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New aspects in myocardial remodeling and dysfunction due to left ventricular pressure overload

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ABBREVIATIONS

BNP – brain natriuretic peptide

bpm – beats per minute

ECM – extracellular matrix

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

HPLC – high pressure liquid chromatography

IEMF – Institute for Experimental Medical Research

MMP – matrix metalloproteinase

SEM – standard error of the mean

SERCA2 – sarcoplasmic reticulum calcium ATPase 2

SMAD – small mothers against decapentaplegic

TAC – transverse aortic constriction

TGF-β – transforming growth factor beta

TIMP – tissue inhibitor of metalloproteinase

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My love for surgery witch I later transferred to mastering experimental animal models was planted at the Department for Cardiothoracic surgery at Sahlgrenska University Hospital, Göteborg, Sweden. There I learned that surgery cannot be learned from the books, but it can “easily” be learned being part of a family of surgical enthusiasts. For wonderfull 4 years and all operated hearts, lungs and other thoracic surgeries I thank all my former colleagues and staff at the department. A special thanks to my mentor **Dr. Mogens Bugge** for believing in me and trusting me in the operating theater. **Dr. Ali Belboul** and **Dr. Donald Roberts** for inviting me to the department. Former heads of the department **Dr. Gunnar Brandrup-Wognsen** and **Dr. Lars Wiklund** for welcoming me to the clinic, present head of the department **Dr. Jakob Gäbel** for letting me make short comebacks during my visits to Göteborg. **Dr. Obaid Al Jassim**, a great friend, and **Dr. Hans Lidén** for my first ever vein harvesting and assistance at open heart surgery. **Professor Anders Jeppsson** for introducing me to my supervisor **Professor Theis Tønnessen**. It was a great honor meeting and working with **Professor Eva W-O Berglin**. The list is long as every single person at the department was so friendly, open and willing to unselfishly share knowledge and surgical skills. Thank you all for letting me learning and living my dream.

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This thesis and all I accomplished so far I dedicate to an extraordinary women, my grandmother **Andja**, my “rodjenka”, and to my nephews **Jelisaveta** and **Filip**. I hope you will find this work motivational for your own dreams and encourage you for higher academic accomplishments.

Love you all!

Biljana Skrbić
Oslo, Norway, august 2016

LIST OF PAPERS

This thesis is from the cardiac research milieu at Oslo University Hospital Ullevål, combining the expertise from the Department of Cardiothoracic Surgery, Department of Cardiology and the Institute for Experimental Medical Research (IEMR). The thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I **Johannes L Bjørnstad, Biljana Skrbic, Ivar Sjaastad, Sigrid Bjørnstad, Geir Christensen, Theis Tønnessen**
A mouse model of reverse cardiac remodeling following banding-debanding of the ascending aorta. *Acta Physiol (Oxf)* 2012;**205**:92-102.

II **Biljana Skrbic, Johannes L. Bjørnstad, Henriette S. Marstein, Cathrine R. Carlson, Ivar Sjaastad, Ståle Nygård, Sigrid Bjørnstad, Geir Christensen, Theis Tønnessen**
Differential regulation of extracellular matrix constituents in myocardial remodeling with and without heart failure following pressure overload. *Matrix Biol* 2013;**32**:133-142.

III **Biljana Skrbic, Kristin V.T. Engebretsen, Mari E. Strand, Ida G. Lunde, Kate M. Herum, Henriette S. Marstein, Ivar Sjaastad, Per K. Lunde, Cathrine R. Carlson, Geir Christensen, Johannes L. Bjørnstad, Theis Tønnessen**
Lack of collagen VIII reduces fibrosis and promotes early mortality and cardiac dilatation in pressure overload in mice. *Cardiovasc Res* 2015;**106**:32-42.

IV **Johannes L Bjørnstad, Biljana Skrbic, Henriette S. Marstein, Almira Hasic, Ivar Sjaastad, William E. Louch, Geir Florholmen, Geir Christensen, Theis Tønnessen**
Inhibition of SMAD2 phosphorylation preserves cardiac function during pressure overload. *Cardiovasc Res* 2012;**93**:100-110.

INTRODUCTION

Heart failure may be defined as a pathophysiological state in which the heart is unable to pump blood at a rate commensurate with the requirements of the metabolizing tissues or can do so only from an elevated filling pressure (1). There are several etiologies that might lead to heart failure. Among these is aortic stenosis with accompanying left ventricular hypertrophy and fibrosis. Aortic stenosis increases left ventricular pressure, which triggers myocardial remodeling (hypertrophy of cardiomyocytes and structural alterations of the ECM). In part, cardiac hypertrophy is recognized as an adaptive response that normalizes wall stress and compensates for increased load. However, pathological hypertrophy might lead to the development of heart failure. To include detrimental changes besides the pathologic growth, the term maladaptive remodeling is often used.

Aortic stenosis is the most frequent valvular heart disease in Norway and the number of patients affected is increasing mainly because of aging. Untreated, severe aortic stenosis leads to progressive heart failure and there is a high risk of sudden death. Up to 90% of patients with critical aortic stenosis die within two years after onset of symptoms of heart failure (2, 3). Aortic valve replacement is the treatment of choice for aortic stenosis and this will often lead to normalization of the myocardial structure (reverse remodeling), but reduced function may persist if preoperative remodeling has been extensive. Excessive myocardial remodeling is associated with high mortality as well as increased operative risk following aortic valve replacement (4).

In daily practice it has been observed that patients with the same degree of aortic stenosis develop myocardial hypertrophy and heart failure of different degrees. Knowledge about the processes taking place during myocardial remodeling due to pressure overload is increasing; however, it is not known why patients with the same degree of aortic stenosis and pressure overload (same gradient across the valve) develop different degrees of myocardial remodeling and heart failure. Different genetics, comorbidity and lifestyle could partially be the answer. However, it has not previously been studied in detail why some patients develop compensated myocardial hypertrophy while some decompensate into heart failure, and how this is regulated. Revealing which mediators play a role in the development of myocardial remodeling and different degrees of heart failure would contribute to an enhanced understanding of these processes.

It has been shown earlier that changes in ECM are likely to affect myocardial compliance and may be important for systolic and diastolic function (5). Collagens are important constituents of the ECM serving as a scaffold for cardiomyocytes and various other cell types within the cardiac tissue (6-8). The collagens consist of several subtypes (9). In addition to the fibrillar collagens there are also non-fibrillar collagens. Of these, collagen type

VIII is stimulating vascular smooth muscle cell migration and matrix metalloproteinase (MMP) synthesis as well as potentially serving as a molecular bridge between different types of matrix molecules (10). It has been suggested that collagen type VIII plays a role in cell migration and differentiation in the developing heart (11). Moreover, collagen type VIII might also play an important role in the organization of other collagens in the ECM and may regulate the interaction between ECM and muscle cells. Thus, different alterations in ECM might explain why some hearts develop hypertrophy only and others progress to heart failure.

Some of the mediators that may affect the function of the heart due to myocardial remodeling have been defined in previous studies (12). Among them, members of the TGF-β superfamily have been shown to be implicated in myocardial remodeling and heart failure (4, 13). We have previously reported that one of its members, activin A, is altered in patients following aortic valve replacement for aortic stenosis (14). The TGF-β superfamily exerts their effects through activation of the TGF-β receptor with a subsequent phosphorylation and activation of TGF-β/SMAD signaling. It has been shown earlier that TGF-β/SMAD signaling may have both protective and deleterious effects on the heart (15-17). Thus, whether activation of TGF-β/SMAD leads to improved or reduced cardiac function remains unclear. Our group has access to the novel pharmaceutical drug SM16, which could help us examine the role of TGF-β signaling in myocardial remodeling and dysfunction. SM16 is a specific inhibitor of activin like kinase 4 and 5, reducing phosphorylation of SMAD2 and SMAD3 (18).

