



Non-coding RNAs as ‘theranostics’ in heart, lung and circulation

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by

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Abstract

The human genome is like a giant library harbouring billions of books, the collective information of life. But, only a few ‘documents’ could be uncovered so far. A new readout of the genome unravelled that the majority of the unexplored genetic information codes for transcripts that escape protein synthesis and carry out various functions in their unique form as RNAs. Such non-coding transcripts, including microRNAs and long non-coding RNAs (lncRNAs), are both key regulators of various cellular processes providing new treatment options for disease, and in body fluids serve as indicators for physiological states and conditions making them of great relevance for diagnostic and prognostic applications. Therefore, non-coding RNAs open up novel ‘theranostic’ avenues for the therapy and detection of cardiovascular and pulmonary disease.

With respect to diagnostics, microRNAs circulating in the plasma of marathon runners were evaluated as potential biomarkers of aerobic exercise capacity in the first part of this thesis. We found that the marathon run increased plasma levels of muscle- and heart-enriched microRNAs (miR-1, miR-133a, miR-206 and miR-208b, miR-499), while levels of inflammatory microRNAs (miR-21, miR-155) remained unchanged. Particularly muscle-related microRNAs remained elevated 24h after the run and strongly correlated with performance parameters, rather than with markers of cardiac and skeletal muscle injury. These data suggest muscle-related microRNAs as indicators for physiological adaptations to strenuous exercise and as potential biomarkers of aerobic exercise capacity.

As a potential therapeutic target, a lncRNA that is involved in the development of heart disease is subject of the second part of this manuscript. Among thousands of lncRNAs we identified a transcript that was strongly induced in hearts and cardiomyocytes of an experimental mouse model for pressure overload-induced cardiac hypertrophy and heart failure. This lncRNA, named cardiac hypertrophy associated transcript (*Chast*), acts downstream of the pro-hypertrophic NFAT signaling pathway and drives pathological changes in the heart when overexpressed. Pharmacological inhibition of *Chast* prevented abnormal growth of the stressed murine myocardium as well as hypertrophic gene response and significantly improved the hearts pump function. We identified a homologous sequence in humans that, in accordance with the mouse model, was significantly induced in cardiac tissue from aortic stenosis patients indicating a functional conservation of *Chast* activity. In brief, this study sets the ground for future preclinical and clinical development of *Chast* inhibitors for the treatment of disease associated to pathological cardiac remodeling, such as cardiac hypertrophy and heart failure.

The third part of this work assesses the susceptibility of mice transgenic for the human SP-D Met(11)Thr polymorphism to allergic airway inflammation and the impact on microRNA expression. We found that neither lung function and morphology, nor pulmonary inflammation discriminate between mice carrying one or the other polymorphic SP-D allele and that murine expression of the human SP-D variants had any impact on the severity of allergic airway inflammation. However, the two microRNAs evaluated (miR-21 and miR-155) were differentially expressed.

Zusammenfassung

Das humane Genom ist vergleichbar einer Bibliothek mit Millionen von Büchern, die gesammelte Information des Lebens. Jedoch sind bisher nur wenige dieser ‚Dokumente‘ enträtselt worden. Eine neue Auslese des Genoms hat gezeigt, dass die Mehrheit der unentdeckten genetischen Information für Transkripte kodiert, die der Proteinsynthese entgehen und ihre Funktion in ihrer einzigartigen Form als RNA ausüben. Solche nicht-kodierenden RNAs, darunter mikroRNAs und lange nicht-kodierende RNAs (lncRNAs), fungieren sowohl als Schlüsselregulatoren verschiedener zellulärer Prozesse, was neue Behandlungsmöglichkeiten für Krankheiten ermöglicht, als auch als in Körperflüssigkeiten detektierbare Indikatoren für physiologische Zustände, was für diagnostische und prognostische Anwendungen bedeutsam ist. Daher ermöglichen nicht-kodierende RNAs neue ‘theranostische‘ Wege für die Therapie und Diagnose kardiovaskulärer und pulmonaler Erkrankungen.

Mit Blick auf neue Diagnostika analysiert der erste Teil dieser Arbeit im Plasma von Marathonläufern zirkulierenden mikroRNAs als potentielle Biomarker für aerobe Ausdauerleistung. Wir konnten zeigen, dass ein Marathonlauf zur Erhöhung der Plasmaspiegel von mikroRNAs führt, die im Muskel und Herzen angereichert sind (miR-1, miR-133a, miR-206 sowie miR-208b, miR-499), jedoch das Niveau von inflammatorischen mikroRNAs (miR-21, miR-155) unverändert bleibt. Speziell die muskelassoziierten mikroRNAs blieben auch 24h nach dem Lauf erhöht, korrelierten stark mit Leistungsparametern und weniger mit kardialen oder muskulären Schadensmarkern. Diese Daten deuten darauf hin, dass muskelspezifische mikroRNAs physiologische Anpassungen an körperliche Anstrengungen indizieren und als Biomarker der aeroben Ausdauerleistung dienen können.

Der zweite Abschnitt des Manuskriptes beschreibt eine lncRNA, die an der Entwicklung von Herzerkrankungen beteiligt ist, als potentielle neue therapeutische Zielstruktur. Unter tausenden von lncRNAs haben wir eine identifiziert, die in Herzen und Kardiomyozyten durch Drucküberlastung in einem Mausmodell für kardiale Hypertrophie und Herzversagen induziert ist. Diese lncRNA, Cardiac hypertrophy associated transcript (*Chast*) genannt, unterliegt der Regulation pro-hypertropher NFAT-abhängiger Signalwege und führt nach Überexpression zu hypertrophen Veränderungen des Herzens. Die pharmakologische Inhibition von *Chast* verhinderte das anormale Wachstum des murinen Myokards unter Stress sowie die hypertrophe Genexpression und verbesserte die Herzfunktion. Wir konnten zum Menschen homologe Sequenz identifizieren, die wie in der Maus, im kardialen Gewebe von Aortenstenosenpatienten erhöht vorlag, was auf eine konservierte *Chast*-Funktion hindeutet. Insgesamt stellt diese Studie die Basis für künftige präklinische und klinische Entwicklungen von *Chast*-Inhibitoren dar, die für die Behandlung von Erkrankungen mit pathologischen kardialen Veränderungen, darunter kardiale Hypertrophie und Herzversagen, dienen könnten.

Der dritte Teil dieser Arbeit analysiert die Anfälligkeit von Mäusen, die ein Transgen für den humanen Met(11)Thr Polymorphismus im SP-D Gen tragen, für eine allergische Entzündungsreaktion der Atemwege und den Einfluss auf die Expression von mikroRNAs. Wir konnten aufzeigen, dass Mäuse mit dem ein oder anderen polymorphen Allel sich weder in der Lungenfunktion und -morphologie, noch in der pulmonalen Entzündungsreaktion unterschieden, und dass die Expression des humanen SP-D in der Maus keinen Einfluss auf den Schweregrad der allergischen pulmonalen Entzündung hat. Dennoch waren die untersuchten mikroRNAs (miR-21 und miR-155) unterschiedlich exprimiert.

1. Introduction

1.1 Non-coding RNAs as promising targets for therapy and diagnosis

Aberrant gene expression is the major origin of diseases. Historically, such processes were known to be mainly driven by proteins. However, less than 2% of the human genome encodes for transcripts that serve as templates for protein synthesis^{1,2}; a crucial finding that reshapes the ‘central dogma’ of life that defines proteins as the functional manifestation of the genetic information and RNA merely as an intermediate of this transfer. The vast majority of the genome harbours an enormous proportion of non-protein-coding RNAs (ncRNAs). It is evident, that this bulk of transcripts exhibits more functions instead of being transcriptional noise or serving as a simple template for protein synthesis. In contrast, ncRNAs represent a largely unexplored territory that is arbitrarily divided into two major classes defined by an operational size threshold: (1) Small RNAs that are shorter than 200 nucleotides include several endogenous RNA species (e.g. Piwi-interacting, small-interfering, or small nuclear RNAs) like the well described microRNAs. (2) Long ncRNAs (lncRNAs) range from 200 to thousands of nucleotides and comprise an extremely heterogeneous population of RNA molecules. Considering the capability of all these transcripts to form three-dimensional structures and to interact with proteins, DNA and other RNA molecules, ncRNAs possess as versatile functions as proteins enabling them to orchestrate most cellular events. It becomes evident that aberrant ncRNA expression or function leads to a disruption of major biological processes causing various human diseases. The role and function of such non-coding transcripts in the pathogenesis and progression of various maladies has an enormous potential for new so called ‘theranostics’ including new therapeutic applications as well as diagnostic and prognostic markers. In the following, microRNAs and lncRNAs should be highlighted.

1.1.1 MicroRNAs

MicroRNAs (miRNAs, miRs) are endogenous, single-stranded RNAs of 18-25 nucleotides in length that regulate the mammalian gene expression by sequence-specific recognition of their target transcripts.³ MiRNAs are mainly transcribed by RNA polymerase II generating a large, mRNA-like precursor (pri-miRNA) that undergoes a multistep processing and maturation⁴ (summarized in Fig. 1): The nuclear microprocessor complex recognizes the pri-miRNA via the RNA-binding protein DGCR8 (DiGeorge syndrome Critical Region 8) and cleaves it into hairpin RNAs mediated by the RNase III endonuclease Drosha.^{5,6} Alternatively, miRtrons (miRNA genes located in introns of protein-coding sequences) can be processed by the splicing machinery.⁷ Resulting pre-miRNAs are transported to the cytoplasm via Exportin-5 and RAN-GTP^{8,9} and cleaved by the RNase III endonuclease Dicer releasing a RNA duplex of around 22 nucleotides.¹⁰ One miRNA strand assembles together with Argonaute proteins (Ago) into the miRNA-induced silencing complex (miRISC).¹¹ Recognition of the target transcript is mainly mediated by complementary nucleotides of the miRNA seed region and sequence stretches within the 3′ untranslated region (3′UTR) of the targeted transcript.¹² Target recognition provokes translational inhibition or mRNA degradation subsequently repressing the

expression of a gene.¹³ The nature of this regulatory mechanism enables miRNAs to regulate hundreds of distinct targets, thus fundamentally influencing genetic programs and cellular processes.

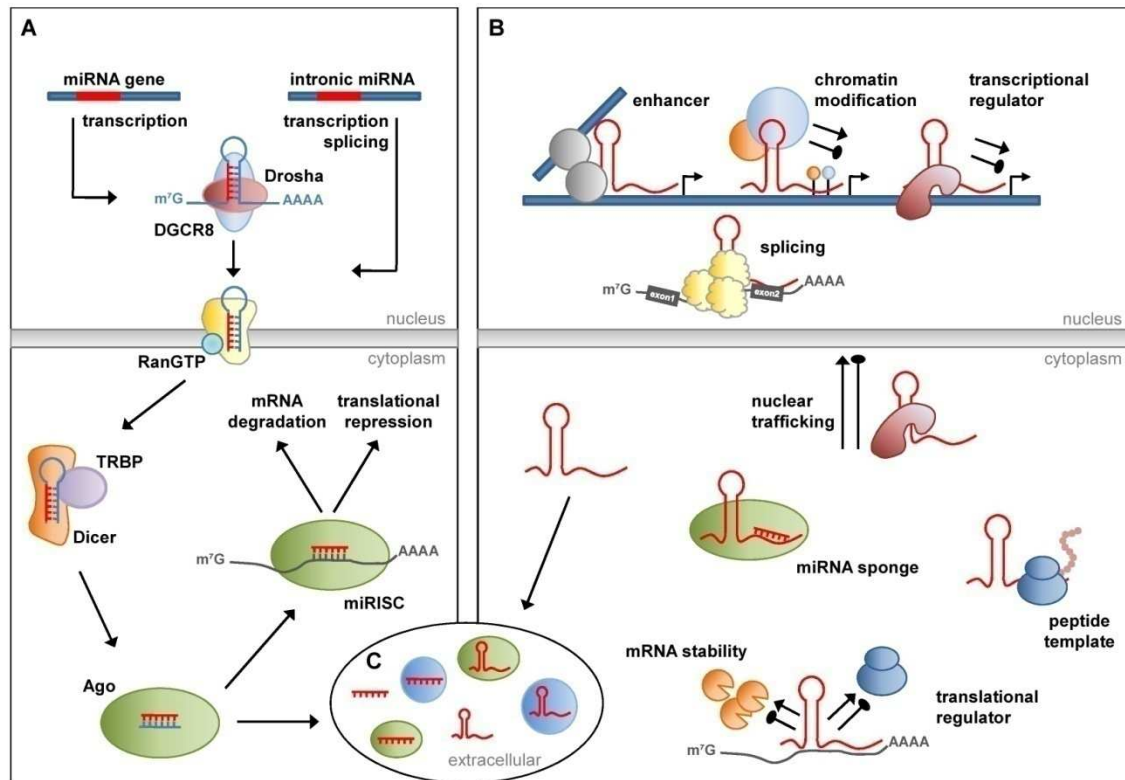


Fig. 1: Biogenesis and function of ncRNAs. (A) MiRNAs are transcribed as longer precursors or derived from introns, and mature via endonucleolytic processing. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) and regulate target transcript expression by degradation or translational repression. (B) LncRNAs control the expression of genes in the nucleus by interacting with DNA, chromatin modifying complexes and/or various transcriptional regulators. Cytoplasmic lncRNAs act as sponges for other transcripts or proteins, serve as protein templates or regulate mRNA degradation and translation. (C) NcRNAs can be secreted into the extracellular space, stabilized in vesicles or proteinous binding partners.

1.1.2 Long non-coding RNAs

LncRNAs represent the majority of the non-coding transcriptome.¹⁴ However, they are the least studied class of ncRNAs, still a heterogeneous group and so far barely defined. In general, the term lncRNA refers to transcripts with at least 200 nucleotides in length that do not template for protein synthesis. The definition by size is an operational, but common biophysical cut-off to discriminate long and short ncRNAs.^{15, 16} The term ‘non-coding’ implies a lack of open reading frames and further coding signatures¹⁷⁻¹⁹, but tends to weaken, since recent findings provide evidence that some lncRNAs encode small peptides.^{20, 21} However, this does not necessarily exclude an alternative function. Peptide encoding lncRNAs might serve both as templates for protein synthesis and as regulatory transcripts.²¹ Another classification of lncRNAs is based on their genomic relation to protein-coding genes: (1) sense lncRNAs cover exons or introns of a coding gene; (2) antisense transcripts overlap to messenger RNA gene sequences on the opposite strand; (3) intergenic lncRNAs are encoded between and

transcribed independently from coding genes; (4) bidirectional lncRNAs arise from promoter regions in opposite direction to protein-coding genes; (5) enhancer RNAs (eRNAs) originate from enhancer regions; (7) circular lncRNAs (circRNAs) form covalently closed loops and are derived from spliced messenger transcripts.²² In general, genomic loci encoding for lncRNAs are similar to those of protein genes. They share the same epigenetic chromatin marks²³, transcriptional regulators and processing machinery including synthesis (usually by RNA polymerase II^{1, 24}), splicing, polyadenylation and capping^{25, 26}.

Compared to protein-coding genes or miRNAs, lncRNAs usually harbour less sequence similarity, but can arise from ultraconserved regions^{24, 27} or syntenic genomic loci²⁷. Conservation might be reflected by secondary structure motifs²⁸, indicating a functional rather than sequence-based interspecies relation. Further, lncRNAs are expressed in lower amounts compared to protein-coding transcripts, but their expression is notably restricted to temporal events or specific tissues.^{1, 29}

In many cases, functions of lncRNAs are determined by their structure, which relies on the primary level on Watson-Crick base pairing and on higher-order secondary arrangements like helices, loops, bulges and pseudoknots³⁰⁻³² that might twist and flip between different forms. Such structures provide a flexible and complex recognition surface enabling the interaction with proteins, DNA or RNA molecules. The function of lncRNAs (reviewed in Geisler *et al.*²² and Devauw *et al.*³³; depicted in Fig. 1) depend on their subcellular localization, since non-coding transcripts are present in the nucleus as well as the cytoplasm.¹ Nuclear lncRNAs are involved in epigenetic and transcriptional regulatory events acting on vicinal genes *in cis* or on distant loci *in trans*. In both cases, lncRNAs associate with chromatin-remodelling complexes or transcription factors and guide such proteins to specific loci provoking either the activation or silencing of gene expression. Examples for such transcriptional regulators are antisense transcripts regulating neighbouring genes on the opposite strand,³⁴ enhancer RNAs, which loop the chromatin to activate their target genes³⁵, or transcripts involved in imprinting like *Xist*, which provokes X-inactivation by spreading over the entire chromosome and induces epigenetic silencing.³⁶ Other lncRNAs directly affect the transcriptional machinery by binding RNA polymerase II.²² Nuclear restricted transcripts like *Neat1* and *Malat1* localize to specific nuclear bodies controlling transcription initiation or termination, RNA editing and processing including splicing as well as nuclear retention of transcripts.^{22, 37, 38} In the cytoplasm, non-coding transcripts act as modulators of mRNA translation, stability or decay.²² Furthermore, lncRNAs serve as endogenous decoys sequestering regulatory factors. Such ‘sponges’ trap transcription factors hampering nuclear trafficking (e.g. lncRNA *NRON*) or compete for the binding of miRNAs attenuating target repression (e.g. *lincMD1*, *HULC* and circRNAs).³⁹⁻⁴¹

1.1.3 Extracellular RNAs

NcRNAs fulfil miscellaneous functions and orchestrate important processes within the cell. Moreover, their physicochemical properties enable these molecules to compactly accumulate information.

Considering that ncRNAs are capable to cross the membrane barrier strongly indicates that the information stored can be transmitted and propagated between neighbouring or distant cells. Indeed, such extracellular or circulating ncRNAs (exRNAs, c-RNAs; shown in Fig. 1) have been detected in blood, plasma, milk, urine and other body fluids.⁴² In general, RNA is immediately degraded in the extracellular environment due to high amounts of nucleases. However, some forms of circulating RNAs seem to be protected from RNase activities during the transport.⁴³ This stabilization is mediated by association to ribonucleoprotein complexes⁴⁴ and lipoproteins⁴⁵ or by encapsulation into membranous vesicles including exosomes, microvesicles, or apoptotic bodies.⁴⁶⁻⁴⁸ In their function as signalling molecules, ncRNAs are seen as emerging intercellular communicators and coordinators of paracrine signalling networks.^{49, 50} Since ncRNA expression patterns are highly correlated with disease states and conditions, extracellular transcripts are of great value for prognostic and diagnostic applications.^{51, 52}

1.2 Cardiovascular and pulmonary disease

Cardiovascular maladies, chronic obstructive (COPD) and inflammatory pulmonary disease predominantly contribute to nearly all causes of morbidity and mortality worldwide.⁵³ These pathologies are closely interrelated (depicted in Fig. 2). Patients with COPD are at elevated risk of cardiovascular disease, whereas the link between inflammatory disease like asthma and cardiovascular risk is less strikingly⁵⁴, but should not be dismissed. Therapeutically, pulmonary and cardiac maladies are challenging, since they are multilayer processes and have been shown to involve ncRNAs.

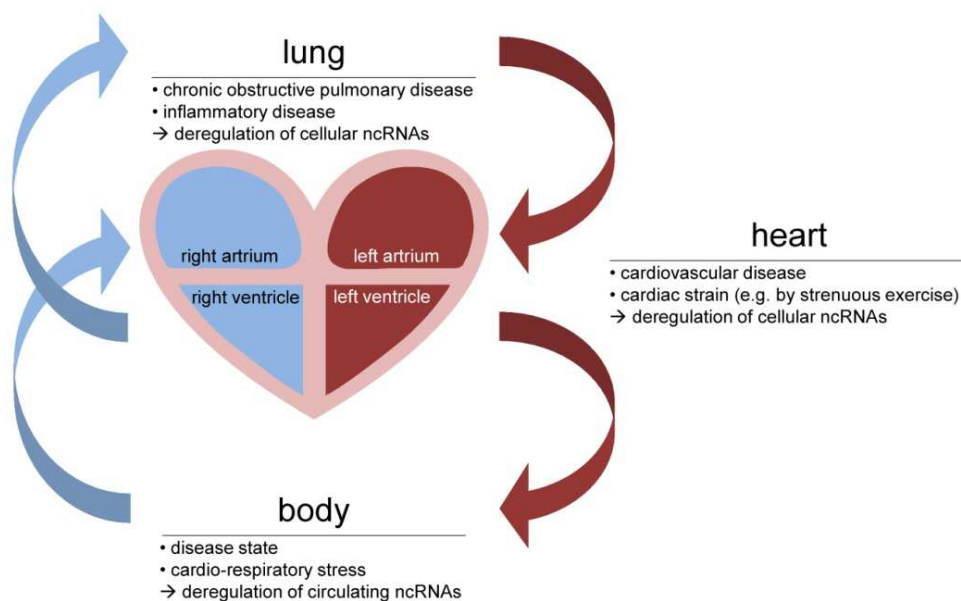


Fig. 2: The complex interaction between the cardiac and the pulmonary system. Cardiovascular and lung disease are closely interrelated and lead to deregulation of ncRNAs. The physiological status of a human being can be sensed by ncRNAs that are stably present in the bloodstream.

1.2.1 Cardiac remodelling

In response to an elevated workload, the heart undergoes a remodelling process to reduce stress and maintain the pump function. Dependent on the nature of the stimulation, cardiac restructuring leads either to a pathological or physiological outcome. Both conditions and types of cardiac remodelling are summarized in Fig. 3.

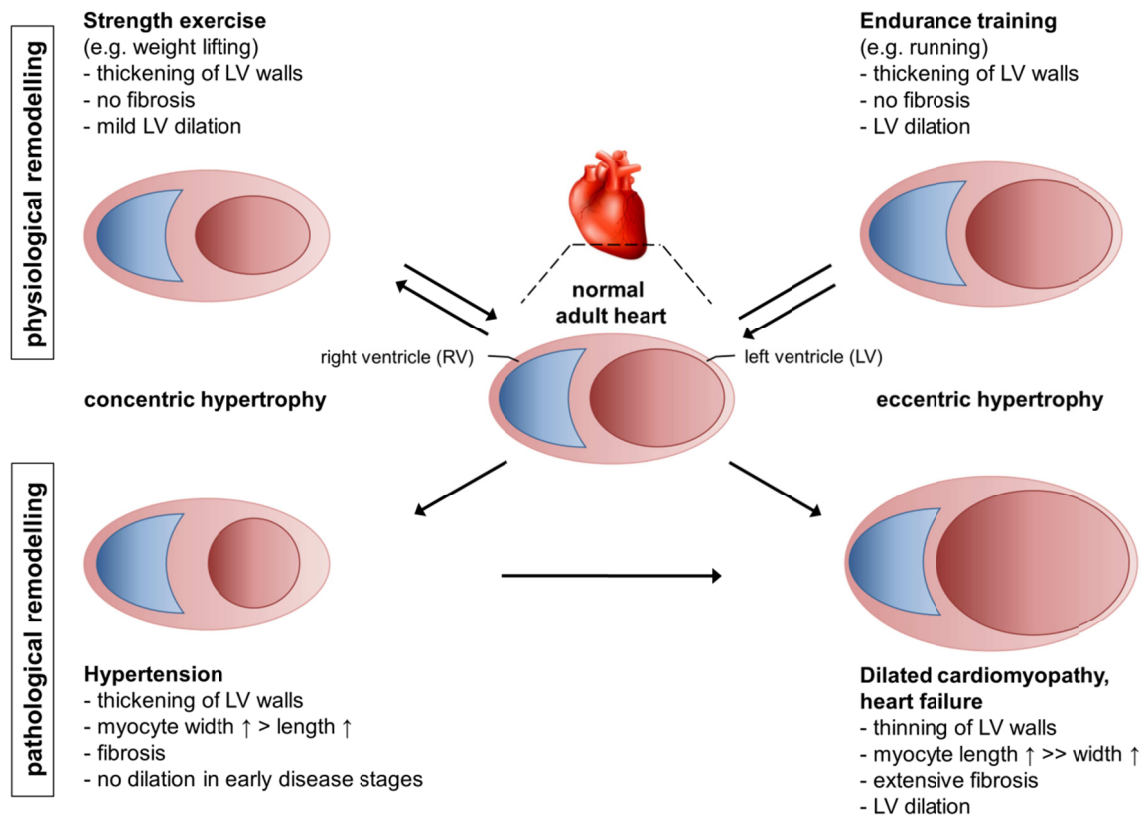


Fig. 3: The heart can increase in size depending on the stimulus resulting in pathological or physiological remodelling. Physiological hypertrophy is an adaptive response to exercise, while pathological hypertrophy is induced by stress signals. Both forms of cardiac growth can be classified by eccentric or concentric growth leading to different morphologies of the heart. Physiological changes are reversible, while pathological hypertrophy is in general unidirectional.

