About Hyaluronan in the Hypertrophic Heart

Studies on coordinated regulation of extracellular matrix signalling

Urban Hellman

Department of Public Health and Clinical Medicine, Medicine
901 87 Umeå
Umeå 2010
How could you describe this heart in words without filling a whole book?

Note written by Leonardo da Vinci beside an anatomical drawing of the heart, c. 1513
# Table of Contents

- **Abstract**
- **List of papers**
- **Abbreviations**
- **Introduction**
  - The heart
  - Cardiac hypertrophy
    - *Fetal gene program*
  - Intercellular signalling
  - Extracellular matrix
  - Glycosaminoglycans
  - Hyaluronan
  - *Structure*
    - Hyaluronan synthesis and catabolism
      - Hyaluronan synthesis
      - Hyaluronidases
      - Hyaluronan turnover
      - Regulation of hyaluronan synthesis
    - Hyaluronan function
      - High molecular weight hyaluronan
      - Low molecular weight hyaluronan
      - Hyaluronan oligosaccharides
      - Intracellular hyaluronan
    - Hyaladherins
      - Hyaluronan receptors
      - Hyalectans
    - Hyaluronan in tissue growth
    - Hyaluronan in the cardiovascular system
      - Cardiac development
      - Myocardial infarction
      - Cardiac vessels
- **Aims**
- **Presentation of papers**
- **Methodology**
  - Rat hypertrophy model
  - Cell culture
  - Stimulation of cells
  - RNA preparation
  - DNA preparation
  - Real-time PCR
  - Hyaluronan purification
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative analysis of hyaluronan</td>
<td>29</td>
</tr>
<tr>
<td>Histochemistry staining for hyaluronan and CD44</td>
<td>29</td>
</tr>
<tr>
<td>Dynamic Light Scattering size analysis of hyaluronan</td>
<td>29</td>
</tr>
<tr>
<td>Microarray gene expression analysis</td>
<td>30</td>
</tr>
<tr>
<td>Microarray gene expression data analysis</td>
<td>30</td>
</tr>
<tr>
<td>Correlation between hyaluronan concentration and gene transcription</td>
<td>31</td>
</tr>
<tr>
<td>Preparation of microvesicles</td>
<td>31</td>
</tr>
<tr>
<td>Cross-talk between cardiomyocytes and fibroblasts</td>
<td>32</td>
</tr>
<tr>
<td>Hyaluronan stimulation of cardiomyocytes</td>
<td>32</td>
</tr>
<tr>
<td>Flow cytometry analysis</td>
<td>33</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>33</td>
</tr>
<tr>
<td>Identification of microvesicular contents</td>
<td>33</td>
</tr>
<tr>
<td>Microvesicular DNA transfer into target fibroblasts</td>
<td>34</td>
</tr>
<tr>
<td>Microvesicular induced effects on target cells</td>
<td>34</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>35</td>
</tr>
<tr>
<td>Paper I and II:</td>
<td></td>
</tr>
<tr>
<td>General characteristics</td>
<td>35</td>
</tr>
<tr>
<td>Quantitative RT-PCR</td>
<td>35</td>
</tr>
<tr>
<td>Quantification of hyaluronan in heart tissue</td>
<td>35</td>
</tr>
<tr>
<td>Histochemical analysis of hyaluronan and CD44</td>
<td>36</td>
</tr>
<tr>
<td>Microarray gene expression</td>
<td>36</td>
</tr>
<tr>
<td>Paper III:</td>
<td>37</td>
</tr>
<tr>
<td>Quantitative analysis of hyaluronan in cell media</td>
<td>37</td>
</tr>
<tr>
<td>Real-time polymerase chain reaction analysis of hyaluronan synthases</td>
<td>37</td>
</tr>
<tr>
<td>Dynamic Light Scattering Size analysis of hyaluronan</td>
<td>37</td>
</tr>
<tr>
<td>Crosstalk between cardiomyocytes and fibroblasts</td>
<td>38</td>
</tr>
<tr>
<td>Hyaluronan stimulation of cardiomyocytes</td>
<td>38</td>
</tr>
<tr>
<td>Paper IV:</td>
<td>39</td>
</tr>
<tr>
<td>Preparation and characteristics of microvesicles</td>
<td>39</td>
</tr>
<tr>
<td>Identification of cardiosomal contents</td>
<td>39</td>
</tr>
<tr>
<td>Cardiosomal DNA transfer into target fibroblasts</td>
<td>39</td>
</tr>
<tr>
<td>Cardiosome induced effects on fibroblasts</td>
<td>39</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td>40</td>
</tr>
<tr>
<td><strong>Conclusions</strong></td>
<td>44</td>
</tr>
<tr>
<td><strong>Sammanfattning på svenska</strong></td>
<td>46</td>
</tr>
<tr>
<td><strong>Acknowledgments</strong></td>
<td>48</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>49</td>
</tr>
</tbody>
</table>
Abstract

Background. Myocardial hypertrophy is a risk factor for cardiovascular morbidity and mortality. Independent of underlying disease, the cardiac muscle strives in different ways to compensate for an increased workload. This remodelling of the heart includes changes in the extracellular matrix which will affect systolic and diastolic cardiac function. Furthermore, signal transduction, molecular diffusion and microcirculation will be affected in the hypertrophic process. One important extracellular component is the glycosaminoglycan hyaluronan. It has been shown to play a major role in other conditions that feature cellular growth and proliferation, such as wound healing and malignancies. The aim of this thesis was to investigate hyaluronan and its role in both an experimental rat model of cardiac hypertrophy as well as in cultured mouse cardiomyocytes and fibroblasts.

Methods. Cardiac hypertrophy was induced in rats by aortic ligation. Hyaluronan concentration was measured and expression of genes coding for hyaluronan synthases were quantified after 1, 6 and 42 days after operation, in cardiac tissue from the left ventricular wall. Localization of hyaluronan and its receptor CD44 was studied histochemically. Hyaluronan synthesis was correlated to gene transcription using microarray gene expression analysis. Cultures of cardiomyocytes and fibroblasts were stimulated with growth factors. Hyaluronan concentration was measured and expression of genes coding for hyaluronan synthases were detected. Hyaluronan size was measured and crosstalk between cardiomyocytes and fibroblasts was investigated.

