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p38 MITOGEN-ACTIVATED PROTEIN KINASE AND TRANSCRIPTION FACTOR GATA-4 IN THE REGULATION OF CARDIOMYOCYTE FUNCTION

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE,
INSTITUTE OF BIOMEDICINE,
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY;
BIOCENTER OULU

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Abstract

Cardiovascular diseases are the leading causes of death in the developed countries and their incidence is not expected to decrease in the future. There is a lifetime risk of one in five of developing heart failure, which still has poor prognosis and current treatments only cover part of the pathophysiology behind this syndrome. Pathological processes contributing to heart failure include cardiac hypertrophy and remodeling, which involves neurohumoral activation, reactivation of fetal genes, impaired Ca^{2+} cycling, increased apoptosis, and increased fibrosis. Intracellular signalling pathways and transcription factors mediating the response to various extracellular stresses have a key role in the regulation of myocardial remodeling and they are investigated in order to develop new approaches for the treatment of heart failure.

The aim of this thesis was to elucidate roles of mitogen-activated protein kinases (MAPKs) and transcription factor GATA-4 in the regulation of cardiomyocyte function in cell cultures, and in hearts *ex vivo* and *in vivo*. The main findings were that (i) Inhibition of p38α MAPK enhanced function of sarco/endoplasmic reticulum Ca^{2+} -ATPase and thus cardiac contractility by increasing phosphorylation of protein phosphatase inhibitor-1 and phospholamban, (ii) p38 MAPK isoforms p38α and p38β regulated promoter activity of B-type natriuretic peptide via distinct pathways, (iii) p38α and p38β MAPKs also had different effects on gene expressions related to fibrosis and hypertrophy, and (iv) p38 and ERK1/2 MAPKs mediated stretch-induced activation of GATA-4 by phosphorylation at Ser 105. GATA-4 also seems to be regulated by ubiquitination.

This study provides novel data of p38 MAPK and GATA-4 in the regulation of cardiomyocyte function. Inhibition of p38α MAPK could be beneficial in the treatment of heart failure. Also GATA-4 is a potential target for treatment of cardiovascular diseases.

Keywords: B-type natriuretic peptide, GATA-4 transcription factor, heart failure, mitogen-activated protein kinases, myocardial contraction, p38 mitogen-activated protein kinase, ventricular remodeling

Kaikkonen, Leena, p38 mitogeeniaktivoituva proteiinikinaasi ja transkriptiotekijä GATA-4 sydänlihassolun toiminnan säätelijöinä.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Farmakologia ja toksikologia; Biocenter Oulu

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Tiivistelmä

Sydän- ja verisuonisairaudet ovat yleisin kuolinsyy länsimaissa, eikä niiden ilmaantuvuus tule vähenemään lähitulevaisuudessa. Elinikäinen riski sairastua sydämen vajaatoimintaan on 20 %, ja sydämen vajaatoiminnan ennuste on edelleen huono. Nykyisillä hoitomuodoilla voidaan puuttua vain osittain sydämen vajaatoiminnan patofysiologisiin mekanismeihin. Sydämen vajaatoiminnan kehittymiseen liittyvät sydänlihaksen liikakasvu ja uudelleenmuovautumisprosessi, johon liittyy neurohumoraalinen aktivaatio, sikiöaikaisten geenien uudelleenilmentyminen, häiriöt solunsisäisessä Ca^{2+}-viestinnässä sekä lisääntynyt ohjelmoitu solukuolema ja sidekudoksen muodostuminen sydämeen. Solunsisäisillä viestinvälitysketjuilla sekä transkriptiotekijöillä, jotka vastaavat solunulkoisten ärsykkeiden välittämisestä solun sisällä, on keskeinen rooli edellämainittujen prosessien säätelyssä. Uusien lähestymistapojen kehittäminen sydämen vajaatoiminnan hoitoon edellyttää myös solunsisäisen viestinvälityksen ja geenien säätelyn mekanismien selvittämistä.

Tämän väitöstyön tavoite oli selvittää p38 mitogeeniaktivoituvan proteiinikinaasin (p38 MAPK) ja transkriptiotekijä GATA-4:n merkitystä sydämen vajaatoiminnan patogeneesissä soluviljelymalleissa. Päälöydöksiä olivat: (i) p38α MAPK -isoformin estäminen paransi kalsiumia solulimakalvostoon pumppaavan SERCA2a:n toimintaa ja sydänlihassolun supistumiskykyä lisäämällä fosfolambaanin ja proteiinifosfataasi-inhibiittori-1:n fosforylaatiota. (ii) p38 MAPK isoformit p38α ja p38β säätelivät B-tyypin natriureettisen peptidin geenin promoottorialuetta erillisten reittien kautta. (iii) p38α ja p38β isoformit vaikuttivat myös eri tavoin sydämen sidekudoksen muodostumiseen ja hypertrofiaan liittyvien geenien ilmentymiseen. (iv) p38 ja ERK1/2 välittävät venytyksen aiheuttaman GATA-4:n aktivaation fosforyloimalla seriini-105 fosforylaatiopaikan. Lisäksi GATA-4:n toimintaa säädellään ubiquitinaation avulla.

Tämä tutkimus tuo uutta tietoa p38 MAPK:n ja GATA-4:n rooleista sydämen vajaatoiminnan kehittymisessä. p38α-isoformin toiminnan estäminen voisi olla hyödyllinen hoitomuoto sydämen vajaatoiminnassa. Myös GATA-4 on potentiaalinen lääkehoidon kohde sydänsairauksien hoidossa.

Asiasanat: B-tyypin natriureettinen peptidi, mitogeeniaktivoituvat proteiinikinaasit, p38 mitogeeniaktivoituva proteiinikinaasi, sydämen vajaatoiminta, sydänlihaksen supistuminen, sydänlihaksen uudelleenmuovautuminen, transkriptiotekijä GATA-4

To my family and friends.

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Oulu, June 2014 Leena Kaikkonen

List of abbreviations

AC	adenylyl cyclase
Ang II	angiotensin II
ANP	atrial natriuretic peptide
AP-1	activator protein-1
AMVM	adult mouse ventricular myocytes
ARVM	adult rat ventricular myocytes
ATF	activating transcription factor
ATP	adenosine triphosphate
BDM	2,3-butandione monoxime
BNP	B-type natriuretic peptide
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CaMKII	Ca^{2+}/calmodulin-dependent kinase
CASQ2	calsequestrin-2
CMF	cardiac myofibroblast
CTGF	connective tissue growth factor
dn	dominant negative
DUSP	dual specificity phosphatase
EGF	epidermal growth factor
EF	ejection fraction
EPO	erythropoeitin
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
GPCR	G-protein coupled receptor
GRK-2	G-protein coupled receptor kinase-2
GSK-3β	glycogen synthase kinase-3β
HAT	histone acetyltransferase
HDAC	histone deacetylase
HF	heart failure
Hsp	heat shock protein
I-1	inhibitor-1
IL	interleukin
I/R	ischemia/reperfusion
JNK	c-Jun N-terminal kinase

LPS	lipopolysaccharide
LV	left ventricle
LVEF	left ventricular ejection fraction
LTCC	L-type calcium channel
MAPK	mitogen-activated protein kinase
MDM2	murine double-minute-2
MK	MAPK-activated protein kinase
MEF2c	myocyte-specific enhancer factor 2c
MHC	myosin heavy chain
MI	myocardial infarction
MKK	MAPK kinase
MMP	matrix metalloproteinase
NCX	sodium-calcium exchanger
NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLK	nemo-like kinase
NRVM	neonatal rat ventricular myocyte
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PE	phenylephrine
PI3K	phosphatidylinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLB	phospholamban
PP1	protein phosphatase-1
PP2A	protein phosphatase-2A
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RyR2	ryanodine receptor-2
SAPK	stress-activated protein kinase
SERCA2a	sarco/endoplasmic reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum
SRF	serum response factor
STAT3	signal transducer and activator of transcription 3
TAB-1	TGF-β-activated protein kinase 1 binding protein 1
wt	wild type

List of original articles

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Kaikkonen L, Magga J, Ronkainen V-P, Koivisto E, Perjes Á, Chuprun JK, Vinge LE, Kilpiö T, Aro J, Ulvila J, Alakoski T, Bibb JA, Szokodi I, Koch WJ, Ruskoaho H & Kerkelä R (2014) p38α regulates SERCA2a function. J Mol Cell Cardiol 67(0): 86–93.

II Koivisto E*, Kaikkonen L*, Tokola H, Pikkarainen S, Aro J, Pennanen H, Karvonen T, Rysä J, Kerkelä R & Ruskoaho H (2011) Distinct regulation of B-type natriuretic peptide transcription by p38 MAPK isoforms. Mol Cell Endocrinol 338(1–2): 18–27.

III Kaikkonen L, Tokola H, Kinnunen S, Tölli M, Välimäki M, Rysä J, Moilanen A-M, Karkkola L, Kerkelä R, Nemer M, Ruskoaho H. Molecular forms and regulation of GATA-4 in mechanical stretch-induced hypertrophy. Manuscript.

*Equal contribution.

In addition, some unpublished data are presented.

Contents

1 Introduction

Cardiovascular diseases are the leading cause of death in the Western countries (McMurray *et al.* 2012, Roger 2013). Heart failure is defined as impaired cardiac pump function, leading to reduced blood flow into other organs and tissues. Heart failure is not a single disease but syndrome following various cardiac pathologies. The main etiological causes of heart failure are systemic and pulmonary hypertension, coronary artery disease, valvular disease and cardiomyopathies. The prevalence of heart failure is 1–2% in developed countries and typically its incidence is higher in elderly people. (McMurray *et al.* 2012). Despite the treatment available, the prognosis of heart failure is still poor. More than 40% of patients with a severe heart failure diagnosis die within five years in the US (Roger 2013). Current treatment of heart failure focuses on improving the prognosis of patient, and relieving symptoms. Currently, the only effective therapies available to positively influence the long-term prognosis involve the inhibition of the neuroendocrine activation that is associated with impaired pumping function (McMurray 2010) and heart transplantation for end-stage heart failure (McMurray *et al.* 2012). Left or bi-ventricular assist device can be used when patient is waiting for heart transplantation (McMurray *et al.* 2012).

Heart failure is usually preceded by left ventricular (LV) hypertrophy and remodelling. Molecular pathways regulating LV hypertrophy and remodeling are investigated in order to develop novel therapies to prevent and reverse pathological LV hypertrophy and LV remodeling (Shah & Mann 2011, van Berlo *et al.* 2013). The hypertrophic response is initially a beneficial adaption to hypertrophic stimuli, trying to maintain pumping capacity by increasing cardiomyocyte size, but eventually it leads to remodeling of the heart tissue (El-Armouche & Eschenhagen 2009, Mudd & Kass 2008). This phenomenon includes e.g. accelerated fibrosis and increased apoptosis. Cardiomyocyte overload induces changes in cardiac gene expression, including an increase in atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) expression, which is considered as a marker of impaired cardiac output (Swynghedauw 1999).

Impaired Ca^{2+} cycling is a hallmark of heart failure at the cellular level. Ca^{2+} is a central messenger in excitation-contraction coupling in the cardiomyocytes. It is stored in the sarcoplasmic reticulum, where it is released upon excitatory stimulus. It is necessary to pump calcium back into the sarcoplasmic reticulum to maintain efficient contractile function. Sarco/endoplasmic Ca^{2+} ATPase (SERCA2a) is the ion pump responsible for Ca^{2+} resequestration to the

sarcoplasmic reticulum. Correction of aberrant Ca^{2+} cycling is a promising new approach for the treatment of heart failure (Kranias & Hajjar 2012).

Mitogen-activated protein kinases (MAPKs) are a family of signaling molecules involved in cardiac hypertrophy and remodeling. p38 MAPK is a stress-activated kinase and its activation is increased e.g. in the ischemic heart (Rose *et al.* 2010). It has been shown to regulate a number of other protein kinases and transcription factors. The exact role of p38 in cardiac hypertrophy and heart failure is yet to be elucidated, but it is considered a potential target for the treatment of cardiac diseases. (Martin *et al.* 2012).

GATA-4 is a transcription factor, which plays an important role in cardiogenesis during embryonic development and regulates the expression of various genes, including ANP and BNP, in the adult heart (Pikkarainen *et al.* 2004, Temsah & Nemer 2005). GATA-4 has cardioprotective effects (Oka *et al.* 2006) and it is also involved in cell differentiation and reprogramming of fibroblasts into functional cardiomyocytes (Gupta *et al.* 2013, Ieda *et al.* 2010, Qian *et al.* 2012, Takeuchi & Bruneau 2009). Thus, GATA-4 is also a potential target for the future treatment of cardiac diseases.

2 Review of literature

2.1 Overview of heart failure

Failing heart is a pump that is not able to pump oxygenated blood into the circulation because its contractile capacity is decreased. Clinically, heart failure can be classified as new (de novo), transient or chronic heart failure (CHF). Acute heart failure, which causes most of hospitalizations with urgent need of therapy, can appear *de novo* or as a result of decompensated CHF. (Tamargo & López-Sendón 2011). Heart failure is defined as systolic or diastolic dysfunction according to ejection fraction (EF). Diastolic heart failure, or heart failure with preserved EF, is usually a consequence of concentric hypertrophy, where left ventricle (LV) volume is normal or reduced. The cross-sectional area of cardiomyocytes grows, which usually results from persistent hypertension or cardiomyopathy. (van Berlo *et al.* 2013). Systolic heart failure, or heart failure with reduced EF, results in impaired contraction during systole. LV volume is usually increased with wall thinning and predominating lengthening of individual myocytes (van Berlo *et al.* 2013). This eccentric growth response may occur for example as a result of myocardial infarction or transition of concentric hypertrophy. In addition, previous viral infection, alcohol abuse, chemotherapy (e.g. doxorubicin or trastuzumab) and idiopathic dilated cardiomyopathy may cause systolic heart failure. Heart failure with reduced EF is the most studied in terms of pathophysiology and treatment. (McMurray *et al.* 2012).

In contrast to pathological hypertrophy, there also exists physiological hypertrophy, which drives the normal growth of the heart from birth to early adulthood, the growth of maternal heart during pregnancy, and the growth of the heart as a result of extreme and/or repetitive exercise (Maillet *et al.* 2013). This physiological hypertrophy differs from the pathological form in coordinated growth in wall and septal thicknesses, where individual cardiomyocytes grow in both length and width, resulting in increased ventricular volume (Maillet *et al.* 2013).

Figure 1 presents the vicious circle of heart failure pathophysiology. Cardiac injury, e.g. myocardial infarction, impairs cardiomyocyte contractile function and cardiac output is decreased. To stabilize blood pressure and cardiac output, the body activates the sympathetic nervous and renin-angiotensin-aldosterone (RAAS) systems. (McMurray 2010). Sympathetic activation leads to the release of

catecholamines, which stimulate β-adrenergic receptors resulting in an increased amount of cyclic adenosine monophosphate (cAMP). This improves cardiac output acutely by increasing heart rate and contraction force. (El-Armouche & Eschenhagen 2009). However, if sympathetic activation is sustained, β-adrenergic overstimulation turns detrimental as a result of peripheral vasoconstriction and cardiomyocyte toxicity due to increased energy consumption, fibrosis, cardiomyocyte hypertrophy, and arrhythmia. Eventually, chronic β–adrenergic stimulation leads to heart failure (El-Armouche & Eschenhagen 2009). Activation of RAAS leads to production of angiotensin II (Ang II), which modulates cardiac contractility, metabolism, and hypertrophic growth and also increases vasoconstriction and release of aldosterone. Activation of RAAS also results in the release of noradrenaline, promotes sodium reabsorption, stimulates vasopressin release, and increases contractility. (Kemp & Conte 2012, Rohini *et al.* 2010).

The expression and levels of various vasodilatory molecules are also increased in the failing heart, including natriuretic peptides, prostaglandins (PGE_2 and $PGEI_2$) and nitric oxide, to balance the excessive vasoconstriction resulting from excessive activation of the adrenergic and renin-angiotensin systems (Mann & Bristow 2005).

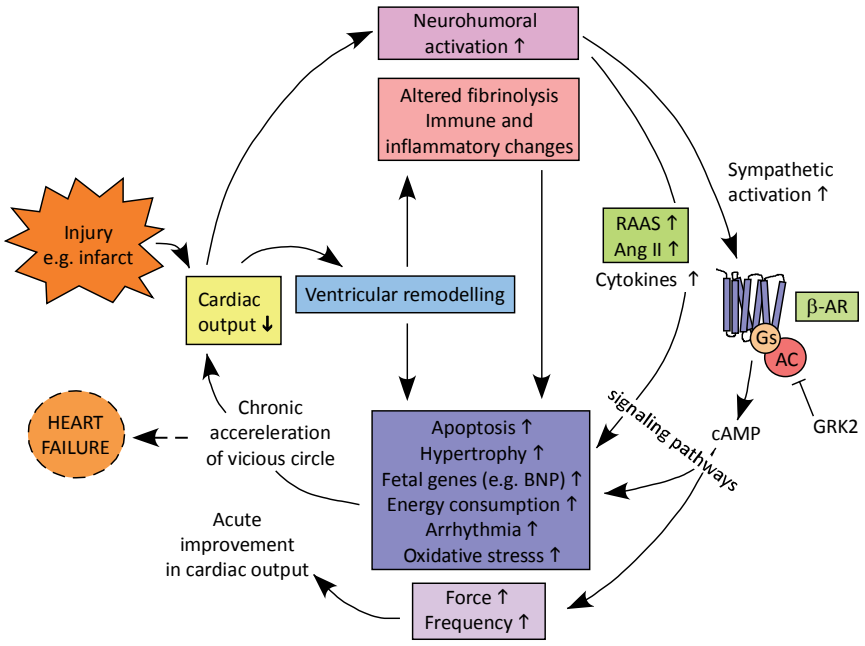

Fig. 1. Vicious circle of heart failure pathogenesis. AC, adenylate cyclase; Ang II, angiotensin II; β-AR, β-adrenergic receptor; BNP, B-type natriuretic peptide; cAMP, cyclic adenosine monophosphate; GRK2, G-protein coupled receptor kinase-2; RAAS, renin-angiotensin-aldosterone system. Modified from El-Armouche & Eschenhagen 2009.

2.2 Cardiac remodeling

Cardiac injury eventually initiates also LV remodeling, which is characterized by changes in LV mass, volume, shape and composition of the ventricle in response to the mechanical stress (stress and strain) and neurohumoral activation (Fig. 1) (Mann *et al.* 2012). This process in the failing cardiomyocytes includes cardiomyocyte hypertrophy, changes in excitation-contraction coupling, progressive loss of myofilaments, β-adrenergic desensitization, abnormal myocardial energetics secondary to mitochondrial abnormalities and altered substrate metabolism and progressive loss and/or disarray of the cytoskeleton (Bernardo *et al.* 2010, Mann *et al.* 2012). Impaired Ca^{2+} cycling and Ca^{2+} homeostasis predispose to contractile dysfunction (Lompré *et al.* 2010). Myocardial defects associated with LV remodeling include myocyte death, in the

form of apoptosis, necrosis, and autophagy, and alterations in the extracellular matrix, which includes matrix degradation by matrix metalloproteinases and increased accumulation of fibrotic tissue (Mann *et al.* 2012).

Cardiac fibroblasts have a key role in the remodeling process, since they can differentiate into the cardiac myofibroblast (CMF) phenotype that exhibits increased migratory, proliferative and secretory properties. The increase in cardiac hemodynamic load also induces the release of pro-inflammatory cytokines (tumor necrosis factor, TNF; interleukin-1β, IL-1β and interleukin-6, IL-6), vasoactive peptides (Ang II; BNP; ANP; endothelin, ET-1; noradrenaline) and profibrotic growth factors (transforming growth factor β, TGF-β, and insulin-like growth factor, IGF) from the fibroblasts. (Tamargo & López-Sendón 2011). Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes facilitating removal of cell and tissue debris and promoting neovascularization, prior to scar formation (Porter & Turner 2009). Pro-inflammatory cytokines negatively regulate cardiac function by activating MMPs, inducing apoptosis and promoting hypertrophy. They may also have direct effect on contractile function (Prabhu 2004, Tamargo & López-Sendón 2011). Mechanical stretch, neurohumoral activation and cytokine production increase the production of reactive oxygen species (ROS) in cardiomyocytes, leading to oxidative injury (Tamargo & López-Sendón 2011).

At the left ventricle level all these changes mentioned above lead to remodelling of the extracellular matrix, left ventricular dilatation and wall thinning (Mann *et al.* 2012, Shah & Mann 2011).

2.3 Signaling in hypertrophy and cardiac remodeling

Cardiomyocyte hypertrophy and transition to heart failure involve a number of signaling pathways in a complex network. Figure 2 presents the time-course of selected signaling pathways and phenotypic endpoints of heart failure. Stress stimuli, including natriuretic peptides, ET-1, Ang II, catecholamines, cytokines and various growth factors, modulate signaling pathways in cardiomyocytes when bound to cell-membrane receptors such as G-protein coupled receptors (GPCRs, $G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\beta\gamma}$), receptor tyrosine kinases, and natriuretic peptide receptors. (Mudd & Kass 2008, Shah & Mann 2011). The central signaling molecules involved in pathological hypertrophy are phospholipase C (PLC), protein kinase C (PKC), PI3K/Akt/GSK-3β, Ca^{2+}/calmodulin-dependent kinase (CaMKII) and calcineurin, MAPKs, which mediate signals of GPCRs, and adenyl cyclase (AC)

together with protein kinase A (PKA), which are mediators of β-adrenergic receptors (Fig. 3) (Frey *et al.* 2004, Maillet *et al.* 2013, Shah & Mann 2011, van Berlo *et al.* 2013). Physiological hypertrophy is driven by activation of specific signaling pathways, such as CAMKII, phosphoinositide 3-kinases (PI3K)/Akt/glycogen synthase kinase 3β (GSK3β), and cyclic GMP (cGMP)-dependent protein kinase pathways (Maillet *et al.* 2013, Mudd & Kass 2008).

Many of the signaling pathways induced by hemodynamic overload are activated within minutes of initiation of the stress and interestingly, a systematic temporal analysis has revealed a double peak of activation for many of these kinases (Fig. 2) (Hoshijima & Chien 2002). Key targets of signaling pathways include transcription factors such as nuclear factor of activated T-cells (NFAT), GATA-4, myocyte enhancer factor-2 (MEF2), Nkx2.5, and serum response factor (SRF), which drive hypertrophic gene programmes (Maillet *et al.* 2013, Mudd & Kass 2008, Shah & Mann 2011). Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), which regulates metabolic remodelling, and nuclear factor kappa light polypeptide gene enhancer in B cells (NFκB), which regulates cell viability, are also indicated to play a role in hypertrophic signaling (Fig. 3) (Shah & Mann 2011).

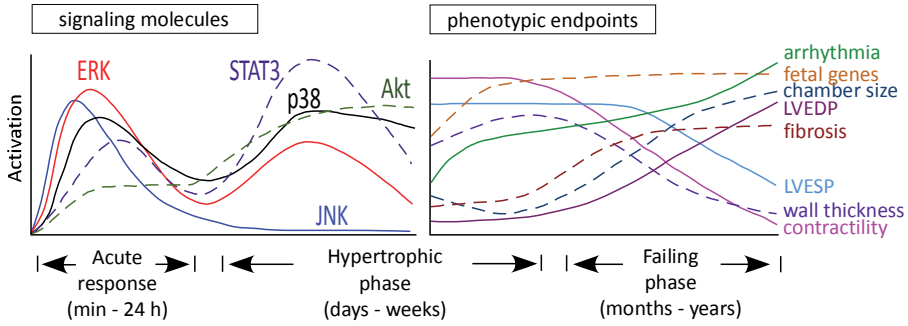

Fig. 2. Schematic activation profiles of various signaling cascades and time-course of phenotypic features of heart failure induced by mechanical stress. ERK, JNK, and p38 are mitogen activated protein kinases (MAPKs). ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; STAT3, signal transducer and activator of transcription 3; LVEDP, left ventricle end-diastolic pressure; LVESP, left ventricle end-systolic pressure. Illustration modified from Hoshijima & Chien 2002.

The acute phase of the hypertrophic response induces activation of expression of immediate-early genes like c-Fos, c-Jun, junB, egr-1, nur77 and cytokine signalling 3 (SOCS3) (Clerk *et al.* 2007, Hoshijima & Chien 2002). This is

followed by re-expression of fetal genes, which is characteristic of pathological hypertrophy (Table 1). These genes include ANP, BNP, β-myosin heavy chain (β-MHC), atrial myosin light chain-1 (MLC-1), and skeletal α-actin (Sk-α-actin). In contrast, many genes normally expressed at high levels in the adult heart are downregulated, including α-MHC, β-adrenergic receptors, and sarco/endoplasmic reticulum ATP-ase 2a (SERCA2a). (Bernardo *et al.* 2010, Swynghedauw 1999).

Table 1. Examples of genes changing during the acute and persistent phases of hypertrophy - heart failure. According to Swynghedauw 1999, Hoshijima & Chien 2002, and Bernardo *et al.* 2010.

