Institute for Experimental Medical Research
Oslo University Hospital and University of Oslo
and
KG Jebsen Cardiac Research Center and
Center for Heart Failure Research, University of Oslo

The extracellular matrix in heart failure:

Proteoglycans and collagens as regulators of inflammation, fibrosis and cardiac dysfunction

Mari Elen Strand, MSc

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Strand ME, Herum KM, Rana ZA, Skrbic B, Askevold ET, Dahl CP, Vistnes M, Hasic A, Kvaløy H, Sjaastad I, Carlson CR, Tønnessen T, Gullestad L, Christensen G, Lunde IG.

FEBS J 2013;280:2228-2247.

II. Shedding of syndecan-4 promotes immune cell recruitment and mitigates cardiac dysfunction after lipopolysaccharide challenge in mice

Strand ME, Aronsen JM, Braathen B, Skrbic B, Kvaløy H, Sjaastad I, Tønnessen T, Christensen G, Lunde IG.

Resubmitted after revision, J Mol Cell Cardiol, 2015.

III. Lumican is increased in experimental and clinical heart failure, and its production by cardiac fibroblasts is induced by mechanical and proinflammatory stimuli

Engebretsen KVT, Lunde IG, Strand ME, Waehre A, Sjaastad I, Marstein HS, Skrbic B, Dahl CP, Askevold ET, Christensen G, Bjørnstad JL, Tønnessen T.

FEBS J 2013;280:2382–2398.

IV. Lack of collagen VIII reduces fibrosis and promotes early mortality and cardiac dilatation in pressure overload in mice

Skrbic B, Engebretsen KVT, Strand ME, Lunde IG, Herum KM, Marstein HS, Sjaastad I, Lunde PK, Carlson CR, Christensen G, Bjørnstad JL, Tønnessen T.

Cardiovasc Res 2015;106:32-42.

Selected abbreviations

AB	aortic banding
ADAMTS	a disintegrin and metalloproteinase domain with thrombospondin motifs
AS	aortic stenosis
α-SMA	alpha smooth muscle actin
AVR	aortic valve replacement
DAMP	damage-associated molecular pattern
DCM	dilated cardiomyopathy
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
GAG	glycosaminoglycan
HFpEF	heart failure with preserved ejection fraction
HPLC	high performance liquid chromatography
HSPG	heparan sulfate proteoglycan
IL	interleukin
KO	knock-out
KS	keratan sulfate
LOX	lysyl oxidase
LPS	lipopolysaccharide
LV	left ventricle
MMP	matrix metalloproteinase
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
SLRP	small leucine-rich proteoglycan
SMAD	small mothers against detrapleigic
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor

Introduction

Heart failure

Heart failure is a major global health issue with high prevalence, morbidity, mortality and socioeconomic impact. Defined as "a pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues",[1] heart failure is a complex, progressive clinical syndrome of multiple etiologies and courses. It can result from any form of cardiovascular disease, including valvular disease, hypertension, ischemic heart disease, myocarditis and cardiomyopathies. Heart failure is the leading cause of hospitalizations and mortality in the Western world. An estimated 23 million people worldwide, including 15 million in Europe, are living with heart failure.[2-4] Both prevalence and mortality are increasing,[3, 5, 6] and today, the 5-year mortality is around 50%.[7-9] The aging population in conjunction with advances in acute cardiac care and management of coronary artery disease[10, 11] is predicted to lead to more patients with risk factors for heart failure.[12-14] Thus, the economic burden and public health impact of heart failure are projected to increase with the rapidly aging population.[5, 9]

Regardless of etiology, the injury or stress inflicted on the heart culminate in changes in its size, shape and function, a process referred to as cardiac remodeling.[15, 16] Cardiac remodeling involves molecular, cellular and extracellular alterations that affect cardiomyocytes, fibroblasts and the extracellular matrix (ECM), however the pattern and type of remodeling is dependent on the underlying pathology. For instance, pressure overload such as seen in patients with aortic stenosis (AS), the most common valvular heart disease in the Western world,[17] results in increased left ventricular (LV) wall thickness with little or no change in chamber size, a process called hypertrophic remodeling.[18] This type of remodeling is not only associated with hypertrophy of individual cardiomyocytes, but also with increased deposition of ECM by activated fibroblasts, a process referred to as cardiac fibrosis.[15] Hypertrophic remodeling is linked to diastolic dysfunction, however if stimuli such as pressure overload persist, the hypertrophic remodeling may progress to thinning of the walls, LV dilatation, systolic dysfunction and failure. Systolic heart failure is the "classical" heart failure diagnosis of patients with reduced ejection fraction (HFrEF). However, it has recently been recognized that many heart failure patients have diastolic dysfunction with normal or preserved EF (HFpEF).[19, 20] Owing to increased awareness, the documented prevalence of HFpEF now exceeds that of HFrEF.[21, 22]

During cardiac remodeling, several responses are activated which provide the heart with a short-term adaptation to stress, however sustained activation of these responses contributes to dysfunction. A well-known example is activation of neurohormonal pathways,[23] which have central roles in progression of heart failure and serve as targets for current therapies. Another example is activation of the innate immune system, which initiates an inflammatory response in an attempt to restore homeostasis and tissue functionality following cardiac injury, hemodynamic stress or infection.[24] Its sustained activation is known to contribute to cardiac dysfunction during for instance pressure overload,[25] however a role for the innate immune system in propagating cardiac dysfunction is currently better understood in diseases such as sepsis.

Sepsis is a systemic inflammatory response syndrome caused by infection that may progress to severe sepsis and septic shock, with high risk of multi-organ failure and death. The mortality rate ranges from 30-80% depending on severity of disease.[26] In the Western world, more than 1 in 1000 people are estimated to have sepsis each year, with 30-50% of cases progressing to more severe stages.[26] Characterized by uncontrolled inflammation, sepsis is, among other severe organ dysfunctions, associated with impaired systolic and diastolic function.[27-29] Importantly, it is the overstimulated immune response, and not the infection in itself, that predominantly contributes to the cardiodepressant effects.[27] Although the pathophysiology of sepsis is of a different nature than heart failure induced by for instance pressure overload, it demonstrates the detrimental effects a dysregulated and sustained inflammatory response can exert on the heart.

That heart failure today is a major health issue with high prevalence, morbidity and mortality reflects that mechanisms regulating remodeling, dysfunction and progression are poorly understood. The initial understanding of heart failure as a clinical syndrome of systolic dysfunction is reflected in current pharmacological treatment strategies, i.e. β-adrenergic blockers and angiotensin-converting enzyme (ACE) inhibitors, providing no cure, but significantly improving morbidity and mortality.[30, 31] Despite increased awareness of diastolic dysfunction in heart failure, there is still lack of treatment and an insufficient understanding of the underlying maladaptive mechanisms. Clinical trials studying conventional heart failure drugs in patients with HFpEF have failed to demonstrate a mortality benefit.[32-34] Offering hope and emphasizing the importance of basic science efforts, however, the novel drug LCZ696, a dual angiotensin receptor-neprilysin inhibitor (ARNi), has provided encouraging results for HFrEF[35] and HFpEF.[36] Although it is premature to conclude about the effect of LCZ696 in HFpEF from the small number of patients included in the PARAMOUNT study,[36]

animal experiments have demonstrated an attenuating effect on cardiac hypertrophy and fibrosis.[37] *The work presented in this thesis is focused on animal models of pressure overload and sepsis, and is aimed at identifying novel molecular players in cardiac inflammation, fibrosis and dysfunction.*

Immune activation in cardiac disease

Inflammation is an adaptive response triggered by infection or injury with the ultimate aim of restoring tissue homeostasis, and involves actions of cellular and humoral components of the immune system.[38] Evolutionary ancient, the innate immune system provides the first line of defense against invading pathogens, and represents a rapidly mobilized and non-specific response. Macrophages, neutrophils and dendritic cells are central cells of the innate immune system, and humoral effectors include the complement system, acute phase reactants, antimicrobial peptides and cytokines.[39] Collectively, these innate immune mechanisms play a crucial role in the initial recognition and killing of pathogens, as well as in activating and shaping the adaptive immune response.[39, 40] Lymphocytes of the adaptive immune system, i.e. T-cells and B-cells, provide a slower, antigen-dependent response that efficiently target the specific pathogen and provide long-lasting protection against reinfection, i.e. immunological memory. Importantly, although the immune system traditionally has been studied in light of infections, inflammatory reactions are frequently associated with pathological conditions devoid of infectious stimuli.[38]

In 1990, Levine *et al.* reported elevated levels of the proinflammatory cytokine tumor necrosis factor (TNF)α in the circulation of heart failure patients, and this landmark paper was the first to link inflammation to heart failure.[41] Since then, increased circulating levels of inflammatory mediators such as cytokines (interleukin (IL)-1β and -6), chemokines (IL-8, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α) and C-reactive protein (CRP), have been demonstrated in heart failure, indicating that immune activation is an inherent feature of the disease.[42] Sources of these inflammatory mediators are believed to be numerous, including cardiac cells as well as circulating immune cells.[43] In the heart, innate immune responses are activated in response to injury, hemodynamic stress or infection, and involve upregulation of cytoprotective factors and initiation of mechanisms to facilitate repair, limit cardiac injury and fight pathogens. While the initial effects may be protective, a dysregulated and persistent inflammatory reaction becomes pathological and contributes to accentuating cardiac damage and dysfunction.[44] The nature of the immune response depends on the initial trigger, and will therefore vary among

etiologies of heart failure.[42, 45] The mechanisms that orchestrate these responses within the heart are still being uncovered, but it is increasingly evident that the innate immune system plays an important role in initiating, integrating and perpetuating the cardiac stress responses during heart failure.

The traditional understanding of the immune system was its functioning in host defense against invading pathogens, which is highly dependent on self-nonself discrimination.[46] Dr. Ilya Metchnikoff, the father of cellular innate immunity, was the first to describe phagocytosis of pathogens by immune cells and to recognize their importance in host defense.[47] Metchnikoff also noted that these cells function as scavengers of damaged host cells, and thus became the first to introduce the concept that the immune system is able to distinguish not only self from nonself, but also intact, healthy self from dead or damaged self.[47] Today, the "danger model"[48] is based on the idea that the innate immune system can be triggered by conserved structural motifs present in danger signals of both infective and endogenous origin, the so-called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively. A classic example of PAMPs is the lipopolysaccharide (LPS) of gram-negative bacteria, which is commonly used experimentally to mimic the effects of innate immune activation during bacterial infection and sepsis. Cardiac DAMPs encompass intracellular molecules released from dying cells, fragments arising from ECM degradation or cytokines released from stressed cardiac cells and activated immune cells.[24, 49] Thus, sterile inflammation can be initiated by the myocardium through DAMPs released during cardiac injury and stress (e.g. pressure overload).

The initiating event of cardiac immune activation is the detection of PAMPs or DAMPs by pattern recognition receptors (PRRs) expressed on cardiac cells or cardiac-residing immune cells. In the heart, the most extensively studied PRRs are the transmembrane toll-like receptors (TLR)-2 and -4[50, 51] and the cytoplasmic nucleotide-binding oligomerization domain-like receptor pyrin containing domain 3 (NLRP3), a component of inflammasomes.[52]

Activation of TLRs induces an inflammatory signaling cascade via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a family of transcription factors with key roles in regulating immune responses. In the failing heart, this multifaceted transcription factor also integrates signaling pathways involved in hypertrophy and survival, thus significantly influences on cardiac remodeling and disease progression.[53] All members of the NF-κB family share a Rel homology domain which mediates DNA binding and dimerization of family members. The p50/p65 complex is the predominant heterodimer in the

heart.[53] Increased levels and/or phosphorylation of these proteins, or reduced levels of the inhibitor of κB (IκBα) in cardiac tissue can be used as indicators of immune activation, and increased activation of NF-κB has been confirmed in the diseased and failing heart of multiple etiologies.[54, 55] Among others, NF-κB responds to TLR activation by inducing proinflammatory gene transcription, including the principal cytokines of innate immunity: TNFα and IL-1β. Thus, the primary outcome of DAMP-induced signaling is the production and release of proinflammatory cytokines.

