalling molecules, and various cell types. With a half-life of approximately 80-120 days in the healthy myocardium, the ECM undergoes a slow rate of turnover at approximately 0.6% per day. In disease conditions, changes in the composition and structure of the ECM in response to environmental cues and/or tissue injury have been shown to play an important role in the process of myocardial remodelling, and the progression of heart failure [249, 253]. Indeed, changes to the cardiac ECM may lead to, or be the result of, various myocardial pathologies, and certainly, the ECM plays a large role in the compensation and decompensation phases of cardiac remodelling [191, 248-250].

1.5.2 Cardiac fibroblasts and myofibroblasts

The importance of cardiac fibroblasts in the heart cannot be understated, as they play a vital role in heart development (the earliest organ to form in the incipient embryo), and in the adaptive/reparative response to stressors pushing the adult heart to failure. Cardiac fibroblasts (CF) constitute 60-70% of the cell population in the myocardium and participate in the pathogenesis of heart disease as the principle regulators of the cardiac ECM. Morphologically, CFs are relatively flat, have spindle

projections, and are localized to the endomysial collagen layers between the much larger cardiomyocytes. Developmentally, CFs originate from proepicardial cells that migrate throughout the embryonic heart, reacting to a delicate balance of signalling factors (e.g. fibroblast growth factor, platelet derived growth factor- β etc..) to undergo epithelial-to-mesenchymal transition (EMT), then differentiation into CFs. There is also growing evidence that embryonic cardiac fibroblasts may originate from endocardial cells and undergo endocardial-to-mesenchymal transition (EndMT) [254]. Embryonic CFs throughout the developing heart begin to construct a comprehensive three dimensional myocardial network, and are responsible for stimulating cardiomyocyte growth and proliferation during ventricular compaction until birth [255, 256]. Interestingly, *in vitro* co-culture of adult cardiomyocytes (ACM) with embryonic CFs induces ACM proliferation, whereas co-culture of ACM with adult CFs induces ACM hypertrophy, consistent with the well established post-natal shift in cardiac growth from embryonic hyperplasia to adult hypertrophy and demonstrating the importance of CF-CM crosstalk [256]. As the fetal heart grows *in utero* it is exposed to a hypoxic, dynamic environment [257]. The principle metabolic requirement of the fetal heart are carbohydrates; it's ability to oxidize fatty acids is limited [257]. The gene pattern expressed during this time, meeting the growing heart's metabolic demands and hemodynamic stresses, is termed the fetal gene program, and is a well known reparative mechanism re-initiated in the heart to compensate for excessive stress in the adult myocardium [210]. Following the maturation of the heart, CFs again play a principle role in the compensatory

mechanism sustaining heart function against the substantial increase in systolic pressures following birth by modifying the ECM to efficiently distribute mechanical stress to the ventricles undergoing severe hypertrophy [258]. This dynamic period lasts for a short time after birth, after which mature CFs lie largely quiescent in the normal heart but continue to regulate basal ECM turnover, maintain signalling interactions with cardiomyocytes, and retain their migratory ability to effectuate a response to stressors [259, 260].

In the progression of heart disease, CFs become active, and along with other precursor cells, differentiate into myofibroblasts (myoCF), a fibroblast-smooth muscle cell hybrid that actively regulates the ECM, become a key source of bioactive agents, and develop contractile apparatus to aid in preventing LV rupture upon loss of cardiomyocytes to apoptosis or necrosis [174, 231, 261]. It has been proposed that oxidative-stress induced cardiomyocyte necrosis is a mechanism preceding and stimulating myoCF differentiation [250, 262]. The current understanding is that myofibroblast (myoCF) progenitor cells include more than just resident fibroblasts; rather, endothelial and epithelial cells (following EndMT and EMT, respectively), smooth muscle cells, and bone-marrow derived cells (e.g. fibrocytes, monocytes) are also now believed to contribute to the pool of pro-myoCF cells [174, 254]. Owing to the diversity of parental cell lineages, myoCF differentiation is a complex process that varies by cell type and stimulus, although AngII, TGF β , and mechanical force are three well established mechanisms [174]. AngII is significantly upregulated with

heart disease, functions through GPCR signalling, and stimulates ERK and p38 signalling [263], and PARP-1 activity [186]. Inhibition of p38 attenuates AngII induced myoCF differentiation [264], while AngII stimulation induces TGF β and Col I expression from CFs [265, 266].

TGF β is a primary and potent mediator of myoCF differentiation that is upregulated and secreted into the ECM by mesenchymal cells, macrophages, monocytes, and resident CFs [174]. TGF β signalling is mediated by the TGF β receptors TGF β RI/RII which initiate SMAD2/3 phosphorylation which, upon activation, complexes with SMAD4. TGF β -SMAD2/3 signalling leads to myoCF differentiation, and α SMA, Col I, Col VI, and MMP expression. TGF β also initiates myoCF differentiation through p38 signalling via TGF β /RII [174].

Interestingly, mechanical stretch has been shown to upregulate a number of signalling cascades including angiotensinogen expression and p38 phosphorylation [267], as well as TGF β expression and activity in cardiac fibroblasts and cardiomyocytes [268-270]. When retained in the ECM, TGF β 1 forms a complex with latent TGF β binding proteins and fibrillin. In the progression of myocardial fibrosis, α SMA mediated myoCF contraction of the increasingly rigid ECM liberates TGF β from this intracellular pool of signalling proteins, inducing a positive feedback loop which further stimulates myoCF differentiation, and secretion of the ECM and myoCF derived cytokines [174].

Rather than being the final step in cardiac remodelling, CFs, myoCFs, and reactive fibrosis are considered integrated, essential players in the adaptive response to pressure overload [271], and indeed, myoCFs have been shown to secrete numerous cytokines heavily involved in cardiac remodelling: TNF α , IL-1 β , IL-6, TGF β , Ang II, ET-1, the NFF (ANP, and BNP), and VEGF are all part of the CF and/or myoCF secretome. Activation of CFs and subsequent upregulation of the myoCF secretome is largely accepted as an early adaptive event to maintain homeostatic cardiac function; however, prolonged presence of myoCF and chronic deposition of collagen in the ECM is considered a detrimental, mal-adaptive mechanism leading to poor clinical outcomes [272].

1.5.3 Collagen synthesis and regulation

The collagen family comprises over 18 distinct isoforms of collagen with diverse physical characteristics whose relative abundance varies from tissue to tissue [273]. Interstitial collagen consists of 3 polypeptide α -chains, composed of large helical domains, which contain a high proportion of hydroxyproline [273]. Collagen type I (Col I) and collagen type III (Col III) are the major structural components in the cardiac extracellular matrix [248]. Col I and III are secreted by CFs and myoCFs as pro-collagen precursors, with N-terminal and C-terminal propeptides that are cleaved leaving short non-helical regions at either end suitable for intermolecular crosslinks [274]. Pro-collagen molecules are released into the extracellular space where the

enzyme lysyl oxidase mediates covalent crosslinking of collagen molecules into rigid, mature fibrils [275].

While Col I accounts for approximately 85% of all collagen in the myocardium [252], the much less rigid Col III comprises the majority of the remaining collagen content and helps confer elasticity to the myocardium. There are numerous methods to quantify the relative ratio of collagen isoforms within the heart (quantitative PCR, western blot, ELISA, masson's trichrome staining), but two of the most exemplary techniques to image the structure of the collagen ECM are picrosirius red staining coupled with polarized light microscopy (picrosirius red detects the high hydroxyproline content in collagen, while polarized light microscopy permits the distinction of the Col I and Col III with high precision [276]), and direct imaging using scanning electron microscopy (SEM). In SEM micrographs, Col I appears thick and rigid, while Col III appears much finer, and is present in significant quantity in the healthy myocardium (see figure 3.1). It is believed that changes in the relative ratios of Col I:Col III, as well as alterations in the organization and cross-linking of the collagen matrix, influence myocardial compliance. Specifically, late-stage LV remodelling in POH is associated with elevated Col I which promotes myocardial stiffness, resulting in impaired contractile function [252, 253]. As discussed previously, diabetic cardiomyopathy (section 1.1.3), and aortic valve stenosis (section 1.3.1) are associated with increased LV fibrosis, decreased LV compliance, and cardiac dysfunction. Microscopic imaging analysis of dilated (idiopathic) cardiomyopathic

hearts showed a significant increase in Col I:Col III ratio compared to normal samples, localized to the endomysial layer surrounding the cardiomyocyte fibrils [277, 278]. Similarly, collagen type I expression [279] are significantly increased in patients with essential hypertension.

In addition to Col I and Col III, the ECM hosts a number of non-fibrillar collagens such as collagen IV, V, and VI, as well as other structural proteins such as laminin, fibrillin, and elastin (table 1.4) that form the *in situ* adhesion substrate for cardiomyocytes and cardiac fibroblasts [273, 280]. Cellular behavior is in part dictated by the relative ratio of these structural proteins in the substrate: Col I and III increase CF proliferation and migration Col VI increases myoCF differentiation and decreases migration, and Col V imparts anti-proliferative effects on smooth muscle cells [281]; Col IV induces smooth muscle cell differentiation [280]; elastin can influence smooth muscle cell proliferation and phenotype [282]. It is hypothesized that the increased expression of the basement membrane collagens (Col IV, V) serves to slow the migration of several cell types, and to aid in maintaining structural integrity in the post-MI heart [280].

Cardiac fibrosis occurs with high incidence in arrhythmic myopathies [261]. Recent data has recognized the importance of non-myocyte reorganization in the pathology of electrophysiological remodelling leading to cardiac arrhythmias [261]. Although CFs are typically considered non-excitabile cells, their depolarized resting membrane potential is lower than that of cardiomyocytes, potentially allowing

electrical signals to pass between cardiomyocytes through intervening CFs with some impedance [261]. However, while the heterogeneity of fibroblasts across different areas of the heart makes definitive conclusions regarding the participation of fibroblasts in electrical signalling elusive [261], expansion of the collagen extracellular matrix in POH and MI has been shown to play a detrimental role in the myocardial electrical coupling [283]. Various patterns of fibrosis (compact, patchy, interstitial, diffuse) have differential impacts on the propagation of electrical signals between myocytes. Patchy or severe fibrosis, where myocytes are separated by acellular regions of collagen, poses the greatest risk for arrhythmias, as electrical propagation is forced to take a circuitous route through the tissue slowing conduction velocities [283]. Fibrosis also promotes uncoordinated triggers originating in aberrant myocytes, which then propagate to and depolarize the few neighbouring myocytes leading to fibrillation [283].

Clearly, the collagen ECM plays a significant, initially adaptive and subsequently detrimental role in the diseased myocardium. In the next section, regulation of the ECM will be discussed.

Table 1.4: Structural proteins in the ECM.
Adapted from [273, 280, 282]

Structural Protein	Location	Role
Collagen I (Col I)	Interstitial	Structural
Collagen III (Col III)	Interstitial	Structural
Collagen IV (Col IV)	Basement membrane	Cell attachment, substrate filter
Collagen V (Col V)	Interstitial	Structural, substrate filter
Collagen VI (Col VI)	Interstitial	Structural
Laminin	Basement membrane	Cell attachment

Elastin	Interstitial, connective tissue	Elastic recoil and resilience
Fibrillin	Interstitial, connective tissue	Elastic recoil and resilience

1.5.3 Matrix metalloproteinases and tissue inhibitors of MMPs

The rate of extracellular matrix turnover in the normal heart is mediated by the concerted effects of i) the matrix metalloproteinases (MMPs) which have the ability to degrade structural proteins, ii) the tissue inhibitors of metalloproteinases (TIMPs) and iii) the continual expression of structural proteins principally by cardiac fibroblasts, with smaller contributions from cardiac myocytes and other cell types (table 1.5) [252].

The expression of matrix metalloproteinases (MMPs) is dramatically upregulated in response to both acute and chronic stresses such as myocardial infarction and hypertension [192, 284]. Patients suffering from dilated cardiomyopathy were shown to have increased ventricular MMP-2 and MMP-9 activity [285], while spontaneously hypertensive rats with heart failure were found to have elevated MMP-2 activity when compared against normotensive controls [286]. By contrast mice lacking MMP-2 or MMP-9 showed a decrease in infarction-induced left ventricular hypertrophy [287, 288], while exogenous MMP-2 treatment was found to dilate and decrease the cardiac tensile strength of ventricular preparations from spontaneously hypertensive rats [289].

MMPs are under transcriptional regulation by a number of factors, although two major *cis*-acting elements are found in the majority of the MMP promoters: polyoma enhancer A binding protein-3 (PEA-3) which interacts with the Ets family of transcription factors, and activator protein-1 (AP-1) which interacts with the Fos and Jun family of transcription factors [253]. The MMP2 promoter, however, lacks both of these elements possibly indicating constitutive expression of the MMP2 protein, although MMP2 expression may be increased in the failing heart after MI [290].

To protect the cell from damage, MMP's are typically synthesized as inactive zymogens, and must undergo post-translational modifications to become proteolytically active[192].Membrane type 1 matrix metalloproteinase (MT1-MMP, also called MMP14) is a zinc dependent MMP that plays an important role in pericellular ECM digestion leading to cell migration and tissue invasion [291]. MT1-MMP is itself synthesized as a pro-peptide, activated through removal of the inhibitory pro-domain in a two-step degradation/cleavage mechanism mediated by the protease furin [292]. Mature, membrane bound MT1-MMP's pericellular activity may be regulated through control of its internalization and trafficking via ubiquitination [293] of its intracellular domain. MT1-MMP's degradative ability may furthermore be supplemented through activation of MMP2 and MMP13, leading to processing of collagen substrates (e.g. collagen IV) that MT1-MMP is incapable of degrading [291] (table 1.5). Cell surface activation of MMP2 requires the homo-dimerization of MT1-MMP which then binds an MMP2-TIMP2 complex leading to cleavage of the MMP2

pro-domain and the liberation of active MMP2 [294, 295]. Recently, we demonstrated that adiponectin stimulates MT1-MMP cell surface localization, MMP2 activation and cardiac fibroblast migration through the phosphorylation of AMPK [108].

Upstream activators of MMP expression include many of the common cytokines involved in the inflammatory response, cardiac dysfunction and metabolic disorders: TNF- α , IL-1 β , ET-1, oxidative stress, sympathetic activation, TGF- β , and mechanical stretch [296]. Accordingly, MMP activity is upregulated in many conditions including diabetic cardiomyopathy, PO and VO, and also in unique animal models such as the SHR [251].

The ECM regulatory function of the TIMPs is primarily as endogenous inhibitors of the MMPs, although, as described above, TIMP2 is involved in the activation of MMP2 via MT1-MMP at the cell surface [253]. As such, a general increase in TIMP expression may serve to limit MMP activity and induce collagen accumulation in the myocardium. Accordingly, in the SHR model TIMP-4 expression is increased in compensated LVH, while the progression of CHF is characterized by an increase in MMP2 activity and TIMP4 expression is decreased in [251]. Similarly, TIMP expression is reduced in end-stage DCM which is associated with increased ECM degradation and LV dilation [251]. Evidence suggests that TIMP2 and 4 may have opposing roles in heart failure. TIMP2, the activator of MMP2, was found to have a much higher specificity than the other TIMPs for MMP2 [294], while TIMP4 has a greater general affinity for MMPs than TIMPs 1 and 2 [297], suggesting that

increased TIMP2 expression may facilitate increased MMP2 activation, while increased TIMP4 may signify an increase in fibrosis.

While MMPs are typically active in the interstitium, TIMP-4 and MMP2 were shown to accumulate within cardiomyocytes following I/R injury [298]. Indeed, active forms of MMP2 and MMP9 have been found intracellularly in cardiomyocytes in patients with dilated cardiomyopathy, which may lead to degradative loss of sarcomeres and impaired contractile function [290].

Table 1.5: ECM substrates of MMPs, and activity change with LVH or CHF. LVH: LV hypertrophy. Defined as increased LVPWd with normal EF. CHF: congestive heart failure. Adapted from [251].

Enzyme	Alternate name	ECM substrate	Δ in LVH	Δ in CHF
MMP1	Collagenase – 1	Collagens (I, II, III, VII, VIII, & X), gelatin, proteoglycan link protein	-	↑
MMP2	Gelatinase A	Collagens (I, IV, V, VII, X & XIV), gelatin, elastin, fibronectin, laminin	↑	↑↑
MMP8	Collagenase – 2	Collagens (I, II, III, V, VII, VIII, & X), gelatin, aggrecan	-	-
MMP9	Gelatinase B	Collagens (IV, V, VII, X, & XIV), gelatin, aggrecan, elastin, fibronectin	↑	↑↑
MMP14	MT1-MMP	Collagens (I, II and III), casein, elastin, fibronectin, gelatin, laminin, proteoglycans	-	↑

1.6 Research Objectives

The rise of the obesity and diabetes epidemic has caused an increase in worldwide cardiovascular risk. There is a great need to determine the endocrine factors linking these serious pathologies.

The adipokine adiponectin has gained significant interest as a potential regulator of cardiac remodelling in heart disease. Accumulating clinical and experimental evidence has suggested that adiponectin may play an important role in the progression of heart failure, but the role of adiponectin in the development of cardiac fibrosis, a negative indicator of heart disease outcome, is currently uncertain. As such, a detailed comparison of changes to the extracellular matrix in adiponectin null and wild-type mice following the induction of pressure overload will contribute to our understanding of adiponectin action in heart disease and help to resolve conflicting data within the field. Furthermore, little is known regarding adiponectin stimulated regulation of the extracellular matrix and cardiomyocyte hypertrophy; insight into adiponectin mediated signalling pathways in cardiac fibroblasts and cardiomyocytes is needed. Lastly, the normal progression of fibrosis following the induction of pressure overload has been established, however little is known regarding changes to the fibrotic myocardium following unloading of the left ventricle. A thorough study of the changes to the extracellular matrix in regressive cardiac remodelling will give valuable insight into the long term clinical consequence of episodes of transient hemodynamic overload and cardiac fibrosis.

The above research objectives lead me to my four studies listed below:

- Study 1:** Adiponectin deficiency alters the progression of ECM remodelling following the induction of pressure overload
- Study 2:** Adiponectin mediates ECM remodelling via the APPL1-AMPK signalling axis in cardiac fibroblasts
- Study 3:** Myocyte Enhancer Factor-2 binding is attenuated in adiponectin deficiency following pressure overload, and is upregulated by adiponectin stimulation of cardiomyocytes
- Study 4:** A detailed analysis of reverse cellular, structural and functional remodelling following aortic banding-debanding

CHAPTER 2: STUDY 1

**ADIPONECTIN DEFICIENCY ALTERS THE PROGRESSION OF ECM
REMODELLING FOLLOWING THE INDUCTION OF PRESSURE
OVERLOAD**

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2.1 Abstract

Adiponectin, circulating levels of which are reduced in obesity and diabetes, has been shown to mediate cardiac remodelling events in response to pressure overload (PO). Fibrosis, a principle feature of PO is the progressive expansion of the extracellular matrix (ECM) which significantly contributes to changes in cardiac size, structure and function leading to heart failure. In this study, we performed a detailed analysis of progressive cardiac ECM remodelling in adiponectin knockout (AdKO) and wild-type (WT) mice following the induction of PO via minimally invasive transverse aortic banding. AdKO mice displayed an increased extent of basal cardiac fibrosis. Expansion of the ECM observed in WT mice after 2 weeks of PO, determined by scanning electron microscopy, Masson's trichrome staining and increased collagen-1 α 1 and 3 α 1 expression, was not observed in AdKO mice until after 4 weeks of PO. Similarly, myocardial expression of principle ECM regulatory genes (collagens, MMPs, and TIMPs) differed between genotypes in response to PO. Circulating Ad levels and myocardial Ad mRNA were reduced after 4 weeks of PO, whereas Ad protein detected in cardiac homogenates was increased at this time. Direct stimulation of primary cardiac fibroblasts with Ad induced a transient increase in total collagen (picrosirius red staining) and collagen III (immunofluorescence) synthesis, as well as enhanced MMP2 activity via gelatin zymography. Ad also enhanced fibroblast migration and attenuated angiotensin-II induced differentiation to a myofibroblast phenotype.

In conclusion, these data indicate that Ad deficiency delays ECM expansion in response to PO. Increased myocardial bioavailability of Ad in WT mice may mediate immediate ECM regulation following PO. The direct effects of Ad on fibroblasts to mediate collagen and MMP expression play an important role in the pathogenesis of cardiac fibrosis.

2.2 Introduction

The physiological significance of myocardial ECM regulation by adipokines in diabetes and obesity has been well established [128, 148, 299]. In particular, regulation of cardiac remodelling by adiponectin is thought to be of great significance [148]. Adiponectin is present at high circulating levels (in the range 2 to 20 ug/ml) in normal individuals but levels are reduced in heart disease, diabetes, and obesity [148, 300]. Recent discovery of heart specific adiponectin production and regulation in various clinical states of heart disease has led to the framing of adiponectin as a 'cardiokine' and an expanded emphasis on the role of adiponectin in the progression of cardiac remodelling [112]. The adiponectin knockout mouse (AdKO) has proven to be an extremely useful model in this regard, and early studies showed that adiponectin deficiency exacerbated pressure overload induced cardiac remodelling [116, 131, 157]. Recently however, similar studies indicate that adiponectin deficiency plays a protective role against the development of adverse cardiac remodelling events leading to heart failure [161-163]. Meta-analysis of clinical studies correlating adiponectin with various heart disease outcomes has been similarly inconclusive [133, 301, 302]. Clearly, more insight into the mechanism of adiponectin action in the pathogenesis of PO induced cardiac remodelling is needed.

Cardiac ECM remodelling plays a critical role in the adaptation to haemodynamic stressors and ultimately in the progression to heart failure [299]. In mouse models, induced left ventricular pressure overload (PO) is commonly

associated with collagen deposition as a reactive response of the myocardium to mitigate cardiovascular decompensation leading to failure. The transition from compensatory ECM support to detrimental fibrosis occurs as differentiated, hypersecretory myofibroblasts persist in the overloaded myocardium [250]. Remodelling of the myocardial collagen matrix is primarily mediated by matrix metalloproteinases (MMPs) [253]. The role of MMP isoforms in heart failure in diabetes and obesity is now well established [128, 303]. During the process of remodelling, MMPs are initially activated to reduce wall stress by increasing fibrillar collagen degradation, allowing LV dilation in response to increased work load. Ultimately, prolonged MMP activation adversely affects cardiac function since the ultrastructural collagen which is initially degraded by MMPs is replaced by poorly structured collagen [304]. Indeed, changes in MMP levels have been described in both human and a variety of animal models of heart failure [303, 304]. Chemical inhibition of MMPs has also been shown to attenuate left ventricular dilation and preserve function after surgical induction of infarction [287] and targeting MMPs is thought to have therapeutic potential although little is known regarding the regulation of collagen and MMPs in the adiponectin deficient heart.

The aim of our study was to conduct a detailed temporal investigation of cardiac ECM remodelling after PO in WT versus AdKO mice. We also investigated changes in cardiac adiponectin expression and content, and study the direct effects of adiponectin on various end points relevant to ECM remodelling in primary cardiac fibroblasts.

2.3 Materials and Methods

Materials

Recombinant full-length adiponectin was produced in-house as previously detailed by [121]. Dulbecco's modified eagle's medium (DMEM), trypsin, antibiotic/antimycotic and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Life Technologies Inc., Burlington, ON, CA). All culture plates were BD Falcon™ brand and purchased from BD Biosciences (Mississauga, ON, CA). Anti-collagen antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). TRIzol® Reagent was purchased from Ambion Inc. (Life Technologies Inc.), Alexa Fluor® 488 goat anti-rabbit antibody was obtained from Molecular Probes (Life Technologies Inc.), and adiponectin primers were obtained from Invitrogen (Life Technologies Inc.). The FITC-labelled goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). VECTASHIELD® mounting medium with DAPI was obtained from Vector Laboratories (Burlington, ON, Canada). RNeasy MinElute Cleanup and RT² First Strand kits were purchased from QIAGEN Inc. (Mississauga, ON, CA). ³H-proline was purchased from Amersham Biosciences (GE Healthcare Lifesciences, Baie d'Urfe, QC, CA). Amicon Ultra-15 Centrifugal Filter Units were obtained from EMD Millipore (Billerica, MA, USA). The Pierce BCA Protein Assay kit was purchased from Thermo Scientific (Fisher Canada, Nepean, ON, CA). The custom fibrosis PCR array was purchased from SABiosciences

(QIAGEN Inc.). LOOK silk black braided non-absorbable 6-0 USP sutures were purchased from Harvard Apparatus (Holliston, MA, USA), titanium ligation clips and microclip ligating applicators used for MTAB were purchased from Teleflex Medical (NC, USA).

Experimental animals

Male C57BL6 (wt) mice (Charles River Laboratories, St. Constant, QC), 6 – 8 weeks of age, and house bred AdKO mice were randomly allocated to treatment groups. Animal facilities met the guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee of York University. Animals were acclimated a minimum of 5 days to a standard housing environment: temperature and humidity-controlled rooms ($21\pm 2^{\circ}\text{C}$, 35-40%), with a daily 12:12h light-dark cycle (lights on at 0700) with access to regular chow diet *ad libitum*.

Minimally Invasive Transverse Aortic Banding

Under general anesthesia (IP Xylazine 0.15mg/g; Ketamine 0.03mg/g), the mouse is kept in a supine position and the fur on the ventral surface removed with a depilatory cream, and the surface sterilized with betadine. A medial cranio-caudal incision is made through the skin from the neck to the bottom of the rib cage, and the neck muscles, fat, and thyroid are retracted to expose the trachea down to the suprasternal notch. An incision is made through the suprasternal notch 2-3 mm down the rib cage. The transverse aorta is visualized under low magnification between the innominate

and left common carotid arteries. A titanium micro-ligation clip is applied across the transverse aorta using banding calipers calibrated to the width of a 26g needle. The sham surgery is performed as outlined above without the placement of the ligation clip. Upon completion of the procedure, the rib cage and skin are closed with silk suture, and the mice injected with Buprenorphine (s.c. 0.05 mg/kg) and placed face down on a warming pad until they awake.

Tissue and serum collection.

Mice were weighed and euthanized using cervical dislocation. Hearts were excised and perfused briefly with KCl to arrest the heart in diastole. Hearts were then weighed and divided for further analysis. Blood samples were collected at time of death, centrifuged (10,000 RPM, 5 min, 4°C) and the serum supernatant collected to analyze adiponectin content.

Scanning electron microscopy.

Heart tissue was fixed in 2% EM grade glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.3 for 1 hour at room temperature, then stored in 0.1M sodium cacodylate buffer, pH 7.3, 0.2M sucrose until further processing. Fixed samples were chemically dehydrated in hexamethylsilazine, mounted on stubs and sputter-coated (Hummer VI Au/Pd 40/60) and examined with a high-resolution scanning electron microscope

(Hitachi S-520) at an accelerating voltage of 20kV equipped with a passive image capture system (Hitachi, Quartz PCI Version 6)

Tissue histology.

Mid-ventricular cross-sections were fixed in 10% formalin solution for 1 hour then stored in 70% ethanol at 4°C until further processing. Fixed heart tissues were dehydrated to xylene and embedded in pure paraffin wax blocks.

Isolation, Culture and Adiponectin Treatment of Neonatal Rat Cardiac Fibroblasts

Neonatal cardiac fibroblasts (CFs) were isolated from 3 – 4 day old Wistar rats as previously described [121]. CFs were used at first passage for myofibroblast differentiation experiments, or passaged twice, grown to 100% confluence (or as otherwise indicated below) and then starved with serum-free DMEM for at least 3 hours prior to treatment with recombinant full-length adiponectin (5.0 µg/ml).

Western Blot Analysis

Cell culture lysis and protein sample preparation was conducted according to methods detailed by [305]. Heart tissue homogenate preparation is detailed [306]. Primary anti-phospho-AMPK α (Thr172), anti-AMPK α , anti-APPL1, and anti- β -actin antibodies were used at 1:1000 dilutions, and appropriate HRP-conjugated secondary antibodies were used at 1:10,000 dilutions. Proteins were detected by

chemiluminescence, quantified by densitometry using Scion Image software (Scion Corp., Frederick, MD, USA) and then normalized to either β -actin or total AMPK protein levels as appropriate.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cultured CFs using TRIzol® Reagent according to the manufacturer's instructions, and purified using the RNeasy MinElute Cleanup Kit to attain an A_{260}/A_{280} ratio between 1.9 and 2.0. First-strand cDNA, synthesized from 1 μ g RNA using the RT² First Strand kit, was used in a custom PCR array comprising 96-well plates pre-coated with primers listed in table 1. Quantitative real-time PCR was conducted using a Chromo4™ Detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, CA) according to cycling conditions outlined by the PCR array manufacturer. Data were analysed using RT² Profiler PCR Array Data Analysis software (Version 3.5; QIAGEN Inc.) and normalized to GAPDH mRNA expression. Adiponectin mRNA expression (forward: 5'-GCAGAGATGGCACTCCTGGA-3'; reverse: 5'-CCCTTCAGCTCCTGTCATTCC-3') was analyzed by quantitative real-time PCR using DyNAmo HS SYBR® Green qPCR kit (Finnzymes, Woburn, MA) with a Chromo4 Detection system and the following cycling conditions: Hot start: 95 °C for 15 minutes; 35 cycles of: 95 °C for 30 seconds, 65°C for 30 seconds, 72 °C for 30 seconds; final extension: 72 °C for 10 minutes.

Wound Scratch Migration Assay

Fibroblast migration in response to adiponectin treatment was assessed using the wound scratch assay as described previously [108]. Fluorescent images were obtained using an Olympus BX51 confocal microscope (Olympus, Seattle, WA, USA) with a 20 x objective, and fibroblast migration was assessed as the closure of the scratch wound in arbitrary length units using Inkscape software (www.inkscape.org).

MMP2 Activity By Gelatin Zymography

Zymographic analysis of conditioned media from CFs grown in 6-well plates was performed as described previously [108]. MMP2 activity was quantified by densitometric analysis of degraded areas using Scion Image software (Scion Corp.).