Acute phase	Hypertrophic-failing phase	
Upregulation	Upregulation	Downregulation
c-Fos	Secreted proteins	Cytoskeletal proteins
c-Jun	ANP, BNP, ET-1,	αMHC, MLC-2,
junB	HB-EGF, TGF-β1,	Cardiac α-actin
egr-1	Periostin	Ion channels/carriers
nur77	Cytoskeletal proteins	SERCA2a, PLB
BNP	βMHC, MLC1a/v,	Kv4.2, 4.3, 1.5
SOCS3	MLC2v, Tropomyosin,	Signaling
	Troponin C, Desmin	ephrin type-A receptor
	Sk-α-actin, Sm-α-actin	Others
	Sarcosin, Desmin	αB-crystallin
	Extracellular matrix	Plasminogen activator
	Fibulin, Fibronectin,	inhibitor-1
	Laminin, Collagen	
	Metabolism/translation	
	Ubiquitin,	
	Creatine kinase,	
	Pyruvate dehydrogenase,	
	Myoglobin,	
	Superoxide dismutase,	
	Aldose reductase	
	Ion-channels/carriers	
	NCX, Kv1.4, VDAC-1	
	Signaling	
	βARK, Adenylyl cyclase,	
	GATA-4, Gαs, Id-1, PKA	
	Others	
	Hsp70	

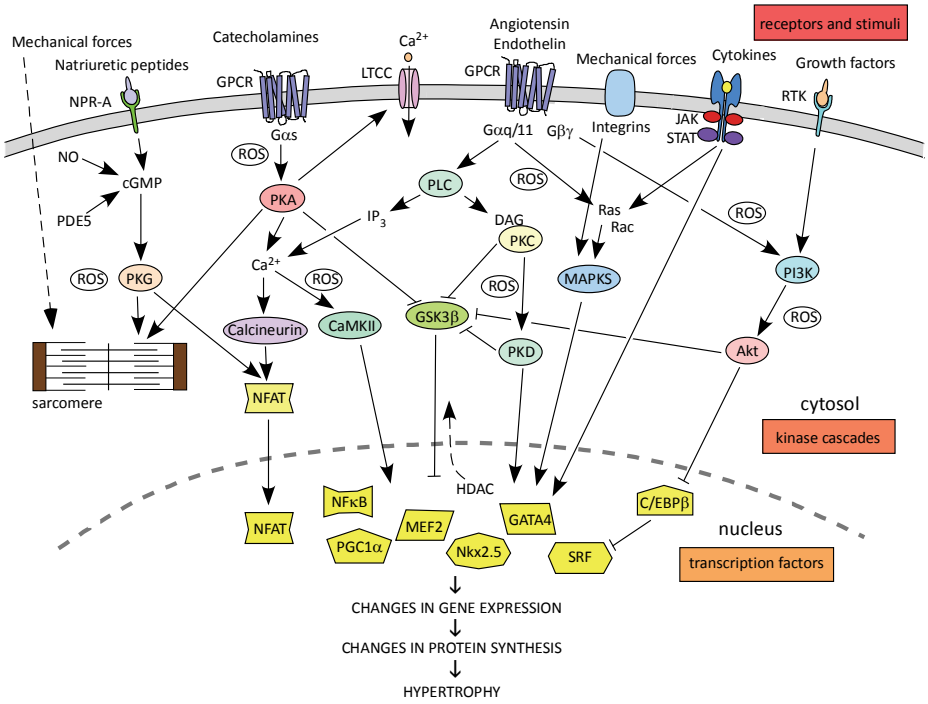

Fig. 3. Signaling cascades in the pathophysiology of heart failure. cGMP, cyclic guanosine monophosphate; CaMKII, Ca^{2+}/calmodulin dependent protein kinase II; C/EBPβ, CCAAT-enhancer-binding protein-β; DAG, diacylglycerol; GPCR, G-protein-coupled receptor; GSK-3β, glycogen synthase kinase-3β; HDAC, histone deacetylases; IP_3, inositol-trisphosphate; JAK, Janus kinase; LTCC, L-type calcium channel; MAPKs, mitogen-activated protein kinase; MEF2, myocyte enhance factor-2; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NPR-A, natriuretic peptide receptor-A; PDE5, phosphodiesterase type-5; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1α; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKD, protein kinase D; PKG, protein kinase G; PLC, phospholipase C; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SRF, serum response factor; STAT, signal transducer and activator of transcription. Modified from Mudd & Kass 2008 and Shah & Mann 2011.

2.4 Regulation of cardiac contractility

2.4.1 Excitation-contraction coupling

Excitation-contraction coupling is the process of converting electrical depolarization of the plasma membrane to the contraction of the cardiomyocyte (Fig. 4). The cardiac action potential depolarizes the plasma membrane, leading to Ca^{2+} entering the cytosol through voltage-gated L-type calcium channels (LTCC). This Ca^{2+} entry triggers a much greater Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptor-2 (RyR2), and together Ca^{2+} influx and release raise the free intracellular Ca^{2+} concentration. This leads to Ca^{2+} binding to the myofilament protein troponin C, which switches on the contraction machinery: actin and myosin form a cross-bridge, tropomyocin is displaced, myofilaments move and the cardiomyocyte contracts. Relaxation is necessary to deactivate the contraction machinery before a new contraction, and for this reason the Ca^{2+} concentration in the cytosol must decline, to allow Ca^{2+} dissociation from troponin. SERCA2a pumps most of the Ca^{2+} back into the SR, but some of the Ca^{2+} is also transported out of the cytosol by the sarcolemmal Na^{+}/Ca^{2+} exchanger (NCX), sarcolemmal Ca^{2+}-ATPase, or mitochondrial Ca^{2+} uniport (Bers 2002, Wehrens & Marks 2004). Abnormalities in SR Ca^{2+} cycling are hallmarks of heart failure and contribute to the pathophysiology and progression of the disease (Gwathmey *et al.* 2013).

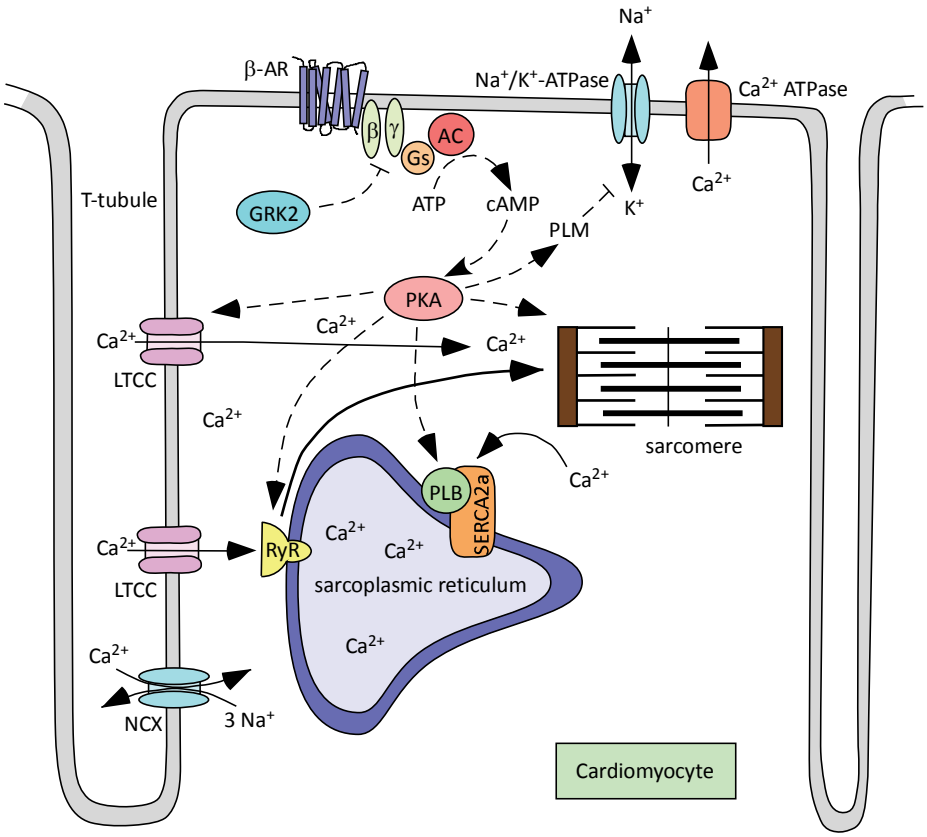

Fig. 4. Ca^{2+} cycling (unbroken line) and β-adrenergic regulation of contractility (dashed line). AC, adenylate cyclase; β-AR, β-adrenergic receptor; GRK2, G-protein coupled receptor kinase-2; LTCC, L-type calcium channel; NCX, sodium-calcium exchanger PKA, protein kinase A; PLB, phospholamban; PLM, phospholemman; SERCA2a, sarco-endoplasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor. Modified from Bers 2002 and Lymperopoulos *et al.* 2013.

2.4.2 β-adrenergic regulation of cardiac contractility

The transmitters of the sympathetic nervous system, noradrenaline and adrenaline, mediate their effects by binding to adrenergic receptors, which belong to the GPCRs family. There are three β-adrenergic receptor (β-AR) subtypes expressed in the human heart: predominant β_1-AR (in healthy, normal myocardium 75–80%), β_2-AR (about 20% of total receptors under normal conditions) and β_3-AR (Rengo

et al. 2011). Stimulation of β_1-ARs and β_2-ARs has a positive inotropic (contractility), chronotropic (frequency), and lusitropic (relaxation) effect, in addition to accelerated impulse conduction and pacemaker activity. Agonist binding to β_1-ARs or β_2-ARs catalyzes the exchange of GTP to GDP on the G_α stimulatory subunit ($G_{\alpha s}$) of the heterotrimeric G-protein, leading to dissociation of the heterotrimer into active $G_{\alpha s}$ and free $G_{\beta\gamma}$ subunits. Active $G_{\alpha s}$ stimulates the effector adenylyl cyclase, which in turn converts ATP into the second messenger adenosine 3´5´-monophosphate (cAMP) (Lymperopoulos *et al.* 2013, Rockman *et al.* 2002). cAMP activates cAMP-dependent protein kinase PKA, which phosphorylates many targets, including RyR, LTCC, protein phosphatase inhibitor-1, phospholamban, troponin I, and phospholemman (Fig. 4). In addition, PKA can cause β-AR desensitization by directly phosphorylating β-ARs, which is independent of the agonist (heterologous desensitization). β_1-ARs and β_2-ARs differ from each other, since the latter can also couple to the adenylyl cyclase inhibitory G-protein (G_i), and this switch from G_s to G_i may happen by PKA phosphorylation of β_2-AR. In addition, overexpression and selective stimulation of β_2-ARs have been shown to exert protective effects on the heart, while β_1-ARs in similar settings result in the opposite effect (Lymperopoulos *et al.* 2013).

GRK2

Dysfunction of β-ARs is a hallmark of heart failure, with β_1-ARs being downregulated. β_2-ARs are non-functional as well, and their signalling is different from that in normal heart. Thus, they seem to be unable to rescue impaired structure and function of the heart (Lymperopoulos *et al.* 2013). The family of G-protein-coupled kinases (GRKs, originally β-adrenergic receptor kinases, β-ARK) consists of seven members (GRK1-7). They phosphorylate agonist-bound receptors (homologous desensitization) and recruit β-arrestin to the receptor. This blocks recoupling of dissociated G-protein subunits, thereby preventing further signaling by a ligand-bound receptor (Fig. 4). GRK2, -3 and -5 are appreciably expressed in the human heart, and GRK2 and -5 are the most abundant (Belmonte & Blaxall 2011). GRK2 and GRK5 levels are elevated in the failing heart (Petrofski & Koch 2003, Ungerer *et al.* 1993). GRK2 has been shown to be upregulated also in several different pathologic conditions, such as ischemia, cardiac hypertrophy and hypertension (Rengo *et al.* 2011). Inhibition of β-adrenergic signaling by long-term β-blocker treatment has been shown to decrease GRK2 levels in mice (Iaccarino *et al.* 1998).

Inhibition of GRK2 has been studied by using β-ARK-ct (GRK2-ct), which binds to $G_{\beta\gamma}$ competing with GRK2. Numerous experiments in experimental HF models have shown that β-ARK-ct offers protection from heart failure, supporting that GRK2 overexpression is detrimental for the heart. (Petrofski & Koch 2003, Rengo *et al.* 2011). For example, β-ARK-ct was shown to improve β-AR responsiveness by preventing $G_{\beta\gamma}$-dependent LTCC suppression in normal and failing cardiomyocytes upon β-AR stimulation (Völkers *et al.* 2011). Interestingly, the same authors reported that β-ARK-ct had indistinguishable effects on ERK1/2, PKCε, p38, ERK5, and Akt activity (Völkers *et al.* 2011). Selective GRK2 ablation in transgenic mice before myocardial infarction (MI) prevented post-MI remodeling and preserved β-AR responsiveness. Ablation of GRK2 ten days after MI enhanced cardiac contractility, decreased remodelling, and increased cell survival. In addition, GRK2 ablation resulted in increased SR Ca^{2+} in myocytes, and GRK2 knockout mice were resistant to adverse alterations and remodeling after MI (Raake *et al.* 2008, Raake *et al.* 2012).

The activity of GRK2 is regulated by phosphorylation. GRK2 Ser670 is phosphorylated by ERK1/2 resulting in attenuated GRK2 activation (Pitcher *et al.* 1999). A recent study also showed that GRK2 localizes to mitochondria after ischemic and oxidative stress, and mitochondrial GRK2 increased Ca^{2+}-induced opening of the mitochondrial permeability transition pore, which is a key step in cellular injury. This localization was dependent on GRK2 phosphorylation at Ser670 by ERK1/2, leading to binding with the heat shock protein 90 (Hsp90) chaperon (Chen *et al.* 2013).

2.4.3 Ca^{2+} cycling in the heart

The amplitude and velocity of Ca^{2+} cycling are regulated by a dynamic balance of phosphorylation and dephosphorylation through kinases and phosphatases (Lompré *et al.* 2010). PKA is a central regulator of many proteins involved in excitation contraction-coupling (Fig. 4). Other kinases contributing to the regulation of contractility are CaMKII and PKC (Bers 2002). Troponin I and myosin binding protein C reduce myofilamental Ca^{2+} sensitivity and thus accelerate relaxation of the myofilaments (Lymperopoulos *et al.* 2013). Calsequestrin is a high-capacity Ca^{2+} binding protein in the SR lumen, regulating Ca^{2+} load (Lompré *et al.* 2010).

SERCA2a and phospholamban

SERCA2a pumps Ca^{2+} from the cytosol into the SR and plays a key role in the cardiomyocyte relaxation process. SERCA is expressed as three isoforms. SERCA2 is the predominant variant, and there are three spliced transcripts known so far, SERCA2a, SERCA2b and SERCA2c, differing at the C-terminus (Kranias & Hajjar 2012). SERCA2a is expressed in cardiac muscle and slow twitch skeletal muscle, and its activity controls both the rate of cytosolic Ca^{2+} removal and the degree of SR Ca^{2+} load. Thus, it has a significant role both in cardiac inotropy and lusitropy. (Bers 2002, Kranias & Hajjar 2012). SERCA2a gene therapy by adeno-associated virus vector-1 (AAV1) has been found to improve myocardial function in animal HF models, and the results of phase I and II trials were promising in patients with end-stage heart failure (Kho *et al.* 2012).

PLB is a key regulator of SERCA2a, which relieves SERCA2a inhibition when phosphorylated either at Ser 16, Thr 17 or Ser 10 by PKA, CaMKII or PKC, respectively (Movsesian *et al.* 1984, Simmerman *et al.* 1986). In addition, cGMP has been shown to phosphorylate PLB *in vitro* at Ser16, but its physiological significance is not clear (Kranias & Hajjar 2012, Mattiazzi & Kranias 2014). *In vivo* experiments have shown that prevention of Ser 16 phosphorylation (by Ser → Ala mutation) attenuates the β-adrenergic response in mammalian hearts, and phosphorylation of Ser 16 is a prerequisite for Thr 17 phosphorylation. However, Thr 17 can be phosphorylated independently of Ser 16 under conditions that activate CaMKII and suppress phosphatase activity, such as increased stimulation frequency of the heart, increased intracellular Ca^{2+}, ischemia/reperfusion injury (I/R-injury), or acidosis (Kranias & Hajjar 2012). SR has been shown to contain intrinsic protein phosphatase activity, which dephosphorylates both PKA- and CaMKII-dependent sites of PLB (Kranias 1985). PLB is also known to be regulated by protein phosphatase-1 via PKA signalling and by the HS-associated protein X-1 (HAX-1). HAX-1 physically interacts with PLB, and the minimal binding region includes the Ser16 and Thr 17 phosphorylation sites of PLB (Kranias & Hajjar 2012, Mattiazzi & Kranias 2014).

Protein phosphatase-1 and -2

Protein phosphatases and protein kinases are molecules having tightly synchronized competition for the delicate balance of protein phosphorylation. Protein phosphatase-1 (PP1), PP2A and calcineurin (or PP2B), distinguished in

the early 1980s, are the most active protein phosphatases in the heart, contributing up to 90% of the dephosphorylation (Heijman *et al.* 2013, Ingebritsen & Cohen 1983). PP1 is a holoenzyme composed of the catalytic subunit (PP1c) and a complex of 200 established or putative regulatory proteins. The PP1 family contains four isoforms encoded by three independent genes. PP1 is ubiquitously expressed in most cell types. PP1 dephosphorylates PLB, making it a reversible inhibitor of SERCA2a. Protein phosphatase inhibitor-1 (I-1) and inhibitor-2 (I-2) are proteins regulating PP1 activity (Heijman *et al.* 2013, Nicolaou *et al.* 2009a). Increased PP1 activity has been observed in end-stage human heart failure. PP1 has been shown to have a negative effect on cardiac function, and thus it is a molecule of interest in the studies of novel therapeutic approaches for the treatment of heart failure (Carr *et al.* 2002).

PP2A can exist either as a dimer or trimer, consisting of catalytic (PP2Ac, two isoforms) and structural scaffold units (PP2A-A, two isoforms) or both units with regulatory subunits, respectively. Regulatory subunits can be grouped into four families, comprised of multiple isoforms and splicing variants with complex nomenclature. PP2A levels are reported to be both elevated and decreased in heart failure (Heijman *et al.* 2013). Cardiac PP2A activity is regulated by an expansive network including thirteen different regulatory subunits with distinct and well defined subcellular localization patterns, differential regulation of all holoenzyme components depending on heart region, species and disease models; and multiple post-translational modifications of the catalytic subunit (DeGrande *et al.* 2013).

Both PP1 and PP2A can be inhibited by okadaic acid but at different concentrations: PP2A is inhibited in the low nanomolar ranges, while PP1 inhibition requires a higher concentration (Herzig & Neumann 2000). Calyculin-A and cantharidin resulted in similar affinity to both PP1 and PP2A with positive inotropic effects (Heijman *et al.* 2013). Fostriecin has been suggested to be a more specific PP2A inhibitor (Walsh *et al.* 1997).

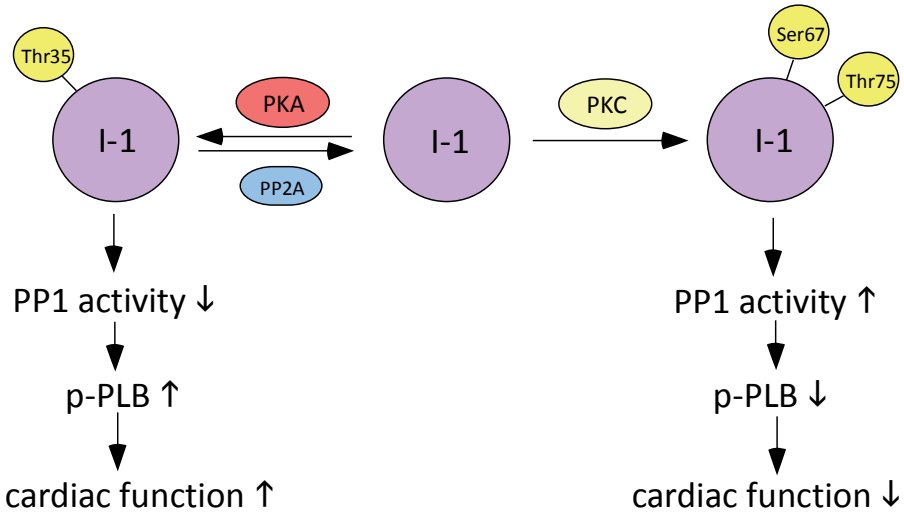

Fig. 5. Inhibitor-1 (I-1) regulates protein phosphatase-1 activity depending on the phosphorylation site of I-1. PLB, phospholamban; PP2A, protein phosphatase-2A. Modified from Nicolaou & Kranias 2009.

I-1 is a cytosolic protein and widely expressed in mammalian tissues, including the heart and cardiomyocytes (Wittköpper *et al.* 2011). In contrast to the elevation in PP1 levels, I-1 levels are decreased in failing human hearts (El-Armouche *et al.* 2004). I-1 has two major phosphorylation sites, Thr35 and Ser67/Thr75, phosphorylated by PKA and PKCα, respectively (Fig. 5) (Braz *et al.* 2004, Carr *et al.* 2002). These phosphorylation sites define the regulatory activity of I-1: when phosphorylated at Thr35, I-1 inhibits PP1 activity, whereas Ser67 and/or Thr75 phosphorylation result in a stimulatory effect on PP1, attenuating the effects of PKA on contractility (Florea *et al.* 2012, Rodriguez *et al.* 2007).

Inducible transgenic mice with controlled expression of active I-1 (T35D) showed enhanced contractile function and, after I/R injury, improvements in contractility and recovery (Nicolaou *et al.* 2009b). Constitutively active I-1 (Ser 67 → Ala) increased PLB and RyR phosphorylation and contractile function in a transgenic mouse model, but adrenergic stimulus and aging caused cardiomyopathy and arrhythmia (Wittköpper *et al.* 2010). On the other hand, constitutive Ser67 and Thr75 phosphorylation of I-1 increased PP1 activity, impaired Ca^{2+} cycling and depressed contractility, and resulted in increased remodeling upon aging (Florea *et al.* 2012). PP2A and calcineurin mediate dephosphorylation of I-1 Thr35 phosphorylation (El-Armouche *et al.* 2006). I-2 is

also an inhibitor of PP1, and it is regulated by GSK-3β or ERK1/2 through Thr72 phosphorylation, or by casein kinase through various phosphorylation sites (Heijman *et al.* 2013, Mingguang *et al.* 2007). Hsp20 has also been identified as another inhibitory PP1 regulator (Qian *et al.* 2011).

2.5 Mitogen-activated protein kinases

Mitogen activated protein kinases (MAPKs) are a highly conserved family of signaling proteins which regulate numerous cellular functions such as gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation. MAPKs are expressed in all eukaryotic cells and they are integrated with a large array of stimuli including environmental stresses, hormones, growth factors, cytokines and agents acting through GPCRs and TGF-β (Fig. 6) (Kyriakis & Avruch 2012).

As shown in Figure 6, MAPKs are typically activated by a canonical three-tiered kinase cascade. MAPK kinase kinases (MAPKKK, MAP3K, MEKK or MKKK) are Ser/Thr kinases, which are activated through and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli (Cargnello & Roux 2011). MAPKKK activates MAPK kinase (MAPKK, MAP2K, MEK or MKK, referred to as MEK and MKK in this work), which phosphorylates the MAPK. Docking and binding partners also regulate the specificity and efficiency of MAPK signaling by helping to bring upstream and downstream signaling components together. Also several non-canonical pathways activate MAPKs independently of MAPK kinases. MAPK activation occurs by phosphorylation of the Thr-X-Tyr motif, which is located in the activation loop of the of kinase subdomain VIII. MAPKs are called proline-directed kinases, since they activate their targets always by phosphorylating their substrates on Ser or Thr followed by a Pro residue. MAPKs have the ability to shuttle between cytoplasm and the nucleus to exert their effects depending on the specific cellular stimuli. (Rose *et al.* 2010).

The MAPK family consists of four conventional pathways including extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAPK, c-Jun NH_2-terminal kinases (JNKs, also called stress-activated protein kinases, SAPKs) and ERK5. Atypical MAPKs include ERK3/4, ERK7 and Nemo-like kinase (NLK). Atypical MAPKs have non-conforming particularities and they are not organized into classical three-tiered kinase cascades (Cargnello & Roux 2011).

MAPK activation is involved in cardiac development and in the pathogenesis of various cardiac diseases. MAPKs may have dual roles in that their activation may lead to both protective and detrimental effects depending on the circumstances (Rose *et al.* 2010).