Cytokines are soluble mediators of inflammation, regulating responses spanning from transcriptional activation to chemotaxis and differentiation of immune cells. As mentioned above, several proinflammatory cytokines are increased in heart failure patients, and their levels relate to disease severity and prognosis.[56, 57] Cytokines influence on heart failure progression by regulating cardiac remodeling and dysfunction, e.g. cardiomyocyte hypertrophy and fibrosis.[45, 58] For instance, TNFα is the most extensively studied cytokine in heart failure, and has been targeted in several clinical trials.[59, 60] Experimental studies suggest that TNFα regulate pleiotropic, and often opposing, responses during heart failure progression, including myocyte hypertrophy, apoptosis and contractility.[42, 45] Additionally, TNFα may influence on diastolic function and fibrosis by modulating both ECM degradation and accumulation, depending on degree of inflammation.[61-63] Also other proinflammatory cytokines, including IL-1β, directly or indirectly regulate fibrotic processes in the heart.[64-66]

An important aspect of any inflammatory reaction is the infiltration of immune cells from the blood to the inflamed tissue. In addition to acting as chemoattractants for immune cells, cytokines facilitate this process by upregulating adhesion molecules, e.g. intercellular adhesion molecule (Icam)1 and vascular cell adhesion molecule (Vcam)1,[67] thus serving as a link between the acute response elicited by cardiac danger signals and the cellular response mediated by the recruited immune cells. Once in the heart, immune cells carry out multiple functions associated with cardiac remodeling and dysfunction. Several immune cell subsets have been implicated in heart failure progression, including cells of the innate and adaptive immune systems.[68, 69] Macrophages are responsible for phagocytosis of dying cardiomyocytes and cytokine secretion, but also coordinate immune responses by regulating T-cell activation during cardiac hypertrophy and remodeling.[70] In pressure-overloaded hearts, macrophage accumulation is related to fibroblasts activation and fibrosis.[71] An increasing body of experimental evidence supports a role for T-cells in ECM remodeling and fibrosis.[72, 73] CD4-positive T helper cells have been shown to regulate pressure overload-induced fibrotic

responses by influencing cardiac fibroblasts to increase collagen production and cross-linking, thus contributing to diastolic dysfunction.[74, 75]

Although the last 25 years of research have revealed a great deal about the role of inflammation in heart failure, mechanisms underlying its initiation and sustained activation remain incompletely understood. The accumulating evidence that inflammation contributes to remodeling and dysfunction dictates that it should be considered a promising therapeutic target. Although therapies targeting TNFα have not shown benefits for heart failure patients, anti-cytokine treatment is a field of ongoing research. Meanwhile, efforts are made to identify novel inflammatory pathways and effectors that can be targeted.[24, 42, 76] *Thus, in this thesis we have investigated novel mechanisms involved in the propagation of the cardiac immune response.*

The cardiac extracellular matrix

Among other processes, cardiac remodeling includes changes in the ECM, a complex network of structural and non-structural molecules that envelopes cardiac cells and blood vessels. The cardiac ECM consists of fibrous matrix proteins (e.g. collagens and elastin), proteoglycans, glycoproteins, proteases, matrikines and a wide array of signaling molecules.[77] The ECM alterations that occur in the dysfunctioning heart are etiology-dependent, and include increased degradation, synthesis or post-translational modifications of matrix molecules. Cardiac fibroblasts, the most abundant cells in the adult mammalian heart, are the main producers of ECM. These are highly plastic cells that respond to mechanical stress, neurohormonal stimuli, inflammatory mediators and growth factors, and are capable of eliciting ECM remodeling in response to changes in the extracellular milieu.[78] With the increased appreciation of ECM remodeling in heart failure progression, research emphasis is currently put on cardiac fibroblasts and ECM.[77, 79] *Several ECM molecules and processes have been investigated in this thesis and are introduced below.*

Fibrosis

Cardiac fibrosis is characterized by accumulation of ECM, mainly collagens I and III, and is present in most cardiac pathologies. Fibrosis can be divided into reparative fibrosis and reactive fibrosis.[80] Reparative fibrosis occurs during scar formation following injury or cell death, e.g. ischemia, whereas reactive fibrosis results in ECM deposition in the interstitium, and is the predominant form of fibrosis in pressure overload. In the early phase of pressure overload, increased collagen deposition is seen alongside cardiomyocyte hypertrophy, and is

considered a compensatory response as the ECM grows to accommodate the increase in muscle mass and to prevent LV dilatation.[81] Collectively, these remodeling processes alter the physical properties of the cardiac tissue and may be sufficient to counteract the altered hemodynamics[81] and also prevent dilatation, thus initially representing a beneficial response. However, progressive fibrotic remodeling resulting from chronic pressure overload is an important pathological step in heart failure progression. Importantly, fibrosis contributes to increased stiffness, impairing relaxation and filling of the LV during diastole and closely associates with diastolic dysfunction in HFpEF.[82] Moreover, fibrosis increases the risk of arrhythmias,[83] and fibrotic remodeling may cause cardiomyocyte slippage, thus affecting systolic function.[84]

Cardiac fibrosis results from changes in the cellular and extracellular environment and is mainly regulated by activated fibroblasts (discussed below). However, several cell populations contribute to the profibrotic state by secreting fibrogenic factors, including immune cells, cardiomyocytes and vascular cells.[84] The alterations in ECM turnover underlying fibrosis result from a disturbance of the balance between synthetic and degradative processes. Thus, in addition to the increased ECM synthesis by cardiac fibroblasts, the levels and activity of the matrix degrading matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), are important determinants of fibrosis. Accordingly, altered levels of several MMPs and TIMPs have been observed in patients and animal models of heart disease, and changes in MMP/TIMP ratio seem to vary between different disease states.[85] For instance, pressure overload is associated with increased levels of TIMPs during the compensated phase, whereas a relative increase in MMPs is observed in parallel with the development of LV systolic dysfunction.[86-88] The family of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteinases is another group of extracellular metalloproteinases with roles in ECM degradation.[89] Their ability to cleave procollagens and proteoglycans[89, 90] suggests that they may influence on cardiac ECM integrity and function during heart failure progression. Interestingly, several members of the ADAMTS family were recently shown to be upregulated in pressure-overloaded hearts,[91] however the role of this family of proteinases in the diseased heart remains to be elucidated. Moreover, other non-structural ECM proteins, i.e. matrikines, have been shown to impact on fibrotic responses in the heart, and represent a rapidly growing field in cardiac research.[92]

There is currently no treatment for cardiac fibrosis. This underlines the importance of improving our understanding of its mechanistic basis to identify targets and at what stages of

heart failure progression interventions would be beneficial. *In this thesis, we have investigated the role of several ECM molecules as novel regulators of the cardiac fibrotic response.*

Syndecans

Syndecans constitute a family of four evolutionary ancient proteoglycans (syndecan-1-4) consisting of a transmembrane core protein with covalently attached heparan sulfate (HS) glycosaminoglycan (GAG) chains, although chondroitin sulfate (CS) or dermatan sulfate (DS) may also be present in syndecan-1 and -3 (Fig. 1A).[93, 94] All cells express syndecans on their surface,[95] and all four syndecans are expressed in the heart.[96] Syndecans interact with a wide range of molecules, including growth factors, ECM proteins, cytokines and pathogens through their GAG chains, thereby regulating biological processes spanning from proliferation and differentiation to wound healing and inflammation.[97] The syndecan core protein is characterized by highly conserved transmembrane and cytoplasmic domains. In contrast, the extracellular domains (ectodomains) are highly divergent in both length and sequence, with the exception of regions of GAG substitution and proteolytic cleavage.[94, 98] The syndecan core protein undergoes enzymatic cleavage which releases the GAG-substituted ectodomains from the cell surface in a process termed syndecan shedding (Fig. 1B). The shed ectodomains function as soluble effectors or inhibitors of the intact, cell-localized proteoglycan. For instance, the shed ectodomains may act as a reservoir of ligands and form gradients that promote chemotaxis.[99] Since syndecans function as coreceptors for adhesion and growth factor receptors, their loss from the cell surface may downregulate signal transduction, affecting proliferation and migration.[100] Shedding of syndecan ectodomains occurs constitutively in some cultured cells, but is accelerated during wound healing and in response to pathological stimuli.[101-104] Indeed, syndecan ectodomains accumulate in wound fluids, consistent with a role in regulating pathophysiological events during inflammation.[102, 105]

Levels of shed syndecan-4 are increased in the circulation of patients with heart disease.[106, 107] Syndecan-4 is the most ubiquitously expressed member of the syndecan family,[95] and is found in the two major cell types of the heart, i.e. cardiac myocytes and fibroblasts, where it is located to cellular attachment sites.[108, 109] Because of its anchoring to the cytoskeleton[110, 111] and the ECM[112] it is believed to function as a mechanosensor. In the heart, syndecan-4 has been shown to be upregulated in disease[96, 113-115] and to mediate mechanical stress-induced remodeling.[114-116] *In this thesis, we investigated regulation of syndecan-4 expression and shedding in cardiac myocytes and fibroblasts, and examined the effects of syndecan-4 shedding on cardiac immune responses.*

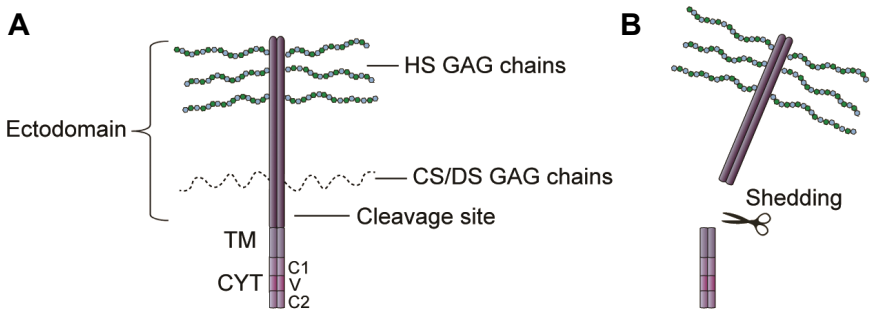

Fig. 1 Schematic of syndecan structure and shedding.
The transmembrane syndecan core protein consists of an extracellular (ectodomain), transmembrane (TM) and cytoplasmic (CYT) domain. The cytoplasmic domain contains two conserved (C1 and C2) and a variable (V) region. The core protein is substituted with heparan sulfate (HS) glycosaminoglycan (GAG) chains, and in some instances chondroitin sulfate (CS) or dermatan sulfate (DS) GAG chains (A). Syndecan ectodomains can be released from the cell surface by proteolytic cleavage at a juxtamembrane site in a process termed shedding (B).

Small leucine-rich proteoglycans

The small leucine-rich proteoglycans (SLRPs) constitute a family of extracellular proteoglycans consisting of a core protein with leucine-rich repeat motifs, N-linked oligosaccharides and one or more GAG side chains.[117] The most widely studied members of the SLRP family are decorin and biglycan, which contain CS or DS, and the keratan sulfate (KS)-substituted fibromodulin and lumican (Fig. 2). The SLRPs were initially considered to be structural components of the ECM capable of binding various types of collagens and to regulate their assembly and organization.[117] However, they are now recognized as key signaling molecules, mediating cell-matrix crosstalk and regulating cell behavior, suggesting their biological activity extends far beyond the ability to bind collagens.[118, 119] Several SLRPs show altered expression levels during pressure overload-induced remodeling,[120, 121] but there is limited knowledge about their roles in the failing heart.