Collagen Quantification and Imaging

Immunofluorescent imaging of intracellular and secreted collagen, and quantification of collagen synthesis (3H-Proline incorporation), and secretion (picrosirius red staining) was performed as described previously [108].

Statistical analysis

Data are expressed as mean values \pm SEM (n), where n represents the number of experiments conducted. Student's *t* tests were used to determine significant differences ($P < 0.05$) between groups. All statistical analyses were conducted using SigmaStat 3.5 Software (Systat Software Inc., San Jose, CA, USA).

2.4 Results

Progression of myocardial fibrosis

The collagen ECM was imaged in ventricular myocardial samples taken from WT mice following MTAB or sham surgery. Scanning electron micrographs following MTAB shows the increasing presence of small fibres from 3 days to 3 weeks, while large collagen bundles are seen 3 and 4 weeks following MTAB surgery (Fig 2.1). Detection of myofibroblasts through alpha-smooth muscle actin (α -SMA) staining was increased in pressure overload heart sections starting 3 days following surgery (Fig 2.2).

Adiponectin deficiency delays the myocardial fibrotic response to pressure overload

AdKO mice exhibit an increased basal fibrosis observable through scanning electron microscopy (Fig 2.4A), but not masson's trichrome staining (Fig 2.3A). This increased presence of Col III-like small fibres was associated with the increased prevalence of myofibroblasts in the sham AdKO myocardium (Fig 2.3B). Pressure overload failed to induce myocardial collagen accumulation in AdKO mice 2 weeks following MTAB surgery (Fig 2.3A). Accordingly, expression of collagen III and IV, MMPs and TIMPs were significantly downregulated in MTAB AdKO mice compared to sham AdKO mice after 2 weeks of PO (Fig 2.5). However, after 4 weeks of PO, myocardial collagen deposition (Fig 2.4A, 2.4B) and the fibrotic gene profile (Fig 2.5)

in AdKO mice was consistent with expected features of cardiac remodelling. Percentage of genes up/down regulated are indicated in pie-charts (Fig 2.5). Pattern of regulation between WT and AdKO is similar 4 weeks after MTAB surgery.

Pressure overload retains adiponectin in the myocardium and induces adiponectin resistance

ELISA detection of adiponectin in circulation was unchanged in WT mice after 2 weeks, and slightly decreased after 4 weeks of PO when compared to respective sham groups (Fig 2.6A). Myocardial adiponectin mRNA was significantly decreased following 2 and 4 weeks of PO, while western blot analysis showed a significant increase in adiponectin protein in myocardial samples after 4 weeks of PO (Fig 2.6B). Expression of the adiponectin receptors (AdipoR1 and AdipoR2) and the adapter protein APPL1 was significantly increased 3 days following MTAB surgery. After 4 weeks of pressure overload, AdipoR1, AdipoR2, APPL1 and APPL2 expression was decreased (FIG 3C).

Adiponectin induces collagen synthesis and secretion from isolated cardiac fibroblasts

Ad treatment significantly increased collagen synthesis (3H-Proline incorporation) and secretion (picrosirius staining) from isolated neonatal cardiac fibroblasts (NCFs) after 6 hours (Fig 2.7A), correlating with the increased detection of intracellular collagen I and III following Ad treatment (Fig 2.7B). Immunostaining

similarly showed increased accumulation of the collagen ECM assembled by cardiac fibroblasts *in vitro* following Ad treatment (Fig 2.7D). Rotated 3-dimensional stacks of collagen stained immunofluorescent images show the localization of the collagen I matrix above NCFs, whereas collagen III appears as thick vertical fibres between NCFs. Adiponectin also stimulated an increase in the active form of MMP2 detected by zymographic analysis of cardiac fibroblast conditioned media (Fig 2.7C).

Adiponectin increases cardiac fibroblast migration and inhibits angiotensin II stimulated fibroblast to myofibroblast differentiation

Ad treatment significantly increased cardiac fibroblast migration as assessed through the wound scratch assay (Fig 2.8B, C), while there was no change in fibroblast proliferation (³H-Thymidine incorporation) following adiponectin treatment (Fig 2.8A). Fibroblast to myofibroblast differentiation, a principle event in the development of cardiac fibrosis, was significantly increased by angiotensin II treatment of isolated cardiac fibroblasts as assessed through increased expression of α -smooth muscle actin (SMA) via western blot analysis and immunofluorescent staining. Adiponectin pre-treatment significantly attenuated angiotensin II induced fibroblast to myofibroblast differentiation (Fig 2.8 D, E).

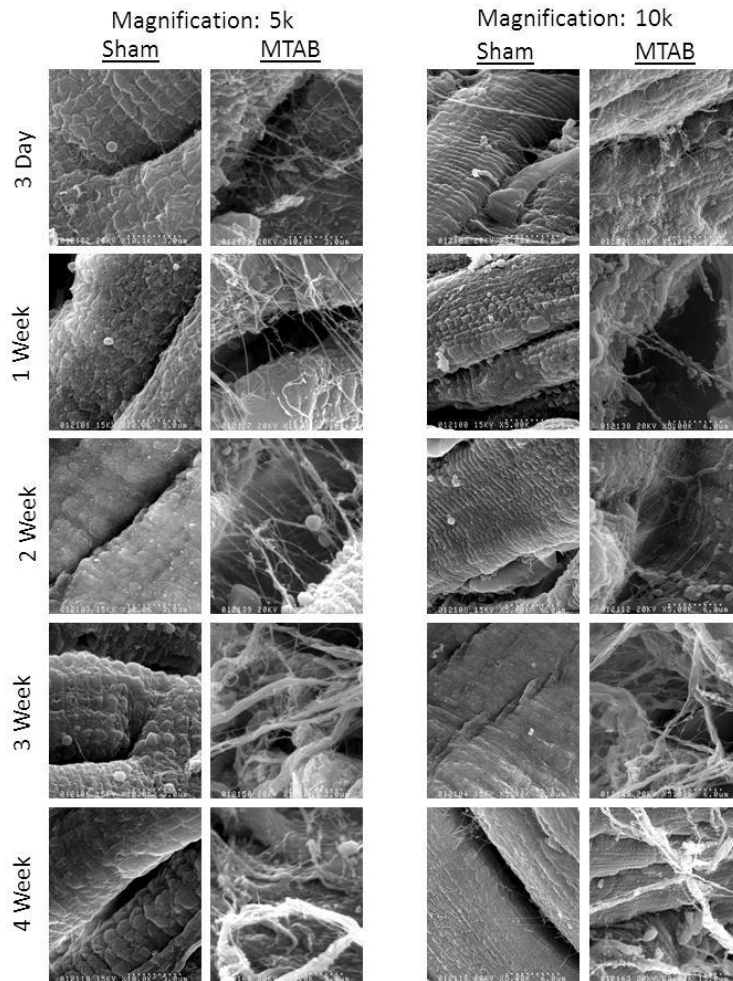


Figure 2.1: Cardiac fibrosis is temporally regulated following PO.

Representative scanning electron micrographs of isolated samples of WT mouse ventricles 3 days, 1, 2, 3, or 4 weeks following sham or MTAB surgery, shown at 5000X or 10000X. Images shown are representative of 5 – 10 images of n = 4 to 6 mice per group

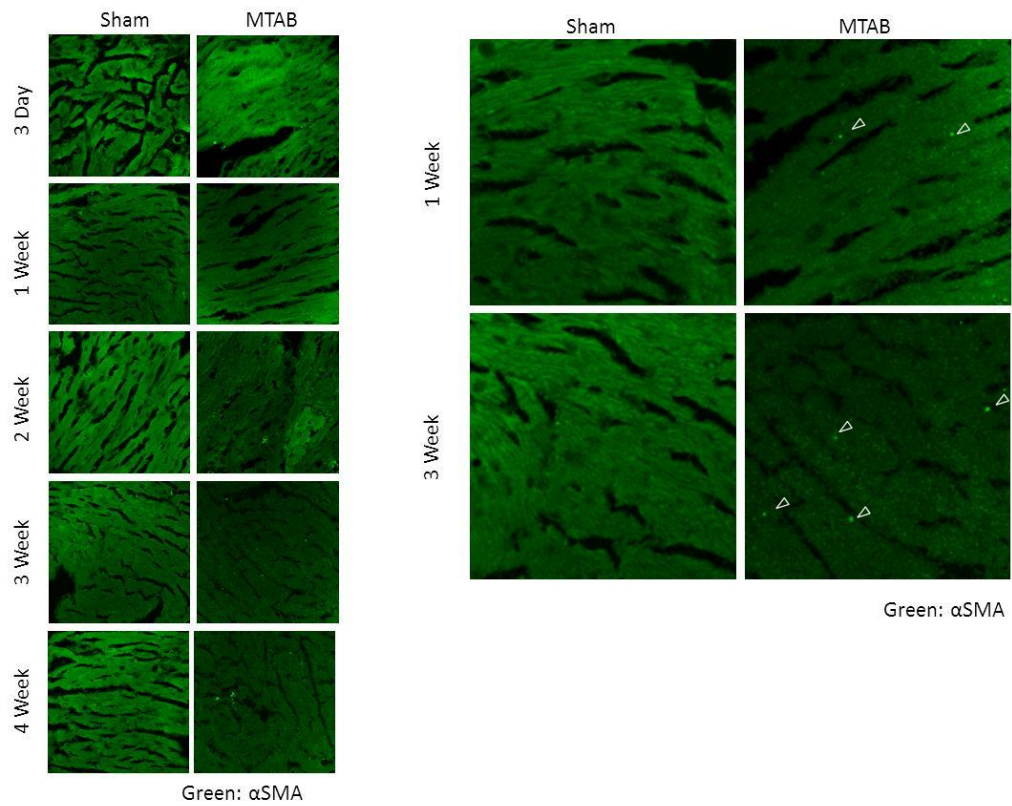


Figure 2.2: Detection of myofibroblasts in the myocardium following PO.

Immunofluorescent staining of α -smooth muscle actin indicating myofibroblasts in histological sections taken from WT mice 3 days, 1, 2, 3, or 4 weeks following sham or MTAB surgery. Myofibroblast positive cells appear bright green. Images shown are representative of 5 – 10 images of $n = 4$ to 6 mice per group. Right side images have been digitally expanded to show α SMA positive cells (white arrow heads).

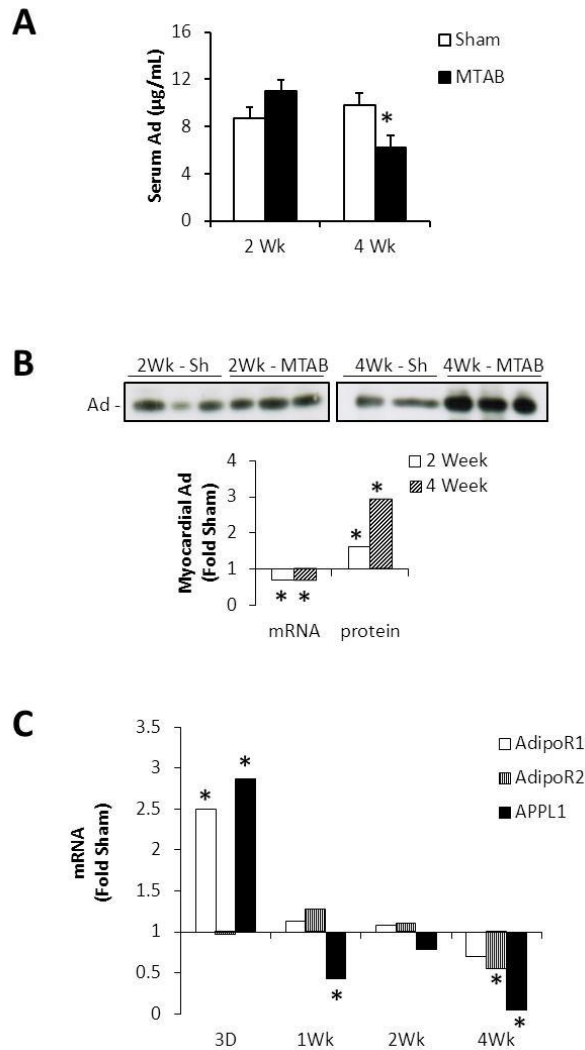


Figure 2.3: PO induces myocardial Ad retention and resistance.

(A) Analysis of serum adiponectin by ELISA. Serum was collected at time of euthanization from AdKO or WT mice 2 or 4 weeks following sham or MTAB surgery. Values are represented as mean of 4 to 6 mice per group \pm sem. (B) Western blot analysis of cardiac homogenate samples from Ad KO or WT mice 2 or 4 weeks following sham or MTAB surgery, quantified in the graph below alongside quantitative real-time PCR analysis of adiponectin mRNA obtained from cardiac homogenates isolated from AdWT mice 2 or 4 weeks following sham or MTAB surgery. (C) Quantitative real-time PCR analysis of AdipoR1, AdipoR2, APPL1, and APPL2 mRNA obtained from cardiac homogenates isolated from WT mice 2 or 4 weeks following sham or MTAB surgery. Values are represented as average C(t) fold sham, where sham is set to 1. Values are average of n = 3 to 5 mice per group \pm sem.

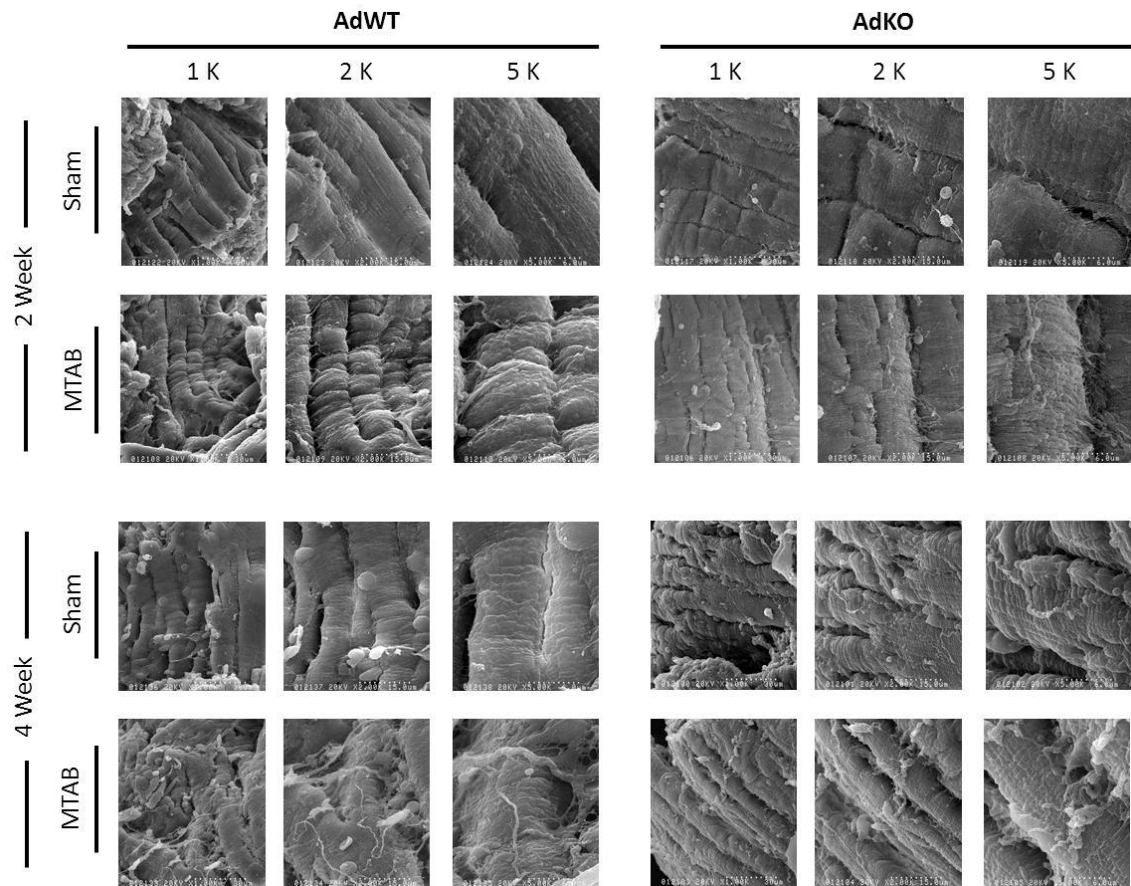


Figure 2.4: Adiponectin deficiency is associated with increased basal fibrosis. Representative scanning electron micrographs of isolated samples of Ad KO or WT mouse ventricles 2 or 4 weeks following sham or MTAB surgery, shown at 2000X (A) or 5000X (B). Images shown are representative of 5 – 10 images of n = 4 to 6 mice per group.

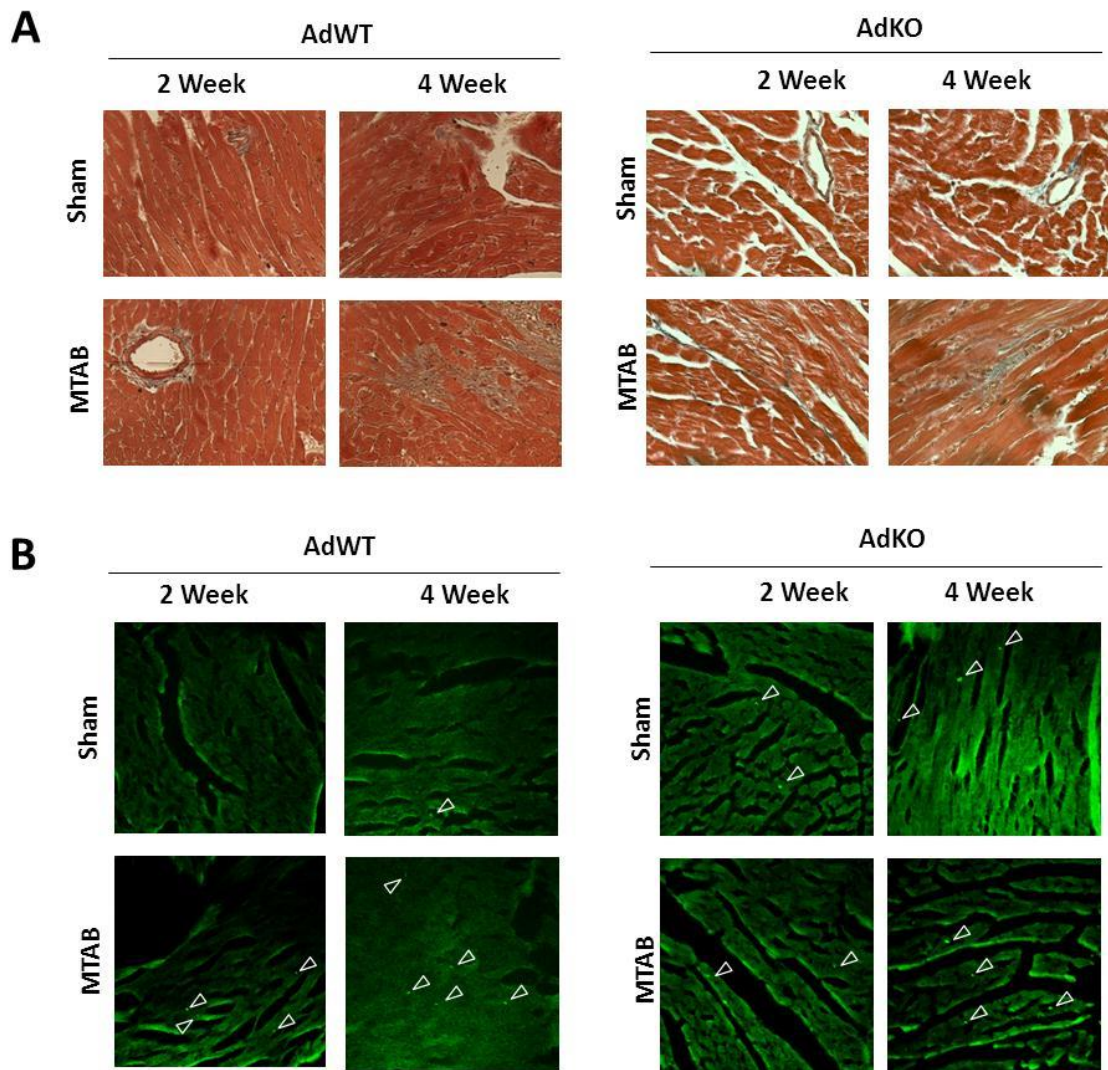


Figure 2.5: Fibrosis and myofibroblast expression is delayed in AdKO mice.

A) Masson's trichrome staining of histological sections taken from AdKO or WT mice 2 or 4 weeks following sham or MTAB surgery. Cardiomyocytes are stained red, nuclei are stained black, and collagen is stained blue. (B) Immunofluorescent α -smooth muscle actin staining of myofibroblasts in histological sections taken from AdKO or WT mice 2 or 4 weeks following sham or MTAB surgery. Cells with strong α -smooth muscle actin staining appear bright green. Arrows indicate example positive cells. Images shown are representative of 5 – 10 images of n = 4 to 6 mice per group.

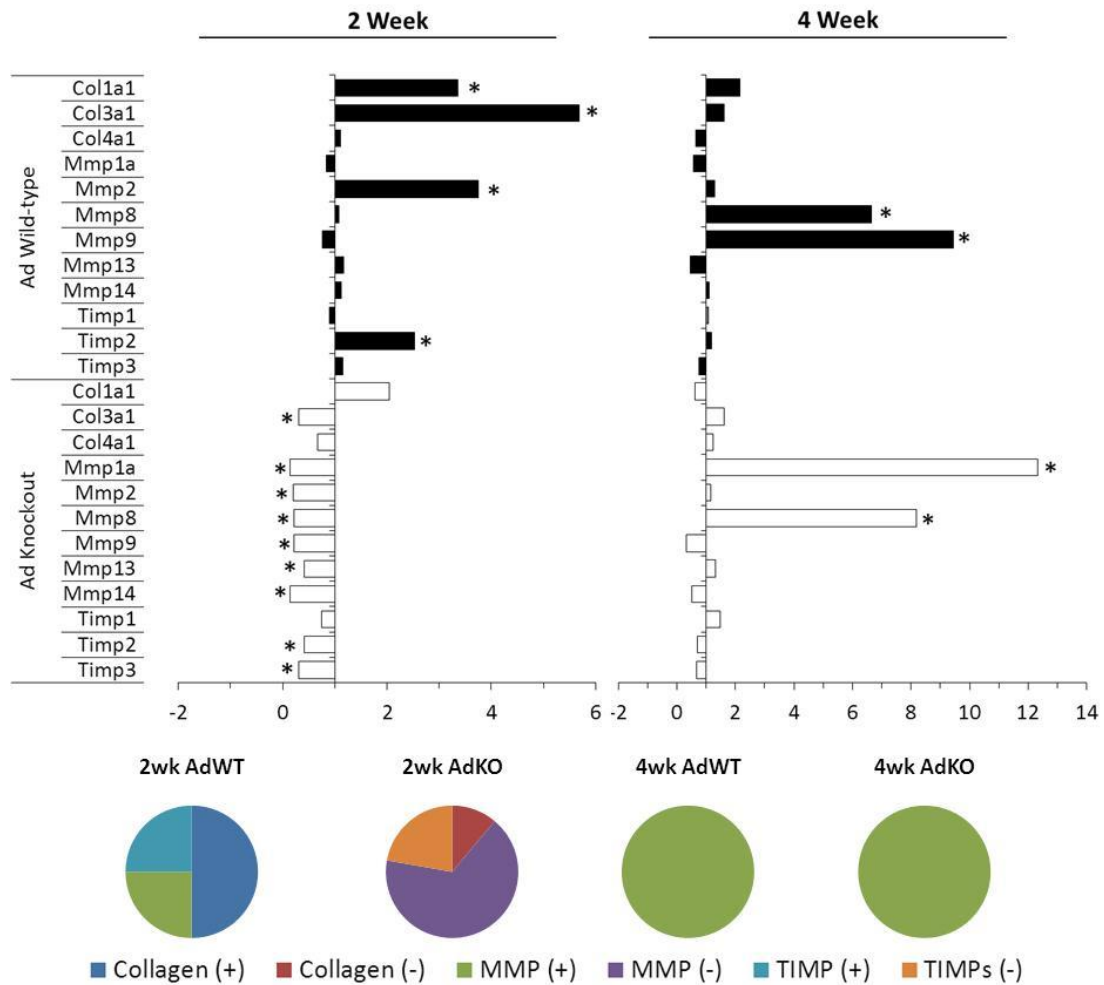


Figure 2.6: Gene regulation of ECM markers following PO.

Analysis of collagen, MMP, and TIMP expression from Ad KO or WT mice 2 or 4 weeks following MTAB surgery, represented as average C(t) value fold sham, where sham is set to 1. Values are average of n = 4 to 6 mice per group. * = p < 0.05

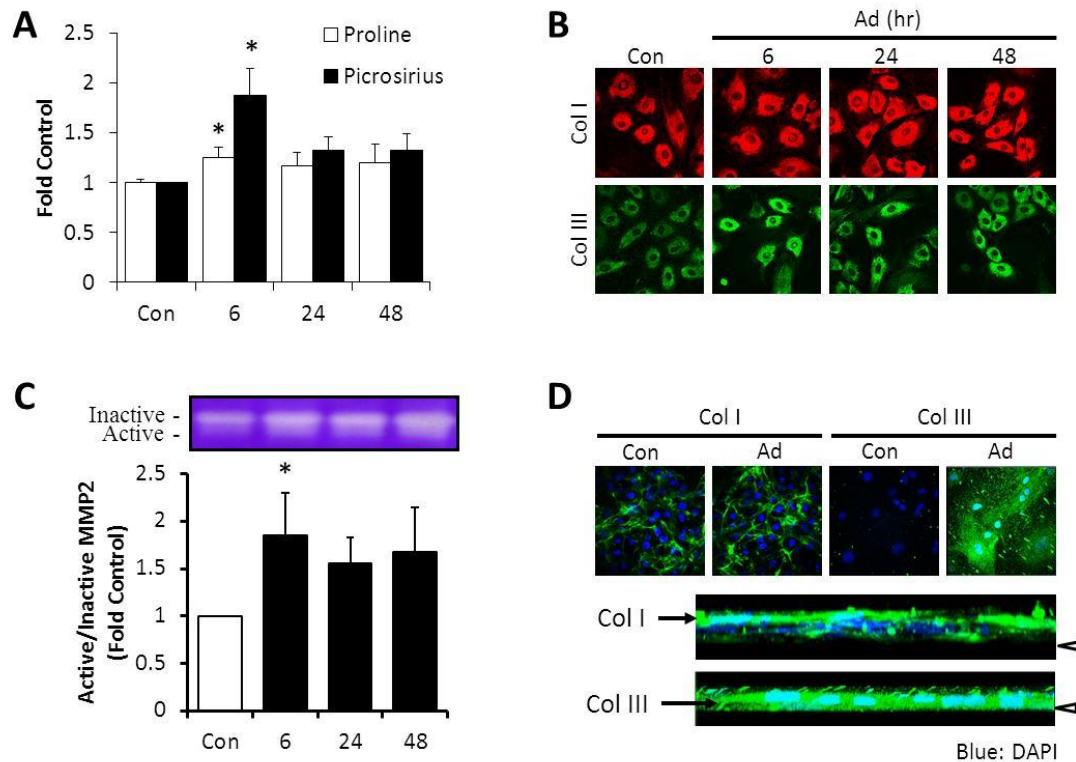


Figure 2.7: Adiponectin Increases Collagen Synthesis and Secretion, and MMP2 activation from Cardiac Fibroblasts.

(A) Intracellular pro-collagen synthesis was assessed by ^3H -proline incorporation following adiponectin treatment ($5 \mu\text{g}/\text{mL}$) for 6, 24, or 48 h. Data represent mean values \pm SEM from $n = 3$ experiments using 3 wells per group for quantification. Total secreted collagen was measured in fibroblast conditioned media following adiponectin treatment for 6, 24 or 48 h by picosirius red staining. Data represented as mean arbitrary units \pm SEM from $n = 7$ experiments. (B) Immunofluorescent images of intracellular collagen I (red) and collagen III (green) synthesized in cardiac fibroblasts at 60x magnification. Cells were treated with adiponectin for 6, 24 and 48 h. Representative images from $n = 3$ experiments are shown. (C) MMP2 activation was analyzed by gelatin zymography in conditioned media collected from 6, 24 and 48 h adiponectin treated ($5 \mu\text{g}/\text{mL}$) fibroblasts. A representative gel, indicating inactive ($\sim 68 \text{ kDa}$) and active ($\sim 62 \text{ kDa}$) MMP2 isoforms, is also shown in (C). MMP2 activity represents the MMP2 active/inactive ratio. Data represent mean values \pm SEM from $n = 3$. (D) Immunofluorescent images of extracellular collagen I (green) and collagen III (red) secreted from cardiac fibroblasts at 60x magnification. Cells were treated with adiponectin for 3 days. Cell nuclei were also stained with DAPI (blue). Representative images from $n = 3$ experiments are shown. Below, 3-dimensional stacks of Ad treated NCFs immunostained for Col-I and Col-III were rotated to show relative vertical orientation of nuclei (DAPI) and collagen (green). Arrow head indicates coverslip.