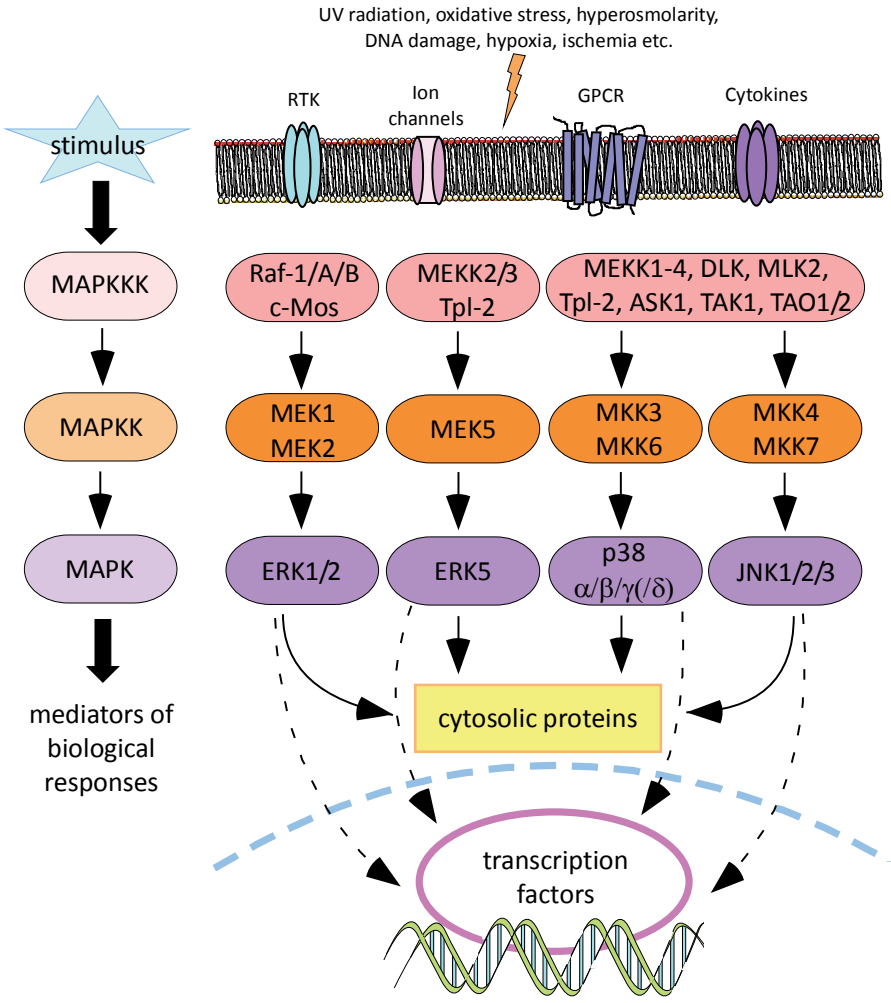

Fig. 6. Conventional MAPK signaling pathways are activated by mitogens, cytokines and cellular stresses. Canonical MAPK signaling cascades are three-tiered sequential phosphorylation events. ERKs mediate responses to growth factors and mitogens, whereas p38s and JNKs mediate extracellular stress responses. Modified from Rose *et al.* 2010 and Cargnello & Proux 2011.

2.5.1 Extracellular signal-regulated kinases

ERK1/2 was the first MAPK to be discovered in the early 1980s as a protein kinase that activated microtubule-associated protein-2 (MAP-2) in response to insulin in 3T3-L1 adipocytes (Sturgill & Ray 1986). ERK1 and ERK2 are individual kinases, but since they share an 83% identical amino acid composition and most of the same signaling activities, they are usually referred to as ERK1/2 or p44/42. ERK1/2 is ubiquitously expressed and it regulates a number of diverse cellular and physiological events. Thus, ERK1/2 also has a significant role in various pathologies including cancer, cardiovascular disease, and diabetes. (Rose *et al.* 2010).

ERK1/2 is activated by growth factors (i.e. platelet-derived growth factor, PDGF; epidermal growth factor, EGF; nerve growth factor, NGF) in response to insulin, serum, ET-1, phenylephrine (PE) and phorbol esters (Bogoyevitch *et al.* 1993, Boulton *et al.* 1990, Rose *et al.* 2010). Receptor tyrosine kinases (RTKs) are principal activators that engage Ras, and Raf-1/A/B are primary MAPKKKs in the ERK1/2 module. MEK1 and MEK2 phosphorylate ERK1/2 in the Thr-Glu-Tyr motif within its regulatory loop (Cargnello & Roux 2011). ERK1/2 activation also occurs in a manner independent of Ras, when it is activated by proinflammatory stimuli including cytokines, lipopolysaccharide (LPS) and other pathogen-associated molecular patterns (PAMPs), oxidized low-density lipoprotein (LDL), and crystalline uric acid (Kyriakis & Avruch 2012). ERK autophosporylation occurs at Thr208 and Thr188 in ERK1 and ERK2, respectively, via protein-protein interaction of ERK1/2 and $G_{\beta\gamma}$ and this results in hypertrophic cardiomyocyte growth (Lorenz *et al.* 2008).

ERK1/2 is distributed throughout the cell and it is able to phosphorylate over 100 substrates in the cytosol (such as death-associated protein kinase, DAPK; tuberous sclerosis complex 2, TSC-2; ribosomal protein S6 kinase, RSK and MAPK-interacting kinase, MNK) and in the nucleus (such as NFAT; Elk-1; MEF2; c-Fos; c-Myc and STAT3, signal transducer and activator of transcription 3) (Cargnello & Roux 2011, Rose *et al.* 2010, Roux & Blenis 2004).

ERK5 is alternatively called also big MAP kinase 1 (BMK1), since it is twice the size of other MAPKs (around 100 kDa). It has a 52% identical amino acid sequence with ERK1/2 and its N-terminal half contains a kinase domain similar to ERK1/2 (Cargnello & Roux 2011). ERK5 is encoded by one gene, but the protein is alternatively spliced into four forms: ERK5a, ERK5b, ERK5c and ERK-T (McCaw *et al.* 2005, Yan *et al.* 2001). ERK5 is ubiquitously expressed, and global

deletion of ERK5 is embryonic lethal due to defects in vascular formation (Hayashi *et al.* 2004). ERK5 has a physiological role at least in cardiac hypertrophy, atherosclerosis, neuronal survival, endothelial cell response to shear stress, and prostate and breast cancers (Hayashi & Lee 2004).

ERK5 is activated by various mitogens and stress stimuli, like hyperosmotic shock, oxidative stress, laminar flow shear stress, and UV irradiation. Upstream kinases of ERK5 are MEKK2/MEKK3 and MEK5, respectively. MEKK2 and MEKK3 are also known to activate MKK3/6 and MKK4/7, thus regulating p38 and JNK activities. ERK5 localizes in the cytoplasm, but translocates to the nucleus upon MEK5 activation or stimulation. Substrates of ERK5 include the MEF2 transcription family, Ets-domain transcription factor (Sap1a), serum- and glucocorticoid –inducible kinase (SGK), connexin 43 (Cx43), and a pro-apoptotic member of the Bcl-2 family (Bad). (Cargnello & Roux 2011, Hayashi & Lee 2004).

PD98059 and U0126 have been developed to inhibit MEK1/2 activity in a non-ATP-competitive way, and thus both inhibitors inhibit ERK1/2 phosphorylation (Alessi *et al.* 1995, Dudley *et al.* 1995, Favata *et al.* 1998). More recently, PD184352 and PD0325901 have entered clinical trials as potential anticancer agents with greater bioavailability MEK1/2 inhibitors (Frémin & Meloche 2010). Both PD98059 and U0126 have been shown to efficiently inhibit also MEK5, followed by ERK5 inhibition, while PD184352 had less of an effect on MEK5 activation (Kamakura *et al.* 1999, Mody *et al.* 2001).

2.5.2 c-Jun-N-terminal kinases

JNKs were discovered in the early 1990s as a cycloheximide-activated microtubule associated protein-2 (MAP-2) kinase from the rat liver that could be affinity-purified on c-Jun bound to beads. There are three distinct genes encoding JNK/SAPKs: JNK1/SAPKβ, JNK2/SAPKα and JNK3/SAPKγ, also called MAPK8, -9 and -10, respectively (Cargnello & Roux 2011, Kyriakis & Avruch 2012). JNKs are more than 85% identical and there are more than 10 spliced forms with molecular weights ranging from 46 to 55 kDa. JNK1 and JNK2 are ubiquitously expressed, while JNK3 is expressed only in neuronal tissues, testis, and cardiomyocytes. They also have functional differences. JNK1 is the major isoform to be activated after stimulation, whereas JNK2 preferentially binds to c-Jun in unstimulated cells and contributes to c-Jun degradation by a ubiquitin-dependent mechanism (Bode & Dong 2007, Raman *et al.* 2007). Like ERKs,

JNKs are involved in a number of different biological processes including cell proliferation, differentiation, apoptosis, cell survival, actin reorganization, cell mobility, metabolism, and cytokine production, thus they have physiological roles in insulin signaling, the immune response and inflammation. This translates into JNK's pathological roles in neurological disorders, arthritis, obesity, diabetes, atherosclerosis, cardiac disease, liver disease, and cancer. (Rose *et al.* 2010).

JNKs, like p38 MAPKs, are activated by various stress stimuli such as heat shock, ionizing radiation, oxidants, genotoxins, I/R injury, mechanical shear stress, vasoactive peptides, proinflammatory cytokines, pathogens, and translational inhibitors (anicomycin, cycloheximide and tunicamycin) (Kyriakis & Avruch 2012). Several MAPKKKs, including MEKK1, mitogen-activated protein kinase kinase kinase 8 (Tpl-2), MLK-1, DLK, TAO1/2, TAK1 and ASK1/2, phosphorylate MKK4/7, which in turn phosphorylate JNKs. JNK is activated by dual phosphorylation of Thr and Tyr residues within a conserved Thr-Pro-Tyr motif in its activation loop (Davis 2000). JNKs have a number nuclear targets, including transcription factors c-Jun, ATF-2, p53, Elk-1, NFAT and activator protein-1 (AP-1) (Davis 2000, Rose *et al.* 2010).

JNKs have been inhibited by two reversible ATP-competitive inhibitors, SP600125 and AS601425 (Cargnello & Roux 2011). However, a study testing their specifity concluded that they had poor selectivity when tested against a panel of purified protein kinases (Bain *et al.* 2007).

2.5.3 Atypical mitogen-activated protein kinases

Atypical MAPKs include ERK3/4, ERK7 and nemo-like kinase (NLK), and their activation does not occur by three-tiered phosphorylation cascades. ERK3 and ERK4 have 73% identical amino acid sequences and their molecular masses are approximately 100 kDa and 70 kDa, respectively. They are considered atypical since their activation loop contains a Ser-Glu-Gly motif (not Thr-X-Tyr) (Coulombe & Meloche 2007). ERK3 mRNA is ubiquitously expressed in mammalian tissues, whereas there is no data on ERK4 gene expression. ERK3 is phosphorylated by ERK3 kinase at Ser 189, located within its activation loop, but no stimuli have been identified that promote ERK3/4 activation (Cargnello & Roux 2011, Coulombe & Meloche 2007). MAPK activated protein kinase MK5 is the only known substrate of ERK3/4 (Åberg *et al.* 2009). ERK3 has been suggested to play a role in a number of biological functions including cell proliferation, cell cycle progression and cell differentiation. ERK3 also has a

significant role during embryogenesis because targeted disruption of the gene encoding ERK3 (*Mapk6*) leads to intrauterine growth restriction and early neonatal death (Cargnello & Roux 2011).

ERK7 was cloned from a rat cDNA library and ERK8 is its human ortholog, and they share a 69% identical amino acid sequence. ERK7 is 45% identical with ERK1, but it has an atypical C-terminal extension that is not present in conventional MAPKs, and the precise role of this extension is not yet known. MAPKKs are not known to be involved in ERK7/8 activation, despite a Thr-Glu-Tyr motif within its activation loop. Both ERK7 and ERK8 seem to be autophosphorylated and none of the classical stimuli activating conventional MAPKs activates ERK7/8. Substrates of ERK7/8 remain elusive; it has been shown *in vitro* that some of classical substrates of conventional MAPKs, such as myelin basic protein (MBP), c-Fos and c-Myc, are also phosphorylated by ERK7 (Cargnello & Roux 2011).

NLK displays 45% amino acid identity with the kinase domain of ERK2 and 38% identity with cyclin-dependent kinase-1 (Cdk1). NLK is considered atypical due to N- and C-terminal extensions not present in conventional MAPKs, and it has a single residue to be phosphorylated in its activation loop. NLK is activated by stimuli of the Wnt pathway and several cytokines. NLK substrates include transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, STAT3, proto-oncogene cMyp, and the β-catenin pathway (Cargnello & Roux 2011, Goedert *et al.* 1997).

2.5.4 p38 mitogen-activated protein kinase

The p38 MAPK family is stress-activated, and p38 was originally isolated as a 38 kDa tyrosine-phosphorylated protein found in LPS-stimulated macrophages (Han *et al.* 1993, Han *et al.* 1994). p38 MAPK family is comprised of four isoforms: p38α, p38β, p38γ, and p38δ. The p38α and p38β isoforms are ubiquitously expressed and they are 74% identical (Jiang *et al.* 1996). p38γ is expressed in the skeletal and cardiac muscle, and it shares 60% identity with p38α (Li *et al.* 1996). It has also been suggested that p38γ is more prominent than p38β in the myocardium (Dingar *et al.* 2010, Marber *et al.* 2011). p38δ is expressed in lungs, kidney, testis, spleen, pancreas, and small intestine, and it is 60% identical with p38α (Martin *et al.* 2012). p38α is necessary for normal embryonic development, since deletion of the p38α gene causes placental and erythroid differentiation defects and leads to embryonic lethality (Mudgett *et al.* 2000, Tamura *et al.* 2000a,

Tamura *et al.* 2000b). Deletion of any other p38 isoforms did not result in phenotype abnormalities in mice (Gerits *et al.* 2007).

p38 is activated by MKK3 and MKK6, and MKK4 is also capable of phosphorylating it (Derijard *et al.* 1995, Raingeaud *et al.* 1996). Non-canonical TGF-β-activated protein kinase 1 binding protein 1 (TAB-1) autophosphorylation also activates p38 (Ge *et al.* 2002, Tanno *et al.* 2003). p38 is a stress-activated protein kinase, which can be activated by such stimuli as oxidative stress, UV radiation, heat, osmotic shock, pathogens, ET-1, Ang II, IL-1, TNF-α, integrins, and GPCRs as well as Rho family GTPases Rac and Cdc42 (Aikawa *et al.* 2002, Cargnello & Roux 2011, Goldsmith & Dhanasekaran 2007, Sugden & Clerk 1998). The phosphorylation motif of p38 is Thr-Gly-Tyr, and its dual phosphorylation leads to local and distant conformational changes that coordinate kinase activation (Martin *et al.* 2012).

p38 isoforms are present both in the cytosol and in the nucleus. Substrates of p38 locate to many cellular compartments. Cytosolic targets of p38 include cytosolic phospholipase A2 (cPLA2), MAPK interacting serine/threonine kinase 1 (MNK1/2), MAPK-activated protein kinases MK2, MK3 and MK5, Hu antigen/embryonic lethal abnormal vision family associated factor X (HuR), B-cell lymphoma 2 (Bcl-2), Caspase-3 and -8, Bax and Tau. Nuclear targets of p38 include activating transcription factor -1/-2/-3 (ATF1/-2/-3), MEF2, ETS domain containing protein like transcription factor (Elk-1), GADD153, Ets1, p53 and NFAT (Cargnello & Roux 2011, Martin *et al.* 2012).

The biological consequences of p38 activation are various. p38 activation is significantly involved in the inflammatory response, especially in the production of proinflammatory cytokines IL-1β, TNF-α and IL-6, in the induction of cyclo-oxygenase-2 (COX-2) and cell adhesion molecules (VCAM-1), and in the expression of intracellular enzymes such as iNOS (Zarubin & Han 2005). p38 also regulates many biological functions such as apoptosis, cell survival, cell cycle regulation, differentiation, senescence, and cell growth and migration. The role of p38 depends on cell type and stimulus, since p38 can respond to over 60 extracellular stimuli. p38 has opposite effects in different conditions and it is challenging to elucidate the exact functional role of p38 (Cargnello & Roux 2011, Rose *et al.* 2010, Zarubin & Han 2005).

p38 activity can be diminished by various protein phosphatases, such as PP2A and PP2C, and also by dual-specificity phosphatases (DUSP-1, -4, -8 and -16, also known as MAPK phosphatases, MKP-1, -2, -5 and -7, respectively) (Kumar *et al.* 2003, Owens & Keyse 2007, Rose *et al.* 2010)

p38 was found to be a target of pyridinyl-imidazole anti-inflammatory drugs. SB203580 compound was the first reported and most extensively characterized of p38 inhibitors (Lantos *et al.* 1984, Lee *et al.* 1994). It has been shown to be highly selective over ERKs and JNKs and it has no effect on p38δ and p38γ isoforms. SB203580 binds to the active sites of p38α and p38β of both the phosphorylated and non-phosphorylated kinase in an ATP-competitive manner (Clark *et al.* 2007, Goedert *et al.* 1997, Young *et al.* 1997). In addition to pyridinyl-imidazole compounds, there are aryl-pyridinyle-heterocycles and non-aryl-pyridinyl compounds used for p38 inhibition. p38 inhibitors can be divided into two groups according to their p38 binding mode: (i) active site or gatekeeping inhibitors (such as SB203580) and (ii) indirect and remote inhibitors of ATP-binding (such as BIRB-796). The p38δ and p38γ isoforms are also sensitive to the latter inhibitors (Clark *et al.* 2007, Martin *et al.* 2012). p38 inhibitors have been widely studied for the treatment of many diseases, especially arthritis and Crohn's disease. Unfortunately, despite the promising results of preclinical studies, these compounds did not provide any further improvement to the traditional anti-inflammatory treatment (Hammaker & Firestein 2010). Additionally, there were adverse effects such as elevated liver enzymes, rash, gastrointestinal disorders, and infections (Coulthard *et al.* 2009). In general, inhibition of MAPKs is challenging due to their ubiquitous expression, various functions depending on the circumstances, and different cellular feedback mechanisms, which may also cause crosstalk and interactions between other signaling pathways (Rose *et al.* 2010).

2.6 p38 regulating cardiac pathology

p38α and p38β were demonstrated to be active in response to ischemia and reperfusion (Bogoyevitch *et al.* 1996). Relatively selective p38 inhibitors have enabled various studies of p38 and thus, it is one of the most investigated kinases in models relevant to cardiac diseases. Its activation has been implicated in both detrimental and protective processes in the stressed myocardium (Bell *et al.* 2008, Clark *et al.* 2007, Steenbergen 2002). This dual role of p38 (and also concerning other MAP kinases) in cardiac pathogenesis can be considered a natural phenomenon, since p38 has a significant role in cardiac development and normal physiology. In addition, interpretation of results may be confusing due to the inhibitors used, since for example SB203580 is known to inhibit both p38α and p38β isoforms, and the isoforms may have different roles and expression patterns

in the heart depending on the circumstances. (Marber *et al.* 2011). On the other hand, controversies could be explained partly by the experimental model performed. For example, there is a difference in myocardial responsiveness between a mouse and a pig model after ischemia reperfusion, in that experimental p38 activation in the mouse contributes to acute cellular injury and death, while there was no causative effect in the pig upon the same injury (Kaiser *et al.* 2005). p38 inhibition has been considered a potential target for the treatment of heart failure, but also hypertrophy and infarction (Kerkela & Force 2006, Marber *et al.* 2011). Table 2 lists transgenic mouse models of different modulations of p38 activity.

Table 2. Review of transgenic mouse models modulating activity of the p38 pathway.

Model	Phenotype	Reference
p38α KO	Embryonic lethality	Adams *et al.* 2000, Mudgett *et al.* 2000, Tamura *et al.* 2000a
Dn-p38α	Enhanced cardiac hypertrophy following hypertrophic stimuli	Braz *et al.* 2003
Dn-p38α	Cardiac hypertrophy, resistant to fibrosis in response to pressure overload	Zhang *et al.* 2003
p38α+/-	Reduced I/R injury	Otsu *et al.* 2003
p38α floxed-KO	Cardiac dysfunction, dilatation, massive fibrosis, apoptosis in response to LV pressure overload	Nishida *et al.* 2004
p38α cardiac-KO	Reduced I/R-injury	Kaiser *et al.* 2004
Dn-p38α	Reduced infarct size, increased LV function, decreased apoptosis	Ren *et al.* 2005
Dn-p38α	Enhanced basal contractile function	Cross *et al.* 2009
Dn-p38β	cardiac hypertrophy, resistant to fibrosis in response to pressure overload	Zhang *et al.* 2003
Dn-p38β	Enhanced basal contractile function, increased I/R-injury	Cross *et al.* 2009
Constitutively active MKK3	Premature death, fibrosis, fetal gene expression ↑, thinned ventricular wall, myocyte atrophy	Liao *et al.* 2001
Constitutively active cardiac-specific MKK3	Increased hypertrophy, fibrosis and contractile dysfunction	Streicher *et al.* 2010
Dn MKK3	Enhanced cardiac hypertrophy following hypertrophic stimuli	Braz *et al.* 2003
Constitutively active MKK6	Premature death, fibrosis, fetal gene expression ↑, reduced end-diastolic ventricular cavity size, no atrophy	Liao *et al.* 2001
Cardiac MKK6 overexpression	Normal morphology and function, reduced I/R injury	Martindale *et al.* 2005
Dn MKK6	Enhanced cardiac hypertrophy following hypertrophic stimuli	Braz *et al.* 2003
Dn MKK6	Reduced I/R-injury	Kaiser *et al.* 2004
DUSP1/4	Cardiomyopathy, diminished contractility and Ca^{2+} handling	Auger-Messier *et al.* 2013

2.6.1 p38 and cardiac hypertrophy

p38 is actived during early ischemia and early reperfusion, and like other kinases, it is likely that kinase activity is cyclic (Hoshijima & Chien 2002). Initial *in vitro*

studies suggested that p38 MAPK activation is sufficient to induce characteristic changes in cardiac hypertrophy and cell death (Wang *et al.* 1998, Zechner *et al.* 1997). However, human samples from patients with compensated hypertrophy did not reveal any p38 activation. On the other hand, in failing hearts all three MAPKs were activated (Haq *et al.* 2001). ET-1, a powerful paracrine mediator of hypetrophy, was shown to induce BNP expression via the p38 pathway and ETS binding site (Pikkarainen *et al.* 2003a). Inhibition of the p38 pathway using small molecule inhibitors (SB203580 and SB202190) or dominant negative p38α (dn-p38α) reduced cardiomyocyte growth in response to hypertrophic stimuli (PE, ET-1, leukemia inhibitory factor, LIF) (Liang & Molkentin 2003, Nemoto *et al.* 1998, Zechner *et al.* 1997). However, a study using intact neonatal cardiomyocytes suggested that p38 inhibitor SB203580 is not sufficient to block ET-1 induced protein synthesis (Choukroun *et al.* 1998). Another study further reported that SB203580 failed to inhibit morphological changes associated with ET-1 or PE-induced cardiomyocyte hypertrophy under 24 h, but it decreased myofibrillar organization and the cell profile at 48 h (Clerk *et al.* 1998). On the other hand, another study showed that SB203580 was capable to diminish Ang II-induced FGF2 upregulation, which is one of the genes upregulated in hypertrophy (Tang *et al.* 2011). In addition, thyroid hormones have been shown to induce cardiac hypertrophy via TGF-β -activated kinase (TAK-1)/p38 *in vitro* (Kinugawa *et al.* 2005).

Targeted p38 activation using a gene-switch strategy with active mutants of MKK3b and MKK6b did not result in significant cardiac hypertrophy *in vivo*. However, p38 activation induced fetal marker genes in the myocardium, interstitial fibrosis, and cardiac failure resulting in premature death (Liao *et al.* 2001). PKCε-knockout mice exhibited increased p38 activation and fibrosis following transverse aortic coarctation (TAC) but no increase in hypertrophy supporting the findings above (Klein *et al.* 2005). Acute p38 activation, by using a tamoxifen-inducible Cre combined with a floxed MKK3bE transgenic line, increased hypetrophy and heart failure together with increased fibrosis (Streicher *et al.* 2010). Mice overexpressing dn-p38α or dn-p38β developed cardiac hypertrophy but both were resistant to cardiac fibrosis in response to pressure overload (Zhang *et al.* 2003). Another study using dn-p38α, dn-MKK3, and dn-MKK6 resulted similarly in increased cardiac hypertrophy in response to various hypertrophic stimuli, but interstitial fibrosis was also increased in dn-p38α and dn-MKK3 mice (Braz *et al.* 2003). A cardiac-specific p38α-knockout (p38α-cKO) manifested with normal cardiac physiology and like control animals, they

developed cardiac hypertrophy in response to LV pressure overload. Supporting the results above, this was accompanied by massive cardiac fibrosis and apoptosis in p38α-cKO mice, leading to cardiac dysfunction and heart dilatation (Nishida *et al.* 2004). These results together suggest that p38 plays a role in pathological remodeling, but is not a key modulator of cardiac hypertrophy.

Thus, the exact role of p38 in cardiac hypertrophy is contradictory, despite the fact that p38 activation has a detrimental effect on cardiac function and normal gene expression both *in vitro* and *in vivo*. Acute activation of p38 is suggested to be prohypertrophic, but chronic activation can suppress hypertrophic growth of the heart (Rose *et al.* 2010).

2.6.2 p38 and apoptosis

Overexpression of p38α with upstream kinase MKK3b has been shown to increase cardiomyocyte death, while dn-p38α suppressed it. On the other hand, wild type p38β (wt-p38β) maintained cell survival ability (Wang *et al.* 1998). Saurin *et al.* showed in cardiomyocyte culture that simulated ischemia activated p38α, and inhibition of p38α by a dn-p38α adenovirus reduced ischemic injury, while p38β was deactivated during ischaemia (Saurin *et al.* 2000). Supporting these results, p38α-deficient cardiomyocytes were also less apoptotic compared to wild type cardiomyocytes (Porras *et al.* 2004).

