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Jaana Rysä

GENE EXPRESSION PROFILING IN EXPERIMENTAL MODELS OF CARDIAC LOAD

FACULTY OF MEDICINE, INSTITUTE OF BIOMEDICINE,
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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in the Auditorium of the Department of Pharmacology and Toxicology, on April 11th, 2008, at 12 noon

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Abstract

Cardiac hypertrophy provides an adaptive mechanism to maintain cardiac output in response to increased workload, and although initially beneficial, hypertrophy eventually leads to heart failure, a major cause of morbidity and mortality in Western countries. The hypertrophic response in cardiac myocytes is accompanied by e.g. activation of signal transduction pathways, such as mitogen-activated protein kinases (MAPKs), and complex changes in gene programming. The purpose of this study was to characterize gene expression patterns in experimental models of cardiac load by using high-throughput DNA microarray technologies.

In the present study, changes in gene expression were evaluated in response to acute pressure overload and prolonged hypertension as well as during the development of left ventricular hypertrophy (LVH) and the transition to diastolic heart failure in an animal model of genetic hypertension, the spontaneously hypertensive rat (SHR). Increased expression of several immediate early genes was seen in response to acute hemodynamic overload *in vivo*. The transition from LVH to diastolic hypertensive heart failure was almost exclusively associated with changes in genes encoding extracellular matrix proteins and their regulatory processes showing the importance of progressive extracellular matrix remodeling.

The effect of p38 MAPK activation on gene expression patterns *in vivo* was elucidated. Cardiac-specific overexpression of p38 MAPK resulted in upregulation of genes controlling cell division and inflammation as well as cell signaling and adhesion. Accordingly, the functional role of p38 MAPK was related to myocardial cell proliferation, inflammation and fibrosis.

Finally, temporal analysis of mechanical stretch induced gene expression changes in neonatal rat cardiomyocyte cultures *in vitro* indicated that mechanical stretch induced complex gene expression profiles, demonstrating that both positive and negative regulators are involved in the hypertrophic process. Many novel stretch responsive genes were identified, and a subset of them may be putative downstream targets of p38 MAPK.

In conclusion, in the present study a number of well-established gene expression changes of cardiac hypertrophy were observed and novel modulators associated with increased cardiac load, such as thrombospondin-4, were identified. The study provides a better understanding of molecular mechanisms associated with increased cardiac load, and may indicate potential targets for novel therapeutic interventions.

Keywords: diastolic heart failure, DNA microarrays, hypertrophy, mitogen-activated protein kinases, stretch

"The temptation to form premature theories upon insufficient data is the bane of our profession."

- Sherlock Holmes, in "The Valley of Fear" by Sir Arthur Conan Doyle

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Abbreviations

AM	adrenomedullin
Ang II	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AP-1	activator protein-1
ATX-R	angiotensin II receptor subtype
ATF	activating transcription factor
AVP	arginine^{8}-vasopressin
BMP	bone morphogenetic protein
BNP	B-type natriuretic peptide
BW	body weight
Caα-A	cardiac α-actin
CaCNL1α1	L-type calcium channel α1c-subtype
CaMK	calcium/calmodulin-dependent kinase
CDK	cyclin-dependent kinase
CDKN	cyclin-dependent kinase inhibitor
cGMP	cyclic guanosine 3',5'-cyclic monophosphate
COL	collagen
COX	cyclo-oxygenase
CPT	carnitine palmitoyl transferase
CT	cardiotropin
CTGF	connective tissue growth factor
cDNA	complementary deoxyribonucleic acid
DAG	diacylglycerol
E/A	peak flow velocity of the early rapid diastolic filling wave (E) to late diastolic filling wave (A)
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal regulated kinase
EST	expressed sequence tag
ET	endothelin
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GADD	growth arrest and DNA damage-inducible gene
GC-A	guanylate cyclase A

G-protein	guanine nucleotide binding protein
GPCR	G protein-coupled protein
GSK	glycogen synthase kinase
HAT	histone acetylase
HDAC	histone deacetylase
Hsp	heat shock protein
IGF	insulin-like growth factor
IKβ	inhibitory κB
IKK	inhibitory κB kinase
IL	interleukin
i.p.	intraperitoneal
IP3	inositol-1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
LacZ	*Escherichia coli* β-galactosidase
LAD	left anterior descending coronary artery
LOXL	lysyl oxidase-like protein
LSD	least significant difference
LV	left ventricle
LVAD	left ventricular assist device
LVEDD	left ventricular end-diastolic dimension
LVEF	left ventricular ejection fraction
LVESD	left ventricular end-systolic dimension
LVFS	left ventricular fractional shortening
LVH	left ventricular hypertrophy
LVW/BW	left ventricular weight to body weight
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MAPKKKK	mitogen-activated protein kinase kinase kinase kinase
MEF	myocyte enhancer factor
MEK	MAPK of ERK kinase
MEKK	MAPK of ERK kinase kinase
MGP	matrix Gla protein
MHC	myosin heavy chain
MIAME	minimal information about a microarray experiment
MKK	MAP kinase kinase

MLC	myosin light chain
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NCX	sodium-calcium exchanger
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NIK	nuclear factor κB-inducing kinase
PAI	plasminogen activator inhibitor
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PDGF	platelet-derived growth factor
PDK	phosphoinositol-dependent kinase
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PKG	cGMP-dependent protein kinase
PLA	phospholipase A
PLC	phospholipase C
PE	phenylephrine
Pol	polymerase
REST	repressor element-1 silencing transcription factor
RTK	tyrosine kinase receptor
RT-PCR	reverse transcriptase PCR
Runx	runt related transcription factor
s.c.	subcutaneous
SD	Sprague-Dawley
SEM	standard error of means
Serca	sarco/endoplasmic reticulum Ca^{2+}-ATPase
Skα-A	skeletal α-actin
SHR	spontaneously hypertensive rat
SNP	single nucleotide polymorphism
SRF	serum response factor
TAC	transverse aortic constriction
TAK	transforming factor-β activating kinase
TGFβ	transforming factor-β
TGFR	transforming factor receptor
TGR	transgenic rat

TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TSP	thrombospondin
UCP	uncoupling protein
UPS	ubiquitin proteosome system
UTR	untranslated region
VEGF	vascular endothelial growth factor
WKY	Wistar Kyoto
WT	wild type
18S	ribosomal 18S

α	alpha
β	beta
ε	epsilon
γ	gamma
κ	kappa

List of original articles

The thesis is based on the following articles, which are referred to in the text by Roman numerals:

I Rysä J, Aro J & Ruskoaho H (2006) Early left ventricular gene expression profile in response to increase in blood pressure. Blood Pressure 15: 375-383.

II Rysä J, Leskinen H, Ilves M & Ruskoaho H (2005) Distinct upregulation of extracellular matrix genes in transition from hypertrophy to hypertensive heart failure. Hypertension 45: 927-933.

III Tenhunen O*, Rysä J*, Ilves M, Soini Y, Ruskoaho H & Leskinen H (2006) Identification of cell cycle regulatory and inflammatory genes as predominant targets of p38 MAPK in the heart. Circ Res 99: 485-93.

IV Rysä J, Tokola H, Aro J, Ilves M, Vuoristo J & Ruskoaho H. Mechanical stretch induced gene expression patterns in cardiac myocytes *in vitro*. Manuscript.

*Equal contribution

Contents

1 Introduction

Heart failure is one of the most important contributors to cardiovascular morbidity and mortality in the Western world (Jessup & Brozena 2003). It can result either from systolic dysfunction, as a result of inadequate pumping activity, or from diastolic dysfunction, which is due to impaired relaxation and therefore improper blood filling (Kostis 2003). Hypertension and myocardial infarction are the two major causes of heart failure. Cardiac hypertrophy provides an adaptive mechanism to maintain cardiac output in response to increased workload, and although initially beneficial, left ventricular hypertrophy (LVH) is an independent risk factor for coronary heart disease, sudden death, heart failure and stroke (Jessup & Brozena 2003, Kostis 2003, Mann & Bristow 2005).

The pathogenesis of cardiac hypertrophy is multifactorial and involves interactions of different cell types including cardiac myocytes, smooth muscle cells, endothelial cells and fibroblasts. Although cardiac myocytes make up only one-third of the total cell number, they account for up to 90% of the cell mass (Jugdutt 2003). Cardiac myocytes have lost their ability to proliferate and growth occurs primarily as a result of an increase in myocyte size (hypertrophy). The hypertrophic response in cardiac myocytes is characterized by morphologic changes that include increase in cell size and protein synthesis, enhanced sarcomeric reorganization as well as changes in gene and protein expression and/or activation of various genes and signal transduction pathways (Heineke & Molkentin 2006, Lorell & Carabello 2000). Among the complex network of parallel signal transduction pathways, mitogen-activated protein kinases (MAPKs) represent one of the central convergence points, since MAPKs are activated by a variety of stimuli and are able to activate diverse cellular processes (Kyriakis & Avruch 2001, Sugden & Clerk 1998b, Wang 2007).

The early genetic response to hemodynamic overload is characterized by the activation of immediate early response genes such as proto-oncogenes *c-fos*, *c-myc*, *c-jun* and *Egr-1* and reactivation of a fetal gene program including atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) (Izumo *et al.* 1988, Magga *et al.* 1994, Ruskoaho *et al.* 1989). This is further accompanied by a subtype switch in contractile protein gene expression such as α-myosin heavy chain (α-MHC) to β-MHC or cardiac muscle α-actin (caα-A) to skeletal muscle α-actin (skα-A) (Sadoshima & Izumo 1997, Sugden & Clerk 1998b, Tokola *et al.* 2001). In addition, the altered expression of genes encoding ion channels, growth factors and metabolic enzymes are well-characterized changes during the

development of cardiac hypertrophy and heart failure (Sadoshima & Izumo 1997, Swynghedauw 1999, Taegtmeyer *et al.* 2005).

Advances in molecular genetics and computational biology have led to the development of high-throughput microarray technologies that can be used to survey genome composition as well as gene expression and regulation on a genomic scale (Trevino *et al.* 2007). Genome-wide expression profiles of experimental models of cardiac hypertrophy as well as human heart failure have been analyzed (Ashrafian & Watkins 2007, Donahue *et al.* 2006, Liew & Dzau 2004, Sharma *et al.* 2005). These studies have identified several novel genes involved in cardiac hypertrophy and heart failure providing new insights into the pathogenesis of these complex diseases.

The aim of the present study was to analyze gene expression patterns in experimental models of cardiac load. More specifically, changes in gene expression were evaluated in response to acute pressure overload as well as to prolonged hypertension. In addition, p38 MAPK dependent gene expression patterns as well as temporal changes in gene expression during mechanical stretch induced hypertrophy were elucidated.

2 Review of the literature

2.1 Systolic and diastolic heart failure

Heart failure is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill or eject blood. Valvular heart disease such as aortic valve stenosis as well as cardiomyopathies caused by genetic and environmental factors can cause heart failure, but in most cases heart failure develops as a result of either prolonged hypertension or coronary artery disease with myocardial infarction (Fig. 1). Heart failure is a continuous and progressive disease, but in the early stages left ventricular structure and function is typically normal. Over time, the pathologic effects of one or more cardiovascular risk factors such as hypertension, obesity, diabetes, smoking, and dyslipidemia may cause the development of left ventricular hypertrophy or myocardial infarction (or both), leading to left ventricular dysfunction and finally to heart failure (Jessup & Brozena 2003, Kostis 2003, Mann & Bristow 2005). The prognosis of heart failure is poor, an annual mortality rate being 10% (Cleland *et al.* 2005).

Heart failure may be due to either left ventricular systolic or diastolic dysfunction. The principal hallmark of patients with predominant systolic dysfunction is a depressed left ventricular ejection fraction (LVEF). When the symptoms and signs of heart failure are accompanied by a preserved LVEF and a predominant abnormality in diastolic function, this clinical syndrome is called diastolic heart failure (or heart failure with preserved ejection fraction). Epidemiologic studies have established that 30-60% of patients with heart failure have a normal ejection fraction (≥50%). (Kitzman *et al.* 2002, Vasan & Levy 2000, Zile *et al.* 2004.)

Systolic and diastolic heart failure have many similar cardiac characteristics, including increased high left ventricular mass, reduced contractility and interstitial fibrosis. The most important difference between them is that systolic heart failure is characterized by low LVEF with large ventricular volume, whereas diastolic heart failure is associated with normal LVEF and small ventricular volume. The underlying pathophysiological abnormalities in diastolic function are caused by a decrease in ventricular relaxation and/or an increase in ventricular stiffness. Clinically patients with diastolic heart failure are elderly, more likely to be a female, usually obese and often have hypertension associated with left

ventricular hypertrophy (LVH). (Kitzman *et al.* 2002, Kostis 2003, Zile *et al.* 2004.)

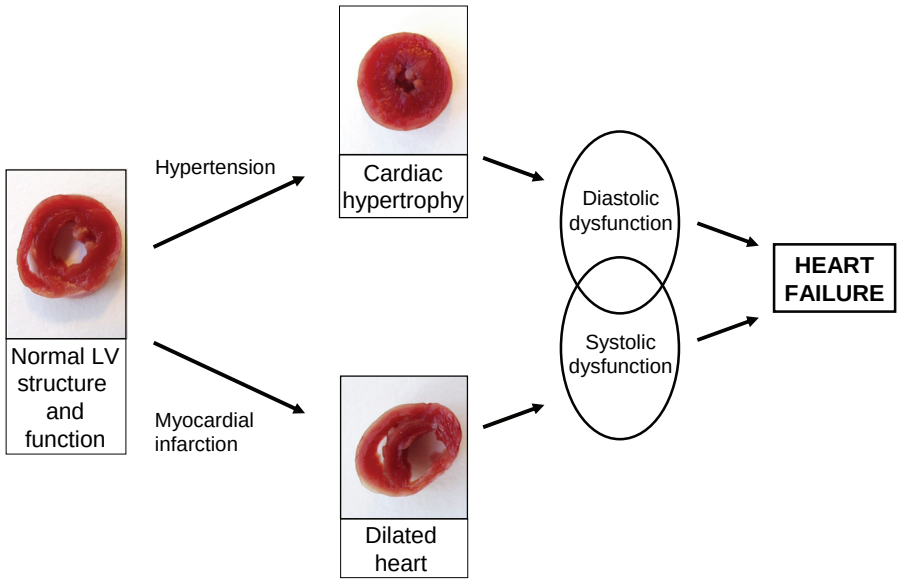

Fig. 1. The development of heart failure. Representative pictures showing control rat heart (on the left), the heart with chronic Ang II infusion (above) and the heart with myocardial infarction (below).

2.2 Cardiac hypertrophy

During embryonic development cardiac myocytes undergo primarily proliferative growth (hyperplasia). By contrast, adult cardiac myocytes are fully differentiated post-mitotic cells and increase in cell size (hypertrophy) is the main response to an increased workload (Lorell & Carabello 2000). Cardiac hypertrophy provides an adaptive response to maintain cardiac output in response to hypertrophic stimuli, which can be extrinsic or intrinsic to the cardiomyocytes. Extrinsic stimuli include pressure overload due to disease such as chronic hypertension or aortic stenosis as well as volume overload, which occurs after myocardial infarction or in aortic and mitral valve regurgitation. Intrinsic stimuli involve genetic abnormalities such as mutations in sarcomeric proteins in familial

hypertrophic cardiomyopathy (HCM). (Lips *et al.* 2003). In the early stages of cardiac hypertrophy, changes in cardiac structure compensate for increased load, whereas in later stages, the heart becomes decompensated, resulting in cardiac failure and increased morbidity and mortality (Fig. 2) (Lorell & Carabello 2000).

Cardiac hypertrophy is defined as an abnormal increase in heart mass and it is functionally, mechanistically and histologically distinguished from normal myocyte growth (Lorell & Carabello 2000). Cardiac hypertrophy appears in different phenotypes, depending on the eliciting stimulus. Hypertrophy can be classified on the basis of chamber morphology as concentric or eccentric. Eccentric hypertrophy is characterized by left ventricular dilation and decreased wall thickness, whereas, in concentric hypertrophy, the ventricular wall is thickened and the left ventricular volume is reduced. Typically, volume overload or myocardial infarction induces eccentric hypertrophy, while chronic hypertension and aortic stenosis elicit the concentric phenotype. However, increased wall stress represents the major stimulus for both phenotypes. In addition to mechanical stretch, a number of neurohumoral factors such as angiotensin II (Ang II) and endothelin-1 (ET-1) are involved in the adaptive process and modify the growth of cardiac myocytes *in vivo*. (Dorn 2007, Lips *et al.* 2003, Lorell & Carabello 2000.)

Not all forms of cardiac hypertrophy are harmful. Developmental hypertrophy and the physiological hypertrophy that occurs in response to exercise or during pregnancy provide an adaptive response without progression to a disease state. In developmental and physiological hypertrophy the growth of cardiac myocytes, and hence the ventricular wall and septum, are comparable with an increase in chamber dimension, whereas in pathological hypertrophy the ventricular wall and septum are thickened with a concomitant decrease in ventricular chamber dimension. (Dorn 2007.)

Remodeling response during cardiac hypertrophy includes increased extracellular matrix deposition, abnormalities in intracellular calcium handling and increased rate of cardiac myocyte apoptosis (Lips *et al.* 2003). At the cellular level, the hypertrophic response of cardiac myocytes is characterized by morphologic changes that include increase in cell size and protein synthesis, accumulation of sarcomeric proteins, reorganization of the myofibrillar structure and complex changes in cardiac gene expression. (Dorn 2007, Lips *et al.* 2003, Lorell & Carabello 2000.)

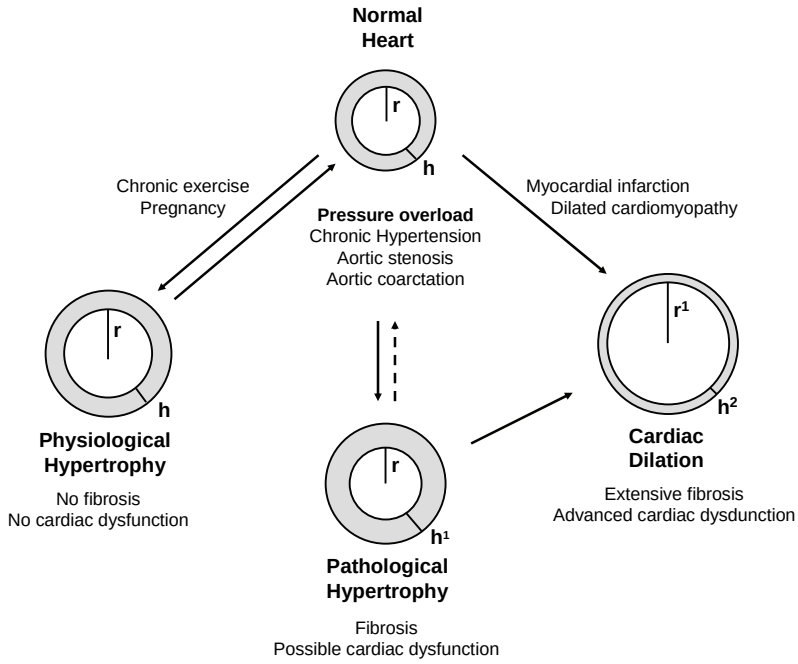

Fig. 2. Stimulus-specific hypertrophic responses of the heart and their effect on ventricular chamber dimensions. In physiological hypertrophy the internal ventricular radius (r) vs. ventricular wall thickness (h) –ratio is the same as in the normal heart. In pathological hypertrophy wall thickness is increased compared to normal heart (h^1>h), and in the dilated heart, the ventricular cavity is enlarged (r^1>r) with reduced wall thickness (h^2<h<h^1). Modified from Dorn 2007 and Diwan & Dorn 2007.

2.3 Intracellular signaling pathways in cardiac hypertrophy

Hypertrophy is initiated and maintained by several factors such as wall stretch, vasoactive peptides, peptide growth factors, hormones and neurotransmitters. Hypertrophic stimuli are able to activate a complex network of parallel signal transduction pathways that eventually lead to biological responses. A number of mediators and signaling pathways of cardiac hypertrophy have been characterized including mitogen-activated protein kinases (MAPKs) and calcineurin–nuclear factor of activated T-cells (NFAT) (Clerk *et al.* 2007, Frey & Olson 2003, Heineke & Molkentin 2006). Overview of the more extensively characterized intracellular signal transduction pathways that coordinate the cardiac hypertrophic response is provided in Figure 3.

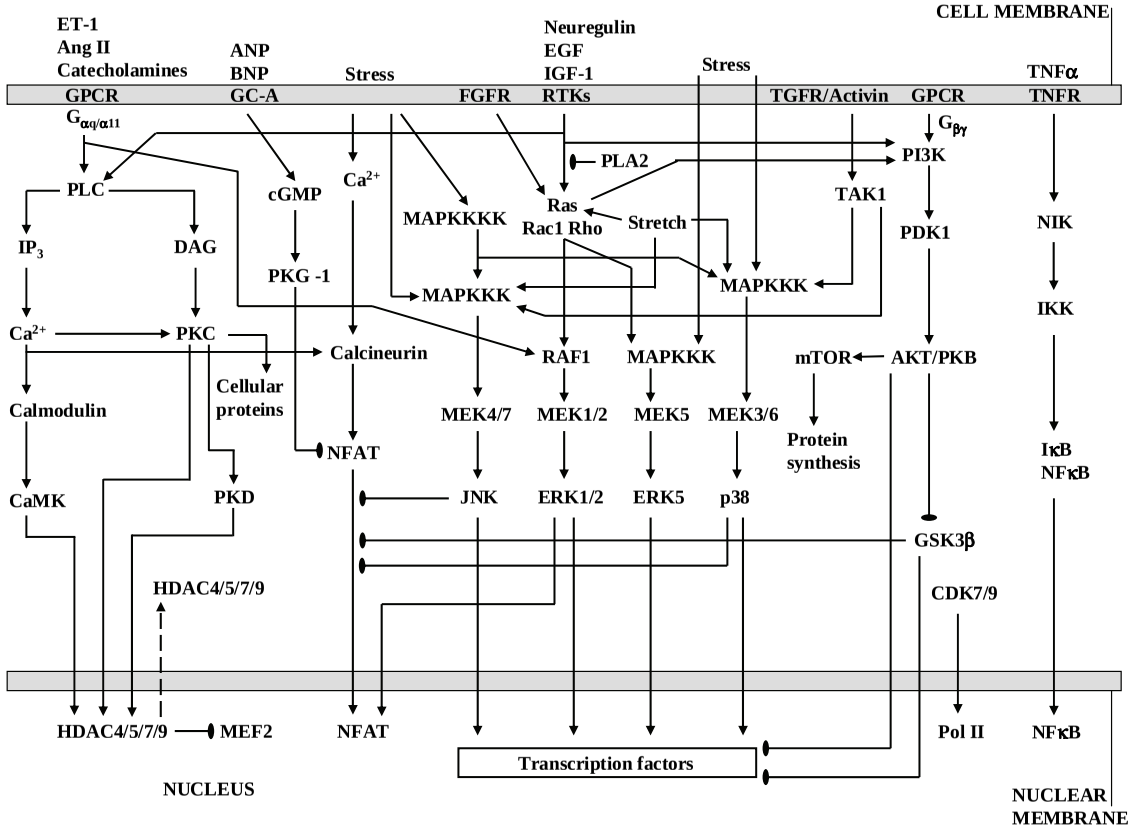

Fig. 3. Schematic view of the intracellular signal-transduction pathways that coordinate the cardiac hypertrophic response. Adapted from Heineke & Molkentin 2006.

2.3.1 Mitogen-activated protein kinases

Fourteen MAPK genes have been identified in the human genome, defining seven different MAPK signaling pathways (Coulombe & Meloche 2007). All eukaryotic cells possess multiple MAPK pathways that provide an important signal transduction system between external stimuli, the nucleus and other intracellular targets (Kyriakis & Avruch 2001). The members of the MAPK family are involved in the regulation of diverse biological processes including cell growth, differentiation, proliferation, and apoptosis. Conventional MAPKs comprise extracellular signal-regulated kinases (ERK 1/2), p38, c-Jun N-terminal kinases (JNKs) and ERK5/big MAP kinase 1 (BMK1), which are all substrates of MAPK kinases (MAPKKs, MKK or MEK). In addition, atypical MAP kinases are known, including ERK 3/4, ERK7 and Nemo-like kinase (NLK) (Coulombe & Meloche 2007).

Conventional MAPK pathways include three conserved three-kinase modules i.e. MAPK, MAPKK, and MAPK kinase kinase (MAPKKK, MEKK). Activation of MAPK signaling pathways consist of a sequence of successively phosphorylating kinases that ultimately result in dual phosphorylation and activation of p38, ERKs and JNKs (Fig. 3). MAPKs are classified into subfamilies based on their sequence homology and mechanism of activation. The ERK cascade is potently activated by hypertrophic stimuli, whereas p38 and JNK are activated by cellular stresses. Importantly, significant overlap and cross-talk exist among different MAPK cascades. (Kyriakis & Avruch 2001, Ravingerova *et al.* 2003.)

Numerous pathophysiological mediators of cardiac hypertrophy such as neurohormones, cytokines and mechanical stretch have been shown to activate different MAPK pathways. In heart, MAPK activation is observed at different stages of heart disease progression, including hypertensive cardiac hypertrophy, hypertrophic and dilated cardiomyopathies, heart failure, ischemic/reperfusion injury as well as inflammatory diseases. (Kyriakis & Avruch 2001, Ravingerova *et al.* 2003, Wang 2007.)

p38 mitogen-activated protein kinase

Four separate genes encoding p38 have been identified (p38α, p38β, p38γ and p38δ), although p38α is the major protein isoform expressed in the adult heart (Chen *et al.* 2001). A number of upstream kinases are involved in the

phosphorylation cascades leading to the activation of p38 including MKK3 and MKK6 at the MAPKK level. Activation of the p38 MAPK pathway has been reported in response to various stress responses such as inflammation, heat, osmotic shock and cytokines (Kyriakis & Avruch 2001). In cardiac myocytes, p38 MAPK is activated by the G protein-coupled receptor (GPCR) –agonists, ET-1, Ang II and phenylephrine (PE) (Sugden & Clerk 1998a). In heart, the functional role of p38 MAPK has been implicated in the regulation of the inflammatory response, energy metabolism, cardiac gene expression, cell proliferation, contractility, myocyte hypertrophy and cell death (Kyriakis & Avruch 2001, Liang & Molkentin 2003, Wang 2007).