AIMS

The main aim of this thesis was to develop an insight into the ECM changes in an experimental model of the pressure-overloaded left ventricle, simulating aortic stenosis in humans.

PAPER I:

The aim in paper I was to establish and standardize a mouse model of reversible left ventricular pressure overload by banding and subsequent debanding of the ascending aorta.

PAPER II:

The aim in paper II was to develop an insight into potential differences in the regulation of myocardial ECM constituents in mice that develop hypertrophy only and in mice that develop signs of heart failure as a response to similar pressure overload and to correlate these findings to left ventricular function and geometry.

PAPER III:

The aim in paper III was to examine the role of collagen VIII on survival and left ventricular dilatation in the acute and more chronic phase of pressure overload *in vivo* and it's role on cardiac fibroblast differentiation and development of fibrosis *in vitro*.

PAPER IV:

The aim in paper IV was to examine the role of SMAD2 signaling in left ventricular function and remodeling in response to pressure overload *in vivo* and in isolated cardiomyocytes *in vitro*, by inhibiting the SMAD2 signaling system pharmacologically with SM16.

METHODOLOGICAL CONSIDERATIONS

ANIMAL MODELS

The use of various mouse models in cardiac research is beneficial for many reasons. Compared to other animal models, mice are small, affordable and require little space in a research animal facility. Moreover, it is often an advantage for studies of gene expression (19) that most lab mice are inbred, reducing genetic variability and inter-individual variability. Furthermore, the mouse genome has been sequenced, and transgenic and knock-out techniques have been developed (20). During the last decades several surgical mouse models have been developed, such as aortic banding inducing left ventricular pressure overload mimicking aortic stenosis and hypertension (21, 22). In the recent years huge progress has been made in developing methods for hemodynamic evaluation in mice, such as echocardiography (23), pressure-volume catheters and cardiac magnetic resonance imaging (24). One could argue that the use of mice in research has limited value for transfer of scientific discoveries to knowledge in human medicine both due to phenotypical and physiological differences. However, in fact 99% of mouse genes have a detectable human homolog (20). To get insight into the processes regulating myocardial remodeling and the development of heart failure, we have established a mouse model of pressure overload by banding of the ascending aorta. In this model, mimicking aortic stenosis in humans, we can explore myocardial changes both on the gene- and protein-level during myocardial remodeling. The model is well established and we obtain a 59% increase in left ventricular weight after 4 weeks of standardized banding. Furthermore, we have established a novel mouse model of reverse myocardial remodeling by banding-debanding of the ascending aorta, mimicking replacement of a stenotic aortic valve in humans. In the past, just a few models of reverse myocardial remodeling have been introduced (25, 26). The use of mouse models also helps us to minimize external confounding factors, which one would meet studying patients. Generally, results from studies in mice should ideally be confirmed by studies in humans.

ECHOCARDIOGRAPHY

Echocardiography is used for characterization of myocardial geometry and function and assessment of heart failure. This method has in recent years been improved by the development of tissue Doppler imaging and strain echocardiography. In humans, echocardiographic examinations are preformed while the patient is awake and due to bigger size of the heart and lower heart rate (approx. 60-70 bpm) one gets better resolution. However, even though the hearts of mice are smaller and the heart rate is higher (approx.

600-700 bpm), echocardiographic examinations are a valuable source of data for hemodynamic characterization of different models and correlation to protein and/or gene expression. In general this is a non-invasive method for measuring cardiac function and structure *in vivo* and gives us the opportunity to perform repeated measurements at different time-points. However, it is a demanding procedure for small animals and one should especially pay attention to how deep the animal is sedated and take measures to preserve body temperature and heart rate, since small changes could affect the results, such as cardiac depression due to deep anesthesia. Sedated animals rapidly loose body temperature and this could lead to decreased cardiac function, influencing or masking the findings. Poorly standardized examinations lead to increased variability, masking potential findings. The echocardiographic investigations in the present studies were performed on a heated table and, when possible, continuously monitoring the body temperature of each mouse. Ideally, all echocardiographic investigations and data analysis should be preformed by a highly trained investigator, well experienced both with human and small animal echocardiography as it was done throughout all projects in this thesis. In the use of different treatment groups and genetically different animals, all investigations have been performed blinded to the different groups.

COLLAGEN VIII KNOCK-OUT

Collagen type VIII deficient mice have been developed by Dr. Bjørn Reino Olsen's research group at Harvard Medical School (27) and kindly provided to our research group by Dr. Ulrike Hopfer (University of Basel, Switzerland). Collagen VIII knock-out mice have been crossed back onto C57BL/6J background for at least 20 generations. As described by Hopfer et al, comparing to C57BL/6J (wild type) mice, collagen type VIII knock-out mice do not express col8α1 or col8α2. They show no major histological differences in most organs, including the heart. They develop normally, are viable and fertile. As the collagen VIII knock-out mice are of comparable appearance, size and behavior it was possible to conduct the experiments described in paper III blinded to the genotype. The knock-out mice were bred at IEMR animal facilities by homozygous intercrosses, and genotype was confirmed by genotyping.

PHARMACOLOGICAL INHIBITION BY SM16

In paper IV we have used SM16, a novel pharmaceutical agent which is a small molecule inhibitor of the TGF-β type I receptor (28). SM16 was generously provided by Dr. Leona Ling at Biogen Idec. Mouse chow formulated with SM16 or standard chow was given to animals from 4 days before operation and until they were sacrificed, one week after aortic banding. SM16 was formulated into chow at a dose of 0.45g SM16/kg chow. At this dose no toxic

effects have been observed, and this dose was recommended by our collaborator at Biogen Idec that provided us with the SM16 (18). We weighed animals and chow pre- and postoperatively and found no differences in chow consumption or animal weight between experimental groups, thus, SM16 did not influenced appetite of the animals.

NON-INVASIVE MEASURING OF BLOOD PRESSURE

Different methods of measuring blood pressure in experimental animals have been described in the literature and are in daily use. Basically, the methods are divided into invasive and non-invasive. There are several advantages and disadvantages in both groups (29). In paper III we measured the blood pressure preoperatively in order to exclude potential hypertension as a possible cause of different early response to pressure overload in the various genotypes. Blood pressure was measured non-invasively, blinded to mouse genotype by the CODA standard tail-cuff blood pressure system (Kent Scientific, Connecticut, US). Measurements were performed on a heated examination table under light sedation and animals spontaneously breathing a mixture of 1.5% isoflurane and 98.5% oxygen on a mask. The blood pressure cuff was placed around the root of the tail. All measurements were performed on the same day, under the same conditions and by the same person in order to minimize external influence on the results.

HANDLING OF ANIMALS

In the present studies the mice were kept in standard animal cages, in rooms with temperature and light regulations (12 hours light/12 hours dark) at animal housing facilities at Oslo University Hospital, Ullevål. Animals had free access to water and food. Pre- and postoperatively there was always a person responsible for following the animal's condition. Imported 6 weeks old wild type animals C57BL/6 had acclimatization of one week prior to operation. Prior to operation, echocardiography investigations, blood pressure measurements and sacrifice, the weight of animal was registered. Anesthesia started at gas chamber with mixture of >5% isoflurane and oxygene as breathing gas. The further form and level of anesthesia was adjusted to the operation, investigation or sacrifice. During operation and sacrifice, animals were intubated and connected to the respirator, while during echocardiographic investigations and blood pressure measurements animals were breathing mixture of isoflurane and oxygene on the mask. The animals recovered in an incubator at 37°C for two hours to prevent heat loss in critical phase. For analgesia, we used an injection of buprenorfin 0,01 ml (0,3 mg/ml) subcutaneously which was repeated in the case where animal were considered to be in pain. If symptoms were not relieved within one hour, the animal was euthanized by the neck break under deep gas anesthesia. During sacrifice,

anesthesia was induced in the same way as during operation. First we made long incision over abdomen and without injuring organs approached by inferior vena cava and obtained blood from it. In the next step the sternum was separated, and the heart together with the lungs was taken out. Lungs, left and right ventricles were weighted, snap frozen in liquid nitrogen and kept at -80°C.

