Inflammation as orchestrator of cardiac disease progression

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Inflammation as orchestrator of cardiac disease progression

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General introduction

Inflammation as orchestrator of cardiac disease progression

Heart Failure

Heart failure (HF) is the end stage of many cardiovascular diseases and is characterized by a decrease in cardiac function and by the inability of the heart to supply the body with adequate amounts of (oxygenated) blood. Patients suffer from shortness of breath, congestion and fatigue, which strongly reduces their quality of life. Its prevalence worldwide is high and is expanding in western society due to increased aging of the population and better survival of acute cardiovascular incidents. Currently about 1-2% of the world's population suffers from HF and amongst elderly this is even 7-10% ^{1, 2}. Half of the people diagnosed with severe HF die within 5 years ^{3, 4}. With this high prevalence, mortality rate and morbidity, HF is a huge burden on our society ⁵. HF derives from a wide range of underlying disease aetiologies, such as a myocardial infarction (MI) or chronic hypertension but also diabetes and obesity ⁶ or viral infections ⁷ can be causes. Although HF treatment using digitalis has been around since the 16th century, the high mortality and morbidity and the expanding prevalence show that there is a desperate need of improved therapies still.

Generally speaking, two types of HF can be distinguished, systolic and diastolic HF. Historically, HF was only suspected when systolic function was impaired and this systolic HF is characterized by a reduced fraction of blood leaving the left ventricle of the heart upon contraction (Ejection Fraction, EF). Normally between 50 and 70% of the blood leaves the left ventricle during systole but, in HF patients with systolic dysfunction this EF is reduced to less than 40%. Patients suffering from diastolic HF, on the other hand, have a normal EF but the filling of the left ventricle during diastole is impaired. Both these types of HF cause similar symptoms in patients, but especially for the latter condition treatment options are limited ⁸. For the development of novel treatment strategies for HF, a more complete understanding of the processes underlying HF development and progression is crucial. The research described in this thesis aims to better understand these pathophysiological processes and the molecular mechanisms underlying them.

Pathophysiology of HF and myocardial structural remodelling

HF is a multifactorial disease that can develop rapidly, for instance after acute myocardial ischemia (AMI) or after viral infection of the cardiac tissue (viral myocarditis, VM). Alternatively, HF develops more gradually by sustained overloading of the heart, secondary to hypertension or valvular disease. The cardiac muscle tries to cope with this acute damage or chronic stress in various ways. These changes are commonly referred to as myocardial structural remodelling and include the enlargement of cardiomyocytes (hypertrophy), excessive deposition of extracellular matrix (fibrosis), and the infiltration of immune cells (inflammation) (Figure 1). Also crucial for the development of cardiac dysfunction are perturbations in cardiac energy metabolism during HF ⁹.

The cardiac muscle contains multiple cell types (cardiomyocytes, fibroblasts, endothelial cells and resident immune cells) that become activated during pathophysiologic stress and contribute to structural remodelling in their own way. Generally speaking, cells are activated after ligand binding to a cell surface or intra-cellular receptor that leads to activation of intra-cellular signal-transduction pathways. Eventually, this results in the activation or translocation of transcription factors towards the nucleus, where they control gene expression. An exciting discovery was the identification of a class of small RNAs that negatively regulate gene expression and these, so-called microRNAs (miRNAs) ^{10, 11} add

additional complexity to the regulation of gene/protein expression and have been shown to regulate pathophysiological remodelling processes associated with HF ¹²⁻¹⁴.

Figure 1. Histological analysis of the myocardium reveals the pathophysiological processes of cardiac inflammation, marked by the infiltration of immune cells, hypertrophy (enlargement of cardiomyocytes) and fibrosis (excessive collagen production). Cross-sections of healthy (above) and diseased (below) mouse hearts are stained with H&E or Picro-Sirius Red.

Cardiac hypertrophy

Cardiac hypertrophy is an adaptive response of the heart to increased workload. It is characterized by enlargement of existing cardiomyocytes leading to ventricular wall thickening ¹⁵ and can either be pathological or physiological. Although short-term hypertrophic remodelling may be adaptive, chronic and continuous activation of this response is detrimental ¹⁶⁻¹⁸. Actually, it is even debated if the development of hypertrophy is beneficial in the early stages of HF ¹⁹, or if it is detrimental at any stage ²⁰. Interestingly, the heart can also develop a physiological hypertrophy in response to exercise (also known as athlete's heart) or pregnancy. There is evidence that these different types of hypertrophy are initiated via distinct molecular signalling pathways ²¹. Stimuli for cardiac hypertrophy can be broadly segregated into biomechanical (stretch-sensitive) and neuro-humoral factors. Among the latter are Angiotensin II (AngII) and Endothelin-1 (ET-1). ET-1 is a paracrine regulator of cardiac hypertrophy secreted by endothelial cells in the microvasculature of the myocardium. In cultured cardiomyocytes of neonatal rats, stimulation with ET-1 induced hypertrophic responses characterized by an increase in protein synthesis and cell size and reactivation of the foetal gene program ²². AngII induces cardiac hypertrophy mainly through its vasoconstrictive effects, leading to an increase in blood pressure ¹⁶, but has also been shown to have a direct pro-hypertrophic effect on cardiomyocytes ²³.

Cardiac fibrosis

Another pathophysiological characteristic of many cardiac diseases is excessive myocardial fibrosis. During the process of cardiac remodelling, not only the cellular component of the myocardium undergoes changes but also the non-cellular part, called the extracellular matrix

(ECM). Fibrosis is the adverse accumulation of collagens and other ECM proteins, which impairs ventricular function and predisposes the cardiac arrhythmias. Increased deposition of these collagens promotes abnormal myocardial stiffness that reduces the cardiac output^{24,25}. During myocardial structural remodelling fibroblasts proliferate and differentiate into myofibroblasts, thereby secreting collagens²⁶. Not only the amount of collagen changes in the diseased heart but also its composition, the degree of crosslinking, and thereby its quality²⁷. The final amount of fibrosis is always the result of a balance between collagen production and collagen degradation. Crucial for collagen degradation are the so-called matrix metalloproteinases (MMPs) that degrade collagen. MMPs in turn, are regulated by a family of tissue inhibitors of metalloproteinases (TIMPs) that counteract the effect of MMPs. The balance between MMP and TIMP activities is crucial for the amount of collagen in the heart. Transforming Growth Factor- β (TGF β) is a pleiotropic cytokine expressed by multiple cell types and is a potent inducer of fibrosis²⁸. TGF β induces the expression of ECM proteins and suppresses the activity of genes encoding the MMPs. In addition, TGF β induces the expression of Type I collagen and of pro-fibrotic genes such as connective tissue growth factor (CTGF)²⁹. TGF β has been shown to be upregulated in patients with HF³⁰.

Cardiac metabolism in HF

The heart is one the most energy demanding organs of the body and at the same time has a limited capacity for storing fuel. The healthy heart derives most of its energy from the oxidation of fatty acids (FA) in a process called oxidative phosphorylation, that takes place inside the mitochondria⁹. This process efficiently generates energy in the form of ATP, required for the rhythmic cardiac muscle contraction. During HF, perturbations in mitochondrial function have been observed⁹. In addition, a shift in cardiac metabolism away from the normal substrate preference of fatty acids towards glucose and towards glycolysis instead of oxidation takes place³¹. Ultimately, this leads to inefficient substrate use and ATP deficiency, a process referred to as “energy starvation”. In this way cardiac metabolic remodelling contributes to HF progression^{32, 33}. Alterations in the expression of proteins involved in substrate uptake and conversion drive the cardiac metabolic remodelling process to a large extent. The transcriptional regulation of many genes involved in cardiac metabolism is under control of the Peroxisome proliferator-activated receptors (PPARs) and the estrogen related receptor- α (ERR α)^{34, 35}. The transcriptional activities of these nuclear receptors are modulated by several co-repressor and co-activator proteins, among which the PPAR γ coactivator-1 α (PGC-1 α). PGC-1 α binds to and co-activates PPAR α and ERR α , leading to increased capacity of FA uptake and oxidation. PGC-1 α is also a powerful initiator of mitochondrial biogenesis. During HF the expression and activation of these crucial regulators of metabolism is reduced.

Inflammation at the heart of structural remodelling?

Damage to the myocardium causes cell death and stress that subsequently initiates an inflammatory response. Necrotic, apoptotic or stressed cells secrete factors that can be recognized by other cells as Damage Associated Molecular Patterns (DAMPs)³⁶. Also pathogens present in the heart can be recognized by cells in a similar way, these are referred to as Pathogen Associated Molecular Patterns (PAMPs). Almost all cell types have specific cell surface pattern recognition receptors (PRRs) for these PAMPs and DAMPs of which the family of Toll-like receptors (TLRs) is the most prominent. Activation of these receptors initiates intra-cellular pathways that result in the expression of pro-inflammatory mediators. The NF κ B pathway is the main pathway involved here and controls expression of a variety of

pro-inflammatory cytokines, such as TNF α . In patients with HF it has been observed that a network of pro-inflammatory mediators (e.g., TNF α , interleukin-1 β , interleukin-6) are expressed³⁷ and inflammation has been shown to have crucial effects on all of the pathophysiological processes described previously³⁸. Pro-inflammatory effectors of the immune system increase pathophysiological stress. In patients with HF, the concentration of the pro-inflammatory cytokines TNF α , IL-6, and IL-1 β correlates with disease severity³⁹. TNF α is an important pro-inflammatory cytokine and exerts strong effects on all cell types present in the heart⁴⁰. Ligation to its receptor (TNFR1) results in activation of the classical NF κ B Pathway, creating a positive feedback mechanism that increases the inflammatory response. Next to the expression of cytokines, activation of the pro-inflammatory pathways via NF κ B also leads to the secretion of specific type of chemotactic cytokines (chemokines) that attract leukocytes from the blood into the tissue. One of these chemokines, MCP-1 (or CCL2) is the major factor responsible for attracting monocytes into the myocardium. Following infiltration these monocytes differentiate into macrophages. These macrophages in turn can polarise into several phenotypes that can either be pro- or anti-inflammatory. Macrophages stimulated by TLR ligands and interferon (IFN)- γ undergo classical M1 activation, while those stimulated by the anti-inflammatory cytokine IL-4 become M2 macrophages⁴¹. M1 macrophages exert acute pro-inflammatory effects, such as the secretion of MMPs and NO, and can be phagocytic and proteolytic. M2 macrophages, on the other hand, are involved in wound healing, tissue remodelling and induce fibrosis. In recent history the cardiac immune response has been studied in pre-clinical models³⁸. It became clear that TNF α had adverse effects in the hearts of both mice⁴² and rats⁴³ and that deletion of TNF α reduced HF pathology in these rodents⁴⁴. Already more than 10 years ago the first clinical trial targeting TNF α in HF patients was performed, using a drug already approved for the treatment of rheumatoid arthritis. The results of this trial proved disappointing as TNF α blockade even led to a worsened clinical outcome in HF patients⁴⁵. This indicated that the exact role of inflammation and the immune system in the disease progression of HF cannot be generalised and that individual effectors of the immune system such as cytokines and chemokines can have either beneficial or detrimental effects depending on the tissue, cell type, disease progression and the underlying HF aetiology. Beneficial processes orchestrated by the immune system are wound healing, debris clearance and pathogen clearance. Numerous immune and non-immune cell types are involved in orchestrating this pathological response. Researchers have yet only started to solve this puzzle, since these responses are complex, depend on the cell type, and are mostly characterized only *in vitro*. Furthermore, the cell types producing these cytokines have not been identified in many cases, and even the target cells of most cytokines have not been fully defined *in vivo*. The complexity is further increased by the temporal changes that occur in the injury and immune responses, leading to an incomplete understanding of the role of these cytokines during disease. Nevertheless, some interesting discoveries have been made recently concerning the effect of individual effectors of the immune system on cardiovascular disease progression. Manipulation of the immune response was proven to successfully prevent progression of HF. For instance, adoptive transfer of immunosuppressive T-regulatory cells⁴⁶ and polarisation of macrophages towards a more anti-inflammatory phenotype attenuated both cardiac hypertrophy and dysfunction^{47,48}.

Inflammation and cardiac hypertrophy

There are numerous examples of studies where blocking the signalling of pro-inflammatory cytokines reduces cardiac hypertrophy^{49 50}. In the heart, cardiac macrophages participate in the development of cardiac hypertrophy⁵¹ by secreting cytokines and growth factors like the pro-hypertrophic IL-6. Macrophages polarised towards M1 are known to increase cardiac hypertrophy and failure after cardiac PO^{47, 48}. Both cardiomyocyte and non-cardiomyocyte cells secrete and respond to numerous cytokines, several pro-inflammatory pathways have been shown to modulate cardiac hypertrophy upon PO in mice, including TNF Receptor 1⁵² and TNF itself⁴⁴, MCP-1^{53 49} and IL-6^{54 50}.

Inflammation and cardiac fibrosis

Inflammation has crucial effects on the balance between the production and degradation of collagens⁵⁵. Tissue infiltrating pro-inflammatory immune cells, like M1 macrophages and neutrophils secrete granules containing MMPs and other factors capable of degrading collagen and other connective tissue components of the ECM⁵⁶. This process facilitates their migration into inflamed tissues. Macrophages are also important effector cells in tissue remodelling and fibrosis and are a main source of MMPs (MMP1-12) as well as their suppressors, the TIMPs. Alternatively activated (M2) macrophages are also a major source of TGFβ, considered the most pro-fibrotic agent. On the other hand, TGFβ has potent anti-inflammatory effects, including the induction regulatory T-cells⁵⁷, and stimulates fibrosis at the same time. These pleiotropic effects on inflammation and fibrosis are clearly shown in TGFβ-deficient mice that exhibit profoundly reduced collagen deposition, while suffering from extensive inflammation and tissue necrosis^{58, 59}. In the murine heart inhibition of TGFβ increased leukocyte infiltration and chemokine expression after MI⁶⁰. Fibroblasts themselves also express PRRs, including the TLRs, and DAMPs activating these receptors can directly promote fibroblast differentiation into collagen-producing myofibroblasts⁶¹.

Inflammation and cardiac metabolism

Metabolic stress is an important inducer of cardiac inflammation. Inflammatory pathways are activated during cardiomyocyte hypertrophy and are attenuated by peroxisome proliferator-activated receptors PPARα and PPARδ^{62, 63}. Reactive oxygen species (ROS) from stressed mitochondria, that are considered DAMPs, have an established role in inflammation and host defence, as they kill intracellular bacteria and have been shown to initiate inflammatory responses. ROS generated during mitochondrial respiration induces the production of pro-inflammatory cytokines. Oka et al.⁶⁴ demonstrated in mice with aortic constriction that mitochondrial DNA that escapes from autophagy causes cardiac inflammation and failure upon recognition by a specific type of TLR (TLR9) that detects extracellular DNA. The polarisation status of macrophages determines their metabolic phenotype⁶⁵. M1 polarised macrophages display a metabolic shift towards anaerobic glycolysis. In contrast, IL-4-activated M2 mouse macrophages show a significant upregulation of mitochondrial fatty acid oxidation⁶⁶. Apart from metabolic stress inducing cardiac inflammation the inverse has also been shown, in that NFκB activation induces metabolic remodelling away from oxidative processes towards a greater reliance on glycolytic metabolism^{63, 67}.

Inflammation in acute VM and chronic HHD

Acute HF and chronic HF are characterised by distinct inflammatory mechanisms and in this thesis two different types of HF aetiologies were studied, namely, Hypertensive Heart Disease (HHD) and Viral Myocarditis (VM). HHD which is associated with chronic, low-grade

inflammation. HDD causes pressure overloading (PO) of the heart and ultimately leads to HF. In western society it is most prevalent amongst elderly woman. In the corresponding mouse model of HDD, hypertension is induced via infusion of AngII using implanted osmotic minipumps. VM is characterized by an acute and fulminant inflammatory response, and VM is an important cause of cardiovascular death in young, otherwise healthy individuals often as a result of common cough/influenza viruses⁶⁸. In a subgroup of patients, VM gives rise to an autoimmune response predisposing to the development of dilated cardiomyopathy (DCM)⁷⁶⁹. In the mouse model of this disease, myocarditis is induced by coxsackievirus B3 (CVB3). Most mouse strains are not susceptible to develop CVB3 induced VM, only specific strains, like C3H or Balb/c mice, develop myocarditis upon CVB3 infection, this is most likely due to differences in immuno-genetic background⁷⁰.

Aims and outline of this thesis

The general aim of this thesis is to understand the role of inflammation in HF and to explore how it can be manipulated in such way that it preserves its crucial role in beneficial processes such as wound healing, debris clearance and pathogen clearance, but that its destructive effects on the cardiac tissue are diminished. To this aim, we have used patient material, different animal models and *in vitro* and *in silico* screening approaches.

Chapter two focusses on the deletion and manipulation of the pro-inflammatory CD40 receptor in an animal model of hypertensive induced HF. CD40 (or TNFRSF5) is a cell-surface receptor belonging to the tumor necrosis factor (TNF) receptor super family and is predominantly expressed by antigen-presenting cells, and its ligation with its ligand-receptor CD40L is a crucial step in the process of antigen presentation. As an important effector of the immune system, the CD40 receptor has been proven to be a critical regulator of the immune response in different chronic inflammatory diseases like atherosclerosis, multiple sclerosis and arthritis⁷¹⁻⁷³. In all of these pathologies the blocking of CD40L-CD40 interactions was found to reduce disease severity. The effect of this receptor on the development of HF, as induced by chronic hypertension, has never been investigated before.

In **chapter three** it is investigated how the acute and fulminant inflammatory response affects cardiac metabolism in VM patients and in a mouse model of CVB3-induced VM. The acute effects of pronounced cardiac inflammation, and VM in particular, on cardiac metabolism are not well studied so far. For this extensive characterisation we made use of cardiac samples of VM patients and the CVB3 mouse VM model. We hypothesize that the fulminant inflammatory response associated with acute VM disturbs cardiomyocyte energy metabolism, thereby compromising cardiac energy metabolism. In addition, it was explored if the manipulation of cardiac metabolism has therapeutic potential.

Over the last decade high-throughput screening has developed into an indispensable tool to identify new therapeutic molecules⁷⁴. In **chapter four** an *in vitro* high-throughput screening approach is applied in which 194 miRNAs, previously identified to be differentially expressed in inflammatory diseases of the heart, were tested for their effects on hypertrophy, fibrosis and inflammation in different cell types. The miRNA field is developing rapidly and the manipulation of their activity via mimics or anti-miRs holds great therapeutic promise^{13, 75}. By transfecting unstimulated and stimulated cardiomyocytes, fibroblasts and bone marrow-derived macrophages (BMDM) with miRNA mimics, we identified novel roles for many miRNAs in the cardiac remodelling process. This research was performed at Cenix bioscience, Dresden, Germany, a company that was renowned internationally for its expertise in high-throughput screening.

In **chapter five** of this thesis an extensive overview was made of preclinical studies dealing with inflammation in cardiac PO. To this end, we identified articles that studied and manipulated cardiac inflammation, fibrosis and hypertrophy upon PO. From almost 300 articles the data was extracted. The dataset obtained was used to define and compare the features of different rodent models of PO and served as input for network analysis, linking phenotypical outcomes to genes and, subsequently to miRNAs that target these genes

Finally, in **chapter six** the findings of the previous chapters are summarized and discussed in broader perspective.

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Inhibition of CD40-CD40 ligand (CD40L) and downstream TRAF signalling reduces angiotensin II induced cardiac hypertrophy

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Abstract

Inflammation is emerging as a crucial modulator of hypertensive heart disease (HHD). Activation of the co-stimulatory CD40 receptor exerts pro-inflammatory effects via macrophages, and serum levels of soluble CD40-ligand are elevated in patients with HHD induced heart failure (HF). Here, we investigated whether CD40-CD40L interactions affect cardiac hypertrophy during HHD and which downstream TNF-receptor associated factor (TRAF) signalling molecules are involved. CD40 knockout (CD40^{-/-}) and wild-type (Wt) mice were treated with Angiotensin II (AngII, 2.5 mg/kg/day) for 4 weeks. Ventricular mass increased significantly in Wt (+31%, P<0.01), but not in CD40^{-/-} mice (+10%, N.S.) upon AngII treatment. In line, the increase in diastolic LV wall thickness and cardiomyocyte cross sectional area was blunted in CD40^{-/-} mice. To determine the CD40-TRAF interactions involved, the effect of site directed mutagenesis in CD40-TRAF binding on cardiac hypertrophy was investigated. Overexpression of intact CD40 in antigen presenting cells exacerbated cardiac hypertrophy upon pressure overload and relative to mice with intact downstream CD40 signalling, disruption of TRAF2/3/5, TRAF6 or both binding sites resulted in significantly reduced hypertrophy (Ventricular mass -24% for CD40-TΔ2, -28% for CD40-TΔ6 and -26% in CD40-TΔ2/6; p<0.05). Administration of CD40^{-/-} macrophage-conditioned medium to cardiomyocytes *in vitro* blunted the hypertrophic response indicating that the secretome of CD40^{-/-} macrophages affects myocyte hypertrophy. In conclusion, inhibition of CD40 in myeloid cells decreases AngII-induced cardiac hypertrophy with direct involvement of the CD40-TRAF2/3/5 and CD40-TRAF6 pathways.

Introduction

The increasing prevalence of heart failure (HF) poses enormous challenges for health care systems worldwide. Hypertension triggers hypertrophic remodelling of the cardiac muscle, ultimately leading to the development of HF. Despite effective medical interventions mortality and morbidity remain substantial. Emerging evidence indicates that the activation of inflammatory processes plays a pivotal role in disease progression during hypertensive heart disease (HHD) ¹. The immune system favours HHD by the release of cytokines stimulating the development of cardiac hypertrophy and fibrosis ²⁻⁴. Manipulation of the immune response may prevent progression of heart failure. For instance, adoptive transfer of immunosuppressive T-regulatory cells ⁵ and polarization of macrophages towards a more anti-inflammatory phenotype attenuate cardiac hypertrophy and dysfunction ^{6, 7}.

CD40 (or TNFRSF5) is a cell-surface receptor belonging to the tumor necrosis factor (TNF) receptor super family and is predominantly expressed by antigen-presenting cells. Its corresponding ligand (CD40L or CD154) is a receptor expressed on T-cells and platelets and can be cleaved into a soluble form (sCD40L). The co-stimulatory dyad CD40L-CD40 is a critical regulator of the immune response in different inflammatory diseases like atherosclerosis, multiple sclerosis and arthritis ⁸⁻¹⁰. Blocking of CD40L-CD40 interactions reduced disease severity. Importantly, serum levels of sCD40L are elevated in patients with HHD and HF ¹¹⁻¹³.

CD40L-CD40 interactions have pleiotropic effects depending on the cell-type and the downstream CD40-signaling pathways involved ¹⁴. The CD40 receptor itself lacks intrinsic signalling activity. Upon activation, it recruits adaptor molecules (TNF Receptor-Associated Factors, or TRAFs) to its cytoplasmic domain to initiate downstream signalling. Ligation of CD40L to CD40 results in trimerization of CD40 and subsequent association of TRAF molecules to its TRAF2/3/5 and/or TRAF6 binding sites, ensuing in nuclear factor κ B (NF κ B) signalling ¹⁵. Downstream signalling via TRAF6 only activates the canonical NF κ B pathway, whilst downstream signalling via TRAF2/3/5 signalling activates both the canonical and non-canonical pathway ¹⁶. In atherosclerosis, disease progression is mediated by CD40-expressing hematopoietic cells and CD40-TRAF6 interactions in macrophages and dendritic cells are specifically required for the pro-atherogenic activity exerted by these cells ¹⁷.

The possible pathophysiological significance of CD40L-CD40-TRAF signalling in the development of HHD has never been addressed. Accordingly, the present study reveals that absence of CD40 in KO mice and deficiency of CD40-TRAF binding in the myeloid cells results in a significant reduction of cardiac hypertrophy.

Material & Methods

Mouse model of HHD

Three months old male CD40 knockout (CD40^{-/-}, n=22), Wt (n=23) and transgenic CD40-Twt (n=16), CD40-TΔ2 (n=14) CD40-TΔ6 (n=13) and CD40-TΔ2/6 (n=14) mice weighing 20 - 30 gram were used (all on a C57Bl6 background¹⁸). Transgenic mice were on a CD40^{-/-} background expressing a chimeric CD40 transgene without (CD40-Twt) or with mutations either at the TRAF2/3/5 (CD40-TΔ2), TRAF6 (CD40-TΔ6) or both binding sites (CD40-TΔ2/6). Expression of this transgene is under the control of the major histocompatibility complex class II (MHC class II) promotor¹⁸. To study the effect of HHD, AngII (Bachem AG, Bubendorf, Switzerland) was dissolved in sterile PBS and was infused subcutaneously (2.5 mg/kg/day) by osmotic mini pumps (ALZET, model 2004, DURECT corporation, Cupertino, USA) for 28 days. During mini pump implantation mice were anesthetized with isoflurane (2.5 Vol% isoflurane / air). In each AngII –treatment group 1-3 (6 to 21%) mice died prematurely due to peri-aortic rupture and bleeding. All knockout and transgenic mice were bred and maintained in the animal facility of Maastricht University. The animals had free access to standard chow (SNIFF, Soest, Germany) and drinking water and were housed in groups of 2-5 mice per cage in a temperature-controlled room with 12h:12h light-dark cycle. This study was approved by the institutional animal research ethics committee and was designed conform the guidelines for the use of laboratory animals formulated in the Dutch law on care and use of experimental animals.

Echocardiographic Assessment of Cardiac Hypertrophy and Blood Pressure Measurement

Cardiac dimensions and function were determined using echocardiography. Mice were anesthetized with isoflurane (2.5 Vol% isoflurane / air) and analysed with a 30-MHz transducer (RMV 707b, VisualSonics, Toronto, Canada). Two-dimensionally targeted B-mode echocardiographic images were recorded at the level of the papillary muscles from the short-axis view. Intraventricular septal (IVS), left ventricular posterior wall thickness (LVPW) and left ventricular internal diameter (LVID) at systole and end-diastole were estimated from these images. From these parameters, fractional shortening (FS) was determined as the percentage decrease of LVID between diastole to systole. Wall thickness was expressed as the mean of IVS and LVPW thickness. Relative wall thickness is determined by dividing wall thickness by the LVID during diastole. Blood pressure was monitored using a tail cuff (CODA, Kent Scientific, Torrington, CT) in awake animals 3 days before sacrifice.

In vitro experiments

Isolation and activation of neonatal ventricular cardiomyocytes.

Rat neonatal ventricular cardiomyocytes (NCMs) were isolated by enzymatic dissociation from 1- to 2-day old neonatal rats. NCMs were plated 2.5×10^5 on gelatinized 6-well plates and cultured as described previously¹⁹. NCMs were primed with IFN γ 100u (Peprotech, London, UK) for 24h and subsequently activated with an agonistic CD40 antibody, 10 μ g/ml (FGK45, Bioceros, Utrecht, The Netherlands) or a Rat IgG isotype control (Santa Cruz Biotechnology, Heidelberg, Germany, Cat. No. SC-2026) for 6 hours to determine inflammatory markers and 24 hours to determine hypertrophic response with 3 replicates per condition.

BMDM Conditioned medium experiment.

Bone marrow derived macrophages (BMDM) were generated as described before²⁰. In brief, bone marrow was isolated from 12 weeks old CD40^{-/-} and Wt mice and cultured in RPMI supplemented with 15% M-CSF containing L929 cell conditioned medium (LCM) to generate BMDMs and lifted for plating at day 8. BMDM conditioned medium was collected after 24h of LPS stimulation and was dialyzed for 24h using a 40 nm membrane against a 10x higher volume of NCM medium. Dialyzed conditioned medium was subjected to NCMs in presence and absence of 10 nM Endothelin-1 (ET-1) (Sigma-Aldrich, Zwijndrecht, The Netherlands, Cat No. E7764) with three replicates per condition. The experiment was repeated three times.

Tissue preparation, histology and immunohistochemistry

After 28 days of AngII infusion and immediately after echocardiography mice were sacrificed by exsanguinations via the abdominal aorta. Blood was collected in heparinized tubes and centrifuged for 15 min at +4°C, obtained plasma was stored at -80°C until analysis. The ventricles, lung, liver, spleen and kidney were dissected, weighed and prepared for further analysis. Atria were removed from the heart and the ventricles were cut into two pieces between base and apex. Transverse midsections of the hearts were fixed in 1% PFA for 4h and embedded in paraffin. The tissue was sectioned in 5 µm thick slices and later processed for Hematoxylin/Eosin, Picrosirius red and Wheat Germ Agglutinin (WGA) staining. The apex of the heart was sectioned into two parts and snap frozen for RNA and protein isolation.

Fibrosis was quantified as Sirius red positive area and peri-vascular fibrosis was excluded from the quantification. WGA staining was performed to determine cardiomyocyte cross sectional area by incubating deparaffinized and rehydrated slides for 30 min with 50 µg/ml FITC labelled Lectin (Sigma-Aldrich, Zwijndrecht, The Netherlands, Cat. No. L4895) and mounting with Vectashield + DAPI (Vector Laboratories, Peterborough, UK, Cat. No. H-1500). All quantifications were performed in a blinded manner using Leica Qwin software.

Immunohistochemistry

Paraffin sections were de-paraffinized and rehydrated. After boiling in a pressure cooker in 10 mM sodium citrate buffer to unmask antigens, the slides were blocked in buffer containing normal Rabbit serum (Dako, Heverlee, Belgium, Cat. No. X0902), incubated with primary antibody Rat-anti-Mouse CD45 (BD Pharmingen, Germany, Cat. No. 550539), and then incubated with biotin-conjugated secondary Rabbit-anti-Rat antibody (Dako, Heverlee, Belgium, Cat. No. E0468). Signal was amplified using TSA kit (PerkinElmer, USA, Cat. No. NEL747A) and visualized using DAB (Sigma-Aldrich, Zwijndrecht, The Netherlands, Cat. No. D5905). Three random photographs were taken at 20X magnification using a LEICA DM2000 microscope and LAS v3.8.0 software. CD45 positive cells per field were counted using ImageJ software.

Analysis of cytokine levels in serum and BMDM conditioned medium

Conditioned medium of unstimulated and LPS (100 ng/ml) stimulated CD40^{-/-} and Wt BMDM was analysed for pro-inflammatory cytokines IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO and TNF-α using V-PLEX Pro-inflammatory Panel 1 (mouse) Kit (MSD, Cat. No. K15048D-1) according to manufacturer's protocol.

Gene expression analysis

Left ventricular tissue was snap frozen in liquid nitrogen and processed for analysis of mRNA content by quantitative PCR. Total RNA was isolated using a RNeasy kit (Qiagen, Venlo, the Netherlands) following manufacturer's protocol. cDNA was prepared using the iScript kit (BioRad Inc., Hercules, CA, USA) using 1 µg RNA of each sample as input. RT-qPCR and SYBR Green (BioRad Inc. Hercules, CA, USA) quantitative RT-PCR analysis was performed on a BioRad iCycler to determine the mRNA levels of the analysed genes. The $\Delta\Delta C_t$ method was used to analyse obtained C_t Values and make the mRNA levels relative to the control groups and corrected for housekeeping gene GAPDH.

Statistical Analysis

Values are presented as MEAN $\pm$ SEM. Data from different groups were analysed using two-tailed Student's *t* testing for Gaussian data when two groups were compared. For comparisons of more than 2 groups, ANOVA in combination with Bonferroni or Dunnett's post testing was applied. For echo-analysis paired comparisons were used between baseline groups and 4 week AngII treated mice. Differences were considered statistically significant at *P* values <0.05.

Results

Absence of CD40 blunts the hypertrophic response after AngII infusion

To assess the role of CD40 in the development of cardiac hypertrophy, CD40 knockout (CD40^{-/-}) and Wild type (Wt) mice were infused with AngII for 4 weeks. Arterial blood pressure rose to a similar extent in both Wt and CD40^{-/-} mice in response to AngII (Supplemental Figure 1). Notably, ventricular mass increased significantly in Wt (+31%, $P<0.01$), but not in CD40^{-/-} mice (+10%, N.S.) in response to AngII treatment (Figure 1a and Table 1). In line, the echocardiographic diastolic LV wall thickness and histological cardiomyocyte cross sectional area (CSA) increased in Wt, but not in CD40^{-/-} mice (Figure 1b-c, Table 2). Also transcript levels of the cardiac hypertrophic markers α -skeletal actin (α -SKA) and atrial natriuretic factor (ANF) remained low in AngII-treated CD40^{-/-} mice, as compared to Wt mice (Figure 1d-e, Supplemental table 1). Fractional shortening (FS) was reduced to a similar extent in Wt and CD40^{-/-} mice following AngII-treatment (Figure 1f, Table 2). Also Sirius red stained interstitial fibrosis or transcript levels of Col1 α and TGF β 1 (both strongly increasing upon AngII infusion) did not differ significantly between Wt and CD40^{-/-} hearts (Figure 2a, Supplemental table 1). Collectively, these findings indicate a blunted hypertrophic response in the absence of CD40. This blunted hypertrophy however did not affect cardiac fibrosis or overall function.

Figure 1. CD40^{-/-} mice have a blunted hypertrophic response after AngII treatment. CD40^{-/-} and Wt were infused with AngII (2.5 mg/kg/day) for 4 weeks. Untreated CD40^{-/-} and Wt mice served as controls. A. Ventricular weight corrected for by body weight at the start of the experiment shows a significant increase in Wt mice after AngII but not in CD40^{-/-} mice. B. Echocardiographic measurements before (white bars) and after 4 weeks (black bars) of AngII treatment showed a strong increase in diastolic wall thickness in AngII treated Wt mice but no significant increase in AngII treated CD40^{-/-} mice. Wall thickness was shown as the average of intra-ventricular septal (IVS) and left ventricular posterior wall thickness (LVPW) in mm during diastole. C. Cardiomyocyte CSA shows a significant increase in Wt but not in CD40^{-/-} mice upon AngII. D. Transcript levels of α -SKA and ANF (panel E.) show a significant increase in Wt but not in CD40^{-/-} mice upon AngII. F. Fractional shortening, determined as a percentage decrease of LVSD between diastole to systole, is significantly decreased in both Wt and CD40^{-/-} mice upon AngII infusion. Error bars represent SEM; * Compared to corresponding sham groups, # Compared to Wt-AngII, † Compared to Baseline values. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

Table 1. Morphometric characteristics of AngII-treated and non-treated Wt and CD40^{-/-} mice.

	Wt (N=11)	Wt AngII (N=16)	CD40 ^{-/-} (N=6)	CD40 ^{-/-} AngII (N=22)
Body Weight Day 0 (g)	25.4±0.5	26.3±0.6	23.5±0.6	24.6±0.4
Body Weight Day 28 (g)	27.2±0.7	27.6±0.6	24.8±0.7	26.0±0.4
Lung (mg)	180±7	215±7	168±7	197±11
Liver (mg)	1064±98	1062±34	1188±41	1015±43
Kidney (mg)	169±4	158±4	169±8	159±6
Spleen (mg)	89.0±7	86.9±4	73.3±6	78.4±3
Ventricles (mg)	115±4	155±6***	119±13	137±4

Data are expressed as means ± S.E.M. For statistical analysis one-way ANOVA test with Bonferroni post-hoc testing was applied. * indicates statistically significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) between AngII-treated and corresponding non-treated mice.

Figure 2. Histological analysis of myocardial tissue after AngII treatment. Panels with Sirius Red, CD45 immunostaining and WGA staining showing the effect of AngII treatment on the myocardium in Wt and CD40^{-/-} mice. A. Interstitial fibrosis is quantified as Sirius Red positive area and shows no difference between Wt and CD40^{-/-} mice. B. Loss of CD40 does not attenuate number of CD45⁺ leukocytes after AngII treatment. Number of leukocytes were determined as CD45⁺ cells using IHC staining. The arrows indicate examples of CD45⁺ cells. Error bars represent SEM; * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$, *Compared to corresponding Sham group.

Table 2. Echocardiographic determination of changes in cardiac dimensions and cardiac performance characteristics of AngII-treated and non-treated Wt and CD40^{-/-} mice.

	Wt	Wt AngII	CD40 ^{-/-}	CD40 ^{-/-} AngII
Heart rate (bpm)				
- 0 days	505±32	502±16	527±14	513±16
- 28 days	485±23	498±21	463±17	530±15
Wall thickness, diastolic (mm)				
- 0 days	0.86±0.02	0.87±0.02	0.86±0.01	0.95±0.03
- 28 days	0.92±0.04	1.08±0.02***	0.81±0.02	0.99±0.02***#
Wall thickness, systolic (mm)				
- 0 days	1.17±0.03	1.19±0.03	1.17±0.04	1.25±0.03
- 28 days	1.20±0.05	1.30±0.02	1.14±0.05	1.20±0.04
Left Ventricular Internal Diameter (mm) Diastolic				
- 0 days	3.67±0.09	3.77±0.05	3.99±0.07	3.72±0.6
- 28 days	3.78±0.08	3.76±0.11	4.24±0.11	3.89±0.09
Left Ventricular Internal Diameter (mm) Systolic				
- 0 days	2.67±0.07	2.75±0.07	2.89±0.05	2.58±0.07
- 28 days	2.87±0.11	3.01±0.11	3.23±0.14	3.12±0.12
Rel. Wall Thickness (Diastolic)				
- 0 days	0.23±0.01	0.23±0.01	0.21±0.01	0.25±0.01*
- 28 days	0.26±0.01	0.31±0.01	0.19±0.01	0.25±0.01#
Fractional Shortening (%)				
- 0 days	27.1±0.99	27.3±1.13	27.7±0.72	30.1±1.44
- 28 days	24.0±1.96	19.9±1.29	24.1±1.49	20.2±1.78

Data are expressed as means ± S.E.M. For statistical analysis one-way ANOVA test with Bonferroni post-hoc testing was applied. * indicates statistically significant difference (* P < 0.05, ** P < 0.01, *** P < 0.001) between AngII-treated and corresponding non-treated mice. # indicates statistically significant difference (# P < 0.05, ## P < 0.01, ### P < 0.001) compared to corresponding Wt mice.

Figure 3. Loss of CD40-TRAF signalling reduces cardiac hypertrophy after AngII treatment. Transgenic CD40-Twt, CD40-TΔ2, CD40-TΔ6 and CD40-TΔ2/6 mice were submitted to AngII (2.5 mg/kg/day) to induce HHD. A. Ventricular weight was corrected for by body weight and shows a significant decrease in CD40-TΔ2, CD40-TΔ6 and CD40-TΔ2/6 mice compared to CD40-Twt controls. B. Cardiomyocytes of mice with disrupted CD40-TRAF binding have reduced CSA after AngII treatment. C. Transcript levels of α-SKA show a trend towards reduced expression in CD40-TΔ2 and CD40-TΔ6 mice and was significantly decreased in CD40-TΔ2/6 mice. D. Echocardiographic measurements before (white bars) and after 4 weeks (black bars) of AngII treatment showed a strong increase in diastolic wall thickness in CD40-Twt mice but no increase in the groups with disrupted CD40-TRAF binding. Wall thickness was shown as mean of intra-ventricular septal (IVS) and left ventricular posterior wall thickness (LVPW) in mm during diastole. E. Fractional shortening was determined as a percentage decrease of LVID between diastole to systole. Disruption of CD40-TRAF binding site does not improve fractional shortening after AngII treatment. Error bars represent SEM; * Compared to CD40-Twt Control, † Compared to corresponding baseline values. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

Both CD40-TRAF6 and CD40-TRAF2/3/5 interactions are implicated in cardiac hypertrophy

To determine which of the CD40-TRAF signal transduction pathways is responsible for the blunted hypertrophic response in CD40^{-/-} mice, transgenic mice with disrupted CD40-TRAF binding were submitted to 4 weeks of AngII infusion. The mice are on a CD40^{-/-} background and express a chimeric CD40 transgene, without (CD40-Twt) or with mutations either at the TRAF2/3/5 (CD40-TΔ2), TRAF6 (CD40-TΔ6) or both binding sites (CD40-TΔ2/6) of the CD40 C-terminus. As transgene expression is under control of the major histocompatibility complex class II (MHC class II) promoter¹⁸, its expression is confined to antigen presenting cells (APCs), mostly macrophages, B-cells and dendritic cells. Baseline body weight, heart weight and serum levels of sCD40L did not significantly differ between the genotypes as was shown previously^{17, 21}.

Baseline echocardiographic measurements revealed only modest differences in cardiac function and dimensions (Table 3). Only in CD40-TΔ2/6 mice, baseline values of wall thickness (LVWd, -11%) and left ventricular internal diameter (LVIDd, +8.5%) significantly changed compared to CD40-Twt control. Upon AngII infusion, ventricular mass was remarkably large (VW/BW Ratio 7.73 ± 0.31) in transgenic mice with intact CD40-TRAF signalling (CD40-Twt) and significantly larger than in Wt mice (VW/BW ratio 5.96 ± 0.20) (Figure 3a and 1a). Disruption of TRAF binding sites resulted in significantly lower cardiac mass after AngII treatment vs. intact CD40-TRAF signalling (VW/BW Ratio -23.9% in CD40-TΔ2, -26.1% in CD40-TΔ6 and -27.8% in CD40-TΔ2/6, $p<0.001$) (Figure 3a, Table 4). Diastolic LV wall thickness, concordantly to the results above, increased significantly in CD40-Twt after

AngII compared to baseline (Figure 3d). In the mutant groups however, the increase in LV wall thickness was completely blunted (Figure 3d, Table 3).

Table 3. Echocardiographic determination of changes in cardiac dimensions and cardiac performance in of transgenic mice with disrupted CD40-TRAF binding treated with angiotensin II (AngII) for 4 weeks.