Lumican, a member of the class II of the SLRP family, is expressed in mesenchymal tissues throughout the body, where it is involved in collagen fibril organization and growth.[122] Mice deficient in lumican develop opacities of the cornea and fragile skin, due to abnormal fibril assembly and altered interfibrillar spacing.[123] Interestingly, recent reports have shown that lumican levels are altered in the diseased heart,[120, 121, 124, 125] indicating that it may regulate ECM structure also during heart failure progression. *In this thesis, we studied lumican in cardiac fibroblasts and failing hearts.*

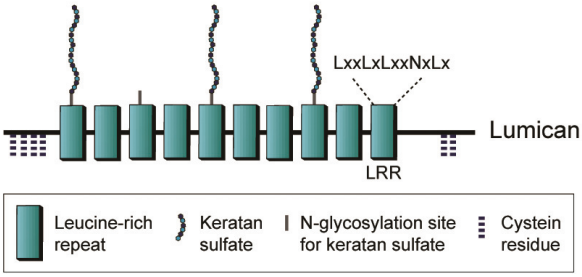

Fig. 2 Schematic of lumican structure.
Lumican core protein contains 10 tandem leucine-rich repeats (LRR) with L representing leucine (or isoleucine), N is usually asparagine, and x is any amino acid. Lumican is substituted with 3-4 keratan sulfate glycosaminoglycan chains at N-linked glycosylation sites in the LRRs. The LRRs are flanked by cysteine residues that can form disulfide bonds.

Collagens

In the heart, the most abundant collagens are the fibrillar collagens I and III, accounting for more than 90% of total collagen.[126] Collagen I assembles into thick fibers that convey tensile strength and structural support, whereas collagen III forms a fine network of fibrils, maintaining elasticity of the ECM.[126] During heart failure progression, as mentioned above, abnormal regulation of the collagen matrix affects mechanical properties, cardiac dimensions and function.[127] Increased degradation of structural collagens causes LV dilatation, and is attributed to the activation of collagen-degrading MMPs in the early phases of remodeling following myocardial infarction (MI) and volume overload.[85] Chronic pressure overload is associated with fibrillar collagen accumulation, i.e. fibrosis, which increases the stiffness of the heart and contributes to diastolic dysfunction.[128, 129] In addition to increased amounts, stiffness is determined by increased collagen cross-linking, which results in mature collagens with high tensile strength.[130] Collagen cross-linking is mediated by the enzyme lysyl oxidase (LOX), a molecule which has received recent attention in heart failure research.[116, 131, 132]

Pressure overload is associated with a switch in collagen isoform expression, and these changes have been shown to relate to hypertrophic remodeling, heart failure and reverse remodeling.[133, 134] In addition to the main structural collagens I and III, the non-fibrillar collagen VIII is differentially regulated in pressure-overloaded, failing hearts.[133] Collagen VIII is found in the ECM of various tissues, where it is believed to function as a bridging molecule between matrix molecules and play a role in tissue remodeling.[135, 136] However, the role of non-fibrillar collagens are less explored than fibrillar collagens. *Here we investigated the role of collagen VIII in pressure overload-induced cardiac dilatation and fibrosis.*

Myofibroblasts and TGF–β1

Under physiological conditions, cardiac fibroblasts have low ECM producing activity. Various pathological stimuli including pressure overload, causes activation of fibroblasts, leading to a phenotype-switch into myofibroblasts, a hallmark of the cardiac fibrotic response.[137] Myofibroblasts are characterized by a contractile, smooth muscle-like phenotype with increased ECM production, supermature focal adhesions (FAs), expression of actin stress fibers, and smooth muscle cell markers such as smooth muscle α-actin (SMA) and SM22 (Fig. 3).[138] The activated myofibroblasts become highly proliferative and invasive, and secrete several growth factors and cytokines that act in auto- and paracrine fashions.

Transforming growth factor (TGF)-β1 is a key profibrotic cytokine and a potent inducer of myofibroblast differentiation.[139, 140] Its levels are highly upregulated during heart failure progression.[139] Upon activation, TGF-β1 binds to its type II receptor which propagates downstream intracellular signaling through the small mothers against decapentaplegic (SMAD) proteins and activates transcriptional responses. Although the role of TGF-β1 and myofibroblasts in pressure overload-induced cardiac remodeling is established, there are major gaps in our knowledge related to how these processes can be targeted to prevent excessive fibrosis and progression of heart failure. *In this thesis, we investigated the roles of lumican and collagen VIII as novel regulators of myofibroblast differentiation and TGF-β1 signaling.*

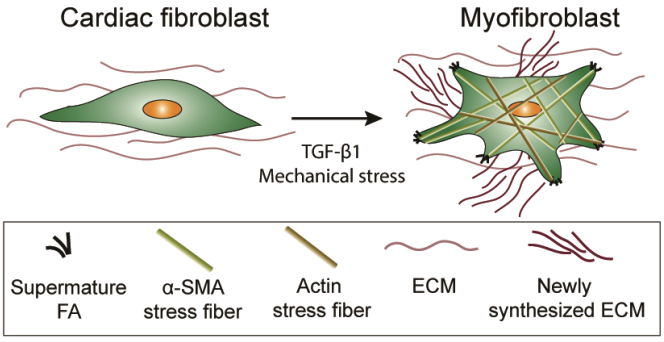

Fig. 3. Differentiation of fibroblasts into myofibroblasts.
Under physiological conditions, cardiac fibroblasts have low extracellular matrix (ECM) producing activity. In response to increased mechanical stress and TGF-β1 signaling, cardiac fibroblasts undergo a phenotypic switch into myofibroblasts. Myofibroblasts are characterized by expression of actin stress fibers, α-smooth muscle actin (α-SMA)-positive stress fibers, supermature focal adhesions (FA) and increased ECM synthesis.

Aims of the thesis

The overall aim of this thesis was to investigate molecular mechanisms underlying cardiac remodeling and progression of heart failure, with focus on proteoglycans and collagens in mediating inflammatory responses, fibrosis and cardiac dysfunction. The specific aims of the separate studies were:

Paper I) Innate immune signaling induces expression and shedding of the heparan sulfate proteoglycan syndecan-4 in cardiac fibroblasts and myocytes, affecting inflammation in the pressure-overloaded heart

- To identify signals and mechanisms responsible for elevated syndecan-4 levels in the pressure-overloaded myocardium.
- To investigate syndecan-4 shedding from cardiomyocytes and fibroblasts.
- To investigate effects of syndecan-4 in recruitment of immune cells to the pressure-overloaded heart.

Paper II) Shedding of syndecan-4 promotes immune cell recruitment and mitigates cardiac dysfunction after lipopolysaccharide challenge in mice

- To study syndecan-4 shedding *in vivo* and its effects on cardiac function, immune cell recruitment and ECM remodeling.
- To investigate whether syndecan-4 can be shed from the human heart.

Paper III) Lumican is increased in experimental and clinical heart failure, and its production by cardiac fibroblasts is induced by mechanical and proinflammatory stimuli

- To investigate left ventricular expression of lumican during pressure overload-induced cardiac remodeling and failure.
- To study regulation and function of increased lumican in cardiac fibroblasts *in vitro*.
- To investigate expression of lumican in failing human hearts.

Paper IV) Lack of collagen VIII reduces fibrosis and promotes early mortality and cardiac dilatation in pressure overload in mice

- To investigate effects of reduced collagen VIII on survival, LV dilatation, myofibroblast differentiation and fibrosis during acute and chronic phases of pressure overload *in vivo*.
- To study effects of collagen VIII on cardiac myofibroblast differentiation in cardiac fibroblasts *in vitro*.

Methodological considerations

The findings presented in this thesis were obtained using animal models of cardiac disease, gain- and loss-of-function experiments *in vivo* and *in vitro*, primary cell cultures from hearts and myocardial biopsies and blood samples from patients. This section discusses methodological considerations of central experiments.

Experimental mouse models

To study cardiac disease, mouse models are widely used and offer several advantages. Mice are small, inexpensive and well characterized. Importantly, 99% of mouse genes have a human homolog,[141] and manipulation of the mouse genome has allowed for extensive mechanistic insight into human heart failure. A great number of surgical, dietary and pharmacological mouse models have been developed to mimic a wide range of cardiac pathologies.

Genetically modified mice

Gain- and loss-of-function studies are valuable tools providing mechanistic insights into cardiac physiology and disease. As heart failure develops, a multitude of proteins become differentially regulated in terms of expression and/or function. Genetically modified mice enable us to investigate the function of these proteins *in vivo*. A protein is deleted (knock-out), altered (knock-in) or introduced (transgenic) through DNA engineering, and the modification can be regulated in a temporal (i.e. inducible) and spatial (i.e. tissue- or cell-specific) manner.

In Paper I and II, we have used homozygous syndecan-4 KO[142] mice to investigate the role of syndecan-4 in cardiac immune responses. We have also utilized nuclear factor of activated T-cells (NFAT)-luciferase reporter mice to examine NFAT transcriptional activity (Paper I).[143] These mice harbor nine copies of an NFAT binding site from the IL-4 promoter inserted upstream of the luciferase gene. To study the effects of collagen VIII in the pressure-overloaded heart, homozygous collagen VIII KO (collagen VIIIα1 and VIIIα2 double-deficient)[144] mice were employed in Paper IV. The two KO models used in this thesis are constitutive KOs, denoting that the genes are disrupted throughout development and in every cell of the adult mouse. Constitutive KOs are associated with features that may preclude the analysis of the phenotype, such as developmental abnormalities or compensatory mechanisms. Of notice, the syndecan-4 and collagen VIII KO mice are viable, fertile and appear phenotypically similar to WT littermates, also with regards to cardiac dimensions and

function at adult age. Importantly, KO and WT mice of the same strain have been used as controls. For our studies, we have employed the genetically modified mice in models of heart failure and LPS challenge and to produce primary cell cultures from neonatal mice, to study the effects of syndecan-4 and collagen VIII KO *in vivo* and *in vitro.*

Mouse model of left ventricular pressure overload

In Paper I, III and IV, pressure overload was induced in mice by banding of the ascending aorta (AB) to mimic AS (Fig. 4) and to study molecular, cellular and extracellular alterations during pressure overload-induced cardiac remodeling. One approach was to follow AB mice over time to investigate time-dependent changes in molecules and processes relevant to heart failure progression (Paper I and IV). Another approach was to stratify AB mice according to degree of heart failure in response to similar flow over the stenosis, both phenomena observed in patients (Paper III).

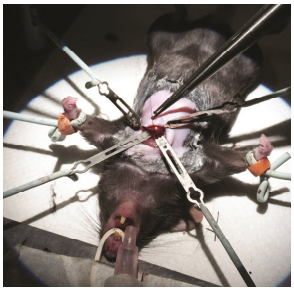

Fig. 4 Experimental mouse model of pressure overload. Mouse subjected to aortic banding to induce left ventricular pressure overload.

The AB model has been shown to be a valid model of the pressure overload-induced hypertrophic remodeling resulting from AS and hypertension.[145] An advantage is the time frame for disease development in mice (i.e. days and weeks), which allows for relatively quick investigations of processes occurring over years in human patients. However, the model has shortcomings that should be considered. Importantly, the mouse model induces an immediate onset of pressure overload, whereas in patients with AS it develops gradually over years. Thus, the changes observed during the acute and early stage of remodeling in mice are likely to differ from that of human disease. The mice utilized are young adults and carry similar genetic background, in contrast to the human patients.