MICROARRAY SCREENING

To study the effects of aortic banding and the development of heart failure, we aimed to examine differences in gene expression in mice with myocardial remodeling only and in mice with heart failure. For this purpose we chose microarray screening. In this method the expression of thousands of genes are monitored simultaneously. Affymetrix GeneChip analyses over 39.000 transcripts on one single array. Microarray is an expensive analysis, which requires that only a small number of animals are analyzed. To interpret biological processes or molecular functions from such a huge amount of data, we used advanced bioinformatics available in the Bioconductor/topGO software. In this way we could examine which biological processes and functional gene groups are most differentially regulated in heart failure due to left ventricular pressure overload. The process of analyzing microarray and topGO data requires close collaboration with statistical experts in the field of bioinformatics. Important findings should be verified either on the transcriptional level (e.g. by QPCR) or by protein analyses (e.g. by western blot).

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

Quantitative real-time polymerase chain reaction (QPCR) analysis of myocardial gene expression is a widely used sensitive method that enables detection and quantification of mRNA expression (30, 31). It may be used for verification of microarray data. To avoid variability in the data, it is of great importance, in the preparation of the tissue for QPCR analysis, to snap freeze tissue in liquid nitrogen (-80°C) with minimum delay, as mRNA is an unstable molecule. Following mRNA isolation, reverse transcriptase is used to create complimentary DNA (cDNA). cDNA is amplified by reaction with a fluorescent reporter and measured in a thermocycler. The cycle at which the fluorescence crosses the threshold is determined, and this corresponds to the level of the mRNA level in the specimen. A small amount of tissue yields sufficient mRNA to perform multiple qPCR analyses. To avoid errors in the analysis of mRNA expression levels, a number of standardization systems have been developed. The most common is quantifying the specific gene studied in relation to another gene called a normalizing or house-keeping gene, which is selected for its almost constant level of expression. qPCR data throughout our project were normalized to glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) transcript abundance to enable a direct comparison of transcript quantity between the different treatment groups. All data were normalized to sham.

WESTERN BLOTTING

Western blot is a well-established analytic method, which is in everyday use for detection of proteins and their levels in a given sample (32, 33). For the Western blot analysis, proteins are isolated from tissue specimens, separated by size using gel electrophoresis, transferred to a membrane and detected using specific antibodies, visualized by chemiluminescence and quantified by densitometry. This method has its limitations. However, there are several methods to avoid false positive or false negative results. For control of protein loading, in each gel we used Coomassie Blue, Vinculin or GAPDH staining. It is of importance to choose specific, preferably monoclonal antibody. Not the least, it is important to use reference groups in order to be able to compare across multiple gels, as the number of lanes in each gel is limited. In our experiments we used reference groups such as sham operated animals or animals fed with standard chow.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

High pressure liquid chromatography (HPLC) is an analytic biochemical technique used in order to quantify the concentration of different molecules (34). In general this method is used to separate the components in a mixture and to identify and quantify each component. The sample to be analyzed passes through a column and the analyte is slowed from physical and chemical interaction with the column. Thus, the time it takes a particular analyte to pass through the column is a fairly specific property of the substance to be quantified. Finally, the analyte is detected by fluorescence. In order to measure the total collagen content in our samples we used the widely accepted method of measuring the hydroxyproline content by HPLC in left ventricular samples (35, 36).

CARDIAC FIBROBLAST CULTURES

Fibroblasts, a type of cells that synthesize ECM, including collagen, play an important role in wound healing and give structural support to tissues. Both neonatal and adult fibroblast cultures are widely used as *in vitro* models to study myocardial fibroblast response (37) and we have established both models in our laboratory. In these models it is possible to study changes in both protein and gene expression in cells isolated from animals with different genotypes or different treatment groups. By stimulation with different substances and/or growth on different media it is possible to study their behavior in physiological and

pathophysiological conditions. It is of high importance that cell cultures are kept under stable conditions at the temperature of 37°C and in a non-infected medium.

CARDIOMYOCYTE CULTURES

In paper IV we stimulated cardiomyocytes isolated from neonatal Wistar rats (38) with TGF-β1 after being preincubated with SM16 or vehicle. Stimulation was initiated following 24h serum starvation according to the established local protocol. Studies of cell cultures make it possible to address cell signaling in a way that is not possible in the intact organ or *in vivo*.

CARDIOMYOCYTE Ca^{++} HANDLING

We have isolated cardiomyocytes from adult mice subjected to aortic banding or sham operation and SM16 or standard chow. The hearts were excised and perfused by a Langendorf setup with a collagenase. The cells were loaded with the fluorescent calcium indicator fluo-4 and field stimulated at 1 Hz. The Ca^{2+} transients were then analyzed by fluorescence measurements (39-41).

STATISTICS

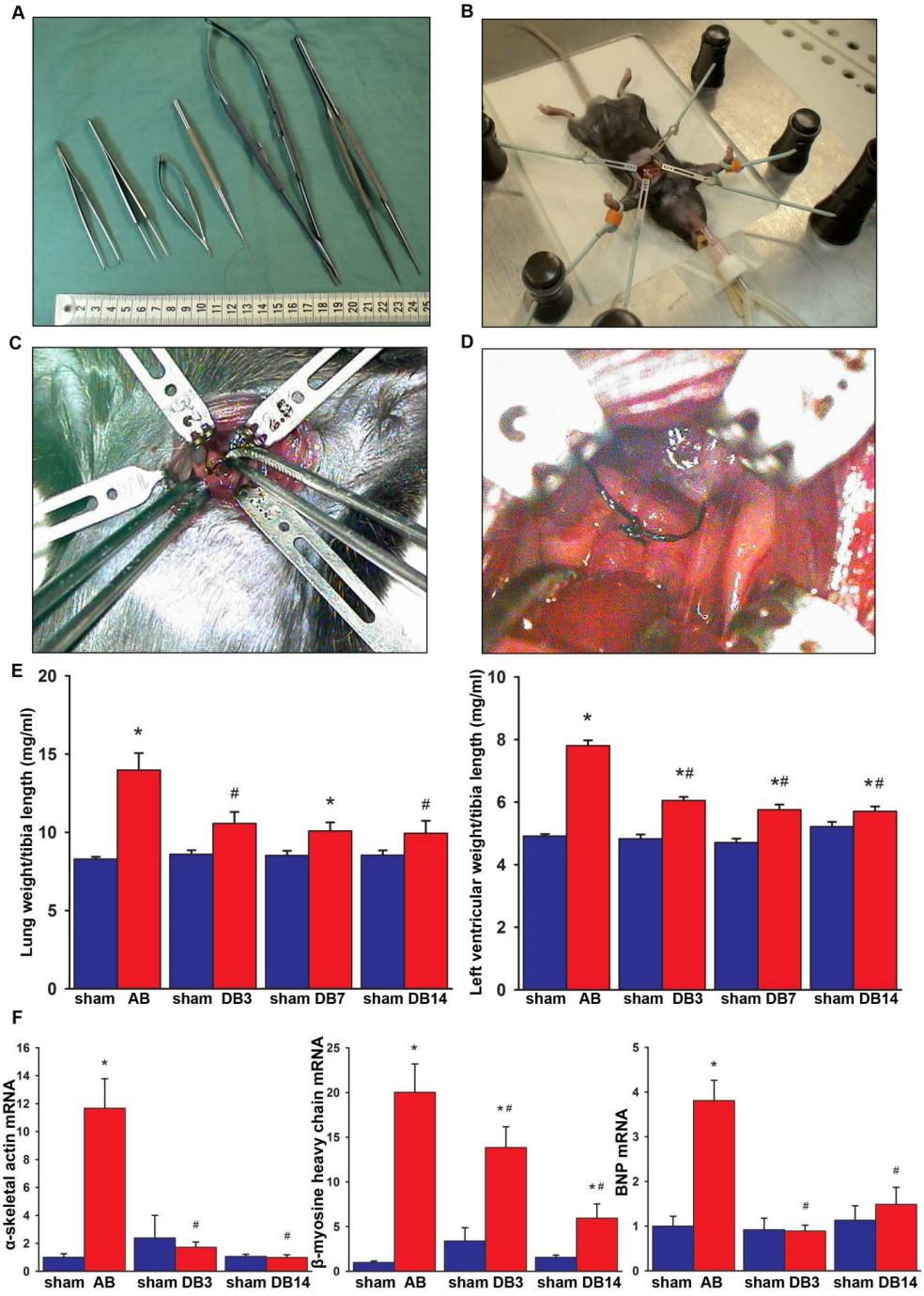
Statistical analyses were performed using Sigma Plot 11 (Systat Software Inc, San Jose, CA). Results are presented as mean ± SEM and a two-tailed significance level of $p \leq 0.05$ was used. Data that did not conform to the assumption of a normal distribution were logarithmically transformed. Parametric statistical methods were used where data conformed to the assumption of normality, and non-parametric methods were used when this criterion was not fulfilled. For analyzing differences between two groups, two-sided Student's t-test or Mann–Whitney Rank Sum Test was used where appropriate. For analyzing differences between several groups, One-way ANOVA or One-way ANOVA on ranks was used when appropriate, and correction for multiple comparisons was done using the Holm-Sidak or Dunn's method, respectively. The relation between two variables was examined and presented with linear regression in paper II. For microarray data in paper II multiple comparisons were corrected for by using FDR according to Benjamini & Hochberg (42), using the R/Bioconductor software. Survival rates in paper III were calculated using Log-rank (Mantel-Cox) test. Two-way ANOVA was used in paper III and IV to study the effects of two different interventions, influence of different genotype in paper III or operation and treatment with either SM16 or standard chow in paper IV.