Several transcription factors can be phosphorylated by p38 MAPK, including NFAT, GATA-4, activating transcription factor-2 (ATF-2), Elk-1 and myocyte enhancer factor-2 (MEF-2) (Chen *et al.* 2001, Garrington & Johnson 1999). At the level of gene expression, activation of p38 MAPK is able to induce hypertrophy associated genes such as ANP and BNP both *in vitro* and *in vivo* (Liang & Gardner 1999, Thuerauf *et al.* 1998, Zechner *et al.* 1997). In addition, p38 MAPK overexpression has been reported to regulate gene expression of Skα-A, cyclo-oxygenase 2 (COX-2) and the Na^{+}-Ca^{2+}-exchanger (NCX) *in vitro* (Degousee *et al.* 2003, Xu *et al.* 2005, Zechner *et al.* 1997) .

In cultured neonatal cardiomyocytes overexpression of constitutively active MKK3 and MKK6 induces cardiac hypertrophy (Wang *et al.* 1998), whereas pharmacological inhibition of p38 MAPK represses the hypertrophic response (Zechner *et al.* 1997). However, transgenic mice overexpressing constitutively active MKK3 or MKK6 showed no signs of cardiac hypertrophy, although increased fibrosis and induction of the hypertrophic gene program was seen (Liao *et al.* 2001). On the contrary, cardiac-specific dominant-negative p38α, MKK3 and MKK6 transgenic mice showed enhanced pressure overload –induced cardiac hypertrophy, revealing an anti-hypertrophic function for p38 MAPK (Braz *et al.* 2003). Furthermore, cardiac-specific p38α conditional knockout animals developed cardiac hypertrophy with massive fibrosis in response to pressure overload (Nishida *et al.* 2004).

Extracellular signal-regulated kinase

ERK 1/2 (also known as p44/42 MAPK) are the classical isoforms of five mammalian ERKs. The ERK pathway is activated by a variety of mitogenic stimuli and generally associated with cell growth and survival. Typically,

stimulation of growth factor receptors leads to activation of the small G-protein Ras, which in turn activates MAPKKK Raf. After activation, Raf phosphorylates and activates MEK 1/2, which in turn activate the MAP kinases ERK 1/2 by phosphorylation of threonine and tyrosine residues. (Coulombe & Meloche 2007, Michel *et al.* 2001, Ravingerova *et al.* 2003.)

In cardiomyocytes ERK 1/2 are activated by hypertrophic stimuli including GPCR-agonists such as ET-1 and phenylephrine, growth factors including fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1) as well as mechanical stretch (Michel *et al.* 2001, Ravingerova *et al.* 2003, Sugden & Clerk 1998b). Activation of ERK 1/2 by GPCR agonists and pressure overload *in vivo* has also been reported (Pellieux *et al.* 2000, Rapacciuolo *et al.* 2001). Once activated, ERK 1/2 phosphorylate a wide array of intracellular targets and transcription factors such as GATA-4 and NFAT (Liang *et al.* 2001, Sanna *et al.* 2005). ERK 1/2 have also been implicated in the phosphorylation of the downstream kinases MAPKAPK-1 and ribosomal S6 kinase (Ravingerova *et al.* 2003).

In cardiac myocytes, ERK 1/2 are involved in hypertrophic growth and gene expression as well as resistance to apoptosis (Liang & Molkentin 2003, Molkentin & Dorn II 2001, Wang 2007). The functional role of the ERK pathway in cardiac hypertrophy has been investigated in several genetically engineered animal models. For example, transgenic mice overexpressing constitutively active MEK-1 developed concentric hypertrophy (Bueno *et al.* 2001). ERK pathway inhibition was reported in dominant negative Raf-1 mice, which demonstrated attenuated hypertrophy and impaired induction of fetal gene expression in response to pressure overload (Harris *et al.* 2004). Furthermore, cardiomyocyte-specific *c-Raf-1* knockout mice developed heart failure without hypertrophy and increased apoptosis (Yamaguchi *et al.* 2004).

c-Jun N-terminal kinase

Three individual JNK genes have been identified (*Jnk1, Jnk2* and *Jnk3*), which together generate ten known isoforms through alternative splicing (Davis 2000). In heart, 46 kDa and 54 kDa proteins are the major isoforms. JNK is activated primarily by cytokines and in response to environmental stress such as radiation, reactive oxygen species, and hyperosmolarity (Davis 2000, Kyriakis & Avruch 2001). In cardiac myocytes, JNK is activated by stress stimuli such as mechanical stretch, osmotic shock and hypoxia as well as by pro-inflammatory cytokines

including interleukin (IL)-1β, tumor necrosis factor α (TNF-α), and Ang II (Michel *et al.* 2001). In cardiac myocytes, the two main targets of JNK phosphorylation are c-Jun and ATF-2 (Sugden & Clerk 1998a).

Although hypertrophic stimulation activates JNK *in vivo*, the role of JNK in the development of cardiac hypertrophy is controversial (Wang 2007). In the first gain-of-function studies in cultured cardiac myocytes, JNK activation led to a hypertrophic phenotype and activation of a fetal gene program (Wang *et al.* 1998). However, in transgenic animal models of JNK activation, the animals developed lethal restrictive cardiomyopathy with a significant induction of fetal gene expression, but no myocyte hypertrophy (Liang & Molkentin 2003, Liang *et al.* 2003, Petrich *et al.* 2004, Sadoshima *et al.* 2002). Possible explanations for these discordant observations are that different JNK isoforms have functional redundancies or dominant-negative mutants of JNK can have effects on other hypertrophy pathways (Wang 2007). In fact, there are studies showing that JNK activates prohypertrophic signaling molecules, such as Akt (Shao *et al.* 2006) and c-Jun (Sugden & Clerk 1998a) as well as negative regulators of hypertrophy such as JunD (Hilfiker-Kleiner *et al.* 2006) and calcineurin (Liang *et al.* 2003).

2.4 Gene expression in cardiac hypertrophy

Molecular remodeling of the myocardium includes qualitative and quantitative changes in gene expression (Table 1). The early (within 1 hour) genetic response to hemodynamic overload is characterized by the activation of immediate early response genes, whose transcriptional induction is independent of new protein synthesis. Well characterized mechanical stretch induced immediate early response genes in the heart include the *fos* family (*FosB, c-fos, fra-1*), *jun* family (*c-jun, JunB, JunD*), *c-myc*, *Egr-1* and *nur77* (Komuro & Yazaki 1993). The members of the *jun* and *fos* families compose the AP-1 (activator protein 1) transcription factor, which is activated by a variety of cellular stresses. In addition to immediate early genes, the heat shock proteins (Hsp70 and Hsp90) are among the other early load-responsive genes that rapidly change their expression in the heart upon hemodynamic overload (Komuro & Yazaki 1993, Sadoshima & Izumo 1997).

Table 1. Characteristic features of cardiac hypertrophic gene expression.

Gene	Change
Proto-oncogenes	
c-fos	↑
c-Jun	↑
JunB	↑
c-myc	↑
Natriuretic peptides	
Atrial natriuretic peptide (ANP)	↑
B-type natriuretic peptide (BNP)	↑
Sarcomeric proteins	
α-myosin heavy chain (α-MHC)	↓
β-myosin heavy chain (β-MHC)	↑
Cardiac α-actin (caα-A)	↓
Skeletal α-actin (skα-A)	↑
Metabolic proteins	
Muscle carnitine palmitoyl transferase 1 (muscle CPT-1)	↓
Liver carnitine palmitoyl transferase (liver CPT-1)	=
Glucose transporter type 1 (GLUT-1; SLC2A1)	=
Glucose transporter type 4 (GLUT-4; SLC2A4)	↓
Pyruvate dehydrogenase kinase, isozyme 4	↓
Proteins involved in intracellular calcium metabolism	
Sarcoplasmic reticulum Ca^{2+}-ATPase 2 (Serca 2A)	↓
Phospholamban	↓
$Na^{+}Ca^{2+}$ exchanger (NCX)	↑
Cytoskeleton and extracellular matrix	
Collagens I and III	↑
Fibronectin	↑
Laminin	↑
Growth factors	
Insulin-like growth factor 1 (IGF-1)	↑
Transforming growth factor β1 (TGFβ1)	↑

↑, indicates increase; ↓, decrease and =, no change in gene expression.

The induction of immediate early genes is followed by activation of a fetal gene program, including upregulation of natriuretic peptides ANP and BNP as well as fetal isoforms of contractile proteins such as skα-A and β-MHC, accompanied by downregulation of genes normally expressed at high levels in the adult heart such as α-MHC (Chien *et al.* 1993). This isoform switch has been proposed to contribute to diminished myofibrillar ATPase activity and impaired contractility in rodents, in which the α-MHC is the predominant isoform (Swynghedauw 1999,

Tardiff *et al.* 2000). The significance of this transition is less certain in human ventricles, where the β-MHC isoform predominates (Swynghedauw 1999). Another characteristic feature of the activation of the fetal gene program includes re-expression of genes that are normally not expressed in adult ventricles such as ANP and atrial myosin light chain (MLC) (Sugden & Clerk 1998b, Swynghedauw 2006). The altered expression of genes involved in calcium transport and binding are also well-characterized changes that occur during the development of cardiac hypertrophy. These changes include downregulation of genes encoding the sarcoplasmic reticulum Ca^{2+}-ATPase 2a (Serca 2a) and phospholamban as well as upregulation of NCX, with consequent abnormalities in calcium handling of the cardiac myocytes (Houser *et al.* 2000, Swynghedauw 1999).

In the overloaded myocardium, metabolic remodeling precedes, triggers and sustains functional and structural remodeling. Generally, acute changes in cardiac load result in post-translational modulation of the activities of regulatory enzymes or proteins, whereas long-term regulation is accomplished through adjustments of gene expression and the amount of enzymes or transporter proteins. The transcript levels of metabolic enzymes change in the same direction as contractile proteins and a switch from adult to fetal isoforms is seen for example in the expression of the isoforms of glucose transporters (GLUTs; also known as facilitated glucose transporter members of the solute carrier family 2) and carnitine palmitoyl transferase 1 (CPT-1). The metabolic consequence is a shift in energy substrate metabolism from fatty acids to glucose as the main fuel of respiration, resulting in improved contractile efficiency of the heart. This switch is accompanied by the downregulation in the mRNA levels of regulatory enzymes affecting the mitochondrial metabolism of glucose and fatty acids i.e. pyruvate dehydrogenase kinase 4, malonyl-CoA decarboxylase and uncoupling protein 3 (UCP-3). (Taegtmeyer *et al.* 2005, Taegtmeyer 2000.)

Myocardial remodeling during the development of cardiac hypertrophy implies an alteration in the extracellular matrix (ECM) composition and distribution as well as changes in the organization and components of the cytoskeleton (Hein *et al.* 2000, Jane-Lise *et al.* 2000). One of the key features of cardiac fibrosis is the increased deposition of the extracellular matrix. The dynamic remodeling of the ECM is controlled by several regulatory mechanisms, including synthesis of ECM components, proteolytic degradation of ECM by matrix metalloproteinases (MMPs) and inhibition of MMP activities by their endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs)

(Deschamps & Spinale 2006, Kassiri & Khokha 2005). Gene expression of collagen I, collagen III, laminin, and fibronectin increase during myocardial remodeling related to cardiac hypertrophy (Swynghedauw 1999). Furthermore, matricellular proteins (e.g. tenascin-C, thrombospondin-1 [TSP-1], TSP-2 and osteonectin) that modulate cell-matrix interactions and cell function may alter ventricular remodeling of the stressed heart (Schellings *et al.* 2004). Expression of matricellular proteins is increased in response to stress and during the left ventricular remodeling of the heart they may induce de-adhesion (i.e. loosening of cell-matrix adherence) (Murphy-Ullrich 2001) and regulate the MMP activity (Spinale 2002). The transformation of cardiac fibroblasts to myofibroblasts and induction of profibrotic mediators, such as TGFβ and plasminogen activator inhibitor 1 (PAI-1), are also characteristic of the cardiac fibrotic process. Myofibroblasts acquire contractile properties similar to smooth muscle cells, and induction of myofibroblast markers, such as α-smooth muscle actin 2 and nonmuscle MHC, have been described during myofibroblast conversion (Powell *et al.* 1999, Tomasek *et al.* 2002).

In loaded myocardium, mechanical stretch stimulates production and/or secretion of a number of growth factors, and the growth factors secreted in response to mechanical stretch are involved in mediating the growth response. In cardiac hypertrophy, increased mRNA expression of various growth factors such as TGFβ1, platelet-derived growth factor (PDGF) and IGF-1 have been reported (Sadoshima & Izumo 1997).

2.5 Regulation of cardiac gene expression

In cardiac hypertrophy, many gene regulatory mechanisms work at either the transcriptional or post-transcriptional level, including chromatin modifications, transcriptional activation by multiple transcription factors, mRNA splicing and mRNA degradation (Fig. 4). However, gene expression can also be controlled at the level of translation and by post-translational modifications including chemical modifications of proteins and proteolysis. (Alberts *et al.* 2002). Recently, microRNAs (miRNAs) have been shown to be involved both in post-transcriptional and translational regulation of gene expression (Bartel 2004). In addition, genetic variation, i.e. differences in the DNA sequence among individuals, can cause significant differences in gene expression (Dermitzakis & Stranger 2006).

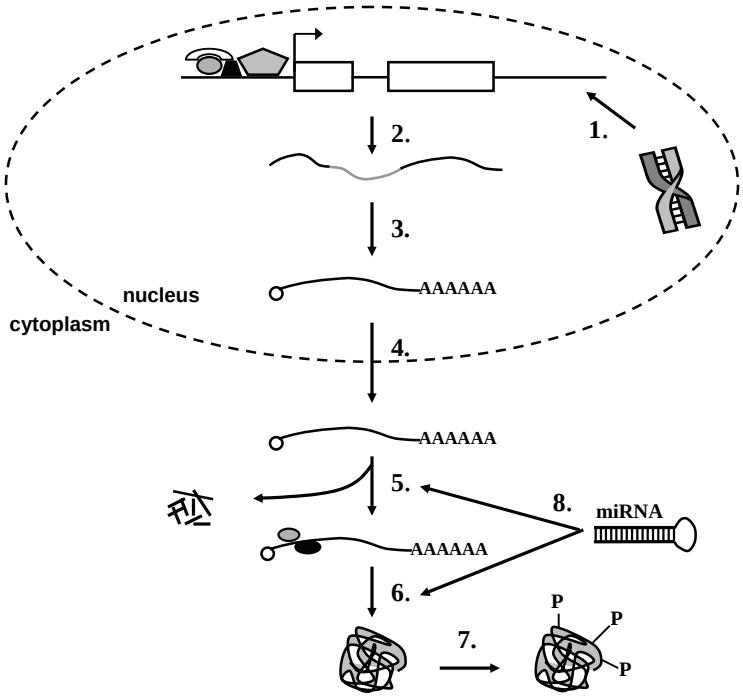

Fig. 4. General overview of the regulation of gene expression. 1, Modification of DNA; 2, Transcription; 3, RNA processing; 4, mRNA transport from the nucleus to the cytoplasm; 6, Translation of protein from the mRNA; 7, Post-translational modification of the protein. 8. MicroRNAs (miRNAs) regulate gene expression by promoting mRNA degradation and inhibiting mRNA translation.

2.5.1 Genetic variation

Genetic variation in humans exists in many forms ranging from single base pair changes to very large cytogenetic alterations involving entire chromosomes. It is estimated that approximately 5% of the human genome is structurally variable (Sharp *et al.* 2006). The genetic abnormalities can underlie or predispose to cardiovascular diseases such as congenital heart disease, hypertrophic and dilated cardiomyopathies and cardiac arrhythmias (Pollex & Hegele 2007). Several cardiovascular disorders result from large cytogenetic changes. The most common genetic cause of congenital heart defects is Down's syndrome, which is caused by trisomy of chromosome 21 (Hoffman & Kaplan 2002). Also copy number variants, characterized as structural variants of intermediate size, are

associated with several cardiovascular diseases including long-QT-syndromes and cardiomyopathies (Pollex & Hegele 2007). Single-nucleotide changes occur widely in the human genome (International HapMap Consortium 2005). Hypertrophic cardiomyopathy is an example of genetic cardiac hypertrophy, caused by a variety of mutations, mainly in genes encoding contractile sarcomeric proteins such as ß-MHC and tropomyosin (Ashrafian & Watkins 2007, Donahue *et al.* 2006). A single nucleotide polymorphism (SNP) -based approach has been used to attempt to identify the genetic basis of cardiovascular diseases. These studies often yield contradictory results, reflecting the inconsistencies and limitations of single-locus candidate gene association studies when examining complex cardiovascular diseases like hypertension and LVH (Pratt & Dzau 1999, Weatherall 1999). Instead of individual SNPs, haplotypes may help to correlate genetic polymorphisms with cardiovascular disease phenotypes (Skelding *et al.* 2007). A haplotype is a group of genetic variants, such as SNPs, closely linked on a single chromosome and inherited as a unit. Recently, novel genes associated with human hypertrophic cardiomyopathy were found by haplotype analysis (Castro *et al.* 2007, Osio *et al.* 2007). SNPs in genes encoding drug targets or drug metabolism pathways can also determine the therapeutic utility of cardiovascular pharmacology. Numerous associations have been reported between selected genotypes and specific responses to cardiovascular drugs (Giacomini *et al.* 2007, Mooser *et al.* 2003). For example, a recent study showed that a polymorphism in the β1-adrenergic receptor altered cardiac function and response to the β-blocker bucindolol in human heart failure (Liggett *et al.* 2006), suggesting the utility of genetic-based treatment in heart failure.

2.5.2 Modification of chromatin structure

The packaging of DNA into chromatin is a central mechanism for the control of gene expression. The basic unit of chromatin is the nucleosome, which comprises DNA wrapped around a histone octamer, creating a tightly packed structure that limits access of genomic DNA to gene regulatory elements, thereby repressing gene expression (Edmondson & Roth 1996, Razin *et al.* 2007). Chromatin structure can be altered by covalent modification of histone proteins, including methylation, ubiquitination, phosphorylation and the best-characterized mechanism, acetylation, catalyzed by histone acetyltransferases (HATs). Generally, histone acetylation enhances transcription. The stimulatory effect of HATs on gene expression is countered in the deacetylation process performed by

histone deacetylaces (HDACs), which promote chromatin condensation and thereby repress transcription. Most of the HATs and HDACs interact also on nonhistone nuclear proteins, such as transcription factors, thus activating or repressing specific genes (Backs & Olson 2006, Sterner & Berger 2000).

Regulation of histone acetylation has been linked to cardiac hypertrophy. The most studied HATs p300 and the closely related CREB (cAMP-response-element-binding) -protein co-activator play important roles in the growth of cardiac myocytes. p300 possesses an intrinsic HAT activity, which is induced during phenylephrine-induced hypertrophy of cardiac myocytes (Gusterson *et al.* 2003). In adult mouse heart, cardiac specific overexpression of p300 results in hypertrophy and heart failure (Yanazume *et al.* 2003). Besides acetylation, p300 is also a transcriptional coactivator of many hypertrophy-responsive transcription factors, such as MEF-2, GATA-4 and serum response factor (SRF) (Backs & Olson 2006). There are three classes of HDACs. Existing data suggest that class I and class II HDACs have opposite roles in the regulation of hypertrophic pathways. Hdac2, a class I HDAC, is required for some hypertrophic responses (Trivedi *et al.* 2007). By contrast, class II HDACs have been shown to repress growth of cardiac myocytes, partly by binding to MEF-2 and inhibiting its activity (Backs & Olson 2006, Zhang *et al.* 2002). In stressed myocardium, the levels of class II HDACs do not appear to change, but instead the stress signals lead to the phosphorylation-dependent nuclear export of class II HDACs from the nucleus to the cytoplasm. This relocation of HDACs enables MEF-2 and other transcription factors to associate with HATs, resulting in increased local histone acetylation with consequent activation of genes involved in cardiac growth (Backs & Olson 2006). Specifically, ANP and β-MHC are among the genes regulated by HATs or HDACs in cardiac myocytes (Kuwahara *et al.* 2001, Zhang *et al.* 2002).

Chromatin modifications have been proposed to be one mechanism involved in the re-expression of fetal genes during hypertrophy. Studies examining the regulation of ANP and BNP genes have revealed the involvement of the repressor element 1-silencing factor (REST; also known as neuron-restrictive silencing factor, NRSF) in repressing the fetal cardiac gene program in the adult heart (Kuwahara *et al.* 2001, Kuwahara *et al.* 2003). REST mediates transcriptional repression by recruiting multiple chromatin-modifying enzymes that remove the post-translational modifications of chromatin at the promoter of the active target gene (Bingham *et al.* 2006). A recent report demonstrated that continued REST

expression attenuates increases in ANP and BNP gene expression in adult rat ventricular myocytes in response to endothelin-1 (Bingham *et al.* 2007).

2.5.3 Transcription factors

Tissue- and development-specific gene expression is regulated by the interplay between chromatin structure and combinatorial interactions between several transcription factors (Edmondson & Roth 1996). Transcription factors are regulatory proteins that bind to DNA at specific regions where they can control the transcription of a particular gene. They may bind to control elements on the gene promoter area or the more distant enhancer elements. Transcription factors can be only minimally active until the appropriate stimulus is present or they can reduce the rate of transcription (so-called repressors). Finally, transcription factors and their co-factors can be regulated through reversible structural alterations such as phosphorylation. (Brivanlou & Darnell 2002.)

A limited number of transcription factors are proposed to coordinate cardiac development and the differentiation of myocytes including the GATA family of transcription factors, MEF-2 transcription factors, and the Csx (cardiac-specific homeobox) or Nkx2-5 transcription factor. Interestingly, many of these transcription factors are re-employed in the adult heart in response to hypertrophic stimuli where they are thought to have fundamental roles in regulating a number of cardiac genes that are differentially expressed in hypertrophied myocardium. Numerous transcription factors have been implicated in stress-dependent gene expression in the heart including AP-1 (Herzig *et al.* 1997, Suo *et al.* 2002, Takemoto *et al.* 1999), GATA-4, MEF-2, SRF, and nuclear factor κB (NF-κB) (Akazawa & Komuro 2003, Oka *et al.* 2007b). Many of these transcription factors are in turn activated through phosphorylation and dephosphorylation events mediated by intracellular signal transducers such as MAPK, calcineurin as well as the kinase-regulated shuttling of class II HDACs (Oka *et al.* 2007b). However, the abundance of most of these transcription factors does not change in stressed myocardium, indicating that their enhanced activity is regulated by post-transcriptional mechanisms (Backs & Olson 2006). A summary of these transcription factors as modulators of the hypertrophic growth in the adult heart is shown in Table 2.

Table 2. Summary of transcription factors as modulators of cardiac disease (modified from Oka *et al.* 2007).

TF	Disease functions in the heart
AP-1	Increased AP-1 binding by pressure overload and hypertrophic agonists
	Dominant negative mutant of *c-jun* inhibits hypertrophy *in vitro*
GATA-4	Overexpression induces cardiomyocyte hypertrophy *in vitro*
	Transgenesis induces progressive cardiac hypertrophy *in vivo*
	Conditional gene targeting reduces hypertrophic response *in vivo*
	Increased cell death/apoptosis in gene-targeted heart *in vivo*
MEF-2	Increased activity by pressure and volume overload-induced cardiac hypertrophy
	Cardiac-specific overexpression of MEF-2A or -2C induces dilated cardiomyopathy
	Overexpression of MEF-2A or -2C induces sarcomere degeneration *in vitro*
NF-κB	Cardiac inflammation (myocarditis) is blocked by overexpression of inhibitory-κB *in vi*vo
	Inhibition with dominant-negative mutant reduces cardiac hypertrophy *in vivo*
	Gene targeting reduces hypertrophic growth induced by angiotensin II *in vivo*
	Super-repressor mutant attenuates cardiac hypertrophy *in vivo*
SRF	Cardiac-specific overexpression induces hypertrophy *in vivo*
	SRF mutant causes postnatal chamber dilation with wall thinning *in vivo*
	Conditional heart-specific deletion promotes progressive heart failure *in vivo*
	Deletion in cardiomyocytes induces disorganization of contractile apparatus
	Activity is inhibited in adult failing heart

TF, transcription factor

AP-1

AP-1 is a complex of transcription factors, composed of homo- and heterodimers of *Jun* (*v-Jun, c-Jun, Jun B, Jun D*), *Fos* (*v-Fos, c-Fos, FosB, Fra1, Fra2*) or activating transcription factor (ATF-2, ATF-3, B-ATF) families (Karin *et al.* 1997). The AP-1 complex binds to a palindromic sequence, TGAC/GTCA, found in numerous genes including ANP, BNP, Skα-A, ET-1 and Ang II receptor subtype 1A genes (Bishopric *et al.* 1992, Grepin *et al.* 1994, Herzig *et al.* 1997, Kawana *et al.* 1995, Kovacic-Milivojevic & Gardner 1993). A number of transcription factors are reported to co-operate with the AP-1 complex, including NFAT, GATA and ETS transcription factors (Wisdom 1999). AP-1 is activated by a variety of stimuli, especially by cellular stresses, including UV-irradiation, DNA damage, growth factors, cytoskeletal rearrangements and pro-inflammatory cytokines (Karin *et al*. 1997, Shaulian & Karin 2002).