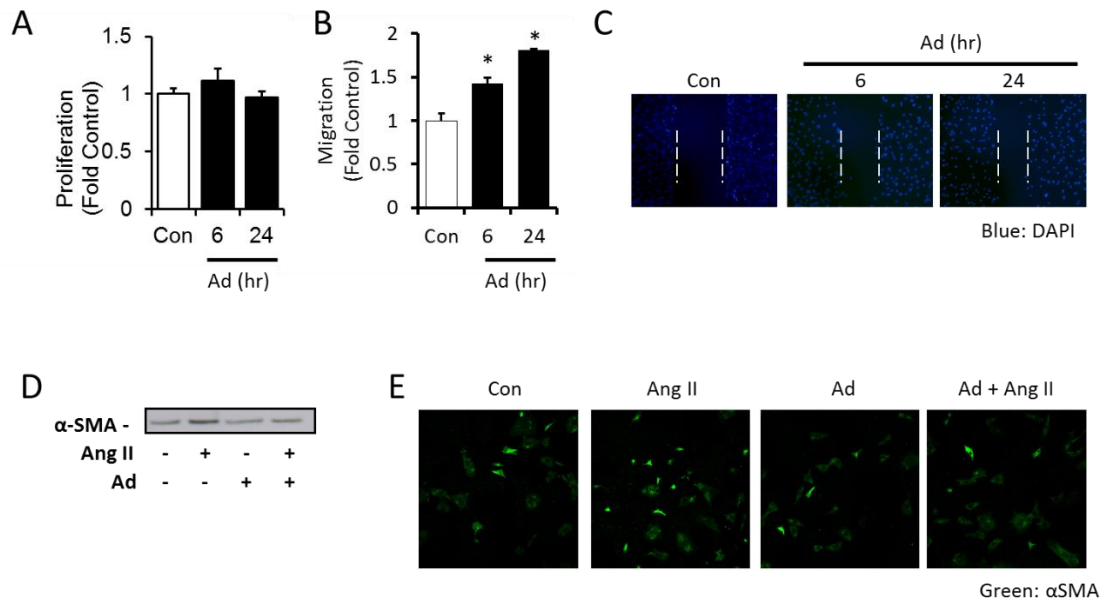


Figure 2.8 Adiponectin increases fibroblast migration, and inhibits fibroblast to myofibroblast differentiation

(A) Fibroblast proliferation was assessed by ³H-thymidine incorporation following 6 or 24 hours adiponectin treatment. Data represent mean values ± SEM from n = 3 experiments. (B) Quantification of cell migration, shown in (C), examined using the wound scratch assay in adiponectin treated (5 µg/mL) cardiac fibroblasts. Cell nuclei were stained with DAPI and imaged using fluorescent microscopy under a 20x objective. Migration was quantified as the reduction of the wound width. Data represent mean values ± SEM from n = 3 experiments, using 7-10 images per group for quantification. *Significant difference (p < 0.05) from untreated control. (D) Western blot analysis of cardiac fibroblast cell lysates treated with adiponectin (5 µg/mL) and/or pre-treated with AngII as indicated. (E) Immunofluorescent staining for αSMA in cardiac fibroblasts treated with adiponectin (5 µg/mL) and/or pre-treated with AngII as indicated.

2.5 Discussion

In this study we sought to characterize the temporal progression of cardiac fibrosis induced by pressure overload in the presence or absence of adiponectin. The well characterized non-ischemic myocardial fibrotic response to pressure overload is the robust increase in Col I/Col III ratio aimed to support the overloaded myocardium and delay the progression towards decompensated heart failure. Whereas previous studies have relied on masson's trichrome staining or collagen mRNA as measures of fibrosis, herein we add a visual temporal analysis of developing myocardial fibrosis using scanning electron microscopy. Our characterization of the progression of fibrosis in WT mice showed an acute increase in small-fibre fibrosis 3 days after MTAB surgery, morphologically consistent with the increased deposition of Col III, followed by the appearance of thick Col I fibres starting 2 weeks after MTAB surgery. Accordingly, there is a significantly increased presence of myofibroblasts from 2 to 3 weeks following MTAB. The conspicuous absence of myofibroblasts 4 weeks after MTAB may be a result of the previously characterized fibroblast apoptotic response [258, 307], a known protective mechanism limiting adverse fibrosis.

MTAB surgery failed to induce myocardial collagen deposition or activation of ECM regulatory genes in the AdKO myocardium, and instead we observed a significant downregulation of MMPs and TIMPs. Indeed, while this may have led to decreased collagen turnover in the heart (basal estimates are 9% per day [273]), previous studies of MMP and TIMP knockout mice have shown these to be cardio-

protective genetic deletions [253]. Suppression of MMP and TIMP expression was alleviated following 4 weeks of PO in AdKO mice, corresponding with increased myocardial fibrosis. In WT mice, Col I and III, and MMP2 were upregulated following 2 weeks of pressure overload, while at 4 weeks, gene expression of MMPs 8 and 9 were enhanced. Changes in MMP expression and activity following acute or chronic pressure overload has been noted previously [308], and is typically associated with increased differentiation of myofibroblasts, and the overall progression of cardiac remodelling.

The presence of α -SMA positive cells (a strong indicator of myofibroblasts) in the myocardium is a common indicator of myocardial stress and decreased ventricular compliance [250], while localization of differentiated myofibroblasts to the peri-infarct area has been associated with decreased cardiac function following MI, as myofibroblasts secrete high levels of MMPs and collagen as part of the innate cardiac repair mechanism [272]. In this study we noted the presence of myofibroblasts in the sham AdKO myocardium which may account for the increased presence of small fibre fibrosis observed via SEM. Typically, immediate expansion of the ECM following PO and MI is associated with adaptive remodelling, contributing to the preservation of cardiac function. Here we observed that fibrosis following 4 weeks of MTAB was reduced in AdKO mice compared to WT mice suggesting a delay in ECM remodelling due to adiponectin deficiency. Accordingly, functional data comparing AdKO and WT mice show that POH and cardiac dysfunction are delayed in AdKO mice subjected to MTAB surgery (chapter 4: study 3). Taken together, we postulate that adiponectin

deficiency permits the presence of myofibroblasts in the normal myocardium, thereby inducing non-pathological small fibre cardiac fibrosis that confers short-term protection of cardiac function following the induction of pressure overload.

To study adiponectin induced fibroblast activation, we treated isolated neonatal cardiac fibroblasts with the potent inducer of myocardial fibrosis angiotensin II (AngII). We found that adiponectin pre-treatment significantly inhibited AngII induced fibroblast to myofibroblast differentiation. Certainly, further study is needed to confirm that the presence myofibroblasts observed in the sham AdKO myocardium is directly due to adiponectin deficiency, however, we now propose that adiponectin deficiency permits AngII induced myofibroblast differentiation in the AdKO heart leading to small fibre fibrosis, observed via SEM, and protection against MTAB induced ECM expansion. Cross-talk between adiponectin and AngII signalling has been previously suggested as previous studies had shown that AngII could induced PARP-1 activation, which in turn downregulates adiponectin mRNA expression in cardiac fibroblasts, suggesting that adiponectin and AngII may share a ying-yang relationship in the heart. Clearly, the adiponectin-AngII balance could prove to be an important feature in the local production and actions of adiponectin and AngII in the remodelling heart, especially with respect to the development of fibrosis.

We had previously demonstrated regulation of matrix metalloproteinase activity in isolated neonatal cardiac fibroblasts by the metabolically potent globular form of adiponectin (gAd) [108], chapter 3:study 2. Interestingly, we noted that while gAd induced significant remodelling of the ECM produced *in vitro* through the upregulation of MMP activity, gAd treatment did not alter collagen gene expression or secretion [108]. In this study we show that recombinant full-length adiponectin, which contains a biologically relevant mixture of all three adiponectin isoforms, significantly induces collagen expression *in vitro*. An understanding of the ability of the different isoforms of adiponectin to mediate either MMP activity or collagen expression from cardiac fibroblasts may be an important factor in delineating adiponectin's role in cardiac remodelling especially since circulating adiponectin levels are known to change with PO and correlate with different physiological outcomes in metabolism and heart disease [112].

Circulating and myocardial transcript levels of adiponectin were reduced following surgery while there was a significant increase in myocardial adiponectin protein detected by western blot analysis, indicating the increased retention, and bioavailability of adiponectin within the fibrotic myocardium [160]. This may be an important mechanism serving to offset observed PO induced adiponectin resistance (i.e. downregulation of AdipoRs and APPL1). The importance of adiponectin accumulation and enhanced bioavailability at sites of cardiac injury [117, 135], as well as the putative liberation of the potent gAd through inflammatory cell derived

leukocyte elastase [80] adds to the complexity of local adiponectin action within the heart. Together, these changes strongly suggest a dynamically changing profile of adiponectin action in the remodelling heart.

Early studies using AdKO mice demonstrated the detrimental effects of adiponectin deficiency on PO induced cardiac remodelling, including exacerbated cardiac hypertrophy and fibrosis, as well as accelerated functional decline [116, 157, 158]. These studies are often cited as evidence of the cardioprotection of adiponectin in light of the emerging complex clinical association of adiponectin with various states of heart failure [133, 301, 302]. However, recent studies in AdKO mice following PO by Stanley instead demonstrate the permissive role of adiponectin in the pathogenesis of adverse remodelling events [161, 162]. The data presented in this chapter adds to accumulating evidence that adiponectin may play a detrimental role in the development of PO induced heart failure. While it remains clear from all studies that adiponectin plays an important role in the response of the heart to PO, the simple view of adiponectin as either cardioprotective or maladaptive must be refined with further detailed analysis of the complex pathogenesis of heart disease.

In conclusion, our studies suggest that adiponectin deficiency delays the progression of PO induced fibrosis. Small fibres in the AdKO myocardium that serve to support the heart against hemodynamic load may be a result of AngII-induced myofibroblast differentiation permitted by adiponectin deficiency. In WT mice,

however, ECM expansion following PO may serve to retain adiponectin within the myocardium to offset adiponectin resistance.

CHAPTER 3: STUDY 2

ADIPONECTIN MEDIATED APPL1-AMPK SIGNALLING INDUCES CELL MIGRATION, MMP ACTIVATION, AND COLLAGEN REMODELLING IN CARDIAC FIBROBLASTS

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3.1 Abstract

Defects in adiponectin action have been implicated in the development of cardiac dysfunction in obesity and diabetes. Cardiac fibroblasts play an important role in regulating extracellular matrix remodelling yet little is known regarding the direct effects of adiponectin on cardiac fibroblasts. In this study, we first demonstrated temporal relocalization of cellular APPL1 in response to adiponectin in primary cardiac fibroblasts and that siRNA-mediated knockdown of APPL1 attenuated stimulation of AMPK by adiponectin. The cell surface content of MT1-MMP and activation of MMP2 were induced by adiponectin and these responses were dependent on AMPK signalling. Enhanced MMP activity facilitated increased fibroblast migration in response to adiponectin which was also prevented by inhibition of AMPK, with no change in cell proliferation observed. Collagen and elastin immunofluorescence demonstrated reorganization of the extracellular matrix in accordance with increased MMP activity, whereas quantitative mRNA analysis, ³H-proline incorporation and picrosirius red assays showed no change in intracellular or extracellular total collagen levels in response to adiponectin. In summary, these data are the first to report the adiponectin stimulated APPL1-AMPK signalling axis in cardiac fibroblasts and characterize MT1-MMP translocation, MMP2 activity and cell migration as functional outcomes. These effects may be of significance in heart failure associated with obesity and diabetes.

3.2 Introduction

The growing epidemic of obesity and diabetes has caused a disturbing and rapid increase in the incidence of heart disease [128, 309]. Extensive studies have indicated that altered circulating levels of adiponectin are associated with cardiac remodelling and dysfunction in obesity and diabetes [128, 148]. The majority of studies indicate a cardioprotective role of adiponectin which is mediated via regulation of multiple myocardial remodelling events [148, 310]. The metabolic, anti-hypertrophic and anti-apoptotic effects of adiponectin are well established and adiponectin has also been shown to mediate anti-fibrotic effects [148, 303].

The cardiac extracellular matrix (ECM) is a highly structured interstitial network of proteins composed mainly of collagens that surrounds the contractile cardiomyocytes. Remodelling of the ECM is widely accepted to have broad implications on cardiac function [250]. Fibrosis is commonly observed in various models of heart disease in rodents and in human failing hearts [311, 312]. Cardiac fibroblasts are the most prevalent cell type in the heart, and regulate ECM dynamics through the expression and regulation of collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs) [249, 253]. Acute and chronic stressors such as myocardial infarction and hypertension have been shown to induce MMP expression and activity to degrade structural ECM proteins. After myocardial infarction, cardiac fibroblasts migrate to the damaged area to participate in repair

[304, 313]. This can initially aid contractile function but renewal of matrix with poorly structured collagen increases myocardial stiffness and contributes to dysfunction. A more detailed understanding of adiponectin's effects and mechanism of action on cardiac fibroblasts is needed.

Many cardioprotective effects of adiponectin have been shown to be mediated via AMPK [314] and recent work in skeletal muscle, cardiomyocytes, endothelial cells and hepatocytes have shown APPL1, an adaptor protein containing an NH2-terminal BAR (Bin/Amphiphysin/Rvs) domain, a PH (pleckstrin homology) domain, a COOH-terminal PTB (phosphotyrosine-binding) domain, and a leucine zipper motif, plays an essential role in activation of AMPK by adiponectin [54, 113, 315-319]. However, adiponectin stimulated APPL1-AMPK signalling in cardiac fibroblasts, and the functional consequences, have not yet been shown.

In the present study, we used primary rat cardiac fibroblasts to examine the direct effects of adiponectin on MMP expression, localization and activities and cell migration. Changes in collagen isoform expression and extracellular collagen content and structure were determined. We also investigated if adiponectin's effects were mediated via AMPK and whether APPL1 was critical for AMPK activation.

3.3 Materials and Methods

Materials

Recombinant globular adiponectin was produced in-house as previously detailed by [121]. Dulbecco's modified eagle's medium (DMEM), trypsin, antibiotic/antimycotic and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Life Technologies Inc., Burlington, ON, CA). All culture plates were BD Falcon™ brand and purchased from BD Biosciences (Mississauga, ON, CA). The MF20 antibody was a kind gift from Dr. J.C. McDermott (York University, Toronto, ON, CA) and von Willebrand factor antibody was purchased from Affinity Biologicals Inc. (Ancaster, ON, CA). Anti-phospho-AMPK α (Thr172), anti-AMPK α , anti-APPL1, anti- β actin primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signalling Technology (New England Biolabs Ltd., Whitby, ON, CA). The anti-collagen antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the anti-elastin antibody was obtained from Elastin Products Company, Inc. (Owenville, M), USA). Western Lightning Plus ECL was obtained from PerkinElmer (Woodbridge, ON, CA). TRIzol® Reagent and all siRNAs used in this study were purchased from Ambion Inc. (Life Technologies Inc.), Lipofectamine® 2000 Transfection Reagent was obtained from Invitrogen (Life Technologies Inc.), and Alexa Fluor® 488 goat anti-rabbit antibody was obtained from Molecular Probes (Life Technologies Inc.). The FITC-labelled goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West

Grove, PA, USA). The MT1-MMP (MMP-14) antibody was purchased from Acris Antibodies Inc. (Cedarlane Labs, Burlington, ON, Canada). VECTASHIELD® mounting medium with DAPI was obtained from Vector Laboratories (Burlington, ON, Canada). RNeasy MinElute Cleanup and RT2 First Strand kits were purchased from QIAGEN Inc. (Mississauga, ON, CA). 3H-proline and 3H-thymidine were purchased from Amersham Biosciences (GE Healthcare Lifesciences, Baie d'Urfe, QC, CA). Amicon Ultra-15 Centrifugal Filter Units and Compound C were obtained from EMD Millipore (Billerica, MA, USA). The Pierce BCA Protein Assay kit was purchased from Thermo Scientific (Fisher Canada, Nepean, ON, CA). The SIGMAFAST OPD kit was purchased from Sigma-Aldrich (Oakville, ON, CA). The custom PCR array for collagens was purchased from SABiosciences (QIAGEN Inc.).

Isolation, Culture and Adiponectin Treatment of Neonatal Rat Cardiac Fibroblasts

Neonatal cardiac fibroblasts (CFs) were isolated from 3 – 4 day old Wistar rats as previously described [121]. Briefly, excised hearts were digested with 0.15% trypsin for 1 hour at room temperature. Trypsin was then neutralized with DMEM containing 10% FBS, and digested cells were centrifuged (10 min at 2000 rpm), resuspended in DMEM containing 10% FBS and 1% antibiotic/antimycotic and plated onto culture plates. Following incubation at 37 °C for 1 hour in a 5% CO₂ atmosphere, cardiomyocytes remaining in suspension were removed, and attached fibroblasts were replenished with complete growth medium described above. Cardiac fibroblast purity was assessed through MF20 (cardiomyocyte) and von Willebrand factor (endothelial

cell) staining [121] which showed less than 1% staining for non-fibroblast cells. CFs were passaged twice, grown to 100% confluence (or as otherwise indicated below) and then starved with serum-free DMEM for at least 3 hours prior to treatment with recombinant globular adiponectin (1.0 µg/ml).

Western Blot Analysis

Cells lysis and protein sample preparation for Western blot was conducted according to methods detailed by [305]. Primary anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-APPL1, and anti-β-actin antibodies were used at 1:1000 dilutions, and appropriate HRP-conjugated secondary antibodies were used at 1:10,000 dilutions.

Proteins were detected by chemiluminescence, quantified by densitometry using Scion Image software (Scion Corp., Frederick, MD, USA) and then normalized to either β-actin or total AMPK protein levels as appropriate.

siRNA Transfection of Cardiac Fibroblasts

CFs were grown to ~30 – 50% confluence in 12-well plates, and then transfected for 4-6 hours with 100 nM control or APPL1 siRNA using Lipofectamine® 2000 Transfection Reagent according to the manufacturer's instructions. The APPL1 siRNA sequence is as follows: GCUUAGUUCUUGUCAUGCAtt. Adiponectin treatment was commenced 48 hours post-transfection and APPL1 knockdown efficiency was assessed by Western blot as detailed above (see Western Blot Analysis).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cultured CFs using TRIzol® Reagent according to the manufacturer's instructions, and purified using the RNeasy MinElute Cleanup Kit to attain an A260/A280 ratio between 1.9 and 2.0. First-strand cDNA, synthesized from 1 µg RNA using the RT2 First Strand kit, was used in a custom PCR array comprising 96well plates pre-coated with primers for collagens-I, -III and -IV. Quantitative real-time PCR was conducted using a Chromo4™ Detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, CA) according to cycling conditions outlined by the PCR array manufacturer. Data were analysed using RT2 Profiler PCR Array Data Analysis software (Version 3.5; QIAGEN Inc.) and normalized to GAPDH mRNA expression.

Wound Scratch Migration Assay

Fibroblast migration in response to adiponectin treatment was assessed using the wound scratch assay. Briefly, a sterile 200 µL pipette tip was used to scratch a vertical line through CFs grown to confluence in 12-well plates. Scratched wells were then thoroughly washed with PBS to remove unattached cells, starved overnight in serumfree DMEM, and then pretreated for 1 hour with DMSO or 20 µM Compound C before adiponectin treatment. Following the indicated adiponectin treatment times, cells were fixed in 90% methanol for 30 min at -20oC, however prior to fixation, 1 or 2 unscratched wells were also freshly scratched with a sterile 200 µL pipette tip to serve as 'fresh scratch' controls. The cells were washed once more with PBS and then

mounted using VECTASHIELD® mounting medium with DAPI. Fluorescent images were obtained using an Olympus BX51 confocal microscope (Olympus, Seattle, WA, USA) with a 20 x objective, and fibroblast migration was assessed as the closure of the scratch wound in arbitrary length units using Inkscape software (www.inkscape.org).

Detection of Cell Surface MT1-MMP

Cell surface MT1-MMP was quantified in intact CFs using an MT1-MMP antibody, which recognizes an extracellular epitope, and the SIGMAFAST OPD kit as previously described [81]. Briefly, adiponectin-treated cells were washed with PBS and fixed on ice for 3 min with 3% paraformaldehyde (PFA). The cells were then washed and incubated for 10 min with 1% glycine to neutralize the PFA, blocked for 30 min in 5% goat serum, incubated on ice for 1 hour with MT1-MMP antibody (1:500 dilution in blocking buffer) and then incubated for 1 hour at 4°C with HRP-linked anti-mouse antibody (1:1000). Cells were then washed with PBS, incubated for 30 min at room temperature with OPD reagent (0.8mL/well), following which 200 µL of 3M HCl was added to stop the reaction. An absorbance reading was taken for 1 mL of each sample at 492nm using a spectrophotometer.

MMP2 Activity By Gelatin Zymography

Media was collected from CFs grown in 6-well plates and concentrated using Amicon Ultra-15 Centrifugal Filter Units following adiponectin treatment at indicated times.

The protein content of the resulting concentrated conditioned media was determined using the Pierce BCA Protein Assay kit, and then equal amounts of protein from media (25 µg) were resolved by SDS-PAGE on a 10% polyacrylamide gel containing 0.3% gelatin. The gel was washed for 1 hour in a solution of 2.5% Triton X-100, briefly rinsed with deionized water, and then incubated for 18 hours at 37 °C in 1M Tris-HCl (pH 7.6) containing 100mM CaCl₂ to activate MMP gelatin degradation. MMP activity was stopped using a 10 min wash in 1M Tris-HCl (pH 7.6) containing 100mM EDTA. Gels were then fixed and stained in a solution containing 50% methanol, 10% acetic acid and 0.25% Coomassie Blue R-250. Areas of gelatin degradation, which were correlated with known molecular weights of MMP2 isoforms, indicated MMP activation. MMP2 activity was quantified by densitometric analysis of degraded areas using Scion Image software (Scion Corp.).

Immunofluorescent Imaging of Endogenous APPL1 and MT1-MMP, and Secreted Collagen and Elastin

CFs were seeded onto 25 mm coverslips. For APPL1 and MT1-MMP imaging, CFs were grown to ~30 - 50% confluence in DMEM containing 10% FBS before adiponectin treatment. For collagen imaging, CFs were initially grown as above for one day, and then cultured for 3 days in DMEM containing 10% goat serum prior to adiponectin treatment. For elastin imaging, CFs were cultured as described above for collagen imaging, however cells were grown in DMEM containing 10% FBS. Following adiponectin treatment at indicated times, cells cultured for APPL1, MT1-MMP and

collagen I imaging were gently washed with PBS, fixed with 3% PFA for 30 min at room temperature, and then incubated with 1% glycine for 10 min at room temperature to quench PFA. Alternatively, cells cultured for elastin imaging were fixed for 30 min with 90% methanol at -20°C. After fixation, the cells were then blocked at room temperature in either 5% goat serum for 1 h for APPL1 and MT1-MMP imaging, 1% horse serum for 30 min for collagen imaging or 1% goat serum for 30 min for elastin imaging, followed by incubation at room temperature with rabbit anti-APPL1 (1:1000 dilution in respective blocking buffer), mouse anti-MT1-MMP (1:200), rabbit anti-collagen I (1:100) or rabbit anti-elastin (1:100) antibody for 1 hour. Cells were then incubated at room temperature with AlexaFluor 488 goat anti-rabbit (1:1000) or FITC-labelled goat antimouse (1:1000) secondary antibody respectively for 1 hour, followed by a final wash with PBS, and mounting on glass microscope slides using VECTASHIELD® mounting medium with DAPI. Immunofluorescent images were obtained using an Olympus BX51 confocal microscope (Olympus, Seattle, WA, USA) with 20x and 60x objectives.

3H-Proline and 3H-Thymidine Incorporation Assays

Pro-collagen synthesis and fibroblast proliferation was assessed by measurement of cellular ³H-proline and ³H-thymidine uptake respectively as previously outlined [320]. Briefly, CFs were treated with or without adiponectin in the presence of ³H-proline or ³H-thymidine (1 µCi/mL final concentration) for the indicated times. At the end of each treatment period, cells were incubated for 30 min with ice-cold 5%

trichloroacetic acid (TCA) at 4°C. The resulting acid precipitate was then solubilized overnight in 0.5 ml of 0.5 N NaOH at 37°C and neutralized with 0.5 ml 0.5 N HCl per well. The radioactivity of each sample was measured in a liquid scintillation counter and corrected for total protein content using the Pierce BCA Protein Assay kit.

Picrosirius Red Detection of Secreted Collagen

CF conditioned media was collected and concentrated following adiponectin treatment, and subsequent protein content was determined as described above (see MMP2 Activity By Gelatin Zymography). Equal amounts of concentrated media (60 µg) was then dried at 37°C in wells of a 96-well plate. To stain collagen, 0.1% picrosirius red solution was added to dried wells for 1 hour at room temperature. Wells were then washed with 10 mM HCl, and the stain was eluted with 0.1 M NaOH for 5 minutes. Eluate absorbance was read at 540nm using a spectrophotometer, and the collagen content of each eluate was quantified based on a collagen standard curve.

Statistical analysis

Data are expressed as mean values \pm SEM (n), where n represents the number of experiments conducted. Student's t tests were used to determine significant differences ($P < 0.05$) between groups. All statistical analyses were conducted using SigmaStat 3.5 Software (Systat Software Inc., San Jose, CA, USA).

3.4 Results

Activation of AMPK by adiponectin was APPL1 dependent

Immunofluorescent imaging of APPL1 localization in CFs revealed a diffuse cytosolic localization under unstimulated conditions (Fig. 3.1A). Adiponectin treatment induced an initial (10 and 15 min) redistribution of APPL1 to the cell membrane where it is likely to directly bind with adiponectin receptors. Changes in APPL1 were clearly time-dependent with nuclear and peri-nuclear localization of APPL1 evident after 30 min (Fig. 3.1A). We next used siRNA to knockdown APPL1 expression (Fig. 3.1B, top) and showed that adiponectin stimulated AMPK phosphorylation was significantly inhibited after APPL1 knockdown (Fig. 3.1B&C). siRNA transfection achieved ~65% APPL1 knockdown as determined by Western blot (data not shown). Of note, we observed a slight increase in basal AMPK phosphorylation after APPL1 knockdown, with no changes in total AMPK expression (Fig. 3.1B).

Adiponectin stimulated MT1-MMP translocation to the cell surface and MMP2 activation, and these effects were mediated via AMPK

To characterize the effects of adiponectin on MMPs in CFs, we first examined cell surface localization of MT1MMP. This was achieved by antibody-based detection of an exofacial epitope in intact cells and quantitative analysis of cell surface MT1-MMP showed over 2-fold increased levels following adiponectin treatment (Fig. 3.2A&B). This observation was supported by qualitative immunofluorescent data

(Fig. 3.2C). To examine mechanisms of this response, we first studied the consequences of reducing APPL1 expression using siRNA and demonstrated that the adiponectin response was significantly blunted (Fig. 3.2A). In addition, after pretreatment of cells with Compound C to inhibit AMPK the adiponectin-stimulated translocation of MT1-MMP to the cell surface was also significantly attenuated (Fig. 3.2B). Zymographic analysis of CF conditioned media demonstrated an increase in MMP2 activation by adiponectin treatment, which was significantly attenuated by pretreatment with Compound C (Fig. 3.2C).

Adiponectin induced cardiac fibroblast migration via AMPK signalling

We performed the wound-scratch assay to measure the effects of adiponectin on CF migration. Adiponectin significantly increased CF migration after 40, 60 and 120 min of treatment (Fig. 3.3A&B). Adiponectin had no significant effect on cell proliferation measured via ³H-thymidine incorporation (Fig. 3.3C). Compound C significantly attenuated adiponectin-stimulated CF migration (Fig. 3.3A&B). There was no significant difference in cell migration between DMSO only, Compound C only, or 'fresh scratch' wells (data not shown).

Adiponectin does not alter collagen synthesis and secretion but induces collagen and elastin remodelling

Under basal conditions, CFs secrete and assemble a collagen I matrix with a wispy and homogenous appearance (Fig. 3.4A). In keeping with the enhanced MMP activity, degradation of this collagen matrix was observed after 1 and 3 hours of

adiponectin treatment (Fig. 3.4A). Interestingly, a restored collagen matrix was observed 24 hours after adiponectin treatment. This newly formed matrix appeared more heterogeneous with larger collagen fibril aggregates, giving a thickened and patchy appearance (Fig. 3.4A). Examination of intracellular pro-collagen synthesis by ³H-proline incorporation (Fig. 3.4B) and total secreted collagen in CF conditioned media measured by picrosirius red staining (Fig. 3.4C) showed no change in response to adiponectin treatment. Using quantitative PCR analysis we also found that expression of collagen-I, -III and -IV isoforms were not altered by adiponectin (Fig. 3.4D). The CF elastin matrix also appeared to be similarly degraded upon adiponectin treatment (Fig. 3.4E).

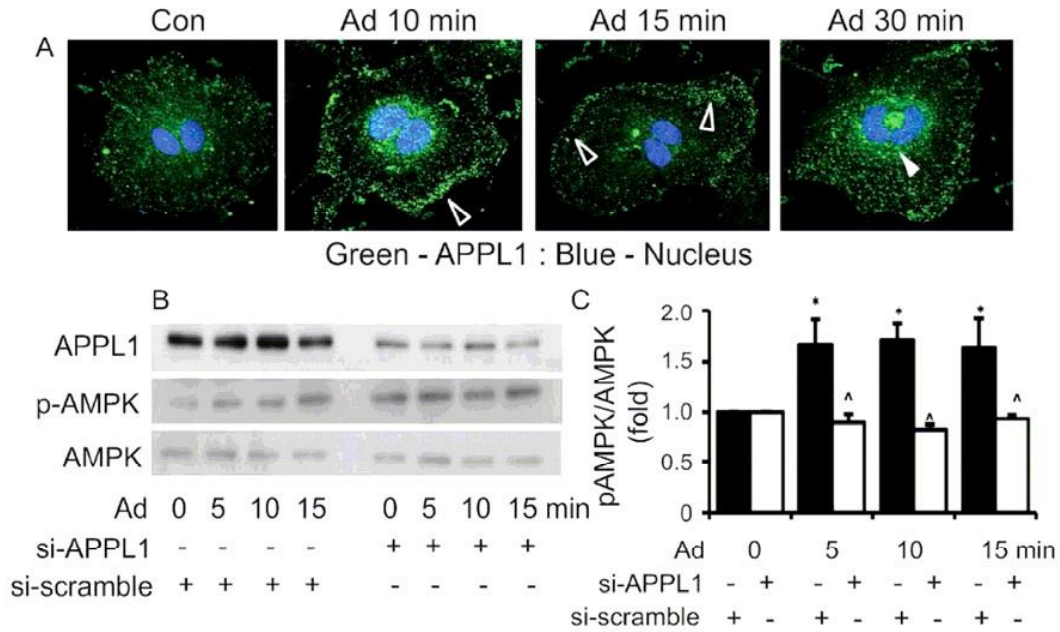


Figure 3.1: Activation of AMPK by adiponectin is APPL1 dependent

(A) Immunofluorescent images of APPL1 (green) in cardiac fibroblasts stimulated with adiponectin (1 $\mu\text{g}/\text{mL}$) for 10, 15 and 30 min. Cell nuclei were also stained with DAPI (blue). Representative images of $n = 3$ experiments are shown. Open arrows indicate membrane localization. Closed arrow indicates peri-nuclear localization. (B) APPL1 or non-specific control siRNA transfected cardiac fibroblasts were treated with adiponectin for 5, 10 and 15 min, and immunoblotted for phospho-AMPK, total AMPK, and APPL1 to verify knockdown efficiency. Representative Western blots are shown from $n = 3$ experiments. (C) Phospho-AMPK was quantified and normalized to total AMPK. Data represent mean values \pm SEM from $n = 3$ experiments. *Significant difference ($p < 0.05$) from control. ^Significant difference ($p < 0.05$) from adiponectin treatment group at corresponding time point.