SUMMARY OF RESULTS

PAPER I

In order to study and hemodynamically evaluate myocardial remodeling and potentially also reverse remodeling, we wanted to establish a mouse model of reversible left ventricular pressure overload by banding and subsequent debanding of the ascending aorta. Our banding-debanding mouse model is described in detail in paper I. Banding of the ascending aorta induced concentric left ventricular remodeling. On average, left ventricular weight was increased by 59% and lung weight was increased by 69%. Increased left atrial diameter suggested elevated left ventricular filling pressure (43) indicative of pulmonary congestion. Systolic and diastolic tissue velocities were used to evaluate the reversible myocardial dysfunction that occurs in banded mice and were on average reduced by 33% and 19% respectively, four weeks after aortic banding. Following aortic banding, increased mRNA expression of cardiac stress markers, α-skeletel actin, β-myosine heavy chain and BNP demonstrated myocardial remodeling. Debanding led to normalization of cardiac stress, thus reverse remodeling.

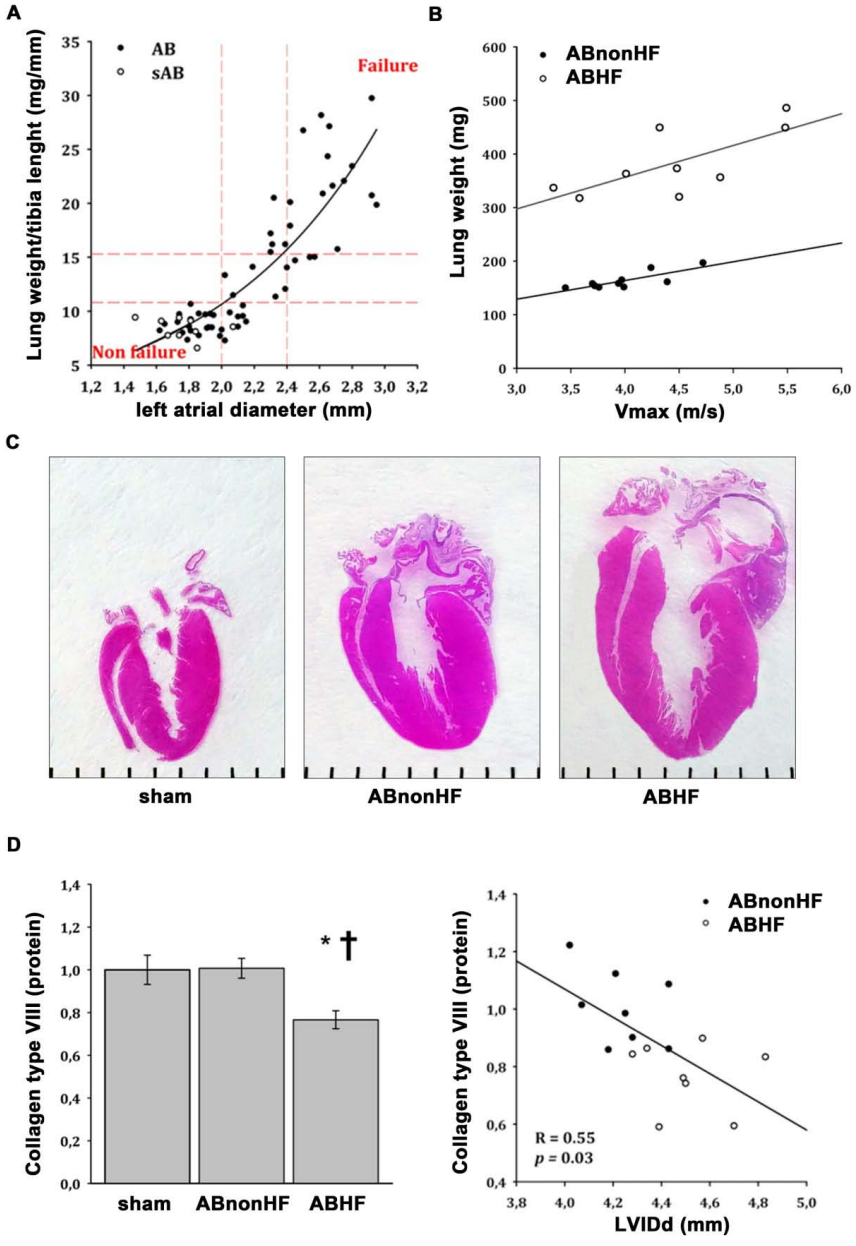
Figure 1. *(A-B) Setup for aortic banding/debanding surgery using small animal retraction system and ultra fine surgical instruments; (C-D) a close-up of banding site. (E) Lung- and left ventricular weight were increased following aortic banding (AB) with regression taking place following aortic debanding (DB); (F) AB increases cardiac stress with regression taking place following DB; * $p \leq 0.05$ vs. sham # $p \leq 0.05$ vs. AB*
Adapted from paper I


A
B
C
D
E
Lung weight/tibia length (mg/ml)
0
5
10
15
20
sham AB
sham DB3
sham DB7
sham DB14
Left ventricular weight/tibia length (mg/ml)
0
2
4
6
8
10
sham AB
sham DB3
sham DB7
sham DB14
F
α-skeletal actin mRNA
0
2
4
6
8
10
12
14
16
sham AB
sham DB3
sham DB14
β-myosine heavy chain mRNA
0
5
10
15
20
25
sham AB
sham DB3
sham DB14
BNP mRNA
0
1
2
3
4
5
sham AB
sham DB3
sham DB14

PAPER II

In paper II we wanted to examine potential differences in the regulation of myocardial ECM constituents in mice with hypertrophy only and with heart failure as response to comparable pressure overload. To identify mice with heart failure we used increased lung weight and left atrial diameter indicative of pulmonary congestion. Despite comparable pressure gradients and cardiac output, mice with heart failure had reduced fractional shortening, systolic and diastolic tissue velocity compared to mice with hypertrophy only. Furthermore, these animals had increased left ventricular internal dimensions both in systole and diastole compared to those without heart failure. Microarray analyses identified 120 differently regulated genes related to the ECM in animals with heart failure compared to those without. Interestingly, mice with heart failure had a 24% reduction of left ventricular collagen VIII protein levels despite increased total left ventricular collagen. This reduction in collagen VIII protein levels correlated negatively with left ventricular dilatation indicating that reduction of collagen VIII could potentially contribute to heart failure development.