Results. Increased concentration of hyaluronan in hypertrophied cardiac tissue was observed together with an up-regulation of two hyaluronan synthase genes. Hyaluronan was detected in the myocardium and in the adventitia of cardiac arteries whereas CD44 staining was mainly found in and around the adventitia. Hyaluronan synthesis correlated to the expression of genes, regulated by transcription factors known to initiate cardiac hypertrophy. Stimulation of cardiomyocytes by PDGF-BB induced synthesis of hyaluronan. Cardiomyocytes also secreted a factor into culture media that after transfer to fibroblasts initiated an increased synthesis of hyaluronan. When stimulated with hyaluronan of different sizes, a change in cardiomyocyte gene expression was observed. Different growth factors induced production of different sizes of hyaluronan in fibroblasts. The main synthase detected was hyaluronan synthase-2. Cardiomyocytes were also shown to secrete microvesicles containing both DNA and RNA. Isolated microvesicles incubated with fibroblasts were observed by confocal microscopy to be internalized into fibroblasts. Altered gene expression was observed in microvesicle stimulated fibroblasts.

Conclusion. This study shows that increased hyaluronan synthesis in cardiac tissue during hypertrophic development is a part of the extracellular matrix remodelling. Cell cultures revealed the ability of cardiomyocytes to both synthesize hyaluronan and to convey signals to fibroblasts, causing them to increase hyaluronan synthesis. Cardiomyocytes are likely to express receptors for hyaluronan, which mediate intracellular signalling causing the observed altered gene expression in cardiomyocytes stimulated with hyaluronan. This demonstrates the extensive involvement of hyaluronan in cardiac hypertrophy.
List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


IV. Hellman U, Ronquist G, Waldenström A. Cardiomyocyte microvesicles convey bioinformatic messages to target cells. Manuscript
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCC5/MRP5</td>
<td>ATP-binding cassette, subfamily C, member 5/ multidrug resistance transporter protein</td>
</tr>
<tr>
<td>ABCC7/CFTR</td>
<td>ATP-binding cassette, subfamily C, member 5/ cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ACTS/Acta1</td>
<td>alpha 1 skeletal muscle actin</td>
</tr>
<tr>
<td>α-MHC/Myh6</td>
<td>alpha-myosin heavy chain</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ANP/Nppa</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>AO</td>
<td>acridin orange</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BGN</td>
<td>biglycan</td>
</tr>
<tr>
<td>β-MHC/Myh7</td>
<td>β-myosin heavy chain</td>
</tr>
<tr>
<td>BNP/Nppb</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>CCN1/CYR61</td>
<td>cystein rich protein 61</td>
</tr>
<tr>
<td>CCN2/CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>CRTL1</td>
<td>cartilage link protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGR1</td>
<td>early growth response 1</td>
</tr>
<tr>
<td>ET1</td>
<td>endothelin 1</td>
</tr>
<tr>
<td>ERBB</td>
<td>erythroblastic leukemia viral oncogene</td>
</tr>
<tr>
<td>ERK1</td>
<td>extracellular signal-regulated kinase1 (MAPK3)</td>
</tr>
<tr>
<td>ERK2</td>
<td>extracellular signal-regulated kinase2 (MAPK1)</td>
</tr>
<tr>
<td>FBN1</td>
<td>fibrillin 1</td>
</tr>
<tr>
<td>FGF2</td>
<td>fibroblast growth factor 2</td>
</tr>
<tr>
<td>FGFR1</td>
<td>FGF receptor 1</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>Gapdh</td>
<td>D-glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GlcUA</td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>D-N-acetylglucosamine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycophosphatidylinositol</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HAS</td>
<td>hyaluronan synthase</td>
</tr>
<tr>
<td>HAPLN</td>
<td>HA and proteoglycan link protein</td>
</tr>
<tr>
<td>HARE</td>
<td>HA receptor for endocytosis</td>
</tr>
<tr>
<td>HBW</td>
<td>heart-to-body weight</td>
</tr>
<tr>
<td>HYAL</td>
<td>hyaluronidase</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early genes</td>
</tr>
<tr>
<td>IL-1B</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JUNB</td>
<td>jun B proto-oncogene</td>
</tr>
<tr>
<td>LTBP2</td>
<td>latent transforming growth factor binding protein 2</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>lymphatic vein endothelium receptor-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor 2</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
</tr>
<tr>
<td>MYC</td>
<td>myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>platelet-derived growth factor BB</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidyl inositol 3-kinase</td>
</tr>
<tr>
<td>RAS</td>
<td>rat sarcoma viral oncogene</td>
</tr>
<tr>
<td>RAS</td>
<td>renin angiotensin system</td>
</tr>
<tr>
<td>RHAMM</td>
<td>receptor for HA-mediated motility</td>
</tr>
<tr>
<td>SP1</td>
<td>specificity protein 1</td>
</tr>
<tr>
<td>SPAM1</td>
<td>sperm adhesion molecule 1</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TGFB</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TP53</td>
<td>tumour protein Tp53</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine-diphosphate</td>
</tr>
</tbody>
</table>
Introduction

The heart

An adult human inhales 6-8 litres of air per minute. This adds up to more than 6 billion tonnes of oxygen inhaled by all humans per year, a lot of it used in the oxidative phosphorylation to form adenosine triphosphate (ATP) in the mitochondria. The oxygen is distributed through the circulatory system, to all tissues and cells in the body, by the heart. After the oxygenated blood from the lungs is collected in the left atrium it passes to the left ventricle, which pumps it out throughout the body. The de-oxygenated blood is collected in the right atrium and is via the right ventricle pumped to the lungs, where carbon dioxide is exchanged for oxygen. This continuous blood flow must be maintained for many decades, which make a flawless heart performance vital. To be able to meet changes in workload, the heart is a dynamic organ that can grow and change in response to altered demands.

Cardiac hypertrophy

The word “hypertrophy” is derived from the Greek hyper (above, more than normal) and trophe (nutrition) and is defined as “the enlargement or overgrowth of an organ or part due to an increase in size of its constitute cells”.

Figure 1. Cardiac response to physiological and pathological stimuli. Principal adaptive physiological growth with maintained cardiac function and maladaptive pathological growth.
Increased cardiac workload will lead to an enlargement of the heart in an attempt to manage the hemodynamic demand. The growth is an adaptive or maladaptive response to physiological or pathological stimuli (figure 1).

In healthy individuals cardiac growth may occur during chronic exercise, pregnancy and maturation. This is commonly referred to as physiological hypertrophy but since this is a normal cardiac growth it has been proposed to be defined as adaptive (figure 2). Growth during exercise and pregnancy is reversible without any adverse effect on cardiac function.

