Novel insights into the calcineurin/NFAT pathway in cardiac hypertrophy

Novel insights into the calcineurin/NFAT pathway in cardiac hypertrophy

ISBN 90-9018810-X

Cover
Original images from Seidman & Seidman, Cell 2001.

Printing Agency
Febodruk BV, Enschede, Nederland

Novel insights into the calcineurin/NFAT pathway in cardiac hypertrophy

Nieuwe inzichten in de calcineurine/NFAT route in cardiale hypertrofie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 9 december 2004 om 16.00 uur

door

Eva van Rooij

PROMOTORES

Prof. Dr. H.J.M.G. Crijns
Prof. Dr. P.A.F.M. Doevendans (Hart-Long Instituut Utrecht)

CO-PROMOTOR

Dr. L.J. de Windt (Hubrecht Laboratorium, KNAW)

BEOORDELINGSCOMMISSIE

Prof. Dr. G.J. van der Vusse (voorzitter)
Prof. Dr. M. Daemen
Dr. Molkentin (Cincinnati Children's Hospital Medical Center)
Prof. Dr. A. Moorman (Academisch Medisch Centrum)
Dr. Y.M. Pinto

Het onderzoek dat aan dit proefschrift ten grondslag ligt is mogelijk gemaakt door een subsidie van de Nederlandse Hartstichting (2000.160)

Het verschijnen van dit proefschrift werd mede mogelijk gemaakt door de Nederlandse Hartstichting en Stichting Hartsvrienden Rescar.
Additionele financiële ondersteuning werd verleend door: J.E. Jurriaanse stichting, St. Jude Medical, Pfizer BV, Astra Zeneca BV, Servier Nederland BV en Arrow Holland Medical Products BV.

Financial support by the Netherlands Heart Foundation and Stichting Hartsvrienden Rescar for the publication of this thesis is gratefully acknowledged.
Additional financial support was granted by: J.E. Jurriaanse foundation, St. Jude Medical, Pfizer BV, Astra Zeneca BV, Servier Nederland BV, and Arrow Holland Medical Products BV.

De uitspraak dat het hart slechts een pomp is
en dus niet de ziel of het symbool van de liefde,
is van dezelfde onverbiddelijke logica
als de bewering dat tranen slechts bestaan uit water en zout
en dus geen teken van verdriet kunnen zijn

- Dr. Jos Roerdink-

CONTENTS

PREFACE

Despite significant progress in the prevention and treatment of cardiovascular diseases, heart failure is still a leading cause of morbidity and mortality in industrial countries. Sustained cardiac hypertrophy, which is defined as an increase in heart size resulting from an increase in cardiomyocyte cell volume, has been recognized as the single most important risk factor for heart failure development, and can be initiated by a wide array of growth factors in response to increased workload, injury, or intrinsic defects in contractile performance. To understand the molecular determinants of the hypertrophic response and to achieve rational drug design to treat heart failure, investigation currently focusses on identifying and characterizing intracellular signal transduction pathways in the heart.

The experiments presented in this thesis topic a signaling pathway that is thought to play a key role in the hypertrophic transcriptional response of the myocyte. This signaling route employs the Ca^{2+}-calmodulin-dependent phosphatase calcineurin and its downstream transcriptional effector Nuclear Factor of Activated T-cells (NFAT).

Compelling evidence for a role of calcineurin as a transducer of hypertrophic signals came from the observation that exposure of cardiomyocytes in tissue culture or rodents to the immunosuppressant drugs, cyclosporine (CsA) or FK-506 blocked the enzymatic activity of calcineurin and partially prevented the ability of cardiomyocytes to undergo hypertrophy in response to angiotensin II, phenylephrine or pressure overload. These drugs interact specifically with the cytoplasmic immunophilin proteins, cyclophilins and FK-506 binding protein-12 (FKBP12), respectively, to form inhibitory complexes that bind the calcineurin A subunit.

Chapter one reviews the studies that employed these substances to block calcineurin-induced cardiomyocyte hypertrophy and the limitations involved using this pharmacological approach. Although several studies indicated these drugs to be potent inhibitors of calcineurin activity in vivo, all these studies were hindered to some degree by intra-and extracardiac side effects of the drugs, especially in vivo. These side effects underline the need to find alternative experimental strategies to further elucidate the (patho)physiological role of calcineurin in the myocardium. A more elegant approach would be to use targeted inhibition of calcineurin or gene targeting of calcineurin in genetically modified mice. Genetic modification provides a superior tool to more specifically evaluate the importance of calcineurin as a regulator of cardiac hypertrophy in vivo. In recent years, different classes of proteins with calcineurin inhibitory properties have been described. These proteins allow a genetic approach to specifically inhibit cardiac calcineurin activity, by using transgenic mice expressing one of these calcineurin-inhibitory proteins in the heart.

One such molecule is modulatory calcineurin interacting protein 1 (MCIP1). MCIP1 blocks calcineurin signaling by binding directly to the catalytic subunit of calcineurin. Transgenic mice with cardiac specific overexpression of the MCIP1 protein driven by the α-MyHC promoter, are protected against several forms of cardiac hypertrophy, which suggest an important role for calcineurin signaling in diverse forms of cardiac hypertrophy. Accordingly, in chapter two the involvement of calcineurin in myocyte hypertrophy after myocardial infarction (MI) is discussed. Calcineurin activity was found to be upregulated after MI and overexpression of the MCIP1 protein resulted in inhibition calcineurin activity, hypertrophic remodeling post-MI, reinduction of fetal gene expression, and attenuated the progression to cardiac dysfunction and heart failure. These results reveal a protective role for MCIP1 in the post-MI heart and suggest that calcineurin is a crucial regulator of postinfarction-induced pathological LV remodeling.

In chapter three, the function and requirement of NFAT factors as primary downstream targets of calcineurin signaling in the heart is further elucidated, the significance of each separate isoform in the heart is evaluated and whether they are functionally redundant. All four members of the NFAT transcription factor family (NFATc1-c4) were found to be expressed in cardiomyocytes. They all showed calcineurin-dependent nuclear translocation, and were able to exert transcriptional activity. Viral mediated gene transfer of a dominant negative NFAT molecule resulted in the blockade of calcineurin induced cardiomyocyte hypertrophy, indicating the necessity of NFAT downstream of calcineurin to produce cardiomyocyte hypertrophy.

Since members of the NFAT transcription factor family are known to be relatively weak activators alone and require nuclear partners, the functional interactions between NFAT and myocyte enhancer factor-2 (MEF-2) proteins in skeletal muscle, led us to investigate whether this is pertinent to the myocardium as well. In chapter four, we discussed our findings that suggest the existence of a regulatory complex formed between MEF2 and NFAT on promoter sequences of several cardiac genes that are known to be upregulated during calcineurin-provoked pathological cardiac hypertrophy. This transcriptional complex also contained chromatin remodeling enzymes with histone acetyl-transferase (HAT) activity that facilitate gene expression by inducing histone acetylation. Since transcriptional activity of MEF2 under unstimulated conditions is actively repressed by class II histone deacetyltransferase (HDAC) activity, the synergistic interaction between NFAT and MEF2 may be under the regulation of the balance between HDAC and HAT activity.

The discovery that the hypertrophic action of calcineurin in cardiomyocytes is partly dependent of MEF2 transcriptional activity, which is actively repressed by HDACs in unstimulated cells, provided us with the hypothesis that systems devoid of HDAC activity may very well prove to be more sensitive to calcineurin activity. Indeed, mice with targeted ablation of HDAC9 demonstrate to be

sensitized to hypertrophic signals and exhibit stress-dependent cardiomegaly. In chapter five we discuss the effect of subjecting HDAC9 deficient mice, which is the predominant HDAC9 isoform in the heart, to MI. Although we were expecting to find a hypertrophy related phenotype, implicate a predominant involvement of angiogenesis, rather than hypertrophic remodeling in the absence of HDAC9.

Chapter six reviews our findings and extrapolates the results described in this thesis to the current knowledge on hypertrophic signaling and transcriptional remodeling to give direction to future research.

Chapter 1 Introduction

Calcineurin and hypertrophic heart disease: novel insights and remaining questions

Orlando F. Bueno, Eva van Rooij, Jeffery D. Molkentin,
Pieter A. Doevendans, Leon J. De Windt

SUMMARY

In the past few years, an emerging body of research has focused on a novel transcriptional pathway involved in the cardiac hypertrophic response. Ever since its introduction, the significance of the calcineurin-NFAT module has been subject of controversy. The aim of this review is to provide both an update on the current status of knowledge and discuss the remaining issues regarding the involvement of calcineurin in hypertrophic heart disease. To this end, the molecular biology of calcineurin and its direct downstream transcriptional effector NFAT are discussed in the context of the genetic studies that established the existence of this signaling paradigm in the heart. The pharmacological mode-of-action and specificity of the calcineurin inhibitors cyclosporine A (CsA) and FK506 is dicussed, as well as their inherent limitations to study the biology of calcineurin. A critical interpretation is given on studies aimed at analyzing the role of calcineurin in cardiac hypertrophy using systemic immunosuppression. To eliminate the controversy surrounding CsA/FK506 usage, recent studies employed genetic inhibitory strategies for calcineurin, which confirm the pivotal role for this signal transduction pathway in the ventricular hypertrophy response. Finally, unresolved issues concerning the role of calcineurin in cardiac pathobiology are discussed based upon the information available, including its controversial role in cardiomyocyte viability, the reciprocal relationship between myocyte Ca^{2+} homeostasis and calcineurin activity and the relative importance of calcineurin in relation to other hypertrophic signaling cascades.

Molecular biology of calcineurin

The calcineurin-NFAT pathway was one of the first signaling paradigms that provided molecular insight how extracellular signals travel from the cell membrane into the nucleus. The precise components of the pathway were defined by working backwards from the T- lymphocyte nucleus to the cell membrane. The regulatory region in the interleukin-2 (IL-2) gene was found to be under control of a transcriptional factor designated Nuclear Factor of Activated T-cells (NFAT), which shuttled between cytoplasmic and nuclear components under influence of a Ca^{2+} signal. Subsequently, nuclear NFAT import was defined to be regulated by dephosphorylation steps catalyzed by the phosphatase calcineurin, which in its turn was subject to regulation by Ca^{2+}-calmodulin binding.[1-3] Finally, calcineurin was identified as the cellular target of the immunosuppressive agents CsA and FK506.[1,2]

Calcineurin itself is a heterodimer composed of two distinct subunits, designated calcineurin A, a 58-59 kDa subunit which contains the catalytic site of the enzyme, and a small ~ 19 kDa calcineurin B subunit which contains the Ca^{2+}-binding regulatory domain of the holoenzyme. Three different mammalian genes, designated α, β and γ, encode calcineurin A, and share substantial homology in the domain encoding the catalytic site. Of these isoforms, calcineurin Aγ displays a testis-restricted expression pattern, while calcineurin Aα and Aβ exist in an overlapping fashion in multiple tissues, including the heart. Multiple alternative splice isoforms of calcineurin Aα and Aβ have been reported.[4] The smaller calcineurin B subunit is encoded by two genes, each having alternative splice isoforms.[5] Only mutant mice lacking calcineurin Aα have been created and these animals display a subtle defect in the antigen-specific T cell response,[6] accumulation of a hyperphosphorylated form of tau in the mossy fibers of the hippocampus, accompanied by cytoskeletal changes and altered synaptic plasticity in the central nervous system.[7,8] The phenotype of the calcineurin Aα knockout is surprising in that it does not reveal the full scale of phenotypic characteristics associated with CsA or FK506 administration, suggesting functional redundancy between the calcineurin Aα and Aβ isoforms. The creation and phenotypic characterization of genetic mouse models mutant for either calcineurin Aβ or calcineurin B will be instrumental for resolving these issues and efforts along this line are in progress.[1]

To date, five genes encoding NFAT complexes have been identified and designated NFATc1 (NFATc or NFAT2), NFATc2 (NFATp or NFAT1), NFATc4 (NFAT3), NFATc3 (NFAT4 or NFATx) and NFAT5, of which only the latter appears to be constitutively nuclear and not subject to regulation by calcineurin.[9,10] The NFATc members share high homology within their DNA binding region, which is related to the Rel domain present in the transcription factor NF-κB.[9] NFATc members transactivate target genes by interacting with other transcription factors

like c-Maf, activator protein-1 (AP-1), and GATA-4.[11 12-15]

Calcineurin physically interacts with NFATc members and dephosphorylates serine residues within SP repeats and the serine-rich region. Dephosphorylation of these residues results in the unmasking of two nuclear localization sequences required for its nuclear import.[16] Nuclear export, on the other hand, is dependent upon rephosphorylation of these same residues by glycogen synthase kinase-3β (GSK3β)[17] and the p38 and c-Jun NH_2-terminal protein kinase (JNK) members of the mitogen-activated protein kinase (MAPK) superfamily.[18,19] The biological significance of the individual NFATc members is rapidly being elucidated due to the availability of murine models deficient for the single NFATc isoforms.[9] Of interest to the cardiovascular field, mice lacking NFATc1 die in utero from failure to develop semi lunar cardiac valves,[20,21] while the combined disruption of NFATc3 and NFATc4 results in embryonic lethality due to abberant vascular patterning.[22]

First evidence for a role of calcineurin in cardiac hypertrophy

Molkentin and colleagues first established the existence of a myocardial calcineurin-NFAT module.[11] Using a yeast-two-hybrid screening of interacting factors for the cardiac embryonic transcription factor GATA-4, a specific interaction was observed between zinc finger domains of GATA-4 with the DNA binding domain of NFATc4 (NFAT3). Since interaction between NFATc4 and GATA-4 drove synergistic activation of a brain natriuretic peptide (BNP) promoter-reporter construct, a specific role for NFATc4 activation and hypertrophic gene induction was suggestive (Figure 1.1).[11]

In line with this hypothesis, CsA (calcineurin inhibition) prevented the hypertrophic growth response of cultured cardiomyocytes in response to humoral factors as angiotensin II (AngII) and phenylephrine (PE).[11] To elucidate the functional significance of the calcineurin-NFATc4 pathway in vivo, several lines of transgenic mice were generated containing either truncated activated mutants of NFATc4 or the calcineurin Aα subunit specifically in the heart. Persistent calcineurin activation was sufficient to promote a hypertrophic response in 11 separate TG founder lines, varying from relatively benign forms of concentric hypertrophy to severe forms of dilated cardiomyopathy and early lethality in low and high copy number TG mice, respectively. Pharmacological inhibition of calcineurin through CsA injections (25 mg/kg/day) resulted in complete prevention of the pathology.[11] NFATc4 TG mice also demonstrated a hypertrophic myopathy associated with re-expression of fetal genes. As a testimony to the specificity of the proposed pathway, CsA was unable to prevent the morphologic pathology of the NFATc4 TG animals, which express a calcineurin-independent form of the transcription factor.[23]

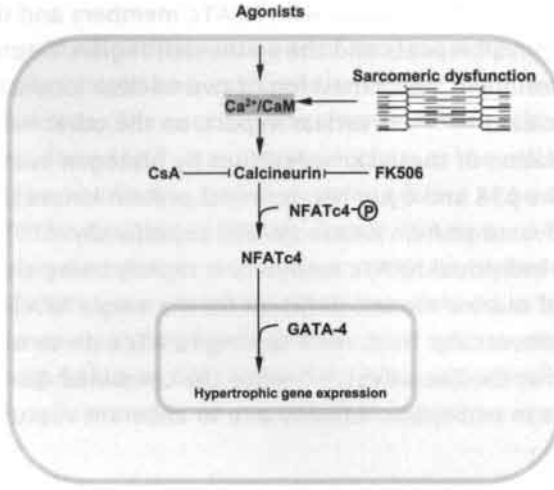


Figure 1.1. Model for calcineurin function in cardiac hypertrophy as postulated by Molkentin and colleagues in 1998.12 In this scheme, Ca2+/calmodulin-dependent phosphatase calcineurin is activated by hypertrophic agonists such as Angiotensin II (AngII) or phenylephrine (PE) or by intrinsic defects, which trigger an increase in intracellular Ca2+. Upon activation, calcineurin dephosphorylates one single nuclear factor of activated T-cell (NFATc) target, which translocates to the nucleus upon activation. NFATc4 (NFAT3) physically associates with the transcription factor GATA-4 to synergistically activate gene transcription of hypertrophic genes such as the brain natriuretic peptide gene (BNP).

This initial study was soon followed by one which analyzed whether calcineurin may also be involved in the ventricular growth response in other animal models of cardiomyopathy and LV hypertrophy. Accordingly, CsA administration prevented hypertrophy in tropomodulin overexpressing mice, a model of dilated cardiomyopathy due to sarcomeric disruption.[24-27] CsA also proved to be effective in TG mice expressing a non-phosphorylatable form of myosin light chain 2v (MLC-2v), which display hypertrophic cardiomyopathy (HCM) as a result of inefficient cross-bridge cycling,[24,28,29] and in mice overexpressing β-tropomyosin, a model of hypertrophic cardiomyopathy and defective calcium handling.[24,30,31] CsA was ineffective, however, in a retinoic acid receptor (RAR) TG driven pathology[24,32], indicating the possibility that calcineurin activation might act as a molecular driving force behind some, but not all forms of ventricular myopathies. The authors were further able to demonstrate activation of calcineurin enzymatic activity concurrent with a 27% increase in indexed heart weight in a rat model of pressure overload created by surgical constriction of the abdominal aorta. CsA administration resulted in complete prevention of calcineurin activation and LV hypertrophy six days following coarctation.[24]

These two initial studies suggested several novel implications for cardiac pathobiology. (1) The existence of a conservational preservation of a calcineurin-

NFATc pathway in the myocardium, such as defined earlier in T lymphocytes, (2) the potential of this transcriptional pathway to activate a fetal gene expression profile consistent with LV hypertrophy (Figure 1.1), (3) pharmacological inhibition of (transgenically driven levels) of activated calcineurin prevents the histopathology resulting from its activation. These initial studies immediately evoked questions related to the significance of this novel pathway in view of the complexity and hierarchy of the multitude of hypertrophic signaling pathways known to govern cardiac hypertrophy. To address these issues the vast majority of these studies, unfortunately, made use of the pharmacological inhibitors of calcineurin. The inconsistent outcome of these studies ignited controversy regarding the requirement of this pathway in the LV hypertrophic response.

Pharmacological inhibitors of calcineurin

Csa (Sandimmune) and FK506 or tacrolimus (Prograf) are the most important immunosuppressive agents used in organ transplantation and in the treatment of diverse immune disorders. These agents produce similar effects on signal transduction pathways in T lymphocytes, however, they do not share similar chemical structure nor do they react with the same target. CsA is a neutral, lipophilic and very hydrophobic, cyclic polypeptide of 11 amino acids extracted from Tolypocladium inflatum Gams. Following oral or intravenous administration, CsA distributes rapidly between blood cells and plasma with an apparent large volume of distribution of 4 to 8 L/kg.[33] CsA accumulates in erythrocytes (50% to 60%) and in leukocytes (10% to 20%), the remainder of the drug is bound to plasma lipoproteins. Tissue drug concentrations depend on actual cyclophilin and lipid content. CsA is metabolized predominantly in the liver by cytochrome P-450IIIA enzymes to more than 30 metabolites. These metabolites do not contribute significantly to the immunosuppressive effects of CsA since the most active ones only retain 10% to 20% of the activity of the parent compound. CsA does not cross the blood-brain barrier, but it crosses the placenta and it can be detected in amniotic fluid and fetal blood. The elimination half-life of CsA has been estimated of approximately 6 hours.[33]

FK506 is a macrolide antibiotic extracted from Streptomyces tsukubaensis. FK506 can be administered orally or intravenously and its elimination half-life is approximately 20 hours. It is extensively metabolized in the liver since less than 1% of the drug is excreted unchanged. FK506 is approximately 100 times more potent than CsA in its calcineurin inhibitory characteristics.

The mechanism of action of these two immunosuppressive agents has been investigated extensively in immune cells. CsA and FK506 bind with high affinity to the ubiquitous cytosolic peptidyl-propyl isomerases cyclophilin and FK506-binding protein-12 (FKBP12), respectively. The complex of CsA-cyclophilin or FK506-

FKBP12 associates with calcineurin and inhibits its phosphatase activity as well as its interaction with a variety of substrates. CsA and FK506 also inhibit the peptidyl-propyl isomerase activity of cyclophilin and FKBP12, but this effect is not involved in the immunosuppressive mechanism of these drugs since CsA analogues with no effect on T cell activation are still able to block the peptidyl-propyl isomerase activity.

It is generally accepted that calcineurin inhibition by CsA and FK506 blocks the dephosphorylation and subsequent nuclear translocation of the NFATc transcription factors.[34,35] However, novel, calcineurin-independent mechanisms of action for CsA and FK506 have recently been proposed. It has been hypothesized that part of the immunosuppressive effects of CsA are mediated through TGFβ1, a cytokine with immunosuppressive effects in diverse cells and tissues.[36,37] However, this is still a matter of controversy since other studies have failed to show an induction of TGF-β1 production during CsA treatment.[38] This issue could potentially be of interest to the current topic in view of the recent demonstration that selective TGFβ-activated kinase (TAK1) activation can result in a cardiomyopathic phenotype in mice.[39] CsA was also found to inhibit the activation of some family members of the mitogen-activated protein kinases (MAPK) in different cell types, although this may still be an indirect response to calcineurin inhibition.[40,41] Chronic CsA administration produces changes in the properties of the sarcoplasmatic reticulum (SR) Ca^{2+}-release channel[42] and in isolated guinea-pig cardiomyocytes alterations in the kinetics of L-type Ca^{2+} channels.[43]

The ryanodine receptor (RyR2) is a multiprotein complex including several phosphatases, kinases, achoring proteins and FKBP12.6. Altered RyR2 channel function has been postulated to play a role in cardiomyopathy, since hyperphosphorylation of the complex was observed in human heart failure biopsies, which results in dissociation of FKBP12.6 from its cognate receptor. These events result in increased Ca^{2+} sensitivity for activation, elevated channel open probability, and impaired myocyte Ca^{2+} homeostasis.[44] Genetic ablation of FKBP12 resulted in severe septum defects and dilated cardiomyopathy in mice.[45] Much less is known about the cardiac biology of the target of CsA, the cyclophilin A-D family.

Accordingly, it is becoming increasingly clear that CsA and FK506 have calcineurin-independent effects in multiple organs, and do not constitute the optimal tool to test for a potential role of calcineurin in the setting of cardiac hypertrophy. If these agents are to be used in a systemic fashion, precaution should be taken for dosage, mode of delivery, and severe extra-cardiac toxicity on major target organs besides the heart muscle (e.g. neural tissue, smooth muscle, kidney), which may influence the outcome of hypertrophy studies.

CsA and FK506: evidence in support of a role for calcineurin in hypertrophy

Endothelin-1 (ET-1) is an established humoral factor that induces cardiac hypertrophy. ET-1 was reported to increase the activity of CaMKII and calcineurin in cultured cardiac myocytes and pretreatment with KN62 (CaMK inhibitor) or CsA strongly suppressed ET-1-induced increases in phenylalanine uptake and in cell size. Pharmacologic inhibition of calmodulin, CaMK or calcineurin or overexpression of dominant-negative mutants of CaMKII and calcineurin strongly suppressed hypertrophic gene activation.[46] Similarly, leukemia inhibitory factor (LIF), a hypertrophic cytokine employing gp130 receptors for transmembrane signaling, enhanced both calcineurin and CaMK activities. KN62 and CsA administration significantly reduced LIF-induced hypertrophy and fetal gene expression, suggesting a crucial role for these Ca^{2+}-activated pathways in LIF-mediated cardiomyocyte growth (Table 1.1).[47]

CsA demonstrated to be highly effective in tropomodulin overexpressing mice, in mice expressing a non-phosphorylatable form of myosin light chain 2v (MLC-2v) and in mice overexpressing β-tropomyosin.[24] An additional genetic model of hypertrophy, the Gqα overexpressing mouse, was found to be partially sensitive to comparable doses of CsA.[48]

Shimoyama and colleagues analyzed the effectiveness of FK506 in a rat model of pressure overload hypertrophy. A drug dosage-effect preceded this study to minimize toxic side-effects on parameters as blood pressure, cardiac hemodynamics, and operative mortality. Accordingly, a dosage of 1 mg/kg/day FK506 injected intramuscularly was found to have negligible side-effects, yet still inhibited LV calcineurin activation. A 3-week treatment regimen on abdominal aortic constricted animals was associated with a near complete prevention of the increase in (indexed) heart size, fibrosis formation, and hypertrophic marker gene expression.[49]

Using the same rat model, Lim and colleagues demonstrated that a 14-day CsA regimen resulted in a dose-dependent prevention of the morphologic, histological and molecular aspects of LV hypertrophy. Although this study suffered from a moderate post-operative mortality (~25%), mortality was unaffected by the drug. One striking and clinically relevant finding demonstrated that treatment of CsA (20 mg/kg/day) was associated with a regression of established myocardial hypertrophy resulting from a 14-day pressure overload.[50]

Hill and colleagues were able to demonstrate a progressive and reproducible hypertrophic response following a 5-week period thoracic aortic banding protocol in mice (45% increase in HW/BW). A relatively high dose of CsA resulted in a near complete blockade of this response (Table 1.1). The authors found no evidence of deleterious effects of CsA on myocardial hemodynamics, the transstenotic pressure gradient, weight gain, physical activity or mortality.[51] Mice

Table 1.1 Studies using CsA and FK506 to analyze the role of calcineurin on the development of cardiac hypertrophy.

Model	Stimulus	Inhibitor	dosage (per day)	Duration	Prevention hypertrophy	Side effects	Genetic strain
Cultured myocytes							
Kato et al. (48)	LIF	CsA	50 ng/ml	1 day	Yes	None	Wistar
Molkentin et al. (12)	PE/AngII	CsA / FK506	500 / 150 ng/ml	3 days	Yes	None	NR
Zhu et al. (47)	ET-1	CsA / dnCnA	500 nM / NA	2 days	Yes	None	Wistar
Boluyt et al. (58)	PE	Fk506	250 ng/ml	1 day	No	HD §	Wistar
Mouse							
Hill et al. (52)	transverse AC	CsA	50 mg/kg	5 weeks	Yes	None	C57Bl/6
Molkentin et al. (12)	ca-CnA TG	CsA	50 mg/kg	14 days	Yes	None	FVB/ICR
Murat et al. (53)	two-kidney one clip	CsA	50 mg/kg	4 weeks	Yes	None	C57Bl/6
Sussman et al. (25)	β-tropomyosin	CsA	30 mg/kg	8 days	Yes	None	FVB
Sussman et al. (25)	MLC-2V*TG	CsA	30 mg/kg	6 weeks	Yes	None	FVB
Sussman et al. (25)	tropomodulin TG	CsA / FK506	30 / 5 mg/kg	2 weeks	Yes	None	FVB
Ding et al. (60)	ascending AC	CsA	50 mg/kg	4 weeks	No	HF	C56Bl/6
Fatkin et al. (56)	αMyHC403 (FHC)	CsA / FK506	30 / 5 mg/kg	5 weeks	No	HD/OCM/AM	C56Bl/6 Sv129
Lim et al. (56)	ca-NFATc4	CsA	30 mg/kg	4 weeks	No	WL	FVB
Meguro et al. (61)	transverse AC	CsA	25 mg/kg	3 weeks	No	HF	CD-1
Sussman et al. (25)	RAR TG	CsA / FK506	30 / 5 mg/kg	6 weeks	No	None	FVB
Muller et al. (59)	transverse AC	CsA	50 mg/kg	3 weeks	No	NR	C56Bl/6
Rat							
Eto et al. (56)	exercise	CsA	20 mg/kg	10 weeks	Yes	None	Wistar
Lim et al. (51)	abdominal AC	CsA	20 mg/kg	2 weeks	Yes	WL	Spraguel/Dawley
Mervaala et al. (55)	angiotensin/renin TG	CsA	5 mg/kg	3 weeks	Yes	None	NR
Oie et al. (57)	myocardial infarction	CsA	50 mg/kg	2 weeks	Yes	HF	Wistar
Sakata et al. (54)	DS hypertension	FK506	1 mg/kg	12 weeks	Yes	DPS	Dahl-Issei
Shimoyama et al. (68)	DS hypertension	FK 506	0.1 / 0.01 mg/kg	6 weeks	Yes	None	Dahl-Issei
Sussman et al. (25)	abdominal AC	CsA	20 mg/kg	6 days	Yes	NR	Spraguel/Dawley
Shimoyama et al. (68)	abdominal AC	FK 506	1 mg/kg	3 weeks	Yes	None	Wistar
Eto et al. (56)	transverse AC	CsA	20 mg/kg	4 weeks	Yes	None	Wistar
Hayashida et al. (64)	DS hypertension	FK506	1 mg/kg	6 weeks	No	PI / WL	Dahl-Issei
Zhang et al. (63)	abdominal AC	CsA	10 mg/kg	4 weeks	No	HF / WL	Spraguel-Dawley
Luo et al. (62)	abdominal AC	CsA / FK506	40 / 4 mg/kg	2 weeks	No	NR	Spraguel-Dawley
Zhang et al. (63)	SHR hypertension	CsA	5 mg/kg	6 weeks	No	None	SHR

LIF, leukemia inhibitory factor; PE, phenylephrine; AngII, angiotensin II; ET-1, endothelin I; HD, hypertrophic development; AC, aortic constriction; ca, constitutively active; RAR, retinoic acid receptor; TG, transgenic; CnA, calcineurin; DS, Dahl salt-sensitive; SHR, spontaneously hypertensive rats; CsA, cyclosporin; NA, not applicable; NR, not reported; HF, heart failure; OCM, obstructive cardiomyopathy; AM, accelerated mortality; WL, weight loss; DBP, decreased blood pressure; PI, pulmonary infection; FHC, familial hypertrophic cardiomyopathy; MLC-2V*, non-phopshorylatable myosin light chain 2V.

§ FK506 induces hypertrophy when used in combination with rapamycin

with renovascular hypertension, created by a two kidney one clip method, also demonstrated substantial LV calcineurin activation and CsA prevented hypertrophy in this model (Table 1.1).[52]

Using the Dahl salt-sensitive rat model, which develops a rapid onset hypertension and both pressure and volume overload hypertrophy, Shimoyama and colleagues demonstrated a substantial reduction in indexed left ventricular weight using only very low doses of FK506 (0.1 and 0.01 mg/kg/day) over a 6 week period of severe hypertension. In fact, cardiac enzymatic calcineurin activity was elevated at any time point examined in this hypertensive model. FK506 also prevented fibrosis deposition and expression of certain fetal-type cardiac genes. Sakata et al. demonstrated LV blockade and prevention to heart failure development in the same model when FK506 (1 mg/kg/day) was administered early (from 8 weeks), but no attenuation of heart failure remodeling when administered late in life (from 17 weeks).[53]

Using TG rats harboring both the human renin and angiotensin genes, Mervaala et al. investigated CsA effectiveness to protect against Ang II-induced myocardial and renal damage. In their model, CsA completely prevented cardiovascular death, decreased 24-hour albuminuria by 90%, lowered systolic blood pressure by 35 mm Hg, and protected against the development of cardiac hypertrophy.[54]

Calcineurin activation may also play a role in distinct hypertrophic stimuli.[55] Following 10 weeks of voluntary exercise training to evoke a physiological adaptive hypertrophy response in rats, indexed LV weight and LV calcineurin activity were increased by 20% and 2.5 fold, respectively, and CsA was effective in preventing both parameters.[55] Post-infarction failure is usually associated with a strong volume overload stimulus. Rats subjected to chronic myocardial infarction (MI) and administered CsA over a 14-day post-MI recovery period demonstrated a significant attenuation of cardiac hypertrophy and α-skeletal actin gene induction. However, the inhibition of hypertrophy led to an increased incidence of LV dilation and reduced hemodynamic performance, suggesting acceleration of the heart failure development in this model.[56]

CsA and FK506: no correlation between calcineurin and cardiac hypertrophy

Boluyt and colleagues demonstrated significant activation of 70-kDa S6 kinase ($P70^{S6K}$) following phenylephrine-stimulation, and rapamycin pretreatment prevented this effect.[57] Rapamycin mediates its effects through specific binding to the intracellular immunophilin FKBP. Since FK506 and rapamycin both bind FKBP and act mutually antagonistic, the authors used FK506 at a 10-fold molar excess to competitively reverse the anti-hypertrophic, rapamycin-mediated effects. This study implicates that FK506 may have both anti-hypertrophic (calcineurin

inhibition) as well as pro-hypertrophic properties, albeit that latter effects only play a role at excess concentrations, and emphasizes the need for correct CsA or FK506 dosage use to study calcineurin biology.

In the initial report of Sussman and coworkers, the RAR overexpressing TG model failed to morphologically respond to CsA.[24] This model was created to study the function of excessive retinoid signaling during cardiac morphogenesis, and resulted in severe heart failure in neonates and juvenile mice when a consititutively activated RAR was driven to the embryonic ventricle by the β-MyHC promoter. Interestingly, postnatal ventricular expression of the same receptor (driven by the α-MyHC promoter) did not result in cardiac pathology, suggesting a critical role for retinoid signaling during developmental stages of ventriculogenesis. Nevertheless, the failure of the RAR model to respond to systemic immunosuppressive therapy suggests that retinoid signaling acts independently from calcineurin during cardiomorphogenesis.

Muller and coworkers found that CsA administration of 25 mg/kg twice daily did not attenuate cardiac hypertrophy in mice with transverse aortic constriction, but, unfortunately, calcineurin activity assays were not provided.[58] Ding and colleagues investigated CsA effectiveness in a murine model of ascending aortic constriction.[59] Morphometric analyses of hearts subjected to this very severe form of pressure overload revealed no statistical differences between non-treated and CsA-treated, banded animals. In fact, calcineurin activity assays revealed a lower enzymatic activity following pressure overload compared to sham operated animals. Given this, one would not expect a significant impact from further CsA administration (calcineurin inhibition), yet the authors reported development of heart failure by drug treatment.[59]

Meguro et al found a substantial prevention of LV hypertrophy by CsA treatment in transverse aortic constricted mice over a period of 3 weeks. However, a disproportionate number of premature deaths in the CsA treated group was observed, all within the first 7 days of the study and accompanied by pleural efflusion. Invasive LV hemodynamic analysis revealed a significant lower ascending as well as abdominal systolic aortic pressure in the presence of comparable trans stenotic pressure gradient, suggesting both an intrinsic myocardial depressive and a distal blood pressure lowering effect by CsA. The authors concluded that inhibition of LV hypertrophy might be of detriment to the heart, and could accelerate decompensation and heart failure.[60]

Luo and colleagues investigated a similar pressure overload model in the rat by constriction of the abdominal aorta as initially employed by multiple groups[24,49,50] and randomized their groups to receive different doses of CsA or FK506.[61] No effect of CsA or FK506 on the development of LV hypertrophy was observed, but a significant increase in mortality was evident with increasing dosages of CsA or FK506. In fact, the highest FK506 dose (4 mg/kg/day) was associated with 90% mortality.[61]

Zhang and colleagues demonstrated that 6 week CsA treatment (5 mg/kg/day) was associated with a significant elevation of blood pressure in the spontaneously hypertensive rat (SHR) model, but this additive blood pressure increase was not associated with increased heart weight.[62] To avoid interpretative complications due to the genetic component of the SHR model, the authors next turned to normotensive rats to address the effect of CsA on pressure overload induced hypertrophy. Two weeks of CsA treatment resulted in a 28% increase in indexed LV weights in abdominal aorta constricted rats compared to a 38% increase in vehicle-treated, banded animals, a difference that was not found to be statistical significant. Four weeks of aortic banding resulted in an increase of 46% in vehicle treated animals, while CsA treated banded animals demonstrated an increase of 27 and 22% at CsA dosages of 10 and 20 mg/kg/day, respectively. Again, the difference was not indicated as statistically significant, even though CsA appeared to have dose-dependent effects on the development of LV hypertrophy. This particular study was also associated with a substantial post-operative mortality, ranging from 33 to 67%, and a reduction in pressure gradients at 4 weeks, most notably in CsA treated groups.[62]

Hayashida and coworkers observed increased LV calcineurin activity in hypertensive hypertrophied Dahl-Issei rat strain, but not during the later congestive heart failure phase. However, a CsA regimen started at 11 weeks of age did not prevent LV hypertrophy, nor heart failure development.[63]

Finally, in a murine model of familial hypertrophic cardiomyopathy (FHC) bearing a knock-in missense mutation in the cardiac myosin heavy chain (αMyHC$^{403/+}$)[64], both CsA and FK506 resulted in accentuated LV hypertrophy and worsening of pathology.[65] Pre-treatment with diltiazem (I_{Ca} antagonist) prevented the exaggerated pathology in αMyHC$^{403/+}$ mice. Long term treatment with a K^+-channel agonist, minoxidil, mimicked the morphologic pattern associated with CsA and FK506 in this model. These changes were attributed to drug-induced elevation of diastolic Ca^{2+} concentration.[65]

Interpreting the CsA and FK506 studies

How can we reconcile these differing accounts on CsA/FK506 effectiveness? By far the easiest explanation would be to assume some level of model-dependency for calcineurin involvement, implicating that other signaling modules may take over in relative importance to the expense of calcineurin. Indeed, it has been postulated that calcineurin inhibition effectively blocks Ang II-mediated cardiac hypertrophy, such as that developing in response to abdominal aortic banding or following reduced renal perfusion in renovascular hypertension. In support of this view, all reports involving models with AngII activation (abdominal aortic constriction[49,50]; renin-angiotensin TG rat[54]; two-kidney-one-clip[52])

reported a higher degree of CsA/FK506 effectiveness (Table I). This view must be an oversimplification, since constriction of the aortic arch (transverse aortic constriction) does not activate the renin-angiotensin system,[66] yet one report still demonstrated near complete prevention of hypertrophy by CsA administration[51] (Table 1.1).

Genetic variability between the animals and their sensitivity to CsA/FK506 may play an underestimated role and coincides with substantial variability in drug dosage. To date, Sprague-Dawley rats, Wistar rats and FVB/N mice exhibited a relatively higher degree of drug tolerance than Dahl-Iwai rats and C57BL/6 mice (Table I). The most striking example of drug tolerance and strain dependency is provided by the studies of Shimoyama and colleagues[49,67], where Wistar rats tolerated 1 mg/kg/day FK506[49], while the same group found a 10-fold lower whole body upper limit tolerance in rats with the Dahl-Iwai background.[67] This contrasts with dosages of up to 4 mg/kg/day FK506 used in Sprague-Dawley rats (40 to 400-fold excess vs Shimoyama et al.[67]) which, not surprisingly, resulted in up to 90% mortality in pressure-overloaded animals. Clearly, the vast variations in genetic background and drug dosage must have influenced the interpretation of calcineurin involvement in cardiac hypertrophy.

Careful inspection further suggests some degree of variance associated with surgical procedure, peri-operative milieu and route of drug administration. A number of studies have reported a high degree of drug-associated premature mortality, up to 90% in drug administered animals, an effect which might have preselected interindividual variations. In fact, careful inspection suggests an inverse relation between high mortality and drug-effectiveness (Table I).

Differences in timing, route and duration of drug administration could be another factor relating to some of the differences between the studies. Ding et al. reported no drug effectiveness following 4 weeks of administration, while Eto et al., employing a similar model of ascending aortic constriction and comparable CsA dosage, demonstrated a clear effect on the early phase (1 week post-surgery), but not on later stages (4 weeks post-surgery) of hypertrophy development. Two groups reported efficient hypertrophy prevention in the Dahl-Salt sensitive model using low doses of FK506 and starting drug administration early in life (6 or 8 weeks),[53,67] while another reported no effectiveness when they started CsA injections at 11 weeks of age.[63] Luo and colleagues reported high mortality and no CsA effectiveness in rats administered the drug in the drinking water.[61] The majority of studies, however, have injected CsA or FK506 subcutaneously (often twice daily) due to its low solubility in aqueous solutions and limited half-life.

A final issue concerns the phenotypic interpretation following drug administration. Meguro et al. reported high mortality in their CsA banded animal groups, interpreted as development of heart failure since a disproportionate number of animals displayed pleural effusion upon autopsy.[60] It is unlikely, however, that heart failure was the true cause of death. Only a 28 and 16%

decrease in LV dP/dt_{max} and LV ejection fraction in CsA-treated, banded vs vehicle-treated, banded animals was observed, respectively.[60] A more plausible explanation for their observations would be enhanced susceptibility to pleural infection in treated animals[67] due to the systemic immunosuppressive therapy.[67]

Conclusively, the vast majority of studies, regardless of their qualitative outcome, were hindered to some degree by intra-and extracardiac side effects of the drugs. Latter notion underscores the problematic situation of using CsA or FK506 as experimental devices, virtually excludes their potential as future treatment options for patients with hypertrophic heart disease, and motivated some research groups to find alternative experimental strategies to elucidate the myocardial (patho)physiological role of calcineurin.

Genetic inhibition of calcineurin

In recent years, different classes of proteins with calcineurin inhibitory properties have been described. AKAP79 (for A-kinase anchoring protein) was the first protein discovered to have calcineurin inhibitory characterstics.[68] AKAP79 acts as a scaffold protein, which binds protein kinase A and protein kinase C besides calcineurin and is thought to anchor multiple classes of signaling modules in the vicinity of substrates to facilitate their proper and timed activation.[68] One interesting class of calcineurin inhibitors are the products of the (Down's Syndrome Critical Region) DSCR1 gene and its relatives, DSCR2 or ZAK14.[69] DSRC1 is located on human chromosome 21 in the so-called Down's syndrome critical region. The small gene products of DSCR1 and DSCR2, designated MCIP1 (for modulatory calcineurin interacting protein-1) and MCIP2, respectively, are remarkably potent inhibitors of calcineurin activity in striated muscle.[70] Interestingly, it was demonstrated that calcineurin activity upregulates the MCIP1 gene product in cardiomyocytes through an intragenic segment in the MCIP1 gene, which includes a dense cluster of consensus NFAT binding sites.[71] MCIP1 may therefore participate in a negative feedback circuit to diminish potentially deleterious effects of unrestrained calcineurin activity in striated muscle cell lineages. Two additional classes of cellular calcineurin inhibitors are Cain (for calcineurin inhibitory protein) and the calcineurin B homologous protein (CHP). Cain (also known as Cabin) is a large 240-kDa protein with multiple binding domains that functions as a scaffolding protein attaching numerous proteins besides calcineurin.[72] Cain is a noncompetetive inhibitor of calcineurin phosphatase activity with a K_i of 440 nM and antagonizes NFATc translocation.[73,74] CHP has a high similarity to the calcineurin B subunit and is able to compete with the calcineurin B subunit for binding the calcineurin A subunit.[75,76]

The identification of specific cellular antagonists of calcineurin have provided an excellent platform to design experimentation that circumvents

the issue of drug specificity and whole body toxicity. This strategy was first reported by Taigen and co-workers, who created adenoviral vectors expressing the specific calcineurin binding domains of the Cain and AKAP79 proteins (designated ΔCain and ΔAKAP, respectively).[77] AdΔCain and AdΔAKAP infection resulted in attenuation of AngII, PE and growth factor-induced calcineurin activity, cardiomyocyte hypertrophy, and atrial natriuretic factor (ANF) expression (Figure 1.2). The same non-competitive calcineurin inhibitors were recently overexpressed in a cardiac-restricted manner in mice. ΔCain and ΔAKAP79 TG animals demonstrated stable transgene expression in the cardiac compartment, reduced cardiac calcineurin activity and a significant attenuation of hypertrophy in response to catecholamine infusion or pressure overload stimuli (Figure 1.2).[78] In the same study, the ΔCain adenovirus was used for viral-mediated gene transfer of the ΔCain peptide into the adult rat myocardium. Overexpression of the ΔCain protein resulted in inhibition of pressure-induced calcineurin activation and cardiac hypertrophy[78] and supports the feasibility to acutely intervene in reactive hypertrophic signaling using gene therapy approaches.[79]

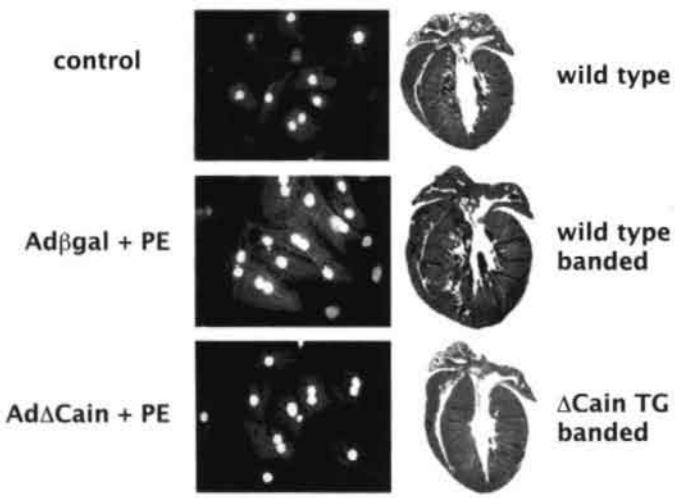


Figure 1.2. Genetic inhibition of calcineurin with calcineurin inhibitory protein (Cain) abrogates the LV hypertrophy response. The left panel summarizes the in vitro results obtained with adenoviral ΔCain gene transfer (AdΔCain) in cardiomyocytes. Phenylephrine (PE) induces a massive increase in cardiomyocyte size in cultures infected with a control adenovirus (Adβgal) compared to uninfected, non-stimulated cells (top left and middle left panels). In contrast, blockade of calcineurin in the presence of ΔCain prevents PE-induced hypertrophy (lower left panel). Cardiomyocytes were visualized by immunocytochemical α-actinin detection, nuclei were visualized with bisbenzamide (white). Hematoxylin-Eosin stained cross-sections demonstrate that similar effects were observed following ΔCain overexpression in vivo. A 14-day pressure overload stimulus resulted in a visible increase in heart weight in wildtype animals, as compared to sham-operated, wildtype mice (top right and middle right panels). Mice overexpressing the non-competetive ΔCain protein in the cardiomyocyte component are protected against this pressure-overload stimulus (lower right panel). **-Full colour image page 177**

As an alternative approach to inhibit calcineurin, transgenic mice expressing a truncated form of human MCIP1 in a cardiac-selective manner were created. Remarkably, unstressed MCIP1 TG animals revealed a 5-10% smaller heart size, establishing a role for calcineurin in normal, developmental myocardial growth. MCIP1 overexpression prevented the massive hypertrophic response, fetal gene induction and progression to dilated cardiomyopathy in the calcineurin TG mouse.[11] MCIP1 TG mice demonstrated resistance towards cardiac hypertrophy as a result of long-term β-adrenergic stimulation and exercise training. Finally, myocardial overexpression of a dominant negative (dn) form of calcineurin provided protection against pressure overload hypertrophy following abdominal aortic banding in mice.[80] The results from these three reports utilizing four distinct genetic strategies to inhibit calcineurin activity (ΔCain, ΔAKAP79, MCIP1 and dn-calcineurin) make it hard to dispute that calcineurin is a required component of hypertrophic signaling following diverse stimuli.[78,80,81]

The existence of viable calcineurin Aα and Aβ somatic knockout mice may provide a means of obtaining further genetic evidence for the involvement of calcineurin in the hypertrophic response. Because the viability of double-null calcineurin Aα and Aβ mice is still uncertain, the establishment of transgenic animals with a dominant-negative calcineurin inhibition based approach[78,80,81] may still prove to be of significant value. A preferential approach would be to identify the critical calcineurin A isoform and ablate it in an inducible, ventricular myocyte cell lineage-dependent fashion, which would also permit definitive insight into the temporal aspects of calcineurin involvement in the distinct stages of heart failure development.

Still many other questions remain and concern calcineurin's precise pathobiological role in terms of biochemical properties, subcellular localization, crosstalk with other notorious hypertrophic signaling modules, its suggested role in myocyte viability and its precise contribution in the progression of human heart failure. The current status of knowledge of these issues is discussed below.

Reciprocal relationship between Ca^{2+} homeostasis and calcineurin activity

One fundamental question relates to the processes leading to myocardial calcineurin activation. How can the cardiac myocyte distinguish between changes in Ca^{2+} that result in calmodulin activation versus the vast fluctuations in Ca^{2+} that occur upon each cycle of contraction and relaxation? Studies in other cell types have demonstrated that NFATc remains nuclear only in response to prolonged, low-amplitude Ca^{2+} signals and is insensitive to transient, high-amplitude Ca^{2+} alterations.[82] Interestingly, the activity of CaMK, another important Ca^{2+}/calmodulin regulated hypertrophic signal transducer,[83] was reported

to be uniquely sensitive to transient, high amplitude Ca^{2+} fluctuations.[82] The differential response of two major Ca^{2+}/calmodulin-regulated hypertrophic signal transducers to fundamentally distinct Ca^{2+} fluctuations may implicate that they fulfill specialized pathophysiological functions within the cardiomyocyte. Whether calcineurin and CaMK also differentially respond to distinct Ca^{2+} alterations in striated muscle cell types and what these functions might be remains to be explored.

Furthermore, the actual source of Ca^{2+} that activates calcineurin (or other Ca^{2+}/calmodulin signaling modules for that matter) is incompletely understood. Studies using nicardipine and verapamil suggest a critical involvement of the L-type Ca^{2+} channel, which may respond downstream of G-protein coupled receptor (GPCR) agonists such as ET-1, AngII or PE[11,46] and gp130 receptor agonists like LIF.[47] LIF enhances intracellular Ca^{2+} transients through an increase in L-type Ca^{2+} current ($I_{Ca,L}$) in adult cardiomyocytes.[84] LIF-mediated activation of $I_{Ca,L}$ resulted in increased calcineurin and CamK activities and nicardipine and verapamil pretreatment fully prevented their activation, suggesting at least one plausible source of Ca^{2+} that activates calcineurin and CaMK.