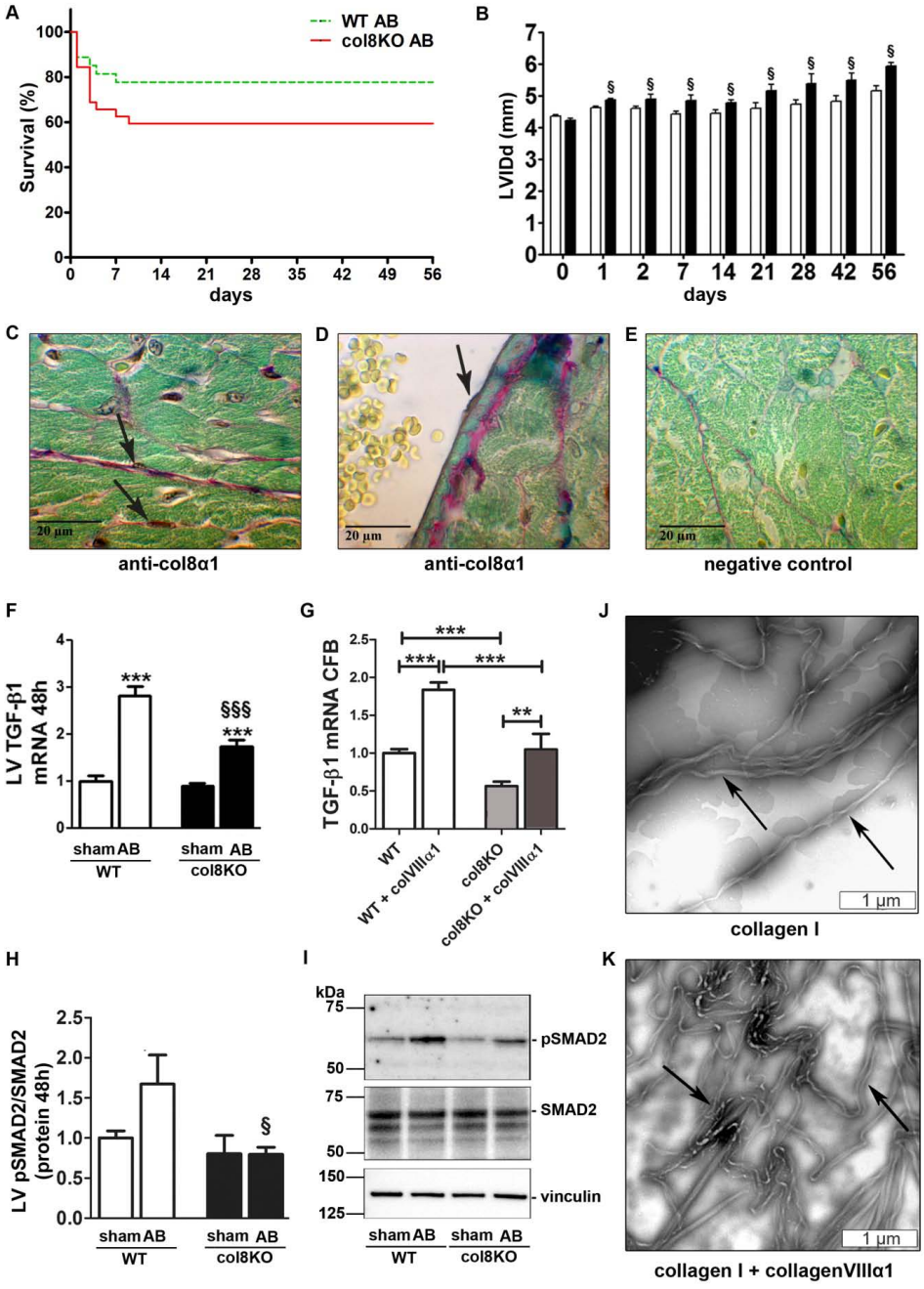
Figure 2. *(A) Despite a comparable degree of pressure overload, only 50% of the mice developed increased left atrial diameter and lung weight indicating pulmonary congestion. (B) Comparable pressure gradients may lead to different degrees of pulmonary congestion (lung weight) and consequently heart failure. (C) Representative longitudinally sectioned hearts (four chambers view), stained with Hematoxylin-eosin. Scale bars 1 mm. (D) Western blot revealed a 24 % reduction in left ventricular collagen VIII protein in ABHF mice compared ABnonHF mice and this correlated negatively with left ventricular dilatation; * p ≤ 0.05 vs. sham † p ≤ 0.05 vs. ABnonHF*
ABnonHF – banded animals with out heart failure; ABHF – banded animals with heart failure; LVIDd – left ventricle internal diameter in diastole;
Adapted from paper II


A
AB
sAB
Failure
Non failure
Lung weight/tibia lenght (mg/mm)
left atrial diameter (mm)
B
ABnonHF
ABHF
Lung weight (mg)
Vmax (m/s)
C
sham
ABnonHF
ABHF
D
Collagen type VIII (protein)
sham
ABnonHF
ABHF
R = 0.55
p = 0.03
LVIDd (mm)

PAPER III

Pressure overload-induced left ventricular dilatation is an important step in transition to heart failure. As we found in paper II that reduction in collagen VIII might be involved in left ventricular dilatation, we examined in paper III the role of collagen VIII in pressure overload-induced remodeling using collagen VIII knock-out mice. Knock-out mice exhibited increased early mortality after aortic banding and increased left ventricular dilatation from day one and over 56 days after aortic banding compared to wild type mice. We studied the changes in gene and protein expression as early as 48 hours after aortic banding and after six weeks. Forty-eight hours after aortic banding, left ventricular mRNA expression of the main fibrillar collagens type I and III was threefold increased in wild type mice. This increase was attenuated in knock-out mice together with reduced expression of the pro-fibrotic cytokine TGF-β, SMAD2 signaling and the myofibroblast markers paxillin (Pxn), alpha smooth muscle actin (α-SMA) and smooth muscle specific protein (SM22). Although less pronounced than in wild type animals, left ventricular collagen protein and mRNA expressions were increased in knock-out animals six weeks after aortic banding. These findings were supported by *in vitro* studies on neonatal cardiac fibroblasts isolated from knock-out mice. *In vitro* studies showed lower expression of TGF-β, Pxn, α-SMA and SM22 in fibroblasts isolated from neonatal knock-out mice. These fibroblasts also had reduced migratory ability possibly due to increased RhoA activity and reduced MMP2 expression. Stimulation with recombinant collagen VIIIα1 increased TGF-β expression and fibroblast migration. Furthermore, we showed that collagen VIII in heart tissue is expressed by and localized to the cardiac fibroblasts and their extracellular surrounding space. Collagen VIII is also present in the cardiac endothelium.