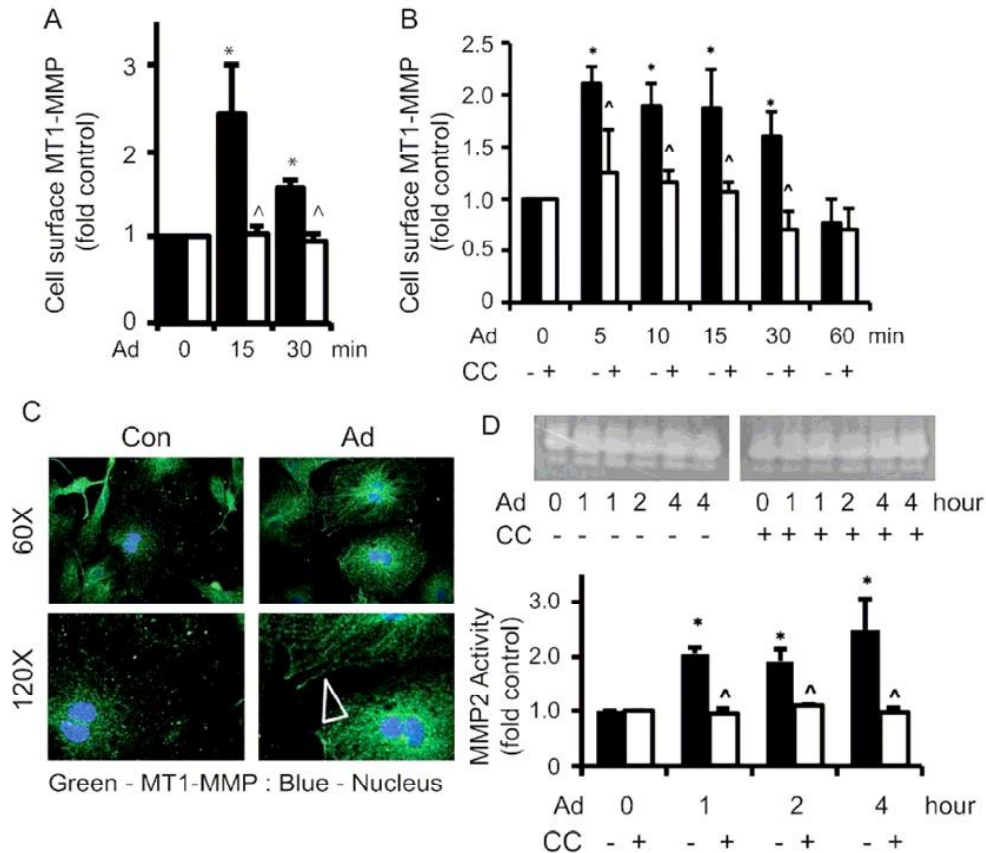


Figure 3.2: Adiponectin stimulates MT1-MMP translocation to the cell surface and MMP2 activation via AMPK

Cell surface MT1-MMP was examined by OPD assay in cardiac fibroblasts that were treated with adiponectin (1 $\mu\text{g}/\text{mL}$) at indicated time points and following (A) knockdown of APPL1 using siRNA versus scrambled control or (B) 1 hour pre-treatment with either vehicle (DMSO) or 20 μM Compound C. Data represent mean values \pm SEM from $n = 3$. (C) Representative immunofluorescent images from $n = 3$ experiments are shown of MT1-MMP (green) localization in adiponectin treated cardiac fibroblasts. Cell nuclei were also stained with DAPI (blue). Open arrow indicates MT1-MMP membrane localization. Top images were taken using a 60X objective, and bottom images were digitally enhanced 2X to achieve 120X magnification. (D) MMP2 activation was analyzed by gelatin zymography in conditioned media collected from 1, 2 and 4 hours adiponectin treated fibroblasts that were pre-treated for 1 hour with either DMSO or 20 μM Compound C. A representative gel, indicating inactive (~ 68 kDa) and active (~ 62 kDa) MMP2 isoforms, is also shown in (C). MMP2 activity represents the MMP2 active/inactive ratio. Data represent mean values \pm SEM from $n = 3$ experiments. *Significant difference ($p < 0.05$) from control. ^Significant difference ($p < 0.05$) from adiponectin treatment group at corresponding time point.

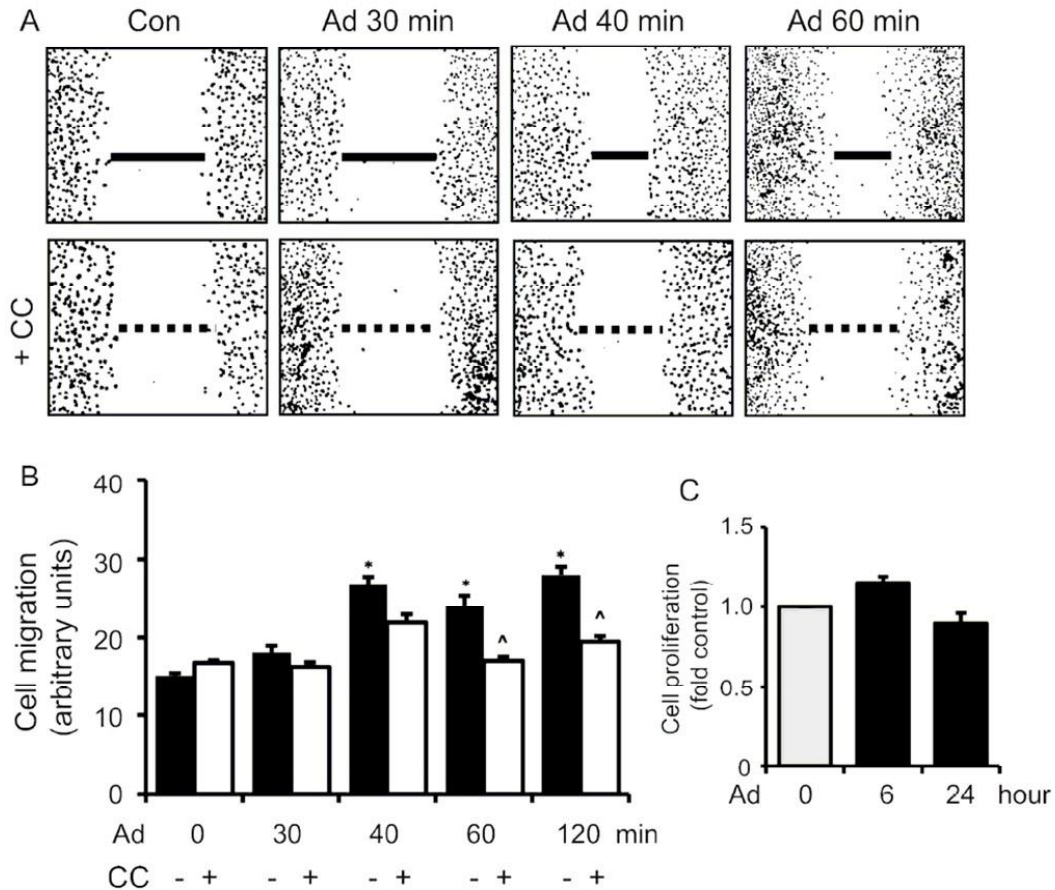


Figure 3.3: Adiponectin induced cardiac fibroblast migration via AMPK signalling.

(A) Cell migration was examined using the wound scratch assay in adiponectin treated (1 $\mu\text{g}/\text{mL}$) cardiac fibroblasts following 1 hour pre-treatment with either DMSO or 20 μM Compound C. Cell nuclei were stained with DAPI and imaged using fluorescent microscopy under a 20x objective. Colours were digitally inverted so that cell nuclei are shown as black against a white background. Cell migration was quantified in (B) as the reduction of the wound width, as denoted by the solid lines in (A). Data represent mean values \pm SEM from $n = 3$ experiments, using 7-10 images per group for quantification. (C) Fibroblast proliferation was assessed by ^3H -thymidine incorporation following 6 or 24 hours adiponectin treatment. Data represent mean values \pm SEM from $n = 3$ experiments. *Significant difference ($p < 0.05$) from untreated control. ^Significant difference ($p < 0.05$) from adiponectin treatment group at corresponding time point.

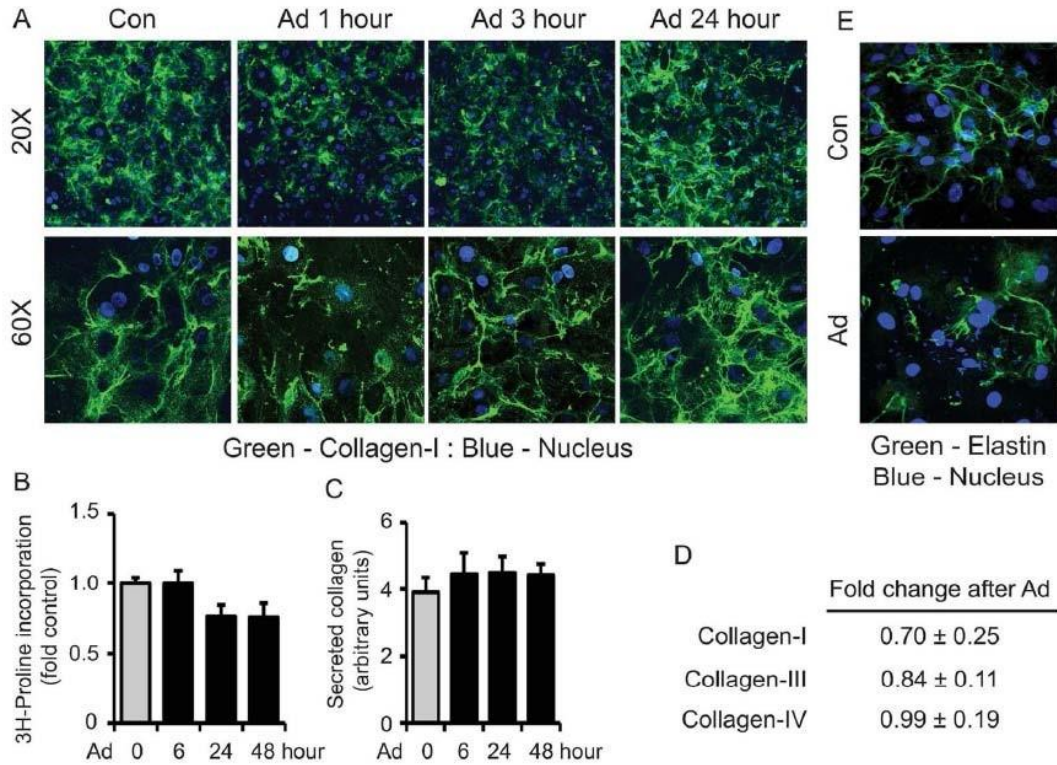


Figure 3.4: Adiponectin does not alter collagen synthesis and secretion but induces collagen and elastin remodelling.

(A) Immunofluorescent images of extracellular collagen I (green) secreted from cardiac fibroblasts at 20x and 60x magnification. Cells were treated with adiponectin (1 μ g/mL) for 1, 3 and 24 hours. Cell nuclei were also stained with DAPI (blue). Representative images from n = 3 experiments are shown. (B) Intracellular pro-collagen synthesis was assessed by ³H-proline incorporation following adiponectin treatment for 6, 24, or 48 hours. Data represent mean values \pm SEM from n = 3 experiments using 3 wells per group for quantification. (C) Total secreted collagen was measured in fibroblast conditioned media following adiponectin treatment for 6, 24 or 48 hours by picrosirius red staining. Data represented as mean arbitrary units \pm SEM from n = 7 experiments. (D) Collagen-I, -III and -IV mRNA expression was examined in cardiac fibroblasts following 24 hours adiponectin treatment by quantitative real-time PCR. Transcript expression was normalized with GAPDH mRNA abundance. Data represent mean values \pm SEM from n = 3. (E) Immunofluorescent images of extracellular elastin (green) secreted from cardiac fibroblasts at 60x magnification. Cells were treated with adiponectin for 3 days. Cell nuclei were also stained with DAPI (blue). Representative images from n = 3 experiments are shown.

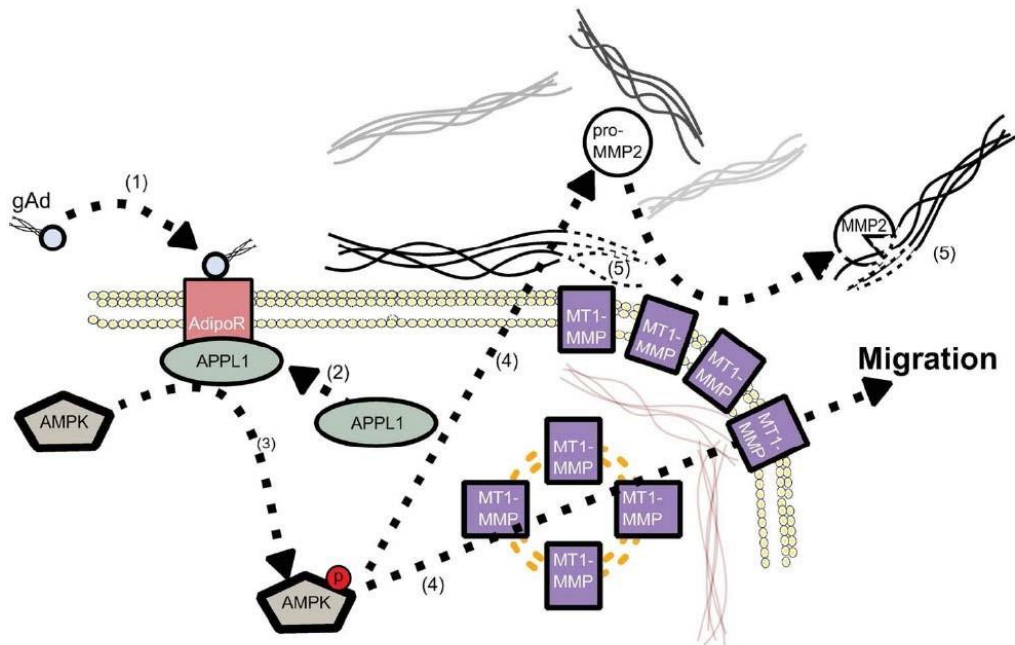


Figure 3.5: Schematic diagram representing the main mechanisms of adiponectin induced APPL1-AMPK signalling, MT1-MMP translocation, MMP2 activation , and cell migration
 The schematic figure depicts an integrative summary of the data presented in this manuscript. (1) adiponectin binding to its receptor, (2) binding of APPL1 to AdipoR and (3) subsequent activation of AMPK (4) MMP isoforms are translocated to the cell membrane (MT1MMP) or secreted (MMP2) and (5) the inactive MMP zymogen which is secreted can be activated by MT1-MMP on the cell surface and facilitate cell migration. Abbreviations shown are for globular adiponectin (gAd) and adiponectin receptor (AdipoR).

3.5 Discussion

In this study, we present evidence for a role of adiponectin-stimulated APPL1-AMPK signalling in MMP activation and cell migration in cardiac fibroblasts. The precise role of adiponectin in heart failure is not fully established, however the majority of studies suggest that adiponectin mediates cardioprotective effects and that lack of adiponectin in obesity and diabetes leads to heart failure [148, 310]. This is strongly supported by studies in adiponectin knockout mice. For example, pressure overload or ischemia/reperfusion induced fibrosis was exaggerated in these mice lacking adiponectin [116, 321]. Furthermore, angiotensin-II induced fibrosis and MMP activity was exaggerated in Ad-KO mice and the enhanced fibrosis in adiponectin knockout mouse studies could be corrected by replenishment of adiponectin [116, 175, 176, 321]. However, little is known regarding direct effects of adiponectin on cardiac fibroblasts. The main function of cardiac fibroblasts is the maintenance of ECM homeostasis to maintain structural integrity and therefore retain optimal heart function [250, 254, 304, 313]. This involves the synthesis and secretion of structural proteins, such as collagen I which accounts for 80-85% of myocardial collagen expression, and MMPs which degrade structural proteins.

In this study we first examined the effect of adiponectin on principal MMP isoforms. MT1-MMP is a membrane type MMP and is translocated to the cell surface by various stimuli [322, 323]. In this location, MT1-MMP can mediate proteolysis of matrix components but, perhaps most importantly, it is an important activator of

other MMP isoforms which are secreted from cells as inactive zymogens [324]. Indeed, cleavage of MMP2 to its active form can be mediated via MT1-MMP and the coordination of MT1MMP and MMP2 activities in various cell types plays an important role in cell migration [325, 326]. In keeping with these dogma we show here that in cardiac fibroblasts adiponectin increased cell surface MT1-MMP levels and also increased MMP2 activity in extracellular media. Furthermore, we observed that adiponectin could enhance cell migration via a mechanism that is likely to be at least partly dependent on MT1MMP and MMP2 mediated effects. Taken together, the ability of adiponectin to coordinate MT1-MMP and MMP2 activities is likely to contribute to the cardioprotective role of this hormone via promoting fibroblast migration to areas of damaged tissue and promoting degradation of existing matrix, established as an initially favourable response to cardiac injury [299, 304].

Primary cardiac fibroblasts in culture form an extracellular matrix which can be visualized with appropriate imaging approaches. We used immunofluorescent detection to show collagen-I degradation occurred after treatment of cells with adiponectin. We also noted reassembly of the collagen matrix upon prolonged culture, yet the organization of this reformed matrix appeared more disorganized and fibrous in nature, which may be analogous to adverse cardiac remodelling [327, 328]. Since there was no change in total collagen synthesis or secreted collagen levels with or without adiponectin treatment, we conclude that degraded collagen is replaced by continuous basal secretion of collagen by cardiac fibroblasts as total MMP activity

subsides. Deficiencies in cardiac elastin have been reported to be involved in various forms of heart failure [299, 329] and overexpression of elastin by gene therapy prevented cardiac dilation [330]. In cardiac fibroblast cultures, we found that enhanced MMP activity in response to adiponectin correlated with elastin degradation although the functional significance of this response would require further investigation.

We studied the mechanisms of adiponectin action in cardiac fibroblasts and have provided evidence for activation of APPL1-AMPK signalling which leads to the ECM related changes described above. The siRNA mediated reduction in APPL1 we achieved in our experiments was not complete, yet it was functionally significant. This may reflect the importance of the balance between APPL1 and APPL2 levels since these isoforms exhibit a yin-yang relationship [120] and the expression of APPL2 is likely to significantly override the remaining APPL1 expression. Many of adiponectin's effects in various cell types have now been shown to be mediated via APPL1 with AMPK as a downstream target [113, 314, 316, 317, 319], yet this is the first demonstration of such a mechanism in cardiac fibroblasts. One previous study in fibroblasts showed that adiponectin induced the expression of interleukin-6 (IL-6) which involved ERK1/2, AMPK and p38 MAPK signalling but that this was not mediated via APPL1 [331]. Other adiponectin signalling targets such as p38 MAPK and ERK1/2 in regulation of MMPs and matrix components in cardiac fibroblasts have yet to be studied.

The effects of adiponectin described here may be mediated via endocrine responses to adipose-derived adiponectin [112]. The presence of a local adiponectin system within the heart has been proposed [88] and suggests that fibroblast or cardiomyocyte derived adiponectin may participate in the maintenance of the cardiac ECM and normal heart function through autocrine and paracrine actions [112]. The concept of the heart producing a range of cardiokines has recently become established [332] and the contribution of effects mediated by locally produced versus circulating adiponectin on cardiac remodelling is worthy of further investigation.

In summary, the data we have presented here establishes that adiponectin signals via APPL1-mediated activation of AMPK to mediate important changes in MMP isoform localization and activity. Subsequently, cell migration is enhanced and structural ECM components are degraded. Study of these events in cardiac fibroblasts further develops our understanding of the diverse functions of adiponectin in the heart and may enhance our appreciation of adiponectin's role in heart failure.

CHAPTER 4: STUDY 3

MYOCYTE ENHANCER FACTOR-2 BINDING IS ATTENUATED IN ADIPONECTIN DEFICIENCY FOLLOWING PRESSURE OVERLOAD, AND IS UPREGULATED BY ADIPONECTIN STIMULATION OF CARDIOMYOCYTES

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4.1 Abstract

Cardiomyocyte hypertrophy in response to pressure overload (POH) is associated with increased heart mass, activation of the myocyte enhancer factor-2 (MEF2) family, and initiation of the fetal gene program indicated by atrial natriuretic factor (ANF) upregulation. The anti-inflammatory, anti-diabetic adipokine adiponectin has been implicated as a regulator of POH induced cardiac remodelling although little is known of adiponectin activated MEF2 and ANF activity. In this study we characterize POH related MEF2 activity in wild-type (WT) and adiponectin knockout (AdKO) mice subjected transverse aortic banding (MTAB). Cardiac hypertrophy was initiated in WT mice 1 week following MTAB surgery. Accordingly, cardiomyocyte diameter, heart weight, ANF mRNA expression, and MEF2 binding activity were significantly increased by MTAB surgery in WT mice. Conversely, MTAB induced hypertrophic changes in Ad-KO mice appear 4 weeks following surgery. ANF mRNA expression was not increased, and MEF2 binding activity was decreased in AdKO MTAB animals compared to sham. Furthermore, treatment of isolated cardiomyocytes with recombinant adiponectin significantly induced MEF2 and ANF reporter activity.

Our data suggests adiponectin targeted MEF2 and ANF activity in the myocardium may regulate cardiac hypertrophy in response to pressure overload.

4.2 Introduction

Hypertrophic changes to the myocardium in response to pressure overload (PO) are characterized by increased cardiomyocyte diameter, left ventricular (LV) posterior wall thickness, and overall heart weight. Pressure overload hypertrophy (POH) is also associated with activation of the fetal gene program, indicated by the upregulation of atrial natriuretic factor (ANF), as well as increased activity of the prohypertrophic myocyte enhancer factor-2 (MEF2) family [218, 333].

Numerous studies have identified the adipocyte derived adipokine adiponectin as a major player in cases of chronic heart failure [148], and recently a study using minimally invasive transverse aortic banding (MTAB) surgery to induce chronic pressure overload implicated adiponectin as playing a permissive role in the development of hypertrophy [162]. Indeed, although adiponectin is known to activate p38 signalling [129], a well characterized inducer of MEF2 activation [224], little is known about adiponectin activation of MEF2 activity in the heart with respect to POH.

In this study we characterize the progression of cardiac hypertrophy in wild-type (WT) and adiponectin knockout (AdKO) mice following MTAB surgery, and show differential MEF2 activation in overloaded wild-type and adiponectin deficient hearts. We also examine POH induced changes of atrogen-1 and MuRF-1, two well characterized anti-hypertrophic genes [334], and lastly look at adiponectin stimulated MEF2 and ANF activity in cardiomyocytes.

4.3 Materials & Methods

Materials

Recombinant full-length adiponectin was produced in-house as previously detailed by [121]. Dulbecco's modified eagle's medium (DMEM), trypsin, antibiotic/antimycotic and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Life Technologies Inc., Burlington, ON, CA). All culture plates were BD Falcon™ brand and purchased from BD Biosciences (Mississauga, ON, CA). TRIzol® Reagent was purchased from Ambion Inc. and Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate was purchased from Molecular Probes (Life Technologies Inc.). Hematoxylin Gill No. 2 stain and Eosin-Y stain were purchased from Sigma-Aldrich (Oakville, ON, CA). RNeasy MinElute Cleanup and RT2 First Strand kits were purchased from QIAGEN Inc. (Mississauga, ON, CA). LOOK silk black braided non-absorbable 6-0 USP sutures were purchased from Harvard Apparatus (Holliston, MA, USA), titanium ligation clips and microclip ligating appliers used for MTAB were purchased from Teleflex Medical (NC, USA).

Experimental Animals.

Wild-type C57BL/6 male mice were purchased from Charles River Laboratories (Montreal, QC, Canada). AdKO (C57BL/6 background) and MEF2 lacZ transgenic

(CD-1 background) mice were routinely bred and genotyped in-house. Compound transgenics were bred by crossing MEF2 lacZ mice with adiponectin homozygous null mice to subsequently create MEF2 lac Z/ Adipo -/- (MEF2 LacZ/AdKO). Experimental animals were housed in a temperature controlled environment under 12h light- 12h dark conditions, and were fed ad-libitum. Animal facilities meet Canadian Council on Animal Care guidelines and all protocols used were approved by the York University Animal Care Committee.

Aortic Banding Surgery.

Under general anesthesia (i.p. xylazine: 0.15 mg/g; ketamine: 0.03 mg/g), hair from the chest was removed and the surgical area disinfected with betadine. A skin incision was made along the midline from the neck to the rib cage and the chest cavity was opened. The rib cage and thymus were retracted to expose the trasverse aorta. A 27g needle is used to calibrate a microclip applicator. A titanium microligation clip is applied between the origins of the innominate and left common carotid arteries, constricting the transverse aorta to the gauge of the needle. The rib cage, muscles, and skin are closed with a 6-0 USP non-absorbable silk suture. The animals are then administered s.c. 0.03 µg/mg Buprenorphine and allowed to recover on a heating pad until fully awake. Sham surgeries are performed as above except the microligation is not applied to the transverse aorta. All mice were monitored after the procedure for normal behaviour.

Echocardiography.

All mice were subjected to transthoracic echocardiographic analysis to measure heart function 2 or 4 weeks following aortic banding or sham surgery. Cardiac function and heart morphology were evaluated using echocardiography (Vevo 2100, VisualSonics). The animals were sedated using 3% isoflurane and maintained with 1~2% isoflurane. The parasternal long axis view (B-mode, M-mode) was obtained and measurements of cardiac structure and function were determined as described previously [335]. The individuals performing echocardiographic analysis of heart function were blinded to surgical group and genotype.

Tissue collection and histology.

Following echocardiography, mice were weighed and euthanized using cervical dislocation. Hearts were excised and quickly perfused with 30mM KCl to induce diastolic arrest. Hearts were then weighed and divided for further analysis. . Mid-ventricular cross-sections of freshly dissected heart tissue were fixed in 10% formalin solution for 1 hour then stored in 70% ethanol at 4oC until further processing. Fixed heart tissues were dehydrated to xylene and embedded in pure paraffin wax blocks.

Scanning electron microscopy. Freshly dissected heart tissue was fixed in 2% EM grade gluteraldehyde in 0.1M sodium cacodylate buffer pH 7.3 for 1 hour at room temperature, then stored in 0.1M sodium cacodylate buffer, pH 7.3, 0.2M sucrose until

further processing. Fixed samples were dehydrated in a graded ethanol series (30-100%) then two steps of hexamethyldisilazane. Coverslips were then mounted, gold sputter coated (Hummer VI Au/Pd 40/60), and imaged using a Hitachi S-520 scanning electron microscope. Images were captured using a passive image capture system (Hitachi, Quartz PCI Version 6).

Gene analysis.

Tissue was frozen then crushed in liquid nitrogen and homogenized in TRIZOL. mRNA was extracted following manufacturer's specifications then processed using the QIAGEN RNeasy clean up kit to attain high purity (260/280 > 1.9, 260/280 between 1.8 to 2.0). Atrogin-1 (Forward: CTTTCAACAGACTGGACTTCTCGA; Reverse: CAGCTCCAACAGCCTTACTACGT), MuRF-1 (Forward: TGTCTGGAGGTCGTTTCCG; Reverse: TGCCGGTCCATGATCACTT), ANF (Forward: GGCTCCGAGGGCCAGCGAGCAGAGCCCCTCA; Reverse: CGTGCCCCGACCCACGCCAGCATGGGTCC), and GAPDH (Forward: TTGCCATCAACGACCCCTTC; Reverse: TTGTCATGGATGACCTTGGC) were analyzed through real-time PCR using the following cycling conditions: 95°C/15 min, followed by 35 cycles of [95°C/30 sec, 55°C/30 sec, 72°C/30 sec], then 72°C/10 min. Melt curve analysis was used to ensure primer specificity. Average Δ Ct values were adjusted by GAPDH then used to calculate fold/sham ratio.

MEF2 binding activity.

MEF2 activity was analyzed *in vivo* and *in vitro* as described previously [336].

Statistics.

Statistical analysis was performed using the Student's t-test and two-way ANOVA followed by Tukey's Multiple-Comparison Test. Data are expressed as mean \pm SE where $p \geq 0.05$ was considered statistically significant

4.4 Results

Four weeks of transverse aortic constriction led to an increase in apparent heart size and a significant increase in p38 phosphorylation in MTAB hearts when compared to sham hearts (Fig 4.1A). Similarly, heart weight to body weight ratio was significantly increased 1 week following MTAB (Fig 4.1B). Wheat germ agglutinin staining in paraffin embedded myocardial cross-sections indicated a significant increase in cardiomyocyte cross sectional area 3 days after MTAB (Fig 4.2A,B). Trans-thoracic echocardiography 2 and 4 weeks following surgery to assess cardiac performance showed a small but non-significant increase in left ventricular end systolic diameter (LVESD) and left ventricular posterior wall thickness (LVPWD),

while there was no apparent change in left ventricular end diastolic diameter (LVEDD). Fractional shortening (FS) was significantly decreased in WT mice after 2 and 4 weeks of PO (Fig 4.3). These functional changes were delayed in AdKO mice to 4 weeks following MTAB surgery.