Another aspect influencing the quality of AB as a model is its reproducibility. However, years of experience and refinement of this method at our institute have resulted in standardized surgical procedures, high reproducibility and low mortality. In our studies, all operations included in each paper were performed by one experienced researcher. To control for effects of the operation itself, sham operation without tightening of the suture around the aorta was performed. Thus, despite its limitations, the AB model closely replicates disease mechanisms of pathological remodeling in human patients and is a valuable tool for studying the pathophysiology of cardiac remodeling and failure in response to pressure overload.

Mouse model of LPS challenge

To investigate the role of syndecan-4 shedding in cardiac immune responses (Paper II), we sought an *in vivo* model where a robust shedding response was present in the heart. LPS is a bacterial endotoxin that induces endotoxin shock with an acute and potent activation of the innate immune system. Our *in vitro* findings in Paper I suggested that LPS prompts high levels of syndecan-4 shedding from cardiac cells. Thus, we used LPS challenge in mice to study syndecan-4 shedding *in vivo*.

LPS challenge elicits a systemic response and dramatic changes in the cardiovascular system,[146, 147] making it difficult to study localized cellular responses without the influence of systemic mediators and effects. However, administration of LPS is a well-established and relatively reproducible laboratory model for studying the basic biology of sepsis and endotoxin shock.[147] Although LPS challenge in rodents fails to reproduce all of the physiologic and immunologic features of human sepsis, it is a valuable tool for evaluating aspects of the innate immune response.[148, 149] In general, immune function is well conserved between mice and men.[150] However, differences exist in immune system development, activation and responses, and should always be considered when extrapolating to human disease.[150] For instance, mice are relatively insensitive to LPS compared to humans and higher doses are required to produce systemic signs in mice (1-25 mg/kg) than in humans (2-4 ng/kg).[148, 151] Since LPS challenge mimics a bacterial infection, the relevance to heart failure comes into question, however enterically-derived LPS has been suggested to sustain a proinflammatory state in the myocardium in chronic heart failure.[152] TLR4, the receptor for LPS, is activated in numerous cardiac pathologies where it responds to endogenous danger signals released during sterile inflammation, i.e. pressure overload and MI.[49, 50] Activated TLR4 during heart failure progression may thus trigger many of the same innate immune cascades that are activated by LPS, indicating that despite differences in duration and magnitude of immune activation, certain disease mechanisms are shared between acute bacterial infections, e.g. LPS challenge, and the chronic inflammatory state of the failing heart.

Evaluation of cardiac function and remodeling

Non-invasive echocardiography was utilized to obtain measurements of cardiac dimensions, e.g. left atrial diameter (LAD), LV internal diameter (LVID), septum and posterior wall thicknesses (IVS and LVPW), and function, e.g. fractional shortening (FS) and EF. This method is standard in cardiac research and has been used in all papers included in this thesis, however echocardiographic examinations in mice are challenging to perform due to their

small size and rapid heartbeat (BPM > 500). Furthermore, as we have done our echocardiographic analyses under light isoflurane sedation, cardiodepressive effects are part of the interpretation. Isoflurane has fewer systemic hemodynamic effects in mice than nonvolatile anesthetics and is the most frequently used agent for surgical intervention and short-term experimentation.[153] The procedure for sedating the mice has been standardized, avoiding deep sedation, and control and experimental mice are investigated together. Recordings are also operator-dependent and subjective. Therefore, all echocardiography included in this thesis was performed by a highly experienced operator blinded to genotype and treatment. Electrocardiographic measurements were recorded in parallel with the echocardiographic examinations, while tail-cuff blood pressure measurements were obtained under similar conditions. To get a more detailed picture of cardiac structure and function, we could have used MRI. MRI is considered the gold standard for *in vivo* cardiac imaging, but it is time-consuming and expensive, whereas echocardiography is relatively fast and cheap. Here, echocardiographic analyses provided sufficient characterization of the mouse cardiac phenotype.

Following sacrifice, hearts and lungs from mice were weighed. Increased heart and lung weights are widely used indicators of hypertrophy and congestive heart failure, respectively. Expression of commonly used cardiac markers of hypertrophy and failure, fibrosis and inflammation were assessed in the myocardial tissue by quantitative real-time polymerase chain reaction (qRT-PCR), histology or high performance liquid chromatography (HPLC). Markers of cardiomyocyte hypertrophy and failure include atrial and brain natriuretic peptide (ANP and BNP), α-skeletal actin (ACTA1) and myosin heavy chain beta (MHC-β). Fibrosis and ECM content were assessed by histological assessments of Sirius Red (collagens), acid fuchsin orange G (AFOG; collagens) and alcian blue (alcianophilic polysaccharides, i.e. GAGs) or hydroxyproline HPLC (collagens). Expression of TNFα, IL-1β, IL-6 and IL-18 mRNA was measured to assess inflammation.

Cell cultures

Primary cardiac cell cultures

After birth, cardiomyocytes generally do not proliferate, and therefore, primary cultures are commonly used in cardiac research. To study cell-specific responses and molecular signaling mechanisms, primary cultures of cardiac myocytes and fibroblasts were isolated from neonatal (1-3 days old) mice and rats. Neonatal cardiac cells are easier to culture and have a greater potential for morphological and phenotypical changes than adult cells. However, some

limitations associated with the use of neonatal cardiomyocyte and fibroblast cultures exist.[154] The cultures can be unpredictable in terms of yield, quality and responsiveness, even when standardized isolation techniques are employed. Like all cell cultures they are non-physiological, and in single cell cultures one will miss the interactions between cell types.

Unlike adult cells, neonatal cardiomyocytes are not fully differentiated. However, they have been shown to respond to hypertrophic, mechanical and paracrine stimuli *in vitro* by inducing gene expression in a similar way to that observed in the diseased, adult heart *in vivo*.[155, 156] Cardiac fibroblasts grown under standard culture conditions undergo a phenotypic switch to myofibroblasts because of the mechanical tension of the rigid substrate.[157, 158] In one way this may preclude functional differences between the two types of fibroblasts if the intention is to study one specific phenotype. On the other hand, these cultures contain fibroblasts at different stages of myofibroblast differentiation, similar to what is observed in the pressure-overloaded heart *in vivo*.[157, 159]

Although neonatal cardiac cell cultures have shortcomings as model systems for the adult heart, they provide a controlled environment to study direct effects of physical or biochemical interventions on a specific cell type and are highly valued in cardiac research. In light of this, we used the primary cell cultures to investigate whether the matrix molecules we found to be upregulated in the diseased heart may act in an auto- or paracrine fashion to convey signaling effects on cardiac cells. These include lumican, collagen VIII and shed syndecan-4 ectodomains. For this purpose, we used recombinant proteins of lumican and collagen VIII, whereas syndecan-4 ectodomains were obtained from a human embryonic kidney (HEK)293 cell line model system (described below).

To investigate roles of syndecan-4 and collagen VIII at the cellular level, primary fibroblast cultures prepared from left ventricles of neonatal syndecan-4 (Paper I) and collagen VIII (Paper IV) KO mice were used. In addition to supporting the *in vivo* findings obtained with these genetically modified mice, *in vitro* studies provide insight into cell-specific, i.e. cardiac myocyte or fibroblast, responses, phenotypes and molecular signaling mechanisms that are masked in whole-tissue experiments. Additionally, adult primary fibroblasts were isolated from the LVs of collagen VIII KO mice to investigate RhoA activity (Paper IV). In Paper I, we used primary cardiomyocyte cultures from NFAT-luciferase reporter mice to examine NFAT transcriptional activity. Luciferase reporter activity is measured as luminescence by adding its substrate, luciferin, to protein lysates from isolated cells.

Cell lines

To compliment loss-of-function studies where the protein of interest has been disrupted, gain-of-function studies examine effects of expressing the protein at higher levels. Transfection with plasmids is a useful tool for introducing modified genes and proteins into cells. Because of low transfection efficiency in primary cardiac cell cultures,[154] the standard and easily transfectable cell line HEK293 was used. One has to keep in mind when interpreting such experiments, artificially high overexpression is resulting from a strong promoter.

In Paper I and II, plasmids containing full-length syndecan-4 cDNA were transfected into cells to investigate constitutive shedding when syndecan-4 levels were increased. In Paper II we capitalized on the elevated levels of syndecan-4 shedding to investigate the function of the shed ectodomains. Conditioned medium was collected from HEK293 cells transfected with syndecan-4 and applied to cultures of cardiac cells. The conditioned medium contained high levels of syndecan-4 ectodomains, and thus worked as a model system for the increased levels of shed syndecan-4 released into the cardiac ECM in response to tissue inflammation, such as seen after LPS challenge. In Paper I, we investigated transcriptional regulation of syndecan-4 by inserting the enhanced green fluorescent protein (EGFP) reporter gene downstream of the syndecan-4 promoter in plasmids. Furthermore, to assess molecular mechanisms regulating syndecan-4 shedding, we produced plasmids expressing syndecan-4 with targeted mutations in domains proposed to interact with shedding enzymes, i.e. syndecan-4 without the juxtamembrane domain (Δ138-145) and syndecan-4 with non-functioning HS GAG chain attachment sites (S44A/S62A/S64A).

Human myocardial biopsies and blood samples

To establish and confirm clinical relevance for our experimental findings, we have utilized myocardial biopsies and blood samples from patients with heart disease. Biopsies included in Paper I and III were obtained from LVs of explanted hearts of patients with end-stage, dilated cardiomyopathy (DCM) undergoing cardiac transplantation. LV biopsies from non-diseased hearts considered, but deemed unsuitable, for transplantation were used as control. Control hearts were obtained from patients whose cause of death was cerebrovascular accidents, none of which had a history of heart disease. Limited access to tissue samples from both patients and controls is an obvious experimental restriction in cardiac research. Human myocardial biopsies are instrumental to the field as they allow for identification of molecular mechanisms and signaling pathways active in the failing heart, and validation of findings from animal models of heart failure.

In Paper II, we investigated shedding of syndecan-4 from the human heart based on venous-arterial differences in blood drawn from the coronary sinus and radial artery, respectively. Open heart surgery is required in order to obtain such samples and we investigated syndecan-4 levels in patients undergoing aortic valve replacement (AVR) surgery due to severe, symptomatic AS. From each patient, blood was drawn from a coronary sinus catheter and from the cannulated radial artery immediately after onset of cardiopulmonary bypass and before clamping of the aorta (Fig. 5). This allowed for analysis of trans-coronary differences in syndecan-4 levels in the same patient, with minimal influence of surgical intervention and ischemia. Thus, the results primarily reflected the release of shed syndecan-4 from the heart due to pressure overload.

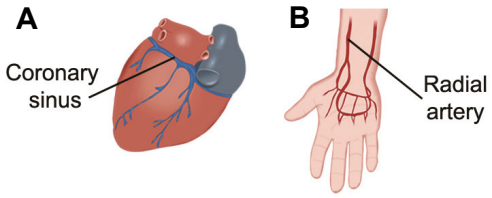

Fig. 5 Illustration of the two sites for blood collection in patients undergoing aortic valve replacement surgery. Venous blood was drawn from the coronary sinus, which collects blood from the cardiac cardiac muscle (A). Arterial blood was drawn from the radial artery (B).