	CD40-Twt (N=16)	CD40-TΔ2 (N=12)	CD40-TΔ6 (N=14)	CD40-TΔ2/6 (N=12)
Heart rate (bpm)				
- 0 days	482±20	516±17	516±14	529±14
- 28 days	557±11	535±18	525±20	515±24
Wall thickness, diastolic (mm)				
- 0 days	1,06±0.03	1,01±0.03	1,00±0.01	0,94±0.04**
- 28 days	1,27±0.05	0,96±0.03***	1,01±0.02***	0,95±0.02***
Wall thickness, systolic (mm)				
- 0 days	1.36±0.02	1.29±0.04	1.28±0.02	1.12±0.02***
- 28 days	1.49±0.06	1.15±0.03***	1.32±0.04*	1.15±0.03***
Left Ventricular Internal Diameter (mm) Diastole				
- 0 days	3.52±0.10	3.52±0.07	3.66±0.90	3.82±0.06*
- 28 days	4.12±0.14	4.18±0.11	3.93±0.12	4.24±0.14
Left Ventricular Internal Diameter (mm) Systole				
- 0 days	2.51±0.07	2.58±0.06	2.57±0.9	2.86±0.06**
- 28 days	3.31±0.15	3.46±0.11	3.02±0.15	3.55±0.17
Rel. Wall Thickness, diastolic				
- 0 days	0.31±0.01	0.29±0.01	0.28±0.01	0.25±0.01**
- 28 days	0.31±0.02	0.23±0.01***	0.26±0.01*	0.23±0.01***
Fractional Shortening (%)				
- 0 days	28.3±1.3	26.5±1.5	30.1±1.3	25.0±1.1
- 28 days	19.9±1.4	17.2±1.5	23.4±1.7	16.2±1.9*

*Data are expressed as means ± S.E.M. For statistical analysis one-way ANOVA test was used. With Dunnett's post-hoc testing vs. CD40-Twt group. * indicates significant differences between CD40-Twt and the groups with disrupted TRAF binding. (* P < 0.05, ** P < 0.01, *** P < 0.001).*

Decreased hypertrophy in mice with disabled CD40-TRAF2/3/5 or/and CD40-TRAF6 binding was confirmed by a blunted increase in cardiomyocyte CSA (Figure 3b). Transcript levels of α -SKA were significantly reduced in CD40-TΔ26 mice and tended to be reduced in CD40-TΔ2 and CD40-TΔ6 mice (Figure 3c, Supplemental table 2). Blood pressure and heart rate did not significantly differ among the 4 groups (Supplemental Figure 2). These findings show that both CD40-TRAF2/3/5 and CD40-TRAF6 signalling are involved in the development of cardiac hypertrophy. Despite attenuation of hypertrophy after AngII treatment, but in line with the result in CD40^{-/-} mice, fractional shortening was not improved by disruption of CD40-TRAF2/3/5 or CD40-TRAF6 interactions (Figure 3e, Table 3). Leukocyte numbers (CD45+ cells)

did not differ significantly in the myocardium of these four groups of transgenic mice (Supplemental Figure 3). Also interstitial cardiac fibrosis and transcript levels of fibrotic markers did not differ significantly between CD40-TΔ2, CD40-TΔ6, and CD40-TΔ2/6 and CD40-Twt mice and Wt mice (Supplemental Figure 3, Supplemental table 2).

Table 4. Morphometric characteristics of transgenic mice with disrupted CD40-TRAF binding all treated with AngII.

	CD40-Twt (N=16)	CD40-TΔ2 (N=12)	CD40-TΔ6 (N=14)	CD40-TΔ2/6 (N=12)
Body Weight Start (g)	25.6±0.7	24.9±0.8	25.0±0.7	24.7±0.5
Body Weight End (g)	25.6±0.4	24.9±0.4	25.1±0.5	25.5±0.4
Lung (mg)	195±9	181±5	203±8	187±10
Liver (mg)	1178±57	1083±51	1130±32	1122±49
Kidney (mg)	163±5	168±7	154±5	165±5
Spleen (mg)	74±3	76±3	86±3*	88±3**
Ventricles (mg)	199±9	151±5***	142±7***	146±7***

*Data are expressed as means ± S.E.M. For statistical analysis one-way ANOVA test was used. With Dunnett's post-hoc testing vs. CD40-Twt group. * indicates significant differences between CD40-Twt and the groups with disrupted TRAF binding. (* P < 0.05, ** P < 0.01, *** P < 0.001).*

CD40 activation indirectly stimulates cardiomyocyte hypertrophy

The number of leukocytes (CD45⁺ cells) in the myocardium increased equally in both Wt and CD40^{-/-} mice after AngII (Figure 2b). However, transcript levels of M1 (iNOS) and M2 (Arginase-1) macrophage markers in the myocardium (Supplemental table 1), suggest a shift towards a more anti-inflammatory M2 phenotype in CD40^{-/-} mice.

Besides immune cells, CD40 is also expressed by cardiomyocytes²². To determine whether activation of CD40 on cardiomyocytes can directly induce hypertrophy, rat NCMs were subjected to an agonistic CD40 (FGK45) antibody or an isotype control, and stimulated with Endothelin-1 (ET-1). Activation of CD40 on NCMs by the agonistic CD40 antibody FGK45 increased the transcript levels of pro-inflammatory genes, MCP-1, MIP-1α and IL-6 after 6h, indicative of successful CD40 ligation (Figure 4b). However, CD40 activation did not induce cardiomyocyte hypertrophy: neither transcript levels of ANP and α-SKA (Figure 4b) nor cell surface area at 24h increased significantly upon FGK45 treatment. To substantiate the notion that CD40 influences macrophage polarization, bone marrow derived macrophages (BMDMs) were generated from Wt and CD40^{-/-} mice and activated with LPS. Conditioned medium of these BMDMs mice was administered to rat neonatal cardiomyocytes (NCMs) and a hypertrophic response was induced by stimulation with ET-1.

The conditioned medium from CD40^{-/-} BMDMs blunted the ET-1 induced hypertrophic response as shown by the reduction in both cell surface area and transcript levels of hypertrophic markers (Figure 4a). Subsequently we determined the levels of several cytokines in the conditioned medium. LPS stimulation induced pronounced increase of all cytokines measured. Although no significant difference in cytokine levels between Wt and CD40^{-/-} LPS treated BMDMs was observed, the pro-inflammatory cytokines IL-1β, TNFα, IL-6 and IL-12 tended to be decreased in CD40^{-/-} BMDM conditioned medium. Moreover, IL-6 levels were significantly lower in the conditioned medium of untreated CD40^{-/-} BMDMs compared to Wt (Supplemental figure 4c).

Figure 4. Conditioned medium of CD40^{-/-} BMDMs blunts hypertrophic response in NCMs. NCMs were cultured in conditioned medium of CD40^{-/-} and Wt BMDMs and stimulated with ET-1. **A.** Phalloidin stained NCMs with 24h ET-1 stimulus or control, quantification of cell surface area shows a reduction cell size for NCMs treated with conditioned medium of CD40^{-/-} BMDMs compared to NCMs treated with conditioned medium of Wt BMDMs. The reduction in size is confirmed by transcript levels of hypertrophic markers α -SKA, ANF and BNP. **B.** Activation of CD40 using an agonistic antibody (FGK45) does not induce hypertrophy on cardiomyocytes. FGK45 does not induce expression of marker genes α -SKA, ANF and BNP alone nor does it affect expression in combination with ET-1 stimulation after 24h. To confirm activation of CD40 on cardiomyocytes by FGK45 transcript levels of marker genes, MCP-1, MIP-1 α and IL-6 were quantified after 6h of activation. Error bars represent SEM; *Compared to corresponding Sham, # Compared to Wt-AngII. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

Discussion

The present study reveals that the absence of the immuno-modulatory co-stimulatory receptor CD40 blunts cardiac hypertrophy upon pressure overload. Also expression of transgenic CD40 in MHC class II⁺ cells exacerbates myocyte hypertrophy, while loss of CD40-TRAF2/3/5 or/and CD40-TRAF6 interactions reduces hypertrophy. Furthermore, hypertrophy is inhibited when cardiomyocytes are exposed to conditioned medium from macrophages lacking CD40. Collectively, these results demonstrate that CD40L-CD40-TRAF interactions in antigen-presenting cells regulate AngII induced cardiac hypertrophy in a paracrine manner.

CD40L-CD40 interactions control cardiac hypertrophy

The current finding that CD40 is involved in cardiac hypertrophy is in line with previous studies that already suggested a potential role for CD40 the development of cardiac hypertrophy in patients, but did not provide biological evidence. CD40L expression on platelets and sCD40L levels in serum positively correlated with increased HF (NYHA) functional class ²³, left ventricular ejection fraction ²⁴ and hypertension ¹¹. Specific pro-inflammatory pathways, including TNF Receptor 1 ²⁵ and TNF itself ²⁶, MCP-1 ^{3, 27} and IL-6 ^{28 29} modulate cardiac hypertrophy upon pressure overload. Although activation of CD40 clearly stimulated cardiomyocyte hypertrophy, the AngII-induced fibrotic response or systolic dysfunction remained unaltered in both CD40^{-/-} mice and mice with mutated CD40-TRAF binding domains (figures 2a and supplemental 3). This suggests that fibrotic remodelling and associated systolic impairment is independent of CD40L-CD40 signalling and contrasts with most other studies in which deletion of other inflammatory activators ^{26, 29} resulted in a blunted fibrotic response and decrease in contractility. Previously, however, some studies have also shown effects on hypertrophy independent of the fibrotic response ⁷ or cardiac function ³⁰.

Notably, in mice with APC-specific expression of CD40 (CD40-Twt mice), AngII induced a far more pronounced hypertrophy (VW/BW ratio 7.73 ± 0.31) as compared to Wt mice (VW/BW ratio 5.96 ± 0.20 , Figures 1a and 3a). This underscores the importance of CD40 in cardiac hypertrophic remodelling. Moreover, as macrophages and dendritic cells are the two most common types of APCs expressing MHC class II in the hypertensive heart, this implies that expression of CD40 in these cell lineages is crucial for CD40-mediated cardiac hypertrophy. In addition to the increased sCD40L levels in the blood of hypertensive patients ¹², AngII has also been shown to directly induce the expression of CD40 on human monocytes and dendritic cells ³¹. This could have an enhancing effect on the mechanism of myeloid CD40 mediated cardiac hypertrophy.

Both CD40-TRAF6 and CD40-TRAF2/3/5 interactions mediate cardiac hypertrophy after AngII induced pressure overload

Whereas in atherosclerosis, disease progression is mainly mediated by APC-specific CD40-TRAF6 signalling ¹⁷, our results here demonstrate that both CD40-TRAF6 and CD40-TRAF2/3/5 interactions are involved in the development of cardiac hypertrophy (Figure 3). Animals that are deficient in either CD40-TRAF6 interactions (CD40-TΔ6 mice) or CD40-TRAF2/3/5 interactions (CD40-TΔ2 mice) are protected from AngII-mediated cardiac hypertrophy. In animals with both CD40-TRAF6 and CD40-TRAF2/3/5 interactions blocked (CD40-TΔ2/6 mice) an expected additional reduction of hypertrophy was absent. This indicates that the pathway downstream of CD40-TRAF signalling involved in hypertrophy is

activated via both TRAF6 and TRAF2/3/5. CD40 can activate NF κ B, via the canonical and the non-canonical NF κ B pathway. Interestingly, downstream CD40 signalling via TRAF6 activates the canonical NF κ B pathway only, whereas CD40-TRAF2/5 interactions activate both pathways¹⁶. Classical NF κ B activation has been shown to mediate cardiac hypertrophy in several mouse models of HF³²⁻³⁵ and leads to expression of pro-inflammatory and pro-hypertrophic cytokines such as IL-6. The blunted hypertrophic response in both CD40-T Δ 2 and CD40-T Δ 6 mice suggests involvement of the canonical pathway here.

Polarization of macrophages lacking CD40L-CD40 signalling

Interestingly, the amount of leukocytes (CD45⁺) was comparable in the myocardium of CD40^{-/-} and Wt mice after AngII. However, we and others^{17, 36}, show that *in vitro* M1 polarization towards pro-inflammatory macrophages is abrogated and skews away from M1 polarization in the absence of CD40 signalling. Polarization towards M1 is known to increase cardiac hypertrophy and failure after pressure overload^{6, 7}. Data on M1 versus M2 marker transcript levels of macrophages in the myocardium are difficult to interpret because of bias from transcripts originating from other cardiac cell types such as cardiomyocytes and fibroblasts. We observed a shift away from the M1 marker in the myocardium of CD40^{-/-} mice, with lower transcript levels of iNOS and IL-12 in particular. Mice with mutated CD40-TRAF binding had lower cardiac transcript levels of M1 marker iNOS and higher levels of Arg-1. However, the fact that CD40^{-/-} BMDM conditioned medium blunted the hypertrophic response in ET-1 treated cardiomyocytes (Figure 3) provides evidence for a change in the secretome of these BMDMs. Analysis of several cytokines in this conditioned medium showed only modest changes (Supplemental figure 4) indicating that the anti-hypertrophic effects of CD40^{-/-} BMDM conditioned medium are not mediated by these cytokines. Moreover, LPS stimulation induces a very pronounced increase in cytokine secretion, perhaps indicating that this trigger is too strong to detect subtler differences in cytokine synthesis between the two genotypes. The significantly lower IL-6 levels, a well-known pro-hypertrophic cytokine²⁹, in unstimulated CD40^{-/-} compared to Wt BMDMs suggests that this might be the case.

Conclusions

We have shown that the absence of CD40L-CD40 signalling modulates cardiac hypertrophy. Furthermore, our results show that both downstream pathways via CD40-TRAF6 and CD40-TRAF2/3/5 are mechanistically involved in in this process. Although cardiomyocyte hypertrophy during pressure overload is a highly complex process involving many cell types and signalling pathways, we provide additional evidence for the importance of myeloid cells in this process. The CD40L-CD40 co-stimulatory dyad offers opportunities for therapeutic intervention, both intra- and extra-cellular, to attenuate cardiac hypertrophy and thereby to treat hypertensive heart disease.

Perspectives

Pathological cardiac hypertrophy is a predictor of cardiac disease progression. CD40L has become an established biomarker of HF in hypertensive patients^{11, 24}. Our data provide biological evidence for a central role of CD40L-CD40-TRAF signalling plays in the development of cardiac hypertrophy. Blocking CD40L interactions has already been subject of clinical trials in various inflammatory diseases such as lupus and rheumatoid arthritis, but thromboembolic events have ended this³⁷. The severe immune-compromising effects of blocking CD40 make long-term treatment difficult. Importantly, compounds specifically

blocking CD40-TRAF6 interactions avoiding these side effects have been developed ³⁸ may have therapeutic potential of targeting the CD40L-CD40-TRAF pathway in the context of HDD.

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Supplementary Figures and Tables

Supplemental figure 1. Heart rate and blood pressure of Wt and CD40^{-/-} mice. Characteristics of Wt and CD40^{-/-} mice upon AngII infusion. A. Heart rate in beats per minute (BPM) does not differ between Wt and CD40^{-/-} mice or after AngII infusion. B. Mean arterial blood pressures were measured using tail cuff method after 26 days of AngII infusion, showing an increase in Wt and CD40^{-/-} mice after AngII treatment. Error bars represent SEM; *Compared to corresponding Sham.

Supplemental figure 2. Heart rate and blood pressure of mice with disrupted TRAF binding. Characteristics of mice with disrupted TRAF binding upon AngII infusion. A. Mean arterial blood pressures were measured using tail cuff method after 26 days of AngII infusion, showing an equal level for all groups. B. Heart rate in beats per minute (BPM) does not differ between groups. Error bars represent SEM; * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ to corresponding baseline group.

Supplemental figure 3. Histological analysis of myocardial tissue of mice with disrupted TRAF binding after AngII treatment. A. Fibrosis is unaffected in mice lacking CD40-TRAF signalling as determined by picrosirius red positive area. B. Loss of CD40-TRAF signalling does not attenuate number of CD45⁺ leukocytes after AngII treatment. Number of leukocytes were determined as CD45 positive cells using IHC staining. Error bars represent SEM; * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$, *Compared to corresponding Sham group

Supplemental figure 4. Cytokine levels in conditioned medium of Wt and CD40^{-/-} BMDM. Levels of TNF α , IL-1 β , IL-6, IL-12, KC, and IL-10 (A-F) were determined in the supernatant of cultured BMDM using a V-PLEX Pro-inflammatory panel. LPS (100 ng/ml) induced expression of all measured cytokines. Only levels of IL-6 in control condition showed a significant difference between the genotypes.

Supplemental table 1. Cardiac mRNA levels of marker genes for hypertrophy, fibrosis and inflammation of Wt and CD40^{-/-} mice treated with vehicle or angiotensin II (AngII) for 4 weeks.

	Ct	Wt N=11	Wt AngII N=16	CD40 ^{-/-} N=6	CD40 ^{-/-} AngII N=22
Hypertrophy					
<i>α-SKA</i>	23.3	1.00±0.47	2.80±0.61*	0.54±0.41	1.62±0.27
<i>ANF</i>	24.2	1.00±0.56	4.87±2.44	0.43±0.20	3.23±1.10
<i>BNP</i>	21.4	1.00±0.19	1.91±0.33	0.69±0.19	1.74±0.25
Fibrosis					
<i>Col1a</i>	27.1	1.00±0.18	2.01±0.33	0.59±0.06	2.54±0.67
<i>TGFb1</i>	28.4	1.00±0.14	1.40±0.18	0.51±0.05	1.76±0.29*
Inflammation / M1 Markers					
<i>iNOS</i>	31.1	1.00±0.55	1.16±0.44	0.59±0.26	0.87±0.29
<i>IL-12</i>	33.9	1.00±0.22	1.07±0.21	1.03±0.31	0.71±0.11
<i>IL-6</i>	33.2	1.00±0.46	1.29±0.33	0.30±0.10	1.25±0.28
<i>IL-1β</i>	32.7	1.00±0.18	0.81±0.10	0.56±0.23	0.85±0.18
<i>IκBα</i>	27.8	1.00±0.20	1.01±0.09	0.79±0.14	1.31±0.16
<i>MCP-1</i>	29.3	1.00±0.42	0.47±0.01	0.34±0.08	0.51±0.05
M2 Markers					
<i>Arg-1</i>	32.5	1.00±0.88	0.22±0.11	0.86±0.42	0.13±0.06
<i>Fizz-1</i>	31.7	1.00±0.20	0.27±0.06**	1.21±0.33	0.32±0.08***
<i>YM-1</i>	34.9	1.00±0.43	1.30±0.35	0.50±0.10	1.04±0.18

Expression levels were measured using qPCR and normalized for GAPDH. Ct values given are mean of Wt non treated samples. Data are expressed as means ± S.E.M. For statistical analysis one-way ANOVA test with Bonferroni post-hoc testing was applied. * indicates statistically significant difference (* P < 0.05, ** P < 0.01, *** P < 0.001) between AngII-treated and corresponding non-treated mice. # indicates statistically significant difference (# P < 0.05, ## P < 0.01, ### P < 0.001) compared to corresponding Wt mice.

Supplemental table 2. Cardiac mRNA levels of marker genes for hypertrophy, fibrosis and inflammation of transgenic mice with disrupted CD40-TRAF binding treated AngII.

	Ct	CD40-Twt (N=16)	CD40-TΔ2 (N=12)	CD40-TΔ6 (N=14)	CD40-TΔ2/6 (N=12)
Hypertrophy					
<i>α-SKA</i>	19.4	1.00±0.27	0.48±0.15	0.47±0.18	0.10±0.03**
<i>ANF</i>	21.4	1.00±0.16	0.84±0.21	0.91±0.22	0.90±0.26
<i>BNP</i>	19.7	1.00±0.14	0.81±0.10	0.72±0.14	0.60±0.14
Fibrosis					
<i>Col1a</i>	23.7	1.00±0.22	0.77±0.19	1.58±0.44	1.28±0.42
<i>TGFb1</i>	25.7	1.00±0.26	1.24±0.69	2.20±0.84	1.42±0.48
Inflammation / M1 markers					
<i>iNOS</i>	28.6	1.00±0.14	0.82±0.20	0.68±0.15	0.93±0.29
<i>IL-6</i>	31.9	1.00±0.37	0.47±0.12	0.74±0.36	0.71±0.17
<i>MCP-1</i>	29.1	1.00±0.14	1.18±0.38	1.85±0.48	1.67±0.37
<i>IL-1β</i>	32.6	1.00±0.21	1.20±0.21	0.94±0.17	3.00±0.70**
<i>IL-12</i>	33.7	1.00±0.25	1.02±0.21	1.66±0.32	1.02±0.16
<i>IkBα</i>	25.3	1.00±0.30	0.59±0.25	1.34±0.27	1.21±0.32
M2 Markers					
<i>Arg-1</i>	34.88	1.00±0.29	5.24±2.58	10.67±6.21	10.49±4.15
<i>Fizz-1</i>	33.2	1.00±0.19	1.81±0.49	2.45±0.72	2.46±0.30
<i>YM-1</i>	33.6	1.00±0.30	0.97±0.28	0.55±0.08	0.60±0.25

Expression levels were measured using qPCR and normalized for GAPDH. Ct values are mean CD40-Twt group. Data are expressed as means ± S.E.M. For statistical analysis one-way ANOVA test was used. With Dunnett's post-hoc testing vs. CD40-Twt group. * indicates significant differences between CD40-Twt and the groups with disrupted TRAF binding. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Inflammation-induced metabolic remodelling of the heart in acute viral myocarditis

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Abstract

Rationale: Abnormalities in cardiac energy metabolism, collectively referred to as cardiac metabolic remodelling, play an important role in the pathophysiology of heart failure. Although inflammation has been shown to interfere with regulatory pathways controlling cardiac metabolism, currently, it is unknown in which way, and to what extent, cardiac metabolism is perturbed in viral myocarditis (VM) and whether metabolic intervention has therapeutic potential in this condition.

Objectives: To delineate the effect of acute VM on cardiac metabolism in both humans and mice and to investigate potential molecular mechanisms underlying these metabolic disturbances. Additionally, we explored if dichloroacetate (DCA), an activator of glucose oxidation, has therapeutic potential in VM.

Methods and Results: Micro-array analysis of cardiac biopsies obtained from myocarditis patients revealed a marked down-regulation of several key pathways involved in oxidative metabolism, indicative of cardiac metabolic remodelling. Moreover, activity levels and protein/mRNA abundance of multiple constituents and molecular regulators of cardiac oxidative metabolism were decreased in Cocksackievirus B3 (CVB3)-induced VM in mice. Furthermore, cardiac NF- κ B signalling was activated in both human and mouse VM and *in vitro* experiments using cultured cardiomyocytes revealed that NF- κ B activation impairs cardiomyocyte oxidative metabolism by interfering with the peroxisome proliferator-activated receptor γ co-activator (PGC-1) network, the main regulatory pathway controlling cellular oxidative metabolism. Surprisingly, DCA treatment failed to restore VM-induced abnormalities in cardiac metabolic gene expression and, in fact, aggravated the VM-induced immune response and cardiac inflammation.

Conclusions: These findings collectively suggest that in acute VM the secretion of pro-inflammatory cytokines by infiltrating leukocytes activates NF- κ B in cardiomyocytes, thereby interfering with the PGC1-mediated control of mitochondrial oxidative metabolism. DCA-treatment was unable to attenuate VM-induced disturbances in cardiac metabolic profile and function.

Introduction

Characterized by an acute, fulminant inflammatory response in the heart, viral myocarditis (VM) is an important cause of death in young, otherwise healthy individuals. In a subgroup of patients VM gives rise to an autoimmune response predisposing to the development of dilated cardiomyopathy (DCM) ^{1, 2}. Current treatment of VM focuses primarily on targeting the innate or acquired immune response ³.

It is well established that cardiac disease is often accompanied by marked perturbations in cardiac energy metabolism, collectively referred to as cardiac metabolic remodelling ⁴. Cardiac metabolic remodelling likely contributes significantly to disease progression ^{5, 6} and appears to be an attractive target for therapeutic intervention ⁷. Stimulation of mitochondrial oxidative metabolism by dichloroacetate (DCA), for instance, was found to improve cardiac function and survival in a rat model of left ventricular hypertrophy and failure ⁸. Although inflammation has been postulated as a potential driver of the metabolic remodelling process ⁹, it is unknown whether disturbances in cardiac energy metabolism are involved in the development of VM-induced cardiac dysfunction.

Previously, it was shown that VM-induced cardiac dilatation upon exposure of mice to Coxsackievirus B3 (CVB3) was associated with reduced mRNA expression levels of genes involved in fatty acid β -oxidation and the mitochondrial respiratory chain ^{10, 11}. Whether or not cardiac metabolism is perturbed in VM in humans is unexplored. Although these studies in mice suggest altered cardiac metabolic gene expression in VM, a comprehensive overview of the cardiac metabolic profile in VM is lacking. Furthermore, these studies do not allow one to discriminate whether these metabolic changes are due to (acute) viral myocarditis *per se*, or whether they arise secondary to the development of DCM over time. If impairments in cardiac oxidative energy metabolism are indeed involved in VM-induced cardiac dysfunction, this would imply that interventions aimed at improving cardiac metabolism may have a therapeutic benefit.

Previously, we and others showed that, both in the heart as well as in skeletal muscle tissue, activation of the inflammatory nuclear factor- κ B (NF- κ B) signalling pathway induced a metabolic shift away from oxidative processes towards a greater reliance on, less efficient, glycolytic metabolism ^{12, 13}. Therefore, we hypothesize that, in both humans and mice, the inflammatory response associated with acute VM ignites NF- κ B-mediated reprogramming of cardiomyocyte energy metabolism thereby compromising cardiac energy status and leading to cardiac dysfunction. To address this, we first performed micro-array analysis on cardiac biopsies of patients with acute VM as well as on cardiac tissue of mice suffering from CVB3-induced VM and found clear evidence for a metabolic shift. Subsequently, we investigated the activity, as well as protein and mRNA levels of multiple constituents and molecular regulators of oxidative metabolism in intact hearts and in cardiac myocytes isolated from mice with CVB3-induced VM. Next, the impact of inflammation and activation of the NF- κ B signalling pathway on metabolic gene expression in cultured cardiomyocytes was assessed in detail. Finally, we explored whether dichloroacetate (DCA), a drug known to stimulate mitochondrial oxidative metabolism, was able to ameliorate VM-induced cardiac injury.

Methods

An expanded Material and Methods is available in the Supplemental material.

Human subjects

Acquisition of human samples and clinical parameters have been described previously ^{14, 15}. In short, all human material was obtained during routine clinical sampling and available for research purposes in accordance with the Declaration of Helsinki and the ethical committee at Maastricht University. Written consent was obtained from all subjects. For micro-array analysis, right ventricular septal biopsies were used from patients with acute myocarditis (n=3). Patients presented with an episode of upper airway or gastro-intestinal infection, followed after 1-3 weeks by chest pain. All myocarditis patients had elevated troponin T plasma levels and immunohistochemically proven cardiac inflammation (n>14 CD45 positive cells/mm²). In all patients, virus presence was demonstrated in cardiac biopsies ^{14, 15}. Coronary artery disease was excluded either by CAG or CT. Controls (n=6) consisted of age-matched patients with unexplained ventricular tachy-arrhythmias but with a normal ejection fraction and the absence of systemic or cardiac inflammation or virus presence at the time of biopsy ^{14, 15}. Cardiac biopsies were immediately snap-frozen for analyses.

Animal studies

All mice were bred and maintained in an open animal facility (University of Leuven, Belgium) and experiments were performed according to the guidelines for the care and use of laboratory animals approved by the institution animal committee of the University of Leuven (ECD 2432013). 4-6 weeks old male wild type C3H mice (Harlan, NL) were injected intraperitoneally (i.p.) with CVB3 virus (Nancy strain, 200µl 5 × 10⁸ PFU, derived from ATCC batch) or saline as previously described ¹⁶. DCA (25mg/kg/day, Sigma) was administered daily via i.p. injection, starting from day 0 of CVB3 infection. Animals were anaesthetized with xylazine (10mg/kg) and ketamine (100mg/kg) prior to sacrifice by cervical dislocation at 7 days post infection and all organs were excised, weighed and taken for further histological and molecular analysis.

Cell culture

Rat neonatal ventricular cardiac myocytes were isolated by enzymatic dissociation from the hearts of 1- to 2-day old neonatal rats. NCMs were plated 1.0x 10⁶ on gelatinized 6-well plates and cultured as described previously ¹⁷. HL-1 cells were kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA), cultured in Claycomb medium (supplemented with 10% FBS, 0.1 mM norepinephrine, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin) at 37 °C and 5% CO₂.

Messenger RNA expression array analysis

Mouse VM samples expression was measured using Affymetrix Mouse Gene 1.0 ST Array. The human total RNA was hybridized to Illumina HumanHT-12 v3.0 Gene Expression arrays. Detailed information about array analyses is available in the methods section in the online data supplement.

Statistics

Data were analysed according to the guidelines of Altman et al. using SPSS (SPSS Inc., Chicago, IL, USA). Non-parametric tests and unpaired t-tests were used when appropriate. Data are represented as the mean $\pm$ SEM. A p-value of less than 0.05 was considered to be significant.

Figure 1. Micro-array analysis reveals down-regulation of oxidative metabolic pathways in the heart during VM. Heat maps of differentially-regulated ($p \leq 0.05$) metabolic genes in right ventricular septal biopsies from patients with viral myocarditis (VM) ($n=3$) and controls ($n=6$) and in cardiac tissue of mice with CVB3-induced VM, 4 and 7 days post inoculation, and sham-treated animals ($n=3$ for all groups) (A). Venn diagram depicting overlap in differentially-regulated metabolic genes in human and mouse VM (B). Echocardiographic assessment of the impact of VM (7 days post-inoculation) on cardiac function in mice (C). * $p \leq 0.05$ compared to control.

Results

Transcriptomic analysis reveals down regulation of oxidative metabolic pathways during acute VM

To identify cellular processes that are affected by VM in both humans and mice, micro-arrays were performed on cardiac biopsies of a previously well-characterized group of VM patients (Table S1)¹⁵ and on cardiac tissue of mice with acute CVB3-induced VM (Figure S1 A, B). Subsequent enrichment analysis revealed that processes related to cardiac energy metabolism were among the most prominently regulated processes in both man and mice (Table S2, S3). Accordingly, heat maps were constructed of all genes that are implicated in glycolysis, the tri-carboxylic acid (TCA) cycle, fatty acid β -oxidation and the electron transport chain (ETC). This revealed that, in the glycolytic pathway, a number of genes were up-regulated while others were down-regulated in both human and mouse (Figure 1A). Remarkably, however, the far majority of genes involved in fatty acid β -oxidation, the TCA cycle and the ETC, was dramatically down-regulated during VM (Figure 1A; Visualization of metabolic pathways in human VM are depicted in Figure S2-S5). Significant overlap in differentially-regulated metabolic genes was observed between species (Fig 1B). Similar to observations in VM patients¹⁵, altered cardiac metabolic gene expression during CVB3-induced VM was associated with a reduced ejection fraction (EF) and a decreased fractional shortening (FS) in absence of cardiac dilatation (Table S1 and Figure 1C). Notably, in mice, reductions in expression levels of constituents of mitochondrial oxidative metabolic pathways were much more pronounced at day 7 post-inoculation compared to day 4 (Figure 1A).

VM-induced metabolic remodelling of the heart

To validate our micro-array data, next we investigated mRNA transcript levels of several genes involved in cardiac oxidative metabolism in a mouse model of CVB3-induced VM and, importantly, subsequently verified whether alterations in the expression levels of these genes were associated with alterations in protein abundance and enzymatic activity of constituents of these metabolic pathways. As depicted in Figure 2A, enzymatic activity of β -hydroxyacyl-CoA dehydrogenase (HAD; rate-limiting enzyme in fatty acid β -oxidation) as well as cardiac mRNA levels of acyl-CoA dehydrogenase for very long chain fatty acids (ACAD-VL) were significantly decreased in VM confirming our micro-array data and further indicating a reduced fatty acid oxidative capacity. Moreover, VM was associated with significant impairments in mitochondrial oxidative phosphorylation as enzyme activity of cytochrome c oxidase (COX) as well as protein and mRNA abundance of several sub-units of complexes involved in oxidative phosphorylation were reduced compared to sham-treated animals (Figure 2B).

In addition to impairments in oxidative metabolic pathways, transcriptomic analysis also indicated an increase in the expression levels of various glycolytic genes in the heart during VM. This was confirmed by increased expression levels of glucose transporter 1 (Glut-1) and of several glycolytic enzymes as assessed by QPCR (Figure 2C). Also, lactate dehydrogenase-1 (LDH1) and the lactate transporter monocarboxylate transporter-4 (MCT-4) were increased expressed during CVB3-induced VM further evidencing activation of a glycolytic gene program (Figure 2D). Notably, transcript abundance of hypoxia-inducible factor 1 α (HIF-1 α ; a pivotal regulator of cardiac glycolytic metabolism) and of known HIF-1 α target genes were increased in VM hearts (Figure 2E) which correlated positively with

glycolytic gene expression (Table. S4). Collectively, these alterations in oxidative and glycolytic metabolic pathways observed during VM were associated with an increase in the phosphorylation status of the energy-sensing molecule adenosine monophosphate kinase (AMPK) suggesting an energy-deprived state of the heart (Figure 2F). In concert, these data indicate that VM-induced metabolic changes observed at the level of the entire transcriptome are reflected by parallel changes at the protein and enzyme activity level and reveal impairments in oxidative metabolism, which appear to be (partly) compensated by HIF-dependent activation of glycolysis.

Altered mitochondrial dynamics in VM

Considering the aberrant expression and activity of several mitochondrial proteins essentially involved in oxidative substrate metabolism, cardiac mitochondrial morphology was assessed by electron microscopy. Although we did not observe prominent changes in mitochondrial ultra-structure 7 days post infection, we did observe altered mRNA expression levels of mitochondrial fusion and fission genes at this time-point (Figure 3 A, B). More specifically, mRNA levels of the mitochondrial fusion gene 1 (*mf1-1*) decreased by approximately 40%, while transcript abundance of the mitochondrial fission gene *FIS1* increased 2-fold suggesting potential alterations in mitochondrial dynamics.

Figure 2. Down-regulation of mitochondrial oxidative metabolic pathways in the heart during CVB3-induced VM. Intact hearts were obtained from mice with CVB3-induced VM ($n=12$) and sham-treated animals ($n=5$). The activity of individual metabolic enzymes was determined and corrected for total protein content (A, B). Western blot was performed to determine protein content of proteins of interest (B, F) and metabolic gene expression was determined by QPCR (A-E). Western blots were corrected for α -tubulin. * $p \leq 0.05$ compared to control.

Figure 3. Disturbed mitochondrial dynamics and impaired PGC-1 signalling during CVB3-induced VM. *C3H* mice were subjected to CVB3-induced VM ($n=12$) or sham-treated ($n=5$) for 7 days. QPCR analysis revealed altered expression levels of mitochondrial fusion and fission genes (A). Electron microscopy images were taken from controls and animals with VM (B). Expression levels of constituents of the PGC-1 signalling network were altered during VM (C, D). Western blots were corrected for α -tubulin. * $p \leq 0.05$ compared to control. Scale bar: 100 μ M.

Peroxisome proliferator-activated receptor γ co-activator molecule 1 (PGC-1) proteins are essential co-activators for a multitude of transcription factors involved in the molecular regulation of mitochondrial biogenesis and dynamics and hence are key determinants controlling cardiac oxidative metabolism¹⁸. Accordingly, PGC-1 α transcript abundance correlated positively with expression levels of various constituents of oxidative phosphorylation and fatty acid β -oxidation in our study (Table S4). Moreover, mRNA levels of PGC-1 α and of its family member PGC-1 β were significantly reduced in the heart during VM as were expression levels of the PGC-1-coactivated transcription factors PPAR- α and PERM1. Protein levels of NRF-1 and Tfam, key regulators of mitochondrial biogenesis¹⁹, were dramatically decreased in VM further evidencing disturbances in the molecular regulation of mitochondrial metabolism. Interestingly, abundance of other PGC-1-co-activated transcription factors was unaltered (ERR- α , Tfam) or increased (NRF-1, NRF-2 α and PPAR- δ) (Figure 3 C-D) suggesting a compensatory cellular response to activate mitochondrial biogenesis.

Figure 4. Cardiomyocyte metabolic gene expression in VM. Cardiomyocytes were isolated from hearts of mice with CVB3-induced VM ($n=8$) and sham-treated animals ($n=8$). QPCR analyses revealed down-regulation of genes involved in oxidative phosphorylation (A, B), fatty acid β -oxidation (C) and PGC-1 signalling (D) while genes involved in glycolytic metabolism (E, F) and HIF signalling (G) were up-regulated. * $p \leq 0.05$ compared to control.

Cardiomyocyte metabolic gene expression is impaired in VM

Importantly, to verify whether the observed changes in metabolic pathways in intact hearts during VM reflect alterations in metabolic gene expression in cardiomyocytes *per se* and are not confined to infiltrating immune cells, cardiomyocytes were isolated from the hearts of animals with or without CVB3-induced VM. CVB3 viral RNA was detectable in cardiomyocytes isolated from animals with VM whereas control cardiomyocytes displayed no detectable levels (Figure S1C). In line with our *in situ* data, cardiomyocytes from VM animals were characterized by significantly reduced expression levels of nuclear- and mitochondrial-encoded sub-units of oxidative phosphorylation complexes as well as decreased ACAD-VL (fatty acid β -oxidation) mRNA abundance (Figure 4A-C). Furthermore, cardiomyocytes from animals with VM displayed decreased transcript levels of constituents of the PGC-1 signalling network as PGC-1 β expression as well as Tfam and NRF-2 α mRNA levels were decreased (Figure 4D). Similar to what was observed in intact hearts, cardiomyocytes isolated from VM hearts also showed increased expression of multiple glycolytic constituents such as Glut-1,

PFKFB3, and MCT-4 (Figure 4 E, F). Although HIF-1 α expression itself was unaltered in cardiomyocytes isolated from VM hearts (data not shown), expression levels of the HIF-1 α target genes CA9 and HMOX were increased compared to control (Figure 4G).

Inflammation-induced activation of NF- κ B signalling impairs cardiomyocyte oxidative metabolism

Having established a cardiomyocyte-specific metabolic remodelling process in VM, we next investigated potential underlying molecular mechanisms. Exposure of cultured neonatal cardiomyocytes to CVB3 virus potently induced expression of genes involved in viral uptake and host response to viral infection but failed to induce any significant decreases in oxidative metabolic gene expression (Figure S6A-E) suggesting that CVB3 virus infection itself does not impair cardiomyocyte metabolic gene expression.

Interestingly, in animals with VM, cardiac expression levels of the pro-inflammatory cytokine tumor necrosis factor α (TNF- α) correlated inversely and significantly with expression levels of various genes involved in fatty acid β -oxidation, oxidative phosphorylation and PGC-1 signalling (Figure 5A-C), suggesting a role for TNF- α in VM-induced cardiac metabolic remodelling.

TNF- α is a known activator of the NF- κ B pathway, a key inflammatory signalling cascade relaying inflammatory signals to the nucleus²⁰. Our micro-array analysis revealed an up regulation in the expression levels of multiple constituents and target genes of the NF- κ B pathway in cardiac biopsies of VM patients as well as in cardiac tissue of mice with VM, in particular 7 days post inoculation (Figure 5D). To confirm these micro-array data, expression levels of various inflammatory cytokines and chemokines, which are known NF- κ B target genes²¹, were analysed by QPCR and found to be dramatically induced during CVB3-induced VM (Figure S7A). Moreover, cardiac expression levels of key constituents of NF- κ B signalling itself (I κ B α , RelA) were increased (Figure 5E) and correlated with the degree of cardiac immune cell influx (Table S4). Immunohistochemical staining for RelA, the main transcriptionally-active subunit of the NF- κ B pathway, showed prominent NF- κ B activation in the cardiomyocytes during CVB3-induced VM (Figure 5F). This was confirmed by increased expression levels of constituents and established target genes of the NF- κ B pathway in cardiomyocytes isolated from the hearts of mice with VM (Figure S7B).

Figure 5. Activation of NF- κ B signalling in cardiomyocytes during VM. Correlations between cardiac TNF- α expression and expression of constituents of fatty acid β -oxidation (A), oxidative phosphorylation (B) and PGC-1 signalling (C) were explored in mice with CVB3-induced VM ($n=12$). Heat map analysis revealed activation of NF- κ B signalling in right ventricular septal biopsies from patients with viral myocarditis (VM) ($n=3$) and in cardiac tissue of mice with CVB3-induced VM ($n=3$) especially 7 days post-inoculation (D). Gene expression of NF- κ B signalling constituents was induced in cardiac tissue of mice with CVB3-induced VM ($n=12$) compared to sham-treated animals ($n=5$) (E). Immuno-histochemical staining of RelA on sections from whole hearts from sham-treated mice ($n=5$) and mice with CVB3-induced VM ($n=9$) demonstrated activation of NF- κ B in cardiomyocytes (F). * $p \leq 0.05$ compared to control.

To further explore a potentially direct detrimental impact of TNF- α and subsequent activation of the NF- κ B pathway on cardiomyocyte oxidative metabolism, we stimulated cultured HL-1 cardiomyocytes with TNF- α . In response to TNF- α , inflammatory gene expression was induced (Figure S8A) and oxidative phosphorylation complex I, II and III sub-unit mRNA expression decreased significantly (Figure 6A). Moreover, TNF- α induced profound reductions in the expression levels of multiple constituents of the PGC-1 signalling network (Figure 6B) and decreased NRF-1 transcriptional activity ($84.4 \% \pm 2.8$ vs $100.0 \% \pm 5.1$; $P \leq 0.05$) and Tfam promoter transactivation ($86.6 \% \pm 4.8$ vs $100.0 \% \pm 4.7$; $P \leq 0.05$) indicating impairments in mitochondrial dynamics and biogenesis.

To examine whether NF- κ B activation as such yielded similar results, we activated NF- κ B signalling in cultured cardiomyocytes by adenoviral over-expression of IKK β , one of the key kinases involved in activation of the NF- κ B pathway²⁰. In HL-1 cardiomyocytes, NF- κ B transcriptional activity as well as mRNA transcript abundance of intra-cellular adhesion molecule (ICAM-1), a known NF- κ B target gene, was potently induced following IKK- β over-expression (Figure S8B). mRNA transcript abundance as well as protein content of oxidative phosphorylation complexes was found to be decreased in response to IKK β -induced NF- κ B activation (Figure 6C, D). Also, IKK- β over-expression was sufficient to decrease PGC-1 α , PGC-1 β , Tfam and PPAR- α transcript abundance similar to what was observed in response to TNF- α (Figure 6E). IKK- β over-expression induced similar changes in oxidative metabolic gene expression in cultured rat neonatal cardiomyocytes (Figure S8C-E).

In concert, this shows that (inflammation-induced) activation of the NF- κ B signalling pathway impairs cardiomyocyte oxidative metabolic gene expression likely through interference with the PGC-1 signalling circuitry. This molecular mechanism may well be involved in VM-induced alterations in cardiac energy metabolism.

DCA fails to restore VM-induced changes in cardiac metabolic gene expression

Given the pronounced impairments in parameters of mitochondrial energy metabolism observed in acute VM, we explored whether systemic administration of DCA, a stimulator of glucose oxidation, would protect against VM-induced impairments in cardiac energy metabolism and thereby improve cardiac function. First, the effectiveness of DCA to upregulate cardiomyocyte oxidative metabolic gene expression was assessed *in vitro* using cultured neonatal cardiomyocytes. In line with previous studies²², the expression of PPAR- α , involved in the regulation of cardiomyocyte oxidative metabolism, was up-regulated by DCA. Moreover, expression of a sub-unit of complex III of the respiratory chain was also up-regulated indicating effectiveness of DCA (Figure 7A).

In vivo, VM resulted in an increased heart weight regardless of DCA treatment and DCA failed to protect against VM-induced cardiac dysfunction (Figure 7B, C). Surprisingly, DCA treatment of VM mice worsened the cardiac phenotype as evidenced by a marked increase in immune cell infiltration and inflammatory gene expression (Figure 7D) as well as more extensive metabolic remodelling at the mRNA level. Notably, spleen weight was also increased in DCA-treated versus vehicle-treated VM mice, suggestive of an exaggerated immune response (Figure 7E-G).