In heart, AP-1 binding is increased by a number of hypertrophic stimuli, including Ang II, isoprenaline, aortic constriction and direct ventricular wall

stress (Cornelius *et al.* 1997, Herzig *et al.* 1997, Suo *et al.* 2002, Takemoto *et al.* 1999). However, due to the different compositions of the AP-1 dimers, the mechanisms regulating AP-1 activity are complex and several signal transduction pathways affect its activity, MAPKs being the central upstream signaling cascade (Karin *et al.* 1997, Shaulian & Karin 2002). The role of the AP-1 complex in the development of cardiac hypertrophy is unclear, but adenoviral production of a dominant negative mutant of *c-jun* inhibits ET-1 and PE -induced hypertrophy in neonatal rat cardiac myocytes (Omura *et al.* 2002).

GATA-4

There are six proteins in the vertebrate GATA family of transcription factors (GATA-1-6). GATA factors consist of a domain of two adjacent zinc fingers and bind to the consensus DNA sequence (A/T)GATA(A/G). GATA-1-3 are regulators of hematopoietic cells, whereas GATA-4-6 are expressed in various mesoderm and endoderm-derived tissues including heart. GATA-4 has an important role in cardiac development and differentiation -specific gene expression. (Molkentin 2000, Pikkarainen *et al.* 2004). GATA-4 regulates basal expression of several cardiac-specific genes including α-MHC, MLC1/3, cardiac troponins C and I, cardiac-restricted ankyrin repeat protein (CARP), ANP, BNP, cardiac NCX, calcineurin A and many others (Molkentin 2000).

GATA-4 plays an essential role in the transcriptional regulation of hypertrophic growth in the adult heart. GATA-4 is re-employed during pathophysiological stress and mediates the induction of genes involved in cardiac hypertrophy such as ANP, BNP and β-MHC. Moreover, various hypertrophic stimuli including pressure overload, ET-1, isoproterenol and phenylephrine activate GATA-4. The transcriptional activity of GATA-4 is regulated by divergent signaling pathways such as glycogen synthase kinase 3β (GSK-3β), ERK and p38 MAPK, suggesting GATA-4 can serve as a convergence point for cardiac hypertrophic signaling. In addition, GATA-4 has been shown to cooperate with other transcription factors such as Csx/Nkx2-5, MEF-2, NFAT and SRF *in vitro*. (Akazawa & Komuro 2003, Molkentin 2000, Pikkarainen *et al.* 2004.) Finally, the transgenic overexpression of GATA-4 in the heart produced cardiac hypertrophy, whereas the cardiac-specific deletion of Gata-4 attenuated hypertrophy in response to both pressure overload and exercise stimulation (Liang *et al.* 2001, Oka *et al.* 2006).

MEF-2

The MEF-2 family comprises four isoforms, referred to as MEF-2(A-D), which form homo- and heterodimers. MEF-2 is a MADS (indicating MCM1, agamous, deficiensis, and SRF) domain containing transcription factor that binds to the concensus sequence CTA(A/T)$_4$TAG present in the 5'-transcriptional regulatory regions of most skeletal and cardiac muscle structural genes including α-MHC, Skα-A, Serca, desmin, dystrophin as well as troponins C, T and I. (Akazawa & Komuro 2003, Oka *et al.* 2007b). MEF-2 interacts with GATA-4 in the transcriptional activation of ANP, BNP, α-MHC and caα-A genes (Morin *et al.* 2001). Hypertrophic stimuli and several cardiac hypertrophic signal transducers such as p38 MAPK and calcineurin have been shown to activate MEF-2 (Akazawa & Komuro 2003, Oka *et al.* 2007b). Several studies have implicated MEF-2 as an important regulator of hypertrophic growth. Cardiac-specific overexpression of MEF-2 in the heart promoted dilated cardiomyopathy and increased hypertrophy following pressure overload induced by aortic constriction (Xu *et al.* 2006). Furthermore, recent studies have provided evidence that MEF-2 functions as a downstream effector of class II HDAC- and Ca^{2+}/calmodulin-dependent protein kinase (CaMK)-dependent cardiac hypertrophy (Lu *et al.* 2000, McKinsey *et al.* 2000a, McKinsey *et al.* 2000b, Passier *et al.* 2000).

NF-κB

The mammalian NF-κB family of transcription factors consists of five proteins: p65/relA, p105/p50 or NF-κB1, p100/p52 or NF-κB2, p68/RelB and p75/c-Rel. These NF-κB subunits can form a variety of homo- and heterodimers in a cell-type specific manner. NF-κB activity is regulated through a cascade of kinases leading to NF-κB translocation to the nucleus and binding to κB sites, such as 5'-GGG(A/G)NN(C/T)CC-3' or 5'-(A/C/T)GGA(A/G)N(C/T)(C/T)CC-3'. NF-κB regulates the gene expression of multiple genes involved in various cellular processes including inflammation, cell adhesion and migration, apoptosis and development. It is activated by proinflammatory cytokines such as TNF-α, UV-irradiation, phorbol esters, free radicals and oxidative stress. (Hall *et al.* 2006, Valen *et al.* 2001.) In heart, NF-κB is involved in cardiac inflammation associated with myocarditis or sepsis (Carlson *et al.* 2003, Haudek *et al.* 2001). Recent evidence indicates that NF-κB is also important as a necessary and sufficient

regulator of cardiac hypertrophy (Gupta *et al.* 2002, Higuchi *et al.* 2002, Kawano *et al.* 2005, Li *et al.* 2004, Purcell *et al.* 2001).

SRF

SRF is a member of the MADS box family of transcription factors. SRF binds to the consensus site $CC(AT)_6GG$, also known as the CArG box, which is found in the promoters of numerous cardiac genes such as ANP, β-MHC, Skα-A, caα-A, MLC-2 and dystrophin, Serca-2 and NCX-1 (Miano 2003, Nelson *et al.* 2005). SRF is shown to interact with other known hypertrophy regulatory factors that influence cellular growth. For example, SRF interacts with Nkx2-5 and GATA-4 to synergistically activate muscle gene expression in heart (Nelson *et al.* 2005, Oka *et al.* 2007b). SRF is a regulator of the hypertrophic response in the adult heart and SRF activity was shown to be inhibited in the failing adult heart (Chang *et al.* 2003, Davis *et al.* 2002). In addition, the overexpression of SRF in the heart induced cardiac hypertrophy with collagen deposition, whereas the cardiac-specific deletion of SRF resulted in disorganization in the contractile apparatus and attenuated expression of sarcomeric proteins (Parlakian *et al.* 2005, Zhang *et al.* 2001b).

2.5.4 Post-transcriptional modifications

Regulation of gene expression at the post-transcriptional level includes processing of the primary RNA transcript (5' capping and 3' polyadenylation), removal of introns by RNA splicing, mRNA stabilization or degradation and transport through the nuclear envelope into the cytosol to be translated (Alberts *et al.* 2002). In cardiac hypertrophy, alternative splicing and control of mRNA stability are widely used mechanisms of post-transcriptional regulation.

RNA splicing

A process whereby multiple transcripts are generated from a single gene is called alternative splicing. In the human genome, 40-80% of human multi-exon genes are predicted to be alternatively spliced. Alternative splicing is one of the most important mechanisms in the generation of protein diversity. (Tress *et al.* 2007.)

In heart, multiple isoforms of several cardiac genes such as the contractile protein gene cardiac troponin T are expressed in the developing, adult and failing

heart (Anderson *et al.* 1995). Interestingly, important cardiovascular drug therapy targets like the Ang II type I receptor (AT1-R) and β1 adrenergic receptor are also regulated via alternative splicing. In humans, there are at least four alternatively spliced ATR1-R mRNAs, which are regulated in a tissue-specific manner. The relative abundance of each AT1-R mRNA splice variant controls AT1-R protein levels and thus the receptor density. These receptor isoforms also have different affinities for Ang II. Consequently, the alternative splicing of AT1-R affects the potency of the Ang II response. (Elton & Martin 2007.) Likewise, at least two alternative spliced transcripts of the human β1-adrenergic receptor have been identified and alterations in the abundance of these transcripts have been reported in heart failure patients with idiopathic dilated cardiomyopathy (Ellis & Frielle 1999).

RNA degradation

Gene expression can be controlled by mRNA degradation. In a study where the gene expression profiles of polyA mRNA (whole-cell) were compared with nuclear run-on assay generated RNA (newly transcribed) in T-cells, up to 50% of transcripts were estimated to be regulated at the level of mRNA stability (Cheadle *et al.* 2005a). The mRNAs of different genes are degraded at various rates and the signal for rapid RNA degradation is often located in the 3' untranslated region (UTR) of the mRNA sequence. Adenylate/uridylate-rich elements (AREs) are the most common determinant of RNA stability in mammalian cells. The size of AREs range from 50 to 150 nucleotides and generally they contain multiple copies of the pentanucleotide AUUUA. AREs are found in the 3'UTRs of many mRNAs that encode proto-oncogenes like *c-fos, c-myc, nur77*, nuclear transcription factors, and cytokines like β-interferon and IL-3. (Chen & Shyu 1995). Furthermore, ARE-directed mRNA degradation is influenced by many exogenous factors, including cytokines, transcription inhibitors and hypertrophic agonists like PE (Chen & Shyu 1995, Hanford & Glembotski 1996).

BNP mRNA contains several AUUUA repeat units in the 3' UTR, which may be involved in the translation-dependent rapid mRNA degradation (Kojima *et al.* 1989). Thus, the unchanged mRNA levels, observed in some experimental models of cardiac overload, may be explained by decreased mRNA stabilization (Magga *et al.* 1997). In addition, activators of protein kinase C (PKC) and MAPKs have been reported to enhance BNP transcript stability in cell culture models of cardiac hypertrophy (Hanford & Glembotski 1996, LaPointe & Sitkins 1993).

Interestingly, it has been shown that different hypertrophic stimuli may use distinct mechanisms to regulate the transcription and mRNA stability of the same gene. Both angiotensin II and phenylephrine downregulate both mRNA and protein levels of the cardiac Kv4.3 potassium channel, but the results suggest that Ang II destabilizes the Kv4.3 mRNA, whereas PE does not affect the rate of Kv4.3 mRNA degradation in neonatal rat cardiac myocytes (Zhang *et al.* 2001a).

2.5.5 MicroRNAs

MicroRNAs (miRNAs) are short (18-25 nucleotides), endogenous, non-coding RNAs that are involved in post-transcriptional and translational regulation of gene expression (Bartel 2004). Primary miRNA transcripts of variable sizes are processed into 70- to 100-nucleotide hairpin-shaped precursors, which in turn are processed into mature miRNAs. MicroRNAs that base pair perfectly with their target sequences result in mRNA degradation whereas miRNAs with an imperfect match to endogenous mRNA target sequences generally repress translation (Kloosterman & Plasterk 2006). According to computational predictions, each miRNA has the potential to target up to 200 genes (Bentwich 2005). MiRNAs can be expressed in a cell-type and tissue specific manner and they have been shown to regulate many fundamental biological processes, including cell growth, differentiation and apoptosis in various species (Kloosterman & Plasterk 2006).

In heart, miRNAs have a role in the regulation of heart morphogenesis (Zhao *et al.* 2005, Zhao *et al.* 2007) and cardiovascular functions *in vivo*, such as conductance of electrical signals, heart muscle contraction and ventricular myocyte hyperplasia and hypertrophy (Care *et al.* 2007, Yang *et al.* 2007a, Zhao *et al.* 2007, van Rooij *et al.* 2006, van Rooij *et al.* 2007). Several miRNAs, such as miR-133 and miR-195, have been shown to be differentially regulated during cardiac hypertrophy and heart failure in mice and humans *in vivo* (Care *et al.* 2007, van Rooij *et al.* 2006). A recent study in transgenic mice has shown that a cardiac specific miR-208 is required for cardiomyocyte hypertrophy and fibrosis (van Rooij *et al.* 2007). In addition, the miR-208, which is encoded by an intron of the α-MHC gene, seems to be involved in mediating the switch from expression of α-MHC to β-MHC during pressure overload or thyroid-hormone induced cardiac growth (van Rooij *et al.* 2007).

Recent DNA microarray studies have identified differential miRNA expression during pressure overload induced cardiac hypertrophy in a mouse model of aortic banding and in transgenic mice expressing activated calcineurin

(Cheng *et al.* 2007, Sayed *et al.* 2007, van Rooij *et al.* 2006) as well as in agonist-induced hypertrophy in cultured cardiomyocytes (Tatsuguchi *et al.* 2007). Many of these miRNAs were also found to be differentially expressed in end-stage human heart failure (Ikeda *et al.* 2007, Thum *et al.* 2007). Interestingly, cardiac transcriptome analysis showed similarities in miRNA expression between fetal and failing human heart tissue suggesting re-expression of fetal miRNAs during heart failure (Thum *et al.* 2007). Moreover, comparison of miRNA profiles among control patients and patients with ishemic cardiomyopathy, dilated cardiomyopathy and aortic stenosis revealed that distinct changes in miRNA expression were associated with different disease etiologies (Ikeda *et al.* 2007).

2.5.6 Translation and post-translational modifications

Hypertrophic growth is accompanied by increases in both protein synthesis and proteolysis rates. However, the more rapid rate of protein synthesis with relation to protein breakdown results in a net increase in protein accumulation (Dorn 2007). There are two common mechanisms that lead to accelerated rates of translation i.e. an increase in the efficiency of translation or in the capacity of protein synthesis (Hannan *et al.* 2003). For example, increased rates of protein synthesis in cardiac myocytes have been shown to correlate with an increase in the activity of translation initiation factors, such as eIF4F, and subsequently to an increased rate of translation initiation, which represents the rate-limiting step of protein synthesis. In addition, increases in ribosomal DNA transcription and in ribosome content have been observed in cardiac hypertrophy (Hannan & Rothblum 1995, Hannan *et al.* 2003)

The mammalian target of rapamycin (mTOR) and the ubiquitin-proteosome system (UPS) have been shown to play an important role in regulating protein turnover (Hedhli *et al.* 2005). mTOR can activate the global ribosomal machinery by controlling the initiation of the translation, but it can also enhance the translational rate of specific transcripts depending on their structure and post-transcriptional modifications (Gingras *et al.* 2001). UPS plays a major role in proteolytic degradation of misfolded or damaged proteins (Hedhli *et al.* 2005). In heart, UPS is involved in protein quality control of ion channels as well as sarcomeric proteins. Accordingly, increased expression of regulatory proteins of UPS have been reported e.g. in patients with idiopathic dilated cardiomyopathy (Willis & Patterson 2006). Both mTOR and UPS control the adaptation of cardiac cell size during cardiac hypertrophy (Hedhli *et al.* 2005, Willis & Patterson 2006,

Zolk *et al.* 2006). For example, it has been shown that rapamycin treatment attenuates the pressure overload induced increase in cardiac cell size and in contractile function (McMullen *et al.* 2004a, Shioi *et al.* 2003), whereas ubiquitin ligases muscle-atrophy F-box (Atrogin-1) and muscle-specific ring finger-1 (MuRF-1) have been shown to attenuate cardiac hypertrophy by interacting with calcineurin and PKC ε, respectively (Arya *et al.* 2004, Li *et al.* 2004).

The dynamic post-translational modification of proteins is important for maintaining and regulating protein structure and function. Among the several hundred different types of post-translational modifications characterized to date, protein phosphorylation plays a prominent role, since it affects an estimated one-third of all proteins and is the most widely studied post-translational modification so far (Cohen 2001, Mann & Jensen 2003). In cardiac hypertrophy, several intracellular signaling pathways ultimately promote changes in gene expression by post-translational modifications of transcription factors, for example phosphorylation through MAPKs or GSK-3 and dephosphorylation through calcineurin (Clerk *et al.* 2007, Heineke & Molkentin 2006). Further, the biological activity of transcription factors is partly regulated by their intracellular localization and phosphorylation/dephosphorylation events modulate their import to the nucleus where they can regulate gene expression. In the case of NFAT transcription factors, calcineurin participates in dephosphorylation of members of the NFAT family, which results in their translocation to the nucleus and the subsequent activation of calcium-dependent genes (Clerk *et al.* 2007). GSK-3 antagonizes the actions of calcineurin by stimulating NFAT nuclear export. In fact, the anti-hypertrophic effects of GSK-3 are explained in part by phosphorylation of pro-hypertrophic transcription factors such as NFAT proteins and GATA-4 (Markou *et al.* 2008). On the other hand, at the level of translation, GSK3 may phosphorylate and reduce the biological activity of eukaryotic initiation factor 2B with a subsequent decrease in protein synthesis (Hardt *et al.* 2004).

2.6 Microarray technologies

Advances in genome projects and in computational biology have led to the development of powerful, high-throughput methods for large-scale genomic research such as serial analysis of gene expression (SAGE) and microarrays. In principle, microarrays contain a series of samples such as DNA, RNA, protein or tissue placed on a solid support. The most commonly employed microarrays are DNA microarrays, which can be used for example to determine the expression

levels of tens of thousands of genes (i.e. gene expression profiling) or to screen hundreds of thousands of SNPs. (Hovatta *et al.* 2005, Seliger 2007.)

2.6.1 DNA microarrays

Initially DNA arrays were based on nylon membranes that are still in use. There are mainly two types of DNA arrays, depending on the type of spotted probes. The traditional microarrays use complementary DNAs (cDNAs), which are obtained by reverse transcription of the mRNAs and printed or spotted onto the slides. The other type of arrays uses small single-stranded oligonucleotides synthesized onto the slide *in situ* by a photolithographic process (Hovatta *et al.* 2005, Trevino *et al.* 2007).

The typical workflow of the DNA microarray experiment is presented in Figure 5. A fluorescent dye is used to label the extracted mRNAs from the tissue or cell samples to be analyzed. Then the labeled samples are purified and hybridized with the DNA array, followed by removal of the non-specific hybrids by washing. Fluorescently labeled microarrays are detected by a (confocal) scanner, which excites the attached fluorescent dye to produce light. The scanner generates a digital image, which is further processed by specialized software to transform the intensity of each spot to a numerical reading, and to estimate the surrounding background noise, which is generally subtracted from the integrated signal. This final signal value is assumed to be proportional to the abundance of the target sequence in the sample to which the probe is directed. This one-dye technology is typically used with oligonucleotide arrays. The cDNA microarrays usually use two different fluorescent dyes i.e. two RNAs (e.g. RNA from diseased and reference samples) are labeled with two different dyes and hybridized competitively on one cDNA microarray. In these assays, the numerical reading is transformed to a ratio equal to the relative abundance of the target sequence from a diseased sample with respect to a reference sample. (Hovatta *et al.* 2005, Trevino *et al.* 2007.)

Several statistical approaches to normalize, filter and analyze gene expression data have been described (Kim *et al.* 2006, Mutch *et al.* 2001). To correct systematic errors that occur during labeling, hybridization and scanning procedures, normalization is performed to generate values that can be compared between experiments. During the data analysis, differentially expressed genes are found using statistical tools. For large-scale data, special correction methods in statistical tests are performed to control the false positive rate. The most common

correction method is the False Discovery Rate by Benjamini and Hochberg (Benjamini & Hochberg 1995). The data can be grouped by clustering, which means finding similarly behaving genes or patterns related to time scale, developmental phase or treatment of the sample. Further analysis of differentially expressed genes consists of data mining, including annotation searches, function predictions, promoter analysis as well as inferring gene regulatory networks from gene expression data (Hovatta *et al.* 2005, Trevino *et al.* 2007).

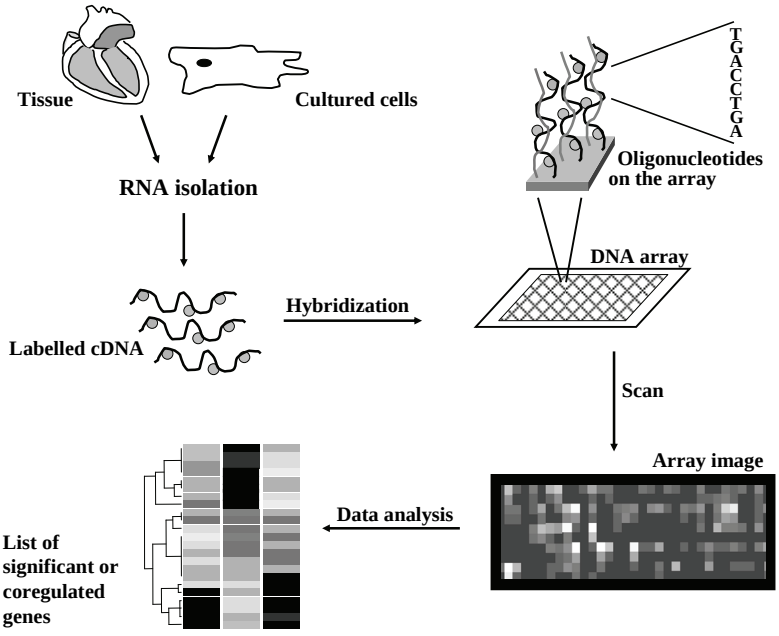

Fig. 5. Schematic representation of a one-dye DNA microarray experiment. First, RNA is isolated from tissue or cells, and then the RNA is labeled with a fluorescent dye and hybridized to a DNA array containing thousands of oligonucleotide probes. Finally, the image is scanned and the signal intensity values are calculated. Data analysis includes transformation of these signals into a list of up- or downregulated genes with statistical significance. Modified from Napoli *et al.* 2003.

Since the first report on DNA microarrays appeared (Schena *et al.* 1995), microarray technology has been criticized for poor reproducibility and lack of standardization. The first cDNA arrays were compromized by restricted sensitivity, limited dynamic range, poor reproducibility and outliers. Continuous improvements have occurred in the scope, power, and cost efficiency as well as in

the achievement of technical consistency and standardization. Minimal Information About a Microarray Experiment (MIAME) standards for microarray experiments have been established to define the minimal amount of information needed to interpret and reproduce a microarray experiment (Brazma *et al.* 2001). Recently, the MicroArray QualityControl (MAQC) project, consisting of participants from academia, government and industry, concluded that results were mostly reproducible among different laboratories (Canales *et al.* 2006, MAQC Consortium *et al.* 2006). Oligonucleotide arrays were found to have better reproducibility and sensitivity when compared to cDNA arrays (Canales *et al.* 2006). The second phase of the MAQC is currently under way and is aiming to establish the applicability of microarray data to clinical settings. Of note, because the published gene expression data is increasing every day, different methods for meta-analysis i.e. integrated analysis of several studies have been developed for making possible the detection of generalities and abnormalities of gene expression in diseases (Choi *et al.* 2003, Elo *et al.* 2005, Kano *et al.* 2005, Rhodes *et al.* 2004, Stevens & Doerge 2005).

DNA microarrays were initially designed to measure gene expression levels. More recent uses of DNA microarrays, however, are not limited to gene expression. DNA microarrays are being used to detect aberrations in methylation patterns (DNA methylation state profiling) (Yan *et al.* 2001), alterations in gene copy number (array-based comparative genomic hybridization) (Pollack *et al.* 1999), alternative RNA splicing (Relogio *et al.* 2005) and pathogens (Conejero-Goldberg *et al.* 2005, Wang *et al.* 2002). The applications of DNA microarrays include analysis of regulatory elements such as chromatin immunoprecipitation on arrays (ChIP-on-chip) (Kim & Nam 2006) and microRNA expression profiling (Buck & Lieb 2004). In addition, genome-wide association studies offer a powerful approach for mapping genes involved in human diseases (Hirschhorn & Daly 2005).

2.7 Gene expression profiling of cardiac diseases

The first attempts to generate large-scale screens of cardiac gene expression used cardiovascular cDNA libraries, containing about 85 000 expressed sequence tags (ESTs) (Hwang *et al.* 1997). *In silico* analysis (computer based comparisons of EST expression profiles among human cDNA libraries) were used to study differential gene expression between the normal and hypertrophied heart (Hwang *et al.* 1997, Hwang *et al.* 2000). Since then, microarray technology has provided

the opportunity for comprehensive molecular and genetic profiling of cardiovascular diseases.

In addition to the gene expression profiles of cardiac hypertrophy and heart failure referred to in detail later, cardiac transcriptomes of animal models including atrial fibrillation (Cardin *et al.* 2007), diabetic cardiomyopathy (Glyn-Jones *et al.* 2007), hypothyroidism (Le Bouter *et al.* 2003), ischemia (Simkhovich *et al.* 2003), ischemic preconditioning (Depre *et al.* 2001, Onody *et al.* 2003, Roy *et al.* 2006, Simkhovich *et al.* 2002, Zingarelli *et al.* 2004, Zubakov *et al.* 2003), obesity related hypertension (Philip-Couderc *et al.* 2003) and right ventricular hypertrophy (Buermans *et al.* 2005) have been determined. Human gene expression profiles of patients with atrial fibrillation (Barth *et al.* 2005, Kim *et al.* 2003, Lamirault *et al.* 2006, Ohki-Kaneda *et al.* 2004, Ohki *et al.* 2005), chronic ischemia (Wang *et al.* 2006), congenital heart disease (Kaynak *et al.* 2003, Sharma *et al.* 2006) coronary artery disease (Chittenden *et al.* 2006, Gabrielsen *et al.* 2007) and giant cell myocarditis (Kittleson *et al.* 2005b) have also been analyzed.

In addition to scientific publications, extensive data sets are available from the Genomics of Cardiovascular Development, Adaptation, and Remodeling, NHLBI Program for Genomic Applications (PGA), at Harvard Medical School (http://www.cardiogenomics.org). CardioGenomics PGA is focused on functional genomics research related to heart, and provides e.g. large, publicly available gene expression profile data sets of mouse models of cardiomyopathies, normal mouse hearts throughout development and human tissues from heart failure patients.

2.7.1 Human heart failure

To date DNA microarrays have been widely used for gene expression profiling of human heart failure (Table 3).

Table 3. Overview of DNA microarray studies of cardiac hypertrophy and heart failure in humans.