Pathological cardiac remodelling

Cardiac remodelling, or left ventricular (LV) remodelling, is a crucial process in cardiovascular diseases that leads to an alteration in size, shape, composition and functions of the myocardium and accompanies ischemic cardiac disease, valvular insufficiency, hypertension, and heart failure, as well as genetic disorders.^{55, 56} This process is initiated by cardiac injury as well as ventricular pressure and/or volume stress provoking a series of initially compensatory morphological, functional, transcriptional, and metabolic adaptations of the heart to meet the increased workload, to minimize wall stress and to maintain contractile function. Pressure overload due to hypertension or aortic stenosis leads to concentric hypertrophy increasing LV mass and wall thickness to normalize cardiac stress. Alternatively, elevation in blood volume increases the chamber radius, causing eccentric hypertrophy (see Fig. 3). This form of heart growth compensates systolic, but not diastolic wall stress.⁵⁶⁻⁵⁸

Prolonged cardiac strain exacerbates maladaptive response resulting in inflammation, thickening of the myocardium, fibrosis, and vascular stiffening finally impairing cardiac function and leading to heart failure.^{49, 55} At the cellular level, the myocardial growth is mainly mediated by cardiomyocytes. The majority of this cell type is postmitotic, and therefore responds to stress either by hypertrophic growth or cell death. Phenotypically, pressure overload increases thickening of cardiomyocytes, volume stress induces longitudinal growth.⁵⁶ This gain in cell size occurs due to elevated sarcomeric assembly and is accompanied by modified gene expression and protein synthesis towards the re-expression of the fetal gene program, unusual Ca^{2+} handling or cell death.⁵⁵

The hypertrophic response is initiated either by neurohumoral and endocrine hormones such as catecholamines, endothelin-1, angiotensin II and insulin-like growth factor 1 sensed through membrane-bound receptors, or by direct detection of mechanical stretch or deformation via an internal sensory system.⁵⁶ Such signals are transduced via signalling pathways including G proteins, protein kinase C, mitogen-activated protein kinases, calcineurin/NFAT, and phosphoinositide 3-kinase.⁵⁶

A crucial hypertrophic signalling pathway is based on two nodal signalling molecules, the serin/threonin phosphatase calcineurin and the transcription factor nuclear factor of activated T cells (NFAT). Cardiac-specific transgenic mice expressing activated forms of calcineurin or NFATc3 have been shown to develop cardiac hypertrophy⁵⁹, while over-expression of the calcineurin inhibitory protein Mcip.1 prevents from the hypertrophic response.⁶⁰ Upon increase of calcium levels, calcineurin is activated via the binding of calcium/calmodulin or by targeted cleavage that binds and dephosphorylates NFAT. The transcription factor translocates to the nucleus, associates with further transcription factors like myocyte enhancer factor 2 (MEF2) and GATA-binding protein (GATA4) to activate transcription of prohypertrophic target genes.^{56, 61} Nuclear translocation and activity of NFAT is opposed by specific phosphorylation by kinases including JNK⁶², p38⁶³, and glycogen synthase kinase 3 β (Gsk3 β)⁶⁴. Further, the calmodulin kinase CaMKII blocks calcium signalling by phosphorylation and inhibition of calmodulin.⁶⁵

In the process of cardiac remodelling, these signalling events provoke changes in the expression of numerous genes that resemble those expression patterns observed during fetal development of the heart.⁶⁶ This 'fetal gene program' includes a transcriptional induction of arterial and brain natriuretic peptide (*ANP* and *BNP*), skeletal α -actin, and a switch from α - to β -myosin heavy chain (α -/ β -*MHC* or *MYH6/7*) expression. Although elements of this altered genetic program might exhibit initially beneficial effects, prolonged activation provokes maladaptive changes of the heart.⁶⁶

Further determinants of the hypertrophic gene program are alterations in the epigenome involving histone modifications and DNA methylation.^{67, 68} DNA methylation is an epigenetic mark that induces transcriptional repression of clustered regions (CpG islands) and is catalysed by DNA methyltransferases (DNMTs). The involvement of such DNA modifications is still in the beginning to be elucidated, unrevealing the methylation profile of diseased and healthy hearts.⁶⁹⁻⁷¹ Initial findings indicate that failing and fetal cardiomyocytes exhibit comparable methylation patterns.⁶⁹ Histone

modifications such as acetylation lead to transcriptional activation through relaxation of the chromatin structure and are regulated by histone acetyltransferases and deacetylases (HDACs).^{67, 68} Class II HDACs play an important role in the development of cardiac hypertrophy. Constitutive activation of such modifiers has been shown to prevent cardiomyocyte growth⁷², while mice lacking HDACs develop cardiac hypertrophy.⁵⁹ Furthermore, these classes of histone modifiers act as repressors of MEF2 and subsequently of prohypertrophic genes. Themselves they are blocked through nuclear export upon hypertrophic conditions.^{72, 73} The outcome of histone methylation depends on the extent and location of the modification.⁶⁷ H3K4 methylation facilitates gene expression, while di- or trimethylation of H3K9 appears repressive. For example, the failing myocardium exhibits differential histone methylation patterns in various gene clusters.^{71, 74}

Physiological cardiac remodelling

Cardiac remodelling occurs not only in response to pathological conditions described above. The myocardium can also undergo adaptive changes, named physiological hypertrophy. The resulting phenotype referred as the ‘athlete’s heart’ fundamentally differs from the failing heart.⁷⁵⁻⁷⁷ Physiological cardiac hypertrophy directs the postnatal growth of the heart from birth to adulthood and represents a beneficial adaptation to physical activity and exhaustive exercise training. This is characterized by preserved and enhanced cardiac function.^{76, 77} Indeed, physiological stimuli in general lead to a mild elevation of left ventricular mass and an enlargement of the chamber (depicted in Fig. 3). Additional changes on the cellular level are cardiomyocyte growth, reorganization of the sarcomere, as well as enhanced protein synthesis.⁷⁵ However, this form of remodelling is neither accompanied by fibrosis nor cardiomyocyte death.⁷⁸ In contrast to pathological hypertrophy, physiological changes of the myocardium are reversible, as the athlete’s heart returns to baseline after prolonged pausing or termination of training.^{79, 80}

On the molecular level, physiological remodelling is initiated by insulin-like growth factor 1 (IGF1) produced by the liver as well as cardiomyocytes in response to exercise.^{81, 82} This factor acts via its corresponding receptor (IGF1 receptor), a receptor tyrosine kinase that subsequently activates a signalling cascade that depends on the phosphoinositide-3 kinase (PI3-K).^{75, 83} Consequently, transgenic mice exhibiting a constitutive activation of this pathway showed increased heart weight with normal to enhanced cardiac function^{84, 85}, while reduced cardiac PI3-K activity blunted the hypertrophic response to exercise.^{84, 86} Another important molecular characteristic of physiological hypertrophy is the absence of the fetal gene program that is normally induced upon pathological conditions. Likewise, genes related to calcium signalling remain unaffected.^{75, 87}

Taken together, these results indicate that physiological cardiac remodelling induced by exercise is likely to be beneficial for the cardiovascular system of healthy individuals. However, under certain conditions, such as inherited cardiac disease, exhaustive exercise can promote detrimental outcomes or sudden cardiac death.⁷⁵ For example, training for the participation in extreme endurance competitions

like a marathon run can cause adverse remodelling that might attenuate benefits of moderate exercise. Further, it can lead to cardiac dysfunctions and elevates the risk for sudden cardiac death.^{88, 89} Cardiovascular stress is reflected by elevated levels of biomarkers that are indicators of cardiac damage including cardiac troponin-I, creatine kinase, brain natriuretic peptide (BNP), and inflammatory marker.^{88, 89}

1.2.2 Inflammatory airway disease

Asthma is a chronic disease of the airway that is accompanied by inflammatory, structural and functional changes leading to airflow limitations. The disease pattern caused by exposure to stimuli like allergens, infections, and air pollutants is determined by chronic inflammation in the airway mucosa and involves persistent infiltration of activated mast cells as well as eosinophils, and a displaced ratio of T_H2 to T_H1 helper cells that entails the release of various pro-inflammatory chemokines and cytokines as well as high levels of immunoglobulin G (summarized in Fig. 4). Structural remodelling of the airway is characterized by epithelial lung fibrosis, thickening of the basement membrane, hypertrophy and hyperplasia of airway smooth muscle cells and elevated secretion of mucus. Thus, asthma is a complex pathology based on a myriad of immunomodulatory cells, inflammatory signals and mediators; a fact that hampers treatment options and efficiency.⁹⁰⁻⁹²

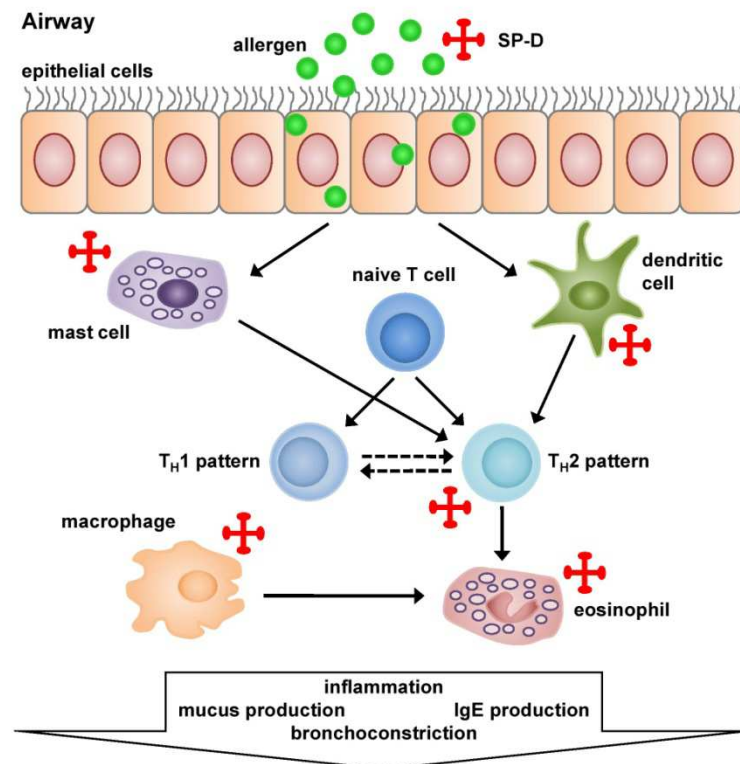


Fig. 4: The role of SP-D in asthmatic airway inflammation. After uptake, dendritic cells present allergens to T cells that proliferate and differentiate into T_H2 helper cells. This population of T cells attracts eosinophils and stimulates IgE production. Mast cells stimulated by allergens are further involved in T_H2 cell differentiation and induce acute asthma attacks. SP-D alters the inflammatory response to allergens by modulating the uptake of such particles and through interactions with inflammatory cells.

One modulator of inflammatory lung disease including asthma is surfactant protein D (SP-D). This protein is part of the lung surfactant, a surface-active heterogeneous layer of proteins and lipids. SP-D belongs to the family of collagenous lectins (collectins) that play a crucial role in the innate immune response. SP-D is involved in the neutralization and clearance of infectious agents as well as the regulation of allergic and inflammatory reactions.⁹³⁻⁹⁵ Collectins share a typical structure comprising an N-terminal collagen-like triple helix, a flexible neck region, and a C-terminal carbohydrate recognition domain. The recognition domain mediates the function of SP-D as a pattern recognition molecule that opsonises and neutralizes infectious as well as allergic agents. The collagenous N-terminal region enables oligomerization into a tetrameric crucifix structure, which characterizes SP-D. It functions as an interaction domain with receptor molecules that are presented by a variety of immune cells in order to initiate clearance mechanisms.⁹⁴ SP-D is mainly secreted by lung type II alveolar epithelial cells, but has been detected in non-pulmonary loci, including trachea, salivary glands, gastrointestinal and genital tract, the heart and the mucosa of other organs.^{94, 95} In the lung, SP-D is an important regulator of the pulmonary homeostasis. Mice lacking SP-D develop chronic inflammation, emphysema, and fibrosis accompanied by an accumulation of surfactant components, infiltration of monocytes and proinflammatory activation of alveolar macrophages.⁹⁶ Commonly, low levels of SP-D in the lung correlate with an increased risk of infections, while over-expression of SP-D seems to be related with chronic inflammatory conditions such as asthma, fibrosis, and COPD.⁹⁷ Genetic changes in the SP-D gene have an impact on protein levels in the lung, as well as on the assembly and the biological function of SP-D.⁹⁸⁻¹⁰⁰ Accordingly, a single nucleotide polymorphism causing an exchange of methionine to threonine at position 11 in the N-terminal region (Met(11)Thr SNP)¹⁰¹ has been associated with prevalence to COPD¹⁰², emphysema¹⁰³, and allergic rhinitis¹⁰⁴. Further, mice expressing the human SP-D variants display a partially reverted phenotype compared to SP-D deficient animals.⁹⁸

Although the relevance of this allelic variant has not been completely evaluated, existing data suggest that SP-D deficiency or dysfunction might play a crucial role in the pathogenesis of asthma.¹⁰⁵ Several studies indicate that an allergic response alters the levels of SP-D in the airway^{106, 107} and that asthma influences the structure of SP-D.¹⁰⁸ Mice lacking the SP-D gene show elevated susceptibility to fungal allergens¹⁰⁹ or ovalbumin challenge^{110, 111}. Functionally, the immunomodulatory properties of this protein are of importance. SP-D alters the cellular response to allergens (see Fig. 4) modulating the uptake of such particles by macrophages, pulmonary epithelial as well as dendritic cells, and subsequently the release of pro-inflammatory chemokines and cytokines.^{112, 113} It inhibits the binding of IgE to dust mite allergens¹¹⁴ or fungal pathogens¹¹⁵. Further, SP-D modulates the function of innate and adaptive immune cells that play an important role in the pathogenesis of asthma.¹⁰⁵ SP-D promotes the antigen uptake and representation by dendritic cells¹¹⁶, hampers IL-2 dependent T-cell proliferation¹¹⁷, allergen-induced lymphocyte proliferation¹¹⁸, and histamine release from sensitized

peripheral blood mononuclear cells of asthmatics¹¹⁸. Further, SP-D inhibits chemotaxis and degranulation of eosinophils.¹¹⁹

In conclusion, SP-D seems to exhibit protective effects in asthma. This protein is involved in the clearance of inhaled allergens, but limits an allergen-induced inflammatory response of macrophages, antigen-presenting cells, T cells and inflammatory cells.¹²⁰ Additional studies are needed to unravel the complex role of SP-D in the pathogenesis of asthma.

1.3 NcRNAs in diagnostics and therapeutics

Symptoms associated with cardiovascular disease can be treated with drugs, medical assist devices or lifestyle changes. But, such treatment strategies can solely improve the cardiac output or enhance the blood flow, and therefore decelerate the disease progression; however, none of these interventions are capable to cure the failing heart.¹²¹ Similarly, therapies of chronic obstructive and inflammatory lung disease like asthma aim to inhibit multiple inflammatory processes and have obtained improvements to patients well-being, but they harbour risks of side effects and inefficiencies.¹²² Accordingly, there is a clear need for new therapeutic targets and diagnostic strategies. Recently, ncRNAs became of increasing interest, not at least because they represent 98% of the so far unexplored territory of the genome. Applications of ncRNAs in theranostics as outlined in the following sections.

1.3.1 MicroRNAs as biomarkers of physiological activity

Physical activity, such as endurance running, can be beneficial for the overall body composition improving metabolism, muscle strength and the respiratory as well as the cardiovascular system.¹²³ Thereby, marathon running is an extreme exertion that implicates metabolic and cardiac stress and effects cardio-respiratory adaptation to ensure sufficient oxygen supply. The response during marathon running was evaluated by various studies. Heart rate^{124, 125}, oxygen uptake rate¹²⁶⁻¹²⁸, as well as biomarkers like creatine kinase^{129, 130}, troponin^{129, 131}, or BNP^{132, 133} serve as indicator for physiological changes. Conventional biomarkers provide limited insights into the adaptive response. Therefore, circulating miRNAs (c-miRNAs) represent an emerging and promising source for diagnostic applications, since these molecules are stably detectable in several body fluids, and therefore easy to access. They can be evaluated with high sensitivity and changes of extracellular miRNAs have been associated with different disease including cardiovascular maladies.⁵¹ Identification of such c-miRNAs particularly regulated by physical activity may provide novel insights into exercise-induced physiological adaptations.

First evidence that acute exercise changes the levels of c-miRNAs came from a study by Baggish *et al.*¹³⁴ following the dynamic regulation of miRNAs related to angiogenesis (miR-20a, miR-210, miR-221, miR-222, miR-328), inflammation (miR-21, miR-146a), cardiac and skeletal muscle contractility

(miR-21, miR-133a), and hypoxia/ischemia adaptation (miR-21, miR-146a, and miR-210). Exhaustive cycling and sustained rowing training for 90 days elevated the plasma levels of miR-20a, miR-21, miR-146a, miR-221, and miR-222. Subsequent studies identified changes especially in muscle- and cardiac specific miRNAs (miR-1, miR-133a, miR-206, miR-208a, miR-208b, and miR-499).^{135, 136} Interestingly, in muscle tissue biopsies the expression of such ‘myomiRs’ were significantly decreased after 12 weeks of aerobic training.¹³⁷ However, exercise seems to induce extracellular concentrations of miRNAs. Accordingly, plasma levels from healthy individuals performing a marathon run or resistance training possessed elevated levels of miR-126 and miR-133, while cycling increased the concentration of miR-126, but not of miR-133.¹³⁸ Such findings were confirmed by two further studies assessing c-miRNA plasma levels of marathon runners.^{139, 140} Baggish *et al.* found that plasma expression patterns of c-miRNAs were different from protein markers, since the miRNA levels immediately returned to pre-exercise levels, while conventional marker remained elevated after 24h after the run.¹³⁹ Further, muscle-specific miRNAs respond to downhill walking¹⁴¹, acute as well as chronic aerobic exercise¹⁴². Aside muscle- and cardiac-specific miRNAs, circulating inflammatory miRNAs seem to play an additional role. Different dose of acute aerobic exercise involved different levels of inflammatory response reflected on the plasma concentration of such ‘c-inflammamiRs’. Only one miRNA was increased after short distance run (10 km), while completing the full marathon distance to an elevation in twelve inflammamiRs. Correlation analysis indicated a close association between c-miRNAs and the regulation of inflammatory cascades.¹⁴³ Other miRNAs that are not related to the heart, muscle or inflammation were identified by microarray analysis, assessing dynamic regulation of miRNAs by endurance training. Immediately after an acute exercise bout, six miRNAs (miR-106a, miR-30b, miR-151-5p, let-7i, miR-652, and miR-151-3p) were significantly down-regulated, representing a potential adaptation to the physical workload. Other c-miRNAs were found to be induced to later time points (miR-338-3p, miR-330-3p, miR-223, miR-139-5p, as well as miR-143 after 1 h, and miR-1 after 3 h).¹⁴⁴

To summarize these findings, exercise has a strong impact on c-miRNA concentrations in the plasma. C-miRNA patterns are dynamic and depend on the type of physical activity, level of eccentric load, training status, and finally exhibit a temporal dynamic. Therefore, circulating miRNAs can be used as multilayer biomarkers: First of all, increased levels of c-miRNAs might serve as indicators for muscle damage and cellular leakage. Muscle-specific miRNAs primarily respond to exercise with higher eccentric component including downhill walking¹⁴¹ and resistance training¹³⁸ provoking injury in the muscle tissue or the endothelial cell layer. However, levels of c-miRNAs change not only after exercise-induced damage, but they are also capable to indicate adaptations to high physiological activity. In several studies no changes of myomiRs were observed, regardless of muscle-damaging exercise protocols.^{134, 145} Further, correlations between miRNA expression and aerobic exercise capacity have been observed^{134, 142, 146}, presumably demonstrating changes in cardiovascular as well as respiratory fitness and exercise-induced adaptations. Additionally, specific c-miRNA patterns might

be useful tools to characterize training modalities.¹⁴⁷ The quantification of c-miRNAs can be further used to determine the risk for cardiovascular disease. Bye *et al.* found that elevated levels of miR-21, miR-210, and miR-222 correlated with low aerobic exercise capacity.¹⁴⁶ Both parameters were associated to cardiovascular disease¹⁴⁸⁻¹⁵⁰, indicating that this correlation observed can be used as potential biomarker.

1.3.2 LncRNAs as therapeutic targets in cardiac hypertrophy and heart failure

In the setting of cardiovascular disease, ncRNA research has largely focused on the role of miRNAs.⁵¹ First evidence that lncRNAs might play an important role in the heart was derived from genome-wide association studies. Interestingly, single nucleotide polymorphisms (SNPs) associated with cardiovascular disease matched to genomic loci that express non-coding RNAs.¹⁵¹ Ishii *et al.* detected an enrichment of genetic variants within a gene coding for the lncRNAs *MIAT* (myocardial infarction associated transcript) that was associated with susceptibility to myocardial infarction. One SNP caused and 1.3-fold induction of *MIAT* expression *in vitro*.¹⁵² Another genetic susceptible locus for coronary artery disease maps to the human chromosome 9p21 harbouring the lncRNAs *ANRIL* (antisense non-coding RNA in the *INK4* locus).^{153, 154} Elevated expression of *ANRIL* was associated with the risk for atherosclerosis and other vascular maladies.^{155, 156} A third example is the lncRNA steroid receptor RNA activator (*SRA*) that seems to be linked to human dilated cardiomyopathy.¹⁵⁷

The pathogenesis of cardiac hypertrophy and heart failure entails a complex and disease-specific change in gene expression that is reflected by alterations in the 'Income'. Accordingly, expression profiling based on microarrays or deep sequencing identified sets of lncRNAs deregulated during cardiac remodelling and in the failing heart. Pressure overloaded left ventricles of mice exhibited 15 differentially expressed lncRNAs in hypertrophied tissue, whereas in tissues undergoing heart failure 135 transcripts were affected.¹⁵⁸ Another study profiled hypertrophied hearts 4 weeks after pressure overload and identified 198 deregulated lncRNAs. Enrichment analysis unravelled an involvement of these transcripts in cardiac muscle hypertrophy.¹⁵⁹ Matkovich *et al.* compared embryonic and hypertrophied adult mouse hearts. Heart development was characterized by a distinct signature of lncRNAs expression that was poorly reflected by the hypertrophied heart.¹⁶⁰ Two other groups assessed the 'Income' of diseased human hearts by deep sequencing-based approaches. Yang *et al.* identified alterations upon severe heart failure and revealed that left ventricular assist device support reversed a subset of deregulated lncRNAs. Interestingly, the expression of lncRNAs and neighbouring protein-coding genes strongly correlated, indicating a potential *cis*-regulatory role of these transcripts.¹⁶¹ Likewise, Di Salvo *et al.* profiled the right ventricular tissue of human failing hearts. They found a large number of RNA transcripts including lncRNAs, pseudogenes and antisense RNAs that were deregulated in failing hearts. Overall 105 lncRNAs were differentially expressed, and this expression was independent from parent coding genes in the case of antisense transcripts and

pseudogenes. *In silico* analysis indicated potential functions of these transcripts as miRNA decoys or binding partners for chromatin modifying complexes.¹⁶²

More comprehensive descriptions of individual lncRNAs in the cardiovascular system highlight the importance of lncRNAs in cardiovascular development. One lncRNA named Braveheart (*Bvht*) was shown to be required for cardiovascular lineage commitment.¹⁶³ This transcript was expressed in early developmental stages of murine embryonic stem cells as well as in the postnatal mouse heart. *In vitro* depletion of *Bvht* hampered the differentiation of stem cells into beating cardiomyocytes. *Bvht* plays an essential role in the expression of crucial cardiac transcription factors determining the cardiovascular cell fate and acts upstream of MesP1, which is a critical regulator of cardiac cell specification. However, the relevance of *Bvht* for the early development of the heart remains open and its functional counterpart in humans has yet to be identified. Another transcript, *Fendrr* (Fetal-lethal non-coding developmental regulatory RNA), was highly expressed in the posterior mesoderm, the origin of the myocardium, and is required for the proper development of the heart and body wall in the mouse. This transcript modulates chromatin signatures of genes related to mesoderm differentiation by binding the histone modifying machinery.¹⁶⁴ Although both lncRNAs are crucial regulators of the cardiovascular development, their implication in cardiac remodelling, heart failure or other cardiovascular diseases remains elusive.

Two hallmark publications have been recently released relating lncRNAs and the pathogenesis of cardiac hypertrophy. Han *et al.*¹⁶⁵ identified a cluster of cardiac-specific lncRNAs that arise in antisense from the locus encoding *Myh7* (myosin heavy chain beta isoform). Pressure-overload induced cardiac stress led to a down-regulation of these myosin heavy chain-associated RNA transcripts (*Myhearts*) mediated by a Brg1-dependent chromatin repressor complex. This is activated by cardiac stress, triggers the pathological switch from *Myh6* to *Myh7* and is associated with hypertrophic cardiomyopathy.¹⁶⁵ Restoring the expression of *Myheart* *in vivo* attenuated the hypertrophic response and protected the heart from failure. Mechanistically, *Myheart* acts by antagonizing its own repressor. The lncRNA binds to Brg1 and prevents this factor to recognize its DNA target sequences, thus inhibiting the activation of the hypertrophic gene program.¹⁶⁵ Interactions between lncRNAs and chromatin remodelling factors are one possible mechanism in the pathogenesis of cardiovascular disease. Such transcripts are not necessarily dependent on proteinous interaction partners. Wang *et al.*¹⁶⁶ identified a natural sponge, or competing endogenous RNA, that regulates expression of miRNAs via specific recognition sites. They found a lncRNA, named cardiac hypertrophy related factor (*Chrf*), that was induced in hypertrophic mouse hearts as well as in the myocardium of heart failure patients. *Chrf* acts as an inducer of cardiomyocyte hypertrophy *in vitro* and apoptosis *in vivo*. Mechanistically, this transcript functions as a molecular sink for miR-489. *Chrf* sequesters the miRNA from its target Myd88, thus favouring cardiac hypertrophy.¹⁶⁶

Although *Myheart* and *Chrf* are potentially conserved in humans and consequently of interest for drug development, the therapeutic value of these findings has not been addressed so far. Therefore, lncRNAs-based treatments of cardiovascular disease are still lacking.

1.3.3 MicroRNAs as regulators of asthma pathogenesis

Allergic asthma is accompanied by the coordinated expression of a myriad of genes that initiate, maintain, and propagate the asthmatic disease response. MiRNAs are involved in the fine-tuning of this transcriptional network and play a critical role in regulating crucial events in the pathogenesis of asthma.^{122, 167-169} First evidence came from profiling studies of miRNAs in lung tissue from mouse models of acute, intermediate, and chronic asthma^{170, 171} as well as from human biopsies¹⁷²⁻¹⁷⁴. These and other studies identified several miRNAs with altered expression including let-7 and miR-200 family members, miR-20b, miR-106a, miR-126, miR-133a, miR-145, miR-146a/b, miR-181a, miR-221, as well as miR-21 and miR-155 (discussed in several reviews^{122, 167, 175}), reflecting changes in the cellular composition of the lung tissue in response to allergic stimuli.¹⁶⁷

MiR-21 has been observed to be among the most induced miRNAs in inflamed tissue of humans^{176, 177} or murine asthma models upon allergic stimuli, including ovalbumin, house dust mite, and IL-3^{177, 178}. These elevated expression levels were mainly observed in macrophages and dendritic cells. MiR-21 targets interleukin-12p35 (IL-12p35) that is involved in balancing T_H1 and T_H2 cytokines. IL-12 repression might contribute to an enhanced T_H2 response observed in asthma.¹⁷⁸ MiR-21 deficient mice showed an altered immune reaction.¹⁷⁹ In an asthma model of ovalbumin challenge, these mice were characterized by lower levels of eosinophils and reduced amount of the T_H2 cytokine IL-4, while the T_H1 cytokine interferon gamma (IFN γ) and IL-2 were elevated, underlining the role of miR-21 in immune polarization.¹⁷⁹ Despite these promising findings, administration of chemically modified oligonucleotide inhibitors antagonizing miR-21 failed to modulate the cytokine production and eosinophil recruitment observed in miR-21 knockout mice.¹⁸⁰ This discrepancy might be due to the oligonucleotide chemistry, dose, time point and route of administration, as well as a general difference between knockout and knockdown strategies.^{169, 175}

Another miRNA with a potential impact in asthma is miR-155. Soujaletto *et al.* analysed inflammatory profiles of patients with current symptoms of allergic rhinitis and found increased levels of miR-155 in nasal mucosa.¹⁸¹ However, in chronic allergic airway disease the group observed a significant down-regulation of the same miRNA.¹⁸² Other studies identified miR-155 as a central regulator of the function of the immune system.^{167, 183} MiR-155 is required for allergic inflammation after allergen challenge in mice and has been shown to determine inflammation in an allergic mouse model by regulating the T_H2 response. Upon ovalbumin challenge, mice lacking miR-155 showed diminished eosinophil inflammation, mucus secretion and T_H2 cell and cytokine levels.¹⁸⁴ Further, miR-155 seems to be involved in the regulation of asthma by effecting dendritic cell and macrophage function. Dendritic cells of miR-155 knockout mice were characterized by limited T_H2 priming

capacity, reduced chemotaxis and IL-1 β secretion and were not capable to promote allergic airway inflammation despite allergen exposure.¹⁸⁵ In human macrophage miR-155 seems to alter the function of these immune cells towards an alternatively activated form that is linked to the T_H2 immune response. This miRNA directly targets the IL-13 receptor that leads to an impaired STAT6 signalling, affected gene expression coupled to IL-13, and subsequently to the asthma-related phenotype of macrophages.¹⁸⁶ Another study linked miR-155 to allergic asthma induced by hyperstretch. The miRNA was induced upon stretch of human bronchial epithelium and seems to regulate IL-8 secretion by targeting the phosphatase SHIP1 (Src homology 2 domain-containing inositol 5-phosphatase 1).¹⁸⁷ Taken together, these findings suggest that miR-21 and miR-155 are potential therapeutic targets in allergic asthma. However, their pharmacological modulation and relation to the asthma modulatory protein SP-D have yet not been investigated.

2. Aims of this thesis

2.1 To assess the diagnostic potential of microRNAs as biomarkers for aerobic exercise capacity

The presence of miRNAs in the bloodstream is a known indicator for physiological adaptations due to disease state or other forms of stress. Such circulating miRNAs change in response to physical activity. The aim of this study was to investigate heart- and muscle-specific miRNAs in plasma of marathon runners, to analyse their relation to conventional biochemical, cardiovascular, and performance indices and to test their potential as biomarkers of aerobic exercise capacity.

2.2 To identify lncRNA candidates for the treatment of cardiac disease

Various reports have already profiled, evaluated, and pharmacologically modulated miRNAs involved in cardiac remodelling and cardiovascular disease. Very few studies discovered lncRNAs as potential therapeutic targets to prevent the pathogenesis of heart failure. We aimed to identify and describe lncRNAs implicated in the development of cardiac hypertrophy and to use these transcripts as potential therapeutic targets for the treatment of cardiac disease.

2.3 To evaluate the impact of the SP-D Met(11)Thr SNP on asthma and inflammamiRs

Previous findings have indicated involvement of either the surfactant protein SP-D or miRNAs in the pathogenesis allergic airway inflammation. A study linking both immunomodulatory factors is still lacking. The objective of this study was to investigate the impact of the Met(11)Thr polymorphism in the SP-D gene on the inflammatory phenotype of an acute model of airway asthma and the expression of the allergic inflammamiRs miR-21 and miR-155.

3. Research articles

3.1 Circulating microRNAs as potential biomarkers of aerobic exercise capacity

Mooren, F. C., **Viereck, J.**, Krüger, K. & Thum, T. Circulating microRNAs as potential biomarkers of aerobic exercise capacity. *Am J Physiol Heart Circ Physiol.* **306**, H557-63 (2014).

3.2 Long non-coding RNA *Chast* promotes cardiomyocyte hypertrophy

Viereck, J., Kumarswamy, R., Foinquinos, A., Xiao, K., Batkai, S., Avramopoulos, P., Kunz, M., Dittrich, M., Maetzig, T., Zimmer, K., Remke, J., Just, A., Fendrich, J., Scherf, K., Schambach, A., Weidemann, F., deWindt, L. J., Engelhardt, S., Dandekar, T. & Thum, T. Long Non-coding RNA *Chast* Promotes Cardiomyocyte Hypertrophy. *Sci Transl Med.* in revision (2015).

The study presented in this work meanwhile has been published as:

Viereck, J.*, Kumarswamy, R.*, Foinquinos, A.*, Xiao, K., Avramopoulos, P., Kunz, M., Dittrich, M., Maetzig, T., Zimmer, K., Remke, J., Just, A., Fendrich, J., Scherf, K., Bolesani, E., Schambach, A., Weidemann, F., Zweigerdt, R., de Windt, L. J., Engelhardt, S., Dandekar, T., Batkai, S. & Thum, T. Long noncoding RNA *Chast* promotes cardiac remodeling. *Sci Transl Med.* **8**, 326ra22 (2016).