HL-1 cardiomyocytes

Figure 6. Activation of NF- κ B signalling impairs cardiomyocyte oxidative phenotype. HL-1 cardiomyocytes were stimulated with TNF- α (10 ng/ml) for 48h and expression levels of genes involved in oxidative phosphorylation (A) and PGC-1 signalling (B) were determined. HL-1 cardiomyocytes were subjected to adenoviral over-expression of IKK β or an adenoviral construct expressing GFP as a control for 48h (C, E) or 72h (D). mRNA and protein expression levels of complexes of oxidative phosphorylation (C, D) and PGC-1 signalling constituents (E) were decreased. Western blots were corrected for α -tubulin. Western blot triplicates for each treatment condition were cut from the same immunoblot as indicated by the black boxes. # $p \leq 0.1$, * $p \leq 0.05$ compared to control.

Figure 7. DCA aggravates VM-induced inflammation and fails to restore cardiac function and metabolic gene expression. Rat neonatal cardiomyocytes were cultured and stimulated with DCA for 24h. mRNA expression levels of PPAR- α and OXPHOS complex III sub-unit were analysed by QPCR (A). C3H mice received a single intraperitoneal (i.p) injection with CVB3 or vehicle. Additionally, all mice received an injection with vehicle or DCA (25 mg/kg/day i.p) for 6 consecutive days. In VM animals, heart weight was increased and cardiac function was impaired regardless of DCA treatment (B, C). DCA potentiated VM-induced increases in cardiac immune cell infiltrate and inflammatory gene expression (D, E) as well as in spleen weight (F). DCA potentiated VM-induced reductions in oxidative metabolic gene expression and inductions of glycolytic gene expression (G). * $p \leq 0.05$ compared to sham-control, # $p \leq 0.05$ compared to VM-control.

Discussion

This is the first study showing that acute VM, in both humans and mice, is associated with metabolic remodelling of the heart. The present findings support the notion that the marked suppression of cardiomyocyte mitochondrial oxidative metabolism is linked to activation of the inflammatory NF- κ B pathway, secondary to paracrine signalling by cytokines secreted by leukocytes infiltrating the myocardium and paracrine/autocrine signalling by inflammatory mediators produced by the cardiomyocytes themselves.

Changes in the energy metabolism of the heart and subsequent altered energetics, resulting in an 'energy-starved' heart, have been postulated to play an important causal role in the development of heart failure ²³. In humans and mice, we found striking similarities indicative for disturbed mitochondrial oxidative energy metabolism in the heart during VM which appeared to be (partly) compensated by activation of glycolysis. The increased phosphorylation status of the cellular energy-sensor AMPK revealed that metabolic remodelling during VM results in an energy-deprived state of the heart, which may well contribute to VM-induced cardiac dysfunction ^{23, 24}. Besides contributing to cardiac dysfunction by providing an insufficient supply of ATP to cardiac myocytes, alterations in mitochondrial metabolic pathways may also have implications beyond ATP production as these pathways also regulate essential, non-ATP-generating anabolic processes that are crucial for maintaining cellular activities ⁴. Importantly, alterations in metabolic gene expression in intact hearts were confirmed in cardiomyocytes isolated from the hearts of these animals, indicating that metabolic remodelling was confined to cardiomyocytes and not confounded by infiltrating immune cells which highly rely on glycolysis for energy generation ²⁵.

During VM, expression levels of TNF- α in the heart correlated negatively with abundance of key constituents and molecular regulators of mitochondrial oxidative metabolism, suggesting that inflammatory cytokines such as TNF- α may act as potential drivers of the cardiac metabolic remodelling process. The observation that stimulation of cultured cardiomyocytes with TNF- α , but not exposure to CVB3 virus *per se*, potentially impaired oxidative metabolic gene expression and its molecular regulation by PGC-1 signalling further strengthened this notion. As expression levels of TNF- α mRNA correlated strongly with the degree of cardiac immune cell influx, infiltrating immune cells likely are the main source of inflammatory cytokines in the heart during VM. However, cardiomyocytes themselves may well contribute to the ensuing inflammatory response in the heart as expression levels of various inflammatory cytokines were increased in cardiomyocytes isolated from hearts of animals with VM. These observations are in line with the observation that cardiac-specific over-expression of TNF- α , a well-characterized model of cytokine-induced cardiomyopathy, resulted in mitochondrial dysfunction and impairments in cardiac oxidative metabolism ²⁶ and demonstrates, in line with other literature ²⁷ that TNF- α is a potent trigger for cardiomyocyte metabolic remodelling and development of cardiac dysfunction, relevant to VM-associated cardiac pathophysiology. Also, in addition to TNF- α , cardiac-specific over-expression of other individual members of the TNF superfamily was sufficient to mediate adverse resulting in a dilated cardiac phenotype and mitochondrial dysfunction ^{28, 29} suggesting a broader involvement of pro-inflammatory mediators and inflammatory signalling.

In this regard, studies have shown that RelA, the main transcriptionally-active sub-unit of the inflammatory NF- κ B signalling pathway, not only impairs transcription of the PGC-1 α gene

itself but also physically interacts with and sequester PGC-1 α protein thereby inhibiting its function in the regulation of cellular oxidative metabolism³⁰⁻³². In addition, activation of NF- κ B signalling has been shown to impair transcription as well as activity of PPAR- α and PPAR- δ isoforms³³⁻³⁵. These studies further highlight the detrimental impact of activation of inflammatory signalling on the molecular regulation of oxidative energy metabolism and are in line with our findings as activation of NF- κ B signalling in cardiomyocytes (*in vitro* as well as *in vivo*) was associated with reductions in expression levels of PGC-1 and PPAR isoforms. Interestingly, while Tfam and NRF-1 protein levels were decreased during VM, mRNA abundance of these key regulators of mitochondrial biogenesis increased suggesting a compensatory cellular response to activate mitochondrial biogenesis in response to cardiac energy depletion, a phenomenon also observed in experimental models of hypertrophic heart disease and heart failure³⁶. Alterations in mitochondrial oxidative metabolic pathways and its molecular regulation by the PGC-1 network were observed within a few days of viral infection suggesting that perturbations in cardiac energy metabolism in VM do not arise secondary to a reduced cardiac function but likely contribute to impairment of cardiac function during disease progression³⁷.

Illustrative of its significance, blockade of the NF- κ B pathway improved cardiac function and survival during TNF- α -induced cardiomyopathy and activation of NF- κ B signalling, by cardiac-specific transgenic over-expression of IKK- β , was sufficient to induce foetal reprogramming of cardiomyocytes, cardiomyopathy and heart failure^{38, 39}. Together with our findings that impaired oxidative metabolism and disturbances in PGC-1 signalling during VM were associated with a massive induction of NF- κ B signalling and that activation of NF- κ B signalling *per se* was sufficient to impair cardiomyocyte oxidative gene expression, this suggests that NF- κ B activation likely constitutes an important etiological factor involved in adverse cardiac metabolic remodelling observed in VM, most likely through down-regulation of the PGC-1 signalling network. Intriguingly, NF- κ B activation has also been shown in other experimental models of heart failure associated with cardiac metabolic remodelling^{40, 41}. In addition, NF- κ B activation during CVB3-induced VM in our study was also associated with activation of a glycolytic gene program and increased expression of HIF-1 α and HIF-1 α target genes. This is in line with current understanding of these pathways as interactions between the inflammatory NF- κ B pathway and the hypoxia-sensitive HIF-1 α pathway, which is known to regulate cardiac glycolytic gene expression⁴², have been described⁴³⁻⁴⁶.

Collectively, these observations are in line with the notion that, besides its well established role in regulating immune and inflammatory responses, the NF- κ B pathway is increasingly being recognized as a central regulator of energy homeostasis via direct interaction with cellular networks that govern glycolysis and respiration⁴⁷.

Given the marked derangements in cardiac energy metabolism observed in the present study and the notion that previous studies showed that correcting cardiac metabolism may offer relief to the diseased heart, we tried to stimulate oxidative metabolism in VM mice directly. Recently, Kato et al. demonstrated that DCA, an activator of the pyruvate dehydrogenase (PDH) complex and, hence, glucose oxidation, improved cardiac function and survival in a rat model of hypertensive heart disease⁴⁸. Unexpectedly, the current study shows that in the setting of acute VM direct stimulation of mitochondrial metabolism with DCA worsened the VM phenotype as reflected by increased leukocyte infiltration, further reductions in metabolic oxidative gene expression and diminished cardiac function. Although the outcome is negative in terms of therapy, this experiment once again

shows the intricate link between inflammation and metabolism ⁴⁷. Notably, administration of DCA to animals with VM also increased spleen weight indicative of an immune-modulatory effect of DCA. This is in coherence with available literature showing that DCA modulates cytokine response toward T-helper 1 lymphocyte function and is able to promote an M1 inflammatory phenotype in macrophages ^{49, 50}. Collectively, these findings suggest that the stimulation of glucose oxidation potentiates pro-inflammatory immune responses and in this way impairs anti-viral responses in VM and further impairs cardiomyocyte oxidative metabolism.

However, it cannot be excluded that DCA also exerts detrimental effects on cardiomyocytes in which oxidative metabolism is markedly affected, as seen in our VM model. It is feasible that stimulating PDH activity with DCA while respiratory chain activity is severely impaired further promotes rather than alleviates mitochondrial dysfunction. This would imply that metabolic therapy in VM demands that metabolism is stimulated in a more balanced way, for instance by stimulating the activity of more upstream regulators of cardiac metabolism, such as PGC-1, either directly or via the targeted inhibition of the NF- κ B pathway. Inhibition of NF- κ B signalling has already been shown to improve outcome in experimental models of myocarditis by attenuating inflammatory responses in the heart and inhibiting viral replication in lymphoid cells ^{51, 52}. It remains to be established, however, if this is through its anti-inflammatory or metabolic effects.

In conclusion, the collective findings support the concept that cytokines, including TNF- α , released from inflammatory cells infiltrating the infected myocardium, lead to the activation of NF- κ B within the cardiomyocyte, ultimately resulting in impairment of mitochondrial oxidative metabolism. It is well feasible that these disturbances in energy generating capacity significantly contribute to the observed depression of cardiac contractile function during acute VM.

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Supplemental methods

Messenger RNA expression array analysis

Mouse VM samples mRNA expression was measured using Affymetrix Mouse Gene 1.0 ST Array. Expression was normalized using RNA from Affymetrix ¹ Bioconductor package and Ensembl mapped custom CDF (version 16) in R. The human total RNA was hybridized to Illumina Human miRNAv2 Expression Panel arrays by ServiceXS (Leiden, The Netherlands). The *lumi* ² package from Bioconductor was used to annotate and quantile normalize the expression data. To assess differential expression, the *limma* ³ package was used and genes with an adjusted p-value of < 0.05 were considered significantly differentially regulated. Pathway enrichment of the differentially regulated genes was done using the *Statistics* tool in PathVisio ⁴ against the curated set of WikiPathway ⁵ mouse pathways.

Pathway visualization

The presented pathways are adaptation of the following WikiPathway pathways: fatty acid β -oxidation (WP1269), glycolysis and gluconeogenesis (WP157) and TCA cycle (WP434). The electron transport chain (WP295) pathway was used as is. The adaptations focus on streamlining the enriched pathways. They are available on WikiPathways as: [new fatty acid], [new TCA] and [new glycolysis]. The visualization of expression data was done through PathVisio. Mouse to human gene mapping was obtained through the BioMart of Ensembl Release 75.

Tissue preparation, Histology and Immunohistochemistry

Directly after sacrifice the hearts were dissected, weighed and prepared for further analysis. Atria were removed from the isolated heart and the heart was cut in two pieces between base and apex. Transverse midsections of the hearts were fixed in zinc fixative (BD Pharmingen) for 48h and later embedded in paraffin, sectioned at 5 μ m, and later processed for hematoxylin / eosin (H&E) staining. The amount of cardiac inflammation was determined by calculating the infiltrate area of 10 random photo's at 10x magnification of an H&E staining using ImageJ program. Paraffin sections were first deparaffinized and rehydrated. The slides were blocked in buffer containing normal Rabbit serum (Dako), incubated with primary antibody Anti-Mouse CD45 (BD Pharmingen), and then incubated with biotin-conjugated secondary Rabbit anti-Rat antibody (Dako). Signal was amplified using ABC kit (Vectastain, Vectorlabs, CA) and visualized using DAB (Sigma-Aldrich).

RelA staining

Paraffin sections were deparaffinized using xylene and rehydrated. Antigen retrieval was performed by boiling the sections which were subsequently blocked using 1% BSA. Sections were exposed to RelA antibody 1/500 diluted in 0.1% BSA overnight at 4°C. After 3 wash steps, a swine anti-rabbit IgG biotin labelled antibody (Dako, E0431) diluted 1/250 in 1% BSA was applied for 1h at room temperature. Signal was amplified using ABC kit (Vectastain, Vectorlabs, CA)

Isolation of adult cardiomyocytes

Adult mouse cardiac cells were extracted by enzymatic digestion using an adjusted method previously described ⁶. Briefly, hearts were dissected and immediately perfused with calcium-free Tyrode solution via a Langendorff perfusion system. After enzymatic digestion

(collagenase I, Worthington, UK) calcium was slowly reintroduced. Freshly isolated, adult cardiomyocytes were lysed directly in RNA lysis buffer for further molecular analysis.

Echo analysis

Mice were anesthetized (1% isoflurane, ecuphar) and echocardiography was performed with a MS400 transducer (18-38 MHz) on a Vevo-imaging station (Vevo2100, VisualSonics). M-mode views, long and short axis B-mode views were obtained and heart rate and LV diameters at end-diastole, end-systole, anterior wall thickness, LV posterior wall thickness in end diastole, were measured, with which fractional shortening and ejection fraction was calculated.

Chemicals and reagents

TNF- α (Calbiochem, Nottingham, United Kingdom) was dissolved in 0.1 % bovine serum albumin (BSA) which also served as a vehicle control (0.005 % final concentration) to a stock of 200 ng/ml. Pre-made adenoviral (AD) constructs (Ad-CMV-IKK β and Ad-CMV-GFP) were purchased from Vector Biolabs (Philadelphia, USA). Viral expression constructs were dissolved in Dulbecco's Modified Eagle Medium (DMEM) with 2 % BSA & 2.5 % Glycerol (1×10^{10} PFU/ml) and further diluted in sterile Hank's Balanced Salt solution (HBSS) (Invitrogen, Leusden, the Netherlands) to a stock concentration of 2×10^8 PFU/ml. A final concentration of 2×10^7 PFU/ml was added to cells.

RNA isolation, cDNA synthesis and QPCR

Directly after sacrifice the hearts were dissected and the apex of the heart was sectioned in two parts and directly frozen in liquid nitrogen for RNA isolation. Left ventricular tissue was snap frozen in liquid nitrogen and processed for analysis of mRNA content by quantitative PCR. For *in vitro* experiments cells were washed with phosphate-buffered saline (PBS) and lysed using RLT buffer (Qiagen, Venlo, the Netherlands) with added 1% β -mercaptho-ethanol. Total RNA was isolated using RNeasy kit (Qiagen, Venlo, the Netherlands) following manufacturers protocol. cDNA was prepared using iScript kit (Biorad Inc., Hercules, CA, USA) using 1 μ g RNA of each sample as input. QPCR analysis was performed on a Biorad iCycler to determine the mRNA levels of the analysed genes.

Electron Microscopy

For determination of ultra-structural mitochondrial morphology, hearts were perfused and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Post fixation was performed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) supplemented with 1.5% potassium ferrocyanide. The samples were then dehydrated and embedded in epon. Ultrathin sections were examined using a Philips CM100 electron microscope.

Transfections and plasmids

For the assessment of NF- κ B transcriptional activity, cells were transfected with a 6kB-TK luciferase plasmid (NF- κ B reporter) as previously described ⁷. For assessment of Tfam promoter activity and NRF-1 transcriptional activity, cells were transfected with mTFA-RC4wt/PGL3 or p4xNRF-1 luciferase reporter construct ⁷⁻⁹. Cells were grown to be 50% confluent and transfected by nanofectin according to manufacturer's instructions (PAA, Pasching, Austria). Cells were harvested by washing 2x with cold PBS and subsequent lysis by adding (100 μ l) 1x Reporter Lysis Buffer (Promega, Madison, WI, USA) and incubation on ice for 10 min. Cell lysates were centrifuged (14000 rpm, 1 min) and supernatants were snap

frozen and stored at -80 °C for later analysis. Luciferase activity was measured according to manufacturer's instructions (Promega) and corrected for β -galactosidase (Tropix, Bedford, MA, USA).

Whole cell lysates and Western blot

Whole heart was powdered and lysed in a whole-cell lysate (WCL) buffer (20 mM Tris, pH 7.4; 150 mM NaCl; 1 % Nonidet P-40; 1 mM DTT; 1 mM Na_3VO_4 ; 1 mM PMSF; 10 $\mu\text{g}/\text{ml}$ Leupeptin and 1 % aprotinin) using a Polytron PT 1600 E (Kinematica AG, Littau/Luzern, Germany). HL-1 cells were harvested in the same buffer by scraping with rubber police men. Crude lysates were incubated on ice for 30 min, followed by a 30 min centrifugation step at 16,000 g and 4 °C. A portion of the supernatant was saved for protein determination, prior to the addition with 4 $\times$ Laemmli sample buffer (0.25 M Tris-HCl pH 6.8; 8 % (w/v) SDS; 40 % (v/v) glycerol; 0.4 M DTT, and 0.04 % (w/v) Bromophenol Blue). The samples were boiled for 5 min at 95 °C and stored at -20 °C. Total protein concentration was assessed with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. For western blotting, 2.0 μg (OXPHOS, GAPDH) or 10 μg (NRF-1, Tfam, AMPK, phospho-AMPK, IKK- β and α -tubulin) of protein was loaded per lane and separated on a 10 % polyacrylamide gel (Mini Protean 3 system; Biorad, Hercules, CA, USA), followed by transfer to a 0.45 μm nitrocellulose membrane (Biorad) by electro-blotting. The membrane was blocked for 1 h at room temperature in 5 % (w/v) non-fat dry milk followed by overnight incubation (4 °C) with a polyclonal antibody specific for OXPHOS proteins, α -tubulin, NRF-1, Tfam, AMPK and phospho-AMPK (all from Cell Signaling, Beverly, MA, USA). Antibodies were all diluted 1/2000 in (w/v) non-fat dry milk. After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Western-blot films were imaged and quantified using the Quantity One analysis software from Bio-Rad.

Enzyme activity assays

Activity of β -hydroxy-acylCoA dehydrogenase (HAD) (EC 1.1.1.35) and cytochrome c oxidase (COX) (EC 1.9.3.1) were measured as described previously ^{7, 10}. Enzyme activity levels were measured at 37°C and at PH 7.3 and were corrected for total protein content which was determined using a detergent compatible protein determination assay (Bio-Rad).

Supplemental figures

Figure S1. CVB3-induced VM in mice. C3H mice were subjected to CVB3-induced VM (n=12) or sham treatment (n=5). The heart and spleen were collected and weighed and QPCR analysis was applied to determine viral load in the heart (A). Immunohistochemistry revealed massive cardiac immune cell influx (B). Cardiomyocytes were isolated from mice with CVB3-induced VM (n=8) and sham-treated animals (n=8) and CVB3 load was determined (C). * $p \leq 0.05$ compared to control.

Figure S4. Pathway analysis of fatty acid β -oxidation in human VM. Red indicates upregulated and green indicates downregulated mRNA levels.

Figure S5. Pathway analysis of glycolysis in human VM. Red indicates upregulated and green indicates downregulated mRNA levels.

Figure S6. CVB3-induced gene expression in cardiomyocytes. Rat neonatal cardiomyocytes were cultured and stimulated with CVB-3 virus at a multiplicity of infection (MOI) of 1.0 for the indicated times. QPCR analyses revealed increased expression of genes involved in viral uptake and replication (A-C) while genes involved in oxidative phosphorylation and fatty acid β -oxidation were unaltered or increased (D, E). * $p \leq 0.05$, # ≤ 0.1 compared to control.

Figure S7. NF-κB activation in cardiomyocytes during CVB3-induced VM. C3H mice were subjected to CVB3-induced VM ($n=12$) or sham treatment ($n=5$). QPCR analysis on intact hearts revealed increased expression of inflammatory mediators in the myocardium (A). Cardiomyocytes were isolated from mice with CVB3-induced VM ($n=8$) and sham-treated animals ($n=8$) and QPCR analyses revealed increased expression levels of constituents and known target genes of the NF-κB pathway (B). * $p \leq 0.05$ compared to control.

Figure S8. Activation of NF- κ B signalling impairs cardiomyocyte oxidative gene expression. HL-1 cardiomyocytes were stimulated with TNF- α (10 ng/ml) for 48h and expression levels of ICAM-1 were determined (A). HL-1 cardiomyocytes were subjected to adenoviral over-expression of IKK β or an adenoviral construct expressing GFP as a control for 48h. IKK β protein was increased and NF- κ B transcriptional activity as well as ICAM-1 mRNA expression was induced (B). Rat neonatal cardiomyocytes were cultured and infected with an adenovirus over-expressing IKK β (Ad-IKK β) for 48h. QPCR analyses were performed and revealed increased inflammatory gene expression (C) and decreased transcript abundance of components of the oxidative phosphorylation chain (D) and constituents of PGC-1 signalling (E). Luciferase activity was corrected for total protein content. Western blots were corrected for tubulin # $p \leq 0.1$, * $p \leq 0.05$ compared to control.

Supplemental tables

Table S1: Clinical characteristics of VM patients.

	Controls	VM
Age	34.2 ± 3.0	28.3 ± 0.33
Ejection Fraction (%)	56.0 ± 3.4	24.3 ± 4.8 *
End Diastolic Dimension	56.6 ± 2.3	53.7 ± 0.3
End Systolic Dimension	39.8 ± 2.4	47.7 ± 1.3 #
Interventricular Septum	8.2 ± 0.5	10.3 ± 0.9 #
Wall Thickness	8.1 ± 0.6	10.0 ± 0.6 #
PVB19 load	77.3 ± 36.3	3105.6 ± 140.9 *
CD45 (/mm ²)	6.2 ± 1.1	27.5 ± 11.6 *

Table S2: Top 10 enriched pathways in VM patients.

Pathway	Enriched	Z Score	p-value
1. Electron Transport Chain	66.67%	8.88	0
2. Oxidative phosphorylation	68.75%	7.02	0
3. Pathogenic Escherichia coli infection	58.49%	5.65	0
4. Fatty Acid Beta Oxidation	54.55%	3.93	0
5. TCA Cycle	64.71%	3.78	0
6. Mitochondrial LC-Fatty Acid Beta-Oxidation	66.67%	3.73	0
7. Fatty Acid Biosynthesis	59.09%	3.7	0
8. TSH signaling pathway	43.94%	3.57	0
9. Focal Adhesion	35.33%	3.28	0.003
10. miR-targeted genes in squamous cell - TarBase	36.75%	2.96	0.003

Table S3: Top 10 enriched pathways in CVB3-induced VM in mice.

Pathway	Enrichment	Z Score	p-value
1. Electron Transport Chain	91.30%	5.51	<0.001
2. Oxidative phosphorylation	97.50%	4.98	<0.001
3. MAPK signaling pathway	78.08%	4.78	<0.001
4. XPodNet - protein-protein interactions in the podocyte	66.15%	4.5	<0.001
4. PodNet: protein-protein interactions in the podocyte	70.71%	4.27	<0.001
5. Insulin Signaling	74.83%	4.04	0.001
6. Apoptosis	81.08%	3.91	<0.001
7. TCA Cycle	93.33%	3.84	0.001
8. Chemokine signaling pathway	73.05%	3.78	<0.001
9. IL-5 Signaling Pathway	80.88%	3.71	<0.001
10. MicroRNAs in Cardiomyocyte Hypertrophy	79.22%	3.65	0.001

Table S4: Correlations in gene expression levels in hearts of mice with CVB3-induced VM (n=12).

	TNF-α	MCP-1	RelA	IκBα
% inflammatory cells	R ² = 0.892 P ≤ 0.000	R ² = 0.923 P ≤ 0.000	R ² = 0.911 P ≤ 0.000	R ² = 0.863 P ≤ 0.000
PGC-1α mRNA	OXPHOS Complex II R ² = 0.854 P ≤ 0.000	ACAD-VL R ² = 0.956 P ≤ 0.000	Glut1 R ² = - 0.861 P ≤ 0.000	PFKFB3 R ² = - 0.810 P ≤ 0.001
HIF-1α mRNA	OXPHOS Complex II R ² = - 0.704 P ≤ 0.011	ACAD-VL R ² = - 0.916 P ≤ 0.000	Glut1 R ² = 0.989 P ≤ 0.000	PFKFB3 R ² = 0.916 P ≤ 0.000

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MicroRNAs act as pleiotropic regulators of heart failure progression

A high-throughput phenotypical screen in three primary cardiac cell types

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In preparation

Abstract

MicroRNAs (miRNAs) have been shown to regulate pathophysiological processes underlying heart failure development, including hypertrophy and fibrosis. Recently, inflammatory miRNAs have been identified to be involved in cardiac hypertrophy and remodelling. The present study aimed to screen the relatively unknown role of a large number of inflammation-related miRNAs in cellular processes underlying cardiac failure. Based on microarray and RNA deep sequencing analyses in cardiac left ventricular samples of rodents and human, we selected 194 miRNAs that were differentially expressed in, and possibly related to, the inflammatory component of cardiomyocyte hypertrophy, fibroblast-mediated collagen production, and macrophage polarization. Following transfection with these miRNA mimics and by means of automated microscopy we quantified: (a) cell size of cardiac myocytes, (b) collagen 1 α 1-positive area in cardiac fibroblast and (c) morphological features associated with polarization status (cell roundness) and nuclear translocation of the NF κ B subunit p65 in primary macrophages. Of the tested miRNAs, 13% significantly modulated cardiomyocyte size (hypertrophy) by more than 2x the standard deviation of controls under unstimulated conditions. Using similar criteria, 32% of the tested miRNAs modulated collagen production by cardiac fibroblasts and 41% modulated macrophage polarization. Notably, 16 miRNAs significantly affected all three cellular processes, including miRNAs with established (miR-199-3p, miR-133b) and unknown (miR-200c-3p, miR-891a-3p) roles in cardiac pathophysiology. In conclusion, we identified roles for previously uncharacterized miRNAs in cardiac remodelling and were able to confirm known and attribute new effects to various more well-known miRNAs, thereby opening new avenues for the treatment of heart failure via miRNA-directed therapeutic approaches.

Introduction

Heart failure (HF), with its high prevalence and mortality rates ¹, is a huge societal burden ². The development of HF is characterized by hypertrophy of cardiac myocytes and excessive extracellular matrix deposition by fibroblasts. More recently it was recognized that HF is associated with a persistent pro-inflammatory state, characterised amongst others by infiltration of activated pro-inflammatory macrophages and polarisation of these macrophages towards a pro inflammatory (M1) phenotype ³. This diverse spectrum of pathological events marks the complexity of HF development.

Specific microRNAs (miRNAs) have been shown to regulate the pathological processes underlying HF development ⁴⁻⁶. MiRNAs represent a class of small (± 22 nucleotides in length) non-coding RNA molecules that negatively regulate target gene expression ^{7, 8}. MiRNA expression levels vary between cell types and have been shown to change under pathological conditions, including cardiac disease ⁹. Recent studies indicate that the effect of miRNAs is not restricted to the cell type(s) in which they are expressed, as they are secreted via extracellular vesicles. In this way, miRNAs can act as paracrine signalling molecules on neighbouring cells ¹⁰⁻¹². Thus, despite various miRNAs associated with HF development being identified, our understanding of their regulating potential and the cardiac cell types in which they exert their function remains incomplete.

Thus, based on previous, published and unpublished studies from our group, we selected 194 miRNAs that were differentially expressed primarily in hearts of HF patients and in different animal models of inflammatory cardiac diseases such as viral myocarditis and acute cardiac rejection ^{6, 13}. Next, we developed a high-throughput phenotypic screening platform to assess their regulatory function in both cardiac myocytes, fibroblasts and macrophages. MiRNA mimics were transfected into primary cultures of neonatal rat cardiac myocytes (nRCMs) and fibroblasts (nRCFs) to test their effects on the hypertrophic and fibrotic response, respectively. In addition, we screened these miRNAs for their influence on the polarization and activation of mouse bone marrow-derived macrophages (BMDMs). The simultaneous testing of this subset of miRNAs in different cell types under unstimulated and stimulated conditions allowed us to assess the role of these miRNAs in the different cellular processes underlying HF development in an integrative manner and to unravel pleiotropic effects of multiple miRNAs.

Materials and Methods

miRNA selection

miRNA expression profiling of patients diagnosed with viral myocarditis (VM), patients which underwent cardiac transplantation (HTX), and three cardiac disease animal models: Cocksackievirus B3 induced-VM, mouse HTX, and the ZSF1 model, resulted in the identification of 194 differentially expressed miRNAs. Several identified miRNAs showed an overlap between the different studied cardiac disease models. Both miRNA strands were included in the screen when the miRNA profiling data did not specify which miRNA strand (3-p/5-p) was measured. Supplemental Table 1 shows all miRNAs selected for *in vitro* validation based on a significantly altered expression in one or more cardiac disease animal models, indicating a possible regulatory role in cardiac disease progression and development.

Isolation and culture of primary cells

Rat neonatal ventricular cardiac myocytes (nRCMs) and rat neonatal cardiac fibroblasts (nRCFs) were isolated by enzymatic dissociation from the hearts of 1-3 day old Wistar rats and cultured as described previously¹⁴. Briefly: After decapitation, neonatal rat pups were dissected using a cut through the sternum and hearts were collected under semi-sterile conditions. Subsequently, atria were removed and the ventricles were divided into 8-12 equal parts. Ventricles were consecutively digested with a mixture of 0.3 mg/ml Collagenase (Sigma #C2674) and 0.3 mg/ml Pancreatin (Sigma # P3292) in ADS buffer (120 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.5 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 20 mM HEPES, 5.6 mM Glucose, pH 7.35) at 37°C. Every 20 min the supernatant was collected, suspended in 10% NBCS, and 10 ml fresh enzyme solution was added to the residual tissue. After 5 rounds of incubation, cell solutions were pooled, centrifuged, and re-suspended in nRCM plating medium (DMEM 11966, 17% M199 medium, 10% HS, 5% NBCS) and pre-plated onto 162cm Corning Costar cell culture flasks (Sigma #CLS3151). After incubation in a humidified 37 °C /5% CO₂ incubator for 1 h, the supernatant, containing mainly nRCMs, was collected and cells therein were counted manually. Adherent nRCFs remaining in the flask were cultured for 2 more days in nRCF medium (DMEM 22320, 10% FBS).

Bone marrow-derived macrophages (BMDMs) were generated as previously described¹⁵, in brief: Bone marrow cells were isolated from 12 week old C57BL/6-N mice and cultured in RPMI supplemented with 15% L929 conditioned medium (containing M-CSF) in petri dishes for 7 days to generate BMDMs. Cells were lifted for plating at day 8 using cell scrapers.

Culture, transfection and stimulation of primary cell culture.

nRCM: nRCMs were seeded into 1% gelatin coated 96-well black, clear bottom, culture plates (Corning #3603) at a density of 40,000 cells/well in 100µl seeding medium. Prior to cell seeding, 0.171µl Lipofectamine 2000 (Invitrogen #11668-019) transfection reagent (diluted 700x in OPTIMEM) was added to the wells in combination with mirVana mimics (Life-Technologies) according to manufacturer's protocol to end up with a final concentration of 10nM. The day after isolation, cells were washed and medium was replaced for 100µl serum-free experimental medium (DMEM 11966, 20% M199 medium) to starve cells for 24h hours. After starvation, cells were stimulated for 72 hours with 5µM phenylephrine (PE) (Sigma #P6126) or treated with PBS as control.

nRCF: nRCFs were lifted using trypsin/EDTA 48 hours after isolation, washed, centrifuged and seeded into uncoated 384 wells µclear plates (Greiner #781092) in 30 µl RCF medium containing 10% FBS. A seeding density of 2000 cells/well was used. After 24h and prior to transfection, cells were washed extensively using an automated washer dispenser (EL406, BioTek) and 30 µl low serum (0.1% FBS) nRCF medium was added. Lipofectamine RNAiMax (Invitrogen) was mixed with OPTIMEM and mirVana mimics (Life-Technologies) according to manufacturer's protocol and 5 µl of the mix was added to the wells, to give a final concentration of 20 nM for mimics and a dilution factor of RNAiMAX of 700x. After 24h of starvation, cells were stimulated with 10 ng/ml TGFβ1 (Peprotech hum. Rec TGFβ1 # 100-21) or vehicle (PBS).

BMDM: BMDMs were lifted using a scraper after 8 days of differentiation. Subsequently, cells were counted and seeded at a density of 2000 cells/well into uncoated 384 wells µclear plates (Greiner #781092) in 30 µl BDMD medium. After 24h, medium was refreshed prior to transfection. Viromer Green (Lypocalyx, Halle, Germany) was mixed with Buffer F and

mirVana mimics (Life-Technologies) according to manufacturer's protocol and 5 μ l of the mix was added to the wells, to give a final concentration of 20 nM mimics and a dilution factor of 1800x for the transfection reagent. 24h after transfection, cells were stimulated with 20 ng/ml IL-4, 20 ng/ml IFN γ (Peprotech), 50 ng/ml LPS (Sigma) or vehicle (PBS).

Immunostaining and Microscopy

All cell types were fixed with 4% paraformaldehyde (45 min) and treated with blocking buffer (15% FBS, 0.3% Triton X-100, 0.03% Saponin in PBS) for 45 min. nRCMs were treated with permeabilisation buffer (3% Triton X-100) for 10 minutes preceding blocking procedure. After blocking, nRCMs were incubated for 3 hours at 37°C with the primary antibodies mouse anti- α -Actinin (1:1000, Sigma, #A7811), and rat anti- α -Tubulin (1:1500, Serotec #MCA77G). nRCFs were treated with rabbit anti-Collagen 1 α 1 (1:500, Abcam #AB34710) and rat anti- α -Tubulin (1:750) antibody overnight at 4°C. BMDMs were treated with rabbit NF κ B p65 antibody (1:300 Santa Cruz #sc-372) and rat anti- α -tubulin (1:750) overnight at 4°C. After primary antibody incubation, nRCMs and nRCFs were incubated with secondary antibodies goat anti-rat AlexaFluor555 (1:750, Life Technologies, #A11006), goat anti-mouse AlexaFluor488 (1:500, Life Technologies, #A11001) and Hoechst (1:6000, Invitrogen #H3570) for 1 hour at room temperature. BMDMs were incubated with secondary antibodies goat anti-rat AlexaFluor555 (1:500), goat anti-rabbit AlexaFluor488 (1:750, Life Technologies, #A11008) and Hoechst for 1 hour at room temperature.

Acquisition & image analysis

For the screening experiments, image acquisition was performed using an ImageXpress Micro automated high-content screening fluorescence microscope (Molecular Devices) at $\times$ 10 magnification with FITC, CY3 and DAPI filter blocks. For the hypertrophy, fibrosis, and inflammation screen, a total of respectively 25, 16, and 9 images were acquired per wavelength per well.

Image analysis was performed using the eCognition software (Definiens, platform (Munich, Germany). Number of nuclei was determined based on Hoechst staining for all three cell types. nRCM and nRCF cell size was quantified using an algorithm that recognized α -tubulin stained area. For nRCFs, collagen1 α 1 positive area was quantified as a percentage of cell area. For BMDMs, cell roundness was determined as a percentage of cells detected with a length–width ratio larger than threshold. NF κ B nuclear translocation was based on p65 positive area in nucleus (Hoechst) and cytoplasm (α -tubulin stained area) and expressed as ratio of nuclear/cytoplasmic p65 staining.

Data analysis and statistics

For all analysed miRNA samples, mean and standard deviation of individual wells over 3 replicate plates was determined. Inter-plate variability was corrected by scaling each data point to the corresponding negative control mean (negative control mimic transfected cells). The effect of miRNA transfection on different read outs was determined via calculation of the log₂ fold change of the sample mean over the negative control mean within the same condition and statistically tested using an unpaired T-Test.

In all three screens, identification of miRNA mimic-induced phenotypical changes was based on a statistically significant ($p < 0.05$) sample mean log₂ fold change of the main read out over negative control, deviating more than 2x the STDEV from the negative control mean. The main readouts per screen were cell size for the hypertrophy screen, number of

nuclei and collagen content for the fibrosis screen, and cell roundness and NFκB nuclear translocation for the inflammation screen. Only hit selection for the NFκB nuclear translocation read out in the inflammation screen was based on statistically significant mean $\log_2$ fold change over negative control, deviating more than 1x the STDEV from the negative control mean.

Results

We assembled a library of 194 miRNA mimics based on their differential expression in the hearts of different animal models of inflammatory cardiac disease, patients diagnosed with viral myocarditis, and patients undergoing cardiac transplantation (Supplemental table 1). To screen for involvement of these miRNAs in cellular processes driving HF, this HF-associated miRNA mimic library was overexpressed in three primary cell types held accountable for pathological cardiac remodelling (Figure 1).

Figure 1. Phenotypical screening using a HF-associated miRNA mimic library was performed in parallel for three different HF-underlying processes using automated image analysis. Effects induced under control and HF-mimicking conditions by miRNA mimics on hypertrophy, fibrosis and inflammation were studied using nRCMs, nRCFs, and BMDMs respectively. Read-outs were cardiac myocyte size, cardiac fibroblast number and collagen content, and macrophage roundness and NFκB translocation.

Hypertrophy screen

MiRNA mimics were transfected into unstimulated and PE-stimulated neonatal rat cardiac myocytes (nRCMs) and cell size was quantified to determine their effect on the hypertrophic response. PE stimulation led to cardiomyocyte hypertrophy, characterized by a 0.30 log₂ fold increase in cell size and increased transcript levels of hypertrophic markers ANF and BNP (Supplementary figure 1). Functional effects induced by transfection of miRNA mimics in nRCMs was first verified using the established anti-hypertrophic miRNA miR-1⁴. Transfection with a miR-1 mimic resulted in a significant reduction of cell size, a 42% reduction in mRNA expression level of direct target CALM2, and prevented the PE-induced upregulation in transcript levels of ANF and BNP (Supplementary figure 1).

The effect of each miRNA on nRCM cell size under unstimulated conditions, relative to negative control-mimic transfected cells (further referred to as negative control), is presented in a volcano plot (Figure 2A). Three miRNAs, miRNA-200c-3p, miR-125a-5p, and miR-92b-3p, significantly increased cell size with a log₂ fold change greater than 2x STDEV of negative control, and 22 miRNAs were found to reduce cell size with a similar fold change

(Figure 2A). Among the latter, miR-486-3p, miR-145-3p, and our positive control miR-1 reduced cell size significantly.

Figure 2. Hypertrophy screen identifies miRNAs that affect cardiac myocyte cell size. Panel A-B shows $\log_2$ fold changes in cell size compared to negative control without (panel A) or with (panel B) PE stimulation. MiRNA mimics that induce a significant effect on cell size, deviating more than 2x STDEV from negative control (indicated by vertical lines) are highlighted in red or blue. The PE-induced increase in cell size after 72h is represented in green in panel A. Panel C shows representative images of the miRNAs with most pronounced effect on cells size compared to negative control, miR-103b and miR-125a-5p. Panel D shows the effect of individual miRNA mimics on cardiac myocyte cell size under both unstimulated (control) and stimulated (PE) conditions. Transfection of mimics for miR-151-3p and -5p reduced cell size under PE-stimulated conditions without having an effect under control conditions (shown in blue). Values represent cardiomyocyte cell size expressed as $\log_2$ fold change, normalised to negative control mimic-transfected cells within the same condition.

PE stimulation triggered the hypertrophic response in nRCMs, inducing a 0.30 log₂ fold increase in cardiac myocyte cell size (Figure 2A). Under PE-stimulated conditions, 8 miRNAs induced an additional significant increase in cell size compared to PE-stimulated negative control. However, none of these miRNAs induced an increase in cell size deviating more than 2x STDEV of negative control (Figure 2B). In contrast, 31 miRNAs were found to significantly reduce the PE-induced increase in cardiac myocyte cell size by more than 2x STDEV of negative control, among which miR-103b, miR-486-3p, miR-151a-3p, miR-145-3p, and miR-891a-3p (Figure 2B). The top 5 miRNAs identified to increase or decrease cardiac myocyte cell size under control conditions are depicted in table 1. Effects induced after miRNA mimic transfection on cell size are shown for both control and PE-stimulated conditions. Combining the effect of the individual miRNA mimics on cardiac myocyte cell size under both unstimulated (control) and stimulated (PE) conditions (Figure 2D) reveals that miR-125a-5p and miR-103b have the most pronounced effect on cell size independent of PE stimulation. Interestingly, both miR-151a-3p and miR-151a-5p decreased cardiac myocyte cell size only in combination with PE-treatment (Figure 2D, shown in blue).

Table 1. Top 5 miRNAs able to increase or decrease cardiac myocyte cell size.

miRNA	Control		PE-stimulated	
	Cell size (Log ₂ Fc)	p-value	Cell size (Log ₂ Fc)	p-value
miR-200c-3p	0,437	0,002	0,200	0,047
miR-125a-5p	0,374	0,001	0,351	0,000
miR-92b-3p	0,339	0,001	0,108	0,441
miR-103b	-0,761	0,001	-0,814	0,003
miR-486-3p	-0,540	0,001	-0,757	0,000
miR-103a-3p	-0,513	0,003	-0,411	0,010
miR-1	-0,500	0,010	-0,260	0,058
miR-214-3p	-0,494	0,005	-0,509	0,001

Table shows the p-value and log₂ fold change in cardiac myocyte cell size under control and PE-stimulated conditions. Only three miRNA mimics increased cell size with more than 2x STDEV of negative control.

Fibrosis screen

To determine the effect of the HF-associated miRNAs on the fibrotic response, collagen production and proliferation of neonatal rat cardiac fibroblasts (nRCFs) were quantified. Transfection of nRCFs with a reported anti-fibrotic control mimic, miR-29b⁵, resulted in a decrease of 93% in transcript levels of direct target collagen-1α1 (COL1α1) (Supplementary figure 2A), confirming the functional effects of introducing the miR-29 mimic. To initiate a pro-fibrotic response cardiac fibroblasts were stimulated with TGFβ. This resulted in a significant upregulation of transcript levels of the fibrosis marker connective tissue growth factor (CTGF) (Supplemental figure 2B), accompanied by an increase in collagen positive area (Figure 3A). Figure 3A shows the log₂ fold change in COL1α1 positive stained cell area (further referred to as collagen content) for each mimic relative to negative control. A large number (51) of miRNA mimics significantly increased the collagen content, including miR-379-3p, miR-151a-5p, and miR-30d-5p. Twelve miRNA mimics decreased collagen content by more than 2x STDEV of negative control, among them are miR-199a-5p (-2.045 p=0.001), miR-103a-2-5p (-1.647 p=0.021) and several members of the miR-29 family: miR-29a-3p (-2.238

p=0.009), miR-29b-3p (-2.381 p=0.000), and miR-29c-3p (-2.163 p=0.000) that are known to target several collagen subtypes ⁵, including COL1 α 1.