In vivo studies are more controversial. *Ex vivo* p38 activation was shown to be transient in response to I/R-injury in Langendorff-perfused rabbit hearts, and p38 inhibition by SB203580 decreased myocardial apoptosis when administered before ischemia or during reperfusion, but no longer when administered 10 min after reperfusion (Ma *et al.* 1999a). A study with p38α+/- mice showed that necrotic injury decreased after ischemia when compared with p38α+/+ mice (Otsu *et al.* 2003). These results are supported by Ren *et al.* and Kaiser *et al.*, who showed that dn-p38α mice had reduced infarct size, less apoptosis, reduced Bcl-X_L deamination, and upregulated Bcl-2 levels, which are associated with better cell survival (Kaiser *et al.* 2004, Ren *et al.* 2005). Another study showed that mice with cardiac-specific overexpression of dn-p38α did not have increased ischemic injury, while cardiac-specific overexpression dn-p38β increased injury after ischemia/reperfusion (Cross *et al.* 2009).

Another study suggested an important role of p38α in cardioprotection, since cardiac-specific p38α knockout resulted in apoptotic cardiomyocyte death in response to pressure overload (Nishida *et al.* 2004). Ischemic preconditioning is a

process where small repeated periods of ischemia elicit cardioprotection against more sustained ischemic periods. p38 activity has been shown to increase during the first cycles of ischemia and reperfusion, but returs to the baseline within 6 cycles (Rose *et al.* 2010). There are studies showing that treatment with SB203580 or SB202190 blocks the cardioprotective effect of preconditioning (Armstrong *et al.* 1999, Nakano *et al.* 2000, Weinbrenner *et al.* 1997). Martindale *et al.* also reported reduced ischemic injury in mice with cardiac restricted overexpression of MKK6 (Martindale *et al.* 2005).

p38α is suggested to be the dominant isoform contributing to infarction, since SB203580 did not reduce either phosphorylation of p38 or infarct size in either homozygous or heterozygous p38α drug-resistant mice (Kumphune *et al.* 2010, Martin *et al.* 2001). The finding by Sicard *et al.* supports these results by showing that the p38β isoform is not necessary for ischemic preconditioning (Sicard *et al.* 2010).

Non-canonical TAB-1-mediated autophosphorylation of p38α has been shown to be involved in the apoptotic process, and it could also explain the inability of SB203580 to reduce infarct injury in p38α drug-resistant mice (Ota *et al.* 2010, Tanno *et al.* 2003). A recent study shows that TAB-1 autoactivation does not induce p38β, and interference with p38α recognition by TAB-1 improves cell viability and function. (De Nicola *et al.* 2013).

The role of p38 in the regulation of cell death is controversial as well. Most of the studies using chemical inhibitors influence both p38α and p38β, which could partly explain the discrepancy in the results. There is evidence that isoforms may have distinct and also context-dependent effect on cell viability (Marber *et al.* 2011).

2.6.3 p38 and cardiac remodeling

p38 activation in mice with cardiac overexpression of MKK3b or MKK6b markedly induced the expression of embryonic marker genes, such as ANP, β-MHC and α-skeletal actin, while SERCA2a, PLB and α-MHC were downregulated. MKK3b(E) overexpression resulted in end-systolic dilation, wall thinning, and myocyte atrophy. MKK6bE overexpression resulted in reduced chamber volume and almost normal end-systolic wall stress. Overexpression of both isoforms compromised diastolic function with elevated chamber stiffness and restrictive filling. (Liao *et al.* 2001). Cardiac-specific dn-p38α mice had markedly increased ventricular systolic function 7 days after MI (Ren *et al.* 2005).

β_2-adrenergic PKA activation is suggested to mediate p38 activation (Zheng *et al.* 2000), and transgenic β_2-AR x dn-p38α mice had reduced fibrosis and apoptosis in addition to rescued left ventricular ejection fraction (LVEF), suggesting that p38α is a critical downstream signal of chronic β_2-AR stimulation (Peter *et al.* 2007). p38 activation also induced the secretion of cytokines, such as IL-6 and TNF-α, and increased fibrosis and expression of matrix metalloproteinase (Li *et al.* 2005). Chemical p38 inhibition by RWJ67657 was shown to increase hypertrophy, attenuate TGF-β1-stimulated collagen synthesis and α-smooth muscle actin (α-SMA) expression in fibroblasts, and also resulted in reduced collagen and α-SMA immunoreactivity post-MI (See *et al.* 2004). In another rat study, p38 inhibition caused no changes either in left ventricular end-systolic diameter or in collagen content, but cytokine expression of TNF and IL-1β were significantly reduced by p38 inhibitor SB239063 after MI (Frantz *et al.* 2007). This discrepancy may be due to different inhibitors used in the studies.

Cardiac myofibroblasts (CMFs) are cells involved in the early adaptive healing process after MI, when they secrete proteolytic matrix metalloproteases (MMPs) and regulate ECM composition. Activation of p38α isoforms by IL-1α was shown to regulate IL-6 and MMP-3 in the CMFs, and thus in part to regulate cardiac remodeling post-MI (Sinfield *et al.* 2013, Turner *et al.* 2010). p38 has been shown to regulate ROS production and thus have an impact on LV function through myofibrillar oxidation (Heusch *et al.* 2010). Tenhunen *et al.* showed that p38 contributes to LV remodeling and heart failure by inducing gene expression resulting in myocardial cell proliferation, inflammation, and fibrosis (Tenhunen *et al.* 2006).

2.6.4 p38 and cardiac contractility

p38 activation has been shown to have a negative inotropic effect on cardiomyocytes (Chen *et al.* 2003, Liao *et al.* 2002, Zechner *et al.* 1997). Szokodi *et al.* showed that simultaneous p38 activation counterbalances the ERK1/2-mediated positive inotropy effect of ET-1, and p38 inhibition augmented ET-1-enhanced contractility (Szokodi *et al.* 2008). Cardiac-specific overexpression of dn-p38α and dn-p38β resulted in enhanced basal contractile function in a Langendorff-perfusion model, but dn-p38β hearts did not recover at the same level as wild type hearts (Cross *et al.* 2009). Long-term (12 week) p38 inhibition by RWJ67657 enhanced fractional shortening after MI (Kompa *et al.* 2008). In addition, the p38α MAPK pathway has been shown to mediate cardiomyocyte

contractile dysfunction in cardiac amyloidosis, and this was suggested to occur via TAB-1 autophosphorylation of p38 (Shi *et al.* 2010).

The exact role of p38 in the regulation of cardiac contractile function is not clear. First, Liao *et al.* suggested that p38 activation reduces the Ca^{2+} sensitivity of myofilaments without changes in $[Ca^{2+}]_i$ homeostasis (Liao *et al.* 2002). However, another study showed that p38 activation downregulated SERCA2a and increased diastolic $[Ca^{2+}]_i$, which activated NF-AT. This prolonged the decay phase of the Ca^{2+} transients (Andrews *et al.* 2003). There are also reports suggesting that p38 activation influences sarcomeric protein translocation and activation (Chen *et al.* 2003) and leads to decreased phosphorylation of α-tropomyosin (Vahebi *et al.* 2007). A recent study showed that activation of p38 targets MK2/3 decreases SERCA2a gene expression and negatively regulates fiber type composition, thus modulating cardiomyocyte function (Scharf *et al.* 2013). Another key regulator of Ca^{2+} homeostasis, the sodium-calcium exchanger (NCX), is upregulated in heart failure, and this has been shown to be mediated by p38α (Menick *et al.* 2007, Xu *et al.* 2009).

2.7 Transcription factor GATA-4

Transcription factor GATA-4 was identified in the early 1990s to be expressed in the heart, gonad, gut and tissues derived from endoderm (Arceci *et al.* 1993, Kelley *et al.* 1993, Laverriere *et al.* 1994). GATA-4 belongs to the GATA family of transcription factors, which consists of six members, GATA-1, -2, -3, -4, -5 and -6. GATA-1, -2 and -3 are prominently expressed in the hematopoietic stem cells and their derivates, regulating differentiation specific gene expression in T-lymphocytes, erythroid cells, and megakaryocytes. GATA-4, -5, and -6 are expressed in various mesoderm and endoderm-derived tissues. (Molkentin 2000, Pikkarainen *et al.* 2004). GATA transcription factors are zinc finger proteins that activate target genes by binding to the consensus sequence (A/T)GATA(A/G) via a DNA-binding domain containing two zinc fingers (Morrisey *et al.* 1997, Temsah & Nemer 2005). GATA-4, -5 and -6 may have some functional redundancy in cardiac promoters, since they bind to similar consensus sequences (Charron *et al.* 1999, Temsah & Nemer 2005). Figure 7 shows protein structure of murine GATA-4.

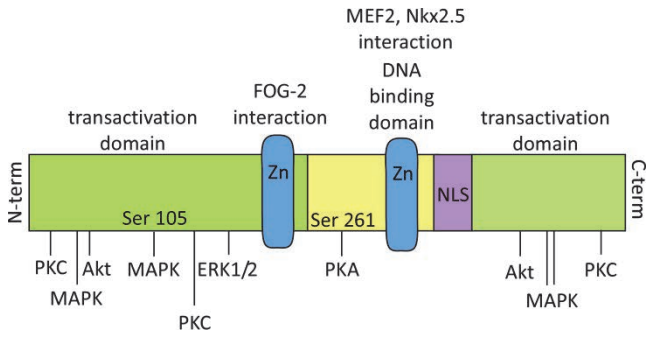

Fig. 7. Protein structure of GATA-4 with functional domains, putative phosphorylation sites, and confirmed MAPK and PKA phosphorylation sites Ser 105 and Ser 261, respectively. FOG-2, friend of GATA-2; MEF2, myocyte enhancer factor-2; NLS, nuclear localization signal. Modified from Temsah & Nemer (2005).

GATA-4 alone and together with its interaction partner friend of GATA-2 (FOG-2) have an essential role in normal ventricular morphogenesis in the embryo. GATA4 -/- embryos died 8.0–9.0 days post coitum (Crispino *et al.* 2001, Heikinheimo *et al.* 1994, Kuo *et al.* 1997, Molkentin *et al.* 1997). Cardiac lineages of differentiating P19 embryonal carcinoma cells express GATA-4 exclusively, and blocking GATA-4 by GATA-4 antisense RNA does not show any marks of cardiac differentiation (Grepin *et al.* 1997, Grépin *et al.* 1995, van der Heyden & Defize 2003). GATA-4 is able to induce cardiogenesis in embryonic stem cells (Grepin *et al.* 1997) and promote a cardiac cell fate from non-cardiogenic cells (Gallagher *et al.* 2012, Ieda *et al.* 2010, Qian *et al.* 2012, Takeuchi & Bruneau 2009). GATA-4 also appears to be involved in the regeneration process (Gupta *et al.* 2013, Kikuchi *et al.* 2010).

GATA-4 induces many target genes that are activated both in cardiogenesis and cardiac hypertrophy, including sarcomeric genes α-MHC, troponin C and I, myosin light chain -3, and ANP, BNP, corin, NCX, acetylcholine receptor-M, cardiac restricted ancyrine repeat protein, adenosine receptor A1, and carnitine palmitoyltransferase-1β (Durocher *et al.* 1997, Oka *et al.* 2006, Pikkarainen *et al.* 2004). Intact GATA-4 binding elements in the promoter regions are necessary for full induction of β-MHC and Ang II receptor genes (Hasegawa *et al.* 1997, Herzig *et al.* 1997). Similarly, GATA-4 binding sites are indispensable for PE-induced rat preproendothelin or BNP and isoproterenol-induced BNP promoter activities. Also, ET-1 has been shown to activate the ANP and BNP promoters in a GATA-4 site-dependent manner (Pikkarainen *et al.* 2004). The mechanical stretch-induced

cardiomyoyte hypertrophic program is mediated by GATA-4 as well (Pikkarainen *et al.* 2003b). GATA-4 and/or GATA-6 signaling may be reinforced by themselves, since they also activate bone morphogenetic protein-4 (BMP-4) and preproendothelin via binding to the GATA-sites of their promoters, and both BMP-4 and ET-1 further activate GATA-4 (Hautala *et al.* 2001, Morimoto *et al.* 2000, Nemer & Nemer 2003).

GATA-4 has been shown to play a key role in cardioprotection. GATA-4 binds to the anti-apoptotic Bcl-2 gene promoter and upregulates Bcl-2 (Kobayashi *et al.* 2006). This was also related to protection from doxorubicin-induced autophagy and cardiomyocyte apoptosis (Kobayashi *et al.* 2010). Erythropoietin (EPO) increases GATA-4 DNA binding activity and BNP secretion in isolated rat hearts, reduces I/R injury, and results in better preserved contractile function (Piuhola *et al.* 2008). Inhibition of GATA-4 by dominant-negative adenovirus abolished EPO-induced cardioprotective effects *in vitro* (Shan *et al.* 2009). Adenoviral GATA-4 overexpression prevented cardiac remodeling in rat hearts after MI; this included myocardial angiogenesis, reduced apoptosis, and stem cell recruitment (Rysä *et al.* 2010). GATA-4 overexpression also protected from hyperglycemia-induced cardiomyocyte death (Kobayashi *et al.* 2007). More recently, overexpression of GATA-4 has been shown to promote bone marrow-derived mesenchymal stromal cell transdifferentiation into the myocardial phenotype via upregulation of insulin-like growth factor-binding protein-4 (IGFBP4) (Li *et al.* 2011).

2.7.1 Posttranslational modifications of GATA-4

Immunoblotted GATA-4 is typically presented as a single band with a molecular size between 44 and 52 kDa. The amino acid sequence predicts GATA-4 to be 44 kDa. Arceci *et al.* reported the predicted molecular size of GATA-4 to be 48 kDa, which is comparable to the molecular sizes of other mouse GATA-binding proteins. If translation begins from a more distal ATG-codon, that would result in an approximately 30 kDa protein, which lacks an intact transactivation domain (Arceci *et al.* 1993). Phosphorylation, acetylation, sumoylation, ubiquitination, Ca^{2+} signaling, redox signaling, and protein-protein interactions are known to regulate GATA-4 so far (Suzuki 2011). To complicate the issue, these modifications may also regulate each other, occurring seldom independently, and their consequences may be different in various conditions. Phosphorylation occurs on one or several serine, threonine, or tyrosine residues, resulting in either

enhancement or inhibition of the activity (Porter *et al.* 2012). Acetylation, ubiquitination, sumoylation, and methylation occur in lysine residues, and only a single modification can be conjugated to a single residue at a time. However, this does not prevent modifications of multiple lysines simultaneously, and thus modifications may occur sequentially or in cascades, where a modification may influence the modification of the neighboring residue (Freiman & Tjian 2003).

Phosphorylation of GATA-4

ERK1/2 and p38 have been shown to phosphorylate GATA-4 at Ser 105 upon ET-1 or PE stimulus (Charron *et al.* 2001, Kerkelä *et al.* 2002, Liang *et al.* 2001), and they also induced GATA-4 DNA binding (Kerkelä *et al.* 2002). Supporting this data, p38 and ERK mediated wall stress-induced GATA-4 DNA binding (Tenhunen *et al.* 2004). The p38 mediated RhoA/Rho kinase (ROCK) pathway has also been shown to potentiate p38 and ERK phosphorylation of GATA-4 (Charron *et al.* 2001, Yanazume *et al.* 2002). Recently, Gallagher *et al.* showed that Ser105 phosphorylation is not necessary for GATA-4-induced cardiogenesis, but it is necessary for synergy with SRF (but not with Tbx5 or Nkx2.5) and has central role in MAPK-mediated hypertrophy and cell survival (Gallagher *et al.* 2012).

GSK-3β was shown to physically associate with GATA-4 and phosphorylate GATA-4 *in vitro*, and GSK-3β overexpression suppressed nuclear expression of GATA; GSK-3β inhibition increased nuclear accumulation, thus GSK-3β may negatively regulate GATA-4 (Morisco *et al.* 2001). PKC has been shown to mediate Ang II-stimulated GATA-4 phosphorylation and DNA-binding, with the putative phosphorylation sites being Ser 419 and Ser 420 (Wang *et al.* 2005). Another phosphorylation site of GATA-4 is Ser 261, which is phosphorylated via erythropoietin-induced ERK1 activation in response to EPO stimulus, leading to increased acetylation and stabilization of GATA-4 (Jun *et al.* 2013b). Of interest, PKA was shown to phosphorylate GATA-4 Ser261 in gonadal cells enhancing transcription of many gonadal marker genes (Tremblay & Viger 2003).

Acetylation of GATA-4

Acetylation occurs via the action of histone acetyltransferases (HATs), and a reversal action is performed by histone deacetylases (HDACs). HDACs influence on cell function, and especially sirtuins (HDAC class III) in particular are

extensively investigated in the field of cardioprotection research (Porter *et al.* 2012). Acetylation of GATA-4 is suggested to make it more resistant to degradation, probably because lysine residues are blocked (Suzuki *et al.* 2004). PE has been shown to increase transcriptional co-activator p300 induced GATA-4 acetylation (Yanazume *et al.* 2003) and also cyclin-dependent kinase-9 (cdk9) has been shown to form a functional complex with GATA-4 and p300 to increase GATA-4 transcriptional activity in cardiac hypertrophy (Sunagawa *et al.* 2010). In contrast to MAPK(ERK)-induced phosphorylation and the compensatory hypertrophic response (Bueno *et al.* 2000), an increase in GATA-4 acetylation in mice overexpressing p300 promoted eccentric dilatation and systolic dysfunction, suggesting that GATA-4 acetylation plays a critical role in the development of myocyte hypertrophy and may contribute to the progression of decompensated heart failure (Yanazume *et al.* 2003). A study by Takaya *et al.* has identified the critical four acetylation sites of GATA-4, which are located between amino acids 311, 318, 320 and 322, and mutation of these sites suppressed hypertrophic responses (Takaya *et al.* 2008). M456-GATA-4 (tetramutant of GATA-4, 4 lysine residues mutated between amino acids 311–322) is almost completely lacking p300-induced acetylation and consistent with the data above, tetramutant GATA-4 resulted in an attenuated hypertrophic response (Takaya *et al.* 2008).

Ubiquitination of GATA-4

Protein quality control is necessary for the maintenance of normal cardiac function, and the ubiquitin-proteasome system (UPS) is a fundamental regulator of protein quality. Cardiac dysfunction is associated with alterations in UPS function (Willis *et al.* 2013). Monoubiquitination of a protein is known to either activate or regulate cellular the location of the target protein, while polyubiquitinated proteins are usually degraded by the 26S proteasome (Freiman & Tjian 2003). Ubiquitin is activated by the ubiquitin-activated enzyme E1 and E2 is a ubiquitin carrier enzyme, which transports ubiquitin to a target protein. E3, a ubiquitin protein ligase, binds ubiquitin to the protein. E3 ligases, which have been reported to regulate cardiac protein turnover, include muscle-ring finger family (MurRF1, -2 and -3), atrogin-1/muscle atrophy F-box (MAFBx), C-terminus of heat shock protein 70-interacting protein (CHIP) and the murine double minute 2 (MDM2). In addition, F-box and leucine-rich repeat protein 22 (Fbxl22), casitas b-lineage lymphoma (c-Cb1), ubiquitin-protein ligase E3A, and

cellular inhibitor of apoptosis (cIAP) have also been described in the heart (Willis *et al.* 2013).

Partial proteasomal inhibition by MG-132 and MG-262 effectively suppressed cardiomyocyte hypertrophy by reducing cell size, inhibiting hypertrophy-mediated induction of RNA and protein synthesis, reducing the expression of several fetal genes, and diminishing BNP promoter transcriptional activation (Meiners *et al.* 2008). The role of GATA-4 ubiquitination is not yet clear; Jun *et al.* has shown that GATA-4 ubiquitination is decreased after erytropoeitin-ERK-induced GATA-4 Ser105 phosphorylation, and thus GATA-4 was more stable under hypoxic conditions (Jun *et al.* 2013a). Another study showed that hyperglycemia-induced GATA-4 reduction was reversed by a proteasome inhibitor. GATA-4 degradation was associated with elevated CHIP E3 ubiquitin ligase levels (Kobayashi *et al.* 2007).

Sumoylation of GATA-4

There is little data of sumoylation of GATA-4. Small ubiquitin-related modifier-1 (SUMO-1) is a 101 amino acid polypeptide (Kim *et al.* 2002). The sumoylation process resembles the ubiquitination process, without directing the target protein automatically toward proteolysis. Sumoylation may for example repress the gene expression (Verger *et al.* 2003) and activate or inactivate its substrate (Perdomo *et al.* 2013). The putative sumoylation site of GATA-4 is lysine 366, and its sumoylation is suggested to maintain GATA-4 occupation in the nucleus, but without any significant effect on GATA-4 DNA binding activity (Wang *et al.* 2004).

2.8 Natriuretic peptides ANP and BNP

Natriuretic peptides were characterized in the 1980s, first atrial natriuretic factor (ANF, also later referred to as ANP) and then brain natriuretic peptide (BNP), names referring to the tissues from which they were isolated (de Bold & Flynn 1983, Sudoh *et al.* 1988). Subsequently, BNP production was also discovered to occur in the mammalian heart atria and ventricles. Other polypeptide hormones expressed in the heart and acting upon the myocardium in a paracrine or autocrine function include C-type natriuretic peptide (CNP), adrenomedullin, proadrenomedullin N-terminal peptide, and ET-1 (Ogawa & de Bold 2014). Both ANP and BNP are mainly synthesized in the cardiac tissue and they are used as

the diagnostic and prognostic markers of cardiovascular diseases (Ruskoaho 2003). Increased expressions of ANP and BNP are considered hallmarks of the hypertrophic response (Bernardo *et al.* 2010). CNP lacks natriuretic properties and it is produced in various tissues, mainly in the vascular endothelium (Ogawa & de Bold 2014).

A typical feature for both ANP and BNP (and CNP) is a 17-amino acid ring formed between two cysteine residues, which is essential for their biological activity. ANP is a 28-amino acid peptide, while BNP is a 32-amino acid peptide. Both peptides have a prohormone form, which is split into the biologically active peptide (McGrath & de Bold 2005, Ruskoaho 2003, Yandle 1994). ANP and BNP are encoded by distinct genes, and the ANP gene is highly conserved among mammalian species (Argentin *et al.* 1985, McGrath & de Bold 2005, Seidman *et al.* 1984).

Both ANP and BNP function predominantly through the natriuretic peptide receptor-A (NPR-A), which is a guanylyl cyclase-coupled receptor and expressed widely throughout the body (Lowe *et al.* 1989, Ogawa & de Bold 2014). Agonist binding to NPR-A increases the amount of cGMP, and its targets include cGMP-dependent protein kinases (PKGs), cGMP-gated ion channels, and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs) (Lincoln & Cornwell 1993). The similar biological actions of ANP and BNP are listed in Figure 8. The net effect of these activities is reduced cardiac preload and afterload and suppressed growth and proliferation (McGrath *et al.* 2005).

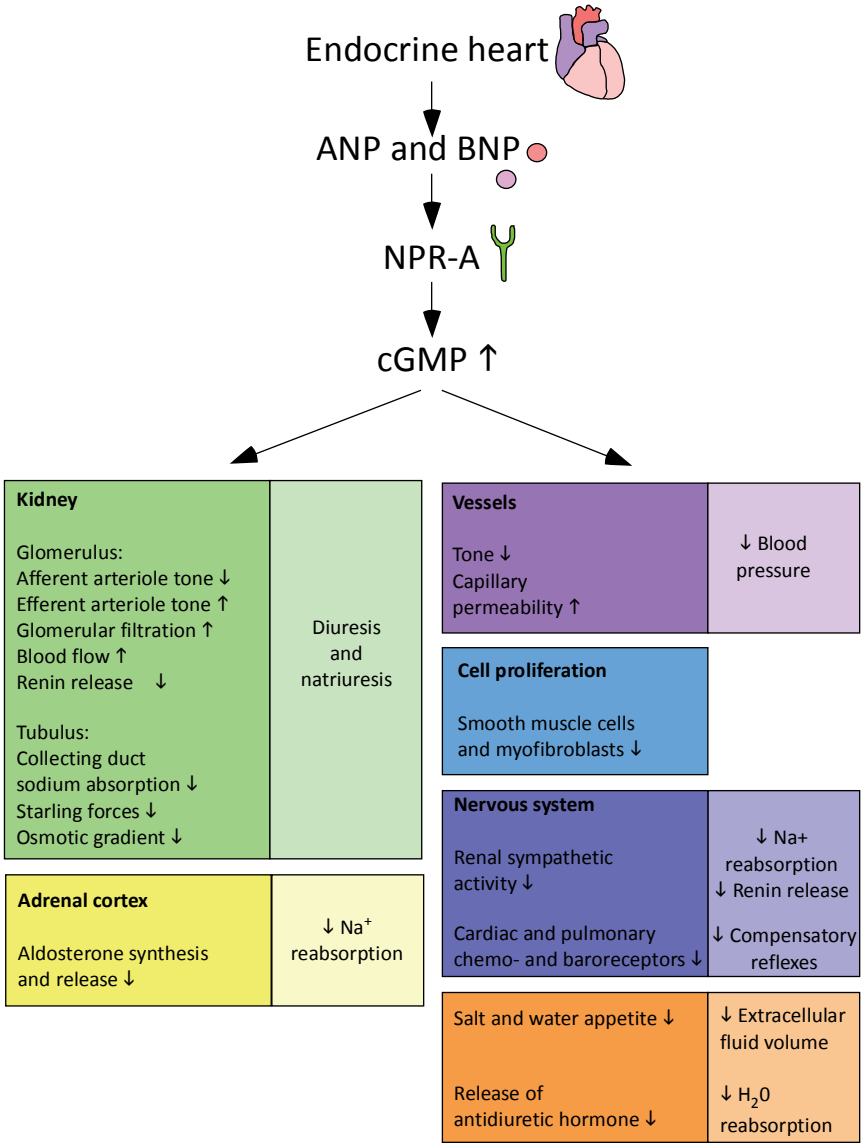

Fig. 8. Biological effects of ANP and BNP. ANP and BNP bind to the NPR-A receptor, which leads to increased cGMP levels. Modified from McGrath *et al.* 2005.