General molecular and cellular biology techniques

In addition to the above-mentioned methods, we have utilized several molecular and cellular biology techniques that are briefly summarized in this section: qRT-PCR was used to measure gene expression. For semi-quantitative analysis of protein expression, immunoblotting (i.e. Western blotting) was performed. Enzyme-linked immunosorbent assay (ELISA) was performed to quantify proteins in medium, cell lysates, serum or plasma. Immunohistochemistry and microscopic imaging were used for analysis of protein localization. The FlexCell tension system was used to subject cells to mechanical stress *in vitro*. Scratch assay was used to measure the migratory capacity of cells. For measurements of matrix metalloproteinase enzymatic activity, zymography was performed. RhoA Activation Assay was used to measure RhoA activity. Electron microscopy was performed to examine *in vitro* collagen assembly.

Experimental approaches for detection of syndecan-4 shedding

One of the main objectives of Paper I and II was to investigate the regulation and role of syndecan-4 shedding, a post-translational syndecan-4 modification. Here the specific

methodology developed and used to detect and quantify shedding at the level of cells, tissue and circulation is described.

Western blot procedure for detection of proteoglycans

When using gel electrophoresis to separate proteins, intact proteoglycans appear as a smear on top of the gel due to the presence of varying amounts of high molecular weight GAG chains.[160] Moreover, the highly negative charge due to sulfation of the GAG chains makes transfer onto blotting membranes difficult. The enzymatic removal of GAG chains prior to gel loading (Fig. 6A) enables the core protein to migrate as discrete bands of specific and expected molecular weights. Enzymatic GAG removal is accomplished by treating protein lysates with the bacterial enzymes heparitinase, chondroitinase, keratinase and PNGase, which cleave HS, CS, KS GAG chains and N-linked oligosaccharides, respectively. In our laboratory, we have established protocols for treating cardiac cell and tissue protein lysates with a mix of deglycosylating enzymes. A method for deglycosylation of syndecans was adapted from Burbach *et al.*[161] to detect and quantify full-length syndecan-4 levels, and the method is detailed in Paper I. Removal of KS GAG chains and N-linked oligosaccharides from lumican[162] was used to identify the lumican core protein in Paper III.

Western blot procedure for detection of syndecan-4 shedding fragments

In our studies, we have developed a method for detection of syndecan-4 shedding from cells and in tissues by immunoblotting, using the calculated expected size of the shedding fragments and specific syndecan-4 antibodies that have been epitope mapped by us[114] (Fig. 6B). Two different syndecan-4 antibodies were applied: one custom made antibody recognizing a cytoplasmic epitope and the other a commercially available antibody recognizing an extracellular epitope of syndecan-4. When probing blots of cell lysates with the antibody recognizing the cytoplasmic epitope of syndecan-4, we detected a 10-15 kDa syndecan-4-specific band that was not detected with an antibody recognizing the extracellular epitope. This fragment had the expected size of the membrane and cytoplasmic part of syndecan-4. Moreover, capitalizing on a number of tagged syndecan-4 plasmid constructs, anti-FLAG and anti-human influenza hemagglutinin (HA) antibodies were utilized in combination with the two syndecan-4-specific antibodies to validate the identity of the 10-15 kDa fragment (Paper I). Thus, this fragment represented the cellular fragment of syndecan-4 remaining in the cell membrane after shedding of the ectodomain, and was used to quantify shedding of syndecan-4 from cells. The set of experiments used to confirm the specificity of

the cellular syndecan-4 fragment is detailed in Paper I. In Paper II, the same approach was used to detect the cellular fragment in LV lysates.

By using an antibody recognizing an extracellular epitope of syndecan-4, a similar methodology can be applied to detect the shed ectodomain in conditioned medium from cell cultures. However, in Paper II, we exploited the N-terminal HA-tag on the plasmid construct of syndecan-4 to confirm the presence of shed ectodomains in conditioned medium from HEK293 cells overexpressing syndecan-4 by using the anti-HA antibody. The main challenge with the methods described in this section is the dependence on antibodies, which will always raise questions regarding specificity. However, by using several and proper controls, we believe that we have demonstrated the validity of the aforementioned method to detect and quantify shedding of syndecan-4 by Western blotting.

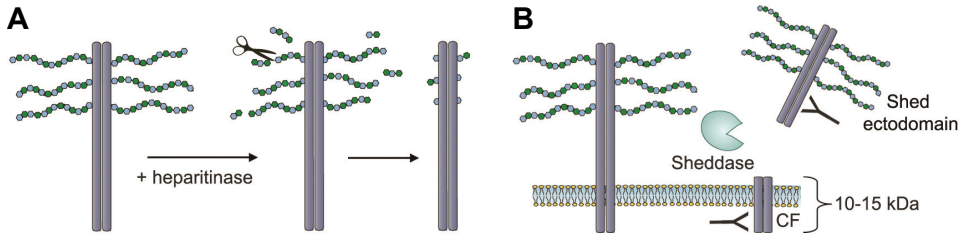

Fig. 6 Illustration of methodology applied to measure syndecan-4 shedding.
Heparitinase treatment of protein lysates allows gel migration and identification of syndecan-4 core protein (A). Epitope-specific antibodies enable the detection of the cellular fragment (CF) and shed ectodomain of syndecan-4 (B).

ELISA for detection of syndecan-4 shedding fragments

Quantification of shed syndecan-4 ectodomains can be obtained by ELISA. In Paper I, this approach was used to measure the levels of shedding in conditioned medium from syndecan-4 overexpressing HEK293 cells. Importantly, this method is well established for detection of circulating syndecan-4, which represents the shed ectodomains, and was used in Paper II to measure syndecan-4 in blood samples from open heart surgery patients and mice challenged with LPS.

Conditions governing blood collection and processing may influence on the concentration of proteins measured in plasma and serum. Therefore, care should be taken when comparing results of blood sample analyses from different experiments. Proteolysis or secretion from cells could alter protein levels in serum samples, which are left to coagulate for 1-2 h before separation from cells, although keeping the samples on ice will slow this process.

In Paper II, our interest was the concentration of syndecan-4 in serum samples from the coronary sinus relative to the radial artery in the same patient. Thus, the two blood samples were collected in parallel from each patient, and only the relative difference between the two was compared between patients. Plasma samples drawn from the LVs of LPS challenged mice (Paper II) were immediately placed on ice, and centrifuged within 15 minutes of collection, to ensure that the measured concentration of shed syndecan-4 closely represented the LV levels *in vivo*.

Ethical considerations

A prerequisite for all research involving use of human samples, patient information and animal experiments is that ethical guidelines are followed. Thus, patient safety and animal welfare are fundamental aspects of the experimental design. For the human myocardial biopsies and blood samples, the protocols were reviewed and approved by the Norwegian regional ethics committee and conformed to the Declaration of Helsinki. Informed written consent was obtained from each patient included and from next of kin for controls. All animal experiments were reviewed and approved by the Norwegian National Animal Research Committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 2011). Care was taken to reduce, replace and refine the use of animals in our studies.

Summary of results

The main results of this thesis are summarized in this section.

Paper I

Innate immune signaling induces expression and shedding of the heparan sulfate proteoglycan syndecan-4 in cardiac fibroblasts and myocytes, affecting inflammation in the pressure-overloaded heart

We showed that syndecan-4 mRNA was increased in the myocardium of mice following 24 h and 1 week of AB, and that its expression correlated with mRNA of proinflammatory cytokines. Accordingly, in cultured cardiac myocytes and fibroblasts, the innate immunity mediators TNFα, IL-1β and LPS induced syndecan-4 mRNA. Bioinformatical and mutational analyses were used to identify a functional site for the proinflammatory NF-κB transcription factor in the syndecan-4 promoter. Interestingly, when assessing syndecan-4 protein by immunoblotting, we found that the same mediators that induced syndecan-4 mRNA, increased shedding of syndecan-4 from cardiac cells. This result was obtained by using an antibody against a cytoplasmic epitope of syndecan-4, which we by several assays showed that detected the cellular fragment of syndecan-4 remaining in cells after shedding of the ectodomains. Transfections with syndecan-4 plasmid constructs harboring mutated enzyme-interaction domains, suggested enzymes dependent on interacting with HS GAG chains to regulate shedding. In cultured cardiac fibroblasts, LPS reduced focal adhesion assembly, suggesting that inflammation-induced shedding affects fibroblast function. We observed recruitment of T-cells to the heart during AB-induced cardiac remodeling, by measuring CD3, CD4 and CD8 mRNA. Importantly, this response was reduced in syndecan-4 KO hearts, accompanied by reduced cardiac function in these mice, suggesting syndecan-4 to regulate T-cell infiltration to the heart. Finally, we showed increased levels of syndecan-4 mRNA and shedding fragments in biopsies from end-stage failing human hearts. Altogether, our data suggested that syndecan-4 and its shedding play a role in the immune response of pressure-overloaded hearts, influencing cardiac remodeling and failure progression.

Paper II

Shedding of syndecan-4 promotes immune cell recruitment and mitigates cardiac dysfunction after lipopolysaccharide challenge in mice

We showed that LPS challenge in mice and cultured cardiac myocytes and fibroblasts increased cardiac syndecan-4 mRNA without altering full-length protein. Elevated levels of

shedding fragments in the myocardium and in blood collected from the heart confirmed increased shedding of syndecan-4 after LPS. We observed a parallel upregulation of ADAMTS1 and 4 and MMP9, suggesting that these shedding enzymes regulate syndecan-4 shedding in LPS-challenged hearts. Cardiac dysfunction was exacerbated in mice unable to shed syndecan-4 (syndecan-4 KO) after LPS challenge, i.e. we demonstrated reduced ejection fraction and diastolic tissue velocities and increased QRS duration compared to WT. The reduced cardiac function was accompanied by reduced expression of immune cell markers CD8, CD11a and F4/80 and the adhesion molecules Icam1 and Vcam1 in syndecan-4 KO hearts, suggesting that syndecan-4 shedding promotes immune cell recruitment to the heart. In agreement with this, expression of adhesion molecules and activation of the innate immunity NF-κB signaling cascade were induced when we exposed cardiac myocytes and fibroblasts to elevated levels of shed heparan sulfate-substituted syndecan-4 ectodomains. In cultured cardiac fibroblasts, syndecan-4 ectodomains regulated expression of ECM constituents associated with collagen synthesis, cross-linking and turnover, suggesting that shedding of syndecan-4 affects ECM remodeling. Finally, we found that syndecan-4 was shed from hearts of AS patients and could be detected in blood, evidenced by elevated levels of shed syndecan-4 in blood from the coronary sinus compared to the radial artery. Collectively, our findings suggested that shedding of syndecan-4 is part of the cardiac innate immune response, promoting immune cell recruitment, ECM remodeling and mitigating cardiac dysfunction in response to LPS.

Paper III

Lumican is increased in experimental and clinical heart failure, and its production by cardiac fibroblasts is induced by mechanical and proinflammatory stimuli

We found that cardiac mRNA and glycosylated protein levels of the SLRP lumican were upregulated in mice with congestive heart failure after 4 weeks of AB (ABHF) and in biopsies from patients with end-stage heart failure. In cultured cardiac fibroblasts, lumican mRNA and secreted protein were increased by mechanical stretch and the proinflammatory cytokine IL-1β. In LVs of ABHF mice, glycosylated lumican correlated with structural collagen I and III, profibrotic TGFβ1 and the collagen cross-linking enzyme LOX. Accordingly, we demonstrated that stimulation with recombinant glycosylated lumican increased NF-κB-dependent collagen I, LOX and TGFβ1 mRNA in cardiac fibroblasts, indicating that lumican affects fibroblast function and fibrosis. In agreement with these findings, we showed that glycosylated lumican increased the levels of the dimeric and cross-linked form of collagen I

and phosphorylation of fibrosis-inducing SMAD3 and decreased the activity of collagen-degrading MMP9. Altogether, our results showed that cardiac lumican was elevated in experimental and clinical heart failure in response to increased inflammation and mechanical stress. Lumican production by cardiac fibroblasts induced alterations in molecules involved in cardiac remodeling and fibrosis.