Figure 3. Effect of miRNA mimics on cardiac fibroblast collagen content and number of nuclei upon transfection. *Vulcano plots showing collagen content and number of nuclei for individual miRNA mimics without (panel A and B) or with (panel C and D) TGFβ stimulation for 72 hours, expressed as log₂ fold change compared to negative control mimic-transfected nRCFs. MiRNA mimics that increase or reduce collagen content are highlighted in red or blue, respectively. As a point of reference, the effects that are reached by 72 hours of TGFβ stimulation of untransfected controls are represented as green triangles in both panels A and B. Panel E shows representative images of miR-199a and miR-379-3p.*

Figure 3B shows the effect of each individual miRNA mimic on the number of nuclei of cardiac fibroblasts. A large fraction of the tested miRNA mimics was able to significantly increase (22) or decrease (95) the number of nuclei with more than 2x STDEV. Interestingly,

41 miRNA mimics were able to alter both cardiac fibroblast collagen content and number of nuclei under baseline conditions. Among these are members of the miR-29 family known for their anti-fibrotic effects ^{5, 16}.

Table 2. Top 5 miRNAs able to increase or decrease collagen content.

miRNA	Control		TGFβ-stimulated	
	Collagen content (Log ₂ f.c.)	p-value	Collagen content (Log ₂ f.c.)	p-value
miR-152-5p	3,141	0,000	2,513	0,000
miR-7-5p	3,086	0,001	2,419	0,001
miR-497-5p	2,325	0,000	2,273	0,000
miR-17-5p	1,966	0,001	2,029	0,001
miR-1	1,889	0,001	2,217	0,001
miR-29b-3p	-2,381	0,000	-2,232	0,004
miR-199a-5p	-2,110	0,000	-2,224	0,087
miR-103a-2-5p	-1,711	0,000	-1,843	0,001
miR-216a-5p	-1,598	0,000	-0,330	0,459
miR-891a-3p	-1,426	0,000	-0,983	0,001

Log₂ fold change in collagen content under control and TGFβ-stimulated conditions and p-values are shown.

Under TGFβ-stimulated conditions, our screen revealed 47 and 10 miRNAs to significantly increase or decrease collagen content, respectively (Figure 3C). In the presence of TGFβ, 20 miRNA mimics increased and 45 decreased the number of nuclei by more or less than 2x STDEV (Figure 3D). The same as under unstimulated conditions, miR101-3p significantly decreased the number of nuclei in nRCFs with more than 2x STDEV in the presence of TGFβ. Mir-101 was shown to inhibit post-infarct cardiac fibrosis by decreasing the proliferation of fibroblasts via targeting Fos ¹⁷. Table 2 lists the top 5 miRNAs with the most pronounced effect on cardiac fibroblast collagen content in both directions. For each miRNA, their effect on collagen content and on the number of nuclei is shown under control and TGFβ-stimulated conditions.

Macrophage polarisation and activation screen in BMDMs

Lastly, we evaluated the effect of the miRNA mimics on macrophage polarisation and activation. Transfection of BMDMs with GAPDH siRNA resulted in a decrease of 93% in GAPDH transcripts levels (Supplementary figure 3A), confirming successful transfection of BMDMs. The effect of individual mimics on macrophage polarisation was quantified under unstimulated (M0), IFNγ (M1) and IL-4 (M2) stimulated conditions. Morphological appearance of macrophages has been shown to be a marker of polarization status in BMDMs ¹⁸, with M1 (pro-inflammatory) macrophages showing a round and M2 (anti-inflammatory) polarized macrophages showing an elongated phenotype. Stimulation of BMDMs with IFNγ resulted in a 27% increase in the number of round classified cells and IL-4 stimulation resulted in a 21% increase in elongated classified cells (Figure 4A). In unstimulated macrophages, four miRNA mimics significantly decreased macrophage roundness with more than 2x STDEV, these are: miR-488-5p, miR-34a-3p, miR-891a-3p, and miR-128-3p. A large number (76) of miRNAs significantly increased roundness (Figure 4A), among them miR-200c-5p, miR-148b-3p, and miR-155-5p, which is known to induce M1 polarisation ¹⁹. Comparable

results were found for macrophages polarised towards M1 (IFN γ) and M2 (IL-4) (Supplemental figure 3B-C). In total, 58 miRNAs were able to significantly alter macrophage roundness in all three polarization conditions. Remarkably, only 2 miRNAs were identified to promote macrophage elongation irrespective of the polarization stimuli, while 56 miRNAs increased macrophage roundness. MiR-155, a well-known pro-inflammatory miRNA, showed a pronounced and significant increase in roundness in all conditions. The top 5 miRNAs able to promote or decrease macrophage roundness, as identified under control conditions, along with their effect on NF κ B nuclear translocation, are shown in Table 3.

Figure 4. Inflammation screen identifies many miRNAs affecting macrophage roundness and NF κ B nuclear translocation. Volcano plots showing log₂ fold change in cell roundness and NF κ B nuclear translocation in control conditions. MiRNA mimics that significantly increased or reduced these readouts according to previously described criteria are highlighted in red or blue, respectively. In panel A, polarization effects on cell roundness in negative control mimic-transfected cells induced by IFN γ and IL-4 stimulation are represented as yellow and green triangles respectively. In panel B, LPS-induced effect on NF κ B nuclear translocation in negative control mimic-transfected cells is represented as a green triangle. Panel C shows representative images of miR-488-5p and miR-146a-3p with pronounced effects on macrophage roundness.

Table 3. Top 5 miRNAs able to increase or decrease macrophage roundness.

miRNA	Roundness (Log ₂ f.c.)	Control		
		p-value	NFκB (Log ₂ f.c.)	p-value
miR-146a-3p	0,404	0,005	0,171	0,212
miR-200c-5p	0,392	0,009	0,154	0,152
miR-30a-3p	0,380	0,002	0,893	0,116
miR-148b-3p	0,378	0,005	0,215	0,053
miR-218-5p	0,378	0,001	0,379	0,121
miR-488-5p	-0,343	0,002	-0,179	0,130
miR-34a-3p	-0,317	0,011	-0,22	0,028
miR-891a-3p	-0,281	0,003	0,05	0,669
miR-128-3p	-0,214	0,014	-0,174	0,101

The table shows the p-value and log₂ fold change in macrophage roundness and NFκB nuclear translocation under control conditions. Only four miRNA mimics reduced roundness with more than 2x STDEV of negative control.

The effect of individual mimics on the activation of BMDMs was quantified using an NFκB nuclear translocation assay either stimulated with- or in absence of LPS. Activation of negative control mimic-transfected BMDMs with LPS resulted in a significant 0.66 log₂ fold increase in NFκB nuclear translocation (Figure 4B). Only miR-30c-2-3p and miR-34a-3p were identified to significantly decrease NFκB nuclear translocation with more than 1x STDEV, while 13 miRNAs were found to significantly increase NFκB nuclear translocation by more than 1x STDEV log₂ fold, including miR-210-3p, miR-590-5p, miR-96-5p, and miR-152-3p. MiR-146b-5p, a positive regulator of the NFκB pathway²⁰, induced the strongest increase in NFκB nuclear translocation. Supplemental figure 3E shows the log₂ fold change in NFκB nuclear translocation for each individual miRNA mimic in macrophages activated with LPS.

Pleiotropic effects of HF-associated miRNAs

The vast majority of miRNAs (86%) affected at least one of the HF-related outcome parameters (Figure 5A). Of the total miRNA-library, 27% of the selected miRNAs significantly altered cardiac myocyte size, more than half (56%) of the tested miRNA mimics were able to significantly affect cardiac fibroblast collagen content, and 43% was identified as modulators of macrophage roundness. The overlap between the different processes is substantial, marking the relatively high number of miRNAs (32%) affecting more than one process. Notably, 16 miRNAs influenced each of the HF-underlying processes to a significant extent (Table 4). Several interesting miRNAs are highlighted in figure 5B, visualizing their effects on all main readouts, illustrating the direction and magnitude of change for each of the studied processes. For example, miR-200c-3p was able to significantly increase cardiac myocyte cell size (0.44 log₂ fold change), fibroblast collagen content (0.88 log₂ fold change), and macrophage roundness (0.375 log₂ fold change). The effects of each individual miRNA mimic in all three screens are shown in supplemental table 1.

Table 4. MiRNAs affecting cardiac myocyte cell size, fibroblast collagen content, and macrophage elongation in parallel.

miRNA	Hypertrophy screen	Fibrosis screen	Inflammation screen
	Cell size (Log ₂ f.c.)	Collagen content (Log ₂ f.c.)	Roundness (Log ₂ f.c.)
let-7c-3p	-0,253	-1,069	0,338
miR-103b	-0,761	0,586	0,310
miR-133b	-0,403	0,819	0,191
miR-145-3p	-0,392	1,025	0,283
miR-152-5p	0,235	3,206	0,291
miR-181b-5p	-0,269	0,975	0,278
miR-199b-5p	-0,235	-1,310	0,239
miR-200c-3p	0,437	0,940	0,375
miR-210-3p	0,285	0,646	0,273
miR-212-5p	-0,375	0,849	0,277
miR-29a-3p	0,195	-2,238	0,085
miR-34a-3p	-0,228	0,773	-0,317
miR-497-5p	-0,443	2,390	0,077
miR-499a-5p	-0,175	-0,651	0,363
miR-891a-3p	-0,482	-1,361	-0,281
miR-9-5p	-0,404	1,406	0,250

Data are expressed as average log₂ fold change in cardiac myocyte cell size, cardiac fibroblast collagen content, and macrophage roundness under non-stimulated control conditions. All 16 miRNA affected the log₂ fold change of these readouts significantly with more than 2x STDEV of negative control.

Figure 5. miRNAs affect multiple HF-related processes. A. The majority (86%) of the miRNAs mimics affect at least one pathological process. Included read outs are cell size, collagen content, and cell roundness, for the hypertrophy, fibrosis, and inflammation screen, respectively. B. Panel B visualises the effect of a selection of miRNA mimics based on their ability to affect all main read outs or based on the usage as known HF-regulating control miRNA (miR-1 and miR-29b-3p). Bar graphs are made relative to the most pronounced miRNA-induced effect per readout. Bars are shown as Mean with SD of relative $\log_2$ fold change * $P < 0.05$ compared to negative control.

Discussion

In the present study, high-throughput phenotypical screening in three different primary cell types was applied to explore the effect of nearly 200 HF-associated miRNAs on three pivotal processes underlying HF development, namely hypertrophy, fibrosis, and inflammation ¹. Novel functions of well-studied miRNAs and less well-known miRNAs putatively involved in HF progression were identified. This comprehensive screening approach unveils the concerted actions of various miRNAs in cardiovascular disease and opens new avenues for the treatment of HF via miRNA-directed therapeutic approaches.

Previously phenotypical screens of miRNAs focussed on a single process, either on myocyte hypertrophy ²¹, inflammation ²², or cell proliferation ²³. These studies often made use of immortalised cell lines, like HL-1 cardiomyocytes and RAW 264.7 macrophages, that have lost some of their cell-type specific characteristics ^{24, 25}. Furthermore, most studies only screened under basal conditions, not including stimuli that mimicked pathological processes ²⁶. By simultaneously screening three primary cell types under basal conditions as well as after stimulation with relevant pathogenic factors, the current approach allows a better understanding of the role of these miRNAs in cardiac pathophysiology.

MiRNAs that regulate cardiomyocyte hypertrophy

We identified various miRNAs which yet have not been described to be involved in cardiac myocyte hypertrophy, and confirmed known anti- or pro-hypertrophic effects of miRNAs, such as miR-1 and miR-19a-3p (Figure 2, Table 1). By targeting calmodulin, miR-1 was shown to attenuate cardiomyocyte hypertrophy through modulation of calcium signalling ⁴. MiR-19a, on the other hand, has been shown to promote cardiac myocyte hypertrophy via targeting the anti-hypertrophic gene atrogin-1. Moreover, miR-19a plasma levels showed a positive correlation with plasma BNP and cAMP levels in HF patients ^{27, 28}. Identification of these well-characterized miRNAs serves as an internal validation of our screening approach.

Transfection of 25 miRNAs mimics into cardiac myocytes altered cell size under unstimulated conditions. MiR-125a-5p, miR-200c-3p, and miR-92b-3p increased cell size, suggesting a pro-hypertrophic action. Cardiac miR-125a-5p is significantly upregulated in hypertrophic and dilated cardiomyopathy patients ²⁹, and miR-92b levels are increased in sera of patients suffering from HF and coronary artery disease ^{30, 31}. Although these findings are suggestive for a role of these miRNAs in cardiac disease, prove for their actual involvement in the regulation of the hypertrophic response is provided in this study. Under PE-stimulated conditions, giving a 24% increase in cell size, 31 miRNAs mimics significantly reduced cell size, suggesting anti-hypertrophic properties. In contrast, none of the miRNAs induced an additional significant increase in cardiomyocyte size in excess of our predefined strict selection criteria of 2 times the standard deviation. Transfection with 13 miRNAs (including miR-148a-3p, miR-145-3p, miR-891a-3p, and miR-486-3p) significantly decreased cell size under both control and PE-stimulated conditions. MiR-486-3p had the most outspoken anti-hypertrophic effect, decreasing cardiomyocyte size by -0.54 and -0.76 log₂ fold under unstimulated and PE-stimulated conditions, respectively.

Interestingly, several miRNAs seemed to have a unique PE-dependent effect. Transfection of miRNA mimics for both miR-151a-3p and -5p did not change cardiomyocyte cell size under unstimulated conditions, but decreased cell size significantly following PE-treatment. This suggests that miR-151a inhibits the hypertrophic response by targeting genes involved in the alpha-adrenergic signalling pathway. A previously performed screen ²¹

identified miR-126 as anti-hypertrophic under basal conditions, while having no effect on cardiac myocyte cell size following PE treatment. In contrast, our screening approach identified miR-126-3p as anti-hypertrophic under both basal and PE-stimulated conditions. It is feasible that these differences are related to the higher dose (50 μ M) and/or shorter duration (48h) of PE treatment used by Jentzsch and co-workers.

MiRNA mimics affecting cardiac fibroblast collagen content and proliferation

Cardiac fibrosis is a well-established predictor of disease progression in HF ³². Cardiac fibroblasts (CFs) proliferate, differentiate towards myofibroblasts, and increase their collagen production during HF disease progression ³³. Cellular proliferation is a commonly used readout for high-throughput screens ²³, mostly in cancer-related cell types, but also in fibroblasts cell lines ³⁴. As cell numbers are equal at the start, nuclei count after 72h of incubation can be considered a surrogate readout for proliferation and apoptosis. Transfection with 22 miRNA mimics increased the number of nuclei. Amongst them are miR-148a-3p, miR-152-3p, miR-19a-3p, and miR-195-3p, suggesting these miRNAs increase the number of fibroblasts in the heart and thereby promote cardiac fibrosis.

Conversely 95 miRNA mimics, including miR-152-5p, miR-15a-5p, miR-16-5p, and miR-195-5p, reduced the number of nuclei, pointing to an anti-proliferative or pro-apoptotic effect of these miRNA's. In this way these miRNAs attenuate the development of cardiac fibrosis. Among the miRNAs with the most outspoken anti-proliferative effect are the members of the miR-148/152 family that were previously described as tumour suppressors ³⁵. MiR-16 which strongly reduced the number of fibroblasts in this screen, was shown to inhibit proliferation of 3T3 fibroblasts in an earlier study ³⁶.

To determine collagen production, we quantified the relative cell area positive for collagen 1 α 1 staining. Various miRNAs showed a pronounced increase in this readout (for instance miR-152-5p and miR-497-5p, miR-152-5p, miR-7-5p, miR-17-5p, miR-1). However, for some miRNAs (miR-152-5p and miR-497-5p) this was accompanied by a significant reduction in cell number, which may confound interpretation of their effect on fibrosis (see limitations of the study). MiRNA mimics that increased collagen content without significantly effecting cell number, are miR-17-5p, miR-181c-5p, and several miR-30 family members. In line, miR-30 was shown to downregulate CTGF, a key pro-fibrotic factor ³⁷. MiRNA mimics that reduced collagen content include miR-21-3p, known for its role in cardiac fibrosis ³⁸, all members of the miR-29 family known to target collagen production ⁵, and miR-24-1-5p, which was shown to reduce cardiac fibrosis after MI ³⁹.

MiRNA mimics regulating BMDM polarisation and activation

Here we present a novel approach for screening macrophage polarisation based on morphological features of BMDMs. Depending on their polarisation status, macrophages exhibit differences in morphology, cytoskeletal organization, functional characteristics, and migrating capacity ⁴⁰. We have screened for phenotypical changes that are specific for M1-M2 polarisation ^{18, 41}. Upon treatment with M1-polarizing stimuli (LPS and IFN γ) macrophages acquire a rounder shape, whereas M2-polarizing stimuli (IL-4) induce an elongated cell shape ^{18, 42}. The extent of cell roundness was shown to correlate with the expression levels of the M1 marker iNOS and cell elongation with expression levels of the M2 marker arginase-1 ¹⁸. Figure 4 confirms that IFN γ stimulation increased the number of round cells, while IL-4 stimulation promoted BMDM elongation (Figure 4 and supplemental figure 3). Although transfection itself induced mild activation of macrophages ⁴³, the screen is able to detect

both positive and negative regulators of macrophage polarisation. However, the sensitivity to detect round classified cells is higher as unstimulated cells have a more elongated morphology, resulting in physical limitations to induce an additional increase in elongation (Figure 4). A decrease in BMDM roundness, indicative of anti-inflammatory M2-polarization occurred after transfection of miR-488-5p, miR-891a-3p, miR-128-3p, and miR-34a-3p that was previously shown to target the IL-6 receptor ⁴⁴. Our screen identified 76 miRNAs inducing an increase in cell roundness related to pro-inflammatory M1 polarization, including miR-146a and miR-155-5p which have been linked to pro-inflammatory activation ^{6, 45, 46}.

Nuclear translocation of NFκB is a crucial step in the coupling of extracellular stimuli to the transcriptional activation of various pro-inflammatory cytokines ⁴⁷. Although the 24h time-point is optimal to detect morphologic changes such as cell roundness, quantification of NFκB nuclear translocation is a more acute process and earlier time-points could potentially be more informative. However, this approach allowed the quantification of more permanent effects since changes in NFκB nuclear translocation were still observed after transfection of miRNA mimics. Decreased nuclear NFκB was observed after transfection with miR-30c-2-3p and miR-34a-3p. MiRNAs increasing nuclear NFκB miR-146b-5p, miR-210-3p, miR-21-3p, and miR-590-5p. In line, silencing of miR-146a and miR-146b in dendritic cells resulted in increased NFκB activation via down-regulation of NFκB inhibitor IκBα ⁴⁸. Ding et al. ²² screened for miRNAs that modulate the NFκB signalling pathway upon TNF stimulation of HEK293 cells, by using a NFκB luciferase reporter. There is significant overlap in the miRNAs identified in their study and the miRNAs affecting nuclear translocation of NFκB in our screen.

Accordingly, this BMDM screen identified several novel miRNAs involved in macrophage polarisation. These results can be extrapolated to all inflammatory pathologies in which macrophages are playing crucial roles (e.g. atherosclerosis, rheumatoid arthritis). Therefore, the miRNAs identified in this screen may also be therapeutically relevant in other diseases with an inflammatory signature.

3p and 5p arms of mimics can show opposite effects

Passenger strands of miRNAs were previously widely neglected ⁴⁹, recently it was shown that most of them are equally functional as the main strand ⁴⁹ and levels were found to be enriched in secreted exosomes of cardiac fibroblasts ¹⁰. Both the -3p and -5p strand of many miRNAs were included in the screen, enabling determination of differences in functionality between both arms. Both miR-152-5p and miR-195-5p have pronounced effect on the number of nuclei and are well known suppressors of cell proliferation ⁵⁰. Strikingly, the -3p and -5p arms of these miRNAs have opposing effects on cardiac fibroblast nuclei counts. MiR-152-5p mimic transfected nRCFs have only 32% of the nuclei counts compared to control transfected cells, while miR-152-3p mimic transfected fibroblasts have 162% of the nuclei compared to control (Supplementary table 1). Similar effects are observed for miR-195 where the -3p arm mimic significantly increased the number of nuclei while transfection with the -5p lead to a significant decrease. Until now, only limited studies have reported opposing effects of both miRNA arms of miR-125a and miR-28 ^{51, 52}. We also observed opposing effects of miR-125a-3p (0.271 log₂ fold change, p=0.000) and -5p (-0.337 log₂ fold change, p<0.001) on proliferation of CFs. It is interesting to speculate on a biological role or mechanism, for example that one arm is destined to leave the cell and targets other cell types, or that it is a potential feedback mechanism to dampen the effect of a miRNA.

Pleiotropic effects of miRNAs

Combining the results of the three screens has led to identification of 16 miRNAs able to affect the main phenotypic read out in each individual cellular screen. For example, miR-210 was able to increase cardiac myocyte cell size, fibroblast collagen content, as well as macrophage roundness. Thus, miR-210 potentially plays an important role in aggravating HF development by promoting cardiac hypertrophy, fibrosis, and inflammation in three cell types simultaneously.

It is important to keep in mind that observed effects were induced after miRNA mimic-library transfection, not taking into account cell type dependent miRNA expression. However, miRNAs have recently emerged as regulators of intercellular communication during physiological and pathological processes in many organs, including the heart^{10, 53, 54}. Secreted extracellular vesicles (EVs) and particularly the exosomal fraction, can carry nucleic acids, including miRNAs, which can be transferred to acceptor cells and inhibit gene expression and regulate biological functions in these cells^{11, 12, 53, 54}. Bang et al.¹⁰ identified a miRNA/exosome-mediated communication mechanism between cardiac fibroblasts and myocytes, contributing to the development of cardiac myocyte hypertrophy. Fibroblast-derived exosomes were enriched with miR-21-3p, which subsequently was transported via exosomes towards neighbouring cardiac myocytes, mediating cellular hypertrophy. In addition, Barile et al.¹¹ showed that miR-210, identified in our screens as a miRNA able to induce significant morphological changes in all three studied cell types, is highly enriched in EVs secreted by cardiac progenitor cells and is able to inhibit apoptosis in HL-1 cardiac myocytes. In our hypertrophy screen, miR-210 was able to significantly increase cardiac myocyte cell size, suggesting that miR-210 potentially plays a role in cardiac myocyte survival and/or growth. Together, these findings suggest that miRNAs function as paracrine signalling mediators, making basal miRNA expression in a given cell type not a prerequisite for a cell-specific effect, since physiological or pathological inter-cell communication overcomes this.

Limitations of the study

In the present study we used human miRNA mimics for transfection into rat and murine primary cells. However, the effect of inter-species differences is limited as sequence analysis showed that 118 out of the 194 miRNAs display a 100% sequence match between human and rodent and only 9 miRNA mimics showed a mismatch in the seed sequence (Supplemental table 2), potentially leading to differences in their actual mRNA-targets.

Regarding the phenotypical readouts, it is important to note that miRNA mimic-induced phenotypical changes are not *per se* reflecting changes in pathological process. For example, an increase in cardiomyocyte size is not per definition related to pathological hypertrophy. This requires additional analysis, for instance via the measurement of *bona fide* hypertrophy markers, like atrial natriuretic factor or skeletal α -actin. This also applies to the relative Col1 α 1 positive fibroblast cell area, the main read out for fibrosis. As shown in the supplemental Figure 2 (panels C-D), various miRNAs gave rise to a significant reduction in cell number, often along with a reduction in cell size, which is reminiscent of a pro-apoptotic effect. The reduction in cell size translates into an increase in relative Col1 α 1 positive area per cell. Theoretically this may lead to the identification of false-positive pro-fibrotic miRNAs, once again indicating that additional analysis is required to classify such miRNAs as truly pro-fibrotic. In a similar way, it can be argued that macrophage roundness not necessarily reflects M1 polarization, as this could also be induced by miRNAs targeting genes involved in the arrangement of the cytoskeleton or apoptosis.

Finally, inclusion of miRNAs in our screen was based on their differential expression in hearts of different rodent models of cardiac disease, patients diagnosed with viral myocarditis, and patients undergoing cardiac transplantation as determined by micro-array analysis or by deep sequencing of small RNAs. Generally speaking, these cardiac diseases are characterized by a marked inflammatory component, resulting in a bias for miRNAs related to inflammation. Nonetheless, many of the selected miRNAs were shown to influence hypertrophy and fibrosis as well, implying that these three processes are intricately linked.

Conclusions

We performed a high throughput phenotypical screen in three relevant primary (cardiac) cell types, thereby identifying miRNAs involved in hypertrophy, fibrosis and inflammation. We conclude that our multi-cell type high-throughput screening approach of unstimulated and stimulated cells, allows in-depth analysis of the effect of HF-associated miRNAs on cardiac pathophysiology. Screening for hypertrophy, fibrosis, and inflammation simultaneously resulted in a complete description of miRNA-induced effects in the development of HF. In this way we confirmed roles of well-known miRNAs, but also identified many miRNAs new to the HF-research field. These miRNAs, especially the miRNAs that affected multiple HF-underlying processes, are interesting candidates for further in-depth investigation as therapeutic targets.

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Supplemental Figures and Tables

Supplemental Figure 1: A) RT-PCR analysis showed that transfection of miR-1 mimic in nRCMs resulted in decreased expression of direct target calmodulin 2 (CALM2). Unpaired t-test $**p<0.01$. Stimulation of nRCMs with PE resulted in upregulation of hypertrophic markers ANF (B) and BNP (C) and transfection of anti-hypertrophic miRNA miR-1 prevented the PE-induced upregulation of both ANF and BNP expression significantly. One-way ANOVA $***p<0.001$.

Supplemental Figure 2: A) RT-PCR quantification showed that transfection of nRCFs with a miR-29b mimic resulted in a significant decrease in mRNA expression of direct target COL1α1. B) Stimulation of nRCFs with TGFβ resulted in increased transcript levels of CTGF. Unpaired T-Test * $P < 0.05$. C-D) Several miRNAs gave rise to a substantial reduction in cell number and simultaneously reduced cell area, suggesting that these miRNAs promote apoptosis. This may have an impact on the relative Col1α1 positive area as well.

Supplemental 3: A) Transfection of BMDMs with a siRNA for Gapdh resulted in a 93% decrease in mRNA levels. Volcano plots showing log₂ fold change in cell roundness in macrophages polarised towards B) M1 (IFN γ) and C) M2 (IL-4) and in D) NF κ B nuclear translocation in LPS-stimulated conditions compared to negative control mimic transfected BMDMs. MiRNAs able to increase and decrease BMDM roundness and NF κ B nuclear translocation are shown in blue and red, respectively.

Supplemental Table 2. MiRNAs that do not have a conserved seed sequence between human and rat.

miRNA	Human sequence	Rat sequence
hsa-miR-222-5p	CUCAGUAGCCAGUGUAGAUCCU	GGCUCAGUAGCCAGUGUAGAU
hsa-miR-450b-3p	UUGGGAUCAUUUUGCAUCCAUA	AUUGGGGACGCUUCGCAUUA
hsa-miR-30b-3p	CUGGGAGGUGGAUGUUUACUUC	CUGGGGAUGUGGAUGUUUACGUC
hsa-miR-345-5p	GCUGACUCCUAGUCCAGGGCUC	UGCUGACCCUAGUCCAGUGC
hsa-miR-146b-3p	UGCCCUUGUGGACUCAGUUCUGG	CCUAGGGACUCAGUUCUGUG
hsa-miR-214-5p	UGCCUGUCUACACUUGCUGUGC	AGAGUUGUUAUGUGUCU
hsa-miR-210-5p	AGCCCUUGCCCACCGCACACUG	AGCCACUGCCCACAGCACACUG
hsa-miR-511-5p	GUGUCUUUUGCUCUGCAGUCA	CAUGCCUUUUGCUCUGCACUC
hsa-miR-135a-3p	UAUAGGGAUUGGAGCCGUGGCG	UGUAGGGAUGGAAGCCAUGAA

Supplemental Table 1. Effects of heart failure-associated miRNA mimics upon transfection, on the main read outs for each screen. Values are represented as relative log₂ fold change over negative control mimic-transfected cells. MiRNAs were selected for high throughput phenotypical screening based on their differential expression in the hearts of patients diagnosed with viral myocarditis (Human VM), patients undergoing cardiac transplantation (Human TX), and in animal models of inflammatory cardiac disease, such as murine viral myocarditis (Mouse VM), murine cardiac transplant (Mouse TX), and rat metabolic syndrome (ZSF1). In the table Mouse VM = A, Mouse TX = B, ZSF1 = C, Human VM = D and Human TX = E.

miRNA	miRNA Profiling	Cardiomyocyte Cell Size		Fibroblast Collagen content		N# of Nuclei		Macrophage Roundness		Nuclear NFkB	
	study	Log2 Fc	Pval	Log2 Fc	Pval	Log2 Fc	Pval	Log2 Fc	Pval	Log2 Fc	Pval
let-7a-5p	A, C	-0,05	0,59	0,58	0,00	0,02	0,58	0,04	0,43	-0,04	0,74
let-7b-3p	A	0,12	0,39	0,17	0,14	-0,15	0,04	0,24	0,05	0,29	0,01
let-7b-5p	A, C	-0,09	0,55	0,36	0,02	-0,02	0,76	0,16	0,09	-0,10	0,60
let-7c-3p	A	-0,25	0,01	-1,13	0,00	-0,09	0,04	0,34	0,00	0,42	0,03
let-7c-5p	A, C	0,12	0,06	0,43	0,02	0,11	0,01	0,08	0,08	-0,08	0,52
let-7d-5p	A	-0,08	0,46	0,43	0,02	0,03	0,39	-0,06	0,05	-0,12	0,37
let-7e-5p	A	-0,09	0,29	1,39	0,00	0,13	0,00	0,16	0,14	0,65	0,37
let-7f-5p	A	-0,02	0,94	0,71	0,00	-0,06	0,20	0,02	0,51	-0,13	0,32
let-7g-5p	A	-0,17	0,06	0,27	0,08	-0,06	0,16	0,02	0,45	-0,15	0,12
miR-1	A	-0,50	0,01	1,89	0,00	-0,56	0,00	0,15	0,05	0,00	0,92
miR-100-5p	A	-0,04	0,77	-0,21	0,67	-0,02	0,78	0,10	0,12	-0,16	0,25
miR-101-3p	A	-0,25	0,00	0,16	0,16	-0,35	0,00	0,25	0,03	0,01	0,86
miR-103a-2-5p	B	-0,12	0,33	-1,71	0,00	-0,35	0,00	0,30	0,00	0,12	0,12
miR-103a-3p	B, C	-0,51	0,00	0,31	0,04	-0,40	0,00	0,12	0,15	0,08	0,50
miR-103b	B	-0,76	0,00	0,52	0,01	-0,39	0,00	0,31	0,01	0,35	0,13
miR-106a-3p	B	0,20	0,18	0,51	0,01	-0,42	0,00	0,07	0,29	-0,03	0,89
miR-106a-5p	B	-0,35	0,02	0,52	0,01	-0,06	0,29	0,22	0,08	0,07	0,65
miR-106b-5p	B	-0,24	0,25	0,62	0,00	-0,21	0,01	0,14	0,06	-0,17	0,31
miR-107	B, C	-0,04	0,76	-0,16	0,88	-0,40	0,00	0,08	0,08	-0,22	0,15
miR-10a-5p	C	-0,05	0,67	-0,74	0,01	0,06	0,02	0,30	0,03	0,14	0,16
miR-10b-5p	A	0,10	0,46	-0,22	0,63	-0,05	0,49	0,24	0,05	0,05	0,75
miR-1224-3p	B, A	0,02	0,57	0,52	0,00	-0,09	0,26	0,08	0,14	0,00	0,93
miR-1224-5p	B, A	-0,29	0,02	-0,36	0,27	0,01	0,72	-0,01	0,70	-0,15	0,11
miR-1246	D	-0,36	0,00	-0,06	0,73	0,08	0,18	-0,08	0,08	-0,07	0,69
miR-125a-3p	B	-0,04	0,58	0,86	0,00	0,27	0,00	0,07	0,07	-0,01	1,00
miR-125a-5p	A, B	0,37	0,00	1,51	0,08	-0,34	0,00	0,06	0,22	0,15	0,14
miR-125b-5p	A, E, C	0,16	0,09	0,71	0,00	-0,20	0,00	0,06	0,30	-0,02	0,99
miR-126-3p	A, B	-0,33	0,02	0,92	0,00	-0,26	0,01	0,02	0,74	0,39	0,04
miR-126-5p	A	-0,18	0,06	-0,12	0,99	0,21	0,00	0,20	0,02	0,05	0,47
miR-128-1-5p	B	0,04	0,31	0,87	0,00	0,03	0,49	-0,03	0,59	-0,15	0,24
miR-128-2-5p	B	0,05	0,66	-0,03	0,70	0,13	0,04	0,02	0,68	-0,24	0,13
miR-128-3p	B	-0,10	0,62	-0,58	0,03	-0,06	0,14	-0,21	0,01	-0,17	0,10
miR-130a-3p	A	-0,40	0,03	0,09	0,27	-0,26	0,00	0,22	0,01	0,19	0,10
miR-130b-3p	A	-0,29	0,09	0,16	0,15	-0,25	0,00	0,19	0,08	0,01	0,88
miR-133a-3p	A	-0,04	0,80	0,73	0,00	-0,13	0,05	0,20	0,05	0,07	0,59
miR-133a-5p	A	0,18	0,19	0,49	0,01	-0,45	0,00	0,03	0,17	-0,11	0,29
miR-133b	A, C	-0,40	0,00	0,75	0,00	-0,10	0,02	0,19	0,00	-0,04	0,79
miR-135a-3p	A	-0,13	0,22	1,00	0,00	-0,13	0,01	0,09	0,09	-0,22	0,12
miR-135b-5p	A, C	-0,07	0,24	-0,59	0,04	-0,46	0,00	0,14	0,12	-0,10	0,48
miR-139-3p	A, C, B	0,02	0,75	0,29	0,04	0,08	0,13	0,07	0,04	0,14	0,36
miR-139-5p	A, C, B	0,01	0,87	-0,39	0,22	-0,11	0,05	0,17	0,00	0,07	0,43
miR-140-3p	C	-0,04	0,66	-0,45	0,15	0,18	0,00	0,09	0,00	-0,09	0,56
miR-142-3p	A, E	-0,39	0,00	-0,44	0,21	-0,16	0,11	0,13	0,11	-0,02	0,96
miR-142-5p	A, E	-0,01	0,95	0,32	0,03	-0,13	0,05	0,11	0,12	-0,06	0,69
miR-143-3p	B, A, C	-0,06	0,46	-0,13	0,99	0,05	0,30	-0,14	0,06	-0,04	0,82
miR-145-3p	B, A	-0,39	0,00	0,96	0,00	-0,27	0,00	0,28	0,04	0,36	0,00
miR-145-5p	B, A	0,09	0,67	0,88	0,00	-0,01	0,92	0,06	0,30	-0,06	0,73
miR-146a-3p	A, B	0,22	0,15	-0,30	0,38	-0,15	0,00	0,40	0,00	0,17	0,21
miR-146a-5p	A, B, C	0,50	0,42	1,07	0,00	0,26	0,00	0,01	0,89	-0,12	0,40
miR-146b-3p	A, B	-0,17	0,02	0,19	0,11	-0,11	0,15	-0,04	0,65	-0,15	0,39
miR-146b-5p	A, B, C	-0,05	0,72	1,01	0,00	0,32	0,00	0,29	0,04	0,61	0,02
miR-148a-3p	B	-0,36	0,04	-0,30	0,45	0,84	0,00	0,19	0,08	-0,08	0,62
miR-148b-3p	C	-0,16	0,05	-0,48	0,10	0,77	0,00	0,38	0,01	0,21	0,05
miR-149-5p	A, E	-0,12	0,30	0,71	0,00	-0,12	0,01	0,22	0,00	0,05	0,59

miR-150-5p	C	-0,06	0,61	0,40	0,02	-0,18	0,00	0,07	0,45	-0,03	0,83
miR-151a-3p	B	-0,15	0,26	-1,03	0,00	-0,33	0,00	-0,11	0,09	-0,04	0,71
miR-151a-5p	A, B	-0,14	0,17	1,51	0,00	-0,10	0,02	-0,07	0,43	0,22	0,05
miR-152-3p	A	-0,24	0,18	-0,18	0,83	0,84	0,00	0,27	0,00	0,27	0,03
miR-152-5p	A	0,23	0,02	3,14	0,00	-1,57	0,00	0,29	0,02	-0,02	0,95
miR-153-3p	A	0,48	0,35	0,39	0,03	-0,49	0,00	0,31	0,00	0,19	0,18
miR-155-5p	A, C, D	-0,12	0,08	0,21	0,09	-0,16	0,04	0,35	0,01	0,20	0,38
miR-15a-5p	B	-0,22	0,19	1,04	0,00	-1,32	0,00	0,09	0,05	-0,09	0,60
miR-15b-5p	C	-0,31	0,05	1,10	0,00	-1,25	0,00	0,22	0,00	0,29	0,00
miR-16-1-3p	B	-0,22	0,07	0,57	0,00	0,03	0,67	0,22	0,00	0,05	0,72
miR-16-2-3p	B	0,13	0,22	0,80	0,00	0,11	0,07	0,18	0,11	0,08	0,49
miR-16-5p	B	-0,46	0,08	1,54	0,00	-1,38	0,00	0,26	0,09	0,04	0,76
miR-17-5p	B	0,08	0,49	1,97	0,00	-0,25	0,01	0,17	0,06	-0,16	0,28
miR-181a-5p	A, B	-0,22	0,20	1,39	0,00	-0,40	0,00	0,33	0,01	0,09	0,62
miR-181b-5p	A, B	-0,27	0,01	0,91	0,00	-0,12	0,03	0,28	0,01	0,18	0,17
miR-181c-5p	A	-0,23	0,16	1,86	0,00	-0,64	0,00	0,27	0,02	0,13	0,41
miR-185-5p	A, C	0,00	0,97	1,28	0,00	-0,47	0,00	0,04	0,55	-0,17	0,27
miR-188-5p	A	0,16	0,00	-0,17	0,88	-0,16	0,00	0,01	0,68	-0,07	0,61
miR-18a-5p	A	-0,09	0,35	-0,35	0,30	-0,09	0,20	0,28	0,00	0,20	0,17
miR-190b	E	0,01	0,85	-0,73	0,02	0,24	0,00	0,25	0,00	0,14	0,18
miR-192-5p	B	-0,11	0,43	0,78	0,00	-0,41	0,00	0,12	0,32	-0,06	0,79
miR-193a-3p	A	0,13	0,37	0,87	0,00	-0,45	0,00	0,20	0,01	-0,02	0,95
miR-194-5p	A	-0,09	0,56	0,44	0,01	-0,30	0,00	0,16	0,04	0,04	0,64
miR-195-3p	B, A	0,06	0,36	0,68	0,00	0,39	0,00	0,37	0,01	0,25	0,32
miR-195-5p	B, A, C	-0,16	0,22	1,64	0,00	-1,37	0,00	0,20	0,07	-0,02	0,98
miR-199a-3p	C	-0,08	0,55	0,13	0,27	0,09	0,13	0,30	0,00	0,21	0,02
miR-199a-5p	C	-0,03	0,68	-2,11	0,00	-0,12	0,09	0,25	0,02	0,18	0,22
miR-199b-5p	A	-0,24	0,01	-1,37	0,00	-0,05	0,25	0,24	0,02	0,02	0,75
miR-19a-3p	A	0,29	0,01	0,82	0,00	0,57	0,00	0,25	0,01	0,17	0,23
miR-19b-3p	B	0,11	0,19	0,12	0,25	0,53	0,00	0,32	0,01	0,35	0,10
miR-200c-3p	E, C	0,44	0,00	0,88	0,00	-0,18	0,00	0,38	0,00	0,37	0,13
miR-200c-5p	E	-0,29	0,13	0,08	0,33	-0,20	0,00	0,39	0,01	0,15	0,15
miR-204-5p	A, D	0,01	0,92	0,09	0,42	0,22	0,00	0,25	0,03	0,19	0,13
miR-208a-3p	A	-0,10	0,51	-0,60	0,03	-0,13	0,02	0,19	0,04	0,93	0,42
miR-208a-5p	A	0,05	0,34	1,07	0,00	-0,33	0,00	-0,03	0,14	-0,11	0,36
miR-208b-3p	C	-0,11	0,28	-0,08	0,87	-0,27	0,00	0,19	0,07	0,24	0,07
miR-20a-3p	C	-0,34	0,01	0,37	0,03	-0,35	0,00	0,07	0,07	-0,14	0,40
miR-20a-5p	C	-0,12	0,42	0,41	0,01	-0,07	0,17	0,05	0,22	-0,18	0,28
miR-20b-5p	A	0,22	0,37	0,77	0,00	-0,08	0,25	0,27	0,00	0,19	0,21
miR-21-3p	B, A	-0,05	0,66	-0,85	0,00	-0,18	0,00	0,04	0,59	0,22	0,02
miR-21-5p	B, A, C	0,17	0,05	0,10	0,25	-0,13	0,05	0,11	0,25	-0,03	0,93
miR-210-3p	A	0,28	0,02	0,58	0,00	0,01	0,79	0,27	0,01	0,45	0,01
miR-210-5p	A	0,00	0,82	0,64	0,00	-0,12	0,02	-0,03	0,64	-0,04	0,80
miR-212-3p	A, D	0,06	0,36	0,22	0,11	-0,12	0,00	0,22	0,08	0,36	0,12
miR-212-5p	A, D	-0,38	0,00	0,78	0,00	-0,61	0,00	0,28	0,02	0,35	0,18
miR-214-3p	C	-0,49	0,00	0,59	0,00	-0,16	0,00	0,05	0,06	0,14	0,22
miR-214-5p	C	0,06	0,49	-0,22	0,78	-0,07	0,19	-0,04	0,16	-0,13	0,39
miR-216a-5p	D	0,06	0,59	-1,60	0,00	-0,06	0,20	0,25	0,00	0,42	0,14
miR-218-5p	C	0,08	0,37	0,34	0,04	-0,08	0,27	0,38	0,00	0,38	0,12
miR-22-3p	B, A	-0,04	0,71	-1,28	0,00	-0,32	0,00	0,07	0,11	-0,14	0,41
miR-22-5p	B, A	-0,20	0,07	0,67	0,00	-0,48	0,00	0,00	0,95	-0,07	0,67
miR-221-3p	B, C	-0,04	0,70	0,53	0,00	0,04	0,31	0,15	0,18	-0,04	0,88
miR-221-5p	B	-0,29	0,00	0,41	0,01	-0,49	0,00	0,23	0,07	0,20	0,27
miR-222-3p	B, A, C	-0,06	0,45	-0,28	0,42	0,05	0,27	0,18	0,07	-0,13	0,23
miR-222-5p	B, A	0,02	0,80	-0,40	0,24	-0,30	0,01	0,28	0,02	0,19	0,18
miR-223-3p	B, A	-0,24	0,00	-0,17	0,83	0,16	0,00	0,28	0,00	0,05	0,64
miR-223-5p	B, A, C	-0,43	0,05	0,40	0,07	-0,43	0,00	0,13	0,00	0,11	0,33
miR-23a-3p	A, B, C	0,07	0,08	1,15	0,00	0,32	0,00	-0,06	0,36	-0,12	0,20
miR-23a-5p	A, B	-0,02	0,90	-0,57	0,04	0,06	0,36	0,27	0,01	0,17	0,47
miR-23b-3p	A, B	-0,08	0,19	1,09	0,00	0,38	0,00	-0,03	0,70	-0,18	0,30
miR-23b-5p	A, B	0,19	0,18	0,43	0,01	-0,83	0,00	-0,06	0,31	-0,19	0,27
miR-24-1-5p	A	-0,28	0,05	-1,03	0,00	-0,23	0,00	0,09	0,30	0,02	0,76
miR-24-2-5p	A, B	-0,19	0,26	-1,26	0,00	-0,16	0,00	0,34	0,01	0,66	0,20
miR-24-3p	A	0,09	0,17	0,89	0,00	0,04	0,40	0,05	0,42	0,00	0,88
miR-26a-5p	A	0,01	0,90	1,41	0,00	0,01	0,86	0,09	0,29	0,08	0,58
miR-26b-5p	A	0,07	0,58	1,18	0,00	0,09	0,07	0,13	0,24	0,05	0,67
miR-27a-3p	A	0,01	0,93	0,19	0,12	0,27	0,00	0,20	0,06	0,20	0,36