3.3 Impact of a Met(11)Thr single nucleotide polymorphism of surfactant protein D on allergic airway inflammation in a murine asthma model

Winkler, C., Bahlmann, O., **Viereck, J.**, Knudsen, L., Wedekind, D., Hoymann, H. G., Madsen, J., Thum, T., Hohlfeld, J. M. & Ochs, M. Impact of a Met(11)Thr single nucleotide polymorphism of surfactant protein D on allergic airway inflammation in a murine asthma model. *Exp Lung Res.* **40**, 154-63 (2014).

Circulating micrornas as potential biomarkers of aerobic exercise capacity

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Mooren FC, Viereck J, Krüger K, Thum T. Circulating micrornas as potential biomarkers of aerobic exercise capacity. *Am J Physiol Heart Circ Physiol* 306: H557–H563, 2014. First published December 20, 2013; doi:10.1152/ajpheart.00711.2013.—Purpose microRNAs (miRs) are crucial intracellular mediators of various biological processes, also affecting the cardiovascular system. Recently, it has been shown that miRs circulate extracellularly in the bloodstream and that such circulating miRs change in response to physical activity. Therefore, the purpose of the current study was to investigate heart/muscle specific and inflammation related miRs in plasma of individuals before, directly after, and 24 h after a marathon run and to analyze their relation to conventional biochemical, cardiovascular, and performance indexes. Male endurance athletes ($n = 14$) were recruited for the study after performing a battery of cardiac functional tests. Blood samples were collected before, directly after, and 24 h after a public marathon run. miR-1, miR-133, miR-206, miR-499, miR-208b, miR-21, and miR-155 were measured using individual Taqman assays and normalized to *Caenorhabditis elegans* miR-39 (cel-39) spike-in control. Moreover, soluble cardiac, inflammatory, and muscle damage markers were determined. As a result, skeletal- and heart muscle-specific miRs showed a significant increase after the marathon. The strongest increase was observed for miR-206. Twenty-four hours after the run, only miR-499 and miR-208b were returned to preexercise levels, whereas the others were still enhanced. In contrast, miR-21 and -155 were not affected by exercise. miR-1, -133a, and -206 correlated to aerobic performance parameters such as maximum oxygen uptake ($\dot{V}O_{2\max}$) and running speed at individual anaerobic lactate threshold (V_{IAS}). miR-1 showed a moderate negative correlation with fractional shortening, whereas miR-133a was positively related to the thickness of intraventricular septum. None of the miRs correlated with cardiac injury markers such as troponin T, troponin I, and pro-brain natriuretic peptide. In conclusion, these findings suggest a potential role for muscle- and heart-specific miRs in cardiovascular adaptation processes after endurance exercise. Moreover, the specific correlation of miR-1, -133a, and -206 to performance parameters indicated their potential role as biomarkers of aerobic capacity.

marathon run; maximum oxygen uptake; cardiovascular system; adaptation

REGULAR PHYSICAL EXERCISE improves aerobic power and is accompanied with an increase of survival in cardiovascular diseases (27). In contrast, low aerobic exercise capacity is a powerful predictor of premature morbidity and higher mortality from all causes, including cardiovascular diseases (6, 18). Therefore, regular dynamic endurance sessions are important parts of preventive exercise programs. Performance gains can be detected using different approaches of which measuring the maximum oxygen uptake ($\dot{V}O_{2\max}$) is considered to be an important method. Thereby, $\dot{V}O_{2\max}$ depends primarily on the

components of oxygen delivery and the oxidative mechanisms of the exercising muscle. However, several recent reports have documented considerable interindividual variations of training responses among participants of longitudinal training studies despite standardized exercise stimuli (7). With $\dot{V}O_{2\max}$ adaptations ranging from 0 to >100%, genetic factors have been proposed to be involved. Consequently, a heritability from improvements in aerobic exercise capacity in response to endurance training has been estimated to ~45–50% (7, 8). Several approaches such as genome wide association studies, RNA expression profiling, and others have been used so far to characterize genetic biomarkers of aerobic exercise capacity, whereas gene regulatory mechanisms including microRNAs (miRs) have been rarely investigated (8, 22, 30).

miRs comprise a class of 19–25 nucleotides, short noncoding RNAs that modulate gene expression on the posttranscriptional level and play important roles in a wide range of physiological and pathological processes (1). Mature miRs are single stranded and derived from immature hairpin precursors. Combined with RNA-induced silencing complex and Argonaute proteins, miRs bind to the 3′-untranslated region (3′-UTR) of their target transcript, provoking translational repression or degradation of the mRNA (1, 5). Importantly, some miRs are expressed in a tissue-specific manner and can be related to specific biological functions. Whereas miR-1, -133, -206, and -499 are highly abundant in both the skeletal muscle and/or the heart, miR-208b is more specific to myocardial tissue (11, 34, 35). Other miRs such as miR-21 and -155 can be related to inflammatory processes (37). As intensive exercise is accompanied by a substantial inflammatory response, it is unsurprising that exhaustive exercise affects the profile of inflammatory miRs in peripheral blood mononucleated cells (29). Recently, it has been demonstrated that the levels of muscle-related miRs (also called “myomiRs”) are altered in response to both acute aerobic and resistance exercise, suggesting a regulatory role during muscle adaptation (17, 28, 32). A role of miRs as alternative biomarkers of muscle damage has also been described (4). Moreover, some exercise-regulated miRs have been found to be involved in the expression of performance-related genes (22). This latter observation has raised the question whether miRs might be important molecular switches that determine exercise-related cellular and molecular adaptation and might function as predictors of future exercise performance.

miRs are also stable outside the cell and were identified as a new class of biomarkers. Incorporated into exosomes (24, 39, 41), microvesicles (12, 20), or protein complexes (2), miRs can therefore be detected in human plasma (25). Recent studies documented changes of such circulating miRs in response to physical activity. Exercise altered the plasma levels of miRs implicated in angiogenesis, skeletal, and cardiac muscle adaptation in an intensity-, type-, and duration-dependent manner

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microRNAs AND EXERCISE CAPACITY

Table 1. General characteristics of marathon runners

Characteristics (n = 14)	Mean ± SD
Age, yr	42.8 ± 6.0
Body mass index, kg/m ²	25.3 ± 2.3
Performance parameters	
VO _{2max} , ml·min ⁻¹ ·kg ⁻¹	48.7 ± 9.6
Running speed at IAS, km/h	13.3 ± 1.1
Marathon time, min	215 ± 30
Echocardiographic data	
Right ventricle, mm	23.6 ± 2.1
Septum, mm	10.0 ± 1.2
LVDD, mm	59.2 ± 6.5
Posterior wall, mm	9.2 ± 2.0
FS, %	33.8 ± 7.0

Values are means ± SD. VO_{2max}, maximum oxygen uptake; LVDD, left ventricular end-diastolic diameter; FS, fractional shortening.

(15, 40). Finally, the potential value of miRs as markers of a low-training status as well as future cardiovascular health was recently suggested (9).

Therefore, we hypothesized that the exercise-induced changes of selective skeletal/heart muscle-related miRs are related to athlete's aerobic exercise performance capacity and may thereby serve as fitness biomarkers. Furthermore, the aim of the study was to test whether the alterations of miRs correlate to conventional soluble cardiac, inflammatory, and muscle damage markers.

MATERIALS AND METHODS

Subjects and study design. Fifteen male subjects were recruited for the study of which, finally, 14 were included. One athlete was excluded from analysis because he did not finish the marathon run. All subjects were endurance runners with normal dietary habits who did not take any medication. They performed regular endurance training, including intensive workouts. However, none of the subjects included into the study was involved in any exercise training for at least 3 days before the run. All subjects passed through a standardized procedure consisting of signing the consent statement, general medical checkup, echocardiography, and spirometry. The investigations were approved by the Ethics Committee of the University conformed to the 1975 Declaration of Helsinki.

Echocardiography. A two-dimensional transthoracic echocardiogram was performed (Hewlett-Packard, Andover, MA). Dimensions of right ventricle, left ventricular end-diastolic and end-systolic diameters, interventricular septum thickness, and posterior wall thickness were measured according to the American Society of Echocardiography guidelines using bidimensional or M-mode images depending on the quality and angulation between the ultrasound beam and the left ventricle.

Spirometry. During the test, subjects were tested for their VO_{2max} using a continuous, progressive exercise test on a treadmill ergometer (Ergo XELG90 Spezial, Woodway, Weil am Rhein, Germany). The initial velocity was 8 km/h, increasing every 3 min by 2 km/h. Respiration parameters were continuously analyzed using Quark b2 (Cosmed, Rome, Italy). Along with a respiratory quotient of 1.05 or higher, a maximal test was considered achieved when the oxygen uptake did not increase more than 2 ml·min⁻¹·kg⁻¹ at the highest level or before the participant left the treadmill. VO_{2max} was measured as liters of oxygen per minute (l/min) and subsequently calculated as VO_{2max} relative to body mass (ml·min⁻¹·kg⁻¹). Moreover, at the end of every performance level (8, 10, 12...km/h) until termination of the exercise test, blood samples were taken from the ear lobe for lactate measurements. Individual anaerobic lactate threshold

was calculated according to the method of Dickhuth using the Ergonizer program (16).

Blood sampling. Blood samples were taken after cannulation of the cubital vein. Resting blood samples were taken 2 days before the marathon because the athletes refused sampling initially before the marathon. However, resting blood sampling was performed in the morning at the same time to avoid diurnal bias. Additional samples were taken after and 24 h after the run.

RNA extraction. Total RNA from plasma samples was prepared using miRNeasy Mini Kit (Qiagen-Sample & Assay Technologies, Hilden, Germany) according to the manufacturer's directions. A standard volume of each plasma sample (200 µl) was supplemented with 5 fmol *Caenorhabditis elegans* miR-39 (cel-miR-39; Sigma-Aldrich, St. Louis, MO) to normalize the results.

Quantification of miRs. A panel of miRs was investigated, which have been recently related either to skeletal/heart muscle (miR-1, -133a, -206, -499, and -208b) or to fibrotic/inflammatory processes such as miR-21 and -155 (13, 37, 38). To quantify the abundance of mature miRs, standard quantitative ("real time") polymerase chain reaction (qRT-PCR) was performed. For that, cDNA of the selected miRs was synthesized with miR-specific RT primers (TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). qRT-PCR was carried out via TaqMan miR assays (Applied Biosystems) in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Each sample was run in duplicates. Cel-miR-39 levels were determined in parallel, using the same type of detection assay (Applied Biosystems) and microRNA expression was normalized to the cel-miR-39 expression.

Leukocyte counts/biochemical measurements. Blood cell counts, hemoglobin, and hematocrit determinations were performed on plasma anticoagulated with ethylenediaminetetraacetate using a semi-automated hematology analyzer (F-820, Sysmex, Norderstedt, Germany). Creatine kinase (CK) and myocardial isoenzyme (CK-MB) as well as lactate dehydrogenase were measured spectrophotometrically according to the guidelines of the International Federation of Clinical Chemistry (Beckman Coulter, Krefeld, Germany). Amino-terminal pro-brain natriuretic peptide (pro-BNP) was analyzed by Electro-ChemiLumineszenz ImmunoAssay (ECLIA; Roche Diagnostics, Mannheim, Germany). Troponin T and troponin I were measured by chemiluminescent microparticle immunoassay (Abbott Park, IL). Interleukin-6 and high-sensitive C-reactive protein (hsCRP) were measured by immunoturbidimetric assay (Beckman Coulter, Krefeld, Germany).

Table 2. Biochemical measurements

	Before	After	After 24 h
Leucocytes (10 ³ /µl)	5.0 ± 0.8	13.4 ± 1.9 ^{a,b}	6.1 ± 1.9 ^c
Lymphocytes (10 ³ /µl)	2.0 ± 0.4	1.4 ± 0.3 ^{a,b}	2.0 ± 0.6
Monocytes (10 ³ /µl)	0.5 ± 0.1	1.1 ± 0.3 ^{a,b}	0.5 ± 0.2
Neutrophils (10 ³ /µl)	2.5 ± 0.5	10.9 ± 1.6 ^{a,b}	3.7 ± 1.2 ^c
Destruction parameters			
CK (U/l)	127 ± 53	419 ± 125 ^{a,b}	598 ± 209 ^c
LDH (U/l)	127 ± 20	208 ± 44 ^{a,b}	153 ± 34 ^c
Cardiac markers			
CK-MB (U/l)	10 ± 4	20 ± 8 ^a	19 ± 8 ^c
Troponin T (pg/ml)	2.2 ± 1.4	28.5 ± 16.9 ^{a,b}	6.8 ± 5.4
Troponin I (ng/ml)	0.004 ± 0.004	0.068 ± 0.060 ^{a,b}	0.020 ± 0.017
Pro-BNP (pg/ml)	15.5 ± 8.7	58.9 ± 30.4 ^a	48.5 ± 32.8 ^c
Inflammatory markers			
hsCRP (mg/l)	0.9 ± 0.6	1.5 ± 0.6 ^b	10.8 ± 5.7 ^c
IL-6 (pg/ml)	BD (set to 1)	8.7 ± 4.1 ^{a,b}	BD (set to 1)

Values are means ± SD. CK, creatine kinase; LDH, lactate dehydrogenase; hsCRP, high-sensitive C-reactive protein; CK-MB, myocardial isoenzyme; pro-BNP, pro-brain natriuretic peptide; IL-6, interleukin-6; BD, below detection threshold. ^aSignificant difference between before/after the run; ^bsignificant difference between after/24 h after the run; ^csignificant difference between before/24 h after the run.

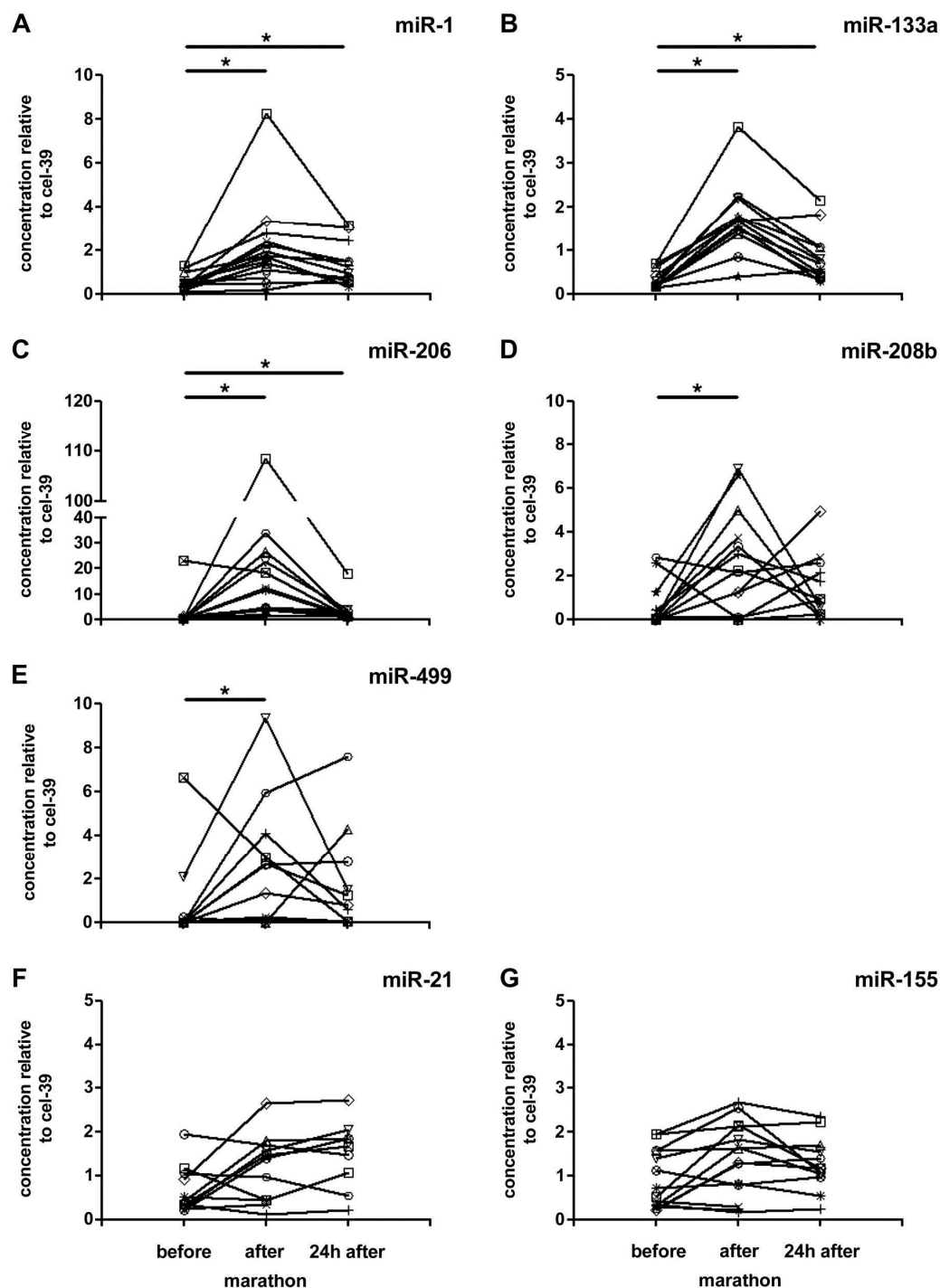


Fig. 1. Effects of a marathon run on various circulatory microRNAs (miRs): miR-1 (A), miR-133a (B), miR-206 (C), miR-499 (D), miR-208b (E), miR-21 (F), and miR-155 (G). * $P < 0.05$, significance between the different time points. cel-39, *Caenorhabditis elegans* miR-39.

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Table 3. Correlations of miRs to aerobic exercise capacity (as indicated by $\dot{V}O_{2\max}$ and V_{IAS}) and cardiac dimensions and function

	$\dot{V}O_{2\max}$		V_{IAS}		IVS		FS	
	R	P	R	P	R	P	R	P
Δ miR-1	0.58	0.03	0.56	0.04	0.29	NS	-0.62	0.02
Δ miR-133a	0.66	0.01	0.69	0.01	0.55	0.05	-0.45	NS
Δ miR-206	0.71	0.004	0.67	0.01	0.43	NS	-0.35	NS
Δ miR-208b	-0.25	NS	0.00	NS	0.31	NS	-0.13	NS
Δ miR-499	-0.11	NS	0.46	NS	0.39	NS	-0.05	NS
Δ miR-21	0.22	NS	-0.11	NS	0.15	NS	-0.50	NS
Δ miR-155	-0.01	NS	-0.30	NS	0.09	NS	-0.41	NS

miR, microRNA; $\dot{V}O_{2\max}$, maximal oxygen uptake; V_{IAS} , running speed at individual anaerobic lactate threshold; IVS, interventricular septum; NS, not significant.

Statistics. GraphPad Prism 5.01 and SPSS 18.0 packages were used. Variables were expressed as means $\pm$ SD and compared with the Kruskal-Wallis test. Pearson's correlation analysis was used to identify any significant relationships. In all cases, $P < 0.05$ was accepted as being significant.

RESULTS

Anthropometric data and performance parameters of the long-distance runners are shown in Table 1. Values for right ventricle and fractional shortening were in the normal range, whereas dimensions of the left ventricle (left ventricular end-diastolic diameter, interventricular septum thickness, and posterior wall thickness) were around the upper level of the normal range as could be expected for runners with a mean training experience of about 7.6 ± 2.3 yr. Athletes performed the marathon distance in a mean time of 215 ± 30 min.

The exercise stimulus resulted in alterations of leucocyte counts, cellular destruction parameters, cardiac markers, and inflammatory marker proteins (Table 2), some of which returned to preexercise levels 24 h later (lymphocytes/monocytes/IL-6), whereas CK and hsCRP continuously increased.

Immediately after the marathon, a significant increase of miR-1, -133a, -206, -208b, and -499 was observed (Fig. 1, A–E). The strongest increase was found for miR-206, which changed nearly 20-fold compared with preexercise levels. Twenty-four hours later, miR-499 and miR-208b were returned to preexercise levels, whereas the others were still enhanced significantly. In contrast, miR-21 (Fig. 1F) and miR-155 (Fig. 1G)

were not affected by exercise. The increase of miR-1, -133a, and -206 was highly correlated among themselves (R values between 0.85–0.90; $P \leq 0.001$), whereas no significant correlation was found in relation to or between the other miRs (R values between -0.17 and 0.49; $P > 0.05$). Only for the fibrosis/inflammation-related miR-21 and -155, a significant correlation was detected ($R = 0.78$; $P \leq 0.01$).

In a next step, we correlated the exercise-induced increase of circulatory miRs to indicators of performance capacity and cardiac function as well as markers of muscle damage and inflammation.

For miR-1, -133a, and -206, moderate to strong correlations to $\dot{V}O_{2\max}$ and running speed at individual anaerobic lactate threshold (V_{IAS}) were found (Table 3, and Fig. 2). miR-1 showed a moderate, negative correlation with fractional shortening, whereas miR-133a was positively related to the thickness of intraventricular septum. In contrast, no correlations were found for miR-499, -208b, -21, and -155.

Finally, no correlations were found between miRs and cardiac biomarkers such as CK-MB, troponin T and I, and pro-BNP (data not shown). There were only discrete moderate correlations of single miRs to parameters of exercise-induced tissue destruction (CK and lactate dehydrogenase) and inflammatory markers (CRP and interleukin-6) (Table 4).

DISCUSSION

An acute bout of endurance exercise induces an increase of heart and skeletal muscle-related circulating miRs. The course of myomiR-1, -133a, and -206 was highly correlated and slightly different from cardio-enriched miR-499 and -208b, suggesting a differential regulation during exercise. In contrast, the fibrosis/inflammation-related miR-21 and -155 showed no significant response to exercise. The most intriguing finding was, however, that the increases of the former three miRs correlated consistently to the athlete's aerobic performance capacity as indicated by $\dot{V}O_{2\max}$ and V_{IAS} . In contrast, their relation to skeletal and cardiac muscle destruction markers was rather limited, which might suggest alternative release mechanisms than just by cell death, thereby emphasizing their potential role as biomarkers of aerobic capacity.

The recent project was intended as a pilot study to test the suitability of circulating miRs as fitness biomarkers of exercise capacity, since miRs have been recently reported as indicators of various pathophysiological conditions such as myocardial infarction, heart failure, or various types of cancers (14, 25, 38). The sample size of the study was therefore limited to 14 participants. On the other hand, the study population was quite

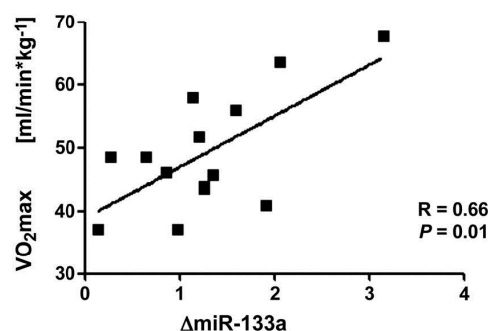


Fig. 2. Exercise-induced increase of miR-133a correlated significantly with athlete's maximal oxygen uptake ($\dot{V}O_{2\max}$).

Table 4. Correlations between miRs and markers of inflammation and cell damage

	CRP		IL-6		LDH		CK	
	R	P	R	P	R	P	R	P
Δ miR-1	-0.42	NS	-0.31	NS	-0.35	NS	0.31	NS
Δ miR-133a	-0.33	NS	-0.53	0.04	-0.23	NS	0.61	0.02
Δ miR-206	-0.53	0.04	-0.29	NS	-0.21	NS	0.46	NS
Δ miR-208b	0.20	NS	-0.19	NS	0.01	NS	0.35	NS
Δ miR-499	-0.06	NS	-0.08	NS	0.39	NS	0.49	NS
Δ miR-21	0.36	NS	-0.24	NS	-0.16	NS	0.26	NS
Δ miR-155	0.48	NS	-0.31	NS	-0.12	NS	0.26	NS

homogeneous with respect to sex (only male subjects), running experience (7.8 ± 2.4 yr), training history (61 ± 19 km/wk), and some performance data (Table 1). To maximize the accuracy of our approach, miR quantitative analysis was restricted to a subset of seven miRs, which have been described previously to be involved either in muscle tissue and cardiac development or inflammatory processes (10, 11). This contrasts to more comprehensive approaches using high-throughput screenings of all miRs, which would have enabled the analysis of specific clusters and/or regulatory patterns.

There are only a few studies available that have investigated the impact of exercise stimulus on miR expression either in tissues or in the circulation. The muscle and/or heart-specific miR-1, -133, -206, -208b, and -499 seem to be involved during skeletal and/or cardiac muscle development, which both are of particular importance for the improvements of $\dot{V}O_{2\max}$ during exercise training programs (11, 13, 35). Specifically, our data suggest that the skeletal muscle-related miR-1, -133, and -206 seem to be more relevant for adaptation of aerobic capacity than the heart-related miR-208b and -499. Most of the studies so far have focused on the intracellular regulation of miR expression in various tissues such as muscle and immune cells (15, 17, 18, 29). During chronic exercise training, a downregulation of skeletal muscle miRs was observed independently whether endurance or strength training was applied (28). Similarly, in models of physiological cardiac hypertrophy, miR-1 and -133 were decreased (10). In contrast, an acute bout of exercise seems to be followed by a transient upregulation of at least miR-1 and -133a in skeletal muscle, which may result in a transient decrease of protein synthesis (28). Likewise, after endurance exercise, a depressed muscle protein synthesis has been described in the early postexercise period in a duration- and intensity-dependent fashion (31). The depleted cellular energy status seems both to impair adaptations of structural muscle proteins and to favor energy restoration and metabolic proteins.

Only three recent reports investigated the exercise-dependent regulation of circulatory miRs including miR-133a as in the current study. Whereas Baggish and coworkers (3) reported no changes of miR-133a, Uhlemann et al. (40) found an increase. In contrast, after resistance exercise Sawada and coworkers (33) found no alterations of miR-11a. These divergent results suggest most likely a differential regulation of miRs with respect to exercise type, duration, and intensity. Whereas the Baggish group (3) used a short exercise test, Uhlemann et al. (40) investigated the effects of a marathon run similar to the actual study. The postexercise increase of circulatory miRs may have different reasons. An apparent increase due to exercise-induced plasma contraction can be excluded as the expression levels were analyzed in relation to cel-miR-39. An upregulation of miRs by de novo miR transcription cannot be excluded under actual conditions. Another explanation might be the release of miRs from destroyed tissue.

Some studies have investigated the potential role of miRs as biomarkers of myocardial infarction or skeletal muscle damage, e.g., miR-1, -133, and -499 (4, 14, 38). Banzet et al. found that changes in circulatory miRs were dependent on the exercise mode. Only eccentric exercise known to induce a high degree of muscle damage was followed by increased plasma levels of miR-133a and -208b. However, two points make this explanation less likely for the current study. First, whereas

miR-1, -133a, and -206 consistently correlate with $\dot{V}O_{2\max}$, only miR-133a moderately correlated with serum CK levels, an indicator for skeletal muscle cell damage. Second, the time course of miRs and CK was completely different. It may, however, be possible that beside necrotic cells, programmed cell death may play a role as apoptotic bodies can contain large amounts of miR (42). Exercise is a well-known inducer of cell apoptosis, e.g., polymorphonuclear cells. We could demonstrate that lymphocyte apoptosis occurs in a similar time course like miR release (26).

Alternatively, it can be speculated that the exercise-induced increase of miRs results from intracellular processing of pre-existing, premature miRs and their subsequent cellular secretion and excretion. A ceramide-dependent machinery for the secretion of miR-enriched exosomes has been recently described (21). But as the definitive underlying release mechanism were beyond the scope of our current study, future studies are necessary to address this question in more detail.

The consisting correlations of miR-1, -133a, and -206 to aerobic capacity raise the question about an epigenetic effect on the well-known variability of exercise performance after training interventions, resulting from complex gene-environment interactions. Several interventional studies reported considerable person-to-person variations of performance gains (7, 19, 23). For example, in the HEalth, RiSk factors, exercise Training And GEnetics (HERITAGE) Family Study, the average increase in $\dot{V}O_{2\max}$ was ~ 400 ml O_2 /min with a standard deviation of ~ 200 ml/min (range, approximately -100 to approximately 1100 ml/min) (7). The heritability estimate of the $\dot{V}O_{2\max}$ training response was about 50%, a level comparable with what has been observed in other family studies and in sets of identical and fraternal twins. This has stimulated the search for appropriate candidate genes which might help to predict gains in performance. In the latest update of the human gene map for physical performance and health-related phenotypes, 165 autosomal, $5\times$ chromosomal, and 17 mitochondrial gene entries and quantitative gene loci were listed (30). Some of these have been recently characterized as genomic predictors of maximal aerobic capacity using different methodological approaches (8, 22, 36).

Therefore, we generated a cumulative list of these significant target genes (see supplementary Table 1 posted along with the online article) and searched whether they are addressed by heart and skeletal muscle-specific miRs. miRs from the heart such as miR-208b and -499 showed with one hit only limited relation to endurance-related genes. In contrast, a significant portion of the genes (about 15–20%) are modulated by either miR-1 or -206, thereby giving an additional, indirect evidence for the relevance of our findings.