Findings in other cell types suggest a reciprocal relation between calcineurin on the one hand, and the RyR, IP3 receptor and L-type Ca^{2+} channel, on the other.[85,86] In line with this notion, we have previously reported that adenoviral expression of calcineurin in neonatal cardiomyocytes resulted in positive inotropy and increased Ca^{2+} transients.[87] In addition, adult myocytes derived from the calcineurin TG mouse exhibited increased $I_{Ca,L}$ amplitude and density and shortened time to half decay compared to wildtype myocytes.[88] Since the total number of L-type Ca^{2+} channel $\alpha 1$ and $\beta 2$ subunits was unaltered, the increased density of $I_{Ca,L}$ is due to an increased fraction of channels that open during the repolarization phase. Interestingly, therapeutic doses of CsA had no effects on $I_{Ca,L}$ in wildtype mice, suggesting that calcineurin indirectly affects L-type Ca^{2+} channel properties. This contrasts observations in the mammalian brain, where calcineurin is the major phosphatase responsible for L-type Ca^{2+} channel inactivation.[89] One plausible explanation for the effects of myocardial calcineurin on the profile of myocyte Ca^{2+} handling may relate to SR Ca^{2+} handling. Indeed, Janssen and coworkers demonstrated that CsA induces sustained SR Ca^{2+} leakage from adult rabbit and human ventricular myocytes at therapeutic doses.[90] Collectively, calcineurin inhibition may have the potential to alter myocardial intracellular Ca^{2+} homeostasis and influence the susceptibility to the occurrence of lethal ventricular arrhythmias.

The finding that CsA exacerbated the hypertrophic response in a murine FHC model[65] has further complicated our understanding of cardiomyocyte Ca^{2+} homeostasis and Ca^{2+}/calmodulin-regulated signaling in the LV hypertrophy response.[91] Fatkin and coworkers observecd an increase in Ca^{2+} transient in wildtype myocytes, and, to a lesser extent, in myocytes from mice carrying

the FHC mutation. Pretreatment with diltiazem, a L-type Ca^{2+} channel blocker, prevented the wosening of the phenotype in mutant mice, suggesting that CsA infavourably modulated $I_{Ca,L}$ properties.[65] Latter interpretation appears to contrast their finding that CsA subtly increased the Ca^{2+} transient of αMyHC$^{403/+}$ myocytes. Moreover, Yatani and coworkers[88] failed to observe a response of CsA on L-type Ca^{2+} channel kinetics in wildtype myocytes, and Janssen and coworkers observed a reduction of the Ca^{2+} transient in human failing trabeculae following CsA administration.[90]

Nevertheless, latter study poses the interesting question whether blockade of calcineurin is of detriment to FHC-associated LV hypertrophy yet effective to reduce acquired forms of LV hypertrophy and dilated cardiomyopathies. The finding that CsA and FK506 proved to be highly effective in rescuing the cardiac phenotype of tropomodulin or MLC-2v transgenic mice,[24] while exacerbating the phenotype of αMyHC$^{403/+}$ animals suggests several possibilities. Alterations at the sarcomere may either induce cardiomyopathy through fundamentally distinct alterations in intracellular Ca^{2+} homeostasis, and/or these studies might have suffered from the short-comings of the particular animal model investigated, and/or the findings are related to calcineurin-unrelated effects of the drugs investigated. Genetic approaches to inhibit calcineurin in these and other models of FHC[92-96] may become helpful in answering these questions.

As a result of these uncertainties regarding momentous calcineurin activation, a major experimental problem relates to the current assessment of cardiac calcineurin activity. The calcineurin activity assay depends upon inclusion of several phosphatase inhibitors to reduce background phosphatase activity and enhance specificity, since different phosphatases besides calcineurin exist in cardiac tissue. One required inhibitor (okadaic acid) also partially inhibits calcineurin activity, leading to an inherent reduction of output. Furthermore, calcineurin is subject to rapid oxidation in vitro, which underscores the notion that calcineurin may be several folds more active in situ compared with purified protein extracts. Thus, regardless whether the complicated technical aspects of the assay are performed correctly, the relevance of the information it provides is still questionable. Other assays are required to objectively monitor instantaneous calcineurin activation in situ.

The identification of MCIP1, as part of a self-promoting negative feedback loop of calcineurin biology, may provide an indirect readout of calcineurin activation status. Before such an assay could be implemented, additional investigation is required to assess whether MCIP1 is specifically induced upon calcineurin (NFATc translocation) activation or whether it is a more general marker of hypertrophy status. Another approach could be to use the intragenic MCIP1 region, which contains an unusual dense cluster of NFAT consensus binding sites,[71] and link this to reporter genes to create transgenic models that provide a constant readout of (myocardial) calcineurin activity. Alternatively,

transgenic models with multimerized NFATc consensus binding sites linked to a reporter output[97] may provide an alternative to monitor the temporal dynamics of calcineurin activation during the progression of LV hypertrophy.

Studies on calcineurin involvement in failing human hearts further underscored the complex pathobiology of calcineurin. Human hypertrophic biopsies revealed that calcineurin activity (as measured by the enzymatic assay) correlated well with absolute calcineurin Aβ protein levels (as measured by immunoblot analyses).[98] It appears that in addition to an acute increase in enzymatic activity of pre-existing protein (Ca^{2+} activation), activity may also be subject to positive feedback mechanisms at the transcriptional and/or translational level. Therefore, future calcineurin assays may simply become based upon assessment of absolute myocardial calcineurin Aβ protein levels.

Calcineurin and cardiomyocyte viability

Mitochondria occupy an unusual large fraction of intracellular volume within adult cardiomyocytes. Multiple death signals impinge upon the mitochondrial membrane potential, resulting in hallmark apoptotic events such as loss of mitochondrial matrix components and activation of caspases. Since cardiomyocytes are limited in their ability to enter the cell cylce reentry, cummulative loss of single cardiomyocytes is regarded as a contributing factor in the genesis of human heart failure.[99] Given this, remarkably few studies have assessed whether calcineurin activity may influence muscle cell viability.

To test whether programmed cell death may contribute to the rapid transition from hypertrophy to overt heart failure in the calcineurin TG mice, calcineurin adenoviral infected cardiomyocytes were tested for their viability. Morphological analyses and TUNEL assays provided evidence that calcineurin activtion afforded protection against 2-deoxyglucose and staurosporine-mediated apoptosis. Moreover, endogenous calcineurin activation through PE stimulation[77] resulted in substantial protected cardiomyocytes from apoptosis, and this effect could be antagonized by targeted inhibition of calcineurin by adenoviral ΔCain gene transfer.[77] The mode of protection was found to be partially associated with NFATc translocation and protein kinase B (Akt) activation. In support of these findings in vitro, it was demonstrated that massively hypertrophic hearts from adult calcineurin TG animals displayed a remarkable level of resistance against ischemia/reperfusion-induced TUNEL laddering. Conversely, CsA treatment did not render cardiomyocytes more vulnerable towards programmed cell death, not even in the apoptosis prone Gqα-overexpressing, cardiomyopathic mouse model, suggesting that endogenous calcineurin activation promotes cardiomyocyte viability.[100] These findings were largely supported by a recent report from Kakita and colleagues, who were able to demonstrate that ET-1-mediated protection of

cardiomyocyte apoptosis requires calcineurin activation, since CsA and FK506 negated ET-1-induced protection against apoptotic cell death and expression of the anti-apoptotic factor Bcl-2.[101]

In contrast, Saito and co-workers demonstrated a 3-fold increase in calcineurin enzymatic activity following isoproteronol exposure concomitant with DNA fragmentation, while CsA and FK506 reversed this effect. TG animals expressing a dominant-negative mutant of calcineurin in the heart were resistant towards isoproteronol-induced cardiomyocyte apoptosis, suggesting that calcineurin may act downstream of catecholamine-induced apoptosis.[102]

Although these findings appear to be in contradiction with one another, it is known that calcineurin can activate opposing pathways that either suppress or induce apoptosis in the same cell type.[103] Indeed, Saito et al. reported that myocardial ischemia/reperfusion (oxidative stress) resulted in significantly more cardiomyocyte apoptosis in their dominant negative-calcineurin TG model than in wildtype littermates[102], supporting the findings of the initial two reports.[100] Conclusively, it seems reasonable to hypothesize that calcineurin-induced hypertrophy protects the heart from apoptotic death, depending upon the actual death signal and (sub)cellular context. The precise molecular mechanisms behind the anti-apoptotic properties of calcineurin are most complex and could well be associated with the initiation of the hypertrophic response itself.[104]

Calcineurin and (human) heart failure

The vast majority of reports to date have remained focussed upon assessing the involvement of calcineurin on the initiation and early progression phase of LV hypertrophy. Although justified in view of the intimate relationship between LV hypertrophy and progression of congestive heart failure, remarkably few studies have directly addressed the more clinically relevant question whether altering the balance of calcineurin activity directly alters the pathogenesis of (experimentally induced) cardiac failure.

Current experimental efforts have been limited to the demonstration that CsA prevents morphological remodeling in the tropomodulin or MLC-2v overexpressing mice with dilated cardiomyopathy,[26,27] and hemodynamic recordings of the rescued phenotypes are still lacking. In light of recent evidence that CsA has direct cardio-depressive actions on rabbit and human cardiomyocytes,[90] an assessment whether genetic calcineurin inhibition per se has negative inotropic/lusitropic effects in these and other valuable murine models of severe heart failure[105-109].

A recent study established the activation of calcineurin in biopsies of patients with hypertrophy secondary to hypertension ("compensatory hypertrophy") and patients with heart failure secondary to coronary artery disease

or idiopathic DCM. One interesting feature of the study is that both increased specific calcineurin activation and calcineurin Aβ protein was evident in patients with "compensatory hypertrophy", while calcineurin activation in the failing hearts seemed to be due to only a relative increase in protein content. These findings were supported by Lim and Molkentin, who reported increased can isoform expression in the failing human heart.[110] In contrast, Tsao and colleagues reported lower CnAβ mRNA levels in human failing hearts, but the probe used was directed against a minor splice isoform of gene of interest.[111]

Due to their substantial extra-cardiac side-effects in humans, the use of CsA or FK506 as treatment options for hypertrophic heart disease in patients is doubtful. In fact, long term CsA treatment is associated with renal toxicity and hypertension, which lead to cardiac hypertrophy in certain subjects. The concept that CsA or FK506 may have direct pro-cardiohypertrophic properties in humans[112] (and calcineurin inhibition associated with myocardial growth) is incorrect. More likely, systemic immunosuppression may induce hypertension in select subjects, which influences myocardial growth secondarily. In addition, the dose of CsA and FK506 required to prevent cardiac hypertrophy in experimental animals studies is approximately 10-fold higher than used to achieve immunosupression in humans, a phenomenon related to a higher myocardial calcineurin content, differential tissue accessibility or the higher metabolic rate of small rodents.

Integrated signal transduction: calcineurin, crosstalk and transcriptional events

Various reports suggest that multiple signaling networks play a role in hypertrophic remodeling.[113] For example, altering the balance of myocardial RSG4 expression, or cardiac introduction of a dominant inhibitory $G_{q\alpha}$ peptide or disruption of the JNK MAPK pathway have been demonstrated to impact on hypertrophic remodeling. How is it possible that inhibition of separate signaling networks can have such dramatic whole organ effects? Clearly, myocardial growth is not regulated by the additive activation of signaling modules, but by a more complex mechanism, involving the activation of multiple (intergrated) transduction pathways.

One aspect that undoubtedly contributes to the integration of cardiac signaling is the interdependence or crosstalk between parallel signaling pathways. We have previously demonstrated that hearts from calcineurin TG mice demonstrated robust JNK MAPK activation, and activation of several PKC isoforms. Adenoviral expression of calcineurin resulted in marked hypertrophy in cultured myocytes[87,100], and was prevented by JNK or PKC inhibition.[87] Also, CsA treatment of pressure overloaded rat hearts prevented PKCα, PKCθ and JNK MAPK activation.[87] Later studies supported this concept of interconnectiveness

between calcineurin, PKC and MAPK factors,[52,114] which coincide with the finding that PKCθ and calcineurin synergize to activate the JNK MAPK pathway in control of the interleukin-2 promoter in T-cells.[115] Collectively, these studies suggest the existence of a conserved, interconnected, regulatory circuit between calcineurin, PKC and JNK MAPK that controls myocardial growth, and it suggests that inhibition of either component may abrogate the entire integrated cascade.[87]

Some level of functional diversity and hierarchy among the multitude of hypertrophic pathways may still be anticipated. Transgenic lines expressing a constitutively activated mutant of the ERK1/2 MAPK-selective activator MEK1 display a physiological form of hypertrophy with increased contractility parameters throughout their complete life span,[116] while activation of another MAPK member, ERK5, results in pure eccentric hypertrophy and heart failure.[117] Transgenic overexpression of protein kinase Cβll (PKCβll) sufficed to result in juvenile lethality and cardiac hypertrophy/failure in mice,[118]but its targeted disruption failed to significantly abrogate pressure overload-induced myocardial growth,[119] suggesting that certain networks are not a required component of the hypertrophic response.

Given the above notions and data from contemporary literature, how should we define the role of calcineurin in cardiac hypertrophy? Given the massive hypertrophic response in calcineurin (and CaMK) TG animals, the overall success of CsA/FK506 studies to inhibit myocardial growth, and the recent demonstration that genetic calcineurin inhibition strategies successfully abrogate hypertrophic growth, it is difficult to dispute that calcineurin plays both a sufficient as well as an indispensable role in transducing hypertrophic signals following most pathophysiological stimuli. In addition, it seems reasonable to assume that calcineurin may be a crucial factor especially during the early phases of hypertrophic heart disease based upon the following notions. (1) The rise in intracellular Ca^{2+} in cardiomyocytes most probably constitutes an early phenomenon during the LV hypertrophy response[120,121] and (2) calcineurin activation was observed predominantly in hearts from patients in a compensatory hypertrophic phase and to a lesser extent in overtly failing patients.[98]

A more fundamental aspect of calcineurin signaling involves the contribution of downstream transcriptional effectors. NFATc4 (NFAT3) is still viewed upon as the critically effector downstream of myocardial calcineurin activation, based upon its ability to interact with GATA-4 and since NFATc4 TG animals display a massive hypertrophic phenotype.[11] However, given the overlapping expression patterns and redundant functions of single NFATc isoforms in other cell types, it is conceivable that some level of NFATc isoform redundancy may also exist in the heart. In that case, a simple genetic loss-of-function approach for each NFATc isoforms may prove to be insufficient to assess whether NFATc activity is the sole downstream transcriptional effector of calcineurin, due to the co-existence of up to four cardiac NFATc family members with largely overlapping functions. Recent evidence indeed suggests that at

least NFATc1 (NFATc) is also present in the ventricular myocyte, can associate with GATA-4 and responds to calcineurin activation, in a manner analogous to NFATc4. Dominant negative NFATc approaches that simultaneously abrogate NFATc function may become useful to circumvent such complications as recently demonstrated for T lymphocytes.[122] Alternative downstream transcriptional effectors may also be responsible for the nuclear events downstream of calcineurin. Recent evidence implicates myocyte enhancing factor-2 (MEF-2), nuclear factor-κB and Elk-1. [83,123]

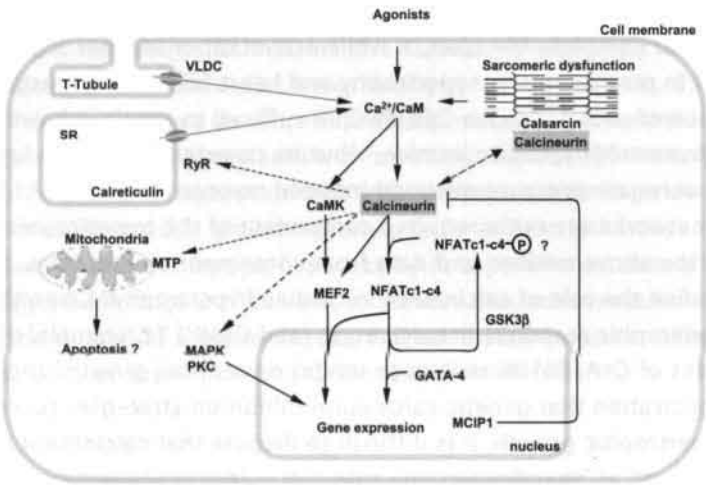


Figure 1.3. Contemporary model for (patho)physiological calcineurin signaling in the cardiac myocyte. Several stimuli can trigger activation of calcineurin, among which intrinsic defects of sarcomere function, multiple humoral factors (e.g. AngII, ET-1, LIF, PE), which directly or indirectly (by transient Ca^{2+} release from SR stores through ryanodine sensitive receptors (RyR) or through L-type Ca^{2+} channels (VLDC) in T-tubuli) elevate free cytosolic Ca^{2+}. The Ca^{2+}/calmodulin complex initiates parallel activation of intracellular signaling targets such as Calmodulin activated protein kinase (CaMK) isoforms, calcineurin, myosin light chain kinase (MLCK) or conventional protein kinase C (PKC) isoforms, each of which have been implicated in cardiomyocyte hypertrophy. Calcineurin differentially alters the activation status of certain members of the mitogen-activated protein kinase (MAPK) or PKC superfamilies, most probably as a reinforcement of cellular signaling. Activated calcineurin may recognize up to four nuclear factor of activated T-cell (NFAT) isoforms in the heart and, in addition, increases myocyte-enhancing factor (MEF2) activation status to direct hypertrophic gene expression remodeling. One particularly interesting genetic target of calcineurin is myocyte enriched calcineurin interacting protein-1 (MCIP1), which acts as a myocyte specific calcineurin inhibitor. Similarly, activition of glycogen synthase kinase 3β (GSK3β counteracts the activation status of NFAT isoforms induced by calcineurin. Calcineurin also positively influences the viability of cardiac muscle cells by effects on the mitochondrial permeability transition (MPT) and anti-apoptotic processes, probably depending upon the actual death signal. Finally, an underrepresented feature of calcineurin function concerns its localization. It has been reported to localize either in the cytosol or nucleus, associated with specialized anchoring proteins,[68] in the vicinity of the sarcoplasmatic reticulum (SR) or close to sarcomeres by its specialized docking protein calsarcin.[124]

Collectively, the present review provided an update on the role of calcineurin in the pathogenesis of cardiac hypertrophy. An emerging concept indicates that interference with distinct hypertrophic signaling pathways may significantly abrogate the LV hypertrophy response.[113] Therefore, future efforts in this field requires a further analyses to identify the key integrated cascades through which maladaptive hypertrophic signals are channeled to achieve the ultimate goal of a better treatment of heart failure. It appears that calcineurin will continue to be the focus of ongoing research, providing us with a platform to understand the complexity of Ca^{2+}/calmodulin signaling on cardiac morphology and function.

REFERENCES

1. Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP- FK506 complexes. Cell. 1991;66:807-15.
2. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T- lymphocyte activation. Nature. 1992;357:695-7.
3. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today. 1992;13:136-42.
4. Chang CD, Mukai H, Kuno T, Tanaka C. cDNA cloning of an alternatively spliced isoform of the regulatory subunit of Ca2+/calmodulin-dependent protein phosphatase (calcineurin B alpha 2). Biochim Biophys Acta. 1994;1217:174-80.
5. Ueki K, Muramatsu T, Kincaid RL. Structure and expression of two isoforms of the murine calmodulin- dependent protein phosphatase regulatory subunit (calcineurin B). Biochem Biophys Res Commun. 1992;187:537-43.
6. Zhang BW, Zimmer G, Chen J, Ladd D, Li E, Alt FW, Wiederrecht G, Cryan J, O'Neill EA, Seidman CE, Abbas AK, Seidman JG. T cell responses in calcineurin A alpha-deficient mice. J Exp Med. 1996;183:413-20.
7. Kayyali US, Zhang W, Yee AG, Seidman JG, Potter H. Cytoskeletal changes in the brains of mice lacking calcineurin A alpha. J Neurochem. 1997;68:1668-78.
8. Zhuo M, Zhang W, Son H, Mansuy I, Sobel RA, Seidman J, Kandel ER. A selective role of calcineurin A alpha in synaptic depotentiation in hippocampus. Proc Natl Acad Sci U S A. 1999;96:4650-5.
9. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol. 1997;15:707-47.
10. Lopez-Rodriguez C, Aramburu J, Rakeman AS, Rao A. NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. Proc Natl Acad Sci U S A. 1999;96:7214-9.
11. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998;93:215-28.
12. Hodge MR, Chun HJ, Rengarajan J, Alt A, Lieberson R, Glimcher LH. NF-AT-Driven interleukin-4 transcription potentiated by NIP45. Science. 1996;274:1903-5.
13. Ho IC, Hodge MR, Rooney JW, Glimcher LH. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell. 1996;85:973-83.
14. Rooney JW, Hoey T, Glimcher LH. Coordinate and cooperative roles for NF-AT and AP-1 in the regulation of the murine IL-4 gene. Immunity. 1995;2:473-83.
15. Cockerill PN, Shannon MF, Bert AG, Ryan GR, Vadas MA. The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. Proc Natl Acad Sci U S A. 1993;90:2466-70.
16. Okamura H, Aramburu J, Garcia-Rodriguez C, Viola JP, Raghavan A, Tahiliani M, Zhang X, Qin J, Hogan PG, Rao A. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol Cell. 2000;6:539-50.
17. Neal JW, Clipstone NA. Glycogen Synthase Kinase-3 Inhibits the DNA Binding Activity of NFATc. J Biol Chem. 2001;276:3666-3673.
18. Gomez del Arco P, Martinez-Martinez S, Maldonado JL, Ortega-Perez I,

Redondo JM. A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp. J Biol Chem. 2000;275:13872-8.
19. Chow CW, Rincon M, Cavanagh J, Dickens M, Davis RJ. Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. Science. 1997;278:1638-41.
20. Ranger AM, Grusby MJ, Hodge MR, Gravallese EM, de la Brousse FC, Hoey T, Mickanin C, Baldwin HS, Glimcher LH. The transcription factor NF-ATc is essential for cardiac valve formation. Nature. 1998;392:186-90.
21. de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, Potter J, Wakeham A, Marengere L, Langille BL, Crabtree GR, Mak TW. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature. 1998;392:182-6.
22. Graef IA, Chen F, Chen L, Kuo A, Crabtree GR. Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell. 2001;105:863-75.
23. Lim HW, De Windt LJ, Mante J, Kimball TR, Witt SA, Sussman MA, Molkentin JD. Reversal of cardiac hypertrophy in transgenic disease models by calcineurin inhibition. J Mol Cell Cardiol. 2000;32:697-709.
24. Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, Colbert MC, Gualberto A, Wieczorek DF, Molkentin JD. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science. 1998;281:1690-3.
25. Sussman MA, Baque S, Uhm CS, Daniels MP, Price RL, Simpson D, Terracio L, Kedes L. Altered expression of tropomodulin in cardiomyocytes disrupts the sarcomeric structure of myofibrils. Circ Res. 1998;82:94-105.
26. Sussman MA, Welch S, Cambon N, Klevitsky R, Hewett TE, Price R, Witt SA, Kimball TR. Myofibril degeneration caused by tropomodulin overexpression leads to dilated cardiomyopathy in juvenile mice. J Clin Invest. 1998;101:51-61.
27. Sussman MA, Welch S, Gude N, Khoury PR, Daniels SR, Kirkpatrick D, Walsh RA, Price RL, Lim HW, Molkentin JD. Pathogenesis of dilated cardiomyopathy: molecular, structural, and population analyses in tropomodulin-overexpressing transgenic mice. Am J Pathol. 1999;155:2101-13.
28. Fewell JG, Osinska H, Klevitsky R, Ng W, Sfyris G, Bahrehmand F, Robbins J. A treadmill exercise regimen for identifying cardiovascular phenotypes in transgenic mice. Am J Physiol. 1997;273:H1595-605.
29. Palermo J, Gulick J, Colbert M, Fewell J, Robbins J. Transgenic remodeling of the contractile apparatus in the mammalian heart. Circ Res. 1996;78:504-9.
30. Muthuchamy M, Boivin GP, Grupp IL, Wieczorek DF. Beta-tropomyosin overexpression induces severe cardiac abnormalities. J Mol Cell Cardiol. 1998;30:1545-57.
31. Muthuchamy M, Grupp IL, Grupp G, O'Toole BA, Kier AB, Boivin GP, Neumann J, Wieczorek DF. Molecular and physiological effects of overexpressing striated muscle beta-tropomyosin in the adult murine heart. J Biol Chem. 1995;270:30593-603.
32. Colbert MC, Hall DG, Kimball TR, Witt SA, Lorenz JN, Kirby ML, Hewett TE, Klevitsky R, Robbins J. Cardiac compartment-specific overexpression of a modified retinoic acid receptor produces dilated cardiomyopathy and congestive heart failure in transgenic mice. J Clin Invest. 1997;100:1958-68.
33. Faulds D, Goa KL, Benfield P. Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. Drugs. 1993;45:953-1040.
34. Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. Identification of

a putative regulator of early T cell activation genes. Science. 1988;241:202-5.
35. McCaffrey PG, Luo C, Kerppola TK, Jain J, Badalian TM, Ho AM, Burgeon E, Lane WS, Lambert JN, Curran T, et al. Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science. 1993;262:750-4.
36. Khanna AK, Cairns VR, Becker CG, Hosenpud JD. Transforming growth factor (TGF)-beta mimics and anti-TGF-beta antibody abrogates the in vivo effects of cyclosporine: demonstration of a direct role of TGF-beta in immunosuppression and nephrotoxicity of cyclosporine. Transplantation. 1999;67:882-9.
37. Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, Shimbo T, Suthanthiran M. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature. 1999;397:530-4.
38. Hughes PE, Alexi T, Walton M, Williams CE, Dragunow M, Clark RG, Gluckman PD. Activity and injury-dependent expression of inducible transcription factors, growth factors and apoptosis-related genes within the central nervous system. Prog Neurobiol. 1999;57:421-50.
39. Zhang D, Gaussin V, Taffet GE, Belaguli NS, Yamada M, Schwartz RJ, Michael LH, Overbeek PA, Schneider MD. TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. Nat Med. 2000;6:556-63.
40. Matsuda S, Shibasaki F, Takehana K, Mori H, Nishida E, Koyasu S. Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation. EMBO Rep. 2000;1:428-34.
41. Kreideweiss S, Ahlers C, Nordheim A, Ruhlmann A. Ca2+-induced p38/SAPK signalling inhibited by the immunosuppressant cyclosporin A in human peripheral blood mononuclear cells. Eur J Biochem. 1999;265:1075-84.
42. Park KS, Kim TK, Kim DH. Cyclosporin A treatment alters characteristics of Ca2+-release channel in cardiac sarcoplasmic reticulum. Am J Physiol. 1999;276:H865-72.
43. Mijares A, Malecot CO, Peineau N, Argibay JA. In vivo and in vitro inhibition of the L-type calcium current in isolated guinea-pig cardiomyocytes by the immunosuppressive agent cyclosporin A. J Mol Cell Cardiol. 1997;29:2067-76.
44. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, Marks AR. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. Cell. 2000;101:365-76.
45. Shou W, Aghdasi B, Armstrong DL, Guo Q, Bao S, Charng MJ, Mathews LM, Schneider MD, Hamilton SL, Matzuk MM. Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. Nature. 1998;391:489-92.
46. Zhu W, Zou Y, Shiojima I, Kudoh S, Aikawa R, Hayashi D, Mizukami M, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I. Ca2+/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. J Biol Chem. 2000;275:15239-45.
47. Kato T, Sano M, Miyoshi S, Sato T, Hakuno D, Ishida H, Kinoshita-Nakazawa H, Fukuda K, Ogawa S. Calmodulin kinases II and IV and calcineurin are involved in leukemia inhibitory factor-induced cardiac hypertrophy in rats. Circ Res. 2000;87:937-45.
48. Mende U, Kagen A, Cohen A, Aramburu J, Schoen FJ, Neer EJ. Transient cardiac expression of constitutively active Galphaq leads to hypertrophy and dilated cardiomyopathy by calcineurin-dependent and independent pathways. Proc Natl Acad Sci U S A. 1998;95:13893-8.

49. Shimoyama M, Hayashi D, Takimoto E, Zou Y, Oka T, Uozumi H, Kudoh S, Shibasaki F, Yazaki Y, Nagai R, Komuro I. Calcineurin plays a critical role in pressure overload-induced cardiac hypertrophy. Circulation. 1999;100:2449-54.
50. Lim HW, De Windt LJ, Steinberg L, Taigen T, Witt SA, Kimball TR, Molkentin JD. Calcineurin expression, activation, and function in cardiac pressure- overload hypertrophy. Circulation. 2000;101:2431-7.
51. Hill JA, Karimi M, Kutschke W, Davisson RL, Zimmerman K, Wang Z, Kerber RE, Weiss RM. Cardiac hypertrophy is not a required compensatory response to short- term pressure overload. Circulation. 2000;101:2863-9.
52. Murat A, Pellieux C, Brunner HR, Pedrazzini T. Calcineurin blockade prevents cardiac mitogen-activated protein kinase activation and hypertrophy in renovascular hypertension [In Process Citation]. J Biol Chem. 2000;275:40867-73.
53. Sakata Y, Masuyama T, Yamamoto K, Nishikawa N, Yamamoto H, Kondo H, Ono K, Otsu K, Kuzuya T, Miwa T, Takeda H, Miyamoto E, Hori M. Calcineurin inhibitor attenuates left ventricular hypertrophy, leading to prevention of heart failure in hypertensive rats. Circulation. 2000;102:2269-75.
54. Mervaala E, Muller DN, Park JK, Dechend R, Schmidt F, Fiebeler A, Bieringer M, Breu V, Ganten D, Haller H, Luft FC. Cyclosporin A protects against angiotensin II-induced end-organ damage in double transgenic rats harboring human renin and angiotensinogen genes. Hypertension. 2000;35:360-6.
55. Eto Y, Yonekura K, Sonoda M, Arai N, Sata M, Sugiura S, Takenaka K, Gualberto A, Hixon ML, Wagner MW, Aoyagi T. Calcineurin is activated in rat hearts with physiological left ventricular hypertrophy induced by voluntary exercise training. Circulation. 2000;101:2134-7.
56. Oie E, Bjornerheim R, Clausen OP, Attramadal H. Cyclosporin A inhibits cardiac hypertrophy and enhances cardiac dysfunction during postinfarction failure in rats. Am J Physiol Heart Circ Physiol. 2000;278:H2115-23.
57. Boluyt MO, Zheng JS, Younes A, Long X, O'Neill L, Silverman H, Lakatta EG, Crow MT. Rapamycin inhibits alpha 1-adrenergic receptor-stimulated cardiac myocyte hypertrophy but not activation of hypertrophy-associated genes. Evidence for involvement of p70 S6 kinase. Circ Res. 1997;81:176-86.
58. Muller JG, Nemoto, S., Laser, M., Carabello, B.A., Menick, D.R. Calcineurin inhibition and cardiac hypertrophy. Science. 1998;282:1007a.
59. Ding B, Price RL, Borg TK, Weinberg EO, Halloran PF, Lorell BH. Pressure overload induces severe hypertrophy in mice treated with cyclosporine, an inhibitor of calcineurin [see comments]. Circ Res. 1999;84:729-34.
60. Meguro T, Hong C, Asai K, Takagi G, McKinsey TA, Olson EN, Vatner SF. Cyclosporine attenuates pressure-overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. Circ Res. 1999;84:735-40.
61. Luo Z, Shyu KG, Gualberto A, Walsh K. Calcineurin inhibitors and cardiac hypertrophy [letter]. Nat Med. 1998;4:1092-3.
62. Zhang W, Kowal RC, Rusnak F, Sikkink RA, Olson EN, Victor RG. Failure of calcineurin inhibitors to prevent pressure-overload left ventricular hypertrophy in rats [see comments]. Circ Res. 1999;84:722-8.
63. Hayashida W, Kihara Y, Yasaka A, Sasayama S. Cardiac calcineurin during transition from hypertrophy to heart failure in rats. Biochem Biophys Res Commun. 2000;273:347-51.
64. Geisterfer-Lowrance AA, Christe M, Conner DA, Ingwall JS, Schoen FJ, Seidman CE, Seidman JG. A mouse model of familial hypertrophic cardiomyopathy.

Science. 1996;272:731-4.
65. Fatkin D, McConnell BK, Mudd JO, Semsarian C, Moskowitz IG, Schoen FJ, Giewat M, Seidman CE, Seidman JG. An abnormal Ca(2+) response in mutant sarcomere protein-mediated familial hypertrophic cardiomyopathy. J Clin Invest. 2000;106:1351-9.
66. Wiesner RJ, Ehmke H, Faulhaber J, Zak R, Ruegg JC. Dissociation of left ventricular hypertrophy, beta-myosin heavy chain gene expression, and myosin isoform switch in rats after ascending aortic stenosis. Circulation. 1997;95:1253-9.
67. Shimoyama M, Hayashi D, Zou Y, Takimoto E, Mizukami M, Monzen K, Kudoh S, Hiroi Y, Yazaki Y, Nagai R, Komuro I. Calcineurin inhibitor attenuates the development and induces the regression of cardiac hypertrophy in rats with salt-sensitive hypertension [In Process Citation]. Circulation. 2000;102:1996-2004.
68. Coghlan VM, Perrino BA, Howard M, Langeberg LK, Hicks JB, Gallatin WM, Scott JD. Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science. 1995;267:108-11.
69. Fuentes JJ, Genesca L, Kingsbury TJ, Cunningham KW, Perez-Riba M, Estivill X, de la Luna S. DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin- mediated signaling pathways. Hum Mol Genet. 2000;9:1681-90.
70. Rothermel B, Vega RB, Yang J, Wu H, Bassel-Duby R, Williams RS. A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. J Biol Chem. 2000;275:8719-25.
71. Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, Bassel-Duby R, Williams RS. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res. 2000;87: E61-8.
72. Youn HD, Grozinger CM, Liu JO. Calcium regulates transcriptional repression of myocyte enhancer factor 2 by histone deacetylase 4. J Biol Chem. 2000;275:22563-7.
73. Sun L, Youn HD, Loh C, Stolow M, He W, Liu JO. Cabin 1, a negative regulator for calcineurin signaling in T lymphocytes. Immunity. 1998;8:703-11.
74. Lai MM, Burnett PE, Wolosker H, Blackshaw S, Snyder SH. Cain, a novel physiologic protein inhibitor of calcineurin. J Biol Chem. 1998;273:18325-31.
75. Lin X, Sikkink RA, Rusnak F, Barber DL. Inhibition of calcineurin phosphatase activity by a calcineurin B homologous protein. J Biol Chem. 1999;274:36125-31.
76. Lin X, Barber DL. A calcineurin homologous protein inhibits GTPase-stimulated Na-H exchange. Proc Natl Acad Sci U S A. 1996;93:12631-6.
77. Taigen T, De Windt LJ, Lim HW, Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci U S A. 2000;97:1196-201.
78. De Windt LJ, Lim HW, Bueno OF, Liang Q, Delling U, Braz JC, Glascock BJ, Kimball TF, del Monte F, Hajjar RJ, Molkentin JD. Targeted inhibition of calcineurin attenuates cardiac hypertrophy invivo. Proc Natl Acad Sci U S A. 2001;98:3322-3327.
79. Choukroun G, Hajjar R, Fry S, del Monte F, Haq S, Guerrero JL, Picard M, Rosenzweig A, Force T. Regulation of cardiac hypertrophy in vivo by the stress-activated protein kinases/c-Jun NH(2)-terminal kinases. J Clin Invest. 1999;104:391-8.
80. Zou Y, Hiroi Y, Uozumi H, Takimoto E, Toko H, Zhu W, Kudoh S, Mizukami

M, Shimoyama M, Shibasaki F, Nagai R, Yazaki Y, Komuro I. Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. Circulation. 2001;104:97-101.
81. Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN, Williams RS. Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A. 2001;98:3328-33.
82. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature. 1997;386:855-8.
83. Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR, Olson EN. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo [comment]. J Clin Invest. 2000;105:1395-406.
84. Murata M, Fukuda K, Ishida H, Miyoshi S, Koura T, Kodama H, Nakazawa HK, Ogawa S. Leukemia inhibitory factor, a potent cardiac hypertrophic cytokine, enhances L-type Ca2+ current and [Ca2+]i transient in cardiomyocytes. J Mol Cell Cardiol. 1999;31:237-45.
85. Cameron AM, Steiner JP, Roskams AJ, Ali SM, Ronnett GV, Snyder SH. Calcineurin associated with the inositol 1,4,5-trisphosphate receptor- FKBP12 complex modulates Ca2+ flux. Cell. 1995;83:463-72.
86. Koide M, Obata K, Iio A, Iida M, Harayama H, Yokota M, Tuan RS. Function of FK506 binding protein (FKBP) in chick embryonic cardiac development. Heart Vessels. 1997;Suppl:7-9.
87. De Windt LJ, Lim HW, Haq S, Force T, Molkentin JD. Calcineurin promotes protein kinase C and c-Jun NH2-terminal kinase activation in the heart. Cross-talk between cardiac hypertrophic signaling pathways. J Biol Chem. 2000;275:13571-9.
88. Yatani A, Honda R, Tymitz KM, Lalli MJ, Molkentin JD. Enhanced Ca2+ channel currents in cardiac hypertrophy induced by activation of calcineurin-dependent pathway. J Mol Cell Cardiol. 2001;33:249-59.
89. Armstrong DL. Calcium channel regulation by calcineurin, a Ca2+-activated phosphatase in mammalian brain. Trends Neurosci. 1989;12:117-22.
90. Janssen PM, Zeitz O, Keweloh B, Siegel U, Maier LS, Barckhausen P, Pieske B, Prestle J, Lehnart SE, Hasenfuss G. Influence of cyclosporine A on contractile function, calcium handling, and energetics in isolated human and rabbit myocardium. Cardiovasc Res. 2000;47:99-107.
91. Frey N, Olson EN. Making matters worse for a broken heart. J Clin Invest. 2000;106:1437-9.
92. James J, Zhang Y, Osinska H, Sanbe A, Klevitsky R, Hewett TE, Robbins J. Transgenic modeling of a cardiac troponin I mutation linked to familial hypertrophic cardiomyopathy. Circ Res. 2000;87:805-11.
93. Sanbe A, Fewell JG, Gulick J, Osinska H, Lorenz J, Hall DG, Murray LA, Kimball TR, Witt SA, Robbins J. Abnormal cardiac structure and function in mice expressing nonphosphorylatable cardiac regulatory myosin light chain 2. J Biol Chem. 1999;274:21085-94.
94. Sanbe A, Nelson D, Gulick J, Setser E, Osinska H, Wang X, Hewett TE, Klevitsky R, Hayes E, Warshaw DM, Robbins J. In vivo analysis of an essential myosin light chain mutation linked to familial hypertrophic cardiomyopathy. Circ Res. 2000;87:296-302.
95. Tardiff JC, Hewett TE, Palmer BM, Olsson C, Factor SM, Moore RL, Robbins J, Leinwand LA. Cardiac troponin T mutations result in allele-specific

phenotypes in a mouse model for hypertrophic cardiomyopathy. J Clin Invest. 1999;104:469-81.
96. Yang Q, Sanbe A, Osinska H, Hewett TE, Klevitsky R, Robbins J. A mouse model of myosin binding protein C human familial hypertrophic cardiomyopathy. J Clin Invest. 1998;102:1292-300.
97. Huang C, Mattjus P, Ma WY, Rincon M, Chen NY, Brown RE, Dong Z. Involvement of nuclear factor of activated T cells activation in UV response. Evidence from cell culture and transgenic mice. J Biol Chem. 2000;275:9143-9.
98. Haq S, Choukroun G, Lim H, Tymitz KM, del Monte F, Gwathmey J, Grazette L, Michael A, Hajjar R, Force T, Molkentin JD. Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. Circulation. 2001;103:670-7.
99. Chien KR. Genomic circuits and the integrative biology of cardiac diseases. Nature. 2000;407:227-32.
100. De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW, 2nd, Kitsis RN, Molkentin JD. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: An apoptosis-independent model of dilated heart failure. Circ Res. 2000;86:255-63.
101. Kakita T, Hasegawa K, Iwai-Kanai E, Adachi S, Morimoto T, Wada H, Kawamura T, Yanazume T, Sasayama S. Calcineurin Pathway Is Required for Endothelin-1-Mediated Protection Against Oxidant Stress-Induced Apoptosis in Cardiac Myocytes. Circ Res. 2001;88:1239-1246.
102. Saito S, Hiroi Y, Zou Y, Aikawa R, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I. beta -adrenergic pathway induces apoptosis through calcineurin activation in cardiac myocytes. J Biol Chem. 2000;275:34528-33.
103. Lotem J, Kama R, Sachs L. Suppression or induction of apoptosis by opposing pathways downstream from calcium-activated calcineurin. Proc Natl Acad Sci U S A. 1999;96:12016-20.
104. Molkentin JD. Calcineurin, Mitochondrial Membrane Potential, and Cardiomyocyte Apoptosis. Circ Res. 2001;88:1220-1222.
105. Arber S, Hunter JJ, Ross J, Jr., Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell. 1997;88:393-403.
106. McConnell BK, Jones KA, Fatkin D, Arroyo LH, Lee RT, Aristizabal O, Turnbull DH, Georgakopoulos D, Kass D, Bond M, Niimura H, Schoen FJ, Conner D, Fischman DA, Seidman CE, Seidman JG, Fischman DH. Dilated cardiomyopathy in homozygous myosin-binding protein-C mutant mice. J Clin Invest. 1999;104:1235-44.
107. Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, Demetris AJ, Feldman AM. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. Circ Res. 1997;81:627-35.
108. Sakata Y, Hoit BD, Liggett SB, Walsh RA, Dorn GW, 2nd. Decompensation of pressure-overload hypertrophy in G alpha q- overexpressing mice. Circulation. 1998;97:1488-95.
109. Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB, Chien KR, Brown JH, Dorn GW, 2nd. Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. Proc Natl Acad Sci U S A. 1998;95:10140-5.
110. Lim HW, Molkentin JD. Calcineurin and human heart failure [letter]. Nat Med. 1999;5:246-7.

111. Tsao L, Neville C, Musaro A, McCullagh KJ, Rosenthal N. Revisiting calcineurin and human heart failure. Nat Med. 2000;6:2-3.
112. Lorell BH, Carabello BA. Left ventricular hypertrophy: pathogenesis, detection, and prognosis. Circulation. 2000;102:470-9.
113. Molkentin JD, Dorn IG, 2nd. Cytoplasmic signaling pathways that regulate cardiac hypertrophy. Annu Rev Physiol. 2001;63:391-426.
114. Zou Y, Yao A, Zhu W, Kudoh S, Hiroi Y, Shimoyama M, Uozumi H, Kohmoto O, Takahashi T, Shibasaki F, Nagai R, Yazaki Y, Komuro I. Isoproterenol activates extracellular signal-regulated protein kinases in cardiomyocytes through calcineurin. Circulation. 2001;104:102-8.
115. Werlen G, Jacinto E, Xia Y, Karin M. Calcineurin preferentially synergizes with PKC-theta to activate JNK and IL-2 promoter in T lymphocytes. Embo J. 1998;17:3101-11.
116. Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN, Molkentin JD. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. Embo J. 2000;19:6341-50.
117. Nicol RL, Frey N, Pearson G, Cobb M, Richardson J, Olson EN. Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. Embo J. 2001;20:2757-67.
118. Bowman JC, Steinberg SF, Jiang T, Geenen DL, Fishman GI, Buttrick PM. Expression of protein kinase C beta in the heart causes hypertrophy in adult mice and sudden death in neonates. J Clin Invest. 1997;100:2189-95.
119. Roman BB, Geenen DL, Leitges M, Buttrick PM. PKC-beta is not necessary for cardiac hypertrophy. Am J Physiol Heart Circ Physiol. 2001;280:H2264-70.
120. Perreault CL, Meuse AJ, Bentivegna LA, Morgan JP. Abnormal intracellular calcium handling in acute and chronic heart failure: role in systolic and diastolic dysfunction. Eur Heart J. 1990;11 Suppl C:8-21.
121. Bentivegna LA, Ablin LW, Kihara Y, Morgan JP. Altered calcium handling in left ventricular pressure-overload hypertrophy as detected with aequorin in the isolated, perfused ferret heart. Circ Res. 1991;69:1538-45.
122. Chow CW, Rincon M, Davis RJ. Requirement for transcription factor NFAT in interleukin-2 expression. Mol Cell Biol. 1999;19:2300-7.
123. Wu H, Naya FJ, McKinsey TA, Mercer B, Shelton JM, Chin ER, Simard AR, Michel RN, Bassel-Duby R, Olson EN, Williams RS. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. Embo J. 2000;19:1963-73.
124. Frey N, Richardson JA, Olson EN. Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. Proc Natl Acad Sci U S A. 2000;97:14632-7.

(Footnotes)

[1] OF Bueno and JD Molkentin. Unpublished observations.

Chapter 2

MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function following myocardial infarction

Eva van Rooij, Pieter A. Doevendans, Harry J.M.G. Crijns, Sylvia Heeneman, Daniel J. Lips, Marc van Bilsen, R. Sanders Williams, Eric N. Olson, Rhonda Bassel-Duby, Beverly A. Rothermel, and Leon J. De Windt

SUMMARY

Pathological remodeling of the left ventricle (LV) following myocardial infarction (MI) is a major cause of heart failure. Although cardiac hypertrophy following increased loading conditions has been recognized as a clinical risk factor for human heart failure, it is unknown whether post-MI hypertrophic remodeling of the myocardium is beneficial for cardiac function over time, nor which regulatory pathways play a crucial role in this process. To address these questions, transgenic (TG) mice engineered to overexpress modulatory calcineurin-interacting protein-1 (MCIP1) in the myocardium were used to achieve cardiac-specific inhibition of calcineurin activation. MCIP1-TG mice and their wildtype (WT) littermates, were subjected to MI and analyzed four weeks later. At 4 weeks after MI, calcineurin was activated in the LV of WT mice, which was significantly reduced in MCIP1-TG mice. WT mice displayed a 78% increase in LV mass following MI, which was reduced by 38% in MCIP-TG mice. Echocardiography indicated marked LV dilation and loss of systolic function in WT-MI mice, whereas TG-MI mice displayed a remarkable preservation of LV geometry and contractility, a pronounced reduction in myofiber hypertrophy, collagen deposition and β-MyHC expression compared to WT-MI mice. Together, these results reveal a protective role for MCIP1 in the post-MI heart and suggest that calcineurin is a crucial regulator of postinfarction-induced pathological LV remodeling. The improvement in functional, structural and molecular abnormalities in MCIP1-TG mice challenges the adaptive value of post-MI hypertrophy of the remote myocardium.

INTRODUCTION

Despite significant progress in the prevention and treatment of cardiovascular disease in the United States in the past two decades, statistics indicate that the incidence and prevalence of heart failure have been increasing steadily, especially in the elderly. Chronic heart failure affects 4.8 million Americans and is the leading cause of hospitalization for people aged 65 years and over. Despite improved medical treatment and intense investigation, heart failure is a leading cause of morbidity and mortality in industrial countries.[1,2]

A fundamental shift in the underlying etiology of heart failure is becoming evident, in which the most common cause of chronic heart failure is no longer hypertension or valvular disease, but rather coronary artery disease (CAD) and long term survival after myocardial infarction (MI).[1] Unfortunately, the prognosis of patients with heart failure and CAD is considerably worse compared to heart failure without CAD.[1] MI induces scar formation and global changes in surviving myocardium, designated post-MI ventricular remodeling. This process consists of an initial wall thinning of the infarcted area, ventricular chamber dilation, side-to-side slippage and eccentric myocyte hypertrophy of the individual myofibers in the noninfarcted (remote) portion of the myocardium.[3] In spite of clinical evidence that the postinfarcted heart often progressively dilates and displays accelerated deterioration of left ventricular function, the early hypertrophic remodeling of the viable portion of the left ventricle (LV) following ischemic damage is considered an adaptive response that compensates for the acute loss of functional myocardium, hence preserving cardiac performance.[4]

Sustained cardiac hypertrophy has been recognized as the single most important risk factor for heart failure development, at least in conditions with increased load such as chronic hypertension or valvular disease, and a powerful predictor for cardiovascular morbidity and mortality.[5] One signaling pathway that links extracellular stimuli to a hypertrophic transcriptional response of the myocyte employs the Ca^{2+}-calmodulin-dependent phosphatase calcineurin and its downstream transcriptional effector Nuclear Factor of Activated T-cells (NFAT).[6,7] Pressure overload hypertrophy studies using genetic mouse models that prevent calcineurin-NFAT activation have confirmed its crucial role in this form of hypertrophic remodeling.[8-11] Whether calcineurin is involved in myocyte hypertrophy post-MI, or whether inhibition of this type of hypertrophic remodeling may have therapeutic potential, remains uncertain. To address these questions, we pursued a genetic approach to evaluate the functional significance of calcineurin in post-MI cardiac remodeling, using a genetic mouse model with mild myocyte-restricted overexpression of MCIP1, which was previously shown to efficiently antagonize cardiac hypertrophy in response to an activated calcineurin transgene, following pressure overload or β-adrenergic stimulation.[10,12] Accordingly, MCIP1-TG mice and their wildtype littermates were randomized to receive either a large

transmural LV infarct or sham operation. We confirmed increased calcineurin activation and substantial hypertrophy in the LV four weeks after MI in WT mice, while TG mice were protected against both endpoints. Remarkably, MCIP1-TG mice also demonstrated preservation of LV geometry and contractility, improved survival, a diminution of β-MyHC expression, and a pronounced reduction in interstitial collagen deposition in the remote myocardium. These findings suggest that calcineurin activation is crucial for maladaptive post-MI remodeling.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

All protocols were approved by institutional guidelines. All surgeries and subsequent analyses were performed in a blinded fashion for genotype. Eight-week old mice from both sexes carrying the human MCIP1 transgene under the control of the αMyHC promoter (an order of magnitude overexpression of MCIP1 at the mRNA level) in a C57BL/6 background (TG)[10] and their non-transgenic littermates (WT) were anesthetized with 2.4% isoflurane and placed in a supine position on a heating pad (37°C). Animals were intubated ventilated with room air using a MiniVent mouse ventilator (Hugo Sachs Elektronik, Germany; stroke volume 250 µl, respiratory rate 210 breaths per minute). MI was induced by permanent ligation of the left coronary artery (LCA) with a 6-0 prolene suture. Occlusion of the LCA was confirmed under a dissecting microscope (Olympus SZ30, Japan) by discoloration of the ischemic area. Sham operated animals underwent the same procedure without occlusion of the LCA.