miR-27b-3p	A, C	-0,19	0,04	-0,15	0,91	0,28	0,00	0,04	0,61	0,04	0,61
miR-29a-3p	A	0,20	0,01	-2,30	0,00	-0,27	0,00	0,08	0,03	0,06	0,64
miR-29b-3p	A	0,07	0,50	-2,45	0,00	-0,20	0,00	0,21	0,09	-0,10	0,44
miR-29c-3p	A	0,00	0,95	-2,23	0,00	-0,16	0,00	0,10	0,34	-0,15	0,27
miR-29c-5p	A	0,07	0,33	0,46	0,01	-0,16	0,04	-0,25	0,34	-0,03	0,96
miR-302a-3p	E	-0,15	0,25	0,68	0,00	0,01	0,89	0,18	0,00	-0,22	0,19
miR-302a-5p	E	0,07	0,52	0,71	0,00	-0,02	0,70	0,09	0,17	0,17	0,14
miR-302c-3p	E	-0,02	0,85	0,61	0,00	-0,03	0,67	0,06	0,11	-0,19	0,23
miR-302d-5p	D	0,22	0,01	1,07	0,00	-0,23	0,01	-0,03	0,68	-0,13	0,37
miR-30a-3p	A	-0,08	0,44	0,82	0,00	-0,12	0,07	0,38	0,00	0,89	0,12
miR-30a-5p	A	0,04	0,75	1,50	0,00	-0,12	0,00	0,07	0,10	0,06	0,41
miR-30b-3p	A	-0,13	0,44	1,38	0,00	-0,40	0,00	0,29	0,00	0,11	0,40
miR-30b-5p	A	0,14	0,25	1,31	0,00	-0,13	0,02	0,05	0,12	0,09	0,29
miR-30c-2-3p	A	-0,13	0,55	0,85	0,00	-0,27	0,01	-0,17	0,06	-0,33	0,01
miR-30c-5p	A	-0,01	0,99	1,16	0,00	-0,16	0,01	0,01	0,88	0,10	0,35
miR-30d-5p	A	-0,08	0,68	1,41	0,00	0,05	0,20	0,05	0,11	0,13	0,28
miR-30e-3p	A, B	0,30	0,06	0,70	0,00	-0,05	0,46	0,28	0,01	0,41	0,08
miR-30e-5p	A, B, C	-0,04	0,79	1,31	0,00	-0,26	0,00	0,06	0,17	0,03	0,73
miR-320a	B	0,19	0,09	0,34	0,05	-0,24	0,02	0,03	0,54	-0,10	0,45
miR-331-3p	A	-0,14	0,12	1,52	0,00	-0,12	0,15	-0,12	0,28	-0,30	0,07
miR-335-5p	A	0,17	0,02	0,51	0,03	-0,27	0,00	0,25	0,07	0,23	0,08
miR-338-3p	A	-0,34	0,01	1,37	0,00	-0,41	0,00	0,16	0,20	0,16	0,26
miR-340-5p	A	-0,14	0,06	0,20	0,25	-0,38	0,00	0,23	0,03	0,05	0,73
miR-342-3p	A	-0,12	0,20	0,10	0,28	0,17	0,02	0,26	0,02	0,29	0,20
miR-345-5p	A	0,10	0,36	0,43	0,08	-0,18	0,01	0,09	0,00	-0,18	0,10
miR-34a-3p	A, B	-0,23	0,02	0,71	0,00	-0,61	0,00	-0,32	0,01	-0,22	0,03
miR-34a-5p	A, B	0,00	0,89	0,79	0,00	-0,42	0,00	0,19	0,00	0,07	0,62
miR-361-3p	B, A	0,08	0,04	1,02	0,00	0,11	0,06	0,07	0,16	-0,13	0,36
miR-361-5p	B, A	-0,45	0,00	-0,36	0,24	-0,24	0,00	0,17	0,04	0,18	0,03
miR-378a-3p	B, A, C	0,01	0,88	1,12	0,00	-0,45	0,00	-0,01	0,86	0,01	0,83
miR-378a-5p	B, A	-0,15	0,21	-0,31	0,43	-0,45	0,00	0,19	0,00	0,00	0,92
miR-379-3p	A	0,00	0,96	1,62	0,00	0,02	0,64	0,16	0,02	-0,19	0,25
miR-379-5p	A	-0,03	0,80	0,25	0,07	-0,66	0,00	0,11	0,06	-0,09	0,54
miR-409-5p	D	-0,15	0,42	-0,55	0,09	-0,37	0,00	0,01	0,64	0,08	0,29
miR-410-3p	E	0,10	0,58	0,29	0,05	-0,16	0,00	0,28	0,00	0,16	0,32
miR-422a	E	-0,09	0,61	-0,50	0,08	-0,60	0,00	0,07	0,43	-0,05	0,76
miR-423-5p	E	-0,14	0,32	0,07	0,31	-0,22	0,01	0,16	0,01	0,17	0,07
miR-425-3p	B	-0,23	0,05	-0,13	0,99	-0,16	0,02	0,08	0,47	-0,11	0,48
miR-425-5p	B	0,04	0,24	1,11	0,00	0,16	0,00	0,14	0,06	0,08	0,55
miR-431-5p	E	0,32	0,00	1,43	0,00	-0,39	0,00	-0,05	0,10	-0,22	0,24
miR-432-5p	D	0,03	0,54	0,09	0,31	-0,35	0,00	0,20	0,01	0,04	0,61
miR-450b-3p	D	0,00	0,96	0,16	0,20	-0,44	0,00	-0,03	0,71	-0,15	0,13
miR-466	A	-0,01	0,98	-0,46	0,12	0,08	0,29	0,33	0,00	0,16	0,28
miR-484	A, C	0,06	0,26	-0,04	0,66	-0,02	0,81	-0,24	0,06	-0,23	0,15
miR-486-3p	B, A	-0,54	0,00	0,09	0,27	-0,26	0,00	0,21	0,03	-0,03	0,92
miR-486-5p	B, A, C	0,00	0,94	0,64	0,00	-0,22	0,00	-0,04	0,60	-0,10	0,48
miR-488-5p	D	0,10	0,29	-1,03	0,00	-0,41	0,00	-0,34	0,00	-0,18	0,13
miR-494-3p	A	0,04	0,64	-0,03	0,64	-0,11	0,10	0,21	0,01	-0,09	0,47
miR-497-5p	A	-0,44	0,03	2,32	0,00	-1,70	0,00	0,08	0,02	-0,04	0,77
miR-499a-5p	A	-0,17	0,03	-0,72	0,01	-0,16	0,00	0,36	0,00	0,14	0,10
miR-511-3p	D	-0,02	0,90	1,09	0,00	0,05	0,45	0,08	0,14	-0,11	0,47
miR-511-5p	D	0,02	0,41	1,03	0,00	-0,11	0,05	0,14	0,05	0,31	0,15
miR-520f-3p	E	0,03	0,78	0,84	0,00	0,35	0,01	0,07	0,17	-0,18	0,19
miR-520f-5p	E	0,16	0,08	-0,05	0,70	0,00	0,93	-0,05	0,18	-0,13	0,27
miR-532-5p	A	-0,14	0,22	-1,07	0,00	-0,29	0,00	0,16	0,03	0,05	0,67
miR-558	D	-0,05	0,52	0,05	0,37	-0,18	0,01	0,13	0,00	-0,06	0,62
miR-582-5p	A	-0,19	0,08	-0,06	0,75	-0,20	0,00	0,26	0,00	0,30	0,00
miR-590-5p	C	0,17	0,00	0,20	0,12	-0,19	0,00	0,33	0,01	0,39	0,01
miR-629-5p	D	-0,05	0,55	1,04	0,00	-0,16	0,02	0,07	0,34	-0,26	0,10
miR-631	D	-0,31	0,01	1,65	0,00	-0,45	0,00	-0,09	0,11	-0,16	0,11
miR-7-5p	A	-0,38	0,01	3,09	0,00	-0,42	0,00	0,04	0,71	0,04	0,79
miR-877-5p	A	-0,30	0,03	1,22	0,00	-0,15	0,11	0,01	0,95	0,09	0,65
miR-889-3p	D	-0,13	0,20	1,03	0,00	0,10	0,10	0,28	0,00	0,16	0,26
miR-889-5p	D	-0,07	0,61	1,04	0,00	-0,79	0,05	0,00	0,93	-0,25	0,08
miR-891a-3p	D	-0,48	0,00	-1,43	0,00	-0,51	0,00	-0,28	0,00	0,05	0,67
miR-891a-5p	D	-0,07	0,50	0,35	0,08	-0,11	0,01	-0,04	0,45	0,04	0,76
miR-892b	D	-0,21	0,03	0,26	0,10	-0,34	0,00	0,02	0,60	-0,10	0,42

miR-9-5p	A	-0,40	0,02	1,34	0,00	0,15	0,02	0,25	0,02	0,05	0,68
miR-92b-3p	D	0,34	0,00	-0,31	0,48	0,08	0,14	-0,15	0,18	-0,10	0,49
miR-96-5p	A	-0,44	0,09	0,48	0,03	-0,39	0,00	0,12	0,11	0,33	0,02
miR-98-5p	A	-0,13	0,09	0,82	0,00	-0,10	0,07	0,23	0,18	0,38	0,46
miR-99a-5p	A	-0,17	0,00	-0,23	0,60	0,02	0,61	0,02	0,78	-0,06	0,60
miR-99b-5p	A, C	-0,17	0,01	-0,13	0,96	0,04	0,49	0,02	0,77	-0,16	0,32

Literature-based gene to phenotype interaction network analysis reveals novel microRNA regulators in heart failure

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Submitted

Abstract

Chronic hypertension and aortic stenosis often lead to pressure overload-induced heart failure (HF). This is associated with maladaptive structural changes, marked by the enlargement of cardiomyocytes (hypertrophy) and excessive collagen deposition (fibrosis). More recently, the role of cardiac inflammation in the aetiology of HF is rapidly gaining attention. It remains to be further established, however, if and how inflammation, hypertrophy and fibrosis influence each other. In cardiac research genetically manipulated mouse models are crucial to study the molecular mechanisms underlying HF. In this study we extracted data from 284 preclinical articles on pressure overload-induced HF published between 2000 and 2014. This unique dataset was subsequently used (1) to investigate the relationship between inflammation, hypertrophy and fibrosis, (2) to generate a gene to phenotype interaction network (GPIN) allowing the identification of critical molecular players in HF development, and (3) to compare the features and define reference values of phenotypical outcome measures of the most commonly used models of pressure overload. By applying a meta-analysis based approach on preclinical studies we demonstrate that during HF development the process of cardiac inflammation is intricately linked with hypertrophy and fibrosis. Furthermore, by extending the dataset-derived GPIN with publically available microRNA targeting data we were able to identify various microRNAs (among which miR-466l, miR-124-3p and miR-30e-5p) predicted to have a large impact on cardiac inflammation, hypertrophy or fibrosis. In conclusion, we present a novel methodology to build and mine a gene-to-phenotype interaction network. This approach enabled us to generate new knowledge based on previously published cardiovascular research.

Introduction

Persistent hypertension and aortic stenosis are major risk factors for the development of heart failure (HF) ¹. Both conditions lead to pressure overload (PO) in the heart and initiate maladaptive cardiac remodelling characterized by enlargement of cardiomyocytes (hypertrophy) ² and increased collagen deposition (fibrosis) ³. More recently, inflammation is recognized as an important player in the development of PO-induced HF ^{4, 5}. Via the infiltration of leukocytes, the activation of resident immune cells, or the activation of pro-inflammatory signalling pathways within cardiomyocytes, inflammation has been shown to influence the development of hypertrophy and fibrosis, and consequently HF progression ^{6, 7}.

Although recognized as a highly valuable tool in clinical science for decades, the application of meta-analyses and systematic reviews to preclinical studies has started only recently ⁸⁻¹⁰. In preclinical cardiac research, rodent models of PO are used routinely to investigate the molecular mechanisms involved in the pathogenesis of non-ischemic HF. Rodent models have been developed in which cardiac PO is being imposed either via surgical intervention (Aortic Constriction, AC) or by pharmacological intervention (Angiotensin II or Isoproterenol infusion). Accordingly, in the present study we performed a systematic literature search for PO-induced HF in rats and mice. Experimental details and outcome parameters (OCPs) were extracted from almost 300 articles and used to explore the relation between inflammation, on the one hand, and hypertrophy and fibrosis on the other.

Genetically modified mouse models are extensively used to investigate the molecular mechanisms involved in the pathogenesis of PO induced heart failure ¹¹. As the genetic manipulation (gain- or loss-of-function) of specific genes implicated in hypertrophy, fibrosis or inflammation forms the core of many studies, this information was used to build a gene to phenotype interaction network (GPIN) from the screened articles. The created network visualizes the effect these specific genes have on the HF processes resembled by the OCPs. Next, as microRNAs (miRNAs) are established negative regulators of gene-expression and have been shown to regulate pathophysiological processes associated with HF ¹²⁻¹⁴, we extended the GPIN with miRNA targeting data. Based on the number of genes in the network that these miRNAs are predicted to target a score was calculated to indicate their relevance. This allows the identification of candidate miRNAs influencing cardiac inflammation, hypertrophy and fibrosis.

In addition, this extensive dataset was used to compare features of frequently used rodent PO models of which, despite their widespread use, the similarities and dissimilarities are ill-defined and reference values for the expected degree of hypertrophy, fibrosis and inflammation are still lacking. This comparison aids the interpretation of performed studies and the design of future PO studies.

In summary, here we present an approach for using existing literature to identify potential novel HF therapeutic targets by creating and mining a Gene to Phenotype interaction network based on a systematic review. Secondly, we explore the relation between inflammation, on the one hand, and hypertrophy and fibrosis on the other. A final goal was to define reference values for pathophysiological parameters, such as hypertrophy, fibrosis and inflammation in different models of hypertensive heart disease.

Methods

Search strategy

Inclusion and exclusion criteria are listed in Table 1. In short, for inclusion a rat or mouse model of cardiac PO had to be used and a numerical quantification of at least 2 of the 3 outcome parameters (OCPs, being hypertrophy, fibrosis or inflammation) had to be given. A wild type control group or sham group also had to be part of the study design. Reviews, editorials and other non-experimental articles were excluded as well as articles in a language other than English. Based on these criteria a search strategy was formulated consisting of different components: disease model, species, selection of at least 2 of the 3 OCPs, organ, and additional filters for language and publication date. The combination of MeSH terms used to conduct the search is provided in the supplemental methods. Subsequently the developed search strategy was used on PubMed in March 2015, covered the years 2000 - 2014 and yielded 581 search results.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
PO inducing intervention	Full text in language other than English
Rat or mouse studies	No original data (review, opinion etc.)
Numerical outcome data for hypertrophy and fibrosis, hypertrophy and inflammation fibrosis and inflammation or all three	Only addressing one of the OCPs of hypertrophy, fibrosis and inflammation
	No full text available
	Lack of proper control groups

Screening abstracts for inclusion

The titles and abstracts of the retrieved articles were reviewed for selection by two authors (WD and GS). Full-text articles were retrieved from abstracts selected for further review. Abstracts of the 581 articles from the PubMed search were screened for relevance to the inclusion criteria (Table 1). Based on the abstract we excluded 231 articles (Figure 1). Based on the full text another 139 articles were excluded. By cross-checking the references of included articles, 73 additional papers were identified as relevant and included in the dataset. Some articles contained more than one study, for example, when TAC and AngII models were used in parallel. In that case, they were dealt with as separate studies, yielding an additional 85 studies, and taking the total amount of studies to 369 (Figure 1).

Figure 1. Flow chart of article selection procedure. Selection based on abstract and full text was performed by two authors. Additional 73 articles were added based on checking reference list in selected papers.

Data extraction

Experimental details and numerical data were extracted from the articles. In case data were presented in the form of figures, we used the freeware program DataThief III (B. Tummers, 2006, <http://datathief.org/>) to convert graphical information into numerical data. Supplemental Table 1 shows all the experimental details and OCPs extracted from the articles, comprising of: basal characteristics of the study the experimental details, OCPs and indications on how these OCPs were measured and presented. Where different measures of an OCP were reported we extracted the one most commonly used. From all studies we extracted mean values for 4 groups:

1. *Control - Sham*. Group that did not receive an experimental treatment and was not subjected to pressure overload (e.g. wild-type, sham-operated mice).
2. *Experimental - Sham*. Group that received the experimental treatment, but was not subjected to pressure overload (e.g. knockout, sham-operated mice).
3. *Control – PO*. Group that did not receive the experimental treatment, but was subjected to pressure overload (e.g. wild-type mice subjected to TAC).
4. *Experimental – PO*. Group that received the experimental treatment and was subjected to pressure overload (e.g. knockout mice subjected to TAC).

From the text of the paper we determined a general conclusion on how the experimental condition affected hypertrophy, fibrosis, cardiac function and inflammation and simplified the conclusions into: reduced (↓), increased (↑) or no significant change (-). These conclusions were not only based on numerical data.

Statistics and subgroup analysis

Data in bar graphs and tables are presented as mean ± SEM unless indicated otherwise. Statistical analyses were performed using GraphPad Prism 5. For multiple group comparisons a one-way ANOVA was performed. Comparisons between two groups were done using unpaired Student's t-tests. A two-tailed P-value of <0.05 was considered as statistically significant. Pearson's r test was used to determine correlations in scatterplots. Fisher's exact test was used for comparison of categorical data.

Gene to phenotype network creation

We combined the effects of genetic manipulations with the effect of the PO model on the OCPs to create gene to phenotype interactions (GPIs). The effects are based on the comparison of the Wt – PO and Experimental – PO group and on the overall conclusion of the study regarding the effect on OCPs. GPIs are directional (always from the gene towards the phenotype) and typed, that is, they describe the overall effect of the gene on an OCP. The type of GPI is either *pro*, *anti* or *neutral*. If the genetic manipulation in a study decreases the expression or function of a gene and the PO model leads to a reduction in the OCP, then the GPI is considered *pro*. For example, when mice with a knockout of the *Tnf* gene (representing a decrease in function of this gene) are subjected to TAC and subsequently cardiac fibrosis is significantly less than in wild-type mice subjected to TAC, then the GPI for *Tnf* and fibrosis is considered *pro* as the study implicates *Tnf* to be pro-fibrotic. The inverse case, over-expression of *Tnf* leading to an increase in fibrosis, is also a combination that leads to a *pro* typed GPI. *Anti* GPIs result from the genetic manipulation and the outcome opposing each other, e.g. a knockout of *IL-10* resulting in an increase in fibrosis. The third type for a GPI is *neutral*: it represents the cases in which the genetic manipulations do not significantly affect a specific OCP. All GPIs are based on the individual studies and do not account for experimental differences between studies. This can lead to contradictory types for GPIs in which the majority type is used for the gene to phenotype interaction network (GPIN), but the confidence in a particular GPI is reduced to reflect the difference between studies. For instance, if three studies result in a *pro* type and one *neutral*, the confidence for the GPI is 0.75 (Figure 2). Table 2 summarizes the rules for typing GPIs. The collection of GPIs from all studies were combined into a single network.

Table 2. Rules for typing gene to phenotype interactions based on general conclusion.

Genetic manipulation of gene of interest	Effect on OCP (general conclusion)	GPI type
down-regulation	Reduction ↓	Pro
up-regulation	Increase ↑	
down-regulation	Increase ↑	Anti
up-regulation	Reduction ↓	
up or down regulation	no significant change	neutral

Enriching the GPIN

In the first step we merged miRNA targeting information into the GPIN. MiRTarbase 4.5 ¹⁵ served as a source for validated miRNA targeting information (9.199 interactions). For predicted miRNA interactions the following databases were used: TargetScan 6.2 (160.413 interactions) ¹⁶, miRDB 5.0 (257.330 interactions) ¹⁷ and DIANA microT-CDS (530.955 interactions) ¹⁸. The miRNA targeting information is consolidated into a single interaction that is weighted based on how many data sources predict a particular interaction.

Figure 2. Workflow for scoring the relevance of miRNAs based on their targets and how those are connected to the OCPs. The score contribution from an OCP via the genes is depicted as the number between brackets (100). In box A, the score from the OCP is multiplied with the confidence of the GPI. Next the score of a gene is weighted based on the miRNA targeting information (box B). The sum of all scores flowing to a miRNA reflects its relevance for the OCP (in this example fibrosis). The schematic example in this figure is not based on the real data.

Identifying heart failure relevant miRNAs

The identification and prioritization of miRNAs relevant to HF is driven by a neighbourhood based network analysis strategy. Herewith, OCPs and genes are connected via GPIs and then via the miRNA targeting interactions to miRNAs. In the first step (Figure 2, box A) all genes connected to an OCP via a GPI are scored. For the gene score the confidence of a GPI is used. This leaves every gene connected with an OCP with one score for each of the OCPs. In the next step (Figure 2, box B) the genes confidence score is transferred to each microRNA targeting a particular gene. The genes confidence score is multiplied with the weight of a miRNA targeting interaction to calculate the contribution of a gene to the score of a miRNA. As one miRNA is often predicted to target multiple genes the transferred scores are accumulated to calculate the relevance scores of a miRNA for each of the OCPs. The final scores are then scaled between 0 and 1 and added to obtain the overall relevance score.

Scripts and Software

The software to build and enrich the GPIN are available at <https://github.com/gsummer>. The algorithm to score microRNA for their relevance regarding the OCPs can be found at <https://github.com/gsummer/netlib-curator>. In short, the GPIN was built iteratively by first adding relevant nodes (all genes from ENSEMBL 81 and mature miRNAs from miRBase 21) and then merging the different interaction databases into the GPIN. cyNeo4j¹⁹ was used to extract sub-networks from the GPIN into Cytoscape²⁰ for visualization.

Results

The selection of articles according to our pre-set criteria (Table 1, Figure 1) yielded a dataset of 369 pressure overload (PO) studies derived from 284 articles published between 2000 and 2014 (listed in supplementary file 1). Most studies (82%) made use of mice, with the number of rat studies steadily decreasing over the years (from 60% in 2000 to 8% in 2014). By far the most commonly applied PO models in mice are aortic constriction (71%) and angiotensin-II infusion (24%, Table 3). As for the rat, more variation in the PO models applied was observed. The main characteristics of the most frequently applied PO models are summarized in the Supplemental Results and Supplementary Table 2.

The majority of studies made use of males (71.3%) and only a limited number of studies used mixed gender or females. Notably, in 16% of the studies gender was not specified. The most commonly used strains were C57BL/6 for mice and Sprague-Dawley for rats (Table 3). Only 79 out of the 206 studies using C57BL/6 mice provided information about which substrain (6-J, 6-N) was used. The average age at the start of the experiment was 10.7 ± 3.8 weeks for mice and 10.6 ± 6.4 weeks for rats and in most studies (31%) the animals were subjected to 28 days of pressure overload.

Table 3. Overview of the study characteristics of the dataset.

PO-intervention	AC (n=243)	AngII (n=82)	ISO (n=12)	SHR (n=15)	Other (n=16)
Mouse (n=306)	71%	24%	3%	-	2%
Rat (n=63)	41%	14%	3%	24%	17%
Total (n=369)	66%	22%	3%	4%	4%
Gender	Male (n=275)	Female (n=19)	Both (n=16)	ND (n=60)	
Mouse (n=306)	71%	5%	5%	19%	
Rat (n=63)	89%	5%	5%	2%	
Total (n=369)	74%	5%	4%	16%	
Mouse strain	C57BL/6 (n=206)	FVB (n=20)	CD-1 (n=6)	Other (n=40)	ND (n=36)
(n=306)	67%	6%	2%	13%	12%
Rat strain	Wistar (n=13)	WKY (n=13)	Dahl (n=5)	SD (n=30)	Other (n=2)
(n=63)	21%	21%	8%	49%	1%

ND = No data / Not indicated, AC = Aortic constriction, AngII = Angiotensin infusion, ISO = Isoproterenol infusion, SHR = Spontaneous hypertensive rats, WKY = Wistar-Kyoto, SD = Sprague Dawley

Aortic constriction or Angiotensin II?

The large number of mouse studies in the dataset (Table 3) provides the statistical power for a more detailed analysis of the phenotype induced by the two most commonly used PO interventions, i.e., trans-aortic constriction (TAC) and AngII infusion.

The effects of TAC and AngII on cardiac hypertrophy, fibrosis and function are shown in Figure 3. To exclude effects of differences in duration on outcome, only studies with a duration of 28 days were included in this analysis. The TAC model shows a more pronounced increase in cardiac mass (+54.9% vs. +31.1%, $P < 0.01$) and cardiomyocyte size (+76.3% vs. +62.1%, N.S.) compared to AngII, indicative of a more pronounced hypertrophic response induced by TAC (Figure 3A-B). As for fractional shortening and collagen area, the dataset reveals no pronounced differences between these two PO models (Figure 3C-D). In

conclusion, this analysis shows that TAC induces more pronounced cardiac hypertrophy compared to AngII infusion in mice, but this is not associated with a concurrent increase in fibrosis, or a more pronounced decline in systolic function.

In the Supplemental Results section of this manuscript an extensive description of PO models is given, including reference values of cardiac phenotypes induced by different PO interventions (Supplemental Table 2). In addition, an overview of the temporal changes induced after PO in mice (Supplemental Figure 1) as well as dose related effects on blood pressure and the PO induced phenotype (Supplemental Figure 2) are provided.

Figure 3. Comparison of cardiac phenotype induced by TAC and AngII in mice. The dataset was filtered to only include TAC ($n=69$) and AngII studies ($n=23$) having a duration of 4 weeks. Data are presented as means and SEM. Showing in panel A. HW/BW ratio. B. Cardiomyocyte cross sectional area (CSA). C. Collagen positive area. D. Fractional shortening. *** $P<0.001$ compared to corresponding Sham, ## $P<0.01$ compared to AngII.

Hypertrophy and fibrosis correlate after PO

Genetically modified mouse models are extensively used to investigate the molecular mechanisms involved in the pathogenesis of PO induced heart failure¹¹. Most studies in our dataset use knockout (KO) or transgenic mice (TG) to target a specific molecular pathway putatively involved either in cardiac hypertrophy, fibrosis or inflammation. Here, we investigated how these three parameters correlate after PO. We calculated fold changes for all OCPs induced by the experimental conditions for each study. Using these fold changes, we explored the relation between these parameters after experimental intervention. As expected, parameters associated with the same pathophysiological process (cardiac hypertrophy), cardiac mass and CSA correlate strongly ($R^2=0.64$, $P<0.001$, Supplemental

Figure 3). By presenting the effects of a genetic manipulation on the level of PO-induced hypertrophy and fibrosis (expressed as fold change) in a scatter plot (Figure 4A), it is immediately evident that the processes of hypertrophy and fibrosis closely correlate ($R^2 = 0.58$, $P < 0.0001$).

Inflammation in relation to cardiac hypertrophy and fibrosis

Next, we combined the general conclusion on inflammation with the conclusion regarding hypertrophy and fibrosis, to determine the relation between these processes. Table 4 shows that changes in inflammation affect the other pathophysiological processes in the same direction, suggesting a close relation between inflammation, on the one hand, and cardiac hypertrophy and fibrosis, on the other.

Because numerical data on inflammation is limited in our dataset and the outcome measures used are diverse, in Figure 4B we used colours to indicate the effect on cardiac inflammation. This clearly demonstrates that inflammation is following the change in both hypertrophy and fibrosis. In addition, in those studies in which leukocyte infiltration was quantified, the fold change in number of macrophages correlated with the fold change in cardiac mass and fibrosis after PO (Figure 4C-D).

Table 4. General outcome of all studies on cardiac hypertrophy, fibrosis and inflammation

Hypertrophy vs. Fibrosis [†]				
	Fibrosis ↑ (n=77)	Fibrosis – (n=49)	Fibrosis ↓ (n=188)	ND (n=55)
Hypertrophy ↑ (n=82)	61	12	1*	8
Hypertrophy - (n=85)	13	24	35	12
Hypertrophy ↓ (n=177)	2*	11	144	22
ND (n=25)	1	2	9	13
Hypertrophy vs. Inflammation[†]				
	Inflammation ↑ (n=27)	Inflammation - (n=24)	Inflammation ↓ (n=108)	ND (n=210)
Hypertrophy ↑ (n=82)	19	9	3*	51
Hypertrophy - (n=85)	7	8	27	43
Hypertrophy ↓ (n=177)	0	4	70	102
ND (n=25)	1	3	7	14
Fibrosis vs. Inflammation [†]				
	Fibrosis ↑ (n=77)	Fibrosis – (n=49)	Fibrosis ↓ (n=188)	ND (n=55)
Inflammation ↑ (n=27)	19	4	1*	4
Inflammation - (n=24)	3	10	4	6
Inflammation ↓ (n=108)	1*	11	87	8
ND (n=210)	56	22	94	37

All studies (rat and mouse) with control and experimental groups after PO (groups 3 and 4 described in methods) are included. Number of studies are indicated per general conclusion: ↑ = increase, – = No significant difference, ↓ = Reduced, ND = No data available. [†] statistically significant according to Fisher's test. Strikingly, a number of studies show opposite changes, e.g., a decrease in inflammation accompanied with an increase in hypertrophy ²¹⁻²³. In a similar way, in two studies the experimental condition affects inflammation and fibrosis in the opposite direction ^{21, 24}. All these studies are marked (). Details of these studies can be found in Supplemental table 4.*

Figure 4. Inflammation correlates with the development of both cardiac hypertrophy and fibrosis. Panel A shows the correlation between the fold change in cardiac mass and the fold change in fibrosis. In panel B the colour indicates the general conclusion on inflammation from this study in the same scatter plot. In panels C and D correlations between fold change in number of macrophages per mm² and hypertrophy and fibrosis are shown.

Gene to Phenotype Interaction Network

The gene to phenotype interaction network (GPIN) we created highlights the complex interactions between the three OCP processes. Our analysis included 253 studies that manipulated individual genes mostly through knockout or TG overexpression. Not all three OCPs were measured in all studies (Supplementary file 1). In fact, as shown in the Venn diagram (Figure 5A) the majority of studies derived from the literature database focused on two OCPs only, mostly fibrosis and hypertrophy. Due to overlaps between the studies this yielded 162 unique genes and mature miRNAs and 330 interactions after consolidating and confidence scoring for the creation of the GPIN. Figure 5B shows the GPIN, with the three OCPs in orange and genes coloured in blue. The *pro* interactions (172) are coloured in red and *anti* (158) in green. As Figure 5A indicates, the effect of manipulation of most genes is measured only on hypertrophy and fibrosis resulting in the majority of genes only affecting these OCPs. It is important to note that this is due to the lack in studies delivering this data instead of a representation of the actual biology at play.

Identifying relevant miRNA regulators

For further analysis of the interplay between the OCPs we merged multiple other interaction data sources (STRING, miRTarBase, TargetScan, miRDB and DIANA microT CDS) into the GPIN to add context (protein-protein interactions) and regulation (miRNA targeting). This increases the size of the network to 23024 nodes (protein coding genes, miRNAs, lncRNAs) and 2,978,343 interactions. We used the GPIN to predict the relevance of individual miRNAs for the OCPs based on their gene targets and on the confidence score of the effect of these genes on the OCPs. This resulted in high scores for several miRNAs, among which, mmu-miR-466l-3p, mmu-miR-124-3p, mmu-miR-30e-5p and mmu-miR-340-5p. Figure 6 shows miRNAs that score for at least one of the OCPs in the top 5% and their targets and OCPs. Table 5 lists the top 20 miRNAs ranked based on the sum of their individual OCP relevance scores.

Table 5. Top 20 miRNAs relevant to pathophysiological processes in PO-induced HF. *The score is based on the weight miRNA targeting interaction to genes in the network affecting the OCPs. As one miRNA targets multiple genes the scores are accumulated to calculate the relevance scores of a miRNA per OCP. The scores are scaled between 0 and 1 and summed for a general relevance score.*

	miRNA	Fibrosis Score	Hypertrophy Score	Inflammation Score	Accumulated Score
1	mmu-miR-466l-3p	1.00	1.00	1.00	3.00
2	mmu-miR-124-3p	0.74	0.82	0.96	2.51
3	mmu-miR-30e-5p	0.46	0.45	1.00	1.92
4	mmu-miR-340-5p	0.58	0.63	0.69	1.90
5	mmu-let-7b-5p	0.54	0.65	0.71	1.90
6	mmu-miR-19b-3p	0.43	0.52	0.86	1.81
7	mmu-miR-384-5p	0.43	0.43	0.91	1.78
8	mmu-miR-19a-3p	0.41	0.50	0.82	1.73
9	mmu-miR-1961	0.50	0.56	0.66	1.72
10	mmu-miR-30d-5p	0.41	0.42	0.87	1.70
11	mmu-miR-30c-5p	0.41	0.42	0.87	1.70
12	mmu-miR-30b-5p	0.41	0.42	0.87	1.70
13	mmu-miR-30a-5p	0.41	0.42	0.87	1.70
14	mmu-let-7e-5p	0.48	0.56	0.64	1.68
15	mmu-miR-325-3p	0.60	0.57	0.48	1.65
16	mmu-let-7d-5p	0.46	0.54	0.64	1.64
17	mmu-let-7i-5p	0.47	0.54	0.62	1.62
18	mmu-miR-98-5p	0.47	0.52	0.62	1.61
19	mmu-miR-27a-3p	0.46	0.50	0.63	1.60
20	mmu-let-7g-5p	0.45	0.52	0.62	1.59

Discussion

The present study provides an unprecedented descriptive overview of pre-clinical cardiac PO studies published during the last 15 years. Animal models of PO-induced HF are vital for advancement of our understanding of the pathophysiology of hypertensive heart disease and aortic stenosis and play a crucial role in the development of new therapies¹¹. By combining the results of 284 research articles we show that inflammation is closely linked with the pathophysiological processes of hypertrophy and fibrosis. In addition, we have constructed a gene-to-phenotype interaction network (GPIN) based on a systematic review of these preclinical studies. This approach of combining a systematic review with network analysis has to our knowledge never been used before. Using published data from articles the effect of individual genes on pathophysiological processes in HF was quantified. We have extended this network with miRNAs known (or predicted) to target the genes and identified miR-4661-3p, miR-124-3p, miR-30e-5p as potential therapeutic targets in PO induced HF. Finally, this dataset provides useful reference values to all researchers studying PO in animal models.

It is unclear to what extent the PO models of AngII infusion and TAC are comparable. One could argue that the AngII model better mimics hypertensive heart disease, while the TAC models shows more resemblance to aortic stenosis. The effects of AngII, however, are not limited to the development of cardiac PO due to increased hypertension only²⁵ as AngII was shown to directly stimulate cardiomyocyte hypertrophy²⁶. Moreover, AngII-induced systemic pro-inflammatory effects can play a role as well²⁷. Analysis of the dataset points out that TAC induces a more profound increase in hypertrophy, as reflected by a larger increase in cardiac mass and cardiomyocyte size, whereas fibrosis and cardiac function do not differ between these PO models. These results indicate that the AngII model is milder in terms of hypertrophy, or alternatively, that the AngII model is characterized by more fibrosis, at a given level of hypertrophy.

We explored the relation between three pathophysiological parameters of PO-induced HF, hypertrophy, fibrosis and inflammation. Our analysis showed that an experimental condition that significantly changes one of the OCPs often induces a parallel change in the other parameters. Noteworthy are the few studies where the pathophysiological parameters are changing in opposite directions. Cai et al.²², for example, showed that inhibition of HSP70 expression enhanced myocardial hypertrophy but suppressed cardiac fibrosis as induced by aortic constriction. Increased hypertrophy is initiated via the role of HSP70 in calcium handling, on the other hand, reduced fibrosis is mediated via a reduction in the inflammatory response as extracellular HSP70 attenuates the conjugation of HSP70 with TLR4, and thereby decreases macrophage infiltration and the inflammatory cytokines expression, which attenuates the pressure overload-induced cardiac fibrosis.

HF has been shown to be associated with inflammation²⁸ and inflammation is believed to play an active role in HF disease progression²⁹. Infiltrating inflammatory cells affect both cardiac hypertrophy and fibrosis⁵. It is interesting to speculate whether the process of inflammation is a cause or a consequence of HF. Regretfully, the number of studies where inflammation is numerically quantified is limited. In contrast to hypertrophy and fibrosis, inflammation is neither stringently defined nor measured in a consistent manner. Next to the infiltration of immune cells, the presence and degree of inflammation is monitored by the secretion of cytokines or the activation of intracellular inflammatory pathways, such as the NFκB pathway. In addition, the diverse set of measurements used to

quantify the inflammatory processes limits the comparability of studies. We found the number of macrophages per mm² of myocardium to be the most frequently used parameter to determine cardiac inflammation. In our dataset only 46 studies (12%) included this measurement. Together, these data support the notion that inflammation correlates with adverse disease progression in HF.

Numerous studies in the dataset target specific components of the inflammatory response, e.g. key pro-inflammatory cytokines such as, TNF α ^{30, 31}, IL-6 ³²⁻³⁴ or IFN γ ^{35, 36}. Deletion of these cytokines in these specific studies leads to a reduction in fibrosis and hypertrophy, thereby supporting the notion that inflammation plays an active role in the development of cardiac fibrosis and hypertrophy after PO. Nevertheless, it remains difficult to distinguish if inflammation is a cause or merely an effect of HF. To our surprise in several studies that manipulated the immune response, inflammation itself was not quantified. For instance, Honsho et al. ³⁷ measured IL-1 β secretion from stretched CF's *in vitro*, but did not measure cardiac inflammation in IL-1 β KO mice subjected to TAC. In other papers, the role of the immune-related interferon regulatory factor (IRF) family members, IRF1 ³⁸, IRF4 ³⁹ and IRF9 ⁴⁰ in HF was explored, without actually quantifying the amount of inflammation in the heart.

Network analysis and miRNAs

Based on the number of genes in the GPIN that miRNAs are predicted to target, we calculated scores for all miRNAs. This novel approach allowed the identification of candidate miRNAs influencing cardiac inflammation, hypertrophy and fibrosis. The microRNA with the highest score for enrichment is mmu-miR-466l-3p. Indeed, it was shown that acute inflammation upregulates miR-466l, which in turn regulates resolution ⁴¹, a process in which inflammation is cleared. Overexpression of miR-466l promotes specialized pro-resolving mediator production ⁴¹ making mmu-miR-466l-3p, identified in our GPIN, an interesting candidate for anti-inflammatory treatment. miR-124-3p, the second highest scorer in in our analysis, was described to control the proliferative, migratory, and inflammatory phenotype of pulmonary vascular fibroblasts ⁴² and to play a role in the pathogenesis of pulmonary arterial hypertension. Moreover, it was shown to suppress the NFAT signalling pathway ⁴³, known to be involved in cardiac hypertrophy. Interesting to mention as well are miR-19 and miR-340. miRNA-19a and 19b are members of the miR-17-92 cluster, which regulates the expression of the ECM proteins CTGF and TSP-1 in ageing-related heart failure as shown by our group previously ⁴⁴. A recent study by Zhu et al. ⁴⁵ used network analysis to identify HF related miRs ⁴⁵, based on expression levels of miRNAs and genes in myocardial biopsies of dilated cardiac myopathy (DCM) patients. Their expression data driven miRNA-Gene-Network identified miR-340 as an important player in HF. This is in line with our GPIN analysis, in which miR-340 was identified as one of the most relevant miRNAs. MiRNAs with highest scores for individual OCPs are putatively important in the development of these pathophysiological processes. However, for many of these miRNAs it remains to be established if they are expressed in the heart and, if so, if their expression changes during HF development. Moreover, our scoring approach favours miRNAs that have many targets. MiR-466l-3p in particular has 3196 predicted target genes in the miRNA targeting databases we integrated in the GPIN. Although potentially interesting as therapeutic target, this considerably increases the chance of unwanted side effects.

Systematic reviews of animal studies and publication bias

Previously others have performed meta-analysis of animal studies, also in the cardiovascular field^{8, 46}. These meta-analyses are based on a much smaller number of studies, and focus on a specific treatment and effect size. A relevant question is to what extent publication bias effects the conclusions of the present study. In animals studies, the number of publications presenting negative results is generally low, as was demonstrated by a systematic review in animal studies of acute ischaemic stroke⁴⁷, where only 2% of the included publications reported no significant effects⁴⁷. In contrast to many other systematic reviews we did not focus on the effect size of a specific treatment or intervention. Generally speaking, that type of analysis is more prone to publication bias⁴⁸. As our PO comparisons and reference values are mostly based on wild-type mice potential effects of publication bias are most likely limited. Moreover, as the articles in our dataset measure more than one outcome parameter the incorporation of neutral data is more common. This is reflected by the relatively high number of studies that report no significant difference in one of the three OCPs we focussed. Nonetheless, the scatterplot in Figure 4A clearly shows two clouds of data points and a void around the area reflecting no change, suggesting that neutral effect sizes are underrepresented⁴⁸. Accordingly, we cannot entirely rule out that there is some effect of publication bias on outcome.

Conclusion

In conclusion, we established a novel approach to identify potential HF therapeutic targets by using existing literature to construct a Gene-to-Phenotype interaction network. In this way we identified several potentially interesting miRNAs. Secondly, we explored the relation between inflammation, on the one hand, and hypertrophy and fibrosis, on the other, and showed that inflammation strongly correlates with the pathophysiological processes of hypertrophy and fibrosis, supporting the concept that inflammation plays a central role in HF development.