Study	Platform	Transcripts	Reference
Failing vs. non failing			
1 ICM, 1 DCM and 2 controls	Hu 6800 (Affymetrix)	~7 100	Yang *et al.* 2000
2 HCM and 2 controls	Custom cDNA array	~10 400	Barrans *et al.* 2001
7 DCM and 5 controls	Custom cDNA array	~10 800	Barrans *et al.* 2002
3 DCM, 2 HCM and 3 controls	Custom cDNA array	~10 300	Hwang *et al.* 2002
8 DCM and 7 controls	Hu 6800 (Affymetrix)	~7 100	Tan *et al.* 2002
2 DCM and 2 controls	UniGem V (Incyte)	~10 200	Boheler *et al.* 2003
10 DCM and 4 controls	Custom cDNA array	~30 300	Grzeskowiak *et al.* 2003
1 ICM, 1 DCM and 2 controls	HG U95A (Affymetrix)	~12 600	Steenman *et al.* 2003
6 DCM and 5 controls	Custom cDNA array	~1 900	Steenbergen *et al.* 2003
5 DCM, 3 ICM and 5 controls	HG U95Av2 (Affymetrix)	~12 600	Kaab *et al.* 2004
9 ICM, 4 DCM and 1 controls	HG U133A (Affymetrix)	~22 000	Yung *et al.* 2004
9 CCC, 7 DCM and 4 controls	Custom cDNA array	~10 400	Cunha-Neto *et al.* 2005
8 IDCM, 7 CAD and 2 controls	Custom cDNA array	~440	Steenman *et al.* 2005
86 ICM, 108 IDCM and 16 controls	HG U133A (Affymetrix)	~22 000	Hannenhalli *et al.* 2006
Pre- and post-left ventricular assist device (LVAD)			
3 DCM and 3 ICM	Hu 6800 (Affymetrix)	~7 100	Blaxall *et al.* 2003b
7 DCM	HG U133A (Affymetrix)	~22 000	Chen *et al.* 2003b
4 ICM, 5 IDCM, 1 HCM and 1 GCCM	Custom cDNA array	~12 800	Chen *et al.* 2003a
8 DCM and 11 ICM	HG U133A (Affymetrix)	~22 000	Hall *et al.* 2004
16 ICM and 32 NICM	HG U133A (Affymetrix)	~22 000	Kittleson *et al.* 2004
10 ICM, 21 NICM and 6 controls	HG U133A (Affymetrix)	~22 000	Kittleson *et al.* 2005a
157 HF, 28 LVAD-supported HF, 14 controls	HG U133A (Affymetrix)	~22 000	Margulies *et al.* 2005
Left ventricular hypertrophy			
36 AS and 29 controls	HG U133A (Affymetrix)	~22 000	Heymans *et al.* 2005)

ICM, ischemic cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; CCC, chronic Chagas cardiomyopathy; CAD, coronary artery disease; IDCM, idiopathic dilated cardiomyopathy; NICM, non-ischemic cardiomyopathy; GCCM, giant cell cardiomyopathy; HF, heart failure; AS, aortic valve stenosis.

Most of the studies compared failing to non-failing hearts, representing end-stage cardiomyopathies, where cardiac explants are from individuals undergoing transplantation and non-failing (i.e. control) hearts are from rejected donor hearts. The challenges of human heart failure microarray studies are the number and the uniformity of patient samples. The anatomical origin of the tissue, etiology, duration and severity of the disease as well as basic clinical variables such as age

and gender can have a significant influence on gene expression (Boheler *et al.* 2003, Kaab *et al.* 2004). Nevertheless, an increase in the expression of genes encoding sarcomeric, ECM and cytoskeletal proteins was seen in almost all DNA microarray analyses between failing and non-failing hearts, underscoring the importance of myocardial matrix remodeling in the failing myocardium. At the level of single genes, ANP, BNP and small leucine-rich repeat proteoglycans (such as lumican, fibromodulin, decorin and biglycans) constituted the most reproducible groups of genes with increased expression in failing hearts. (Barrans *et al.* 2002, Boheler *et al.* 2003, Hannenhalli *et al.* 2006, Hwang *et al.* 2000, Hwang *et al.* 2002, Steenman *et al.* 2003, Tan *et al.* 2002, Yang *et al.* 2000, Yung *et al.* 2004). The role of matrix remodeling at the level of collagen synthesis and degradation was further elucidated in chronic pressure overload-induced left ventricular hypertrophy (Heymans *et al.* 2005). The comparison between gene expression profiles between aortic stenosis and control samples without LVH, showed that increased expression of TIMP-1 and TIMP-2 was related to the degree of interstitial fibrosis (Heymans *et al.* 2005).

In the other approach, gene expression profiles before and after placement of a left ventricular assist device (LVAD) have been compared (Table 3). A LVAD is a mechanical pump-type device, surgically implanted to give circulatory support in end-stage human heart failure. Importantly, LVAD support can result in beneficial reverse remodeling both in myocardial structure and function (Burkhoff *et al.* 2006). Studies comparing pre- and post-LVAD gene expression offer a possibility to study the effect of mechanical unloading in paired human left ventricular samples. However, the use of myocardial biopsies in these studies raises additional problems with the site of tissue acquisition and often requires RNA amplification. Interestingly, the studies comparing pre- and post-LVAD gene expression profiles, showed differential expression in genes associated with metabolism (Blaxall *et al.* 2003b) and vascular organization (Hall *et al.* 2004) as well as downregulation in mRNA levels of cytokines (Chen *et al.* 2003b) and natriuretic peptides (ANP and BNP) (Chen *et al.* 2003a).

Altogether, these studies demonstrate that mechanical support of a failing myocardium can induce significant changes at the level of gene expression. However, similar gene expression patterns have been identified both in the failing and LVAD-unloaded hearts (Margulies *et al.* 2005). Comprehensive analysis comparing transcriptomes of non-failing hearts with those of failing and LVAD-supported hearts, suggested that the alterations in gene expression after LVAD are distinct from a return toward the normal state and may not represent a simple

reversal of changes observed during the disease progression (Margulies *et al.* 2005).

To screen transcription factors regulating myocardial gene expression in human heart failure, gene expression data was integrated with genome sequence data in a computational *in silico* approach (Hannenhalli *et al.* 2006). First, differentially expressed genes between failing and non failing human hearts were determined and then -5-kb of the 5'-flanking genome sequences of these heart failure genes were analyzed for overrepresented transcription factor binding sites. The study indicated that several well known cardiac transcription factor families identified in murine models of cardiac hypertrophy and heart failure, such as MEF-2, NKX, NFAT, GATA and Forkhead Box (FOX) were associated also with human heart failure.

In summary, despite large differences in the array design and experimental conditions, the results are often complementary and implicate a common gene expression profile of the failing heart. DNA microarrays have facilitated the delineation of a better cardiomyopathy classification (Ashrafian & Watkins 2007, Donahue *et al.* 2006, Sanoudou *et al.* 2005), since genomic profiling has indicated that different forms of cardiomyopathy, including dilated cardiomyopathy (Cunha-Neto *et al.* 2005, Hwang *et al.* 2002), ischemic and nonischemic cardiomyopathy (Kittleson *et al.* 2004), alcoholic (Tan *et al.* 2002), hypertrophic (Hwang *et al.* 2002), and Chagas' cardiomyopathy (Cunha-Neto *et al.* 2005) can be distinguished by their molecular phenotypes. In addition, some studies have investigated the possibility of using gene expression profiling to predict the prognosis after LVAD placement, although the results are somewhat controversial. In the study of Blaxall *et al.* (2003) DNA microarrays were used to predict recovery after placement of a LVAD. However, the results were not confirmed in the re-analysis by another research group, demonstrating the influence of the statistical method used (Hall *et al.* 2004). In another study, gene expression patterns of nonfailing hearts were compared with hearts of cardiomyopathy patients who required LVAD before cardiac transplantation and those who did not have an LVAD before transplantation. The study implied that non-LVAD patients resembled nonfailing patients more than their failing counterparts, who required an LVAD before cardiac transplantation (Kittleson *et al.* 2005a). In conclusion, although there are some promising studies supporting the role of DNA microarrays in cardiovascular disease diagnostics and prognostics, the technique still has some limitations for routine clinical use.

2.7.2 *In vivo models of cardiac hypertrophy and heart failure*

Animal models of human cardiovascular diseases permit studies e.g. of cardiac hypertrophy and chronic heart failure in various stages of the disease as well as investigation of the mechanisms of pathogenesis and the effects of drug interventions (Muders & Elsner 2000). Rat and mouse are the most widely-used animal models in cardiovascular research, and can not be fully replaced by cell culture models, since *in vitro* models do not allow investigating the integrative function of the heart in the whole organism.

Physiological hypertrophy

Chronic exercise training is known to cause a number of adaptations in the heart resulting in improved cardiac performance and physiological hypertrophy (Dorn 2007). Several studies have used DNA microarrays to evaluate the effect of exercise in rats at the level of gene expression (Table 4). Interestingly, despite the clear evidence of left ventricular hypertrophy in response to the training program, no induction of a fetal-gene program was observed (Diffee *et al.* 2003, Iemitsu *et al.* 2005, Strom *et al.* 2005). In fact, a significant decrease in the expression levels of ANP, skα-A and GLUT-1 was seen in the trained ventricular tissue after 11 wks of exercise (Diffee *et al.* 2003).

Table 4. Overview of gene expression profiling studies of physiological hypertrophy.

Study	Platform	Transcripts	Reference
Exercise (treadmill) for 11 wk	RG U34A (Affymetrix)	~8 800	Diffee *et al.* 2003
Exercise for 8 wk (treadmill)	Atlas Glass microarray 3.8.I Rat Clontech)	~3 800	Iemitsu *et al.* 2005
Exercise (treadmill) for 6 wk vs. Dahl salt-sensitive rats (6% NaCl diet for 6 and 15 wk)	RG U34A (Affymetrix)	~8 800	Kong *et al.* 2005
Exercise (treadmill) for 7 wk	RG U34A (Affymetrix)	~8 800	Strom *et al.* 2005

The differences between genetic responses of pathological and physiological hypertrophy were evaluated in Dahl salt-sensitive rats (Kong *et al.* 2005). Physiological hypertrophy was generated by daily exercise whereas pathological hypertrophy was induced by feeding a sodium diet. Despite the development of a comparable degree of hypertrophy in both models, there were significant differences at the gene expression level. An increase in the stress genes was

characterized in pathological hypertrophy. Furthermore, distinct changes in ANP and BNP gene expression were seen in the two conditions. Upregulation of ANP gene expression was seen in physiological hypertrophy (2.3-fold), a greater one in pathological hypertrophy (6.1-fold) and the biggest induction of ANP mRNA levels was noted in heart failure (14.3-fold). Interestingly, there was a significant increase in BNP mRNA levels during the pathological hypertrophy but not in physiological hypertrophy. The expression levels of several known hypertrophic response genes were changed in both physiological and pathological hypertrophy, including phospholamban, GSK3β and UCP-2 (Kong *et al.* 2005). There were an almost equal number of genes that showed altered expression predominately in pathological hypertrophy or in both pathological and physiological hypertrophy. The number of genes that changed only in physiological hypertrophy was 50 percent smaller, and included genes involved in metabolism and cellular growth, such as members of the IGF/EGF signaling pathway (Kong *et al.* 2005).

Hypertensive rat strains

Several DNA microarray analyses of hypertensive rat strains have been done (Table 5).

Table 5. Overview of the gene expression profiling studies in hypertensive rat strains.

Study	Platform	Transcripts	Reference
Dahl salt-sensitive rats			
High (8%) vs. low (0.3%) NaCl diet for 6, 8, 11, 13 and 15 wk	RG U34A (Affymetrix)	~8 800	Ueno *et al.* 2003
Dahl salt-sensitive rats			
6% NaCl diet for 6 and 15 wk	RG U34A (Affymetrix)	~8 800	Kong *et al.* 2005
SHR vs. WKY rats	RAE230A (Affymetrix)	~16 000	Cerutti *et al.* 2006
TGR(mRen2)27 rats vs. nontransgenic littermates	RAE230A (Affymetrix)	~16 000	Cerutti *et al.* 2006
Lyon hypertensive rats vs. Lyon low blood pressure control rats	RAE230A (Affymetrix)	~16 000	Cerutti *et al.* 2006

TGR, transgenic rat

To determine gene expression patterns associated with LVH, left ventricular gene expression profiles of three hypertensive rat models: spontaneously hypertensive rat (SHR), Lyon hypertensive rat (LH), transgenic rat TGR(mRen2)27 and their respective age-matched controls (Wistar-Kyoto rat [WKY] and Lyon low blood pressure [LL] control strains as well as $TGR^{-/-}$ nontransgenic littermates) were

studied (Cerutti *et al.* 2006). SHR and LH are genetically derived hypertensive inbred rat strains, whereas TGR(mRen2)27 is a transgenic rat carrying an additional mouse renin gene (Ren2) (Mullins *et al.* 1990). The rats were studied at the age of 12 months, during the early stage of LVH, before the development of any heart dysfunction. Interestingly, only one gene, sialyltransferase 7A, was overexpressed in all three hypertensive rat strains compared with their respective controls, whereas a large number of genes (~200) showed differential expression both in SHR vs. WKY and in LH vs. LL rats. The correlation analysis between gene expression data and the LVW/BW index revealed a large set of genes whose expression was correlated with the LVW/BW index, including known genes associated with cardiac remodeling such as TIMP-1, TGFβ2 and Down's syndrome critical region homolog 1 (Cerutti *et al.* 2006). Finally, the differentially expressed genes, which correlated with the LVW/BW index, were localized along the chromosomes. This analysis identified chromosomal regions that are often located within blood pressure and cardiac mass quantitative trait locis (QTLs) identified in SHR or LH rats. Thus, a set of genes whose expression correlates with the LVW/BW index at an early stage of LVH, seems to be located within restricted chromosomal regions involved in cardiovascular diseases (Cerutti *et al.* 2006).

In Dahl salt-sensitive rats upregulation of the stress genes such as genes involved in inflammation (arachidonate 12-lipoxygenase), oxidative stress, and acute stress response (heat shock proteins) were identified during the development of cardiac hypertrophy. Genes associated with the apoptosis pathway also showed significantly altered levels of expression. During decompensated heart failure, there was even more impressive activation of stress genes. Moreover, during heart failure there was a high activation of inflammatory response genes, such as the pancreatitis associated protein and arachidonate 12-lipoxygenase (Kong *et al.* 2005).

On the other hand, the amount of sodium in the diet causes alternative genetic responses in Dahl salt-sensitive rats. In the study of Ueno *et al.* 2003, Dahl salt-sensitive rats were fed either a high- or a low-sodium diet for 15 weeks. During the time-course studied, rats with a high-sodium diet developed cardiac hypertrophy, which subsequently progressed to heart failure, whereas rats with a low-sodium diet developed only a modest degree of hypertrophy. As expected, expression levels of many genes were dependent on the amount of sodium in the diet. For example the left ventricular expression profiles of ANP, β-actin, MHC,

lipoxygenase, aldolase A and α-tubulin differed markedly between high-sodium and low-sodium rats (Ueno *et al.* 2003).

Transgenic animals

Transgenic and knockout animal models are important tools to investigate the physiological roles of individual genes in the development of cardiac hypertrophy and heart failure, and a large number of genetically engineered mouse and rat models with altered cardiac function have been generated. Transgenic studies have implicated a variety of genes to be involved in the hypertrophic process. Such genes include calmodulin (Gruver *et al.* 1993), L-type voltage-dependent calcium channel (Muth *et al.* 2001), PKCβ2 (Wakasaki *et al.* 1997), calcineurin (De Windt *et al.* 2001) and periostin (Oka *et al.* 2007a). However, transgenic approaches have limitations, especially because of the interdependence of genes and the capacity of animals to develop compensatory mechanisms to maintain fundamental physiological functions (Chu *et al.* 2002).

Several gene expression profiling studies of transgenic and knockout models exhibiting cardiac hypertrophy have been analyzed. These include the ablation of the atrial natriuretic peptide precursor (Wang *et al.* 2003), muscle LIM protein (Blaxall *et al.* 2003a), nitric oxide synthases 1 and 3 (Cappola *et al.* 2003) as well as the overexpression of Akt (protein kinase B) (Cook *et al.* 2002, Schiekofer *et al.* 2006), IGF-receptor (McMullen *et al.* 2004b) and dominant negative MEF-2 (van Oort *et al.* 2006). Furthermore, the effect of the diverse mutations in the α-tropomyosin gene have been evaluated (Prabhakar *et al.* 2003, Rajan *et al.* 2006) as well as gene expression profiles of TGR(mRen2)27 rats (Cerutti *et al.* 2006, Kurdi *et al.* 2004, Schroen *et al.* 2004) and double transgenic rats [dTGR(hAOGEN-hREN)] harboring both the human angiotensinogen and renin genes (Wellner *et al.* 2005). These gene expression profiling studies of transgenic and knockout models of cardiac hypertrophy have provided insights into the underlying genetic determinants of the hypertrophic response. For example, the proapoptotic mitochondrial protein Nix was identified based on DNA microarray analysis of Gq-transgenic hearts, (Aronow *et al.* 2001) and subsequent studies demonstrated an important role of Nix in the transition from hypertrophy to heart failure (Yussman *et al.* 2002).

Interestingly, there was only little in common when gene expression profiles of four transgenic strains and their non transgenic littermates were compared (Aronow *et al.* 2001). The striking finding was that when transgenic mouse

models of protein kinase C-ε activation peptide (ψεRACK), $G\alpha_q$, calcineurin and calsequestrin were compared, there was no single differentially expressed gene in common among these four models of cardiac hypertrophy. In fact, only ANP was coregulated in the three pathological models ($G\alpha_q$, calsequestrin and calcineurin). These results may be due to compensatory changes in response to gene perturbations. Alternatively the disease profiles of these models are not appropriately phenotyped and staged (Glueck *et al.* 2001). The comparison of left ventricular gene expression profiles of three hypertensive rat models, SHR, LH and TGR(mRen2)27 revealed similar results, since only one gene was similarly regulated in all three hypertensive rat strains, whereas a large number of parallel gene expression changes were observed in two inbred strains (Cerutti *et al.* 2006). Furthermore, cardiac transgenesis with a tetracycline transactivator was shown to cause significant effects on myocardial gene expression and function (McCloskey *et al.* 2005). Thus, caution is needed when interpreting gene expression profiling results of transgenic and knockout models.

Pharmacological models

Pharmacologic inducers of hypertrophy such as Ang II and isoproterenol have been used to study the hypertrophic gene expression program *in vivo* (Table 6). One of the first DNA microarray studies of cardiac hypertrophy employed two hormone-induced hypertrophy models in mice (Friddle *et al.* 2000). The gene expression profiles were investigated during the induction and regression of Ang II and isoproterenol induced cardiac hypertrophy. The study lists the gene expression changes observed in both models, but the apparent drug-specific effects on gene expression were not reported. Of the genes, which showed altered expression during the induction phase of cardiac hypertrophy, equal numbers showed increases and decreases in expression. Well-established hypertrophy associated genes such as ANP and BNP were among the upregulated genes, whereas the expression of genes participating in energy metabolism was decreased. Genes that altered both during induction and regression periods of the cardiac hypertrophy belonged to several functional classes, but the genes that showed altered expression only during the regression of hypertrophy were mainly unknown transcripts (Friddle *et al.* 2000).

Table 6. Overview of gene expression profiling studies of pharmacological induction of cardiac hypertrophy *in vivo*.

Study	Platform	Transcripts	Reference
Isoproterenol (7 d) and Ang II (2 wk) infusion in mice	Custom cDNA array	~3 000	Friddle *et al.* 2000
Ang II infusion for 24 h and 2 wk in mice	Custom cDNA array	~27 000	Larkin *et al.* 2004
Norepinephrine infusion for 0.5, 1, 2, 3 and 7 days in SD rats	GF300 cDNA array (Invitrogen)	~5 300	Li *et al.* 2003
3,5-diiodothyropropionic acid (Ditpa) administration and Ang II infusion for 2 wk in rats.	RG U34A (Affymetrix)	~8 800	Strom *et al.* 2004

When the effect of acute (24 h) and chronic (2 wk) Ang II treatments on cardiac gene expression were investigated, the majority of the differentially expressed genes responded to acute Ang II treatment (Larkin *et al.* 2004). Both acute and chronic treatments resulted in decreased expression of mitochondrial metabolic genes, including GLUT-1, GLUT-4, muscle CPT-1 and UCP-3. Similarly, increased expression of genes involved in protein translation and ribosomal activity, such as three translation initiation factors, was seen following both acute and chronic Ang II treatments. Acute Ang II treatment increased expression of genes involved in oxidative stress, including two superoxidase dismutases, and amino acid metabolism, whereas chronic treatment increased expression of cytoskeletal and ECM genes, such as fibronectin, osteonectin and a wide range of collagens. In addition, chronic Ang II treatment also resulted in decreased expression of genes involved in fatty acid metabolism. (Larkin *et al.* 2004). The findings of this study may provide new insights into possible mechanisms of hypertension induced tissue damage related to activation of the renin-angiotensin-aldosterone system.

The cardiac myocyte-specific changes in gene expression during norepinephrine-induced cardiac hypertrophy were studied in a model, in which cardiomyocytes were isolated from the left ventricle by collagenase digestion after continuous norepinephrine infusion *in vivo* (Li *et al.* 2003). The clustering analysis revealed that several enzymes involved in energy metabolism, including carnitine octanolyltransferase, ATP synthase subunit c and glycogen phosphorylase were elevated at the early stage of norepinephrine infusion, whereas downregulation of several genes encoding cell signaling molecules such as connexin 43 and glypican 3 were seen at the late stage of infusion. (Li *et al.* 2003).

The study of Strom *et al.* (2004) analyzed gene expression profiles between different models of cardiac hypertrophy, including aortic banding, myocardial infarction, an arteriovenous shunt as well as angiotensin II and thyroxin analogue induced hypertrophy. The differences in genetic response were seen primarily between surgical models and the pharmacological models, which could be explained by the selective nature of hormone stimulation as compared to more complex stimuli in surgical models (Strom *et al.* 2004).

Surgical models

Surgical models, such as ligation of the left anterior descending coronary artery (LAD), pressure overload via aortic banding, and volume overload via an aorto-caval fistula have been used to study the molecular physiology of cardiac hypertrophy. To date, several microarray studies using murine models of experimental myocardial infarction have been published (Table 7).

Table 7. Overview of the DNA microarray studies of experimental myocardial infarction.

Study	Platform	Transcripts	Reference
24 h after LAD ligation in rats	Atlas mouse cDNA array (Clontech)	~600	Lyn *et al.* 2000
1, 3, 7 d and 2, 4, 12 wk after LAD ligation in mice	cDNA array (Synteni Inc.)	~1 100	Sehl *et al.* 2000
2, 4, 8, 12, 16 wk after LAD ligation in rats	cDNA array (Incyte Pharmaceuticals)	~4 300	Stanton *et al.* 2000
8 wk after LAD ligation in rats with and without captopril treatment	RG U34A (Affymetrix)	~8 800	Jin *et al.* 2001
1 h after ligation of LAD in mice	Gene Discovery cDNA array (Genome systems Inc.)	~18 400	Kitakaze *et al.* 2001
4 wk after LAD ligation in rats; RV vs. LV was compared	Custom cDNA array	~13 800	Chugh *et al.* 2003
1 wk after LAD ligation in mice	MG 11k, A and B arrays (Affymetrix)	~12 500	Mirotsou *et al.* 2003
1 wk after LAD ligation in mice	Atlas Mouse 1.2 cDNA array (Clontech)	~1 200	Finsen *et al.* 2004
3 and 9 wk after LAD ligation in rats	RG U34A (Affymetrix)	~8 800	Strom *et al.* 2004
1, 4, 24, 48 h, 1 wk, 8 wk after LAD ligation in mice	MG U74Av2 array (Affymetrix)	~12 700	Tarnavski *et al.* 2004
24 h, 28 d after LAD ligation in rats	10K Uniset Array (Amersham)	~9 900	LaFramboise *et al.* 2005
¼, 1, 4, 12, 24, 48 h after LAD ligation in mice	MG U74A, B, C v2 arrays (Affymetrix)	~37 000	Harpster *et al.* 2006

RV, right ventricle; LV, left ventricle; LAD, left anterior descending coronary artery.

However, direct comparison of these studies is difficult, because of the variation regarding the number of replicates, experimental design, microarray platform and statistical analysis that has been used. Temporal gene expression profiles have been analyzed during post-infarction remodeling ranging from 1 hour up to 16 weeks after myocardial infarction. In addition, the chamber-specific ventricular gene expression patterns (Chugh *et al.* 2003) and the effect of angiotensin converting enzyme (ACE)-inhibition on cardiac gene expression have been studied in an experimental rat model of myocardial infarction (Jin *et al.* 2001).

Taken together, DNA microarray studies of cardiac remodeling after myocardial infarction have confirmed the region-specific differences in gene expression in the heart as well as different transcriptional alterations during the acute and late phase of post-infarction remodeling (Harpster *et al.* 2006, LaFramboise *et al.* 2005, Mirotsou *et al.* 2003, Tarnavski *et al.* 2004). As expected, the largest number of differentially expressed genes was measured in the infarct zone in comparison to the surviving free wall or the interventricular septum of the infarcted myocardium (Harpster *et al.* 2006, LaFramboise *et al.* 2005, Mirotsou *et al.* 2003). During the immediate post-infarction period (at 24 hours), down-regulation of genes involved in signal transduction, transcription, metabolism, inflammation, proteolysis, cell proliferation and ion channel/transport in the infarct-zone was matched by reciprocal activation of these genes in the remaining left ventricle. Furthermore, the activation of genes associated with matrix remodeling (such as MMPs 9, 12 and 23) and cell proliferation was seen in the remote zone of the left ventricle at day one, but in the infarct zone the activation of these genes was seen not until 4 weeks after LAD ligation (LaFramboise *et al.* 2005). Notably, LAD ligation did not induce a significant acute phase response in the infarct zone during the first 24 hours. On the contrary, the inflammatory response as well as activation of detoxification and anti-oxidant genes was seen in the remote zone within 24 hours, and in the infarcted area by day 28 (LaFramboise *et al.* 2005).

Transcription factor AP-1 was found to be central in the early (within 48 hours) transcriptional regulation of post-myocardial infarction, since the biological processes controlled by AP-1 activation constituted approximately 30% of total number of differentially expressed genes after acute myocardial infarction (Harpster *et al.* 2006). In addition, the regulation of nitric oxide/polyamine metabolism was altered in the early post-ischemic events (Harpster *et al.* 2006).