ANP and BNP are continuously secreted from the atria under normal conditions (Ogawa & de Bold 2014). First, it was shown that wall stress induced ANP secretion (Ruskoaho *et al.* 1987, Ruskoaho *et al.* 1986), and wall stretch was also

the predominant stimulus controlling the synthesis and release of BNP from the atria and ventricles (Kinnunen *et al.* 1993, Magga *et al.* 1994, Mäntymaa *et al.* 1993). PE stimulus results in discoordinated increases in ANP and BNP secretion and gene expression, while stretch and ET-1 stimuli significantly increase BNP gene expression alone (Bruneau & De Bold 1994, Bruneau *et al.* 1997, Mäntymaa *et al.* 1990, Ogawa *et al.* 1999). BNP secretion is suggested to mimic the rapid induction of proto-oncogenes in response to hemodynamic stress (Magga *et al.* 1994, Mäntymaa *et al.* 1993). In addition, stretch-stimulated atrial ANP and BNP release are suggested to be intracellular Ca^{2+} regulated (Laine *et al.* 1994, Laine *et al.* 1996).

Regulation of the BNP gene promoter

Many of the stimuli that induce secretion of natriuretic peptide, such as ET-1, PE, angiotensin and mechanical stress, signal through G-protein-coupled receptors and activate the Ras/c-Raf-1 pathway and the phosphoinositide pathway, which cause recruitment and activation of effector molecules such as MAPKs (McGrath & de Bold 2005). Transcription factors regulating the BNP promoter include AP-1, GATA-4, and NF-κB (Liang & Gardner 1999, Pikkarainen *et al.* 2003a, Pikkarainen *et al.* 2004, Tokola *et al.* 2001). Il-1β activation of p38 was shown to be partially involved in BNP activation, targeting to the proximal M-CAT element of the BNP promoter (He & LaPointe 1999). Mechanical stress induced activation of p38 was also shown to increase BNP promoter activation through activation of transcription factor NF-κB; ERK was also involved but without NF-κB recruitment (Liang *et al.* 2000). Kerkelä *et al.* showed distinct roles of ERK1/2 and p38 in ET-1 induced and GATA-4-mediated regulation of the BNP gene, suggesting that p38 activation is necessary for BNP gene expression (Kerkelä *et al.* 2002). Supporting these results, ET-1 induced BNP activation was shown to be mediated via p38 pathway (Pikkarainen *et al.* 2003a). On the other hand, mechanical stretch induced ERK1/2 activation was shown to increase BNP promoter activity via an M-CAT binding site (Koivisto *et al.* 2011).

3 Aims of the research

The aim of this study was to characterize molecular pathways involved in cardiac remodeling, contractility, and hypertrophy. Experiments were mainly performed in neonatal and adult cardiomyocytes by utilizing adenoviral modulation or chemical inhibition of signaling pathways together with various hypertrophic stimuli. Specific aims were

1. To identify potential mechanisms underlying p38 mediated negative inotropy.
2. To investigate the roles of p38α and p38β in regulating genes associated with cardiac hypertrophy and remodeling.
3. To study the effect of mechanical stretch on molecular forms of GATA-4 and elucidate the regulatory mechanisms of GATA-4.

4 Materials and methods

4.1 Materials

4.1.1 Antibodies

Table 3. List of antibodies used for immunoblots and immunoprecipitation.

Manufacturer	Antibody (product number)	Type of antibody
Abcam	p-GRK2 S670 (ab78353)	Primary
Badrilla Ltd.	p-PLB Ser16 (A010-12)	Primary
	p-PLB Thr17 (A010-13)	Primary
	PLB (A010-14)	Primary
Biosource	p-GATA-4 Ser105 (44-948)	Primary
Cell Signalling Technology	p-ATF-2 (#9221S)	Primary
	DARPP32 Thr34 (Inhibitor-1 Thr35) (#2304)	Primary
	p-eIF2α Ser51 (#3597)	Primary
	p-ERK1/2 Thr202/Tyr204 (#9106)	Primary
	Acetylated lysine (#9441)	Primary
	p-p38 Thr180/Tyr182 (#9211)	
	p38 (#9212)	Primary
	Ubiquitin (#3933)	Primary
	SUMO-1 (#4930)	Primary
	anti-IgG HRP-linked rabbit (#7074)	Secondary
	anti-IgG HRP-linked mouse (#7076)	Secondary
Life Technologies/Invitrogen	Alexa Fluor 680 (A21076)	Secondary
Millipore	GAPDH (MAB374)	Primary
	p-p38 Thr180/Tyr182 (AB3828)	Primary
Rockland Immunochemicals	IRDye 800 (611-732-107)	Secondary
Santa Cruz Biotechnology	GATA-4 (sc-9053)	Primary
	GRK-2 (sc-562)	Primary
	MKK3 (sc-959)	Primary
	MKK6 (sc-1992)	Primary
	p38α (sc-728)	Primary
Dr. James Bibb	Inhibitor-1 Ser67	Primary

4.1.2 Cell culture reagents

Cell culture reagents [bovine serum albumin, $CaCl_2$, Dulbecco's Modified Eagle's medium/F-12 (DMEM/F-12), DMEM, phosphate buffered saline (PBS), insulin-transferrin sodium-selenite media supplement (ITS), laminin L-glutamine, penicillin-streptomycin, sodium pyruvate, 3'-3'-5'triiodotyronine, 2,3-butandione monoxime (BDM), dimethyl sulphoxide (DMSO), endothelin-1 (ET-1), and phenylephrine (PE)] were from Sigma-Aldrich (St. Louis, MO). Collagenase type II was from Biochemical Corporation (Lakewood, NJ). αMEM supplemented with Hank's salt, αMEM supplemented with Earle's salt, HEPES, and Optimem were from Invitrogen (Life Technologies, Carlsbad, CA, USA). [^{3}H]-leucine was from GE Healthcare (Waukesha, WI, USA).

4.1.3 Biomolecules and pharmacological agents

SB203580, BIRB796, PD98059, and SP600125 were from LC Laboratories (Woburn, MA). MG-262 was from Enzo Life Sciences (New York, NY). Fostriecin was from Tocris (Bristol, UK). FuGENE6 transfection reagent was from Roche Molecular Biochemicals (Mannheim, Germany) and Lipofectamine 2000 transfection reagent was from Invitrogen (Life Technologies, Carlsbad, CA, USA). Human recombinant purified GATA-4 protein (TP310945) was from OriGene (Rockville, MD, USA).

4.1.4 Adenoviral vectors and plasmids

Dominant-negative (dn) and wild-type (wt) adenoviruses coding p38α and p38β MAPKs, the adenoviruses of their upstream kinases, constitutively active MKK3b and MKK6b, and a control adenovirus coding β-galactosidase LacZ were all driven by cytomegalovirus immediately early promoter and all adenoviruses were generated as previously described (Han *et al.* 1997, Huang *et al.* 1997, Jiang *et al.* 1996, Wang *et al.* 1998). All of these adenoviruses were generously supplied by Dr. Veli-Matti Kähäri from the University of Turku, Finland.

Wild-type GRK2 (wt GRK2), truncated GRK2 adenovirus βARKct, and mutated GRK2 S670A adenoviruses have been described previously (Chen *et al.* 2013, Hehir *et al.* 1996, Vinge *et al.* 2007, White *et al.* 2000). GRK2 adenoviruses were generously supplied by Dr. Leif Erik Vinge, Oslo University, Norway and Dr. J. Kurt Chuprun, Temple University, Philadelphia, USA.

Adenoviruses coding GATA-4 and NLS-LacZ were generated as described earlier (Charron *et al.* 2001). The plasmid expressing mouse GATA-4 (pMT2-GATA-4) and the empty pMT2 plasmid were generous gifts from D.B. Wilson (Department of Pediatrics, St. Louis Children's Hospital, MO, USA) (Arceci *et al.* 1993).

The following BNP promoter constructs were generated as reported earlier: (Δ-534/+4) BNP promoter luciferase construct (rBNP-luc), rBNP promoter construct containing two site-directed mutations of the proximal GATA-4 sites (BNP GATAMut), rBNP promoter construct containing a site-directed mutation at the AP-1 binding site (BNP AP-1Mut), and rBNP promoter with a mutation at the EBS site (Pikkarainen *et al.* 2002, Pikkarainen *et al.* 2003a).

4.1.5 Experimental animals

Neonatal (2–4 days old) Sprague-Dawley rats (SD-rats) of both genders, as well as adult male SD-rats weighing 300–350 g, were used for *in vitro* experiments (cell culture). Adult rats were used also for *in vivo* and *ex vivo* experiments. All animal protocols were approved by the Animal Use and Care Committee of the University of Oulu and the National Animal Experiment Board. All animals were from the colony of the Center for Experimental Animals at the University of Oulu.

4.2 Experimental protocols

4.2.1 Neonatal cardiomyocyte culture (I, II, III)

Neonatal rat ventricular cardiomyocytes (NRVMs) were used as an experimental model of hypertrophic adult myocardium, since many fetal genes are actived in NRVMs in response to various hypertrophic stimuli, mimicking the hypertrophic response in the adult myocardium. NRVMs were prepared from 2- to 4-day-old Sprague-Dawley rats (SD-rats). Rats were sacrified by quick decapitation and the thorax was opened to remove ventricles. Ventricles were rinsed out in 1×PBS and then cutted into small pieces in collagenase (2 mg/ml and 25 mM $CaCl_2$ in 1×PBS). Cell dissociation was performed by repeated incubations in collagenase at +37 °C The incubations were approximately 5 min (collected supernatant discarded), 15 min, 25 min, 25 min and 15 min. After each incubation, except first incubation, the collected supernatant was filtered (100 μm) to remove cellular

debris. Finally, the cell suspension was centrifuged twice (5 min, 1000 rpm), and the supernatant was discarded and replaced with fresh DMEM/F-12 medium, including 10% fetal bovine serum, 2.56 mM L-glutamine, and penicillin-streptomycin (100 IU/ml) after both centrifugations. Isolated cells were pre-plated onto 100 mm culture dishes (3–4 hearts/plate) for 2 hours to remove fibroblasts, since they attach faster than cardiomyocytes. Cell suspension (unattached myocytes with some fibroblasts) from pre-plated wells was re-plated at a density of 1.8–2×10^5/cm^2 and incubated in DMEM/F-12 supplemented with 10% fetal bovine serum overnight before changing to serum-free culture media (CSFM; DMEM/F-12, 2.5 mg/ml bovine serum albumin, 1 μM insulin, 2.56 mM L-Glut, 32 nM selenium, 2.8 mM sodium puryvate, 5.64 μg/ml transferrin 1 nM T3, 100 IU/ml penicillin-streptomycin). CSFM was used in all experiments except siRNA transfection, which was done in Optimem. The media was changed every 24 hours.

When appropriate, agonist treatment by ET-1 (100 nM for 1- 24 hours) or PE (100 μM for 15 minutes) was added to culture medium on the third or fourth day of culture. Pharmacological inhibitors (10 μM SB203580, 10 μM PD98059, 10 μM SP600125, 1 μM BIRB-796, 1 nM okadaic acid, 1 μM Fostriecin, 25 nM MG-262) were added one hour prior to stimulus or on the third day of culture.

After experiments, wells were rinsed twice with cold 1×PBS and quickly frozen in −70 °C.

Adenoviral infection and plasmid transfection

Adenoviruses were added to the culture medium approximately 18–24 hours after plating and incubated for 24 hours. The virus concentration was 1 to 4 MOI (depending on the designed experiment). If both plasmids and adenoviruses were used, adenoviruses were added to the culture medium 6–8 hours after the plasmid insertion and incubated for 24 hours.

Plasmid transfection was conducted with a plasmid containing intact -534 BNP or mutated -534 BNP –luc-constructs along with pRL-TK control plasmids (1 μg and 0.5 μg, respectively, on 6-well plates and 0.25 and 0.125 μg on 24-well plates). pRL-TK plasmid was used as a control vector of BNP-transfected cells in each sample to equalize for transfection efficacy. FUGENE^{TM}6 (Roche Applied Science, Penzberg, Germany) was used as a transfection reagent. The data are presented as a luciferase activity/pRL-TK activity ratio. The reporter gene analysis was performed according to Dual-Luciferase® Reporter Assay System

protocols. Cells were lysed with cell lysis buffer and aliquots of cell lysates were assayed for luciferase activity and control plasmid enzymes. The Dual-Luciferase® Reporter Assay System and pRL-TK control expression plasmid were from Promega Co. (Fitchburg, WI, USA). Luminescence was measured by luminometer (Luminoskan RS, from Thermo Labsystems).

Mechanical stretch

For stretch experiments, cells were initially plated on collagen I-coated flexible bottomed BioFlex plates (Flexcell) after normal pre-plating. Mechanical stretch was introduced to cells by applying cyclic vacuum suction (0.5 Hz, 10–21%, sinusoidal waveform) under the flexible bottomed plates by computer-controlled equipment (Fig. 9.) (Flexercell Strain Unit FX-5000, Flexcell, Hillsborough, NC, USA).

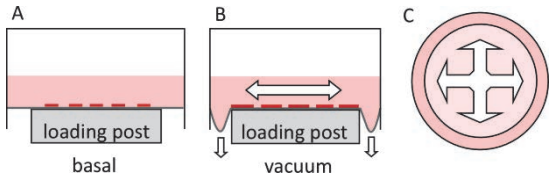

Fig. 9. Equibiaxial mechanical stretch is produced by vacuum suction under the flexible bottomed plate from the sides of a cylindrical loading post. A) Basal, b) vacuum, and c) top view.

RNA interference

RNAi is a mechanism by which exogenous RNA (siRNA) directs destruction of specific mRNA leading to downregulation of the cellular gene expression at the level of mRNA stability (Ulvila *et al.* 2010). Specific p38α siRNAs (p38α1 and p38α2, 5'-CAACCUCGCUGUGAAUGAA-3' and 5'-GCUUACCGAUGACCACGUU-3', respectively, from Ambion, used in a 1:1 combination) and negative control siRNA (Cat#4390844, Ambion) were transfected into the cardiomyocytes using Lipofectamine2000 (Invitrogen) as the transfection reagent 18–24 h after initial plating. The siRNA concentration was 100–150 nM. Cells were incubated in OPTI-mem (Invitrogen) for 18 h, and thereafter the cells were incubated in CSFM.

4.2.2 Adult cardiomyocyte cell culture (I)

Adult mouse and rat ventricular cardiomyocytes were cultured as described previously (Martini *et al.* 2008, O'Connell *et al.* 2003, Zhou *et al.* 2000). Adult mouse ventricular cardiomyocytes (AMVMs) were isolated from 8–12 week old male C57/BL6 mice by Ca^{2+}-low retrograde perfusion, and the hearts were enzymatically digested by Tyrode's-collagenase type 2 with BDM. Briefly, mice were anesthetized with isoflurane (4%), hearts were quickly removed and the aorta was cannulated above the aortic valve for retrograde perfusion at a flow rate of 3 ml/min for 10 min. After perfusion, atrial appendices and fibrous tissue were cut off and ventricles were cut into small pieces with scissors and collected in a tube. Tissue fragments were gently mechanically dispersed, and after digestion the filtered cell suspension was centrifuged at 20 × g. The subsequent pellet was resuspended in modified Tyrode's solution and Ca^{2+} was gradually reintroduced equal to that in cultivating medium (αMEM supplemented with Hank's salt containing 5% FBS, insulin-transferrin-selenium, 10 mM BDM, 2 mM L-Glutamine and penicillin-streptomycin). Cells were plated on laminin-coated wells (10 µg/ml) at a density of $1–1.5\times10^4$ cardiomyocytes/cm^2 and incubated in a humidified atmosphere at 37 °C containing 2% CO_2. Ca^{2+}-transient measurements were performed on the same day.

Adult rat ventricular cardiomyocytes were used for experiments requiring overnight incubation, since they stay viable longer than adult mouse cardiomyocytes. ARVMs were isolated from 8–12 weeks old male SD-rats according to the protocol above with some modifications. Rats were anesthetized with CO_2, decapitated, and aorta was cannulated above the aortic valve before removing the heart. Retrograde perfusion was begun with Tyrode's solution at a flow rate of 5.5 ml/min for 1 min before switching to Tyrode's with collagenase type 2 and BDM. The total perfusion time was 20 min at a flow rate of 10 ml/min. After Ca^{2+} reintroduction, resuspension medium was αMEM supplemented with Earle's salt containing 5% FBS, 10 mM HEPES insulin-transferrin-selenium (Invitrogen), 10 mM BDM, 2 mM L-Glutamine, and penicillin-streptomycin. ARVMs were plated onto laminin-coated wells as well and incubated in a humidified atmosphere at 37 °C containing 5% CO_2. After a 2 h incubation, cells were gently washed to remove non-attached cells and a fresh serum-free medium (containing 0.01% BSA) was added to the wells. According to the experimental setting, adenoviruses were then added at 100 MOI. Experiments with adult

cardiomyocytes were performed after an overnight incubation, approximately 20 h after plating.

4.2.3 Isolated perfused rat heart preparation (I)

For isolated rat heart perfusions we used male 7-week-old Sprague-Dawley rats. As described previously, rats were anesthetized with CO_2, decapitated, and hearts were rapidly excised and subjected to retrograde perfusion by the Langendorff technique as described previously (Szokodi *et al.* 2008).

4.2.4 COS-1 cell culture (III)

COS-1 cells were maintained in DMEM containing 10% FBS. Cells were plated onto 6-well plates and transfected with 1 μg of PMT-2-GATA-4 expression plasmid using FuGENE6 reagent. The medium was changed 6–8 hours after transfection. When applicable, MG-262 (25 nM) was added to the medium. Cells were harvested and subjected to nuclear protein extraction 12–72 hours after transfection.

4.2.5 Isolation and analysis of RNA (I, II, III)

RNA extraction from cardiomyocytes was performed with Trizol reagent following the manufacturer's protocol (Invitrogen, Life Technologies) using the Phase Lock Gel System (Eppendorf AG, Hamburg, Germany). For cDNA synthesis, the First-Strand cDNA Synthesis Kit (GE Healthcare, Waukesha, WI, USA) was used according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) with TaqMan chemistry (Life Technologies) was used for RNA analysis on an ABI 7300 sequence detection system (Applied biosystems, Life Technologies) as previously described (Majalahti-Palviainen *et al.* 2000, Tenhunen *et al.* 2006). The primers and fluorogenic probes used in qRT-PCR are presented in Table 4.

Table 4. Sequences of rat primers and probes used for qRT-PCR analysis (sequences 5' to 3').

Gene	Forward	Reverse	Probe
18S	TGGTTGCAAAGCTGAAACT TAAAG	AGTCAAATTAAGCCGC AGGC	CCTGGTGGTGCCCTTCC GTCA
ANP	GAAAAGCAAACTGAGGGC TCTG	CCTACCCCCGAAGCAGCT	TCGCTGGCCCTCGGA GCCT
BNP	TGGGCAGAAGATAGAC CGGA	ACAACCTCAGCCCGTC ACAG	CGGCGCAGTCAGTCGC TTGG
β-MHC	GCTACCCAACCCTAAGG ATGC	TCTGCCTAAGGTGCTGTT TCAA	TGTGAAGCCCTGAGACCT GGAGCC
bFGF	CCCGGCCACTTCAAGGAT	GATGCGCAGGAAGAAGCC	CCAAGCGGCTCTACTGCAA GAACGG
MMP-9	CCGCCAACTATGACCAGG ATAA	AGTTGCCCCCAGTTACA GTGA	TGTATGGCTTCTGTCCTAC TCGAGCCGA
MMP-2	CATGAAGCCTTGTTTACC ATGG	TGGAAGCGGAACGGA AACT	TGGCAATGCTGATGGACA GCCC
CTGF	CGCCAACCGCAAGATTG	CACGGACCCACCGAAGAC	CACTGCCAAAGATGGTGCA CCCTG
COL1A1	CCCCTTGGTCTTGGA GGAA	GCACGGAAACTCCAGC TGAT	CTTTGCTTCCCAGATGTCC TATGGCTATGATG
aFGF	ATGGCACCGTGGATGGG	TTTCCGCACTGAGCT GCAG	AGGGACAGGAGCGACCAG CACATTC
PDGF-A	CGAGCGACTGGCTCGAA	GAGTCTATCTCCAAGAGTC GCTGG	TCAGATCCACAGCATCCGG GACC
p38α	CCTGCGAGGGCTGAAGTA TATAC	GCGAGGTTGCTGGGCTTT	CTCGGCTGACATAATCCAC AGGGACC
p38β	CTGAGCGATGAGCATGT TCAG	CCGCCGAGTGGATATAC TTCA	TCCTTGTCTACCAGCTGCT GCGTGG
PLB	AAGTCTGTCGCCACCGCA	TGGTGGAGGGCCAGGTT	CCTGCACCATGCCAACG CAGC
SERCA2a	CAGCCATGGAGAACG CTCA	CGTTGACGCCGAAGTGG	ACAAAGACCGTGGAGGAG GTGCTGG
CASQ2	AAGGAGCATCAAAGACC CACC	TCGTCTTCCCATGTTTCA AACA	CGTCGCTTGCGCCCA GAGG
NCX	CTCTTGTTTACCCATGTTG ACCATAT	GAGCCAGTACATTCAGTGG TTTCA	TGCAGATACAGAGGCAGA AACAGGAGGAA

4.2.6 Protein synthesis (II)

Protein synthesis was analyzed by measuring [^{3}H] leucine incorporation as described previously (Berk *et al.* 1989). Briefly, cells were cultured on 24-well plates according to the normal protocol until the third day, when the medium was replaced with CSFM supplemented with [^{3}H] leucine (5 μCi/ml) for 24 hours. Cells were lysed and processed for measurement of incorporated [^{3}H] leucine using liquid scintillation counter.

4.2.7 Protein extraction (I, II, III)

Total protein extractions from cardiomyocytes were performed as described earlier (Kerkelä *et al.* 2002). Briefly, cells were lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, and 1 mM Na_3VO_4 (pH 7.5) supplemented with 1 mM dithiothreitol (DTT, 1:1000), protease inhibitors (1:100) and phosphatase inhibitors (1:100, Sigma-Aldrich, St. Louis, MO, USA). Cells were scraped and lysates were vortexed for 15 s and centrifuged (10,000 RPM, 20 min, +4 °C). Protein concentration was determinated with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were boiled in 1×SDS for 5 min and either loaded directly on SDS-PAGE or frozen in −20 °C for later use. Nuclear and cytosolic proteins were extracted as described earlier (Koivisto *et al.* 2011).

4.2.8 Western blot analyses (I, II, III)

Protein extracts were matched for protein concentration (10–45 μg), loaded on to an SDS-PAGE gel, and transferred to nitrocellulose filters. The membranes were blocked in blocking buffer, either Li-Cor and tris-buffered-saline (TBS) 1:1 or 5% milk-TBS-tween (depending on the detection method). The membrane blots were incubated with primary antibodies (1:200–1:3000 in 0.5%-milk-TBS-tween or Li-Cor-TBS) overnight at 4 °C.

For chemiluminescence detection, the secondary antibodies were anti-rabbit and anti-mouse IgG HRP-linked used at a concentration of 1:2000 in 0.5% milk-TBS-tween. Chemiluminescence reagents (ECL plus, RPN2132) were purchased from GE Healthcare (Pittsburgh, PA, USA).

For infrared detection, the secondary fluorescent antibodies were Alexa Fluor 680 from Invitrogen (Life Technologies Ltd, Paisley, UK) and IRDye 800 from Rockland Immunochemicals (Gilbertsville, PA, USA). Antibody binding was detected by the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

4.2.9 Protein kinase activity assay (II)

The protein kinase activity assay was performed as described earlier (Kerkelä *et al.* 2002), and all reagents were purchased from Cell Signalling (Danvers, MA, USA). After the experiment, myocytes were scraped into 200 µl of cell lysis buffer. The phosphorylated p38 was immunoprecipitated with an immobilized antibody at 4 °C. Following overnight incubation on a rotator shaker, the immunoprecipitates were washed twice with cell lysis buffer containing inhibitors after centrifugations (10 000 rpm), and then once more with a kinase buffer. The pellets were incubated for 30 min at 30 °C in a kinase buffer containing 200 µM ATP and 2 mg/ml ATF-2 fusion protein as a substrate (50 µl/sample). The samples were placed on ice to stop the reactions. After adding 10 µl of 5×SDS, the samples were boiled, microcentrifuged, and analyzed by Western blotting for phosphorylated ATF-2.

4.2.10 Immunoprecipitation (III)

Immunoprecipations were done according to the manufacturer's protocol of Pierce's Crosslink IP kit (26147, Thermo Scientific, Rockford, IL). Nuclear protein samples (5–25 µg) were incubated with GATA-4 (sc-9053) antibody (10 µg) overnight. Immunoprecipates were loaded on to an SDS-PAGE gel like total protein samples.