Transthoracic echocardiography

Three days and four weeks following MI, the mice were re-anesthetized with 2.4% isoflurane and placed on a heating pad (37°C). Echocardiography was performed using a Sonos 5500 ultrasound machine (Hewlett-Packard) with a 12 MHz transducer applied parasternally to the shaved chest wall. For optimal resolution, the transducer was covered with a surgical latex finger filled with ultrasound transmission gel to obtain a standoff of 0.5-0.7 cm and used at a depth setting of 2 cm. The transducer was maneuvered to obtain both live 2-dimensional images in a parasternal long-axis and short axis view and 2D guided M-mode tracings crossing the anterior wall, LV cavity and posterior wall. Images were analyzed offline using EnConcert Image Diagnosis Application (Philips, Netherlands). All parameters were averaged from 4 cardiac cycles.

Invasive pressure measurement

After the animals were anesthetized with urethane, a 1.4F high-fidelity micromanometer catheter (Millar Instruments) was introduced into the right carotid artery, advanced into the left ventricle, and pressure measurements were performed as described previously.[13]

Northern blot analysis

Northern blot analysis for MCIP1 was performed with 20 µg of total RNA in each lane and probed in Ultrahyb (Ambion, Austin, TX) with a ^{32}P-labeled DNA fragment encompassing the exon 4, 5' splice variant of murine MCIP1 (Radprime, Invitrogen). Northern blot analysis for ANF, β-MyHC and 18S was performed as previously described.[14] Signals were detected using a phosphor imaging screen

(Biorad), and quantified with Quantity 1 software (Biorad).

Immunoprecipitation and Western blot analysis

To detect LV calcineurin Aβ abundance, 500 µg LV extract was immunoprecipitated with a pan-calcineurin A subunit antibody (AB1695, Chemicon), and probed with an anti-calcineurin Aβ antibody (sc-6124, Santa Cruz). To analyze the phosphorylation status of NFAT, 500 µg of LV protein extract was immunoprecipitated with either anti-NFATc1 (sc-13033, Santa Cruz) or anti-NFATc3 (sc-8321, Santa Cruz), and Western blotted with an anti-phospho-serine antibody (61-8100, Zymed). Immunoreactivity was detected with Chemiluminescence (ECL, Amersham Pharmacia Biotech).

Calcineurin activity assay

Phosphatase activity was measured using the calcineurin (PP2B) Assay kit (cat # 20700, Calbiochem) according to the manufacturer's instructions. Calcineurin activity was measured as the dephosphorylation rate of a synthetic phoshopeptide substrate (RII peptide).

Histological analysis

Four weeks after MI all animals were anesthetized, the hearts were arrested in diastole and perfused antegradely at physiologic pressures with PBS containing 1 mg/ml sodium nitroprusside and 5% formalin. Heart tissue was fixed in 3.7% formaldehyde, cut either (1) longitudinally and perpendicular to the infarcted area and aortic root, or (2) transversally, and embedded in paraffin. Four µm sections were cut and stained with AZAN, laminin, haematoxylin and eosin (H&E), or Sirius red.[15] Longitudal AZAN stained sections were used to determine infarct size. A computerized morphometric system (Quantimet 570, Leica, The Netherlands) was utilized to calculate the percentage infarcted tissue of total left ventricular tissue. Laminin and Sirius red stained sections were used to determine myocyte hypertrophy or to visualize interstitial/perivascular collagen amount, respectively. Myocyte fiber size was assessed by digital surface measurement of approximately 300 cells per animal in 4-6 animals per group. Only myofibers with a centrally positioned nucleus were included. The amount of collagen was determined as the percentage of left ventricular tissue stained positive for Sirius red. Since the presence of collagen was more intense towards the apex, measurements were presented for both the whole septum and the apical halve of the septal wall.

Statistical Analysis

The results are presented as means ± SEM. Statistical analyses were performed by using INSTAT 3.0 software (GraphPad, San Diego) and ANOVA followed by Tukey's post-test when appropriate. Statistical significance was accepted at a P value < 0.05.

MCIP1 overexpression attenuates postinfarction-induced calcineurin activation

After MI, the heart undergoes an adaptive response that is accompanied by eccentric hypertrophy of the noninfarcted myocardium. Although calcineurin is activated and intimately involved in pressure overload-induced hypertrophy,[8,9,12,16] it is uncertain whether calcineurin is also activated during postinfarction remodeling.

To explore this, calcineurin (PP2B) activity was first measured as the dephosphorylation rate of a synthetic phosphopeptide substrate. Calcineurin activity was elevated by 35% in the remote myocardium of WT-MI mice ($P < 0.05$). In contrast, total calcineurin activity was decreased in TG-MI hearts by 26% compared to WT-MI (Figure 2.1A).

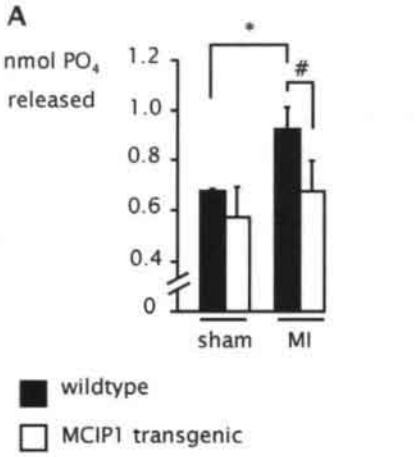


Fig. 2.1A Cardiac extracts for the indicated groups were assayed for Cn activity, expressed as nmol released phosphate. MCIP1 transgenic mice displayed lower Cn activity both under basal conditions and following infarction. Data are expressed as means ± SEM from n = 3-5 animals per group. * $P < 0.05$ vs. sham operated group, # $P < 0.05$ vs. WT-MI group.

LV calcineurin Aβ abundance has previously been shown to correlate with LV PP2B activity.[17] Calcineurin Aβ immunoreactivity was increased by 19% in WT-MI compared to the corresponding sham group ($P < 0.05$), while this increase amounted to only 6% TG-MI animals (N.S.; Figure 2.1B).

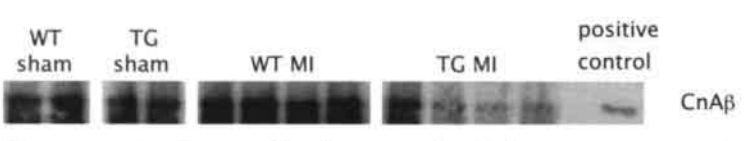


Fig. 2.1B Representative Western blot demonstrating CnAβ expression for indicated groups.

Since activated calcineurin dephosphorylates members of the NFAT family, which in turn drive expression of the MCIP1 exon 4 splice isoform via an upstream cluster of NFAT binding sites,[18] the phosphorylation status of two cardiac NFAT members and expression level of the MCIP1 exon 4 splice isoforms was determined. As expected, endogenous NFATc1 and NFATc3 was found to be in a hyperphosphorylated state in both sham groups, consistent with relatively low calcineurin activity. In contrast, the levels of both phospho-NFATc1 as well as NFATc3 decreased by 51% and 16% ($P < 0.05$) in WT-MI mice, respectively, consistent with relatively higher endogenous PP2B activity. In contrast, the levels of phosphorylated NFATc1 and NFATc3 in TG-MI mice revealed a less pronounced decrease by 16% ($P < 0.05$) and 1% (N.S.), respectively (Figure 2.1C), suggestive of calcineurin inhibition.

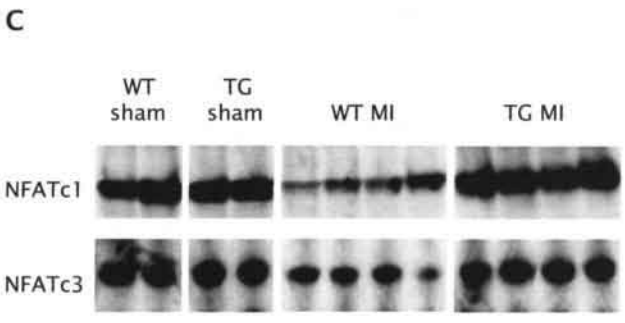


Fig. 2.1C Representative Western blot using an antibody against phospho-serine for the level of hyperphosphorylated NFATc1 and NFATc3. The data demonstrate decreased phospho-NFAT levels following MI in wildtype mice, while MCIP1 transgenic mice have less pronounced decreases in phospho-NFAT levels compared to corresponding sham group.

Expression of the MCIP1 exon 4 isoform provides an additional indication of myocardial calcineurin activity in vivo (see discussion). The MCIP1 exon 4 specific probe was designed to the untranslated 5' region of the endogenous mouse 2.2 kb mRNA, and therefore does not detect transcripts from the MCIP1 transgene. Northern blot analysis indicated an approximate 9-fold induction of the 5' exon 4 splice variant of MCIP1 expression after MI in WT hearts, while in TG-MI hearts, only a 2-fold induction was observed (Figure 2.1D and discussion). Collectively, these results indicate that calcineurin is activated in the LV following MI, and that MCIP1 overexpression efficiently counteracts the activation characteristics of cardiac calcineurin

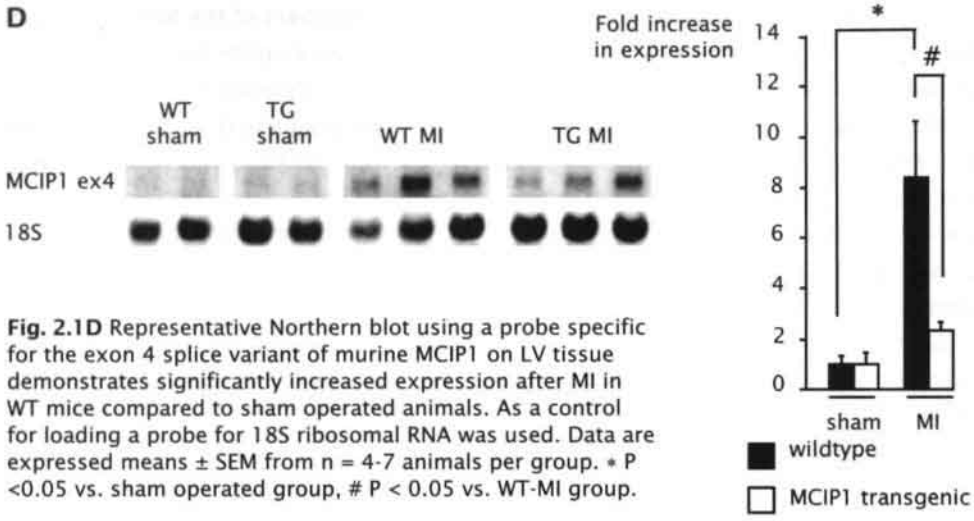


Fig. 2.1D Representative Northern blot using a probe specific for the exon 4 splice variant of murine MCIP1 on LV tissue demonstrates significantly increased expression after MI in WT mice compared to sham operated animals. As a control for loading a probe for 18S ribosomal RNA was used. Data are expressed means ± SEM from n = 4-7 animals per group. * P <0.05 vs. sham operated group, # P < 0.05 vs. WT-MI group.

MCIP1 overexpression attenuates LV hypertrophy after MI

No significant difference in infarct size was observed between MCIP1-TG animals and their non-transgenic littermates (WT-MI 41 ± 3% vs. TG-MI 38 ± 4%: Figure 2.2A).

A more prominent increase in the HW/BW ratio was observed in the WT-MI group (48%) compared to MCIP1-TG MI mice (24%; P < 0.05). The hypertrophic remodeling was more pronounced in the LV and this was reflected in LVW/BW and LVW/TL ratios, which both showed a 77% and 78% increase WT-MI mice, and only a 36% and 41% increase in the MCIP1-TG groups, respectively (Table 2.1 and Figure 2.2B).

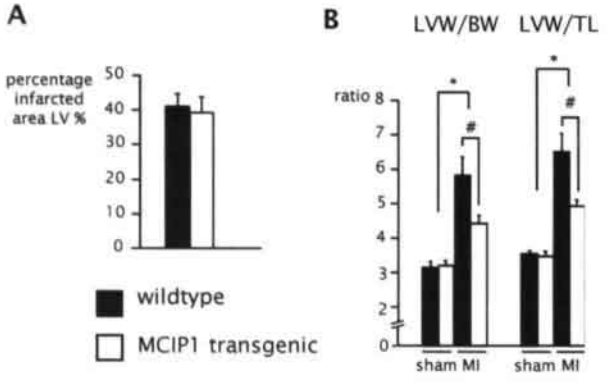


Fig. 2.2AB (A) Morphometric analyses of AZAN sections indicate no significant difference in infarct size between the wildtype and MCIP1 transgenic mice (n = 6 in both groups). (B) After MI an increase in LVW in the wildtype mice is evident (n = 10), which is significantly blunted in the MCIP1 transgenic mice (n = 12). * P <0.05 vs. sham operated group, # P < 0.05 vs. WT-MI group.

Measurement of septal wall thickness in histological sections further supported these findings (WT-MI 1.1 ± 0.1 mm vs. TG-MI 0.8 ± 0.2 mm; $P < 0.05$). Interestingly, in both genotypes females exhibited less pronounced hypertrophy than their male littermates (data not shown). Atrial weight (AW) and right ventricular weight (RVW) increased to a similar extent in both WT-MI and MCIP1-TG animals (Table 2.1).

Laminin-staining demonstrated an 81 ± 2% increase of the cross sectional area of myocytes in the spared myocardium in the WT-MI group, while TG-MI mice only demonstrated a 29 ± 7% increase relative to corresponding, noninfarcted, control groups (Figure 2.2C). Conclusively, these findings indicate that MCIP1 overexpression protects the myocardium against postinfarction hypertrophy.

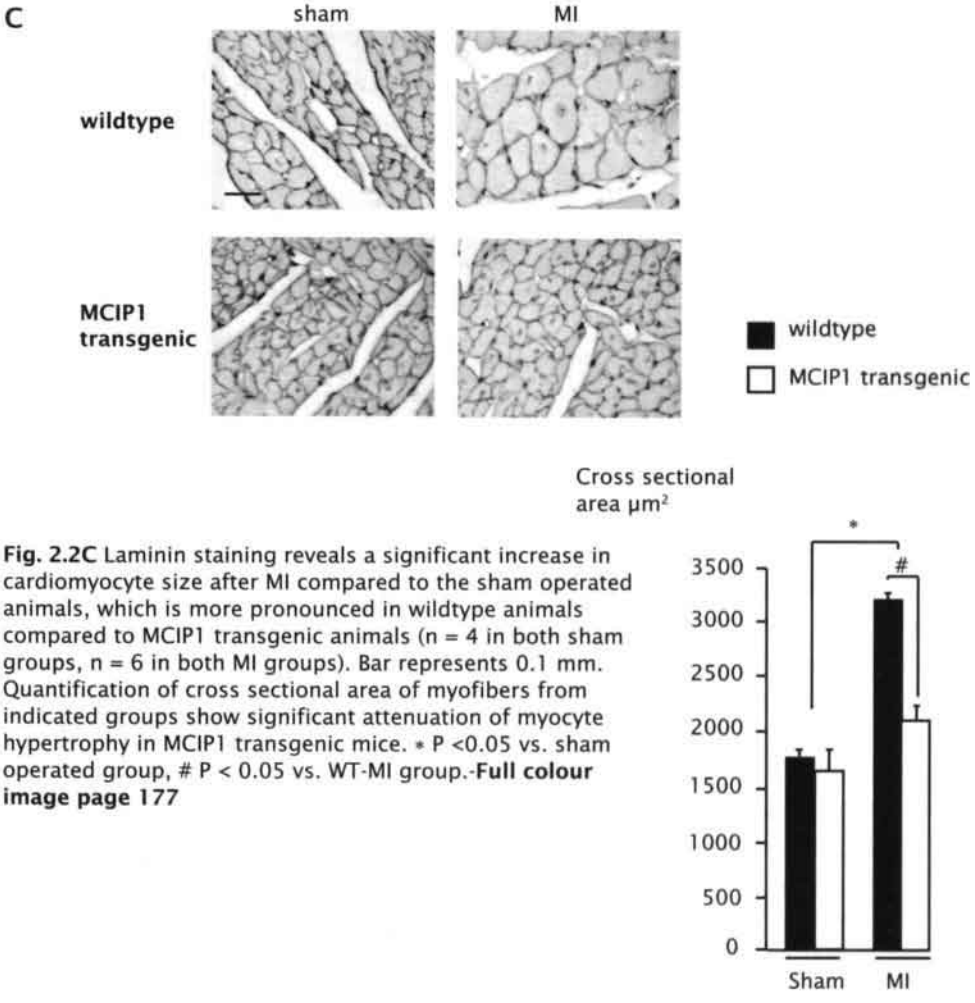


Fig. 2.2C Laminin staining reveals a significant increase in cardiomyocyte size after MI compared to the sham operated animals, which is more pronounced in wildtype animals compared to MCIP1 transgenic animals (n = 4 in both sham groups, n = 6 in both MI groups). Bar represents 0.1 mm. Quantification of cross sectional area of myofibers from indicated groups show significant attenuation of myocyte hypertrophy in MCIP1 transgenic mice. * $P < 0.05$ vs. sham operated group, # $P < 0.05$ vs. WT-MI group.-**Full colour image page 177**

Table 2.1.
Morphological characteristics in wildtype and MCIP1 TG mice after sham operation and 4 weeks after myocardial infarct

	sham		myocardial infarction		
	WT	TG	WT	TG	% change tg vs. wt 4 weeks post-MI
n	13	12	10	12	
HW, mg	114 ± 2	112 ± 4	169.6 ± 9*	143.7 ± 5*†	-20.5
LVW, mg	77 ± 2	76 ± 5	137 ± 10*	106 ± 6*†	-38.5
RVW, mg	19.5 ± 2	21.8 ± 3	28.1 ± 2*	27.3 ± 3	-18.8
Lung weight, mg	150 ± 4	153 ± 3	162 ± 5	152 ± 4	N.S.
Liver weight, mg	909 ± 33	880 ± 34	1042 ± 50	981.5 ± 49	N.S.
Atria weight, mg	10.9 ± 0.8	10.5 ± 0.5	15.7 ± 2.2	15.9 ± 1.4	N.S.
TL, mm	21.5 ± 0.1	21.9 ± 0.2	21.5 ± 0.2	21.7 ± 0.1	N.S.
BW, g	23.8 ± 0.7	22.9 ± 0.4	23.9 ± 1.0	23.5 ± 0.6	N.S.
HW/BW, mg/g	4.8 ± 0.1	4.9 ± 0.1	7.1 ± 0.4*	6.1 ± 0.3*†	-23.4
HW/TL, mg/mm	5.3 ± 0.1	5.1 ± 0.1	7.9 ± 0.5*	6.6 ± 0.3*†	-19.7

Data are expressed as means ± SEM. TG, transgenic; HW, heart weight; LVW, left ventricular weight; RVW, right ventricular weight; TL, tibial length; BW, body weight.
* indicates P<0.05 vs corresponding sham group; † indicates P<0.05 vs wildtype postinfarction group.

Preservation of cardiac geometry and function in MCIP1 TG mice after MI

To examine the impact of MCIP1-mediated attenuation of postinfarction hypertrophy on hemodynamic behavior, all groups were subjected to 2D and M-mode echocardiography three days and four weeks after MI, since hypertrophic remodeling in mice starts three days after the ligation of the coronary artery.[19] Representative images of M-mode recordings are shown in Figure 2.3A. Already 3 days after infarct, an increase in LVID and decrease in contractility was evident in both MI groups compared to sham operated animals, with no functional or geometric differences between WT-MI and TG-MI (Table 2.2). In contrast, four weeks after MI, a thickening of the posterior wall in diastole (PWthd) and a further increase in LVID was visible in WT-MI mice, suggestive of progressive LV dilation (Table 2.2). These geometric changes were accompanied by a further functional deterioration in WT-MI mice as indicated by progressive decreases in fractional shortening (FS) and ejection fraction (EF) compared to the corresponding sham group (Figure 3B). The presence of the MCIP1 transgene resulted in a significant reduction of PWthd and LVID in response to MI, and a preservation of FS (Table 2.2 and Figure 2.3B) and EF (Table 2.2).

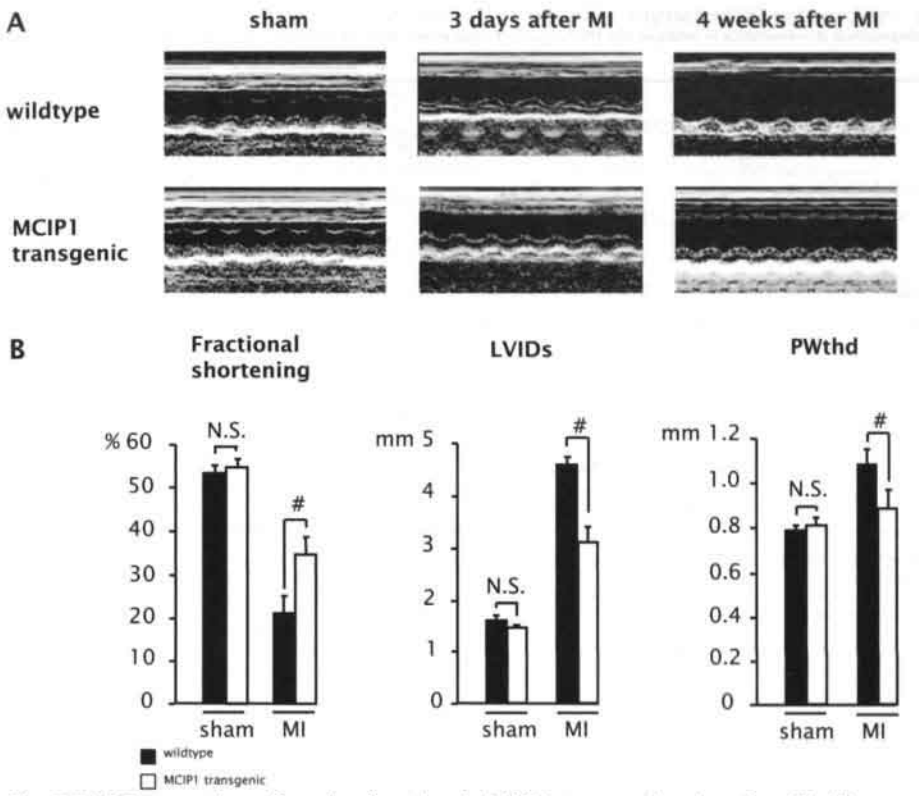


Fig. 2.3AB Preservation of cardiac function in MCIP1 transgenic mice after MI. (A) Representative M-mode images of sham or infarcted wildtype and MCIP1 transgenic mice 3 days and 4 weeks after MI. Data demonstrate comparable initial LV dilation in both groups, with progressive dilation and loss of contractile behaviour in wildtype mice, which was substantially attenuated in MCIP1 transgenic mice. (B) Bargraph representation of FS, LVIDs, and PWthd indicate that the presence of the MCIP1 transgene attenuates functional and morphological deterioration after MI (n = 5 in both sham groups; n = 7-8 in MI groups). * P <0.05 vs. sham operated group, # P < 0.05 vs. WT-MI group.

To further evaluate the hemodynamic profile of all experimental groups, invasive pressure measurements were performed. A relatively lower systolic baseline function was observed in sham-TG compared to sham-WT mice (Table 2.3). At present we have no explanations for this difference. More importantly, the response of both genotypes to MI was clearly different. In the WT-MI group a dramatic decrease in LV dP/dt_{max}, LV dP/dt_{min}, LVESP and LVDP was observed ($P < 0.05$). In contrast, TG-MI mice showed a marked preservation of these parameters, and this level of preservation correlates well with the echocardiographic observations (Table 2.2 and Figure 2.3).

Taken together, these results indicate that MCIP1 overexpression specifically alters the late phase of post-MI remodeling rather than the acute response to the infarct itself, and efficiently counteracts the geometric and functional deterioration after MI.

Table 2.2.
Echocardiographical characteristics in wildtype and MCIP1 TG mice after sham operation and after myocardial infarction.

	sham		3 days after MI		4 wks after MI			
	WT	TG	WT	TG	WT	TG	% change tg vs. wt 3 days post-MI	% change tg vs. wt 4 weeks post-MI
n	7	8	7	6	12	11		
HR	400 ± 18	402 ± 17	388 ± 12	390 ± 15	406 ± 11	401 ± 12	N.S.	N.S.
PWthd, mm	0.68 ± 0.05	0.67 ± 0.06	0.73 ± 0.05	0.73 ± 0.02	1.09 ± 0.08*	0.82 ± 0.04†	N.S.	-38
PWths, mm	1.39 ± 0.07	1.29 ± 0.06	1.31 ± 0.03	1.28 ± 0.07	1.50 ± 0.18	1.30 ± 0.07	N.S.	N.S.
LVIDd, mm	3.52 ± 0.12	3.21 ± 0.07	4.44 ± 0.13*	4.34 ± 0.13*	5.56 ± 0.14*	4.69 ± 0.24*†	N.S.	-12
LVIDs, mm	1.68 ± 0.08	1.52 ± 0.04	3.11 ± 0.11*	3.10 ± 0.10*	4.36 ± 0.12*	2.98 ± 0.12*†	N.S.	-64
% EF	62.75 ± 2.3	58.5 ± 2.8	35.5 ± 3.4*	32.5 ± 1.5*	26.0 ± 2.5*	36.3 ± 4.8*†	N.S.	21
% FS	52.2 ± 2.0	52.6 ± 1.4	29.3 ± 1.4*	29.7 ± 1.3*	21.6 ± 2.3*	36.9 ± 1.8*†	N.S.	29

Data are expressed as means ± SEM. Tg, transgenic; PWthd, posterior wall thickness in diastole; PWths, posterior wall thickness in systole; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole;
FS, left ventricular fractional shortening calculated as (LVIDd-LVIDs)/LVIDd
EF, left ventricular ejection fraction

* indicates P<0.05 vs corresponding sham group; † indicates P<0.05 vs wildtype 4 weeks postinfarction group.

Table 2.3.
Hemodynamic characteristics in wildtype and MCIP1 TG mice after sham operation or myocardial infarction.

	wildtype			MCIP1 transgenic		
	sham	MI		sham	MI	
n	4	5	% change vs. corresp. sham group	5	6	% change vs. corresp. sham group
LV dP/dt_{max}, mm Hg.s-1	9,338 ± 481	6,202 ± 903	-34	5,524 ± 886	5,402 ± 830	-2
LV dP/dt_{min}, mm Hg.s-1	-6,645 ± 672	-4,681 ± 517	-30	-5,128 ± 651	-4,832 ± 630	-6
LVDP, mmHg	93.5 ± 4.9	72.9 ± 4.7	-22	66.4 ± 6.2	66.2 ± 5.8	0
LVESP	94.6 ± 5.3	76.9 ± 4.3	-19	67.1 ± 6.1	67.3 ± 6.0	0
LVEDP	1.1 ± 1.0	4.1 ± 1.5	372	0.8 ± 0.3	3.1 ± 0.6	388

Data are expressed as means ± SEM. LV dP/dt_{max}, first maximal derivative of left ventricular pressure;
LV dP/dt_{min}, first minimal derivative of left ventricular pressure; LVDP, left ventricular developed pressure;
LVESP, left ventricular end systolic pressure; LVEDP, left ventricular end diastolic pressure

Overexpression of MCIP1 improves survival after myocardial infarction

The survival rates of WT and MCIP1-TG mice were evaluated up to 4 weeks after MI. The survival of WT-MI mice was 89% and 61% at 1 and 4 weeks after surgery, while MCIP1-TG MI mice displayed a survival of 90% and 79%, respectively (Figure 2.3C). Differences in survival became evident in the last 3 weeks before sacrifice (Figure 2.3C), and was more pronounced in male littermates (data not shown). These observations correlate well with the fact that infarct sizes were equal in both groups, and that infarct healing is completed within 7 days after MI in mice.[15] The combined observations suggest that MCIP1 overexpression has no influence on the early process of infarct healing, but attenuates hypertrophic LV remodeling that starts ~ 3-7 days after MI in mice, and improves postinfarction survival.

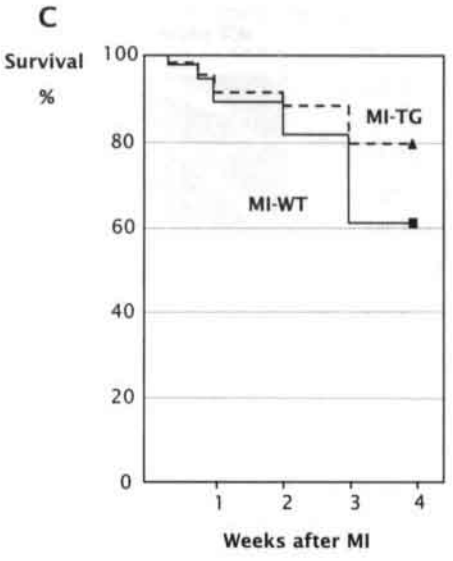


Fig. 2.3C Early post-MI mortality was comparable between wildtype and transgenic mice subjected to MI, but became significantly higher for wildtype mice in the last three weeks before sacrifice (WT-MI, n = 51; TG-MI, n = 43).

Attenuated interstitial collagen deposition after calcineurin inhibition in MI

H&E staining confirmed the presence of extensive myofiber hypertrophy and disarray in WT-MI mice (Figure 2.4A), accompanied by a patchy presence of cellular infiltrates in the interstitial compartment (indicated by arrowheads). These histological alterations in response to infarction were nearly absent in TG-MI hearts similar to both sham operated groups (Figure 2.4A). In the noninfarcted area, reactive fibrosis, such as perivascular and interstitial fibrosis, was observed in a gradient with more pronounced presence of collagen deposition towards the infarct. WT-MI mice exhibited the most severe fibrotic alterations, both expressed as percentage of the complete septum (10%) as well as the lower apical half of the septal wall (16%), and this was substantially attenuated in MCIP1-TG MI mice (Figure 2.4B). These results indicate that the presence of the MCIP1 transgene led to a significant reduction of the major structural alterations of the remote myocardium in the postinfarcted heart.

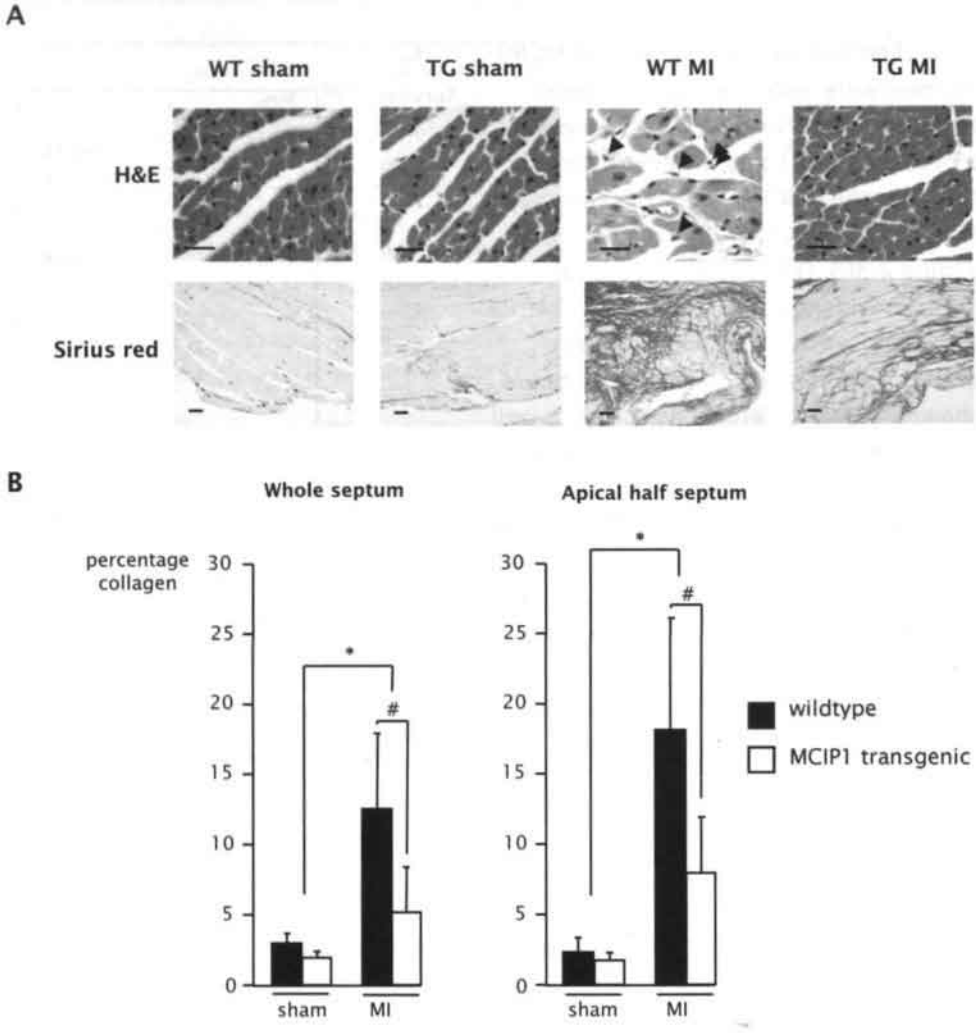


Fig. 2.4AB MCIP1 transgenic animals are protected against MI-induced structural remodeling. (A) Representative images of histological staining for the indicated groups. Upper panels are H&E stained images, lower panels Sirius red stained images (Bar indicates 0.1 mm). H&E stained images reveal remarkable myocyte hypertrophy, myofiber disarray and cellular infiltrates (arrowheads) in a section from a representative postinfarcted wildtype mouse. In contrast MCIP1 transgenic mice are largely protected against these structural alterations. Sirius red staining indicates low levels of collagen deposition in the sham operated mouse hearts from both phenotypes, a massive accumulation of fibrillar collagen (interstitial and perivascular fibrosis) after infarction in wildtype mice, which is substantially attenuated in MCIP1 transgenic mice. -**Full colour image page 180** (B) Tissue content of fibrillar collagen in septum (remote myocardium) is significantly more upregulated in wildtype animals than in MCIP1 transgenic mice, a difference that is even more pronounced in the lower half of the septal wall (n = 2 in both sham groups; n = 6 in both MI groups). * P <0.05 vs. sham operated group, # P < 0.05 vs. WT-MI group.

Cardiac gene expression in infarcted hearts with calcineurin inhibition

We examined the gene expression profile of genes activated during postinfarction hypertrophy. At 4 weeks after MI an induction of both ANF and β-MyHC was observed in WT-MI hearts relative to corresponding sham operated mice, indicating the activation of a "fetal cardiac gene program". ANF expression was similar in both TG-MI and WT-MI hearts. In contrast, the induction of β-MyHC expression was significantly blunted in TG-MI hearts compared to WT-MI hearts (Figure 2.5). These results indicate that the transcriptional response of the MCIP1 transgenic animals is different than that of wildtype animals during MI-induced LV remodeling.

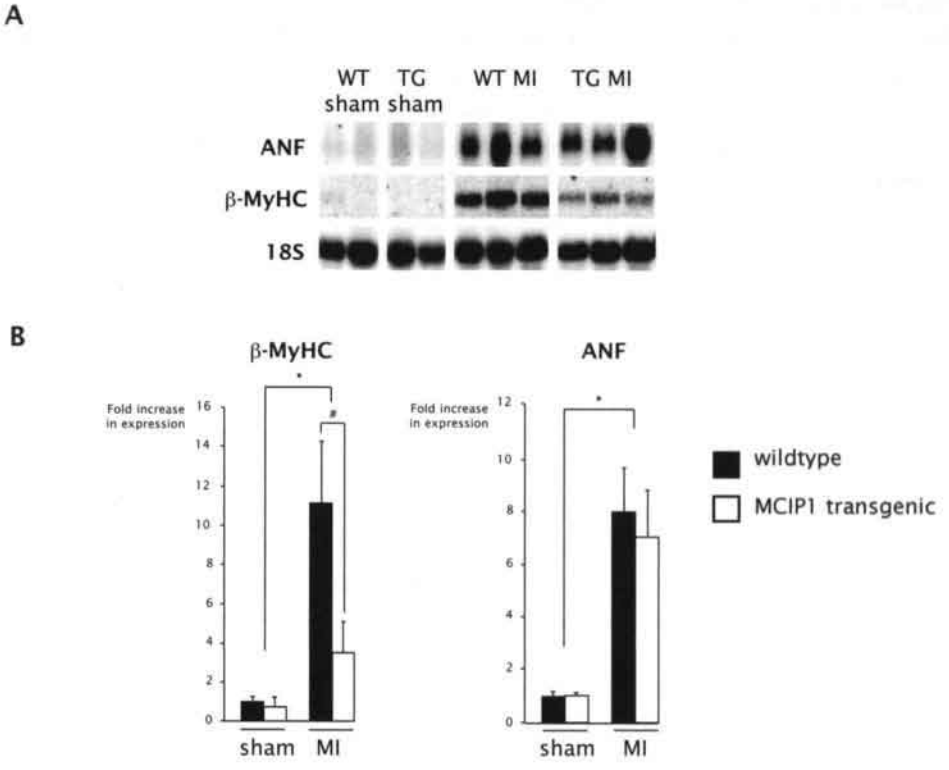


Fig. 2.5AB MI-induced fetal gene expression is differentially affected by MCIP1 overexpression.
(A) Representative Northern blot for ANF (upper panels), β-MyHC (middle panel) and 18S ribosomal RNA from indicated experimental groups. Data indicate strong upregulation of ANF and β-MyHC following infarction in wildtype mice, and lower β-MyHC expression in postinfarcted transgenic animals. (B) Quantification of Northern blot analyses indicates significant upregulation of ANF and β-MyHC gene expression after MI in wildtype mice. Only MI-evoked elevation of β-MyHC is significantly blunted in the MCIP1 transgenic mice. * $P < 0.05$ vs. sham operated group, # $P < 0.05$ vs. WT-MI group.

DISCUSSION

Suppression of postinfarction hypertrophic remodeling by MCIP1

The involvement of calcineurin as a transducer of pro-hypertrophic signals in the heart has gradually gained more acceptance. The use of genetically modified mice with reduced or enhanced calcineurin activity in the heart has confirmed the pathological role of this enzyme in cardiac muscle cell biology.[8-11,16,20] Most studies, however, have focused on the function of calcineurin in hypertrophy development following acute increases in loading conditions (e.g. aortic banding procedures) or agonist infusion protocols. The potential role of calcineurin in LV remodeling following ischemic loss of functional myocardium has remained ill defined. In view of the increasing proportion of heart failure patients with coronary artery disease as a primary etiology, [1] elucidation of the regulatory pathways controlling postinfarction remodeling will have both fundamental and therapeutic avenues.

Preceding the present study, three groups have used rat models of myocardial infarction (MI) in combination with systemic delivery of the calcineurin inhibitor cyclosporine A (CsA) to address the role of calcineurin in post-MI remodeling. All three groups found a correlation between CsA administration and reduced postinfarct hypertrophy.[21-23] CsA toxicity and potential calcineurin-independent effects of CsA can confound interpretation of these studies.[20] Our findings from the present study using MCIP1 transgenic mice are in agreement with the rat studies insofar as the attenuation of hypertrophy is concerned. However, in contrast to some of the CsA studies, long term prognosis of the MCIP1 transgenics is improved compared to wildtype rather than in the CsA treated rats which showed a more rapid progression toward heart failure. The MCIP1-TG mice allowed us to achieve cardiac-specific inhibition of calcineurin without the concern of secondary drug effects or systemic toxicity.[20]

Quantification of calcineurin phosphatase activity from tissue lysates using biochemical assays is problematic, due to the dynamic, calcium/calmodulin-dependent regulation of activity, the sensitivity of calcineurin to oxidation of the Fe-Zn active center, and does not reflect the proportion of the enzymatic calcineurin pool that is actually in an active state. We could detect both increased phosphatase activity and more abundant presence of the calcineurin Aβ isoform following MI in wildtype mice. We also used the phosphorylation status of endogenous NFAT and expression levels of the NFAT-responsive exon 4 splice variant of MCIP1 as criteria to monitor calcineurin activity.[10] These assays were in line with increased calcineurin activation in the postinfarcted heart. The present study confirms the earlier documented calcineurin activation profile in the postinfarcted LV, but our interpretation of its value in LV remodeling differs in

several respects from other studies.

Postinfarction hypertrophy is maladaptive in nature

Myocardial hypertrophy is traditionally viewed as a long-term adaptive response of the cardiac muscle to either altered mechanical loading conditions (e.g. resulting from valvular disease or hypertension) or decreased performance due to loss of contractile units (e.g. after MI).[4] Following this interpretation, increased wall thickness serves as the means to restore wall stress in line with the law of Laplace.[4] Recent insights into the particulars of the hypertrophic phenotype, however, have demanded a more nuanced interpretation of this phenomenon of "compensatory hypertrophy". First, ventricular hypertrophy is demonstrably a risk factor for cardiovascular mortality in humans.[5] Second, beyond just increased mass, the specific long-term transcriptional responses to increased load entail a myriad of quantitative and qualitative changes in cardiac gene expression that are reminiscent of fetal cardiac myocytes. In patients with cardiac failure, functional improvement related to treatment with β-blockers is correlated with beneficial changes in myocardial gene expression, most prominently exemplified by a correction in the mRNA expression level of the β-MyHC gene.[24] In the present study we also noted a pronounced decrease in β-MyHC gene expression and no relative change in ANF expression in infarcted transgenic mice compared to their wildtype counterparts (Figure 2.5). Of note, in the original description of MCIP1 overexpressing mice [10] this divergence between ANF vs β-MyHC fetal gene expression was also evident. ANF and its receptor have been reported to possess potent antihypertrophic activity [25,26], while recombinant human BNP has been shown to improve decompensated congestive heart failure in patients [27]. It will become of interest to investigate the specific contribution of natriuretic peptides in the calcineurin-NFAT axis. Conclusively, Laplace's Law, although conceptually sound, does not take into account any qualitative alterations of the wall, and only incompletely explains the phenotypic particulars of heart enlargement.

Third, several studies using genetically engineered mice with markedly blunted growth responses to pressure overload appear to be protected from adverse effects of stress signaling and heart failure progression.[12,28] In fact, Esposito et al. subjected normal and hypertrophy resistant mice to aortic constriction and observed that hemodynamic function and overall cardiac geometrics were better preserved in hypertrophy-resistant mice than in wildtype mice, despite the inability of the transgenic mice to correct wall stress.[28] Hence, the value of normalizing wall stress through hypertrophic myocardial growth is dispensable for the preservation of cardiac function in the face of a long-term hemodynamic burden.[29] The findings in the present study fortify the conclusions that postinfarction-, and load-induced reactive signaling and hypertrophic

remodeling share fundamental similarities and may be equally deleterious in nature.[29] One outspoken observation in postinfarcted MCIP1 overexpressing mice was their marked inhibition of interstitial collagen deposition. It is important to note, that the MCIP1 transgene is targeted exclusively to the cardiac muscle cell population, which suggests a crucial role and hierarchy for the cardiomyocyte population in governing cardiac fibroblast proliferation and collagen production through paracrine regulation of latter cell type.

Dual roles of MCIP1 in calcineurin signaling

While we interpret the protective effects of MCIP1 on post-MI cardiac pathology to reflect the reduction in calcineurin activity in the hearts of MCIP1-TG mice, it should also be noted that our recent studies have revealed positive and negative effects of MCIP1 on calcineurin activity. It is clear that high levels of MCIP1 expression can inhibit calcineurin activity through direct association with the catalytic subunit of the enzyme. Paradoxically, however, MCIP1 knockout mice, which are viable, show a reduction in cardiac calcineurin activity and a diminished hypertrophic response to pressure overload and chronic adrenergic stimulation.[30] We have attributed the latter findings to a permissive role of MCIP1 in activation of calcineurin, possibly via a chaperone or protein folding function of MCIP1. It is also formally possible that MCIP1 evokes activities through cellular effectors in addition to calcineurin. Further confirmation of the precise roles of MCIP1 and calcineurin in post-MI hypertrophy and remodeling will be provided by studies of these processes in calcineurin-TG mice and in knockout mice lacking calcineurin and MCIP1.

Acknowledgements
We thank Peter Willemsen and Nicolle Bitsch for excellent technical assistance.

REFERENCES

1. Gheorghiade M, Bonow RO. Chronic heart failure in the United States: a manifestation of coronary artery disease. Circulation. 1998;97:282-9.
2. Cleland JG, Khand A, Clark A. The heart failure epidemic: exactly how big is it? Eur Heart J. 2001;22:623-6.
3. Pfeffer MA, Braunwald E, Moye LA, Basta L, Brown EJ, Jr., Cuddy TE, Davis BR, Geltman EM, Goldman S, Flaker GC, et al. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators. N Engl J Med. 1992;327:669-77.
4. Sadoshima J, Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. Annu Rev Physiol. 1997;59:551-71.
5. Mathew J, Sleight P, Lonn E, Johnstone D, Pogue J, Yi Q, Bosch J, Sussex B, Probstfield J, Yusuf S. Reduction of cardiovascular risk by regression of electrocardiographic markers of left ventricular hypertrophy by the angiotensin-converting enzyme inhibitor ramipril. Circulation. 2001;104:1615-21.
6. Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF, Molkentin JD. Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol. 2002;22:7603-13.
7. van Rooij E, Doevendans PA, de Theije CC, Babiker FA, Molkentin JD, de Windt LJ. Requirement of nuclear factor of activated T-cells in calcineurin-mediated cardiomyocyte hypertrophy. J Biol Chem. 2002;277:48617-26.
8. De Windt LJ, Lim HW, Bueno OF, Liang Q, Delling U, Braz JC, Glascock BJ, Kimball TF, del Monte F, Hajjar RJ, Molkentin JD. Targeted inhibition of calcineurin attenuates cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A. 2001;98:3322-7.
9. Zou Y, Hiroi Y, Uozumi H, Takimoto E, Toko H, Zhu W, Kudoh S, Mizukami M, Shimoyama M, Shibasaki F, Nagai R, Yazaki Y, Komuro I. Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. Circulation. 2001;104:97-101.
10. Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN, Williams RS. Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A. 2001;98:3328-33.
11. Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, Richardson JA, Hill JA, Olson EN. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A. 2002;99:907-12.
12. Hill JA, Rothermel B, Yoo KD, Cabuay B, Demetroulis E, Weiss RM, Kutschke W, Bassel-Duby R, Williams RS. Targeted inhibition of calcineurin in pressure-overload cardiac hypertrophy. Preservation of systolic function. J Biol Chem. 2002;277:10251-5.
13. Doevendans PA, Daemen MJ, de Muinck ED, Smits JF. Cardiovascular phenotyping in mice. Cardiovasc Res. 1998;39:34-49.
14. Degens H, Gilde AJ, Lindhout M, Willemsen PH, Van Der Vusse GJ, Van Bilsen M. Functional and metabolic adaptation of the heart to prolonged thyroid hormone treatment. Am J Physiol Heart Circ Physiol. 2003;284:H108-15.
15. Creemers E, Cleutjens J, Smits J, Heymans S, Moons L, Collen D, Daemen M,

Carmeliet P. Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. Am J Pathol. 2000;156:1865-73.
16. Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, Lorenz JN, Molkentin JD. Impaired cardiac hypertrophic response in Calcineurin Abeta -deficient mice. Proc Natl Acad Sci U S A. 2002;99:4586-91.
17. Haq S, Choukroun G, Lim H, Tymitz KM, del Monte F, Gwathmey J, Grazette L, Michael A, Hajjar R, Force T, Molkentin JD. Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. Circulation. 2001;103:670-7.
18. Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, Bassel-Duby R, Williams RS. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res. 2000;87: E61-8.
19. Hwang MW, Matsumori A, Furukawa Y, Ono K, Okada M, Iwasaki A, Hara M, Miyamoto T, Touma M, Sasayama S. Neutralization of interleukin-1beta in the acute phase of myocardial infarction promotes the progression of left ventricular remodeling. J Am Coll Cardiol. 2001;38:1546-53.
20. Bueno OF, van Rooij E, Molkentin JD, Doevendans PA, De Windt LJ. Calcineurin and hypertrophic heart disease: novel insights and remaining questions. Cardiovasc Res. 2002;53:806-21.
21. Deng L, Huang B, Qin D, Ganguly K, El-Sherif N. Calcineurin inhibition ameliorates structural, contractile, and electrophysiologic consequences of postinfarction remodeling. J Cardiovasc Electrophysiol. 2001;12:1055-61.
22. Youn T, Piao H, Kwon J, Choi S, Kim H, Park D, Kim D, Kim Y, Cho M. Effects of the calcineurin dependent signaling pathway inhibition by cyclosporin A on early and late cardiac remodeling following myocardial infarction. Eur J Heart Fail. 2002;4:713-8.
23. Oie E, Bjornerheim R, Clausen OP, Attramadal H. Cyclosporin A inhibits cardiac hypertrophy and enhances cardiac dysfunction during postinfarction failure in rats. Am J Physiol Heart Circ Physiol. 2000;278:H2115-23.
24. Lowes BD, Gilbert EM, Abraham WT, Minobe WA, Larrabee P, Ferguson D, Wolfel EE, Lindenfeld J, Tsvetkova T, Robertson AD, Quaife RA, Bristow MR. Myocardial gene expression in dilated cardiomyopathy treated with beta-blocking agents. N Engl J Med. 2002;346:1357-65.
25. Calderone A, Thaik CM, Takahashi N, Chang DL, Colucci WS. Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. J Clin Invest. 1998;101:812-8.
26. Kishimoto I, Rossi K, Garbers DL. A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy. Proc Natl Acad Sci U S A. 2001;98:2703-6.
27. Colucci WS, Elkayam U, Horton DP, Abraham WT, Bourge RC, Johnson AD, Wagoner LE, Givertz MM, Liang CS, Neibaur M, Haught WH, LeJemtel TH. Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group. N Engl J Med. 2000;343:246-53.
28. Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ, Rockman HA. Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. Circulation. 2002;105:85-92.
29. Sano M, Schneider MD. Still stressed out but doing fine: normalization of wall stress is superfluous to maintaining cardiac function in chronic

pressure overload. Circulation. 2002;105:8-10.
30. Vega RB, Rothermel BA, Weinheimer CJ, Kovacs A, Naseem RH, Bassel-Duby R, Williams RS, Olson EN. Dual roles of modulatory calcineurin-interacting protein 1 in cardiac hypertrophy. Proc Natl Acad Sci U S A. 2003;100:669-674.