Pathological cardiac hypertrophy is induced by stress signals, e.g. long standing hypertension, neurohormonal activation, myocardial infarction, valvular heart disease, hypertrophic and dilated cardiomyopathy. This conditions may initially be adaptive to normalize wall stress and preserve contractile performance. However sustained myocardial hypertrophy predisposes the individual to heart failure, arrhythmia and sudden death. Pathological cardiac hypertrophy has a severe prognosis and is a predictor of progressive heart disease. It is a common condition which makes it a major health problem worldwide.

Based on the ratio of left ventricular wall thickness to left ventricular chamber dimension, hypertrophic growth develops in two ways. Concentric hypertrophy is caused by chronic pressure overload in which addition of contractile sarcomeres in parallel results in increased cell and wall thickness with a reduced left ventricular volume. Eccentric hypertrophy is mainly due to volume overload in which addition of sarcomeres in series cause cell

![Proposed criteria for classifying cardiac hypertrophy](image-url)
elongation, dilation and sometimes thinning of the heart wall. In both types of growth development, the increase in cardiomyocyte size and cardiomyocyte disarray is accompanied by an increase in the number of cardiac fibroblasts, causing fibrosis and increased myocardial stiffness. This is followed by further hemodynamic overload and hypertrophy resulting in a detrimental cycle of cardiac enlargement and myocyte loss. This can contribute to diastolic dysfunction and predisposition to arrhythmias. Increase and activation of fibroblasts with subsequent fibrosis have been associated to increase of circulating hormones such as angiotensin II (Ang II) and endothelin 1 (ET1) and cytokines/proteins such as transforming growth factor β (TGFβ), connective tissue growth factor CCN2 (CTGF) and platelet-derived growth factor (PDGF).

**Fetal gene program**

After birth, when the heart is exposed to an oxygen rich environment, there is a shift of substrates for energy provision from lactate and glucose oxidation to fatty acid oxidation. This shift is accompanied by expression of “adult” isoforms of proteins. However, the ability to re-activate the fetal gene program is preserved in the heart. The heart senses hypertrophy-inducing stimuli either directly through biomechanical stretch sensitive receptors, like the integrins, or neurohormonal stimuli, like angiotensin II (AngII), endothelin-1 and adrenalin. This leads to the expression of immediate early genes (e.g. Jun, Fos, Myc, Egr1) and genes considered to be markers for the fetal gene program, Nppa, Nppb, Myh7 and Acta1 (coding for atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and α1 skeletal muscle actin (ACTS)). Hormones, cytokines, chemokines and peptide growth factors in the circulation or the extracellular matrix interacts with G-protein-coupled receptors (GPCRs), tyrosine kinase receptors, serine/threonine receptors and gp130 linked receptors, thus activating intracellular signalling pathways. Intracellular transduction through phosphorylation and dephosphorylation events are mediated by an array of pathways, e.g. the mitogen-activated protein kinase (MAPK) pathway, the calcineurin-nuclear factor of activated T-cells (NFAT) circuit, the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, the renin angiotensin system (RAS) and the JAK-STAT pathway (Janus Kinase - Signal Transducer and Activator of Transcription).

A number of transcription factors coordinate cardiac development and differentiation of myocytes. Many of these, such as myocyte enhancer factor 2 (MEF2), GATA4, NFAT, serum response factor (SRF), Nkx2.5, nuclear factor κB (NFκB), Hand1/2, EGR1 and CREB are re-activated during cardiac
hypertrophy by the signal transduction pathways mentioned above. Their involvement in the fetal gene program is exemplified by GATA4, which is a transcriptional regulator of ANP, BNP, α-MHC and β-MHC. EGR1 has also been shown to be a transcriptional mediator of cardiac hypertrophy.

Pressure and/or volume overload, will through extracellular factors via intracellular pathways that affect gene expression and protein translation, ultimately alter myocyte shape to adopt to the increased burden of the heart (figure 3).

**Intercellular signalling**

The myocardial cells can receive signals in several different ways. One way is through direct contact with another cell, where the signal molecule is bound to the membrane of the signalling cells or with gap-junction connexons, where the cytoplasms of the two cells are connected through a continuous aqueous channel.

Cells can also secrete signal molecules. This includes paracrine signalling, which only affects local cells, and endocrine signalling, where endocrine cells secrete hormones into the bloodstream, thus transmitting the signal throughout the body.

Intercellular communication can also be facilitated by exocytosis of membrane microvesicles called exosomes. They are about 40-100 nm in
diameter and are secreted when multivesicular bodies, containing exosomes, fuse with the cell membrane and release their content into the extracellular environment. Exosomes can be released by most cell types in vitro and they have also been found in vivo in several body fluids. Exosomes can contain proteins, RNA, microRNA and DNA. Both mRNA and microRNA have been shown to be functional after transfer to target cells and protein content may be linked to functions associated with the originating cell-type. Detection of tumor cell-derived exosomes in blood, with tumor cell biomarkers, suggests a role of exosomes in diagnosis and therapeutic decisions.

**Extracellular matrix**

Different tissues differ not only by their cell types. Their extracellular environment is built up by a complex network of proteins and polysaccharides constituting the extracellular matrix (ECM). Cells synthesize and secrete the components locally, where they assemble in a network.

Glycosaminoglycans (GAGs) and fibrous proteins (e.g. collagens, fibronectin, fibrillin, laminin, elastin) are the most abundant classes of molecules in the ECM. The diverse forms of tissues are enabled by variations of the ECM composition. It varies from rock hard to soft and transparent and the ECM can be the major component, as in cartilage, or a minor component, as in the brain. The ECM serves as physical structure and a scaffold for cell adhesion and cell movements. It also transduces signals into cells, thus regulating cellular functions and can bind soluble growth factors, regulating their distribution, activation and presentation to cells.