4.2.11 Protein phosphatase 2A assay (I)

Protein phosphatase 2A (PP2A) activity of cultured neonatal cardiomyocytes was determined by measuring dephosphorylation of the phosphopeptide (R-K-pT-I-R-R) according to the manufacturer's protocol (Ser/Thr Phosphatase Assay Kit 1, #17-127, Millipore, Billerica, MA, USA).

4.2.12 Detection of cell death (II)

Apoptotic cell death can be differentiated from necrotic cell death by determination of histone-complexed DNA fragments. To determine apoptosis in cultured cardiomyocytes, cytoplasmic histone-associated DNA fragments were analysed using the ELISA^{PLUS} Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol.

To determine necrosis in cultured cardiomyocytes, the release of adenylate kinase from ruptured cells into the cell culture medium was carried out with the bioluminescent ToxiLight® Bioassay kit (Lonza Rockland Inc, Rockland, ME, USA) kit according to the manufacturer's instructions.

4.2.13 Adenoviral gene transfer in vivo (II, III)

Intramyocardial gene transfer by local virus injection is an efficient site-specific method for gene delivery (Moilanen *et al.* 2011). Male Sprague-Dawley rats weighing 250–300 g were used for cardiac gene transfer of recombinant adenoviruses (LacZ, wild-type p38α or wild-type p38β, constitutively active MKK3b or MKK6b, GATA-4). A total of 1×10^9 pfu (plaque-forming units) of adenovirus in a 100 µl volume was injected with a Hamilton precision syringe directly into the anterior wall of the left ventricle as previously described (Tenhunen *et al.* 2006). After three days (I) or two weeks (III), the animals were killed and from the removed hearts, the cardiac chambers were separated for left ventricular tissue samples. Samples were immersed in liquid nitrogen and stored at −70 °C for later analysis. RNA extraction was performed as previously described (Tenhunen *et al.* 2004) to measure mRNA levels of ANP, CTGF, bFGF, and MMP-9 by RT-QPCR.

4.2.14 Ca^{2+}-transient measurements (I)

Isolated adult mouse or rat cardiomyocytes were plated onto 35 mm glass-bottommed laminin-coated (10µg/ml) dishes. Serum-free media was replaced with BDM-free Tyrode's solution containing 1.25 mM $CaCl_2$. When applicable, ARVMs were infected by adenoviruses 18 h prior to measurement. Similarly, SB203580 (10 µM) was added 1 h before loading the cells, and DMSO was added to control cells in the same volume. The measurement protocol was modified from previous studies (Korhonen *et al.* 2009, Rapila *et al.* 2008). Loading of cells

was performed either with 2 μM Fluo-4-acetoxymethyl (AM)-ester (#14201, Invitrogen) or 5 μM Rhod2-AM (#1245MP Invitrogen) for 20 min at 37 °C, followed by rinsing and pre-incubation (Fluo-4-AM) for 15 min before measurement. Rhod2-labeled cells were measured immediately after rinsing, because staining accumulates inside the nucleus. During the measurement, cells were paced at 0.5 Hz 40–50 V (pulse duration 1 ms). The fluorescence signals were recorded with Zeiss Cell Observer SD (Carl Zeiss, Germany) spinning disc confocal microscope (Fluo-4 excitation: 488 nm, emission: 500–550 nm; Rhod2 excitation: 561 nm, emission: 598–660 nm; 40×/0,75 objective) with a sampling rate of 100 frames/sec. Axiovision and OriginPro were used for analyses of Ca^{2+}-transients. Ca^{2+}-transients are expressed as a background subtracted F/F_0 –ratio, where F means the background subtracted fluorescence intensity and F_0 is the background subtracted minimum fluorescence value measured from each cell at rest.

4.2.15 Cell shortening measurements (I)

Freshly isolated ARVMs were treated with SB203580 (10 μM) or DMSO for 1 h prior to measurements for analysis of cell shortening. Measurements were performed immediately after plating to avoid efficient attaching to the bottom of the well. However, for studies utilizing adenoviruses, cells were plated onto 35 mm glass-bottommed laminin-coated dishes to keep them viable until measurement. Adenoviral infection was performed 18 h prior to measurements. Cells were paced at 0.5 Hz 20–30 V (pulse duration 5 ms) during measurement. Single cell contraction videos were recorded with a Zeiss Cell Observer SD (Carl Zeiss, Germany) using brightfield with a 63×/1.46 objective and a sampling rate of 60–70 frames/sec. Maximal cell shortening between relaxation and contraction was measured by Zen 2012 (Carl Zeiss, Germany).

4.2.16 Particle image velocimetry (I)

Particle image velocimetry (PIV) analysis (Tseng *et al.* 2012) was performed for neonatal cardiomyocytes, which are confluent cultures and beat in multiple directions, while adult cardiomyocytes beat only one-way. Isolated NRVMs were plated onto 35 mm glass-bottommed laminin-coated dishes, and adenoviruses were added in serum-free medium the next day after plating according to the normal protocol. When applicable, SB203580 (10 μM) was added 4 hours before

measurement. Cells were incubated on the microscope on-stage incubator (+37 °C, 5% CO2) and paced at 1 Hz 15 V to ensure identical conditions. Confluent regions were recorded for particle image velocimetry by Zeiss Cell Observer SD (Carl Zeiss, Germany) microscope using a 63×/1.46 objective and differential interference contrast imaging at the speed of 30 fps. Fields were analyzed by two-dimensional vector analysis with the ImageJ (Fiji) PIV-plugin (Tseng *et al.* 2012).

4.2.17 DNA microarray analysis (III)

DNA microarray analysis for stretched cardiomyocyte samples was performed as previously described (Rysä *et al.* 2005). Briefly, the quality and integrity of the isolated RNA was monitored by gel electrophoresis. Total RNA (n=5 in each group) was used as a template for synthesizing cDNA and making biotinylated cRNA according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). cDNA was reverse-transcribed from 2 µg of total RNA with a T7-(dT)24-primer by means of the One-cycle cDNA synthesis kit (Affymetrix). The DNA purification was done using the GeneChip Sample Cleanup Module (Qiagen). The cRNA was prepared and biotin-labeled by *in vitro* transcription (Affymetrix) and fragmented before hybridization. The biotinylated cRNA was hybridized to the GeneChip Rat Expression Set 230_2.0 Arrays, which contains approximately 30,000 rat transcripts. After hybridization GeneChips were washed and stained with streptavidin-phycoerythrin (Molecular Probes). Staining signals were amplified by biotinylated anti-streptavidin (Vector Laboratories) and streptavidin-phycoerythrin by using an Affymetrix Fluidics station according to the standard protocol. GeneChip Scanner 3000 with GeneChip Operating Software (GCOS) v. 1.2 (Affymetrix) was used in scanning. Affymetrix CEL files were imported into GeneSpring 7.2 software (Silicon Genetics) and Robust Multichip Average (RMA) normalization was performed.

4.3 Statistical analyses

Results are presented as mean ±S.E or S.D. Data were analyzed with Student's t test between two groups and for multiple experimental groups with a one-way analysis of variance followed by LSD or the Bonferroni post hoc test. Differences were considered statistically significant at the level of $p < 0.05$. Statistical analyzes were performed with SPSS software (IBM SPSS statistics, Armonk, NY, USA).

5 Results and discussion

5.1 p38 isoforms in the regulation of cardiac contractility (I)

5.1.1 p38 inhibition and phosphorylation of phospholamban

p38 MAPK is known to be a negative regulator of cardiac contractility, but the exact cellular mechanisms have not been clear. There is evidence from several prior studies using both genetical and pharmacological approaches that p38 inhibition enhances cardiomyocyte contractile function (Auger-Messier *et al.* 2013, Bellahcene *et al.* 2006, Cross *et al.* 2009, Kerkelä *et al.* 2010, Vahebi *et al.* 2007). A previous study suggests that Ca^{2+}-sensitivity of myofilaments is increased upon p38 inhibition (Liao *et al.* 2002). Forced p38 activation has been shown to enhance dephosphorylation of α-tropomyosin and decrease ATPase activity (Vahebi *et al.* 2007). However, p38 inhibition in isolated perfused rat hearts resulted in increased phosphorylation of PLB at Ser 16 (Szokodi *et al.* 2008). In addition, a study using mice deficient in both dual specificity phosphatases showed that p38 activation results in decreased cardiac contractility and in defective Ca^{2+}-handling (Auger-Messier *et al.* 2013).

To determine the role of p38 MAPKs in controlling cardiac calcium regulatory proteins, the effect of p38 inhibition on phospholamban phosphorylation was studied first. p38 inhibitor treatment (SB203580, 10 μM) increased phosphorylation of PLB at Ser 16 in neonatal rat ventricular myocytes (Fig. 10A). G_q –agonist endothelin-1 (ET-1, 100 nM) had also a modest enhancing effect on phospholamban Ser 16 phosphorylation. The threonine 17 phosphorylation site of phospholamban was not affected by SB203580. To study the roles of the p38α and p38β isoforms, neonatal rat ventricular cardiomyocytes were infected with adenoviruses encoding either for dominant negative p38α (dn-p38α) or dominant negative p38β (dn-p38β). This resulted in increased phosphorylation of phospholamban at Ser 16 by dn-p38α, whereas dn-p38β had no significant effect (Fig. 10B). Total phospholamban protein levels were not affected by either of the dn-p38 isoforms. Further, p38α depletion by RNAi in neonatal cardiomyocytes enhanced Ser 16 phosphorylation of phospholamban (Fig. 10C). p38α and p38β overexpression by wild type isoform specific adenoviruses together with their upstream kinases, MKK3 and MKK6, respectively, significantly decreased phospholamban protein levels.

Overexpression of p38α also markedly reduced phosphorylation of phospholamban at Ser 16 (Fig. 10D). Forced p38α and p38β activation also reduced mRNA levels of phospholamban, SERCA2a, and calsequestrin, whereas sodium-calcium exchanger mRNA levels were not changed.

Treatment of adult rat ventricular myocytes (ARVMs) with SB203580 (10 μM) caused a marked increase in PLB phosphorylation at Ser16. Treatment with another p38 inhibitor BIRB796 (1 μM) markedly enhanced PLB phosphorylation at Ser16, similar to findings with the structurally different p38 inhibitor SB203580 (Fig. 10E). Overexpression of dn-p38α enhanced phospholamban Ser16 phosphorylation, similarly to results in neonatal cardiomyocytes.

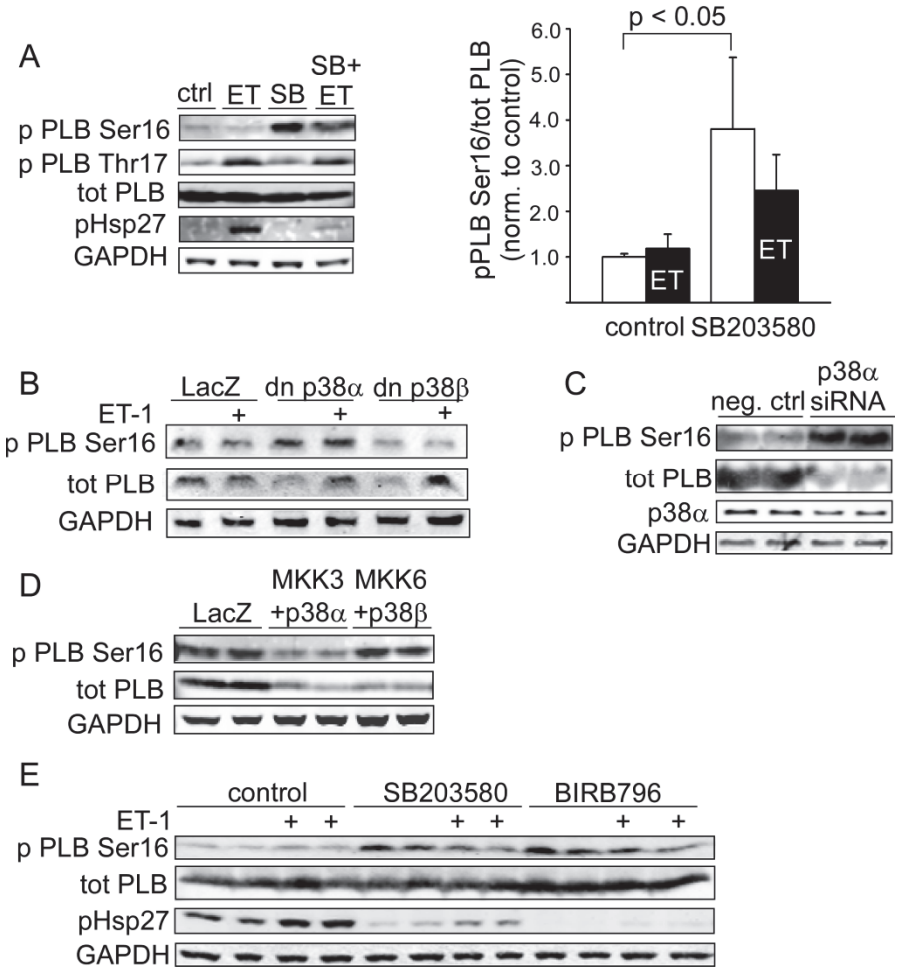

Fig. 10. Effects of p38 inhibition on PLB phosphorylation. Immunoblot analyses of Ser16 phosphorylation and total PLB (A-E) and immunoblot analysis of PLB phosphorylation at Thr17 (A). A) NRVMs treated either with vehicle (DMSO), ET-1 (100 nM, 1h), SB203580 (10 μM) or ET-1 + SB203580. Phosphorylated Hsp27 was used as a p38 inhibition control and GAPDH as the loading control (mean ±SEM, n=8–12). B) NRVMs infected either with dn-p38α (2 MOI), dn-p38β (2 MOI) or LacZ (2 MOI). C) NRVMs transfected with negative control or p38α siRNA. Immunoblot of p38α as a silencing control. D) NRVMs overexpressing LacZ, MKK3+p38α or MKK6+p38β, virus dose 2+2 MOI in each well. E) ARVMs treated either with vehicle (DMSO), SB203580 (10 μM), or BIRB796 (1 μM) alone or in combination with ET-1 (100 nM).

5.1.2 p38 inhibition and SERCA2A function

Phospholamban plays a key role in the regulation of SERCA2a function. SERCA2a is a protein responsible for pumping Ca^{2+} back into the sarcoplasmic reticulum after cardiac contraction. Approximately 92% of the released Ca^{2+} is removed from cytosol by SERCA2a (Bers 2002). The PLB phosphorylation status thus associates with functional alterations in cardiac contractility (Bers 2008). Since SERCA2a expression and function are attenuated in failing hearts, SERCA2a gene transfer is a promising new approach for the treatment of heart failure (Kho *et al.* 2012).

Zheng *et al.* showed that p38 inhibitor SB203580 had a positive effect on β_2-AR-mediated contractility, but they did not find any changes in Ca^{2+}-handling upon p38 inhibition (Zheng *et al.* 2000). On the other hand, it has been also shown that forced p38 activation *in vitro* and *in vivo* suppresses SERCA2a expression and impairs diastolic Ca^{2+} uptake and cardiomyocyte contractility (Andrews *et al.* 2003, Liao *et al.* 2002).

Since p38 inhibition enhanced phospholamban Ser 16 phosphorylation, the effect of p38 inhibition on SERCA2a function was studied further by analyzing Ca^{2+} cycling and cell shortening in adult cardiomyocytes. p38 inhibitor SB203580 (10 μM) significantly decreased Ca^{2+} -transient decay time ($\tau = 270 \pm 29$ ms vs. 203 ± 50 ms, $p < 0.01$, Fig.11A), but Ca^{2+} -transient amplitude was not affected by p38 inhibition. Overexpression of dn-p38α in ARVMs resulted in a marked decrease in Ca^{2+}-transient decay time compared to LacZ infected cells ($\tau = 336 \pm 98$ ms vs. 149 ± 55 ms, $p < 0.001$, Fig. 11B). However, dn-p38α had no effect on Ca^{2+}-transient amplitude. This suggests that p38 inhibition does not affect the function of ryanodine receptors or L-type Ca^{2+}-channels.

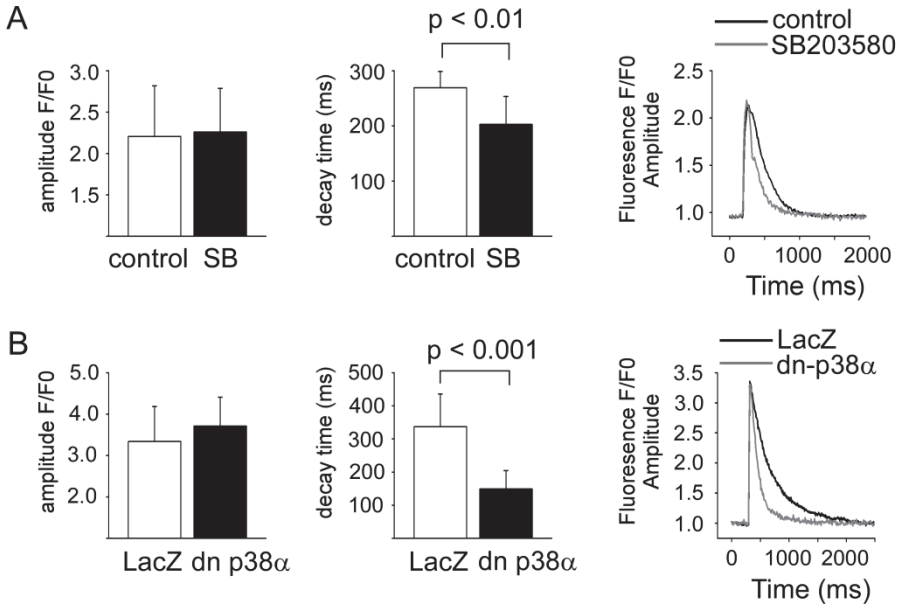

Fig. 11. Inhibition of p38 induces shortening of the Ca^{2+} decay time. Ca^{2+}-transient average amplitudes, decay times (ms), and representative fluorescence signals measured from Fluo-4-loaded cells at 0.5 Hz pacing frequency in adult cardiomyocytes treated either with vehicle (DMSO) or SB203580 (10 μM) (A) or infected with LacZ or dn-p38α (100 MOI) adenoviruses (B). Data is shown as mean ±SD; Ca^{2+}-transients of 11–29 cardiomyocytes were measured for each group.

ET-1, an activator of G_q-dependent-signaling, was sufficient to increase phosphorylation of PLB upon both SB203580 treament and dn-p38α in neonatal and adult cardiomyocytes. Analysis of Ca^{2+}-cycling upon ET-1 (100 nM) revealed a significant decrease in Ca^{2+} -decay time also in cells co-treated with ET-1 and SB203580 compared to cells treated with ET-1 alone (τ = 332 ± 29 ms vs. 187 ± 38 ms, $p < 0.001$). Similar results were also obtained with ET-1 stimulated dn-p38α infected cardiomyocytes (τ = 220 ± 78 ms vs. 139 ± 33 ms, $p < 0.001$). These data further suggest a crucial role for p38α in the mediation of the contractility response.

Cardiomyocyte contractility analysis in ARVMs showed that treatment with SB203580 (10 μM) significantly enhanced cell shortening (6.1 ± 2.7% vs. 9.0 ±2.9%, $p < 0.05$, Fig. 12A). Also dn-p38α alone markedly increased cell shortening compared to LacZ infected cells (7.1 ± 1.6% vs. 4.9 ± 1.8%, $p < 0.05$, Fig. 12B). Particle image velocimetry analysis in SB203580 treated neonatal

cardiomyocytes did not result in any significant effect on contractility, but dn-p38α significantly increased contractility compared with LacZ infected neonatal cardiomyocytes. Thus, consistent with increased PLB phosphorylation in neonatal and adult cardiomyocytes, these data show that inhibition of p38α enhances SERCA2a function as showed by a significant decrease in Ca^{2+}-transient decay time and increased cell shortening. Noteworthy, these results also persisted when cells were stimulated with G_q-agonist ET-1. Since ET-1 activates both p38α and p38β isoforms, these results suggest p38α has a key role in the regulation of the lusitropic response.

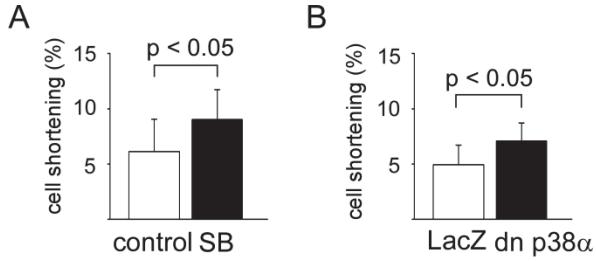

Fig. 12. Analysis of cell shortening in ARVMs. A) Freshly isolated cardiomyocytes were treated with either SB203580 (10 μM) or DMSO (control). B) ARVMs were infected with dn-p38α (100 MOI) or LacZ (100 MOI, control). A total of 7–11 myocytes were measured for each group from two different hearts. Data is shown as mean ±SD.

5.1.3 Effects of p38 inhibition on PP1 activity

PKA phosphorylates PLB at Ser 16 and it is dephosphorylated by protein phosphatase -1 (PP1) (Kranias & Hajjar 2012). Inhibitor-1 (I-1) suppresses PP1 activity, when phosphorylated at Thr 35 by PKA. On the other hand, PP1 activity is enhanced by Ser 67 phosphorylation of I-1, which is a proline-directed site (Bibb *et al.* 2001). There are no previous data of the effect of p38 on I-1. Studies in NRVMs showed that dn-p38α overexpression did not change phosphorylation of I-1 at Ser67, but markedly increased phosphorylation of I-1 at Thr 35 (Fig. 13). Instead, dn-p38β overexpression modestly increased Ser 67 phosphorylation of I-1, but it had no effect on Thr 35 phoshorylation. Similarly, p38α siRNA potentiated I-1 Thr 35 phosphorylation. Also, infusion of SB203580 in isolated rat hearts resulted in increased Thr 35 phosphorylation with no effect on Ser 67 phosphorylation. A previous study of isolated perfused rat hearts showed

increased phosphorylation of PLB at Ser 16 upon SB203580 treatment (Szokodi *et al.* 2008). These findings provide further support for the key role of p38α, but not p38β, as a negative regulator of cardiac contractile function.

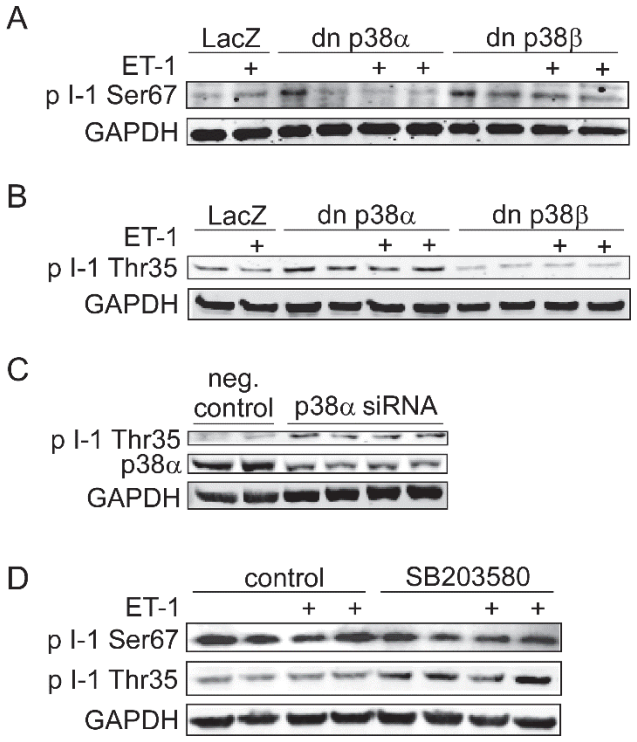

Fig. 13. Inhibition of p38α increases phosphorylation of inhibitor-1 (I-1) at Thr 35. Immunoblot analyses of I-1 phosphorylation at Thr 35 and Ser 67. A-B) Neonatal cardiomyocytes infected with either dn-p38α or dn-p38β (2 MOI). C) p38α depleted by p38α siRNA (150 nM). D) Langendorff perfused hearts, treated with either vehicle, SB203580 (3 μM), ET-1 (3 nM) or SB203580+ET-1 (3 μM + 3 nM).

5.1.4 p38 inhibition attenuates PP2A activity

Protein phosphatase 2A (PP2A) has been shown to dephosphorylate Thr 35 of I-1 (El-Armouche *et al.* 2006). I-1 Thr 35 is not known to be a direct target of p38. Immunoblot analysis of the effects of p38 inhibitor SB203580 (10 μM) and PP2A inhibitor okadaic acid (OA, 1 nM) on PLB Ser 16 phosphorylation and I-1 Thr 35 phosphorylation, revealed that both inhibitors increased PLB Ser 16

phosphorylation and I-1 Thr 35 phosphorylation similarly. Treatment of cells with SB203580 and OA also resulted in a comparable decrease in direct PP2A activity also, when measured by PP2A assay. In addition to PLB, phosphorylation of eIF2α at Ser 51, a known PP1 target (Boyce *et al.* 2005), was also modestly increased by both SB203580 and okadaic acid. Additionally, a specific PP2A inhibitor fostriecin (Weinbrenner *et al.* 1998) resulted in increased PLB Ser 16 and I-1 Thr 35 phosphorylation. In addition to I-1, phosphorylation of other known PP2A targets, cardiac troponin I (cTnI) at Ser 22/23 (Deshmukh *et al.* 2007) and ERK (Junttila *et al.* 2008), were increased upon p38 inhibition with SB203580. There is prior evidence suggesting that activation of p38α induces dephosphorylation of ERK1/2 by PP2A (Liu & Hofmann 2004, Westermarck *et al.* 2001). It is known that PKA-induced cTnI phosphorylation promotes relaxation and it may also have a pivotal role in the positive inotropic response (Layland *et al.* 2004). p38α has been shown to affect cellular localization of PP2A, since $p38\alpha^{-/-}$ cells have higher levels of PP2A in the nucleus and lower levels in the membranes (Zuluaga *et al.* 2007). Thus, p38 inhibition may contribute to the enhanced inotropic response *in vivo* also by enhanced cTnI phosphorylation.