References

(references combined and included with supplemental material)

Supplementary Methods

Detailed search terms and strategy:

Disease model for pressure overload:

pressure overload [TIAB] or TAC[TIAB] or (aortic constriction[TIAB]) or Angiotensin II[TIAB] or Angiotensin II[MeSH] or (ANG II[TIAB]) or ((spontaneous and hypertensive) or (spontaneously and hypertensive))

Species:

Mouse [mesh] OR Rat [MeSH]

Selection of at least 2 of the three outcome parameters:

Fibrosis and inflammation: (((fibrosis[MeSH]) OR fibrosis[tiab]) and (inflammation[MeSH] or inflammation[tiab] or (inflammatory response[tiab])))

Fibrosis and hypertrophy: (((fibrosis[MeSH]) OR fibrosis[tiab]) and ((Cardiomyopathy, Dilated[mesh]) or (Hypertrophy, Left Ventricular[mesh]) or cardiac hypertrophy[MeSH]))

Inflammation and hypertrophy: ((inflammation[MeSH] or inflammation[tiab] or (inflammatory response[tiab])) and ((Cardiomyopathy, Dilated[mesh]) or (Hypertrophy, Left Ventricular[mesh]) or cardiac hypertrophy[MeSH]))

Organ:

heart [MeSH]

Filters for publication date and language:

Date (01-01-2000 until 31-12-1014) and (English).

The combined search term used in PubMed:

((((Mouse [mesh] OR Rat [MeSH]) AND (((fibrosis[MeSH]) OR fibrosis[tiab]) and (inflammation[MeSH] or inflammation[tiab] or (inflammatory response[tiab])))) OR (((fibrosis[MeSH]) OR fibrosis[tiab]) and ((Cardiomyopathy, Dilated[mesh]) or (Hypertrophy, Left Ventricular[mesh]) or cardiac hypertrophy[MeSH])) OR ((inflammation[MeSH] or inflammation[tiab] or (inflammatory response[tiab])) and ((Cardiomyopathy, Dilated[mesh]) or (Hypertrophy, Left Ventricular[mesh]) or cardiac hypertrophy[MeSH]))))) AND (pressure overload [tiab] or (aortic constriction) or TAC[tiab] or Angiotensin II[tiab] or Angiotensin II[mesh] or (ANG II[tiab]) or (spontaneous and hypertensive) or (spontaneously and hypertensive)) AND heart [MeSH])) AND ("2000/01/01"[Date - Publication] : "2014/12/31"[Date - Publication])

Data analysis and processing

Our approach was based on the methods of a meta-analysis, using a similar search protocol and strict inclusion criteria. In the data extraction, processing and visualisation we deviated from the approach used in a meta-analysis, by visualizing outcome parameters extracted from individual studies as data points in our plots instead of using forest plots. Standard deviations or other statistical measures of variance were not extracted from the studies, because it was not feasible to obtain these values from the graphics in many papers in a reliable way. Moreover, we felt that the sheer number of studies allows statistical analysis based on the mean values extracted.

Normalization of numerical parameters

In the various studies similar OCPs were assessed, normalized and presented in different ways. Cardiac mass for example was presented normalized for either body weight (BW) or for tibia length (TL). Cardiomyocyte size was usually presented as cross sectional area (CSA, μm^2), but sometimes also as diameter (μm). OCPs of cardiac mass, cardiomyocyte size and fibrosis were normalized to allow direct comparison of these outcome measures between the different studies in our analyses.

Cardiac Mass: In the literature cardiac mass was presented as: HW/BW ratio (mg/g), LVw/BW ratio (mg/g), HW/TL ratio (mg/mm), LVw/TL ratio (mg/mm), Absolute HW (mg), Absolute LVw (mg) and as relative increase vs. SHAM. All these values were converted to HW/BW ratio, being the most frequently used measure. Corrections for TL towards BW were

calculated using a correction factor based on historical data from the Jackson laboratory phenome database ⁴⁹ (<http://phenome.jax.org/>).

Cardiomyocyte size: Cardiomyocyte size was most often presented as CSA (μm^2), but also as myocyte diameter (μm) or as Arbitrary Units (AU). Myocyte diameter was converted to CSA based on the area of a circle and scaled based on the mean SHAM level to mean CSA SHAM level ratio. We have corrected Myocyte diameter μm into CSA by using the following formula: $(\text{Diameter} / 2)^2 \times \pi \times 1.7$. Based on the area of a circle but multiplied by a factor 1.7 to scale the mean of SHAM groups levels to the mean CSA SHAM levels ($244 \mu\text{m}^2$).

Fibrosis area: Reported baseline values for the percentage fibrosis in the myocardium ranged from 0% to 12.3%. Studies reporting a very low percentage fibrosis at baseline, show a very large relative increase after PO. To avoid artificially high values for the relative increase when baseline fibrosis areas approached zero, baseline values below 1% were set to 1%. An exception was made when values after PO were lower than 2% to avoid creating an artificial decrease after PO.

Macrophages per mm^2 : The only inflammation related parameter of which numerical data was extracted is the number of macrophages per mm^2 determined by immuno-histochemical staining. Although different markers for macrophages were used (For mice: CD68, Mac-2, Mac-3, F4/80 and for Rat: ED-1) we did not distinguish between them in the analysis.

Supplemental Table 1. Overview of study details and outcome parameters (OCs) extracted.

General information	Experimental details	Extracted outcome data
Name first author	Species of Animals	Hypertrophy Measure of cardiac mass (HW/BW) Measure of cardiomyocyte size (CSA) Measure of wall thickness (LVPWd) General conclusion ($\uparrow \downarrow -$)
	Gender	
	Strain	
Year of publication	Duration	
	Age at start	
	Number of animals used	Cardiac Function Measure of cardiac function (EF/FS%) Measure of Blood pressure (SBP) General conclusion ($\uparrow \downarrow -$)
Title	Use of KO/TG animals	
	Gene ID of manipulated	
PubMed ID	gene	
	PO model	
Journal	Infused dose (AngII / ISO)	Fibrosis Measure of collagen/fibrotic area (%) General conclusion ($\uparrow \downarrow -$)
	Size of constriction (AC)	
Brief description		
		Inflammation Macrophages number (cells per mm^2) General conclusion ($\uparrow \downarrow -$)

Outcome parameters are mostly numerical data, except for the general conclusions on the articles on the processes hypertrophy, function, fibrosis and inflammation.

Supplemental Results

Introduction

Understanding of the pathogenesis of HF requires a detailed characterisation of the underlying pathophysiological processes and of the different disease aetiologies, such as hypertensive heart disease and aortic stenosis. Rodent models are extensively used to investigate the molecular mechanisms involved in the pathogenesis of PO-induced heart failure ¹¹. In this supplemental section, our dataset, composed of 369 studies, in which over 16 thousand animals were used in total, was used to compare features of frequently used rodent PO models (Supplemental Table 2). Despite extensive use of these preclinical HHD models, the similarities and dissimilarities are ill-defined and reference values for the expected degree of hypertrophy, fibrosis and inflammation have not been reported so far. Moreover, the combined studies in our dataset allow a detailed analysis of the temporal changes induced by PO in the mouse heart. Collectively, this provides deeper insight into the interpretation of performed studies and can improve the design of future PO studies, thereby potentially reducing animal numbers needed in the future.

Supplemental table 2. Overview of most common mouse and rat models of pressure overload.

Pressure overload model		Brief description	Variables	Species	(dis)advantages
Aortic constriction	AAC	Banding of the abdominal aorta	Size of constriction	Rat	Induces potent hypertrophic response. Invasive surgery.
	TAC	Banding of the transverse aorta	Size of constriction	Mouse	Induces potent hypertrophic response. Invasive surgery.
Angiotensin II infusion		Infusion via osmotic mini-pumps	Dose and Duration	Mouse, Rat	Systemic elevation of BP, No invasive surgery
Isoproterenol infusion		Infusion via osmotic mini-pumps	Dose and duration	Mouse, Rat	No invasive surgery, hypertrophy along with myocardial damage.
Salt-diet in salt sensitive rats		Salt added to diet / drinking water	Duration, genetic background	Rat	Clinically relevant model, Gradual onset of hypertension.
Genetic background		Genetic trait associated with hypertension.	Genetic background, transgene, age	Rat	No invasive surgery, or pharmacological intervention. Gradual onset of hypertension. Control animals often a problem.

Results and Discussion

Cardiac features of rats and mice

Analysis revealed baseline differences in several outcome parameters (OCPs) between rat and mice. In Supplemental Table 3 we defined reference values for (patho)physiological parameters, such as hypertrophy, fibrosis, cardiac function, blood pressure and inflammation in both rat and mouse. Strikingly, average baseline cardiomyocyte surface area (CSA) values reported for mice are lower for mice than for rat. Reported baseline values for fractional shortening ($48 \pm 12\%$) can be used as reference values. Baseline systolic blood pressure (SBP) is somewhat higher in rats than mice. Differences in collagen area at baseline and after PO between species were not observed. The number of infiltrated macrophages shows an enormous variation between studies, and is therefore difficult to compare. Because of the limited number of rat studies and the variation in the PO models applied (Table 4), we focused on mice studies only for further analysis.

Supplemental Table 3. Overview of reference values of outcome parameters in baseline and PO conditions for wild-type rat and mouse of all strains.

Parameter	Rat		Mouse	
	Sham	PO	Sham	PO
HW/BW Ratio (mg/g)	2.9 ± 0.49	4.13 ± 0.93	4.6 ± 0.7	6.9 ± 1.4
CSA (μm^2)	308.2 ± 114.3	538.7 ± 242.8	244 ± 15	400 ± 25
Fractional Shortening (%)	48.2 ± 12.5	N/A	48.6 ± 12.6	N/A
LVPW(d) (mm)	1.5 ± 0.4	2.0 ± 0.5	0.9 ± 1.3	1.2 ± 1.4
Fibrosis (% area)	2.2 ± 2.1	8.4 ± 3.5	1.9 ± 1.7	7.9 ± 2.9
Macrophages per mm^2	48.8 ± 103.0	421.1 ± 2005.4	42.1 ± 111.4	232.6 ± 669.2
SBP (mm Hg)	118.6 ± 15.5	N/A	105.9 ± 12.4	N/A

Values are represented as the mean with standard deviation. N/A: Values that are irrelevant without subgroup analysis due to large variation in study design.

Cardiac hypertrophy, but not fibrosis, correlates with PO duration

Supplemental Figure 1 shows the fold change for OCPs between sham and PO conditions plotted against the duration of the experiment. Cardiac mass correlates positively with the duration of the experiment, ($R^2 = 0.33$, $P < 0.0001$), characterized by a rapid increase in cardiac mass in the first weeks after PO which seems to stabilize after 21 days (Supplemental Figure 1A). Cardiomyocyte size shows a more or less similar pattern, albeit with far more variation between data points (Supplemental Figure 1B). Interestingly, no correlation was observed between the degree of fibrosis and the duration of the experiment (Supplemental Figure 1C). Cardiac function was found to decline ($R^2 = 0.19$, $P < 0.0001$) as a function of the duration of the experiment (Supplemental Figure 1D). It is noteworthy, however, that some studies (13%) actually report an increase in fractional shortening upon PO, suggesting a compensatory response in systolic cardiac function under some conditions.

Supplemental figure 1. Temporal changes in cardiac phenotype in mice after PO. Mouse studies with TAC and AngII up to a duration of 63 days are included. A. Scatterplot showing fold increase in cardiac mass between sham and PO plotted against the duration of the experiment. B. Scatterplot showing fold change in cardiomyocyte size between sham and PO plotted against the duration of the experiment. C. Shows fold change in fibrosis for durations (binned). D. Scatterplot showing fold change in Fractional shortening between sham and PO plotted against the duration of the experiment. The dotted line indicates no difference in fold change.

Previously, a number of experimental studies provided data on the time course of changes in hypertrophy, fibrosis and inflammation after induction of PO⁵⁰⁻⁵². This allows a comparison of serial measurements performed by others, with the data generated in this systematic review. Histological examination of cardiac tissue at 3, 7 and 28 days after TAC by Xia et al.⁵⁰ indicates that cardiac mass as well as other parameters of hypertrophy increase with duration of the experiment. The infiltration of macrophages as well as cardiac fibrosis already peak at 7 days⁵⁰. Interestingly, in our analysis the amount of fibrosis shows no linear increase either (Supplemental figure 1C). It is interesting to speculate whether the balance between collagen synthesis and degradation shifts as a function of time, as collagen degradation is induced by the infiltrating pro-inflammatory cells⁵³. Well in line with our analysis (Supplemental figure 1D) cardiac function (fractional shortening) showed a steady linear decrease over time, becoming significant only after 28 days in the study of Xia and colleagues⁵⁰. Rizzi and colleagues have studied temporal changes in cardiac matrix metalloproteinase activity after 15, 30 and 75 days of PO⁵¹. They reported a progressive increase in cardiac hypertrophy in time and measured highest collagen area at 15 days, indicating once more that collagen is degraded in later stages of disease. Stewart et al., on

the other hand investigated fibroblast function after PO and showed collagen content to increase continuously 7, 14 and 28 days after abdominal aortic constriction ⁵². Others have studied the variety in the phenotype induced by TAC ⁵⁴ demonstrating that mice have lower ejection fraction at 3 or 9 weeks post-TAC, but that only a 28% of these mice develop HF.

The strength of our approach to characterize the PO-induced phenotype based on published articles is that the combination of results from hundreds of different labs corrects for the large variation in between labs due to animal handling, housing and other factors. The large number of studies combined gives a more accurate description of the PO induced phenotype. This allows better prediction of model variation and effect sizes, thereby reducing the number of animals needed for future studies.

Supplemental figure 2. PO-induced cardiac phenotype in different mouse strains and used doses of AngII. A. HW/BW ratios of TAC and AngII studies in different mice strain before and after PO. B. Percentage fibrosis in TAC and AngII studies in different mice strain before and after PO. C. Effect of AngII dose on blood pressure (SBP) showing only a modest and non-significant rise with increasing AngII doses.

Strains, dosing, blood pressure and other variables

Clear phenotypical differences between the various mouse strains used in PO studies are not observed. Cardiac mass and fibrosis increased to a similar extent in C57BL/6, Balb/c, FVB and CD-1 mice (Supplemental Figure 2A-B). Supplemental Figure 2C shows the relation between the dose of AngII infused and the measured systolic blood pressure (SBP). Surprisingly, an AngII dose of 1 mg/kg/day suffices to achieve a pronounced increase in blood pressure (SBP) and the effect of higher doses on blood pressure seems limited.

There are many differences in experimental setup between studies. Studies vary in the use of strains, anaesthetics, size of constriction and AngII dose, etc., making comparisons difficult. This heterogeneity potentially explains differences in outcome. For example, it has been demonstrated that AngII infusion leads to dilated cardiomyopathy (DCM) in Balb/c mice, but not in C57BL/6 mice ⁵⁵. Notably, C57BL/6 mice of a different substrain (-J or -N substrain) have been shown to respond different to TAC ⁵⁶. Survival and cardiac function were significantly lower in the -N mice compared with -J mice ⁵⁶, stressing the importance of defining which substrain is actually used (Table 3). Even the type of mini-pump used for AngII infusion can lead to differences in blood pressure ⁵⁷. In order to enable better comparison of different studies, it would be useful to introduce further standardization of animal experimental conditions.

Conclusion

Systematic review of the literature shows that cardiac mass and cardiomyocyte size increase predominantly in the first weeks after PO, whereas cardiac function continues to decrease steadily over the next weeks. Surprisingly, the level of fibrosis hardly increases with time. The obtained reference values help to predict the model variation and effect sizes which, in turn, can help to reduce the number of animals needed in future studies. The current analysis also highlights the importance of detailed and proper documentation of all experimental conditions in order to increase reproducibility and interpretation of animal studies.

Supplemental figure 3. Correlation of fold change induced by the experimental condition between OCPs of hypertrophy cardiac mass and cardiomyocyte cross-sectional area (CSA).

Supplemental table 4. Articles with general conclusions that show opposite directions for the pathophysiological processes of cardiac hypertrophy, fibrosis and inflammation

General conclusion	Author and year	PubMed ID
Hypertrophy ↑ Fibrosis ↓	Cai et al., 2010	20542874
Hypertrophy ↓ Fibrosis ↑	Honsho et al., 2009	19834007
	Kazakov et al., 2012	22106415
Hypertrophy ↑ Inflammation ↓	Frolova et al., 2012	22362893
	Cai et al., 2010	20542874
	Ares-Carrasco, 2009	19820199
Hypertrophy ↓ Inflammation ↑	Liu et al., 2008	18490734
Inflammation ↑ Fibrosis ↓	Liu et al., 2008	18490734
Inflammation ↓ Fibrosis ↑	Frolova et al., 2012	22362893

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Supplemental File 1. Overview of all included studies.

* article contains multiple studies. Pm ID = PubMed ID. Gene = The Ensembl name of the manipulated gene and the direction of manipulation: ↓ Down (e.g. knockout) ↑ = up (e.g. transgenic). General conclusions regarding cardiac hypertrophy (H), fibrosis (F) and inflammation (I) are indicated as follows: reduced (↓), increased (↑) or no significant change (-) ND = No data / Not determined.

First Author	Year	Title	Pm ID	S	gene	H	F	I
Muller	2000	NF-kappaB inhibition ameliorates angiotensin II-induced inflammatory damage in rats.	10642297	R		↓	ND	↓
Akishita*	2000	Inhibitory Effect of Angiotensin II Type 2 Receptor on Coronary Arterial Remodeling after Aortic Banding in Mice	11015348	M	<i>Agtr2</i> ↓	↑	↑	ND
Akishita*	2000	Inhibitory Effect of Angiotensin II Type 2 Receptor on Coronary Arterial Remodeling after Aortic Banding in Mice	11015348	M	<i>Agtr2</i> ↓	-	↑	ND
Koyanagi	2000	Role of monocyte chemoattractant protein-1 in cardiovascular remodeling induced by chronic blockade of nitric oxide synthesis.	11056100	R	<i>Ccl2</i> ↓	ND	-	↓
Sakata	2000	Calcineurin Inhibitor Attenuates Left Ventricular hypertrophy, Leading to Prevention of Heart Failure in Hypertensive Rats	11056104	R		↓	-	ND
Uozumi	2001	gp130 plays a critical role in pressure overload-induced cardiac hypertrophy.	11262406	M	<i>Il6st</i> ↓	↓	ND	ND
Knowles	2001	Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A-deficient mice.	11306601	M	<i>Npr1</i> ↓	↑	↑	ND
Ichihara	2001	Angiotensin II Type 2 Receptor Is Essential for Left Ventricular Hypertrophy and Cardiac Fibrosis in Chronic Angiotensin II-Induced Hypertension	11457756	M	<i>Agtr2</i> ↓	↓	↓	ND
Rapacciuolo	2001	Important role of endogenous norepinephrine and epinephrine in the development of in vivo pressure-overload cardiac hypertrophy.	11527648	M	<i>Dbh</i> ↓	↓	ND	ND
Eickels*	2001	17β-Estradiol Attenuates the Development of Pressure-Overload Hypertrophy	11560859	M		↓	-	ND
Eickels*	2001	17β-Estradiol Attenuates the Development of Pressure-Overload Hypertrophy	11560859	M		↓	ND	ND
Moser*	2002	Predominant activation of endothelin-dependent cardiac hypertrophy by norepinephrine in rat left ventricle.	11959681	R		-	ND	ND
Moser*	2002	Predominant activation of endothelin-dependent cardiac hypertrophy by norepinephrine in rat left ventricle.	11959681	R		-	ND	ND
Baumgarten*	2002	Load-Dependent and -Independent Regulation of proinflammatory Cytokine and Cytokine receptor Gene Expression in the Adult mammalian heart	11994254	M		ND	ND	ND
Baumgarten*	2002	Load-Dependent and -Independent Regulation of proinflammatory Cytokine and Cytokine receptor Gene Expression in the Adult mammalian heart	11994254	M		ND	ND	-
Baumgarten*	2002	Load-Dependent and -Independent Regulation of proinflammatory Cytokine and Cytokine receptor Gene Expression in the Adult mammalian heart	11994254	R		ND	ND	ND
Jeron	2002	Systemic immunosuppression fails to suppress cardiac cytokine induction in pressure overload hypertrophy in rats.	11999344	R		-	ND	↓
Kuwahara	2002	Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats.	12093782	R	<i>Tgfb1</i> ↓	-	↓	ND
Sadoshima	2002	The MEK1-JNK pathway plays a protective role in pressure overload but does not mediate cardiac hypertrophy.	12122119	M	<i>Map3k1</i> ↓	-	ND	↑
Nakayama	2003	Cardiac-specific overexpression of a high Ca2+ affinity mutant of SERCA2a attenuates in vivo pressure overload cardiac hypertrophy.	12424227	M	<i>Atp2a1</i> ↑	↓	ND	ND
Cingolani*	2003	Regression of hypertensive myocardial fibrosis by Na(+)/H(+) exchange inhibition.	12574110	R		↓	↓	ND
Cingolani*	2003	Regression of hypertensive myocardial fibrosis by Na(+)/H(+) exchange inhibition.	12574110	R		↓	↓	ND
Cingolani*	2003	Regression of hypertensive myocardial fibrosis by Na(+)/H(+) exchange inhibition.	12574110	R		↓	-	ND
Kuwahara	2003	Roles of intercellular adhesion molecule-1 in hypertensive cardiac remodeling.	12624002	R	<i>Icam1</i> ↓	-	↓	↓
Zhang	2003	The role of the Grb2-p38 MAPK signaling pathway in cardiac hypertrophy and fibrosis.	12639989	M	<i>Grb2</i> ↓	↓	↓	ND
Hu	2003	Minimally invasive aortic banding in mice: effects of altered cardiomyocyte insulin signaling during pressure overload.	12738623	M	<i>Insr</i> ↓	-	↑	ND
Wang*	2003	Effects of pressure overload on extracellular matrix expression in the heart of the atrial natriuretic peptide-null mouse.	12756220	M	<i>Nppa</i> ↓	-	-	ND
Wang*	2003	Effects of pressure overload on extracellular matrix expression in the heart of the atrial natriuretic peptide-null mouse.	12756220	M	<i>Nppa</i> ↓	↑	↑	ND
Wang*	2003	Effects of pressure overload on extracellular matrix expression in the heart of the atrial natriuretic peptide-null mouse.	12756220	M	<i>Nppa</i> ↓	↑	↑	ND
Okumura	2003	Disruption of type 5 adenylyl cyclase gene preserves cardiac function against pressure overload.	12904575	M	<i>Adcy5</i> ↓	-	ND	ND
Takahashi	2003	Angiotensin II-induced ventricular hypertrophy and extracellular signal-regulated kinase activation are suppressed in mice overexpressing brain natriuretic peptide in circulation.	14621189	M	<i>Nppb</i> ↑	↓	↓	ND
Ichinose	2003	Pressure overload-induced LV hypertrophy and dysfunction in mice are exacerbated by congenital NOS3 deficiency.	14644766	M	<i>Nos3</i> ↓	↑	↑	ND
Diep	2004	PPARα activator fenofibrate inhibits myocardial inflammation and fibrosis in angiotensin II-infused rats	14871557	R		-	↓	↓
Kuwahara	2004	Hypertensive myocardial fibrosis and diastolic dysfunction: another model of inflammation?	14967845	R	<i>Ccl2</i> ↓	-	↓	↓
Rasoul	2004	Antifibrotic effect of Ac-SDKP and angiotensin-converting enzyme inhibition in hypertension.	15076166	R		-	↓	↓
Chen	2004	Heat shock treatment suppresses angiotensin II-induced activation of NF-kappaB pathway and heart inflammation: a role for IKK depletion by heat shock?	15087290	R		ND	↓	↓
Hikoso	2004	Pressure overload induces cardiac dysfunction and dilation in signal transducer and activator of transcription 6-deficient mice.	15492321	M	<i>Stat6</i> ↓	-	↑	↑
Gao	2005	Regression of pressure overload-induced left ventricular hypertrophy in mice.	15665058	M		ND	ND	ND

Kuster	2005	Mineralocorticoid receptor inhibition ameliorates the transition to myocardial failure and decreases oxidative stress and inflammation in mice with chronic pressure overload.	15687129	M		-	↓	↓
Kawano*	2005	Blockade of NF-kappaB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II.	15921667	M	<i>Nfkb1</i> ↓	-	ND	ND
Kawano*	2006	Blockade of NF-kappaB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II.	15921667	M	<i>Nfkb1</i> ↓	↓	ND	-
Ha	2005	Reduced cardiac hypertrophy in toll-like receptor 4-deficient mice following pressure overload	15967420	M	<i>Tlr4</i> ↓	↓	-	↓
Hara	2005	Augmented cardiac hypertrophy in response to pressure overload in mice lacking the prostaglandin I2 receptor.	15983244	M	<i>Ptgir</i> ↓	↑	↑	ND
Kassiri	2005	Combination of tumor necrosis factor-alpha ablation and matrix metalloproteinase inhibition prevents heart failure after pressure overload in tissue inhibitor of metalloproteinase-3 knock-out mice.	16037568	M	<i>Timp3</i> ↓	↑	↑	ND
Ha	2006	Blockade of MyD88 attenuates cardiac hypertrophy and decreases cardiac myocyte apoptosis in pressure overload-induced cardiac hypertrophy in vivo.	16199478	R	<i>Myd88</i> ↓	↓	↓	↓
Peng	2005	Angiotensin-converting enzyme inhibitors: a new mechanism of action.	16216963	R		-	↓	↓
Nagata	2006	Mineralocorticoid receptor antagonism attenuates cardiac hypertrophy and failure in low-aldosterone hypertensive rats.	16505208	R		↓	↓	↓
O'Connell	2006	Alpha1-adrenergic receptors prevent a maladaptive cardiac response to pressure overload.	16585965	M	<i>Adra1a</i> ↓, <i>Adra1b</i> ↓	-	↑	ND
Kong*	2006	Suppression of class I and II histone deacetylases blunts pressure-overload cardiac hypertrophy.	16735673	M	<i>Hist1h3a</i> ↓	↓	-	ND
Kong*	2006	Suppression of class I and II histone deacetylases blunts pressure-overload cardiac hypertrophy.	16735673	M	<i>Hist1h3a</i> ↓	↓	ND	ND
Gao	2006	Inhibition of mTOR reduces chronic pressure-overload cardiac hypertrophy and fibrosis.	16877971	M		↓	↓	ND
Empel	2006	EUK-8, a Superoxide Dismutase and Catalase Mimetic, Reduces Cardiac Oxidative Stress and Ameliorates Pressure Overload-Induced Heart Failure in the Harlequin Mouse Mutant	16904556	M	<i>Aifm1</i> ↓	↑	↑	ND
Franco	2006	Eplerenone prevents adverse cardiac remodelling induced by pressure overload in atrial natriuretic peptide-null mice.	16922805	M	<i>Nppa</i> ↓	↑	↑	ND
Bisping	2006	Gata4 is required for maintenance of postnatal cardiac function and protection from pressure overload-induced heart failure.	16983087	M	<i>Gata4</i> ↓	↑	↑	ND
Sakamoto	2006	Upregulation of heat shock transcription factor 1 plays a critical role in adaptive cardiac hypertrophy.	17095722	M	<i>HSF1</i> ↑	↓	↓	ND
Ren	2007	Beta3 integrin deficiency promotes cardiac hypertrophy and inflammation.	17184791	M	<i>Itgb3</i> ↓	↑	ND	↑
Harada	2007	Diacylglycerol kinase zeta attenuates pressure overload-induced cardiac hypertrophy.	17251681	M	<i>Dgkz</i> ↑	↓	↓	↓
Sun	2007	Tumor necrosis factor-alpha mediates cardiac remodeling and ventricular dysfunction after pressure overload state	17353445	M	<i>Tnf</i> ↓, <i>Il1b</i> ↓	↓	↓	↓
Duhaney	2007	Peroxisome proliferator-activated receptor alpha-independent actions of fenofibrate exacerbates left ventricular dilation and fibrosis in chronic pressure overload.	17353509	M		↑	↑	ND
Buys	2007	Cardiomyocyte-restricted restoration of nitric oxide synthase 3 attenuates left ventricular remodeling after chronic pressure overload.	17416602	M	<i>Nos3</i> ↓	↑	-	ND
Sanada*	2007	IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system.	17492053	M	<i>Tmed1</i> ↓	↑	↑	↑
Sanada*	2007	IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system.	17492053	M	<i>Il33</i> ↑	↓	↓	↓
Nishioka	2007	Eplerenone attenuates myocardial fibrosis in the angiotensin II-induced hypertensive mouse: involvement of tenascin-C induced by aldosterone-mediated inflammation.	17513943	M		ND	↓	↓
Oka	2007	Genetic manipulation of periostin expression reveals a role in cardiac hypertrophy and ventricular remodeling.	17569887	M	<i>Postn</i> ↓	↓	↓	ND
Coles*	2007	Classic interleukin-6 receptor signaling and interleukin-6 trans-signaling differentially control angiotensin II-dependent hypertension, cardiac signal transducer and activator of transcription-3 activation, and vascular hypertrophy in vivo.	17591976	M	<i>Il6</i> ↓	↓	ND	ND
Coles*	2007	Classic interleukin-6 receptor signaling and interleukin-6 trans-signaling differentially control angiotensin II-dependent hypertension, cardiac signal transducer and activator of transcription-3 activation, and vascular hypertrophy in vivo.	17591976	M	<i>Il6st</i> ↑	-	ND	ND
Freeling*	2008	Neostigmine and pilocarpine attenuated tumour necrosis factor alpha expression and cardiac hypertrophy in the heart with pressure overload.	17872965	R		↓	ND	↓
Freeling*	2008	Neostigmine and pilocarpine attenuated tumour necrosis factor alpha expression and cardiac hypertrophy in the heart with pressure overload.	17872965	R		↓	ND	↓
Ruiz-Hurtado	2007	LA419, a novel nitric oxide donor, prevents pathological cardiac remodeling in pressure-overloaded rats via endothelial nitric oxide synthase pathway regulation.	17984372	R		↓	↓	ND
Müller	2008	Pressure-induced cardiac overload induces upregulation of endothelial and myocardial progenitor cells.	18006457	M		↓	ND	ND
Sharma	2008	Novel anti-inflammatory mechanisms of N-Acetyl-Ser-Asp-Lys-Pro in hypertension-induced target organ damage.	18178715	M		-	↓	↓
Smeets	2008	Cardiac hypertrophy is enhanced in PPAR alpha-/- mice in response to chronic pressure overload.	18187461	M	<i>Ppara</i> ↓	↑	-	↑
Pillai	2008	Activation of SIRT1, a class III histone deacetylase, contributes to fructose feeding-mediated induction of the alpha-myosin heavy chain expression.	18192211	M		↓	↓	ND
Kiriazis	2008	Knockout of beta(1)- and beta(2)-adrenoceptors attenuates pressure overload-	18193078	M	<i>Adrb1</i> ↓	↓	↓	↓

		induced cardiac hypertrophy and fibrosis.						
Nonaka-Sarukawa	2008	Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats.	18205252	R	<i>I110</i> ↑	↓	↓	ND
Oudit	2008	Loss of PTEN attenuates the development of pathological hypertrophy and heart failure in response to biomechanical stress.	18281373	M	<i>Pten</i> ↓	↓	↓	ND
Xu	2008	Ecto-5'-nucleotidase deficiency exacerbates pressure-overload-induced left ventricular hypertrophy and dysfunction.	18391093	M	<i>Nt5e</i> ↓	↑	↑	↑
Giampietri	2008	c-Flip overexpression reduces cardiac hypertrophy in response to pressure overload.	18398344	M	<i>Cflar</i> ↑	↓	-	↓
Mito	2008	Myocardial protection against pressure overload in mice lacking Bach1, a transcriptional repressor of heme oxygenase-1.	18426999	M	<i>Bach1</i> ↓	↓	↓	ND
Moens	2008	Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy.	18474817	M		↓	↓	ND
Liu	2008	Bacillus Calmette-Guérin and TLR4 agonist prevent cardiovascular hypertrophy and fibrosis by regulating immune microenvironment.	18490734	M		↓	↓	-
Caglayan*	2009	Differential Roles of Cardiomyocyte and Macrophage Peroxisome Proliferator-Activated Receptor γ in Cardiac Fibrosis	18511847	M	<i>Pparg</i> ↓	↑	-	↑
Caglayan*	2008	Differential Roles of Cardiomyocyte and Macrophage Peroxisome Proliferator-Activated Receptor γ in Cardiac Fibrosis	18511847	M	<i>Pparg</i> ↓	↑	↑	↑
Foronjy	2008	Transgenic expression of matrix metalloproteinase-1 inhibits myocardial fibrosis and prevents the transition to heart failure in a pressure overload mouse model.	18633185	M	<i>Mmp1a</i> ↑	↓	↓	ND
Gallo	2008	Inhibition of class I histone deacetylase with an apicidin derivative prevents cardiac hypertrophy and failure.	18697792	M		↓	↓	ND
Copaja Soto	2008	Early expression of monocyte chemoattractant protein-1 correlates with the onset of isoproterenol-induced cardiac fibrosis in rats with distinct angiotensin-converting enzyme polymorphism	18957386	R		-	-	-
Di Zhang	2008	Cross-Talk Between Mineralocorticoid and Angiotensin II Signaling for Cardiac Remodeling	18981328	M	<i>NR3C2-003</i> ↑	↑	↑	-
Xia*	2008	Characterization of the inflammatory and fibrotic response in a mouse model of cardiac pressure overload.	19030868	M		ND	ND	ND
Xia*	2008	Characterization of the inflammatory and fibrotic response in a mouse model of cardiac pressure overload.	19030868	M		ND	ND	ND
Xia*	2008	Characterization of the inflammatory and fibrotic response in a mouse model of cardiac pressure overload.	19030868	M		ND	ND	ND
Molina*	2009	Improvement in hemodynamic performance, exercise capacity, inflammatory profile, and left ventricular reverse remodeling after intracoronary delivery of mesenchymal stem cells in an experimental model of pressure overload hypertrophy.	19065545	R		ND	ND	ND
Molina*	2009	Improvement in hemodynamic performance, exercise capacity, inflammatory profile, and left ventricular reverse remodeling after intracoronary delivery of mesenchymal stem cells in an experimental model of pressure overload hypertrophy.	19065545	R		ND	ND	ND
Molina*	2009	Improvement in hemodynamic performance, exercise capacity, inflammatory profile, and left ventricular reverse remodeling after intracoronary delivery of mesenchymal stem cells in an experimental model of pressure overload hypertrophy.	19065545	R		ND	ND	-
Molina*	2009	Improvement in hemodynamic performance, exercise capacity, inflammatory profile, and left ventricular reverse remodeling after intracoronary delivery of mesenchymal stem cells in an experimental model of pressure overload hypertrophy.	19065545	R		ND	ND	-
Donaldson	2009	Estrogen Attenuates Left Ventricular and Cardiomyocyte Hypertrophy by an Estrogen Receptor -Dependent Pathway That Increases Calcineurin Degradation	19074476	M		-	ND	ND
Tang*	2008	Lysosomal cysteine peptidase cathepsin L protects against cardiac hypertrophy through blocking AKT/GSK3beta signaling.	19096818	M	<i>Ctsl</i> ↑	↓	↓	ND
Tang*	2008	Lysosomal cysteine peptidase cathepsin L protects against cardiac hypertrophy through blocking AKT/GSK3beta signaling.	19096818	M	<i>Ctsl</i> ↑	↓	↓	↓
Nagayama	2009	Sildenafil stops progressive chamber, cellular, and molecular remodeling and improves calcium handling and function in hearts with pre-existing advanced hypertrophy caused by pressure overload.	19130990	M	<i>Pde5a</i> ↓	↓	↓	ND
Lee*	2011	The transcription factor Eya2 prevents pressure overload-induced adverse cardiac remodeling.	19272299	M	<i>Eya2</i> ↑	↓	↓	↓
Lee*	2010	The transcription factor Eya2 prevents pressure overload-induced adverse cardiac remodeling.	19272299	M	<i>Eya2</i> ↑	↓	↓	ND
Lee*	2009	The transcription factor Eya2 prevents pressure overload-induced adverse cardiac remodeling.	19272299	M	<i>Eya2</i> ↑	-	-	ND
Li	2009	MyD88-dependent nuclear factor-κB activation is involved in fibrinogen-induced hypertrophic response of cardiomyocytes	19342961	R		ND	ND	ND
Levick	2009	Cardiac Mast Cells Mediate Left Ventricular Fibrosis in the Hypertensive Rat Heart	19398662	R		-	↓	↓
Cai	2009	Crocetin protects against cardiac hypertrophy by blocking MEK-ERK1/2 signalling pathway.	19413885	M		↓	↓	↓
Bian*	2009	Cellular repressor of E1A-stimulated genes attenuates cardiac hypertrophy and fibrosis	19413895	M	<i>Creg1</i> ↓	↓	↓	↓
Bian*	2009	Cellular repressor of E1A-stimulated genes attenuates cardiac hypertrophy and fibrosis	19413895	M	<i>Creg1</i> ↓	↓	↓	↓
Kvakan	2009	Regulatory T cells ameliorate angiotensin II-induced cardiac damage.	19470887	M		↓	↓	↓
Hikoso	2009	The IκB Kinase β/Nuclear Factor κB Signaling Pathway Protects the Heart From Hemodynamic Stress Mediated by the Regulation of Manganese Superoxide	19478205	M	<i>Ikbkb</i> ↓	↑	↑	ND

		Dismutase Expression						
Wu	2009	Liver X receptors are negative regulators of cardiac hypertrophy via suppressing NF-kappaB signalling.	19487338	M	<i>Nr1h3</i> ↓	↑	ND	ND
Kayama	2009	Cardiac 12/15 lipoxygenase-induced inflammation is involved in heart failure.	19546247	M	<i>Alox15</i> ↓	↓	ND	↓
Nakamura*	2009	Critical role of apoptosis signal-regulating kinase 1 in aldosterone/salt-induced cardiac inflammation and fibrosis.	19581502	M	<i>Map3k5</i> ↓	-	↓	↓
Nakamura*	2009	Critical role of apoptosis signal-regulating kinase 1 in aldosterone/salt-induced cardiac inflammation and fibrosis.	19581502	M	<i>Map3k5</i> ↓	-	↓	↓
Li	2009	Nrf2 protects against maladaptive cardiac responses to hemodynamic stress.	19592468	M	<i>Nrf2</i> ↓	↑	↑	ND
Zelaryan	2009	NF-kappaB activation is required for adaptive cardiac hypertrophy.	19620128	M	<i>Nfkbia</i> ↓	-	-	ND
Satomi-	2009	Deficiency of nectin-2 leads to cardiac fibrosis and dysfunction under chronic pressure overload.	19667252	M	<i>Pvrl2</i> ↓	-	↑	ND
Kobayashi	2009	Exaggerated blood pressure variability superimposed on hypertension aggravates cardiac remodeling in rats via angiotensin II system-mediated chronic inflammation.	19704105	R		↑	↑	↑
Kudo		Connective tissue growth factor overexpression in cardiomyocytes promotes cardiac hypertrophy and protection against pressure overload.	19707545	M	<i>Ctgf</i> ↑	ND	-	ND
Panek	2009	Adverse ventricular remodeling and exacerbated NOS uncoupling from pressure-overload in mice lacking the beta3-adrenoreceptor.	19766235	M	<i>Adra2b</i> ↓	↑	↑	ND
Moens	2009	Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes.	19820199	R		↑	-	↓
Ares-Carrasco	2009	Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways.	19834007	M	<i>Il1b</i> ↓	↓	ND	ND
Honsho*	2009	Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways.	19834007	M	<i>Il1b</i> ↓	↓	↑	ND
Kratsios	2010	Antioxidant amelioration of dilated cardiomyopathy caused by conditional deletion of NEMO/IKKgamma in cardiomyocytes	19850942	M	<i>Ikbkg</i> ↓	↑	↑	↑
Li*	2009	Evidence for the importance of adiponectin in the cardioprotective effects of pioglitazone.	19933924	M	<i>Adipoq</i> ↓	↑	-	ND
Li*	2009	Evidence for the importance of adiponectin in the cardioprotective effects of pioglitazone.	19933924	M		↓	↓	ND
Levick	2010	Sympathetic nervous system modulation of inflammation and remodeling in the hypertensive heart.	20048196	R		↓	↓	-
Kimura*	2010	Targeted deletion of the extracellular signal-regulated protein kinase 5 attenuates hypertrophic response and promotes pressure overload-induced apoptosis in the heart.	20075332	M	<i>Mapk7</i> ↓	↓	↓	ND
Kimura*	2010	Targeted deletion of the extracellular signal-regulated protein kinase 5 attenuates hypertrophic response and promotes pressure overload-induced apoptosis in the heart.	20075332	M	<i>Mapk7</i> ↓	↓	↓	ND
Shen	2010	Tetrandrine blocks cardiac hypertrophy by disrupting reactive oxygen species-dependent ERK1/2 signalling.	20105174	M		↓	↓	↓
Yan	2010	Breviscapine protects against cardiac hypertrophy through blocking PKC-alpha-dependent signaling.	20127712	M		↓	↓	↓
Zhang	2010	C-reactive protein promotes cardiac fibrosis and inflammation in angiotensin II-induced hypertensive cardiac disease.	20157054	M	<i>Crp</i> ↑	ND	↑	↑
Liu	2009	Allicin protects against cardiac hypertrophy and fibrosis via attenuating reactive oxygen species-dependent signaling pathways	20185286	M		↓	↓	↓
Shimano	2010	Adiponectin deficiency exacerbates cardiac dysfunction following pressure overload through disruption of an AMPK-dependent angiogenic response.	20206634	M	<i>Adipoq</i> ↓	↑	↑	ND
Tang	2010	Adenylyl cyclase 6 deletion reduces left ventricular hypertrophy, dilation, dysfunction, and fibrosis in pressure-overloaded female mice.	20359598	M	<i>Adcy6</i> ↓	↓	↓	ND
Liao	2010	Krüppel-like factor 4 regulates pressure-induced cardiac hypertrophy.	20433848	M	<i>Klf4</i> ↓	↑	↑	ND
Kompa	2010	Chronic urotensin II receptor antagonist treatment does not alter hypertrophy or fibrosis in a rat model of pressure-overload hypertrophy.	20452383	R	<i>Uts2b</i> ↓	-	-	ND
Haudek*	2010	Monocytic fibroblast precursors mediate fibrosis in angiotensin-II-induced cardiac hypertrophy.	20488188	M	<i>Ccl2</i> ↓	-	↓	-
Haudek*	2010	Monocytic fibroblast precursors mediate fibrosis in angiotensin-II-induced cardiac hypertrophy.	20488188	M	<i>Ccl2</i> ↓	-	↓	↓
Haudek*	2010	Monocytic fibroblast precursors mediate fibrosis in angiotensin-II-induced cardiac hypertrophy.	20488188	M	<i>Ccl2</i> ↓	-	↓	↓
Cai*	2010	Intracellular or extracellular heat shock protein 70 differentially regulates cardiac remodelling in pressure overload mice.	20542874	M	<i>Hspa1b</i> ↓	↑	↓	↓
Cai*	2011	Intracellular or extracellular heat shock protein 70 differentially regulates cardiac remodelling in pressure overload mice.	20542874	M	<i>Hspa1b</i> ↓	↓	↓	↓
Ai	2010	Silibinin attenuates cardiac hypertrophy and fibrosis through blocking EGFR-dependent signaling.	20564207	M		↓	↓	ND
Huang	2010	Tumor suppressor A20 protects against cardiac hypertrophy and fibrosis by blocking transforming growth factor-beta-activated kinase 1-dependent signaling.	20585109	M	<i>Tnfrsf10b</i> ↑	↓	↓	ND
Mohammed	2010	Mineralocorticoid accelerates transition to heart failure with preserved ejection fraction via "nongenomic effects".	20625113	M		↑	↑	ND
Heineke	2010	CIB1 is a regulator of pathological cardiac hypertrophy.	20639889	M	<i>Cib1</i> ↓	↓	↓	ND
Sumitomo-	2010	Heparin cofactor II protects against angiotensin II-induced cardiac remodeling via attenuation of oxidative stress in mice.	20660821	M	<i>Serpind1</i> ↓	-	↑	ND
Ueda	2010	Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice.	20697155	M	<i>Nr3c2</i> ↓	↓	↓	↓
Usher	2010	NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart.	20713697	M	<i>Nox4</i> ↓	↓	↓	ND
Kuroda								