Accordingly, in contrast to WT mice, AdKO mice do not display an increase in heart weight or cardiomyocyte cross sectional area 2 weeks after MTAB, but instead show a similar level of cardiac hypertrophy when compared to WT mice following 4 weeks of pressure overload (Fig 4.4A,B). Cardiomyocyte linearity was impaired in WT mice following MTAB (Fig 4.4C).

To investigate the role of MEF2 binding following pressure overload, MEF2-lacZ mice were crossbred with WT and AdKO mice. MEF2 activity, shown through ex-vivo x-gal staining of heart crosssections was increased in WT mice following MTAB surgery. MEF2 binding was noticeably absent in AdKO MTAB mice despite a slight increase in MEF2 binding in AdKO sham vs WT sham mice (Fig 4.5A). PCR analysis of mRNA levels in heart homogenates showed the significant upregulation of ANF in WT MTAB hearts (fold sham), while MuRF-1 mRNA was downregulated in both AdKO and WT animals, and Atrogin-1 was significantly decreased in AdKO mice while increased in WT mice. ANF mRNA was significantly downregulated in AdKO animals after 4 weeks of MTAB (Fig 4.5B, C). Adiponectin treatment was found to significantly increase MEF2 and ANF binding activity in isolated neonatal cardiomyocytes (Fig 4.5D).

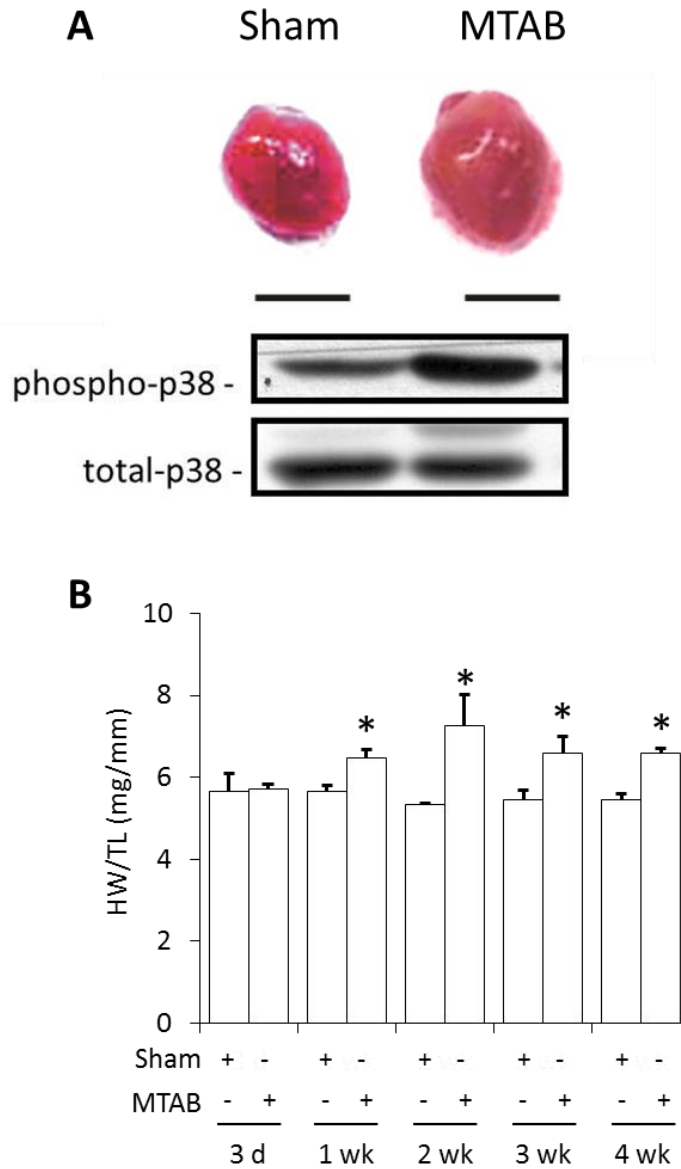


Figure 4.1: MTAB increases heart weight and p38 phosphorylation.

A) Representative whole heart images taken immediately following euthanization after 4 weeks of sham or MTAB surgery. Below is representative western blot analysis of total and phosphorylated p38 following 4 weeks of sham or MTAB surgery. n = 4 to 6 mice per group.

B) Total wet heart weight adjusted for body weight at time of euthanization (mg/g) quantified in mice euthanized 3 days, 1, 2, 3, and 4 weeks following sham or MTAB surgery. n = 4 to 6 mice per group \pm SEM. * = p < 0.05.

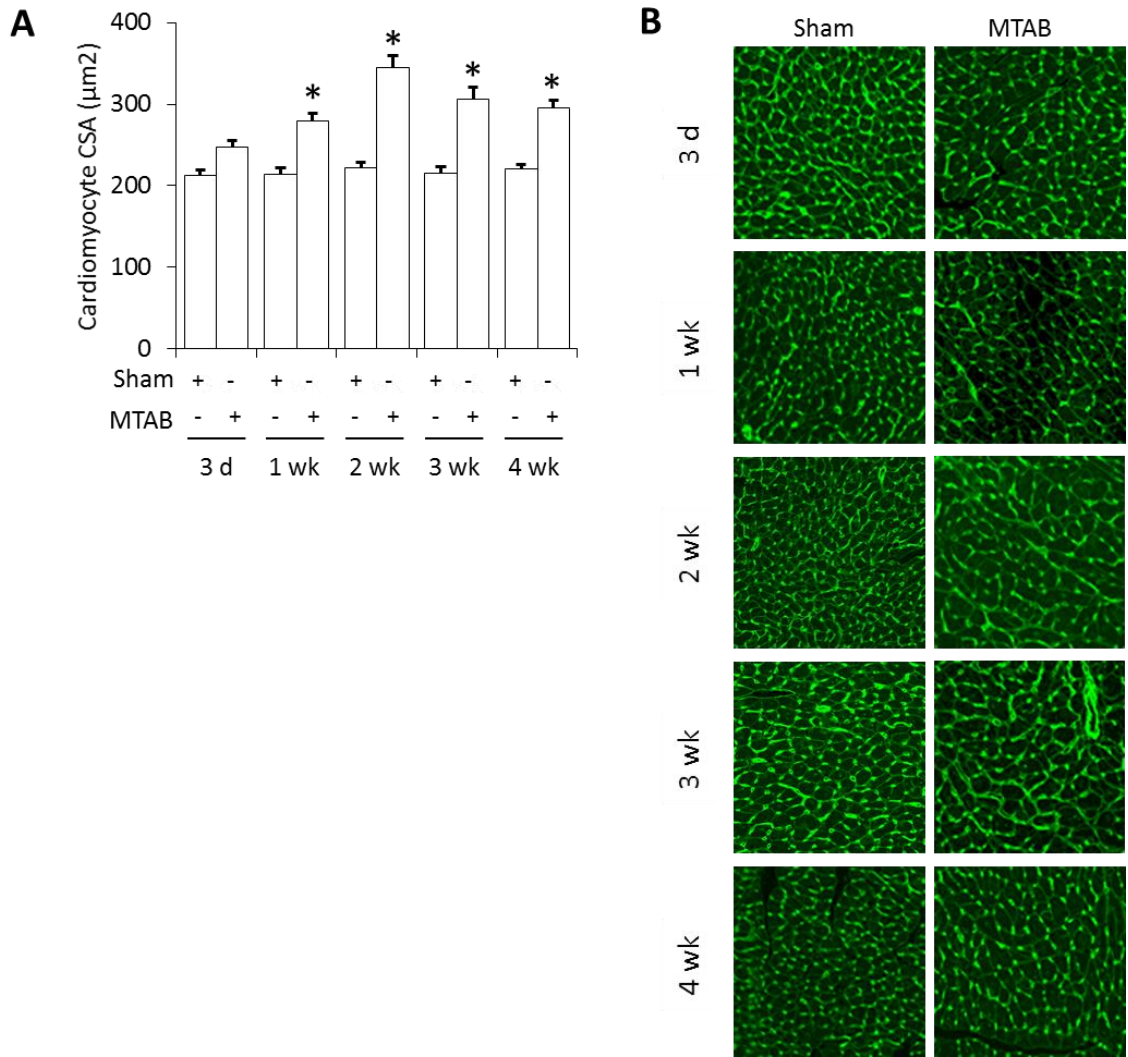


Figure 4.2: Cardiomyocyte cross-sectional area after MTAB.

A) Cardiomyocyte cross-sectional area from WT mice 3 days, 1, 2, 3, or 4 weeks following MTAB surgery. $n = 4$ to 6 mice per group \pm SEM. * = $p < 0.05$. B) Representative immunofluorescent wheat-germ agglutinin staining of myocardial histological cross-sections taken from WT mice following sham or MTAB surgery.

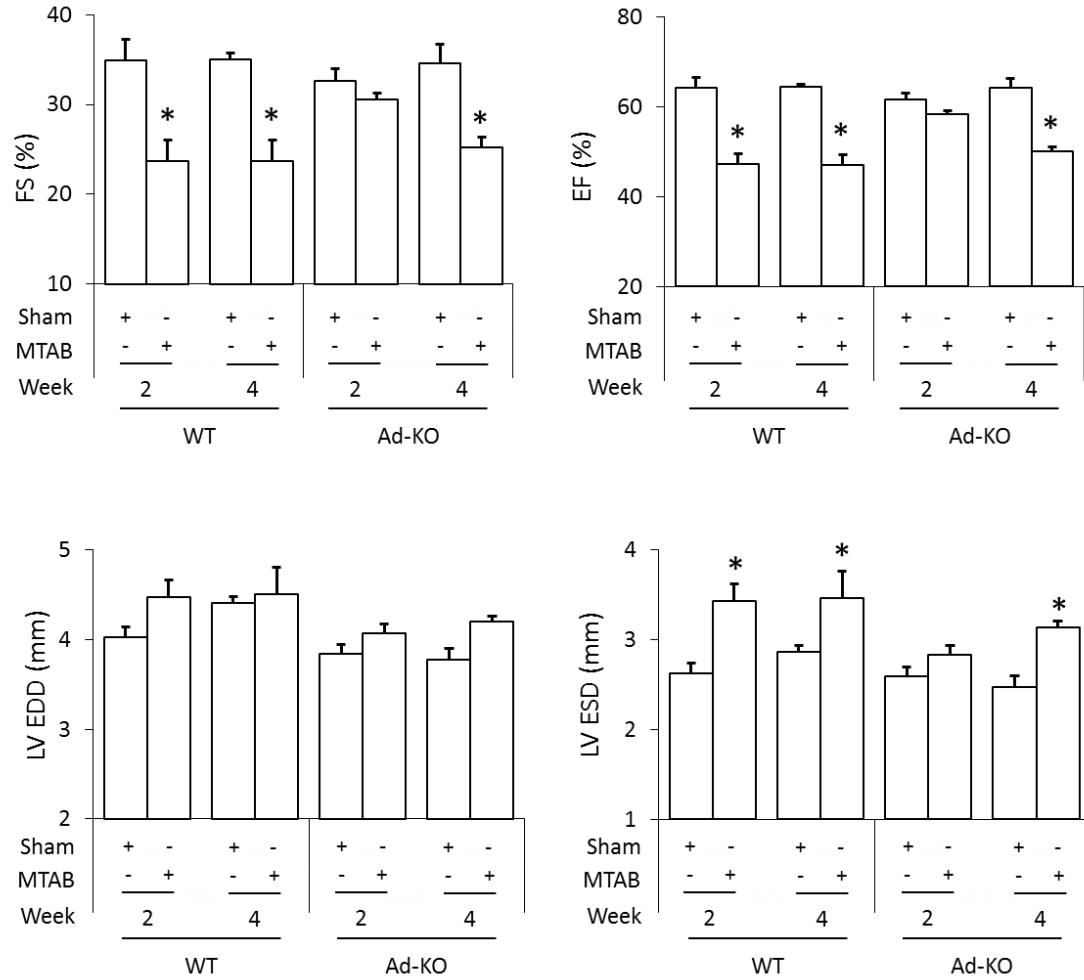


Figure 4.3: Echocardiographic analysis of AdKO and WT mice.

Echocardiographic analysis of cardiac structure (LV EDD: left ventricular end diastolic diameter; LVESD: left ventricular end systolic diameter) and cardiac function (FS: Fractional shortening; LVPWD: left ventricular posterior wall dimension) of AdKO and WT mice 2 or 4 weeks following sham or MTAB surgery. Values are average of n = 4 to 7 mice per group \pm sem. * = p < 0.05 vs respective sham.

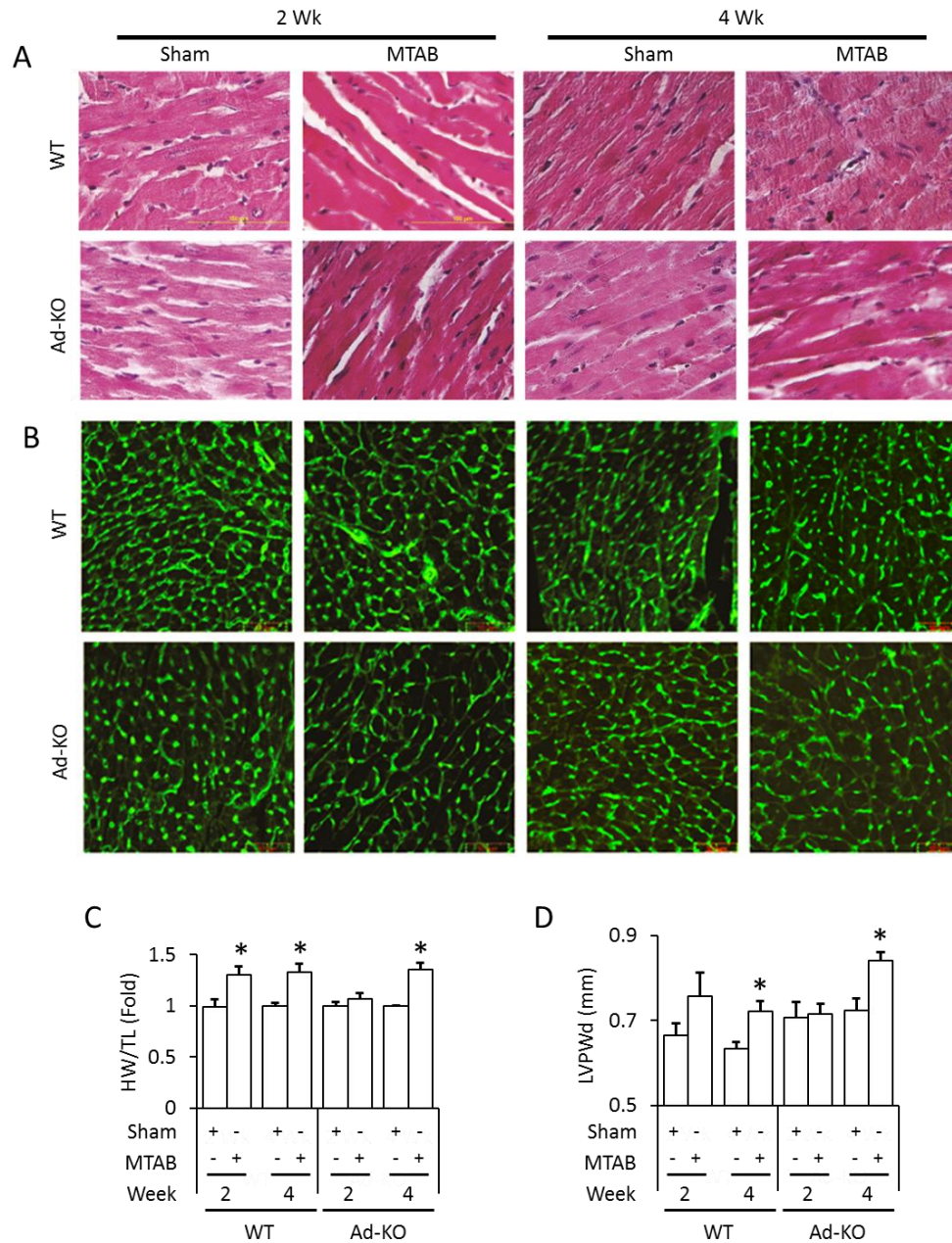


Figure 4.4: Cardiac hypertrophy is delayed in adiponectin deficient mice.

(A) Longitudinal sections of LV stained with H&E. (B) Immunofluorescent wheat germ agglutinin staining for cardiomyocyte membrane in LV cross-sections. 5 – 10 images of n = 4 to 6 mice per group. Cardiac hypertrophy assessed through heart weight (HW) to tibial Length (TL) ratio (C), and LV posterior wall dimension (D) in sham or MTAB animals after 2 and 4 weeks of PO.

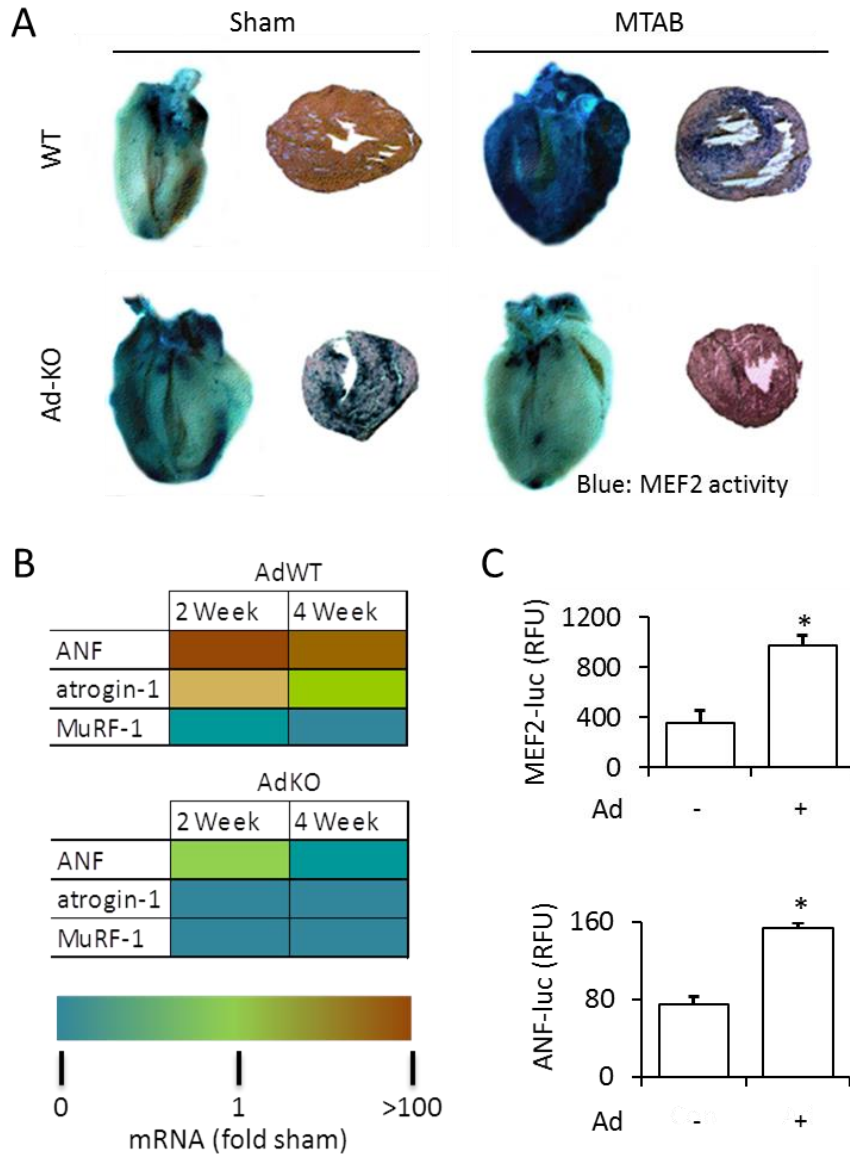


Figure 4.5: MEF2 activity is increased in WT mice following MTAB

(A) Representative longitudinal or cross-sectional scans of the myocardium following β -gal staining, indicating areas of MEF2 activity. (B). Heat map of quantified ANF, atrogen-1, and MuRF-1 mRNA isolated 2 or 4 weeks following surgery. Data is represented as relative change in mRNA, associated with change in colour. Values were calculated as $C(t)$, adjusted with GAPDH, fold sham over MTAB. (C) Relative MEF2 and ANF binding activity using MEF2-luciferase and ANF-luciferase reporter assays $n \geq 3$. * = $p \leq 0.05$ vs control.

4.5 Discussion

Early MTAB studies using AdKO mice clearly demonstrated that adiponectin deficiency exacerbated cardiac fibrosis and hypertrophy in the progression of POH [116, 156]. However, recent studies have indicated that adiponectin may play a permissive role in cardiac hypertrophy, and that adiponectin deficient mice may be protected from PO [161, 162]. In the data presented here, we show that WT mice develop LVH faster than AdKO mice. We also show that MEF2 activity is upregulated in WT hearts following MTAB, but is also upregulated in AdKO sham animals. Lastly we demonstrate that adiponectin treatment induces MEF2 and ANF activity in cardiomyocytes.

Our previous data indicated that adiponectin deficiency may delay the onset of cardiac fibrosis (see chapter 2: study 1). The data presented here suggests that cardiac hypertrophy in AdKO mice is similarly delayed following MTAB surgery. Of note, we may conclude that the surgical technique performed in the study was successful since WT mice subjected to MTAB surgery exhibited common features of cardiac hypertrophy: increased posterior wall thickness, cardiomyocyte hypertrophy, and increased heart weight. Furthermore, the individuals performing echocardiographic analysis of heart function were blinded to surgical group and genotype as much as possible. The observed changes to cardiac performance in WT mice (decreased fractional shortening, increased LV ESD) were stable from 2 to 4 weeks following surgery indicating mild PO and the lack of progressive decline.

Conversely, MTAB failed to induce functional change or hypertrophic remodelling in AdKO mice until 4 weeks after surgery indicating that, as was noted previously, the progression of remodelling following the induction of pressure overload is altered in the absence of adiponectin.

Our data aligns with recent findings regarding the permissive role of adiponectin in the pathogenesis of POH. While the discrepancies between these studies and the first data published using this model may be explained by differences in mouse diet and the gut micro-biota, variation in knockout models from different inbred colonies, or simply subtle differences in animal facilities and surgical technique, we sought to elucidate the transcriptional activity targeted by adiponectin in POH as a means to understand the specific mechanisms targeted by adiponectin following MTAB.

MEF2 is a family of transcription factors known to play a potent hypertrophic role in the pathogenesis of heart failure and is strongly associated with the fetal gene program (see section 1.4.2). We crossbred AdKO mice with MEF2 reporter mice and subjected them to MTAB surgery. MEF2 activity was significantly increased in hypertrophic WT hearts and downregulated in AdKO mice following MTAB surgery. Accordingly, we found that adiponectin significantly increased MEF2 and ANF activity in isolated neonatal rat cardiomyocytes.

Taken together, these data implicate adiponectin as a mediator of hypertrophic changes to the myocardium following MTAB surgery through MEF2 and

ANF activation. It is important to note however that, while cardiac hypertrophy is a negative clinical indicator of heart disease, hypertrophic remodelling is in fact an adaptive mechanism upregulated in order to compensate the overloaded LV against increased systolic wall stress (see section 1.4.1). Indeed, our mice failed to show a progressive decline in cardiac function potentially indicating that while the AdKO mice experienced a delay in the onset of hypertrophy, WT cardiac function was maintained in the adaptive phase of remodelling.

Indeed, O'Shea et al. showed that 6 weeks of PO induces cardiac hypertrophy in both AdKO and WT mice although the pattern of remodelling was different between the two strains. WT mice were shown to exhibit signs of dilated cardiomyopathy, while AdKO mice presented with the more protective concentric hypertrophic phenotype [162].

We also note that MEF2 activity was significantly increased in AdKO sham animals. This data correlates with our findings in chapter 2: study 1 that AdKO sham animals exhibit a subtle increase in small fibre fibrosis. However, we found no functional differences between sham AdKO and WT mice. From this we speculate upon the existence of a compensatory mechanism upregulated in AdKO mice linking fibrosis and MEF2 activity in the basal state that may confer protection against PO.

From our data and those of other groups, it is clear that adiponectin is a cardio-important adipokine, but the exact role of adiponectin in the progression of heart failure remains controversial. Here we have presented novel findings that adiponectin

can activate MEF2 and ANF, two common markers of the fetal gene program. Whether or not this plays a vital role in the actions of adiponectin in heart failure requires further study.

CHAPTER 5: STUDY 4

REVERSE CELLULAR, STRUCTURAL AND FUNCTIONAL REMODELLING FOLLOWING AORTIC BANDING-DEBANDING

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5.1 Abstract

Clinical unloading of the left ventricle through either aortic valve replacement or implantation of a left ventricular assist device (LVAD) has revealed the regressive capacity of the hypertrophic heart to regain normal cardiac function. This has been mimicked in mouse aortic constriction models of pressure overload (PO) hypertrophy using debanding surgery to unload the LV, initiating the regression of cardiac remodelling. The importance of the extracellular matrix (ECM) and changes which occur upon PO induced compensatory LV remodelling is well understood, although the consequences of debanding on ECM structure and LV function remains to be elucidated. In this study, we used minimally invasive transverse aortic banding (AB) in C57BL/6 mice and performed temporal analysis of cardiac remodelling up to 4 weeks and then 2 weeks after debanding. Echocardiography showed that PO-induced changes at 4 weeks were consistent with compensatory concentric hypertrophy (increased LV mass and LVPWD; normal EF and FS), and were reversed upon LV unloading. Speckle tracking analysis indicated a significant increase in LV endocardial radial strain in AB hearts which was restored to normal following debanding. Scanning electron microscopy revealed that thick collagenous fibres observed after 4 weeks of PO were lost following debanding. However, the ECM retained an expansive and disorganized network of thin fibres. Gene array analysis showed that AB and debanding induced opposing trends on mRNA expression of MMP, TIMP, and collagen isoforms. Taken together, our data show that LV unloading

leads to normalization of LV function, hypertrophy and, gross fibrosis. However, the structure of the debanded ECM may have implications concerning the heart's response to future myocardial stress.

5.2 Introduction

Pressure overload induces pathological long term remodelling of the left ventricle with changes in hypertrophy and fibrosis and a concomitant decline in cardiac function [337]. However, it is well known that an initial compensatory remodelling also occurs, which serves to preserve ventricular function. Beta-blockers, ACE inhibitors and angiotensin II receptor antagonists (ARBs) have been successful in delaying the progression to maladaptive remodelling. Congestive heart failure (CHF) patients have benefitted from aggressive surgical techniques such as valve replacement and left ventricular assist device (LVAD) implantation, both of which directly alleviate pressure overload (PO) of the left ventricle [233, 234]. Indeed, accumulating clinical and experimental evidence has demonstrated the restoration of cardiac function and regression of left ventricular remodelling following unloading of the left ventricle, a process now commonly termed reverse remodelling [235, 236].

Left ventricular overload in mouse models using aortic constriction leads to features commonly observed in compensatory concentric hypertrophy: left ventricular hypertrophy (LVH), and increased posterior wall thickness (LVPWD). LV unloading through removal of the constricting aortic band (debanding), results in reverse remodelling of the left ventricle, restoration of normal cardiac function [237-239], activation of a unique gene expression profile [240] and increased autophagic flux [242], as long as debanding is performed before the heart enters decompensated failure [241]. Debanding was also shown to differentially regulate the expression of

collagen isoforms in the heart [239, 247], but considering the global impact of cardiac fibrosis in altering ventricular geometry, inducing diastolic dysfunction, and disrupting electrical synchronicity [253, 283, 307], a clearer structural picture of the 3-dimensional architecture of collagen networks in the debanded myocardium is needed.

There is a strong association between obesity, heart failure, and circulating levels of the adiponkine adiponectin [148]. Recently, a study in CHF patients showed a significant decrease in adiponectin levels, and also in the principle adiponectin signalling target AMPK. Upon LVAD implantation, AMPK activation and adiponectin levels were restored [338].

In this study, we aimed to image the collagen extracellular matrix following unloading of the left ventricle, and compare LV strain between overloaded and debanded animals. We also characterized changes in cardiac adiponectin sensitivity before and after aortic debanding. Unloading of the LV undergoing compensatory remodelling resulted in the loss of myofibroblasts within the myocardium. This correlated with different trends of MMP, TIMP and Col expression between the banding and respective sham group. Thick fibre fibrosis and LV strain were also significantly attenuated following debanding. However, the morphology of the post-banded ECM suggests incomplete collagen regression and a fibrous structure typically associated with poor clinical outcome.

5.3 Materials and Methods

Materials

Anti-phospho-AMPK α (Thr172), anti-AMPK α , anti-APPL1, anti- β -actin primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signalling Technology (New England Biolabs Ltd., Whitby, ON, CA). Western Lightning Plus ECL was obtained from PerkinElmer (Woodbridge, ON, CA). TRIzol[®] Reagent was purchased from Ambion Inc. (Life Technologies Inc.), and Alexa Fluor[®] 488 goat anti-rabbit antibody was obtained from Molecular Probes (Life Technologies Inc.). The FITC-labelled goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). VECTASHIELD[®] mounting medium with DAPI was obtained from Vector Laboratories (Burlington, ON, Canada). RNeasy MinElute Cleanup and RT² First Strand kits were purchased from QIAGEN Inc. (Mississauga, ON, CA). ³H-proline was purchased from Amersham Biosciences (GE Healthcare Lifesciences, Baie d'Urfe, QC, CA). The Pierce BCA Protein Assay kit was purchased from Thermo Scientific (Fisher Canada, Nepean, ON, CA). The custom fibrosis PCR array was purchased from SABiosciences (QIAGEN Inc.).