**Glycosaminoglycans**

GAGs are highly negatively charged, unbranched polysaccharides consisting of repeating disaccharide units. The members of the GAG family are defined according to their disaccharide structure and number and location of sulphate groups; hyaluronan, chondroitin sulphate, dermatan sulphate, heparin sulphate and keratin sulphate. GAGs form gels at very low concentrations and are highly osmotic due to their negative charge and attract large amounts of water. This enables the ECM to withstand compressive forces. All GAGs except HA are attached to a core protein, forming proteoglycans. Proteoglycans have an important role in cell-cell signalling. They can bind growth factors, proteases and protease inhibitors and thus block the activity of the protein, provide a reservoir of the protein for delayed release and present the protein to cell surface receptors.
Hyaluronan

In 1934, Karl Meyer and John Palmer isolated from bovine vitreous humour a previously undescribed polysaccharide\(^\text{18}\). They named it hyaluronic acid from hyaloid (vitreous) and uronic acid. However, in 1894, Carl Thore Mörner isolated a “mucin” from the vitreous humour, presumably consisting of protein contaminated hyaluronan\(^\text{19}\). The name hyaluronan (HA) was introduced in 1986. HA is the only GAG that is unsulphated and not forming proteoglycans by binding to a core protein. HA is present in all vertebrates and most tissues differentiate in an ECM where HA is a major constituent. It can also be found in the pericellular coat called glycocalyx. Skin tissue harbours about 50% of the total amount of HA in the body and it is also a major part of, for example, the vitreous humour of the human eye and synovial joint fluid\(^\text{20}\).

Structure

HA is an unbranched polysaccharide which assumes a stiffened random coil in solution. It is a polydisperse population of molecules at varying chain lengths, occupying a large hydrated volume, up to 1000 times greater than its own dry volume. HA is soluble up to very high concentrations and shows no evidence of chain-chain association\(^\text{21}\). The chemical structure of repeating D-glucuronic acid and D-N-acetylglucosamine disaccharides was solved in the 1950s (figure 5)\(^\text{22}\). The number of repeated disaccharides can reach 10\(^5\), representing a molecular mass of \(~20,000\) kDa\(^\text{23}\).

\[\text{GlcUA} \quad \text{GlcNAc}\]

\[\text{GlcUA} \quad \text{GlcNAc}\]

Figure 4. Hyaluronan structure. HA is composed of repeating disaccharides of D-glucuronic acid (GlcUA) and D-N-acetylglucosamine (GlcNAc) linked by a glucuronic \(\beta(1\rightarrow 3)\) bond. The disaccharide units are then linearly polymerized by hexosaminidic \(\beta(1\rightarrow 4)\) linkages.
**Hyaluronan synthesis and catabolism**

**Hyaluronan synthesis**

Most cell types that have been investigated can synthesize HA. One exception is erythrocytes and the ability of cardiomyocytes to synthesize HA has never been investigated.

In contrast to the other GAGs, which are synthesized and covalently linked to core proteins in the Golgi apparatus and secreted by exocytosis, HA is synthesized on the cytosolic side of the cell membrane. In mammals there are three hyaluronan synthases (HASs) with multiple transmembrane domains, which utilize UDP-glucuronic acid (uridine-diphosphate) and UDP-N-acetylglucosamine to assemble the HA chain. The growing chain is transported through the cell membrane into the extracellular space. It has been shown that the transporter proteins ATP-binding cassette, subfamily C, member 5 (ABCC5/MRP5) and member 7 (ABCC7/CFTR) possess the ability to translocate HA to the ECM through the cell membrane. Atomic force microscopy observations of HA have revealed its capability to form many different conformations, e.g. extended chains, relaxed coils, condensed rods. HA can also form fibers/cables, networks and stacks through self-association.

**Hyaluronidases**

HA is degraded by a group of enzymes called hyaluronidases (HYALs) through hydrolysis. There are six HYAL-like sequences found in the human genome. **Hyali**, 2 and 3 are clustered on chromosome 3p21.3. **Hyal4**, **HyalP1** and **Spam1** (sperm adhesion molecule 1) are clustered on chromosome 7q31.3. **HyalP1** is a pseudogene, transcribed but not translated in humans. **SPAM1** codes for PH-20, the enzyme that facilitates penetration of a sperm through the HA-rich cumulus mass surrounding the ovum, necessary for fertilization. In mouse, **Hyal1** is often co-transcribed with **Hyal3**.

Three of the enzymes can be GPI (glycophosphatidylinositol) -linked to the outer cell membrane, HYAL2, HYAL4 and PH-20, but they also exist in free processed form. HYAL1, HYAL2 and PH-20 have known enzymatic activities but no enzymatic activity of HYAL3 and HYAL4 on HA has been detected, so far. However HYAL-4 have been shown to have hydrolytic activity on chondroitin sulfate.

HYAL1 and HYAL2 are the major HYALs in somatic tissue. In a complex with the HA receptor CD44 and HYAL2, HA is bound and then cleaved by HYAL2 to 20kDa fragments, about 50 disaccharide units. The complex is internalized in caveolin and flotillin rich caveolae, delivered to endosomes.
and then the HA fragments are degraded by HYAL1 in lysosomes to tetra- and hexasaccharides followed by degradation to monosaccharides by exoglycosidases (figure 5)\textsuperscript{31}.

Figure 5. A putative metabolic pathway of HA degradation. HA is bound to CD44 and internalized. Hyaluronidase-2, -1 and exoglycosidases are subsequently degrading HA to monosaccharides.

**Hyaluronan turnover**

It has been estimated that almost a third of all HA in the human body is degraded in 24 h. The half time of HA in the blood is between two and five minutes. In the epidermis it is between one and two days and in cartilage about one to three weeks\textsuperscript{32, 33}. A large proportion of HA is captured by receptors on reticulo-endothelial cells in lymph nodes and in the liver, which internalize and degrade HA in lysosomes.

**Regulation of hyaluronan synthesis**

The HASs differs in enzymatic properties. HAS3 is the most catalytically active, followed by HAS2, and then HAS 1, the least active \textsuperscript{34}. It has been shown that they also differ in the size of HA synthesized. HAS3 synthesized the smallest, 100-1000 kDa whereas HA synthesized by HAS1 and HAS2 ranged between 200-2000 kDA. It has later been shown that all HASs are capable of synthesizing high molecular sizes of HA, depending on cell type and regulatory factors\textsuperscript{35}. The HASs are using a cytosolic pool of substrate and synthesis may be regulated by availability of the UDP-sugars\textsuperscript{36, 37}. The level of HA synthesis is also regulated by the expression of the HAS genes and the
subsequent translation of HAS proteins. HAS expression is regulated by several growth factors and cytokines, such as PDGF-BB, TGFB, FGF2 and IL-1B.

**Hyaluronan function**

*High molecular weight hyaluronan*

High molecular size HA, defined as 400-2000 kDa (200-10000 saccharides), is extracellular and space-occupying. It has several regulatory and structural functions. In the fluid of the joint capsule it is a lubricant and shock absorber. In the vitreous humour of the eye, it is a space-occupying material. It also functions in organizing the ECM.