An overview of DNA microarray studies of *in vivo* pressure and volume overload models of cardiac hypertrophy and heart failure are given in Table 8.

Table 8. Overview of left ventricular gene expression profiles of pressure and volume overload models of cardiac hypertrophy and heart failure.

Study	Platform	Transccripts	Reference
Pressure overload models			
TAC in mice for 1 wk	MG 11k, A and B arrays (Affymetrix)	~12 500	Mirotsou *et al.* 2003
TAC in mice (48 h, 10 d and 3 wk)	MG U74Av2 (Affymetrix)	~12 500	Zhao *et al.* 2004
TAC in rats for 2 wk, then constriction relief (1, 3, 7 d)	CodeLink Uniset Rat I Bioarray (Amersham)	~9 900	Yang *et al.* 2007b
TAC in mice (1 d and 30 wk; both sexes).	MG U74Av2 (Affymetrix)	~12 500	Weinberg *et al.* 2003
Ascending aortic banding for 6, 12, 16 and 30 wk in rats	RG U34A (Affymetrix)	~8 800	Strom *et al.* 2004
Abdominal aortic banding for 28 d in rats	RG U34A (Affymetrix)	~8 800	Miyazaki *et al.* 2006
TAC in mice for 10 and 30 wk	MG U74Av2 (Affymetrix)	~12 500	Mirotsou *et al.* 2006
Volume overload models			
Aorto-caval shunting for 3 and 8 wk in rats	RG U34A (Affymetrix)	~8 800	Strom *et al.* 2004
Aorto-caval shunting for 28 d in rats	RG U34A (Affymetrix)	~8 800	Miyazaki *et al.* 2006

TAC, transverse aortic constriction.

In time series studies of development of cardiac hypertrophy induced by transverse aortic constriction (TAC), the largest number of differentially expressed genes have been associated both with acute (Weinberg *et al.* 2003) and chronic (Zhao *et al.* 2004) phases of the hypertrophic process. In the study of Zhao *et al.* (2004), the largest numbers of upregulated transcripts were in the functional groups related to metabolism, cell growth and cell communication. For example, at 48 hours upregulation of BNP and at 10 days upregulation of Ki-67 antigen mRNA levels were seen in response to pressure overload. The gene expression levels of skα-A, biglycan, periostin and TSP-1 were elevated from 10 days up to 3 weeks during the pressure overload. Notably, the induction of mRNA levels of several immediate early response genes such as *Egr-1, Egr-2, Jun B* and *c-fos* was seen only after 10 days of pressure overload (Zhao *et al.* 2004).

Temporal dependence as well as gender dependence of the left ventricular genomic response to pressure overload was studied in male and female mice at day one and 30 weeks after TAC (Weinberg *et al.* 2003). Upregulated genes were predominant in both males and females during acute and chronic pressure overload, although distinct expression profiles with no overlap were seen during acute and chronic overload. ANP, BNP and connective tissue growth factor

(CTGF) were among the genes co-regulated in males and females during the chronic overload. Several genes, including those encoding the serine protease inhibitor, lipocalin, LIM protein, eukaryotic translation initiation factor eIF-4AI, TSP-1, catalase and caveolin, showed differential degrees of upregulated expression between males and females. Furthermore, immunity, inflammation and stress response genes were strongly represented in clusters upregulated in males, but unchanged in females, during acute pressure overload (Weinberg *et al.* 2003).

The study of Mirotsou *et al.* (2006) identified genes with expression patterns that correlate with parameters of structure and function of left ventricular hypertrophy. Expression profiles were tested for significant correlation e.g. with measurements of left ventricular weight vs. body weight ratio (LVW/BW) and LVESD in a mouse model of TAC. Importantly, gene expression profiling results were validated in two independent data sets. ANP had the highest correlation for LVW/BW (i.e. an index of cardiac hypertrophy), whereas amyloid β-precursor protein binding 1 showed the highest negative correlation to LVW/BW. The gene with the highest correlation to LVESD (an index of left ventricular chamber size and dilation) was periostin, whereas mitochondrial isocitrate dehydrogenase had the highest negative correlation to LVESD. In this study, several genes encoding secreted proteins were found to be associated with hypertrophy, such as biglycan, fibulin-2, matrix Gla protein (MGP), lumican, CTGF, follistatin-like 1 and decorin. However, further studies are needed to evaluate whether the proteins encoded by these genes may be candidates for novel clinical biomarkers of cardiac hypertrophy and heart failure (Mirotsou *et al.* 2006).

The effect of mechanical unloading and regression of cardiac hypertrophy was investigated in a TAC-model by relieving the constriction after significant cardiac hypertrophy had developed (Yang *et al.* 2007b). Fifty-two genes were induced during the regression period. Calgranulin B (S100 calcium binding protein A9) and a transcriptional cofactor eyes absent 2 homologue (*eya2*) were among the putative negative regulators of cardiac hypertrophy.

The effect of the AT1-R antagonist losartan on pressure overload induced cardiac gene expression was studied in a model of suprarenal abdominal aortic constriction (Li *et al.* 2003). The study showed that inhibition of hypertrophy with losartan was accompanied by a dose dependent normalization in myocardial gene expression. For instance, upregulation of ANP and collagen α1 as well as downregulation of genes participating in fatty acid β-oxidation by aortic constriction retained a nearly normal expression level following losartan treatment. However, mRNA levels of the αB crystalline -related protein and

urokinase were not affected by losartan administration, and gene expression of the ribosomal protein L5 was induced by losartan treatment (Li *et al.* 2003).

Another study compared the differences in gene expression profiles between pressure overload and volume overload induced cardiac hypertrophy (Miyazaki *et al.* 2006). The genes that were upregulated in both forms of hypertrophy included BNP, ANP, skα-A and metallothionein-1 (MT-1), whereas downregulation of genes belonging to functional groups of calcium ion binding and the basic-leucine zipper transcription factors were seen. On the other hand, selective upregulation of genes that bind to actin, such as tropomyosin-4, thymosin-4 and transgelin, was seen in the volume overload group suggesting that actin–binding proteins may contribute to the formation of the eccentric morphology induced by volume overload (Miyazaki *et al.* 2006).

To identify common pathways underlying cardiac remodeling, gene expression patterns were evaluated in two different mouse models of cardiac hypertrophy and remodeling i.e. TAC and myocardial infarction (Mirotsou *et al.* 2003). In addition, regional (i.e. left ventricular free wall vs. septum) differences in myocardial gene expression were investigated. Hierarchical clustering of differentially expressed transcripts revealed four main gene clusters that exhibited differential expression patterns in these models. One cluster of genes exhibited a greater response in the TAC-model than in the myocardial infarction model, including ANP, BNP as well as genes involved in the immune response, cytoskeletal organization and biogenesis. The other cluster was enriched for cell cycle and signal transduction genes, whose upregulation was seen in the myocardium after myocardial infarction, but little or no change in gene expression levels were noted in the TAC-model. ECM genes and genes involved in cell adhesion, such as different collagen isoforms, tenascin, periostin, biglycan lysyl oxidase and fibronectin were upregulated in the left ventricular free wall and the septum of the TAC model and in the left ventricular free wall during post-infarction remodeling. Similarly, the cluster enriched for enzyme encoding genes showed downregulation in both models. Overall, the gene expression profiling results suggest that the ECM plays an important role in the response to cardiac hypertrophy. In addition, the study identified several novel genes such as mesenchyme homeobox 1 (Mox-1), nitrogen and perforin not previously associated with hypertrophy (Mirotsou *et al.* 2003).

To identify a common genetic hypertrophic response, gene expression patterns of different models of cardiac hypertrophy were compared, including aortic banding, myocardial infarction, an aorto-caval shunt and pharmacological

induction of hypertrophy (Strom *et al.* 2004). The data suggest the existence of some common feature of hypertrophic remodeling of the myocardium including activation of growth-inducing molecules such as TGFβ-signaling proteins and several ribosomal proteins as well as activation of multiple signaling molecules including those related to the phosphatidylinositol (PI)-pathway and Ca^{2+}-handling. In addition, enhanced remodeling through ECM and cytoskeletal systems as well as induction of defense and inflammation proteins related to coagulation and lymphocyte activation seemed to be essential attributes of hypertrophic remodeling. Furthermore, downregulation of genes involved in energy metabolism suggests a shift away from lipids as the main energy source. (Strom *et al.* 2004).

2.7.3 In vitro models of cardiac hypertrophy

Cardiac cell cultures have been used to study gene expression profiles in response to mechanical stretch, environmental stressors, hypertrophic agonists and growth factors (Table 9).

Table 9. Overview of gene expression profiling studies of cell culture models of cardiac hypertrophic growth.

Treatment and cell type	Platform	Genes	Reference
Insulin-like growth factor-1 in NRCM	Atlas rat 1.2 cDNA array (Clontech)	~1 200	Liu *et al.* 2001
Fibronectin- induced hypertrophy in NRVM	RAE230A (Affymetrix)	~7 000	Chen *et al.* 2004
TGFβ and BNP for 24 and 48h in primary human fibroblasts	Custom cDNA array	~8 600	Kapoun *et al.* 2004
Mechanical stretch for 24 h in NRCM	RG U34A (Affymetrix)	~8 800	Boerma *et al.* 2005
Mechanical stretch for 6 h and olmesartan in NRVM	RG U34A (Affymetrix)	~8 800	Ohki *et al.* 2003
ET-1 or 1-azakenpaullone for 1 h in NRVM	Rat Expression Set 230_2.0 (Affymetrix)	~30 000	Markou *et al.* 2008

NRVM, neonatal rat ventricular myocytes; NRCM, neonatal rat cardiac myocytes.

In the study of Boerma *et al.* gene expression profiles of neonatal cardiac myocytes and fibroblasts were studied after ionizing and ultraviolet radiation as well as mechanical stress in order to characterize the general cellular pathways activated in response to different stressors (Boerma *et al.* 2005). These general stress response genes included genes involved in oxidative stress such as heme

oxygenase-1 and thioredoxin reductase-1. Cyclic mechanical stretch for 24 hrs induced cell-type specific gene expression profiles in neonatal rat cardiac myocytes and fibroblasts, since differentially expressed genes were mostly upregulated in cardiomyocyte cultures, whereas in cultures of fibroblasts the majority of genes were downregulated in reponse to mechanical stretch. A large number of upregulated genes in cardiomyocyte cultures were involved in cholesterol biosynthesis. Notably, a higher number of genes involved in ECM formation, such as collagens, fibronectin and laminin, were differentially expressed in response to mechanical stretch in cardiomyocyte cultures than in cultures of fibroblasts (Boerma *et al.* 2005).

The effect of RNH-6270, the active metabolite of the AT1-R antagonist olmesartan, was studied on mechanical stretch induced gene expression in cultured neonatal rat ventricular myocytes (Ohki *et al.* 2003). Altogether, RNH-6270 did not have a major effect on mechanical stretch induced gene expression. RNH-6270 suppressed gene expression of nine genes (e.g. monoamine oxidase B and retinol-binding protein) and gene expression of twenty-one mechanically-suppressed genes was restored by RNH-6270, including Bcl-Xα, arylamine N-acetyltransferase, transcription factor Sp-1 as well as the major acute phase α1 protein, which carries the sequence for bradykinin (Cole *et al.* 1985). Further studies are needed to determine whether these molecular alterations play a role in mediating the beneficial effects of AT1-R blockers.

Insulin-like growth factor-1 (IGF-1) can affect multiple pathways in the heart and also has an important role in the development of the hypertrophic response (Heineke & Molkentin 2006, Ren *et al.* 1999). DNA microarrays were used to study the effect of IGF-1 on gene expression in cardiomyocytes (Liu *et al.* 2001). IGF-1 modulated the expression of several functional categories, such as cell cycle, cellular respiration and mitochondrial function. Pharmacological inhibitors of ERK and PI3 kinase were used to determine whether IGF-1 regulation of gene expression involves activation of PI3 kinase and ERK signaling pathways. The results showed that the majority of IGF-1 regulated genes require the activation of both ERK and PI3 kinase suggesting that PI3 kinase and ERK coordinately mediate the transcriptional regulatory effects of IGF-1 in cardiac myocytes (Liu *et al.* 2001).

Gene expression changes associated with fibronectin-induced cardiac myocyte hypertrophy were studied in cultured neonatal rat ventricular myocytes (Chen *et al.* 2004). Cardiac myocytes plated on fibronectin displayed several features of hypertrophy and the induction of known hypertrophy markers such as

ANP and BNP was seen. Fibronectin induced several metabolic pathways including a number of enzymes of cholesterol biosynthesis, fatty acid biosynthesis and the mitochondrial electron transport chain. In addition, fibronectin induced the expression of genes encoding ribosomal proteins, translational factors and the ubiquitin proteasome pathway. Interestingly, no increase was seen in the expression of ECM genes such as biglycan, collagens, fibronectin and TIMP-1, whose increased expression have been described during the development of cardiac hypertrophy *in vivo*. Instead, elevated expression of fibrosis-promoting peptides such as CTGF and SPARC (Secreted Protein Acidic and Rich in Cysteine) was noted. (Chen *et al.* 2004). Whether the secretion of CTGF and SPARC by cardiac myocytes could be responsible, at least in part, for upregulation of the ECM genes seen during the development of cardiac hypertrophy *in vivo*, remains to be studied.

Inhibition of GSK-3β has been implicated in cardiac myocyte hypertrophy in response to endothelin-1 or phenylephrine (Haq *et al.* 2000, Morisco *et al.* 2000). The effects of the GSK-3β inhibitor 1-azakenpaullone on gene expression were studied in cardiac myocytes, and the gene expression profiles induced by 1-azakenpaullone were compared with those induced by endothelin-1 (Markou *et al.* 2008). Azakenpaullone was found to promote significant changes in gene expression, but the overall response was less than with endothelin and there was little overlap between the genes identified. Thus, the results suggest that although GSK-3β may contribute to cardiac hypertrophy, it does not contribute extensively to the hypertrophic genetic response induced by endothelin-1 (Markou *et al.* 2008).

The effect of BNP on the TGF-β induced profibrotic process was studied in primary human fibroblasts incubated in the presence or absence of TGFβ and BNP (Kapoun *et al.* 2004). cDNA microarray analysis revealed that BNP treatment inhibited TGFβ-induced effects on gene expression resulting in a reduction of TGFβ-regulated mRNAs related to fibrosis (e.g. collagen 1, fibronectin, CTGF, PAI-1, and TIMP-3), myofibroblast conversion (α-smooth muscle actin 2 and nonmuscle MHC), proliferation (PDGF-A, IGF-1 and FGF-18), and inflammation (COX-2, IL-6, TNF-α induced protein 6, and TNF superfamily member 4). Taken together, the results suggest that BNP may function as an antifibrotic factor during myocardial remodeling in cardiac hypertrophy.

3 Aims of the research

The aim of this study was to characterize cardiac gene expression profiles in experimental models of cardiac load. More specifically, the objectives were:

1. To identify early load-responsive genes in response to acute pressure overload in conscious normotensive rats
2. To investigate changes in gene expression during the development of left ventricular hypertrophy and the transition to diastolic heart failure in an experimental animal model of genetic hypertension
3. To study the effect of p38 MAPK activation on cardiac gene expression using adenovirus-mediated intramyocardial gene transfer
4. To examine temporal gene expression patterns during the mechanical stretch induced hypertrophy in neonatal rat ventricular myocytes

4 Materials and methods

A summary of experimental protocols is presented in Table 10.

Table 10. Summary of the experimental models and methods.

Study	Experimental model	Methods
I	Pressure overload (AVP or Ang II) *in vivo*	Cloning
		cDNA array
		Hemodynamic measurements
		Northern Blot
II	Genetic rat model of hypertension: SHR	Cloning
		DNA microarray
		Echocardiography
		Northern Blot
		Real time quantitative RT-PCR
III	Adenovirus-mediated gene transfer *in vivo*	DNA microarray
		Echocardiography
		Electrophoretic mobility shift assay
		Immunohistochemistry
		Kinase activity assays
		Northern Blot
		Real-time quantitative RT-PCR
		Western blot
IV	Cardiac myocyte cell culture and	DNA microarray
	mechanical stretching	Northern Blot
		Real-time quantitative RT-PCR
		Western blot

AVP, arginine^{8}-vasopressin; Ang II, angiotensin II, RT-PCR, reverse transcriptase polymerase chain reaction; SHR, spontaneously hypertensive rat.

4.1 Experimental animals

Male 2- to 3-month-old Sprague-Dawley (SD) rats weighing from 250 to 300 g (I, III) and newborn, 2- to 4-day-old SD rats of both sexes (IV) from the colony of the Centre of Experimental Animals at the University of Oulu were used. Male 12-, 16- and 20-month old SHR of the Okamoto-Aoki strain and their age-matched WKY rats from the colony of the Centre of Experimental Animals at the University of Oulu, were also used (II). The SHR strain was originally obtained from Møllegaards Avslaboratorium, Skensved, Denmark.

All rats were kept in plastic cages with free access to tap water and regular rat chow in a room with a controlled 40% humidity and a temperature of 22 °C. A controlled 12-hour environmental light cycle was maintained. The experimental designs were approved by the Animal Care and Use Committee of the University of Oulu. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

4.1.1 Spontaneously hypertensive rats (II)

The SHR is a well-established animal model of genetic hypertension with many features in common with human essential hypertension (Trippodo & Frohlich 1981). The SHR was originally introduced by Okamoto and Aoki (Okamoto & Aoki 1963), developed by selective inbreeding of WKY rats exhibiting elevated blood pressure. In the SHR model, systolic blood pressure becomes markedly elevated when the rats are around 2 months of age, followed by a relatively long period of stable, compensated left ventricular hypertrophy with progressive increases in LV volume and fibrosis (Bing *et al.* 2002, Boluyt & Bing 2000). After the age of 18 months, cardiac hypertrophy is found in all SHR (Boluyt & Bing 2000).

At approximately 18 months of age, male SHR begin to develop signs of impaired heart function whereas in the female SHR evidence of dysfunction appears to be delayed by a few months (Pfeffer *et al.* 1982)). The development of heart failure in SHR is associated with marked myocardial fibrosis, increased passive stiffness, and impaired contractile function (Bing *et al.* 2002, Boluyt & Bing 2000). Mean age of male SHR with congestive heart failure is approximately 21 months (Bing *et al.* 2002, Doggrell & Brown 1998). Rats are observed to develop tachypnea and labored respiration with pathologic features of heart failure such as pleural effusions, pulmonary edema and left atrial thrombi (Bing *et al.* 2002, Boluyt & Bing 2000). The SHR has been extensively used to study the pathophysiology of hypertension; however, the genetic mechanisms responsible for increased blood pressure in this model remain to be defined.

4.2 Experimental design in conscious rats

4.2.1 Acute pressure overload (I)

The rats were anesthetized with 0.26 mg/kg fentanyl citrate, 8.25 mg/kg fluanisone, (Hypnorm, Janssen-Cilag) and 4.1 mg/kg midazolam (Dormicum, Roche) intraperitoneally (i.p.). A PE-60 catheter was inserted into the abdominal aorta through the right femoral artery for the measurement of hemodynamics as previously described (Ruskoaho *et al.* 1989). Briefly, PE-50 catheters were placed into the left femoral vein for administration of drugs. All catheters were exteriorized behind the neck, filled with heparinized (150 IU/ml) saline, and plugged with a stainless pin. The rats were housed individually in experimental cages after the operation and had free access to food and and water. The day after the operation, the arterial and right atrial catheters were attached to pressure transducers and mean arterial pressure (MAP) and heart rate were recorded and analyzed with Ponemah data acquisition software (Gould Instrument System Inc.). The venous catheter was connected to a syringe or an infusion pump (B-Braun Perfusor ED, Braun Melsungen AG) for infusions. During the infusion and measurement the animals were conscious and freely moving. The experiment was preceded by 30 minutes' acclimatization. AVP (Peninsula Laboratories) at a dose of 0.05 µg/kg per minute or vehicle (0.9% NaCl) was infused intravenously (i.v). at 37.5 µL/min for 30 min and 4 hours. At the end of the experiments, the rats were decapitated, the thoracic cavity was opened, and the heart was removed. Left ventricular tissue samples were blotted dry, weighed, immersed in liquid nitrogen, and stored at -70 °C for further analysis.

4.2.2 Angiotensin II induced hypertension (I)

Angiotensin II (Sigma Chemicals) (33 µg/kg/h) or vehicle (0.9% NaCl) was administered through subcutaneously implanted osmotic minipumps (Alzet, Durect corporation) in conscious rats for 6, 12 and 72 hours, and 2 weeks as described before (Foldes *et al.* 2001). The osmotic minipumps were placed during inhalation anesthesia with isoflurane (Baxter International Inc.). The rats were given 0.2 mg/kg buprenorphine hydrochloride (Temgesic, Schering-Plough) s.c. for post operative analgesia. In a separate series of experiments, vehicle (0.9% NaCl), Ang II (33 µg/kg/h), losartan (400 µg/kg/h) (a generous gift from Merck), or Ang II in combination with losartan was administered for 6 hours (Lako-Futo

et al. 2003). After the experiment, left ventricular tissue samples were obtained as described above.

4.3 Adenoviral gene transfer *in vivo* (III)

Recombinant adenoviruses containing the coding regions of the constitutively active MKK3b (RAdMKK3bE) and wild-type (WT) p38α (RAdp38α) genes driven by the cytomegalovirus immediate early promoter were generated as previously described (Wang *et al.* 1998). The recombinant replication-deficient adenovirus RAdlacZ, which contains the *Escherichia coli* β-galactosidase (LacZ) gene was used as a control virus. The recombinant adenoviruses were generously supplied by Dr. Veli-Matti Kähäri, University of Turku, Finland.

Adenovirus-mediated gene transfer into the left ventricle was performed as a local intramyocardial injection as previously described (Szatkowski *et al.* 2001). Male SD rats weighing 250-300 g were anesthetized with 250 µg/kg medetomidine hydrochloride (Domitor, Orion Pharma) i.p. and 50 mg/kg ketamine hydrochloride (Ketalar, Pfizer) i.p. A left thoracotomy and pericardial incision was performed. Recombinant adenovirus (8×10^8pfu) in a 100 µl volume was injected using a Hamilton precision syringe directly into the anterior wall of the left ventricle, the heart was repositioned in the chest, and the incision was closed. After the operation the anesthesia was partially antagonized with 1.5 mg/kg atipamezole hydrochloride (Antisedan, Orion Pharma) i.p. and the rats were given 0.05-0.2 mg/kg buprenorphine hydrochloride (Temgesic, Schering-Plough) s.c. for post operative analgesia.

4.4 Echocardiography (II-III)

Transthoracic echocardiography was performed using the Acuson Ultrasound System (Sequoia^{TM} 512) and a 15-MHz linear transducer (15L8) (Acuson, MountainView, California, USA). Before examination, rats were sedated with 50 mg/kg ketamine (Ketalar, Pfizer) i.p. and 10 mg/kg xylazine (Rompun, Bayer) i.p. Using two-dimensional imaging, a short axis view of the left ventricle at the level of the papillary muscles was obtained, and a two dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle was obtained. Left ventricular end-systolic (LVESD) and end-diastolic (LVEDD) dimensions as well as the thickness of the interventricular septum (IVS) and posterior wall (PW) were measured from the M-mode tracings. The left

ventricular fractional shortening (FS) and ejection fraction (EF) were calculated from the M-mode LV dimensions using the following equations: LVFS (%) = {(LVEDD-LVESD) / LVEDD} x 100, LVEF (%) = {$(LVEDD)^3$ – $(LVESD)^3$ / $LVEDD^3$} x 100. For evaluation of left ventricular diastolic function, mitral flow was recorded from an apical four-chamber view. Measurements of peak flow velocity of the early rapid diastolic filling wave (E) and late diastolic filling wave (A) were made and the E/A ratio was calculated. An average of three measurements of each variable was used. After the echocardiography, cardiac tissue samples were obtained as described above.

4.5 Cell culture (IV)

Neonatal rat ventricular myocytes were prepared from 2-4 day old SD rats as previously described (Pikkarainen *et al.* 2003). Briefly, after digestion of ventricular tissue with collagenase (2 mg/ml), the cell suspension was pre-plated for 30-45 minutes. The non-attached myocyte-enriched cell fraction was plated at a density of $2x10^5$ /cm^2 on flexible bottomed collagen I-coated 6-well elastomere plates (Bioflex, Flexcell International Corporation), and cultured overnight with Dulbecco's modification of Eagle's medium/Ham's F12 medium (DMEM/F12) containing 10% fetal bovine serum, and thereafter in complete serum free medium (CSFM). The cells were exposed for 15 minutes to 48 hours to cyclic mechanical stretch as previously described (Pikkarainen *et al.* 2003). Stretch was introduced to cells by applying a cyclic vacuum suction under the flexible bottomed plates by computer-controlled Flexercell Strain Unit equipment FX-3000 (Flexcell). The vacuum varied in two-second cycles at a level sufficient to promote cyclic 10 to 25% elongation of the cardiomyocytes at the point of maximal distension of the culture surface. Within each culture the stretch was started stepwise for experimental groups and finished at the same time. For the microarray analysis, experiments were carried out in two separate sets each having their own control. The first set contained the experiments of 1, 4 and 12 hours, and the second set 24 and 48 hours of stretch. After the experiments the cells were washed twice with phosphate-buffered saline (PBS) and quickly frozen at -70 °C or lysed in protein lysis buffer.

4.6 Isolation and analysis of RNA (I-IV)

Total RNA from left ventricular tissue samples was isolated by the guanidine thiocyanate-CsCl method as previously described (Magga *et al.* 1994). From cultured myocytes total RNA was isolated from the TRIzol cell extracts following the manufacturer's protocol (Invitrogen) by using the Phase Lock Gel system (Eppendorf).