Chapter 3

Requirement of Nuclear Factor of Activated T-cells in calcineurin mediated cardiomyocyte hypertrophy

Eva van Rooij, Pieter A. Doevendans, Chiel C. de Theije, Fawzi A. Babiker, Jeffery D. Molkentin and Leon J. De Windt

SUMMARY

The calcium-activated phosphatase calcineurin has been implicated as critical intracellular signal transducer of cardiomyocyte hypertrophy. Although previous data suggested nuclear factor of activated T cell (NFAT) as it's sole transcriptional effector, the absolute requirement of NFAT as a mediator of calcineurin signaling has not been examined in the heart. We therefore investigated the expression and activation profile of NFAT genes in the heart. Four members (NFATc1-c4) are expressed in cardiomyocytes, elicit nuclear translocation upon calcineurin activation, and are able to drive transactivation of cardiac promoter luciferase constructs. To define the necessary function of NFAT factors as hypertrophic transducers, a dominant negative NFAT construct was created, encompassing part of the N-terminal region of NFATc4 containing a conserved calcineurin-binding motif. Cotransfection of this construct dose-dependently abrogated promoter activation, irrespective of the NFAT isoform used, while a control construct with the calcineurin-binding motif mutated displayed no such effects. Adenoviral gene transfer of dominant negative NFAT rendered cardiomyocytes resistant towards all aspects of calcineurin or agonist-induced cardiomyocyte hypertrophy, while adenoviral gene transfer of the control construct had no discernable effect on these parameters. These results indicate that multiple NFAT isoforms are expressed in cardiomyocytes where they function as necessary transducers of calcineurin in facilitating cardiomyocyte hypertrophy.

INTRODUCTION

Heart failure is a leading cause of morbidity and mortality in industrial countries, affecting over 10 million Americans and Western Europeans, with a 5-year mortality approaching 50 percent despite current medical therapy.[1,2] These mortality figures reflect the lack of biologically efficacious therapies directed against the underlying disease processes that lead to maladaptive left ventricular remodeling and, ultimately, failure itself. In response to a plethora of intra- and extracardiac stimuli, cardiomyocytes exhibit cellular enlargement or hypertrophy as a compensatory adaptation to increased ventricular wall stress.[3] However, sustained cardiac hypertrophy is the single most important risk factor for the development of heart failure.[4,5] Since intracellular signaling pathways are thought to both initiate and perpetuate the cardiac hypertrophic response and its transition to dilated failure, recent investigation has attempted to identify key regulatory factors with the goal of defining novel therapeutic targets.[3]

One recently characterized intracellular signaling pathway that links extracellular stimuli to a hypertrophic transcriptional response employs the phosphatase calcineurin and its downstream transcriptional effector Nuclear Factor of Activated T-cells (NFAT). Four of the five NFAT proteins (NFATc1, NFAT2 or NFATc; NFATc2, NFAT1 or NFATp; NFATc3, NFAT4 or NFATx; NFATc4 or NFAT3) reside in the cytoplasm in unstimulated cells, but quickly translocate to the nucleus in response to stimulation that promote Ca^{2+} mobilization.[6] The Ca^{2+}-calmodulin activated phosphatase calcineurin physically interacts with NFAT members within the cytoplasm, where it directly dephosphorylates multiple serine residues within the NH_2-terminal regulatory domain of NFAT, resulting in the unmasking of two nuclear localization sequences required for nuclear import.[7-9]

Calcineurin-NFAT signaling has been implicated as a critical regulator of the cardiac hypertrophic growth response. Molkentin et al. generated several lines of transgenic mice expressing activated mutants of either calcineurin or NFATc4 in a cardiac-selective manner, which developed robust hypertrophy that quickly transitioned to ventricular dilation and overt heart failure.[10,11] The identification of calcineurin as a signaling factor has attracted considerable interest, in part due to the demonstration that the calcineurin inhibitory drugs cyclosporine (CsA) and FK506 were shown to abrogate the cardiomyopathic response in several, but not all, rodent models of congenital and acquired forms of hypertrophic heart disease (reviewed in [12,13]). A central role for calcineurin in the cardiac hypertrophic response was substantiated by the observation that hearts from transgenic mice expressing either MCIP1, a dominant negative calcineurin mutant or the calcineurin inhibitory domains of Cain or AKAP79, were largely resistant to pleiotropic, hypertrophic stimuli.[14-16] More recently, calcineurin Aβ gene targeted mice were generated and shown to be defective in mounting a cardiac hypertrophic response due to pressure overload or agonist infusion.[17]

While a large number of studies have convincingly demonstrated the importance of calcineurin as a hypertrophic mediator, the importance of NFAT factors downstream transducers have not been evaluated in cardiomyocytes.

In the present study we demonstrate the presence of all four, calcineurin-sensitive members of the NFAT family (NFATc1, c2, c3 and c4) in the ventricular cardiomyocyte cell lineage. All four isoforms displayed calcineurin-dependent nuclear translocation and the ability to transactivate cardiac promoters. To simultaneously inhibit all myocardial NFAT factors in an effort to effectively examine their necessary function as hypertrophic transducers, a dominant negative NFAT strategy was developed. Dominant negative NFAT dose-dependently abrogated calcineurin-NFAT dependent transactivation of MCIP1 and BNP promoter luciferase constructs. Adenoviral-mediated gene transfer of dominant negative NFAT in cultured cardiomyocytes efficiently inhibited calcineurin- and agonist-induced cardiomyocyte hypertrophy. Taken together, these data demonstrate a previously unexpected level of redundancy of the downstream targets of calcineurin and establish their requirement in pathophysiological signaling in the cardiomyocyte.

EXPERIMENTAL PROCEDURES

Reporter constructs and expression vectors

Expression vectors containing a constitutively activated mutant of calcineurin Aα (CnAα), NFATc1, c3 or c4 were described previously [10,11] pEFBOS-HA-NFATp, a vector containing an N-terminal HA-tagged full length murine NFATc2, was generously provided by Laurie Glimcher (Harvard, Boston). pCG-GATA-4, a vector with full-length rat GATA-4 was generously provided by Anton Moorman (AMC, Amsterdam). pCDNA3-NFATc4(Ala mut), an expression vector containing the first 130 NH_2-terminal aa of human NFATc4 with the conserved PxIxIT box mutated to Ala residues (AxAxAA) was described previously and a generous gift from Dr. Roger Davis (Harvard, Boston). A constitutively activated flag-tagged NFATc3 clone was PCR-generated (fw 5'-GGTGGGTCAGGCCTTGGCCTT, rv 5'-TTAGAGCCCATCAGATCTTCC) and lacks the first 315 N-terminal aa of the published human NFATc3/NFATx sequence (PubMed U85429). The fragment was cloned into the EcoRI site of the pECE vector to include a N-terminal Flag-tag to generate pECE-(Δ315)NFATc3. A construct encompassing aa 3 through 191 of murine NFATc4 was PCR generated (fw 5'-GCCGCAAGCTGCGAGGATGAG, rv 5'-GACGGCTCGGGCTGAAGA) and subcloned as an EcoRI fragment into the pECE vector to incorporate an N-terminal Flag-epitope to generate pECE-NFATc4(PxIxIT). A human BNP promoter-luciferase construct was obtained by cloning an 1800 bp PCR-amplified fragment of the proximal human BNP promoter sequence from genomic DNA (fw 5'-GTAG AAACACCTTGTGATCAC, rv 5'-GGGACTGCGGAGGCTGCT) into the HindIIII site of pGL3 (Promega) to generate pGL3-hBNP(1800)Luc. Site-directed mutagenesis was performed using the Quickchange-XL kit from Stratagene. Two GATA sites centered at -116 (10) were consecutively mutated to CCTC using the following primers (mutated sequences small case, only forward primer sequences shown) fw-1 5'-GCCCGGAATGTGGCTcctcAAT AGAGATAACCCTGCAT and fw-2 5'-GGCTcctc AATCAGAcctcACCCTGATGGCAGG to generate pGL3-(ΔGATA)-hBNP(1800)Luc. Subsequently, the NFAT site centered at -927 [10] was mutated in pGL3-(ΔGATA)-hBNP(1800)Luc using the following primer (mutated sequences in small case, only forward primer sequence shown) fw 5'-CTATCCTTTTGtgaagaaTCCTG to generate pGL3-(ΔNFATΔGATA)hBNP(1800)-Luc. An MCIP1-luciferase reporter, containing a 904 bp intragenic sequence encompassing the third intron of the human MCIP1 (DSCR1) gene [18], was PCR generated from genomic DNA (fw 5'-CAACCTCTGGCATAAAT, rv 5'-CTTGAGCTGGTGCTTATAAA) and cloned as a HindIII fragment in pGL3 to generate pGL3-hMCIP1(Int3)Luc. This reporter is identical as described previously.[18] All new PCR generated constructs were amplified using Accutaq high fidelity system (Sigma) and confirmed by diagnostic restriction and double strand sequencing analysis.

Tissue culture and transient transfection assays

Adult mouse ventricular myocytes were isolated as described previously [19], pelleted by centrifugation and stored at -80°C. Isolation and culture of neonatal rat ventricular cardiomyocytes was performed as described before in detail.[20] Low passage COS-7 and HEK 293 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS. COS-7 cells were grown in 12-well plates and transfected using 5 µl FuGENE 6 reagent (Roche, Indianapolis) and a total of 2 µg DNA, consisting of the above luciferase reporter constructs, in the presence or absence of expression vectors for ΔCnA, NFATc1, c2, c3 or c4, pECE-NFATc4(PxIxIT), pCDNA3-NFATc4(Ala mut), pECE-(Δ315)NFATc3, or pCG-GATA4 as indicated. In addition, 20 ng of pRL-CMV (Promega), an expression vector containing the renilla luciferase gene under control of a CMV promoter, was included in each experiment to correct for transfection efficiency (see below). Empty expression vector was used to normalize DNA amount. The cultures were harvested for luciferase activities 48 hours after transfection. Fifty µl of cell extract (100 µl) was assayed for luciferase activity for 3 s in a Biocounter M1500 luminometer (Lumac, Netherlands) using the Dual Luciferase assay system (Promega), where firefly luciferase activity is normalized for renilla luciferase activity to control for variations in transfection efficiency according to the manufacturer's procedures.

RT-PCR and Northern blot analysis

Total RNA was isolated from the indicated murine tissues or cell types using Trizol reagent (Invitrogen). Presence of NFATc1, c2, c3 or c4 mRNA in adult C57BL/6 murine ventriculocytes was analyzed by RT-PCR using primers specific for the individual NFAT isoforms as described before.[21] Northern blot hybridizations on size-fractionated total RNA (10 µg) from indicated tissues were performed as described previously.[22] To obtain probes specific for the NFAT isoforms, the mRNA sequences of murine NFATc1 through c4 were aligned (using ClustalW software) and primers designed to the 3' untranslated regions showing no or minimal overlap (Primers: NFATc1 fw 5'-GATGCTGAACCTGAGACGCC, rv 5'-GCCACCAGCCAGTCTGGTGT; NFATc2 fw 5'-ATTGCTATCTTAGTAAAAT CAAGG, rv 5'-TAATCTGAA AGCAAGA; NFATc3 fw 5'-GGTGATGAGAGACACTCC TCTCCC, rv 5'-ATCATATAAAAGTACCTA; NFATc4 fw 5'-CCGCACAGCCTCACT GATGT, rv 5'-GCCACCGCTCCTTCCTCC). The isoform-specific probes were randomly labeled with ^{32}P-dCTP (Dupont de Nemours NV, Brussels Belgium), added to the blots and incubated in Rapid Hyb hybridization solution (Amersham) at 58°C. Stringent post-hybridization wash conditions were used. Filters were exposed to PhosphoImager screens (Biorad) and analyzed using Quantity 1 (Biorad) and Adobe Photoshop 6.0 software. The intensity of the18S ribosomal RNA band detected with a radiolabeled 18S probe was used as a quantitative control.

Western blot analysis

The method used is a minor modification of a recently described protocol.[23,24] In brief, protein extracts were lysed in ice-cold buffer (0.5% NP40, 150 mM NaCl, 0.5 mM EDTA, 10 mM Tris-HCl pH 8.0, 2 µg/ml leupeptin, 10 µg/ml PMSF (Sigma), 2 µg/ml soybean trypsin inhibitor). Protein concentration in lysates was determined using a protein dye assay (Biorad) followed by separation on gradient gels (Biorad), and transferred to PVDF membrane (Biorad). Filters were blocked for 1 hour at room temperature using 10% non fat dry milk dissolved in Tris buffered saline with 0.1% Triton-x (Sigma), TBST. Primary antibodies included rabbit polyclonal anti-NFATc1 (Santa Cruz, H-110), mouse monoclonal anti-NFATc2 (Santa Cruz, 4G6-G5), rabbit polyclonal anti-NFATc3 (Santa Cruz, M75), rabbit polyclonal anti-NFATc4 (Santa Cruz, H-74) and mouse monoclonal anti-flag (Sigma, F-3165). Anti-NFATc1-c3 were diluted 1:200 and anti-NFATc4 was diluted 1:1000 in blocking buffer (5% non fat dry milk dissolved in TBST). Membranes were incubated with primary antibodies overnight at 4°C. Secondary antibodies included swine anti-rabbit peroxidase or rabbit anti-mouse peroxidase (DOKA, Denmark) and were used at a dilution of 1:2000 in blocking buffer and incubated for 2 hours at room temperature. Signals were detected with an Enhanced Chemiluminescence kit (ECL, Amersham) and analyzed using Adobe photoshop 6.0 software.

Generation of recombinant, replication-deficient adenoviruses

The adenovirus expressing β-galactosidase with a nuclear localization signal (Adβgal) was a generous gift from Mark Sussman (Children's Hospital, Cincinnati). The adenovirus expressing an activated mutant of calcineurin (AdCnA) was described and characterized previously.[11,24] AdNFATc4(PxIxIT) and AdNFATc4(Ala mut), replication-deficient adenoviruses expressing either Flag-tagged NFATc4(PxIxIT) or NFATc4(Ala mut), were generated by subcloning PCR amplified fragments (fw 5'-CCAGAAGTAGTGAAGC, rv 5'-ATGATCATTACTTA TCTA and fw 5'-AGCGGCAGCCAACATG, rv 5'-GCATTTAGGT GACACTAT, respectively) as Xba I fragments into the adenoviral shuttle vector pACCMVplpA, using either pECE-NFATc4(PxIxIT) or pCDNA-NFAT3(Ala mut) as templates. The recombinant shuttle vectors were cotransfected with pJM17 in HEK 293 cells to produce initial recombinant adenovirus lysates. Procedures for plaque purification, expansion and titering the replication-deficient adenovirus and infection of cardiomyocytes were performed as described previously.[11,24] Cardiomyocytes were infected with indicated adenoviruses at an MOI 100 for 2 hr, and cultured in serum-deficient medium either or not in the presence of Endo-1 (100nM; Sigma) or CT-1 (1nM).

Immunocytochemistry

Fixed cultured cardiomyocytes underwent immunocytochemistry as previously described in detail.[11,20,24] To visualize the subcellular localization of

NFATc1, c2 and c3, primary, isoform-specific antibodies (See Western blots) were used at a dilution of 1:400 followed by corresponding anti-mouse or anti-rabbit Oregon green labelled secondary antibody incubation (Molecular Probes) at a dilution of 1:400. Cells were washed with phosphate buffered saline/0.1% NP40 including bisbenzimide (Sigma) to visualize nuclei. For visualization of cardiomyocyte size, sarcomeric organization and perinuclear ANF expression, the primary antibody included polyclonal anti-rat ANF (Peninsula laboratories), followed by secondary anti-rabbit Oregon green (Molecular Probes) conjugated antibody and a phalloidin Texas Red conjugated antibody (Molecular probes), all used at a dilution of 1:400. An epifluorescence microscope (Eclipse E800, Nikon) was used to visualize the cells at a 400x magnification. Quantitation of cardiomyocyte cell surface area was performed on digitized images using NIH image software. At least 50 cardiomyocytes in 10 to 20 fields were examined in three independent experiments.

Statistical analysis

The results are presented as mean values ± standard error of the mean (SEM). Statistical analyses were performed using InStat 3.0 software (GraphPad Software Inc., San Diego, CA) and ANOVA followed by Bonferroni's post-test when appropriate.

RESULTS

Presence of four NFAT members in ventricular cardiomyocytes.

RT-PCR analysis was employed to investigate which members of the NFAT transcription factor family are present in ventricular cardiomyocytes, and hence, might function as calcineurin effectors. Using RNA from adult mouse ventricular cardiomyocytes, transcripts of the expected size for NFATc2, c3 and c4 were easily detected following a limited number of amplifications (Figure 3.1A). NFATc1 was also detectable albeit at lower levels than the other isoforms (Figure 3.1A, top left panel, lane 2). As a control for RT quality, a GAPDH RT-PCR was performed, which resulted in robust product in RT material (data not shown). These data suggest that transcripts of all NFAT isoforms are present in the ventricular muscle cell lineage.

To extend these results, Northern blot analyses for the different NFAT isoforms were performed on RNA isolated from several murine muscle types, including the individual cardiac chambers (right and left ventricle, atria), three skeletal muscle types (diaphragm, quadriceps and gastrocnemius) and visceral smooth muscle (stomach) using NFAT isoform-specific probes. The NFATc4-specific probe yielded the most intense signal in both cardiac chambers, displaying two prominent transcripts, one of 6.0 and one of about 4.5 kb, with the latter giving the most intense signal. The same two transcripts were present throughout all muscle types, albeit at lower levels (Figure 3.1B, lower panel). NFATc3 gave two transcript sizes, with a smaller one of about 4.8 kb being the most intense in all muscle types, but most prominently expressed in the cardiac ventricular chambers. Two transcripts for NFATc2 were also detected, which were each expressed at comparable levels in all tissues tested. NFATc1 showed three different transcripts, with the smallest transcript (2.0 kb) being expressed in the left ventricle and the two larger transcripts being expressed at roughly equivalent levels in most other tissues analyzed (Figure 3.1B, upper panel). All blots were probed for 18S to verify quality and equal loading of RNA (data not shown). Collectively, these data indicate that transcripts for all four calcineurin-regulated NFAT factors are present in cardiomyocytes, with those for NFATc3 and NFATc4 being present at the highest levels.

To verify whether these transcripts were also efficiently translated into their respective protein products, a series of Western blots were performed on total protein lysate from cultured neonatal rat ventriculocytes using isoform-specific antibodies. Tissue extract of rat brain and rat thymus (data not shown) served as a positive control. Figure 3.1C demonstrates that signals for all four calcineurin-regulated NFAT factors were obtained in cardiomyocytes, albeit at differing intensity, and with products ranging in mass from 70 kDa to 200 kDa. For NFATc1

and NFATc2 discrete protein signals were observed, while for NFATc3 and NFATc4 multiple bands in the range of 100 kDa to 200 kDa were observed. This may reflect generation of proteins by alternatively spliced transcripts (see Figure 3.1B) and/or by differential phosphorylation states of the NFATc3 and NFATc4 proteins. Although the different affinities of the separate antibodies used do not allow for direct comparison of signal intensity, it is interesting to note that the relatively higher intensity of the protein signals for NFATc3 (Figure 3.1C) correlates with its relatively high signal in the Northern blot analysis (Figure 3.1B). Conclusively, RT-PCR, Northern blot and Western blot analyses all point towards the existence of all four calcineurin-regulated NFAT isoforms in the ventricular cardiomyocyte.

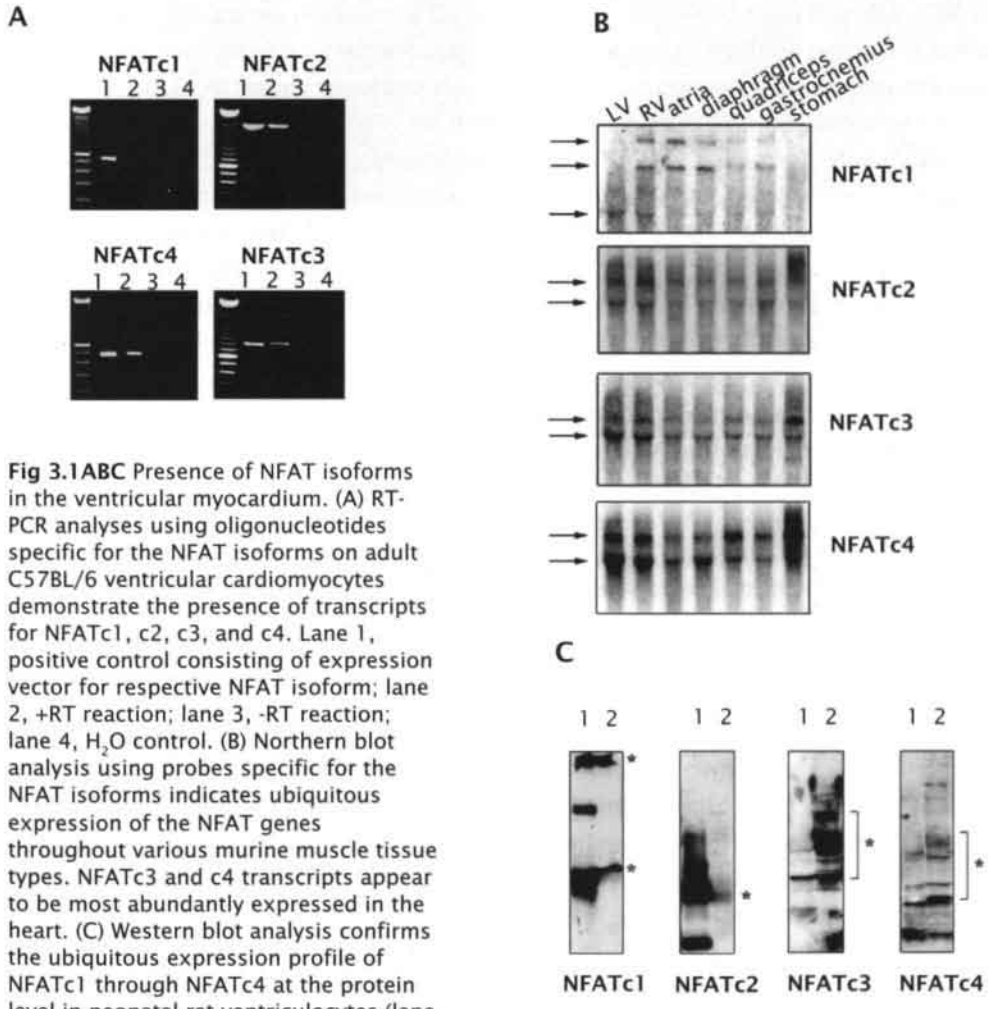


Fig 3.1ABC Presence of NFAT isoforms in the ventricular myocardium. (A) RT-PCR analyses using oligonucleotides specific for the NFAT isoforms on adult C57BL/6 ventricular cardiomyocytes demonstrate the presence of transcripts for NFATc1, c2, c3, and c4. Lane 1, positive control consisting of expression vector for respective NFAT isoform; lane 2, +RT reaction; lane 3, -RT reaction; lane 4, H_2O control. (B) Northern blot analysis using probes specific for the NFAT isoforms indicates ubiquitous expression of the NFAT genes throughout various murine muscle tissue types. NFATc3 and c4 transcripts appear to be most abundantly expressed in the heart. (C) Western blot analysis confirms the ubiquitous expression profile of NFATc1 through NFATc4 at the protein level in neonatal rat ventriculocytes (lane 2 in each panel). Lane 1 constitutes a positive control of rat brain tissue extract (Santa Cruz sc-2392).

Nuclear translocation of all cardiac NFAT isoforms upon calcineurin activation

NFAT transcription factors are dephosphorylated upon activation of the Ca^{2+}/CaM dependent phosphatase calcineurin, resulting in unmasking of their nuclear localization signals permitting nuclear import. To verify that NFATc isoforms could be activated by calcineurin in cardiac myocytes, we performed immunocytochemistry for each NFAT factor at baseline or after infection with an adenovirus expressing a constitutively activated form of calcineurin (AdCnA) or after stimulation with Endo-1. Cardiomyocytes were DAPI stained to visualize the nuclei and facilitate observation of nuclear localization (Figure 3.2B, D, F and H, left and right panel). NFATc1, c2 and c3 were easily detectable using their respective antibodies and displayed a predominant cytosolic localization in unstimulated cardiomyocytes (Figure 3.2A, left and right panel, NFATc1, c3, and data not shown for NFATc2). AdCnA infection resulted in nuclear accumulation of each NFAT isoform in nearly 100% of the myocytes evaluated. Stimulation with the agonist Endo-1 for 12 hrs resulted in efficient NFATc1 and c3 nuclear translocation in about 70% of cardiomyocytes (Figure 3.2E, left and right panel). Similar findings were obtained for NFATc2 (data not shown). To control for the specificity of the antibodies used, the primary isoform-specific antibody was omitted, which resulted in background fluorescence (Figure 3.2G, left and right panel). All cells examined were also positive for sarcomeric actin using phalloidin staining, thereby confirming their identity as cardiomyocytes (data not shown). These results indicate that NFATc1, c2 and c3 are equally sensitive to endogenous calcineurin activation as demonstrated priorly for NFATc4 [10] and point towards a potential contribution for all NFAT members in calcineurin signaling in the ventricular cardiomyocyte.

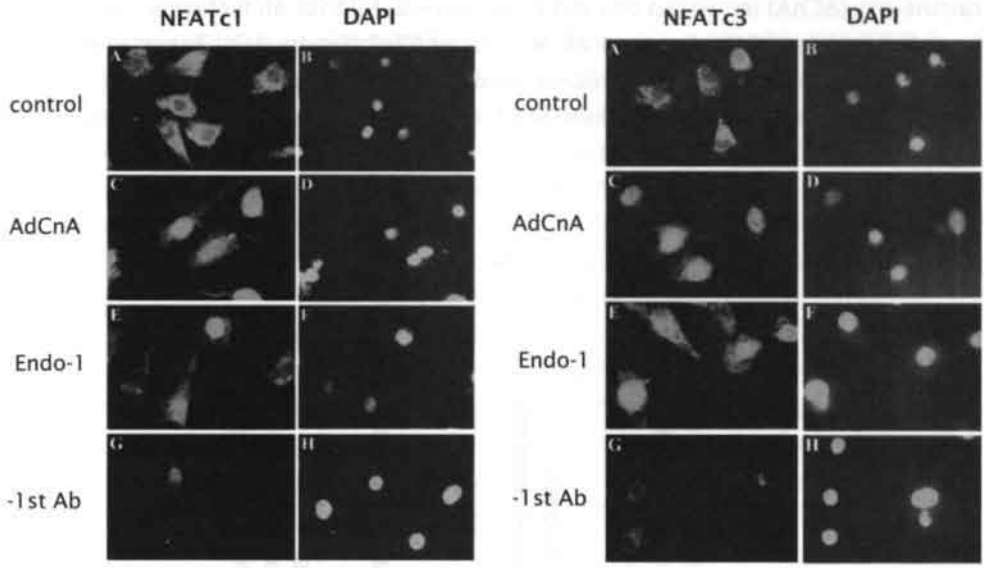


Fig. 3.2 Calcineurin-dependent nuclear translocation of NFATc1 and c3 in cardiomyocytes. Cultured cardiomyocytes were either left unstimulated (A, B), stimulated with AdCnA (C, D), or endothelin-1 (Endo-1) (E, F) and immunostained for subcellular localization of NFATc1 (left Panel) or c3 (right Panel). Nuclei were stained with bisbenzemide (B, D, F, H). Under serum free conditions, NFATc1 and NFATc3 were localized cytoplasmatically (A). Following stimulation with either AdCnA (C) or Endo-1 (E) both isoforms translocated to the nucleus. Panels G and H represent negative controls by ommittance of the primary antibody. **-Full colour image page 179**

All cardiac NFAT isoforms participate in MCIP1 induction

To explore whether the calcineurin-mediated, nuclear import of the cardiac NFAT isoforms was associated with their ability to participate in transcriptional activity of cardiac-specific, calcineurin-responsive promoters, a series of transient cotransfection assays were carried out. Recently, a novel gene was characterized that is present at low levels under physiological conditions in the heart, but undergoes dramatic upregulation following calcineurin activation in the heart.[15,18,25] Remarkably, the gene product itself is a highly specific inhibitor of calcineurin and the gene was therefore designated modulatory calcineurin interacting protein-1 (MCIP1). It is thought that MCIP1 participates in a negative feedback loop to prevent the deleterious effects of unrestrained activation of the enzyme in the ventricular myocyte.[15,18,25] Analysis of the gene structure revealed intron 3 to harbor multiple NFAT consensus sites and was found to be uniquely sensitive to calcineurin-NFAT activation.[18] Cotransfection of hMCIP1(Int3)Luc with expression vectors for the individual NFAT isoforms only slightly induced transcriptional activity (Figure 3.3).

Addition of a construct expressing a constitutively activated mutant of calcineurin (ΔCnA) increased this induction several fold for all isoforms, ranging from 3.5 fold for NFATc4 to over 20 fold for NFATc2 (Figure 3.3). Taken together, these results suggest that all myocardial NFAT members are able to induce transcriptional activation of the human MCIP1 calcineurin-responsive enhancer region.

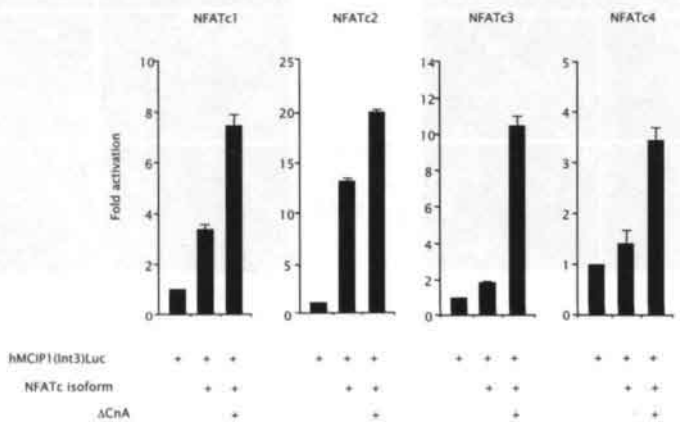


FIg. 3.3 Activation of the human MCIP1-promoter by cardiac NFAT isoforms. COS-7 cells were transiently transfected with a luciferase reporter gene linked to an intragenic segment proximal of exon 4 of the human MCIP1 gene (hMCIP1(Int3)Luc) either or not in the presence of expression vectors for NFATc1, c2, c3, c4, or activated calcineurin (ΔCnA) as indicated. Forty-eight hours later, cells were harvested and luciferase activity was determined. The hMCIP1(Int3)Luc construct proved to be exceptionally sensitive to NFATc activation, independent of the isoform studied. The data represent the mean ± SEM of four independent experiments and are presented as fold activation compared to a control with hMCIP(Int3)Luc alone.

Synergistic activation of the BNP gene by NFAT and GATA4

It was previously demonstrated that the BNP gene promoter is regulated by a distal NFAT sequence element in cooperation with calcineurin and GATA4.[10] To assess whether the additional cardiac NFAT members are also capable of synergizing with GATA4 in regulating this promoter, the hBNP(1800)Luc reporter was tested in the presence or absence of GATA4, ΔCnA and expression vectors for the individual NFAT isoforms. GATA4 alone markedly upregulated hBNP(1800)Luc to about 17 fold over baseline (Figure 3.4A), confirming the previously documented sensitivity of this gene to GATA factors.[26,27] Each individual NFAT isoform demonstrated relatively weak activation in the presence of ΔCnA (Figure 3.4A). In contrast, cotransfection of any single NFAT isoform in the presence of ΔCnA and GATA4 resulted in robust upregulation of hBNP(1800)Luc, ranging from 25 to 50 fold induction depending upon the NFAT member studied (Figure 3.4A). These results indicate that all NFAT members can participate in synergistic activation of the BNP gene in conjunction with GATA4.

A

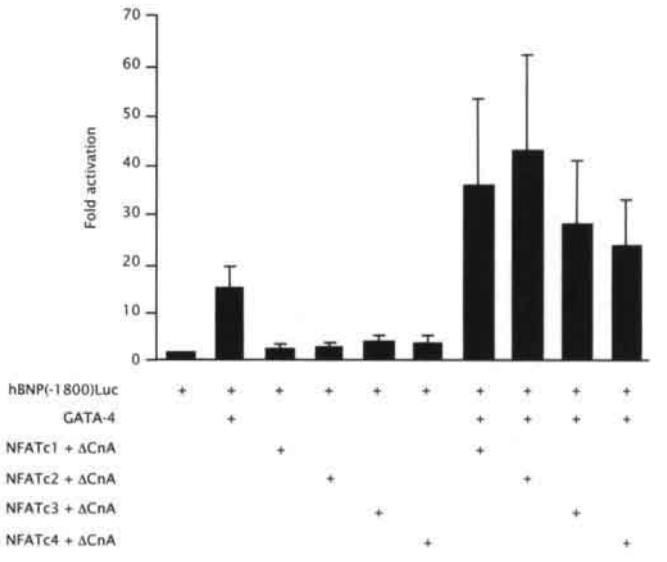


Fig. 3.4A Cardiac NFAT and GATA-4 synergistically activate the human BNP promoter. COS-7 cells were transiently transfected with a luciferase reporter gene linked to an 1800 bp flanking region of the human BNP promoter (hBNP(1800)Luc) either or not in the presence of expression vectors encoding the individual NFAT isoforms, activated calcineurin (ΔCnA), or GATA4, as indicated. Forty-eight hours later, cells were harvested and luciferase activity was determined. As reported previously for NFATc4 (10), NFATc1, c2 and c3 were also able to transactivate the hBNP(1800)Luc construct synergistically with GATA-4. Luciferase values represent the mean ± SEM of three independent experiments and are presented as fold activation compared to a control with hBNP(1800)Luc alone.

To test the specificity of this interaction and whether functional binding sites for either factor are required in the synergistic activation of the BNP reporter, a series of hBNP(1800) promoter-luciferase mutants were generated and examined. As a control, the activity of the wildtype hBNP(1800)Luc is shown at the left panel in Figure 3.4B. The two GATA binding sites centered around -116 bp were mutated, which rendered the BNP promoter construct insensitive to GATA4, but not to activation by NFATc3 or c4 (Figure 3.4B, middle panel). Next, in the context of the GATA-mutated reporter (ΔGATA)hBNP(1800)Luc, the distal NFAT site at -927 was mutated, which showed no activation in the presence of NFAT and/or GATA4 (Figure 4B, right panel). These data indicate that the synergistic activation pattern initially described for NFATc4 with GATA4 in control of the BNP gene is fully conserved among each myocardial-expressed NFAT and that this activation profile is critically dependent upon the presence of both intact NFAT and GATA4 binding sites,

B

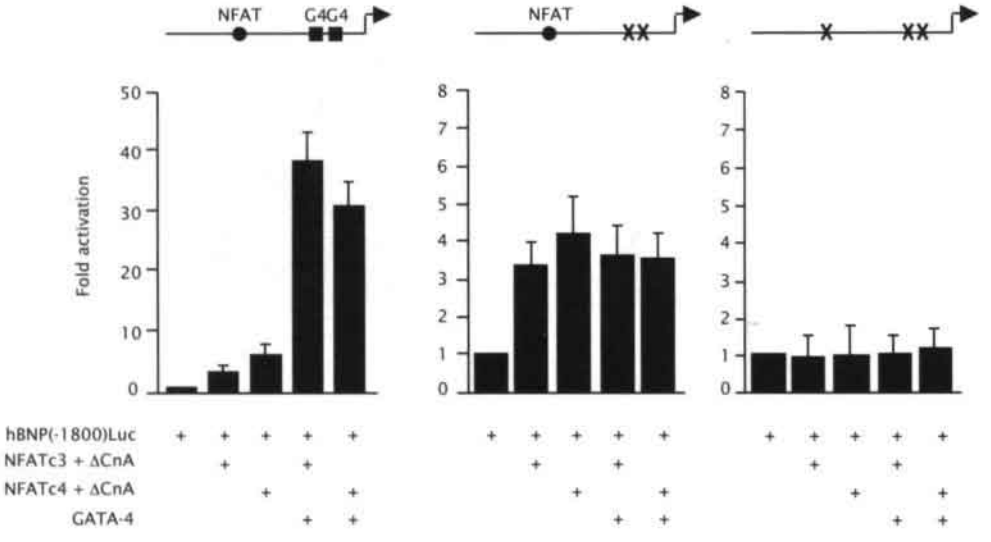


Fig. 3.4B COS-7 cells were transfected with either the wildtype hBNP(1800)Luc reporter (left panel), the GATA-mutated (ΔGATA)hBNP(1800)Luc reporter (middle panel) or the GATA and NFAT site mutated (ΔNFAT, ΔGATA)hBNP(1800)Luc reporter either or not in the presence of expression vectors encoding the most cardiac NFAT isoforms NFATc3 or c4, activated calcineurin (ΔCnA), and GATA4. Forty-eight hours later, cells were harvested and luciferase activity was determined. Luciferase values represent the mean ± SEM of three independent experiments and are presented as fold activation compared to a control with either hBNP(1800)Luc reporter alone.

Dominant negative NFAT inhibits calcineurin-mediated MCIP1 gene expression

Because all four members of the NFAT transcription factor family are present in cardiomyocytes, a dominant inhibitory strategy was developed that targets NFAT activation. Such a strategy would bypass gene redundancy issues to permit evaluation of NFAT's role as a calcineurin effector in the heart. Several independent groups have demonstrated that constructs consisting of only the NH_2-terminal domain of NFAT can interfere with NFAT-mediated transcription in a dominant inhibitory fashion. This region includes the Ser-rich region (SRR) and three conserved Ser-Pro repeats (SP boxes A, B, and C) (Figure 3.5A). The SP boxes represent major sites of interaction of NFAT with calcineurin in vitro,[28] and sites of NFAT phosphorylation in vivo have been identified in the SRR.[28-30] Previous studies identified the conserved Pro-Xaa-Ile-Xaa-Ile-Thr (PxIxIT) box (residues 114 to 119 in NFATc4) as the region that confers inhibitory NFAT transcriptional activity. To globally inactivate all NFAT factors, we generated a construct encoding amino acid residues 3-191 of the N-terminus from NFATc4 that contains this calcineurin *interacting region* (Figure 3.5A).

A

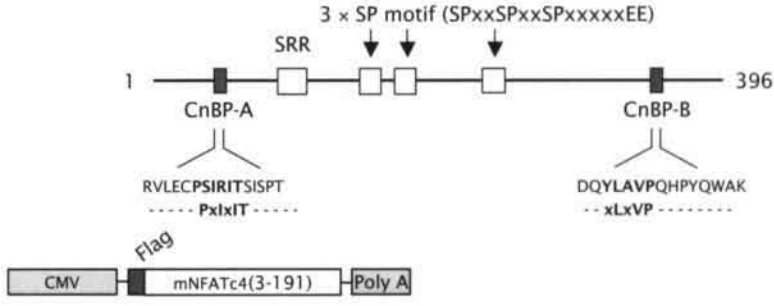


Fig. 3.5A Schematic representation of the NH_2-terminal homology region of NFATc4 with conserved domains depicted. Below a schematic representation of the dominant negative NFATc4(PxIxIT) construct driven by a CMV promoter, encompassing the first 191 aa residues, including the conserved calcineurin docking PxIxIT domain.

Cotransfection assays in COS-7 cells were performed using this construct, each of the NFATc isoforms, and the hMCIP1(Int3)-luciferase reporter plasmid (Figure 3.3B). Overexpression of ΔCnA and NFATc1 through c4 each induced robust NFAT transcriptional activity (Figure 3.5B). However, expression of the dominant negative NFATc4(PxIxIT) construct, the NH_2-terminal NFATc4 homology domain (residues 3 to 191), dose-dependently inhibited transcription mediated by each NFAT isoforms (Figure 3.5B). In the absence of full length NFAT isoforms, transcriptional activity was not observed in either the absence or the presence of NFATc4(PxIxIT) (data not shown). These data indicated that the NH_2-terminal NFAT homology domain interferes in a dominant inhibitory fashion with NFAT-mediated regulation of a cardiac responsive promoter, regardless of the NFAT isoform studied.

B

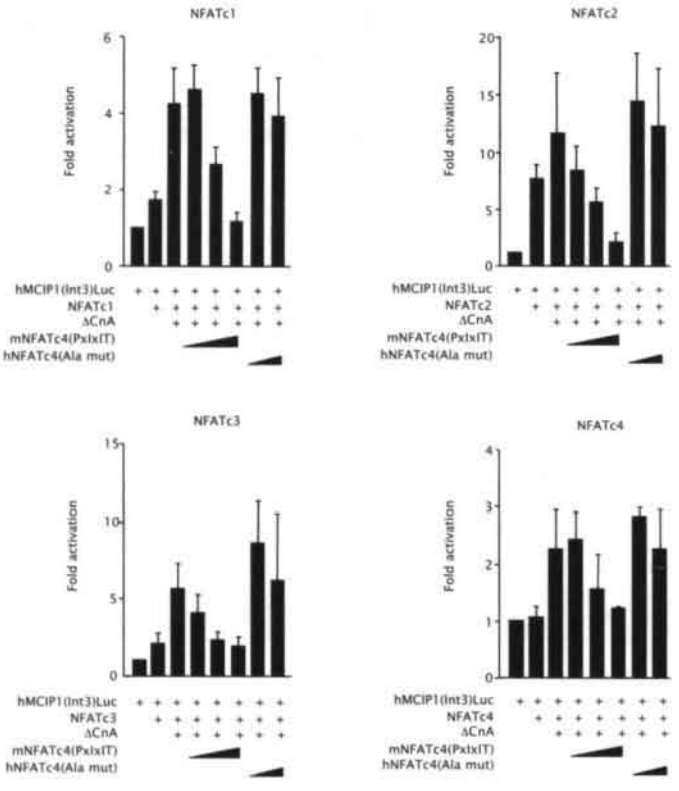


Fig. 3.5B Transfection experiments in COS-7 cells using the hMCIP(Int3)Luc reporter indicates that increasing amounts of dominant negative (0.2, 0.4, and 0.6 μg) NFATc4(PxIxIT) dose-dependently inhibits NFAT-mediated transcriptional activity exerted by each individual NFAT isoform and in the presence of activated calcineurin (ΔCnA). No inhibitory effects are seen when the control construct NFATc4(Ala mut), in which the PxIxIT is replaced with Ala residues, is cotransfected (0.2 and 0.6 μg) with the hMCIP(Int3)Luc reporter and the NFAT isoforms in the presence of ΔCnA. Luciferase values represent the mean ± SEM of three independent experiments and are expressed as fold activation compared to a control with hMCIP1(Int3)Luc alone.

To test whether our dominant inhibitory NFATc4(PxIxIT) construct was dependent upon the presence of an intact PxIxIT box and to exclude issues regarding cytotoxicity, a similar NH_2-terminal NFATc4 construct, NFATc4(Ala mut) was included in cotransfection assays. In this construct, the conserved PxIxIT box residues were mutated to Ala residues to generate an AxAxAA box, which is now ineffective in blocking calcineurin interaction. As anticipated, co-expression of this mutant construct displayed no inhibitory effect on NFAT-mediated induction of the hMCIP1(Int3)Luc reporter (Figure 3.5B). These data indicate that the PxIxIT box mediates the dominant negative action of our NFATc4(PxIxIT) construct.

Dominant negative NFAT inhibits calcineurin-mediated cardiomyocyte hypertrophy

To investigate the requirement of NFAT activation in calcineurin-mediated cardiomyocyte hypertrophy, we generated two replication-deficient adenoviral vectors expressing either the dominant negative NH_2-terminal NFAT construct NFATc4(PxIxIT) or the control construct NFATc4(Ala mut) under control of the CMV promoter (Figure 3.6A).

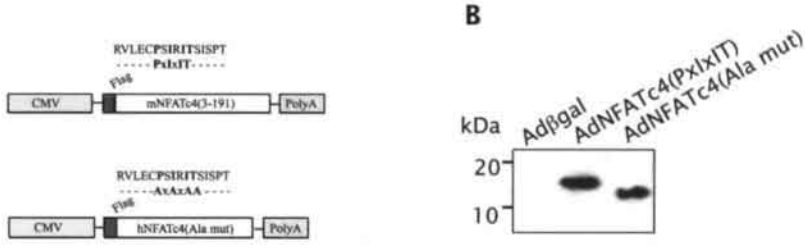


Fig. 3.6AB (A) Schematic representation of dominant negative NFATc4(PxIxIT) and the control NFATc4(Ala mut) constructs expressed as adenoviral vectors. (B) Western blot analysis using an anti-Flag antibody on COS-7 cell lysates infected with either Adβgal, the dominant negative NFAT adenovirus AdNFATc4(PxIxIT) or the control virus AdNFATc4(Ala mut) at an MOI of 100.

Infection of COS-7 cells with either AdNFATc4(PxIxIT) or AdNFATc4(Ala mut) at an MOI of 100 resulted in robust expression of polypeptide fragments of ~19 and ~16 kDa, respectively, which were easily detectable on the basis of their Flag-immunoreactivity (lane 2 and 3, Figure 3.6B). Conversely, Adβgal infection resulted in the absence of any proteins reactive for the anti-Flag antibody (lane 1, Figure 3.6B). Taken together, these results demonstrate that AdNFATc4(PxIxIT) and AdNFATc4(Ala mut) are correctly expressed and should represent an effective way to inhibit NFAT activity in cultured neonatal cardiomyocytes.

Cardiomyocytes were first infected with the control adenovirus Adβgal (Figure 3.6C, panel A-C), AdNFATc4(PxIxIT) (Figure 6C, panel D-F) or AdNFATc4(Ala mut) (Figure 3.6C, panel G-I). After 24 hrs, the cultured cells were then stimulated with the hypertrophic agonist cardiotrophin-1 (CT-1) or Endo-1 (data not shown), by infection with the activated calcineurin-expressing adenovirus, or left untreated for 24 hrs (Figure 3.6C). The data demonstrate that only AdNFATc4(PxIxIT) infection prevented cardiomyocyte hypertrophy in response to AdCnA or CT-1 (Figure 3.6C, panel E and F). Adenoviral infection with either Adβgal (Figure 3.6C, panel B and C) or the control adenovirus AdNFATc(Ala mut) (Figure 3.6C, panel H and I) had no discernable effects on either AdCnA or agonist-induced sarcomeric deposition and cellular enlargement. Importantly, neither Adβgal, AdNFATc4(PxIxIT) or AdNFATc4(Ala mut) infection induced cardiomyocyte apoptosis nor did it affect the morphology (Figure 3.6C, panel A, D and G) and viability of unstimulated cells (data not shown).