HA networks show a high resistance towards water flow, thus forming flow barriers in tissue, even though water can freely diffuse in the network. Movements of macromolecules are also hindered in a HA network, whereas low-molecular weight molecules can diffuse more easily. The HA chains are constantly moving in the solution and thus, the pore sizes in the network change, allowing molecules to pass with different degrees of retardation depending on their volume.

High molecular HA has been shown to be anti-inflammatory and immunosuppressive. It also suppresses angiogenesis and synthesis of HA.

*Low molecular weight hyaluronan*

Low molecular size HA ranges between 10 kDa and 500 kDa. These molecules share their biological functions with HA oligosaccharides, such as induction of NFκB, increase of cyto- and chemokines and increase of nitric oxide.

*Hyaluronan oligosaccharides*

HA sizes up to 10 kDa refers to as oligosaccharides, which have many novel functions that are not shared by either low or high molecular weight HA. When HA oligosaccharides of various sizes bind to cell surface receptors, such as CD44 and RHAMM (receptor for HA-mediated motility), they mediate intracellular signal transduction pathways which affect gene expression and cell function. In several cases they suppress the actions of high molecular HA, e.g. they stimulate angiogenesis and can induce an inflammatory response. HA oligosaccharides are also found in most malignancies, where they facilitate tumour cell motility and invasion.
It is unknown if the smaller sizes of HA is only derived from degradation of high molecular HA or if the HASs can be regulated to synthesize them.

**Intracellular hyaluronan**

HA seems to be internalized for more reasons than to be degraded. HA has been detected in various cell types, e.g. smooth muscle cells, endothelial cells, epithelial cells and fibroblasts. The variation of morphology and distribution suggests different purposes for HA inside the cells. It has been detected both in the cytoplasm and the nucleus. Intracellular HA may be involved in growth regulation and mitosis\textsuperscript{57, 58}. The increase of both extracellular and intracellular HA is simultaneous during mitosis and proliferation. How HA is transported to the cytoplasm and nucleus is unknown, however the possibility that it is synthesized and deposited directly into the cytoplasm, instead of being extruded to the outside of the cell, has not been completely ruled out.

**Hyaladherins**

HA can bind to different proteins. These proteins with HA binding domains have been named hyaladherins. Differences in their tissue expression, cell localisation and regulation explain how HAs simple structure can display such a wide range of functional activities. Most of the HA receptors and binding proteins are members of the link module superfamily\textsuperscript{59}. These HA receptors have one link module and all other link module superfamily members possess two link modules in tandem.

**Hyaluronan receptors**

HA-receptor interactions mediate at least three important physiological processes; signal transduction, HA internalization and pericellular matrix assembly\textsuperscript{60, 61}. There are four HA cell surface receptors, mediating intracellular signalling, with the link module motif; lymphatic vein endothelium receptor-1 (LYVE-1), stabilin-1, stabilin-2 and CD44.

LYVE-1, a homologue to CD44 with unique transmembrane and cytoplasmic domains, is expressed by lymphatic vein endothelium and internalizes HA\textsuperscript{62}.

Stabilin-1 has a possible role in angiogenesis\textsuperscript{63}. Stabilin-2, or HARE, (HA receptor for endocytosis) clears HA from vascular and lymphatic circulations\textsuperscript{64}.

CD44 is a transmembrane glycoprotein, coded in humans by a gene with 19 exons. Exons 6-14 are alternatively spliced and can generate a multitude of variant CD44 isoforms\textsuperscript{65, 66}. The most common form, CD44s/CD44H
includes none of the variant exons but all isoforms contain the HA-binding link module. CD44 has been shown to be involved in many biological functions, e.g. retention and endocytosis of HA, angiogenesis, tumour invasion and metastasis, adhesion and rolling of lymphocytes and also cell migration. The main ligand of CD44 is HA, however collagen, fibronectin, osteopontin and several other molecules can also bind to CD44.

RHAMM (receptor for HA-mediated motility) is a cytoplasmic protein with the ability to translocate outside the cell via unknown routes. As an extracellular HA-binding protein, RHAMM contributes to normal wound healing. The HA-CD44-RHAMM complex mediates, through the intracellular domain of CD44, an increased expression of CD44 and increased activation of ERK1/2, thus activating migration and invasion functions.

**Hyalectans**

Also parts of the link module superfamily are the hyalectans or leclicans, a family of large aggregating chondroitin sulphate proteoglycans. They consist of versican, aggrecan, neurocan and brevican and they all have the HA-binding link module motif at the N-terminal of their core protein. The middle part of the core protein is a GAG-binding region.

The hyalectan binding to HA is stabilized by a link protein, also possessing the link module motif, which binds HA and another part binds the hyalectan. There are four members in the HA and proteoglycan link protein (HAPLN) family (HAPLN1-4). HAPLN1, or cartilage link protein (CRTL1) is predominantly expressed in cartilage. HAPLN2 and HAPLN4 are expressed in the brain and central nervous system and HAPLN3 is widely expressed.

Versican is the hyalectan that is the most versatile in structure and tissue distribution. Versican binds many other molecules than HA and in vitro studies suggest that versican is involved in cell adhesion, proliferation and migration, thus playing a role in development and maintenance of the ECM.

Aggrecan and HA form huge complexes in the chondrocyte ECM (figure 6), providing cartilage with its load bearing properties. Some of these aggregates are bound to the cell surface via CD44.

Neurocan and brevican are expressed in brain and the nervous system and found in HA complexes in the ECM.
**Hyaluronan in tissue growth**

The ability of HA to co-regulate cell behaviour during embryonic development, healing processes, inflammation and tumour development makes HA very important in tissue growth. HA concentration and organization changes when tissues and organs differentiate. Cells divide and migrate in an ECM rich in HA\textsuperscript{79, 80}. HA promotes proliferation by providing a hydrated pericellular zone that enables cell rounding during mitosis. Inhibition of HA synthesis leads to cell cycle arrest at mitosis before cell rounding and detachment\textsuperscript{57}.