4.6.1 Northern blotting (I-IV)

For Northern Blot analyses 20 µg of total RNA from left ventricular tissue or 5 µg total RNA from cultured myocytes were separated by agarose-formaldehyde gel electrophoresis and transferred to a MAGNA nylon membrane (Osmonics Inc.) as previously described (Magga *et al.* 1994).

Probes for Northern hybridization were made by the reverse transcriptase polymerase chain reaction (RT-PCR) technique. The PCR products were cloned into pCR®2.1-TOPO or pCR®2.1 –cloning vectors (Invitrogen). Sequencing showed that the probes correspond to bases 522-1232 of Bcl-X (GenBank accession number U72350), 118-467 of collagen I α1 (ColIα1; Z78279), 1808-2212 of CTGF (AF120275), 737-1058 of *Egr-1* (NM_012551), 11-603 of epidermal fatty acid-binding protein (E-FABP; U13253), 3961-4470 of fibronectin-1 (FN-1; NM_019143), 260-662 of growth arrest and DNA damage inducible protein 45 (GADD45α; L32591), 536-950 of Hsp-70 (L16764), 1286-1708 of immediate early transcription factor NGF1B (Nur77; U17254), 782-1283 integrin β1 (U12309), 1-440 of MGP (NM_012862), 587-806 of PAI-1 (M24067), 1036-1506 of TSP-4 (X89963), transforming growth factor stimulated clone-22 (TSC-22; L25785) and 180-590 of TIMP-1 (L31883).

These probes and previously amplified cDNA probes for rat BNP, skα-A, caα-A, α-MHC, β-MHC and ribosomal 18S RNA (Lako-Futo *et al.* 2003), rat EST clone UI-R-E0-bu-h-10-0-UI (corresponding GeneBank accession number AA800844) and full length rat ANP (Flynn *et al.* 1985) were random primer -labeled with [α^{32}P]-dCTP, and the membranes were hybridized and washed as described previously (Magga *et al.* 1994) except that after hybridization the membranes were washed at +63 °C and exposed to Phosphor screens (Eastman Kodak). The results were visualized and quantified with Molecular Imager FX equipment (Bio-Rad Laboratoires) using QuantityOne software (Bio-Rad

Laboratoires). The signals of each mRNA were normalized to 18S in each sample to correct for potential differences in loading and/or transfer.

4.6.2 Real time quantitative RT-PCR (II-IV)

Rat acidic FGF (aFGF), α-MHC, adrenomedullin (AM), AT1-R), basic FGF (bFGF), β-MHC, BMP-2, caα-A, cardiotrophin-1 (CT-1), *c-fos*, collagen III α1 (ColIIIα1), corin, ET-1, endothelin receptor type A (ETA-R), IL-6, L-type calcium channel α1c-subunit (CaCNL1α1), osteopontin (OPN), platelet-derived growth factor A (PDGF-A), Serca 2a, skα-A, TGFβ1, TGFβ2, vascular endothelial growth factor A (VEGF-A), xanthine dehydrogenase (XDH) and ribosomal 18S RNA were measured by real-time quantitative RT-PCR using TaqMan chemistry on an ABI 7700 Sequence Detection System (Applied Biosystems) as previously described (Majalahti-Palviainen *et al.* 2000). The cDNA was synthesized from 0.5 µg of left ventricular total RNA. The sequences of the forward (F) and reverse (R) primers and for fluorogenic probes for RNA detection are shown in Table 11. The results were normalized to 18S RNA quantified from the same samples.

Table 11. The forward and reverse primers for real time quantitative RT-PCR.

Gene	Primers	Fluorogenic Probe
aFGF	(F) ATGGCACCGTGGATGGG	AGGGACAGGAGCGACCAGCACATTC
	(R) TTTCCGCACTGAGCTGCAG	
α-MHC	(F) GCAGAAAATGCACGATGAGGA	TAACCTGTCCAGCAGAAAGAGCCTCGC
	(R) CATTCATATTTATTGTGGGATAGCAAC	
AM	(F) CATTGAACAGTCGGGCGAGT	CCCATTGGCGCCTGCGGA
	(R) CAGGGTGCGAAGCTCTCTG	
ANP	(F) GAAAAGCAAACTGAGGGCTCTG	TCGCTGGCCCTCGGAGCCT
	(R) CCTACCCCCGAAGCAGCT	
AT1-R	(F) GTGGCCAAAGTCACCTGCA	CATCTGGCTGATGGCTGGCTTGG
	(R) GTGGATGACAGCTGGCAAACT	
bFGF	(F) CCCGGCCACTTCAAGGAT	CCAAGCGGCTCTACTGCAAGAACGG
	(R) GATGCGCAGGAAGAAGCC	
β-MHC	(F) GCTACCCAACCCTAAGGATGC	TGTGAAGCCCTGAGACCTGGAGCC
	(R) TCTGCCTAAGGTGCTGTTTCAA	
BMP-2	(F) ACACCGTGCTCAGCTTCCAT	ACGAAGAAGCCATCGAGGAACTTTCAGAA
	(R) GTCGGGAAGTTTTCCCACTCA	
Caα-A	(F) GGGCCCTCCATTGTCCA	CGCAAGTGCTTCTGAGGCGGCTAC
	(R) GCACAATACTGTCGTCCTGAGTG	
c-fos	(F) GGCTGAACCCTTTGATGACTTC	TGTTTCCGGCATCATCTAGGC
	(R) GGGCAGTCTCCGAGCCA	
COLIIIα1	(F) AGCTGGCCTTCCTCAGACTTC	TTCCAGCCGGGCCTCCCAG
	(R) GCTGTTTTTGCAGTGGTATGTAATG	
Corin	(F) CCCAGTGGACATATCTGTGGC	TGTCCTCATTGCCAAGAAGTGGGTCC
	(R) TTCAAAGCAATGGGCAACTGT	
CT-1	(F) TTGTCCCCTGGTGGCAAG	CCACCCACCCTCAGGACCTTCCTT
	(R) TTGCCTACAAAGGAACTGGTGG	
ET-1	(F) ATGGACAAGGAGTGTGTCTACTTCTG	CACCTGGACATCATCTGGGTCAACACTC
	(R) GGGACGACGCGCTCG	
ETA-R	(F) GGAATGGGAGCTTGCGG	TTGCCCTCAGCGAACACCTCAAGC
	(R) TTTGCCACCTCTCGACGC	
IL-6	(F) ATATGTTCTCAGGGAGATCTTGGAA	CAGAATTGCCATTGCACAACTCTTTTCTCA
	(R) TGCATCATCGCTGTTCATACAA	
CaCNL1α1	(F) TTGACAATGTTCTGGCAGCC	TGATGGCCCTCTTTACCGTCTCCACC
	(R) TCTGGCCACCCCTCGA	
OPN	(F) AATCGCCCCCACAGTCG	TGTCCCTGACGGCCGAGGTGA
	(R) CCTCAGTCCGTAAGCCAAGC	
PDGF-A	(F) CGAGCGACTGGCTCGAA	TCAGATCCACAGCATCCGGGACC
	(R) GAGTCTATCTCCAAGAGTCGCTGG	
Serca 2a	(F) CAGCCATGGAGAACGCTCA	ACAAAGACCGTGGAGGAGGTGCTGG
	(R) CGTTGACGCCGAAGTGG	
Skα-A	(F) TCCTCCGCCGTTGGCT	CATCGCCGCCACTGCAGCC
	(R) AATCTATGTACACGTCAAAAACAGGC	
TGFβ1	(F) CATCGACATGGAGCAGGTGA	ACGGAAGCGCATCGAAGCCATC
	(R) TTGGACAGGATCTGGCCAC	
TGFβ2	(F) ACCTTTTTGCTCCTGCATCTG	TCCCGGTGGCGCTCAGTCTGT
	(R) GTCGAGGGTGCTGCAGGTA	
VEGF-A	(F) GATCCGCAGACGTGTAAATGTTC	TGCAAAAACACAGACTCGCGTTGCA
	(R) TTAACTCAAGCTGCCTCGCC	
XDH	(F) CCTCCAGGGATTCCGGAC	TTTGCCAAGGATGGTGGGTGCTG
	(R) TGGGTTGTTTCCACTTCCTCC	
18S	(F) TGGTTGCAAAGCTGAAACTTAAAG	CCTGGTGGTGCCCTTCCGTCA
	(R) AGTCAAATTAAGCCGCAGGC	

4.7 Gene expression profiling

To examine gene expression patterns cDNA arrays and DNA oligonucleotide arrays were used. A summary of expression profiling experiments is presented in Table 12.

Table 12. Summary of the gene expression profiling studies.

Study	Experimental model	Time point	n	Platform	Transcripts
I	Acute pressure overload in conscious animals (AVP-infusion)	30 min and 4 hours	4	ATLAS rat cDNA expression array	~600
II	SHR- and age-matched WKY rats	12-, 16-, and 20 months	4*	Rat Genome U34A	~8 800
III	p38 MAPK overexpression in vivo	3 days after gene transfer	5	Rat Expression Set 230_2.0	~30 000
IV	Cyclic mechanical stretch of cardiomyocytes	1, 4, 12, 24 and 48 hours	5	Rat Expression Set 230_2.0	~ 30 000

*n=3 in 20 months old WKY rats. DNA arrays were obtained from Clontech (I) and Affymetrix (II-IV).

4.7.1 cDNA expression arrays

Analysis of differential gene expression was done by using ATLAS rat cDNA expression arrays (Clontech) consisting of 588 known rat genes as described by the manufacturer. RNA preparations were digested with DNase I (Amersham Biosciences) to avoid any contamination by genomic DNA. The quality and integrity of the isolated RNA was monitored by gel electrophoresis determination in the presence of RNA standards (Promega).

For cDNA synthesis 5 µg of total RNA isolated from left ventricles (n=4 in each group) was used. Briefly, ^{33}P-labeled first-strand cDNA probes were synthesized with Superscript® II (Invitrogen) in the presence of gene-specific primers (Clontech) and RNase inhibitor (Ambion) according to the manufacturer's protocol and purified by Sephadex G-50 (Amersham Biosciences). Hybridization of the cDNA probes to ATLAS™ membranes was carried out overnight at 68 °C in ExpressHyb (Clontech) and washed according to the manufacturer's protocols. The membranes were exposed to Phosphor screens (Eastman Kodak) and the results were analyzed with Molecular Imager FX equipment (Bio-Rad Laboratories).

The intensity values for each of the 588 double spotted cDNAs were quantified by QuantityOne software (Bio-Rad Laboratories). Normalization

among arrays was performed by dividing the background-subtracted signal for each spot on a blot by the average intensity of all of the spots on that blot. Then the average was taken of each duplicate cDNA spot to represent the gene expression level of that gene. Finally, the average signal of four different hybridizations (i.e. four different RNA-samples) was calculated and used in further analyses as the specific expression level of that gene in each treatment. A threshold value for the lowest reliable expression level was introduced to filter out unreliable low intensity data. Ratios between gene expression levels between different treatments were then calculated (representing the fold-change of mRNA levels). Genes were defined as differentially expressed if the fold-change was at least 1.5-fold and statistically significant (Student's t-test; $P<0.05$).

4.7.2 Oligonucleotide arrays (II-IV)

The quality and integrity of the isolated RNA was monitored by gel electrophoresis. The biotinylated cRNA probes were prepared and hybridized according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly, cDNA was reverse-transcribed from 5 µg (II), 7 µg (III) or 2 µg of total RNA by using Superscript II (Invitrogen)(II) or a One-cycle cDNA synthesis kit (Affymetrix) (III-IV) with a T7-(dT)24-primer. The cRNA was prepared and biotin-labeled by in vitro transcription (Enzo Biochemical; II or Affymetrix; III-IV) and fragmented before hybridization. The biotinylated cRNA was hybridized to the GeneChip Rat Expression Set 230_2.0 Arrays (III-IV). After hybridization, the GeneChips were washed and stained with streptavidin-phycoerythrin (Molecular Probes). The staining signal was amplified by biotinylated anti-streptavidin (Vector Laboratories) and a second staining with streptavidin-phycoerythrin using an Affymetrix Fluidics station was carried out according the standard protocol. An Affymetrix scanner (Agilent) with Affymetrix Microarray Suite 5.0 software (II) or a GeneChip Scanner 3000 with GeneChip Operating Software (GCOS) v. 1.2 (Affymetrix) (III-IV) was used in scanning. The probe quality was judged by the 3'/5' hybridization ratio for the set of housekeeping genes on the Affymetrix chip. The ratio obtained was <2 and comparable among all hybridized samples.

Data analysis

In study II, raw data analysis was performed using Affymetrix software. Default analysis parameters provided by the software were used for background and noise calculations. Global scaling was applied to allow comparison of gene signals across multiple arrays. Absolute calls (present, marginal and absent) and signal values (serving as a relative indicator of RNA abundance) for each assayed gene were calculated by Affymetrix algorithms. Normalization, filtering, and cluster analysis of the data were performed with the GeneSpring 4.2.1 software (Silicon Genetics). Each gene was normalized to itself by making a synthetic positive control for that gene, and dividing all measurements for that gene by this positive control, assuming it was at least 0.01. This synthetic control was the median of the gene's expression values over all the samples. To increase the stringency and the quality of data analysis, we defined an expressed gene by requiring at least three Affymetrix “present” calls out of four replicates. Genes were defined as differentially expressed if the fold-change was at least 1.5-fold and statistically significant (Welch *t*-test; $P<0.05$). To identify hypertrophy-associated genes, the genes that fulfilled the filtering criteria in both strains were eliminated from the final analysis.

In studies III-IV, Affymetrix CEL-files (raw data files) were imported into GeneSpring 7.2 software (Silicon Genetics) and Robust Multichip Average (RMA) normalization was performed. RMA consists of background adjustment, quantile normalization, and summarization by median polishing. Genes were defined as differentially expressed if the fold change was at least 2.0-fold and statistically significant ($P<0.05$, Welch *t*-test (III) or One-way ANOVA (IV) followed by Benjamini and Hochberg false discovery rate).

Differentially expressed genes (II-IV) were classified on the basis of biological function using a modified version of a previously established classification scheme (Adams *et al.* 1995). Gene Ontology (www.geneontology.org) was used to make an initial assignment of functional classification. For a portion of the genes, the function could be predicted by BLASTn searches. Because GeneOntology output classifications did not necessarily address a specific pathophysiological condition or experimental treatment, the PubMed database (www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed) was searched for each gene and the functional assigment was confirmed or adjusted under one of the following broad biological classifications: apoptosis, cell adhesion, cell division, cell signaling/communication, cell structure/motility,

cell/organism defense, channel/transport proteins, inflammatory and immune response, biosynthesis/metabolism, protein synthesis/turnover/posttranslational modification, regulation of transcription, translation factors, unclassified and expressed sequence tags (ESTs) as well as other unknown transcripts.

The gene expression profiling data comply with the MIAME standard and all the published data sets are available at the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) (Barrett *et al.* 2007) with the accession numbers GSE2116 (II) and GSE3866 (III).

To identify potential interactions between p38-regulated genes (III), the gene list generated by microarray analysis was analyzed using PathwayAssist software 3.0 (Stratagene).

4.8 Protein extraction (III-IV)

For the total protein extracts, the left ventricular tissue samples were broken in liquid nitrogen, and homogenized for 10 minutes in lysis buffer consisting of 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1 mM ethylene diaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mmol/L β-glycerophosphate, 1 mM Na_3VO_4, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 1 mM dithiothreitol (DTT) and 10 μg/mL each of leupeptin, pepstatin and aprotinin. The tissue homogenates were centrifuged at 2000 rpm for 1 minute at +4 °C. The total protein fraction was separated by the addition of 5 x nuclear extraction buffer (NEB) (100 mM Tris-HCl [pH 7.5], 750 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5% Triton X 100, 12 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 5 mM Na_3VO_4) to the tissue homogenate, followed by centrifugation at 12500 rpm for 20 minutes. The supernatant was frozen in liquid nitrogen and stored at –70 °C until assayed. To extract the nuclear fraction, the supernatant from the first centrifugation was incubated on ice for 15 minutes, NP-40 was added, and the nuclei were collected by centrifugation at 12 500 rpm for 30 seconds. The pellet was resuspended in a solution containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 2 mM benzamidine, 1 mM PMSF, 50 mM NaF, 1 mM DTT, 3 μg/mL 1-chloro-3-tosylamido-7-phenyl-2-butanone (TPCK), 3 μg/mL L-1-tosylamido-2-phenylethyl chloromethyl ketone (TLCK), and 10 μg/mL of each leupeptin, pepstatin and aprotinin. The samples were incubated at +4 °C for 30

minutes, centrifuged at 12 500 rpm for 5 minutes and the resulting supernatants frozen in liquid nitrogen and stored –70 °C until assayed.

For the total protein extracts, the cultured cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4) supplemented with 20 µg/ml leupeptin, 2 µg/ml pepstatin, 20 µg/ml aprotinin, 1 mM PMSF, 50 mM NaF, 1 mM DTT, 6 µg/ml TPCK, and 6 µg/ml TLCK. The lysate was cleared by 10 min centrifugation at +4 °C and the supernatant was transferred to a new tube as the total protein extract.

The protein concentrations of each sample were determined colorimetrically by using Protein Assay (Bio-Rad Laboratories).

4.9 Kinase activity assays (III)

The assays for measuring p38 MAPK, ERK and JNK activity were performed according to the manufacturer's instructions (Cell Signaling Technology). p38 MAP kinase activity was measured with an immunocomplex kinase assay by using ATF-2 and Elk-1 as substrates. Briefly, the tissues were homogenized and the supernatants were immunoprecipitated with immobilized phospho-p38 monoclonal antibody, which binds to the active, phosphorylated form of p38α. The immunoprecipitated sample is then presented to its substrate, ATF-2 or Elk-1 fusion protein in an *in vitro* kinase reaction with the kinase buffer and ATP. Finally, the samples were analyzed by western blotting for phospho-Elk-1 or phospho-ATF-2 (Cell Signaling Technology).

ERK activity was measured with an assay similar to the p38 kinase activity assay, except that the protein was immunoprecipitated with immobilized phospho-p44/42 monoclonal antibody, Elk-1 fusion protein was used as a substrate, and the samples were analyzed by western blotting for phospho-Elk-1 (Cell Signaling Technology).

For the JNK assay, the c-Jun fusion protein was used to pull down active JNK. The samples were analyzed by western blotting for phospho-c-Jun (Cell Signaling Technology).

4.10 Western blotting (III-IV)

For western blot analysis, protein samples (18-30 µg) were loaded on sodium dodecyl sulfate –polyacrylamide (SDS-PAGE) gels and transferred to Optiran Ba-

S 85 nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked in 1-5% non-fat milk and incubated with anti-phospho-p38, anti-p38, anti-phospho p44/42, anti-p44/42, anti-phospho-JNK and anti-JNK specific antibodies (Cell Signaling Technology and Santa Cruz Biotechnology) in 0.5-1% milk in Tris-buffered saline – 0.05% Tween 20 overnight at +4 °C. After incubation with horseradish peroxidase –conjugated anti-rabbit, anti-mouse or anti-goat IgG secondary antibody (Cell Signaling Technology and Santa Cruz Biotechnology), the proteins were detected by enhanced chemiluminesence reagents (ECL Plus™, GE Healthcare Life Sciences) and quantified using a hyperfilm MP (GE Healthcare Life Sciences) (III) or a Fuji LAS-3000 luminescent image analyzer (Fujifilm) (IV). For a second western blot, the membranes were stripped for 30 min at +60 °C in stripping buffer containing 62.5 mmol/L Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 100 mmol/L mercaptoethanol. The films were scanned and the results were analyzed with QuantityOne software (Bio-Rad Laboratories)

4.11 Gel mobility shift assays (III)

Double-stranded synthetic oligonucleotide probes corresponding to GATA or AP-1 binding sequences of the rat BNP promoter, SRF binding sequence of the rat c-fos promoter, MEF2 binding sequence of the mouse muscle creatine kinase promoter or oligonucleotides with a NF-κB consensus binding sequence were sticky end -labeled with [α^{32}P]-dCTP by Klenow enzyme. The sequences of the oligonucleotide probes used for the gel mobility shift assay are provided in Table 13.

Table 13. Oligonucleotide probes for gel mobility shift analysis.

Probe	Sequence (5'→3')	Reference
AP-1	GGAAGTGTTTTTGATGAGTCACCCCA	Hautala *et al.* 2001
GATA	TGTGTCTGATAAATCAGAGATAAC-CCCACC	Hautala *et al.* 2001
MEF-2	AGCTCGCTCTAAAAATAACCCTGTCCC	Martin *et al.* 1993
NFκB	AGTTGAGGGGACTTTCCCAGGCCA	Tenhunen *et al.* 2004
SRF	ACAGGATGTCCATATTAGGACATCTGCG	Morin *et al.* 2001
OCT-1	GATCCGAGCTTCAAATTATTTGCATAAGCGATTGA	Kemler *et al.* 1989

OCT-1, octamer-1

Binding reactions consisted of 20 μg of nuclear protein and 2 μg of poly-(dI-dC)(dI-dC) in a buffer containing 10 mM HEPES pH 7.9, 1 mM $MgCl_2$, 50 mM

KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, 0.025% NP-40, 0.25 mM PMSF and 1 mM of each aprotinin, leupeptin and pepstatin. The binding reaction mixtures were first pre-incubated on ice for 10 minutes, the labeled probe was added and then the reaction mixtures were incubated for 20 minutes at room temperature. Protein-DNA complexes were separated by non-denaturing gel electrophoresis on a 5% polyacrylamide gel in 0.5 x Tris-borate-EDTA (TBE) buffer at 4 °C. The gels were dried and exposed to Phosphor screens (Eastman Kodak). The results were visualized and quantified with Molecular Imager FX equipment (Bio-Rad Laboratories) using QuantityOne software (Bio-Rad Laboratories). The specificity of the transcription factor binding was confirmed by competition experiments with 10- and 100-fold molar excesses of unlabeled oligonucleotides with mutated binding sites as well as by supershift experiments using specific antibodies (Santa Cruz Biotechnology) against GATA-4, GATA-5, GATA-6, the members of AP-1 complex (*c-fos, c-Jun, Jun B* and *Jun D*), NF- κB (p50 and p65) and SRF. In addition, DNA binding activity assays of the Octamer-1 (Oct-1) transcription factor were run in parallel to control the specificity of the altered DNA binding activity. Competition and supershift experiments were performed by preincubating the nuclear extract with 1 μg of the appropriate antibody for 20 minutes at room temperature before the binding reaction.

4.12 Histological and image analysis (III)

For histological analysis, the left ventricles were fixed in 10% buffered formalin solution and embedded in paraffin. Sections 5-μm-thick were cut and stained with hematoxylin and eosin, Masson's trichrome or Sirius red. The total fibrotic area was measured from the Masson's trichrome-stained histological sections using a digital image analysis system (MCID/M4 with software version 3.0, Imaging Research).

For the Ki-67 antigen, GATA-4, p38 and IL-6 immunohistochemistry was carried out by using anti-Ki-67 antigen (DakoCytomation), anti-GATA-4 (Santa Cruz Biotechnology), anti-IL-6 (R&D Systems) or anti-p38 (Cell Signaling Technology) specific antibodies and a commercial immunoperoxidase system was used to visualize bound antibody.

4.13 Statistical analysis

Statistical analysis was performed by unpaired Student's *t*-test or One-way Analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test for multiple comparisons. A value of $P<0.05$ was considered statistically significant. Results are expressed as mean ± standard error of the mean (SEM).

5 Results

5.1 The effect of acute cardiac overload on left ventricular gene expression (I)

5.1.1 Characterization of pressure overload model

To study the very early gene expression changes in response to pressure overload, an *in vivo* model of intravenous administration of AVP for 30 minutes and 4 hours in conscious normotensive rats was used. MAP rose rapidly and reached a maximum value within 15 minutes during AVP infusion, and was associated with a significant decrease in heart rate (Table 14). Both MAP and heart rate remained unchanged in the vehicle-treated animals. At the level of gene expression, a significant 7.9-fold ($P<0.001$) increase in left ventricular *Egr-1* mRNA levels was seen already after 30 minutes of AVP infusion, followed by 8.1-fold ($P<0.001$) upregulation of BNP mRNA levels at 4 hours.

Table 14. Mean arterial pressure and heart rate in response to vehicle- and AVP-infusions in conscious Sprague-Dawley rats.

Time	0 min	15 min	30 min	1 h	2 h	4 h
Mean Arterial Pressure, mmHg						
Vehicle (n=13)	110±2	110±2	111±2	111±3	108±2	108±2
AVP (n=11)	114±2	161±2*	158±3*	154±3*	155±2*	155±2*
Heart Rate, bpm						
Vehicle (n=13)	415±8	383±11	387±11	385±9	403±6	416±10
AVP (n=11)	413±8	281±12*	259±9*	242±14*	225±14*	243±14*

Results are mean ± SEM. *$P<0.001$ vs. 0-minute time point (One-way ANOVA followed by LSD post hoc test).

5.1.2 Gene expression changes in response to acute pressure overload

Atlas™ cDNA expression membranes were used to identify left ventricular gene expression changes in response to acute pressure overload produced by 30 minute and 4 hour infusions of AVP. Of the 588 genes analyzed, 7 genes were significantly increased and one gene decreased after 30 minutes of AVP infusion, whereas the expression of 11 genes was elevated and the expression of 3 genes

was down-regulated after 4 hours infusion of AVP. Figure 6 shows the number of differentially expressed genes (at least 1.5-fold increase vs. control rats; $P<0.05$) organized into groups representing their known biological functions. Selected cDNA expression array results were confirmed by comparison with mRNA levels obtained by Northern blot analysis. Fold changes in mRNA levels were similar as measured by either cDNA expression array or Northern analysis.