Experimental animals

Male C57BL6 (WT) mice (Charles River Laboratories, St. Constant, QC), 6 – 8 weeks of age were randomly allocated to treatment groups. Animal facilities met the

guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee of York University. Animals were acclimated a minimum of 5 days to a standard housing environment: temperature and humidity-controlled rooms ($21\pm 2^{\circ}\text{C}$, 35-40%), with a daily 12:12h light-dark cycle (lights on at 0700) with access to regular chow diet *ad libitum*.

Minimally Invasive Transverse Aortic Banding and Debanding

Under general anesthesia (IP Xylazine 0.15mg/g; Ketamine 0.03mg/g), the mouse is kept in a supine position and the fur on the ventral surface removed with a depilatory cream, and the surface sterilized with betadine. A medial cranio-caudal incision is made through the skin from the neck to the bottom of the rib cage, and the neck muscles, fat, and thyroid are retracted to expose the trachea down to the suprasternal notch. An incision is made through the suprasternal notch 2-3 mm down the rib cage. The transverse aorta is visualized under low magnification between the innominate and left common carotid arteries. A titanium micro-ligation clip is applied across the transverse aorta using banding calipers calibrated to the width of a 26g needle. The sham surgery is performed as outlined above without the placement of the ligation clip. Upon completion of the procedure, the rib cage and skin are closed with silk suture, and the mice injected with Buprenorphine (s.c. 0.05 mg/kg) and placed face down on a warming pad until they awake.

The debanding procedure was performed as above but titanium band was removed in debanded animals and not removed in sham-debanded animals.

Tissue collection.

Mice were weighed and euthanized using cervical dislocation. Hearts were excised and perfused briefly with KCl to arrest the heart in diastole. Hearts were then weighed and divided for further analysis.

Scanning electron microscopy.

Freshly dissected heart tissue was fixed in 2% EM grade glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.3 for 1 hour at room temperature, then stored in 0.1M sodium cacodylate buffer, pH 7.3, 0.2M sucrose until further processing. Fixed samples were dehydrated in a graded ethanol series (30-100%) then two steps of hexamethyldisilazane. Dried samples were attached to coverslips were then mounted, gold sputter coated (Hummer VI Au/Pd 40/60), and imaged using a Hitachi S-520 scanning electron microscope. Images were captured using a passive image capture system (Hitachi, Quartz PCI Version 6).

Tissue histology.

Mid-ventricular cross-sections were fixed in 10% formalin solution for 1 hour then stored in 70% ethanol at 4°C until further processing. Fixed heart tissues were dehydrated to xylene and embedded in pure paraffin wax blocks.

Western Blot Analysis

Cell culture lysis and protein sample preparation was conducted according to methods detailed by [305]. Heart tissue homogenate preparation is detailed [306]. Primary anti-phospho-AMPK α (Thr172), anti-AMPK α , anti-APPL1, anti-AdipoR1, anti-AdipoR2, and anti- β -actin antibodies were used at 1:1000 dilutions, and appropriate HRP-conjugated secondary antibodies were used at 1:10,000 dilutions. Proteins were detected by chemiluminescence, quantified by densitometry using Scion Image software (Scion Corp., Frederick, MD, USA) and then normalized to either β -actin or total AMPK protein levels as appropriate.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cultured CFs using TRIzol[®] Reagent according to the manufacturer's instructions, and purified using the RNeasy MinElute Cleanup Kit to attain an A_{260}/A_{280} ratio between 1.9 and 2.0. First-strand cDNA, synthesized from 1 μ g RNA using the RT² First Strand kit, was used in a custom PCR array comprising 96-well plates pre-coated with primers listed in table 1. Quantitative real-time PCR was conducted using a Chromo4[™] Detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, CA) according to cycling conditions outlined by the PCR array manufacturer. Data were analysed using RT² Profiler PCR Array Data Analysis software (Version 3.5; QIAGEN Inc.) and normalized to GAPDH mRNA expression. Adiponectin, AdipoR1, AdipoR2, APPL1, APPL2, GAPDH mRNA expression

(primers, T_m listed in table 2) were analyzed by quantitative real-time PCR using DyNAmo HS SYBR® Green qPCR kit (Finnzymes, Woburn, MA) with a Chromo4 Detection system and the following cycling conditions: Hot start: 95 °C for 15 minutes; 35 cycles of: 95 °C for 30 seconds, annealing temp (table 2) for 30 seconds, 72 °C for 30 seconds; final extension: 72 °C for 10 minutes.

Statistics

Data are expressed as mean values \pm SEM (n), where n represents the number of experiments conducted. Student's *t* tests were used to determine significant differences ($P < 0.05$) between groups. All statistical analyses were conducted using SigmaStat 3.5 Software (Systat Software Inc., San Jose, CA, USA).

5.4 Results

Blood flow dynamics are impaired by AB and restored by debanding

To validate the AB and debanding surgeries we studied blood flow dynamics of the transverse aorta as measured from the origin of the innominate artery using pulse-wave (Fig 5.1A top row) and colour Doppler (Fig 5.1A bottom row) echocardiography. Banding of the transverse aorta between the innominate and left carotid arteries (Fig 5.1A) significantly reduced aortic peak gradient (TAPG) and peak velocity (TAPV) by 40% and 20%, respectively, throughout 4 weeks of PO (Fig 5.1B&C). TAPG and TAPV were restored 1 week following debanding surgery, and

resulted in significantly higher values compared to sham 2 weeks after debanding (Fig 5.1B&C).

Reverse remodelling of compensatory hypertrophy is induced by aortic debanding

Common characteristics of pressure overload induced cardiac hypertrophy (increased total heart weight (HW), left ventricular mass (LV mass) and left ventricular posterior wall thickness (LVPWd)), are indicators of progressive cardiac remodelling initiated as a compensatory response to maintain cardiac function. AB surgery significantly increased HW to body weight ratio after 4 weeks (Fig 5.2A), LV mass after 1 and 2 weeks (Fig 5.2B), and LVPWd after 2, 3 and 4 weeks (Fig 5.2C). Measures of cardiac function, including ejection fraction, fractional shortening and cardiac output remained unchanged after up to 4 weeks of PO, and followed by 1 or 2 weeks of debanding (Table 4.1). Changes to HW, LVPWD, and LV mass were ameliorated following debanding surgery (Fig 5.2A,B&C).

LV strain is increased by PO

Speckle tracking analysis of segmental LV strain shows an increase in radial (Fig 5.3a), but not longitudinal (Fig 5.3b), strain following PO. This change was ameliorated by debanding surgery. Segmental radial strain analysis (time-to-peak) shows regionalized difference in mid- anterior vs posterior wall strain. After 4 weeks

of PO, there was a significant delay in time-to-peak radial strain in the anterior mid-ventricular endocardial wall (121 ms) as compared to the posterior mid-ventricular endocardial wall (35 ms). This difference was reduced after debanding (43 ms and 73 ms respectively) (Fig 5.3c). Sequential analysis of individual sham and AB mice shows an increase in radial strain rate between 3 and 4 weeks after MTAB surgery. Strain rate is attenuated following unbanding (Fig 5.3d).

Structural and molecular analysis of cardiac fibrosis after AB and debanding

Initial analysis of 3-dimensional collagen architecture by scanning electron microscopy demonstrated increased collagen deposition after 4 weeks of PO compared to sham and that this was greatly reduced in debanded animals (Fig 5.4a). However, using a cell maceration technique to remove non-fibrous myocardial tissue, the absence of thick fibres, and disorganization of thin fibres in the debanded cardiac ECM is apparent (Fig 5.4b). Molecular analysis of collagen, MMP and TIMP isoform mRNA expression revealed a distinct fibrotic profile between banded and debanded animals. Collagens 3a1 and 4a1, as well as TIMPs 2 and 3 were preferentially upregulated in MTAB animals, while MMPs 8, 9, and 13 were upregulated after debanding (Fig 5.5). Collagen 4a1 levels decreased slightly after PO and were not altered by debanding, however MMPs 2, 8, 9, 13 and 14 were all reduced only after PO (Fig 5.5). The increased presence of α -smooth muscle actin (α SMA) positive cells,

indicative of myofibroblasts (white arrows), after PO suggests a phenotype of more myofibroblasts, a trend which was lost in debanded animals (Fig 5.6).

Adiponectin sensitivity and pressure overload

MTAB induced a slight reduction in AdipoR1 and APPL1 gene and protein expression, while AdipoR2 expression was significantly attenuated compared against the sham myocardium. There was no detectable difference in gene expression of the adiponectin receptors or APPL1 between debanded and sham-debanded mice. Western blot analysis showed a decrease in AdipoR2 and APPL1 protein in MTAB animals compared to sham animals, while no difference was noted between debanded and sham-debanded mice. AMPK and p38 phosphorylation was decreased in MTAB animals.

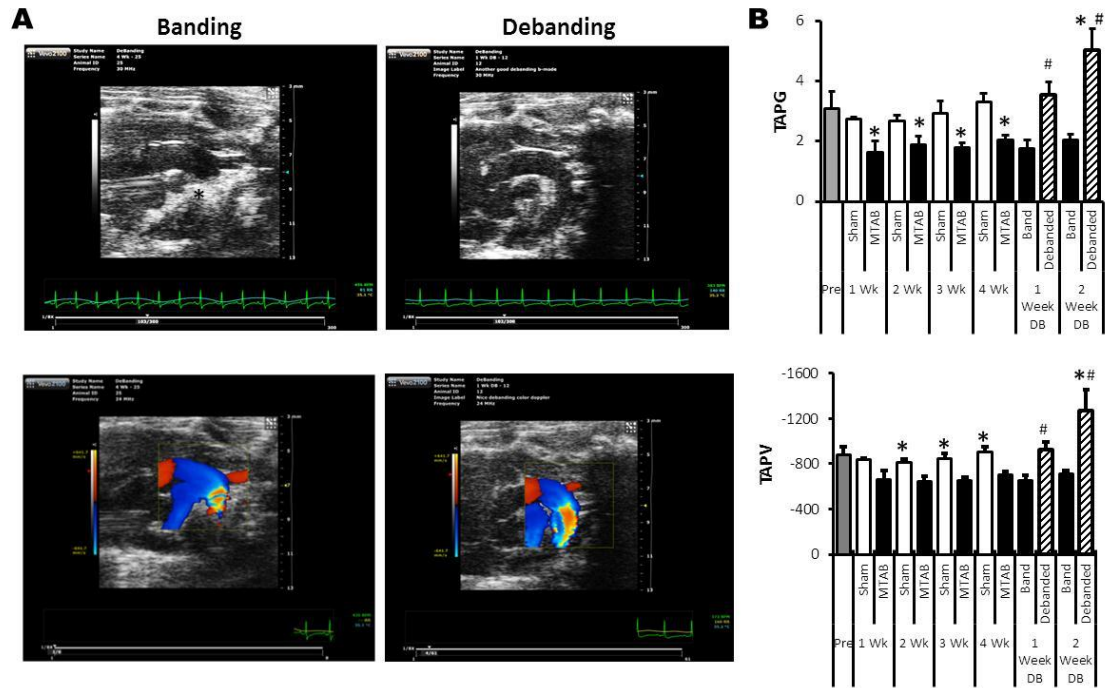


Figure 5.1: Transverse Aorta Blood Flow Dynamics in Banded and Debanded Mice

(A) Transthoracic echocardiographic imaging shows placement of transverse aortic band (*) between innominate and subclavian arteries (top left) results in impaired blood flow as shown through colour Doppler imaging (lower left). Debanded transverse aorta displays no residual narrowing of vessel (top right), and normal blood flow (lower right). Representative images of $n = 7$ animals per group. Red colour indicates blood moving to top of image; Blue colour indicates blood moving to bottom of image. (B) Pulse-wave doppler quantification of transverse aorta peak velocity (TAPV) and transverse aorta peak gradient (TAPG) of sham (white), banded (black), and debanded (striped) animals. $n \geq 4$ per group; * = $p \leq 0.05$ vs Sham. # = $p \leq 0.05$ vs band.

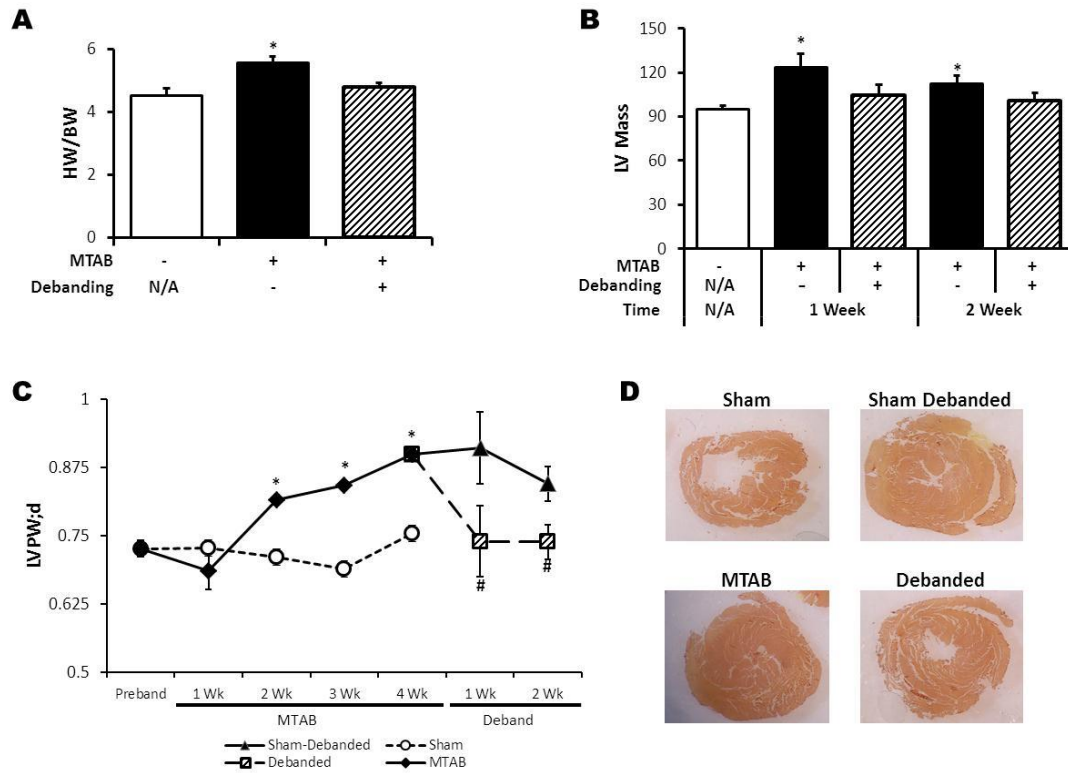


Figure 5.2: MTAB induced pressure overload hypertrophy is attenuated following debanding

(A) Gross wet heart weight of sham (white), banded (black), and debanded (striped) animals immediately following euthanization and brief cardiac perfusion with KCl. (B) Left ventricular posterior wall thickness (LVPWD) measured through long axis m-mode echocardiography in prebanded (grey), sham (white), banded (black), and debanded (striped) mice. (C) Corrected Left ventricular mass (LV Mass) measured through long axis m-mode echocardiography in sham (white), banded (black), and debanded (striped) mice. (D) Representative picosirius red stained transverse sections of sham, banded, sham-debanded, and debanded hearts. All quantification: $n \geq 4$ per group; * = $p \leq 0.05$.

Table 5.1: Echocardiographic assessment of cardiac function

		2 Wk		4 Wk		1 Wk DB		2 Wk DB	
Parameter	Surgery:	Sham	MTAB	Sham	MTAB	Sham-Debanded	Debanded	Sham-Debanded	Debanded
Heart Rate	BPM	457 +/- 39	434 +/- 17	425 +/- 12	447 +/- 11	429 +/- 23	453 +/- 9	452 +/- 13	434 +/- 8
Diameter:s	mm	2.99 +/- 0.05	2.93 +/- 0.15	3.06 +/- 0.2	2.93 +/- 0.1	2.91 +/- 0.18	2.91 +/- 0.19	3.14 +/- 0.15	3.04 +/- 0.23
Diameter:d	mm	4.21 +/- 0.02	4.04 +/- 0.13	4.23 +/- 0.17	4.07 +/- 0.09	4.28 +/- 0.1	4.18 +/- 0.15	4.33 +/- 0.09	4.26 +/- 0.18
Volume:s	uL	34.77 +/- 1.38	34.79 +/- 4.25	37.29 +/- 5.77	33.83 +/- 3.03	34 +/- 5.21	34.22 +/- 5.27	39.83 +/- 4.45	38.86 +/- 6.71
Volume:d	uL	78.91 +/- 1.06	73.08 +/- 5.35	80.38 +/- 7.5	73.58 +/- 3.82	82.68 +/- 4.75	78.77 +/- 6.51	84.71 +/- 4.16	82.93 +/- 8.18
Stroke Volume	uL	44.13 +/- 2.44	38.28 +/- 1.79	43.1 +/- 2.92	39.75 +/- 1.46	48.68 +/- 2.79	44.56 +/- 1.7	44.88 +/- 1.49	44.07 +/- 3.04
Ejection Fraction	%	55.96 +/- 2.36	54.21 +/- 2.68	54.11 +/- 3.52	54.67 +/- 1.85	59.98 +/- 4.34	58.35 +/- 3.3	53.68 +/- 3.16	55.64 +/- 4.34
Fractional Shortening	%	28.95 +/- 1.57	27.93 +/- 1.66	27.83 +/- 2.17	28.13 +/- 1.14	32.28 +/- 2.99	30.82 +/- 2.2	27.74 +/- 1.99	29.32 +/- 2.88
Cardiac Output	mL/min	20.09 +/- 0.75	16.5 +/- 0.88	18.3 +/- 0.96	17.76 +/- 0.83	20.77 +/- 1.29	20.15 +/- 0.61	20.3 +/- 0.95	18.96 +/- 1.17

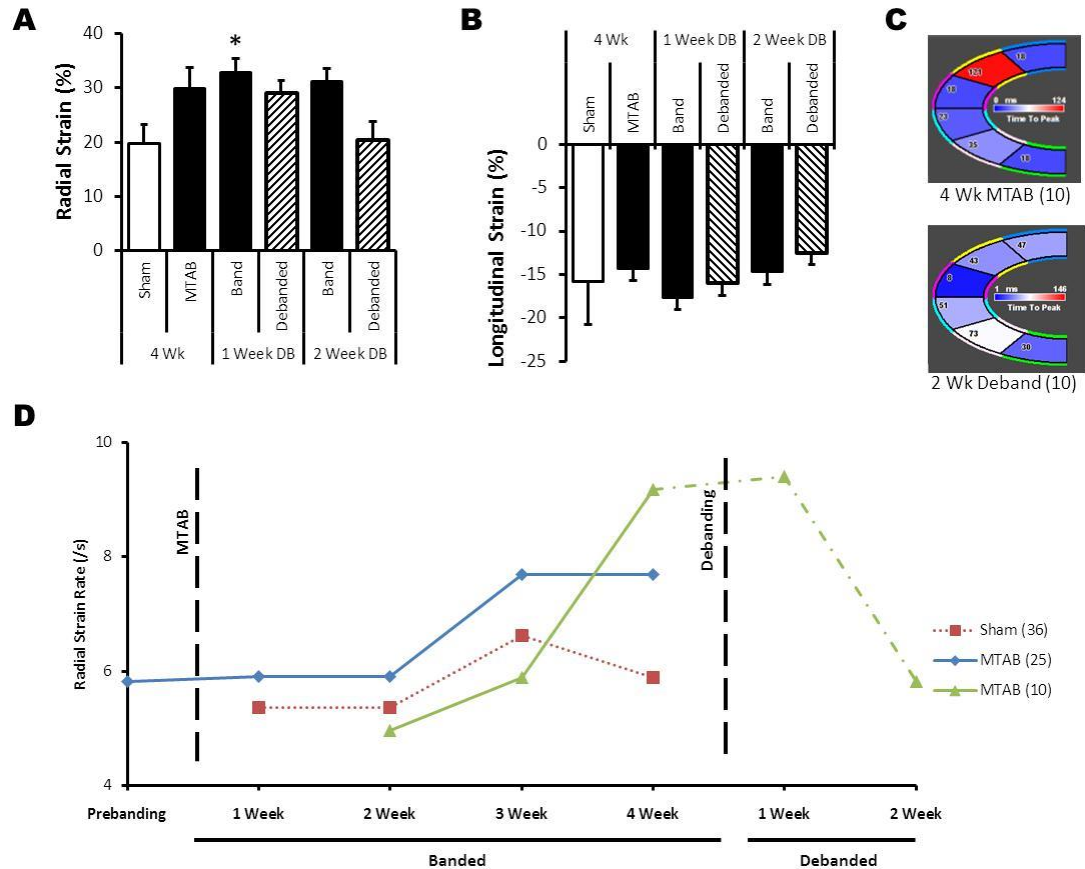


Figure 5.3: Left ventricular strain analysis

(A) Average radial strain of whole left ventricle from long axis m-mode echocardiography in sham (white), banded (black), and debanded (striped) mice. (B) Average longitudinal strain of whole left ventricle from long axis m-mode echocardiography in sham (white), banded (black), and debanded (striped) mice. (C) Time-to-peak analysis (longitudinal strain) from long axis m-mode echocardiography from mouse 10 after 4 weeks of banding (above), and 2 weeks following debanding (below). Increased time-to-peak is represented as heat map. Anterior wall is towards top of image, posterior wall is towards bottom of image. (D) Average radial strain rate of whole left ventricle from long axis m-mode echocardiography from individual mice (mouse ID indicated in brackets) for study duration.

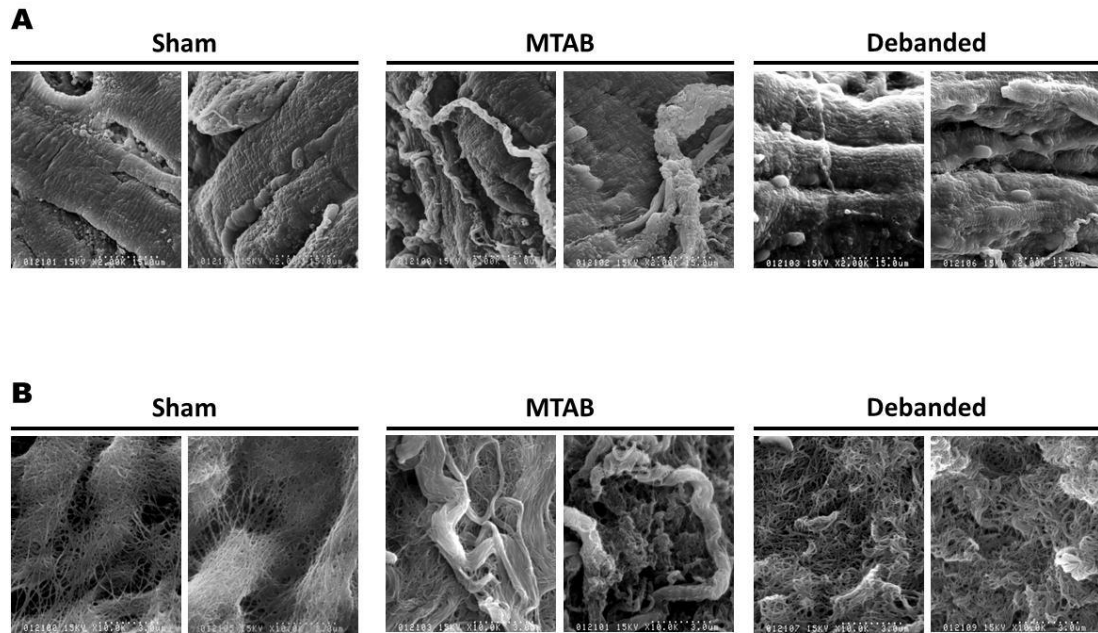


Figure 5.4: Debanding induces reverse remodelling of the ECM

(A) Representative scanning electron micrographs of ventricle samples obtained from 4 wk sham, 4 wk MTAB and 2 wk debanded animals. Thick collagen fibres may be seen in abundance in the MTAB images. (B) Representative scanning electron micrographs of ventricle samples obtained from 4 wk sham, 4 wk MTAB and 2 wk debanded animals. Non-fibrous tissue was digested and washed out of samples after fixation as described in materials and methods section. Thick collagen fibres may be seen in abundance in MTAB images. Thin fibres appear more numerous and disorganized in Debanded images compared against Sham.

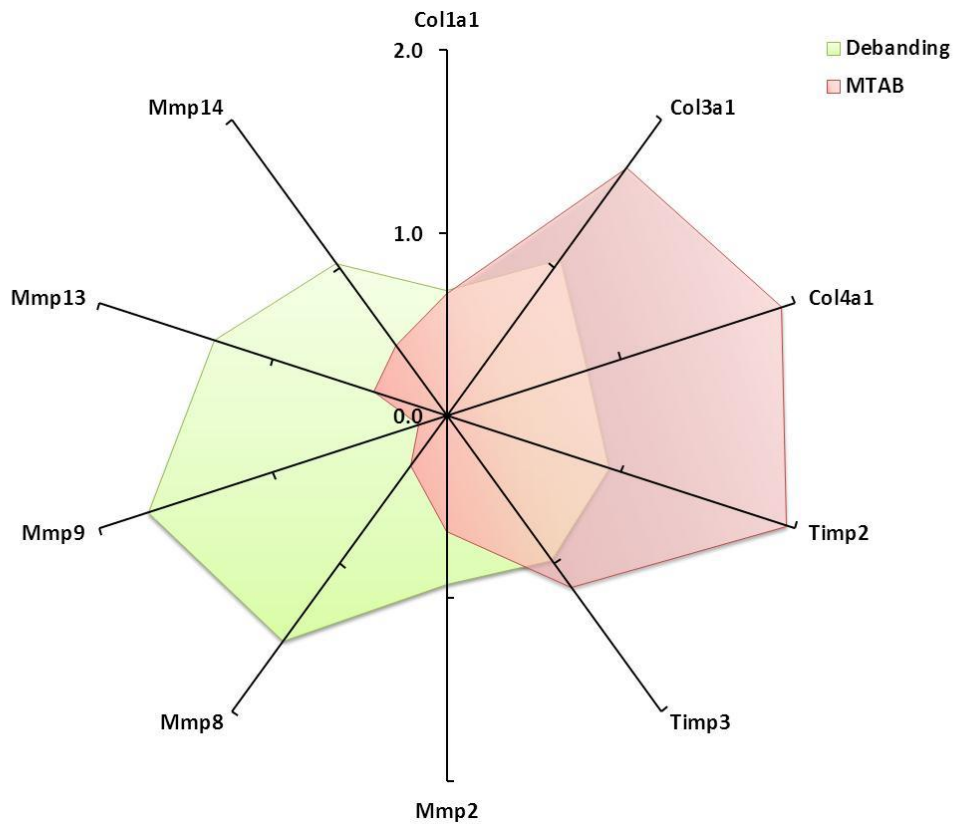


Figure 5.5: Expression of fibrosis markers

PCR array analysis of Collagen, TIMP, and MMP mRNA isolated from Sham, MTAB, Sham-Debanded, and Debanded mice. Each axis corresponds to fold-change of header gene compared to respective sham. MTAB values (solid line) represented as fold C(t) vs. sham; Debanding values (green) represented as fold C(t) vs. sham-debanding (red). Downregulation shown as values < 1.0. $n \geq 4$ per group.

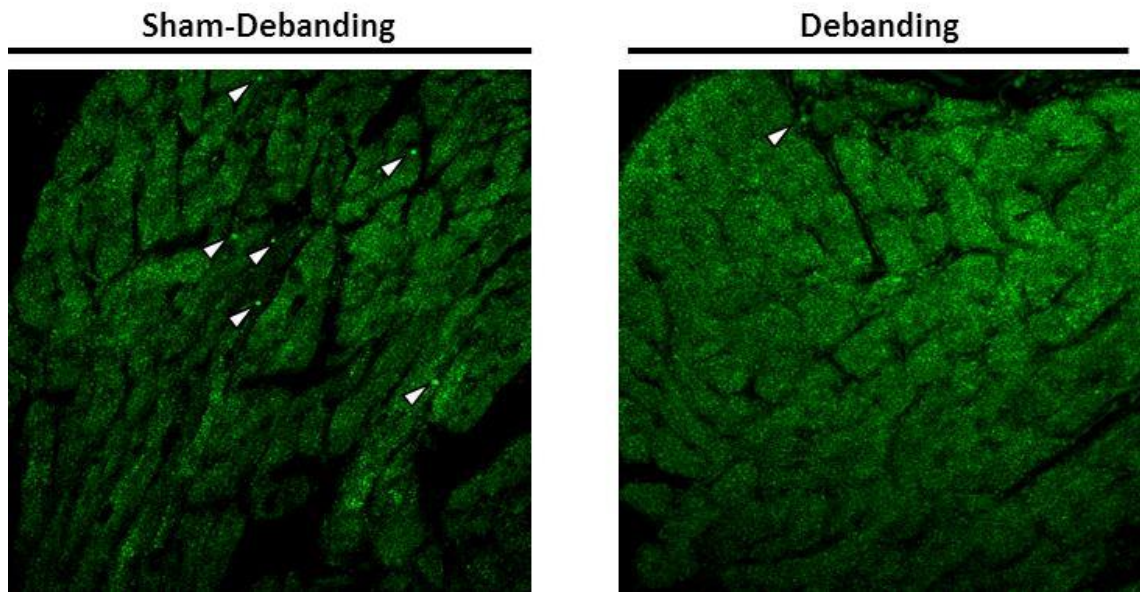


Figure 5.6: Myofibroblasts expression is lost following debanding

Representative immunofluorescent images of transverse ventricular sections obtained from 4 wk MTAB, 2 wk Sham-Debanded, and 2 wk debanded animals. Increased α -smooth muscle actin (α SMA) staining can be seen in MTAB and Sham-Debanded images. Little α SMA staining is seen in Debanded images.