HA concentration may be increased in malignant tumours compared to benign and normal tissue. In some tumours HA levels are predictive of malignancy\textsuperscript{81}. Malignant cells have the ability to survive in anchorage-independent conditions in which normal cells would undergo apoptosis\textsuperscript{82}. Cells require cell-survival signals from growth factors and ECM components, such as fibronectins, laminins and collagens, mediated through integrins. HA strongly promotes anchorage-independent growth\textsuperscript{83-86} and malignant cells resistance to growth arrest and apoptosis is dependent on HA-CD44 interaction\textsuperscript{87, 88}. Through remodelling of the ECM, a growth-adapted cellular environment can be created that induces cells for survival and proliferation. Increased HAS expression and subsequent HA synthesis caused increased metastasis formation or growth of tumours in xenograft models of fibrosarcoma, prostate, colon and breast cancer\textsuperscript{83, 89-91}. In contrast, reduced HAS levels suppresses tumour growth\textsuperscript{84, 92}.

Another example of tissue growth where HA is involved is wound healing\textsuperscript{93} and there are more similarities between wounds and tumours. Tumours, in particular carcinomas activate a latent wound healing program.

![Figure 6. Extracellular matrix aggregates of HA and proteoglycans.](image-url)
but in a prolonged and over expressed manner. Most genes that regulate the wound healing process are also important regulators of cancer growth\textsuperscript{94}.

In adult wound healing, tissues are restored but with the addition of fibrosis and scar. In contrast, fetuses heal skin wound without leaving a scar. The fetal wound matrix contains huge amounts of HA which remains longer than in adult wounds. There are also more HA receptors on fetal cells than on adult cells. This might cause the fetal wound matrix to be more permissive for fibroblast migration and thus accelerate repair and avoid fibrosis and scar tissue\textsuperscript{95}.

**Hyaluronan in the cardiovascular system**

**Cardiac development**

The retinoic acid and the neuregulin/ERBB (erythroblastic leukemia viral oncogene) signalling are two major pathways involved in formation of the trabeculated myocardium during cardiac development. The nuclear retinoic acid receptor has been found to be a transcription factor regulating the *Has2* gene\textsuperscript{96}. Mice embryos without *Has2* expression (*Has2*\textsuperscript{-/-}) synthesize no HA and display severe cardiac and vascular abnormalities, caused by impaired transformation of the cardiac endothelium to mesenchyme, and die during embryonic development\textsuperscript{97}. This phenotype is rescued by activating the ERBB signalling pathway, either through addition of exogeneous HA or active RAS (rat sarcoma viral oncogene). This shows that for a normal cardiac development, HA signalling mediated through the ERBB and RAS signalling pathways is necessary\textsuperscript{98, 99}.

HA signalling also mediates MAP3K1 (MEKK1) phosphorylation by CD44, which subsequently activates both ERK1/2 (extracellular signal-regulated kinase1, MAPK3/extracellular signal-regulated kinase2, MAPK1) and NFκB. This induces differentiation in epicardial cells, which plays an important role in the formation of coronary vasculature\textsuperscript{100}.

HA demonstrates an active role in regulation of cardiac development and analysis of HAPLN1, and two versican deficient mouse models demonstrate similar cardiac malfunctions in the models\textsuperscript{101}. This suggests that a complete HA-HAPLN1-versican complex is necessary for normal cardiac development.

Endocardial cushion development and remodelling of the atrioventricular septal complex during cardiogenesis is regulated by CCN1/CYR61 (cystein rich protein 61) and CCN2/CTGF (connective tissue growth factor) and HA-induced signalling\textsuperscript{102}.

HYAL2-deficient mice develop right or left atrium dilation and enlargements of valves. Left ventricular cardiomyocytes displayed significant
hypertrophy in acutely affected mice. This demonstrates the importance HA degradation in the heart\textsuperscript{103}.

Neonatal CD\textsubscript{44} null mice have shown the necessity of CD\textsubscript{44} for development of right ventricular hypertrophy induced by hypoxia, indicating a role of CD\textsubscript{44} in the hypertrophic process\textsuperscript{104}.

\textit{Myocardial infarction}

The increased presence of HA after myocardial infarction has been shown in rats indicating involvement of HA in the healing process\textsuperscript{105}. Injection of a HA-based hydrogel into the epicardium of the infarcted area in rat hearts, increased wall thickness, reduced infarcted area, increased vasculature and improved cardiac function was achieved, compared to controls\textsuperscript{106}.

\textit{Cardiac vessels}

As mentioned above, short HA fragments have been found to induce angiogenesis, via induction of endothelial cell proliferation in contrast to high molecular size HA\textsuperscript{107}. The localisation of HA in the vessels differ. Arteries and veins differ in localisation of HA in the vessel walls and the vessels of newborn rats contain more HA compared to adult rats\textsuperscript{108}. In adult human aorta, HA is found in all three wall layers\textsuperscript{109}. 
The overall aim of this thesis was to investigate if the GAG hyaluronan is part of the hypertrophic process in the heart.

The aims were:

- To investigate the temporal expression of the genes coding for the HASs, the growth factor FGF2 and their receptors CD44 and FGFR1, in an experimental rat model of cardiac hypertrophy.

- To study the correlation between myocardial HA concentration and gene expression over time.

- To investigate if cardiomyocytes are capable of synthesizing HA.

- To study possible cell signalling between fibroblasts and cardiomyocytes leading to HA synthesis and if so, in which manner the signal is transduced.

- To study if the size of the HA produced can be related to its biological effects.

- To investigate if cardiomyocytes posses intercellular communication capability via exocytosis of exosomes.
Presentation of papers

Paper I
In this study the expression of the genes coding for HAS 1, 2, 3, CD44, fibroblast growth factor 2 (FGF2) and FGF receptor1 (FGFR1) as well as histological evidence for an increase of HA and CD44 were investigated in an experimental rat model of cardiac hypertrophy.

The abdominal aorta was banded to induce cardiac hypertrophy and mRNAs, prepared from heart tissue, were analysed after 1, 6 and 42 days. Total concentration of myocardial HA was quantified. HA and CD44 were investigated histochemically.

Paper II
To further investigate the role of hyaluronan and regulation of its synthesis in the same experimental rat model of cardiac hypertrophy as in paper I, quantitative measurements of myocardial hyaluronan concentration was correlated to gene transcription in hypertrophic cardiac tissue. Factor analysis was used to study this correlation over time.

Paper III
Cardiomyocytes capability to synthesize HA was studied and as well as if the different HA synthases are activated in the different cell types.

Mouse cardiomyocytes (HL-1) and fibroblasts (NIH 3T3) were cultivated in absence or presence of the growth factors FGF2, PDGF-BB and TGFB2. HA concentration was quantified and the size of HA was estimated using dynamic light scattering. Cardiomyocytes were also stimulated with HA to detect presence of HA receptors mediating intracellular signalling which affect gene transcription.