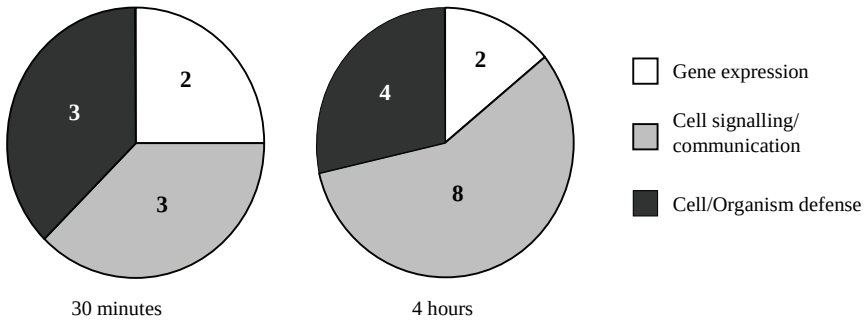

Fig. 6. Number of differentially expressed genes in functional groups after 30 minutes and 4 hours of AVP-infusion.

5.1.3 Changes in gene expression in Ang II-induced hypertension

To confirm the changes in gene expression observed by the cDNA array analysis in another model of pressure overload, cardiac gene expression of GADD45α, PAI-1, E-FAPB and Bcl-X was examined in Ang II-induced hypertension in conscious rats. In this model, continuous administration of Ang II increases MAP within 3 hours and MAP remains significantly elevated throughout the 2-week period (Foldes *et al.* 2001). After 6 hours, administration of Ang II resulted in an increase in left ventricular gene expression of GADD45α, PAI-1, E-FAPB and Bcl-X, similar to that of Egr-1, Hsp-70, NGF1-B and BNP, the genes known to be rapidly upregulated in response to pressure overload. Longer infusions of Ang II for 3 days to 2 weeks did not increase GADD45α, PAI-1, E-FAPB and Bcl-X mRNA levels. The AT1-R blocker losartan completely abolished the Ang II-induced activation of GADD45α gene expression at 6 hours, similarly to BNP mRNA levels, whereas the Ang II-induced change in the left ventricular gene expression of Bcl-X was reduced 40% ($P<0.01$). The small Ang II-induced increase in E-FABP gene expression was not significantly inhibited by losartan.

5.2 Gene expression profiles during development of cardiac hypertrophy and diastolic heart failure (II)

5.2.1 Progression of left ventricular hypertrophy and expression of hypertrophy-associated genes

To study the effect of chronic hypertension on left ventricular gene expression, an experimental model of genetic hypertension, SHR, was used (Bing *et al.* 2002, Care *et al.* 2007, Trippodo & Frohlich 1981). In young SHR, MAP is already significantly elevated and continues to increase with aging as previously reported (Kinnunen *et al.* 1992, Kuoppala *et al.* 2003). The increase in pressure overload is associated with a progressive LVH, as reflected by an increased LVW/BW ratio (Table 15).

Table 15. Body and cardiac weights and echocardiography data of SHR compared to normotensive WKY rats.

Variable	Group	12 months	16 months	20 months
Body weight (g)	WKY	399 ± 14	415 ± 8	449 ± 8†
	SHR	407 ± 20	391 ± 6§	374 ± 13‖
LVW (mg)	WKY	765 ± 28	778 ± 82	921 ± 22*
	SHR	1.037 ± 43‖	1.166 ± 51‖	1.288 ± 70*‖
LVW/BW (mg/g)	WKY	1.92 ± 0.06	1.87 ± 0.17	2.05 ± 0.06
	SHR	2.55 ± 0.07#	2.98 ± 0.15#	3.48 ± 0.21†#
Septum (diastole) (mm)	WKY	1.9 ± 0.1	2.1 ± 0.1*	2.0 ± 0.1
	SHR	2.2 ± 0.1§	2.4 ± 0.1§	2.7 ± 0.1‡#
Left ventricle (diastole) (mm)	WKY	8.1 ± 0.3	8.1 ± 0.6	7.8 ± 0.2
	SHR	8.3 ± 0.4	8.1 ± 0.6	8.1 ± 0.6
Left ventricular posterior wall	WKY	1.9 ± 0.1	2.1 ± 0.1	2.0 ± 0.1
(diastole) (mm)	SHR	2.1 ± 0.1	2.4 ± 0.1*§	2.7 ± 0.1‡#
Fractional shortening (%)	WKY	38 ± 2	47 ± 2*	43 ± 1
	SHR	38 ± 1	54 ± 2‡§	42 ± 1
Ejection fraction (%)	WKY	73 ± 2	82 ± 2*	79 ± 2
	SHR	73 ± 1	89 ± 1†§	78 ± 1

The values are expressed as mean ± SEM (n= 4-11/group).

* $P<0.05$, † $P<0.01$, ‡ $P<0.001$ vs. 12 months, one-way ANOVA followed by LSD post hoc test.

§ $P<0.05$, || $P<0.01$, # $P<0.001$ SHR vs. WKY, Student's *t*-test.

Echocardiography revealed increased thickness of the left ventricular wall and features of diastolic dysfunction, characterized by a decreased E/A-ratio and prolonged LV-IVRT in 16-month old SHR. At 20 months, the E/A-ratio was

significantly increased above normal suggesting development of diastolic heart failure. In contrast, no significant change in left ventricular systolic function was seen. Thus, old SHR had heart failure with a preserved LVEF, abnormal left ventricular diastolic properties and marked left ventricular hypertrophy.

To characterize the experimental model at the gene expression level, mRNA levels of ANP, different isoforms of contractile proteins, collagens and factors promoting growth and fibrosis were measured. A progressive increase was seen in the β-MHC/α-MHC-ratio and in the skα-A/caα-A-ratio as well as in gene expression levels of ANP, collagen IIIα1, collagen Iα1, ET-1 and TGFβ1 in SHR with aging.

5.2.2 DNA microarray analysis of diastolic heart failure

To identify genes that are associated with the transition of LV hypertrophy to diastolic heart failure, the left ventricular gene expression profiles from 12-, 16- and 20-months old SHR were compared with profiles seen in WKY. The number of genes that were up- or downregulated at least 1.5-fold (P<0.05) in the left ventricle among the SHR age groups are seen in Figure 7. Selected DNA microarray results were confirmed by other quantitiative gene expression platforms, and similar fold-changes in mRNA levels were obtained when measured with either DNA microarray or Northern/quantitative PCR.

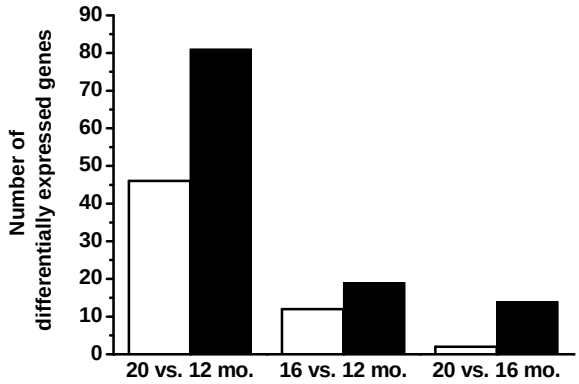

Fig. 7. Number of genes downregulated (white columns) or upregulated (black columns) during the development of cardiac hypertrophy and heart failure in SHR.

When the time course of the progression of LVH and diastolic heart failure was examined, DNA microarray analysis identified 127 genes that showed differential expression. Comparison of left ventricular RNA profiles from 20- and 12-month old SHR identified 61 known genes and 20 ESTs, whose expression was up-regulated more than 1.5-fold, whereas expression of 31 known genes and 15 ESTs was down-regulated more than 1.5-fold. Several well-characterized changes of the hypertrophic gene expression response in SHR were identified. Upregulation of ANP, collagen I, collagen III, fibronectin-1, and osteopontin mRNA levels were seen as well as downregulation of genes encoding proteins involved in fatty acid and energy metabolism such as acyl-CoA dehydrogenase. Noteworthy, gene expression of fibronectin-1 and thrombospondin-4 was upregulated in all time periods, whereas there was no single gene whose gene expression was downregulated in all groups.

Comparison of left ventricular RNA profiles during development of LVH (16-months vs. 12-months old SHR) showed that the expression of 13 known genes and 6 ESTs was elevated >1.5-fold and expression of 12 genes (9 known genes and 3 ESTs) was down-regulated >1.5-fold. Most of the enhanced genes encoded ECM proteins, whereas the majority of the repressed genes encoded metabolic enzymes.

After the development of diastolic dysfunction at the age of 16 months (20-month vs. 16-month old SHR), only 9 known genes and 5 ESTs were up-regulated and 2 genes were down-regulated (1 known gene and one EST) more than 1.5-fold. The majority of up-regulated genes encode ECM proteins including TSP-4, biglycan, procollagen 1α1, fibronectin-1, α-tropomyosin 2 and γ-crystallin.

In addition, gene expression profiling identified several genes not previously associated with the development of hypertensive cardiac hypertrophy or diastolic heart failure, such as MGP, TSP-4 and the EST-sequence AA800844 (similar to LOXL-1).

5.3 The role of p38 MAPK in the regulation of cardiac gene expression (III)

5.3.1 Activation of p38 MAPK by adenovirus-mediated gene transfer

To study the genes regulated by p38 MAPK in the heart, adenovirus-mediated intramyocardial gene transfer was used to locally increase p38 MAPK activity *in*

vivo. To achieve maximal upregulation of p38 kinase activity, the adenoviruses encoding constitutively active upstream kinases MKK3bE and MKK6bE as well as adenoviruses encoding WTp38α and WTp38β were examined in different combinations. The strongest p38 activation was observed with co-injection of MKK3bE ($6x10^8$ infectious units) and WTp38α ($2x10^8$ infectious units). The control animals were injected with adenovirus expressing the *Escherichia coli* β-galactosidase (*Lac Z*) ($8x10^8$ infectious units). The combination of MKK3bE and WTp38α produced a significant increase in left ventricular p38 MAPK activity 3 days after injection, whereas no change was observed in the ERK1/2 or JNK activity. The specificity of the MKK3bE and WTp38α gene transfer was further confirmed by an ATF-2 based kinase assay, showing a corresponding activation in p38 kinase activity.

5.3.2 The effect of p38 MAPK activation on cardiac function

To determine the effects of p38 MAPK overexpression on cardiac function, echocardiographic analysis was performed 3 days and 1 week after gene transfer (Table 16).

Table 16. Body and cardiac weights and echocardiography data 3 days and 1 week after MKK3bE + WTp38α gene transfer.

Variable	Group	3 days	1 week
LV weight/body weight (mg/g)	Lac Z	3.0 ± 0.1	3.0 ± 0.1
	p38	3.1 ± 0.1	3.2 ± 0.1**
Interventricular septum diastole (mm)	Lac Z	1.6 ± 0.1	2.0 ± 0.1
	p38	1.6 ± 0.1	2.7 ± 0.3*
Left ventricle diastole (mm)	Lac Z	8.1 ± 0.3	8.1 ± 0.3
	p38	8.1 ± 0.2	7.9 ± 0.2
Posterior wall diastole (mm)	Lac Z	1.6 ± 0.1	1.7 ± 0.1
	p38	1.6 ± 0.1	1.6 ± 0.1
Ejection fraction (%)	Lac Z	81 ± 2	74 ± 5
	p38	80 ± 2	69 ± 3
Fractional shortening (%)	Lac Z	45 ± 2	40 ± 4
	p38	44 ± 2	34 ± 3
E/A ratio	Lac Z	-	3.4 ± 0.5
	p38	-	5.2 ± 0.4*

Lac Z indicates *Lac Z* -injected and p38 indicates the MKK3bE + WTp38α-injected group. The values are expressed as mean ± SEM. *P<0.05, **P<0.01 vs Lac Z (Student's *t*-test)

Cardiac function of the MKK3bE + WTp38α-treated animals was similar to that of *Lac Z*-injected group 3 days after gene transfer as assessed by echocardiography. One week after gene transfer, the measurements showed preserved cardiac function in the MKK3bE + WTp38α–treated animals, as no statistically significant changes in left ventricular diameter, fractional shortening or ejection fraction were observed. The diameter of the interventricular septum was significantly increased and moreover, the E/A-ratio in MKK3bE + WTp38α-treated animals was raised suggesting development of diastolic dysfunction. The LVW/BW-ratio was also increased in the MKK3bE + WTp38α-treated animals in comparison to that of *Lac Z*-injected group 1 week after gene transfer

5.3.3 Histological analysis of morphological changes caused by p38 MAPK overexpression

To determine the morphological changes caused by p38 MAPK overexpression, histological analysis was performed one week after MKK3bE + WTp38α gene transfer. The hematoxylin-eosin stained histological sections showed a notable thickening of the anterior wall and a massive infiltration of inflammatory cells in the MKK3bE + WTp38α-treated hearts. Furthermore, Masson's trichrome and Sirius Red stainings showed large stained areas in the p38 MAPK overexpressing hearts indicating massive cardiac fibrosis one week after gene transfer. Only minor fibrotic lesions and inflammatory cell infiltration were observed 3 days after gene transfer.

To further illuminate the cell types affected by p38 MAPK overexpression, immunohistochemistry with a specific p38 MAPK antibody was performed, showing that p38 MAPK immunostaining was localized on cardiac myocytes.

5.3.4 The effect of p38 MAPK activation on cardiac transcription factors

To study the effect of p38 MAPK activation on cardiac transcription factors, gel mobility shift assays were used to analyze the DNA-binding activities of selected transcription factors 3 days after MKK3bE + WTp38α gene transfer. EMSAs using oligonucleotide probes containing the binding sites for AP-1, GATA-4, NF-κB, MEF-2 and SRF were performed and significant increases in AP-1 (4.5-fold), GATA-4 (1.6-fold), NF-κB (1.7-fold) and SRF binding (1.4-fold) were observed

whereas no change in MEF-2 binding was seen in MKK3bE + WTp38α–treated hearts in comparison to that of the *Lac Z*-injected group.

To confirm the specificity of the DNA binding reactions, supershift and competitor analysis using specific antibodies and competitor DNAs were performed. An antibody-induced supershift was seen for GATA-4 but not for GATA-5 or GATA-6 complexes, and GATA-4 DNA binding was effectively inhibited by unlabeled self DNA but unaffected by non-related competitor DNA Oct-1 or competitor DNA containing a mutated GATA site. Similarly AP-1, MEF-2, NF-κB and SRF binding were attenuated by their respective unlabeled self DNAs but not by mutated or non-related competitor DNAs. Antibody induced supershifts were observed for AP-1, MEF-2 NF-κB and SRF, demonstrating that the complexes bound by oligonucleotide probes contained these proteins.

5.3.5 The effect of p38 MAPK activation on cardiac gene expression

To identify genes that are regulated by p38 MAPK in the heart *in vivo*, the LV gene expression profiles three days after MKK3bE + WTp38α gene transfer were compared with those of *Lac Z*-treated animals. The microarray analysis identified 264 transcripts that were upregulated more than 2-fold in the left ventricle overexpressing p38 MAPK compared to *Lac Z* -injected hearts according to selection criteria (≥2-fold change; $P<0.05$). The percentages of upregulated p38 MAPK target genes in different functional groups are shown in Figure 8.

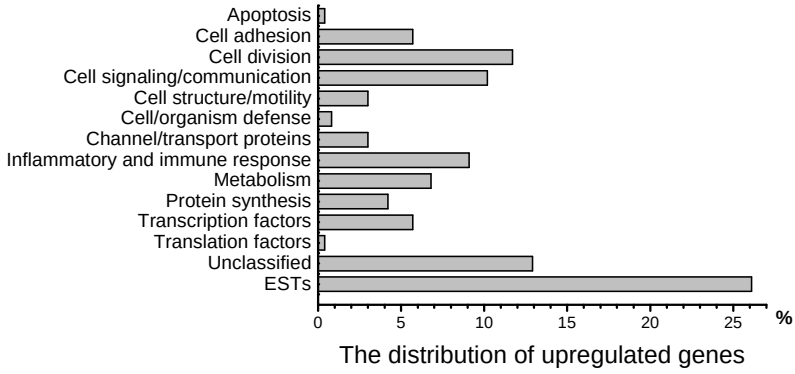

Fig. 8. The distribution of upregulated p38 MAPK target genes according to functional classification.

The major functional groups of known genes affected by p38 MAPK overexpression were genes involved in cell division (31 genes), cell signaling/communication (28 genes) and inflammatory and immune response (23 genes). Most of the transcripts upregulated in the p38 MAPK overexpressing hearts were genes with unknown function or ESTs. Of note, only two genes (pyruvate dehydrogenase kinase 4 and the gap junction membrane channel protein-α1) were down-regulated more than 2-fold by p38 MAPK overexpression in the rat left ventricle.

The individual genes upregulated by p38 MAPK included critical regulators of the cell cycle (e.g. cyclins A2, B1 and B2, kinesin family member-23, and polo-like kinases), fundamental inflammation-associated genes (e.g. IL-6 and toll-like receptor) and well-known fibrosis related factors (e.g. selectin E, CTGF, tenascin C and osteopontin). Although the DNA microarray analysis identified several genes that have previously been suggested as cardiac p38 MAPK target genes (e.g. COX-2, ANP, and histone H3), most of the genes have not previously been known to be regulated by p38 MAPK, including transcription factors ATF-3, runt related transcription factor 1 (Runx1) and Krox20 (also known as *Egr-2*).

Finally, the complete list of 266 differentially expressed genes was loaded into PathwayAssist. Of those genes, 73 were recognized by the software, and 39 of them were found to have pathway relationships in published research literature. A central signaling molecule in this interaction map was IL-6, but also chemokine (C-C motif) ligand-2 (CCL2), myelocytomatosis oncogene and cyclin dependent kinase inhibitor 1A (CDKN1A) were central nodal points in this interaction network.

5.3.6 Confirmation of DNA microarray results

DNA microarray results were compared with mRNA levels obtained by Northern blot analysis or real-time quantitative RT-PCR, and similar fold changes in mRNA levels were observed. To further confirm DNA microarray results revealing upregulation of genes involved in cell proliferation and the inflammatory response as major targets of p38 MAPK activation, immunohistochemistry with the Ki-67 antigen and IL-6 specific antibodies were performed. IL-6 immunostaining was localized on cardiac myocytes, whereas inflammatory and endothelial cells were stained with Ki-67. Furthermore, a large number of Ki-67 positive cells were observed in the histological sections of

MKK3bE + WTp38α -treated hearts, whereas only a few Ki-67 positive cells were seen in *Lac Z* –injected hearts.

5.4 The effect of mechanical stretch on gene expression patterns in cardiac myocytes *in vitro* (IV)

5.4.1 Activation of cardiac hypertrophic gene program and MAPK-pathways by mechanical stretch

To validate the *in vitro* mechanical stretch model of cultured cardiac myocytes, gene expression levels of the immediate early genes c-fos, BNP and ANP as well as activation of three main MAPK-pathways were measured. The highest induction of *c-fos* mRNA levels was seen after one hour of mechanical stretch (2.4-fold, $P<0.01$), whereas ANP and BNP mRNA levels peaked at 24 to 48 hours (5.6 ± 1.0-fold, $P<0.01$ and 3.6 ± 0.4, $P<0.001$, respectively) after the start of cyclic mechanical stretch. Significant activation of the p38 MAPK, ERK and JNK–pathways was seen in response to mechanical stretch as analyzed by western blot. The ratio of phosphorylated to total p38 MAPK increased transiently, peaking at 4 hours (2.4-fold, $P<0.001$), whereas the activation of ERK and JNK was more rapid, peaking already at 15 to 30 minutes (3.3-fold, $P<0.01$, and 3.1-fold, $P<0.001$, respectively).

5.4.2 DNA microarray analysis of temporal gene expression changes in response to mechanical stretch

To identify temporal gene expression patterns in response to mechanical loading, gene expression profiles were evaluated at 1, 4, 12, 24 and 48 hours after the start of cyclic mechanical stretch in cardiac myocytes *in vitro*. DNA microarray analysis identified altogether 167 different genes that were upregulated and 147 genes that were downregulated at least 2-fold ($P<0.05$) when comparing gene expression patterns of stretched cells with unstretched control cells at 1, 4 and 12 h after the start of the experiments (Fig. 9). After 24 and 48 hours of cyclic mechanical stretching, DNA microarray analysis revealed a total of 632 genes that showed altered gene expression (218 upregulated and 414 downregulated genes) in stretched cells compared with unstretched control cells. Selected microarray results were confirmed by Northern blot analysis or real-time quantitative RT-

PCR, and similar fold changes in mRNA levels were observed as measured by both microarray and Northern/RT-PCR.

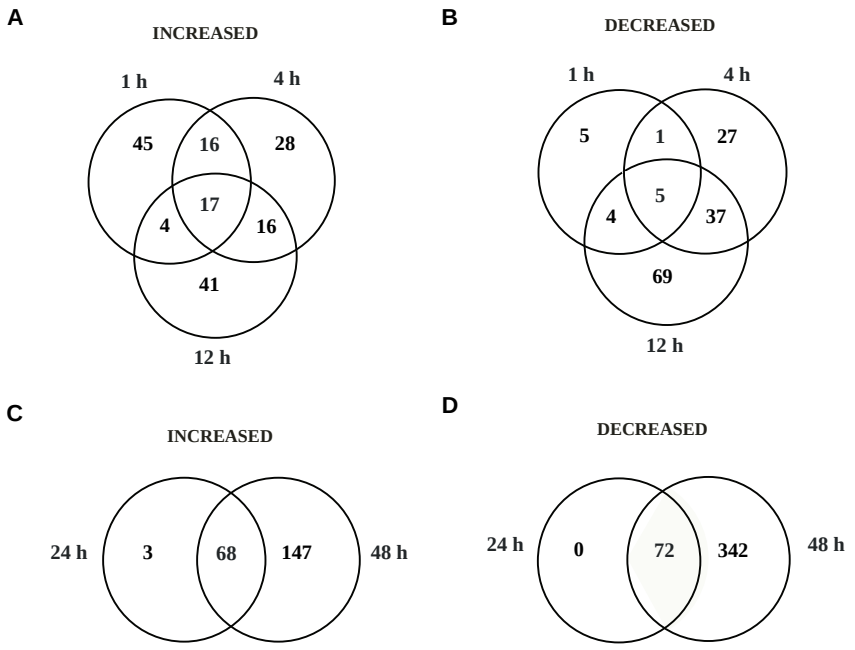

Fig. 9. The effect of cyclic mechanical stretch on gene expression patterns in cardiac myocytes *in vitro*. Venn diagrams showing the number of genes up- or down-regulated at least 2-fold during different time points (A-D). Overlapping parts of the circles represent genes that show differential gene expression at the time points indicated.

1-12 h

After 1 hour of cyclic mechanical stretch, 82 genes were upregulated and only 15 genes downregulated more than 2-fold in stretched cells compared to unstretched cardiac myocytes (Fig. 9). The largest functional group induced by mechanical stretch was genes encoding transcription factors, including well-known factors of the early hypertrophic genetic response like components of the AP-1 complex (*c-fos*, *Jun-B*, *Fra-1*) and early growth response 1 (*Egr-1*) (Sadoshima & Izumo 1997). A number of upregulated genes were also related to cell

signaling/communication, including epiregulin and amphiregulin and growth differentation factor 15.

After 4 hours 147 genes showed altered expression in response to mechanical stretch (77 genes increased and 70 genes decreased more than 2-fold; Fig. 9). At four hours, the majority of changes at the gene expression level were seen in the cell signaling/communication and biosynthesis/metabolism groups. These two functional groups of known genes were also the largest groups that were altered after 12 hours, when expression of 78 genes was increased and 115 was decreased more than 2-fold in stretched cells compared with unstretched control cells. In the stretched cells the expression of genes involved in amino acid and nucleotide metabolism such as arginosuccinate synthetase and cytosolic branched-chain aminotransferase 1 was increased from 4 up to 48 hours.

24-48 h

Gene expression of 71 genes was upregulated and 72 genes downregulated at 24 hours, whereas expression of 215 genes was upregulated and 414 downregulated at 48 hours, when comparing gene expression patterns of stretched cells with unstretched control cells (Fig. 9). Upregulation of genes encoding structural and motility genes such as fibulin 2 was seen 24 hours after the start of mechanical stretch and a number of genes encoding channel and transport proteins including several solute carriers and ion channels were differentially expressed at 24-48 hour time points. After 48 hours of mechanical stretch, increased expression of genes that encode sarcomeric skeleton proteins (e.g. α1-actinin, tubulins β2b, β2c, β3 and β6) were seen as well as upregulation of genes involved in protein modification (e.g. PAI-1) and cell adhesion (tenascin C, integrins αL and αV). At 48 hours, more genes were downregulated than upregulated in the biosynthesis/metabolism group, and e.g. gene expression of muscle creatine kinase, GLUT-4 and pyruvate dehydrogenase kinase 4 was downregulated. Also gene expression levels of many chemokines (e.g. CCL6, CCL9, CXCL5 and CXCL12) were downregulated after 48 hours of stretching. In addition, gene expression of a number of genes encoding channel and transport proteins including several solute carriers and ion channels were differentially expressed at the 24-48 hour time points.

Mechanical stretch -activated gene expression

Gene expression of inflammation-associated genes, such as IL-6 was upregulated throughout the entire time course. Several genes related to calcium signaling, such as modulatory calcineurin-interacting proteins (MCIP1 and MCIP2), were also shown to be differentially regulated. Interestingly, several cell cycle regulators were among the differentially expressed genes in response to mechanical stretch such as CDKN1A and 1C, cyclin D1, G0/G1 switch gene 2 and polo-like kinase 2. However, only a few genes were altered in functional groups involved in apoptosis and protein translation. Notably, most of the altered transcripts were genes with unknown function or ESTs suggesting that several mechanical stretch-activated genes are still undiscovered.

5.4.3 Comparison of gene expression patterns induced by mechanical stretch in vitro and p38 MAPK overexpression in vivo

Gene expression patterns altered in response to mechanical stretch in cardiac myocytes *in vitro* (IV) were compared with those altered by p38 MAPK overexpression in heart *in vivo* (III) to find potential new p38 MAPK -regulated targets of the mechanical stretch induced hypertrophic program. Over 70 different genes, including tenascin C, IL-6, tubulin β3, CDKN1A and TGFβ2, were found to have significantly altered expression in both experimental models.