Figure 3. *(A-B) Compared to wild type mice (WT), collagen VIII knock-out mice (col8KO) have increased mortality and early left ventricular (LV) dilatation following aortic banding (AB). (C-E) Immunohistochemical detection of collagen VIIIα1 (arrow) showed that collagen VIIIα1 is localized to the ECM (C) of cardiac fibroblasts and the endothelium (D) of the vessel wall in the heart. Scale bars 20μm as indicated; (F-G) Collagen VIII plays an important role in the activation of TGF-β as shown both in in vivo studies on banded mice (AB) and in in vitro studies on cardiac fibroblasts (CFB). (H) Collagen VIII deficiency reduces TGF-β signaling following AB in mice. (I) Representative immunoblots of p-SMAD2 and SMAD2 protein 48hrs after AB. (J-K) Collagen VIII plays an important role in promoting collagen I (arrows) fiber formation. Scale bars 1μm as indicated; Data are presented as mean ± SEM; ** p≤0.01 *** p≤0.001 AB vs. sham or as indicated by lines, § p≤0.05 §§§ p≤0.001 KO vs. WT.*
Adapted from paper III

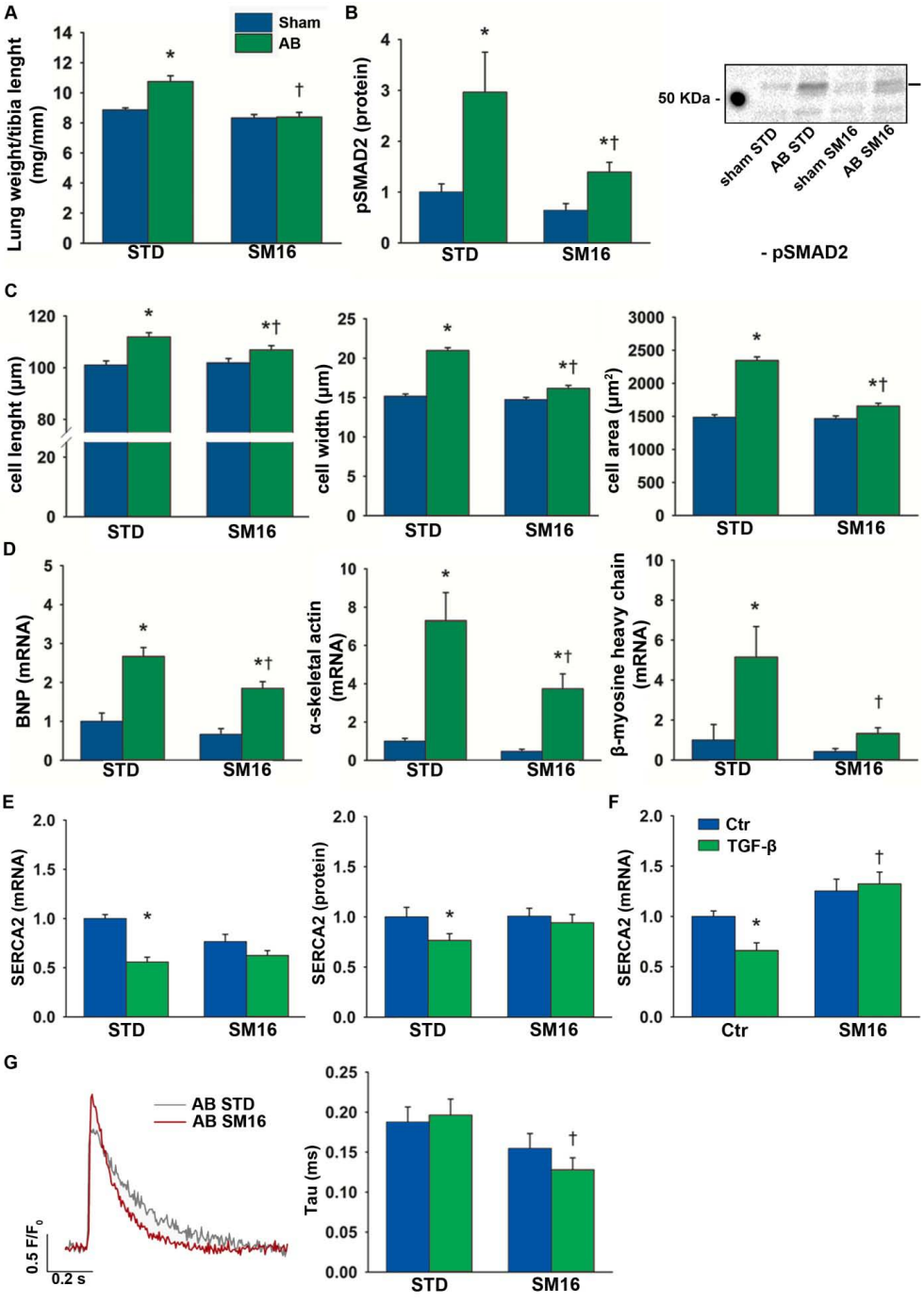

A
WT AB
col8KO AB
Survival (%)
days
B
LVIDd (mm)
days
C
anti-col8α1
D
anti-col8α1
E
negative control
F
LV TGF-β1 mRNA 48h
sham AB
WT
sham AB
col8KO
G
TGF-β1 mRNA CFB
WT
WT + colVIIIα1
col8KO
col8KO + colVIIIα1
J
collagen I
H
LV pSMAD2/SMAD2 (protein 48h)
I
kDa
pSMAD2
SMAD2
vinculin
K
collagen I + collagenVIIIα1

PAPER IV

Intracellular TGF-β signaling is mediated by SMAD2/3. In mice subjected to aortic banding we studied the effects of SMAD2/3 signaling in pressure overload inhibiting SMAD2/3 by SM16. Left ventricular pressure overload by aortic banding led to reduced cardiac function as demonstrated by echocardiographic measurements and the presence of pulmonary congestion and increased SMAD2 phosphorylation. SM16 inhibited phosphorylation of SMAD2, improved cardiac function and attenuated the fetal gene shift. Furthermore, SM16 inhibited aortic banding induced cardiomyocyte hypertrophy that led to 38% increased cell width, 11% increased cell length and 58% increased cell area. We were not able to evaluate possible effects of SMAD2 on cardiac fibrosis, as left ventricular collagen protein levels were not increased at the one-week time-point after aortic banding, examined in this study. Following aortic banding, left ventricular sarcoplasmic reticulum calcium ATPase 2 (SERCA2) was reduced while SM16 treatment preserved the levels of this important calcium handling protein. The levels of SERCA2 correlated inversely to lung weight, indicating an association between reduced SERCA2 levels and pulmonary congestion. Furthermore, SM16 enhanced cytosolic Ca^{2+} removal in cardiomyocytes isolated from banded mice. *In vitro* studies of neonatal rat cardiomyocytes investigated a role for TGF-β/SMAD signaling in regulating SERCA2. SM16 prevented TGF-β1 induced phosphorylation of SMAD2 leading to reduced SERCA2 mRNA expression.