Paper IV

Lack of collagen VIII reduces fibrosis and promotes early mortality and cardiac dilatation in pressure overload in mice

We demonstrated that mice deficient in the non-fibrillar collagen VIII exhibited increased mortality 3-9 days after AB and increased LV dilatation from day one. Immunohistochemistry revealed that LV collagen VIII was localized to cardiac fibroblasts, their surrounding extracellular space and in cardiac endothelium. LV expression of the main structural collagens (collagen I and III) was increased in WT mice 48 h after AB, but unaltered inn collagen VIII KO mice. We found reduced SMAD2 signaling and reduced expression of profibrotic TGF-β1and the myofibroblast markers α-SMA, Pxn and SM22 in collagen VIII KO hearts compared to WT 48 h after AB. Six weeks after AB, LV collagen I and III mRNA and total collagen protein measured by hydroxyproline HPLC were increased to a lesser extent in collagen VIII KO mice compared to WT. Consistently, cultured cardiac fibroblasts from collagen VIII KO mice showed reduced expression of TGF-β1, α-SMA, Pxn and SM22 compared to WT. Interestingly, we demonstrated that stimulation with recombinant collagen VIII rescued the expression of TGF-β1 in collagen VIII KO cardiac fibroblasts, and upregulated TGF-β1 mRNA in WT cells. The migratory capacity was reduced in cardiac fibroblasts from collagen VIII KO mice compared to WT. Our findings indicated that this was due to increased Rho activity and reduced MMP2 expression, and not due to reduced proliferation, which was increased in collagen VIII KO fibroblasts. Finally, we showed that recombinant collagen VIII rescued the migratory ability of collagen VIII KO cardiac fibroblasts and increased migration in WT cells. We concluded from our data that lack of collagen VIII reduces myofibroblast differentiation and fibrosis and promotes early mortality and LV dilatation in response to pressure overload in mice.

Discussion

Syndecan-4: an effector of cardiac immune responses?

In the first two papers of this thesis, the role of syndecan-4 in cardiac immune responses was investigated. Syndecans are the major source of cell surface HS. HS is known to bind and regulate a number of molecules and processes related to inflammation, e.g. immune cell maturation, activation and chemotaxis and wound healing.[163] Thus, it is not surprising that syndecans influence on key events of the inflammatory cascade in tissue injury and wound healing. Of note, studies using mice deficient in syndecan-4 have revealed an important role for this particular member in host defense responses.[164]

Syndecan-4 is rapidly upregulated in the LVs of mice after AB as demonstrated in Paper I, in line with previous findings by us[96, 115] and others,[113] showing elevated syndecan-4 in LVs of mice following AB and MI. Of notice, syndecan-4 expression peaks as early as 6 h after AB (unpublished observations, expression measured at 1 h-18 weeks of AB). In Paper I, we found that TNFα, IL-1β and LPS directly induce syndecan-4 expression in cultured cardiac myocytes and fibroblasts through NF-κB, consistent with a parallel upregulation of syndecan-4, TNFα and IL-1β in pressure-overloaded hearts. Our findings of TNFα, IL-1β and LPS as regulators of syndecan-4 expression in the heart are in line with studies from non-cardiac tissues showing syndecan-4 to be upregulated by inflammatory cytokines and TLR agonists, e.g. bacterial products.[165-167] Moreover, *in vitro* mechanical stress induced syndecan-4 expression in cardiomyocytes. Since comparable experiments have demonstrated that cyclic mechanical stretch enhances NF-κB activity and production of TNFα and IL-1β,[66, 168] it is possible that autocrine signaling by mechanical stress-induced cytokines and not the mechanical signal *per se* triggers syndecan-4 expression in response to pressure overload. Syndecan-4 was first reported to be an immediate-early gene activated by mechanical stress during tissue injury in smooth muscle cells,[169, 170] and our findings in Paper I support a role for syndecan-4 in the acute responses to increased mechanical stress induced by LV pressure overload. Considering that syndecan-4 is induced within 1 h in gastric epithelial cells and macrophages challenged with bacteria or bacterial products,[167] syndecan-4 appears to also be part of the primary host response to bacterial infection. In agreement with these findings, we showed that cardiac syndecan-4 was induced by LPS challenge *in vivo* (Paper II). Thus, in light of what is known from other tissues and the rapid upregulation of syndecan-4 in the heart by key mediators of innate immunity, we propose the novel concept of syndecan-4 as an effector of the cardiac immune response.

A role for syndecan-4 in immune responses has been indicated by previous studies revealing inflammation-related phenotypes in syndecan-4 KO mice.[171-174] Consistent with our findings in LPS-challenged hearts, syndecan-4 expression is rapidly induced in lungs by administration of LPS and *Streptococcus pneumonia*.[172, 174] In these mouse models of bacterial pneumonia, increased levels of neutrophils and chemokines were found in the bronchoalveolar lavage (BAL) fluid of syndecan-4 KO mice.[172, 174] In studies by Nikaido *et al*.[172] and Ishiguro *et al*.,[171] lack of syndecan-4 was associated with increased mortality to bacterial challenge and increased levels of circulating proinflammatory cytokines, possibly due to the ability of syndecan-4 to bind and modulate the inhibitory effect of TGF-β1 on IL-1β production in macrophages.[171] We, however, found no differences in TNFα and IL-1β synthesis between WT and syndecan-4 KO hearts following LPS challenge (Paper II). This is in contrast to the pressure-overloaded heart, where expression of several proinflammatory cytokines was increased in syndecan-4 KO mice during LV remodeling (Paper I). In the liver, syndecan-4 was shown to protect against inflammation-induced injury by binding and modulating the effect of another immunoregulatory cytokine, i.e. osteopontin.[173] Consistently, these studies show that upregulation of syndecan-4 in response to inflammatory stimuli limits the extent of inflammation and tissue injury. Of note, the mechanisms whereby syndecan-4 mediates its protective effects, ranging from neutrophil migration[172, 174] to direct modulation of cytokines,[171, 173] appear to vary between the different tissues and the nature of the inflammatory reaction. Our findings in Paper I and II suggested that syndecan-4-mediated protection against inflammation-induced cardiac dysfunction was related to the infiltration of immune cells to the heart (discussed below).

In the heart, TNFα and IL-1β are important mediators of the sterile inflammation activated during pathological conditions,[175] whereas LPS is a component of bacterial cell wall and the prototypical stimulus for inflammatory responses induced by bacterial infections.[176] However, the immune response elicited by LPS is by and large prompted by activation of NF-κB and production of TNFα and IL-1β,[177] signifying that despite being different in origin, magnitude and duration, several molecules and processes are shared between sterile inflammation and bacterial infections. Our results indicate that induction of syndecan-4 is a prime example of a shared feature of innate immune activation. Another well-known example of this is TLR4, the principal receptor for LPS.[178] The fundamental role of TLR4 in pathogen recognition and activation of innate immunity is signified by the awarding of the 2011 Nobel Prize in Physiology or Medicine to the scientists discovering its *Drosophila melanogaster* homolog[179] and its role in LPS-induced sepsis.[178] At present, a growing body of evidence

links this receptor to remodeling and failure of the heart in a number of cardiac pathologies,[180-183] where TLR4 is activated by endogenous DAMPs released during sterile inflammation.[49-51] We showed that TLR4 inhibition completely abolished LPS-induced syndecan-4 expression in cardiac myocytes and fibroblasts. Considering the involvement of TLR4 in initiating immune responses during hemodynamic stress, we speculate that syndecan-4 expression is mediated through TLR4 in pressure-overloaded hearts. One could imagine that the evolutionary old syndecans[184] have co-evolved with TLRs and that syndecan-4 is an integral part of the innate immune response induced by TLR4. The efficacy of the innate immune system is reflected in the fact that most organisms survive with this system alone; the adaptive immune system has evolved only in vertebrates.[185] Nonetheless, the discovery of TLRs resolved a long standing question in immunology, namely how pathogen-derived signals activate the innate immune system and thus lead to the priming of the adaptive immune response mediated by cells of the adaptive immune system, such as the T-cells.

One consequence of innate immune activation in the heart is recruitment of immune cells from the blood to the cardiac tissue. HS GAG chains are involved in essentially every stage of immune cell transmigration through the vessel wall, thereby regulating their recruitment into the tissue.[163, 186, 187] Thus, we hypothesized that HS-carrying syndecan-4 mediates innate immune responses by regulating infiltration of immune cells. Indeed, we found that recruitment of immune cells to the heart was attenuated in syndecan-4-deficient mice following AB (Paper I) and LPS challenge (Paper II). Thus, this effect of syndecan-4 appeared to be independent of the stimulus being AB or LPS, reflecting a shared mechanism. However, whether this response was due to a direct role of HS on syndecan-4 in promoting immune cells transmigration or other mechanisms or both, was not elucidated. Collectively, findings in paper I and II indicated that syndecan-4 is an effector of cardiac immune responses, serving as a link between innate immune activation and the responses mediated by cells of the immune system, e.g. T-cells, recruited to the heart in response to pathological stimuli.

Syndecan-4 shedding in cardiac inflammation: regulation, consequences and relevance to human cardiac disease

An important finding in this thesis was that not only increased expression, but also shedding of syndecan-4 from cardiac myocytes and fibroblasts was induced by inflammatory mediators associated with both pressure overload and heart failure, i.e. TNFα and IL-1β, and bacterial infections, i.e. LPS. Shedding is a post-translational modification whereby syndecans are cleaved, releasing soluble, biologically active ectodomains. Syndecan shedding is highly

regulated and dependent on the so-called sheddase enzymes.[188] In Paper I and II, we showed that inhibition of NF-κB reduced syndecan-4 shedding from cardiac myocytes and fibroblasts. However, whether a reduction in syndecan-4 synthesis contributed or whether activation of the responsible sheddase was dependent on NF-κB-mediated signaling was not elucidated. Interestingly, many cytokines that are known to activate NF-κB, also induce expression of several sheddases, e.g. MMPs.[85, 137] Thus, the attenuation of syndecan-4 shedding subsequent to NF-κB inhibition likely results from a dual role of this multifaceted transcription factor, regulating both syndecan-4 and the responsible sheddases.

In Paper I, we found that shedding of syndecan-4 appears to be regulated by enzymes dependent on the presence of HS GAG chains. Interestingly, HS has been shown to bind and modulate the activity of several sheddases.[189, 190] Contrary to our findings for syndecan-4, shedding of syndecan-1 is suppressed by the presence of HS GAG chains,[191] consistent with the acceleration of shedding of this proteoglycan by heparanase.[192] The juxtamembrane domain of syndecan-4 has been shown to be sensitive to proteolytic cleavage *in vitro*,[98] however we observed only a slight reduction in constitutive shedding of syndecan-4 after removal of this domain. This is in line with the findings of Rodríguez-Manzaneque *et al.* showing that removal of the juxtamembrane domain had no effect on ADAMTS1-induced shedding of syndecan-4.[193] In fact, this sheddase was found to cleave syndecan-4 close to the first HS chain attachment site. Interestingly, the juxtamembrane domain was shown to be essential for both constitutive and accelerated shedding of syndecan-1, as mutations in the amino acid sequence in this site blocked shedding both *in vitro* and *in vivo*.[194] Altogether these studies demonstrate that several mechanisms govern the shedding of syndecans, and that findings from *in vitro* settings do not necessarily reflect the processes occurring *in vivo*.