Whereas most studies in this area have been performed with athletes, similar findings have also been reported for patients with chronic diseases such as heart failure, hypertension, etc. For example, the HERITAGE Family Study reported person-to-person variability for the blood pressure responses to exercise (mean decrease, 7 mmHg; range from $+3$ to -25 mmHg) (7). In clinical practice, about 25% of hypertensive patients can be classified as non- or low-responders regarding the blood pressure decreasing effects of regular exercise training. As low cardiorespiratory fitness is a powerful predictor of morbidity and cardiovascular mortality, there is a great need to understand how the exercise stimulus is turned into fitness (18). The

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investigation of the role of miRs seems to be a promising approach to improve our understanding about the underlying mechanisms as suggested also by a recent study from Bye et al. (9). They could demonstrate that resting plasma levels of miR-210, -222, and -21 were higher in healthy individuals with low $\dot{V}O_{2\max}$ (9). Further longitudinal studies, however, are required to confirm these actual approaches. It seems worthwhile to include additional miRs, tissue biopsies, and exercise/training regimes to get more detailed and differentiated insights into this topic. But it can be expected to be helpful on our way into the era of individualized, optimized, and therefore more efficacious health recommendations and preventive training programs.

In summary, our data demonstrate the exercise-dependent regulation of heart and skeletal muscle-related circulating miRs. The potential suitability of selective miRs such as miR-1, -133a, and -206 as novel biomarkers of aerobic exercise capacity is suggested by their high correlation to standard performance parameters. The limited association between miRs and biochemical markers of cardiac function and skeletal muscle damage indicates their independence as biomarkers and might suggest specific cellular release mechanisms.

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DISCLOSURES

All authors have completed the International Committee of Medical Journal Editors uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf and declare the following: no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous three years, and no other relationships or activities that could appear to have influenced the submitted work.

AUTHOR CONTRIBUTIONS

F.C.M., K.K., and T.T. conception and design of research; F.C.M., J.V., and K.K. performed experiments; F.C.M., J.V., K.K., and T.T. analyzed data; F.C.M., J.V., K.K., and T.T. interpreted results of experiments; F.C.M., J.V., K.K., and T.T. prepared figures; F.C.M., J.V., and K.K. drafted manuscript; F.C.M., K.K., and T.T. edited and revised manuscript; F.C.M., J.V., K.K., and T.T. approved final version of manuscript.

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Long Non-coding RNA *Chast* Promotes Cardiomyocyte Hypertrophy

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One Sentence Summary: Inhibition of long non-coding RNA *Chast* prevents pressure-overload induced cardiac hypertrophy

Abstract: Recent studies have highlighted that long non-coding RNAs (lncRNAs) play an important role in cardiac development. However, understanding of the role of lncRNAs in cardiac diseases is limited. Global lncRNA expression profiling indicated that several lncRNA transcripts are deregulated during pressure overload-induced cardiac hypertrophy in mice. Using stringent selection criteria, we identified *Chast* as a potential lncRNA candidate that can influence cardiomyocyte hypertrophy. Cell fractionation experiments indicated that *Chast* is specifically upregulated in cardiomyocytes *in vivo* in transverse aortic constriction (TAC)-operated mice. Viral over-expression of *Chast* is sufficient to induce cardiomyocyte hypertrophy *in vitro* and *in vivo*. Importantly, GapmeR-mediated silencing of *Chast* attenuated TAC-induced cardiac hypertrophy. In accordance with data from the mouse studies, *CHAST* homolog in humans is significantly upregulated in hypertrophic heart tissue from aortic stenosis patients. These results indicate that *Chast* can be a potential target to prevent cardiac hypertrophy and highlight a general role of lncRNAs in heart diseases.

Introduction

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs that are larger than 200 nucleotides (nt) in length and do not generally encode proteins. lncRNAs regulate pathways by diverse mechanisms including chromatin modification [1], transcriptional [2], post-transcriptional regulation [3] or by influencing sub-cellular trafficking [4,5]. The role of lncRNAs in the heart has just begun to be unravelled. A lncRNA, *Braveheart (Bvht)*, was recently shown to be crucial for cardiac development [6] while another lncRNA *Fendrr* was shown to control chromatin modifications and thereby controlling developmental signalling in the heart [7]. Recent evidence also suggested that lncRNAs may also have a functional role in adult cardiovascular system [8-10] and have a strong diagnostic potential as biomarkers in heart failure patients [11]. These findings highlight that lncRNAs can be targeted to develop novel class of therapeutics to treat heart diseases. Indeed, a recent study has shown that lncRNA *Myheart (Mhrt)* protects against pathological cardiac hypertrophy.

In the present study we performed whole genome lncRNA profiling to identify differentially expressed lncRNAs in hypertrophic mouse hearts. Subsequent functional studies identified that lncRNA ENSMUST00000130556, which is referred in this manuscript as *Cardiac hypertrophy associated transcript (Chast)*, plays an important role in pressure-overload induced cardiac hypertrophy and served as a novel powerful treatment target.

Results

LncRNA expression pattern is altered during cardiac pressure overload

To investigate lncRNA transcriptome changes during pressure overload-induced cardiac hypertrophy, we performed a lncRNA microarray analysis in RNA isolated from sham or transverse aortic constriction (TAC)-operated mouse hearts. The majority of lncRNAs represented on the array (19,427 out of 31,423 lncRNAs) were detectable and several lncRNAs were deregulated in hypertrophic hearts (**Fig. 1A, B**); 1,237 lncRNAs were upregulated upon TAC operation while 1,623 lncRNAs were down-regulated. To avoid detection of primary messenger RNAs in quantitative PCRs, we excluded lncRNA transcripts that are located within a protein-coding gene in sense orientation. We next selected 22 highly deregulated (11 up-regulated, 11 down-regulated) candidates for validation in independent

quantitative PCR (qPCR) experiments. Out of these 22 candidates, transcripts were consistently detected in all tested samples for at least 13 lncRNAs (6 up- and 7 down-regulated). QPCR analyses indicated that 4 lncRNAs were significantly deregulated (1 up and 3 down-regulated) in TAC operated hearts (**Fig. 1C**, **Supplemental Fig. S1**). We next focussed on the upregulated lncRNA ENSMUST00000130556 as inhibition of this lncRNA in cardiac hypertrophy might have a therapeutic benefit and we refer to this lncRNA as *Cardiac hypertrophy associated transcript* (*Chast*). Expression of *Chast* is temporally regulated between 4 to 13 weeks after left ventricular pressure-overload, with a peak expression level at 6 weeks (**Fig. 1D**). At this time point (4-6 weeks) compensated cardiac function is translated to decompensation (**Fig. 1D**). Interestingly, cellular fractionation experiments revealed that *Chast* was specifically upregulated in the cardiomyocyte fraction of 6 week TAC-operated hearts (**Fig. 1E**). To validate that *Chast* is expressed as a full length transcript, we performed 3' and 5' RACE experiments in RNA isolated from murine hearts and confirmed that *Chast* is expressed as a single transcript of 823 nt long with two exons, which is similar to the Ensembl transcript ENSMUST00000130556 (**Fig. 1F**). To confirm that *Chast* is a non-coding RNA, the protein-coding potential of the full-length transcript was analysed using the Protein Coding Potential Calculator [12]. Similar to other bona fide lncRNAs *Xist*, *Braveheart* and *Fendrr*, *Chast* received low coding potential scores. Known protein coding genes *Gapdh*, *ActB*, α -MHC, *troponin T* served as positive controls (**Supplemental Fig. S2A**). Further, we applied the Sequence Frame Translator and the NCBI ORF Finder and identified four open reading frames that were small (< 100 amino acids; **Supplemental Fig. S2B**). To rule out the possibility, that *Chast* encodes one of these peptides, we over-expressed the transcript in HL-1 cardiomyocytes and analysed the resulting peptides by high resolution mass spectrometry. None of the peptides corresponding to potential ORFs were identified.

Chast induces cardiomyocyte hypertrophy

Expression of *Chast* was induced upon stimulation of HL-1 cardiomyocytes with prohypertrophic factors such as phenylephrine (PE) and isoproterenol (ISO) (**Fig. 2A**). Lentiviral over-expression of *Chast* induced hypertrophic growth in HL-1 cells (**Fig. 2B-D**). Importantly, silencing of *Chast* significantly attenuated PE+ISO induced hypertrophic response in HL-1 cells (**Fig. 2E-G**). Consistent

with this, expression of the cardiac stress marker atrial natriuretic peptide (*ANP*) was increased either by PE+ISO treatment or upon *Chast* over-expression while GapmeR mediated suppression of *Chast* prevented PE+ISO induced *ANP* over-expression (**Fig. 2D, G**). These results highlight that *Chast* is involved in a prohypertrophic growth of cultured cardiomyocytes. To delineate the mechanism of action of *Chast*, we measured *Chast* expression in RNA extracted from different cellular compartments of cardiomyocytes. As expected, *Gapdh* and *ActB* were predominantly found in cytosol, while known epigenetic modulators such as *Xist* and *Neat1* lncRNAs were predominantly found associated with chromatin. Interestingly, *Chast* was found to be ubiquitously present in cytosol, nuclear soluble and chromatin associated forms (**Supplemental Fig. S3**). This indicates that *Chast* has the possibility to modulate both transcriptional and post-transcriptional processes. In order to identify cardiac relevant genes that are modulated by *Chast*, we performed global microarray mRNA expression analysis after GapmeR-mediated suppression of *Chast* in the murine HL-1 cardiomyocytes. Results indicated that *Chast* suppression has a strong effect on HL-1 cardiomyocyte transcriptome (**Supplemental Fig. S4A**). KEGG analysis revealed that pathways related to muscle development, metabolic processes, Wnt signalling and cardiomyopathies are among the biological processes affected by *Chast* silencing (**Supplemental Fig. S4B, C**).

Chast expression is regulated by the prohypertrophic nuclear factor of activated T-cells (NFAT)

Bioinformatic analysis revealed that the gene encoding *Chast* has at least one binding site for prohypertrophic transcription factor NFAT (**Supplemental Fig. S5A; Supplemental Table 1**). To test if expression of *Chast* is regulated by NFAT, we treated HL-1 cardiomyocytes with the pharmacological NFAT inhibitor 11R-VIVIT. As anticipated, expression of *Chast*, along with another known NFAT target *Mcip1.4* was significantly down-regulated upon NFAT inhibition (**Supplemental Fig. S5B**). To confirm whether *Chast* is regulated by NFAT activation, we tested *Chast* expression in hearts of calcineurin transgenic (TG) mice [13] where NFAT is constitutively active. Interestingly, calcineurin TG mice displayed high *Mcip1.4* levels and an about 3 fold increased *Chast* expression compared to wild type animals (**Supplemental Fig. S5C**). Finally, binding of the transcription factor

NFAT to the *Chast* promoter was also verified by chromatin immune-precipitation (CHIP) assays (**Supplemental Fig. S5D**).

In vivo targeting of Chast ameliorates cardiac hypertrophy

To test if prohypertrophic effects of *Chast* in cultured HL-1 cells have any translational relevance, we performed *in vivo* gain and loss of function experiments. For cardiac over-expression of *Chast*, we created AAV9 viral particles where *Chast* is expressed under control of cardiac troponin T promoter (**Fig. 3A, B**). *In vivo* over-expression of *Chast* induced hypertrophic growth in the heart accompanied by increased cardiomyocyte size (**Fig. 3C-E**). Expression of cardiac stress markers like *BNP* and β -*MHC* was also increased upon *Chast* over-expression (**Fig. 3F**). Importantly, GapmeR-mediated *in vivo* silencing of *Chast* ameliorated pressure-overload (TAC) induced cardiac hypertrophy (**Fig. 4A-C**). Consistent with this, mice treated with GapmeRs against *Chast* showed improved ejection fraction (EF) and smaller systolic and diastolic volumes (**Fig. 4D**). Additionally, expression of cardiac stress markers (*ANP*, *BNP* and β -*MHC*) was also relatively reduced in TAC+GapmeR treated animals when compared to TAC animals (**Fig. 4E**). We next tested if *Chast* is conserved in humans. An alignment of the mouse *Chast* sequence to human ESTs revealed existence of a transcript that has a similar sequence (66% homology) and structure compared to mouse *Chast* originating from chromosome 17 (NC_000017.11|64783199-64783552; Homo sapiens chromosome 17, GRCh38.p2 Primary Assembly); **Supplemental Fig. S6A-D**). Importantly, similar to pressure-overloaded mouse models, expression of *Chast* was strongly upregulated in hypertrophic cardiac tissue of patients with aortic stenosis compared to healthy hearts (**Supplemental Fig. S6E**), highlighting the translational relevance of our mouse *in vitro* and *in vivo* findings.

Discussion

Although the majority of the human genome is transcribed, less than 2% of it encodes for proteins. MicroRNAs and lncRNAs are among important classes of functional RNAs whose role in several biological processes has been extensively studied. Although functions of microRNAs and their translational importance are well studied in cardiac diseases, such studies are relatively scarce for cardiac lncRNAs except from few reports on their role in cardiac development. One recent study has identified that the lncRNA *Mhrt*, which originates from MYH7 locus, is cardioprotective, and restoration of *Mhrt* levels protects heart from hypertrophy and failure. In the present study we identified that the lncRNA *Chast* is pro-hypertrophic and *in vivo* targeting of *Chast* could ameliorate pressure over-load induced cardiac hypertrophy.

Using 3' and 5' RACE experiments, we validated that *Chast* is expressed as a full length transcript. *Chast* has several 'stop' codons and MS analysis revealed that over-expression of *Chast* in HL-1 cardiomyocyte cell line did not show any ORF activity suggesting that *Chast* is a legitimate non-coding RNA. Interestingly, *Chast* is not a cardiac-specific lncRNA. However, its cell-type specific expression is very sensitive to pressure-overload (*in vivo*) or pro-hypertrophic stimuli such as phenylephrine and isoproterenol (*in vitro*). Consistent with this, we found that *Chast* expression, at least in part, is induced by pro-hypertrophic transcription factor NFAT. Over-expression of *Chast* was sufficient to induce hypertrophic growth and cardiac stress markers *in vitro* and *in vivo*. More importantly, prevention of *Chast* induction in pressure over-loaded hearts using GapmeR mediated approach also prevented hypertrophic growth of the hearts. We identified a novel EST in the human genome whose sequence and secondary structure are similar to mouse *Chast*. Interestingly, according to our findings in pressure over-loaded mice hearts, *CHAST* is significantly upregulated in aortic stenosis patients with cardiac hypertrophy. Thus, these results highlight that *Chast* is a pro-hypertrophic lncRNA and *in vivo* targeting of this lncRNA could be beneficial in cardiac hypertrophy. Our study sets the ground for future clinical development of *Chast* inhibitors for the treatment of pathological cardiac hypertrophy.

Materials and Methods

Study Design

The aims of this study were to identify lncRNAs that play an important role in the development of cardiomyocyte hypertrophy and to translate these findings into a therapeutic approach. By transcriptional profiling of hypertrophic hearts we derived the lncRNA *Chast* as a potential therapeutic target. Loss and gain of function studies were used to assess the influence of *Chast* on the growth and the hypertrophic gene program of cardiomyocytes *in vitro*. Quantitative real-time PCR (qRT-PCR) and measurements of the cell surface area were applied to quantify cardiac hypertrophy. Experiments were reproduced in at least three independent experiments as stated in the figure legends. For *in vivo* experiments, mice were randomly assigned to treatments groups and, wherever applicable, treatment conditions kept blinded until the statistical analysis. Group sizes were chosen on the basis of previous experiences. Pharmacological inhibition of *Chast* by locked nucleic acid longRNA GapmeRs (in the following designated as GapmeRs) injection was used to illustrate the effect of *in vivo* repression on cardiac function and the hypertrophic gene program. Cardiac hypertrophy was quantified heart-weight-to-tibia-length ratio, echocardiography and qRT-PCR-based measurements of RNA expression changes.

Animal experiments

All animal experiments were performed in accordance with the relevant guidelines and regulations with the approval of the responsible local and national authorities.

To generate cardiac pressure overload, male C57BL/6N mice (8- to 10-week old) were subjected to transverse aortic constriction (TAC) as describe previously [14]. The sham-operated animals underwent the same procedure without constriction of the aorta. After different time points after the surgery as indicated in the manuscript, hearts were excised and frozen in liquid nitrogen or fractionated as described below. The same procedure was applied for the collection of tissues from healthy mice.

Adult (8- to 10-week old) male C57BL/6N mice were once treated by tail vein injection with 0.5+E12 copies of AAV9 either harbouring the *Chast* sequence or an empty vector. 20 mg/kg GapmeR-*Chast*

or GapmeR-negative control A (both provided by Exiqon, Vedbaek, Denmark) in saline (0.9% NaCl) as carriage medium were delivered weekly by i.p. injection. After six or five weeks respectively, cardiac functions and dimensions were assessed by echocardiography and hemodynamic measurements and the hearts were removed for biochemical analysis.

Heart tissues of calcineurin transgenic mice and their wildtype littermates were kindly provided by Prof. Dr. Leon de Windt (Academic Hospital Maastricht, Maastricht, The Netherlands).

Human tissue sampling

This study was performed with the approval of the institutional ethical committee. We analysed cardiac tissue from patients undergoing heart transplantation because of end-stage heart failure ($N=23$) and, for comparison, healthy adult human hearts ($N=21$). Immediately after explantation, biopsies were removed from the left ventricle and frozen in liquid nitrogen.

RNA isolation

Total RNA of tissues and cultured cells was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) or TriFast method (Pqlab, Erlangen, Germany) according to the manufacturer's instructions. Quantification and quality control were performed with Synergy HT Reader (BioTek, Winooski, VT) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively.

qRT-PCR analysis

For microarray validation and quantitative detection of lncRNAs and mRNAs, complementary DNAs were synthesized with the iScript Select cDNA synthesis kit (Bio-Rad, Hercules, CA) and quantitative real-time PCR analysis (qRT-PCRs) were performed using specific primers and the iQ SYBR Green mix (Bio-Rad) according to the manufacturer's protocol. Gene-specific expression levels were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) or β -actin (*ActB*). Used primer sequences were given in **Supplemental Table 2**.

Cell culture, transfection and treatment

Murine HL-1 cardiomyocytes were kindly provided by Prof. Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA) and cultured in Claycomb medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) as described before [15]. Cells were transiently transfected with GapmeRs (*Chast*: 5'TGGATTTGGAGGTTGG3'; negative control A: AACACGTCTATACGC; Exiqon, Vedbaek, Denmark) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

To mimic cardiomyocyte hypertrophy *in vitro*, HL-1 cells were cultured in standard medium and then shifted to standard medium with low serum (1% serum) supplemented with 100 μ M phenylephrine and 100 μ M isoproterenol (both from Sigma-Aldrich, St. Louis, MO) or PBS as control. Cell exposure to the hypertrophic stimuli was stopped after 48 h of incubation at 37°C in 5% CO₂.

In NFAT inhibition experiments, the cell-permeable peptide 11R-VIVIT (Calbiochem, Billerica, MA) at a concentration of 1 μ M was added to HL-1 cells in standard medium with low serum for 48h.

Cell size measurement *in vitro* and in heart samples

To mimic hypertrophic microenvironment, HL-1 cells were cultured in standard medium and shifted to medium with low serum (1%), in addition of 100 μ M phenylephrine and 100 μ M isoproterenol for 48 h. For measurement of cell size, cells were fixed with 4% paraformaldehyde and stained with 10 μ g/ml Phalloidin-Tetramethylrhodamine B isothiocyanate and 5 μ g/ml 4',6-Diamidin-2-phenylindol (DAPI) (both from Sigma-Aldrich, St. Louis, MO). Cryo sections of the left ventricular myocardium were visualized by wheat germ agglutinin stain coupled to Alexa Flour 488 (Invitrogen, Carlsbad, CA). The cell surface area of cardiomyocytes was calculated using the NIS-Elements BR 3.2 package (Nikon Instruments Inc, Tokyo, Japan).

Statistical analysis

For statistical analysis GraphPad 6 (GraphPad Software, La Jolla, USA) was used. Results are given as mean $\pm$ SEM. Statistical comparison among two groups were evaluated by two-tailed unpaired Student's *t*-test. For comparison of more than two groups one-way ANOVA corrected with the Bonferroni post-test was performed. In all cases, $P<0.05$ was considered as being statistically significant.

Additional procedures are described in the online supplements.

Supplementary Materials

Additional methods

Cardiac cell fractionation

Microarray-based lncRNA profiling

Transcriptome profiling after *Chast* deregulation

Rapid amplification of cDNA ends (RACE)

Generation of transgenic HL-1 cell lines stably over-expressing *Chast*

Subcellular fractionation

Promoter analysis

Chromatin immunoprecipitation (ChIP)

Protein coding potential

RNA folding and sequence-structure conservation

Identification of potential homologous human transcripts

Supplemental Figures

Fig. S1. Cardiac left ventricular pressure-overload leads to a down-regulation of several candidate lncRNAs.

Fig. S2. *Chast* is a long non-coding RNA.

Fig. S3. *Chast* is located in all subcellular fractions of cardiomyocytes.

Fig. S4. GapmeR-mediate silencing of *Chast* alters the transcriptome of HL-1 cells.

Fig. S5. The prohypertrophic transcription factor NFAT is an upstream regulator of *Chast* expression.

Fig. S6. *Chast* has a functional homolog in humans.

Supplemental Tables

Supplemental Table S1. Transcription factor binding site prediction for the *Chast* promoter.

Supplemental Table S2. Oligonucleotide primers used in this study.

Supplemental references

References and Notes

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Author contributions

TT, RK and JV devised the study, designed the experiments and wrote the manuscript. JV performed and analysed the experiments with exception of the *in vivo* mouse experiments (done by SB, AF, KZ, and JR). Bioinformatic analysis and microarray evaluation were done by TD, KX, MD, and MK. Lentiviral over-expression vectors were provided by AS and TM, while SE and PA supplied the adenoviral constructs. Tissue samples from calcineurin transgenic mice and human aortic stenosis and healthy hearts were provided by LdW and FW, respectively. AJ, JF and KS supported in experimental work.

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Competing interests

JV, RK and TT have filed patents in the field of cardiovascular lncRNAs diagnostics and therapeutics.

Figures:

Fig. 1

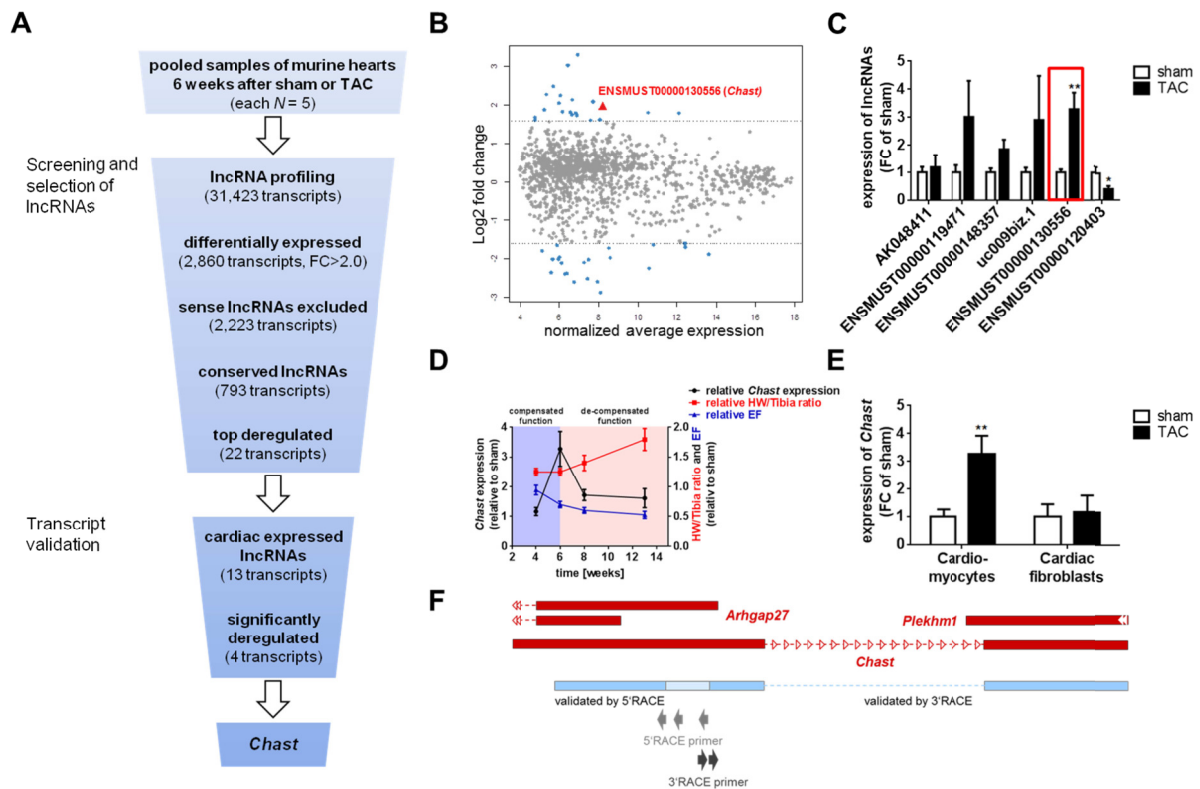


Fig. 1. Cardiac hypertrophy associated transcript (*Chast*) is induced by pressure-overload. (A)

Selection strategy of lncRNAs derived from microarray-based lncRNA profiling. (B) MA-plot (M = log ratio, A = average mean) of differentially regulated lncRNAs (FC>1.5 indicated by symbols signs and dashed line; *Chast* was highlighted in red). (C) Validation of lncRNAs induced by transverse aortic constriction (TAC; *Chast* is highlighted by a red box) (N=6). (D) Time course of *Chast* expression (black) compared to heart-weight-to-tibia ratio (red) and ejection fraction (EF, blue) (N=5-8), each relative to sham controls. (E) *Chast* expression in heart cell fractions after transverse aortic constriction (N=6). (F) Graphical representation of 3' and 5' rapid amplification of cDNA ends (RACE). Data are presented as mean $\pm$ SEM. *P* values are determined by Student's *t*-test, **P*<0.05; ** *P*<0.01. FC = fold change.

Fig. 2

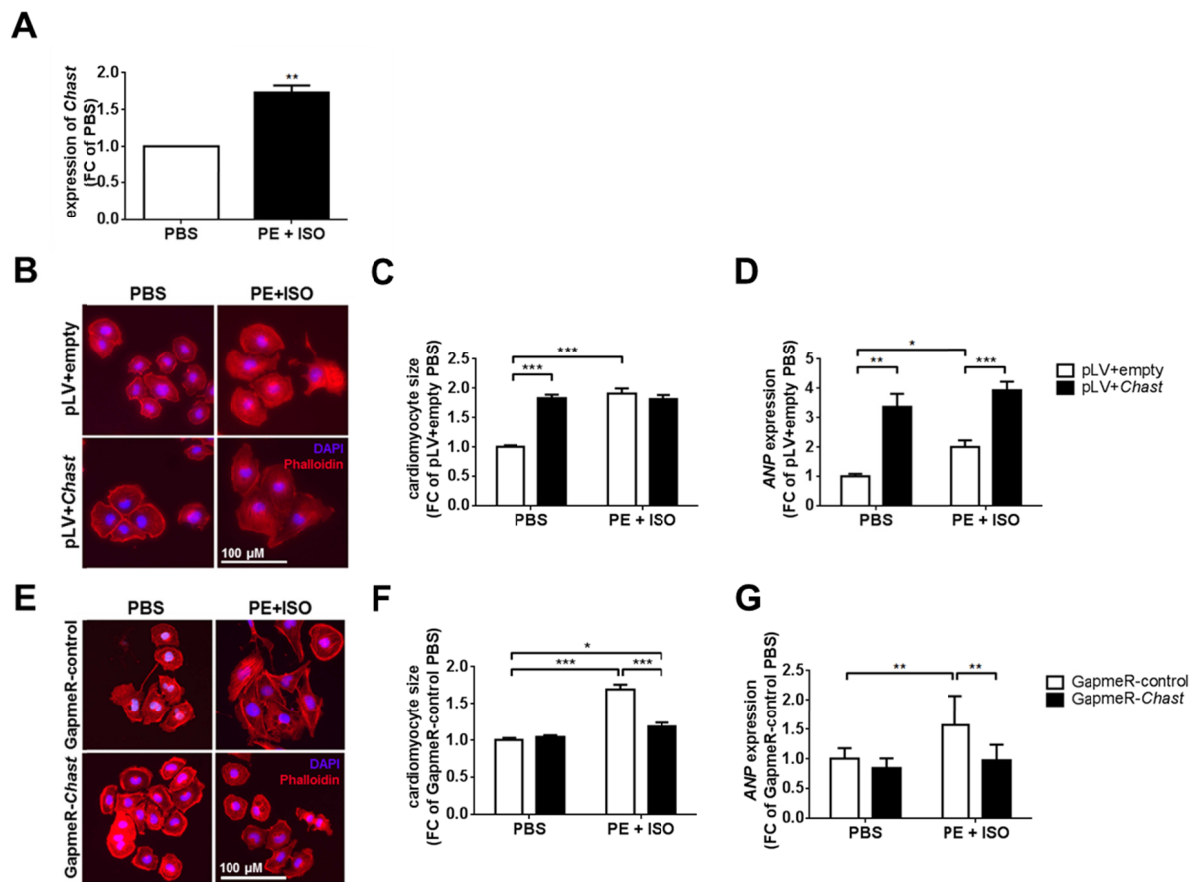


Fig. 2. *Chast* induces cardiomyocyte hypertrophy *in vitro*. (A) Expression levels of *Chast* after stimulation of HL-1 cardiomyocytes with isoproterenol (ISO) and phenylephrine (PE) (100 μ M each, 48h). Cardiomyocyte size after GapmeR-mediated silencing (B, C) or lentiviral over-expression (pLV+; E, F) of *Chast* after PE and ISO treatment. Effects of *Chast* deregulation on the expression of cardiac stress marker ANP (D, G). *N*=at least three independent experiments. Data are presented as mean $\pm$ SEM. *P* values are determined by one-way ANOVA with Bonferroni correction, **P*<0.05; ***P*<0.01; ****P*<0.001. FC = fold change, PBS = phosphate buffered saline, DAPI = 4',6-diamidino-2-phenylindole.