In summary, as shown in Figure 14, these data suggest that the enhanced lusitropic response upon p38 inhibition *in vitro* occurs via decreased PP2A activity, which increases I-1 Thr 35 phosphorylation and results in decreased PP1 activity. Finally, the phosphorylation of PLB is increased and the function of SERCA2a is enhanced. However, there may be also other yet unknown mechanisms contributing to the inotropic response upon p38 inhibition.

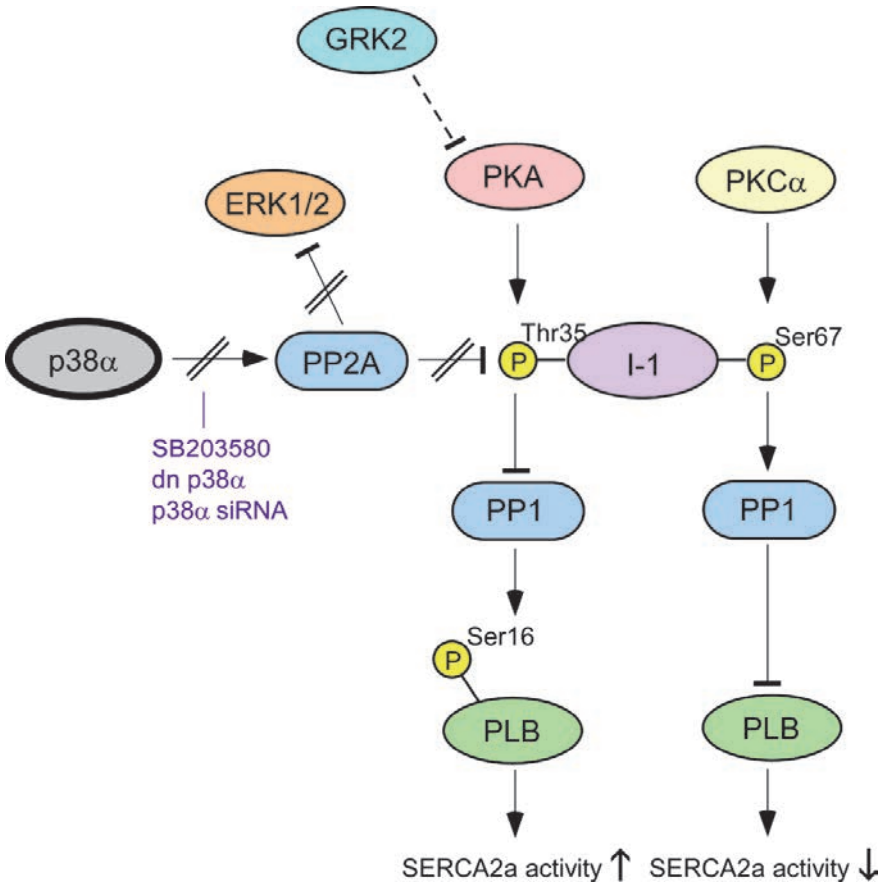

Fig. 14. Regulation of PLB by inhibition of p38α. A schematic view of effects of p38α inhibition on signalling elements regulating PLB phosphorylation. ERK1/2, extracellular signal regulated kinase-1/2; GRK2, G-protein coupled receptor kinase-2; I-1, protein phosphatase inhibitor-1; PKA, protein kinase A; PKCα, protein kinase C alpha; PP1, protein phosphatase-1; PP2A, protein phosphatase-2A; PLB, phospholamban; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}ATP-ase.

5.1.5 p38 inhibition and GRK2

The current data did not indicate any holistic PKA activation upon p38 inhibition, since there were no signs of either RyR or L-type calcium channels (LTCC) activation according to the Ca^{2+}-transient amplitude. GRK2, which is the major cardiac isoform of G-protein coupled receptor kinases (GRKs) and elevated in

patients with heart failure (Ungerer *et al.* 1993), was employed as a physiological tool to modulate PKA activity in cardiomyocytes. Overexpression of βARK-ct (carboxy terminus of GRK2), which has been shown to simulate GRK2 depletion, increased the phosphorylation of PLB at Ser 16 in NRVMs. However, there was no synergistic effect of p38 inhibition and βARK-ct on PLB Ser 16 phosphorylation. GRK2 overexpression by an adenovirus encoding wild type GRK2 (wt-GRK2) attenuated PLB phosphorylation induced by p38 inhibition. As shown in Figure 15A, analysis of Ca^{2+}-transients in the adult rat cardiomyocytes overexpressing wt-GRK2 revealed attenuation in Ca^{2+}-transient amplitude compared to LacZ infected cells (2.27 ± 0.37 vs. 1.93 ± 0.4, $p < 0.01$) and shortening of the decay time of the Ca^{2+} transient (τ = 133 ± 43 ms vs. 105 ± 25 ms, $p < 0.05$). p38 inhibition had no beneficial effect on Ca^{2+}-transient decay time in cardiomyocytes overexpressing GRK2, which was consistent with the attenuated response of PLB Ser16 phosphorylation upon p38 inhibition (Fig. 15B and Fig 16C). Thus, these data suggests that p38 inhibition does not rescue Ca^{2+}-transient decay time when PKA signaling is suppressed by GRK2 overexpression.

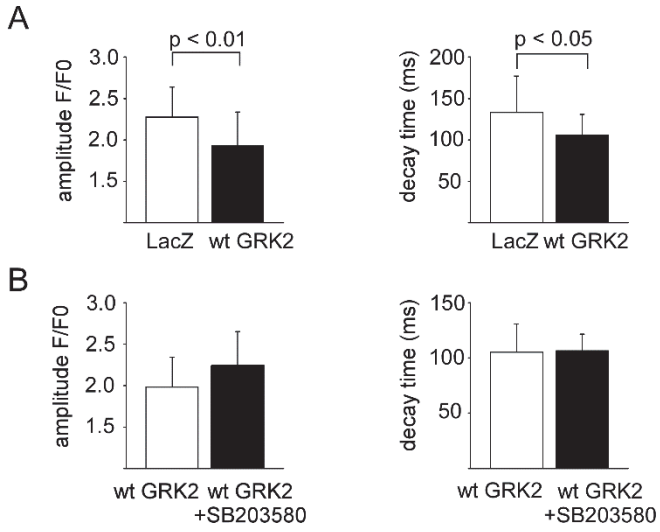

Fig. 15. Intrinsic PKA activity is required for the p38 inhibition response. Averaged amplitude and decay times (ms) of intracellular Ca^{2+}-transients measured with Rhod2. Adult rat cardiomyocytes were infected either with LacZ or wt-GRK2 and treated either with vehicle (DMSO) or SB203580 (10 μM) 1 h prior to measurement. A total of 13–57 cardiomyocytes from a minimum two hearts were measured in each group (mean ±SD), the total amount of virus was 100 MOI in all experiments.

Further, the effects of p38 inhibition on GRK2 activation were studied. GRK2 phosphorylation at Ser670 is catalyzed by ERK, and it leads to GRK2 transportation into the mitochondria (Chen *et al.* 2013, Pitcher *et al.* 1999). p38 inhibition with SB203580 or dn-p38α were also sufficient to increase Ser 670 phosphorylation of GRK2 in adult cardiomyocytes (Fig. 16A-B). To assess the functional significance of Ser 670 of GRK2 upon p38 inhibition, we utilized wt-GRK2 and Ser 670 phosphorylation resistant mutated GRK2-S670A adenoviruses in adult cardiomyocytes. While wt-GRK2 overexpression attenuated the PLB Ser 16 phosphorylation induced p38 inhibition, overexpression of GRK2-S670A had no effect on it (Fig. 16C). Ser670 phosphorylation of GRK2 thus appears to regulate GRK2 function, but does not play a role in the inotropic response to p38 inhibition.

Noteworthy, p38 inhibition enhanced ERK phosphorylation (Fig. 16D). This was not attenuated by GRK2 overexpression, suggesting it is independent of PKA activity. p38 may regulate ERK via a PP2A-dependent mechanism (Junttila *et al.* 2008).

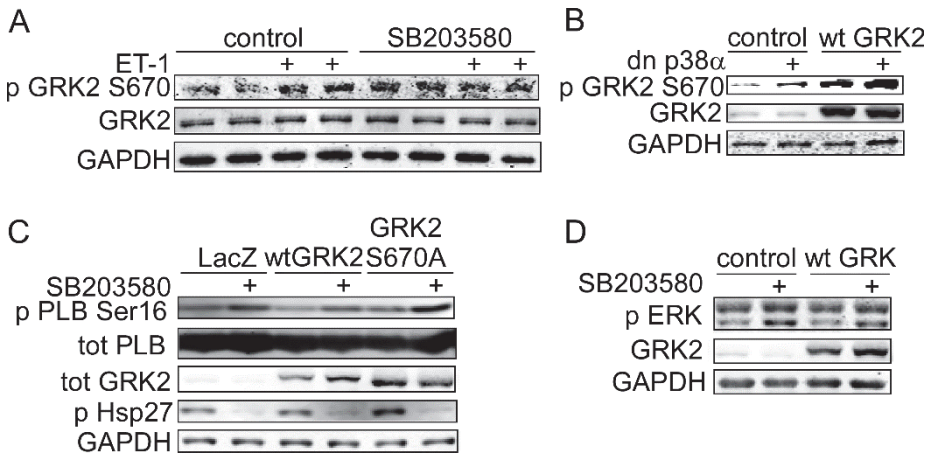

Fig. 16. p38 inhibition enhances GRK2 phosphorylation. A-B) Immunoblot analyses of phosphorylation of GRK2 at Ser670 in adult rat cardiomyocytes. A) ARVMs were treated either with vehicle (DMSO) or SB203580 (10 µM) and stimulated with ET-1 (100 nM) for 1 h. B) ARVMs were infected either with LacZ or wt-GRK2 and when needed in combination with dn-p38α. C) Immunoblot analysis of Ser 16 phosphorylation and total PLB in ARVMs infected either with LacZ, wt-GRK2 or GRK2S670A and treated either with vehicle (DMSO) or SB203580 (10 µM). Phosphorylated Hsp27 was used as the p38 inhibition control. D) Immunoblot analysis of ERK1/2 phosphorylation, ARVMS were infected either with LacZ or wt-GRK2 and treated either with vehicle (DMSO) or SB203580 (10 µM). The total amount of virus was 100 MOI in all experiments. ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; GRK2, G-protein coupled receptor kinase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hsp27, heat-shock protein 27; PLB, phospholamban.

5.1.6 Therapeutical use of p38 inhibition in cardiac diseases

The current data indicate that p38 inhibition enhances SERCA2a function and thus improves cardiac contractile function. SERCA2a is a Ca^{2+} pump ATPase that requires hydrolysis of 1 mole of ATP to transfer 2 moles of Ca^{2+} into the SR (Inesi *et al.* 1988). Previous studies have suggested that p38 inhibition enhances contractility by increasing Ca^{2+}-sensitivity and forced p38 activation increased dephosphorylation of α-tropomyosin (Liao *et al.* 2002, Vahebi *et al.* 2007). A recent study also showed that downstream targets of p38, MK2/3, negatively regulated SERCA2a gene expression (Scharf *et al.* 2013). Despite some controversy on the role of p38 in cardiac pathology, several studies have shown that inhibition of p38 during ischaemia protects the heart from injury (Marber *et*

al. 2011, Martin *et al.* 2012, Rose *et al.* 2010). Of note, p38 isoforms may have distinct roles in cardioprotection. Heterozygous p38α mice showed reduced infarct size and necrotic injury following I/R compared to wild type mice (Otsu *et al.* 2003). Similarly, overexpression of dn-p38α decreased ischeamic p38 activation and also diminished ischemic lethality in cardiomyocytes (Saurin *et al.* 2000). Further, in a more recent study, it was elegantly shown that inhibitor resistant p38α abolishes the protective effects of p38 inhibition on I/R injury (Kumphune *et al.* 2010). The use of p38 inhibitors has been studied for the treatment of inflammatory diseases, especially in Crohn's disease, rheumatoid arthritis, chronic obstructive pulmonary disease, and psoriaris (Coulthard *et al.* 2009), without any significant benefit compared to traditional treatments (Martin *et al.* 2012). Thus far, in the cardiovascular field, p38 inhibitor trials are focused on atherosclerosis, hypercholesterolemia-induced endothelial dysfunction, or following percutaneous coronary intervention. Preliminary clinical trials suggest that the observed beneficial effects of p38 inhibition are based on improved vascular function and suppression of systemic and perhaps local inflammation (Martin *et al.* 2012). Losmapimod (GW856553, inhibiting both p38α and β) is currently being investigated for the treatment of non-ST-elevation myocardial infarction, with primary endpoints related to safety in this high-risk population and substudies assessing also infarct size and coronary vasoregulation (Melloni *et al.* 2012). However, data from the current study suggests that inhibition of p38α may not be desired in the treatment of ischemic heart disease, when oxygen supplies are decreased and energy consumption should be limited. On the other hand, there are promising studies of SERCA2a gene therapy in the treatment of heart failure and diastolic dysfunction (Gwathmey *et al.* 2013, Kho *et al.* 2012). A study investigating myocardial dysfunction in meningococcal sepsis showed decreased IL-6 levels and enhanced cardiac contractility upon SB203580 treatment (Pathan *et al.* 2011). p38α inhibition could be another useful strategy to enhance SERCA2a function and therefore cardiac diastolic function. Isoform specific inhibitors for p38 could provide more interpretable results in the clinical trials. Another possible mechanism to circumvent drawbacks of clinical pharmacological inhibitors of p38 could be to interfere with non-canonical TAB-1 phosphorylations of p38α. This needs more studies about TAB-1-mediated responses in cardiac pathology (De Nicola *et al.* 2013). Further studies of p38 and regulation of contractility should also consider the role of the p38β and p38γ isoforms.

5.2 p38 isoforms in the regulation of cardiac gene expression

5.2.1 p38 isoforms and contractility genes (I)

Forced activation of p38α and p38β alone and together with their upstream kinases MKK3 and MKK6, respectively, resulted in almost complete abolishment of PLB mRNA (Fig. 17A). These results are consistent with the data obtained from PLB immunoblot analysis. SERCA2a and calsequestrin mRNA levels were also significantly decreased by activation of either of the p38 MAPK pathways. Sodium-calcium exchanger (NCX) mRNA levels were not changed by either p38α or p38β alone, but MKK3+p38α together suppressed then significantly. However, dominant negative viruses also had an effect on mRNA levels of contractility proteins: overexpression of dn-p38α decreased SERCA2a mRNA levels significantly, and dn-p38β decreased PLB, SERCA2a, CASQ2 and NCX mRNA levels (Fig. 17B). Previously, dn-p38α and dn-p38β cardiac-specific transgenic mice also showed decreased cardiac SERCA2a gene expression (Zhang *et al.* 2003). In the current study, depletion of p38α by p38α siRNA resulted in a modest decrease in PLB mRNA levels, but had no effect on SERCA2a mRNA levels. Pharmacological p38 inhibition with SB203580 (10 μM, 24 h) had no effect on mRNA levels of PLB, SERCA2a, CASQ2, or NCX. These results thus suggest a possible non-kinase role for p38 in regulating its downstream targets, since overexpression of either wild type or dominant negative p38, but not chemical p38 inhibition or p38α depletion by RNAi, decreased SERCA2a expression. There is evidence that yeast Hog1, which is a mammalian p38 homolog, binds to chromatin and has a more structural role by facilitating the formation of transcriptional complexes in addition to just phosphorylating transcription factors and other targets (de Nadal & Posas 2010). Hog1 does not bind just to the promoter but also to coding regions; however to date there is no evidence for a similar role of p38 in mammalian cells (de Nadal *et al.* 2011). p38 has been shown to be recruited to the chromatin via its interaction with transcription factors such as Elk-1, AP-1, and NFκB. In addition, the recruitment of the RNA polymerase II complex to the target promoters requires p38 activity (Ferreiro *et al.* 2010). Thus, overexpression of active or inactive p38 kinases may exert effects via non-kinase functions of kinases.

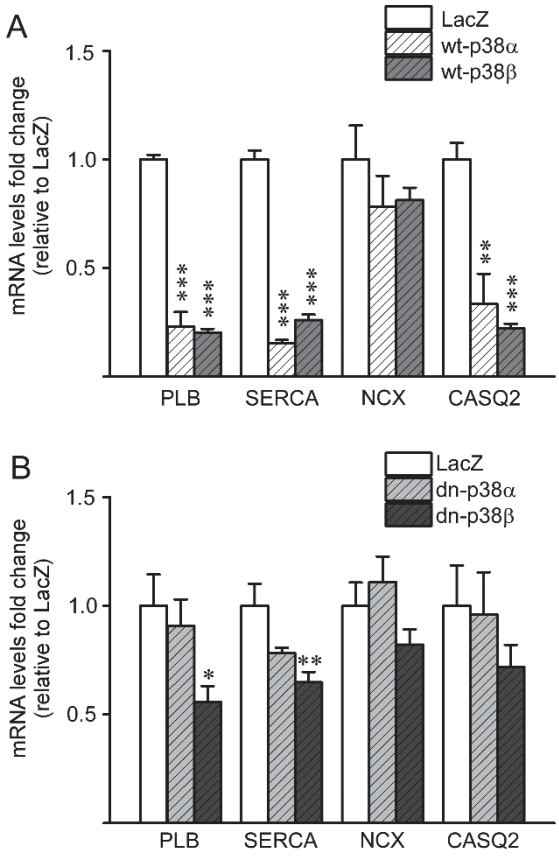

Fig. 17. Effects of p38 isoforms on contractility genes. Neonatal cardiomyocytes were infected with adenoviruses encoding either wild-type p38α and p38β (A) or dominant-negative p38α and p38β isoforms (B). The virus dose was 2 MOI in each group. mRNA levels were normalized to 18S quantified from the same samples and results are presented relative to a LacZ control. (mean±SEM, n=3–10, *p < 0.05, **p < 0.01, ***p < 0.001 vs. LacZ). PLB, phospholamban; SERCA2a, sarcoplasmic reticulum Ca^{2+} ATPase; NCX, sodium-calcium exchanger; CASQ2, calsequestrin-2.

5.2.2 p38 isoforms regulating genes related to cardiac hypertrophy (II)

B-type natriuretic peptide (BNP), atrial natriuretic peptide (ANP), and β-myosin heavy chain (β-MHC) are genes related to cardiac hypertrophy. To study the effect of p38 isoforms on their regulation, wt-p38α and wt-p38β adenoviruses

combined with adenoviruses encoding their upstream activators MKK3b(E) and MKK6b(E), respectively, were used in NRVMs. It has been shown that the upstream kinase effectively activating signaling via p38β is MKK6, not MKK3 (Jiang *et al.* 1996). MKK3, however, is a potent upstream activator of p38α (Raingeaud *et al.* 1996). Further, co-expression of activated MKK3 and p38β does not further increase the ATF-dependent signaling compared to p38β alone (Jiang *et al.* 1996). Thus, to activate the p38 isoforms, the most specific and efficacious method is to combine MKK6 with p38β and MKK3 with p38α. In addition, since the upstream kinases used in the current study are both constitutively active, simultaneous overexpression of either of the wild type p38 isoforms enables more robust activation of the desired isoform. Importantly, neither MKK3 nor MKK6 has an effect on ERK or JNK signalling (Derijard *et al.* 1995, Han *et al.* 1996).

Interestingly, only MKK6+p38β increased ANP and BNP mRNA levels significantly (3.5-fold, $p < 0.001$ and 2.8-fold, $p < 0.01$, respectively) in NRVMs. MKK3+p38α did not induce ANP or BNP mRNA levels in NRVMs, but significantly decreased β-MHC mRNA levels (by 78%, $p < 0.001$).

In vivo gene transfer of MKK3b(E), MKK6b(E), wt-p38α, or wt-p38β into the left ventricular r wall of adult SD-rats resulted in increased ANP mRNA levels three days post-injection in the MKK6+p38β group (2.3-fold, $p < 0.05$). Other combinations (MKK3+p38α, MKK6+p38α and MKK3+p38β) modestly enhanced ANP mRNA transcription, but the changes were not significant. BNP mRNA levels were augmented by MKK3+p38α (2.1-fold, $p < 0.05$), by MKK6+p38α (2.4-fold, $p < 0.05$), and by MKK6+p38β (2.2-fold, $p < 0.01$). Forced activation of either p38α or p38β had no effect on β-MHC gene expression.

In vitro and *in vivo* data of increased ANP and BNP mRNA levels were consistent in the MKK6+p38β groups. MKK3+p38α did not increase either ANP or BNP mRNA levels *in vitro*, but there was a marked increase in BNP mRNA levels *in vivo*. This suggests that p38β may have a more significant role in regulating cardiac hypertrophy. Wang *et al.* have shown p38β to mediate hypertrophic response (Wang *et al.* 1998). However, it is noteworthy, that when the amount of protein synthesis, another hallmark of hypertrophy (Sugden & Clerk 1998), was measured, both p38 isoforms alone and combined with their upstream kinases significantly induced protein synthesis measured by [^{3}H] leucine incorporation in NRVMs (Fig. 18). In addition, overexpression of

upstream activators of p38, MKK3b(E), and MKK6b(E), alone increased protein synthesis.

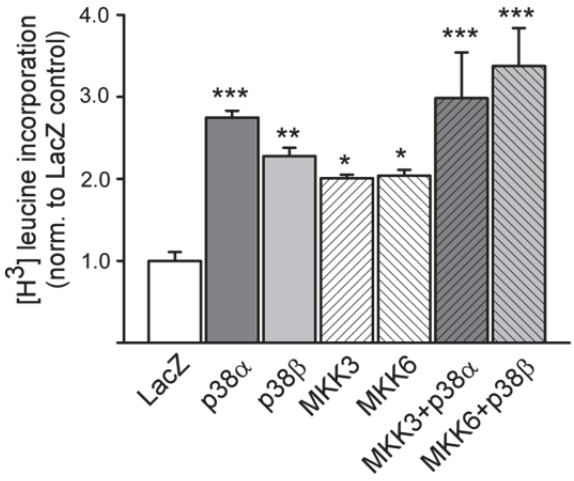

Fig. 18. Analysis of protein synthesis in neonatal cardiomyocytes. [^{3}H] leucine incorporation was analyzed in cardiomyocytes overexpressing p38α and p38β isoforms and their upstream kinases. Cells were infected with recombinant adenoviruses, and [^{3}H] leucine was added to the culture medium. Protein synthesis was determined by detecting incorporated [^{3}H]leucine. Mean ± SEM, n=12, *p < 0.05, **p < 0.01, ***p < 0.001 vs. LacZ.

5.2.3 p38 isoforms regulating fibrosis-related and growth factor genes (II)

Cardiac fibrosis is a central feature of heart failure (for a review, see (Swynghedauw 1999). Prior data suggests that p38 MAPK is also involved in the regulation of fibrosis (Tenhunen *et al.* 2006), and pharmacological p38 inhibition by SB203580 and FR167653 resulted decreased fibrosis in dilated cardiomyopathy hamsters (for review see (Clerk & Sugden 2006, Kyoi *et al.* 2006). However, the roles of p38α and p38β isoforms in fibrosis process have not been clear. As shown in Figure 19, MKK3b(E)+wt-p38α significantly increased CTGF, bFGF, and MMP-9 mRNA levels (3.0-, 2.0- and 3.3-fold, respectively) *in vitro*, whereas MKK6b(E)+wt-p38β had no effect. Forced activation of either p38 isoforms had no effect on MMP2-2 or COL1A1 mRNA levels. Both p38 isoforms significantly reduced aFGF gene expression (both to 0.4-fold). MKK6b(E)+wt-

p38β resulted in a significant decrease in PDGF-A mRNA levels (0.7-fold), while MKK3+wt-p38α had no effect on them.

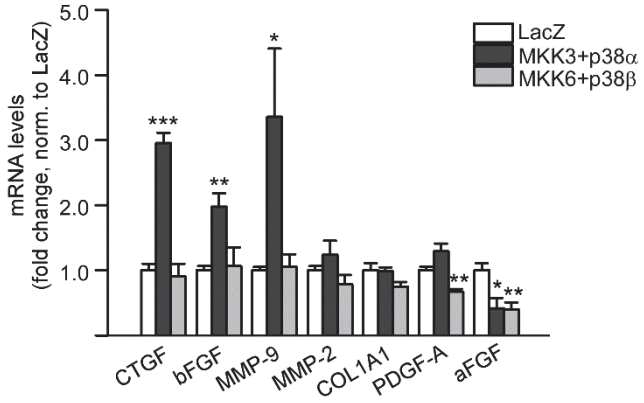

Fig. 19. p38 isoforms have distinct effects on the expression of fibrosis-related genes. Neonatal cardiomyocytes were infected with adenoviruses encoding either MKK3b(E)+wt-p38α or MKK6b(E)+wt-p38β, mRNA levels were determined by qRT-PCR and normalized to 18S RNA, presented here relative to a LacZ control. The virus dose was 2+2 MOI. Mean±SEM, n=4–6, *p <0.05, **p < 0.01, ***p < 0.001 vs. LacZ. CTGF, connective tissue growth factor; bFGF, basic fibroblast growth factor; MMP-9/-2, matrix metalloprotease-9/-2; COL1A1, collagen type 1 alpha1; PDGF-A, platelet-derived growth factor-A; aFGF, acidic fibroblast growth factor.