Serpi	2010	Vascular endothelial growth factor-B gene transfer prevents angiotensin II-induced diastolic dysfunction via proliferation and capillary dilatation in rats.	20733007	R	<i>Vegfb</i> ↑	ND	↓	ND
Song	2010	mTOR attenuates the inflammatory response in cardiomyocytes and prevents cardiac dysfunction in pathological hypertrophy.	20861467	M	<i>mTOR</i> ↓	↓	↓	↓
Del Re	2010	Proapoptotic Rassf1A/Mst1 signaling in cardiac fibroblasts is protective against pressure overload in mice.	20890045	M	<i>Rassf1</i> ↓	↑	↑	ND
Li*	2010	Cellular FLICE-inhibitory protein protects against cardiac remodeling induced by angiotensin II in mice.	20975036	M	<i>Cflar</i> ↓	↑	↑	ND
Li*	2010	Cellular FLICE-inhibitory protein protects against cardiac remodeling induced by angiotensin II in mice.	20975036	M	<i>Cflar</i> ↑	↓	↓	ND
Patrick*	2010	Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice.	20978354	M	<i>Mir21</i> ↓	-	-	ND
Patrick*	2011	Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice.	20978355	M	<i>Mir21</i> ↓	-	-	ND
Harding	2011	Lack of microsomal prostaglandin E synthase-1 reduces cardiac function following angiotensin II infusion.	21193590	M	<i>Ptges</i> ↓	-	-	ND
Sopko	2010	Bone marrow support of the heart in pressure overload is lost with aging.	21203577	M		↑	ND	-
Nagai*	2011	C-reactive protein overexpression exacerbates pressure overload-induced cardiac remodeling through enhanced inflammatory response.	21220701	M	<i>Crp</i> ↑	↑	-	↑
Nagai*	2011	C-reactive protein overexpression exacerbates pressure overload-induced cardiac remodeling through enhanced inflammatory response.	21220701	M	<i>Crp</i> ↑	-	↑	↑
Skoumal	2011	Parthenolide inhibits STAT3 signaling and attenuates angiotensin II-induced left ventricular hypertrophy via modulation of fibroblast activity.	21223972	R	<i>Stat3</i> ↓	↓	↓	↓
Givvimani*	2011	Hydrogen sulfide mitigates transition from compensatory hypertrophy to heart failure.	21233344	M		ND	↓	ND
Givvimani*	2011	Hydrogen sulfide mitigates transition from compensatory hypertrophy to heart failure.	21233344	M		ND	↓	ND
Shimano	2011	Cardiac myocyte-specific ablation of follistatin-like 3 attenuates stress-induced myocardial hypertrophy	21245136	M	<i>Fstl3</i> ↓	↓	↓	ND
Chen	2011	Omega-3 fatty acids prevent pressure overload-induced cardiac fibrosis through activation of cyclic GMP/protein kinase G signaling in cardiac fibroblasts.	21282499	M		↓	↓	↓
Liu	2011	Deprivation of MKK7 in cardiomyocytes provokes heart failure in mice when exposed to pressure overload.	21284947	M	<i>Map2k7</i> ↓	↑	ND	↑
Lothar*	2011	Ablation of mineralocorticoid receptors in myocytes but not in fibroblasts preserves cardiac function.	21321305	M	<i>Nr3c2</i> ↓	↑	-	-
Lothar*	2011	Ablation of mineralocorticoid receptors in myocytes but not in fibroblasts preserves cardiac function.	21321305	M	<i>Nr3c2</i> ↓	-	-	-
Qi	2011	Angiotensin II infusion-induced inflammation, monocytic fibroblast precursor infiltration, and cardiac fibrosis are pressure dependent.	21373977	M		↓	↓	↓
Zhang	2011	HSP75 protects against cardiac hypertrophy and fibrosis.	21381076	M	<i>Trap1</i> ↑	↓	↓	ND
Aceros*	2011	Moxonidine improves cardiac structure and performance in SHR through inhibition of cytokines, p38 MAPK and Akt	21426316	R		↓	↓	↓
Aceros*	2011	Moxonidine improves cardiac structure and performance in SHR through inhibition of cytokines, p38 MAPK and Akt	21426316	R		↓	ND	↓
Ren	2011	Proinflammatory protein CARD9 is essential for infiltration of monocytic fibroblast precursors and cardiac fibrosis caused by Angiotensin II infusion.	21436792	M	<i>Card9</i> ↓	-	↓	↓
Gao	2011	High salt intake accelerated cardiac remodeling in spontaneously hypertensive rats: time window of left ventricular functional transition and its relation to salt-loading doses.	21529316	R		↑	↑	ND
Liu	2011	Lentivirus mediated IL-17R blockade improves diastolic cardiac function in spontaneously hypertensive rats.	21530504	R	<i>Il17ra</i> ↓	↓	↓	ND
Waehre	2011	Lack of chemokine signaling through CXCR5 causes increased mortality, ventricular dilatation and deranged matrix during cardiac pressure overload.	21533157	M	<i>Cxcr5</i> ↓	↑	↑	-
Zhu	2011	The TIR/BB-loop mimetic AS-1 prevents cardiac hypertrophy by inhibiting IL-1R-mediated MyD88-dependent signaling	21533832	M		↓	↓	↓
Xu	2011	CCR2 mediates the uptake of bone marrow-derived fibroblast precursors in angiotensin II-induced cardiac fibrosis.	21572015	M	<i>Ccr2</i> ↓	-	↓	ND
Peng	2011	Angiotensin II-induced dilated cardiomyopathy in Balb/c but not C57BL/6J mice.	21602297	M		-	↑	ND
Ehrentraut	2011	The toll-like receptor 4-antagonist eritoran reduces murine cardiac hypertrophy	21613426	M	<i>Tlr4</i> ↓	↓	ND	↓
Usui*	2011	Endogenous muscle atrophy F-box mediates pressure overload-induced cardiac hypertrophy through regulation of nuclear factor-kappaB.	21617130	M	<i>Fbxo32</i> ↓	↓	↓	↓
Usui*	2011	Endogenous muscle atrophy F-box mediates pressure overload-induced cardiac hypertrophy through regulation of nuclear factor-kappaB.	21617130	M	<i>Fbxo32</i> ↓	↓	ND	ND
Yan*	2011	Cardiac-specific mindin overexpression attenuates cardiac hypertrophy via blocking AKT/GSK3β and TGF-β1-Smad signalling.	21632881	M	<i>Spon2</i> ↑	↓	↓	ND
Yan*	2011	Cardiac-specific mindin overexpression attenuates cardiac hypertrophy via blocking AKT/GSK3β and TGF-β1-Smad signalling.	21632881	M	<i>Spon2</i> ↑	↓	↓	ND
Xu	2011	IkBβ attenuates angiotensin II-induced cardiovascular inflammation and fibrosis in mice.	21646597	M	<i>Nfkbia</i> ↓	ND	↓	↓
Mandl	2011	Puma deletion delays cardiac dysfunction in murine heart failure models through attenuation of apoptosis.	21670227	M	<i>Bbc3</i> ↓	-	↓	ND
Garlie*	2011	Tumor necrosis factor receptor 2 signaling limits β-adrenergic receptor-mediated cardiac hypertrophy in vivo.	21691899	M	<i>Tnfrsf1a</i> ↓	-	-	↓
Garlie*	2011	Tumor necrosis factor receptor 2 signaling limits β-adrenergic receptor-mediated cardiac hypertrophy in vivo.	21691899	M	<i>Tnfrsf1a</i> ↓	↓	ND	ND
Garlie*	2011	Tumor necrosis factor receptor 2 signaling limits β-adrenergic receptor-mediated cardiac hypertrophy in vivo.	21691899	M	<i>Tnfrsf1b</i> ↓	↑	-	-
Garlie*	2011	Tumor necrosis factor receptor 2 signaling limits β-adrenergic receptor-mediated cardiac hypertrophy in vivo.	21691899	M	<i>Tnfrsf1b</i> ↓	-	ND	ND

		cardiac hypertrophy in vivo.							
Chan	2011	Chronic β -adrenoceptor Antagonist Treatment Controls Cardiovascular Remodeling in Heart Failure in the Aging Spontaneously Hypertensive Rat	21709583	R		↓	↓	ND	
Steinbusch*	2011	Absence of fatty acid transporter CD36 protects against Western-type diet-related cardiac dysfunction following pressure overload in mice.	21712535	M	<i>Cd36</i> ↓	↑	ND	↑	
Steinbusch*	2011	Absence of fatty acid transporter CD36 protects against Western-type diet-related cardiac dysfunction following pressure overload in mice.	21712535	M	<i>Cd36</i> ↓	-	ND	↓	
Iyer	2011	Inhibition of inflammation and fibrosis by a complement C5a receptor antagonist in DOCA-salt hypertensive rats.	21753735	R	<i>CSar1</i> ↓	↓	↓	↓	
Castoldi	2011	MiR-133a Regulates Collagen1A1: Potential Role of miR-133a in Myocardial Fibrosis in Angiotensin II-Dependent Hypertension	21769867	R		↓	↓	ND	
Georgescu	2011	Decreased metalloprotease 9 induction, cardiac fibrosis, and higher autophagy after pressure overload in mice lacking the transcriptional regulator p8.	21775709	M	<i>p8</i> ↓	-	↓	↓	
Kanellakis	2011	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ regulatory T cells suppress cardiac fibrosis in the hypertensive heart.	21785365	M		↓	↓	↓	
Buckley	2011	Mice lacking functional TRPV1 are protected from pressure overload cardiac hypertrophy.	21814047	M	<i>Trpv1</i> ↓	↓	↓	↓	
Shu	2011	Gastrodin protects against cardiac hypertrophy and fibrosis.	21833534	M		↓	↓	ND	
Carnevale	2011	Placental Growth Factor Regulates Cardiac Inflammation Through the Tissue Inhibitor of Metalloproteinases-3/Tumor Necrosis Factor α Converting Enzyme Axis: Crucial Role for Adaptive Cardiac Remodeling During Cardiac Pressure Overload	21900081	M	<i>Pgf</i> ↓	↓	↓	↓	
Xia	2011	Endogenous thrombospondin 1 protects the pressure-overloaded myocardium by modulating fibroblast phenotype and matrix metabolism.	21947471	M	<i>Thbs1</i> ↓	↑	-	-	
Rosenblatt-Velin	2012	Cardiac dysfunction and impaired compensatory response to pressure overload in mice deficient in stem cell antigen-1.	21957128	M	<i>Sca1</i> ↓	↑	ND	ND	
Zou	2011	Ryanodine receptor type 2 is required for the development of pressure overload-induced cardiac hypertrophy.	21986507	M	<i>Ryr2</i> ↓	↓	↓	ND	
Zhou	2011	Activating transcription factor 3 deficiency promotes cardiac hypertrophy, dysfunction, and fibrosis induced by pressure overload.	22053207	M	<i>Atf3</i> ↓	↑	↑	ND	
Finckenberg	2012	Caloric restriction ameliorates angiotensin II-induced mitochondrial remodeling and cardiac hypertrophy.	22068868	R		↓	↓	ND	
Zou*	2012	Qiliqiangxin inhibits the development of cardiac hypertrophy, remodeling, and dysfunction during 4 weeks of pressure overload in mice.	22075750	M		↓	↓	↓	
Zou*	2012	Qiliqiangxin inhibits the development of cardiac hypertrophy, remodeling, and dysfunction during 4 weeks of pressure overload in mice.	22075750	M		↓	↓	↓	
Zou*	2012	Qiliqiangxin inhibits the development of cardiac hypertrophy, remodeling, and dysfunction during 4 weeks of pressure overload in mice.	22075750	M		↓	↓	↓	
Zou*	2012	Qiliqiangxin inhibits the development of cardiac hypertrophy, remodeling, and dysfunction during 4 weeks of pressure overload in mice.	22075750	M		↓	↓	↓	
Kazakov	2012	Endothelial nitric oxide synthase of the bone marrow regulates myocardial hypertrophy, fibrosis, and angiogenesis.	22106415	M	<i>Nos3</i> ↓	↓	↑	ND	
He	2012	In vivo and in vitro protective effects of pentamethylquercetin on cardiac hypertrophy.	22183794	R		↓	↓	↓	
Gaspar-Pereira	2012	The NF- κ B subunit c-Rel stimulates cardiac hypertrophy and fibrosis.	22210479	M	<i>Rel</i> ↓	↓	↓	ND	
Martin-Fernández	2012	Spiroglactone prevents alterations associated with cardiac hypertrophy produced by isoproterenol in rats: involvement of serum- and glucocorticoid-regulated kinase type 1.	22327331	R		↓	↓	↓	
Frolova	2012	Thrombospondin-4 regulates fibrosis and remodeling of the myocardium in response to pressure overload.	22362893	M	<i>Thbs4</i> ↓	↑	↑	↓	
Adiarto	2012	ET-1 from endothelial cells is required for complete angiotensin II-induced cardiac fibrosis and hypertrophy.	22365964	M	<i>Edn1</i> ↓	↓	↓	ND	
Bian*	2012	Disruption of mindin exacerbates cardiac hypertrophy and fibrosis.	22367478	M	<i>Spon2</i> ↓	↑	↑	ND	
Bian*	2012	Disruption of mindin exacerbates cardiac hypertrophy and fibrosis.	22367478	M	<i>Spon2</i> ↓	↑	↑	ND	
Wei	2012	Endothelial expression of hypoxia-inducible factor 1 protects the murine heart and aorta from pressure overload by suppression of TGF- β signaling.	22403061	M	<i>Hif1a</i> ↓	-	↑	ND	
Shang*	2012	Involvement of reactive oxygen species and JNK in increased expression of MCP-1 and infiltration of inflammatory cells in pressure-overloaded rat hearts.	22470108	R		↓	↓	ND	
Shang*	2012	Involvement of reactive oxygen species and JNK in increased expression of MCP-1 and infiltration of inflammatory cells in pressure-overloaded rat hearts.	22470108	R		↓	↓	ND	
Kanellakis	2012	A pro-fibrotic role for interleukin-4 in cardiac pressure overload.	22492684	M	<i>Il4</i> ↓	↓	↓	ND	
Wu	2012	Hexokinase II knockdown results in exaggerated cardiac hypertrophy via increased ROS production.	22517678	M	<i>Hk2</i> ↓	↑	↑	ND	
Park	2012	The chemical chaperone 4-phenylbutyric acid attenuates pressure-overload cardiac hypertrophy by alleviating endoplasmic reticulum stress.	22525677	M		↓	↓	ND	
Oka	2012	Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure	22535248	M	<i>Dnase2a</i> ↓	↑	↑	↑	
Yang	2012	Serum-glucocorticoid regulated kinase 1 regulates alternatively activated macrophage polarization contributing to angiotensin II-induced inflammation and cardiac fibrosis.	22556335	M	<i>Sgk1</i> ↓	↓	↓	↓	
Wang	2012	Promyelocytic leukemia zinc finger protein activates GATA4 transcription and mediates cardiac hypertrophic signaling from angiotensin II receptor 2.	22558183	M	<i>Zbtb16</i> ↓	↓	↓	ND	
Han	2012	Reciprocal interaction between macrophages and T cells stimulates IFN- γ and MCP-1 production in Ang II-induced cardiac inflammation and fibrosis.	22567105	M	<i>Ifng</i> ↓	-	↓	↓	
Ma	2012	Macrophage-stimulated cardiac fibroblast production of IL-6 is essential for TGF β /Smad activation and cardiac fibrosis induced by angiotensin II.	22574112	M	<i>Il6</i> ↓	-	↓	↓	
Valente	2012	CKS (Act1 or TRAF3IP2) mediates Angiotensin-II-induced Interleukin-18 expression, and Nox2-dependent cardiomyocyte hypertrophy.	22575763	M	<i>Traf3ip2</i> ↓	↓	↓	↓	
Xiao	2012	Augmented cardiac hypertrophy in response to pressure overload in mice lacking	22606234	M	<i>Adgrl4</i> ↓	↑	↑	ND	

		ELTD1.							
Baicu	2012	Effects of the absence of procollagen C-endopeptidase enhancer-2 on myocardial collagen accumulation in chronic pressure overload.	22610170	M	<i>Pcolce2</i> ↓	↓	↓	↓	ND
LaRocca*	2012	CXCR4 gene transfer prevents pressure overload induced heart failure	22668785	M	<i>Cxcr4</i> ↑	↓	ND	ND	ND
LaRocca*	2012	CXCR4 gene transfer prevents pressure overload induced heart failure	22668785	M	<i>Cxcr4</i> ↑	↓	ND	ND	ND
Verma*	2012	Interleukin-10 treatment attenuates pressure overload-induced hypertrophic remodeling and improves heart function via signal transducers and activators of transcription 3-dependent inhibition of nuclear factor-κB.	22705886	M	<i>Il10</i> ↓	↑	↑	↑	
Verma*	2012	Interleukin-10 treatment attenuates pressure overload-induced hypertrophic remodeling and improves heart function via signal transducers and activators of transcription 3-dependent inhibition of nuclear factor-κB.	22705886	M	<i>Il10</i> ↑	↓	↓	↓	
Yang	2012	Carboxyl terminus of heat shock protein 70-interacting protein inhibits angiotensin II-induced cardiac remodeling.	22717542	M	<i>Stub1</i> ↑	-	↓	↓	
Garcia	2012	Interferon-γ ablation exacerbates myocardial hypertrophy in diastolic heart failure.	22730392	M	<i>Ifng</i> ↓	↑	-	-	
Kolwicz	2012	Cardiac-specific deletion of acetyl CoA carboxylase 2 prevents metabolic remodeling during pressure-overload hypertrophy.	22730442	M	<i>Acacb</i> ↓	↓	↓	ND	
Hamad	2012	Cardioprotection of controlled and cardiac-specific over-expression of A(2A)-adenosine receptor in the pressure overload.	22792196	M	<i>Adora2a</i> ↑	↓	↓	↓	
Takatsu*	2012	Comparison of the effects of cilnidipine and amlodipine on cardiac remodeling and diastolic dysfunction in Dahl salt-sensitive rats.	22796710	R		↓	↓	↓	
Takatsu*	2012	Comparison of the effects of cilnidipine and amlodipine on cardiac remodeling and diastolic dysfunction in Dahl salt-sensitive rats.	22796710	R		↓	↓	↓	
Meems	2012	The vitamin D receptor activator paricalcitol prevents fibrosis and diastolic dysfunction in a murine model of pressure overload.	22800987	M	<i>Vdr</i> ↑	-	↓	ND	
Liu	2012	Depletion of endogenous kallistatin exacerbates renal and cardiovascular oxidative stress, inflammation, and organ remodeling.	22811485	R		-	↑	↑	
Lai*	2012	Pressure overload-induced cardiac remodeling and dysfunction in the absence of interleukin 6 in mice	22825686	M	<i>Il6</i> ↓	-	-	ND	
Lai*	2012	Pressure overload-induced cardiac remodeling and dysfunction in the absence of interleukin 6 in mice.	22825686	M	<i>Il6</i> ↓	-	-	-	
Xu	2012	Chronic administration of hexarelin attenuates cardiac fibrosis in the spontaneously hypertensive rat.	22842067	R		↓	↓	ND	
Izumiya	2012	Chronic C-Type Natriuretic Peptide Infusion Attenuates Angiotensin II-Induced Myocardial Superoxide Production and Cardiac Remodeling.	22848833	M		↓	↓	ND	
Zhou*	2012	Stem cell antigen 1 protects against cardiac hypertrophy and fibrosis after pressure overload.	22851736	M	<i>Sca1</i> ↓	↑	↑	ND	
Zhou*	2012	Stem cell antigen 1 protects against cardiac hypertrophy and fibrosis after pressure overload.	22851736	M	<i>Sca1</i> ↑	↓	↓	ND	
Gold	2012	Determining the absolute requirement of G protein-coupled receptor kinase 5 for pathological cardiac hypertrophy: short communication.	22859683	M	<i>Grk5</i> ↓	↓	↓	↓	
Nagai*	2012	Eicosapentaenoic acid suppresses adverse effects of C-reactive protein overexpression on pressure overload-induced cardiac remodeling.	22875408	M	<i>Crp</i> ↑	↑	↑	↑	
Nagai*	2012	Eicosapentaenoic acid suppresses adverse effects of C-reactive protein overexpression on pressure overload-induced cardiac remodeling.	22875408	M	<i>Crp</i> ↑	-	↑	↑	
Cittadini	2012	SOCS1 gene transfer accelerates the transition to heart failure through the inhibition of the gp130/JAK/STAT pathway	22875468	R	<i>Socs1</i> ↑	-	↑	↑	
Mohamed	2012	Targeted disruption of Hspa4 gene leads to cardiac hypertrophy and fibrosis.	22884543	M	<i>Hspa4</i> ↓	↑	↑	ND	
Yang	2012	Coronary artery remodeling in a model of left ventricular pressure overload is influenced by platelets and inflammatory cells.	22916095	M	<i>Rag1</i> ↓	-	-	ND	
Velten*	2012	Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice.	22977006	M		-	↓	↓	
Velten*	2012	Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice.	22977006	M		↓	↓	↓	
Velten*	2012	Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice.	22977006	M		↓	↓	↓	
Velten*	2012	Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice.	22977006	M		↓	-	↓	
Velten*	2012	Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice.	22977006	M		-	ND	ND	
Velten*	2012	Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice.	22977006	M		↓	ND	ND	
Sekinishi	2012	Periodontal pathogen Aggregatibacter actinomycetemcomitans deteriorates pressure overload-induced myocardial hypertrophy in mice.	23038095	M		↑	↑	ND	
Tanaka	2012	Inhibition of IκB phosphorylation prevents load-induced cardiac dysfunction in mice.	23042949	M		↓	↓	↓	
Markó	2012	Interferon-γ signaling inhibition ameliorates angiotensin II-induced cardiac damage.	23108651	M	<i>Ifng</i> ↓	↓	↓	↓	
Westermann	2012	Selective PDE5A inhibition with sildenafil rescues left ventricular dysfunction, inflammatory immune response and cardiac remodeling in angiotensin II-induced heart failure in vivo.	23117837	M	<i>Pde5a</i> ↓	↓	↓	↓	
Watanabe	2013	Chronic depletion of glutathione exacerbates ventricular remodelling and dysfunction in the pressure-overloaded heart.	23129588	M	<i>Gclm</i> ↓	↑	↑	ND	
Ma	2012	Proteasome inhibition attenuates heart failure during the late stages of pressure overload through alterations in collagen expression	23142711	R		-	↓	↓	
Koga	2013	Macrophage migration inhibitory factor antagonizes pressure overload-induced	23144312	M	<i>Mif</i> ↓	↑	↑	-	

		cardiac hypertrophy.							
Boite	2012	Postnatal ablation of Foxm1 from cardiomyocytes causes late onset hypertrophy and fibrosis without Exacerbating Pressure Overload-Induced Cardiac Remodeling	23144938	M	<i>Foxm1</i> ↓	-	ND	-	
You	2013	Olmesartan attenuates cardiac remodeling through DLL4/Notch1 pathway activation in pressure overload mice.	23188126	M		↓	↓	ND	
Pillai	2013	Nampt secreted from cardiomyocytes promotes development of cardiac hypertrophy and adverse ventricular remodeling.	23203961	M	<i>Nampt</i> ↓	↓	↓	ND	
Huang	2013	Effect of oxyamrine, the active component from Radix Sophorae flavescentis (Kushen), on ventricular remodeling in spontaneously hypertensive rats.	23211799	R		↓	↓	ND	
Zong	2013	Baicalin protects against cardiac hypertrophy through blocking MEK-ERK1/2 signaling.	23225340	M		↓	↓	ND	
Westphal*	2012	Effects of estrogen, an ERα agonist and raloxifene on pressure overload induced cardiac hypertrophy.	23227210	M	<i>Esr1</i> ↑	-	↓	ND	
Westphal*	2012	Effects of estrogen, an ERα agonist and raloxifene on pressure overload induced cardiac hypertrophy.	23227210	M	<i>Esr1</i> ↑	-	↓	ND	
Westphal*	2012	Effects of estrogen, an ERα agonist and raloxifene on pressure overload induced cardiac hypertrophy.	23227210	M	<i>Esr1</i> ↑	-	-	ND	
Yu*	2013	Genetic and pharmacological inhibition of galectin-3 prevents cardiac remodeling by interfering with myocardial fibrogenesis.	23230309	M	<i>Gal3</i> ↓	-	↓	ND	
Yu*	2013	Genetic and pharmacological inhibition of galectin-3 prevents cardiac remodeling by interfering with myocardial fibrogenesis.	23230309	M	<i>Gal3</i> ↓	-	↓	ND	
Yu*	2013	Genetic and pharmacological inhibition of galectin-3 prevents cardiac remodeling by interfering with myocardial fibrogenesis.	23230309	R	<i>Gal3</i> ↓	-	↓	-	
Morgan*	2013	Soluble epoxide hydrolase inhibition does not prevent cardiac remodeling and dysfunction after aortic constriction in rats and mice.	23232840	M		-	-	ND	
Morgan*	2013	Soluble epoxide hydrolase inhibition does not prevent cardiac remodeling and dysfunction after aortic constriction in rats and mice.	23232840	R		-	-	ND	
Sun	2013	Bone morphogenetic protein-4 mediates cardiac hypertrophy, apoptosis, and fibrosis in experimentally pathological cardiac hypertrophy.	23248151	M	<i>Bmp4</i> ↓	↓	↓	ND	
Manso	2012	Talin1 has unique expression versus talin 2 in the heart and modifies the hypertrophic response to pressure overload.	23266827	M	Talin1 ↓	↓	↓	ND	
Okamoto	2012	FHL2 prevents cardiac hypertrophy in mice with cardiac-specific deletion of ROCK2.	23271052	M	<i>Rock2</i> ↓	↓	↓	ND	
Yang	2013	Inhibition of farnesyl pyrophosphate synthase attenuates angiotensin II-induced cardiac hypertrophy and fibrosis in vivo.	23277274	M	<i>Fdps</i> ↓	↓	↓	ND	
Fujita	2013	Atrial natriuretic peptide exerts protective action against angiotensin II-induced cardiac remodeling by attenuating inflammation via endothelin-1/endothelin receptor A cascade.	23277455	R	<i>Nppa</i> ↑	-	↓	↓	
Wang	2013	Reduced cardiac fructose 2,6 biphosphate increases hypertrophy and decreases glycolysis following aortic constriction.	23308291	M	<i>Pfkfb2</i> ↓	↑	↑	ND	
Li	2013	Apocynin attenuates oxidative stress and cardiac fibrosis in angiotensin II-induced cardiac diastolic dysfunction in mice.	23334241	M		↓	↓	ND	
Duerrschmid*	2013	TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis	23337087	M	<i>Tnfrsf1a</i> ↓	↓	↓	↓	
Duerrschmid*	2013	TNF receptor 1 signaling is critically involved in mediating angiotensin-II-induced cardiac fibrosis	23337087	M	<i>Tnfrsf1b</i> ↓	-	-	↓	
Dai	2013	IKKi Deficiency Promotes Pressure Overload-Induced Cardiac Hypertrophy and Fibrosis	23349709	M	<i>Ikbke</i> ↓	↑	↑	ND	
Tamaki*	2013	Metastasis-associated protein, S100A4 mediates cardiac fibrosis potentially through the modulation of p53 in cardiac fibroblasts.	23352991	M	<i>S100a4</i> ↓	-	↓	-	
Tamaki*	2013	Metastasis-associated protein, S100A4 mediates cardiac fibrosis potentially through the modulation of p53 in cardiac fibroblasts.	23352991	M	<i>S100a4</i> ↓	-	↓	ND	
Zouein	2013	Role of STAT3 in angiotensin II-induced hypertension and cardiac remodeling revealed by mice lacking STAT3 serine 727 phosphorylation.	23364341	M	<i>Stat3</i> ↓	↓	-	↓	
Suzuki*	2013	Long pentraxin PTX3 exacerbates pressure overload-induced left ventricular dysfunction.	23372656	M	<i>Ptx3</i> ↓	↓	↓	ND	
Suzuki*	2013	Long pentraxin PTX3 exacerbates pressure overload-induced left ventricular dysfunction.	23372656	M	<i>Ptx3</i> ↑	↑	↑	ND	
Engelbrechtsen	2013	Decorin, lumican, and their GAG chain-synthesizing enzymes are regulated in myocardial remodeling and reverse remodeling in the mouse.	23412898	M		ND	ND	ND	
Zhou	2013	Paeoniflorin attenuates pressure overload-induced cardiac remodeling via inhibition of TGFβ/Smads and NF-κB pathways.	23417833	M		↓	↓	↓	
Chen*	2013	Vinexin-b protects against cardiac hypertrophy by blocking the Akt-dependent signalling pathway	23429936	M	<i>Sorbs3</i> ↓	↑	↑	ND	
Chen*	2013	Vinexin-b protects against cardiac hypertrophy by blocking the Akt-dependent signalling pathway	23429936	M	<i>Sorbs3</i> ↑	↓	ND	ND	
Rifki	2013	RalGDS-dependent cardiomyocyte autophagy is required for load-induced ventricular hypertrophy.	23473774	M	<i>Ralgds</i> ↓	↓	ND	ND	
Zhang	2013	Triptolide protects rat heart against pressure overload-induced cardiac fibrosis.	23541609	R		-	↓	↓	
Park	2013	Targeted ablation of the histidine-rich Ca(2+)-binding protein (HRC) gene is associated with abnormal SR Ca(2+)-cycling and severe pathology under pressure-overload stress.	23553082	M	<i>Hrc</i> ↓	↑	↑	ND	
Jiang*	2013	Role of interferon regulatory factor 4 in the regulation of pathological cardiac hypertrophy.	23589561	M	<i>Irf4</i> ↓	↓	↓	ND	
Jiang*	2013	Role of interferon regulatory factor 4 in the regulation of pathological cardiac hypertrophy.	23589561	M	<i>Irf4</i> ↑	↑	ND	ND	
Karakikes	2013	Therapeutic cardiac-targeted delivery of miR-1 reverses pressure overload-induced cardiac hypertrophy and attenuates pathological remodeling.	23612897	R	<i>Mir1b</i> ↑	↓	↓	ND	
Deng	2013	Indole-3-carbinol protects against pressure overload induced cardiac remodeling	23625645	M		↓	↓	ND	

		via activating AMPK- α .							
Ganesan	2013	MIR-378 controls cardiac hypertrophy by combined repression of mitogen-activated protein kinase pathway factors.	23625957	M	<i>Mir378</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Deng	2013	Hesperetin protects against cardiac remodelling induced by pressure overload in mice.	23719775	M		$\downarrow$	$\downarrow$	$\downarrow$	ND
Misaka	2013	Deficiency of senescence marker protein 30 exacerbates angiotensin II-induced cardiac remodelling.	23723062	M	<i>Rgn</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Chen	2013	Intermedin suppresses pressure overload cardiac hypertrophy through activation of autophagy.	23737997	M		$\downarrow$	$\downarrow$	$\downarrow$	ND
Liu	2013	A Novel Immunomodulator, FTY-720 Reverses Existing Cardiac Hypertrophy and Fibrosis From Pressure Overload by Targeting NFAT (Nuclear Factor of Activated T-cells) Signaling and Periostin	23753531	M		$\downarrow$	$\downarrow$	$\downarrow$	
Wei	2013	Smad7 inhibits angiotensin II-induced hypertensive cardiac remodelling.	23761400	M	<i>Smad7</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	
Jiang	2013	Role for granulocyte colony stimulating factor in angiotensin II-induced neutrophil recruitment and cardiac fibrosis in mice.	23761490	M	<i>Csf3</i> $\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$	
Ikeda	2013	Deletion of phd2 in myeloid lineage attenuates hypertensive cardiovascular remodeling.	23778187	M	<i>Egln1</i> $\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$	
Li*	2013	Alpha-calcitonin gene-related peptide is protective against pressure overload-induced heart failure	23816470	M	<i>Calca</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	
Li*	2013	Alpha-calcitonin gene-related peptide is protective against pressure overload-induced heart failure	23816470	M	<i>Calca</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	
Wang	2013	Pirfenidone attenuates cardiac fibrosis in a mouse model of TAC-induced left ventricular remodeling by suppressing NLRP3 inflammasome formation.	23839341	M		$\downarrow$	$\downarrow$	$\downarrow$	
Tanaka	2013	The overexpression of Twinkle helicase ameliorates the progression of cardiac fibrosis and heart failure in pressure overload model in mice.	23840758	M	<i>Peo1</i> $\uparrow$	-	$\downarrow$	$\downarrow$	ND
Zhang	2013	Fibulin-2 deficiency attenuates angiotensin II-induced cardiac hypertrophy by reducing transforming growth factor- β signalling.	23841699	M	<i>Fbln2</i> $\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Jia	2013	Inhibition of platelet activation by clopidogrel prevents hypertension-induced cardiac inflammation and fibrosis.	23887740	M		$\downarrow$	$\downarrow$	$\downarrow$	
Wei	2013	Deficiency of Smad7 enhances cardiac remodeling induced by angiotensin II infusion in a mouse model of hypertension.	23894614	M	<i>Smad7</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	
Konstandin	2013	Fibronectin contributes to pathological cardiac hypertrophy but not physiological growth.	23912225	M	<i>Fn1</i> $\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Heymans*	2013	Macrophage microRNA-155 promotes cardiac hypertrophy and failure.	23956210	M	<i>Mir155</i> $\downarrow$	$\downarrow$	-	$\downarrow$	
Heymans*	2013	Macrophage microRNA-155 promotes cardiac hypertrophy and failure.	23956210	M	<i>Mir155</i> $\downarrow$	$\downarrow$	-	$\downarrow$	
Zong	2013	NOD2 deletion promotes cardiac hypertrophy and fibrosis induced by pressure overload	23958879	M	<i>Nod2</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Pedram*	2013	ER β selective agonist inhibits angiotensin-induced cardiovascular pathology in female mice.	23970786	M	<i>Esr1</i> $\downarrow$	-	-	-	ND
Pedram*	2013	ER β selective agonist inhibits angiotensin-induced cardiovascular pathology in female mice.	23970786	M	<i>Esr1</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Windak	2013	The AP-1 transcription factor c-Jun prevents stress-imposed maladaptive remodeling of the heart.	24039904	M	<i>Jun</i> $\downarrow$	-	$\uparrow$	$\uparrow$	ND
Jiang*	2013	Interferon regulatory factor 9 protects against cardiac hypertrophy by targeting myocardin	24144649	M	<i>Irf9</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Jiang*	2013	Interferon regulatory factor 9 protects against cardiac hypertrophy by targeting myocardin	24144649	M	<i>Irf9</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Jiang*	2013	Interferon regulatory factor 9 protects against cardiac hypertrophy by targeting myocardin	24144649	M	<i>Irf9</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Jiang*	2013	Interferon regulatory factor 9 protects against cardiac hypertrophy by targeting myocardin	24144649	M	<i>Irf9</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Higashikuni	2013	Toll-like receptor-2 mediates adaptive cardiac hypertrophy in response to pressure overload through interleukin-1B upregulation via nuclear factor- κ B activation	24249711	M	<i>Tlr2</i> $\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$	
Netsu	2014	Midkine exacerbates pressure overload-induced cardiac remodeling	24291499	M	<i>Mdk</i> $\uparrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Liu	2014	Regulator of G protein signaling 3 protects against cardiac hypertrophy in mice.	24375609	M	<i>RGS3</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
Jiang*	2014	Interferon regulatory factor 7 functions as a novel negative regulator of pathological cardiac hypertrophy.	24396025	M	<i>Irf7</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	
Jiang*	2014	Interferon regulatory factor 7 functions as a novel negative regulator of pathological cardiac hypertrophy.	24396025	M	<i>Irf7</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	
Zhang*	2014	Dickkopf-3 attenuates pressure overload-induced cardiac remodelling.	24413772	M	<i>Dkk3</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Zhang*	2014	Dickkopf-3 attenuates pressure overload-induced cardiac remodelling.	24413772	M	<i>Dkk3</i> $\uparrow$	$\downarrow$	$\downarrow$	$\downarrow$	ND
van Bilsen	2014	Hypertension is a conditional factor for the development of cardiac hypertrophy in type 2 diabetic mice.	24416343	M		$\uparrow$	-	-	
Zhang	2014	Antagonist of C5aR prevents cardiac remodeling in angiotensin II-induced hypertension.	24419904	M	<i>Hc</i> $\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$	
Wang	2014	Double-stranded RNA-dependent protein kinase deficiency protects the heart from systolic overload-induced congestive heart failure.	24463368	M	<i>Eif2ak2</i> $\downarrow$	-	$\downarrow$	$\downarrow$	
Maejima*	2014	Muscle-specific RING finger 1 negatively regulates pathological cardiac hypertrophy through downregulation of calcineurin A.	24526353	M	<i>Trim63</i> $\downarrow$	$\uparrow$	ND	ND	
Maejima*	2014	Muscle-specific RING finger 1 negatively regulates pathological cardiac hypertrophy through downregulation of calcineurin A.	24526353	M	<i>Trim63</i> $\downarrow$	$\uparrow$	$\uparrow$	$\uparrow$	ND
Pereira	2014	GLUT1 deficiency in cardiomyocytes does not accelerate the transition from compensated hypertrophy to heart failure.	24583251	M	<i>Slc2a1</i> $\downarrow$	$\uparrow$	-	-	ND
Songstad	2014	Effect of transverse aortic constriction on cardiac structure, function and gene expression in pregnant rats.	24586871	R		-	-	-	ND
Jang	2014	Imatinib mesylate attenuates myocardial remodeling through inhibition of platelet-derived growth factor and transforming growth factor activation in a rat model of hypertension.	24591339	R		$\downarrow$	$\downarrow$	$\downarrow$	ND

Ma*	2014	The requirement of CD8+ T cells to initiate and augment acute cardiac inflammatory response to high blood pressure.	24600037	M	<i>Ccl2</i> ↓	ND	↓	↓
Ma*	2014	The requirement of CD8+ T cells to initiate and augment acute cardiac inflammatory response to high blood pressure.	24600037	M	<i>Cd8a</i> ↓	ND	↓	↓
Jiang*	2014	Interferon regulatory factor 1 is required for cardiac remodeling in response to pressure overload.	24732887	M	<i>Irf1</i> ↓	↓	↓	ND
Jiang*	2014	Interferon regulatory factor 1 is required for cardiac remodeling in response to pressure overload.	24732887	M	<i>Irf1</i> ↑	↑	↑	ND
Jiang*	2014	Interferon regulatory factor 1 is required for cardiac remodeling in response to pressure overload.	24732887	R	<i>Irf1</i> ↓	↓	↓	ND
Wang	2014	Nrf2 enhances myocardial clearance of toxic ubiquitinated proteins.	24747945	M	<i>Nrf2</i> ↑	↓	↓	ND
Li*	2014	Caspase recruitment domain 6 protects against cardiac hypertrophy in response to pressure overload.	24777975	M	<i>Card6</i> ↓	↑	↑	ND
Li*	2014	Caspase recruitment domain 6 protects against cardiac hypertrophy in response to pressure overload.	24777975	M	<i>Card6</i> ↑	↓	↓	ND
Li*	2014	Caspase recruitment domain 6 protects against cardiac hypertrophy in response to pressure overload.	24777975	M	<i>Card6</i> ↑	↓	↓	ND
Kritzer	2014	The scaffold protein muscle A-kinase anchoring protein β orchestrates cardiac myocyte hypertrophic signaling required for the development of heart failure.	24812305	M	<i>Akap6</i> ↓	↓	↓	ND
Bracey	2014	Mitochondrial NLRP3 protein induces reactive oxygen species to promote Smad protein signaling and fibrosis independent from the inflammasome.	24841199	M	<i>Nlrp3</i> ↓	-	↓	ND
Demos-Davies*	2014	HDAC6 contributes to pathological responses of heart and skeletal muscle to chronic angiotensin-II signaling.	24858848	M	<i>Hdac6</i> ↓	-	-	ND
Demos-Davies*	2014	HDAC6 contributes to pathological responses of heart and skeletal muscle to chronic angiotensin-II signaling.	24858848	M	<i>Hdac6</i> ↓	-	-	-
Li	2014	γδT Cell-Derived Interleukin-17A via an Interleukin-1β-Dependent Mechanism Mediates Cardiac Injury and Fibrosis in Hypertension	24866139	M	<i>Il17a</i> ↓	ND	↓	↓
Wang	2014	5TNF-α and IL-1β neutralization ameliorates angiotensin II-induced cardiac damage in male mice.	24877626	M	<i>Tnf</i> ↓, <i>Il1b</i> ↓	↓	↓	↓
Liu	2014	Endoplasmic reticulum stress sensor protein kinase R-like endoplasmic reticulum kinase (PERK) protects against pressure overload-induced heart failure and lung remodeling.	24958502	M	<i>Eif2ak3</i> ↓	↑	↑	ND
Endo	2014	18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload-induced maladaptive cardiac remodeling.	25049337	M		↓	↓	↓
Cardin*	2014	Differences in cell-type-specific responses to angiotensin II explain cardiac remodeling differences in C57BL/6 mouse substrains.	25069667	M		-	-	↓
Cardin*	2014	Differences in cell-type-specific responses to angiotensin II explain cardiac remodeling differences in C57BL/6 mouse substrains.	25069667	M		-	-	↑
Patrucco	2014	Roles of cGMP-dependent protein kinase I (cGKI) and PDE5 in the regulation of Ang II-induced cardiac hypertrophy and fibrosis.	25139994	M	<i>Prkg1</i> ↓	-	-	ND
Bernardo	2014	Therapeutic silencing of miR-652 restores heart function and attenuates adverse remodeling in a setting of established pathological hypertrophy.	25145628	M	<i>Mir652</i> ↓	↓	↓	ND
Yang	2014	Granule cargo release from bone marrow-derived cells sustains cardiac hypertrophy.	25239803	M	<i>Ugr2b</i> ↓	↓	-	-

General Discussion

Inflammation ignites cardiac disease progression

Understanding the molecular mechanism underlying cardiac disease is crucial to improve diagnosis and develop novel treatment strategies. In this context, the role of inflammation in the pathophysiology of cardiac disease, and heart failure (HF) in particular, is receiving widespread attention and it is becoming evident that inflammation plays a pivotal role in the progression of this disease ^{1, 2}. Besides that, patients suffering from inflammatory pathologies with increased blood cytokine levels, such as rheumatoid arthritis, have an increased risk to develop HF ³. Several key studies have shown levels of the inflammatory cytokines TNF α , IL-1 β and IL-6 to be elevated the blood ⁴ as well as in the myocardium ⁵ of HF patients. In addition, plasma concentrations of TNF α and IL-6 predict survival chances in HF patients ⁶. Collectively, these observations indicate that inflammatory mechanisms play a prominent role in disease progression in HF.