Fig. 3

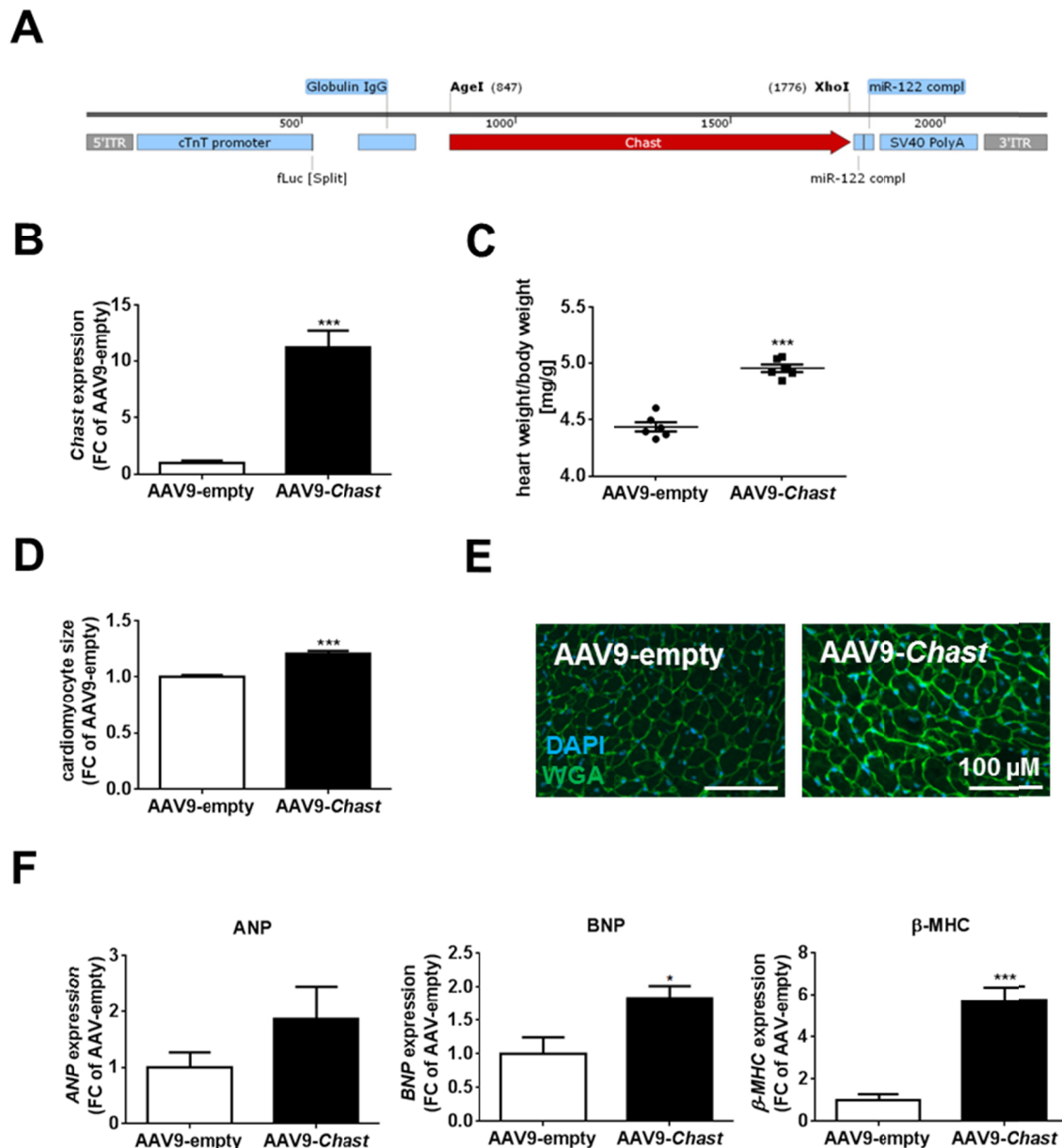


Fig. 3. *In vivo* over-expression of *Chast* promotes cardiac hypertrophy in mice. (A) Map of the cardiomyocyte-specific AAV9 vector used. (B) Elevation of *Chast* levels in cardiomyocyte fraction six weeks post injection compared to the control virus (AAV9-empty). (C) Heart-weight-to-Tibia-length ratio. (D, E) Determination of the cardiomyocyte diameter. (F) Expression of hypertrophic marker genes *ANP*, *BNP*, and *β-MHC*. *N*=6. Data are presented as mean ± SEM. *P* values are determined by Student's *t*-test, **P*<0.05; ***P*<0.01; ****P*<0.001. FC = fold change, DAPI = 4',6-diamidino-2-phenylindole, WGA = wheat germ agglutinin.

Fig. 4

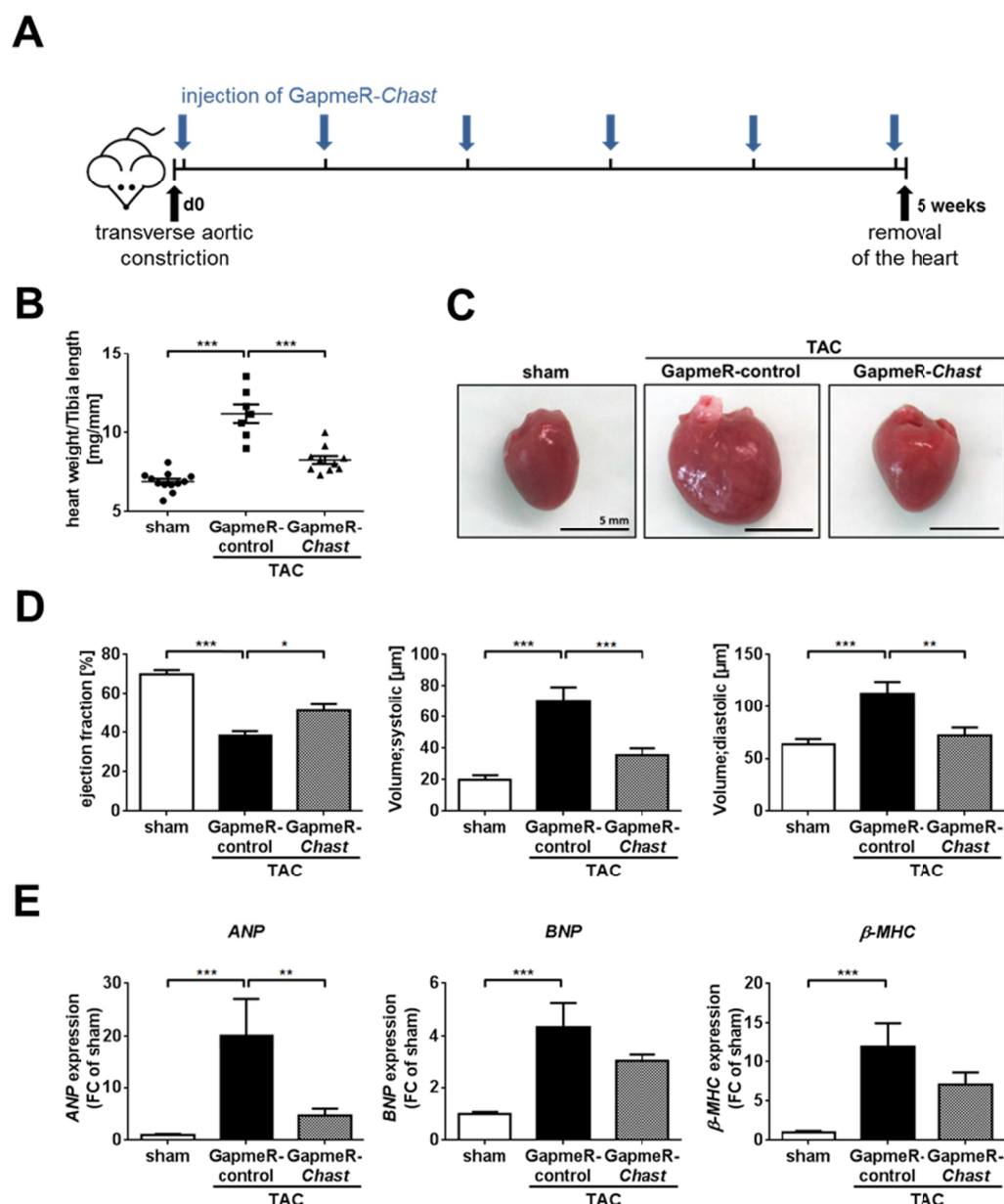


Fig. 4. Pharmacological inhibition of *Chast* in vivo ameliorates cardiac hypertrophy. (A)

Overview of the experimental setup. **(B)** Heart-weight-to-tibia-length ratio of sham mice and TAC-operated animals treated either with GapmeR-control or GapmeR-*Chast*. **(C)** Representative photographs of mouse hearts after respective treatments. **(D)** Echocardiographic analysis of cardiac dimensions and function. **(E)** Expression levels of hypertrophic marker genes *ANP*, *BNP*, and β -*MHC*. $N=6-8$. Data are presented as mean $\pm$ SEM. P values are determined by one-way ANOVA with Bonferroni correction, $*P<0.05$; $**P<0.01$; $***P<0.001$. FC = fold change, PBS = phosphate buffered saline.



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Online supplements

Long Non-coding RNA *Chast* Promotes Cardiomyocyte Hypertrophy

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Supplements

Supplemental Material and Methods

Cardiac cell fractionation

Adult mouse cardiomyocytes were isolated according to the previously described retrograde perfusion method [1] that was slightly modified and expended to also obtain cardiac fibroblasts. Adult mice were injected with 0.1 ml heparin (500 I.E./ml, i.p.) to prevent coagulation and anesthetized with 3% isoflurane and 100% O₂ (1 L/min) in an anaesthesia chamber. Hearts were cannulated through the aorta and retrograde perfused with prewarmed perfusion buffer (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄·7H₂O, 0.032 mM phenol red, 12 mM NaHCO₃, 10 mM KHCO₃, 10 mM HEPES, 30 mM taurine, 0.1% glucose, 10 mM 2,3-butanedione monoxime (BDM)) two times for 3 min. Then the buffer was switched to prewarmed digestion buffer (perfusion buffer supplemented with 700 U/ml collagenase II (Worthington, Lakewood, NJ) and 12.5 μM CaCl₂) for 20 min. After removing the atria, the ventricles were transferred to a small beaker containing 2.5 ml digestion buffer and cut into several small pieces. The tissue was then gently dissociated with a 1 ml syringe for 1 min and for another 3 min after addition of 2.5 ml stop buffer (perfusion buffer containing 10% FBS and 12.5 μM CaCl₂). The resulting cell suspension was filtered through a 100 μM cell strainer and supplemented with plating medium (MEM supplemented with 10% FBS, 2 ng/ml vitamin B12, 4.2 mM NaHCO₃, 100 U/ml penicillin:100 μg/ml streptomycin). Then cardiac myocytes and fibroblasts were separated as follows. Cardiomyocytes were sedimented by gravity for 10 min at 4°C. The supernatant containing the non-myocyte fraction was centrifuged at 1500 rpm for 5 min and the cell pellet dissolved in plating medium. Then cardiac fibroblasts were separated by preplating at 37°C and 1% CO₂ for 45 min. After separation, each cell fraction was washed with PBS, pelleted at 3000 rpm for 5 min and flash frozen in liquid nitrogen.

Microarray-based lncRNA profiling

For microarray analysis, RNA of five mouse heart tissue samples from sham or TAC groups 6 weeks after surgery were pooled. These samples were subjected to global lncRNA profiling using the

Arraystar Mouse LncRNA Microarray v 2.0 (ArraystarInc, Rockville, MD) that allows a simultaneous detection of 31,423 lncRNAs and 25,376 coding transcripts. Agilent Feature Extraction software (version 11.0.1.1) was used to analyse acquired array images. Quantile-normalization and subsequent data processing were performed with the GeneSpring GX v12.1 software package (Agilent Technologies). After quantile-normalization of the raw data, lncRNAs that at least two out of two samples have flags in Present or Marginal (“All Targets Value”) were chosen for further data analysis. Differentially expressed lncRNAs were identified through filtering on “Fold Change”. Correspondence analysis has been performed on the top 10% lncRNAs with the highest variance across all groups. Differentially expressed lncRNAs were depicted as mean average (MA) plot representing the differential expression (log FC) against the average expression. Three fold up- or down-regulated candidates are highlighted in blue.

To identify lncRNA candidates with a strong impact in cardiac hypertrophy, we followed the subsequent strategy (Figure 1A): we first exclude all lncRNAs that were annotated as “sense overlap” and focused on transcripts with similarity to human sequences based on a NCBI BLAST search (selection criterion for conservation: mouse_lncRNA_blast_hg19_genome: score ≥ 100 & evalue $\leq 2e-3$; all sequences transcribed from UCRs were also denoted as “conserved”). Among those conserved lncRNAs, eleven transcripts with either the highest or eleven with the lowest fold change were selected for further analysis. The expression of transcripts was confirmed in cDNA samples generated from RNA that was pretreated with DNase I (RNase-Free DNase Set, Qiagen, Hilden, Germany) prior reverse transcription and compared to untreated samples. Differential expression of lncRNAs was next validated by qRT-PCR (see below).

Transcriptome profiling after *Chast* deregulation

To obtain first impression on effects of modulation of *Chast* expression in cardiomyocytes, we performed a global mRNA expression profiling. RNA was obtained from HL-1 cells after GapmeR mediated silencing of *Chast* and subjected to microarray analyses (Mouse GE 4x44K v2 Microarray, Agilent Technologies, Santa Clara, CA). For genes with more than one Probe on the array the feature with the maximal variance has been selected as representative. A moderated T-Test has been used to

detect differentially expressed transcripts comparing the over-expression and knockdown groups to their corresponding controls. This was calculated from a linear model (limma) using the moderated t statistics as implemented in the eBayes function which has been specifically developed for the analysis of small sample size experiments.[2] By exploiting information across genes it delivers more stable results than a conventional t-test. Both resulting *P*-Values have been aggregated using a second order statistics as described in Dittrich *et al.*[3] and implemented in Beisser *et al.* [4]. All *P*-Values have been multiple testing corrected using the Benjamini Hochberg procedure [5]. Corrected *P*-Values < 0.05 have been considered significant.

In total, 527 differentially expressed genes were selected (criteria: normalized intensity > 200; *P*-value <0.02; FDR>0.1) Cluster 3.0 [6] was used for hierarchical clustering and deregulated genes represented in a heatmap applying Java Treeview [7].

Rapid amplification of cDNA ends (RACE)

Full length sequence of *Chast* was isolated from RNA of mouse hearts applying 5' and 3' rapid amplification of cDNA ends (RACE) according to the manufacturer's specifications (Invitrogen, Carlsbad, CA). Oligonucleotides used for RACE, nested PCR, and PCR are presented in **Supplemental Table S2**. RACE products were separated by agarose gel electrophoresis, purified from the gel (Qiagen, Hilden, Germany), and analysed by DNA sequencing.

Generation of transgenic HL-1 cell lines stably over-expressing *Chast*

To facilitate simultaneous over-expression of *Chast* in combination with a selectable marker, bidirectional lentiviral vectors (pLV+) containing an antisense oriented expression unit for *Chast* (ENSMUST00000130556) and a sense oriented expression unit for eGFP-2A-Puro were cloned. Control vectors were devoid of *Chast* (pLV+empty). Bidirectional vectors were produced according to standard procedures [8] by transient transfection of HEK293T cells with plasmids encoding for the lentiviral vector, lentiviral gag/pol, Rev, VSVg and NovB2. The latter helps to increase bidirectional vector titre by protection of viral genomic RNA against dicer mediated cleavage [8,9]. Viral

supernatants were harvested 36 h post transfection and transduced into HL-1 cells. After the initial puromycin selection, transgenic cells were cultured and treated as described above.

Subcellular fractionation

Fragmentation of HL-1 cells into cytoplasmatic, nuclear-soluble and chromatin-associated fractions was performed as described before [10].

Promoter analysis

Potential protein interaction partners were searched by regulatory elements using Nsite [11] and predicted with the software catRAPID [12], and examined against the whole mouse reference nucleotide-binding proteome. All sequences were taken from the Ensembl and NCBI database. The promoter sequence was generated from the eukaryotic promoter database [13], where upstream sequences (-1000 bp) of the murine *Chast* and two homologs in human were analysed for putative common transcription factor binding sites applying PROMO [14] (Parameter: Maximum matrix dissimilarity rate less or equal 15%).

Chromatin immunoprecipitation (ChIP)

HL-1 cardiomyocytes were crosslinked with 1% formaldehyde and a subsequent ChIP analysis was performed according to the supplier's protocol (MAGnifyChIP; Invitrogen, Carlsbad, CA) with the NFATc3 antibody (sc-8405 X; Santa Cruz Biotechnology, Heidelberg, Germany). Fold enrichment was quantified using qRT-PCR and primer pairs listed in **Supplemental Table S2**. Data were normalized to the IgG negative control, compared to an unrelated intronic region of the *Chast* sequence.

Protein coding potential

To assess the presence of encoded peptides we analysed the protein-coding potential of lncRNA candidates using the ORF detection program Sequence 6 Frame Translator [15], which were then

mapped against the STRING protein database (<http://string-db.org/>). Furthermore, we used the NCBI ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and the Coding Potential Calculator [16]. HL-1 cardiomyocytes stably over-expressing *Chast* (pLV+*Chast*) or harbouring the control vector (pLV+empty) were collected by trypsinization, washed with 1x PBS, and lysed using CellLytic M (Sigma-Aldrich, St. Louis, MO) for 15 min at 4°C. The lysate was cleared from the cellular debris by centrifugation at 15,000 g for 15 min at 4°C. Proteomics approach was applied to check the expression of proteins or peptides. 20 µg of the protein lysate was separated by 10-20% gradient Tris-Tricine gel for peptides and small proteins (Bio-Rad, Hercules, CA) in SDS-PAGE buffer. Proteins were visualized by mass spectrometry-compatible silver stain (Thermo Fischer Scientific, Waltham, MA). Protein samples <15 kDa were in gel-digested using trypsin and peptides analysed by a high resolving LC-MS system at the Institute for Toxicology (Hannover Medical School, Hannover, Germany) applying shot-gun proteomics and data-depended analysis. Resulting data were searched against an in-house database containing the predicted ORFs and several known contaminant proteins. Proteins of about 1 fmol abundance are detected using this approach.

RNA folding and sequence-structure conservation

RNAalifold [17] was applied to generate the consensus RNA secondary structure. Multiple alignment of *Chast* and two homologs were performed using Clustal Omega [18].

Identification of potential homologous human transcripts

The expression of potential human homologs was assessed by strand-specific gene expression analysis. RNA of human heart samples was converted into cDNA according to the gene-specific protocol of the iScript Select cDNA synthesis Kit (Bio-Rad, Hercules, CA) applying reverse primers listed in **Supplemental Table 1**. cDNA synthesis reaction without addition of reverse transcriptase served as internal control to rule-out any possible amplification of contaminating DNA.

Supplemental Figures

Fig. S1

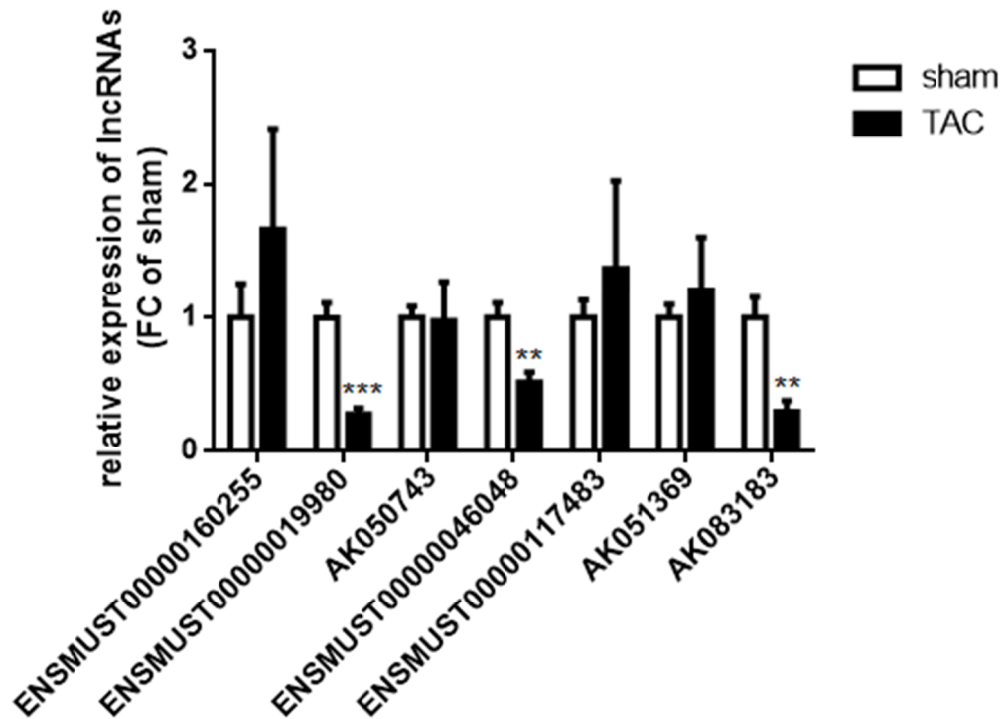


Fig. S1. Cardiac left ventricular pressure-overload leads to a down-regulation of several candidate lncRNAs. Validation of lncRNAs repressed upon by transverse aortic constriction (TAC). $N=6$. Data are presented as mean $\pm$ SEM. P values are determined by Student's t -test, * $P<0.05$; ** $P<0.01$; *** $P<0.001$. FC = fold change.

Fig. S2

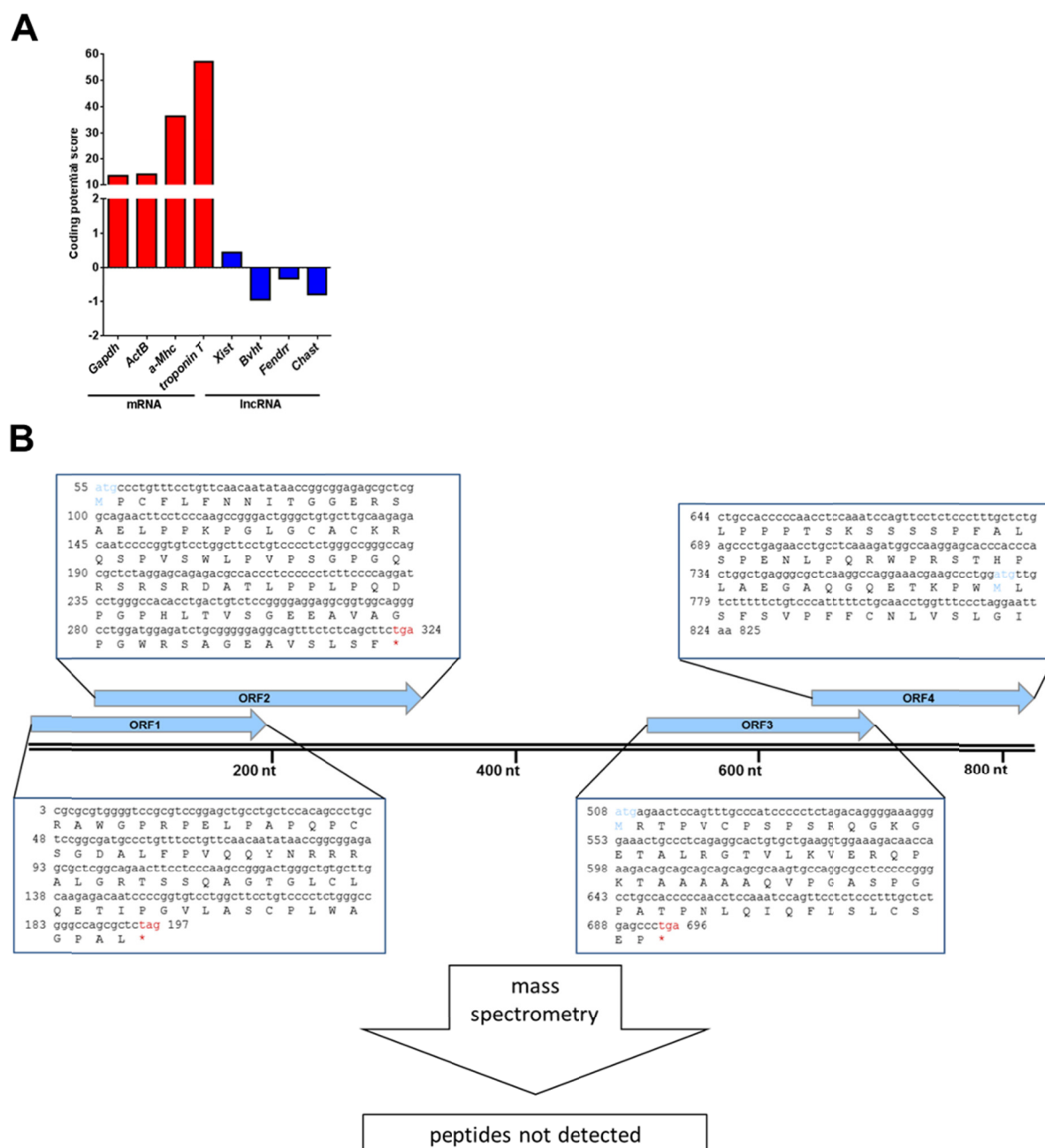


Fig. S2. *Chast* is a long non-coding RNA. Coding potential scores of known mRNAs (*ActB*, *Gapdh*, α -MHC, and *troponin T*) or lncRNAs (*Xist*, *Braveheart* (*Bvht*), and *Fendrr*) compared to *Chast*. (A) Presentation of the open reading frames (ORFs) potentially encoded by the *Chast* sequence. (B)

Fig. S3

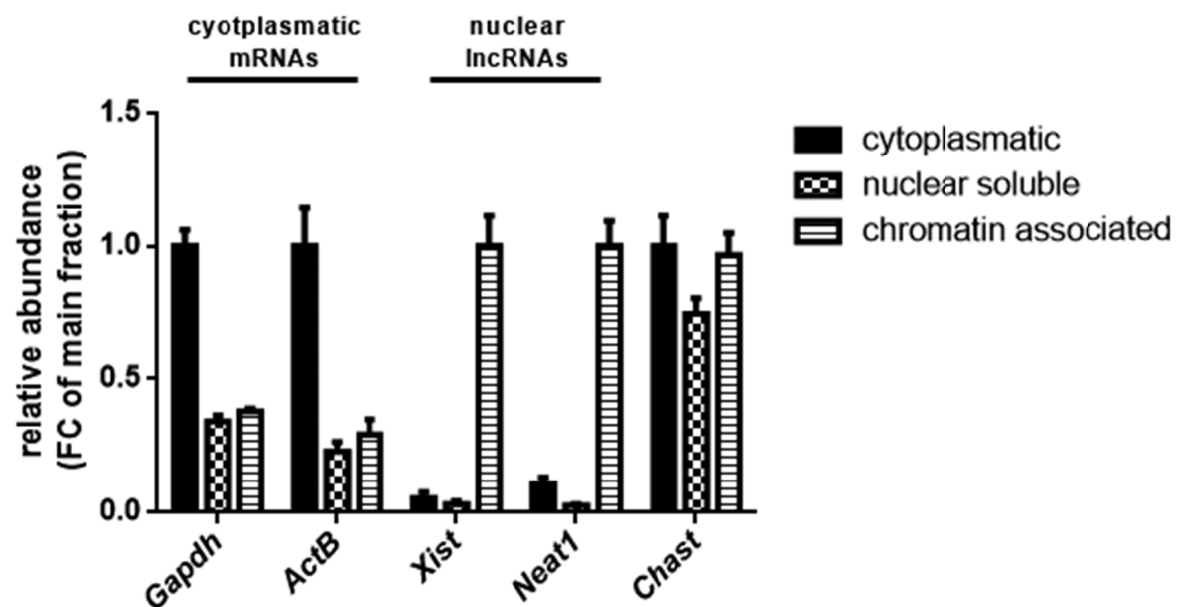


Fig. S3. *Chast* is located in all subcellular fractions of cardiomyocytes. Subcellular abundance of *Chast* in HL-1 cells compared to mRNAs (*Gapdh*, *ActB*) and lncRNAs (*Xist*, *Neat1*). *N*=at least three independent experiments. Data are presented as mean $\pm$ SEM.