6 Discussion

6.1 Temporal gene expression profiles in response to increased cardiac load

The development of cardiac hypertrophy and heart failure is a dynamic and complex process, driven by a combination of genetic, epigenetic and environmental factors (Liew & Dzau 2004, Lorell & Carabello 2000). With the emergence of the DNA microarray technique, it is possible to have a more comprehensive characterization of the hypertrophic response at the level of gene expression than in traditional single-gene studies (Liew & Dzau 2004, Liew 2005). However, since cardiac hypertrophy is a progressive disease, the number and character of the genes that are differentially expressed depends on the stage of the hypertrophic process. This was taken into account in studies I, II and IV, since gene expression profiles in response to hemodynamic overload *in vivo* and mechanical stretch *in vitro* were studied at several time points. The AVP-induced pressure overload model facilitated the study of very early transcriptional changes in response to an increase in blood pressure in conscious rats, whereas an animal model of genetic hypertension, SHR, provides a well-established model of essential hypertension for gene expression studies of cardiac hypertrophy. Wall stress represents the major stimulus for cardiac hypertrophy (Lorell & Carabello 2000) and therefore the effect of mechanical stretch on gene expression was investigated in cardiac myocytes. The *in vitro* cyclic mechanical stretch model of cultured neonatal cardiomyocytes provides an appropriate model to study molecular mechanisms of stretch-activated cardiomyocyte hypertrophy. Mechanical stress induces pathophysiological effects in cardiomyocytes in a time-dependent manner, and the changes resemble those of cardiac overload-induced hypertrophy *in vivo* (Pikkarainen *et al.* 2003, Sadoshima & Izumo 1997).

Comprehensive gene expression profiles of LVH have been analyzed taking into account factors such as divergent causes of cardiac hypertrophy, gender, and temporal progression toward cardiac failure (Mirotsou *et al.* 2003, Mirotsou *et al.* 2006, Strom *et al.* 2004, Weinberg *et al.* 2003). However, direct comparison of these studies is difficult, and even in the aortic banding model of pressure overload induced hypertrophy, differences in the degree of constriction, rat/mouse strain, time points studied as well as issues related to the DNA microarray analysis itself, may cause substantial variations in gene expression. Thus, despite

the large amount of data generated by gene expression profiling studies, is still unclear whether different cardiac hypertrophy phenotypes are a result of divergent transcriptional responses or if a common hypertrophic gene program exists (Cerutti *et al.* 2006, Mirotsou *et al.* 2003, Strom *et al.* 2004). However, some common features of the hypertrophic response seem to exist. These were confirmed in the present studies, in which novel genes were also identified.

In agreement with previous studies (Izumo *et al.* 1988, Sadoshima & Izumo 1997), the increased expression of several immediate early genes such as proto-oncogenes and heat shock proteins were seen in response to acute hemodynamic overload *in vivo* as well as to mechanical stretch in cardiomyocyte cultures *in vitro*. In the present study, PAI-1 was also among the early load-responsive genes to increased cardiac load. A hallmark feature of cardiac hypertrophy is re-activation of a fetal gene expression program (Izumo *et al.* 1988, Sadoshima & Izumo 1997, Sugden & Clerk 1998b). According to DNA microarray studies of failing human hearts with different cardiomyopathies, different experimental models of cardiac hypertrophy and heart failure, including genetic animal models of hypertrophy, the induction of ANP and/or BNP seem to be the most predominant change at the gene expression level (Aronow *et al.* 2001, Barrans *et al.* 2002, Boheler *et al.* 2003, Friddle *et al.* 2000, Hannenhalli *et al.* 2006, Hwang *et al.* 2000, Hwang *et al.* 2002, Mirotsou *et al.* 2006, Miyazaki *et al.* 2006, Steenman *et al.* 2003, Strom *et al.* 2004, Tan *et al.* 2002, Weinberg *et al.* 2003, Yang *et al.* 2000, Yung *et al.* 2004). Furthermore, studies of fetal gene expression in physiological and pathological hypertrophy have revealed that upregulation of the fetal gene program and especially ANP and/or BNP gene expression is not observed in exercise-induced hypertrophy, although some controversial results exist (Diffee *et al.* 2003, Iemitsu *et al.* 2005, McMullen & Jennings 2007, Strom *et al.* 2005).

Another key feature of cardiac hypertrophy is downregulation of genes involved in fatty acid metabolism, observed also in the present study. Blunted expression of acyl-CoA-dehydrogenase, muscle creatine kinase, pyruvate dehydrogenase kinase 4 and GLUT-4 are associated with an altered metabolism of the heart in hypertrophy and failure, similar to the downregulation of myoglobin gene expression, reflecting a switch in cardiac substrate selection from fatty acid to glucose utilization (Flogel *et al.* 2005, Taegtmeyer *et al.* 2005).

Cardiac hypertrophy and heart failure have a morphological basis, i.e. structural alterations in myocyte structure and accumulation of interstitial fibrosis represent central features of left ventricular remodeling (Jane-Lise *et al.* 2000,

Swynghedauw 1999, Swynghedauw 2006). Accordingly, in the present study many fundamental genes that encode contractile proteins (e.g. MHC), sarcomeric skeleton proteins (e.g. a-actinin, tubulins), and ECM composition (e.g. collagens, fibulin) were actively regulated also at the transcriptional level. In addition, gene expression of factors promoting growth and fibrosis, such as ET-1 and TGFβ1, were activated during the hypertrophic process.

The development and maintenance of myocardial hypertrophy involves a coordinate response between several major gene programs. Calcium is a direct mediator of electrical activation, ion channel gating and excitation-contraction coupling, and directly activates a variety of signaling pathways. Calcium-dependent signaling is also involved in alterations in gene expression, and some of the key calcium regulatory proteins are regulated at the level of gene expression during hypertrophy and heart failure (Heineke & Molkentin 2006, Houser *et al.* 2000). The present study identified e.g. modulatory calcineurin-interacting proteins (MCIPs) and calpains as putative mediators of calcium-induced pathways mediating mechanical stretch induced hypertrophic signaling.

The present study is consistent with the other DNA microarray studies, identifying several cell-cycle regulators such as cyclins to be differentially expressed in failing human hearts and in experimental models of cardiac hypertrophy (Cerutti *et al.* 2006, Strom *et al.* 2004, Yung *et al.* 2004)). The role of mitotic activity of myocytes is a subject of debate. However, the transcriptional regulation of genes related to the cell-cycle is most likely implicated in other cardiac cell types, and accordingly e.g. in the active remodeling of the myocardial matrix, accompanied by an increased turnover of cellular content. Obviously, these cell-cycle regulators may possess some novel, undefined roles in regulating functions other than cell division.

In summary, small changes within regulatory networks rather than large changes in a single molecular complex may be common to different hypertensive models. In addition, regulation at the level of gene expression seems to be an interplay between several positive and negative regulators of cardiac hypertrophy (Hardt *et al.* 2004). DNA microarrays have suggested a wide array of changes in gene expression are associated with the hypertrophic response. The next step could be to carry out integrated analysis of several gene expression studies to expand the knowledge of the molecular mechanisms of cardiac hypertrophy and heart failure. Also the use of pathway analysis tools could be useful for generating hypothesis for future studies.

6.2 Gene expression patterns during development of LVH and diastolic heart failure

Gene expression profiles associated with left ventricular hypertrophy and diastolic heart failure were studied in SHR, which have alterations in both active relaxation and passive compliance characterizing the diastolic dysfunction (Bing *et al.* 2002, Boluyt & Bing 2000). A key finding of study II was that there was no extensive shift in gene expression patterns when diastolic dysfunction progressed to diastolic heart failure. Even though DNA microarray analysis was extended to include genes that were upregulated at least 1.5-fold, a surprisingly small number of genes showed altered expression. Instead, most of the significant changes in gene expression developed over the whole 8-month follow-up period, associated both with the hypertrophic process and the development of heart failure. Thus, the adaptive response to chronic pressure overload seems to be associated with small, long-term changes at the level of gene expression.

The main functional groups implicated in the development of LVH were genes related to metabolism, cell signaling and cell structure. During the development of left ventricular hypertrophy enhanced expression of genes involved in ECM deposition and turnover was noted as well as downregulation of genes involved in fatty acid metabolism. However, the transition to heart failure seems to be mainly a consequence of increased ECM composition leading to thickening of left ventricular walls, myocardial stiffness and abnormal relaxation. Other DNA microarray studies of left ventricular remodeling associated with pressure overload induced hypertrophy have also highlighted the role of the extracellular matrix in the development of diastolic heart failure (Mirotsou *et al.* 2003, Mirotsou *et al.* 2006). Although changes in calcium homeostasis, myofilament contractile proteins, and cardiomyocyte cytoskeleton proteins have been associated with the development of diastolic heart failure (Kass *et al.* 2004, Zile & Brutsaert 2002), they do not seem to be significantly regulated at the gene expression level in diastolic heart failure caused by pressure-overload hypertrophy.

6.2.1 Novel genes associated with diastolic heart failure

DNA microarray analysis of hypertensive cardiac hypertrophy identified several novel and potentially important genes such as TSP-4 and lysyl oxidase like

(LOXL) protein that are associated with hypertrophy and the development of diastolic heart failure.

Thrombospondin-4

The highest upregulation in gene expression levels during the development of left ventricular hypertrophy in SHR was displayed by TSP-4 and it was one of the few genes that showed marked upregulation also after development of diastolic dysfunction (i.e. after 16 months). Northern analysis confirmed the DNA microarray results, since a progressive increase in mRNA levels was seen in SHR with aging, whereas mRNA levels were almost undetectable in the left ventricle of WKY rats. Thrombospondins 1-5 are multidomain matricellular proteins that function as regulators of various cell interactions including myocardial matrix remodeling (Schellings *et al.* 2004, Spinale 2002). Not much is known about TSP-4, which is expressed e.g. in heart, skeletal muscle and brain (Arber & Caroni 1995, Lawler *et al.* 1993). Increased thrombospondin-4 mRNA levels have been previously reported in patients with end-stage dilated cardiomyopathy (Tan *et al.* 2002) and coronary artery disease (Gabrielsen *et al.* 2007), in hypertensive rat strains (Cerutti *et al.* 2006), as well as after experimental myocardial infarction in rats (Jin *et al.* 2001). TSP-4 was implicated in a core set of genes with consistent differential expression in four different models of cardiac hypertrophy as assessed by DNA microarrays, suggesting that TSP-4 may play an important role in mediating ventricular remodeling (Strom *et al.* 2004). According to the studies II-IV, neither p38 MAPK activation nor mechanical stretch alter TSP-4 mRNA levels, so the molecular mechanisms involved in activation and regulation of TSP-4 gene expression remain to be studied.

Lysyl oxidase-like protein

Increased LOXL mRNA levels were measured in SHR during progression of LVH. Lysyl oxidase and the related protein LOXL are extracellular copper enzymes, which catalyze the formation of lysine-derived cross-links in fibrillar collagens and elastin (Csiszar 2001). Increased LOXL protein levels have been measured in several models of fibrosis, suggesting that LOXL plays an important role in remodeling the extracellular environment during development and fibrotic processes (Hayashi *et al.* 2004, Kim *et al.* 1999). Increased LOXL mRNA levels have been measured in response to various hypertrophic stimuli, since LOXL was

coregulated during ventricular remodeling following myocardial infarction (Mirotsou *et al.* 2003) as well as in response to pressure and volume overload induced cardiac hypertrophy (Mirotsou *et al.* 2003, Miyazaki *et al.* 2006). It has been shown that inhibition of collagen cross-linking results in a reduction of fibrillar collagen deposition in the myocardium (Kato *et al.* 1995). Thus upregulation of LOXL may play an important role in the remodeling process of LVH affecting collagen deposition and consequently ventricular stiffness during the development of diastolic heart failure.

6.3 The role of p38 MAPK in regulating cardiac gene expression

Although p38 MAPK plays an important role in numerous biological processes and seems to have a central position in hypertrophic signaling (Kyriakis & Avruch 2001, Sugden & Clerk 1998b, Wang 2007), the understanding of p38 MAPK dependent gene expression has remained uncompletely defined. In previous studies gene expression profiles of pharmacological p38 MAPK inhibition has been determined in transformed follicular lymphoma cells and in cultured cardiomyocytes (Engel *et al.* 2005, Lin *et al.* 2004). In addition, transcription profiling of transgenic MKK3b adult mouse hearts has been performed following MKK3b transgene induction by tamoxifen (Mitchell *et al.* 2006). However, pharmacological inhibitors have systemic effects *in vivo* and the lack of specificity of the inhibition causes off-target effects to other kinases as well (Wang 2007). On the other hand, in transgenic animals the direct effects of p38 MAPKs on gene expression are difficult to distinguish from the secondary ones, such as the effect of hemodynamic changes (Liao *et al.* 2001). Furthermore, cardiac transgenesis with tamoxifen induction itself may have significant effects on myocardial gene expression as reported with transgenesis using the tetracycline transactivator (McCloskey *et al.* 2005). Thus local intramyocardial adenovirus-mediated overexpression of p38 MAPK provided an appropriate method to study the direct effect of p38 MAPK activation on gene expression at a time point when no functional or structural changes were observed and all cardiac cell types are affected by p38 MAPK activation.

6.3.1 p38 MAPK regulated transcription factors

Cardiac transcription factors play a predominant role in regulating gene expression during the development of LVH and heart failure (Akazawa &

Komuro 2003, Oka *et al.* 2007b). Many of these transcription factors are in turn activated through phosphorylation mediated by intracellular signal transducers such as MAPKs (Oka *et al.* 2007b). The p38 MAPK is known to phosphorylate e.g. GATA-4 and components of the AP-1 transcription factor (Pikkarainen *et al.* 2003). Adenovirus-mediated p38 MAPK overexpression resulted in activation of DNA-binding activities of AP-1, GATA-4, NF-kB and SRF transcription factors suggesting that gene expression patterns controlled by p38 MAPK may be a result of transcriptional control through these transcription factors, although p38 MAPK overexpression may affect other intracellular signaling molecules as well. In addition, the impact of p38 MAPK activation on gene expression may be a consequence of the interaction of AP-1, GATA-4, NF-kB and SRF with other transcription factors.

6.3.2 p38 MAPK regulated cardiac gene expression

DNA microarray analysis of cardiac specific overexpression of p38 MAPK revealed 264 genes that were upregulated more than 2-fold, and the majority of which were related to cell division, inflammation, cell signaling, cell adhesion and transcription. Activation of the p38 MAPK pathway has been reported in response to inflammation, and p38 MAPK has been implicated in regulating cell cycle and division. Thus, gene expression patterns identified in the present study correlated with the known functions of p38 MAPK. In histological analysis of p38 MAPK-treated hearts, fibrosis and infiltration of inflammatory cells were not seen until one week after gene transfer, suggesting that upregulation of genes related to inflammation and fibrosis preceded the functional changes. In addition, the activities of AP-1 and NF-kB were upregulated by p38 MAPK overexpression, which agrees with the gene expression data, since both of these factors are known to be involved in inflammatory responses as well as in control of the cell cycle and fibrosis (Manabe *et al.* 2002). Furthermore, this finding is in agreement with the studies in dTGR(hAOGEN-hREN), which indicate that AP-1 and NF-kB transcription factors are involved in initiating chemokine and cytokine expression associated with angiotensin II –induced end-organ damage (Luft *et al.* 1999).

Based on histological staining, the majority of the proliferating cells were inflammatory cells. Chronic inflammation is characteristic also for heart failure and proinflammatory cytokines have several effects on left ventricular remodeling, influencing cardiac contractility, cardiomyocyte hypertrophy as well as apoptosis and necrosis (Mann 2003). The present study underscores the role of p38 MAPK

in the regulation of inflammatory pathways *in vivo*, similar to previous reports (Li *et al.* 2005, Park *et al.* 2007). The comparison of p38 MAPK activated gene expression profiles to differentially expressed genes in SHR showed only minor similarities. Thus, although p38 MAPK is implicated in the regulation of fibrosis, p38 MAPK does not seem to contribute to regulation of ECM gene expression in SHR.

6.3.3 p38 MAPK regulated genes in response to mechanical stretch

p38 MAPK controlled a wide array of genes at the transcriptional level in the heart *in vivo*. By comparing gene expression profiles of p38 MAPK overexpression *in vivo* to differentially expressed genes regulated by cyclic mechanical stretch in cardiomyocytes *in vitro*, a number of genes that mechanical stretch may activate via p38 MAPK were identified (IV). Temporal analyses have shown a double peak activation for p38 (Hoshijima & Chien 2002) and similar biphasic activation was seen in p38 MAPK regulated genes in response to mechanical stretch, as measured by a number of differentially expressed genes. In cardiomyocytes, the p38 MAPK predominant targets are, according to gene expression pattern comparison analysis, transcription factors and cell signaling molecules. The present results suggest that mechanical stretch activates gene expression of e.g. ANP, tenascin C, tubulin β3, CDKN1A, PAI-1 and TGFβ2 at least partly via p38 MAPK (Fig. 10).

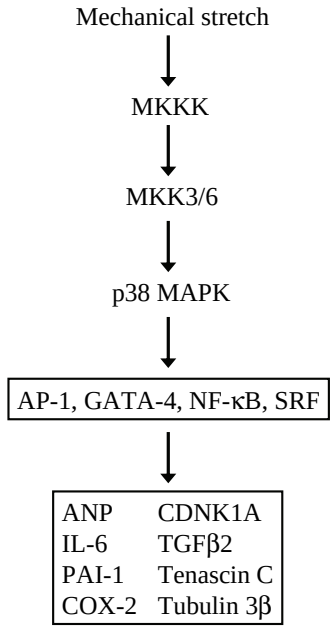

Fig. 10. Schematic presentation of the results of current studies focusing on the role of p38 MAPK in regulation of mechanical stretch-activated gene expression in cardiac myocytes.

6.3.4 Novel p38 MAPK regulated targets associated with mechanical stretch induced hypertrophic response

DNA microarray analysis of mechanical stretch induced gene expression changes in cardiomyoyte cultures identified several novel genes such as PAI-1 and CDKN1A, which are putatively regulated by p38 MAPK.

Plasminogen activator inhibitor-1

PAI-1 is a member of serine protease inhibitor superfamily, and the major physiological inhibitor of tissue and urokinase plasminogen activators. Plasminogen activators (PA) and PAI-1 play key roles in regulation of fibrinolysis *in vivo* and in the proteolytic degradation of the extracellular matrix (Saksela &

Rifkin 1988, Vassalli *et al.* 1991). PAI-1 is elevated acutely in thrombotic events (such as deep vein thrombosis) but is also associated with angiogenesis and vascular remodeling in chronic diseases, i.e. atherosclerosis, cardiac hypertrophy and myocardial infarction (Bloor *et al.* 1997, Juhan-Vague & Alessi 1993, Thompson *et al.* 1995). Increased PAI-1 mRNA levels have been reported e.g. during left ventricular remodeling in several experimental models of cardiac hypertrophy (Strom *et al.* 2004), after acute muscle contraction (Chen *et al.* 2002) as well as after cyclic strain in human umbilical vein endothelial cells (HUVECs) (Frye *et al.* 2005).

PAI-1 gene expression was upregulated in the old SHR suggesting altered extracellular matrix degradation in the hypertrophic left ventricle. However, PAI-1 mRNA levels were also markedly elevated in response to acute pressure overload *in vivo*, the induction of PAI-I mRNA being greater than that observed for *Hsp-70* or *c-jun*. Similarly, upregulation of PAI-1 gene expression was seen already after 1 hour of mechanical stretch in cultured cardiac myocytes. Thus, PAI-1 gene expression mimics the rapid induction of proto-oncogenes and it seems to be a major stress-regulated gene. In addition, the present data implies that mechanical stretch could activate PAI-1 gene expression via p38 MAPK in cardiac myocytes. However, the precise role of PAI-1 as a regulator of stress-induced cascades in heart remains to be studied.

CDKN1A

CDKN1A (p21Cip1/Waf1) is an important regulator of the cell cycle, which inhibits CDKs, arresting cells in the G1 phase of the cell cycle (Sherr & Roberts 1995). Increased levels of CDKN1A have been reported also in failing human hearts (Steenman *et al.* 2003, Tan *et al.* 2002, Yung *et al.* 2004) and during left ventricular modeling in cardiac hypertrophy (Mirotsou *et al.* 2003). Upregulation of CDKN1A gene expression was seen in hearts overexpressing p38 MAPK and in stretched cardiomyocytes. Even though CDKN1A has been shown to directly inhibit hypertrophy in cardiomyocytes (Tamamori *et al.* 1998), it is more likely that CDKN1A is implicated in cell cycle progression in other cardiac cell types than cardiac myocytes. In late-stage human hearts, cellular proliferation may be related to inflammatory response and fibrosis, whereas in neonatal cardiomyocyte cultures some fibroblasts remain, although their numbers are low (<3-5%) (Boerma *et al.* 2005). On the other hand, neonatal cardiac myocytes have some features of fetal cells (Simpson & Savion 1982), thus some activity of genes

related to mitosis can be expected. However, cell cycle regulators such as CDKN1A may have new, undefined roles in cells. CDKN1A was found to repress cardiomyocyte apoptosis induced by hypoxia (Hauck *et al.* 2002), thus increased expression of this antiapoptotic gene may contribute to myocyte survival. Furthermore, CDKN1A has been reported to bind to the promoter of the *myc* gene and inhibit its activation together with other transcription factors (Vigneron *et al.* 2006).

6.4 Limitations of DNA microarray methodology

Microarray studies have been criticized as confounded by a number of sources of variation ranging from technical errors to experimental design and biological variation. When the technical performance of DNA microarrays was assessed in the MAQC project, inter- and intraplatform reproducibility of gene expression measurements were reported among the different laboratories (Canales *et al.* 2006, MAQC Consortium *et al.* 2006). However, regardless of the technical reliability and the huge amount of data that is being generated in gene expression profiling experiments, it is difficult to perform direct comparisons of different microarray studies, because of the issues related to e.g. different platforms, statistical methods and gene profiling of tissues containing mixed cell populations.

It should be remembered that DNA microarrays do not measure gene expression but signal intensity, and the assumption that the quantity of mRNA in the sample represents the degree of transcription of that gene, has been done (Hovatta *et al.* 2005). Thus, the probe design and selection must be optimized and validated for maximal sensitivity and specificity. In Affymetrix microarrays, multiple probes are designed near the 3' end of the mRNA sequence. This 3' end limitation makes it impossible to identify transcripts with undefined 3' ends and truncated or alternatively spliced transcripts. It has recently become possible to use exon arrays i.e. whole transcript arrays, in which probesets cover the entire RNA transcripts, providing a more accurate view of gene expression (Abdueva *et al.* 2007). In addition, new high-throughput sequencing techniques provide a new tool also for gene expression profiling (Margulies *et al.* 2005).

In the studies I-IV, gene expression analyses were optimized by avoiding inter-animal pooling of samples, using at least four technical replicates per sample and no target RNA amplification was done. The first generation cDNA arrays (I) suffered from a lack of sensitivity and limited dynamic range as well as a modest number of genes spotted on the filters. DNA microarray results should be

validated by another quantitative gene expression technique such as Northern blot or quantitative RT-PCR. In studies I-IV, similar fold changes in mRNA levels were measured by both DNA array and real-time quantitative RT-PCR or Northern blot. The most common sources of variability in comparing DNA microarray results with other quantitative gene expression platforms are differences in probe sequence and thus the target location (Canales *et al.* 2006).

An important issue in analyzing DNA microarray data is the choice of statistical tests. However, a recent report indicates that in identifying differentially expressed genes, fold-change ranking plus a nonstringent *P* cutoff can be successful in identifying reproducible gene lists, instead of relying heavily on the statistical significance (Canales *et al.* 2006, MAQC Consortium *et al.* 2006). On the other hand, once generated, the DNA microarray data can be re-analyzed by using a broad variety of algorithms to extract biologically relevant information. For example Gene Set Enrichment Analysis (GSEA) (Mootha *et al.* 2003, Subramanian *et al.* 2005) could be used to re-analyze the data of the studies II-IV, since GSEA is designed to detect modest but coordinate changes in the expression of groups of functionally related genes.

Finally, DNA microarrays define gene expression at the steady-state RNA transcript level and it should remembered that gene expression is regulated at many levels and regulation of mRNA stability contributes significantly to the observed changes in gene expression as measured by DNA microarrays (Cheadle *et al.* 2005b). For example, integrating gene and protein expression patterns would provide more in-depth insight into molecular mechanisms of cardiac hypertrophy and heart failure, since the transcript levels provide little predictive value with respect to protein expression (Gygi *et al.* 1999, Tuomisto *et al.* 2005). However, despite its limitations, DNA microarrays are a powerful tool to gain a more comprehensive, genome wide understanding of changes at the level of gene expression as well as to identify new targets for diagnosis and treatment of cardiac hypertrophy and heart failure.

7 Conclusions

In the present study, cardiac gene expression profiles were studied in experimental models of cardiac load. A number of novel modulators associated with increased cardiac load were identified, and well-established gene expression changes of cardiac hypertrophy were observed.

1. The increased expression of several immediate early genes such as proto oncogenes and heat shock proteins were seen in response to acute hemodynamic overload *in vivo*.
2. The transition from left ventricular hypertrophy to diastolic hypertensive heart failure was almost exclusively associated with changes in genes encoding extracellular matrix proteins and their regulatory processes showing the importance of progressive ECM remodeling in predisposing to failure.
3. Cardiac-specific overexpression of p38 MAPK resulted in upregulation of the genes controlling cell division, inflammation as well as cell signaling and adhesion. Accordingly, the functional role of p38 MAPK in the adult heart was related to myocardial cell proliferation, inflammation and fibrosis.
4. Temporal analysis of mechanical stretch induced gene expression changes in neonatal rat cardiomyocyte cultures indicated that mechanical stretch induced complex gene expression profiles, demonstrating that both positive and negative regulators are involved in the hypertrophic process. Many novel stretch responsive genes were identified, and a subset of them may be putative downstream targets of p38 MAPK.

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II Rysä J, Leskinen H, Ilves M & Ruskoaho H (2005) Distinct Upregulation of Extracellular Matrix Genes in Transition from Hypertrophy to Hypertensive Heart Failure. Hypertension 45(5):927-933.

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