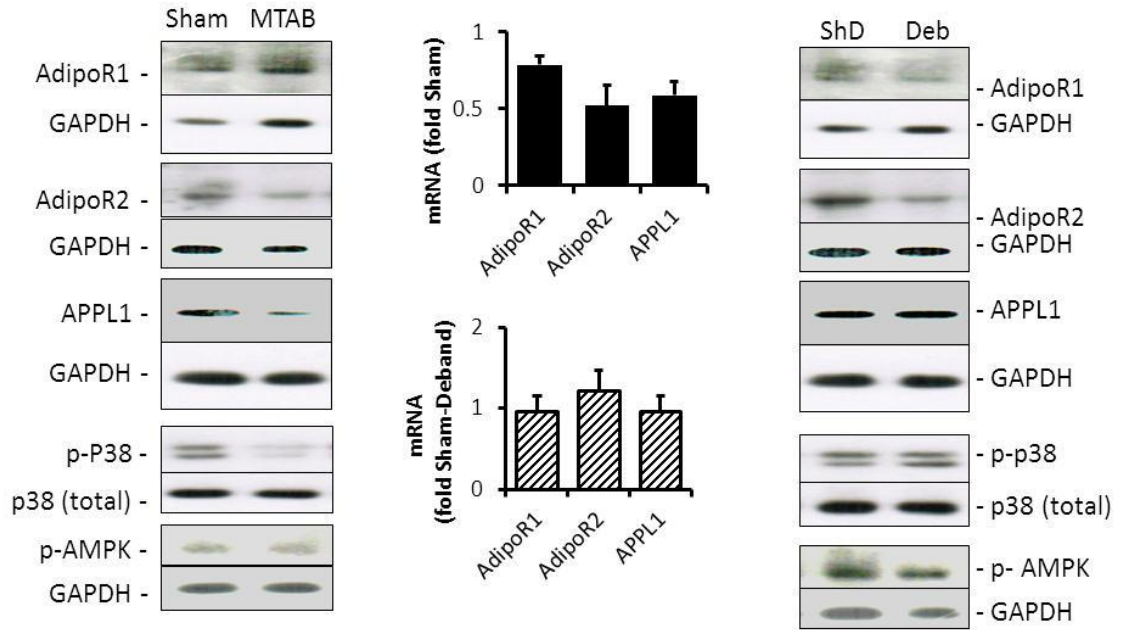


Figure 5.7: Adiponectin signalling

Middle graphs: Quantitative PCR analysis of AdipoR1, AdipoR2, and APPL1 are shown as fold MTAB over Sham (black bars), or fold Deband over Sham-Deband (striped bars). $n \geq 4$. * = $p \leq 0.05$ vs sham. # = $p \leq 0.05$ vs sham-deband. Western blot analysis of AdipoR1, AdipoR2, APPL1, phospho-p38, and phospho-AMPK with relevant loading control shown below.

5.4 Discussion

Cardiac remodelling was classically viewed as a linear progression of geometric changes to the left ventricle concomitant with the development of fibrosis following a myocardial infarction, ultimately ending in heart failure. In fact, there is now accumulating evidence with respect to non-ischemic heart disease that the remodelled heart may undergo a reversal of maladaptive changes resulting in normalized cardiac function. Here we have presented evidence indicating that features of reverse remodelling extend to the structure of the extracellular matrix, highlighting the dynamic capabilities of the myocardium in actively regulating pro- and anti-fibrotic pathways to define the myocyte support structure.

After 4 weeks of banding, blood flow dynamics of the transverse aorta were significantly impaired while LV mass and LVPWD were increased without changes to EF or FS. These data are consistent with PO induced compensatory cardiac remodelling involving concentric hypertrophy. In this background, debanding resulted in supra-normal blood flow across the transverse aorta and initiated regression of hypertrophic changes of the left ventricle. The decoupling of aortic blood flow and LV geometry may represent a transient phenomenon as myocyte contractile force exceeds hemodynamic load, implying a pressure ‘underload’ condition in which the contractile capacity of the left ventricle exceeds its pressure and volumetric demands. The maintenance of cardiac function through both overload and underload highlights the dynamic, compensatory abilities of the heart, but also masks the

underlying changes to cardiac structure, especially with respect to the extracellular matrix, which may have long term consequences to cardiac function.

The initiation of cardiac fibrosis in PO serves a different function from the fibrotic response in MI. The increased deposition of collagen in a heart undergoing compensatory concentric hypertrophy establishes a stress-tolerant collagen scaffold to enhance systolic force generation, prevent diastolic over filling, and protect against myocyte slippage. As a result, the expanded ECM serves in a highly supportive role in PO stress, a distinct contrast to the necessarily final, reparative role an infarct scar plays in MI to replace lost myocytes and maintain LV structural integrity [250, 307]. Our previous temporal analysis of MTAB induced fibrosis using scanning electron microscopy (chapter 2) showed the increased presence of ECM small fibres after 1 week, followed by the appearance of thick, disorganized fibres after 4 weeks of pressure overload, characteristic of established models of cardiac fibrosis. We found the upregulation of pro-fibrotic processes following MTAB was a sharp contrast to the lesser degree of fibrosis associated with LV unloading, implying the activation of anti-fibrotic pathways following debanding and may be a reflection of increased MMP gene expression. A finer analysis of the collagen ECM structure between our surgical groups using a cell-maceration technique to remove all non-fibrous material reveals a collagen network following debanding lacking the thick collagen strands commonly associated with cardiac fibrosis, but instead containing more (in apparent number) highly disorganized thin collagen fibres than the normal myocardium. There may be

negative electrophysiological consequences to this pattern of enhanced fibrosis as exacerbated cardiac fibrosis has been shown to disrupt cardiomyocyte electrical coupling, leaving the myocardium vulnerable to impaired signalling (e.g. arrhythmias) [283]. The pattern of expanded fibrosis in debanded mice correlates with previous findings in patients following LVAD implantation [339], and increased fibrosis in non-ischemic cardiac disease (e.g. aortic stenosis) is associated with increased risk of death due to cardiac failure [202, 244, 245].

Aortic debanding has been associated with an increase in LC3-II, an indicator of autophagic flux [242]. Since hypertrophy of individual cardiomyocytes plays a principle role in the hypertrophic response to pressure overload, correlating changes in autophagy and atrophy with changes in overall cardiomyocyte cross-sectional area in our model of banding and debanding is a necessary and pending addition to this study.

The association of adiponectin, the metabolically active adipokine, with various stages of heart disease is now well established [148] although the direct relationship between circulating adiponectin levels and prospective patient outcome remain elusive [38]. Indeed, the picture of a local adiponectin system, regulated independently from adipocyte derived adiponectin, is emerging as expression of adiponectin from cardiomyocytes has been shown both *in vitro* [85, 340, 341] and in congestive heart failure patients [88], and previously we demonstrated adiponectin mediated regulation of the ECM through stimulation of cardiac fibroblasts [108].

However, when considering adiponectin as a therapeutic for heart failure, recent evidence that adiponectin sensitivity may be impaired by Angiotensin II signalling [182], the status of adiponectin sensitivity in non-ischemic cardiomyopathies needs to be addressed. Clinically, adiponectin sensitivity was recovered in patients with advanced heart failure following LVAD implantation [338]. Our data shows the downregulation of the adiponectin receptors AdipoR1 and AdipoR2, as well as the intracellular binding protein APPL1 following MTAB surgery, which may indicate the desensitization of the myocardium to adiponectin stimulation, correlating with the depressed activation of the well known adiponectin targets AMPK and p38. There was no detectable change in adiponectin sensitivity between sham-debanded and deband animals. The issue of adiponectin sensitivity within the overloaded myocardium takes on greater importance considering the continued and expanded use of pioglitazone and rosiglitazone, PPAR γ agonists, in heart failure patients. Indeed, cardiomyopathic adiponectin resistance must be appreciated as a potential factor limiting adiponectin's therapeutic efficacy.

Although we and others have demonstrated the efficacy in unloading the left ventricle through debanding surgery for the study of reverse remodelling, other novel methods have yielded similar findings. Heterotopic transplantation was shown to achieve atrophic remodelling of the rat heart [342], while the injection of mesenchymal stem cells was shown to significantly improve hemodynamic performance, reverse LV remodelling, and improve exercise tolerance following aortic

banding [343], while increasing EF and capillary density in a model of doxorubicin-induced heart failure [344].

The data presented here highlights the ability of the heart to adapt to a prolonged, transient increase in LV pressure. However, while cardiac function is maintained throughout this phase of adaptive remodelling, non-symptomatic adverse remodelling of the collagen ECM may have a largely influence the structure of the ECM following unloading. The increased fibrosis noted following LV unloading may seriously impact future risk of cardiovascular mortality. This suggests that more attention should be paid to the mechanisms governing fibrosis. Therapeutics designed to limit non-pathological fibrosis may hold long term benefits to individuals at high risk of developing heart disease.

CHAPTER 6: CONCLUSION

6.1 Summary of research

The rise of the obesity and diabetes epidemics has caused an increase in worldwide cardiovascular risk [128, 309]. As a result, focus has been placed on finding factors linking these diseases. Adiponectin is one of the major secreted factors from adipose tissue, and exists in circulation at a relatively high concentration [39-41]. Numerous studies have demonstrated a negative relationship between circulating adiponectin levels and diseases such as obesity and T2D [42-44]. However, the data linking circulating adiponectin and various stages of heart disease are less clear, and indeed, there is much controversy as to the predictive value of circulating adiponectin levels on heart failure outcomes [38, 133]. Similarly, there is some conflicting data regarding the role that adiponectin plays in pressure overload models of heart disease [116, 156, 161, 162].

Animal studies have suggested adiponectin influences fibrosis in models of heart failure [116, 118]. Adiponectin regulation of cardiomyocyte metabolism has been well characterized [112], but less is known about adiponectin mediated regulation of the extracellular matrix. For the studies presented here, I chose to focus on the regulation of the extracellular matrix by adiponectin *in vivo*, and adiponectin signalling mechanisms regulating effects in isolated cardiac fibroblasts.

I first characterized the pattern of progressive fibrosis in the heart following the induction of pressure overload by transverse aortic banding surgery in wild-type mice. Collagen accumulation in the myocardium was characterized by an early increase in

small fibre collagens, followed by the appearance of thick, disorganized collagen fibres. At this time there was also an increased presence of hypersecretory myofibroblast cells in the heart, as well as an overall increase in heart size. Comparing the pattern of remodelling in wild-type to that in adiponectin deficient mice, I noted the delayed onset of fibrosis in the latter group. This correlated with a delay in cardiac dysfunction. From this study it was clear that the progression of fibrosis in heart disease is influenced by the presence or absence of adiponectin. I then sought to determine which adiponectin stimulated signalling pathways mediated changes in extracellular matrix components in cardiac fibroblasts, the cell type primarily responsible for mediating fibrosis in the heart.

I used primary cardiac fibroblasts and found that adiponectin treatment activated the APPL1-AMPK signalling pathway, leading to increased cell surface localization of the membrane-bound ECM degrading enzyme MT1-MMP [174, 291]. An adiponectin-stimulated AMPK-dependent signalling mechanism also significantly increased fibroblast migration and activation of MMP2, another important regulator of the ECM that is closely associated with cardiac fibrosis. I theorized that MT1-MMP and MMP2 activation would regulate the ECM assembled by fibroblasts in culture. Accordingly, I found acute adiponectin stimulation resulted in a degraded collagen and elastin ECM in culture. Interestingly, the matrix was subsequently reassembled in a disorganized pattern, similar to the patterns noted in the first *in vivo* study.

Knockdown of APPL1 in fibroblasts inhibited adiponectin stimulated AMPK activation and MT1-MMP localization. To our knowledge, this study presents the first documented evidence that the intracellular AdipoR binding protein APPL1 can mediate remodelling of the extracellular matrix. The *in vitro* studies also firmly established that adiponectin action on fibroblasts is a potentially important mechanism in the progression of heart failure.

Previous *in vivo* and *in vitro* data [85, 116], as well as my *in vivo* studies, have suggested that adiponectin may regulate cardiac hypertrophy in PO, but the transcriptional mechanisms involved were still unclear. Through collaboration with the McDermott lab, WT or AdKO were crossbred with transgenic MEF2-lacZ reporter mice, and then subjected to PO. We found that MEF2 activity was upregulated in WT mice following PO, but this trend was absent in AdKO mice. Accordingly, adiponectin stimulation increased MEF2 expression, as well as that of ANF, a MEF2 downstream target and key indicator of the pro-hypertrophic fetal gene program. This study provides the first evidence that adiponectin directly targets the pro-hypertrophic transcription factor MEF2, and stimulates the fetal-gene program through ANF upregulation.

In 2012 a clinical study in heart failure patients suggested the existence of adiponectin insensitivity in the heart which could be reversed by unloading of the left ventricle [338]. In my final animal study, I had three goals: a) to establish a surgical model of LV unloading in our lab; b) to evaluate markers of adiponectin signalling

before and after LV overload; c) to image the extracellular matrix before and after unloading using the scanning electron microscope. Similar to the initial *in vivo* study, MTAB induced the accumulation of thick collagen fibres in the myocardium. These fibres were absent following unloading and replaced by disorganized thin fibres. The data regarding adiponectin sensitivity in the myocardium was not very conclusive and requires follow-up studies, although LV unloading significantly decreased LV wall strain and regressed LV hypertrophy.

6.3 Future Experiments

Work on chapters 1 and 3 is finishing. The final manuscripts are being edited for submission in May 2014.

Future work will continue to characterize adiponectin signalling in cardiac fibroblasts. Other studies have indicated the role of p38-MAPK and ERK in adiponectin stimulated signalling in cardiac fibroblasts [331]. Using pharmacological inhibitors and siRNA-mediated knockdown, the importance of these signalling molecules in adiponectin mediated ECM remodelling will be established. Furthermore, a clearer understanding of the functional differences between AdipoR1 and AdipoR2 in cardiac fibroblasts is necessary, as well as a characterization of the endogenous APPL1 inhibitor, APPL2 [120].

Through related honours thesis projects that I supervised, we have preliminary data showing the upregulation of adiponectin transcription from cardiomyocytes and cardiac fibroblasts by the TZD rosiglitazone. Furthermore, we found that AngII is able to attenuate this effect, confirming previous findings that AngII could downregulate adiponectin expression via PARP-1 [185, 186]. Conversely, we have data indicating that adiponectin pre-treatment can inhibit AngII induced fibroblast to myofibroblast differentiation *in vitro*. In order to establish if adiponectin's effects in this system are through a general inhibition of cell differentiation, or through targeted disruption of AngII signalling, we will follow up by using another potent inducer of myofibroblast differentiation: TGFbeta. The relative status of adiponectin sensitivity between cardiac fibroblasts and myofibroblasts is unknown. We will conclude this study by examining the expression of various components of the adiponectin signalling pathway (i.e. AdipoR1, AdipoR2, APPL1, APPL2), and compare adiponectin stimulated AMPK and p38-MAPK phosphorylation in cardiac fibroblasts and myofibroblasts.

As highlighted in my introduction, the yin-yang relationship between adiponectin and AngII in the heart may prove to be a very important balance that needs to be considered in heart disease models and in the development of therapeutics.

Sample analysis for the reverse remodelling manuscript (chapter 4) is almost complete. However, the role of autophagy in the process of reverse remodelling following unloading of the LV has been suggested [242]. We will analyze TEM samples

and tissue homogenates generated from our debanding experiments to establish the presence of autophagy and mitophagy as part of our reverse remodelling model. Furthermore, we will expand this study in two ways: 1) study the changes to the ECM following debanding in mice exhibiting signs of decompensated heart failure (EF < 30%); 2) contrast these findings to the pattern of reverse remodelling in adiponectin deficient mice.

6.4 Conclusion

Collectively, my studies show that adiponectin is an important player in the response of the heart to pressure overload, especially as a regulator of the ECM. My *in vivo* studies show that cardiac fibrosis and hypertrophy are delayed following MTAB surgery by adiponectin deficiency, and I propose that this is conferred through a fibrosis-dependent cardioprotective mechanism. Furthermore, through establishing the APPL1-AMPK signalling axis in cardiac fibroblasts, and identification of MEF2-ANF targeting in cardiomyocytes, my work offers an expanded understanding of adiponectin signalling in the heart. Lastly, I show that ECM expansion in PO induced compensatory, non-pathological hypertrophy is incompletely resolved following LV unloading. This finding may have serious implications in the long term prognosis of hypertensive patients with respect to fibrosis induced diastolic dysfunction and progressive heart failure.

Along with our upcoming exciting data sets, we hope to provide significant insight into the actions of adiponectin in cardiac remodelling, as well as to introduce new potential targets in the development of therapeutics for heart disease.

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STATEMENT OF CONTRIBUTION

Chapter 2

K. Dadson contributed to planning all experiments and conducted all surgeries. S. Tuerdi aided in tissue collection and electron microscope sample preparation. This chapter was written by K. Dadson and edited by Dr. G. Sweeney and is being prepared to be submitted for publication.

Chapter 3

K. Dadson contributed to planning and conducted all experiments except siRNA targeted knockdown of APPL1 performed by H. Chasiotis, and PCR analysis performed by S. Wannaiampikul. This chapter was co-written by K. Dadson and H. Chasiotis, edited by Dr. G. Sweeney, and is published in *Journal of Cellular Biochemistry*.

Chapter 4


K. Dadson contributed to planning all experiments and conducted all surgeries. S. Tuerdi and K. Dadson jointly planned all analysis except echocardiography analysis conducted through collaboration with N. Polidovitch, S. Beca., and Dr. P. Backx; MEF2-lacZ/AdKO crossbreeding, and MEF2 and ANF reporter experiments were performed in collaboration with S. Hashemi, J. Zhao, and Dr. J. McDermott. This chapter was written by K. Dadson and edited by Dr. G. Sweeney, and is being prepared to be submitted for publication.

Chapter 5


K. Dadson contributed to planning and conducted all experiments and surgeries. Some echocardiography analysis was performed by V. Kovacevic as part of her undergraduate honours thesis project under the supervision of K. Dadson. Western blot analysis was performed by Palanivel R. This chapter was written by K. Dadson and edited by Dr. G. Sweeney.

Appendix C

This review paper was co-written by K. Dadson, Dr. Y. Liu, and Dr. G. Sweeney, and is published in *Frontiers in Endocrinology*.



J. Keith E. Dadson (PhD Candidate)



Dr. Gary Sweeney (PhD Supervisor)

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APPENDIX A: LIST OF PUBLICATIONS

1. Dadson K, Chasiotis H, Wannaiampikul S, Tungtrongchitr R, Xu A, Sweeney G. Adiponectin Mediated APPL1-AMPK Signalling Induces Cell Migration, MMP Activation, and Collagen Remodelling in Cardiac Fibroblasts. *J Cell Biochem.* 2013 Nov 20. doi: 10.1002/jcb.24722. [Epub ahead of print]
2. Dadson K, Liu Y, Sweeney G. Adiponectin action: a combination of endocrine and autocrine/paracrine effects. *Front Endocrinol (Lausanne).* 2011 Nov 8;2:62.
3. Vu V, Dadson K, Odisho T, Kim W, Zhou X, Thong F, Sweeney G. Temporal analysis of mechanisms leading to stimulation of glucose uptake in skeletal muscle cells by an adipokine mixture derived from primary rat adipocytes. *Int J Obes (Lond).* 2011 Mar;35(3):355-63.
4. Fang X, Fetros J, Dadson KE, Xu A, Sweeney G. Leptin prevents the metabolic effects of adiponectin in L6 myotubes. *Diabetologia.* 2009 Oct;52(10):2190-200.
5. Zinman LH, O'Connor PW, Dadson KE, Leung RC, Ngo M, Bril V. Sensitivity of repetitive facial-nerve stimulation in patients with myasthenia gravis. *Muscle Nerve.* 2006 May;33(5):694-6.

APPENDIX B: PUBLICATIONS IN PREPARATION

1. Dadson K, Turdi S, and Sweeney G. Adiponectin deficiency alters the progression of ECM remodelling following the induction of pressure overload
2. Dadson K, Turdi S, Hashemi S, Zhao J, Polidovitch N, Beca S, McDermott J, Backx P, and Sweeney G. Myocyte Enhancer Factor-2 Binding is Attenuated in Adiponectin Deficiency Following Pressure Overload, and is Upregulated by Adiponectin Stimulation of Cardiomyocytes

APPENDIX C: REVIEW ARTICLE



Adiponectin action: a combination of endocrine and autocrine/paracrine effects

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The widespread physiological actions of adiponectin have now been well characterized as clinical studies and works in animal models have established strong correlations between circulating adiponectin level and various disease-related outcomes. Thus, conventional thinking attributes many of adiponectin's beneficial effects to endocrine actions of adipose-derived adiponectin. However, it is now clear that several tissues can themselves produce adiponectin and there is growing evidence that locally produced adiponectin can mediate functionally important autocrine or paracrine effects. In this review article we discuss regulation of adiponectin production, its mechanism of action via receptor isoforms and signaling pathways, and its principal physiological effects (i.e., metabolic and cardiovascular). The role of endocrine actions of adiponectin and changes in local production of adiponectin or its receptors in whole body physiology is discussed.

Keywords: adiponectin, metabolic syndrome, cardiovascular disease

INTRODUCTION

ADIPONECTIN: REGULATION OF ITS EXPRESSION AND POST-TRANSLATIONAL MODIFICATION

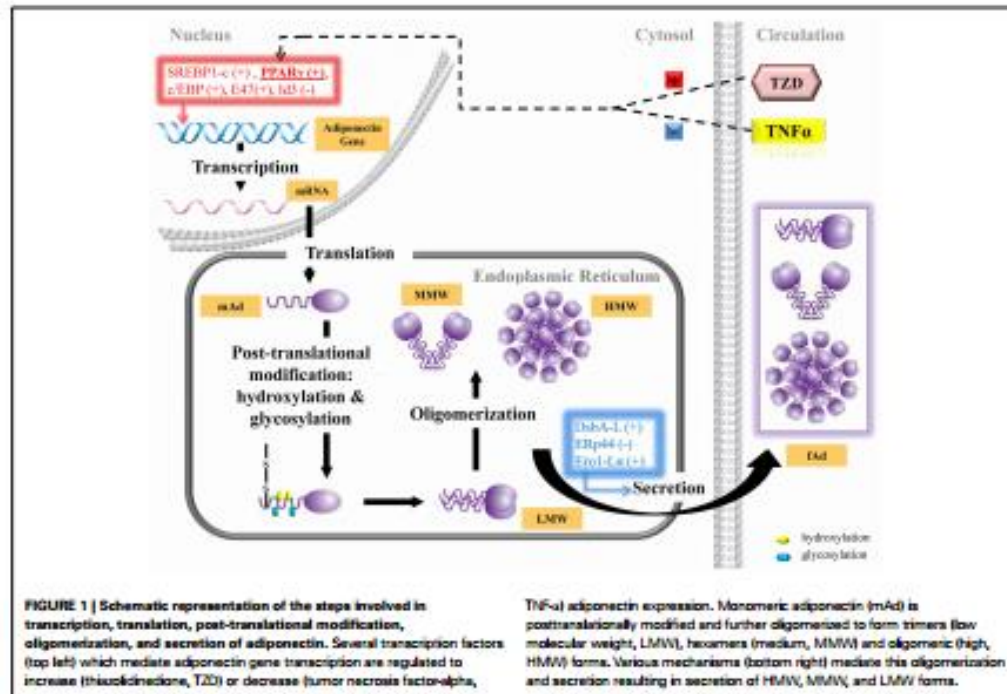
Adiponectin was discovered as an adipocyte-derived 30 kDa secretory protein, which consists of an amino-terminal signal sequence followed by a collagenous domain and a carboxyl-terminal globular domain (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996). Adiponectin is transcriptionally regulated by peroxisome proliferator-activated receptor γ (PPAR γ), C/EBP, SREBP, E47, and Id3 protein (Fajas et al., 1998; Osborne, 2000; Motoshima et al., 2002; Yilmaz et al., 2004; Doran et al., 2008; Figure 1). Drugs like rosiglitazone and pioglitazone, belonging to the thiazolidinedione (TZD) class of PPAR γ agonists, have been clinically and experimentally proven to be potent inducers of adiponectin expression (Tsuchida et al., 2005; Phillips et al., 2008; Liu et al., 2009) and indeed many of the metabolic and cardioprotective effects of rosiglitazone or pioglitazone are absent/decreased in mice lacking adiponectin (Li et al., 2010; Tao et al., 2010; Zhou et al., 2010). Therefore, elevated adiponectin expression is a critical mechanism of action in mediating beneficial effects of this drug class. Regulation of SREBP-1c is another well known mechanism activating adiponectin transcription while more recently Id3 and E47 were demonstrated as novel regulators of this SREBP-1c-mediated adiponectin expression in adipocytes. E47 potentiates SREBP-1c-mediated adiponectin promoter activation and this is inhibited upon interaction with Id3. Decreased Id3 levels increased adiponectin expression and Id3-null mice had increased adiponectin expression in visceral fat tissue and serum (Doran et al., 2008).

Extensive post-translational modification plays a vital role for assembling adiponectin to form its functional oligomeric complexes (Wang et al., 2008; Simpson and Whitehead, 2010). The

initiation step of adiponectin multimerization involves the non-collagenous globular domain forming trimers (Waki et al., 2003). Subsequently, the disulfide bond formed via Cys39 (mouse) or Cys36 (human) is critical for adiponectin to form higher molecular weight multimers based on its trimeric form (Tsao et al., 2003). Post-translational modification including hydroxylation and glycosylation of the four conserved lysine residues (lys68, lys71, lys80, lys104) within the collagenous domain of adiponectin are required for the formation of HMW oligomeric complex (Wang et al., 2002, 2006). The disulfide bond A oxidoreductase-like protein (DsbA-L) was found to positively regulate the process of adiponectin multimerization. The secretion of adiponectin is specifically regulated by endoplasmic reticulum (ER) proteins ERp44 and Ero1-L α . The covalent bond formed between ERp44 and the thiol group of Cys39 on adiponectin retains adiponectin in ER while the disulfide bond formed between ERp44 and Ero1-L α releases adiponectin (Anelli et al., 2003; Wang et al., 2007; Schraw et al., 2008). Adiponectin exists abundantly in the plasma and circulates in its HMW (oligomer), MMW (hexamer), and LMW (trimer) oligomeric forms (Waki et al., 2003). The combination of these oligomeric forms is often referred to as full-length adiponectin (fAd). An additional circulating form of adiponectin, the albumin binding LMW, has been identified subsequently (Ebinuma et al., 2006). Moreover, upon protease cleavage the globular domain of fAd (referred to hereafter as gAd) can be liberated. Although significant circulating levels are not observed, gAd is proposed to be cleaved locally by specific tissues or at sites of inflammation (Fruebis et al., 2001; Waki et al., 2005).

ADIPONECTIN RECEPTORS AND RECEPTOR ADAPTOR PROTEINS

Adiponectin exerts many of its cellular effects principally through binding to two receptor isoforms with seven



putative transmembrane domains. These adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) isoforms have distinct distribution patterns in various tissues (Yamauchi et al., 2003a, 2007; Kadowaki et al., 2007, 2008). It was shown that skeletal muscle cells bound gAd more avidly than fAd and suppression of AdipoR1 expression with siRNA reduced high-affinity gAd binding (Yamauchi et al., 2003b). Conversely, suppression of AdipoR2 expression with siRNA largely reduced fAd binding, but only modestly reduced globular adiponectin binding. Collectively, available data indicates that AdipoR1 is a high-affinity receptor for gAd and a low-affinity receptor for fAd, whereas AdipoR2 is an intermediate-affinity receptor for fAd and gAd. Since AdipoR1 is the predominant form expressed in skeletal muscle, while AdipoR2 is predominantly expressed in liver, this correlated with the fact that gAd exerts its insulin mimetic and insulin-sensitizing effect more effectively compared to fAd in skeletal muscle and vice versa (Yamauchi et al., 2002). T-cadherin, was also found to competitively bind only the hexameric and HMW forms of adiponectin (Hug et al., 2004; Asada et al., 2007; Chan et al., 2008). Although T-cadherin lacks an intracellular domain (Hug et al., 2004), various studies have suggested the involvement of this protein in mediating functional effects of adiponectin. These include cardioprotective effects (Denzel et al., 2010), anti-atherosclerotic effects in vasculature (Takeuchi et al., 2007; Andreeva et al., 2010), anti-diabetic

effects in skeletal muscle (Hug et al., 2004) as well as anti-fibrotic effects in liver (Asada et al., 2007).

Several adiponectin receptor binding proteins have now been identified (Buechler et al., 2010; Heiker et al., 2010). The first and best characterized is adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain, and leucine zipper motif (APPL1; Mao et al., 2006a). Other more recently identified adaptor proteins which have been suggested to be involved in adiponectin's intracellular signal transduction include activated protein kinase C1 (RACK1; Xu et al., 2009), ER protein 46 (ERp46; Charlton et al., 2010), and protein kinase CK2 β (Heiker et al., 2009). Among the adaptor proteins, only APPL1 associates with both AdipoR1 and AdipoR2 while RACK1, ERp46, and CK2 β bind to AdipoR1. APPL1 interacts with the intracellular region of adiponectin receptors through its PTB domain (Mao et al., 2006a) and sequentially activates downstream signaling. It has now been shown that APPL1 plays an important role in mediating many of adiponectin's effects, including metabolic effects in liver, muscle, and endothelial cells (Kobayashi et al., 2004; Mao et al., 2006a; Cheng et al., 2007, 2009; Chandrasekar et al., 2008; Wang et al., 2009a; Zhou et al., 2009; Cleasby et al., 2011; Xin et al., 2011) as well as cardioprotective effects (Fang et al., 2010; Park et al., 2011). The interaction between RACK1 or CK2 β and AdipoR1 was indicated by yeast two-hybrid studies and confirmed

in cells by colocalization and coimmunoprecipitation (Heiker et al., 2009; Xu et al., 2009). RNAi-mediated RACK1 knockdown prevented adiponectin regulated glucose uptake in HepG2 cells (Xu et al., 2009). Pharmacological inhibition of CK2 β attenuated adiponectin signaling in skeletal muscle cells (Heiker et al., 2009). Coimmunoprecipitation also confirmed the interaction between ERp46 and AdipoR1, but not AdipoR2, and interestingly the suppression of ERp46 expression resulted in increased cell surface AdipoR1 levels and enhanced adiponectin stimulated phosphorylation of AMP kinase (AMPK) but reduced phosphorylation of p38-mitogen-activated protein kinase (MAPK; Charlton et al., 2010).