Fig. S4

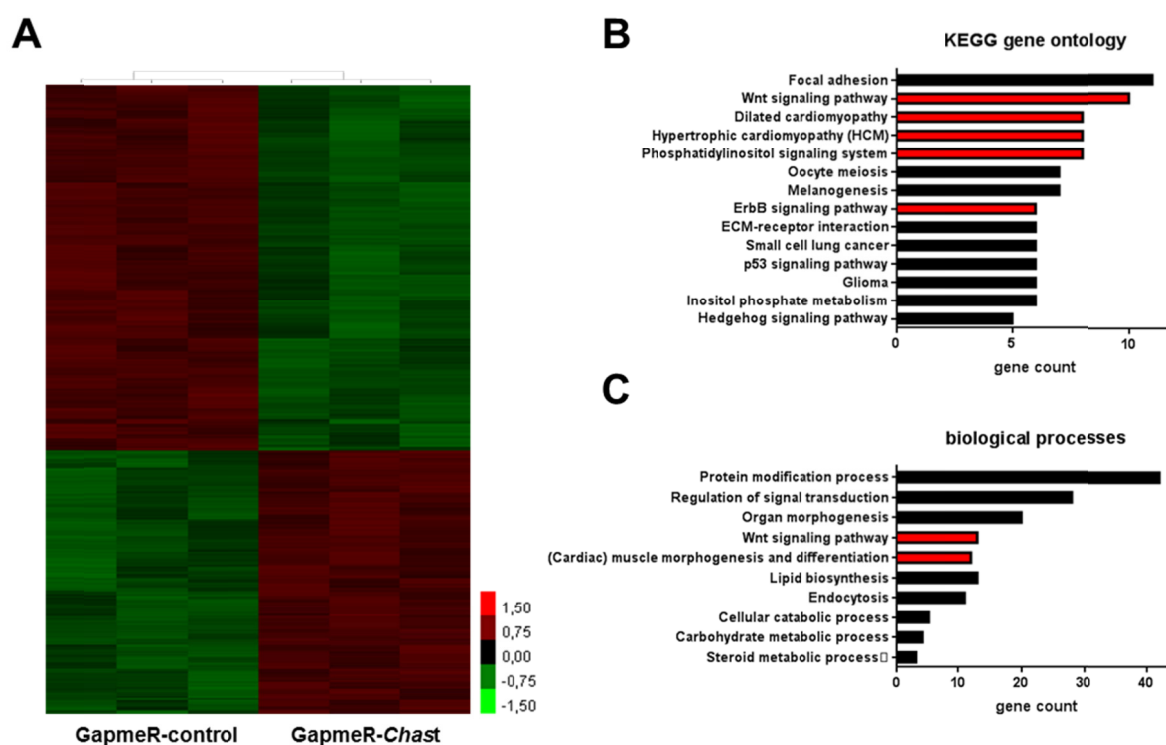


Fig. S4. GapmeR-mediate silencing of *Chast* alters the transcriptome of HL-1 cells. Heatmap representing differentially regulated transcripts after treatment with GapmeR-*Chast* compared to GapmeR-control. (A) KEGG gene ontology analysis (B) and biological processes (C) regulated by *Chast*. Hypertrophy-associated pathways are highlighted in red. *N*=three independent experiments.

Fig. S5

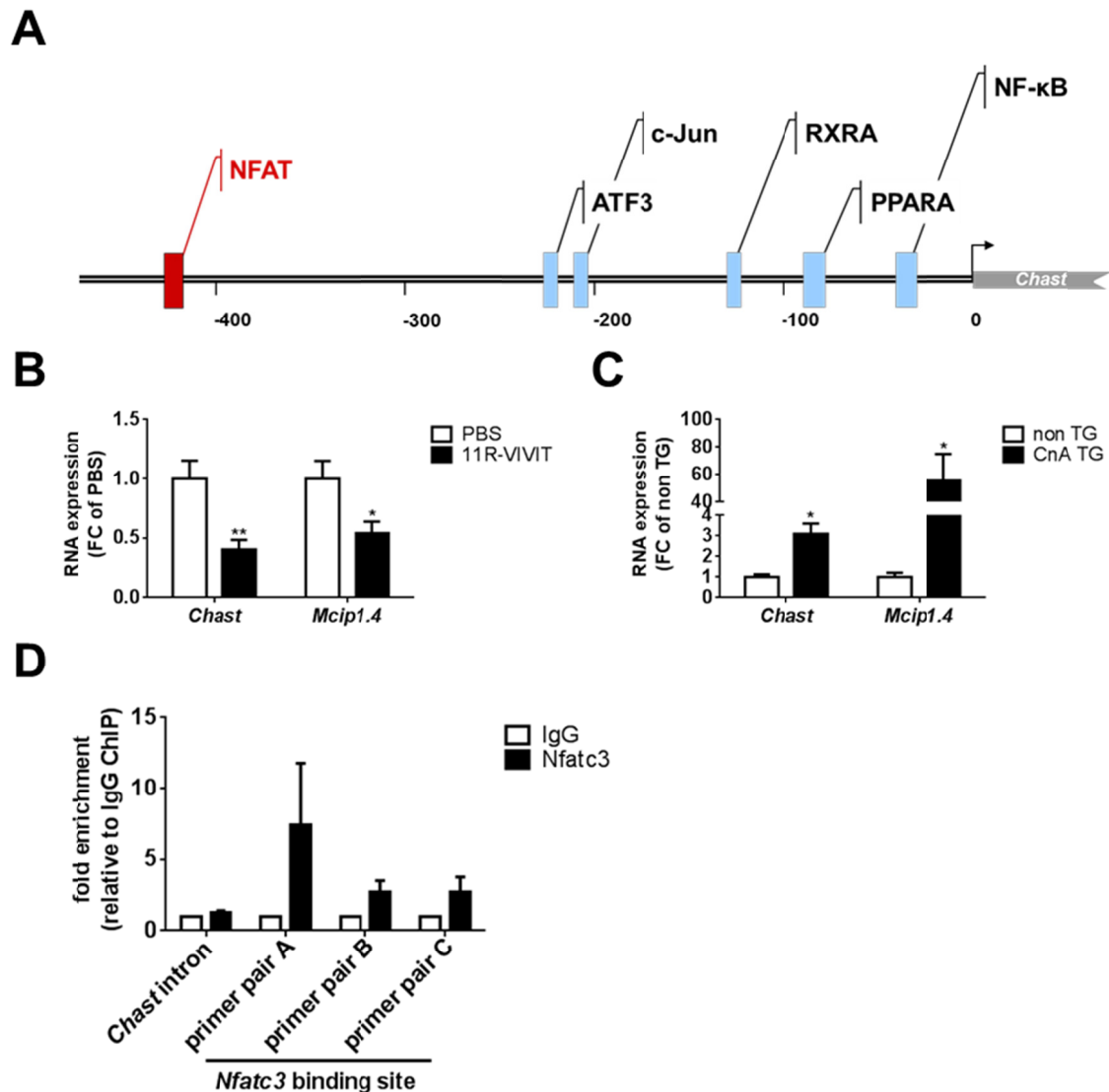


Fig. S5. The prohypertrophic transcription factor NFAT is an upstream regulator of *Chast* expression. Transcription factor binding site prediction. (A) Gene expression of *Chast* and *Mcip1.4* after treatment of HL-1 cells with the pharmacological NFAT inhibitor 11R-VIVIT (1 μ M, 48 h; N =at least three independent experiments) (B) or in heart tissue of calcineurin transgenic (CnA TG) mice compare to wildtype littermates (non TG) (N =4) (C). QRT-PCR after chromatin immunoprecipitation (ChIP) with three different primer pairs surrounding the potential NFAT binding site in comparison to a control primer targeting the *Chast* intron. Data are presented as mean $\pm$ SEM. P values are determined by Student's t -test, * $P < 0.05$; ** $P < 0.01$; FC = fold change, PBS = phosphate buffered saline.

A

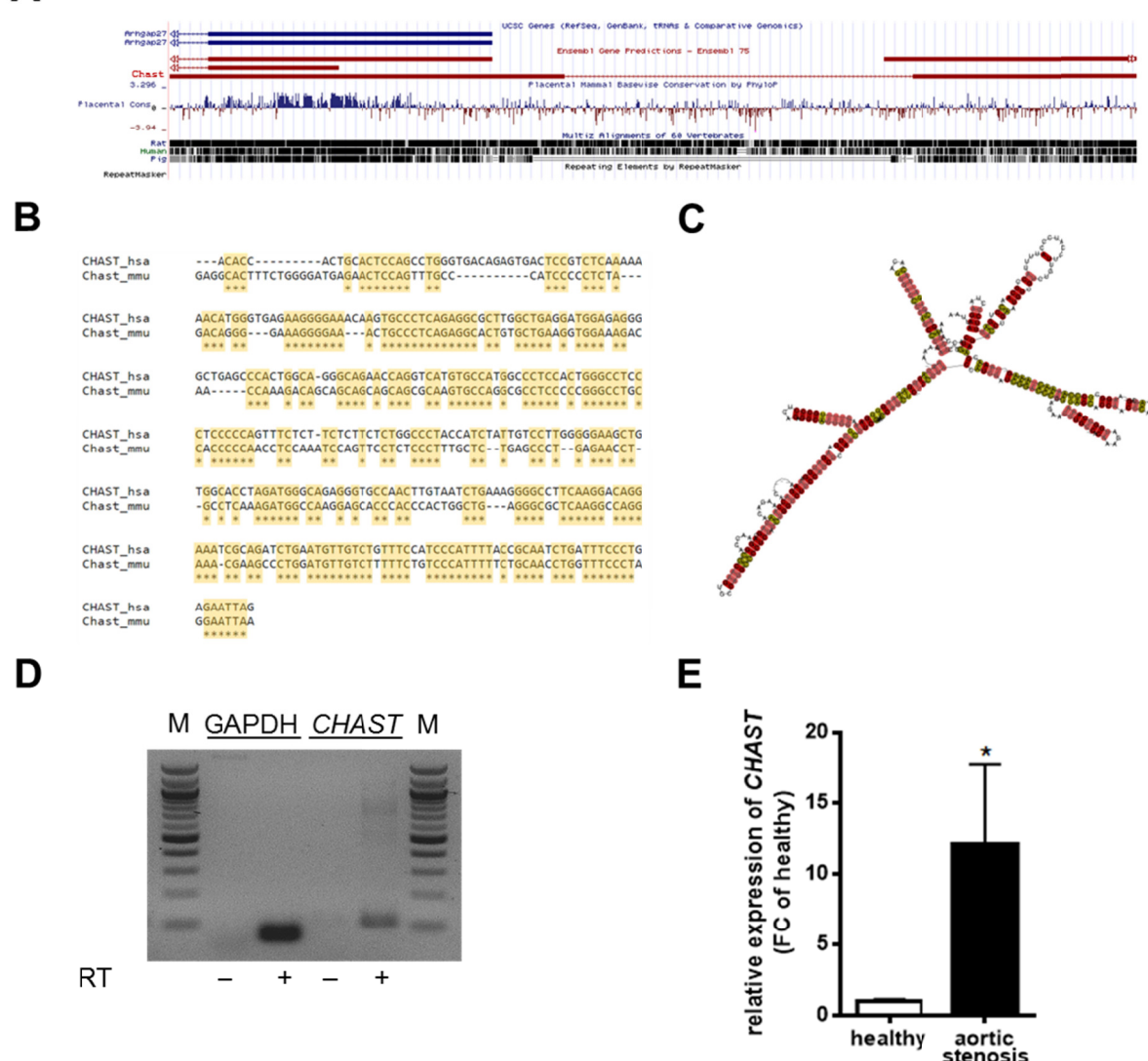


Fig. S6. *Chast* has a functional homolog in humans. UCSC Browser-derived representation of the genomic locus of *Chast* indicating conserved regions. **(A)** Pairwise alignment of the human and murine *Chast* sequence by Clustal Omega. **(B)** Consensus structure of human *CHAST* and mouse *Chast* derived from LocARNA (red indicates there is only one possible pair, ochre means two possible pairs). **(C)** Amplification of the human *CHAST* homolog by gene-specific PCR based on RNA from total human heart tissues (each primer was tested with or without reverse transcriptase (RT)). **(D)** Expression of human *CHAST* in healthy donor heart tissues ($N=23$) or in patients with aortic stenosis ($N=21$). Data are presented as mean $\pm$ SEM. P values are determined by Student's t -test, $*P<0.05$. FC = fold change.

Supplemental Tables

Supplemental Table S1. Transcription factor binding site prediction for the *Chast* promoter.

TF	TFBS ID	Location	Reference
ATF3	T01313	-227...-220	[19]
c-Jun	T00133	-211...-205	[20]
NFAT	T01945	-428...-419	[21]
NF-κB	T00593	-41...-31	[22]
PPAR-α:RXR-α	T05221	-89...-79	[23]
RXR-α	T01345	-130...-124	[24]

Supplemental Table S2. Oligonucleotide primers used in this study.

qRT-PCR		
Primer name	Primer sequence (forward)	Primer sequence (reverse)
Mmu_GAPDH	5'TTCACCACCATGGAGAAGGC3'	5'GGCATGGACTGTGGTCAATGA3'
Mmu_ActB	5'ATCAAGATCATTGCTCCTCCTG3'	5'ATCAAGATCATTGCTCCTCCTG3'
Mmu_Xist	5'TCATCCGCTTGCCTTCATAG3'	5'GAGATCAGTGTGGCTAAATCAGA3'
Mmu_Neat1	5'TGGCCCCCTTTTGTTCATTAGC3'	5'TGGAAGGCCATTGTTTCAGG3'
Mmu_ANP	5'CCTGTGTACAGTGCCTGTGTC3'	5'CCTAGAAGCACTGCCGTCTC3'
Mmu_BNP	5'CTGAAGGTGCTGTCCAGAT3'	5'GTTCTTTTGTGAGGCCTTGG3'
Mmu_β-MHC	5'TCTCCTGCTGTTTCCTTACTTGCT3'	5'CAGGCCTGTAGAAGAGCTGTACTC3'
Mmu_Mc1p1.4	5'CTGCACAAGACCGAGTT3'	5'TGTTTGTCTGGGATTGG3'
Mmu_ENSMUST00000130556 (<i>Chast</i>)	5'CCACTGACCCTCATCCTTGT3'	5'CCCAGAAAGTGCCTCCTTGT3'
Mmu_AK048114	5'GAACCCGGGTCTAACCTAGC3'	5'AGCTGCTCTTGGGAAAAACA3'
Mmu_ENSMUST00000119471	5'TGGACTACGACATGGTGCAT3'	5'TGTGCTTTGTAAAGCCACAG3'
Mmu_ENSMUST00000148357	5'CCATCGGAAAGATTTGGTG3'	5'GGAGGTCGGAGATATGCTGA3'
Mmu_uc009biz.1	5'TGGTTTGGTTTGGTTTGA3'	5'TCCACTGGCAGAAAACAATG3'
Mmu_ENSMUST00000120403	5'ACAATCTGCCACACACGTA3'	5'TTCAGGATGCCCCATTTCAG3'
Mmu_ENSMUST00000160255	5'GGCTGAGTGGGTCTTGGC3'	5'TGGGGCACTCTCCATTGT3'
Mmu_ENSMUST0000019980	5'CCCTGTTGAAGCTTGCTAGG3'	5'TTACTCGGCTCTCTGGGAAA3'
Mmu_AK050743	5'CTCTGAGCAAACGTCCTTCC3'	5'AGGATGCTGCGATTCACTTT3'
Mmu_ENSMUST00000046048	5'CGATCCAAATGATTCCAAGC3'	5'CATCCTCCCAGTCTTTCCAA3'
Mmu_ENSMUST00000117483	5'TTCTCAAGCAGCAAAATGG3'	5'TCGATGATGTCAGCTTTCCA3'
Mmu_AK051369	5'ACCAGAATTGGTCTGTCATC3'	5'ACCTGAAAGCTTGGAGGACA3'
Mmu_AK083183	5'CCAGAGTGGCTTAGACTGC3'	5'TTAAACCCCTGGCTTGTGG3'
Hu_GAPDH	5'CCAGGCGCCCAATACG3'	5'CCACATCGCTCAGACACCAT3'
Hu_17:62843244-62843536 (<i>CHAST</i>)	5'GCAGAGGGTGCCAACCTGTA3'	5'TCTCAGGGAAATCAGATTGCGG3'

RACE	
Primer name	Primer sequence
5'RACE-GSP1- <i>Chast</i>	5'ACAAGGATGAGGGTCAGTGG3'
5'RACE-GSP2- <i>Chast</i>	5'CCCGCAGATCTCCATCCAG3'
5'RACE-GSP3- <i>Chast</i>	5'TATACCATGGCGGAGACAGTCAGGTGTGG3'
3'RACE-GSP1- <i>Chast</i>	5'CCACTGACCCTCATCCTTGTG3'
3'RACE-GSP2- <i>Chast</i>	5'TATACCATGGGAAGTCAACGGCCACTCTGCAG3'

Chromatin immuno precipitation (ChIP)	
Mmu_ <i>Chast</i> _intron_fwd	5'CTAGTGTGCGAGGGCTAAGG3'
Mmu_ <i>Chast</i> _intron_rev	5'GTGGAGGGAAAGAGCTCACC3'
Mmu_ <i>Chast</i> - <i>Nfat</i> _P2a_fwd	5'TGCTCCCTGAATCAGAACT3'
Mmu_ <i>Chast</i> - <i>Nfat</i> _P2a_rev	5'TGTCTGTGAGCGTCCCTG3'
Mmu_ <i>Chast</i> - <i>Nfat</i> _P2b_fwd	5'TGAGCCCTTCACAGCAAGAT3'
Mmu_ <i>Chast</i> - <i>Nfat</i> _P2b_rev	5'CCCTGTCTGTGAGCGTCC3'
Mmu_ <i>Chast</i> - <i>Nfat</i> _P2c_fwd	5'GCGGAGTTGGCTGTCTTGTG3'
Mmu_ <i>Chast</i> - <i>Nfat</i> _P2c_rev	5'CCTGTCTGTGAGCGTCCCTG3'

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ORIGINAL ARTICLE

Impact of a Met(11)Thr single nucleotide polymorphism of surfactant protein D on allergic airway inflammation in a murine asthma model

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ABSTRACT

The surfactant-associated proteins SP-A and D are pattern recognition molecules with collectin structure. A single nucleotide polymorphism (SNP) exchanging a methionine (Met) for a threonine (Thr) in the amino-terminal SP-D domain influences the oligomeric structure and function of the protein. In this study, we investigated the susceptibility of mice transgenic for the human SP-D Met(11)Thr SNP to allergic airway inflammation and consequences for microRNA (miRNA, miR) expression. Mice expressing either human Met or Thr SP-D were sensitized and challenged with ovalbumin (OVA) in an acute model of allergic asthma. The influence of the SP-D polymorphism on the allergic airway inflammation was evaluated by lung function measurement, pulmonary inflammation parameters, morphological analysis and miRNA expression. Airway hyperresponsiveness, allergic inflammation, and mucus metaplasia were not significantly different between mice expressing one or the other allelic variant of SP-D. OVA sensitization and challenge led to significant airway hyperresponsiveness in wildtype mice and significantly lower eosinophil numbers and interleukin 5 levels in Thr SP-D mice. OVA challenge induced an up-regulation of miR-21 and 155 in Thr SP-D mice and a downregulation of miR-21 in Met SP-D mice. Our results show that murine expression of human polymorphic SP-D variants does not significantly influence the severity of allergic airway inflammation. MiR-21 and 155 are differentially regulated in transgenic mice in response to allergic inflammation. Further studies are required to elucidate the impact of this SNP on inflammatory conditions of the lung.

KEYWORDS allergic asthma, microRNAs, protein multimerization, single nucleotide polymorphism, surfactant protein D

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INTRODUCTION

The surfactant protein D is a hydrophilic protein, which belongs to the family of collagenous lectins, termed collectins. These proteins are composed of a carbohydrate recognition domain (CRD) connected via a neck region with a collagenous domain that ends in an amino-terminal lectin domain. SP-D units form cruciform dodecamers that in turn assemble to a "fuzzy ball"-like multimeric structure (SP-D) [1, 2].

Three single nucleotide polymorphisms (SNP) are described that result in an exchange of amino acids in the protein. A SNP in the SP-D gene switching ATG to ACG causes an exchange of a methionine (Met) for a threonine (Thr) at position 11 in the amino-terminal region of the protein [3]. This SP-D SNP influences assembly, function and concentration of SP-D [4–6]. In addition to trimers and dodecamers Met SP-D forms higher order multimers while Thr SP-D predominantly forms trimers. The oligomeric structure in turn influences the collectin function of the protein. The high molecular weight form of SP-D reveals a higher binding affinity to aggregate gram-positive and gram-negative bacteria, Influenza A virus and mannan [5, 6]. Via binding of their head domains to signal-regulatory protein (SIRP)-1 α SP-D multimers appear to silence pro-inflammatory mediator production while trimers bind with their tail domain to Calreticulin/CD91 and appear to induce mediator production [7, 8]. SP-D concentration decreases with the number of C alleles with highest levels in individuals with the T/T allele and lowest in individuals with the C/C allele; SP-D serum levels are 1035.2 ± 88.6 ng/mL for the Met and 744.1 ± 86.5 ng/mL for the homozygous Thr genotype (concentration average $\pm$ SEM) [5, 9]. The frequency of this SNP is reported for a healthy Danish population. 47.1% of individuals are heterozygous for the Met(11)Thr SNP, 35.4% carry the homozygous Met and 17.5% the Thr SP-D genotype [5].

Genetic association studies point to a higher susceptibility to allergic rhinitis [10], emphysema [11], chronic obstructive pulmonary disease [12] and pneumonia-associated multiple organ dysfunction syndrome and acute respiratory distress syndrome [9] in individuals with the Thr mono- or biallelic genotype. Interestingly, patients who receive lung allografts survive longer and are less susceptible to lung allograft dysfunction when they are homozygous for the Met SP-D variant [13]. In contrast to reports associating the Thr SP-D variant to lung disease a higher frequency of the Met SP-D variant has been found in infants hospitalized for treatment of severe respiratory syncytial virus bronchiolitis [14]. Finally, there is no association between asthma and SP-

D SNPs in a study on 322 asthmatic children [15] (for review see [16, 17]).

The murine SP-D gene on chromosome 14 shows 76% identity to the human homologue located on chromosome 10 [18, 19]. Mice deficient in SP-D develop an emphysema-like morphology of the lung; they show an accumulation of foamy macrophages in the alveolar lumen and hyperplasia and hypertrophy of alveolar epithelial type II (AE II) cells [20, 21]. Stereological exploration of a Met(11)Thr SNP mouse model revealed that mice expressing human SP-D variants exhibit a partially normalized phenotype compared to SP-D $^{-/-}$ mice [4].

The aim of the present study was to investigate the impact of this human Met(11)Thr SNP on the inflammatory phenotype and on expression of two microRNAs (miRs) involved in allergic inflammation in a murine model of OVA-induced allergic airway inflammation.

MATERIAL AND METHODS

Animals

C57BL/6.Cg-*Sftpd*^{tm1Haw} SP-D deficient knock-out mice, C57BL/6.Cg-*Sftpd*^{tm1HawZDW}Tg(*Rosa26-SFTPD*^{rs721917-T})Haw mice expressing the human Met and C57BL/6.Cg-*Sftpd*^{tm1HawZDW}Tg(*Rosa26-SFTPD*^{rs721917-C})Haw mice expressing the human Thr variant of SP-D (referred to as SP-D $^{-/-}$ and Met resp. Thr SP-D mice) were a kind gift of Jens Madsen, University of Southampton, United Kingdom. The generation of the mouse model is described in a previous paper [4]. C57BL/6 wildtype (wt) mice were purchased from Charles River, Germany. Mice were supplied with food and water ad libitum. Met mice (12 male/20 female) were 12 to 13 weeks, Thr mice (13 male/19 female) 12 to 17 weeks, SP-D $^{-/-}$ (14 male/15 female) 4 to 46 weeks, and wt mice (12 male/20 female) 7 to 9 weeks old.

Induction of an Allergic Airway Inflammation

Wt, SP-D $^{-/-}$, Met SP-D, and Thr SP-D mice were sensitized according to the standard acute OVA model as described before [22]. Briefly, 10 μ g OVA (Sigma-Aldrich, Steinheim, Germany) adsorbed to 1.5 mg aluminum hydroxide (alum) in 200 μ L phosphate-buffered saline per mouse were injected intraperitoneally on days 0, 14, and 21. Control animals were sham-sensitized with 1.5 mg alum adsorbed to saline, accordingly. All mice including controls were challenged with 1% OVA aerosol for 20 minutes on two consecutive days (day 27, 28). All

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animal experiments were conducted following approval of the appropriate authority (Bezirksregierung Hannover).

For details about the measurement of pulmonary function see supplemental material and references [22, 23].

Analysis of Pulmonary Inflammation

Mice were euthanized directly after lung function measurement 24 hours after the last OVA aerosol challenge and lungs were lavaged two times with 0.8 mL of 0.9% NaCl containing protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany).

Cytospin preparations from bronchoalveolar lavage (BAL) cells were stained according to the Pappenheim standard protocol. Analysis of cytokines in the cell-free supernatant was performed using a Th1/Th2 10-plex from MSD (Meso Scale Discovery, Rockville, USA). Human SP-D in BAL and serum was determined by ELISA measurements (Biovendor, Heidelberg, Germany).

Stereological Analysis

Stereological analysis was performed according to the recommendations published by Hsia *et al.* [24]. First, we applied systematic uniform random sampling (SURS) to sample large to medium sized airways within alcian blue periodic acid Schiff reagent (AB-PAS) stained Paraplast-embedded tissue sections. We estimated epithelial mucus content using the stereology toolbox software newCast (Visiopharm, Version 3.6.5.0, Hørsholm, Denmark). Slides were examined using the 40 $\times$ objective. All stereological measurements were performed by the same, blinded investigator. For further details regarding fixation and removal of the lungs and the stereological investigation see supplemental material.

RNA Isolation and microRNA Reverse Transcription Polymerase Chain Reaction

RNA was isolated from lung parenchyma of wt, SP-D^{-/-}, Met and Thr mice using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Mature miRNAs were detected by reverse transcription polymerase chain reaction in a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, USA) applying TaqMan MicroRNA Assays specific for miR-21 and miR-155 (Applied Biosystems, Foster City, USA). Each sample was run in du-

plicates. The small nuclear RNA (snoRNA202) was amplified as an internal control.

Statistical Analysis

Statistical analysis of functional, morphological, and miRNA data were performed using ANOVA and Newman-Keuls test for posthoc multiple comparisons (GraphPad Prism software, La Jolla, USA). Functional data were log transformed before analysis. Variables were expressed as mean $\pm$ SD. A probability value of $P < .05$ was accepted as being significant.

RESULTS

Impact of the Human Met(11)Thr SP-D Polymorphism on Allergic Airway Inflammation

Sensitization and challenge of wt animals with OVA led to increased airway resistance in response to methacholine challenge compared to sham-sensitized animals ($P < .05$) (Figure 1A). However, no significant changes were observed comparing the airway hyperreactivity between animals expressing either the human Met SP-D or Thr SP-D polymorphism. Compared to wt control mice OVA-sensitized mice carrying the human Thr SP-D polymorphism revealed significantly lower total cell numbers after induction of the acute allergic airway inflammation ($P < .05$), these mice also possessed a lower percentage of eosinophils ($P < .05$) (Figure 1B) and absolute eosinophil numbers ($P < .05$, data not shown). Comparing the mice harboring the Thr polymorphism to the mice carrying the Met SP-D variant differences regarding the eosinophilic inflammation were not observed.