Paper IV
Microvesicles from media of cultured cardiomyocytes derived from adult mouse heart were isolated by differential centrifugation and preparative ultracentrifugation, followed by characterization with transmission electron microscopy and flow cytometry.

Identification of microvesicular mRNA and DNA was performed and transfer of cardiac microvesicles into target fibroblasts was analyzed with gene expression analysis and confocal microscopy.
Methodology

**Rat hypertrophy model**
To obtain tissue from hypertrophied hearts, a cardiac hypertrophy rat model was used. A titanium clip with 0.15 mm inner diameter was put around the aorta just proximal to the renal arteries. Age-matched control rats were sham operated and subjected to exactly the same procedure except for placing the clip around the aorta. The rats were sacrificed at 1, 6 and 42 days postoperatively. To determine if cardiac hypertrophy occurred in the aortic banded animals the heart-to-body weight (HBW) ratio was calculated. The hearts were excised after pentobarbital anaesthesia and immediately washed in NaCl 0.9%, weighed and placed in liquid nitrogen or RNAlater (Qiagen). (Paper I, II)

**Cell culture**
HL-1, a cell line derived from adult mouse heart, displaying phenotypic features typical of adult cardiomyocytes, was used. Cardiomyocytes plated in T-75 flasks coated with fibronectin (Sigma)-gelatin (Fisher Scientific) were maintained in Claycomb Medium (JRH. Biosciences). During culture, the medium was changed routinely every 24 h.
Fibroblasts, NIH 3T3 cells (LGC Standards AB) were cultured and passaged following the standard procedure in Dulbecco’s modified Eagle’s medium (DMEM, Fisher Scientific).

HL-1 and NIH3T3 cells were passaged twice per week. All cultures were kept in an atmosphere of 95% air-5% CO2, 37ºC and at a relative humidity of approximately 95%. (Paper III, IV)

**Stimulation of cells**
NIH 3T3 cells alone and HL-1 cells alone were plated at a concentration of 0.3×10^6 cells/mL into 6-well plates. In a monolayer co-culture of HL-1 with NIH 3T3, 0.24×10^6 HL-1 cells/mL were mixed with 0.06×10^6/mL of NIH 3T3 cells to a 6-well plate. After 72 h, cells were grown to confluence and all media were replaced with serum-free and antibiotic-free media for 24 h. In the treatment groups, cells were then stimulated with FGF2 (5ng/mL, 10ng/mL), PDGF-BB (50ng/mL, 100ng/mL) and TGFB2 (5ng/mL, 10ng/mL) (Biosource, Invitrogen), respectively, six replicates of each. Plates with no growth factor addition were used as controls. After 24 h, the media from each growth condition was collected and the cells were then harvested and placed in RNAlater (Qiagen). (Paper III)
**RNA preparation**

For each rat group, total RNA was isolated from heart tissue from 6 aortic banded rats and 6 sham operated rats, using the RNeasy Fibrous Tissue Kit (Qiagen). (Paper I and II)

For each cell group, total RNA was isolated from three wells using the RNeasy Mini Kit (Qiagen). (Paper III)

Total RNA was isolated with RNEASY Mini Kit (Qiagen) from cardiomyocytes (n=2) and microvesicular pellets (n=2) prepared from 18 mL Claycomb medium after 48 h incubation with cardiomyocytes. (Paper IV)

The concentration of the RNA was measured in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) and the integrity of the RNA was analyzed in a 2100 Bioanalyser (Agilent Technologies Inc.). Omniscript RT Kit (Qiagen) was used to synthesize cDNA. (Paper I). Aliquots of total RNA were converted to biotinylated double-stranded cRNA according to the specifications of the Illumina Totalprep RNA Amplification Kit (Ambion). (Paper II-IV)

**DNA preparation**

DNA was isolated with GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) from a microvesicular pellet prepared from 18 mL Claycomb medium after 48 h incubation with cardiomyocytes. To add a poly-T tail, DNA was incubated with 25 µL 100 mM dGTP (Gibco BRL, Life Technologies) and terminal deoxynucleotidyl transferase (TdT) (Invitrogen) for 30 min at 37ºC, according to manufacturer’s protocol. The constructed cDNA was purified and transcribed to synthesize biotinylated cRNA with Illumina Totalprep RNA Amplification Kit (Ambion). (Paper IV)

**Real-time PCR**

Relative quantitation of gene expression changes was performed using an Applied Biosystems Prism 7900HT Sequence Detection System according to the manufacturer’s specifications.

The gene-specific minor groove binder probes were FAM labelled and the GAPDH-specific minor groove binder probe was VIC labelled. All samples were run in triplicates and amplification were analyzed using Applied Biosystems Prism Sequence Detection Software version 2.2-2.3 Relative quantification was calculated according to the comparative \( C_T \) method (Applied Biosystems) using a statistical confidence of 99.9%. The amount of target gene mRNA, normalized to an endogeneous control and relative to a calibrator, is given by \( 2^{-\Delta\Delta C_T} \). The gene expression fold change of the aorta
banded animals is the average \(2^{-\Delta C_T}\) value relative to the average \(2^{-\Delta C_T}\) value for the sham operated animals. (Paper I-III)

Rat cDNA-specific TaqMan Gene Expression Assays for Has1, Has2, Has3, CD44, Fgf2, Fgfr-1, Gata4, alpha-myosin heavy chain (Myh6) and beta-myosin heavy chain (Myh7) from Applied Biosystems were used in paper I. The rat D-glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene was used as an endogeneous control. (Paper I)

Relative quantification of mRNA expression of four genes differentially expressed was performed to confirm the validity of the microarray expression data in paper II. Rat cDNA-specific TaqMan Gene Expression Assays for Csgp2, Itgb1, Ccn2/Ctgf, and Tgfb2 from Applied Biosystems were used in the study. The rat Gapdh gene was used as an endogeneous control. (Paper II)

Mouse cDNA-specific TaqMan Gene Expression Assays for Has1, Has2 and Has3 from Applied Biosystems were used in paper III. The mouse Gapdh gene was used as an endogenous control. (Paper III)