CIRCULATING ADIPONECTIN: REGULATION AND CHANGES IN NORMAL AND DISEASE STATES ADIPONECTIN ISOFORMS, THEIR CIRCADIAN RHYTHM AND CLEARANCE

Adiponectin circulates in the concentration range of \sim 3–30 μ g/ml in healthy individuals, with a lower level in male compare to female which is mainly attributed to lower amounts of hexameric HMW form (Xu et al., 2005; Wang et al., 2008). Studies on the metabolism and clearance of adiponectin have yielded variable results with a half-life of approximately 75 min reported recently based on tracking fluorescently labeled recombinant adiponectin in the circulation (Halberg et al., 2009) while a previous study (as the study performed in rabbit using recombinant human adiponectin) found HMW adiponectin had a half-life of 13 h and LMW a half-life of 17.5 h (Peake et al., 2005). A recent study indicated the important role of posttranslational modifications, including sialylation which modifies the O-linked glycans on Thr residues with sialic acid, in the regulation of adiponectin's half-life (Richards et al., 2010). Indeed, enzymatic removal of the sialic acid from adiponectin accelerates its clearance from circulation (Richards et al., 2010). Like many other metabolic hormones adiponectin, especially its HMW form, is regulated by the biological clock and shows circadian rhythms with a reduction occurring during the night (Froy, 2007; Cano et al., 2009; Gomez-Abellan et al., 2010; Scheer et al., 2010; Tan et al., 2011). The endocrine effects of adipose-derived adiponectin are conventionally believed to regulate many physiological processes, and many studies have established strong correlations between circulating adiponectin levels and various disease states.

ADIPONECTIN IN OBESITY AND DIABETES

A decreased plasma adiponectin level has been found in patients with obesity and type 2 diabetes despite the increasing mass of adipose tissue (Arita et al., 1999; Ouchi et al., 2001; Weyer et al., 2001; Matsuda et al., 2002; Daimon et al., 2003; Spranger et al., 2003; Ryo et al., 2004; Liu et al., 2007). Moreover, many studies have shown that instead of the absolute total circulating level of adiponectin, the ratio between HMW and total adiponectin can more accurately predict insulin resistance and development of features of the metabolic syndrome (Araki et al., 2006; Hara et al., 2006; Katsuki et al., 2006; Liu et al., 2007; Hamilton et al., 2011). However, although HMW is often referred to as most biologically active for this reason, there remains a lack of direct metabolic studies have been conducted using only the HMW form of adiponectin.

Proinflammatory cytokines, in particular tumor necrosis factor α (TNF α), are considered to be a principal cause of the reduction in circulating adiponectin seen in obese/diabetic patients (Ouchi et al., 2003a,b; Takemura et al., 2007). Nevertheless, it is important to balance these strong clinical correlations with consideration of whether alterations in adiponectin are always a cause or, in some cases, a consequence of disease states and this will be highlighted below.

ADIPONECTIN IN CARDIOVASCULAR DISEASE

A substantial amount of evidence indicates a potential pathophysiological contribution of adiponectin in cardiovascular disease (Shimura, 2010; Xu et al., 2010; Hui et al., 2011; Okamoto, 2011). Clinical studies have generally identified negative correlations between plasma adiponectin levels and various aspects of cardiovascular disease such as atherosclerosis, myocardial infarction, heart failure, endothelial dysfunction and hypertension, and established as an independent risk factor for these. The HMW form of adiponectin has been generally regarded as the best predictor of cardiovascular outcome yet, interestingly, a recent study also identifies trimeric LMW adiponectin as a potentially useful biomarker in cardiovascular disease (Hamilton et al., 2011). Adiponectin knockout mice have been particularly informative in terms of elucidating the interaction between adiponectin and cardiovascular injury, with exaggerated degrees of induced cardiovascular defects typically observed in these mice which can be corrected by adiponectin replenishment. Additionally, several single nucleotide polymorphisms (SNPs) have been identified in the adiponectin gene that consequently cause a reduction of its serum concentration (Menzaghi et al., 2002, 2007; Tanko et al., 2005; Yang and Chuang, 2006). For example, clinical data has shown evidence of the correlation between adiponectin gene +45 and +276G SNPs with obesity, type 2 diabetes, insulin resistance, cardiovascular disease, and hypertension (Mousavinasab et al., 2006; Loos et al., 2007; Yang et al., 2007; Doolley et al., 2008). Adiponectin exerts its beneficial cardiovascular influence via targeting many cell types and inducing antioxidative, metabolic, anti-fibrotic, anti-apoptotic, anti-inflammatory, and vasodilator activities (Xu et al., 2010; Hui et al., 2011). The concept of adiponectin resistance, potentially due to changes in expression of AdipoRs and APPL1/2, may be an underestimated factor in the development of CVD (Lau et al., 2011).

ADDITIONAL DISEASE PROCESSES RELATED TO CHANGES IN ADIPONECTIN LEVELS

An extremely wide variety of physiological processes have been found to be regulated by adiponectin and, as a consequence, changes in circulating adiponectin have been implicated in various clinical settings (Kadowaki et al., 2008; Wang and Scherer, 2008; Yamauchi and Kadowaki, 2008; Shetty et al., 2009; Brochu-Gaudreau et al., 2010; Chiarugi and Fiaschi, 2010). Correlations have been established between changes in circulating adiponectin levels and cancer (Kelesidis et al., 2006; Jarde et al., 2011; Paz-Filho et al., 2011), hepatic fibrosis (Dogru et al., 2010), reproductive events (Michalakis and Segars, 2010), bone mass density (BMD; Napoli et al., 2010), and inflammation (Fantuzzi, 2008). Some of these are discussed in more detail below.

ADIPONECTIN PHYSIOLOGY AND EVIDENCE FOR CONTRIBUTION OF ENDOCRINE AND LOCAL EFFECTS

SKELETAL MUSCLE

Adiponectin has clearly been shown to regulate glucose and fatty acid metabolism in skeletal muscle, principally via studies which have used animal models with enhanced or suppressed circulating adiponectin or used recombinant forms of the protein to treat cells *in vitro* or skeletal muscle *ex vivo*. Cell based *in vitro* studies show adiponectin can increase both basal and insulin-stimulated glucose uptake by promoting GLUT4 translocation to the cell membrane (Ceddia et al., 2005; Fang et al., 2005, 2009; Mao et al., 2006a) and increase fatty acid uptake and oxidation (Tomas et al., 2002; Yoon et al., 2006) through the activation of AMPK, p38-MAPK, and PPAR α pathways (Yamauchi et al., 2002, 2003b; Yoon et al., 2006). Animal model studies *in vivo* correlate well with these observations as systemic infusion, adenoviral-based delivery or genetic overexpression of adiponectin can successfully correct high-fat diet-induced insulin resistance in skeletal muscle and decrease serum TG and FFA levels (Yamauchi et al., 2001, 2005; Maeda et al., 2002; Combs et al., 2004). Although it is very well accepted that adiponectin mediates beneficial metabolic effects in skeletal muscle, the precise underlying molecular mechanisms were uncovered in more detail recently. Adiponectin can increase skeletal muscle mitochondrial mass and oxidative capacity, at least in part via inducing extracellular Ca^{2+} influx and subsequently activating the Ca^{2+} /calmodulin-dependent protein kinase beta (CaMKK β)-AMPK-Sirt1-*peroxisome proliferator-activated receptor gamma coactivator-1alpha* (PGC1 α) pathway (Iwabuchi et al., 2010). Previous studies also showed transgenic mice overexpressing adiponectin had improved insulin sensitivity whereas adiponectin knockout mice exhibit some degree of insulin resistance and decreased expression of PGC1 α and PPAR γ (Civitarese et al., 2006; Kadowaki et al., 2006). Most recently, Scherer's group identified another important mechanism underlying adiponectin's beneficial metabolic effects, namely enhanced ceramide catabolism in skeletal muscle (Holland et al., 2011).

The prevailing assumption was that the metabolic effects of adiponectin in skeletal muscle were due to endocrine effects of adipocyte-derived adiponectin (Figure 2). Importantly, emerging evidence (Delaigle et al., 2004, 2006; Krause et al., 2008; Liu et al., 2009; Amin et al., 2010; Jortay et al., 2010; Van Berendoncks et al., 2010) suggests that adiponectin can also be expressed and secreted by skeletal muscle and thus may also be classified as a myokine (Pedersen and Febbraio, 2008) which exerts its effect locally. We have shown an increased level of adiponectin mRNA, intracellular and secreted protein in response to rosiglitazone treatment *in vitro*; and subsequently verified that this skeletal muscle produced adiponectin exerted functional metabolic effects including enhanced insulin-stimulated Akt phosphorylation and glucose uptake (Liu et al., 2009). In agreement with this, Leff's group also demonstrated PPAR γ -mediated skeletal muscle adiponectin production which mediated autocrine effects to improve insulin sensitivity and could protect against high-fat diet-induced insulin resistance *in vivo* (Amin et al., 2010). It has been suggested that skeletal muscle adiponectin content increases in response to certain inflammatory conditions and obesity in an attempt at providing local anti-inflammatory and antioxidative

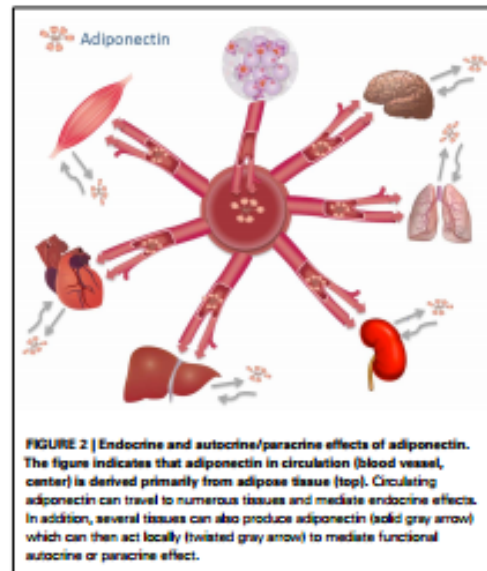


FIGURE 2 | Endocrine and autocrine/paracrine effects of adiponectin. The figure indicates that adiponectin in circulation (blood vessel, center) is derived primarily from adipose tissue (top). Circulating adiponectin can travel to numerous tissues and mediate endocrine effects. In addition, several tissues can also produce adiponectin (solid gray arrow) which can then act locally (twisted gray arrow) to mediate functional autocrine or paracrine effect.

protection (Delaigle et al., 2004, 2006; Jortay et al., 2010). Indeed, the globular form of adiponectin mediates potent metabolic effects in skeletal muscle (Fruebis et al., 2001; Tomas et al., 2002; Ceddia et al., 2005; Chen et al., 2005; Fang et al., 2005; Mao et al., 2006a) and it is conceivable that elevated local amounts of gAd are produced in inflamed tissue by elastase enzyme derived from infiltrating inflammatory cells (Waki et al., 2005). Finally, autocrine effects of gAd have recently been identified in the regulation of skeletal muscle cell differentiation (Fiaschi et al., 2009, 2010).

Local effects of adiponectin may also be determined by changes in expression of its receptor isoforms and signaling intermediates, although relatively little is known on this topic to date. Weight loss induced by either exercise or diet together with exercise enhance the expression of adiponectin receptor mRNA in skeletal muscle of humans and animal models (Vu et al., 2007; Christiansen et al., 2010). A study in non-diabetic Mexican Americans with or without a family history of Type 2 diabetes concluded that skeletal muscle expression levels of both AdipoR1 and AdipoR2 correlated positively with insulin sensitivity (Civitarese et al., 2004). Hyperglycemia and hyperinsulinemia can both alter AdipoR expression in muscle cells and consequently adiponectin sensitivity (Fang et al., 2005). Although it is generally believed that enhancing AdipoR-APPL1 signaling is beneficial (Mao et al., 2006a; Cheng et al., 2007, 2009; Saito et al., 2007; Zhou et al., 2009; Fang et al., 2010; Cleasby et al., 2011), recent studies have identified that the abundance of APPL1 mRNA is significantly higher in muscle of type 2 diabetic individuals (Holmes et al., 2011). Bariatric surgery corrected hyperglycemia and this was correlated with increased circulating adiponectin and skeletal muscle AdipoR1 expression with reduced APPL1 content (Holmes et al., 2011).

CARDIOVASCULAR

As described above, many studies have established correlations between circulating adiponectin levels and various cardiovascular outcomes and the underlying mechanisms are now well understood. For example, adiponectin is now established as a cardio-protective adipokine as it mediates beneficial effects on cardiac remodeling events such as energy metabolism, hypertrophy, fibrosis, and apoptosis (Shibata et al., 2004, 2005, 2007; Liao et al., 2005; Palanivel et al., 2007; Fang et al., 2010; Li et al., 2010; Tao et al., 2010; Shimano et al., 2011). Anti-inflammatory, vasodilator, and anti-atherosclerotic effects confer further beneficial influences of adiponectin on the cardiovascular system (Fantuzzi, 2008; Brochu-Gaudreau et al., 2010; Xu et al., 2010; Hui et al., 2011).

Mice lacking adiponectin have been particularly informative in establishing the cardioprotective role of adiponectin, with numerous studies in these mice demonstrating an exaggerated response of the heart to cardiac stress (Shibata et al., 2004, 2005, 2007; Liao et al., 2005; Li et al., 2010; Tao et al., 2010; Shimano et al., 2011), which was attenuated upon restoration of circulating adiponectin. The ability of adiponectin to counteract deterioration in cardiac function was mediated by metabolic, anti-apoptotic, anti-fibrotic, and anti-hypertrophic effects (Shibata et al., 2004, 2005, 2007; Tao et al., 2007; Wang et al., 2009b; Fang et al., 2011; Park et al., 2011). Adiponectin has been shown to regulate fatty acid β -oxidation in the heart. In cell based *in vitro* studies of isolated cardiomyocytes, adiponectin was shown to stimulate the phosphorylation of AMPK, IRS1, and Akt (T308 and S473) correlating with the regulation of glucose and fatty acid uptake and metabolism (Palanivel et al., 2007), and to target cofilin to mediate remodeling of the actin cytoskeleton leading to the translocation of lipoprotein lipase (LPL) to the cell surface (Ganguly et al., 2011). Adiponectin was also shown to stimulate the phosphorylation of acetyl coenzyme A carboxylase (ACC), as well as to induce CPT-1 expression and activation through AMPK (Li et al., 2007a). Recently (Fang et al., 2010), we demonstrated that adiponectin increases fatty acid uptake, CD36 translocation, and insulin-stimulated glucose transport as well as Akt phosphorylation in isolated adult cardiomyocytes, and enhances fatty acid oxidation in conjunction with AMPK and ACC phosphorylation in the isolated working heart. However, despite an increase in fatty acid oxidation and myocardial oxygen consumption, adiponectin increased hydraulic work, and maintained cardiac efficiency (Fang et al., 2010).

The phosphorylation of AMPK was shown to attenuate norepinephrine induced cardiomyocyte hypertrophy and ERK phosphorylation (Shibata et al., 2004), angiotensin II induced NF- κ B activation and hypertrophy (Wang et al., 2011), and also shown to fully (Shibata et al., 2005), or minimally (Wang et al., 2009a) attenuate hypoxia-reoxygenation induced apoptosis. Adiponectin was shown to attenuate hypoxia-reoxygenation induced apoptosis in H9C2 cells through the AdipoR1/APPL1 signaling pathway (Park et al., 2011). Cardiac fibrosis is associated with impaired cardiac function, and there are numerous studies demonstrating the exaggerated fibrotic response of the heart to cardiac stress in the absence of adiponectin (Shibata et al., 2004, 2005, 2007; Liao et al., 2005; Li et al., 2010; Tao et al., 2010; Shimano et al., 2011). Very

few studies have directly investigated regulation of extracellular matrix components by adiponectin *in vitro*. Cardiac fibroblasts express AdipoR1 (Huang et al., 2009) and treatment of adult rat fibroblasts with gAd was shown to increase IL-6 expression and secretion via the activation of AMPK, p38-MAPK, and ERK1/2 (Fan et al., 2011).

Although an overwhelming amount of data indicates numerous beneficial effects of adiponectin, there is also some contradictory evidence from clinical and experimental studies on the cardioprotective role of adiponectin. For example, recent clinical data have positively correlated high levels of adiponectin with mortality and severity in patients with congestive heart failure (Shimura, 2010). Adiponectin knockout mice subjected to long-term pressure overload suggested that under chronic stress, adiponectin deficiency preserves oxidative capacity and cardiac function despite an increase in cardiac hypertrophy compared to wild-type mice, suggesting that adiponectin may in fact be playing a permissive role in long-term cardiac dysfunction (O'Shea et al., 2010).

Both cardiomyocytes (Pineiro et al., 2005; Ding et al., 2007) and epicardial adipose tissue (Gormez et al., 2011; Hirata et al., 2011) can produce adiponectin and thus increase autocrine/paracrine bioavailability. Indeed, epicardial adipose-derived adiponectin was recently identified as a predictor of positive outcome following cardiac surgery (Kourliouros et al., 2011), although it should be noted that expression of adiponectin from epicardial adipose tissue has been shown to be lower than that from subcutaneous adipose tissue (Bambace et al., 2011). Similarly, cardiomyocytes produce relatively small amounts of adiponectin (Pineiro et al., 2005; Ding et al., 2007), yet these are likely to be sufficient for locally mediated effects. A number of studies have found that cardiac adiponectin levels are altered in various cardiomyopathies. For example, a recent study has shown in patients with dilated cardiomyopathy that adiponectin expression was decreased sixfold and this was mirrored in immunohistochemical analysis of endomyocardial biopsies (Skurk et al., 2008). Additionally, the accumulation of adiponectin within the myocardial tissue following stress through leakage from the vascular compartment could also serve to increase the local supply of bioavailable adiponectin and serve to compensate for the inflammatory induced downregulation of both local and systemic adiponectin expression (Ouchi et al., 2000; Shibata et al., 2007; Fujita et al., 2008). Interestingly, adiponectin has also been shown to accumulate in atherosclerotic plaques and whether this is causative or protective against progression of atherosclerosis is still incompletely resolved, although the latter seems most likely (Li et al., 2007b; Cai et al., 2010; Reynolds et al., 2010).

Myocardial adiponectin resistance (Saito et al., 2007; Kollias et al., 2011; Ma et al., 2011) may necessarily be the first target in developing adiponectin-based therapeutics in the treatment of cardiovascular disease. Overall, circulating or local adiponectin levels tend to correlate negatively with cardiovascular disease incidence and prognosis, however since many cardiovascular events are progressive in nature there may in some cases be a temporal compensatory increase in adiponectin expression, particularly within the affected tissue. Thus, several paradoxical observations

have been reported in the literature and it is likely that the timing of targeting adiponectin therapeutically will be vital to its success in the cardiovascular arena (Dembinski, 2010).

LIVER

Regulation of hepatic glucose and fatty acid metabolism plays an important role in the ability of adiponectin to improve whole body energy homeostasis (Kadowaki and Yamauchi, 2005; Fang and Sweeney, 2006; Kadowaki et al., 2007). For example, low levels or defects in adiponectin action correlate with steatosis, hepatomegaly, and local inflammation associated with various liver diseases. The intracellular signaling mechanisms via which adiponectin mediates effects in hepatocytes are similar to those in muscle (Wang et al., 2009c), with one apparent exception being a more important role of the AdipoR2 isoform in mediating the effects of adiponectin in liver (Yamauchi et al., 2003b, 2007; Yamauchi and Kadowaki, 2008). Adiponectin is known to exert its effects in the liver primarily through the activation of the AMPK and PPAR α pathways. In addition to the well characterized insulin-sensitizing and insulin-like effects of adiponectin in the liver, low serum adiponectin levels have been associated with high TNF α levels and the presence of non-alcoholic fatty liver disease (NAFLD) independent of insulin resistance (Hui et al., 2004; Aygun et al., 2006; Polyzos et al., 2011). In particular, adiponectin has been shown to mediate anti-fibrotic effects through the activation of AMPK in hepatic stellate cells (Adachi and Brenner, 2008), anti-apoptotic effects via PI3K and AMPK activation in hepatocytes (Jung et al., 2009), and to be anti-inflammatory through the inhibition of TNF α induced hepatotoxicity (Sennello et al., 2005). A role for T-cadherin has also been proposed in mediating the effects of adiponectin on liver fibrosis (Asada et al., 2007). Furthermore, circulating adiponectin levels have been found to be downregulated in morbidly obese patients with non-alcoholic steatohepatitis (NASH) compared to individuals with simple steatosis (Uribe et al., 2008; Ma et al., 2009), while a paradoxical increase in serum adiponectin levels were detected in patients with cirrhosis, independent of insulin resistance (Tietge et al., 2004; Kaser et al., 2005). It is worth bearing in mind that increased adiponectin levels in liver cirrhosis may reflect reduced hepatic clearance of adiponectin and/or a compensatory increase toward the overwhelming production of proinflammatory cytokines in cirrhosis. Thus, a potentially detrimental contribution of adiponectin as NAFLD progresses to cirrhosis must be considered (Polyzos et al., 2010).

Hepatic AdipoR1 and AdipoR2 mRNA expression levels were higher in insulin-resistant subjects, perhaps reflecting a compensatory mechanism for reduced plasma adiponectin (Felder et al., 2010). Liver fibrosis in individuals infected with hepatitis C virus was associated with hyperadiponectinemia and, interestingly, reduced AdipoR1 expression (Corbetta et al., 2011). A study in humans showed no change in AdipoR expression in patients with NASH (Uribe et al., 2008), but an important role for changes in AdipoR expression was also shown in a study using a high-fat and high-cholesterol diet in obese fa/fa Zucker rats to induce NASH was associated with decreased AdipoR1 and AdipoR2 expression (Matsumami et al., 2011). In addition, liver expression and localization of adiponectin were increased in wild-type mice in response

to carbon tetrachloride induced hepatofibrosis (Yoda-Murakami et al., 2001).

LUNG

Respiratory complications are often observed in obese individuals (Ford, 2005; Shore, 2010). Importantly, decreased serum adiponectin levels correlate with poor lung function in asthma and chronic obstructive pulmonary disease (COPD, independent of adiposity (Sood et al., 2008; Stanciu et al., 2009; Sutherland et al., 2009; Chan et al., 2010; Thyagarajan et al., 2010). Indeed, clinical treatment of COPD with corticosteroids and antibiotics improved lung function concomitant with elevated circulating adiponectin levels and a decrease in systemic inflammatory markers such as IL-6 and TNF α (Krommidas et al., 2010). Note, it is possible that the increased adiponectin levels may result from diminished IL-6 and TNF α after treatment. Adiponectin knockout mice exhibited progressive alveolar enlargement and endothelial cell apoptosis, which was attenuated by adenoviral administration of adiponectin (Nakanishi et al., 2011). Continuous infusion of adiponectin via subcutaneously implanted osmotic pumps to replenish decreased levels was found to attenuate ovalbumin-induced airway inflammation in mice through the attenuation of inflammatory cell influx, corresponding with a reduction in IL-13 and IL-5 (Shore et al., 2006). Furthermore, chronic allergic airway inflammation and pulmonary vascular remodeling are also exacerbated in adiponectin deficient mice (Medoff et al., 2009; Nakagawa et al., 2009; Summer et al., 2009). Nevertheless, a double-blind randomized clinical trial found that asthmatic patients exhibited only a modestly beneficial effect in the late asthmatic response to inhaled allergen challenge after 28 days of rosiglitazone treatment to increase serum adiponectin levels (Richards et al., 2010).

Although the studies described above focused on endocrine effects of adiponectin, it is again important to consider the potential effects of locally produced adiponectin in the lung. Adiponectin was found to be localized to the murine pulmonary vascular endothelium under normal (Summer et al., 2009) or hypoxic (Nakagawa et al., 2009) conditions and bronchoalveolar fluid contained low levels of adiponectin (Summer et al., 2008). Adiponectin was overexpressed in the bronchoalveolar lavage (BAL) fluid of COPD patients and in a multimeric distribution profile differing from that found in serum (Zhu et al., 2010). Interestingly, these findings correlated with the increased localization of AdipoR1 to the airway epithelial cells of COPD patients (Miller et al., 2009).

OTHERS

Numerous effects of adiponectin have been established in other peripheral tissues (Kadowaki et al., 2008), besides the obvious autocrine effects on adipocytes themselves (Wu et al., 2003). For example, the longstanding complication of nephropathy in obesity and diabetes has naturally led to studies on the pathophysiological role of adiponectin in this process (Chen et al., 2004; Srivastava, 2006; Stenvinkel, 2011). Increased circulating adiponectin levels are found in predialysis patients with end stage renal disease (ESRD; Shen et al., 2007; Stenvinkel, 2011) and adiponectin suggested to be a predictive factor for the progression of chronic kidney disease in men (Kollerits et al., 2007). General consensus

based on available literature indicates that adiponectin is renoprotective (Abe et al., 2010), for example via attenuating pathological progression toward renal fibrosis and glomerular hypertrophy (Ohashi et al., 2007). Even in the absence of a stressor, adiponectin deficient mice exhibited segmentally fused podocyte processes, increased albumin leakage into the urine (albuminuria), and kidney oxidant stress when compared to wild-type controls, while treatment with adiponectin reduced the degree of albumin permeability of a podocyte monolayer *in vitro* (Sharma et al., 2008).

Obesity is strongly associated with increased BMD due not only to the increased mechanical load, but also to adipocyte-derived hormonal factors mediating the cross-talk between adipocytes and bone (Confavreux et al., 2009). As such, there is accumulating and contradictory evidence indicating that adiponectin plays a role in bone maintenance and metabolism (Lenchik et al., 2003; Bozic et al., 2010; Barbour et al., 2011). Specifically, *in vitro*, adiponectin has been found to decrease osteoclast differentiation and bone-resorption activity (Oshima et al., 2005) via APPL1-mediated Akt1 suppression (Tu et al., 2011), while increasing osteoblast proliferation and differentiation via an AdipoR1 dependent p38-MAPK/JNK signaling pathway (Luo et al., 2005), suggesting that adiponectin positively influences bone growth. However, additional studies in adiponectin deficient or hyperadiponectinemic examining bone mass and fragility yielded some paradoxical observations (Oshima et al., 2005; Williams et al., 2009; Mitsui et al., 2011). Indeed, another study reported no abnormality in bone mass or turnover in adiponectin knockout mice or adiponectin transgenic mice overexpressing globular adiponectin (Shinoda et al., 2006). Interestingly, adiponectin has been found to be expressed by bone forming osteoblasts (Bermer et al., 2004; Shinoda et al., 2006) indicating a potential complex autocrine, paracrine, and endocrine role of adiponectin in mediating bone density.

Adiponectin also has centrally mediated effects, such as regulation of food intake and energy expenditure (Qi et al., 2004; Kadowaki et al., 2008). Although adiponectin was reportedly unable to cross the blood brain barrier (Spranger et al., 2006), it was found in the cerebrospinal fluid of rats (Qi et al., 2004) and humans (Neumeier et al., 2007) although at significantly lower levels and with different oligomeric profile than that in peripheral circulation (Ebinuma et al., 2007). Adiponectin mRNA expression and localization within the CNS has now been shown (Rodríguez-Pacheco et al., 2007; Psilopanagioti et al., 2009). Several studies have documented the functionality of adiponectin (Rodríguez-Pacheco et al., 2007) *in vitro*, and through

intracerebral injection (Hoyda et al., 2009a; Iwama et al., 2009; Park et al., 2011), and also central expression of adiponectin receptors (Hoyda et al., 2009b).

ADIPONECTIN ACTION AS A THERAPEUTIC TARGET

The rationale for targeting adiponectin is based on the well documented beneficial physiological actions of adiponectin spanning diabetes, inflammation, cardiovascular diseases, and cancer and it is expected that studies in animal models will translate well to human physiology in the case of adiponectin (Mao et al., 2006b; Zhu et al., 2008; Shetty et al., 2009; Wang et al., 2009; Marette and Sweeney, 2011). Adiponectin-based therapeutics would have potentially wide-ranging applications in markets with widespread demographics. In diabetes alone there are still significant unmet therapeutic needs despite an annual global market value of around \$30 billion. Synthesis and administration of recombinant forms of adiponectin is generally not a viable therapeutic approach due to the cost of synthesizing correctly posttranslationally modified bioactive forms and the disadvantage of the route of administration, although the recombinant globular domain of adiponectin is in pre-clinical trials for Merck and Proteomix have a highly glycosylated form of adiponectin in pre-clinical trials. There are several reports of compounds which increase adiponectin expression and secretion, although these increases tend to be very modest at non-supra-physiological doses (Zhu et al., 2008). The commonly prescribed TZD class of anti-diabetic agents act at least in part via elevating adiponectin (Pajvani et al., 2004; Kubota et al., 2006; Li et al., 2010). A more attractive therapeutic option would be the discovery of small molecule compounds which mimic or enhance adiponectin action. Presently, a small molecule adiponectin-mimetic developed by Rigel Pharmaceuticals that improves insulin sensitivity in a diabetic mouse model is in pre-clinical development and Kadowaki's group have also identified such a compound. However there are no potent and specific adiponectin-based therapeutics which are clinically available yet. Based on the information in this review article it is interesting to consider the future possibility of combining adiponectin-based therapeutics with tissue- or cell-specific delivery approaches.

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