Cardiac gene transfer *in vivo* with wt-p38α in combination with either MKK3b(E) or MKK6b(E) resulted in significant increase in CTGF mRNA levels. MKK6b(E)+wt-p38α significantly induced bFGF and MMP-9 mRNA levels. MKK6b(E)+p38β significantly increased CTGF mRNA levels, but other fibrosis-related genes were not affected upon p38β overexpression.

Data from this study indicates that p38 isoforms regulate fibrosis-related genes distinctly. CTGF levels were upregulated by forced activation of MKK3b(E)+wt-p38α both *in vitro* and *in vivo*. Previous studies have shown that CTGF increases in response to several hypertrophic stimuli, like ET-1, PE, angiotensin II, growth factors, and mechanical stretch (Daniels *et al.* 2009, Matsui & Sadoshima 2004). Other fibrosis-related factors, MMP-9 and bFGF, were also upregulated upon forced overexpression of MKK3b(E)+wt-p38α *in vitro*. Interestingly, overexpression of MKK6b(E) together with wt-p38α increased MMP-9 and bFGF mRNA levels *in vivo*. MMP-9 is a protein contributing to the

re-organization of the extracellular matrix (Spinale *et al.* 2013). Overexpression of MKK6b(E)+wt-p38β reduced the mRNA levels of growth factors PDFG-A and aFGF *in vitro*. The latter was decreased also by MKK3b(E)+wt-p38α overexpression. This suggests that p38α induces fibrosis-related factors, while p38β typically may have inhibiting effects on the transcription of growth factors. Fibrosis is a one of the central processes involved in cardiac remodeling, and these results indicate that p38α may critically regulate the plasticity of the heart. There is prior evidence from *in vivo* studies showing that overexpression of MKK3b or MKK6b increased cardiac fibrosis (Liao *et al.* 2001), and transgenic mice overexpressing MKK3b showed increased cardiac interstitial fibrosis and contractile dysfunction (Streicher *et al.* 2010). Additionally, transgenic mice expressing dn-p38α had reduced cardiac fibrosis (Zhang *et al.* 2003). In contradiction to these data, there are results from an *in vivo* study utilizing local overexpression of MKK3b+wt-p38α by adenoviral injection in SD-rats: p38α activation was reported to decrease cardiac fibrosis and reduce apoptosis (Tenhunen *et al.* 2006). Nishida *et al.* have also shown in a pressure overload model that cardiac-specific p38α knockout mice showed dilated cardiomyopathy, increased fibrosis, and apoptosis (Nishida *et al.* 2004). These controversial results may be partly explained by different experimental settings.

5.3 p38 isoforms in cardiac cell death (II)

Apoptosis and necrosis are also essential parts of cardiac pathology, and p38 MAPKs are known to regulate cardiomyocyte viability. Ma *et al.* have shown that p38 inhibition by SB203580 reduced apoptosis in isolated perfused rabbit hearts (Ma *et al.* 1999b). An additional study also suggests that p38α may be pro-apoptotic, while p38β may be anti-apoptotic (Wang *et al.* 1998). We utilized MKK3b(E), MKK6b(E), wt-p38α, and wt-p38β adenoviruses *in vitro* to analyze apoptotic cell death by ELISA. Overexpression of either the p38α or p38β isoform induced apoptosis (1.5- and 1.6.-fold, respectively). Similar results were observed with forced activation of p38α by MKK3b(E)+wt-p38α and with activation of p38β by MKK6b(E)+wt-p38β, whereas overexpression of either MKK3b(E) or MKK6b(E) alone did not enhance apoptosis. Additionally, necrotic cell death was induced significantly by the MKK3b(E)+wt-p38α overexpression when measured by release of adenylate kinase.

These results do not suggest any difference between p38α and p38β isoforms in regulating apoptosis in cultured cells. One possible explanation for the

contradiction between current data and previous results by Wang *et al.* (1998), is the use of upstream kinases: Wang *et al.* used MKK3b(E) for both p38α and p38β. In our experiments, the combination of MKK3b(E) and wt-p38β did not result in apoptosis but MKK6b(E) + wt-p38β had a similar effect as MKK3b(E) +wt-p38α. Thus, there were no differences between p38α and p38β isoforms in the regulation of cell death *in vitro*.

5.4 Downstream mediators of p38 isoforms regulating BNP gene activity (II)

To define the roles of p38 isoforms in the regulation of BNP gene transcription, BNP promoter activation was analyzed *in vitro*. Overexpression of both wt-p38α and wt-p38β significantly increased transcription of a rat (Δ-534/+4) BNP promoter luciferase construct (rBNP-luc, $p < 0.001$ and $p < 0.01$, respectively). Treatment of cardiomyocytes with ET-1, a known hypertrophic stimulus (Shubeita *et al.* 1990), resulted in a 2.3-fold increase in BNP promoter activity. Interestingly, dn-p38α had no effect on rBNP promoter activity, but dn-p38β completely diminished ET-1-induced activation.

To further study BNP reporter activation, mutated rBNP promoter constructs were utilized. Mutation of the proximal GATA-4 binding sites (BNP GATAmut) significantly decreased wt-p38β-induced rBNP promoter activity. However, BNP GATAmut had no effect on wt-p38α-induced rBNP promoter activation. Another transcription factor, AP-1, is also known to co-operate with GATA-4 in BNP gene activation in pressure-overloaded hearts (Herzig *et al.* 1997). Interestingly, induction of the BNP promoter by wt-p38α was abolished when the AP-1 binding site was mutated, while wt-p38β-induced activation was not affected. Overexpression of upstream kinases MKK3b(E) and MKK6b(E) alone significantly augmented BNP promoter activity. Modulation of either the GATA- or AP-1 site significantly suppressed MKK6b(E) induced activation, but had no effect on MKK3b(E)-induced activation of the BNP promoter. Thus, these results suggest that p38α activates the BNP promoter via AP-1, p38β via GATA-4, and MKK3b(E) is able to activate the BNP promoter independently of GATA-4 and AP-1.

5.5 GATA-4 in mechanical stretch (III)

The significant role of GATA-4 in cardiac development and in cardiac pathologies is well established. Regulators of GATA-4 are also studied extensively, but the exact roles of some of the regulatory mechanisms remain to be elucidated. In this study, the focus was to elucidate the role of the molecular forms of GATA-4 and posttranslational modifications of GATA-4 in mechanical stretch.

5.5.1 Molecular forms of GATA-4

The known molecular size of GATA-4 is 44 kDa, calculated according to the amino acid sequence. The regarded functional form typically presented in immunoblots is 52 kDa. GATA-4 gene transfer to hearts *in vivo* and to cultured cardiomyocytes by an adenovirus overexpressing GATA-4 resulted in an increase in the 52-kDa functional form of GATA-4 as well as an increase in the 48-kDa form (Fig. 20A). *In vitro* gene transfer by GATA-4 adenovirus for 72 hours resulted in markedly increased GATA-4 bands of 72, 52, 48 and 44 kDa (Fig. 20B). Mechanical stretch of NRVMs increased the total amount of 52-kDa GATA-4, peaking at 12 h (1.9-fold, $p < 0.05$) and decreasing at 24 h and 48 h. Similar data was observed concerning the 48- and 44-kDa forms (3.5-fold, $p < 0.01$ and 2.3-fold, $p < 0.01$, respectively). The 72-kDa band increased during longer stretch after 12 h, being significantly increased at 48 h (1.6-fold, $p < 0.05$).

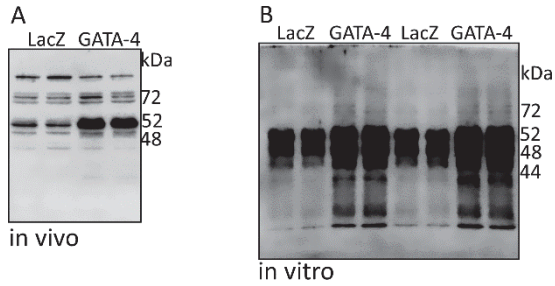

Fig. 20. Representative GATA-4 immunoblots of GATA-4 gene transfer *in vivo* at 2 weeks (A) and *in vitro* (B). A) LacZ and GATA-4 adenoviruses (1×10^9 pfu) were injected into the anterior wall of the left ventricle. B) Neonatal ventricular cardiomyocytes were infected with LacZ or GATA-4 adenoviruses (2 MOI) for 72 hours. Nuclear proteins were used for immunoblots.

The most common form of GATA-4 is 55 kDa, and there are only sparse data about other molecular forms of GATA-4. Wang *et al.* reported that PIAS1 (protein inhibitor of STAT1), a sumoylation ligase E3, enhanced sumoylation of GATA-4 by intensifying the band at 65 kDa, with the occasional emergence of a band at 90 kDa (Wang *et al.* 2004). Analysis of the molecular forms of GATA-4 most often shows a band at 72 kDa, a strong band at 52 kDa, and a weaker band at 48/44 kDa. *In vivo* and *in vitro* gene transfer with GATA-4 adenovirus showed similar results, except in *in vivo* the band at 48 kDa was more intense compared to the 44 kDa band. GATA-4 antibody recognized an intense band at 52 kDa and lower bands in the human purified recombinant protein immunoblot, whereas no band at 72 kDa was detectable. Neither antibody against ubiquitin nor acetylated lysine produced any detectable bands when the purified human recombinant protein was analyzed. This may be due to the purification process and ubiquitin or acetylation moieties may not be present following purification. Mechanical stretch was employed as hypertrophic stimulus in NRVMs, which resulted in an increase in the 52 kDa band that peaked at 12 h. Similar data were observed concerning the 48-kDa and 44-kDa bands, whereas the 72-kDa band increased at 48 h.

COS-1 cells have no endogenous GATA-4 production. As shown in Figure 21, analysis of COS-1 cells transfected with GATA-4 plasmid for 24 h and 48 h showed that, there were detectable GATA-4 bands at 52, 44, and 38 kDa. Analysis of samples immunoprecipitated with GATA-4 antibody also revealed a 72-kDa band. A 72-kDa and a 52-kDa band were also detected by ubiquitin and sumoylation antibodies from immunoprecipitated samples.

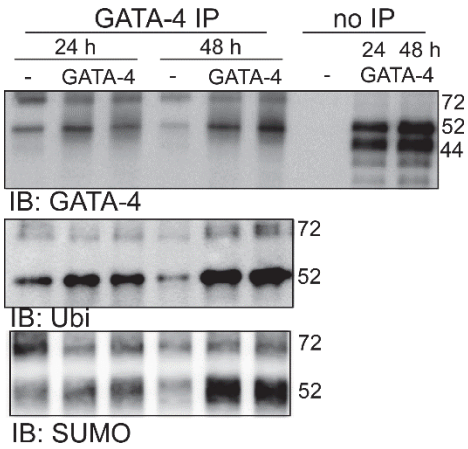

Fig. 21. GATA-4 molecular forms in COS-1 cells after GATA-4 plasmid transfection. COS-1 cells were transfected with GATA-4 plasmid for 24 h and 48 h. Indicated samples were immunoprecipitated with GATA-4 antibody. Representative immunoblots of GATA-4, ubiquitin, and SUMO-1 antibodies.

Thus, it could be hypothesized that the 52 kDa band represents the functional GATA-4 form and could be monoubiquitinated (44 kDa GATA-protein and 8.5 kDa ubiquitin moiety) and the approximately 72 kDa band could be polyubiqitinated (44 kDa and three ubiquitin moieties).

5.5.2 Phosphorylation of GATA-4

Mechanical stretch has been shown to increase GATA-4 DNA binding activity (Pikkarainen *et al.* 2003b). GATA-4 is known to be phosphorylated at Ser105 by ERK1/2 and p38 in cardiomyocytes (Kerkelä *et al.* 2002, Liang *et al.* 2001). Also, the stability of GATA-4 is enhanced by Ser105 phosphorylation (Suzuki 2003). Phosphorylation of another GATA-4 site by ERK, Ser 261, has been shown to enhance erythropoietin-ERK-induced GATA-4 acetylation. GATA-4 Ser105 mutation, on the other hand, had no effect on acetylation (Jun *et al.* 2013a, Jun *et al.* 2013b). Prior studies indicate that phosphorylation and acetylation are the main mechanisms to increase DNA-binding activity and transcriptional activity of GATA-4 (Takaya *et al.* 2008, Yanazume *et al.* 2003). In the current study, the time course of GATA-4 phosphorylation was studied during mechanical stretch. Mechanical stretch of cultured cardiomyocytes increased phosphorylation of GATA-4 at Ser105, peaking at 15 min (3.8-fold, $p < 0.01$). This band was seen at

the 52 kDa level. There are previous data showing that MAPKs, ERK1/2, p38, and JNK are also activated by mechanical stretch (Koivisto *et al.* 2011). ERK-inhibitor PD98059 (10 μM) and p38 inhibitor SB203580 (10 μM) decreased stretch-induced phosphorylation of GATA-4 (0.58-fold, $p < 0.01$ and 0.63-fold, $p < 0.05$, respectively), whereas JNK inhibitor SP600125 (10 μM) had no effect on it. In summary, these data show that mechanical stretch induced GATA-4 phosphorylation at Ser105 peaking at 15 min and phosphorylation was attenuated by chemical inhibitors of ERK1/2 and p38.

5.5.3 Acetylation of GATA-4

GATA-4 acetylation by transcriptional co-activator p300 has been shown to be induced upon PE treatment (Yanazume *et al.* 2003). Recently, cyclin-dependent kinase-9 (cdk9) has also been shown to form a functional complex with GATA-4 and p300 to increase GATA-4 transcriptional activity in cardiac hypertrophy (Sunagawa *et al.* 2010). GATA-4 acetylation has been suggested to transform GATA-4 to be more resistant to degradation, probably because lysine residues are blocked (Suzuki *et al.* 2004). In our studies, the acetylation profile of GATA-4 was not affected in NRVMs in response to mechanical stretch. Thus, phosphorylation appears to be more important regulator of GATA-4 activity than acetylation in mechanical stretch.

Microarray analysis of mechanically stretched NRVMs revealed some changes in the expression of genes related to acetylation. Hopx is a small homeodomain factor that interacts with histone deacetylase 2 (Hdac2) and together they mediate deacetylation of GATA-4 leading to impaired ability of GATA-4 to transactivate cell cycle genes (Trivedi *et al.* 2010). Interestingly, Hopx was downregulated during mechanical stretch, but Hdac2 levels were stable. This result is yet to be confirmed by qRT-PCR. Gene expression of histone deacetylases and sirtuins were not changed in response to mechanical stretch.

5.5.4 Ubiquitination and sumoylation of GATA-4

Similar to acetylation and ubiquitination, sumoylation occurs at lysine residues. The process is quite similar to ubiquitination, but it does not direct the target protein toward proteolysis. Sumoylation may for example repress gene expression (Verger *et al.* 2003) and activate or inactivate its substrate (Perdomo *et al.* 2013). GATA-4 sumoylation is known to enhance GATA-4 transcriptional activity and

the major sumoylation site is lysine 366 (Wang *et al.* 2004). Data by Wang *et al.* indicated that GATA-4 sumoylation enhances GATA-4 transcriptional activity and may modulate GATA-4 nuclear localization (Wang *et al.* 2004). On the other hand, Belaguli *et al.* showed that GATA-4 activation and nuclear localization are independent of sumoylation in intestinal epithelial cells (Belaguli *et al.* 2012). In the present study, Western blot analysis with sumoylated antibody recognized a band at 52 and 72 kDA in immunoprecipitated COS-1 cells transfected with GATA-4 plasmid (Fig. 21). On the other hand, mechanical stretch did not affect gene expression of SUMO-1 in the microarray analysis. The role and significance of GATA-4 sumoylation in response to mechanical stretch remain to be established.

Ubiquitin is a 76-amino acid moiety, which is 8.5 kDa in size. Monoubiquitination may activate a protein or tag it for delivery to other cellular compartments, thereby modulating its activity (Willis *et al.* 2010). Polyubiquitination of a target protein directs it to proteosomal degradation. Ubiquitination ligases (E3 ligases) are enzymes that interact with ubiquitin-conjugating enzymes (E2 ligases) to transfer activated ubiquitin to the target substrate. The known E3 ligases specific to cardiac diseases are muscle ring finger-1 (Murf-1), CHIP (carboxy-terminus of Hsp70-interacting protein), muscle atrophy F-box/atrogin-1 (MAFBx) and murine double-minute-2 (MDM-2) (Willis *et al.* 2010). Interestingly, Wang *et al.* reported that GATA-4 was not ubiquitin targeted (Wang *et al.* 2004). Another study revealed that hypoxia induces GATA-4 ubiquitination, which is prevented by EPO-ERK-induced Ser 105 phosphorylation (Jun *et al.* 2013a). Mutation of GATA-4 at Ser105 resulted in unaffected ubiquitination upon ERK-activation (Jun *et al.* 2013a). Kobayashi *et al.* showed that hyperglycemia-induced GATA-4 depletion was reversed completely by MG-262 (Kobayashi *et al.* 2007). Hyperglycemia did not activate the ubiquitin-proteasome system (UPS), measured by UPS reporter, nor did it increase peptidase activities or protein expression of proteosomal units. The ubiquitin E3 ligase CHIP was increased in hyperglycemia, its overexpression decreased GATA-4 protein levels and CHIP knockdown by siRNA prevented GATA-4 depletion in hyperglycemia (Kobayashi *et al.* 2007).

DNA microarray analysis was used to assess if there were any changes in gene expression of these four ligases and their isoforms following mechanical stretch for 1, 4, 12, 24 and 48 hours. Gene expression of MDM2 was increased after 12 h of mechanical stretch. MDM2 is a critical regulator of tumor suppressor p53 and it has been shown to attenuate cardiac hypertrophy and promote cell

survival in cardiomyocytes (Toth *et al.* 2006). Interestingly, GRK2 has been shown to be an MDM2 target for ubiquitination (Salcedo *et al.* 2006). Gene expressions of other E3 ligases were not changed in mechanical stretch.

Chaperones are proteins which prevent protein misfolding and refold denaturated proteins and/or target them for degradation. Heat shock proteins 70 and 90 (Hsp70, Hsp90) are activated during stress but are abundant also without stress, whereas Hsp27 and alphaB-crystallin (CryAB) are increased in response to stress (Willis & Patterson 2010). Hsp27 and CryAB are expressed in the cardiac and skeletal muscles. In the current study, only the Hsp70 gene expression was increased in response to the mechanical stretch. Kee *et al.* has shown Hsp70 to be increased in cardiac hypertrophy (Kee *et al.* 2008).

MG-262, a boronic peptide acid, inhibits proteosomal function. Proteosomal inhibition is supposed to cause accumulation of ubiquitinated proteins (Powell 2006). First, proteasome inhibitor MG-262 (25 nM) together with GATA-4 plasmid was added to COS-1 cells in order to study if the proteasome inhibitor has an effect on GATA-4 molecular forms. Time points of interest were 12, 24, 48, and 72 hours and both nuclear and cytosolic protein fractions were analyzed. As shown in Figure 22A, at 12 h there was little GATA-4 production in the nucleus. GATA-4 expression was detectable at the 24 h time point, but it was not affected by MG-262 treatment. Interestingly, after 48 h and 72 h, the increase in the 44 kDa and 72 kDa bands disappeared in MG-262 treated groups. In the nuclear fractions, the ratio of 72/52 kDa bands decreased significantly in MG-262 treated cells at 48 and 72 hours, while there was a significant increase in the ratio of 52/44 kDa bands at 72 hours. The nucleus/cytosol-ratio of the 52 kDa GATA-4 band tended to increase at 48 h and decrease at 72 h in the MG-262 groups, suggesting that the nucleus may be saturated with 52 kDa GATA-4 because it is not degraded upon proteasome inhibition.

Finally, proteasome inhibitor MG2-262 was added to the cardiomyocytes upon mechanical stretch. Results were similar with COS-1 cell responses. As shown in Figure 22B, MG-262 treatment increased the GATA-4 52 kDa band at 24 h without stretch, but there was no increase in MG-262 treated stretched cells. On the other hand, MG-262 treatment significantly decreased the 52 kDa band at 48 h. Proteosomal inhibition tended to decrease 72 kDa bands with and without mechanical stretch at 48 h. The 44 kDa molecular form of GATA-4 showed a significant decrease only at 48 h in non-stretched cardiomyocytes. Interestingly, microtubule-associated protein 1 light chain 3B (LC3B), an autophagy marker (Kabeya *et al.* 2000, Kabeya *et al.* 2004), increased significantly in non-stretched

MG-262 treated samples when compared to the control group and tended to increase in MG-262 treated stretched cells as well. This suggests that due to proteosomal inhibition, ubiquitinated proteins may be degraded by autophagosomes. The 72 kDa band could be ubiquitinated GATA-4, and it is increased upon MG-262 treatment but autophagial degradation may balance the overloading of ubiqitinated proteins.

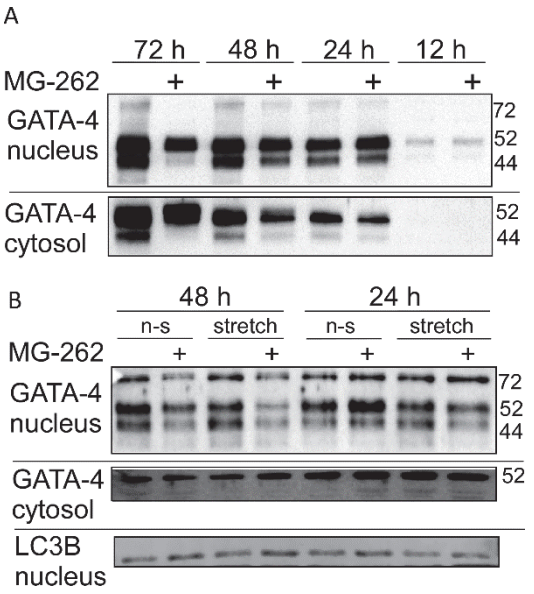

Fig. 22. Immunoblot analysis of the molecular forms of GATA-4 and LC3B. Proteosomal inhibitor MG-262 (25 nM) reduces the 72 kDa band in COS-1 cells (A) and cardiomyocytes (B). COS-1 cells were transfected with GATA-4 plasmid for 12–72 h. Cardiomyocytes were stretched (0.5 Hz, cyclic 10–21% elongation) for 24–48 h.

These results suggest that there is more than one molecular form of GATA-4 and posttranslational modifications may have an effect on the molecular forms seen in the immunoblots.

6 Conclusions

In the present study, signaling pathways involved in the regulation of cardiac function and gene expression were investigated in cultured cardiomyocytes, and in hearts *ex vivo* and *in vivo*.

1. Chemical and genetic inhibition of p38α MAPK induced phosphorylation of phospholamban. This enhanced SERCA2a function and diastolic Ca^{2+} uptake, improving cardiac relaxation and thus contractile function. GRK2 overexpression by blunting intrinsic PKA activation diminished the p38α inhibitory response to the contractility. Mechanistically, p38α inhibition reduced PP2A activity, which enhanced phosphorylation of PKA target I-1 at Thr 35 and inhibited PP1 activity. p38α inhibition did not induce holistic PKA activation. In contrast, p38β inhibition decreased PLB and I-1 Thr 35 phosphorylation. These findings provide a novel mechanism for regulation of cardiac contractility upon p38 inhibition and suggest that p38α MAPK inhibitors could be considered for treatment of diastolic heart failure in particular.
2. p38α and p38β MAPK isoforms mediated BNP promoter activation via distinct pathways. p38β increased BNP promoter activity via GATA-4 transcription factor, whereas p38α increased the BNP promoter activity via AP-1 transcription factor. Upstream kinases of p38, MKK3 and MKK6, also activated the BNP promoter differently: MKK6 acted via both p38α and p38β, while MKK3 was able to activate the BNP promoter at least partly independently of p38 isoforms. p38α upregulated the expressions of genes related to cardiac fibrosis, whereas p38β was involved in the regulation of hypertrophy-associated genes ANP and BNP. In summary, this data suggest that p38 MAPK isoforms seem to have distinct roles in the regulation of cardiac gene expression.
3. GATA-4 was regulated by phosphorylation at Ser 105 by p38 and ERK1/2 MAPKs in response to mechanical stretch. In addition to the proposed functional GATA-4 molecular form of 52 kDa, adenovirus-mediated gene delivery and mechanical stretch increased the 48/44 and 72 kDa molecular forms. Moreover, GATA-4 may be regulated by ubiquitination in addition to ERK/p38 phosphorylation in response to mechanical stretch.

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Original articles

I Kaikkonen L, Magga J, Ronkainen V-P, Koivisto E, Perjes Á, Chuprun JK, Vinge LE, Kilpiö T, Aro J, Ulvila J, Alakoski T, Bibb JA, Szokodi I, Koch WJ, Ruskoaho H & Kerkelä R (2014) p38α regulates SERCA2a function. J Mol Cell Cardiol 67(0): 86–93.

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Original publications are not included in the electronic version of the dissertation.

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