C

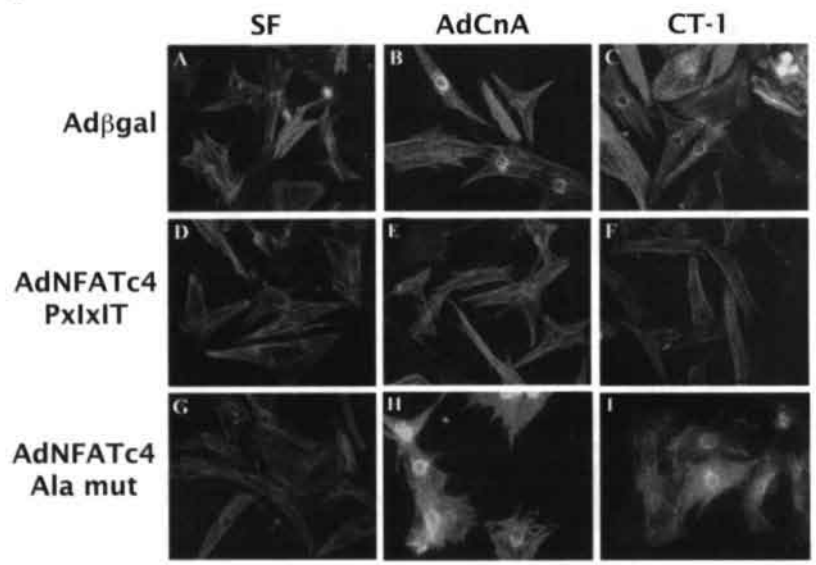


Fig. 3.6C Representative images of immunostained cardiomyocytes infected with the indicated adenoviruses either or not in combination with the hypertrophic agonist CT-1. Phalloidin/ ANF double staining demonstrates less cellular enlargement, sarcomeric organization and perinuclear ANF staining in the presence of AdNFATc4(PxIxIT) following AdCnA infection or agonist stimulation. -**Full colour image page 179**

Quantitation of cardiomyocyte hypertrophy was performed by video edge detection on large numbers of myocytes (Figure 3.6D). Serum free (SF) cultured cardiomyocytes demonstrated to have a similar cell surface area (1153 ± 87 μm^2) as Adβgal infected, SF cultured cardiomyocytes (1061 ± 69 μm^2). In agreement with previous studies, AdCnA, CT-1 or Endo-1 treatment (20,24) resulted in a more than 2-fold increase in cell surface area (2295 ± 112, 2274 ± 104 and 2072 ± 151 and μm^2, respectively, $P<0.01$ vs SF). Prior infection with Adβgal and subsequent stimulation with AdCnA, CT-1 or Endo-1 resulted in comparable cardiomyocyte hypertrophy as priorly SF cultured myocytes (2080 ± 89, 2455 ± 155 and 2347 ± 118 μm^2, respectively, $P<0.01$ vs SF). AdNFATc4(PxIxIT) infection completely abrogated the pro-hypertrophic effects of AdCnA, CT-1 or Endo-1 treatment to 1147 ± 52, 1193 ± 56 and 1184 ± 92 μm^2, respectively ($P < 0.01$ vs AdCnA, CT-1 and Endo-1, P = NS vs SF). In sharp contrast, AdNFATc4(Ala mut) infection prior to AdCnA infection or treatment with CT-1 or Endo-1 had no effects on the morphological alterations of these prohypertrophic stimuli (2384 ± 116, 2905 ± 224 and 2476 ± 171 μm^2, respectively; P = NS vs AdCnA, CT-1 or Endo-1). The data demonstrate that adenoviral dominant negative NFAT transfer was able to prevent the hypertrophic remodeling of cardiomyocytes following calcineurin activation.

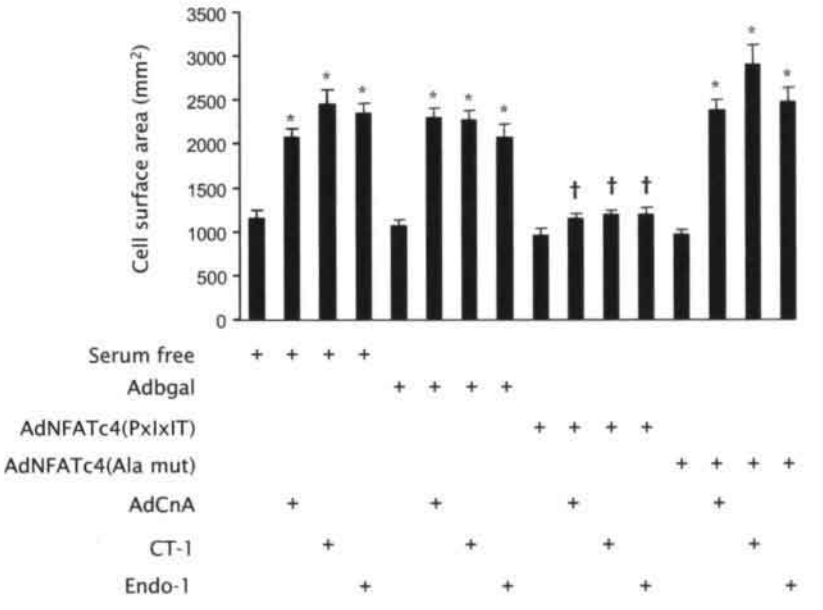


Fig. 3.6D Cell surface areas were quantified for each of the indicated conditions, demonstrating that inactivation of NFAT signaling abrogates cardiomyocyte hypertrophy in response to an activated calcineurin mutant, Endo-1 or CT-1 stimulation. Data in D represent the mean ± SEM of three independent experiments. * indicates $p < 0.05$ vs serum free conditions, † indicates $p < 0.05$ vs Adβgal followed by AdCnA infection.

Increased ANF expression is a hallmark of cardiac hypertrophy and is readily detected by immunocytochemistry as perinuclear staining.[31,32] Serum-free cultured cardiomyocytes infected with either Adβgal, AdNFATc4(PxIxIT) or AdNFATc4(Ala mut) were stimulated with agonist or the activated calcineurin-expressing adenovirus and scored for the numbers of cells with perinuclear ANF expression (Figure 3.6C). The data demonstrate that only AdNFATc4(PxIxIT) infection blocked ANF expression in response to the pro-hypertrophic stimuli investigated (Figure 3.6C, panel E and F). Taken together, the results indicate that dominant negative NFAT abrogated ANF expression following calcineurin activation or agonist stimulation in cultured cardiomyocytes.

DISCUSSION

Overlapping expression of NFAT isoforms in the heart

One unexpected finding of the present study is that the ventricular cardiomyocyte population contains all four, calcineurin-sensitive NFAT isoforms described in the literature to date (reviewed in.[6,33,34] All NFAT members of the transcription factor family are expressed in multiple isoforms, generated by alternative splicing.[35-38] The results in the present study support this notion (Figure 3.1B). The existence of multiple splice isoforms has been shown in detail for NFATc2 and c4 in T lymphocytes and other cells,[35-37] and it has been shown that all spliced isoforms elicit transactivation of NFAT-responsive promoters, albeit with slightly differing efficiencies.[38] The observation that cardiomyocytes express each of the 4 calcineurin-regulated NFAT family members, which themselves undergo differential splicing, justifies the dominant negative strategy employed here to inhibit NFAT-mediated transcriptional activation.

Although initially characterized in T-cells, almost all tissues in the mammalian organism express one or more NFAT family member. For example, NFATc2 is somewhat restricted in expression to immune cells and skeletal muscle, while NFATc3 expression is enriched in thymocytes and skeletal muscle cells, but also present at lower levels in various other tissues. NFATc1 and NFATc4 appear to be expressed in a more ubiquitous pattern,[6,39-44] where they influence development, proliferation, and differentiation of a number of mammalian tissues.[33,45] The data in the present study confirm this ubiquitous expression pattern of NFAT members throughout several muscle types, supporting the function of the calcineurin-NFAT signaling pathway regulating cardiac hypertrophy, skeletal muscle myogenesis and fiber-type specification, and smooth muscle cell proliferation and vessel remodeling.[33,45-50]

Crucial role for NFAT signaling in cardiomyocyte hypertrophy

The NFAT dominant inhibitory approach employed here specifically blocked the ability of calcineurin or agonist stimuli to promote nuclear accumulation and transcriptional activation of endogenous or overexpressed NFAT factors. Our approach utilized overexpression of the NFAT NH_2-terminal calcineurin docking domain containing the conserved sequence Pro-Xaa-Ile-Xaa-Ile-Thr (PxIxIT box).[51] It should be noted that NFATc4(PxIxIT), the dominant negative NFAT construct used in the present study, encompasses aa residues 3 to 191 of human NFATc4, while the "internal control" construct, NFATc4(Ala mut), was slightly shorter and encompasses residues 2 to 130 of human NFATc4. Although it would have been

formally more correct to use dominant negative and control constructs of the same length, it is highly unlikely that the differing phenotypic effects observed between the dominant negative construct and the control construct may be due to this slight difference in length. Indeed, Chow et al. have clearly demonstrated that the dominant inhibitory action of N-terminal portions of NFAT only depends upon the presence of the PxIxIT box, which encompasses residues 114 to 119 in human NFATc4, rendering relative length of truncated NFAT constructs beyond residue 119 in this particular context irrelevant.[28]

To address whether NFAT signaling is required for (calcineurin-mediated) cardiomyocyte hypertrophy, two model systems are routinely employed; cultured cardiomyocytes and genetically altered mice. In this study we employed adenoviral-mediated gene transfer in cultured cardiomyocytes to circumvent potential difficulties associated with gene targeting such as isoform redundancy or compensatory changes in gene expression. For example, gene targeting of individual NFAT family members did not reveal a widespread defect in the ability of T cells to proliferate or generate cytokines such as IL-2 [28,52-55], even though the calcineurin-NFAT paradigm was established as a regulator of interleukin-2 gene transcription. Germane to our study, Chow et al. established the involvement of NFAT activity in regulating in IL-2 expression using a similar dnNFAT molecule.[28] This dnNFAT molecule selectively inhibited NFAT transcription activity by interfering with the activation-induced nuclear import of NFAT and the active component of this inhibitor correspondent to the PxIxIT box located in the conserved NH_2-terminal homology region of NFAT.[28]

In addition to gene targeting, transgenesis in the mouse could be employed as a means of blocking NFAT activation through overexpression of the dominant negative NFAT protein domain in the heart. However, exhaustive attempts to generate dominant negative NFAT transgenic mice failed, presumably due to embryonic or early post-natal lethal effects associated with complete NFAT inhibition in the heart (De Windt and Molkentin, unpublished observations). Indeed, NFATc3 X NFATc4 double null mice die late during embryogenesis with severe vascular abnormalities.[47]

Another documented approach to abrogate NFAT signaling is the use of kinases that directly phosphorylate NFAT transcription factors, thus antagonizing nuclear accumulation. For example, glycogen synthase kinase-3β (GSK-3β) is a serine/threonine protein kinase with many targets, including at least two NFAT proteins.[55] In addition, GSK-3β has been identified as a critical negative modulator of cardiomyocyte hypertrophy by directly antagonizing the prohypertrophic effects of activated calcineurin.[52] Recently, GSK-3β was also proven to be capable of inhibiting hypertrophic signaling in the intact myocardium.[53] Transgenic mice expressing a constitutively activated form of GSK-3β in cardiomyocytes displayed a severely blunted hypertrophic response to chronic β-adrenergic stimulation, pressure overload, and the actions of the calcineurin transgene.[53] However,

GSK-3β also inhibits GATA-4 function in cardiomyocytes,[54] suggesting that GSK-3β likely also inhibits the hypertrophic response through NFAT-independent mechanisms. Nevertheless, we favor the interpretation that calcineurin-NFAT signaling is a dominant regulatory pathway for cardiac hypertrophy, and likely the germane mechanism underlying the anti-hypertrophic effect of GSK-3β. Indeed, cardiomyocytes infected with adenoviruses expressing truncated forms of either the calcineurin inhibitory protein Cain/cabin-1 or AKAP79 [20], which target and inhibit calcineurin itself [56-58], also showed a severe attenuation of myocyte hypertrophy in vitro. The combined observations suggest a pivotal role for calcineurin-NFAT signaling in cardiomyocyte hypertrophy.

Cardiac NFAT signaling: functional redundancy or functional specification?

Although this study establishes that NFAT activity is required for both calcineurin as agonist-induced cardiomyocyte hypertrophy, the present data await extrapolation to the in vivo situation. As discussed above, we were unsuccessful in generating cardiac-specific transgenic mice expressing this dominant negative NFAT protein. These observations suggest that NFAT factors are crucial during developmental maturation of the myocardium. However, it is not known if all NFAT factors contribute to the myocyte growth response through a generalized mechanism, or if individual isoforms play specific functions. For example, NFATc1 gene targeted mice die during embryonic development due to defects in heart valve formation.[59,60] With respect to the adult heart and the regulation of hypertrophic growth, we have recently targeted the NFATc4 gene in the mouse. Surprisingly, NFATc4-null mice did not show a defect in their ability to mount a hypertrophic response.[10,61] By contrast, NFATc3-null mice did show a significant attenuation of hypertrophy following diverse stimuli.[61] Collectively, these observations suggest that several NFAT isoforms might play critical regulatory roles in the adult myocardium. Indeed, here we observed that NFATc3 is abundantly present in ventricular myocytes (Figure 3.1).

Alternatively, it is possible that certain NFAT factors have specified to fulfill various pathophysiological roles in the heart, in addition to or even excluding hypertrophic signaling. For example, we have demonstrated that adenoviral expression of NFATc4 rendered cardiomyocytes less susceptible to staurosporine or oxidative stress-induced apoptosis.[11] Moreover, Kakita et al. demonstrated that NFATc1 plays a crucial role in endothelin-1 mediated protection against oxidative stress-induced apoptosis in cardiomyocytes.[62] Since NFATc4 apparently plays a minor role in cardiac hypertrophic signaling [61], yet signals a pro-survival phenotype [11] it is possible that certain NFAT factors have highly specified functions in the heart. To more firmly establish the functional hierarchy

or potential inter-isoform specific roles between the individual myocardial NFAT members in the heart, generation of mouse models with loxP flanked alleles for the four documented NFAT genes is warranted.

Acknowledgements

We thank Marc Sussman, Antoon Moorman, Roger Davis and Laurie Glimcher for reagents, Joep Brinkman and Jodil Willems for technical assistance in adult cardiomyocyte isolation, and Victor Thijssen for graphical assistance. This work was supported by the Netherlands Heart Foundation (NHS 99-114 and NHS 2000-160) and the Interuniversitary Cardiology Institute Netherlands (to P.A.D); National Institutes of Health (NIH) grants HL60562 and HL07382 and a Scholar Award from the Pew Foundation (to J.D.M.); and an American Heart Postdoctoral Fellowship (Ohio Valley Affiliate), a Young Investigator's Award in Cardiology from the Bekales Foundation and grant NWO 902-16-275 from the Netherlands Foundation for Scientific Research (to L.J.D.W.).

[1] Abbreviations are: ANF, atrial natriuretic factor; βgal, βgalactosidase; BNP, brain natriuretic factor, CnA, calcineurin; CT-1, cardiotrophin-1; Endo-1, endothelin-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCIP1, myocyte enriched calcineurin inhibitory protein, NP40, nonidet P40; NFAT, Nuclear Factor of Activated T-cells; RT, reverse transcriptase

REFERENCES

1. Cleland, J. G., Khand, A., and Clark, A. (2001) Eur Heart J 22, 623-626.
2. http://www.americanheart.org/statistics.
3. Hunter, J. J., and Chien, K. R. (1999) N Engl J Med 341, 1276-1283.
4. Chien, K. R. (1999) Cell 98, 555-558.
5. Lorell, B. H., and Carabello, B. A. (2000) Circulation 102, 470-479.
6. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu Rev Immunol 15, 707-747
7. Aramburu, J., Garcia-Cozar, F., Raghavan, A., Okamura, H., Rao, A., and Hogan, P. G. (1998) Mol Cell 1, 627-637.
8. Aramburu, J., Yaffe, M. B., Lopez-Rodriguez, C., Cantley, L. C., Hogan, P. G., and Rao, A. (1999) Science 285, 2129-2133.
9. Garcia-Cozar, F. J., Okamura, H., Aramburu, J. F., Shaw, K. T., Pelletier, L., Showalter, R., Villafranca, E., and Rao, A. (1998) J Biol Chem 273, 23877-23883.
10. Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) Cell 93, 215-228.
11. De Windt, L. J., Lim, H. W., Taigen, T., Wencker, D., Condorelli, G., Dorn, G. W., 2nd, Kitsis, R. N., and Molkentin, J. D. (2000) Circ Res 86, 255-263
12. Molkentin, J. D. (2000) Circ Res 87, 731-738.
13. Bueno, O. F., van Rooij, E., Molkentin, J. D., Doevendans, P. A., and De Windt, L. J. (2002) Cardiovasc Res 53, 806-821.
14. Zou, Y., Hiroi, Y., Uozumi, H., Takimoto, E., Toko, H., Zhu, W., Kudoh, S., Mizukami, M., Shimoyama, M., Shibasaki, F., Nagai, R., Yazaki, Y., and Komuro, I. (2001) Circulation 104, 97-101.
15. Rothermel, B. A., McKinsey, T. A., Vega, R. B., Nicol, R. L., Mammen, P., Yang, J., Antos, C. L., Shelton, J. M., Bassel-Duby, R., Olson, E. N., and Williams, R. S. (2001) Proc Natl Acad Sci U S A 98, 3328-3333.
16. De Windt, L. J., Lim, H. W., Bueno, O. F., Liang, Q., Delling, U., Braz, J. C., Glascock, B. J., Kimball, T. F., del Monte, F., Hajjar, R. J., and Molkentin, J. D. (2001) Proc Natl Acad Sci U S A 98, 3322-3327.
17. Bueno, O. F., Wilkins, B. J., Tymitz, K. M., Glascock, B. J., Kimball, T. F., Lorenz, J. N., and Molkentin, J. D. (2002) Proc Natl Acad Sci U S A 99, 4586-4591
18. Yang, J., Rothermel, B., Vega, R. B., Frey, N., McKinsey, T. A., Olson, E. N., Bassel-Duby, R., and Williams, R. S. (2000) Circ Res 87, E61-68.
19. Schaap, F. G., Binas, B., Danneberg, H., van der Vusse, G. J., and Glatz, J. F. (1999) Circ Res 85, 329-337
20. Taigen, T., De Windt, L. J., Lim, H. W., and Molkentin, J. D. (2000) Proc Natl Acad Sci U S A 97, 1196-1201
21. Ranger, A. M., Gerstenfeld, L. C., Wang, J., Kon, T., Bae, H., Gravallese, E. M., Glimcher, M. J., and Glimcher, L. H. (2000) J Exp Med 191, 9-22.
22. De Windt, L. J., Willemsen, P. H., Popping, S., Van der Vusse, G. J., Reneman, R. S., and Van Bilsen, M. (1997) J Mol Cell Cardiol 29, 2095-2106
23. Lim, H. W., De Windt, L. J., Steinberg, L., Taigen, T., Witt, S. A., Kimball, T. R., and Molkentin, J. D. (2000) Circulation 101, 2431-2437.
24. De Windt, L. J., Lim, H. W., Haq, S., Force, T., and Molkentin, J. D. (2000) J Biol Chem 275, 13571-13579.
25. Rothermel, B., Vega, R. B., Yang, J., Wu, H., Bassel-Duby, R., and Williams, R. S. (2000) J Biol Chem 275, 8719-8725.
26. Hautala, N., Tenhunen, O., Szokodi, I., and Ruskoaho, H. (2002) Pflugers Arch 443, 362-369.

27. Marttila, M., Hautala, N., Paradis, P., Toth, M., Vuolteenaho, O., Nemer, M., and Ruskoaho, H. (2001) Endocrinology 142, 4693-4700.
28. Chow, C. W., Rincon, M., and Davis, R. J. (1999) Mol Cell Biol 19, 2300-2307.
29. Beals, C. R., Clipstone, N. A., Ho, S. N., and Crabtree, G. R. (1997) Genes Dev 11, 824-834.
30. Zhu, J., Shibasaki, F., Price, R., Guillemot, J. C., Yano, T., Dotsch, V., Wagner, G., Ferrara, P., and McKeon, F. (1998) Cell 93, 851-861.
31. Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J., and Chien, K. R. (1998) J Biol Chem 273, 2161-2168.
32. Wang, Y., Su, B., Sah, V. P., Brown, J. H., Han, J., and Chien, K. R. (1998) J Biol Chem 273, 5423-5426.
33. Crabtree, G. R., and Olson, E. N. (2002) Cell 109 Suppl, S67-79.
34. Graef, I. A., Chen, F., and Crabtree, G. R. (2001) Curr Opin Genet Dev 11, 505-512.
35. Hoey, T., Sun, Y. L., Williamson, K., and Xu, X. (1995) Immunity 2, 461-472
36. Imamura, R., Masuda, E. S., Naito, Y., Imai, S., Fujino, T., Takano, T., Arai, K., and Arai, N. (1998) J Immunol 161, 3455-3463
37. Luo, C., Burgeon, E., and Rao, A. (1996) J Exp Med 184, 141-147.
38. Chuvpilo, S., Avots, A., Berberich-Siebelt, F., Glockner, J., Fischer, C., Kerstan, A., Escher, C., Inashkina, I., Hlubek, F., Jankevics, E., Brabletz, T., and Serfling, E. (1999) J Immunol 162, 7294-7301
39. Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R., and Williams, R. S. (1998) Genes Dev 12, 2499-2509.
40. Delling, U., Tureckova, J., Lim, H. W., De Windt, L. J., Rotwein, P., and Molkentin, J. D. (2000) Mol Cell Biol 20, 6600-6611
41. Friday, B. B., and Pavlath, G. K. (2001) J Cell Sci 114, 303-310.
42. Horsley, V., Friday, B. B., Matteson, S., Kegley, K. M., Gephart, J., and Pavlath, G. K. (2001) J Cell Biol 153, 329-338.
43. Kegley, K. M., Gephart, J., Warren, G. L., and Pavlath, G. K. (2001) Dev Biol 232, 115-126.
44. Olson, E. N., and Williams, R. S. (2000) Cell 101, 689-692.
45. Olson, E. N., and Williams, R. S. (2000) Bioessays 22, 510-519.
46. Boss, V., Abbott, K. L., Wang, X. F., Pavlath, G. K., and Murphy, T. J. (1998) J Biol Chem 273, 19664-19671.
47. Graef, I. A., Chen, F., Chen, L., Kuo, A., and Crabtree, G. R. (2001) Cell 105, 863-875.
48. Stevenson, A. S., Gomez, M. F., Hill-Eubanks, D. C., and Nelson, M. T. (2001) J Biol Chem 276, 15018-15024.
49. Suzuki, E., Nishimatsu, H., Satonaka, H., Walsh, K., Goto, A., Omata, M., Fujita, T., Nagai, R., and Hirata, Y. (2002) Circ Res 90, 1004-1011.
50. Wada, H., Hasegawa, K., Morimoto, T., Kakita, T., Yanazume, T., Abe, M., and Sasayama, S. (2002) J Cell Biol 156, 983-991.
51. Park, S., Uesugi, M., and Verdine, G. L. (2000) Proc Natl Acad Sci U S A 97, 7130-7135
52. Haq, S., Choukroun, G., Kang, Z. B., Ranu, H., Matsui, T., Rosenzweig, A., Molkentin, J. D., Alessandrini, A., Woodgett, J., Hajjar, R., Michael, A., and Force, T. (2000) J Cell Biol 151, 117-130.
53. Antos, C. L., McKinsey, T. A., Frey, N., Kutschke, W., McAnally, J., Shelton, J. M., Richardson, J. A., Hill, J. A., and Olson, E. N. (2002) Proc Natl Acad Sci U S A 99, 907-912.

54. Morisco, C., Seta, K., Hardt, S. E., Lee, Y., Vatner, S. F., and Sadoshima, J. (2001) J Biol Chem 276, 28586-28597
55. Hardt, S. E., and Sadoshima, J. (2002) Circ Res 90, 1055-1063
56. Sun, L., Youn, H. D., Loh, C., Stolow, M., He, W., and Liu, J. O. (1998) Immunity 8, 703-711.
57. Lai, M. M., Burnett, P. E., Wolosker, H., Blackshaw, S., and Snyder, S. H. (1998) J Biol Chem 273, 18325-18331.
58. Coghlan, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M., and Scott, J. D. (1995) Science 267, 108-111
59. Ranger, A. M., Grusby, M. J., Hodge, M. R., Gravallese, E. M., de la Brousse, F. C., Hoey, T., Mickanin, C., Baldwin, H. S., and Glimcher, L. H. (1998) Nature 392, 186-190.
60. de la Pompa, J. L., Timmerman, L. A., Takimoto, H., Yoshida, H., Elia, A. J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B. L., Crabtree, G. R., and Mak, T. W. (1998) Nature 392, 182-186.
61. Wilkins, B. J. D. W., L.J., Bueno, O.F., Braz, J.C., Glascock, B.J., Kimball, T.F., Molkentin, J.D. Mol Cell Biol (In Press)
62. Kakita, T., Hasegawa, K., Iwai-Kanai, E., Adachi, S., Morimoto, T., Wada, H., Kawamura, T., Yanazume, T., and Sasayama, S. (2001) Circ Res 88, 1239-1246

Chapter 4

Regulation of cardiac gene expression and hypertrophy by MEF2-dependent recruitment of Nuclear Factor of Activated T-cells and p300 histone acetyltranferase

Eva van Rooij, Ralph J. van Oort, Anne-Sophie Armand,
and Leon J. De Windt

SUMMARY

One central pathway that transduces prohypertrophic signals in the cardiac muscle cell employs the Ca^{2+} activated phosphatase calcineurin (CnA), but to date, little work has focused on the downstream Ca^{2+}-activated transcriptional circuits that regulate hypertrophy. Members of the Nuclear Factor of Activated T-cells (NFAT), the transcriptional effectors of Ca^{2+}-calcineurin signaling, are relatively weak activators alone and require nuclear partners such as AP-1, C/EBP, GATA-4 and the transcriptional coactivator p300 to allow maximal induction of target genes. Ca^{2+} induction of a subset of cardiac genes and hypertrophy in cardiac muscle cells has been mapped to response elements recognized by the MEF2 family of transcription factors. Herein, we report transcriptional regulation of genes by a combinatorial mechanism involving proteins of the NFAT and MEF2 families. Transient co-expression of MEF2C or -A, NFATc3, p300 and constitutively activate CnA was sufficient to recapitulate the selective induction of a subset of genes during cardiac hypertrophy. *In vivo*, MEF2 and NFATc3/c4 formed a complex containing histone acetyltransferase (HAT) activity on distal MEF2 *cis*-elements in the β-*myosin heavy chain* (β-MyHC) and *connective tissue growth factor* (CTGF) genes, which are both elevated during pathological cardiac hypertrophy. Using viral gene transfer of dominant negative MEF2, which prevents binding of MEF2 on its corresponding *cis*-elements, we demonstrate that MEF2 is a required component for cardiomyocytes to undergo Ca^{2+}/CnA-induced hypertrophy. Taken together, these data provide evidence for the existence of a transcriptional regulatory complex that integrates the Ca^{2+}-activated CnA/NFAT signaling pathway with the myogenic transcriptional activity of MEF2.

INTRODUCTION

Cytoplasmic members of the Nuclear Factor of Activated T-cells (NFATc1-c4, Hugo Nomenclature Committee http://www.gene.ucl.ac.uk/nomenclature/) represents a family of Ca^{2+}/calcineurin (CnA)-dependent transcription factors that are activated by sustained, low-amplitude Ca^{2+} signals that provoke activation of the heterodimeric phosphatase CnA.[1] Most NFAT isoforms are constitutively expressed and exist as inactive, cytosolic phosphoproteins. CnA binds NFAT on conserved motif and dephosphorylates serines residues within SP repeats and serine rich motifs in the amino-terminus of NFAT family members, which unmasks a nuclear localization sequence, triggering their nuclear translocation.

Although originally thought to be largely restricted to cells of the immune system, NFAT family members have since been shown to play a role in other cell types. In non-immune cells, NFAT has been shown to regulate heart valve development,[2,3] control differentiation of skeletal myocytes into slow- or fast-twitch fiber types [4] and contribute to the development of hypertrophy in cardiac and skeletal myocytes.[5,6]

DNA binding by NFAT proteins is quite weak and therefore NFAT family members probably rarely act alone, but rather need transcriptional partners to bind DNA. NFAT engages in direct protein-protein interactions and/or influences transcription synergistically with transcription factors such as Maf, ICER, and p21SNFT that belong to the same basic region-leucine zipper (bZIP) family as AP-1;[7-9] the zinc finger GATA factors, and EGR;[5,10] and the helix-loop-helix domain proteins Oct, HNF3, and IRF-4.[11,12] Thus, cooperative binding of NFAT proteins with diverse transcription factors makes Ca^{2+} signaling dependent on coincident activation of other signaling pathways in the transcriptional control of diverse target genes.

Ca^{2+} responsiveness of a number of promoters/enhancers has also been mapped to another set of response elements recognized by the MADS (MCM-1, agamous, deficiens, serum response factor) box transcription factor MEF2, which binds to response elements bearing the consensus sequence $CTA(A/T)_4TAG$. CnA activates Nur77 in a MEF2-dependent manner in lymphocytes. CnA was shown to enhance the DNA-binding activity of MEF2A by its direct dephosporylation.[13] Alternatively, CnA has also been reported to activate the Nur77 promoter in T-lymphocytes by promoting nuclear translocation of NFATc2, which in its turn forms a ternary complex with MEF2.[14] Following this premise, NFAT may act as a transcriptional activator for MEF2. More recently, a number of studies have elegantly shown that a network of distinct transcriptional coactivators and repressors modulates MEF2 function. MEF2 is actively repressed by class II histone deacetylases (HDACs) in unstimulated cells,[15] while chromatin remodeling enzymes with histone acetyl-transferase (HAT) activity, such as p300 and GRIP-1, have been found to associate with MEF2,[16] were they are thought to facilitate gene

expression by inducing histone acetylation.

Here we report the identification of a subset of MEF2-responsive cardiac genes specifically upregulated following CnA activation. Transient co-expression of MEF2, activated NFAT and p300 recapitulated the selective induction of genes in CnA transgenic hearts. Multiple conserved, muscle regulatory motifs were found within the distal regulatory regions of β-MyHC and CTGF, consisting of NFAT sites, E-boxes, and CArG motifs surrounding a conserved MEF2 site. This cluster of regulatory elements display striking similarity to the enhancer of the mouse *muscle creatine kinase* gene.[17] The specific interaction of a MEF2/NFAT/p300 transcriptional complex on the MEF2 site was demonstrated using chromatin immunoprecipitation (ChIP) assays. The requirement of MEF2 for CnA signaling was demonstrated by a dominant negative MEF2, which abrogated the characteristic hypertrophic remodeling in cultured cardiomyocytes. Our results are consistent with a model wherein MEF2 controls cardiac gene expression by acting as a primary channel for Ca^{2+}-activated NFAT signaling and chromatin remodeling.

EXPERIMENTAL PROCEDURES

Recombinant plasmids

Expression vectors for NFATc1-c4 or an activated mutant of CnA were described previously.[1] pCDNA3 vectors containing human MEF2A, or -C were generously provided by Eric Olson. Expression vectors containing hemaglutinin (HA) epitope tagged full length p300 or CBP under control of the CMV promoter (pCMVβ-p300-HA and pCMVβ-CBP-HA) were kindly provided by Richard Eckner. The mouse βMyHC promoter, containing a genomic fragment from position -5500 to position +1 relative to the transcription start site (generous gift of Jeff Robbins), was cloned as a *BamHI - HindIII* fragment into the *BglII - HindIII* site of pGL3-Basic (Promega). The rat CTGF promoter containing a genomic fragment from position -3000 to +1 relative to the transcription site cloned adjacent to luciferase in pGL3 (Promega) and was a kind gift from Roel Goldschmeding. A CMV-driven expression vector for murine DSCR1 was generated by RT-PCR on total RNA isolated from adult mouse heart tissue using Trizol reagent (Invitrogen) and SuperscriptII Reverse Transcriptase (Invitrogen), subcloned into the *EcoRI* site of pECE to incorporate a NH_2-terminal FLAG epitope tag and PCR cloned into the *HindIII* site of pCDNA3 (Invitrogen) to create pCDNA3-mMCIP1. The 3×MEF2-Luc plasmid, generously provided by Eric Olson, contains three copies of a high-affinity MEF2 binding site from the MCK enhancer inserted upstream of pGL2 (Promega). Cytomegalovirus (CMV) promoter driven eukaryotic expression plasmid (pCMV-Tag5A; Invitrogen) coding for a COOH-terminal myc epitope-tagged polypeptide corresponding to aa 2 through 117 from human MEF2 was PCR generated from pCDNA3-hMEF2C using Accutaq high fidelity system (Sigma) followed by ligation into the *BamHI* site of pCMV-Tag5A and confirmed by diagnostic restriction and sequencing analysis. The vector pCMV-Tag5A-MEF2(R24L) expressing a dominant negative MEF2 was constructed using site-directed mutagenesis from the parent plasmid by replacing the argenine residue at position 24 by leucine using site-directed mutagenesis (Quickchange-XL kit; Stratagene) and the following forward primer (mutations bold and underlined): 5'-cgt aac aga cag gtg aca ttt aca aag ttg aaa ttt ggg ttg atg. Deletion fragments encoding either full length or domains of hMEF2C or hNFATc3/x1 were PCR-generated to create NH_2-terminal fusions with the yeast DNA-binding domain of Gal4 by ligation into the *BamHI* or *SalI* site of pBIND (Promega). The luciferase reporter pG5-Luc (Promega) multimerized binding sites (5x) for GAL4 inserted upstream to luciferase. Detailed information about plasmids and oligo sequences is available upon request.

Cell culture, transfections and luciferase assays

Low passage COS7 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS. 2 x 10^5 COS7 cells seeded in 24-well plates and transfected at 50-60% confluency with a total of 2 μg DNA in DMEM containing 2% FBS with 1.5 μl

FuGENE 6 reagent (Roche, Indianapolis) per μg DNA. pDM-lacZ (Promega; 0.2 μg), was included in each experiment to correct for transfection efficiency. The cells were washed 48 hours after transfection with phosphate-buffered saline and lysed with 100 μl of Reporter Lysis Buffer (Roche), centrifuged at 15,000 g for 5 min and fifty μl of lysate assayed for luciferase activity using the Luclite luciferase reporter gene assay kit (Packard Instruments, Meriden, CT) according to the manufacturer's protocol on a 96-well Topcount liquid scintillation counter (Packard Instruments, Meriden, CT). Twenty μl of cellular lysate was used for measuring β-galactosidase activity. Isolation and culture of neonatal rat ventricular cardiomyocytes was performed as described before in detail. [18]

Coimmunoprecipitation assays

For coimmunoprecipitation experiments, 5×10^5 COS7 cells grown in 6-well dishes were transfected using 1.5 μl FuGENE 6 reagent per μg DNA with expression vectors (1 μg each) encoding the indicated (see Figure 2) Gal4- or Flag epitope-tagged hMEF2C or hNFATc3/x expression vectors. Twenty-four hours later, cells were harvested in 500 μl lysis buffer (PBS containing 0.5% NP-40, 150 mM NaCl, 0.5 mM EDTA, 10 mM Tris-HCl, 2 μg/ml leupeptin, 10 μg/ml PMSF, 2μg/ml soybean trypsin inhibitor and protease inhibitors; Complete, Boehringer Mannheim). Cells were subjected to brief sonication, and cellular debris removed by centrifugation. Full-length hMEF2C or hNFATc3 was immunoprecipitated using anti-pan-MEF2 or anti-NFATc3 polyclonal antibody (sc-10794 and sc-8321; Santa Cruz, respectively) and 30 μl protein A/G agarose (Santa Cruz). Precipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and sequentially immunoblotted with monoclonal antisera against Gal4 (sc-510, Santa Cruz Biotechnology). Signals were detected with an Enhanced Chemiluminescence kit (ECL, Amersham) and analyzed using Adobe photoshop 6.0 software.

Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation was carried out using the Upstate Biotechnology ChIP assay kit as per the manufacturer's instructions. Briefly, neonatal rat ventricular myocytes either left untreated or infected with adenovirus expressing an activated mutant of calcineurin (AdCnA)[18] on 10 cm dishes containing 5×10^6 cells were crosslinked by adding $1/10^{th}$ volume of formaldehyde solution (3.7 %) to the media at room temperature for 20 min with gentle mixing. Soluble chromatin was obtained by sonicating cellular lysates to shear genomic DNA, clarifying by centrifugation (10 min at 14,000 x g at 4°C), diluting 10-fold in ChIP dilution buffer precleared with protein A beads. Equal amounts of soluble chromatin from each sample were immunoprecipitated with 10 μg of one of the following antibodies: anti-acetylated histone H3 antibody (#06-599, Upstate Biotechnology); monoclonal antisera against NFATc2 (sc-7296, Santa Cruz) or NFATc3 (sc-8405, Santa Cruz); pan-MEF2 polyclonal antibody

(sc-10794, Santa Cruz). Following immunoprecipitation and immobilization of immunocomplexes, the lysates were put at 65°C for four hours to reverse the formaldehyde crosslinks. Associated DNA was purified by the Qiaex PCR purification kit. PCR was carried out using specific primers to the promoter regions for rat β-MyHC, and CTGF or mouse DSCR1. For primer sequences see Supplemental Table 1.

Real time PCR and primer design

Primers were targeted against c-jun, ANF, CTGF, desmin, α-MyHC, β-MyHC, MLC2a, MLC2v, α-SkA, Glut4, MCK, MCIP1Ex4, and L7. The primers were specific for mouse sequences (www.ensembl.org) and selected based on the following requirements: i) primer melting temperature of ~60°C, ii) GC-content of ~55%, iii) preferably no G at 5' end, iv) avoid runs of more than 3 identical nucleotides, and v) amplicon length of ~100 nucleotides. Specificity was checked with the Basic Local Alignment Search Tool (BLAST) and the specific melting point of the amplicons was analyzed using Biorad Dissociation curve software (iCycler, Biorad). All primer sets were tested for PCR efficiency and alternative primers were designed in case they fell outside the 5% efficiency range ($3.14 \leq \text{slope} \leq 3.47$).

Ventricular tissue samples from wildtype and calcineurin transgenic mice were homogenized using an Ultrathurax in 1 ml TRIzol reagent (Invitrogen) to isolate total RNA according to the instructions provided by the manufacturer. Three μg of the RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). In order to avoid the amplification of genomic DNA, PCR primers were chosen so that the amplification product covered several exons of the gene of interest. Real-time PCR using the BioRad iCycler (Biorad) and fluorescence detection was performed in 96-well plates using SYBR Green. PCR amplification was performed (in duplicate) as a singleplex reaction in a total reaction volume of 25 μl. The PCR reaction was performed in a 25 μL volume consisted of 400 nM forward and reverse primers (Supplemental Table 2), 40 ng cDNA, 12.5 μl 2×SYBR Green PCR master mix (Biorad). The PCR was cycled between 95 °C/30 s and 60 °C/30 for 40 cycles, following an initial denaturation step at 95 °C for 3 min. Amplification products were routinely checked using dissociation curve software (Biorad), and transcript quantities were compared using the relative C_t method, where the amount of target normalised to the amount of endogenous control (L7) and relative to the control sample is given by $2^{-\Delta\Delta Ct}$. Real time PCR results were verified by electrophoresis of the reverse transcribed material in 1.2% agarose gels and visualized under UV illumination after ethidium bromide staining.

Recombinant adenoviruses.

COOH-terminal Myc-tagged MEF2 encompassing residues 1-117 and incorporating the R24L mutation[19] was cloned into the *NotI* site of pAdTrack-CMV

viral shuttle vector (generous gift of Bert Vogelstein, John Hopkins University, Baltimore) and recombined in BJ5183 bacteria (Stratagene) to obtain an E1-E3-deleted adenoviral bicistronic vector to generate AdMEF2(R24L), which expresses both MEF2(R24L) and GFP under separate CMV promoters. AdGFP was generated as described previously.[20] An adenovirus expressing an activated mutant of calcineurin (AdCnA) was described previously.[21]

Immunocytochemistry.

Myocytes were prepared as described previously.[18,21] To visualize cardiomyocyte size and sarcomeric organization, cells were stained with phalloidin-Texas Red (Molecular probes) at a dilution of 1:400 and nuclei visualized with Vectashield/DAPI (H-1200, Vector Laboratories). An epifluorescence microscope (Axiovert 135M Zeiss) was used obtain digitized images at a 400x magnification and quantitaty cardiomyocyte cell surface area using NIH image software on 80-100 GFP-positive cardiomyocytes in 10 to 20 fields in three independent experiments.

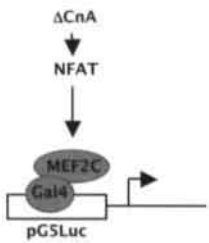
Statistical analysis.

The results are presented as mean values ± standard error of the mean (SEM). Statistical analyses were performed using InStat 3.0 software (GraphPad Software Inc., San Diego, CA) and ANOVA followed by Bonferroni's post-test when appropriate.

NFAT mediates activation of MEF2 by calcineurin.

NFAT is a known substrate for calcineurin (CnA) and translocates from the cytosol into the nucleus upon dephosphorylation by CnA, making it a likely candidate transcriptional effector of CnA-dependent MEF2 activation. To date, NFATc2 has been shown to mediate such an interaction with MEF2, but the functional significance of NFATc2 in the heart, unlike NFATc3, has not been addressed so far.[18,22] To verify the activation profile of MEF2 by calcineurin, we ectopically expressed Gal4-MEF2C and pG5-Luc in the absence or presence of the four NFAT isoforms, NFATc1, -c2, -c3, and -c4. Reporter gene activation was increased by expression of either NFAT isoform, and was increased further by the presence of a constitutively active form of CnA (ΔCnA), which dephosphorylates NFAT even in the absence of a Ca^{2+} signal (Figure 4.1A). These data suggest all four NFAT isoforms can stimulate MEF2 transcriptional activity (Figure 4.1A). Since, of all NFAT isoforms, NFATc3 demonstrated the most potent activation potential of MEF2C, and this NFAT isoform was demonstrated to be required component of CnA signaling in the heart,[22] we concentrated on this NFAT isoform for the remainder of the study.

A



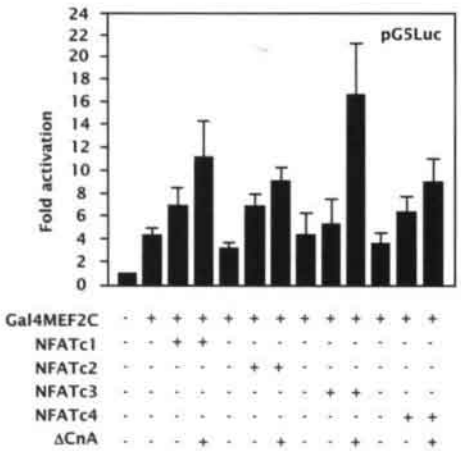


Fig 4.1A COS7 cells were transiently transfected with a Gal4 luciferase reporter construct, and a Gal4-MEF2C fusion protein either or not in the presence of expression vectors for NFATc1, c2, c3, c4, activated calcineurin (ΔCnA). The data demonstrate that MEF2 activity increased in the presence of NFAT, regardless of the isoform transfected.

p300 is a member of a family of coactivators involved in the regulation of transcription and chromatin, and was shown to directly interact with either NFAT or MEF2.[16,23-25] To test the possibility that the MEF2/NFAT complex can activate transcription by its ability to recruit p300, we expressed Gal4-MEF2C, NFATc3 and ΔCnA in the presence of pG5-Luc, which gave a 15-fold increase in the reporter gene activity, while addition of p300 further enhanced this level to 25-fold activation (Figure 4.1B). These findings were confirmed by co-transfecting Flag-MEF2C, NFATc3 and ΔCnA, but now using a reporter with luciferase under control of multimerized MEF2 binding sites. In this case, presence of Flag-MEF2C, NFATc3 and ΔCnA resulted in a 10-fold increase of the reporter construct, whereas presence of p300 resulted in a 30-fold activation (Figure 4.1C). Collectively, these data indicate that all NFAT isoforms are able to stimulate MEF2-dependent gene expression, and that presence of the co-activator p300 potentiates this effect, most likely by ternary complex formation.

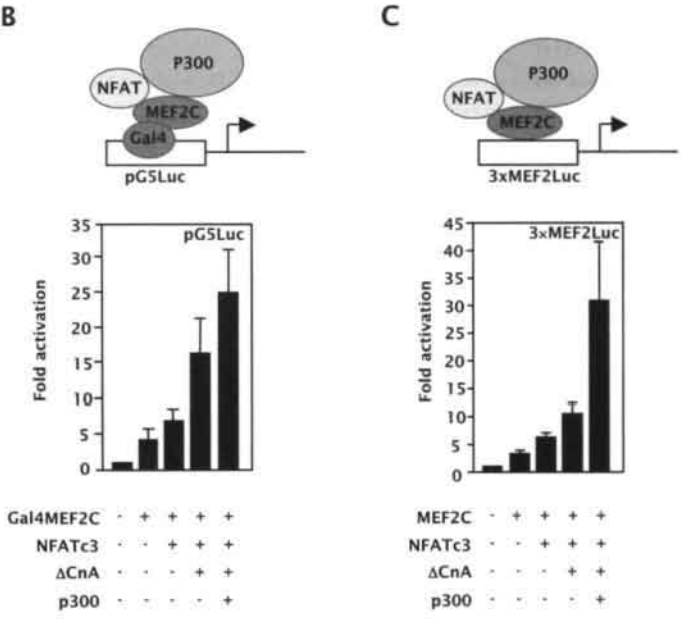
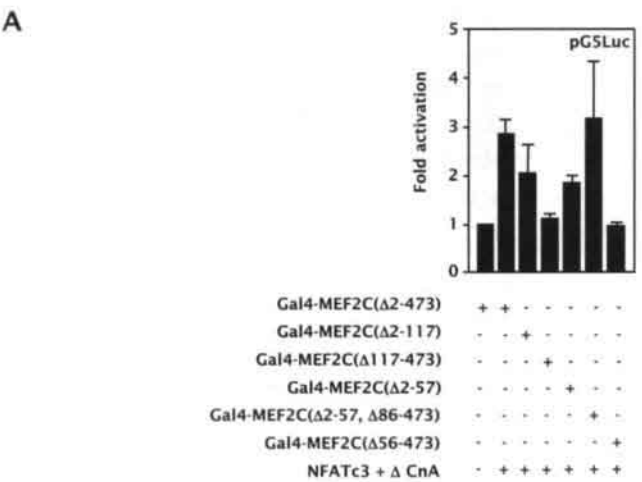


Fig. 4.1BC NFAT and p300 synergistically activate MEF2 transcriptional activity. (B) Transfection experiments using a Gal4 reporter indicate that the MEF2/NFAT complex is more robustly activated by addition of p300. (C) A multimerized MEF2 reporter was synergistically activated by the MEF2/NFAT/p300 complex.

Minimal domains involved in protein-protein interaction between NFAT and MEF2.

Recently, interacting domains on MEF2D and NFATc2 were mapped to the MADS-box DNA binding in MEF2D and the C-terminal transactivation domain (C-TAD) of NFATc2. To test whether these domains were conserved in distinct MEF2 and NFAT isoforms, various truncation fragments of MEF2C were fused to Gal4. Co-expression of Flag-NFATc3 and ΔCnA in the presence of the pG5-Luc

reporter demonstrated efficient activation in the presence of full length MEF2C, and deletion fragments harboring the MADS box (Figure 4.2A). In contrast, MEF2C mutants lacking respectively the MADS box and MEF2 domain, or only the MADS box (see Figure 2C for schematic representation of the various MEF2 mutants used), were unable to transactivate the pG5-Luc reporter in the presence of NFATc3 (Figure 4.2A).



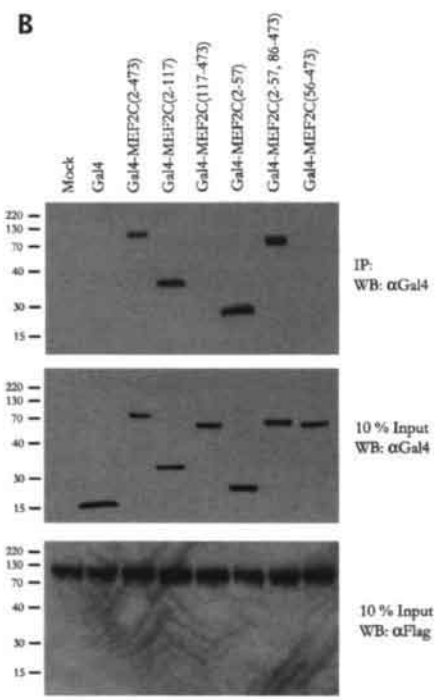


Fig 4.2AB Mapping of NFATc3 and MEF2C interacting domains. (A) COS7 cells were transfected with deletion mutants of Gal4-MEF2C fusions with or without Flag-NFATc3. The data show that the sensitivity of MEF2C to NFATc activation depends on the MADS-box in MEF2C. The data represent the mean ± SEM of four independent experiments and are presented as fold activation compared to Gal4-MEF2C fusions without NFATc3. (B) Protein extracts from COS7 cells transfected with empty vectors (Gal4), Flag-NFATc3 and/or Gal4-MEF2C deletion constructs were immunoprecipitated using an anti-NFATc3 antibody, and subjected to Western blotting using an anti-Gal4 antibody (top panel). The lower two panels are loading controls for Gal4-MEF2C fusions or Flag-NFATc3.

C

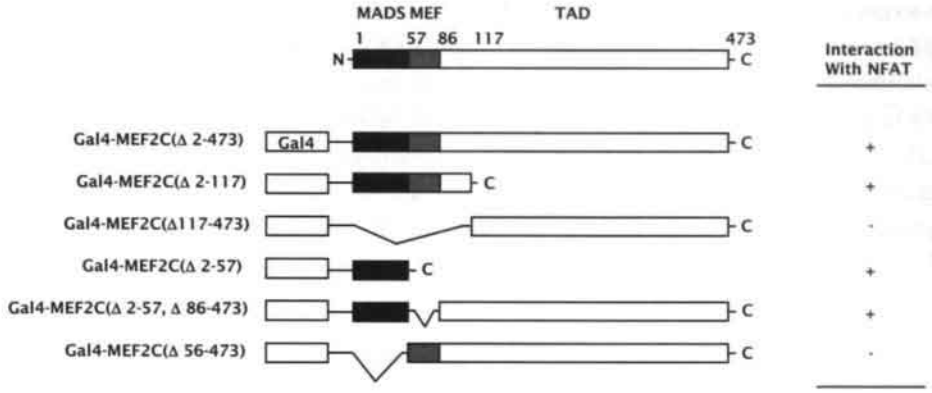


Fig. 4.2C Schematic overview of the Gal4-MEF2C deletion constructs and their ability to bind NFATc3.