The alleviated eosinophilic inflammation was also reflected by lower interleukin (IL)-5 levels in the BAL in OVA-sensitized transgenic mice, with lowest levels in Thr SP-D mice compared to wt mice ($p < 0.001$) (Figure 2A). IL-4 and further cytokines reflecting a Th2-biased inflammation like IL-10, IL-2 were not found to be significantly different between OVA-sensitized groups. Human SP-D levels in BAL and serum were evaluated in Met SP-D and Thr SP-D mice, but no differences were observed neither with regard to the inflammation-driven regulation of pulmonary SP-D nor generally between both mice strains (Figure 2B). Interestingly, serum levels in these humanized mouse strains were found to be 300 times higher compared to levels in the pulmonary compartment. Analysis of additional parameters characteristic for the allergic inflammation

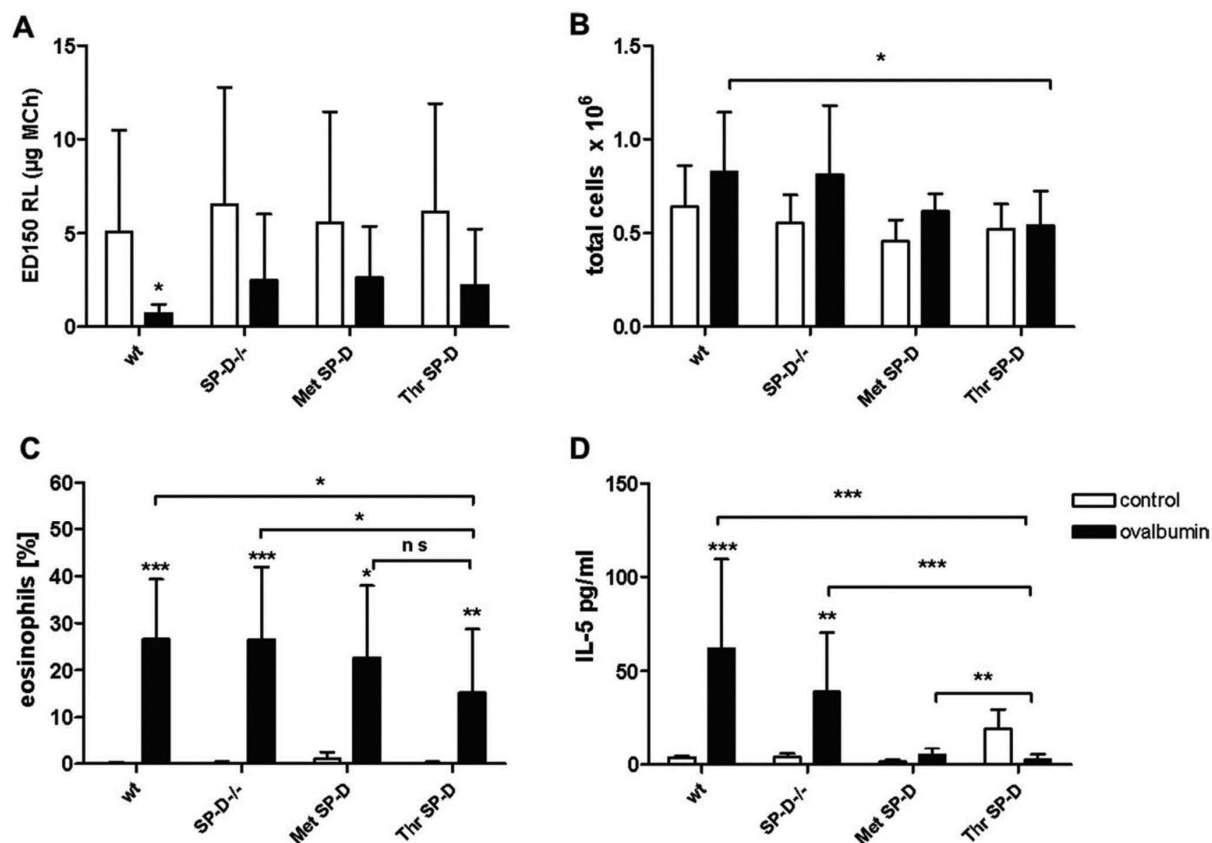


FIGURE 1. Measurement of airway hyperresponsiveness and pulmonary inflammation. (A) Inhaled dose of MCh in μg required to increase pulmonary resistance (R_L) by 150% above baseline ($\text{ED}_{150} R_L$) measured in anesthetized, orotracheally intubated, and spontaneously breathing mice ($n = 16$). Characterization of the cellular inflammation in the BAL fluid assessing (B) total cell counts and (C) eosinophils in% and (D) IL-5 ($n = 10$). Data are presented as mean + SD, * $P < .05$, ** $P < .01$, *** $P < .001$, ns nonsignificant, OVA-sensitized group compared to the respective control group or as otherwise indicated by lines.

like OVA-specific IgE levels in serum, the number of Th2-polarized CD4⁺ lymphocytes in draining lymph nodes and the allergen-specific proliferation of these cells also revealed no differences between OVA-sensitized Met SP-D and Thr SP-D mice (data not shown).

Impact of the Human Met(11)Thr SP-D Polymorphism on Height and Mucus Content of Airway Epithelium

In response to OVA sensitization and challenge mice developed mucus metaplasia in the airway epithelium (Figure 3A). Mucus metaplasia was restricted to large and medium sized airways while small distal airways were devoid of mucus (Figure 3B,C). Furthermore, eosinophils in modest numbers infiltrated the peribronchial and especially the perivascular connective

tissue (Figure 3D). Quantification of epithelial mucus demonstrated a significant increase in wt mice for estimation of volume to surface ratio (V_s) ($P < .05$; Figure 4B) and volume density of mucus (V_v) ($P < .05$; data not shown). Estimation of the ratio of surface area covered by epithelial mucus to total epithelial surface area (S_s) revealed a significant rise in epithelial mucus in all OVA-sensitized groups of mice, compared to the wt control group ($P < .05$; data not shown). A significant difference in epithelial mucus content between groups of mice carrying the SP-D Met(11)Thr SNP was not observed. Because estimation of the total height of epithelium (volume to surface ratio) revealed no significant difference between OVA and sham-sensitized mice (Figure 4A) the rise in epithelial mucus content is apparently not accompanied by a substantial hypertrophy of the respiratory epithelium.

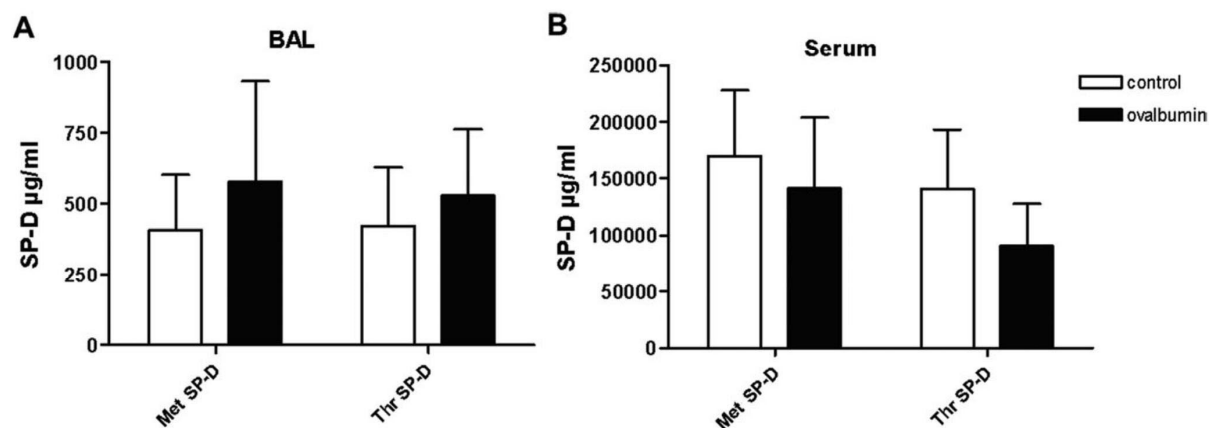
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FIGURE 2. Human SP-D in BAL (A) and serum (B), ($n = 10$). Data are presented as mean + SD.

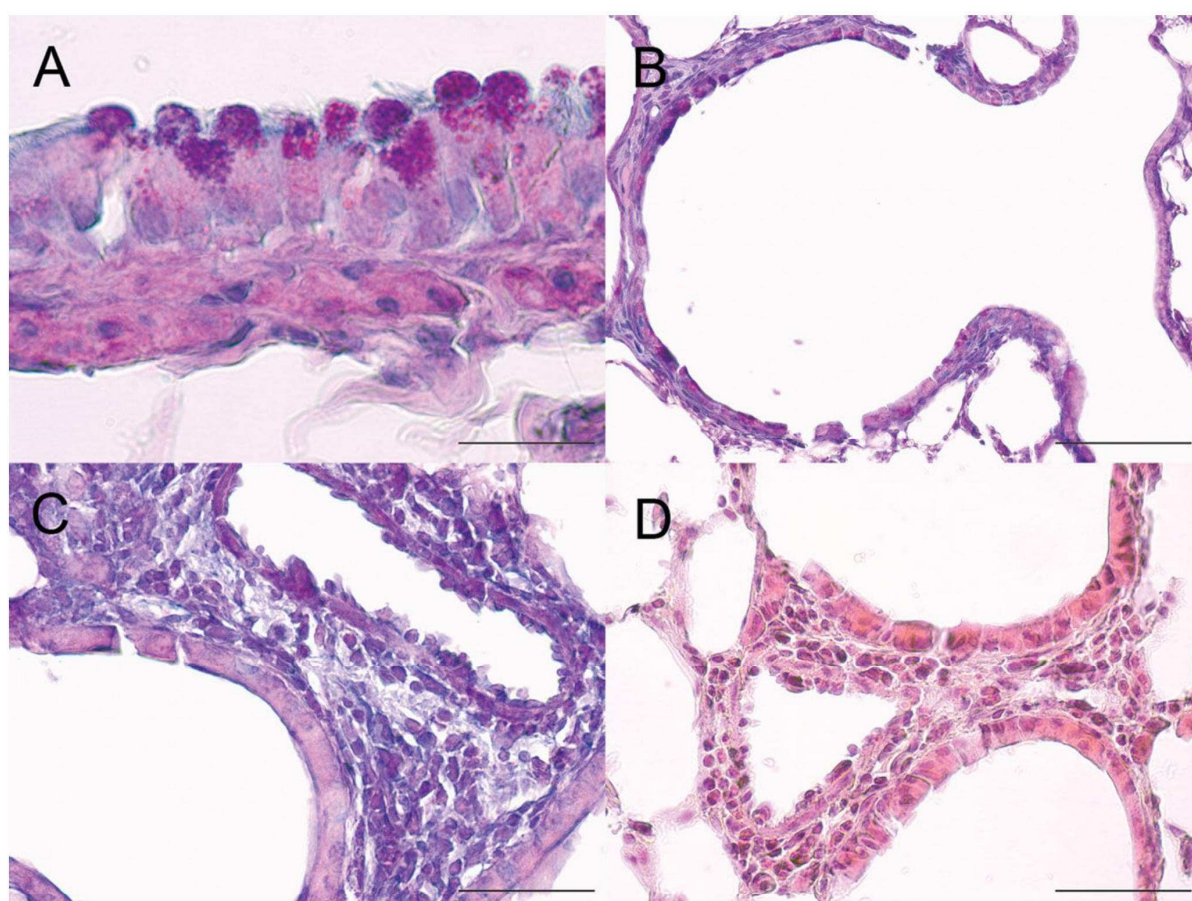


FIGURE 3. Histology of lung tissue. AB-PAS (A-C) and HE (D) staining in wt (A, C, D) and Thr SP-D (B) mice. (A) proximal airway with apical AB-PAS staining in nonciliated cells (scale bar 20 μm), (B) midlevel airway with AB-PAS positive cells branching into small distal airways that are AB-PAS negative (scale bar 100 μm). (C) and (D): distal airway and adjacent small blood vessel with eosinophilic infiltration of perivascular and peribronchial tissue (scale bar 50 μm).

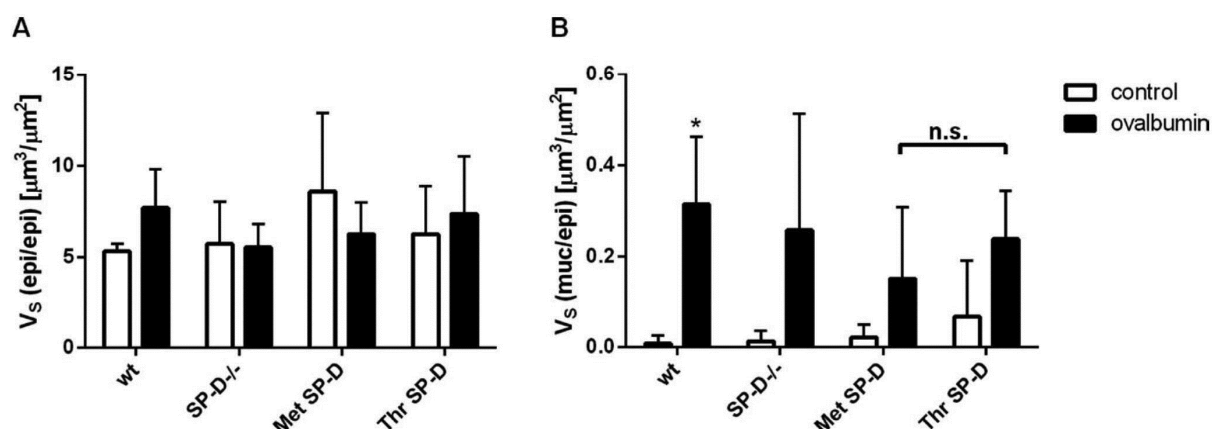


FIGURE 4. Quantitative microscopy. (A) Epithelial volume per surface area of airway epithelium (representing mean epithelial layer thickness), (B) Mucus volume per surface area of airway epithelium (wt $n = 5$, SP-D^{-/-} $n = 3$, Met SP-D $n = 6$, Thr SP-D $n = 6$). Data are presented as mean + SD, ** $P < .05$, n.s. nonsignificant, OVA-sensitized group compared to the respective control group or as otherwise indicated by lines.

Impact of the Human Met(11)Thr SP-D Polymorphism on miR-21 and miR-155 Expression

SP-D^{-/-} animals had higher miR-21 levels and miR-21 was decreased by OVA challenge. In contrast, OVA challenge of Thr SP-D mice led to a significant increase in miR-21 and miR-155 expression, whereas in Met SP-D mice OVA challenge resulted in a decreased expression of miR-21. In OVA-sensitized mice harboring, the Thr SNP levels of both miRs were elevated compared to the wt, knock-out, and animals with the Met variant (for individual P -values see Figure 5A and B).

DISCUSSION

In this study, we investigated the impact of the human SP-D Met(11)Thr SNP on the inflammatory phenotype in OVA-induced allergic airway inflammation and miRNA-related effects. We hypothesized that mice expressing allelic variants of the SP-D show different degrees of allergic inflammation because allelic SP-D variants differ in their microbial binding affinity, they distinctively modulate expression of inflammatory mediators and seem to increase the susceptibility to certain airway diseases like allergic rhinitis [5–8, 10].

Assessing bronchial hyperreactivity, we found that wt OVA-sensitized mice developed significant airway hyperresponsiveness in response to challenge. OVA-sensitized SP-D^{-/-} and transgenic mice responded to methacholine in a comparable way to wt mice but the

differences compared to groups of sham-sensitized mice did not reach significance.

Investigation of cells in the BAL revealed that Thr SP-D mice exhibited lower total cell numbers and a lower percentage of eosinophils in the BAL compared to wt mice. We did not observe a significant difference between mice expressing one or the other allelic variant of SP-D. Comparing SP-D^{-/-} and wt mice some authors report a higher susceptibility of SP-D^{-/-} mice to allergic inflammation [25, 26] while others describe a lower number of eosinophils and airway hyperreactivity in SP-D^{-/-} mice in response to *Aspergillus fumigatus* challenge [27]. We observed an inflammatory phenotype not significantly different in SP-D^{-/-} compared to wt mice.

With regard to cytokine levels, transgenic mice showed significantly lower IL-5 levels in response to OVA challenge, with lowest levels in Thr mice compared to wt mice. Maturation and differentiation of eosinophils is controlled by IL-5 [28]. Thus, lower IL-5 levels parallel lower eosinophil numbers in Thr SP-D mice. Furthermore, IL-5 levels in these mice did not change substantially to allergen challenge; on the contrary, they resembled levels seen in wt sham-sensitized mice. Surprisingly, sham-sensitized Thr SP-D mice had significantly higher IL-5 levels than sham-sensitized wt mice; we have no explanation for this finding yet. Unlike IL-5, IL-4 levels rose significantly in response to allergen challenge in Thr SP-D mice, suggesting that there was no general Th2 cytokine dysregulation. Further investigations will be required to elucidate IL-5 regulation in mice transgenic for the SP-D Met(11)Thr SNP.

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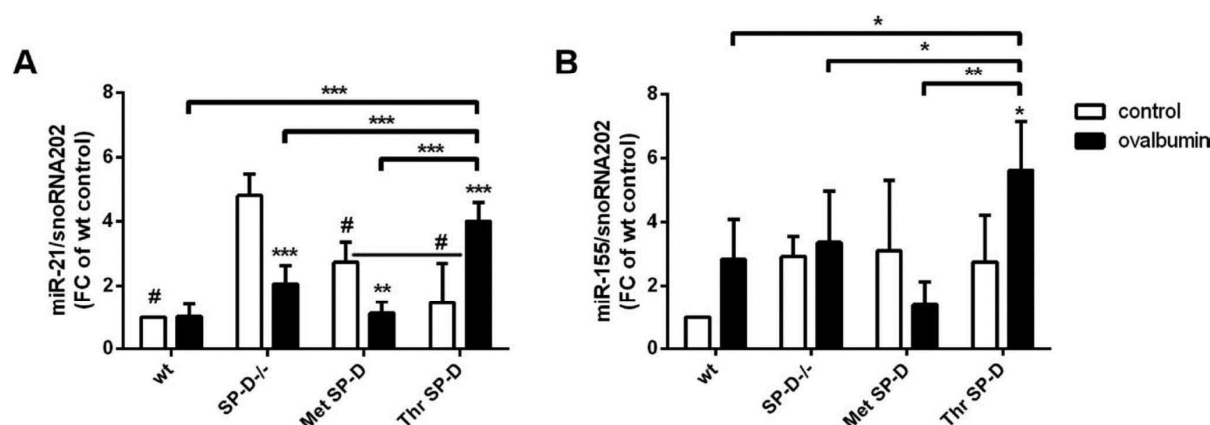


FIGURE 5. Expression patterns of miR-21 (A) and miR-155 (B). Relative miRNA levels in lung tissue ($n = 4$). MiRNA expression is normalized to snoRNA202. FC: fold change. Data are presented as mean $\pm$ SD, * $P < .05$; ** $P < .01$; *** $P < .001$, compared to the respective control groups or as otherwise indicated by lines; # $P < .001$ compared to SP-D-/- control group.

Quantifying epithelial mucus content we did not observe a significant difference between groups of transgenic mice in response to allergen challenge. Compared to wt mice SP-D deficiency had no significant influence on epithelial mucus content, either. It has been shown that intratracheal administration of rat SP-D in mice significantly reduces the number of PAS-positive cells in the airway epithelium [29]. In an *Aspergillus fumigatus* challenge model, epithelial mucus content is not significantly lower in SP-D-/- compared to wt mice [27]. It is possible that mice deficient in SP-D are not entirely deficient in innate immunity. SP-A with its multiple CRDs arranged in a bouquet-like structure may provide a kind of immunological rescue for SP-D-/- mice, helping to clear allergen particles and to lower pro-inflammatory mediators by binding to SIRP-1 α .

With regard to SP-D levels, healthy humans homozygous for Met SP-D possess almost 50% more SP-D in the serum than individuals homozygous for Thr SP-D [5]. Presumably due to ROSA26-driven SP-D expression SP-D serum concentrations in this study were higher than those previously published [5]. The Met/Thr SP-D ratio seen in murine serum samples was similar to the ratio observed in humans. Data on Met and Thr SP-D concentrations in the BAL have not yet been reported; in this study, mean SP-D BAL levels in both groups of transgenic sham-sensitized mice were approximately the same. As far as we can judge from SP-D serum levels, ROSA26-promoted SP-D expression in this mouse model successfully reproduces constitutional differences in human SP-D levels.

In this SP-D Met(11)Thr SNP mouse model, Met SP-D did not alleviate the allergic inflammation com-

pared to Thr SP-D. Results from studies investigating the composition of SP-D under challenging conditions might provide an explanation for this result. Allergen challenge models demonstrate that allergic inflammation (and smoking) can disrupt SP-D multimers and impair protein function [30–33]. Thus, in our asthma model in the course of airway inflammation, large multimers of Met SP-D might have been degraded to SP-D trimers, thereby partly neutralizing its superior microbial binding affinity and silencing of pro-inflammatory mediator production. The oligomerization of SP-D in this SP-D Met(11)Thr SNP mouse model has been documented in unchallenged mice [4]. We did not investigate the oligomerization status in response to allergen challenge and therefore it is possible that postinflammatory modification of SP-D occurred, affecting our results.

MiRs are short single-stranded noncoding RNAs that silence expression of coding genes. MiR-21 is linked to various disease conditions. In myocardial disease, for example, miR-21 appears to promote cardiac fibrosis and hypertrophy and miR-21 silencing by antagomirs has therapeutic effects [34]. Furthermore, miR-21 is upregulated in allergic diseases like allergic asthma, eosinophilic esophagitis and atopic dermatitis [35, 36]. Thus, we hypothesized that allergic inflammation might modulate miRNA expression levels in a SP-D Met(11)Thr SNP mouse model. Our results show that OVA sensitization and challenge is followed by an upregulation of miR-21 and 155 in Thr SP-D mice. In contrast, in Met SP-D mice miR-21 is significantly downregulated implicating different effects of SP-D SNPs on miRNA regulation. Surprisingly, miR-21 levels did not differ between OVA-sensitized and sham-sensitized wt mice

in response to challenge. Our results demonstrate that miR-21 and 155 are differentially regulated in mice transgenic for a human SP-D Met(11)Thr SNP in response to allergen challenge.

CONCLUSION

This study evaluates for the first time the effect of the human SP-D Met(11)Thr SNP in a mouse model of allergic airway inflammation. We could not find a significant difference in the inflammatory phenotype between mice carrying one or the other polymorphic SP-D variant while miRNA expression was differentially regulated. Future investigations of this mouse model under different challenging conditions will be required to further elucidate the impact of this SNP on airway and lung diseases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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SUPPLEMENTARY MATERIAL

Fixation, Removal, and Sampling of Mice Lungs

Briefly, mice were sacrificed, the abdomen was opened and the inferior vena cava exposed. We

introduced an intravenous canula and immediately started a preflush perfusion with 5 to 10 mL of a heparinized 0.9% NaCl solution at a hydrostatic pressure of 30 cm H₂O. At the time most of the blood was removed from the circulation the preflush perfusion was stopped. The trachea was exposed and via a small incision a canula was introduced and fixed with a ligature. The lung was then inflated with a hydrostatic pressure of 13 cm H₂O. In the beginning, the pressure was increased slowly to 25 cm H₂O, lowered to 10 cm H₂O, again increased to 25 cm H₂O and finally set to 13 cm H₂O. Then, the perfusion was carried on with 5 mL of a mixture of 1.5% paraformaldehyde (PFA, Merck, Germany) and 1.5% glutaraldehyde (GA, Agar Scientific, United Kingdom) in 0.15 M 2-[4-(2 Hydroxyethyl)-1-piperazinyl]-ethanysulfonic acid sodium salt (HEPES, Merck, Germany) buffer. The lung was removed and finally immersed in fixative for several days.

Subsequently, the lung was embedded in agar (Agar-Agar, Kobe I, Roth, Germany) and prepared for IUR sectioning. The block was placed onto an equally spaced clock and cut at a random angle. With the cut plane at the bottom the block was placed onto a cosine-weighted clock and again cut at a random angle. The cut plane that results is isotropic. Slabs of equal thickness cut with a random start parallel to this plane are isotropic uniform random (IUR). 1.5 mm thick IUR slabs were cut using a tissue slicer and every third slab randomly allocated to paraffin embedding (Surgiplast, Leica, Germany). Paraffin blocks were cut at 3 μ m and stained with AB-PAS and haematoxylin eosin (HE).

Stereological Assessment

Stereology was performed according to the recommendations by Hsia [24]. Mucus content is expressed by volume density of mucus, that is, volume of mucus per volume of epithelium [$\mu\text{m}^3/\mu\text{m}^3$] estimated by point counting and calculated as follows

$$V_V(\text{muc}, \text{epi}) = \frac{\sum P(\text{muc})}{\sum P(\text{epi})}$$

where $P(\text{muc})$ and $P(\text{epi})$ are points of a stereological test point grid hitting AB-PAS positive mucus resp. epithelium.

Volume density of mucus normalized to the surface area the epithelium covers, that is, volume of mucus per surface area of epithelium [$\mu\text{m}^3/\mu\text{m}^2$] is calculated as follows

$$V_S(\text{muc}, \text{epi}) = \frac{\sum P(\text{muc}) \cdot l/p}{2 \cdot \sum I(\text{epi})}$$

where $P(\text{muc})$ are points hitting mucus, $I(\text{epi})$ are intersections of test lines with the epithelium and l/P is the length per point of a combined test line and point grid. $V_s(\text{epi}, \text{epi})$ represents mean epithelial layer thickness.

The ratio of total surface area to surface area covered by epithelial mucus was estimated by intersection counting and calculated as follows

$$S(\text{muc}/\text{epi}) = \frac{I(\text{muc})}{I(\text{epi})}$$

where $I(\text{epi})$ and $I(\text{muc})$ are intersections of a line grid with epithelial mucus resp. with the epithelium.

Measurement of Pulmonary Function

A 24 hours after the final OVA aerosol challenge, pulmonary function was measured invasively to assess AHR as described previously [22, 23]. Briefly, mice were intubated orotracheally and placed in supine position in temperature-controlled body plethysmographs (type 871, HSE-Harvard Apparatus, March-

Hugstetten, Germany). Pulmonary resistance (RL) was calculated from the measured differences in transpulmonary pressure and tidal respiratory flow over each entire breath cycle under isoflurane anesthesia (by HEM 4.2 software, Notocord Systems, Croissy, France). After recording baseline values for 2 min, AHR to aerosolized methacholine chloride (MCh, from 5% solution; Sigma, Deisenhofen, Germany) was determined using a Bronchy type III generator (Fraunhofer ITEM) under continuation of lung function measurement. The total dose inhaled via the orotracheal tube (in μg) was calculated and controlled by a computerized feedback dose control system (Fraunhofer ITEM; [23] based on the continuously measured respiratory minute volume and photometrical aerosol concentration. The mice were exposed to increasing MCh dose steps of 0.063, 0.125, 0.3, 0.5, 1, 2, 4, 8, and 16 μg . From individual dose effect plots (delta% RL vs. MCh dose) the effective inhalation dose in μg MCh required to produce a 150% increase in RL was evaluated for each animal ($\text{ED}_{150\text{RL}}$).

4. Statement of contribution

4.1 Circulating microRNAs as potential biomarkers of aerobic exercise capacity

In this study, I was involved in the conceptual design and selection of candidate miRNAs. Plasma samples, runner characteristics and biochemical analysis were provided by FC Mooren and K Krüger. I prepared RNA from the plasma samples and performed real-time PCR-based quantification of miRNA expression. Further, I analysed potential target genes of candidate miRNAs provided in the online supplements. Finally, I supported manuscript writing.

4.2 Long non-coding RNA *Chast* promotes cardiomyocyte hypertrophy

I conceived this study, designed the experiments and wrote the manuscript with the help of T Thum and R Kumarswamy. I identified candidate lncRNAs, performed and analysed the *in vitro* experiments including hypertrophy assays, real-time PCR quantification of gene expression, cloning, immunohistochemistry, and chromatin immunoprecipitation. For the performance and evaluation of *in vivo* experiments, as well as for the generation of tissue and primary cell samples I was supported by S Batkai, A Foinquinos, K Zimmer, and J Remke. I supported in bioinformatic analysis of profiling data sets and transcript specific examinations that were mainly conducted by T Dandekar, K Xiao, M Dittrich, and M Kunz. My experimental work was further assisted by A Just, J Fendrich, and K Scherf.

4.3 Impact of a Met(11)/Thr single nucleotide polymorphism of surfactant protein D on allergic airway inflammation in a murine asthma model

In this study, I performed RNA isolation from murine lungs and miRNA expression analysis by real-time PCR. In addition, I helped with the manuscript preparation.

5. Discussion

The discovery of ncRNAs and the elucidation of their function is an important step forward in the understanding of the molecular biology of physiological and pathological adaptations. This knowledge is mainly based on transcriptome profiling studies unveiling transcripts that have been considered as noise of gene expression for a long time. Such insights enable to extensively map the genome, to improve the understanding of its composition and to identify novel targets for diagnosis and therapy. Novel ‘theranostics’ harbour the potential to complement or replace traditional biomarker and pharmaceuticals.

5.1 Circulating microRNAs as biomarkers for aerobic exercise capacity

MiRNAs are important regulators of gene expression, also affecting the cardiovascular system. Recent findings indicate that miRNAs are not only restricted to processes within the cell, but also circulate in the bloodstream. Further, their extracellular levels change in response to stress including disease conditions as well as exercise activity, providing a valuable diagnostic tool to monitor the physiological state of a human being. A marathon run is an extreme exertion that implicates cardio-respiratory stress as well as physiological adaptations. However, less is known about the involvement of miRNAs in the body’s response to such an acute bout of endurance exercise. Therefore, we assessed the effect of a marathon run on a selected panel of circulating miRNAs.