Figure 4. *(A) The lung weight increase due to left ventricular pressure overload is abolished by SM16. (B) Pressure overload increases phosphorylation of SMAD2 (pSMAD2) and SM16 reduces pSMAD2. (C) SM16 reduces cardiomyocyte hypertrophy. (D) SM16 reduces cardiac stress. (E) Left ventricular SERCA2 mRNA and protein expressions are reduced due to aortic banding (AB) and SM16 attenuates this reduction. (F) In isolated cardiac myocytes SM16 inhibited TGF-β mediated downregulation of SERCA2. (G) SM16 increases cytosolic Ca^{++} removal following aortic banding; * p ≤ 0.05 vs. sham or control, † p ≤ 0.05 vs. AB STD (standard chow) or stimulation with TGF-β.*

Adapted from paper IV


A
Sham
AB
Lung weight/tibia lenght (mg/mm)
STD
SM16
B
pSMAD2 (protein)
50 KDa -
sham STD
AB STD
sham SM16
AB SM16
- pSMAD2
C
cell lenght (µm)
cell width (µm)
cell area (µm²)
D
BNP (mRNA)
α-skeletal actin (mRNA)
β-myosine heavy chain (mRNA)
E
SERCA2 (mRNA)
SERCA2 (protein)
F
Ctr
TGF-β
G
AB STD
AB SM16
0.5 F/F0
0.2 s
Tau (ms)

DISCUSSION

In paper I we have established a hemodynamically well characterized banding-debanding mouse model for studying mechanisms of myocardial remodeling and potential reverse remodeling due to pressure overload. In all papers banding of the ascending aorta was carefully standardized and echocardiographic measurements showed comparable Vmax in operated animals whereas cardiac output and heart rate were not significantly different, suggesting comparable degree of aortic constriction in all experimental groups. In paper II we have observed that despite comparable pressure gradients, only 50% of the mice developed signs of cardiac dysfunction with increased left atrial diameter and increased lung weight, indicating pulmonary congestion and heart failure. We identified collagen genes among the most altered group of genes in left ventricular tissue taken from banded animals with and without heart failure. Interestingly, we found a 24% reduction in left ventricular collagen type VIII protein levels in mice that developed heart failure. Collagen VIII protein levels correlated negatively with left ventricular dilatation. This finding led us to study the role of non-fibrillar collagen VIII in the heart by using collagen VIII knock-out mice. In paper III we have demonstrated an important novel finding that lack of collagen VIII disrupts the acute adaptation of the heart to increased pressure overload, influencing survival and development of left ventricular dilatation during cardiac remodeling. *In vivo* findings in paper III were accompanied by a reduced expression of collagen I and III, TGF-β signaling and myofibroblast differentiation. *In vitro*, neonatal cardiac fibroblasts lacking collagen VIII showed reduced expression of TGF-β and myofibroblast markers and at the same time reduced migratory ability compared to cardiac fibroblast isolated from neonatal wild type mice. Stimulation of fibroblasts with recombinant collagen VIIIα1 increased TGF-β expression and migration and we suggest that collagen VIII might also act as a signaling molecule in the heart directly affecting the function of cardiac fibroblasts. The profibrotic cytokine TGF-β activates SMAD2/3 signaling. As previously both deleterious and cardioprotective effects have been attributed to SMAD2/3 signaling, we studied in paper IV the effects of pharmacological inhibition of this signaling system. We found that the novel drug SM16 attenuated phosphorylation of SMAD2 and preserved cardiac function in pressure overload. Furthermore, we suggest beneficial effects on cardiomyocyte Ca^{2+} handling as the possible mechanism for this cardioprotective effect.

COMPARATIVE MEDICINE

As stated in the introduction, aortic stenosis is the most frequent valvular heart disease leading to left ventricular pressure overload and myocardial remodeling, which may lead to

the development of heart failure. Most human studies of the surgical treatment of heart disease are to a large extent observational, often with significant heterogeneity. In the last couple of decades a number of different animal models have been developed to study heart disease. The use of large animal models, such as non-human primates and pigs has given us important physiological knowledge and more recently small animal models have been widely used to study the mechanisms of heart disease (21, 22). To understand the basic mechanisms behind myocardial remodeling it is important to use a well-characterized model with minimal genotypical and phenotypical differences, such as inbred lab mice (44). In the later years increased development and use of genetically modified mice has become a powerful tool for experimental research, especially for studying disease mechanisms. Furthermore, the development of refined methods for characterization of animal models such as echocardiography, blood pressure measurement, catheterization, telemetry, magnetic resonance imaging (MRI) etc. have increased the number of well characterized animal models available.

The model of transverse aortic constriction (TAC) is a widely used model of pressure overload simulating aortic stenosis (45-47). While technically rather easy to perform, this model has its limitations as banding of the aorta arch leads to increased pressure overload not only in the left ventricle but also in the right side of the brain and right upper limb of the animal. This could possibly influence the mobility of the animal, the behavior and cause comorbidity and indirectly affect the degree of clinical signs of illness, including heart failure, in operated animals. This includes deeper / faster respiration (strained respiration), reduced movement in the cage, bristling fur (hedgehog character), edema and stereotypic behavior. Banding of the ascending aorta in mice, however probably reflects humans with aortic stenosis better than TAC. In ascending aortic banding the acutely induced left ventricular pressure overload is stable over time with less potential for development of collateral circulation as seen in TAC. Discussing the use of experimental aortic banding model, one has to consider the fact that aortic stenosis in humans is a valvular constriction located subcoronarly, while ascending aortic banding in animals is a supracoronary constriction of the aorta. This, of course, influences the pressure in the coronary arteries, thus influencing the perfusion of the myocardium and possibly affecting the behavior of the myocardium in pressure overload. This has been studied in pigs (48) and the authors conclude that aortic stenosis can be studied equally well with experimental valvular and supracoronary aortic constriction. Another fact when discussing the use of experimental aortic banding as a model for aortic stenosis in humans is that aortic stenosis in humans largely develops over time and is a more chronic disease, while aortic banding in mice represents acute left ventricular pressure overload that is stable over time. The model of banding of the ascending aorta, which we have used, is the model of cardiac failure more correct, but is more technically

demanding. Approaching the ascending aorta comes with the risk of mechanical damage of the aorta itself, its wall and not least other anatomical structures of the heart, especially the pulmonary artery. In ascending aortic banding, the mice are more sensitive to a banding that is a little too tight, than what is the case for TAC. Furthermore, by establishing a hemodynamically well characterized banding-debanding mice model, we have contributed to the ability to study the mechanisms of reverse remodeling, which have previously not been studied as much as remodeling due to pressure overload.

Taken together, all benefits and limitations of animal models and their use in translational research give reason to conclude that the use of well characterized animal models followed by at least observational studies in humans give us valuable knowledge about physiological and pathophysiological processes that provides us a solid base for development of treatment and prevention.

FIBRILLAR COLLAGENS IN PRESSURE OVERLOAD

Collagen fibrils are ECM components, which provide structural support for the myocardial tissue, and their type and organization in the tissue determines the mechanical properties of the tissue (49). There are several subtypes of collagens (9). Fibrillar collagens type I and III are predominant subtypes in the heart, and the knowledge of their role in the heart is increasing. It has previously been suggested that pressure overload increases protein levels of collagen types I and III in the left ventricle. A role in maladaptive remodeling and cardiac dysfunction has been suggested for collagen type I, while an increase in collagen type III has been associated with adaptive remodeling (8). In pressure overload accumulation of collagens in the ECM, i.e. fibrosis, is thought to contribute to impaired cardiac function and heart failure (50) by increasing ECM stiffness of the myocardium. The increased collagen amounts and concentric remodeling seen in the wild type mice during early response to aortic banding may lead to increased cardiac stiffness and counteract development of left ventricular dilatation in the acute phase. We and others have previously reported that collagen isoforms are differently expressed during myocardial remodeling irrespective of heart failure (51, 52). Interestingly, we found in paper II that although total collagen content in the left ventricle was increased in animals with heart failure compared to those without, there was no difference in collagen I or III protein levels between the two groups. Since we found no differences in the levels of fibrillar collagens, the idea that other collagen isoforms might play an important role in the development of heart failure to the different extent came into focus. Interestingly, in paper III mice lacking non-fibrillar collagen VIII developed left ventricular dilatation early after aortic banding. mRNA expression of fibrillar collagens was attenuated, but total collagen protein content was not altered at 48 hours. These results

suggest that lack of synthesis of fibrillar collagen proteins in pressure overload again might not be the main reason for the mortality or early left ventricular dilatation observed in the collagen VIII knock-out mice.