The aim of this thesis was to better understand the role of the immune system in HF and to explore how it can be manipulated in such way that it preserves its crucial role in beneficial processes such as wound healing and pathogen clearance, but that its destructive effects on the cardiac tissue are diminished. All four experimental chapters of this thesis have touched upon this goal in different ways and will be discussed in the context of inflammation in this chapter.

Since the role of inflammation in the disease progression of HF was first described several decades ago ⁷, pre-clinical papers have appeared in which immuno-modulatory approaches were tested in animal models of HF. At the same time, in the mid-nineties, the wide availability of knockout (KO) and transgenic (TG) mice increased the toolset available to manipulate the inflammatory response. The first pre-clinical papers to elaborate on inflammation in HF focussed on the role of TNF α ⁸⁻¹⁰, considered to be one of the most important pro-inflammatory cytokines. Swiftly, the focus broadened to other pro-inflammatory effectors, including numerous cytokines and chemokines. For instance, treatment of hypertensive rats with a MCP-1 neutralising anti-body resulted in improvement of cardiac diastolic function ¹¹. Subsequently more studies showed that modulation of inflammatory processes influences cardiac pathology upon pressure overload (PO) induced HF ¹²⁻¹⁶.

Cardiac stress results in myocardial inflammation, reflected by infiltration of multiple immune cell populations into the myocardium ⁵. The synthesis and release of chemokines by cardiac cells attracts myeloid cells like monocytes, macrophages ^{17, 18}, neutrophils ¹⁸, as well as lymphocytes ¹ to the heart. Each of these leukocyte populations exerts specific effects on the pathophysiological processes underlying HF development ¹. Moreover, these cell types are bearing specific subtypes that can either be pro- or anti-inflammatory, and therefore harmful or beneficial in disease progression. For instance, T-regulatory cells have been shown to ameliorate AngII induced cardiac damage ¹⁹ and inhibit cardiac fibrosis after TAC ²⁰. In contrast, another lymphocyte cell type, Th17, was shown to be deleterious in cardiac disease ²¹. Studies in both patients and animals indicated a relationship between pro-inflammatory markers and pressure-overload induced hypertrophy and fibrosis ⁵. The question is if immune activation *per se* is detrimental in the chronically pressure overloaded heart. In this thesis we focussed on the role of inflammation in two different HF disease aetiologies, hypertensive heart disease (HHD) and viral myocarditis (VM). HHD causes pressure overloading (PO) of the heart and ultimately leads to HF and is associated with chronic, low-grade inflammation. VM, however, is caused by a viral infection of the heart and initiates an acute and severe cardiac inflammatory response.

An adverse role for CD40 activation in the pressure-overloaded heart

An important effector of the immune system, the CD40 receptor, was left unstudied in the context of HHD induced HF. Our study, described in **chapter two**, shows that the absence of the co-stimulatory molecule receptor CD40 in myeloid cells blunts cardiac hypertrophy upon Angiotensin-II (AngII) infusion. Thereby we have shown once more that intervening with the cardiac immune response is a potential way to inhibit the progression of cardiac disease. Furthermore, we have shown involvement of both CD40 downstream signalling pathways using TRAF2/3/5 and TRAF6 in the development of cardiac hypertrophy. Both these pathways are known to activate the classical NF κ B pathway ²², previously described as a crucial mediator of cardiac hypertrophy ²³⁻²⁵.

An interesting finding is that deletion of CD40 reduced hypertrophy, but not fibrosis, suggesting that separate pathways are responsible for these two processes. Previously, it was shown that alternatively activated (M2) macrophages can induce fibrosis via TGF β secretion ²⁶. The deletion of CD40 induces more alternatively activated (M2) macrophages ²⁷, that in turn, induce more fibrosis, possibly counteracting the reduction in fibrosis associated with a reduction in disease phenotype in the CD40 KO mice.

Interestingly, the ligand of the CD40 receptor, CD40 ligand (CD40L), has become an established biomarker of HF in hypertensive patients ^{28, 29}. But in contrast to other cardiovascular diseases such as atherosclerosis ²⁷, no functional role for CD40L-CD40 signalling in the development of HHD was described and we are the first to report on these effects. In addition, blocking CD40L-CD40 interactions has already been subject of clinical trials in inflammatory diseases ³⁰ and a specific compound blocking CD40-TRAF6 signalling has been developed ^{31, 32}. This gives opportunities for targeting CD40L-CD40 signalling as therapeutic approach for HHD.

Our research on the CD40 receptor in HHD still leaves several questions unanswered. For example, it remains unclear what paracrine factors secreted from CD40-activated macrophages promote myocyte hypertrophy. Detailed proteomic analysis of the secretome of activated KO and Wild type (Wt) macrophages could answer this question. Alternatively, miRNAs secreted in exosomes by these myeloid cells could be responsible for the pro-hypertrophic effect. This type of inter-cellular signalling using miRNAs has been shown previously between cardiac fibroblasts and cardiomyocytes ³³, where miR-21-3p derived from exosomes of fibroblasts induced hypertrophy in cardiomyocytes ³³.

The differences between AngII infusion and other models inducing cardiac PO, such as transverse aortic constriction (TAC), should be considered also. The pharmacological model of AngII infusion induced marked hypertension in our study, but hypertrophy induced in this model may not be limited to pressure overloading alone, as AngII can stimulate cardiomyocyte hypertrophy directly. Moreover, previously described systemic pro-inflammatory effects of AngII may play a role as well ³⁴. A surgical model like TAC induces PO with more local effects only. Consequently, the AngII model mimics HHD in patients, whilst TAC represents better aortic stenosis in otherwise healthy patients.

Metabolic changes during acute cardiac inflammation in viral myocarditis

In **chapter three** of thesis we demonstrated that acute and fulminant inflammation in itself leads to perturbations in cardiac metabolism. Our data show that acute VM, in both humans and mice, is associated with extensive metabolic remodelling of the heart. These findings provide evidence that suppression of cardiomyocyte mitochondrial oxidative metabolism is controlled by inflammatory mechanisms, and here specifically by activation of the inflammatory NF κ B pathway. In this mechanism paracrine signalling by cytokines secreted from leukocytes that infiltrate the myocardium plays a crucial role (Figure 1).

Acute viral myocarditis

Figure 1. Representation of our findings on the effects of acute inflammation on the metabolic phenotype of the heart. Upon infection with CVB3 virus monocytes and macrophages infiltrate the heart and secrete pro-inflammatory cytokines that, in turn, activate NF κ B in cardiomyocytes. NF κ B inhibits the expression and activity of metabolic regulators PGC-1 α and PPARs. This leads to impaired mitochondrial oxidative capacity and reduced cardiac function.

To investigate if a metabolic therapy would ameliorate VM-induced cardiac injury, we treated mice with dichloroacetate (DCA), a drug known to stimulate glucose oxidation via the activation of the pyruvate dehydrogenase (PDH) complex. Previously, it was demonstrated that DCA improved cardiac function and survival in a rat model of HHD³⁵. Strikingly, in our VM model DCA worsened the VM phenotype as reflected by a marked increase in leukocyte infiltration. Notably, administration of DCA to these animals also increased spleen weight indicative of immune-modulatory effects of DCA. This is in line with literature showing that DCA modulates the immune response by skewing macrophage polarisation towards M1 and inducing T-helper 1 lymphocyte function³⁶. These immune-modulatory effects of DCA may override the beneficial effects of DCA on cardiac metabolism. It is unclear how this increase in inflammation affects the function of the heart at later stages of the disease. Possibly, increased M1 and Th1 function benefits the anti-viral response in the heart, allowing complete viral removal and therefore complete recovery. On the other hand, pronounced inflammation induces more irreversible tissue damage, ultimately leading to the development of dilated cardiomyopathy (DCM).

This brings us to the general question on how inflammation in VM should be understood. Different mouse strains show variable susceptibility to CVB3-induced VM,

possibly due to differences in their immuno-genetic background. Likewise, some people develop a myocarditis during a CVB3 or PVB19 infection while the majority does not. Also here the immuno-genetic background, gender, previous infections and other factors may play a role ³⁷. Patients that do develop myocarditis sometimes completely recover, leaving no visible damage to the heart, while other patients develop dilated cardiomyopathy over time. In this light, the question can be posed whether inflammation is beneficial or harmful in CVB3-induced VM. Most likely, pronounced pro-inflammatory activation does more harm than good to the myocardial tissue, but is an inevitable consequence of the viral infection in the heart ³⁷.

An interesting question is, if inflammation is inducing adverse perturbations in cardiac metabolism, why this mechanism has evolved? It is likely that these metabolic perturbations serve a function in the immune response. Previously, macrophage-mediated inflammation and insulin resistance were shown to be linked ³⁸. This crosstalk between the immune system and metabolism is conserved among species ³⁹ suggesting that inflammation induced metabolic perpetuations are beneficial under specific circumstances. One such scenario is an acute infection, where it provides a mechanism for diverting nutrients away from non-essential functions, such as storage, towards immunity by raising glucose levels to “fuel” the highly glycolic leukocytes ⁴⁰. In this way metabolic changes can aid the anti-pathogen response.

What distinguishes adverse from beneficial inflammation during HF?

The results presented in chapter two and three of this thesis suggest that inflammation is playing a deleterious role in HF, and that targeting the cardiac immune response is a good strategy to prevent disease progression. Finding a therapy that effectively reduces the intensity and duration of the immune response, without interfering with wound healing and reparative mechanisms remains a challenge. Although there have been several clinical trials addressing this (see ⁴¹ for review), no such adequate therapy has yet emerged. The difficulty to discriminate between beneficial and harmful inflammation and the pleiotropic effects of many cytokines are possible reasons for this. For instance the cytokine IL-4 is considered to have anti-inflammatory effects, but at the same time was shown stimulate fibrosis ⁴².

In the heart a competent cardiac immune response is mounted after acute damage induced by myocardial infarction (MI). Necrotic and damaged cardiomyocytes release DAMPs and are in turn cleared by phagocytic cells. In the overloaded heart it is less clear what initiates the inflammatory response. There are indications that mitochondria become damaged in the pressure-overloaded heart and are degraded by autophagy ⁴³. A recent study by Oka et al. ⁴⁴ shows that mtDNA from these stressed mitochondria escapes autophagy and is recognized by Toll-like receptor (TLR) 9, thereby initiating cytokine release. Haemodynamic stress is not limited to cardiac disease but also occurs during exercise ⁴⁵. In this context it is worth mentioning that endurance athletes who develop physiological hypertrophy, also show increased IL-6 levels in the blood after a bout of exercise ⁴⁶. Moreover, it has been shown that activation of the NFκB pathway is needed for development of physiological cardiac hypertrophy in mice ⁴⁷. Considering this, it is unlikely that elevated cytokine levels *per se* initiate adverse cardiac remodelling during PO, but rather their continuous presence induced by the sustained haemodynamic stress ⁵. This continuous pro-inflammatory activation prevents resolving of inflammation and leads to accumulation of leukocytes in the heart. Elimination of these deleterious immune populations from the myocardium could be achieved by preventing their migration, or by skewing their polarisation towards anti-

inflammatory phenotypes. In this perspective intervening in CD40 signalling seems a smart strategy, as it was shown previously to affect the infiltration of leukocytes³⁰, macrophage polarisation^{27,48} and activation of lymphocytes³⁰.

The complex immune response of the heart: Alternative research approaches needed?

Because cardiac inflammation is such a complex process, it is difficult to draw conclusions from studies that manipulate one single effector by for example knockdown of a specific cytokine or chemokine. Moreover, the redundancy in function of many cytokines and chemokines⁴⁹, possibly limits the effect of these single gene KO studies on the disease phenotype. Indeed, in most cases HF is not a single loss-of-function genetic abnormality. Therefore, a better approach might be to target miRNAs instead of protein coding genes. Because miRNAs target multiple genes involved in pathophysiological pathways and repress, but not completely ablate transcript levels of their targets⁵⁰, they exert more subtle effects. Therefore, miRNAs are able to fine-tune molecular processes involved in HF development, including inflammation. A disadvantage of the use of miRNAs is that the high number of targets per miRNA makes potential side-effects difficult to predict.

In **chapter four** of this thesis a high-throughput phenotypical screening was applied to explore the effect of 194 HF-associated miRNAs on three pivotal cell-type specific processes underlying HF development, namely hypertrophy, fibrosis, and inflammation⁵¹. This comprehensive screening approach unveiled the effects of various miRNAs in cardiovascular disease and opened new avenues for the treatment of HF via miRNA-directed therapeutic approaches. By performing this screen in three cell types, we discovered novel roles for miRNAs in hypertrophy, fibrosis and inflammation, and we confirmed the effects of well-known “inflammatory” miRNAs such as miR-155^{17,52}. Additionally, we identified miRNAs new to the HF research field. Several miRNAs affected all of the pathophysiological processes investigated. For example, transfection with mimics of miR-200c-3p and miR-210-3p increased cell size in cardiac myocytes (hypertrophy), increased collagen content in cardiac fibroblasts (fibrosis), and it skewed macrophages towards M1 polarization. MiR-891a-3p mimics, on the other hand, influence these processes in the other, more beneficial direction. Both these results open up therapeutic opportunities in HF, either by blocking (miR-200c-3p, miR-210-3p) or delivering (miR-891a-3p) these miRNAs *in vivo*.

New insights using published pre-clinical studies

Animal models are vital for advancement of our understanding of the pathophysiology of HF and therefore the development of novel therapies. With the wide availability of KO and TG mice a plethora of studies addressing the molecular pathways of HF has been published over the past decades (chapter five of this thesis). Although many of these studies have shown promising results in the protection against HF in animal models, disappointingly, success in the translation of these studies into clinical practice remains limited². In combination, however, these pre-clinical studies are the puzzle pieces that give clues about the complex molecular and cellular pathways involved in the pathogenesis of HF. In **chapter five** we have combined almost 300 of these studies, thereby providing an unprecedented overview of pre-clinical cardiac PO studies published in the last 15 years. Using this dataset, we could show that inflammation closely correlates with the pathophysiological processes of hypertrophy and fibrosis and that, whenever the degree of hypertrophy or fibrosis changes, there is a parallel change in the degree of inflammation. Conversely, actively decreasing or increasing inflammation by targeting genes involved in the molecular processes underlying

inflammation, almost always leads to a comparable change in hypertrophy and fibrosis. Previously, network analysis has been used to link expression levels of messenger RNA and microRNAs in dilated cardiomyopathy (DCM) in a miRNA-Gene-Network⁵³. In chapter five we have constructed a gene to phenotype network (GPIN) based on our systematic review of preclinical studies. Using published data, we determined the effect of individual genes on, what we consider, key pathophysiological processes in HF. This network was extended with miRNAs predicted to target the genes that are linked to phenotypes. By using a scoring system for miRNAs targeting these genes, we identified several miRNAs as crucial regulators of pathophysiological processes underlying HF disease progression. The fact that the relatively unknown miR-466l-3p turned out to be the miRNA with the highest cumulative impact on the three outcome parameters, warrants further investigation into its role in PO induced HF. We also identified miR-340, previously identified in DCM patients by network analysis⁵³, to be involved in PO-induced HF. Interestingly, several miRNAs identified in chapter five also displayed pronounced phenotypes in the *in vitro* the screen described in chapter four, thereby conforming their importance in HF disease progression once more. Transfection with miR-19a-3p induced significant increases in all readouts, except NFκB nuclear translocation. MiR-340-5p significantly increases macrophage roundness and decreases both the number of fibroblasts as well as cardiomyocyte cell size. In conclusion, the meta-analysis-based GPIN has proven to be an insightful novel approach to generate new “knowledge” from published data and awaits future application to other animal models of human cardiovascular disease.

Conclusion and future perspectives

The research in this thesis has led to a better understanding of the immune response during HF and created several openings on how to manipulate this response. In chapter two we show that CD40L-CD40-TRAF signalling modulates cardiac hypertrophy. Moreover, we provided additional evidence for the importance of myeloid cells in the development of cardiac hypertrophy. The CD40L-CD40 co-stimulatory dyad offers opportunities for the treatment of HHD. The study described in chapter three supports the concept that cytokines, including TNF-α, released from inflammatory cells infiltrating the myocardium, lead to the activation of NFκB in cardiomyocytes, ultimately resulting in impairment of mitochondrial oxidative metabolism. These metabolic perturbations are likely to contribute to the cardiac contractile dysfunction during acute VM. In chapter four we performed a high throughput screen in three relevant primary cell types simultaneously, thereby identifying miRNAs involved in hypertrophy, fibrosis and inflammation. In this way, not only we confirmed roles of well-known miRNAs, but also identified many miRNAs new to the HF-research field. Finally, we established a novel approach to identify potential HF therapeutic targets in chapter five. Using existing literature, a Gene to Phenotype interaction network was created that allowed the identification of novel miRNAs putatively involved in HF pathogenesis. The vast amount of miRNA data in this thesis is a gold-mine for future miRNA research and might ultimately lead to novel therapies.

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Summary

Heart failure (HF) is the incapability of the heart to supply the body with sufficient levels of oxygenated blood. With an increasing number of patients, HF is currently one of the biggest healthcare challenges worldwide. The pathophysiology of HF is marked by structural changes in the heart such as the enlargement of cardiac myocytes (hypertrophy) and production of excessive collagen (fibrosis) in the heart. Inflammation has been shown to play a crucial role in the initiation of these processes. The goal of this thesis was to increase our understanding of the inflammatory response during HF and to explore how it can be manipulated in a way that it preserves its crucial role in beneficial processes such as wound healing, debris clearance and pathogen clearance, but that its destructive effects on the cardiac tissue are diminished. The research in this thesis is described in four experimental chapters and creates several openings on how to manipulate the inflammatory response during HF.

A common cause of HF is cardiac pressure overload. This is often induced by hypertension. **Chapter two** focuses on the deletion and the genetic manipulation of the pro-inflammatory CD40 receptor in an animal model of angiotensin-II induced hypertension. CD40 is a receptor expressed on the cell surface of several types of inflammatory cells. The CD40 receptor was shown to be a critical regulator of the immune response in different chronic inflammatory diseases like atherosclerosis, multiple sclerosis and arthritis. In all these pathologies blocking of CD40 interactions was found to reduce disease severity. The effect of this receptor on the development of HF and cardiac hypertrophy, as induced by chronic hypertension, was never investigated before. Here we show that CD40 signalling modulates cardiac hypertrophy and we demonstrated, using mice with a mutated CD40 receptor, that the intracellular TRAF signalling molecules are involved in this mechanism. Moreover, we provided additional evidence for the importance of myeloid cells in the development of cardiac hypertrophy. These results indicate that the CD40 receptor and its interaction partners offer opportunities for the development of treatment strategies against HF.

Myocarditis is an acute inflammation of the myocardium, which is often caused by a viral infection and is a common cause for sudden cardiac death in young adults. The infiltration of inflammatory cells into the myocardium represents a hallmark of the disease. In **chapter three** we investigated how this acute and fulminant inflammatory response affects cardiac metabolism. For this study we used cardiac samples of viral myocarditis patients and a mouse model of virus-induced myocarditis. The acute effects of pronounced cardiac inflammation, and viral myocarditis in particular, on cardiac metabolism were not well studied so far.

We elaborately describe how the metabolism of the heart changes during this acute inflammation. A healthy cardiomyocyte generates its energy mainly by burning (oxidation) fatty acids. We observed that cardiomyocytes switch to the use of glucose instead of fatty acids as a substrate during this acute inflammation. This, in turn, leads to an inefficient energy supply. On top of this, we observed that the activity of the oxidative mechanism is reduced which further disturbs the energy supply of the cardiomyocytes. In conclusion, from our study the concept arises that cytokines, including TNF- α , released from inflammatory cells infiltrating the myocardium, lead to the activation of NF- κ B in cardiomyocytes, ultimately resulting in impairment of mitochondrial oxidative metabolism. These metabolic

perturbations are likely to contribute to the cardiac contractile dysfunction during acute viral myocarditis.

Over the last decade, the way in which new molecules for potential therapies are identified has changed substantially. Where previously a specific hypothesis formed the basis of a new study, it is nowadays more common to work with large data sets and select interesting new molecules based on so-called high-throughput screens. In these screens a large number of different molecules can be tested on their ability to change the properties of cultured cells. The experimental work and the analysis are fully automated in these screens in order to limit the variation in the results.

In **chapter four** an *in vitro* screening approach is applied in which 194 microRNAs, previously identified to be differentially expressed in inflammatory diseases of the heart, were tested for their effects on hypertrophy, fibrosis and inflammation in different cell types. We have designed and performed this screen at a specialised company, Cenix BioScience in Dresden, Germany. These three pathophysiological processes have been studied in three relevant primary cell types. To study the effects of these microRNAs on the hypertrophic response we used primary cardiomyocytes, to determine the effect on collagen production we used primary fibroblasts and to study the effects of these microRNAs on the inflammatory response we have used macrophages that were cultured from the bone marrow of mice. By transfecting microRNAs into these cells in both unstimulated and stimulated conditions, we could quantify the role of these different microRNAs in cardiac hypertrophy, fibrosis and inflammation. Thereby, we could not only confirm roles of well-known microRNAs, but also identify novel roles of microRNAs in HF development.

In preclinical HF research there are several commonly used rodent models of cardiac pressure overload that mimic hypertension or aortic stenosis in humans. The last decades a plethora of studies focusing on the genetic manipulation of mice in these models has been published. In **chapter five**, we have made a comprehensive overview of these studies. Thereby focussing on the role of inflammation during pressure overload of the heart. To this end, we have selected studies that intervened in one of the pathophysiological processes of HF. For the analyses in this chapter we extracted the data from 284 articles and combined them into a large dataset. Subsequently, this dataset was used to compare features, and to define reference values of the most commonly used preclinical pressure overload models. By analysing the phenotypes of these models we could show that aortic constriction induces more pronounced cardiac hypertrophy than angiotensin-II infusion.

Next, we used this dataset to identify novel microRNA targets for HF therapy. As the genetic manipulation of specific genes implicated in hypertrophy, fibrosis or inflammation forms the core of most studies in our dataset, the reported effect these genetic manipulations have on HF-related processes was used to build a gene-to-phenotype interaction network (GPIN). As microRNAs are established negative regulators of gene expression and have been shown to regulate pathophysiological processes associated with HF, we extended the GPIN with microRNA targeting data. This allowed the identification of microRNAs influencing cardiac inflammation, hypertrophy and/or fibrosis. Some of these microRNAs are already known in the cardiovascular field, while others have not been linked to cardiac disease previously. Accordingly, this network analysis can be used as a starting point for future preclinical studies specifically addressing the role of these microRNAs in HF. We present a novel approach to build and mine a gene to phenotype interaction network

based on a meta-analysis. Our meta-analysis-based network analysis approach enables us to generate new insights from published cardiovascular research and the newly created methodology will also be of value for other areas of research.

The research presented in this thesis shows that inflammatory processes significantly contribute to the worsening of the disease phenotype in different aetiologies of HF and created several openings to manipulate this response, for example by targeting the CD40 receptor and its interaction partners or through the delivery of microRNAs. Despite these promising new findings, future translational research is needed to further confirm the potential of these results.

Samenvatting

Hartfalen is een aandoening waarbij het hart niet meer in staat is voldoende zuurstofrijk bloed rond te pompen om in de behoeftes van het lichaam te voorzien. Het is het eindstadium van vele cardiovasculaire aandoeningen. Met een alsmaar toenemend aantal patiënten vormt hartfalen één van de grote uitdagingen voor de gezondheidszorg wereldwijd. Het doel van dit proefschrift is de rol van ontsteking (inflammatie) in het ontstaan en het verloop van hartfalen beter te begrijpen. Daarnaast willen we onderzoeken op welke manier de inflammatoire respons kan worden gemanipuleerd zodat de goedaardige onderdelen van dit proces intact blijven, zoals wondherstel en het opruimen van dode cellen en ziekteverwekkers, terwijl de schadelijke effecten op het hartweefsel worden verminderd. Het onderzoek beschreven in dit proefschrift bevat vier experimentele hoofdstukken waarin dit doel is nagestreefd.

Een veel voorkomende oorzaak van hartfalen is drukoverbelasting van het hart, vaak wordt dit veroorzaakt door een te hoge bloeddruk (hypertensie). **Hoofdstuk twee** van dit proefschrift beschrijft onderzoek in muizen waarbij de pro-inflammatoire CD40 receptor is weggehaald of de signaalfunctie ervan is aangepast in een diermodel waarbij hypertensie is geïnduceerd door de muizen te behandelen met angiotensine-II, dat een bloeddruk verhogende werking heeft. De CD40 receptor is aanwezig op de buitenkant van bepaalde witte bloedcellen en vervult een belangrijke functie bij het opstarten van een inflammatoire respons. Het is reeds aangetoond dat deze receptor een cruciale rol speelt bij het ontstaan van inflammatoire aandoeningen zoals atherosclerose, multipale sclerose en reumatische artritis. Bij al deze aandoeningen heeft het blokkeren van de CD40 receptor geleid tot verbeteringen in het ziektebeeld. Of de CD40 receptor ook een rol speelt bij de inflammatoire processen tijdens hartfalen was echter nog niet eerder onderzocht. In hoofdstuk twee van dit proefschrift laten we zien dat de CD40 receptor een rol speelt bij het ontstaan van een pathologische toename in grootte van de hartspier, ook wel cardiale hypertrofie genoemd. Verder laten we zien dat witte bloedcellen een cruciale rol spelen in dit mechanisme en dat de zogenaamde TRAF-moleculen belangrijk zijn in het signaleringsmechanisme binnen in de cel. Dit betekent dat de CD40 receptor en zijn interactie partners een goed aanknopingspunt bieden voor het ontwikkelen van nieuwe medicijnen tegen hartfalen.

Myocarditis is een acute ontsteking van de hartspier die vaak wordt veroorzaakt door een virusinfectie, maar ook een bacteriële infectie of bepaalde parasieten kunnen deze ziekte initiëren. Het treft vooral jonge gezonde mensen. In het **derde hoofdstuk** van dit proefschrift hebben we onderzocht hoe zo'n hevige acute ontsteking aan de hartspier (myocarditis) het metabolisme van het hart beïnvloedt. Daarvoor hebben we gebruik gemaakt van patiëntmateriaal en muizen die we hebben geïnfecteerd met een virus wat myocarditis veroorzaakt. De acute effecten van een hevige ontsteking van de hartspier op het cardiale-metabolisme waren tot op heden nog nooit bestudeerd.

We beschrijven op een uitgebreide manier hoe het metabolisme van het hart tijdens acute ontsteking veranderd. Een gezonde hartspiercel genereert zijn energie grotendeels door verbranding (oxidatie) van vetzuren, maar tijdens de acute ontsteking schakelt deze cel over op het gebruik van glucose i.p.v. vetzuren als substraat. Daarnaast is de activiteit van het oxidatieve mechanisme verminderd. Dit alles leidt tot een minder efficiënte

energievoorziening voor de hartspeer. Het belangrijkste concept beschreven in hoofdstuk drie is dat cytokines, afgegeven door infiltrerende immuuncellen in het hart, bijdragen aan de activatie van de pro-inflammatoire transcriptie factor NFκB in hartspeerzellen. Dit leidt uiteindelijk tot vermindering van het oxidatieve mechanisme in de mitochondriën van deze cellen. Het is zeer waarschijnlijk dat deze vermindering bijdraagt aan de vermindering van de contractie van het hart tijdens acute virale myocarditis.

De manier waarop nieuwe moleculen voor mogelijke therapieën tegen hartfalen kunnen worden geïdentificeerd is erg veranderd de laatste jaren. Waar vroeger vaak een specifieke hypothese aan de basis stond van een onderzoek, wordt tegenwoordig met grote datasets gewerkt en kunnen interessante nieuwe moleculen op basis van zogenaamde high-throughput screens worden geselecteerd. In deze screens kan van een groot aantal verschillende moleculen worden getest of ze bepaalde eigenschappen van gekweekte cellen kunnen beïnvloeden. In **hoofdstuk vier** is een dergelijke screen beschreven die door ons is uitgevoerd bij het bedrijf Cenix BioScience in Dresden, Duitsland. In deze screen hebben we gebruik gemaakt van microRNAs. Dit zijn kleine RNA-moleculen die de expressie van hun target genen kunnen verminderen. Voor deze studie hebben we 194 microRNAs op voorhand geïdentificeerd op basis van differentiële expressie in het hart tijdens inflammatoire hartziekten. De opzet van deze screen was om te kijken wat de effecten van deze microRNAs zijn op drie cruciale processen betrokken bij het ontstaan van hartfalen, namelijk: hypertrofie (toename in grootte van hartspeerzellen), fibrose (overmatige productie van bindweefsel) en inflammatie. Deze drie pathofysiologische processen zijn onderzocht in verschillende relevante celtypen die we direct uit weefsel hebben geïsoleerd. Zo hebben we hartspeerzellen gebruikt om het effect van deze microRNAs op hypertrofie te bekijken en fibroblasten om productie van bindweefsel te bestuderen. Voor het bestuderen van de effecten van microRNAs op inflammatoire processen hebben we macrofagen opgekweekt uit het beenmerg van muizen. Door deze microRNAs middels transfectie, in ongestimuleerde en gestimuleerde cardiomyocyten, fibroblasten en macrofagen te brengen hebben we de rol van deze verschillende microRNAs in cardiale hypertrofie, fibrose en inflammatie in kaart gebracht. Daarbij hebben we niet alleen bestaande functies van eerder beschreven microRNAs kunnen bevestigen, maar ook nieuwe microRNAs kunnen identificeren die een rol in hartfalen spelen.

In **hoofdstuk vijf**, ten slotte, hebben we een uitgebreide analyse gemaakt van dierstudies waarin is gekeken naar inflammatie tijdens drukoverbelasting van het hart. Hiervoor hebben we studies geselecteerd waarbij werd ingegrepen in een van de pathofysiologische processen tijdens hartfalen. Voor de analyses in dit hoofdstuk hebben we uit 284 wetenschappelijke artikelen alle data gehaald en deze gecombineerd tot één grote dataset. Deze dataset hebben we vervolgens gebruikt om het effect van een verandering in één van de pathofysiologische processen op de andere te bekijken, een belangrijke conclusie hieruit was dat de mate van inflammatie sterk correleert met de mate van hypertrofie en fibrose.

Daarnaast hebben we met behulp van de dataset verschillende eigenschappen van deze diermodellen beter gedefinieerd en met elkaar vergeleken. Een interessante uitkomst daarbij was dat de chirurgische techniek van het afbinden van de aorta een sterkere hypertrofie induceert dan de farmacologische techniek waarbij muizen worden blootgesteld aan angiotensine II.

Vervolgens hebben we de dataset gebruikt om fenotypische gegevens en genen met elkaar te verbinden middels een netwerkanalyse, ook wel een Gene-to-Phenotype-Network (GPIN) genoemd. Vervolgens hebben we microRNA target informatie aan dit netwerk toegevoegd zodat we per microRNA een geaccumuleerd effect op de ziekteprocessen van hartfalen hebben kunnen berekenen. Hiermee hebben we kunnen voorspellen wat voor effect elke microRNA heeft op de processen van hypertrofie, fibrose of inflammatie. Daaruit kwam naar voren dat micro-RNA-466l een zeer sterk effect had op genen die vervolgens deze drie processen kunnen beïnvloeden. Het is daarom zeer de moeite waard deze en andere door ons geïdentificeerde microRNAs nader te onderzoeken.

Het onderzoek gepresenteerd in dit proefschrift laat zien dat inflammatoire processen in belangrijke mate bijdragen aan het verergeren van het ziektebeeld bij verschillende ziekteprocessen die tot hartfalen kunnen leiden. Daarnaast heeft het geleid tot een beter begrip van de inflammatoire respons en verschillende mogelijkheden gecreëerd om deze respons te manipuleren, voornamelijk via microRNAs. Ondanks deze hoopvolle nieuwe bevindingen is er nog veel toekomstig onderzoek nodig om het potentieel van de door ons geïdentificeerde microRNAs verder te bevestigen.

Valorization

Relevance

Heart failure (HF) is the end stage of many cardiovascular diseases. It is accompanied by severe symptoms that decrease the patients' quality of life. Half of the patients diagnosed with HF die within five years. Patient numbers have increased in the recent years, mainly because of the aging population and the increasing number of people with obesity. At this time, a targeted therapy or treatment with drugs is still lacking, therefore, the development of novel treatment strategies is an absolute necessity. For this, a better understanding of the pathology of this disease is crucial and that is exactly what the research described in this thesis has focused on.

In recent years it has become increasingly evident that HF is associated with increased inflammation in the heart, and there is increasing evidence that inflammatory factors play an active role in the pathology of many cardiovascular diseases. We have provided clues for the development of new drugs that interfere with the inflammatory response of the heart. In particular, we have shown that the CD40 receptor and its binding partners offer interesting opportunities for this and we could identify microRNAs with important roles in the pathology of HF.

We have also shown that inflammation plays a major role in the development of cardiac hypertrophy and demonstrated that severe acute inflammation interferes with the metabolism of the cardiomyocytes.

Target groups

The research described in this thesis can be of use for different groups outside the academic world. The identification of microRNAs as potential targets could be used to develop drugs for HF therapy by the pharmaceutical industry. In addition, the method of selection of microRNAs that we developed in chapter five can be applied more widely and possibly microRNAs can be selected for further *in vivo* studies based on our data. The microRNA research field grows rapidly and the delivery of microRNA mimics now offers exciting therapeutic possibilities. However, it is important to map the precise effects of all these microRNAs, including all of the pathophysiological processes taking place in the failing heart as we did here.

The results generated in this thesis are only of indirect value to patients. Despite the fact that many of the findings in this thesis point to a role of inflammation in HF it is difficult to translate these results into medical practice. Because the animal models used do not directly represent the clinical practice additional translational research approaches must be initiated.

Activities, Products & Innovation

Several of the microRNAs identified by us *in vitro* or *in silico* could be used for the development of new drugs for HF therapy. The results of the screen can be used outside the cardiology field. For example, to study muscle growth or fibrosis in other organs such as kidney or lung. Our methodology of microRNA identification through network analysis can be applied in a much wider context. Therefore, we have made the source of our methodology publicly available. Direct translation of our results into products is difficult to predict at this time. We do offer our expertise in setting up a network analysis based on a meta-analysis.

Planning & Realisation

Several difficulties could arise in the process of developing medicines based on microRNAs. Delivery of microRNA mimics in vivo remains difficult and the many target genes make side effects difficult to predict. Indeed, despite many attempts, there are very few successful microRNA based drugs developed so far. Other results we have obtained are simply too far removed from direct application. One possible problem is that the steps that are required to get our results closer to the clinical practice cannot be put in action because it requires big investments.

Valorisatie

Relevantie

Hartfalen is het eindstadium van vele cardiovasculaire aandoeningen. De ernstige symptomen zorgen ervoor dat patiënten een verminderde kwaliteit van leven ervaren. Bijna de helft van de mensen met de diagnose ernstig hartfalen sterft binnen vijf jaar. Het aantal mensen dat lijdt aan hartfalen neemt de laatste jaren toe. Dit komt onder meer door de vergrijzende bevolking en door een toename van het aantal mensen met een ongezonde levensstijl, waaronder zwaarlijvigheid. Op dit moment ontbreekt voor hartfalen een gerichte therapie of behandeling met medicijnen, waardoor het ontwikkelen van nieuwe behandelingsstrategieën hoogst wenselijk is. Hiervoor is een beter begrip van de pathologie van dit syndroom cruciaal en dat is dan ook waar het onderzoek beschreven in dit proefschrift op gericht is.

De laatste jaren is het steeds duidelijker geworden dat hartfalen gepaard gaat met een verhoogde aanwezigheid van ontstekingsfactoren in het hart. Ook komt er steeds meer bewijs dat deze ontstekingsfactoren een actieve rol spelen in de pathologie van de vele cardiovasculaire aandoeningen die uiteindelijk tot hartfalen kunnen leiden. Met het onderzoek beschreven in dit proefschrift hebben we nieuwe aanknopingspunten geleverd voor het ontwikkelen van medicijnen die ingrijpen in de werking van deze ontstekingsmechanismen. We laten onder meer zien dat de CD40 receptor en de bijbehorende signaaleiwitten een belangrijke rol spelen bij het ontstaan van cardiale hypertrofie, suggererend dat deze eiwitten wellicht goede aangrijpingspunten voor therapie bieden. Daarnaast hebben we verschillende microRNAs kunnen identificeren die een belangrijke rol hebben in de pathologische processen die een rol spelen bij het ontstaan van hartfalen. Ook laten we zien dat hevige acute ontstekingen het metabolisme van de hartspeer flink kunnen verstoren. Deze kennis is erg waardevol bij de ontwikkeling van nieuwe medicijnen tegen hartfalen.

Doelgroepen

De onderzoeksresultaten beschreven in dit proefschrift kunnen van betekenis zijn voor verschillende groepen buiten de academische wetenschap. De door ons geïdentificeerde microRNAs hebben potentie als medicijnen voor de behandeling van hartfalen. Dit is in eerste instantie interessant voor de farmaceutische industrie. Deze microRNAs kunnen worden gebruikt voor verder in vivo onderzoek, de medicijnen die daar dan hopelijk uit voort komen zijn uiteraard in het belang van de patiënten.

Het microRNA onderzoeksveld neemt snel in grootte toe en het nabootsen van microRNAs met zogenaamde mimics biedt inmiddels interessante therapeutische mogelijkheden. Daarom is het belangrijk om van alle interessante microRNAs de precieze effecten in kaart te brengen, ook op alle relevante pathofysiologische processen, zodat eventuele bijwerkingen beter kunnen worden ingeschat. Door de rol van microRNAs in verschillende pathofysiologische processen tegelijk te bestuderen hebben we hieraan bijgedragen.

Ondanks dat de vele bevindingen in dit proefschrift die op een rol van ontstekingsmechanismen in hartfalen duiden, naast de al aanwezige bewijzen, is het moeilijk om de resultaten van dit basale onderzoek op korte termijn in de medische praktijk toe te passen.

Activiteiten, Producten & Innovatie

De resultaten van de screen beschreven in Hoofdstuk 4 zijn ook relevant voor buiten de cardiologie, bijvoorbeeld in het onderzoek naar spiergroei (hypertrofie) of fibrose in andere organen zoals de nieren en longen. De door ons ontwikkelde methodologie, identificatie van microRNAs door middel van netwerkanalyse kan veel breder worden toegepast, zelfs buiten de medisch biologische wetenschap. Daarom is de broncode van onze methodologie openbaar beschikbaar gemaakt. Directe vertaling van onze resultaten in concrete producten is op dit moment lastig vorm te geven, hooguit het aanbieden van de expertise in het opzetten van een netwerkanalyse gebaseerd op een meta-analyse.

Planning & Realisatie

Eventuele risico's bij het proces van ontwikkelen van medicijnen op basis van microRNAs zijn dat het nog steeds erg lastig is om de microRNA mimics op de juiste plek in het lichaam te krijgen. Ook het feit dat microRNAs vaak vele genen tegelijk kunnen reguleren maakt de kans op ongewenste bijwerkingen groot.

Mede daardoor zijn er ondanks recente pogingen nog maar weinig succesvolle medicijnen op basis van microRNAs ontwikkeld. Andere punten uit het proefschrift staan te ver af van directe toepassing. Een mogelijk probleem is dat de stappen die nodig zijn om onze resultaten dichterbij de klinische praktijk te krijgen niet kunnen/zullen worden gezet omdat het te grote investeringen vereist.

Dankwoord

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For over a year of my PhD time I have lived in the beautiful city of Dresden where I had the chance to work at Cenix Bioscience. Regretfully I also had to experience the sad ending of the company. Despite this misfortune my time at Cenix was one big nice adventure that learned me a lot about (1) High throughput screening (2) Working in a company compared to academia (3) What nice a city Dresden is when living in the Neustadt (4) That you can make beautiful hikes in Sächsische Schweiz (5) and I've learned a lot about the Germans. The hard work there has also yielded nice results as described in chapter four of this book.

For this I would like to thank all people at **Cenix**, also for giving me a warm welcome in Dresden and a great time. I should name everyone, because all of you made my stay a great and informative time. But in particular I need to thank: **Birte** and **Kerstin** for all the help in the design of the screens and the interpretation of the results. **Alex**, **Kartini**, **Sarah** thanks

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Biography

Wouter Derks was born on the 5th of May 1984 in Heide, near Venray, the Netherlands. After finishing his secondary education with profile nature, technology and health, he started to study biology at Wageningen University. In Wageningen he specialised in animal biology and immunology. He completed a thesis on the role of chemokines in the immune system of teleost fish. Later, Wouter went to perform research internships at the INRA institute near Paris and at the INSERM institute in Montpellier, France. After receiving his MSc degree from Wageningen University, Wouter commenced his PhD program in the lab of prof. dr. S. Heymans at Maastricht University, Department of Cardiology, in January 2011 in a cooperation with prof. dr. E. Lutgens and supervised by dr. M. van Bilsen.

During his PhD he studied the role of the immune response on the development of cardiac disease using pre-clinical animal models. He worked on the role of the CD40 receptor in hypertensive heart disease and studied the effect of acute inflammation in the heart induced by viral myocarditis. From February 2014 until March 2015 Wouter has worked at Cenix Bioscience GmbH, Dresden, Germany, in the context the European CardiomiR project, there he developed high-throughput screening assays to identify microRNAs involved in inflammatory cardiac pathology.

List of Publications

Submitted: Inhibition of CD40-CD40 ligand (CD40L) and downstream TRAF signalling reduces angiotensin II induced cardiac hypertrophy. Derks W, Carai P, Boon L, Lutgens E, Van Bilsen M, Heymans S.

In preparation: Inflammation-induced metabolic remodelling of the heart in acute viral myocarditis. Remels A*, Derks W*, Verhees K, Kelders M, Summer G, Theije C, Heymans S, Papageorgiou A, van Bilsen M

*contributed equally

In preparation: MicroRNAs act as pleiotropic regulators of heart failure progression - A high-throughput phenotypical screen in three primary cardiac cell types. Verjans R*, Derks W*, Korn K, Sönnichsen B, Van Bilsen M, Schroen B, Heymans S.

*contributed equally

Submitted: Literature-based Gene to Phenotype Interaction Network analysis reveals novel microRNA regulators in Heart Failure. Derks W*, Summer G*, Coenen R, Heymans S, Van Bilsen M.

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Hypertension is a conditional factor for the development of cardiac hypertrophy in type 2 diabetic mice. van Bilsen M, Daniels A, Brouwers O, Janssen BJ, Derks WJA, Brouns AE, Munts C, Schalkwijk CG, van der Vusse GJ, van Nieuwenhoven FA. PLoS One. 2014 Jan 9;9(1)

Diversification of IFN γ -inducible CXCb chemokines in cyprinid fish. van der Aa LM, Chadzinska M, Derks W, Scheer M, Levraud JP, Boudinot P, Lidy Verburg-van Kemenade BM. Dev Comp Immunol. 2012 Oct;38(2)