5.1.1 Running a marathon alters plasma levels of heart- and muscle-specific miRNAs

Our findings revealed that performing a marathon run elevates the levels of muscle and heart-enriched miRNAs in the circulation, while inflammatory miRNAs showed no response. The prolonged increase of the myomiRs (miR-1, -133a, and -206) up to 24 h was highly correlated and slightly deviates from the course cardiomiRs (miR-499 and -208b), indicating an independent regulatory mechanism during exercise. The circulating myomiRs were well correlated with markers of aerobic exercise capacity including maximum oxygen uptake (VO_{2max}) and individual aerobic lactate threshold (V_{IAS}), while none of the miRNAs was associated to cardiac damage markers. These results suggest that myomiRs and cardiomiRs are involved in the physiological adaptation to strenuous exercise and indicate their potential value as biomarkers of aerobic exercise capacity.

Our findings are in line with previous and subsequent studies. Uhleman *et al.*¹³⁸ found a comparable increase of miR-133 in the circulation of subjects directly after finishing a marathon. Gomes *et al.*¹⁴⁰ assessed the levels of all three myomiRs (miR-1, -133a, and -206) and again identified an elevation in the plasma of runners finishing a half-marathon. Accordingly, Baggish *et al.*¹³⁹ investigated the expression of miR-1, -133a, -499, and -208 and showed that all these miRNAs were immediately elevated after the marathon. Taken together, these data indicate that a specific type of exercise exhibits a typical pattern of deregulated circulating miRNAs. This pattern can change with respect to exercise

type, duration and intensity.^{134, 138, 141, 143, 147} Inflammatory miRNAs analysed in our study were not affected by the marathon run. Circulating levels of miR-155 in response to exercise have not been investigated so far, but other studies indicate dynamic changes of miR-21. A single bout of exhaustive cycling or rowing training for 90 days induced miR-21 in plasma.¹³⁴ This miRNA was elevated and correlated with the fitness level of subjects¹⁴⁶ and exhibits different expression patterns after endurance and resistance training.¹⁴⁷ Further, miR-21 was decreased in response to chronic endurance training.¹⁴⁴ Again, several factors may explain the discrepancies between the current and other studies, including training level, mode and extent of exercise, methodological and experimental design, as well as differences in age, genetics and environmental background of the participants.¹⁸⁸ But in general, inflammamiRs seem to be dynamically regulated after a marathon run¹⁴³ or other bout of acute endurance exercise¹⁴⁴ and could be of interest for further investigations.

Our study exhibits another limitation with respect to the sample size. To date, larger cohorts of marathon runners or individuals performing exercise have not been assessed. Therefore, the recent work is in line with the efforts of other authors. This study was conceived as a pilot study with the intention to explore the potential of circulating miRNAs as biomarkers for exercise performance and fitness, aside their well-established role as biomarkers for cardiovascular disease among others.¹⁸⁹ Therefore, the number of participants was limited to 14 male endurance athletes. However, the participants showed homogenous characteristics according to sex, running experience and training history, as well as further performance parameter. To increase the informative value of this report, we performed repeated measures and compared them with the resting state. The candidate miRNAs were selected regarding their specificity to heart and skeletal muscle as well as inflammation¹⁹⁰⁻¹⁹³ and, to prevent an impact of haemolysis, because the miRNAs selected are not present in blood cells. Deregulation of miRNAs as an effect of exercise-induced plasma concentration was circumvented by normalizing the expression to an exogenous spike-in control. Thus, the experimental setup provided better accuracy of the results.

5.1.2 Circulating miRNAs are indicators for the runners performance

All the findings of our and other studies together emphasize the potential role of circulating miRNAs as markers of exercise physiology. In most cases, the cell source for circulating miRNAs remains speculative. Although several miRNAs have been reported to be either specifically or enriched in the heart or the skeletal muscle^{193, 194}, the majority of miRNAs are expressed in several tissues and cell types although to different extent¹⁹⁵. They can be secreted into the extracellular space, including the blood stream. For example, miR-133a is highly expressed in heart and skeletal muscle¹⁹³, but also present in liver¹⁹⁶, lung¹⁹⁷, or brown adipose tissue¹⁹⁸. Accordingly, a direct identification of the source of exercise-induced miRNAs in the circulation remains challenging. The elevation of myomiRs and cardiomiRs can be explained by cellular leakage with destroyed tissue following strenuous exercise. Several studies addressed the potential role of such miRNAs as biomarkers of an infarcted heart¹⁹⁹ or

injured skeletal muscle¹³⁸. Additionally, since exercise can induce apoptosis, miRNAs can be released from apoptotic cells.¹³⁵ Uhleman *et al.*¹³⁸ identified a potential relation between exercise-induced elevation of miRNAs in the bloodstream and muscular damage. Banzet *et al.*¹⁴¹ found that especially eccentric exercise, that is associated with a high degree of muscle damage, led to an increase of circulating muscular miRNAs. However, this explanation is not transferrable to the current study, since the myomiRs correlated with makers of exercise performance rather than with plasma levels of the classical muscle damage marker creatine kinase. MiRNA levels were stable over a longer period. This makes a release by cell rupture less likely, since such a sudden event would lead to potentially insufficient packed or shielded RNAs that, with respect to high levels of RNase activity in the bloodstream, would be rapidly degraded.⁴³ In addition, miRNAs investigated and levels of creatine kinase followed different time courses, a finding that is in line with the observation from Baggish *et al.*¹³⁴. Another explanation of the elevated miRNA levels in circulation might be due to a general induction of miRNA biogenesis pathways in response to exercise, suggesting an association between exercise and an enhancement of muscle function.²⁰⁰ A potential contribution of apoptotic or necrotic events cannot be entirely excluded, since apoptotic bodies are a common source of miRNAs⁴⁵ and exercise is a well-described inducer of cell apoptosis in e.g. lymphocytes²⁰¹.

Aside the source of exercise-induced miRNAs, the uptake from the circulation into certain recipient cells remains open. Several reports have shown that miRNAs enclosed in exosomes can be transferred from cell to cell, where they alter the gene expression and the biological function, accordingly.^{49, 202} For example, miR-486 was found to be decreased immediately after a single bout of cycling exercise and during the resting state over 4 weeks.¹⁴² The same group found that miR-486 levels tended to increase in the skeletal muscle of exercising mice and indicating a potential uptake mechanism from the circulation to muscular cells enhancing glucose uptake.²⁰³

Considering the myriad of targets and subsequent functions of one miRNA, the effect of its release on the donor cell cannot be disregarded. A few studies have investigated the impact of exercise on miRNA expression within muscle tissues. MyomiRs and cardiomiRs seem to be involved in the development of heart and skeletal muscle that in turn impact performance parameter during physiological training.^{190, 191, 193} Our data suggest that the myomiRs miR-1, -133a, and -206 seem to be of particular relevance for physiological adaptations to exercise. Within the muscle tissue, the expression of miRNAs have been reported to be down-regulated upon chronic exercise¹³⁷ or due to physiological hypertrophy²⁰⁴, but induced by an acute bout of exercise¹³⁷. Altered miRNA expression pattern involve a transient decrease of protein synthesis¹³⁷ that has been observed in the adaptive phase after exercise performance²⁰⁵. This might enhance energy restoration and metabolic processes rather than structural protein synthesis. To address this question, we generated a list of endurance-related genes and analysed potential targeting by the candidate myomiRs or cardiomiRs. Heart-related miRNAs such as miR-208b and -499 seem not to be associated with endurance-related genes. Compared to the myomiRs miR-1 and -206, a significant portion of the genes are potential targets.

As the release and uptake of exercise-induced circulating miRNAs is not clear, the mechanism of clearance is less studied. Of course, the uptake itself is one potential mechanism how miRNAs might be removed from the bloodstream as it has been described for miR-486.²⁰³ Another possibility is an increased degradation by the kidney or liver. Alternatively, nucleolytic degradation might occur.¹³⁹ So far, this aspect of circulating miRNAs remains speculative and is an exciting question that should be addressed in future.

5.1.3 Conclusion

In summary, our findings demonstrate that a marathon race affects the plasma levels of heart and skeletal muscle-related miRNAs. The limited correlation between miRNAs and indicators of muscle tissue damage or cardiac dysfunction suggests a release mechanism independent from cellular leakage and a value as potential stand-alone biomarker related to aerobic fitness. In addition, the plasma profile of circulating miRNAs such as miR-1, -133a, and -206 and their strong association to conventional performance parameters point a possible application as biomarkers for aerobic exercise capacity. Potentially, our data suggest an adaptive molecular mechanism to strenuous exercise that would be interesting to be evaluated in more detail by future studies.

5.2 LncRNA *Chast*: a novel therapeutic lncRNA target for the treatment of cardiac disease

LncRNAs are an emerging class of non-coding regulators that recently have been implicated to play a crucial role in the pathophysiology of the heart. However, the underlying mechanisms and the value of lncRNAs as therapeutic targets remain largely unknown. To address this question we aimed to identify non-coding transcripts that are involved in the development of pathological hypertrophy and to translate these findings into a therapeutic strategy.

5.2.1 Pathological hypertrophy affects the cardiac ‘lncome’ and alters *Chast* expression

To unravel changes in the lncRNAs transcriptome (‘lncome’) upon pressure overload-induced cardiac hypertrophy and to identify candidate transcripts for therapeutic application, we performed and validated an array-based lncRNA profiling. Although RNA sequencing provides advantages such as de novo detection of transcripts independent of prior annotations, differentiation of isoforms or genetic variants²⁰⁶, microarrays are still a common and well established method. Furthermore, microarray data allow better detection and sensitivity for low-abundance transcripts^{207, 208}, a general feature of lncRNAs^{1, 29}. For higher accuracy of the microarray validation, we excluded lncRNAs that overlap in sense to protein-coding genes and tested their presence in heart samples. Finally, we focused on *Chast*, since this lncRNA was significantly induced in pressure-overloaded mouse hearts and specifically in cardiomyocytes, indicating a potential involvement in cardiac hypertrophy. Therefore, we assumed

that inhibition of this transcript might provide a therapeutic benefit, as it has been shown for other RNA species (e.g. miRNAs^{209, 210}).

5.2.2 *Chast* is a non-coding transcript

An important feature of lncRNAs is their non-coding nature implying a lack of translational processing of such transcripts. However, recent studies indicate that lncRNAs can associate with ribosomes¹⁸ and some of these transcripts serve as template for small peptides²⁰. To address this question, we analysed *Chast* regarding translational signatures. Indeed, the sequence harboured putative open reading frames and stop codons, but mass spectrometry based analysis of cardiomyocytes over-expressing *Chast* indicated that none of the predicted peptides was detected suggesting *Chast* as a true non-coding RNA. Aside the non-coding nature of lncRNAs, which determines their function as RNA, the subcellular localization within a cell can provide further insights into the transcripts biology.²¹¹ In contrast to messenger RNAs that are predominantly found in the cytosol, the compartment of translation, lncRNAs themselves have to reach their specific target site making their localization within the cell much more essential. In the nucleus, lncRNAs can be involved in the regulation of proximal or distal DNA stretches, in the cytosol, miRNAs can be possible targets.²¹¹ Interestingly, *Chast* was found to be present in all compartments of cardiomyocytes, indicating potential function on different target sites.

5.2.3 *Chast* is involved in the regulatory network of cardiac hypertrophy

Comparable to the cardiomyocytes-specific induction by cardiac stress *in vivo*, *Chast* expression was sensitive to hypertrophic conditions *in vitro*. This induction seems to be mediated by the activity of the pro-hypertrophic transcription factor NFAT that was shown to be a regulatory element of the *Chast* promoter and its expression. Further, enhanced expression of *Chast* was sufficient to provoke hypertrophic growth and to reactivate cardiac stress markers in the heart as well as in cardiomyocytes. The underlying mechanism and targets downstream of *Chast* activity are currently speculative. To identify cardiac relevant genes that are modulated by *Chast*, we performed mRNA expression profiling of cardiomyocytes with depleted *Chast* expression. Gene ontology analysis demonstrated that pathways related to *Wnt* signalling, muscle development and cardiomyopathy were affected. Further studies are needed to confirm the relation between *Chast* and critical genes involved in the pathways mentioned. A more detailed mechanism can be assumed considering the genomic localization of *Chast*. This transcript is encoded opposite to *Ahrgap27* (Rho GTPase activating protein 27) and *Plekhm1* (Pleckstrin homology domain-containing family M member 1) suggesting that *Chast* is a natural antisense transcript acting in a potential *cis* regulatory manner. Although *Chast* has been recently annotated as *Ahrgap27* antisense gene (*Ahrgap27-os1*), preliminary data (not shown) do not indicate a relation among these two genes. In general, *Ahrgap27* is poorly expressed in heart tissue as well as cardiomyocytes. Neither pressure-overload induced cardiac hypertrophy nor deregulation of

Chast had an impact on *Ahrgap27* expression (data not shown), suggesting an independent expression of both genes. In contrast, *Plekhm1* was significantly down-regulated in hypertrophic mouse hearts and induced upon *Chast* repression (data not shown), indicating that *Chast* might regulate the expression of *Plekhm1*. This protein is of interest, since it has been described recently as an endolysosomal adaptor that links autophagic and endocytic pathways at the lysosome.^{86, 212} Autophagy is a catabolic self-degradative process maintaining the cellular homeostasis and plays a crucial role in cardiac hypertrophy.^{210, 213} Therefore, targeting of *Plekhm1* might explain prohypertrophic effects mediated by *Chast*. Further studies are planned to address this potential mechanism. Since *Chast* is not restricted to the nucleus, but also found in the cytosol, suggests another mode of action. As lncRNA *Chrfa66*, *Chast* might act as an endogenous competing RNA regulating the abundance and function of miRNAs. Preliminary data (not shown) based on computational predictions and experimental RNA-RNA interaction studies indicate that miRNAs potentially bind to *Chast*. The mechanistic consequence remains to be elucidated. Despite the mechanistic limitations of this study, our recent data involving *Chast* as an actor in pathological hypertrophy indicate that this transcript is an interesting candidate for therapeutic applications.

5.2.4 Pharmacological inhibition of *Chast*: a beneficial and translational therapy

To examine if the prohypertrophic effect of *Chast* observed *in vitro* and *in vivo* harbours any translational potential, we applied *Chast* inhibitors as possible treatment option for cardiac hypertrophy. Since to date lncRNAs-based treatments are still lacking²¹⁴, this strategy is based on miRNA therapeutics that have been established successfully. Indeed, an oligonucleotide antagonist against miR-122 for the treatment of hepatitis C already reached clinical trial phase IIa.^{214, 215} In cardiovascular research, pharmacological inhibition of miRNAs are used to prevent cardiac hypertrophy and heart failure.^{209, 210} Since these therapeutics are mainly based on the recruitment of the cytosolic RNA interference machinery, they are not applicable for lncRNAs that are localized to and function in the nucleus; that is also true for *Chast*. To fully block *Chast* activity in both cellular compartments, we applied GapmeRs, single-stranded hybrid oligonucleotides harbouring a central DNA stretch flanked by locked nucleic acids. Target lncRNA recognition in the nucleus recruits RNase H and induces nucleolytic degradation.²¹⁶ Recently, this chemistry has been applied to silence the lncRNAs *Malat1* in an *in vivo* model of cardiovascular disease.²¹⁷ GapmeR-mediated silencing of *Chast* *in vivo* circumvented the induction of *Chast* and subsequently abrogated pressure-overload induced hypertrophic growth and gene expression accompanied by an improved heart function. Therefore, we assume that pharmacological inhibition of *Chast* is of therapeutic value for the treatment of cardiovascular disease.

Moreover, the translational potential of this study is underlined by the identification of an EST in the human genome which exhibits several analogies to the mouse transcript. Despite lacking an overall sequence identity, a specific stretch of the murine sequence harbours a relative high sequence

homology to the human *CHAST* and both transcripts seem to share a similar secondary structure fold. In general, lncRNAs lack strong conservation on the sequence level.^{24, 27} Homology is suggested to be largely reflected by secondary structure motifs, rather than by the primary sequence.²¹⁸ Functional interactions with DNA, RNA or proteins are another potential indicator for evolutionary relations among transcripts.²⁸ For example, the X chromosome inactivating lncRNA *Xist* shows poor similarities on the sequence level, but its unique function is well conserved among species.³⁶ Accordingly, as observed in murine pressure-overloaded hearts, expression levels of the human *CHAST* were significantly elevated in myocardial tissue of aortic stenosis patients, thus indicating a possible conservation of the murine *Chast* function. Although further details of this interspecies relation need to be established, our study provides the basis for prospective clinical improvements of *Chast* therapeutics as a new treatment options for cardiovascular disease.

5.2.5 Conclusion

Based on genome-wide lncRNA profiling we identified the novel lncRNA *Chast* that was upregulated by cardiac stress. This transcript promotes pathological hypertrophy in cultured cardiomyocytes and hypertrophic heart growth in mice. Consequently, *in vivo* targeting of *Chast* protected from cardiac hypertrophy and the development of heart failure. Identification of a potential *Chast* homolog in humans sets the ground for future lncRNA interference therapies for the treatment of pathological cardiac hypertrophy and heart failure.

5.3 The impact of SP-D polymorphism on asthma and inflammamiR expression

Less is known about the impact of the SP-D Met(11)Thr polymorphism on the inflammatory phenotype of allergic airway inflammation. To address this issue, we analysed the susceptibility and inflammatory response of SP-D deficient and humanized mice carrying on of the polymorphic alleles to allergic asthma. Further, we evaluated related effects on the expression of two miRNAs involved in inflammation.

5.3.1 None of the polymorphic SP-D variants influenced the severity of allergic inflammation

Assessing lung function, pulmonary inflammation and lung morphology upon asthma challenge, we did not observe significant differences between mice harbouring either the human Met or the Thr allele. These findings might reflect an insufficient function of the transgenic human Met SP-D upon asthma challenge. Differences in oligomerization due to the SP-D polymorphism have been investigated in unchallenged mice, only.⁹⁸ Other studies have shown that exposure to allergens or smoke impairs the multimeric structure, and thus the immunomodulatory properties of SP-D.^{108, 219} In our asthma model, the challenge with ovalbumin might have provoked depolymerisation of SP-D to

trimers diminishing its protective effects in allergen-induced inflammatory response. Therefore, assessing the oligomerization state of Met or Thr SP-D upon allergic challenge might be of interest in further studies.

5.3.2 InflammamiRs were differentially regulated by the Met(11)Thr SP-D polymorphism

We assessed the impact of the human SP-D Met(11)Thr polymorphism on the expression of miR-21 and miR-155. Both miRNAs have been described as modulators of the polarized inflammatory response in allergic asthma.^{179, 184} Under basal conditions, SP-D deficient mice and animals harbouring one of the polymorphic alleles displayed at least a tendency of elevation in both miRNA levels, indicating a potential relation between SP-D and these inflammamiRs. SP-D has been shown to have a regulatory effect in asthma by inhibiting the allergen-induced response.^{111, 120} Consequently, impaired SP-D function might lead to an exacerbation of the inflammatory response. This can be due to elevated miR-21 and miR-155 levels observed in this study, since both miRNAs have been shown to contribute to the regulation of allergic airway inflammation.^{179, 184} Further, acute asthma challenge led to a significant induction of both miRNAs in lungs of transgenic mice expressing the Thr variant, while in Met mice and SP-D deficient animals miR-21 was significantly reduced. This finding indicates a differential regulation of miRNA expression potentially as a consequence of the SP-D polymorphism. The underlying mechanism remains elusive. Upon ovalbumin challenge, wt mice tended to elevated expression of miR-155 that is in line with previous observations.¹⁸⁴ In contrast to other studies¹⁷⁸, miR-21 levels did not discriminate between asthmatic and non-asthmatic wt mice. This might be due to different genetic models applied in our and the former study. For example, genetic drift in several substrains of C57BL6 mice causes genomic and phenotypic differences.^{220, 221} An interesting question would be to identify the cell type in the lung that is the source as well as contributes to the differential regulation of the miRNAs; an open matter that will be addressed in future.

5.3.3 Conclusion

In conclusion, we assessed the susceptibility of transgenic mice harbouring the human SP-D Met(11)Thr polymorphism in an acute model of allergic asthma. Although airway hyperreactivity, allergic inflammatory response, and mucous metaplasia did not discriminate between mice carrying one or the other polymorphic SP-D variant, the expression of the inflammamiRs miR-21 and miR-155 were differently regulated. Further studies are needed to elucidate the influence of this polymorphism on inflammatory response in the lung.

6. Future perspectives of ncRNA ‘theranostics’

NcRNAs including miRNAs and lncRNAs add a new layer of complexity to the molecular architecture of physiological and pathological adaptations in human beings. However, this complexity of the transcriptome opens a myriad of possibilities for diagnostic markers and ‘druggable’ targets. NcRNAs are potential biomarkers, because they are easily accessible in biological fluids. Their highly specific expression patterns can be used for diagnosis as well as for therapy. These emerging applications are limited by the understanding of the biology of such transcripts. Recent studies focus on deregulated ncRNAs and apply pharmacological inhibition of upregulated transcripts or gene therapeutic approaches to enhance the expression of down-regulated ones. With respect to reliable delivery systems, effectivity of such therapeutics and techniques to reduce off-target effects, these strategies are still challenging. Further, they do not consider that physiological and pathological adaptations might be caused by structural and subsequent functional changes of ncRNA activities that are poorly understood so far. Nevertheless, a better knowledge of ncRNAs and an overcome of current hurdles, will improve diagnostic and therapeutic applications.

6.1 NcRNAs as biomarkers of aerobic exercise capacity

Circulating miRNAs harbour great potential as biomarkers, not only as diagnostics and prognostics for disease, but also to indicate, monitor, and optimize the training status of athletes. Accessing miRNAs as well as mRNAs and lncRNAs that circulate in the bloodstream due to exercise induced cell damage or adaptations would provide a plethora of biomarkers to monitor and determine risk factors as well as the fitness level of sporting subjects. Therefore, an additional approach would be to perform a profiling covering the whole circulating transcriptome. A challenging question for the future is to identify the source of such circulating transcripts as well as their effect on recipient cells.

6.2 NcRNAs as therapeutic targets in cardiovascular disease

Based on our current findings it is apparent that the lncRNA *Chast* is an important regulator of pathological remodelling in the heart and that *Chast* inhibition provides a promising strategy for future therapies. Decoding the mechanism of *Chast* activity in cardiomyocytes and other cardiac cells is an important question that will be addressed in the future. Assessing the role of *Chast* and the outcome of pharmacological inhibition in other models of cardiac remodelling would provide a more detailed understanding of the lncRNA and its role in the heart. This involves application of approaches with clinical relevance modulating *Chast* expression either when cardiac remodelling already has been established or in the remodelling phase after myocardial infarction. Identifying *Chast* homologs in other vertebrates would enable large animal studies to develop therapeutics to counteract cardiac remodelling in human hearts.

6.3 NcRNAs as regulators in asthma and SP-D deficiency

Our study identified inflammamiRs that were differentially regulated in SP-D deficient mice and animals harbouring one of the SP-D polymorphisms. Elucidating the role of such miRNAs contributing to each SP-D phenotype is an interesting future question. A successive approach would be to access miRNA deregulation by pharmacological inhibition as well as using a genetic knockout model deficient in SP-D as well as miR-21. Identification of the pulmonary cell type contributing to the miRNA deregulation would be of interest as well.

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Publications and presentations

Research article

- 1) **Viereck, J.**, Kumarswamy, R., Foinquinos, A., Xiao, K., Batkai, S., Avramopoulos, P., Kunz, M., Dittrich, M., Maetzig, T., Zimmer, K., Remke, J., Just, A., Fendrich, J., Scherf, K., Schambach, A., Weidemann, F., deWindt, L. J., Engelhardt, S., Dandekar, T. & Thum, T. Long Non-coding RNA *Chast* Promotes Cardiomyocyte Hypertrophy. *Sci Transl Med.* in revision (2015).

The study presented in this work meanwhile has been published as:

Viereck, J.*, Kumarswamy, R.*, Foinquinos, A.*, Xiao, K., Avramopoulos, P., Kunz, M., Dittrich, M., Maetzig, T., Zimmer, K., Remke, J., Just, A., Fendrich, J., Scherf, K., Bolesani, E., Schambach, A., Weidemann, F., Zweigerdt, R., de Windt, L. J., Engelhardt, S., Dandekar, T., Batkai, S. & Thum, T. Long noncoding RNA *Chast* promotes cardiac remodeling. *Sci Transl Med.* **8**, 326ra22 (2016).

- 2) Winkler, C., Bahlmann, O., **Viereck, J.**, Knudsen, L., Wedekind, D., Hoymann, H. G., Madsen, J., Thum, T., Hohlfeld, J. M. & Ochs, M. Impact of a Met(11)Thr single nucleotide polymorphism of surfactant protein D on allergic airway inflammation in a murine asthma model. *Exp Lung Res.* **40**, 154-63 (2014).
- 3) Mooren, F. C.*, **Viereck, J.***, Krüger, K. & Thum, T. Circulating microRNAs as potential biomarkers of aerobic exercise capacity. *Am J Physiol Heart Circ Physiol.* **306**, H557-63 (2014).

Review article

- 1) **Viereck, J.**, Kumarswamy, R. & Thum, T. Long noncoding RNAs as inducers and terminators of vascular development. *Circulation.* **131**, 1236-8 (2015).
- 2) Foinquinos, A., **Viereck, J.** & Thum, T. MicroRNA-Mediated Regulation of Cardiovascular Differentiation and Therapeutic Implications. *MicroRNA in Regenerative Medicine*, edited by Sen C. K., 1075-1091 (2015).
- 3) Kunz, M., Xiao, K., Liang, C., **Viereck, J.**, Pachel, C., Frantz, S., Thum, T. & Dandekar, T. Bioinformatics of cardiovascular miRNA biology. *J Mol Cell Cardiol.* **14**, 00387-3 (2014).
- 4) **Viereck, J.**, Bang, C., Foinquinos, A. & Thum, T. Regulatory RNAs and paracrine networks in the heart. *Cardiovasc Res.* **102**, 290-301 (2014).

Talks

- 1) 81. Jahrestagung der DGK, Deutsche Gesellschaft für Kardiologie, 8th April 2015, Mannheim (Germany) – ‘Long non-coding RNA *Chast* promotes cardiomyocyte hypertrophy’
- 2) 81. Jahrestagung der DGK, Deutsche Gesellschaft für Kardiologie, 8th April 2015, Mannheim (Germany) – ‘The H19 long non-coding RNA is linked to pathological cardiac hypertrophy’
- 3) 79. Jahrestagung der DGK, Deutsche Gesellschaft für Kardiologie, 4th April 2013, Mannheim (Germany) – ‘Marathon running affects plasma profiles of heart- and muscle-specific microRNAs’

Poster

- 1) The Non-Coding Genome, EMBO|EMBL Symposium, 20th October 2015, Heidelberg (Germany) – ‘Inhibition of long-noncoding RNA *Chast* prevents pressure-overload induced cardiac hypertrophy’
- 2) The Non-Coding Genome, EMBO|EMBL Symposium, 19th October 2015, Heidelberg (Germany) – ‘The H19 long-noncoding RNA is linked to pathological hypertrophy’
- 3) International Symposium 50 Years of Scientific Excellence at the Hannover Medical School, Hannover Medical School, Hannover (Germany), 25th September 2015 – ‘Inhibition of long non-coding RNA *Chast* prevents pressure-overload induced cardiac hypertrophy’
- 4) 2nd World Congress on Acute Heart Failure, European Society of Cardiology, 25th May 2015, Seville (Spain) – ‘Long non-coding RNA *Chast* promotes cardiomyocyte hypertrophy’
- 5) 2nd World Congress on Acute Heart Failure, European Society of Cardiology, 25th May 2015, Seville (Spain) – ‘The H19 long non-coding RNA is linked to pathological cardiac hypertrophy’
- 6) Mechanisms of cardiovascular diseases and the road to therapy – a dialogue between basic and translational science, MHH-Klinik für Kardiologie und Angiologie, 12th September 2014, Hannover (Germany) – ‘Long non-coding RNA *Chast* promotes cardiomyocyte hypertrophy’
- 7) 3rd DZL symposium, Deutsche Zentrum für Lungenforschung, 8th May 2014, Hannover (Germany) – ‘The role of surfactant protein D in fibrotic lung remodelling’
- 8) 80. Jahrestagung der DGK, Deutsche Gesellschaft für Kardiologie, 24th April 2015, Mannheim (Germany) – ‘Circulating microRNAs as potential biomarkers of aerobic exercise capacity’
- 9) 80. Jahrestagung der DGK, Deutsche Gesellschaft für Kardiologie, 26th April 2015, Mannheim (Germany) – ‘The role of surfactant protein D in fibrotic lung remodelling and implications for the heart’
- 10) The Non-Coding Genome, EMBO|EMBL Symposium, 10th October 2013, Heidelberg (Germany) – ‘Circulating non-coding RNAs as potential biomarkers of aerobic exercise capacity’

Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently, and that I have not submitted it at any other university worldwide.

Hannover, November 2015

Janika Viereck