NON-FIBRILLAR COLLAGEN VIII IN THE HEART

In contrast to collagen I and III, collagen VIII is a non-fibrillar collagen. In paper III we have demonstrated that collagen VIII is synthesized by cardiac fibroblasts *in vitro* and is localized in fibroblasts and the surrounding ECM tissue of mouse hearts *in vivo*. As previously shown (53) collagen VIII is also localized to the inner vessel wall/endothelium/endothelial cells. Its role in the heart has not been studied previously. In paper II we found reduced protein levels of collagen VIII in animals with heart failure compared to those without. Even more interestingly, we found a negative correlation between left ventricular collagen VIII levels and left ventricular dilatation, leading us to the hypothesis that collagen VIII might play a protective role in the transition from concentric to eccentric left ventricular hypertrophy with left ventricular dilatation that is seen in the progression to heart failure. This is further supported by earlier studies describing collagen VIII as a molecular bridge between other ECM components (10). A role in tissue repair (54-56), angiogenesis and tissue remodeling has also been suggested (57). Collagen VIII directs adhesion of ECM components and absence of collagen VIII may affect tissue integrity both directly and indirectly. We have shown by *in vitro* findings in paper III that collagen VIII modifies the structural organization of collagen I in the myocardium. Furthermore, it seems that recombinant collagen VIIIα1 increases collagen I fiber formation when mixed *in vitro*. This might support a role for collagen VIII in facilitating ECM interactions that may prevent left ventricular dilatation in the early phase of pressure overload. Cardiac fibroblasts have a leading role in the synthesis, deposition and degradation of collagens in the heart tissue (58). During pressure overload cardiac fibroblasts differentiate into their activated form, myofibroblasts, a process promoted by TGF-β signaling. This leads to increased fibrosis and stiffness of the heart, which may counteract cardiac dilatation. Reduced TGF-β signaling in animals lacking collagen VIII suggests a mechanism for attenuated collagen synthesis and increased left ventricular dilatation. *In vitro* attenuated expression of TGF-β in cardiac fibroblasts lacking collagen VIII could be rescued by stimulation with recombinant collagen VIIIα1 suggesting a direct effect of collagen VIII on cardiac fibroblast function. At the same time we have demonstrated in paper III that collagen VIII not only affects fibroblast differentiation, but also migration and possibly also acting as a signaling molecule.

TGF-β/SMAD SIGNALING IN LEFT VENTRICULAR PRESSURE OVERLOAD

In the pathophysiological process of hemodynamic stress, the myocardium reacts by secreting different growth factors and cytokines (12) contributing to myocardial remodeling that may lead to cardiac dysfunction. The TGF-β superfamily is a large group of cytokines involved in different pathological processes, among them inducing collagen synthesis (59), cellular proliferation and differentiation (60, 61). Members of the TGF-β superfamily activate SMAD proteins and TGF-β is reported to be the main stimulus for phosphorylation of SMAD2/3. TGF-β plays a critical role in deleterious cellular and extracellular changes in the myocardium due to pressure overload (62, 63). A role for TGF-β/SMAD signaling in collagen synthesis and cardiac fibrosis has previously been reported (64-66) with either beneficial or deleterious effect. TGF-β forms a complex with ALK4 and 5 leading to phosphorylation of SMAD2 and 3. SM16 is a small molecule acting as an inhibitor of ALK4 and 5 and in that way leading to reduced SMAD2/3 phosphorylation (28). In paper IV we subjected mice fed with SM16 formulated chow to aortic banding. Aortic banding leads to increased SMAD2 phosphorylation, which interestingly was attenuated by SM16 treatment in our study. Aortic banding did not lead to increase in phosphorylation of SMAD3. Furthermore, SM16 treatment preserved cardiac function in aortic banded animals, as cardiac stress and pulmonary congestion were abolished. Even though SM16 treatment significantly reduced collagen transcription, the collagen protein level was not altered one week after induced pressure overload. Left ventricular pressure overload reduces transcription and protein levels of SERCA2 (67, 68) and this was attenuated by SM16 treatment. SERCA2 is critical for calcium homeostasis in cardiomyocytes. We isolated cardiomyocytes from mice treated with SM16. SM16 treatment increased cytosolic Ca^{2+} removal following aortic banding possibly due to improved SERCA2 function. *In vitro* studies on neonatal rat cardiomyocytes stimulated with TGF-β1 showed that SM16 treatment inhibited SMAD2 phosphorylation and downregulation of SERCA2. We demonstrated that SMAD2 signaling, in fact affects cardiomyocyte hypertrophy and behavior in pressure overload and that inhibition of SMAD2 potentially could represent a base for development of new treatment in order to improve cardiac function in patients with heart failure.

CONCLUSIONS

PAPER I:

We have established a mouse model of reversible left ventricular pressure overload by banding-debanding of the ascending aorta, and this model is hemodynamically well characterized.

PAPER II:

We have revealed that alterations in ECM gene expression is one of the most evident transcriptional change during the early phase of myocardial remodeling and development of heart failure. We suggest that left ventricular dilatation may be related to reduced levels of left ventricular collagen type VIII.

PAPER III:

We demonstrated the novel finding that collagen VIII is important for the early adaptation of the heart to pressure overload affecting survival and development of left ventricular dilatation. We suggest a role for collagen VIII in the regulation of ECM structure and fibrosis, as mice lacking collagen VIII exhibit attenuated TGF-β signaling. This may disrupt the normal myofibroblast differentiation and fibrosis formation in response to pressure overload.

PAPER IV:

We reported for the first time that pharmacological inhibition of SMAD2 signaling by SM16 preserves cardiac function in left ventricular pressure overload and this inhibition was also verified in isolated cardiomyocytes. As a possible mechanism, we suggest that SM16 has beneficial effects on cardiomyocyte Ca^{2+} handling due to prevention of SMAD2-mediated downregulation of SERCA2.

PERSPECTIVES

By understanding the mechanisms behind cardiac remodeling, reverse remodeling and development of heart failure due to left ventricular pressure overload, we will hopefully be able to help reduce the risk of death for patients with aortic stenosis and heart failure, choose the right timing for operation and maybe develop treatment enhancing reverse remodeling. Moreover, such knowledge will be useful in studying other cardiovascular disorders, such as in heart failure due to high blood pressure which might be due to many of the same mechanisms as in aortic stenosis.

The novel finding of a role for collagen VIII in the regulation of ECM structure and fibrosis in left ventricular pressure overload opens a door for further studies on collagen VIII and its role in the heart. Considering previous research on collagen VIII in other organs and especially its role in wound healing, it could possibly be of interest to study its role in a myocardial infarction model.

Furthermore, our mouse model of reverse cardiac remodeling is a base for further studies of reverse remodeling, an important process representing a healing potential of diseased myocardium.

ETHICS

All participants handling animals in the studies described in this thesis are board certified lab animal researchers and IEMR is an approved animal-lab facility. Animal care, surgery and hemodynamic evaluation are well established in our institution and the involved participants have the necessary experience. We have used an animal model because there are still no simulation programs for the mechanisms that we wanted to study, and *in vitro* models cannot give answers to our hypothesis. The operation method has been refined so that mortality in experienced hands has been reduced to 10% during banding and/or debanding of the ascending aorta. The anesthesia method is refined to be less damaging to the heart both during the operation, blood pressure measurements and echocardiographic investigations. We have used a minimum number of animals for our experiments and we have used the maximum of tissue from each operated animal. Echocardiography and data analyses were performed by well-experienced investigators, which minimized the time under anesthesia and at the same time the stress on the animal. Sacrifice of the animals was performed in deep anesthesia. During the operations and postoperatively there was always a person responsible for observing the animal's condition. The animals developing heart failure following aortic banding were usually in remarkably good shape, without clinical signs of heart failure, cachexia or pathologic inactivity. The very few animals that developed clinical signs of heart failure were observed more often or sacrificed immediately and excluded from the study. The project had obtained the necessary permission for animal experiments registered at www.fdu.no (FDU 2507, FDU 3170, FDU 3310, FDU 1443, FDU 118).

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