In Paper II, we observed a parallel upregulation of syndecan-4 and three sheddases proposed to regulate its shedding,[193, 195] i.e. ADAMTS1, ADAMTS4 and MMP9. Interestingly, a similar correlation was found during AB-induced remodeling (unpublished observations), further supporting a role for these enzymes in regulating syndecan-4 shedding in the heart. Although several sheddases are found to induce shedding *in vitro*,[98] there seems to be a discrepancy between the activity of these enzymes *in vitro* and what they do *in vivo*.[196, 197] As we observed in Paper II, not all sheddases are upregulated at the same time and/or in the cardiac tissue, and there is likely to be a time-, cell- and tissue-specific regulation of sheddases, ultimately leading to some limitation in terms of which enzymes may mediate the shedding response. This is evident from studies where inhibition of one or a small number of sheddases is found to profoundly affect shedding levels of a particular syndecan.[195, 198, 199] For

instance, specific knockdown of MMP9 by siRNA completely blocked TNFα-induced syndecan-4 shedding from human glomerular endothelial cells.[195] In WT mice, shedding of syndecan-1 is increased in bleomysin-treated lungs, however the pattern of syndecan-1 staining remained unchanged and no syndecan-1 was detected in BAL fluid in MMP7 KO mice.[199] However, Pasqualon *et al*. showed that in cultured lung epithelial tumor cells, constitutive shedding of overexpressed syndecan-1 was significantly reduced in ADAM17 KO mice.[198] These reports demonstrate that the sheddase responsible for shedding of a specific syndecan may depend not only on cell and tissue type, but also on the pathophysiological setting. In light of this, it is likely that more than one sheddase may regulate syndecan-4 shedding during cardiac inflammation, however that which sheddases are involved depends on the etiology and nature of the immune response.

Shedding of syndecan ectodomains is accelerated in response to pathological stimuli.[101-104] Thus, when studying the function of syndecans in disease, the picture is complicated by the release of their soluble ectodomains, and one major challenge is to separate effects of cell-associated syndecan-4 from those of the bioactive ectodomains shed in response to inflammation. In Paper II, we capitalized on the robust syndecan-4 shedding response induced by LPS (Paper I) to study the consequences of syndecan-4 shedding from cardiac cells *in vivo*.

Following LPS challenge, cardiac dysfunction was exacerbated and infiltration of immune cells attenuated in syndecan-4 KO mice. Since LPS increased shedding of syndecan-4 without altering levels of full-length protein, these effects could be attributed, at least in part, to the lack of shed ectodomains in the syndecan-4-deficient mice. The shed ectodomains of syndecans are believed to direct immune cells to inflamed tissues by forming chemoattractive gradients of GAG-bound chemokines.[199] Adding another mechanism, in Paper II we demonstrated that the shed ectodomains of syndecan-4 can act on cardiac myocytes and fibroblasts directly, inducing NF-κB signaling and expression of cellular adhesion molecules, i.e. Icam1 and Vcam1. Since Icam1 and Vcam1 are critically involved in regulating immune cell infiltration to tissues during inflammation,[71, 200, 201] we speculate that this mechanism contributes to the attenuated recruitment of immune cells to hearts of mice lacking syndecan-4. Moreover, we found that the transcriptional response in cardiac cells was dependent on the presence of HS chains on the syndecan-4 ectodomains. One possible explanation for this finding is that the shed ectodomains of syndecan-4 facilitate transportation of HS-binding cytokines through the ECM.[202] Alternatively, HS GAG-substituted syndecan-4 may represent a novel danger signal, adding to the growing list of proteoglycans that can function as

DAMPs.[203] Whether it is indirect via bound signaling molecules or a direct interaction with cellular receptors, the paracrine effect of syndecan-4 ectodomains may contribute to enhanced intercellular communication between the different cell populations of the heart, i.e. cardiac myocytes, fibroblasts and immune cells, a topic that is receiving increased focus.[204-206]

Matsui *et al*. previously reported increased mortality due to cardiac rupture in syndecan-4-defienct mice after MI.[113] Consistent with our findings from the AB and LPS models, this was associated with an impaired inflammatory response, evidenced by reduced numbers of immune cells in the infarct region. Based on our findings in Paper II, these effects of syndecan-4 could be facilitated, at least in part, by its ectodomains. Interestingly, adenoviral administration of the syndecan-4 ectodomain into the circulation resulted in increased mortality due to cardiac rupture, similar to what was observed in syndecan-4 KO mice.[113] Herein, the shed form of syndecan-4 appears to act as a dominant-negative inhibitor of intact syndecan-4. Although contradictory to our findings in Paper II, which indicate a protective role for shed syndecan-4, these results could be explained by the different pathological settings of the two studies, i.e. MI vs. LPS challenge, or duration, i.e. hours vs. days-weeks. Moreover, as only the circulatory levels of syndecan-4 ectodomains were reported after adenoviral administration,[113] the potential beneficial effect of the shed ectodomains may be dependent on their localization to the cardiac tissue. In line with this, sustained cardiac overexpression of syndecan-4 after intramyocardial administration of adenovirus containing the full-length molecule, which, based on our findings in Paper I and II, is likely to increase levels of shedding, was found to be protective in rat hearts post-MI.[207]

Our findings in Paper II are the first to shed light on the functional effects of increased syndecan-4 shedding in the heart, indicating that during an acute inflammatory response, the increased shedding of syndecan-4 represents a protective mechanism that mitigates cardiac dysfunction and ensures a properly coordinated immune response by promoting recruitment of immune cells. Our findings indicate that ADAMTS1, ADAMTS4 and MMP9 might regulate cardiac syndecan-4 shedding, however experiments using specific inhibitors, siRNA or KO mice for these sheddase should be carried out to determine their roles. Moreover, *in vivo* studies combining heart failure mouse models with targeted disruption, e.g. blocking the responsible sheddases or genetically engineering a shedding-resistant syndecan-4, or enhancement, e.g. overexpression of syndecan-4 ectodomain, of syndecan-4 shedding in the heart would provide further insights into the functional consequence of this process during cardiac disease.

Circulating biomarkers are valuable diagnostic and prognostic tools in heart disease and several biomarkers show clinical importance by providing information regarding pathogenesis, diagnosis or risk assessment and stratification, e.g. BNP, N-terminal (NT) pro-BNP, CRP and troponin T and I.[208] Biomarkers reflect diverse pathological processes occurring during the development and progression of heart disease.[209] Syndecan-4 was recently listed as one of the emerging biomarkers displaying potential promise for future clinical use.[210] This assessment is based on a few studies in relatively small patient cohorts showing elevated circulating levels of syndecan-4. Measurements in 11 patients revealed that plasma levels of syndecan-4 increased with acute MI, peaking 2-3 weeks after onset.[106] Based on analysis of 45 heart failure patients, increased circulating syndecan-4 was found to correlate negatively with EF and positively with LV mass and end systolic and diastolic diameters, suggesting syndecan-4 as a biomarker of remodeling.[107, 211]

Circulating syndecan-4 represents shed ectodomains, indicating that shedding is accelerated in patients with cardiac disease. The increased levels of cell-localized syndecan-4 shedding fragments in myocardial biopsies from patients with end-stage heart failure (Paper I), suggest that syndecan-4 shedding occurs in the failing human heart. Indeed, in Paper II, we were able to confirm that syndecan-4 was shed from hearts of patients with AS. Importantly, we demonstrated that elevated shedding from the heart itself could, at least in part, contribute to the increased levels of circulating syndecan-4 in patients with cardiac pathology. In view of our findings showing that inflammatory signaling is a significant stimulus of syndecan-4 shedding, we speculate that syndecan-4 is a biomarker that can provide information about the inflammatory state in the myocardium. Moreover, based on the elevated levels of syndecan-4 in the blood after LPS challenge in mice (Paper II), syndecan-4 might hold promise as a biomarker for sepsis or bacterial infections. Interestingly, a recent report supports this. Serum levels of syndecan-4 were elevated in patients with acute pneumonia and correlated negatively with pneumonia severity score.[172] Furthermore, higher syndecan-4 levels on admission correlated to improvement after short-term antibiotic therapy.

In conclusion, recent literature suggests syndecan-4 to be a promising biomarker for cardiac disease, and work presented in this thesis supports this concept. Due to the regulation of syndecan-4 during cardiac remodeling and inflammation[96, 113-115], it is likely that syndecan-4 as a biomarker can provide information about the pathogenesis of heart failure, especially with regard to inflammation in the myocardium.

The multiple roles of syndecan-4 in cardiac remodeling

Elucidating the roles of syndecans in cardiac pathology represents a relatively new field in cardiac research. In recent years, studies by our group[96, 114-116] and others[113, 207, 212] have investigated syndecan-4 in cardiac disease in mice and men. These studies have highlighted a role for syndecan-4 in regulating not only cardiac inflammation, but also remodeling, e.g. hypertrophy and fibrosis, and function following pressure overload and MI. Common to inflammation, fibrosis and hypertrophy, their sustained activation have deleterious consequences and contribute to failing of the heart.

In cardiac myocytes and fibroblasts, syndecan-4 functions as a mechanosensor, inducing hypertrophic and fibrotic remodeling in response to increased mechanical stress.[114, 115] Herein, dephosphorylation of serine179 in the cytoplasmic domain of syndecan-4 (V-region) acts as a switch to activate the calcineurin-NFAT pathway. In the setting of AB, syndecan-4 KO mice show attenuated hypertrophy and fibrosis, and develop LV dilatation and premature failure. Myofibroblast differentiation was reduced after AB and MI in syndecan-4 KO mice,[113, 115] likely due to reduced mechanosensing in syndecan-4-deficient cardiac fibroblasts.[115] Accordingly, collagen synthesis, collagen cross-linking, LOX expression and activity were attenuated in syndecan-4 KO mice following AB, accompanied by reduced passive tension[115, 116] In Paper II, we showed that shed ectodomains of syndecan-4 induced expression of LOX in cardiac fibroblasts. Taken together with the findings by Herum *et al*. showing that syndecan-4 ectodomains enhance collagen cross-linking *in vitro*,[116] it seems that shed syndecan-4 could have both direct and indirect effects on collagen cross-linking, indicating that syndecan-4 shedding could influence on cardiac stiffness and diastolic dysfunction.

In addition to creating soluble effectors that could influence on remodeling, shedding is believed to abrogate down-stream signaling of the intact syndecan. It seems likely that shedding would disrupt the mechanosensing properties of syndecan-4, and thus it could be speculated that syndecan-4 shedding is a regulatory mechanism to dampen the mechanical stress-induced hypertrophic and fibrotic responses mediated by the full-length molecule. However, a peptide consisting of the membrane and cytoplasmic parts of syndecan-4 retained the ability to induce NFAT activation in cardiomyocytes.[114] Moreover, a recent study showed that the corresponding fragment arising from syncecan-1 shedding retained some of the function of full-length syndecan-1 in terms of intracellular signaling, and that shedding by ADAM17 in fact promoted migration.[198] In line with this, ADAMTS1-induced shedding of syndecan-4 was shown to reduce focal adhesions and promote migration of fibroblasts, thus

resembling the phenotype of syndecan-4-deficient cells.[193] Consistently, LPS-induced shedding of syndecan-4 resulted in loss of focal adhesions in cardiac fibroblasts (Paper I), indicating that shedding of syndecan-4 may be a mechanism whereby these cells regulate their motility, i.e. adhesion and migration, an important aspect of their function. However, there is currently limited knowledge about the regulation and role of syndecan-4 shedding during pressure overload-induced remodeling. Based on our findings in Paper I, it is likely that the inflammatory response activated by pressure overload induces shedding of syndecan-4. Indeed, although levels of full-length syndecan-4 is upregulated in pressure-overloaded hearts,[96, 114] we have observed increased levels of syndecan-4 shedding fragments during AB-induced remodeling (unpublished observations), consistent with increased shedding in patients with AS (Paper II). Therefore, it would be of interest to investigate in more detail how shedding is regulated and how it affects syndecan-4-mediated responses in the pressure-overloaded heart.