To verify the transfection results, Flag-NFATc3, ΔCnA in the presence or absence of various Gal4-MEF2C deletion constructs were co-expressed in COS7 cells, and immunoprecipitated with an anti-NFATc3 antibody. The presence of Gal4-MEF2C deletion mutants was detected by immunoblotting against Gal4. Among the NFATc3-interacting clones detected were full length MEF2C (Figure 4.2B, lane 3) and mutants that harbored the MADS box domain (Figure 4.2B, lane 4, 6, and 7). MEF2C-mutants lacking either the MADS box and MEF2 domain (Figure 4.2B, lane 5) or the MADS box alone (Figure 4.2B, lane 8) were unable to interact with Flag-NFATc3.

Conversely, to map the domain on NFATc3 that interacts with MEF2, four Gal4-NFATc3 fusions were constructed, harboring either full length NFATc3, the N-terminal regulatory domain, the Rel Homology Domain (RHD), or the C-TAD. COS7 cells transiently transfected with pG5-Luc, Gal4-NFATc3 mutants and Flag-MEF2C demonstrated efficient transactivation when either full length NFATc3 or the C-TAD of NFATc3 linked to Gal4 (Figure 4.2D). In contrast, Gal4 constructs lacking the C-TAD showed no discernable activation of pG5-Luc (Figure 4.2D).

D

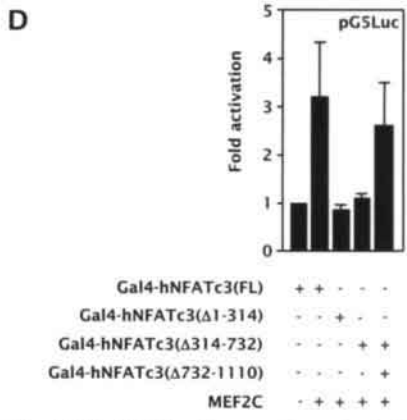


Fig. 4.2D COS7 cells were transfected with deletion mutants of Gal4-NFATc3 fusions with or without Flag-MEF2C. The data show that the sensitivity of NFATc3 to MEF2C-dependent activation depends on the C-TAD in NFATc3. The data presented as fold activation compared to Gal4-NFATc3 fusions in the absence of MEF2C.

Next, Flag- MEF2C, with either of the four distinct Gal4-NFAT fusions were co-expressed in COS7 cells, followed by co-immunoprecipitation with a pan-MEF2 antibody. Presence of the distinct Gal4-NFAT fusion proteins was scored by Gal4 immunoreactivity. Among the interacting proteins detected were full length NFATc3 (Figure 4.2E, lane 3) and the C-TAD of NFATc3 coupled to Gal4 (Figure 4.2E, lane 6). These data are in line with the transient transfection assays from Figure 4.2D. Collectively, these findings indicate that specific interactions exist between the MADS box of MEF2C with NFATc3, on the one hand, and the C-TAD of NFATc3 with MEF2C, on the other (Figure 4.2C and 4.2F).

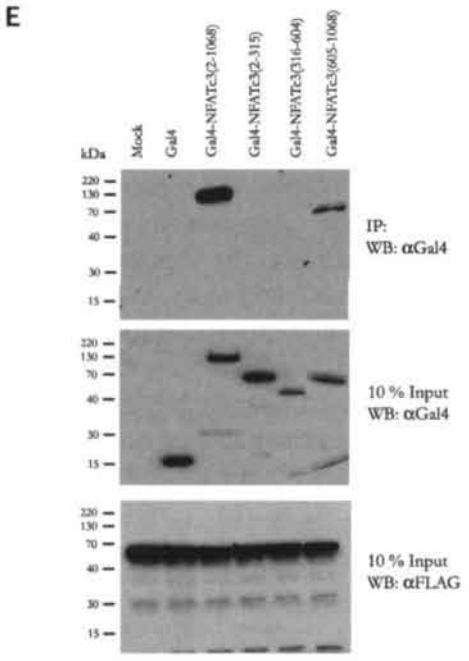
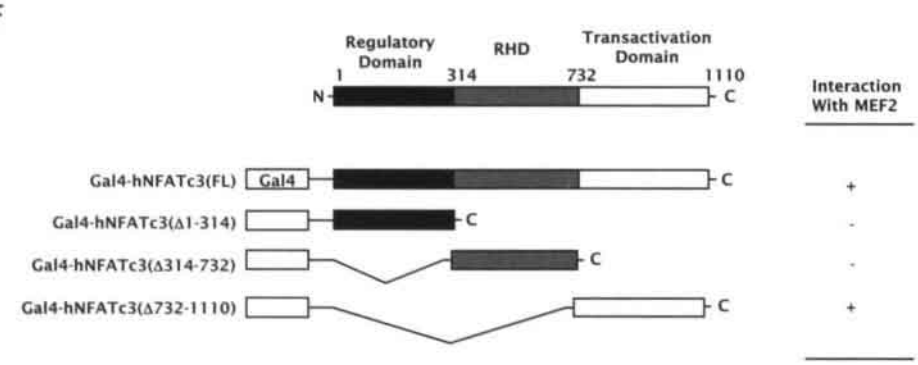


Fig 4.2EF Mapping of NFATc3 and MEF2C interacting domains. (E) Protein extracts from COS7 cells transfected with empty vectors (Gal4), Gal4-NFATc3 deletion constructs and/or Flag-MEF2C were immunoprecipitated using an anti-MEF2 antibody, and subjected to Western blotting using an anti-Gal4 antibody (top panel). The lower two panels are loading controls for Gal4-NFATc3 fusions or Flag-MEF2C. (F) Schematic overview of the Gal4-NFATc3 deletion constructs and their ability to bind MEF2C.



Cardiac MEF2-NFAT complex formation and gene expression in vivo.

To assess the significance of MEF2/NFAT/p300 complex formation in terms of cardiac gene expression, transcript levels of a subset of genes were determined by quantitative PCR in 1 month-old wildtype and CnA transgenic hearts.[5] Transgenic hearts displayed a clear upregulation of transcripts for β-MyHC, *the atrial isoform of the regulatory myosin light chain-2* (MLC2A), *atrial natriuretic factor* (*ANF*), CTGF, and α-*skeletal actin* (αSkA) (Figure 4.3A). Interestingly, the expression levels of several other genes, such as *c-jun, desmin, GLUT-4* and *MCK*, previously reported to be MEF2-regulated,[26] displayed no discernable changes in expression level between transgenic and wildtype hearts. As the upregulated genes roughly fell into two classes (sarcomeric components; β-MyHC, MLC2A, αSKA; and signaling molecules; ANF, CTGF) we selected one gene with the highest transcript upregulation from each class (β-MyHC and CTGF) for further analysis.

A

Fold change realtime PCR	
C-jun	-1.1
ANF	6.1
CTGF	11
Desmin	1.6
α-MyHC	-1.3
β-MyHC	187.7
Mlc2a	59.4
Mlc2v	-1.5
α-SkA	6.0
Glut4	-3.1
MCK	-1.2
MCIP1Ex4	6.5
L7	1

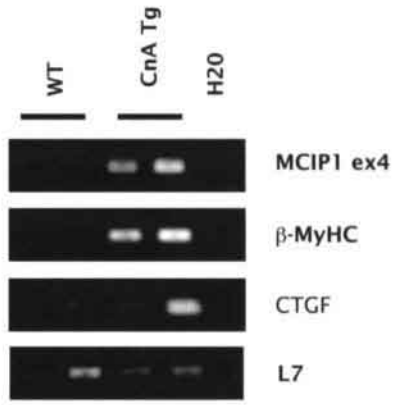


Fig. 4.3A Calcineurin activates MEF2-dependent genes. Quantitive real time PCR indicate that several MEF2 regulated genes are upregulated in calcineurin transgenic hearts. These data were verified for β-MyHC and CTGF by regular RT-PCR.

To define a regulatory mechanism involving NFAT and MEF2 in the induction of β-MyHC and CTGF, large promoter regions for mouse, rat, and human were analyzed and aligned for cross species-conservation. The promoter region of the cardiac β–MyHC gene has been studied in various species, and a number of potential regulatory elements within 300 bp of the transcriptional start site have been identified. However, results from transgenic studies suggest that far-upstream regulatory elements, including a consensus MEF2 site, reside in the 5' flanking region of the β-MyHC gene.[27] A fragment of β-MyHC gene between -2600

bp and -2350 bp relative to the transcriptional start site in the murine sequence, displayed a remarkable conservation with the corresponding rat and human sequence and harbored multiple regulatory sequences, which have been shown to be important for muscle-specific gene expression (Figure 4.3B). These include three E-boxes (consensus CANNTG), one activator protein (AP)-2 site (data not shown), one CArG motif, one CT/ACCC box and one A/T-rich MEF-2 binding site ([2444]TTATTTATAG), which is completely homologous with the consensus MEF2 binding sequence (C/T)TA(T/A)$_4$TA(G/A).

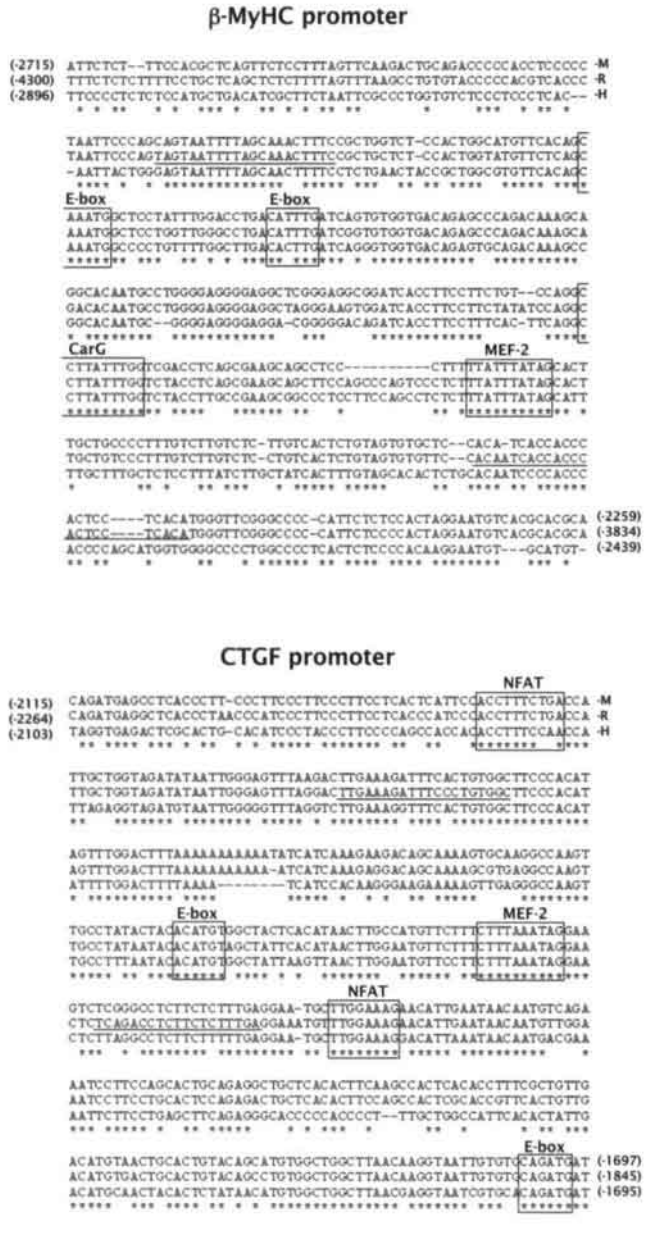


Fig 4.3B Aligment of the mouse, rat and human sequence of part of the promoter region for β-MyHC and CTGF, indicating conserved regions containing muscle-specific regulatory elements.

Also for the CTGF gene a highly conserved region was found between -2075 bp and -1715bp relative to the transcriptional start site of the mouse CTGF gene, containing NFAT binding sites, E box consensus sites, and a MEF2 binding site. The A/T-motif in the CTGF gene ([1889]TCTTTAAATAG) differs from the MEF2 A/T-rich binding site by two single nucleotide substitutions (underlined) although the motif binds endogenous MEF2 (Figure 4.3B).

C

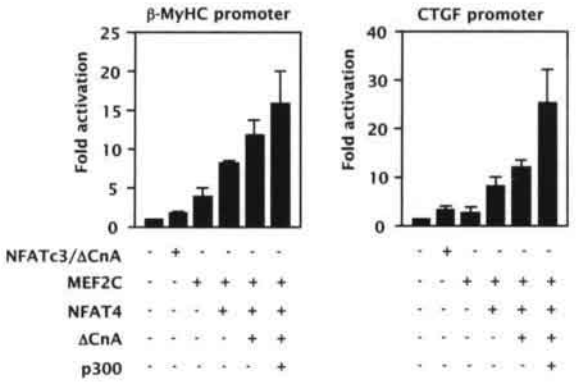


Fig. 4.3C COS-7 cells transiently transfected with β–MyHC and CTGF luciferase reporters proved to be sensitive to combined stimulation with MEF2C and activated NFATc3.

To test whether MEF2/NFAT complex formation suffice to activate these genes, we first tested reporter constructs containing the upstream regulatory sequence of β-MyHC and CTGF driving luciferase. Only a modest increase in reporter gene activation was observed in response to co-expression of NFATc3 and ΔCnA (data not shown). As expected, the presence of MEF2C or MEF2A (data not shown) induced transcriptional activation of both reporter constructs. However, a synergistic activation pattern was observed when Flag-NFATc3 and ΔCnA was co-transfected with MEF2 and this effect was even further enhanced in the presence of the transcriptional cofactor p300 (Figure 4.3C).

To determine whether a MEF2/NFAT complex binds to the A/T-rich motifs in the β-MyHC and CTGF regulatory sequences *in vivo*, we performed ChIP assays using anti-MEF2, anti-NFATc2, anti-NFATc3 and anti-acetylated histone H3 antibodies. For these experiments, neonatal rat ventricular myocytes were either infected with an adenovirus expressing ΔCnA (AdΔCnA) or not, and 24 hrs later, soluble, fragmented chromatin was subjected to immunoprecipitation with indicated antibodies. No antibody (Figure 4.3D, lane 9) or nonspecific rabbit IgG (data not shown) were used as negative controls. Input chromatin served as a positive control (Figure 4.3D, lane 10).

As a positive control for the ability to detect NFAT bound to its regulatory sequence *in vivo*, when immunoprecipitated chromatin with antibodies against NFATc2 or c3 was subjected to PCR with primers spanning multiple NFAT sites in the intragenic (intron 3) region of DSCR1 (see table 4.1 for primer sequences), a 295-bp band was detected in the amplicons only after AdΔCnA stimulation (Figure 4.3D, lanes 5 and 6) and not in serum-free cultured cardiomyocytes (Figure 4.3D, lanes 1, 2), nor in the antibody control (Figure 4.3D, lane 9), confirming that NFAT sites were occupied by NFATc2/c3 *in vivo*. Interestingly, NFAT occupation of the regulatory sequence was accompanied by acetylation of the intragenic region, suggesting that NFAT recruits p300 activity following calcineurin activation (Figure 4.3D, lanes 8).

When immunoprecipitated chromatin was subjected to PCR with primers spanning the A/T-rich motifs in β-MyHC or CTGF, specific 273 bp and 151 bp-bands were obtained after AdΔCnA stimulation with all antibodies, indicating that a complex consisting of MEF2/NFAT and HAT activity (indicative of p300 presence) occupied the MEF2 binding sites. Intriguingly, under serum free conditions, weak MEF2 immunoreactivity was also detectable, which supports the notion that MEF2 may be present in an actively repressed state in the absence of calcineurin activity. Collectively, these data support the notion that a complex consisting of MEF2, NFAT, and p300 accumulates on selected A/T-rich motifs in genes following calcineurin activity *in vivo*.

D

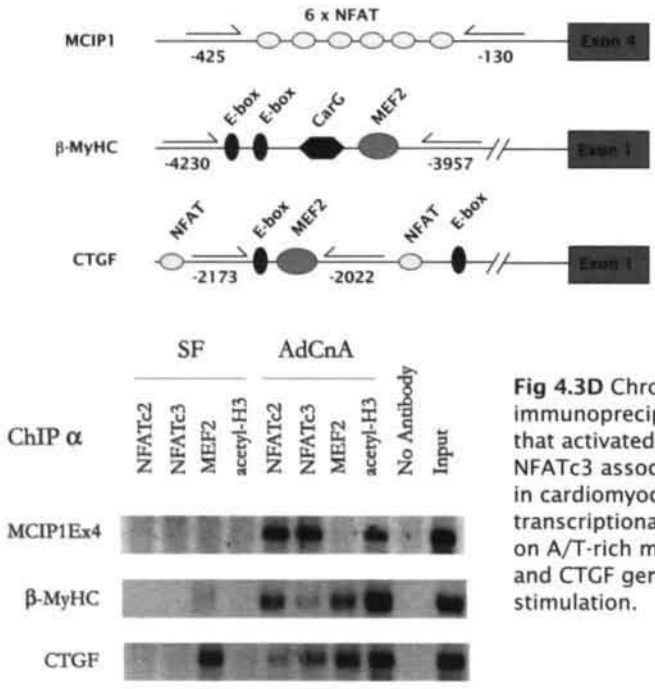


Fig 4.3D Chromatin immunoprecipitation demonstrates that activated NFATc2 and NFATc3 associates with MEF2 in cardiomyocytes to form a transcriptionally active complex on A/T-rich motifs in the β-MyHC and CTGF genes upon calcineurin stimulation.

Table 4.1 ChIP primers

	Forward primer	Reverse primer
MCIP1	5' gctgtttacagccacagaccttc 3'	5' gacagcaaattcctgagtca3'
β-MyHC	5' tagtaattttagcaaactttcg 3'	5' tgtgaggagtgggtggtgattgt 3'
CTGF	5' ttgaaagatttcactgtggc 3'	5' ctcaaagagaagaggtctga 3'

MEF2 is required for MEF2-NFAT cooperative cardiac gene activation.

To assess the requirement of MEF2 downstream of calcineurin-mediated gene expression, we created a dominant negative MEF2 construct by placing the first 117 amino acid residues from MEF2C with a terminal myc-epitope tag in a vector under control of the CMV promoter. Additionally, we replaced the Arginine residue at position 24 to Leucine (R24L), which creates a non-DNA binding mutant form of MEF2 (Figure 4.4A).[19] The effectiveness of the dominant negative MEF2 construct, MEF2(R24L), was confirmed by co-transfecting MEF2C or MEF2A with increasing amounts of the MEF2(R24L) construct using 3×MEF2-Luc reporter as a readout, which dose-dependently abrogated MEF2-dependent transcription activity (Figure 4.4A).

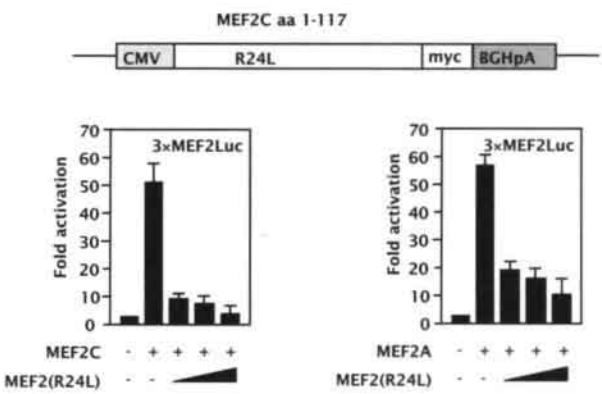


Fig 4.4A Schematic representation of the dominant negative MEF2 construct (MEF2(R24L)). COS7 cells were transiently transfected with a multimerized MEF2 reporter in the presence of either expression plasmids containing MEF2A or MEF2C and increasing amounts of MEF2(R24L).

Next, it was tested whether MEF2(R24L) was capable of inhibiting the synergistic transcriptional activation of β-MyHC-Luc and CTGF-Luc. For both cardiac gene promoter constructs, overexpression of MEF2(R24L) dose-dependently decreased the activation induced by the presence of MEF2C or MEF2A (data not shown), NFATc3, ΔCnA and p300. Likewise, co-transfection of an expression vector for DSCR1, a protein inhibitor that antagonizes NFAT signaling,[28] also dose-dependently decreased reporter activity of either promoter constructs, indicating that NFAT activation is required for calcineurin induced activation of β-MyHC and CTGF (Figure 4.4B). Taken together, these findings imply that both MEF2 and NFAT are required components in the transcriptional activation of β-MyHC and CTGF following calcineurin stimulation.

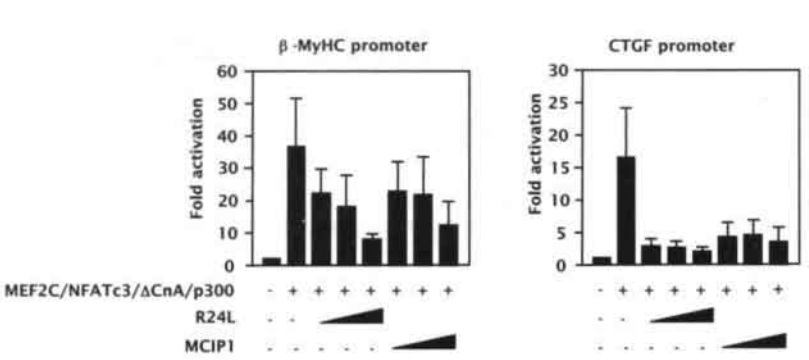


Fig. 4.4B MEF2(R24L) and MCIP1 dose-dependently inhibited transcriptional activation of β-MyHC and CTGF promoter constructs.

MEF2 is required downstream of calcineurin-induced cardiomyoyte hypertrophy

Apart from inducing a distinct gene expression profile in cardiac myocytes, activation of calcineurin in myocytes is associated with hypertrophic remodeling. To investigate the requirement of MEF2 in calcineurin-mediated cardiomyocyte hypertrophy, we generated two replication-deficient adenoviral vectors expressing either the dominant negative MEF2(R24L) construct and GFP under separate CMV promoters (Figure 4.5A), or GFP alone. COS7 cells infected with AdMEF2(R24L) at m.o.i. of 0.4, 0.8 or 1.0 demonstrated increasing immunoreactivity for Myc-epitope tag (derived from the MEF2(R24L) construct) and GFP (Figure 4.5B, lane 1, 2, and 3 upper panel). Conversely, AdGFP infection, even at a higher m.o.i., only resulted in strong GFP expression and no myc immuno reactivity (Figure 4.5B, lane 4). Taken together, these results demonstrate that AdMEF2(R24L) and AdGFP are correctly expressed and should represent effective devices to inhibit MEF2 activity in primary cardiomyocyte cultures.

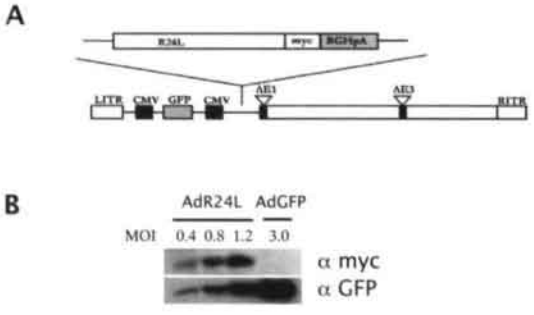


Fig. 4.5AB (A) Schematic representation of the adenoviral vector expressing MEF2(R24L). (B) Western blot analysis with an anti-Myc or anti-GFP antibody on COS7 cell lysates infected with either AdGFP, or AdMEF2(R24L) to confirm gene transfer.

To monitor the change in cell size or sarcomere organization, sarcomeric actin was stained with Phalloidin (Figure 4.5C, panels a, c, e) and nuclei were counterstained with DAPI. Cardiomyocytes were infected with the control adenovirus AdGFP (Figure 4.5C, panels a-b), or infected with AdMEF2(R24L) (Figure 4.5C, panels c-d). After 24 h, the cells were either left untreated (Figure 4.5C, panels a-c) or infected with AdΔCnA for 24 h (Figure 4.5C, panels b-d). The data demonstrate that only AdMEF2(R24L) pre-infection abrogated cardiomyocyte hypertrophy in response to AdΔCnA (Figure 4.5C, panels d). No discernable effects on AdΔCnA-induced cellular enlargement were observed when cells were pre-infected with AdGFP (Figure 4.5C, panels b).

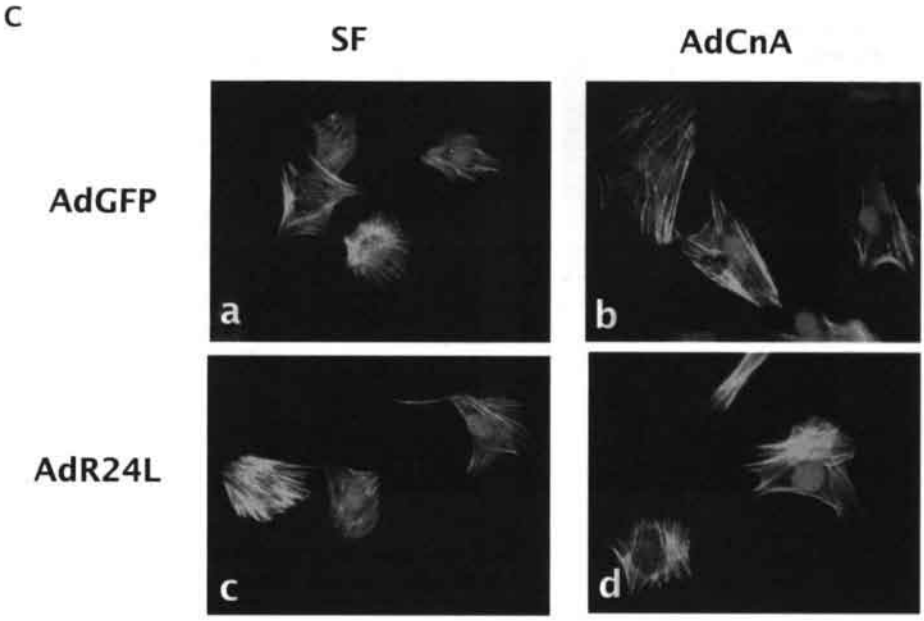


Fig. 4.5C Representative images of immunostained cardiomyocytes infected with the indicated adenoviruses. Phalloidin staining indicates less hypertrophy and sarcomere organization in the presence of AdMEF2(R24L) following AdΔCnA infection. -**Full colour image page 180**

Quantitation of cardiomyocyte hypertrophy was performed by video edge detection on large numbers of GFP-positive myocytes (Figure 4.5D). In agreement with our previous findings, AdΔCnA resulted in a more than 2-fold increase in cell surface area (3478±140 μm^2 vs. 1601±101 μm^2, $p<0.01$ versus AdGFP). AdMEF2(R24L) infection abrogated, but not completely inhibited, the prohypertrophic effects of AdΔCnA (2272±76 μm^2, $p<0.05$ versus AdΔCnA, $p<0.05$ versus AdGFP). The data demonstrate that MEF2 is involved in some, but not all, aspects of hypertrophic remodeling of cardiomyocytes following calcineurin activation.

D

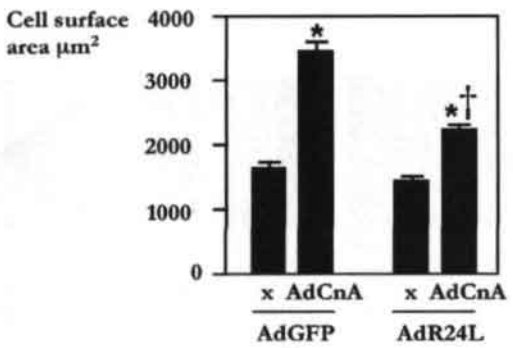


Fig. 4.5D Quantitation of cell surface areas demonstrate that inactivation of MEF2 signaling represses cardiomyocyte hypertrophy in response to AdΔCnA. These data represent the mean ± SEM of three independent experiments. * indicates $p < 0.05$ vs serum free conditions, † indicates $p < 0.05$ vs SF with AdCnA infection.

DISCUSSION

MEF2 transcription factors play key roles as regulators of cardiac gene expression.[26] The data in this study provide evidence that, in cardiac myocytes, NFAT proteins are actively recruited by MEF2 to target promoters in selected genes. This observation is reminiscent of the cooperative interaction between MEF2 proteins and myogenic basic-helix loop helix (bHLH) factors in skeletal muscles,[29] and MEF2 proteins with GATA factors in cardiac muscles,[30] and suggest that MEF2 factors interact with a variety of transcription factors to potentiate gene expression. Given the co-expression of MEF2 and NFAT in smooth muscle, skeletal muscle, neuronal and hematopoetic cells, the MEF2-dependent pathway described in this work may provide a molecular paradigm for Ca^{2+}/calmodulin signaling in many target cells.[14]

In addition to the calcineurin-NFAT signaling pathway, calmodulin-dependent kinases (CamK) have been reported to enhance MEF2 transcriptional activity through relieving their repression by class II histone deactylases (HDACs).[26] Whether this alternative modulation of MEF2 activity by CamK is complementary to the mechanism described in this study remains to be elucidated. The cooperative pathway between MEF2, NFAT and histone acetyltransferases described here is, however, distinct from the molecular mode of action of CamK to increase MEF2 transcriptional activity, suggesting that calcium signals can activate MEF2 via two separate Ca^{2+} signaling paradigms; one utilizing CamK to relieve the active repression of MEF2 by HDAC, and one employing calcineurin to induce cytoplasmic-nuclear translocation of a transcriptional partner of MEF2 to potentiate transcription of a subset of MEF2-dependent genes. In fact, transgenic CamK activity in the heart is associated with a remarkably similar pathophysiological outcome [31] as that resulting from transgenic calcineurin activation,[5] suggesting that MEF2 functions as a unique integration target to affect transcription in the heart. In this respect it is interesting to note that in response to intracellular Ca^{2+} elevation, the activity of CamK proceeds calcineurin activation.[32] It is tempting to speculate that this early CamK activity may serve to relieve the active HDAC repression of MEF2, sequentially followed by calcineurin-induced nuclear translocation of NFAT, which allows (further) potentiation of MEF2 transcriptional activity. Whether such temporal aspects in Ca^{2+} signaling also exist in the cardiac muscle cell should be explored in the future.

With respect to the nature of the target genes regulated by the MEF2/NFAT/p300 complex in the heart, a relative large number of genes were fetal isoforms of sarcomeric components, most notably β-MyHC, α-SKA and the MLC2a. Elevation of transcripts for these genes are a general characteristic of cardiac hypertrophy (hence their denotation as "hypertrophic markers"), and their upregulation is generally observed at the expense of their adult corresponding isoforms (α-MyHC, α-cardiac actin and MLC2v, respectively). Prior seminal work on the functional

consequence of selective replacement experiments of the adult α-MyHC by the fetal β-MyHC isoform indicated that this was associated with a dominant negative effect on myofibrillar ATPase activity and reduced force generating capacity of the sarcomere.[33] Based upon our data, it is tempting to speculate that the MEF2/NFAT interaction mediates remodeling of the sarcomere and profound functional deterioration observed in calcineurin transgenic mice.

CTGF is a secreted protein implicated in multiple cellular events including angiogenesis, skeletogenesis and wound healing.[34] It is a member of the CCN family of secreted proteins, named after CTGF, cysteine-rich 61 (CYR61), and nephroblastoma overexpressed (NOV) proteins. CTGF is expressed in and secreted from both cardiac myocytes and fibroblasts.[35] Secreted CTGF signals in part through the binding of BMP4/2 and TGF-β1 in the extracellular space, through its Chordin-like domain, leading to inhibition of BMP signaling, and activation of TGF-β1. TGF-β1 is a well-known inducer of extracellular matrix (ECM) components such as collagen and fibronectin, also in the heart. The CTGF gene may participate in this event, as its promoter contains a TGF-β response element[34] aside from a functional MEF2 site (this study). In support of this view, collagen deposition and anchorage-independent growth induced by TGF-β1 in fibroblasts can be inhibited by adding neutralizing antibodies against CTGF,[34] indicating a synergistic relationship between CTGF and TGF-β1. Our results indicate that a MEF2/NFAT complex controls CTGF expression downstream of calcineurin activity, and suggest a potential crosstalk between calcineurin and TGF-β1 governing auto-paracrine signaling controlling fibroblast proliferation, ECM deposition and excessive fibrosis, which are all characteristic for the calcineurin transgenic cardiomyopathy.[5]

In conclusion, our data demonstrate the existence of a MEF2/NFAT regulatory transcriptional pathway controlling genes such as CTGF and β-MyHC in cardiac muscle cells. The documented function of the select target genes of this transcriptional circuitry may open new avenues to explain parts of the maladaptive characteristics associated with calcineurin activity in the heart.

Acknowledgements

We thank Chiel de Theije and Victor Thijssen for excellent technical assistance and members of the lab for fruitful discussions. This work was supported by grants 2000-160 from the Netherlands Heart Foundation (NHS) and 902-16-275 and 912-04-054 from the Netherlands Foundation for Health Research and Development (ZonMW) to L.J.D.W.

REFERENCES

1. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. 2003;17:2205-32.
2. Ranger AM, Grusby MJ, Hodge MR, Gravallese EM, de la Brousse FC, Hoey T, Mickanin C, Baldwin HS, Glimcher LH. The transcription factor NF-ATc is essential for cardiac valve formation. Nature. 1998;392:186-90.
3. de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, Potter J, Wakeham A, Marengere L, Langille BL, Crabtree GR, Mak TW. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature. 1998;392:182-6.
4. Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, Williams RS. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev. 1998;12:2499-509.
5. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998;93:215-28.
6. Musaro A, McCullagh KJ, Naya FJ, Olson EN, Rosenthal N. IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. Nature. 1999;400:581-5.
7. Ho N, Gullberg M, Chatila T. Activation protein 1-dependent transcriptional activation of interleukin 2 gene by Ca2+/calmodulin kinase type IV/Gr. J Exp Med. 1996;184:101-12.
8. Bodor J, Bodorova J, Gress RE. Suppression of T cell function: a potential role for transcriptional repressor ICER. J Leukoc Biol. 2000;67:774-9.
9. Bower KE, Zeller RW, Wachsman W, Martinez T, McGuire KL. Correlation of transcriptional repression by p21(SNFT) with changes in DNA.NF-AT complex interactions. J Biol Chem. 2002;277:34967-77.
10. Decker EL, Nehmann N, Kampen E, Eibel H, Zipfel PF, Skerka C. Early growth response proteins (EGR) and nuclear factors of activated T cells (NFAT) form heterodimers and regulate proinflammatory cytokine gene expression. Nucleic Acids Res. 2003;31:911-21.
11. Bert AG, Burrows J, Hawwari A, Vadas MA, Cockerill PN. Reconstitution of T cell-specific transcription directed by composite NFAT/Oct elements. J Immunol. 2000;165:5646-55.
12. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. J Exp Med. 2002;195:1003-12.
13. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. Embo J. 2001;20:6414-23.
14. Youn HD, Chatila TA, Liu JO. Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. Embo J. 2000;19:4323-31.
15. Zhang CL, McKinsey TA, Olson EN. The transcriptional corepressor MITR is a signal-responsive inhibitor of myogenesis. Proc Natl Acad Sci U S A. 2001;98:7354-9.
16. Sartorelli V, Huang J, Hamamori Y, Kedes L. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. Mol Cell Biol. 1997;17:1010-26.
17. Jaynes JB, Johnson JE, Buskin JN, Gartside CL, Hauschka SD. The muscle

creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. Mol Cell Biol. 1988;8:62-70.
18. van Rooij E, Doevendans PA, de Theije CC, Babiker FA, Molkentin JD, de Windt LJ. Requirement of nuclear factor of activated T-cells in calcineurin-mediated cardiomyocyte hypertrophy. J Biol Chem. 2002;277:48617-26.
19. Molkentin JD, Black BL, Martin JF, Olson EN. Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. Mol Cell Biol. 1996;16:2627-36.
20. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A. 1998;95:2509-14.
21. De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW, 2nd, Kitsis RN, Molkentin JD. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: An apoptosis-independent model of dilated heart failure. Circ Res. 2000;86:255-63.
22. Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF, Molkentin JD. Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol. 2002;22:7603-13.
23. Garcia-Rodriguez C, Rao A. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). J Exp Med. 1998;187:2031-6.
24. Avots A, Buttmann M, Chuvpilo S, Escher C, Smola U, Bannister AJ, Rapp UR, Kouzarides T, Serfling E. CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. Immunity. 1999;10:515-24.
25. Kawamura T, Ono K, Morimoto T, Akao M, Iwai-Kanai E, Wada H, Sowa N, Kita T, Hasegawa K. Endothelin-1-Dependent Coactivator of Nuclear Factors of Activated T Lymphocytes Signaling Associates With Transcriptional Coactivator p300 in the Activation of the B Cell Leukemia-2 Promoter in Cardiac Myocytes. Circ Res. 2004.
26. Naya FS, Olson E. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Curr Opin Cell Biol. 1999;11:683-8.
27. Huang WY, Chen JJ, Shih N, Liew CC. Multiple muscle-specific regulatory elements are associated with a DNase I hypersensitive site of the cardiac beta-myosin heavy-chain gene. Biochem J. 1997;327 (Pt 2):507-12.
28. Rothermel B, Vega RB, Yang J, Wu H, Bassel-Duby R, Williams RS. A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. J Biol Chem. 2000;275:8719-25.
29. Molkentin JD, Olson EN. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci U S A. 1996;93:9366-73.
30. Morin S, Charron F, Robitaille L, Nemer M. GATA-dependent recruitment of MEF2 proteins to target promoters. Embo J. 2000;19:2046-55.
31. Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR, Olson EN. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest. 2000;105:1395-406.
32. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. Nature. 1998;392:933-6.
33. Tardiff JC, Hewett TE, Factor SM, Vikstrom KL, Robbins J, Leinwand LA.

Expression of the beta (slow)-isoform of MHC in the adult mouse heart causes dominant-negative functional effects. Am J Physiol Heart Circ Physiol. 2000;278:H412-9.
34. Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol. 2002;4:599-604.
35. Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF- beta in cardiac fibroblasts and cardiac myocytes: a potential role in heart fibrosis. J Mol Cell Cardiol. 2000;32:1805-19.

Chapter 5

An epigenetic pathway controlling cardiac angiogenesis following cardiac injury in a gender dependent manner

-preliminary data

Eva van Rooij, Jens Fielitz, Roel van der Nagel, Victor Thijssen, Harry J. Crijns, Rhonda Bassel-Duby, Eric N. Olson, and Leon J. De Windt

SUMMARY

Upon an ischemic insult the heart undergoes a maladaptive remodeling process involving dilation, hypertrophy of the non-infarcted area and collagen deposition, which can eventually evolve into heart failure. Previously, we were able to implicate the calcium/calmodulin-dependent phosphatase calcineurin as a dominant player in the hypertrophic remodeling of the remote myocardium following myocardial infarction (MI). In addition, we recently discovered coordinate transcriptional synergy between the main downstream target of calcineurin, NFAT, and the myogenic transcription factor MEF2 to be present in cardiomyocytes. Since MEF2 transcriptional activity is repressed by class II histone deacetylases (HDACs), we speculated that HDAC ablation would render mice hypersensitive to MI-induced hypertrophic remodeling of the remote myocardium. Since HDAC9 is highly expressed in the heart and very effective in suppressing hypertrophy in vitro, we subjected these mice to MI. Due to potential gender specific effects of HDAC activity, we included a specific focus on the pathophysiological response of either sex to MI.

Our data indicate that HDAC9 ablation in the adult heart results in severely increased mortality in males after MI compared to wild type animals. Strikingly, female HDAC9-null mice show an enhanced survival compared to wild type mice accompanied by a better maintenance in function. Deleterious left ventricular remodeling was largely absent in these animals due to the presence of a distinct epicardial layer of surviving cells in the infarct area. Gene expression analysis on the infarcted areas of female wild type and HDAC9-deficient mice revealed an upregulation of angiogenetic factors, such as VEGF and angiopoietin in the HDAC9-null background. Histological examination clearly indicated a strong angiogenic response, resulting in cardiomyocyte cell survival specifically in the infarct zone in the HDAC9 knockout females. These data suggest an interaction between HDAC9 and estrogen that induces neo-angiogenesis in response to hypoxia. This enhanced angiogenic response protects these HDAC-null females from maladaptive remodeling after MI, which leads to sustained improvement in cardiac function and survival.

INTRODUCTION

Heart failure secondary to ischemic cardiomyopathy is the primary cause of cardiovascular mortality, since it induces expansion and alterations in the infarcted and non-infarcted regions of the heart that impinges upon cardiac function. This remodeling process involves side-to-side slippage of myocytes in the myocardium occurring in association with ventricular dilation and is responsible for wall thinning. The resulting increase in internal load is thought to promote further stress, dilation and hypertrophy of the non-infarcted area.[1] Although neo-angiogenesis within the infarcted tissue is also an integral component of the remodeling process, the newly developed microvascular network is unable to support the greater demands of the hypertrophied myocardium, causing progressive loss of viable tissue, infarct extension and fibrous replacement.[2] To date, relatively little is still known about the molecular pathways governing remodeling of the infarct area and remote myocardium in the course of ischemic cardiomyopathy.

Previously, we were able to implicate the calcium/calmodulin-dependent phosphatase calcineurin as a dominant player in the hypertrophic remodeling of the remote myocardium following MI.[3] Calcineurin functions through dephosphorylation of its downstream transcription factor Nuclear Factor of Activated T-cells (NFAT), enabling nuclear translocation and transcriptional activation.[4] There is evidence from other cell types that NFAT may directly interact with the myogenic transcription factor Myocyte Enhancer Factor 2 (MEF2). MEF2 is a MADS (MCMI, agamous, deficiens, serum response factor) box transcription factor that is expressed during the development of all three muscle lineages, but is also activated during cardiac hypertrophy.[5,6] Additionally, both NFAT and MEF2 require interaction with the transcriptional coactivator p300 for their full transcriptional activity, a coactivator that possesses intrinsic histone acetyl transferase activity to promote a transcriptionally active chromatin configuration.[7,8] Recently, we established a coordinate transcriptional synergy between MEF2 and NFAT to be present in cardiomyocytes (van Rooij *et al.*, submitted).

Histone acetyltransferases (HATs), like p300, catalyze the acetylation of core histones of nucleosomes, resulting in chromatin relaxation and thereby allowing access of the transcriptional machinery to specific regions of DNA. The activity of HATs is antagonized by histone deacetylases (HDACs), which deacetylate histones and transcription factors, resulting in chromatin condensation and transcriptional repression.[9] In mammalian cells, HDACs comprise three distinct classes on the basis of protein structure and homology to yeast HDACs. Class I (HDAC 1, 2, 3, 8, and 11) and III HDACs (which form a structurally distinct class of NAD-dependent enzymes) are ubiquitously expressed, whereas class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) are highly enriched in striated muscles and brain.[10] Class II HDACs contain

a unique amino-terminal extension that is responsive to cytoplasmic signals, regulating its association with the myogenic family of MEF2 transcription factors.[11-13] Because histone HATs and HDACs compete for the same binding site on MEF2, class II HDACs may also indirectly inhibit binding of HATs to MEF2 and subsequent histone acetylation and transcription.[14]

To follow-up on the importance of transcriptional synergy between MEF2 and NFAT in cardiac remodeling, we subjected mutant mice lacking class II HDAC9 to myocardial infarction (MI). We focused on this isoform because MITR, the primary product of the HDAC9 locus, was highly effective in suppressing hypertrophy in vitro.[15] Since HDAC9 knockout mice are sensitized to hypertrophic stimuli and exhibit stress-dependent cardiomegaly, we speculated that these mice would be hypersensitive to MI-induced hypertrophic remodeling to the remote myocardium. Since a previous report on class II HDAC mutant mice indicated a possible gender difference,[9] and possible interactions between HDACs and the estrogen receptor have been reported,[16-20] we included a specific focus on the pathophysiological response of either sex to MI.

Here we show that ablation of HDAC9 in the adult heart, as expected, resulted in severely increased mortality in male HDAC9 knockout animals compared to their male wild type counterparts. Strikingly, however, female HDAC9 knockout mice showed a significantly decreased mortality after MI versus wild type mice coupled to a better maintenance in function. This effect was attributable to a decrease in deleterious left ventricular remodeling due to the presence of a distinct epicardial layer of surviving cells in the infarct area of the HDAC9 knockout females. Microarray comparison between female wild type and HDAC9-deficient infarcts revealed an upregulation of an angiogenetic cluster of genes, such as VEGF and angiopoietin in the HDAC9-null background, which was confirmed by quantitative PCR analysis. Histological examination revealed new blood vessel formation in the infarct zone in the HDAC9 knockout females, resulting in cardiomyocyte cell survival. Since estrogen is known to positively influence endothelial cell growth important in angiogenesis,[21] these data support the existence of a gender-dependent, epigenetic pathway involving HDAC, that regulates neo-angiogenesis in response to hypoxia. This enhanced angiogenic response leads to sustained improvement of cardiac function in female subjects, which predominates over a MEF2-dependent pathway controlling hypertrophic remodeling in the remote myocardium.

EXPERIMENTAL PROCEDURES

Surgical procedures

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Five-month old HDAC9 knockout (KO) mice and wild type (WT) mice of either sex were anesthetized with 2.4% isoflurane and placed in a supine position on a heating pad (37°C). Animals were intubated with a 19G stump needle and ventilated with room air using a MiniVent mouse ventilator (Hugo Sachs Elektronik, Germany; stroke volume 250 µl, respiratory rate 210 breaths per minute). Via left thoracotomy between the fourth and fifth ribs, the left anterior coronary artery (LCA) was visualized under a microscope and ligated using a 6-0 prolene suture. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Leica) by discoloration of the occluded distal myocardium. Sham operated animals underwent the same procedure without occlusion of the LCA. All surgeries and subsequent analyses were performed in a blinded fashion for genotype.

Transthoracic echocardiography

Three days and 21 days following MI two-dimensional echocardiography was performed in conscious mice using the fully digital Vingmed System (GE Vingmed Ultrasound, Horten, Norway) and a 11.5-MHz linear array transducer as previously described.[22] Briefly, cine loops and still images were digitally stored for subsequent analysis using the EchoPac software (GE Vingmed Ultrasound). Two-dimensional short-axis views of the LV at the level of the tip of the papillary muscle were recorded with a typical frame rate of 263/s. Left ventricular (LV) parameters and heart rates were obtained from M-mode interrogation in a short-axis view. M-mode tracings were used to measure posterior wall thicknesses at end-diastole and end-systole (PWthd, PWths, respectively), and LV internal diameter (LVID)was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). The data were analyzed by a single observer blinded to the murine genotype. LV fractional shortening (FS) was calculated according to the following formula: FS (%) = [(LVIDd -LVIDs)/LVIDd] x100.

RNA extraction and RT-PCR analysis

Total RNA from the infarcted area was isolated using Trizol (Invitrogen). A 10 µg aliquot representative of three animals per sample group was then analyzed on Affymetrix U74Av2 microarrays. A subset of differentially expressed RNAs was further characterized by quantitative real time PCR. Briefly, 2 µg RNA from each sample was used to generate cDNA using Super Script II reverse transcriptase per manufacturer's specifications (Invitrogen Life Technologies Inc., Burlington, Ontario, Canada). Real time PCR was cycled between 95 °C/30 s and 60 °C/30 for

40 cycles, following an initial denaturation step at 95 °C for 3 min. Amplification products were routinely checked using dissociation curve software (Biorad), and transcript quantities were compared using the relative Ct method, where the amount of target normalised to the amount of endogenous control (cyclophilin) and relative to the control sample is given by $2^{-\Delta\Delta_{Ct}}$. Real time PCR results were verified by electrophoresis of the reverse transcribed material in 1.2% agarose gels and visualized under UV illumination after ethidium bromide staining.

Histology and histochemistry

Three weeks after MI heart tissue was incubated for 30 minutes in Krebs buffer (118 mM NaCl, 4.7 mM KCL, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 11mM glucose) to arrest the heart in diastole, fixed in 3.7% paraformaldehyde, and embedded in paraffin. Sections were sampled from apex to base at 1.0 mm intervals and stained with hematoxylin and eosin to visualize infarcted area and stained with sirius red to visualize fibrosis.

Capillary-density analysis

Multiple paraffin sections were prepared from both sham and MI operated group of either sex of both WT and HDAC9 knockout mice (n = 3-6 in each group). Endothelial cells were immunohistochemically stained to examine the capillary density. All sections were pretreated with 1% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated for 45 minutes with 5% BSA. Capillary endothelium was identified by staining sections with a rat anti-rabbit Willebrand Factor (vWF) mAb that cross-reacts with mouse vWF (Abcam, UK). Additionally, lectin histochemistry was performed as described previously[23,24] with minor modifications. The sections were incubated with biotinylated *Griffiona simplicifolia* lectin (Vector Laboratories, UK) for 2 hours at room temperature. Subsequently all sections were stained for immunoperoxidase using a commercially available kit (HRP DakoCytomation, Denmark). Final color products were developed using a solution containing 3,3'-diaminobenzidine (DAB) and the sections were counterstained with eosin.

Statistical Analysis

The results are presented as means ± SEM. Statistical analyses were performed by using INSTAT 3.0 software (GraphPad, San Diego) and ANOVA followed by Tukey's post-test when appropriate. Statistical significance was accepted at a P value < 0.05.