In Paper II, we showed that shed syndecan-4 reduced the expression of the structural collagens I and III in cardiac fibroblasts, indicating that increased levels of syndecan-4 shedding could mediate the reduced collagen production observed in LVs following LPS challenge. These results suggest that the soluble ectodomains play an attenuating and opposing role to the full-length syndecan-4, likely reflecting that syndecan-4 exerts different functions depending on the level and nature of the inflammatory response. Importantly, shed syndecan-4 shifted the MMP/TIMP balance towards ECM degradation, indicating that inflammation-induced shedding of syndecan-4 may have profound effects on ECM remodeling, and thus, cardiac function, during infection and inflammation. Moreover, as MMPs are known to facilitate migration of immune cell into tissues,[213] the ability of syndecan-4 ectodomains to induce MMP expression may represent another mechanism whereby syndecan-4 shedding promotes immune cell recruitment to the heart.

In both the AB and LPS models, syndecan-4 affected the recruitment of T-cells to the heart. Interestingly, T-cells have been shown to not just shape inflammatory responses, but also influence on cardiac remodeling. $CD4^+$ T-cells promote collagen accumulation and cross-linking in pressure-overloaded hearts.[72, 75] However, some T-cell subsets, e.g. regulatory T-cells, have been shown to attenuate cardiac hypertrophy and fibrosis in a number of cardiac pathologies.[214-216] Hence, by modulating the cellular immune response, syndecan-4 shedding indirectly influences on remodeling.

In conclusion, it is becoming increasingly clear that syndecan-4 is an important regulator of cardiac remodeling following injury, stress and infection and an integral mediator

of inflammatory, fibrotic and hypertrophic processes. Although syndecan-4 deficiency reduces stiffness and hypertrophy of the heart,[114, 116] cardiac dysfunction is more pronounced in syndecan-4 KO mice in response to pathological conditions spanning from AB[114] and MI[113] to LPS challenge (Paper II), suggesting that syndecan-4 exerts a complex, but ultimately protective role during cardiac pathology. In light of the effects of shed syndecan-4 ectodomains on the expression of ECM molecules and recruitment of immune cells, we propose the novel concept that shedding of syndecan-4 plays an important and multifaceted role in inflammation-induced cardiac remodeling.

The small leucine-rich proteoglycan lumican in heart failure: a novel regulator of fibrotic signaling?

In Paper III, lumican, a member of the SLRPs, was investigated in cardiac fibroblasts and failing hearts. A common feature of genetically modified mice with disrupted genes encoding members of the SLRP family of ECM-localized proteoglycans, e.g. decorin, biglycan, lumican and fibromodulin, is the impaired growth or organization of collagen fibers, resulting in a disorganized ECM of various connective tissues.[123, 217-219] Previous work from our group has shown that reduced levels of fibromodulin, decorin and lumican are associated with a loosely packed ECM following AB.[120] Collectively, these results suggest that SLRPs are important regulators of ECM structure in the heart.

Although information about the function of lumican in normal and diseased hearts is scarce, a few studies by our group[120, 121, 220] and others[124, 125] have shown that lumican levels are altered in the remodeling heart. Importantly, protein levels of lumican correlated positively with lung weight during pressure overload-induced remodeling, suggesting an association between lumican and development of congestive heart failure.[121] Consistent with this, we found that protein levels of glycosylated lumican[221] were increased only in mice that had undergone transition to congestive heart failure, characterized by increased lung weight and LAD. Also, in agreement, lumican levels were elevated in LVs of patients with end-stage heart failure. The consequences of increased lumican in the failing heart remain unknown. In view of lumican regulating collagen fibril assembly and ECM organization,[123, 222] the upregulation of lumican could be a compensatory mechanism counteracting LV dilation. This would suggest a protective role of lumican during heart failure progression of pressure-overloaded hearts, and in our group, we are currently testing this hypothesis by subjecting lumican KO mice[123] to AB (unpublished results and ongoing work).

Studies over the past decade have revealed that lumican and other SLRPs can modulate tissue responses as matrikines,[118] i.e. molecules released from the ECM, mediating cell-matrix crosstalk and signaling. In line with this, stimulation with recombinant glycosylated lumican induced fibrotic signaling in cardiac fibroblasts, e.g. activation of SMAD3 signaling, increased expression of collagen I, LOX and TGF-β1 and reduced activity of MMP9. It has been suggested that SLRPs regulate cross-linking of collagens by directing LOX to the proper sites.[223] In Paper III, increased formation of the dimeric and cross-linked form of collagen I was observed after stimulation with lumican, possibly due to increased activity of LOX. This finding suggests that lumican increases collagen cross-linking, ultimately leading to reduced turnover of collagens and a stiffer heart.

We showed that, similar to syndecan-4, IL-1β and mechanical stress induced expression of lumican by cardiac fibroblasts, but unlike the rapid upregulation of syndecan-4, levels of lumican are increased at a later stage of remodeling. Newly synthesized lumican is released into the pericellular space, however at the present time it is not known whether the newly synthesized lumican is binding to collagens in the ECM or act primarily as a profibrotic signaling molecule or both. Studies using lumican KO mice will provide insights into the mechanisms and consequences related to the role of lumican in the heart.

The leucine-rich repeats of the lumican core protein contain potential interaction sites for TLRs and CD14.[224, 225] However, unlike the SLRP biglycan which acts as an endogenous ligand for TLR4 and TLR2,[226] lumican appears to have an indirect effect on receptor-mediated signaling by presenting other ligands to their respective receptors. Interestingly, lumican mediates interaction between LPS and CD14, thereby activating the TLR4 pathway in macrophages.[227] Our findings showing that inhibition of NF-κB attenuated the transcriptional effects of recombinant glycosylated lumican are consistent with lumican signaling through TLR4 also in cardiac fibroblasts. However, multiple signaling pathways converge on NF- κB activation, and thus, the involvement of other receptors cannot be ruled out. Since TLR4 has been shown to regulate hypertrophic and fibrotic responses in pressure overload,[180] it is tempting to speculate that lumican could mediate signaling through TLR4 in the pressure-overloaded heart.

Collagen VIII in the pressure-overloaded heart: a structural component and a novel profibrotic signaling molecule?

In Paper IV, the role of collagen VIII in pressure overload-induced remodeling was investigated. Collagen VIII is a non-fibrillar, short-chain collagen consisting of collagen VIII

α1 and α2 chains.[228, 229] Recent work from our group showed that collagen VIII is altered in mice with and without heart failure developed from pressure overload[133] and increased during reverse remodeling following relief of pressure overload.[134] Specifically, collagen VIII protein is reduced in mice with heart failure, and levels of collagen VIII correlate negatively with degree of LV dilatation, indicating that collagen VIII affects tissue integrity in the heart and the transition from hypertrophy to dilatation.[133] In Paper VI, we showed that collagen VIII is involved in acute adaption of the heart to pressure overload and that its presence is crucial for survival and protection from dilation during the acute phase of remodeling. The finding of no further increase in mortality as the pressure overload stabilized, suggests that collagen VIII is vital for the ability of the heart to withstand the sudden or initial increase in mechanical load induced by AB. The mechanisms behind the protective role of collagen VIII remain undefined, however based on our findings and the existing knowledge about the function of collagen VIII in other cells and tissues, several hypotheses may be postulated.

Collagen VIII can direct adherence to ECM molecules, such as the structural collagen I,[230] and is suggested to serve as a molecular bridge between different ECM molecules.[136] In Paper IV, we showed that collagen VIII accelerated the formation of collagen I fibers *in vitro*. Thus, these findings suggest that collagen VIII modifies tissue integrity directly and that its function in protecting against dilatation might be structural, preventing "sliding" of collagen fibers.

Several recent studies indicate that collagen VIII is more than just a structural component of the ECM. Rather, it can regulate tissue remodeling by mediating fibrotic signaling and processes in smooth muscle cells.[230-232] Thus, we speculated that collagen VIII could regulate the integrity of the LV indirectly by promoting fibrotic remodeling by cardiac fibroblasts. In line with this, we showed attenuated TGF-β1 signaling, reduced production of structural collagens I and III and decreased myofibroblast differentiation after pressure overload in mice lacking collagen VIII. Considering the crucial role of TGF-β1 in promoting differentiation of fibroblasts into myofibroblasts,[139, 140] the attenuated myofibroblast differentiation and collagen synthesis was likely due to reduced TGF-β1-induced signaling in collagen VIII-deficient cardiac fibroblasts. Collagen VIII has previously been shown to facilitate migration of smooth muscle cells.[230, 232, 233] Consistently, the migratory capacity was markedly reduced in collagen VIII KO cardiac fibroblast. Thus, collagen VIII appeared to mediate not only differentiation of fibroblasts into myofibroblasts, but also important aspects of myofibroblast function. A novel finding in Paper IV was the ability of recombinant collagen to rescue the phenotype of collagen VIII-deficient cardiac fibroblasts, i.e. expression

of TGF-β1 and migration, demonstrating that collagen VIII can act as a signaling molecule. Thus, a possible scenario is that collagen VIII is released from the ECM in response to pressure overload to induce profibrotic responses in cardiac fibroblasts.

During chronic pressure overload, prolonged myofibroblast activation results in increased collagen production and fibrosis, ultimately leading to myocardial stiffening and dysfunction.[137, 234] However, myofibroblasts differentiation may represent a beneficial response in the acute adaptation to increased load, counteracting LV dilatation. Indeed, we demonstrated a protective role for collagen VIII during the early phase of pressure overload that could be related to modulation of the fibrotic response exerted by myofibroblasts. Alternatively, collagen VIII might have a more direct effect on the structural integrity of the LV by its ability to bridge components of the ECM. Thus, further elucidation of the mechanisms underlying the protective effect of collagen VIII is needed to evaluate the potential of this collagen isoform as a target for therapy.

Concluding remarks and future perspectives

While historically considered a structural scaffold, it is now appreciated that the ECM is a highly dynamic structure mediating cellular responses during physiological and pathological conditions. Chronic inflammation is a hallmark of heart failure, and is closely linked to the extensive ECM remodeling observed. Reciprocally, the ECM is a source of bioactive molecules that can regulate tissue responses such as fibrosis and inflammation, and under normal conditions, these processes fine-tune the biochemical and structural status of the heart. The results presented in this thesis show that the transmembrane proteoglycan syndecan-4 and the ECM-localized proteoglycan lumican and collagen VIII are active components of the cardiac ECM, influencing on inflammation, fibrosis and dysfunction during heart failure progression. In addition to their established roles of binding structural ECM components, our investigations have revealed that these molecules also exert roles as signaling molecules subsequent to their synthesis or release from cells or the ECM, to mediate inflammatory and fibrotic signaling. Common to these processes is that, despite initially part of an adaptive response to stress, injury or infection, sustained activation may have deleterious consequences and contribute to cardiac dysfunction and failure. Thus, further investigations into the role of syndecan-4, lumican and collagen VIII during the adaptive and maladaptive stages of cardiac remodeling are needed. Although their potential as therapeutic targets or biomarkers is yet to be determined, the findings of this thesis support a concept of an important role for these molecules in heart failure and thus, continued research in this field.

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