HDAC9 ablation increases post-MI survival in female mice

Myocardial infarction elevates the workload on the heart and instigates adverse cardiac remodeling, comprising dilation of the infarcted area, hypertrophy of the remote myocardium and collagen deposition in the interstitium. Due to the cooperative interaction between the cardiac transcription factors NFAT and MEF2 in regulating hypertrophic gene expression profile, we speculated that mice lacking the MEF2 repressor HDAC9 would rapidly develop an excessive cardiac hypertrophic response in the remote myocardium after MI. Since we previously reported that MI-induced hypertrophy of the remote myocardium is maladaptive, we expected rapid deterioration of HDAC9 knockout mice after MI compared to their wild type counterparts.

When we monitored the survival rates up to three weeks after MI, survival after MI was 63% for males and 64% for female mice in the wild type background. Although, a dramatic decreased survival was evident in HDAC9 knockout males compared to wild type mice (36%), female HDAC9-deficient mice displayed remarkably improved postinfarction survival (85%) (Fig. 5.1).

	survival after surgery	survival 3 weeks after MI	survival (%)
wild type	22	14	63.6
male	8	5	62.5
female	14	9	64.7
HDAC9 KO	24	15	62.5
male	11	4	36.4
female	13	11	84.6

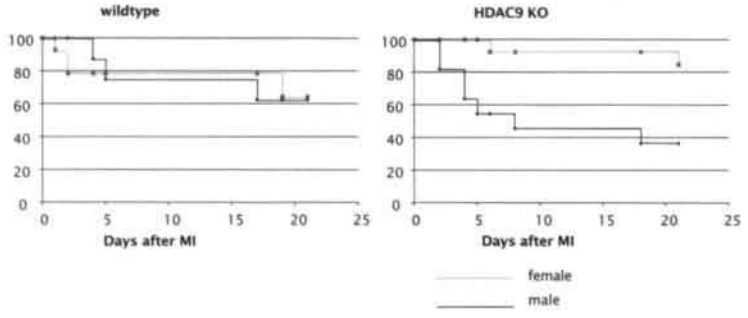


Fig. 5.1 HDAC9 knockout females show an enhanced survival after MI. Post-MI mortality was comparable between male and female WT mice. Although male HDAC9 knockout animals suffered from an elevated mortality compared to wild type, contrarily the females showed a highly increased survival after MI.

HDAC9 KO females maintain cardiac function after myocardial infarction

To further investigate the evident gender-dependent response after MI in the HDAC9 null background, we serially analyzed cardiac function and geometry following infarct. At baseline, heart rate and fractional shortening in female HDAC9 knockout mice was significantly lower as for female WT mice. The force-frequency relationship, which indicates a positive correlation between heart rate and contractile force, might partially explain the lower fractional shortening for the HDAC9 knockout. Strikingly, while heart rate in WT female animals significantly decreased after MI, heart rate in HDAC9 knockout animals displayed a progressive increase until, three weeks after MI, it reached a level comparable to WT heart rate (Table 5.1 and Fig. 5.2).

A

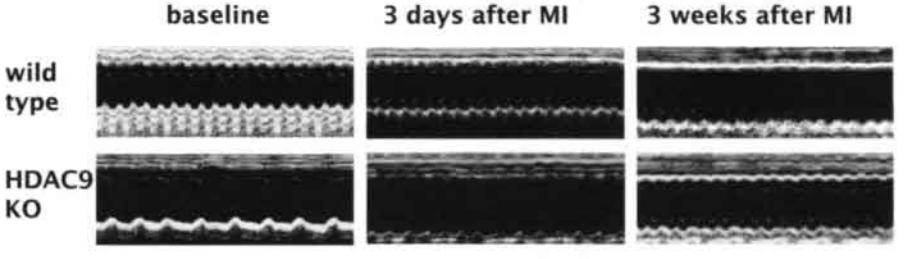


Fig. 5.2A Cardiac function after MI better maintained in female HDAC9 knockout mice. Representative M-mode images of sham or infarcted WT and HDAC9 kncokout females 3 days and 3 weeks after MI. Data demonstrate comparable initial LV dilation in both groups, with progressive dilation and loss of contractile behaviour in WT mice, which was substantially attenuated in HDAC9 KO mice. (n=5-12 per group)

Three weeks after MI wild type animals showed a severe dilation and deterioration in function. Since the morphology of the HDAC9 knockout females was better maintained after MI, their hearts dilated significantly less, and fractional shortening remained better. Three weeks after MI the HDAC9 knockout females even showed a significantly enhanced contractility compared to WT females (Table 5.1 and Fig 5.2B). This increase might partially find it explanation in the progressive increase in heart rate in female HDAC9 knockout mice after MI. Remarkably, three weeks after surgery we were also able to detect some regeneration of contractile force of the infarcted wall, which also contributes to the enhanced contractility (Fig. 5.2A).

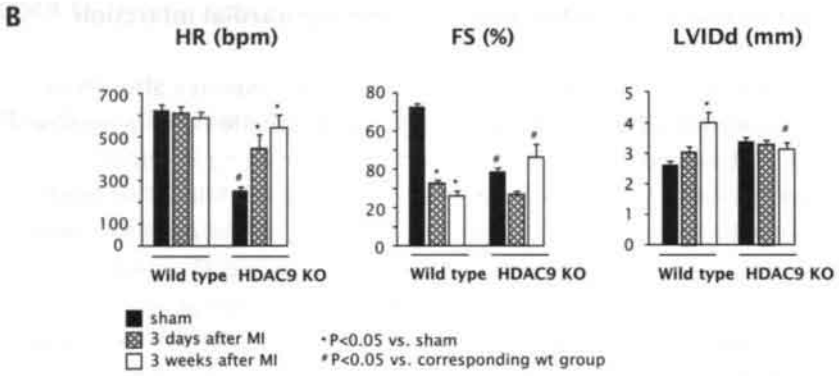


Fig. 5.2B Bargraph representation of HR, FS, and LVIDd. Although HDAC9 kncokout females showed a depressed HR at baseline, after MI it became elevated to a comparable level as for WT mice. FS and LVIDd indicate that HDAC9 knockout females show a functional and morphological deterioration after MI (n = 5 in both sham groups; n = 8-12 in MI groups). * P <0.05 vs. sham operated group, # P < 0.05 vs. corresponding WT group.

Table 5.1 Echocardiographical characteristics in wild type ans HDAC9 KO female mice after sham operation and after myocardial infarction.

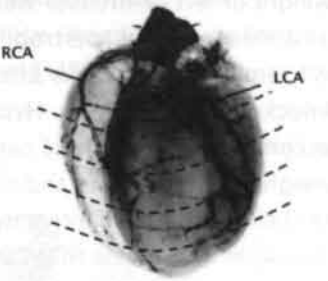
	wild type			HDAC9 KO			
	sham	3 days post MI	3 weeks post MI	sham	3 days post MI	10 days post MI	3 weeks post MI
n	5	10	8	5	12	12	10
HR	630 ± 26	614 ± 25	592 ± 20	258 ± 12#	453 ± 57	549 ± 41*	594 ± 53*
PWthd, mm	0.81 ± 0.04	0.74 ± 0.04	0.95 ± 0.09	0.83 ± 0.07	0.86 ± 0.04	0.90 ± 0.05	0.92 ± 0.03
PWths, mm	1.31 ± 0.05	1.30 ± 0.04	1.45 ± 0.05	1.30 ± 0.07	1.32 ± 0.06	1.44 ± 0.07	1.47 ± 0.06
LVIDd, mm	2.62 ± 0.07	3.07 ± 0.12	4.02 ± 0.28*	3.42 ± 0.07	3.31 ± 0.08	3.54 ± 0.17	3.14 ± 0.17#
LVIDs, mm	0.71 ± 0.04	2.14 ± 0.09*	3.08 ± 0.31*	2.06 ± 0.04#	2.40 ± 0.08	2.46 ± 0.22	2.03 ± 0.30#
% FS	73.3 ± 1.5	33.0 ± 1.1*	26.5 ± 2.3*	39.6 ± 1.2#	27.4 ± 1.2	31.3 ± 4.2	46.8 ± 6.1#

Data are expressed as means ± SEM. PWthd, posterior wall thickness in diastole; PWths, posterior wall thickness in systole; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in diastole; FS, left ventricular fractional shortening calculated as (LVIDd-LVIDs)/LVIDd.

*P<0.05 vs. sham
#P<0.05 vs. corresponding wt group

Attenuated post-infarction remodeling in HDAC9 KO females

Histological examination showed the HDAC9 knockout females to be protected against cardiac remodeling after MI. H&E stained sections indicated the infarct to span the entire free wall of the left ventricle for both WT and HDAC9 knockout mice.



Although occlusion of the LAD deprived the same circumferential left ventricular area of oxygen in WT and knockout hearts, the HDAC9 knockout female myocardium showed reduced dilation due to a non-transmural infarct. These sections also clearly showed extensive hypertrophy of the remote myocardium in the wild type animals in response to MI, which was absent for the HDAC9 knockout females (Fig. 5.3A).

A

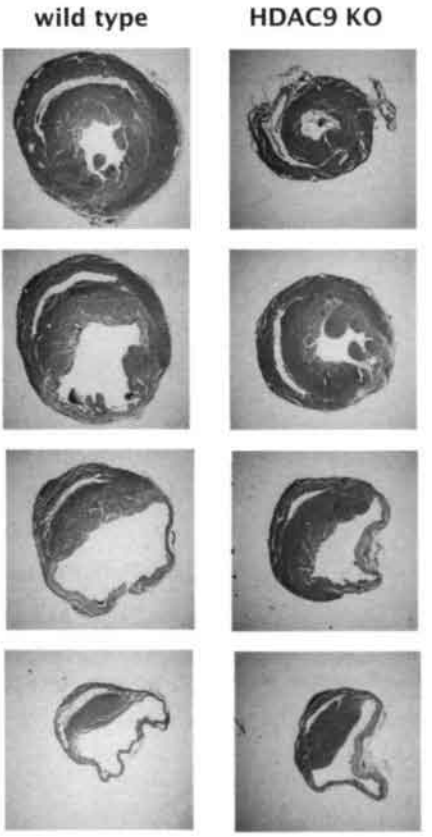


Fig. 5.3A Reduced cardiac remodeling in HDAC9 knockout females after MI. (A) Morphometric analyses of H&E transversal sections from apex to base indicate no significant difference in infarcted left ventricular circumference between wild type and HDAC9 knockout females, with more dilation and an increased hypertrophic response in the wild type mice. - **full colour image page 181**

Geometric analysis confirmed these data: the ratio between the heart weight or left ventricular weight and either the body weight or the tibia length as a measure for hypertrophy, showed a severe hypertrophic response for the WT females three weeks after MI, while this response was lacking in the HDAC9 knockout female group. Hypertrophic remodeling in WT female mice was also accompanied by signs of cardiac failure, as indicated by the increase in atrial weights, lung weights and right ventricular weights (Table 5.2). In contrast, due to the lack of maladaptive hypertrophy these features of cardiac failure were absent in the female HDAC9 knockout mice. Due to the low survival rate in HDAC9 knockout animals this comparison could not be made between male wild type and knockout animals.

Table 5.2 Echocardiographical characteristics in wild type ans HDAC9 KO female mice after sham operation and after myocardial infarction.

	wild type		HDAC9 KO	
	sham	3 weeks post MI	sham	3 weeks post MI
n	5	9	5	11
BW operation, g	26.0 ± 0.9	26.2 ± 0.7	25.1 ± 1.7	25.5 ± 0.9
BW, g	26.3 ± 1.0	24.0 ± 0.6	25.3 ± 1.7	24.4 ± 0.9
HW, mg	134.6± 3	196.1 ± 13*	141.8± 6	158.8 ± 8#
LVW, mg	90.5 ± 2.6	111.5 ± 7.0*	88.0 ± 4.0	99 ± 2.6
RVW, mg	**24.5 ± 0.9**	**35.0 ± 3.8**	**22.5 ± 0.3**	**23.0 ± 0.8#**
Atria weight, mg	**12.0 ± 0.6**	**26.8 ± 6.7***	**11.5 ± 0.9**	**13.2 ± 1.0#**
Lung weight, mg	**173.8 ± 9.3**	**256.3 ± 36.5***	**157.4 ± 8.9**	**153 ± 16.0#**
Liver weight, mg	1286 ± 49	1161 ± 41	1123 ± 54	1034 ± 50
TL, mm	17.4 ± 0.1	17.5 ± 0.1	16.9 ± 0.3	17.5 ± 0.1
HW/BW, mg/g	5.1 ± 0.2	8.1 ± 0.4*	5.6 ± 0.2	6.5 ± 0.3#
HW/TL, mg/mm	**7.8 ± 0.2**	**11.2 ± 0.7***	**8.4 ± 0.7**	**9.0 ± 0.4#**
LVW/BW, mg/g	3.3 ± 0.0	4.7 ± 0.2*	4.0 ± 0.2	3.9 ± 0.1#
LVW/TL, mg/mm	**5.2 ± 0.2**	**6.4 ± 0.4***	**5.3 ± 0.2**	**5.6 ± 0.1#**

Data are expressed as means ± SEM. HW, heart weight; LVW, left ventricular weight; RVW, right ventricular weight; TL, tibia length; BW, body weight.

*P<0.05 vs. sham
#P<0.05 vs. corresponding wt group

Sections stained for sirius red indicated the presence of reactive fibrosis in the noninfarcted regions of the left ventricle with more pronounced presence of collagen deposition towards the infarct. However, WT mice showed a more severe collagen deposit than the HDAC9 knockout females (Fig. 5.3B).

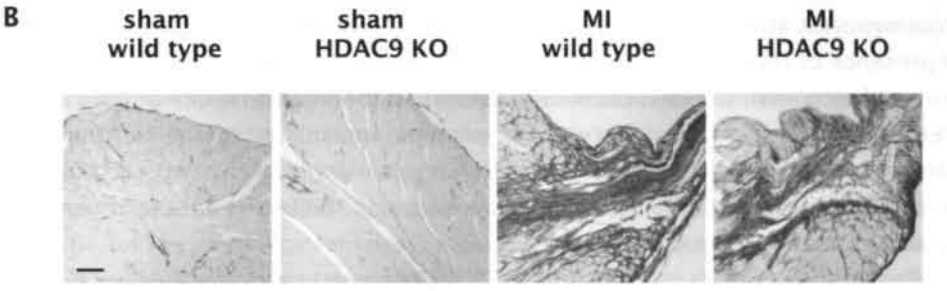


Fig. 5.3B Sirius red staining indicates low levels of collagen deposition in the sham operated mouse hearts from both phenotypes. Although massive accumulation of fibrillar collagen (interstitial and perivascular fibrosis) is present after infarction in wild type mice, this response is substantially attenuated in female HDAC9 knockout mice. Bar indicates 0.1 mm. - **full colour image page 181**

These data indicate that HDAC9 ablation in female mice leads to a significant reduction of the major structural alterations of the remote myocardium in the postinfarcted heart, and results in a significant cardioprotective effect against myocardial infarction.

Increased expression of angiogenic factors in hypoxic cardiac region of HDAC9 KO females

More detailed examination of the infarcted area in the HDAC9 knockout females clearly indicated the presence of a distinct layer of surviving cardiomyocytes at the epicardial surface of the infarcted region (Fig. 5.4). Due to the presence of this additional layer of muscle cells the integrity of the infarcted area was better maintained after MI and prevented the heart to dilate in the same extent as the WT mice. To explore this phenotype, we isolated total RNA from the infarcted area and analyzed gene expression levels using an Affymetrix U74Av2 microarray. Based on the microarray data, expression levels of a subset of genes was also examined by realtime PCR.

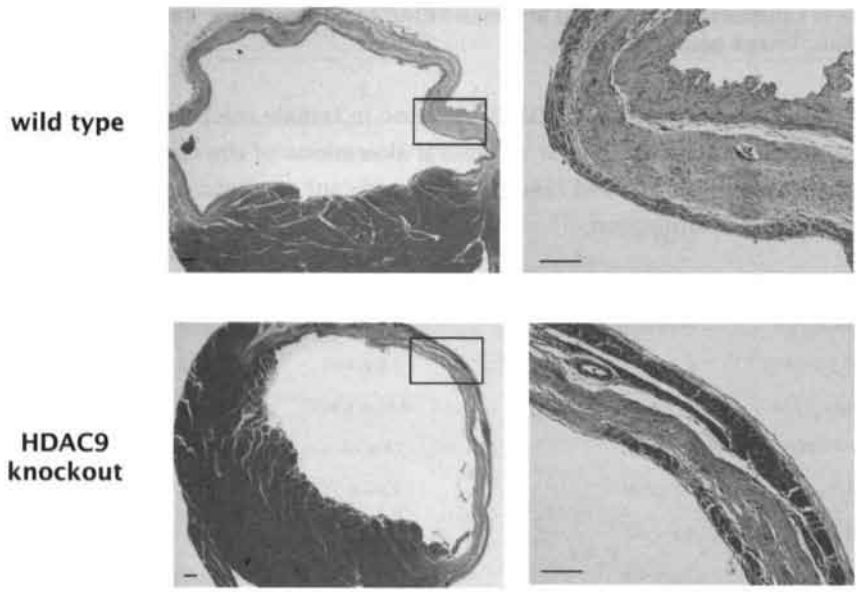


Fig. 5.4 Additional epicardial layer of surviving cells in infarcted area of female HDAC9 knockout mice. H&E stained sections show the presence of an additional layer of survival cells at the epicardial surface of the infarcted area in the female HDAC9 knockout animals compared to wild WT. Bar indicates 0.1 mm. - **full colour image page 182**

The presence of estrogen in pre-menopausal women has been shown to have beneficial effects on the cardiovascular system. Although the exact mechanism is still unknown, estrogens appear to facilitate angiogenesis and collateral vessel formation.[21] In addition, hypoxia also induces a well defined angiogenic response directly after myocardial infarction.[25,26] Both hypoxia and estrogens have been shown to stimulate the synthesis of the major angiogenic factor called vascular endothelial growth factor (VEGF-A).[26-29]

Angiogenic profiling of the infarcted area indicated that several genes known to be involved in angiogenesis were highly expressed in the infarcted area of the HDAC9 knockout females (Table 5.3 and Fig. 5.5A). VEGF-A and its receptors (VEGFR1 and VEGFR2) were found to be most highly upregulated (85, 67 and 101 fold vs. wild type females, respectively). This is in line with the fact that VEGF-A induces overexpression of VEGFR1 and VEGFR2 to create a loop allowing endothelial cells to become responsive and activated.[30] Angiopoietins (Ang1 and Ang 2) and the Tie2 receptor constitute an additional endothelial cell-specific angiogenic system. It has been suggested that the Ang1-Tie2 system regulates the stability and maturation of vessel structures, and Ang2 functions as a natural antagonist for Tie2.[31] Previously Ang2, which was 10 fold upregulated in the infarcted area of the HDAC9 knockout females, was shown to be upregulated by hypoxia and angiogenic cytokines, including VEGF.[32,33]

Table 5.3 Primers angiogenic profiling

Forward target	Reverse primer 5'-3'	Forward primer 5'-3'
Cyclophilin	AGCTAGACTTGAAGGGGAATG	ATTTCTTTTGACTTGCGGGC
β-actin	GGAGGAGCTGGAAGCAGCC	GAAGCTGTGCTATGTTGCTCTA
Angiopoietin-1	GCAAGGCTGATAAGGTTATGA	AGCTACCAACAAACAACAGCA
Angiopoietin-2	TTCTTCTTTACGGATAGCAAC	AGCCACGGTCAACAACTGC
Angiopoietin-3	GCAGTTGTTCCCTCTTCTCTT	AACAGGGCCCTGGAGACC
VEGF-A	CTCCAGGGCTTCATCGTTA	CAGAAGGAGAGCAGAAGTCC
VEGF-B	TGCCCATGAGTTCCATGC	CCCAGTTTGATGGCCCCA
VEGF-C	TTTAAGGAAGCACTTCTGTGTGT	GTAAAAACAAACTTTTCCCTAATTC
VEGF-D	GGTGCTGAATGAGATCTCCC	GCAAGACGAGACTCCACTGC
VEGF-R1	GCTGCTTGGAGATCTCACTG	TCAGCAGCTCAAGTGTCACC
VEGF-R2	TTCCAGATGCTGGGCAAGTC	ATGACATCTTGATTGTGGCAT
VEGF-R3	TGCATGCTGGGTGGACTATCA	GCAGGAGGAGGAAGAGGAGC
TIE-1	AATGGCAGACCAGGCAATC	CCCCACTGGTCTCCTTTAG
TIE-2	GTTGACTCTAGCTCGGACTGT	GAAGTCGAGAGGCGATCCC
bFGF	CACATTTAGAAGCCAGTAATCT	CCCGACGGCCGCGTGGAT

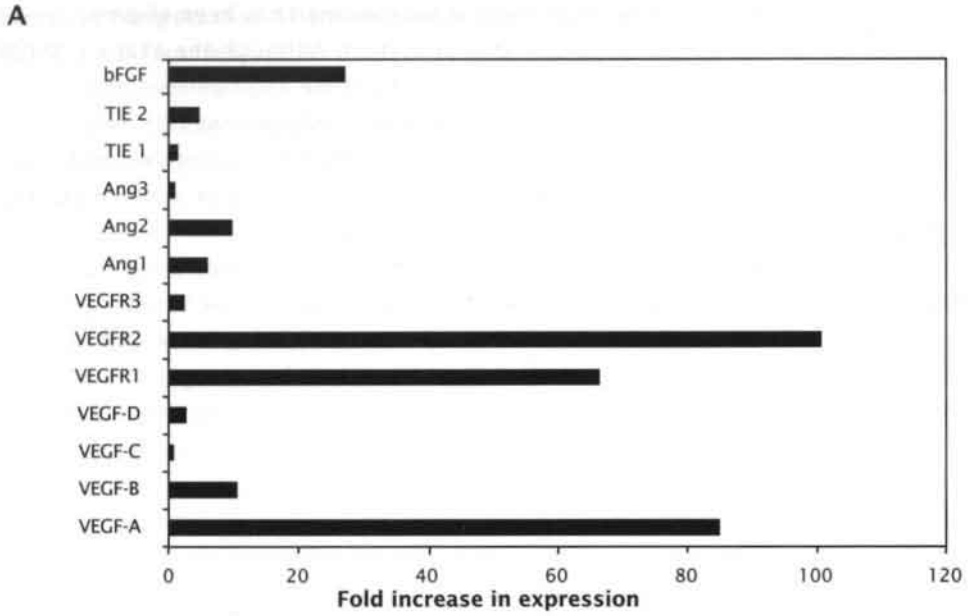


Fig. 5.5A Hypoxia induces an enhanced angiogenic respons in the HDAC9 knockout females after MI. Quantitative PCR analysis of angiogenic factors indicate a severly increased angiogenic response in the infarcted region of the HDAC9 knockout females versus the WT female mice.

Interestly, the expression levels of the angiogenic factors tested, did not show any significant differences in the sham operated animals between either sex of wild type and HDAC9 knockout animals, nor did they differ between male and female HDAC9 knockout mice (data not shown). Although it is known that VEGF and angiopoietins play complementary and coordinate roles during vascular remodeling in normal tissue, our data indicate that these systems also interact during hypoxia-induced angiogenesis in the heart of HDAC9 knockout female mice.

Conclusively, gene expression profiling indicated upregulation of angiogenic factors under hypoxic conditions in HDAC9 knockout females, and implies an interaction between HDAC, estrogen signaling and hypoxia in the induction of new vessel formation.

Enhanced vessel formation in infarcted region HDAC9 KO females

To visualize the vasculature we stained transversal sections of the heart for von Willebrand Factor and lectin. Von Willebrand factor (vWF) is a glycoprotein produced uniquely by endothelial cells, and is routinely used to identify vessels in tissue sections. Although we were unable to detect vWF in transversal sections of sham operated mice, there were vWF positive vessels present in the infarcted area of both WT and HDAC9 knockout females with a higher abundance in the HDAC9 knockout females (Fig 5.5B). Although vWF is heterogeneously distributed throughout the vasculature, previously it was reported that fibroblast growth factor-2 and VEGF synergistically up-regulate expression of vWF mRNA and protein in cultured endothelial cells. The local upregulation of these angiogenic factors in the infarcted area might explain the presence of the vWF positivity in a subset of vessels exclusively in the infarcted region, especially for the HDAC9 knockout females.

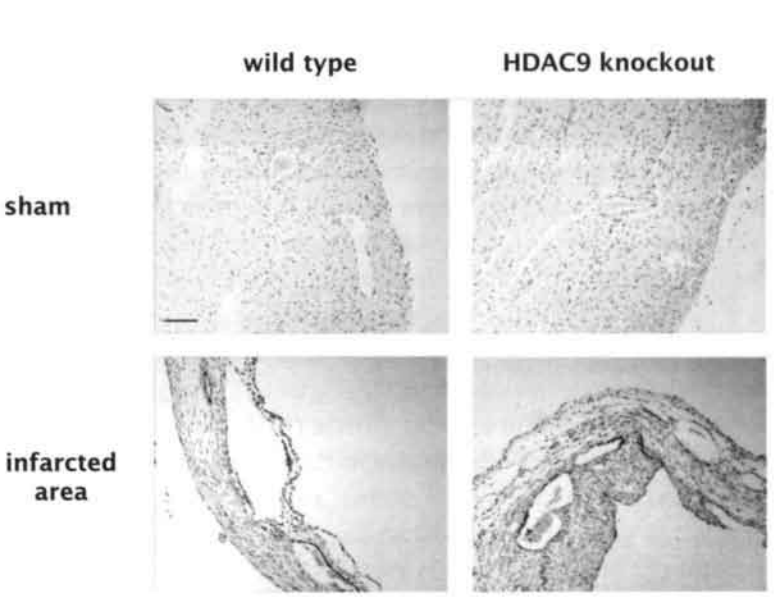


Fig 5.5B vWF stained images reveal only vWF positive vessels in the infarcted regions, with a higher abundancy in the infarcted area of the female HDAC9 knockout mice. Bar indicates 0.1 mm. - **full colour image page 182**

Additionally, we used *Griffonia Simplicifolia* lectin I (GS-I) as an endothelial marker. There was a regular distribution of capillaries around cardiomyocytes in both sham operated groups. Three weeks after MI the border zone of the infarcted region contained regions of low vascularity in the WT animals, which was even more decreased in the infarct itself. However, both the border zone and the infarcted region of the HDAC9 knockout females appeared to be highly vascular, with enlarged, thin-walled vessels (Fig. 5.5C). This is in line with the severe upregulation of VEGF-A in this area, since a characteristic feature of VEGF-A mediated angiogenesis in a variety of models is the generation of dilated enlarged vessels.[34-36] Although the sections need to be quantified extensively, these data indicate a severely enhanced VEGF-A -induced angiogenic response in the infarcted region of the HDAC9 knockout females, which is likely to be responsible for the cardioprotective effect after myocardial infarction.

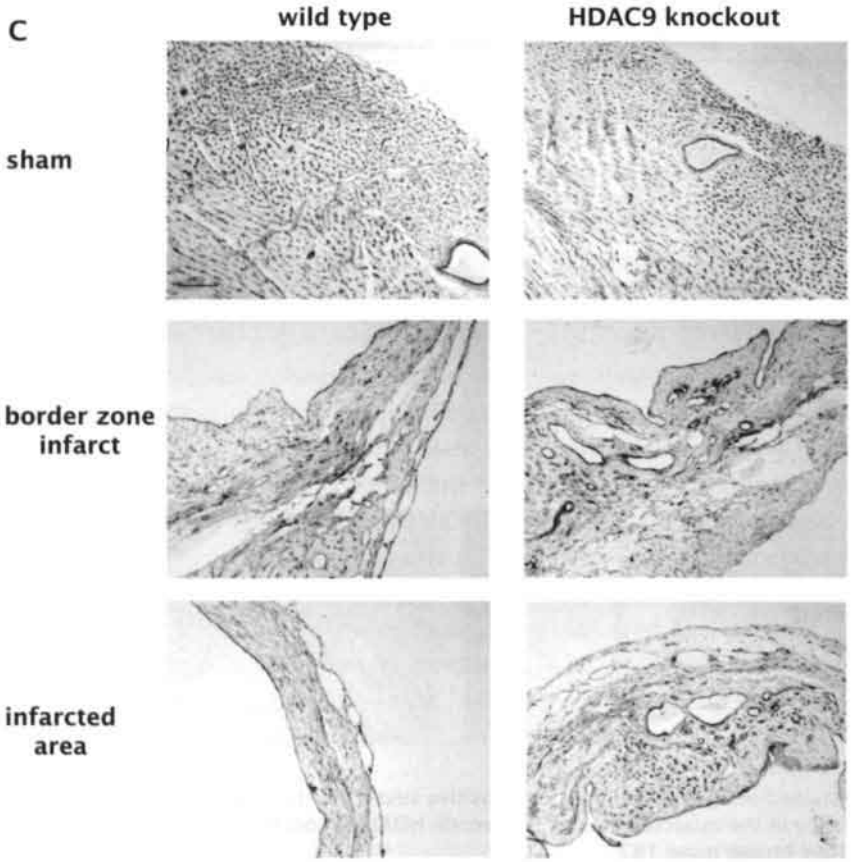


Fig. 5.5C Sections stained for lectin an equal distribution of capillaries surrounding cardiomyocytes in the non-infarcted areas, while the ischemic region shows irregular patterning of vasculature with additional and enlarged vessels in the border zone and the infarcted region of the HDAC9 knockout females compared to the WT animals. Bar indicates 0.1 mm. - **full colour image page 183**

DISCUSSION

Heart failure secondary to ischemic cardiomyopathy is the primary cause of cardiovascular mortality. When the myocardium is deprived of blood, a protective response is triggered to improve myocardial perfusion by the formation of new capillaries (angiogenesis) and by the enlargement of pre-existing collateral vessels (arteriogenesis). Although neo-angiogenesis within the infarcted tissue is also an integral component of the remodeling process after myocardial ischemia, the newly developed microvascular network is unable to support the greater demands of the hypertrophied myocardium, resulting in progressive loss of viable tissue, infarct extension and fibrous replacement.[2]

Animal studies evaluating gender difference collectively demonstrated that estrogen exerts significant benefits on the cardiovascular system and showed that estrogen replacement therapy in postmenopausal women reduces morbidity and mortality associated with cardiovascular disease by ~50%. [37-39] The cardioprotective effect of estrogen is partly due to its role in angiogenesis. Estrogens are known to promote new vessel formation both in vitro and in vivo [40], and have been postulated to be pro-angiogenic in ischemic tissues. Although the mechanisms involved in the pro-angiogenic effects of estrogen are probably multifactorial,[41-45] it includes upregulation of VEGF-A, its receptors and additional angiogenic proteins, which were all found to be highly upregulated in our study.

The biological actions of estrogens are mediated by the intracellular estrogen receptor (ER) α and β that function as transcription factors in response to ligand binding. Interestingly, current models of ER action suggest that it modulates the rate of transcription initiation through interactions with the basal transcription machinery and through the alterations in the state of chromatin organization at the promoter of target genes via the recruitment of a variety of cofactors. Coactivators, such as cAMP-response element binding protein (CREB)-binding protein (CBP), p300 and p300/CBP-associated factor (pCAF) possess intrinsic acetyltransferase (HAT) activity capable of modifying the chromatin organization of the target genes.[46] In response to estrogen binding, there is a cyclic recruitment of co-activators leading to hyperacetylation of target genes, which decondenses nucleosomes and facilitates the binding of transcription factors to the underlying DNA regulatory elements, leading to more efficacious gene activation.[47,48] This temporal presence of acetyltransferases is followed by a transient wave of deacetylation, suggesting the presence of histone deacetylases (HDAC) at the ER target promoters even under inducing conditions in the presence of ligands. In the last few years several reports have indicated that coregulators of ER, including repressor of estrogenreceptor activity (REA), ligand-dependent corepressor (LcoR), metastatic-associated protein 1 (MTA1) and receptor interacting protein (RIP)140, recruit HDAC to nuclear receptor target genes in the

presence of estrogen. [16-18,20]

This interaction between the estrogen receptor and HDAC may provide a plausible mechanism for the profoundangiogenic response observed in the HDAC9 knockout female infarct under hypoxic conditions. Based on these data it is tempting to speculate that ablation of HDAC9 removes the transcriptional repressive effect on the estrogen receptor, thereby enhancing the estrogen-induced upregulation of angiogenic genes. Since there were no significant differences in angiogenic gene expression between WT and HDAC9 knockout animals at baseline, nor between HDAC9 knockout male and female mice, the angiogenic response is apparently triggered by the local ischemia, instead of constituting a more generalized phenomenon in the HDAC9 knockout females.

It will be of great additional value to unravel which hypoxia related factors are responsible for the cardioprotective phenotype of the HDAC9 knockout females. Although to date several angiogenic molecules have been indentified to be involved in hypoxia regulated angiogenesis, hypoxia-inducible factor 1 (HIF-1α) might be a good candidate to interact in some way with the estrogen receptor, since it is a transcriptional activator of VEGF.[25] VEGF-A and its receptors are critically involved in the regulation of pathological blood vessel growth in the adult during various angiogenesis-dependent diseases associated with tissue hypoxia, and were found to be highly upregulated in the infarcted tissue of the HDAC9 knockout females. Since VEGF synthesis is modulated not only by hypoxia, but also by steroid hormones, our data reveal a novel complex interplay between estrogen- and hypoxia-dependent pathways driving VEGF expression.

Additionally, these results indicate a possible mechanism for stimulation of collateral vessel formation in ischemic tissue by removal of the ER repressor HDAC. Conclusive data on estrogens involvement on remodeling of the collateral vasculature and thereby remodeling of the heart in response to an ischemic event, will possibly result from further studies in the HDAC9 knockout mice on the effects of gonadectomy and estrogen replacement previous to MI. Eventually, therapeutic stimulation of angiogenesis by the ovarian steroid hormone in the absence of its transcriptional repressor HDAC, could be beneficial for the treatment of ischemic heart disease.

Acknowledgements

We thank Cheryl Nolen and Jeroen Korving for excellent technical assistance. EvR was supported by the Hein JJ Wellens Foundation, and NWO.

REFERENCES

1. Paul S. Ventricular remodeling. *Crit Care Nurs Clin North Am.* 2003;15:407-11.
2. Battegay E. [Angiogenesis--mechanisms and therapeutic approaches]. *Schweiz Rundsch Med Prax.* 1995;84:118-21.
3. van Rooij E, Doevendans PA, Crijns HJ, Heeneman S, Lips DJ, van Bilsen M, Williams RS, Olson EN, Bassel-Duby R, Rothermel BA, De Windt LJ. MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction. *Circ Res.* 2004;94:e18-26.
4. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell.* 1998;93:215-28.
5. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. *Embo J.* 2001;20:6414-23.
6. Youn HD, Chatila TA, Liu JO. Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. *Embo J.* 2000;19:4323-31.
7. Sartorelli V, Huang J, Hamamori Y, Kedes L. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. *Mol Cell Biol.* 1997;17:1010-26.
8. Garcia-Rodriguez C, Rao A. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). *J Exp Med.* 1998;187:2031-6.
9. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays.* 1998;20:615-26.
10. McKinsey TA, Zhang CL, Olson EN. Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev.* 2001;11:497-504.
11. Lu J, McKinsey TA, Zhang CL, Olson EN. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell.* 2000;6:233-44.
12. Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann N Y Acad Sci.* 2003;983:84-100.
13. Black BL, Olson EN. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol.* 1998;14:167-96.
14. McKinsey TA, Zhang CL, Olson EN. Signaling chromatin to make muscle. *Curr Opin Cell Biol.* 2002;14:763-72.
15. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell.* 2002;110:479-88.
16. Mellgren G, Borud B, Hoang T, Yri OE, Fladeby C, Lien EA, Lund J. Characterization of receptor-interacting protein RIP140 in the regulation of SF-1 responsive target genes. *Mol Cell Endocrinol.* 2003;203:91-103.
17. Wei LN, Farooqui M, Hu X. Ligand-dependent formation of retinoid receptors, receptor-interacting protein 140 (RIP140), and histone deacetylase complex is mediated by a novel receptor-interacting motif of RIP140. *J Biol Chem.* 2001;276:16107-12.
18. Fernandes I, Bastien Y, Wai T, Nygard K, Lin R, Cormier O, Lee HS, Eng

F, Bertos NR, Pelletier N, Mader S, Han VK, Yang XJ, White JH. Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms. *Mol Cell.* 2003;11:139-50.
19. Kurtev V, Margueron R, Kroboth K, Ogris E, Cavailles V, Seiser C. Transcriptional regulation by the repressor of estrogen receptor activity via recruitment of histone deacetylases. *J Biol Chem.* 2004;279:24834-43.
20. Mazumdar A, Wang RA, Mishra SK, Adam L, Bagheri-Yarmand R, Mandal M, Vadlamudi RK, Kumar R. Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. *Nat Cell Biol.* 2001;3:30-7.
21. Rubanyi GM, Johns A, Kauser K. Effect of estrogen on endothelial function and angiogenesis. *Vascul Pharmacol.* 2002;38:89-98.
22. Oie E, Clausen OP, Yndestad A, Grogaard HK, Attramadal H, E Ol. Endothelin receptor antagonism attenuates cardiomyocyte apoptosis after induction of ischemia in rats. *Scand Cardiovasc J.* 2002;36:108-16.
23. Orgad U, Alroy J, Ucci A, Merk FB. Histochemical studies of epithelial cell glycoconjugates in atrophic, metaplastic, hyperplastic, and neoplastic canine prostate. *Lab Invest.* 1984;50:294-302.
24. Alroy J, Goyal V, Skutelsky E. Lectin histochemistry of mammalian endothelium. *Histochemistry.* 1987;86:603-7.
25. Wurzel J, Goldman BI. Angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med.* 2000;343:148-9.
26. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med.* 2000;342:626-33.
27. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature.* 1992;359:843-5.
28. Levy AP, Levy NS, Loscalzo J, Calderone A, Takahashi N, Yeo KT, Koren G, Colucci WS, Goldberg MA. Regulation of vascular endothelial growth factor in cardiac myocytes. *Circ Res.* 1995;76:758-66.
29. Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Harkonen PL. Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. *Mol Cell Endocrinol.* 1999;149:29-40.
30. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J.* 1999;13:9-22.
31. Takagi H, Koyama S, Seike H, Oh H, Otani A, Matsumura M, Honda Y. Potential role of the angiopoietin/tie2 system in ischemia-induced retinal neovascularization. *Invest Ophthalmol Vis Sci.* 2003;44:393-402.
32. Mandriota SJ, Pyke C, Di Sanza C, Quinodoz P, Pittet B, Pepper MS. Hypoxia-inducible angiopoietin-2 expression is mimicked by iodonium compounds and occurs in the rat brain and skin in response to systemic hypoxia and tissue ischemia. *Am J Pathol.* 2000;156:2077-89.
33. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem.* 1999;274:15732-9.
34. Rosenstein JM, Mani N, Silverman WF, Krum JM. Patterns of brain angiogenesis after vascular endothelial growth factor administration in vitro and in vivo. *Proc Natl Acad Sci U S A.* 1998;95:7086-91.
35. Pettersson A, Nagy JA, Brown LF, Sundberg C, Morgan E, Jungles S, Carter

R, Krieger JE, Manseau EJ, Harvey VS, Eckelhoefer IA, Feng D, Dvorak AM, Mulligan RC, Dvorak HF. Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/ vascular endothelial growth factor. *Lab Invest.* 2000;80:99-115.

36. Drake CJ, Little CD. 1 the de novo formation of blood vessels. *J Histochem Cytochem.* 1999;47:1643.
37. Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med.* 1991;20:47-63.
38. Isles CG, Hole DJ, Hawthorne VM, Lever AF. Relation between coronary risk and coronary mortality in women of the Renfrew and Paisley survey: comparison with men. *Lancet.* 1992;339:702-6.
39. Farhat MY, Lavigne MC, Ramwell PW. The vascular protective effects of estrogen. *Faseb J.* 1996;10:615-24.
40. Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK, Schnaper HW. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation.* 1995;91:755-63.
41. Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab.* 1996;81:3112-8.
42. Greb RR, Heikinheimo O, Williams RF, Hodgen GD, Goodman AL. Vascular endothelial growth factor in primate endometrium is regulated by oestrogen-receptor and progesterone-receptor ligands in vivo. *Hum Reprod.* 1997;12:1280-92.
43. Krasinski K, Spyridopoulos I, Asahara T, van der Zee R, Isner JM, Losordo DW. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation.* 1997;95:1768-72.
44. Ferrara N. VEGF: an update on biological and therapeutic aspects. *Curr Opin Biotechnol.* 2000;11:617-24.
45. Hyder SM, Stancel GM, Chiappetta C, Murthy L, Boettger-Tong HL, Makela S. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. *Cancer Res.* 1996;56:3954-60.
46. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell.* 2000;103:843-52.
47. Glass CK, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* 2000;14:121-41.
48. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell.* 2002;108:465-74.

Chapter 6 Discussion

Cardiac hypertrophy: the good, the bad and the ugly

Cardiac hypertrophy; the good, the bad, and the ugly

Cardiac hypertrophy is defined by an increase in heart size and/or myofibrillar volume without a change in myocyte number in response to increased workload, injury, or intrinsic defects in contractile performance. These changes trigger a cardiac remodelling process in which the heart becomes abnormally enlarged, which can eventually lead to heart failure and sudden death in humans.

Traditionally, it has been widely believed that hypertrophy occurs as an adaptive response as a consequence of pressure overload or due to an increase in demand during endurance exercise and pregnancy, and is termed "compensatory" on the premise that it facilitates ejection performance by normalizing systolic wall stress while maintaining a normal organization of cardiac structure. In the longer term, beneficial, "compensated" hypertrophy may transition into maladaptive "decompensated" hypertrophy and heart failure, a process during which the same signaling routes are thought to be active.[1]

In the last few years, the general tendency to view hypertrophy as a single entity with similar morphological and molecular manifestations, has shifted towards the belief that the hypertrophic response of the myocyte is directed by the extracellular signal that reaches the cell. Cardiac hypertrophy is initiated by a wide array of endocrine, paracrine, and autocrine growth factors, which all activate a different subset of intracellular signaling cascades. The cellular response, which can either be genomic or non-genomic, is the combined resultant of the activated signaling routes and directs the cell towards a predisposed fate. Despite the complexity of this signaling network and the discrepancy between the different types of hypertrophy, a rough distinction can be made between 'good' physiological hypertrophy with normal organization of cardiac structure and normal or enhanced cardiac function, and 'bad' pathological hypertrophy, which is associated with an altered pattern of cardiac gene expression, fibrosis, cardiac dysfunction, and increased morbidity and mortality.

Several research groups have applied transgenesis in an attempt to elucidate the role of the distinct signaling cascades involved in different types of hypertrophy. Elucidation of these distinct signaling cascades that play a role in these two different forms of hypertrophy, might raise the prospect of modulating pathological hypertrophic growth, while preserving the heart's ability to adapt to an increase in physiological demands.

Calcineurin's involvement in pathological hypertrophy

In 1998 Molkentin et al. first identified the calcium-dependent phosphatase calcineurin as a key mediator of cardiac hypertrophy.[2] Cardiac specific overexpression of an activated form of calcineurin revealed to be sufficient to induce a robust hypertrophic response in transgenic mice and to eventually result in a failing phenotype of the myocardium. Due to the apparent pathological phenotype, calcineurin has gained a lot of interest. The studies presented in the thesis have been dedicated to elucidate the precise mode of action and physiological or pathological role of calcineurin in the hypertrophic growth process of the heart.

Acute myocardial infarction induces left ventricular hypertrophy, and eventually gives rise to congestive heart failure.[3] Although the role of calcineurin in pressure overload hypertrophy has already been established,[4-8] pharmacological inhibition of calcineurin failed to clearly define its involvement in cardiac remodeling after myocardial infarction.[9-12] We were able to identify calcineurin as a key player in the remodeling process of the remote myocardium following myocardial infarction. Our data indicated that calcineurin-induced hypertrophic remodeling leads to a predisposition to maladaptivity, characterized by fibrosis and chamber dilation, fueling the transition to overt heart failure. The use of a genetic mouse model with a mild myocyte-restricted overexpression of Modulatory Calcineurin Interacting Protein 1 (MCIP1) to effectively block calcineurin activity specifically in the heart,[13] enabled us to indicate that inhibition of calcineurin activity is able to largely prevent these characteristics (Chapter 2). These findings not only indicate that calcineurin plays a significant role in hypertrophic remodeling of the heart after MI, but also show that inhibition of calcineurin and subsequent hypertrophy may be of therapeutic benefit.

Since the MCIP1 transgene is under the transcriptional control of the α-MyHC promoter, which is activated at birth,[14] MCIP1 continuously inhibits calcineurin activity. This directly prevents the myocardium from a hypertrophic response after exposure to myocardial infarction, and favours the idea that cardiac hypertrophy caused by activation of calcineurin is maladaptive in nature.

Wilkins et al. recently confirmed the existence of a divergence between active signaling routes for different forms of cardiac hypertrophy by showing that voluntary wheel running and swimming resulted in significant cardiac hypertrophy without activation of calcineurin-NFAT signaling.[8] Although in most models of pathological hypertrophy studied to date, inhibition of calcineurin-NFAT signaling has yielded either a reduction in the hypertrophic response and/or a delay in the progression from hypertrophy to heart failure, calcineurin proved to play no role in regulating physiological or adaptive growth of the myocardium.

The calcineurin/NFAT signaling route

The involvement of the calcineurin/NFAT signaling in maladaptive cardiac hypertrophy intrigued us to more specifically unravel the calcineurin/NFAT signaling route. In the initial description of calcineurin's involvement in cardiac hypertrophy, calcineurin was shown to dephosphorylate the transcription factor Nuclear Factor of Activated T-cells c4 (NFATc4) in cardiomyocytes, enabling it to translocate to the nucleus and activate cardiac transcription.[2] However, although several lines of transgenic mice expressing activated mutants of NFATc4 in a cardiac-selective manner showed NFATc4 to be sufficient to induce robust hypertrophy, loss of NFATc4 did not compromise the ability of the myocardium to undergo hypertrophic growth, which excluded the absolute requirement of NFATc4 in the hypertrophic process. In contrast, NFATc3-null mice appeared to be partially deficient in their ability to undergo cardiac hypertrophy in response to calcineurin activation.[15] Together these observations established NFATc3 to be a critical downstream mediator of calcineurin-regulated hypertrophy in the heart and validated the original hypothesis that the calcineurin-regulated hypertrophic responses require NFAT effectors in vivo.

The high degree of homology for all calcineurin-sensitive NFAT isoforms together with the observation the NFATc3-null mice were unable to completely block calcineurin-induced hypertrophy[15] led us to hypothesize that this might be a result of functional redundancy by other NFAT isoforms. Since only simultaneously abrogation of all NFAT-dependent transcriptional activity renders cardiomyocytes resistant to calcineurin- and agonist-induced hypertrophy (Chapter 3), partial blockade of calcineurin activity in the NFAT-null mice might be due to functional redundancy between NFAT isoforms.

Due to the ability of the NFAT isoforms to, at least in part, compensate for each others function, in vivo confirmation of our findings would be possible by generating transgenic mice overexpressing the dominant negative NFAT protein in the heart. However, exhaustive attempts to generate dominant negative NFAT transgenic mice using the α-MyHC promoter were unsuccessful due to embryonic or early post-natal lethality, suggesting that NFAT factors are crucial during developmental maturation of the myocardium. Inducible transgenic systems to temporally regulate expression of the dominant negative NFAT construct should be applied to bypass these developmental problems that arise from blocking all cardiac NFAT isoforms at a neonatal stage. This temporal regulation will expand our ability to dissect the effect of blocking transcriptional activity NFAT within the hypertrophic, adult heart.

Transcriptional synergy between MEF2 and NFAT

Despite the wealth of information linking calcineurin and the progression of cardiac hypertrophy, to date little is known of the exact mechanism by which calcineurin promotes growth of the cardiac myocyte through activating NFAT gene targets. Although NFAT is a relatively weak transcriptional activator alone and only a limited amount of genes involved in hypertrophy have been characterized to harbor NFAT binding sequences in their regulatory promoter regions, the severity of the cardiac phenotype in the calcineurin transgenic animals indicates the relevance of this signaling route.

Assembly of synergistic transcription factors and coactivators on promoters or enhancers results in a DNA-bound complex containing several proteins, that overall is more efficient in inducing transcription than any of its individual components alone. There is evidence from other cell types that NFAT may directly interact with the myogenic transcription factor Myocyte Enhancer Factor 2 (MEF2). MEF2 is a MADS box transcription factor that is expressed during the development of all three muscle lineages, but is also activated during cardiac hypertrophy.[16,17] Additionally, both NFAT and MEF2 require interaction with the transcriptional coactivator p300 for their full transcriptional activity, a protein that is also known to possess intrinsic histone acetyl transferase activity to promote a transcriptionally active chromatin configuration.[18,19] Due to the importance of MEF2 in regulating the differentiation of myogenic lineages, we hypothesized that if MEF2 would be downstream of calcineurin, it may, at least in part, explain the dramatic remodeling effects of calcineurin in the heart.

Both *in vitro* and *in vivo* we were able to demonstrate the existence of a transcriptional regulatory complex in cardiomyocytes involving proteins of the NFAT and MEF2 families in the presence of the transcriptional coactivator p300, that integrate the Ca^{2+}-activated CnA/NFAT signaling pathway with the myogenic transcriptional activity of MEF2. We demonstrated MEF2 to be a required component for cardiomyocytes to undergo Ca^{2+}/CnA-induced hypertrophy, as adenoviral overexpression of a dominant negative MEF2 abrogated the characteristic hypertrophic remodeling in cultured cardiomyocytes (Chapter 4). These findings expand the significance of calcineurin signaling, since they prove calcineurin to be able to induce expression of not only genes containing NFAT binding sites, but also genes containing MEF2 binding sequences in their promoter regions.

Interestingly, we only found a distinct subset of MEF2 sensitive genes to be upregulated in calcineurin transgenic mice. This indicates that the synergistic interaction between MEF2 and NFAT is not a general mechanism, but limited to distinct genes. It will be very challenging to elucidate the mechanism for the gene specificity of this synergy between MEF2 and NFAT. Currently, we are

in the process of crossing inducible dominant negative MEF2 transgenic mice with mice overexpressing a constitutively activated form of calcineurin. These triple transgenic mice will indicate whether we can inhibit or even reverse the calcineurin-induced pathological, hypertrophic phenotype of the heart. At the same time, microarray analysis on cardiac tissue of these mice will provide us with detailed insights into the MEF2 sensitive genes that are involved in hypertrophic calcineurin signaling.

Transcriptional control of HDAC

Histone acetylation/deacetylation represents a central mechanism for the control of gene expression. Histone acetyltransferases (HATs), like p300, catalyze the acetylation of core histones of nucleosomes, resulting in chromatin relaxation and transcriptional activation. The activity of HATs is antagonized by histone deacetylases (HDACs), which deacetylate histones and transcription factors, resulting in chromatin condensation and transcriptional repression.[20] HDACs are divided into three classes, of which class II HDACs (HDACs 4, 5, 6, 7, 9, and 10) contain a unique amino-terminal extension that mediates association with MEF2 factors through which it respresses its transcriptional activity.[21,22]

Due to the coordinate interaction between NFAT, MEF2 and p300 and the increase in calcineurin activity after MI, we hypothesized that HDAC9 knockout animals, which have been shown to be highly sensitive to hypertrophic stimuli and spontaneously develop cardiac hypertrophy with advanced age,[23] would be prone to develop cardiac hypertrophy and failure after MI due to calcineurin activation. Since previously a gender difference was reported for mice overexpressing a constitutively active class II HDAC,[24] both sexes were included in this study.

Strikingly, although MI resulted in severe deterioration in contractile function and increased mortality for the male HDAC9 knockout animals compared to wild type animals, female HDAC9 knockout animals showed less deleterious remodeling, resulting in better maintained cardiac contractility and structure. More detailed examination showed this effect to be attributable to an enhanced angiogenic response in the infarct area of the HDAC9 knockout female mice, rather than the result of sensitized MEF2 signaling (Chapter 5). A possible explanation for this phenomenon might lay in the transcriptional repression the estrogen receptor through HDAC.[25] one might speculate that removal of HDAC9 might sensitize the estrogen receptor, thereby enhancing the angiogenic effects estrogen signaling has been reported to provoke.

It will be very interesting to see whether ovariectomy in these female mice will result in the apparent failing phenotype of the male HDAC9 knockout mice, and whether estrogen replacement can rescues these ovarectamized females.

Additionally these results also indicate the necessity to specifically

target the calcineurin/ NFAT pathway. Our speculation that HDAC ablation would sensitize MEF2 signaling through the MEF2/NFAT interaction, thus enhance calcineurin induced hypertrophic process after MI, appeared premature. Although the severe mortality for the male HDAC9 knockout mice might probably be related to enhanced MEF2 signaling, the elevation in survival for the female HDAC9 knockout mice indicates an interaction between HDAC and estrogens. This indicates that the complexity and diversity of the signaling cascades involving HDAC obviously interferes with the specifity of the outcomes, and a more defined approach should be taken to give concluding results.

Experimental pitfalls and future therapeutic prospects

The data presented in this thesis suggest that calcineurin inhibition would be desirable in treating certain forms of maladaptive hypertrophy. However, before calcineurin can function as a clinical target there are several experimental and therapeutic drawbacks we need to overcome.

An extensive amount of research has focused on elucidating the role of calcineurin in pathologic hypertrophy, but, up to date, still no reliable, standardized method has been developed to measure calcineurin activity in vivo. Quantification of calcineurin phosphatase activity from tissue lysates using biochemical assays is experimentally problematic, due to the dynamic, calcium/ calmodulin-dependent regulation of activity, and the sensitivity of calcineurin to oxidation of the Fe-Zn active center.[26] At the same time this assay does not reflect the proportion of the enzymatic calcineurin pool that is actually in an active state in vivo, but only indicates the amount of maximally activatable calcineurin present in a specific tissue homogenate. Due to these flaws, we used several other methods in parallel to monitor calcineurin activity, such as absolute protein levels of the Aβ subunit,[27] the phosphorylation status of endogenous NFAT,[28] the transcriptional activity of NFAT,[8] specifically the exon 4 splice isoform of endogenous MCIP1,[13] and biochemical phosphatase enzyme assays. Although all these methods reflect calcineurin activity, optimization of a direct measurement of calcineurin activity would be preferential.

Although there are a lot of benefits from using genetically modified animal models to fully explore their phenotypes at the whole organ and whole animal level, the extension of cardiovascular physiological methodologies to the mouse is imperative.

Several animal studies have demonstrated that pharmacological or genetic inhibition of calcineurin by overexpressing peptide inhibitors are beneficial in preventing cardiac hypertrophy and failure (all reviewed in [29,30]). The many physiological roles of calcineurin signaling, such as in the immune response, cause the existing calcineurin inhibitory drugs to have a relatively toxic profile.

Therefore novel therapeutic agents would be needed to target calcineurin more specifically in the heart. Given the complexity and redundant nature of the signaling network that drive cardiac pathogenesis, a potentially more efficacious therapy would be to target downstream elements, like NFAT, in the pathological calcineurin signaling cascades. However, even if it can be achieved to design a drug that specifically target calcineurin's main downsteam effector NFAT, there should be some caution in the application of this pharmacological tool. Calcineurin inhibition may render the heart more susceptible to apoptotic cell death,[31] and although the exact cardioprotective mechanism of calcineurin against myocyte apoptosis is unknown, it appears to require NFAT activation.[32]

Conclusively, our findings as well as those from others, suggesting a pivotal involvement of calcineurin in several forms of pathological hypertrophy in cell culture, rodents and patients, suggest that calcineurin may be a valid target for therapeutic intervention in patients. Recent advancements in understanding calcineurin signaling, its interconnectivity with other hypertrophic signaling pathways, as well as discoveries of effectors of calcineurin activity with cardiac muscle specificity, should offer new entry points for more appropriate drug design for the treatment of hypertrophic heart disease and heart failure.

REFERENCES

1. Grossman W, Jones D, McLaurin LP. Wall stress and patterns of hypertrophy in the human left ventricle. J Clin Invest. 1975;56:56-64.
2. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998;93:215-28.
3. Paul S. The pathophysiologic process of ventricular remodeling: from infarct to failure. Crit Care Nurs Q. 1995;18:7-21.
4. Zou Y, Hiroi Y, Uozumi H, Takimoto E, Toko H, Zhu W, Kudoh S, Mizukami M, Shimoyama M, Shibasaki F, Nagai R, Yazaki Y, Komuro I. Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. Circulation. 2001;104:97-101.
5. De Windt LJ, Lim HW, Bueno OF, Liang Q, Delling U, Braz JC, Glascock BJ, Kimball TF, del Monte F, Hajjar RJ, Molkentin JD. Targeted inhibition of calcineurin attenuates cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A. 2001;98:3322-7.
6. Hill JA, Rothermel B, Yoo KD, Cabuay B, Demetroulis E, Weiss RM, Kutschke W, Bassel-Duby R, Williams RS. Targeted inhibition of calcineurin in pressure-overload cardiac hypertrophy. Preservation of systolic function. J Biol Chem. 2002;277:10251-5.
7. Saito T, Fukuzawa J, Osaki J, Sakuragi H, Yao N, Haneda T, Fujino T, Wakamiya N, Kikuchi K, Hasebe N. Roles of calcineurin and calcium/calmodulin-dependent protein kinase II in pressure overload-induced cardiac hypertrophy. J Mol Cell Cardiol. 2003;35:1153-60.
8. Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, Jones F, Kimball TR, Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. Circ Res. 2004;94:110-8.
9. Cai Q, Baxter GF, Yellon DM. Reduction of infarct size in isolated rat heart by CsA and FK506: possible role of phosphatase inhibition. Cardiovasc Drugs Ther. 1998;12:499-501.
10. Deng L, Huang B, Qin D, Ganguly K, El-Sherif N. Calcineurin inhibition ameliorates structural, contractile, and electrophysiologic consequences of postinfarction remodeling. J Cardiovasc Electrophysiol. 2001;12:1055-61.
11. Oie E, Bjornerheim R, Clausen OP, Attramadal H. Cyclosporin A inhibits cardiac hypertrophy and enhances cardiac dysfunction during postinfarction failure in rats. Am J Physiol Heart Circ Physiol. 2000;278:H2115-23.
12. Youn T, Piao H, Kwon J, Choi S, Kim H, Park D, Kim D, Kim Y, Cho M. Effects of the calcineurin dependent signaling pathway inhibition by cyclosporin A on early and late cardiac remodeling following myocardial infarction. Eur J Heart Fail. 2002;4:713-8.
13. Rothermel BA, McKinsey TA, Vega RB, Nicol RL, Mammen P, Yang J, Antos CL, Shelton JM, Bassel-Duby R, Olson EN, Williams RS. Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A. 2001;98:3328-33.
14. Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. J Biol Chem. 1991;266:24613-20.
15. Wilkins BJ, De Windt LJ, Bueno OF, Braz JC, Glascock BJ, Kimball TF,

Molkentin JD. Targeted disruption of NFATc3, but not NFATc4, reveals an intrinsic defect in calcineurin-mediated cardiac hypertrophic growth. Mol Cell Biol. 2002;22:7603-13.
16. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. Embo J. 2001;20:6414-23.
17. Youn HD, Chatila TA, Liu JO. Integration of calcineurin and MEF2 signals by the coactivator p300 during T-cell apoptosis. Embo J. 2000;19:4323-31.
18. Garcia-Rodriguez C, Rao A. Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). J Exp Med. 1998;187:2031-6.
19. Sartorelli V, Huang J, Hamamori Y, Kedes L. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. Mol Cell Biol. 1997;17:1010-26.
20. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays. 1998;20:615-26.
21. Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. Histone deacetylases: unique players in shaping the epigenetic histone code. Ann N Y Acad Sci. 2003;983:84-100.
22. Lu J, McKinsey TA, Zhang CL, Olson EN. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Mol Cell. 2000;6:233-44.
23. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell. 2002;110:479-88.
24. Czubryt MP, McAnally J, Fishman GI, Olson EN. Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. Proc Natl Acad Sci U S A. 2003;100:1711-6.
25. Kurtev V, Margueron R, Kroboth K, Ogris E, Cavailles V, Seiser C. Transcriptional regulation by the repressor of estrogen receptor activity via recruitment of histone deacetylases. J Biol Chem. 2004;279:24834-43.
26. Bogumil R, Namgaladze D, Schaarschmidt D, Schmachtel T, Hellstern S, Mutzel R, Ullrich V. Inactivation of calcineurin by hydrogen peroxide and phenylarsine oxide. Evidence for a dithiol-disulfide equilibrium and implications for redox regulation. Eur J Biochem. 2000;267:1407-15.
27. Lim HW, De Windt LJ, Steinberg L, Taigen T, Witt SA, Kimball TR, Molkentin JD. Calcineurin expression, activation, and function in cardiac pressure-overload hypertrophy. Circulation. 2000;101:2431-7.
28. van Rooij E, Doevendans PA, Crijns HJ, Heeneman S, Lips DJ, van Bilsen M, Williams RS, Olson EN, Bassel-Duby R, Rothermel BA, De Windt LJ. MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction. Circ Res. 2004;94:e18-26.
29. Bueno OF, van Rooij E, Molkentin JD, Doevendans PA, De Windt LJ. Calcineurin and hypertrophic heart disease: novel insights and remaining questions. Cardiovasc Res. 2002;53:806-21.
30. Leinwand LA. Calcineurin inhibition and cardiac hypertrophy: a matter of balance. Proc Natl Acad Sci U S A. 2001;98:2947-9.

31. De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW, 2nd, Kitsis RN, Molkentin JD. Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: An apoptosis-independent model of dilated heart failure. Circ Res. 2000;86:255-63.
32. Pu WT, Ma Q, Izumo S. NFAT transcription factors are critical survival factors that inhibit cardiomyocyte apoptosis during phenylephrine stimulation in vitro. Circ Res. 2003;92:725-31.

SUMMARY

The heart consists of terminally differentiated cells, meaning that they have lost their ability to divide. When the heart becomes exposed to growth factors in response to increased workload, injury, or intrinsic defects in contractile performance, the cardiac muscle cells therefore start to grow, which is called cardiomyocyte hypertrophy. In 1998 Molkentin et al. first identified the calcium-dependent phosphatase calcineurin as being a key mediator in this process of cardiomyocyte hypertrophy.

As soon as the role of calcineurin in cardiomyocyte hypertrophy became well established, pharmacological tools where applied to begin to determine whether inhibition of calcineurin activity *in vivo* might be an effective means of preventing cardiac hypertrophy. In chapter 1 we review the use of the pharmacological calcineurin inhibitors CsA and FK-506 to prevent hypertrophy in response to a variety of pathological stimuli, and discuss the opportunities to use genetically modified animals.

In chapter 2 we investigate calcineurin's involvement in left ventricular hypertrophy as a consequence of acute myocardial infarction. We were able to identify calcineurin as a key player in the maladaptive remodeling process of the remote myocardium following myocardial infarction, characterized by fibrosis and chamber dilation, resulting in overt heart failure. The use of a genetic mouse model with a mild myocyte-restricted overexpression of Modulatory Calcineurin Interacting Protein 1 (MCIP1) to effectively block calcineurin activity specifically in the heart, enabled us to indicate that inhibition of calcineurin activity is able to largely prevent these pathological characteristics.

Taken the apparent importance of calcineurin signaling in maladaptive hypertrophy, in chapter 3 we attempted to further elucidate the downstream substrate of calcineurin, the transcription factor nuclear factor of activated T-cells (NFAT). Our data indicate both the presence and transcriptional activity of all four, calcineurin-sensitive members of the NFAT family (NFATc1, c2, c3 and c4) in the ventricular cardiomyocyte cell lineage. A dominant negative approach enabled us to simultaneously abrogate NFAT-dependent transcriptional activity and rendered cardiomyocytes resistant to calcineurin- and agonist-induced cardiomyocyte hypertrophy, indicating the requirement of NFAT in cardiomyocyte hypertrophy.

Despite the wealth of information linking calcineurin and the progression of cardiac hypertrophy, little is known of the exact mechanism by which calcineurin promotes growth of the cardiac myocyte. In chapter 4 we were able to both *in vitro* and *in vivo* indicate the existence of a transcriptional regulatory complex in cardiomyocytes involving proteins of the NFAT and MEF2 families of transcription factors. This interaction integrates the Ca^{2+}-activated CnA/NFAT signaling pathway with the myogenic transcriptional activity of MEF2, that is

required for cardiomyocytes to fully undergo Ca^{2+}/CnA-induced hypertrophy.

In chapter 5 we investigated cardiac remodeling after MI in mice lacking the histone deactylase, HDAC9. Since the transcriptional activity of MEF2 is repressed by histone deacetylases, and due to the interaction between NFAT and MEF2, we hypothesized that HDAC9-null animals would be hypersensitive to myocardial infarction induced calcineurin activation and would therefore ultrarapid develop cardiac hypertrophy and failure. However, although MI resulted in an severe deterioration in contractile function and increased mortality for the male HDAC9 knockout animals, surprisingly, compared to wild type animals, the female HDAC9 knockout animals showed less remodeling, which resulted in a better maintained cardiac contractility and structure. More detailed examination showed this effect to be attributable to an enhanced angiogenic respons in the infarcted area of the HDAC9 knockout females.

In chapter 6 the major findings of this thesis and future directions for research on calcineurin signaling are discussed. Our data emphasize the significance of the calcineurin/NFAT pathway in cardiomyocyte hypertrophy, reveal its maladaptive character and unravel the downstream effectors, and might possibly attribute to future therapeutic drug design for the treatment of hypertrophic heart disease.

SAMENVATTING

Het hart bestaat uit terminaal gedifferentieerde cellen, wat betekent dat zij het vermogen om te delen zijn verloren. Zodra het hart, als gevolg van een toename in werklast of intrinsieke defecten in contractiliteit, wordt blootgesteld aan groeifactoren, beginnen de hartspiercellen te groeien, wat cardiomyocyt hypertrofie wordt genoemd. In 1998 ontdekte Molkentin *et al.* dat de calcium afhankelijke fosfatase calcineurine een belangrijke rol speelt in dit proces van cardiomyocyt hypertrofie.

Zodra de rol van calcineurine in cardiomyocyt hypertrofie een geaccepteerd gegeven werd, begon men farmacologische substraten te gebruiken om te bepalen of inhibitie van calcineurine activiteit *in vivo* een effectieve manier is om cardiale hypertrofie te voorkomen. In hoofdstuk 1 bediscussiëren we het gebruik van farmacologische inhibitie van calcineurine met behulp van CsA en FK-506 om hypertrofie als gevolg van een verscheidenheid aan pathologische stimuli te voorkomen en zetten we de mogelijkheden om genetisch gemodificeerde dieren te gebruiken uiteen.

In hoofdstuk 2 onderzoeken we de betrokkenheid van calcineurine in hypertrofie van het linker ventrikel als gevolg van een acuut myocard infarct. Wij zijn in staat gebleken calcineurine aan te wijzen als een belangrijke factor in het proces van maladaptieve remodellering van het niet-geïnfarceerde deel van het hart na een hartinfarct, wat wordt gekarakteriseerd door fibrose, dilatatie en uiteindelijk hartfalen. Het gebruik van een genetisch gemodificeerde muis die Modulatory Calcineurin Interacting Protein 1 (MCIP1) in hartspiercellen tot over expressie brengt om calcineurine activiteit specifiek in deze cellen te blokkeren, heeft ons in staat gesteld aan te tonen dat inhibitie van calcineurine activiteit deze maladaptieve verschijnselen grotendeels voorkomt.

Doordat calcineurine klaarblijkelijk een belangrijke rol speelt in pathologische hypertrofie, hebben we in hoofdstuk 3 getracht de onderliggende effector van calcineurine, de transcriptie factor 'nuclear factor of activated T-cells (NFAT)', verder uit te diepen. Onze data tonen aan dat alle vier calcineurine gevoelige leden van de NFAT familie van transcriptiefactoren (NFATc1, c2, c3 en c4) aanwezig zijn in ventriculaire hartspiercellen en tevens transcriptioneel actief zijn. Een dominant negatieve aanpak, waarmee we simultaan alle NFAT-afhankelijke transcriptionele activiteit kunnen blokkeren, resulteerde in cardiomyocyten die niet langer een hypertrofe respons vertoonden in reactie op calcineurine of andere hypertrofe agonisten, wat aangeeft dat NFAT vereist is in cardiomyocyt hypertrofie.

Ondanks de veelheid aan informatie over calcineurine en de ontwikkeling van cardiale hypertrofie, is er nog maar weinig bekend over het mechanisme waarmee calcineurine groei van hartspiercellen bewerkstelligt. In hoofdstuk 4

tonen wij zowel *in vitro* als *in vivo* het bestaan van een transcriptioneel regulerend complex in hartspiercellen tussen NFAT eiwitten en MEF2 factoren aan. Deze interactie integreert de Ca^{2+}-geactiveerde CnA/NFAT signaleringsroute en de myogene transcriptionele activiteit van MEF2, welke in hartspiercellen vereist is om een volledige Ca^{2+}/CnA geïnduceerde hypertrofe respons te vertonen.

In hoofdstuk 5 hebben we de cardiale remodellering na een myocard infarct onderzocht in muizen die de histon deacetylase 9 missen (HDAC9 knockout). Omdat de transcriptionele activiteit van MEF2 wordt onderdrukt door histon deacetylases en door de interactie tussen NFAT en MEF2, was onze hypothese dat deze HDAC9 knockout muizen hypersensitief zouden zijn voor activatie van calcineurine als gevolg van het myocard infarct en daardoor extra snel hypertrophy zouden ontwikkelen en in falen gaan. Hoewel het infarct in de mannelijke HDAC9 knockout muizen resulteerde in een verslechtering van cardiale contractiliteit en een verhoogde mortaliteit ten opzichte van wild type muizen, vertoonden de vrouwelijke HDAC9 knockout muizen verrassend genoeg een verminderde remodellering, wat resulteerde in behoud van contractiliteit en structuur. Meer gedetailleerd onderzoek toonde aan dat dit effect toe te schrijven is aan een verbeterde angiogene respons in het infarct gebied van de vrouwelijke HDAC9 knockout muizen.

In hoofdstuk 6 bediscussiëren we de belangrijkste bevindingen van dit proefschrift en de toekomstige richting voor het calcineurine onderzoek. Onze data benadrukken de significante bijdrage van de calcineurine/NFAT route in hypertrofie van de hartspiercel, onthullen het maladaptieve karakter en ontrafelen de onderliggende effectors en kunnen mogelijk een bijdrage leveren aan de ontwikkeling van toekomstige therapeutische drugs voor de behandeling van hypertrofe hartziekten.

CURRICULUM VITAE

De auteur van dit proefschrift, Eva van Rooij, is op 1 juli 1977 in Eindhoven, Nederland, geboren. Nadat zij haar diploma behaalde aan het Eckart College in Eindhoven, is zij begonnen met de studie Gezondheidswetenschappen aan de universiteit van Maastricht, waarbij zij de biologische richting koos. Tijdens deze periode heeft zij zich voornamelijk gericht op de moleculaire biologie. Nadat zij hiervoor haar academisch diploma behaalde, is zij begonnen aan een AIO-project bij de afdeling Cardiologie van de universiteit Maastricht, waarvan zij de laatste twee jaar op het Hubrecht laboratorium in Utrecht heeft gewerkt. In 2004 ontving zij subsidies van de Hein J. J. Wellens stichting en NWO voor een werkbezoek aan het laboratorium van Prof. Eric N. Olson, Universiteit van Texas Southwestern Medical Center, Dallas. Eva ontving tevens dit jaar van NWO een beurs om als postdoctoraal onderzoeker op het laboratorium van Prof. Eric N. Olson te gaan werken.

The author of this thesis, Eva van Rooij, was born the first of July 1977 in Eindhoven, the Netherlands. After graduating at the Eckart College in Eindhoven, she started her academic training in Biological Health Science at the University of Maastricht. During this period she her main focus was molecular biology. After obtaining her academic degree, she started a PhD project at the department of Cardiology at the University Hospital Maastricht, of which she worked the last two years at the Hubrecht laboratory in Utrecht. In 2004 she received grants from the Hein J. J. Wellens foundation and NWO to visit the laboratory of Prof. Eric N. Olson, University of Texas Southwestern medical Center, Dallas. This year Eva also obtained a fellowship from NWO to work as postdoctoral research scientist at the laboratory of Prof. Eric N. Olson.

LIST OF PUBLICATIONS

Papers

1. **Eva van Rooij**, Ralph J. van Oort, Anne-Sophie Armand, Leon J. De Windt Regulation of cardiac gene expression and hypertrophy by MEF2-dependent recruitment of Nuclear Factor of Activated T-cells and p300 histone acetyltranferase. Submitted.

2. **Eva van Rooij**, Pieter A. Doevendans, Harry J. G. M. Crijns, Sylvia Heeneman, Daniel J. Lips, Marc van Bilsen , R. Sanders Williams, Eric N. Olson , Rhonda Bassel-Duby, Beverly A. Rothermel, and Leon J. De Windt. MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function following myocardial infarction; Circ Res. 2004 Feb 20:94(3):18-26 Epub 2004 Jan 22.

3. **Van Rooij E**, Doevendans PA, de Theije CC, Babiker FA, Molkentin JD, de Windt LJ. Requirement of nuclear factor of activated T-cells in calcineurin-mediated cardiomyocyte hypertrophy. J Biol Chem. 2002 Dec 13;277(50):48617-26.

4. Bueno OF, **van Rooij E**, Molkentin JD, Doevendans PA, De Windt LJ. Calcineurin and hypertrophic heart disease: novel insights and remaining questions. Cardiovasc Res. 2002 Mar;53(4):806-21.Review.

Bookchapter

1. Orlando F. Bueno, **Eva van Rooij,** Daniel J. Lips, Pieter A. Doevendans and Leon J. De Windt. Cardiac Signaling: the Good, the Bad and the Ugly. In: Cardiovascular Genomics; new pathophysiological concepts. Ed. PA Doevendans, Kluwer Academics.

ACKNOWLEDGEMENTS / DANKBETUIGINGEN

Voordat ik echt mijn laatste punt ga zetten achter deze uiteenzetting van mijn wetenschappelijke werk van de afgelopen jaren, moet ik bekennen dat ik deze prestatie niet alleen heb verricht. Zowel professioneel als sociaal zijn er verschillende personen geweest die me hebben bijgestaan.

Allereerst wil ik Professor Pieter Doevendans bedanken. Pieter, jij bent degene geweest die mij de kans heeft geboden mezelf te introduceren in de wereld van het cardiologisch onderzoek. Nu, enkele jaren later, heb ik oprecht begrip voor jouw gedrevenheid.

Zonder de hulp van Leon de Windt was ik niet gekomen waar ik vandaag ben. Leon, dankzij jouw intensieve begeleiding en wetenschappelijke input heb ik mijn promotie-onderzoek succesvol kunnen uitvoeren. Hoewel ik de afgelopen jaren op het gebied van calcineurine jouw kennis aardig heb weten te evenaren, heb ik enkel de hoop ooit het wetenschappelijk overzicht te krijgen waarover jij beschikt.

Grote waardering heb ik tevens voor Professor Harry Crijns. Vanaf het moment dat de verhoudingen duidelijk werden, heeft U alles in het werk gesteld om mijn traject zo soepel mogelijk te laten verlopen. Zowel Uw wetenschappelijke als politieke ondersteuning hebben een fundamentele bijdrage geleverd aan het tot stand komen van mijn proefschrift.

I am most grateful to prof. Eric Olson for giving me the opportunity to visit his laboratory at Southwestern Medical Center in Texas. My stay in Dallas certainly refreshed my enthusiasm for research and I am very much looking forward to my time as researcher in your lab.

Allerlei problematische perikelen die het wetenschappelijke leven met zich meebrengt, zijn een stuk gemakkelijker te verduren met steun van naaste collega's. Vanes, jij en ik vormden al snel een front tegen alle beslommeringen die we onderweg zijn tegengekomen. Ik had je graag bijgestaan tijdens jouw eindspurt. Daan, Ralph, Roel, Ruben, Sonja en de rest van de (oud-) collega's en de Hubrechtmensen van 'the first floor' ook jullie bedankt voor de samenwerking en de gezellige avondjes.

Zonder familie en vrienden had ik deze klus ook niet kunnen klaren. Mijn ouders, Martien en Jacqueline, zonder jullie had ik dit nooit bereikt. Van kinds af aan hebben jullie mij in alles geholpen en mij het doorzettingsvermogen gegeven om te komen waar ik vandaag ben. Ik vrees alleen wel dat het van thuis meegekregen zangtalent mijn collega's af en toe ook wel tot waanzin heeft gedreven. Mijn zus Sylvia en mijn broer Martien, draag ik een ontzettend warm hart toe. Syl, dankzij jou weet ik dingen beter te relativeren en in het juiste perspectief te plaatsen. Martien, jongen, samen met jou 2 handen op 1 buik. Van jouw eerlijkheid en directheid kunnen velen nog wat leren.

Ann en Bert, jullie ook ontzettend bedankt voor de steun en begrip die ik de afgelopen jaren van jullie als mijn schoonouders heb ontvangen. Verder ben ik geweldig dankbaar voor mijn fijne familie. Het wekelijks samenkomen bij oma maakt iedere zaterdagmiddag weer een klein Brabants feestje.

Ook zonder de support van mijn vriendinnen had ik niet gekund. Is en An, al een eeuwigheid zijn we samen en waarschijnlijk 'for life'. Super fijn om mensen te hebben op wie je altijd terug kan vallen. De meiden uit Maastricht, stuk voor stuk bedankt. Klaar, in onze tijd als huisgenoten heb jij mij door heel wat promotie-problemen heen weten te slepen. Bij jou kon ik altijd mijn ei kwijt en daarna 'hup, tranen drogen en wat wijn erin'. Maartje, ook jij bedankt voor jouw deel aan mental-coaching op de momenten dat ik weer eens te strak stond. En natuurlijk mijn paranimf Noor, jouw zorgzaamheid en eindeloze begrip maken je een fantastische vriendin.

Maar, mijn allergrootste dankbaarheid en respect gaat uit naar mijn liefde, Marc. Onvoorwaardelijk heb jij naast mij gestaan en je kranig verweerd tegen alle stress-geïnduceerde 'mood swings' en onrustige nachten. Door jouw begrip voor mijn keuze om mijn onderzoekservaring in het buitenland uit te breiden, ben ik je, zo mogelijk, alleen nog maar meer gaan waarderen.

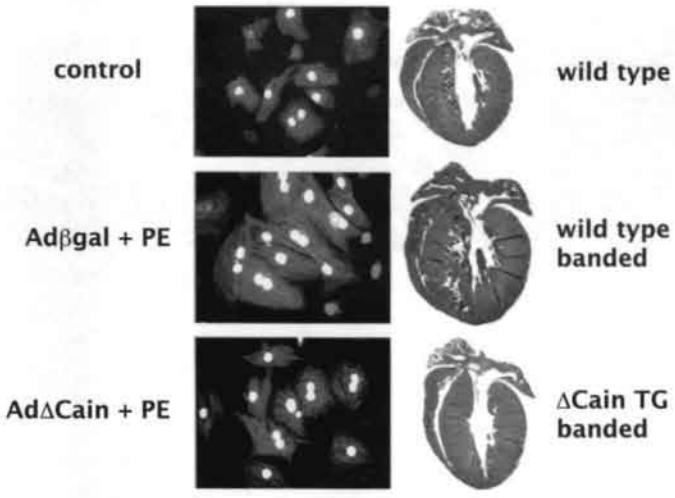


Figure 1.2. Genetic inhibition of calcineurin with calcineurin inhibitory protein (Cain) abrogates the LV hypertrophy response. The left panel summarizes the in vitro results obtained with adenoviral ΔCain gene transfer (AdΔCain) in cardiomyocytes. Phenylephrine (PE) induces a massive increase in cardiomyocyte size in cultures infected with a control adenovirus (Adβgal) compared to uninfected, non-stimulated cells (top left and middle left panels). In contrast, blockade of calcineurin in the presence of ΔCain prevents PE-induced hypertrophy (lower left panel). Cardiomyocytes were visualized by immunocytochemical α-actinin detection, nuclei were visualized with bisbenzamide (white). Hematoxylin-Eosin stained cross-sections demonstrate that similar effects were observed following ΔCain overexpression in vivo. A 14-day pressure overload stimulus resulted in a visible increase in heart weight in wildtype animals, as compared to sham-operated, wildtype mice (top right and middle right panels). Mice overexpressing the non-competetive ΔCain protein in the cardiomyocyte component are protected against this pressure-overload stimulus (lower right panel).

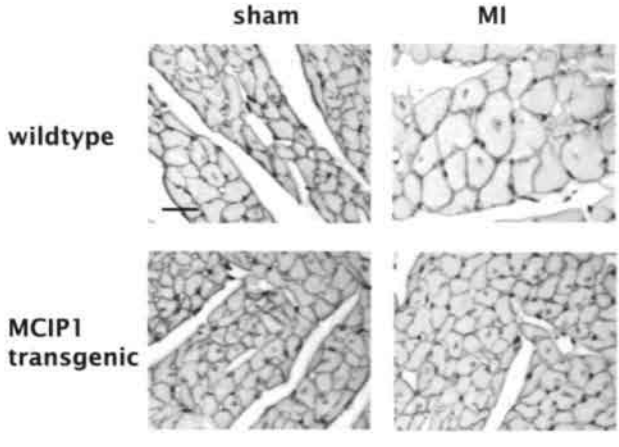


Fig. 2.2C Laminin staining reveals a significant increase in cardiomyocyte size after MI compared to the sham operated animals, which is more pronounced in wildtype animals compared to MCIP1 transgenic animals (n = 4 in both sham groups, n = 6 in both MI groups). Bar represents 0.1 mm.

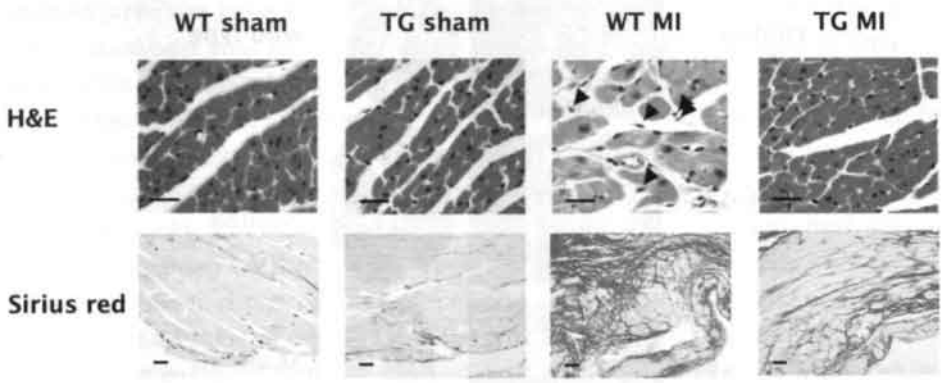


Fig. 2.4A MCIP1 transgenic animals are protected against MI-induced structural remodeling. Representative images of histological staining for the indicated groups. Upper panels are H&E stained images, lower panels Sirius red stained images (Bar indicates 0.1 mm). H&E stained images reveal remarkable myocyte hypertrophy, myofiber disarray and cellular infiltrates (arrowheads) in a section from a representative postinfarcted wildtype mouse. In contrast MCIP1 transgenic mice are largely protected against these structural alterations. Sirius red staining indicates low levels of collagen deposition in the sham operated mouse hearts from both phenotypes, a massive accumulation of fibrillar collagen (interstitial and perivascular fibrosis) after infarction in wildtype mice, which is substantially attenuated in MCIP1 transgenic mice.

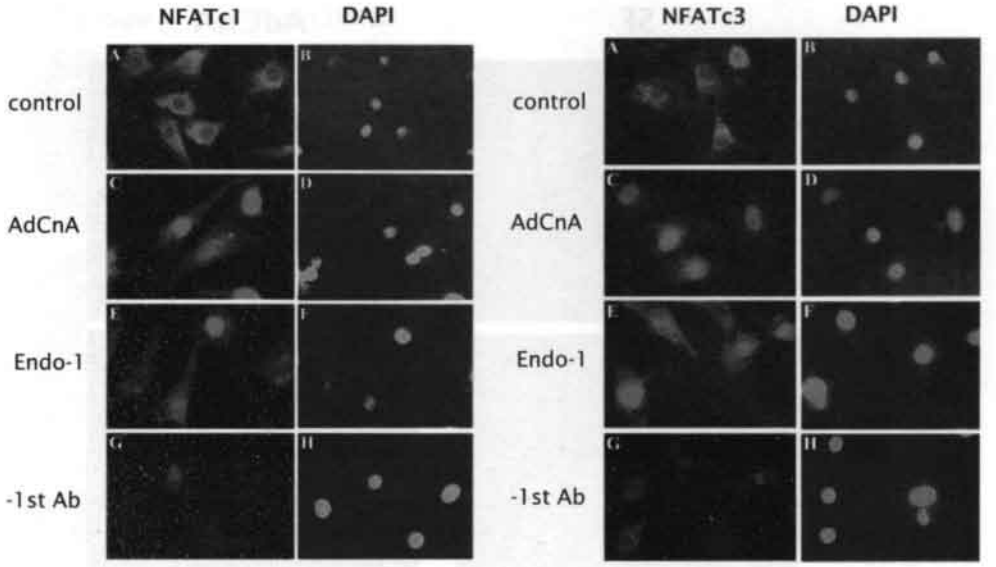


Fig. 3.2 Calcineurin-dependent nuclear translocation of NFATc1 and c3 in cardiomyocytes. Cultured cardiomyocytes were either left unstimulated (A, B), stimulated with AdCnA (C, D), or endothelin-1 (Endo-1) (E, F) and immunostained for subcellular localization of NFATc1 (left Panel) or c3 (right Panel). Nuclei were stained with bisbenzemide (B, D, F, H). Under serum free conditions, NFATc1 and NFATc3 were localized cytoplasmatically (A). Following stimulation with either AdCnA (C) or Endo-1 (E) both isoforms translocated to the nucleus. Panels G and H represent negative controls by ommittance of the primary antibody.

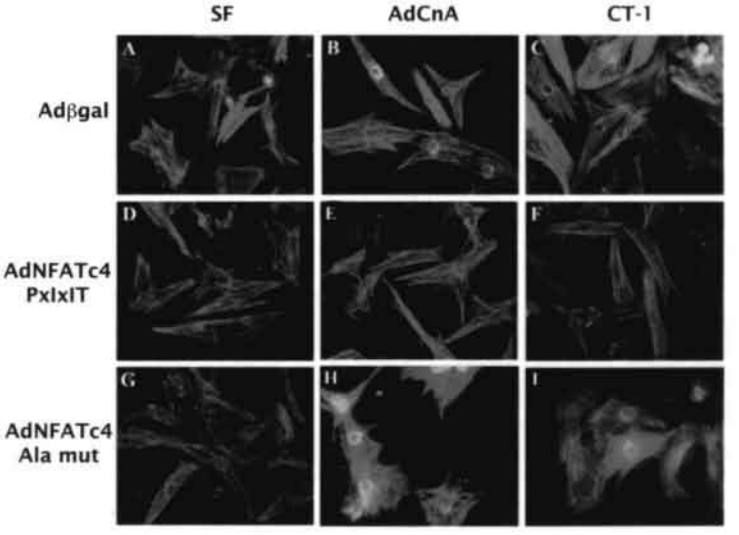


Fig. 3.6C Representative images of immunostained cardiomyocytes infected with the indicated adenoviruses either or not in combination with the hypertrophic agonist CT-1. Phalloidin/ANF double staining demonstrates less cellular enlargement, sarcomeric organization and perinuclear ANF staining in the presence of AdNFATc4(PxIxIT) following AdCnA infection or agonist stimulation.

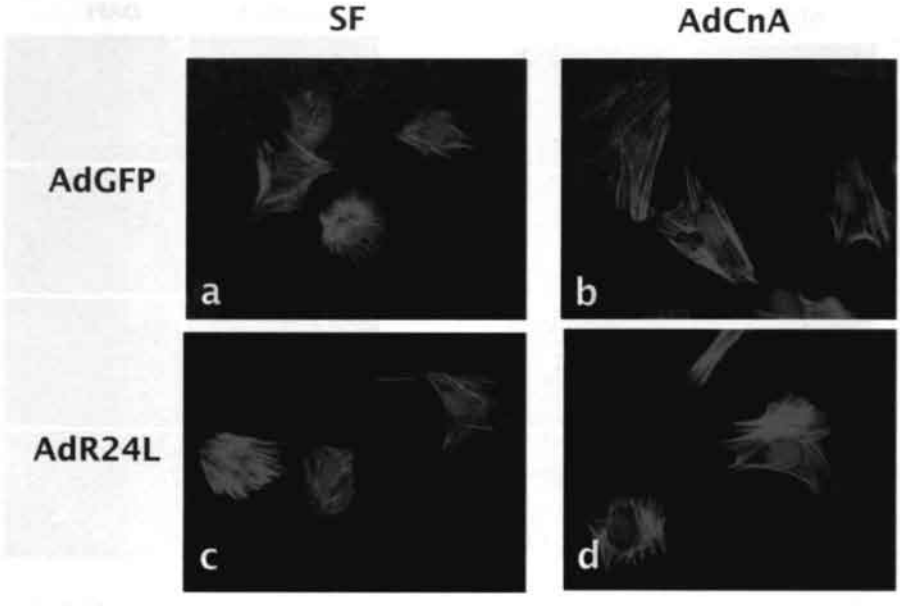
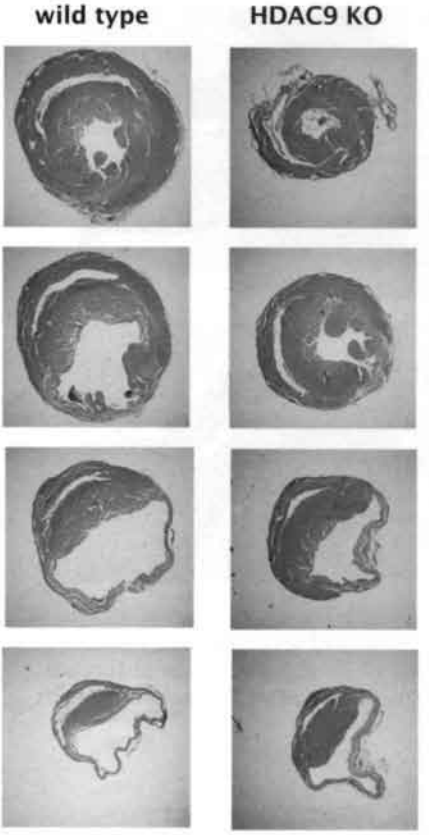


Fig. 4.5C Representative images of immunostained cardiomyocytes infected with the indicated adenoviruses. Phalloidin staining indicates less hypertrophy and sarcomere organization in the presence of AdMEF2(R24L) following AdΔCnA infection.



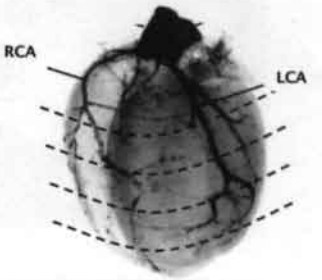


Fig. 5.3A Reduced cardiac remodeling in HDAC9 knockout females after MI. (A) Morphometric analyses of H&E transversal sections from apex to base indicate no significant difference in infarcted left ventricular circumference between wild type and HDAC9 knockout females, with more dilation and an increased hypertrophic response in the wild type mice.

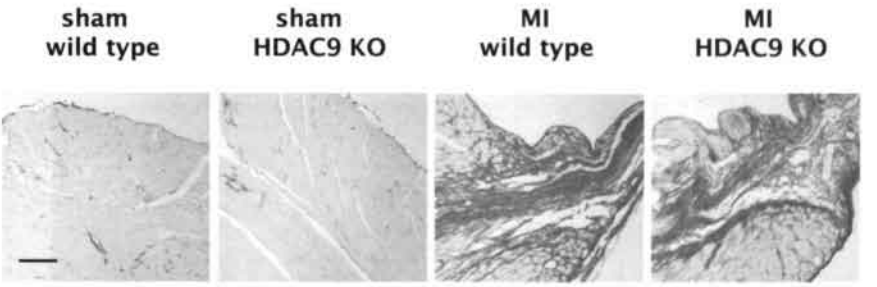


Fig. 5.3B Sirius red staining indicates low levels of collagen deposition in the sham operated mouse hearts from both phenotypes. Although massive accumulation of fibrillar collagen (interstitial and perivascular fibrosis) is present after infarction in wild type mice, this response is substantially attenuated in female HDAC9 knockout mice. Bar indicates 0.1 mm.

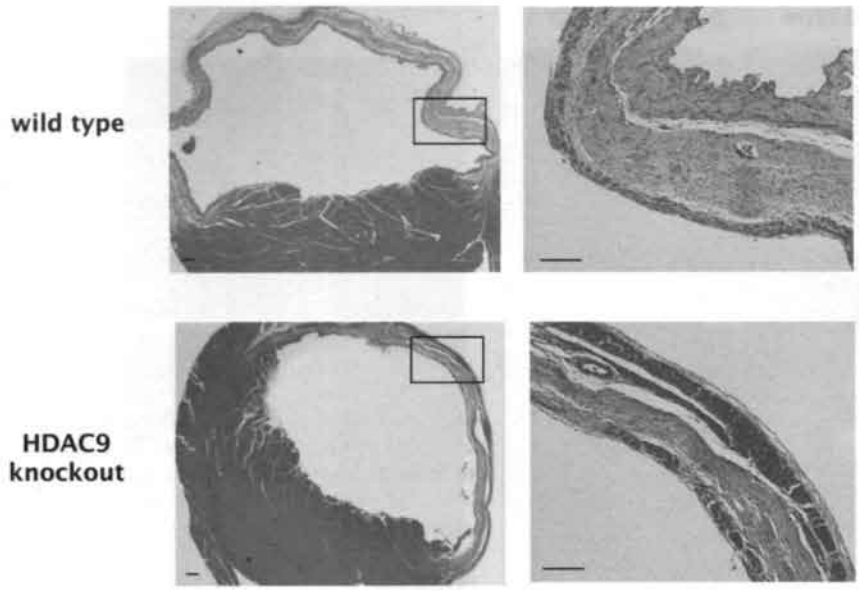


Fig. 5.4 Additional epicardial layer of surviving cells in infarcted area of female HDAC9 knockout mice. H&E stained sections show the presence of an additional layer of survival cells at the epicardial surface of the infarcted area in the female HDAC9 knockout animals compared to wild WT. Bar indicates 0.1 mm.

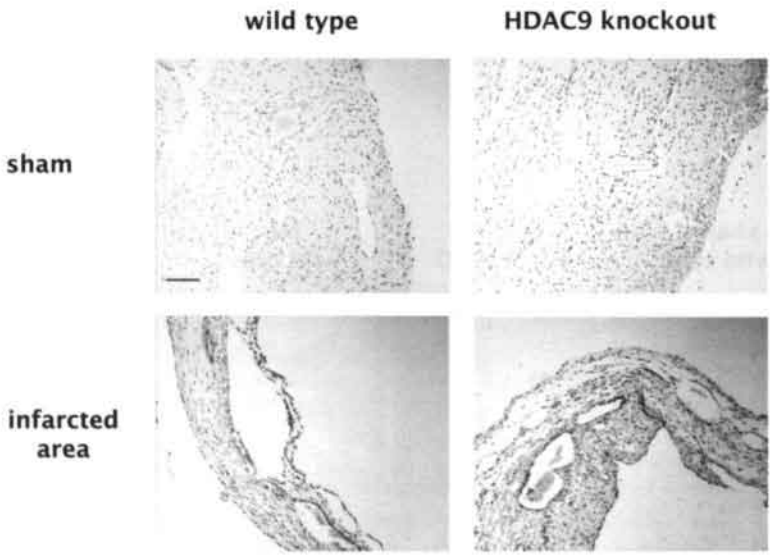


Fig 5.5B vWF stained images reveal only vWF positive vessels in the infarcted regions, with a higher abundancy in the infarcted area of the female HDAC9 knockout mice. Bar indicates 0.1 mm.

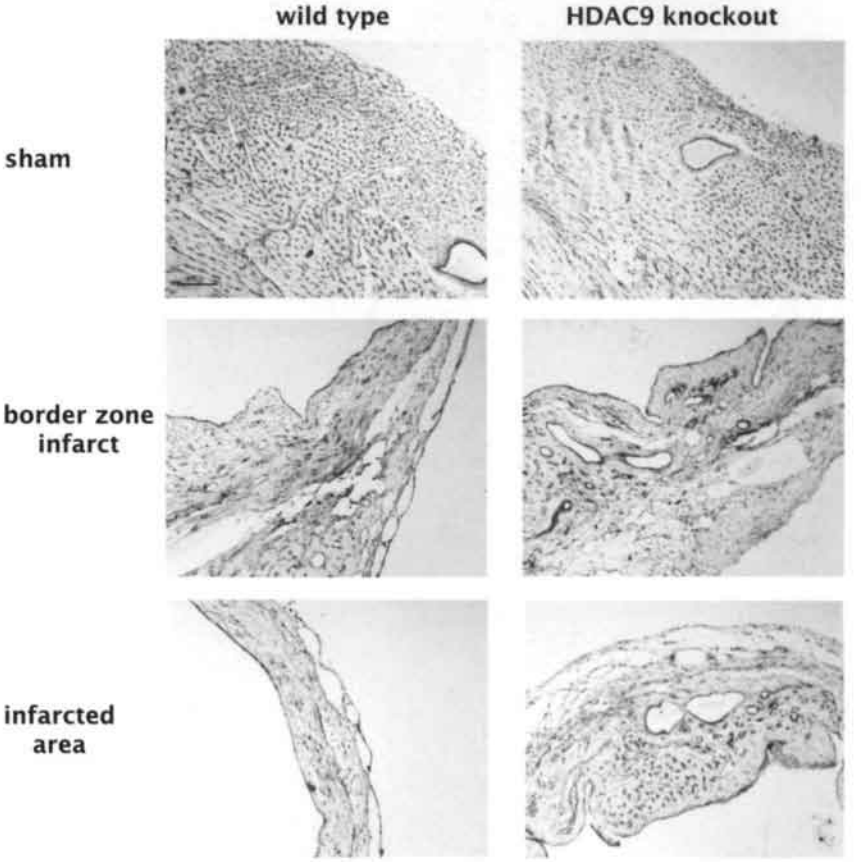


Fig. 5.5C Sections stained for lectin an equal distribution of capillaries surrounding cardiomyocytes in the non-infarcted areas, while the ischemic region shows irregular patterning of vasculature with additional and enlarged vessels in the border zone and the infarcted region of the HDAC9 knockout females compared to the WT animals. Bar indicates 0.1 mm.