**Hyaluronan purification**

Heart tissue

The wet weight of the rat heart tissue samples was measured before being dried in a rotary vacuum pump for 3 hours. Dry weights were measured, and then the samples were homogenized. The samples were suspended in a solution of pronase, 5 Units/ml (Pronase from Streptomyces griseus, BioChemica, Fluka). Thereafter, 0.1 units protease per mg tissue was added to each tissue sample before they were incubated in a water bath at 55ºC for 16 hours. The temperature was then raised to 100ºC, and samples were boiled for 10 minutes. 50-100µl phenylmethanesulfonyl fluoride (BioChemika, Fluka) was added, and samples were centrifuged for 15 minutes at 15,000 G. The supernatant was diluted 20-100 times in PBS. (Paper I, II)

Cell media

Hyaluronan was purified from cell media of cultured cardiomyocytes and fibroblasts prior to size analysis. Four mL of cell media were concentrated to 50 µL using 10 kDa cut-off Ultra Cell filter-unit (Millipore) by 15 min centrifugation at 4,000 x g. A total digestion of proteins, present in the growth medium, was achieved using pronase (Boehringer Mannheim) at a concentration of 2 mg/mL in each sample. The protein digestion was allowed to continue for 72 h at 40ºC and subsequently quenched by increasing the temperature to 100ºC for 3 minutes. Digested peptides and amino acids, as
well as CaCl2, were removed using a 10kDa cut-off Ultra Cell filter-unit by 3 x 15 min centrifugation at 4,000 x g.

Desalted HA samples were loaded on a DEAE-FF anion-exchange column with a bead volume of 1 mL (GE-Life Science). HA was eluted from the column with 8 column volumes of 0.4 M NaCl 20 mM TRIS-HCl pH 7.4

The eluted fraction was collected and concentrated to approximately 1.5 mL. The volume of the concentrated samples was further reduced to 30 µL using a 0.5 mL 10 kDa cut-off Ultra Cell filter-unit. The buffer was changed by 3 steps of 15 min centrifugation followed by dilution with 0.5 mL of 100 mM NaCl in D2O using the same concentration device. The purity of the samples was controlled by SDS-page, for detection of residual proteins after pronase digestion, and by NMR to detect any other impurities, such as lipids and DNA. (Paper III)

Quantitative analysis of hyaluronan

HA samples purified from rat heart tissue and cell media from cell cultures were analyzed for HA concentration using an enzyme-linked binding protein assay (Corgenix). Absorbance was read at 450 nm with correction at 650 nm on a spectrophotometer (Multiscan Ascent, Thermo Labsystems). All analyses were performed with SPSS statistical analysis package (version 13.0, SPSS Inc.). Data were expressed as mean±S.D. Differences between two groups were compared using Mann-Whitney U test. Statistical significance was set to P<0.05. (Paper I-III)

Histochemistry staining for hyaluronan and CD44

Hearts from the animals used for gene expression analysis were also used for histochemical analysis with the addition of 2 hearts in the 6 and 42 days groups. Approximately 2 mm thick slices were dissected just below the bi- and tricuspid valves and fixed in glutaraldehyde, dehydrated and embedded in paraffin. The HA and CD44 stainings were performed on serial sections. For the localization of HA, the same hyaluronan binding protein probe as in the quantification analysis, was used (Corgenix). For the localisation of CD44 a purified mouse anti-rat (Pgp-1, H-CAM) monoclonal antibody (Pharmingen) was used. All slides were blindly evaluated by two of the authors. For photo documentation a Canon EOS 10D camera and a 100 mm macro lens with accessories were used (Canon Inc.). (Paper I)

Dynamic Light Scattering size analysis of hyaluronan

The size of HA synthesized by fibroblasts, cardiomyocytes or a co-culture of the two cells types was estimated by Dynamic Light Scattering (DLS). The
Z-average value, which is the mean hydrodynamic diameter weighted against the intensity of the DLS signal, was used to estimate the mean hydrodynamic size of the different HA molecules. Due to the hygroscopic properties of HA, the hydrodynamic diameter of HA is highly dependent on the buffer and salt conditions used in the DLS measurements. Therefore, the hydrodynamic diameters of HA purified from cell media were compared with those of HA with known molecular weights of 70 kDa, 450 kDa and 2420 kDa (Hyalose, L.L.C.) recorded at identical conditions.

Each HA sample was intensively shaken at 4°C for 20 minutes, to dissolve possible entanglements. To sediment remaining entanglements, the samples were centrifuged for 20 minutes at 14,000 rpm, 50 µL of sample was added into a disposable low volume cuvette with a 10 mm path length. DLS measurements were conducted at 20°C using a Nano Zetasizer (Malvern Instruments) equipped with a HeNe-laser with a wave length of 633 nm. Before each measurement the temperature was allowed to equilibrate for 10 minutes. The back scattered light was detected at an angle of 173 degrees. For each cell condition the light scattering was measured for 200 seconds with 10 replicate measurements. The DLS data was analyzed using the Dispersion Technology Software v.5.10 (Malvern). Z-average, the mean intensity weighted diameter, was collected for each HA sample. Mean values and standard deviations from each measurement were calculated and compared using GrapPad Prism v.5. Outliers in Z-average were detected and rejected by calculation of the Dixon’s Q ratio, using $P = 0.05^{114}$. (Paper III)

**Microarray gene expression analysis**

Aliquots of total RNA were converted to biotinylated double-stranded cRNA with the Illumina Totalprep RNA Amplification Kit (Ambion). The labelled cRNA samples were hybridized to RatRef-12 Expression Beadchip (paper II), MouseRef-8 v2 (paper III, IV) (Illumina, San Diego, CA, USA), incubated with streptavidin-Cy3 and scanned on the Illumina Beadstation GX (Illumina, San Diego, CA, USA). (Paper II-IV)

**Microarray gene expression data analysis**

To determine differentially expressed genes, microarray data were analyzed using Illumina Beadstudio software (version 3.2.1). Intensity data were normalized using Beadstudios cubic spline algorithm. A number of filtering steps were applied to avoid false positives. Significant differential expression was calculated using the Beadstudio software by applying multiple testing corrections using Benjamini and Hochberg False Discovery Rate (FDR) $^{115, 116}$. The gene expression fold change was calculated as the average signal value of aorta ligated animals/stimulated cells relative to the
average signal value of the sham operated animals/control cells. A significant up-regulation was defined as a foldchange ≥1.5 and a significant down-regulation was defined as foldchange ≤0.67. Statistical significance was set to \( P < 0.05 \). To avoid selecting genes with high foldchange due to low signal intensity a minimum signal intensity value was utilized. For up-regulated genes the signal intensity was set at >50 in the ligated aorta group/stimulated cells, 2.5 times the highest background signal. For down-regulated genes the signal intensity was set at >50 in the sham group/control cells. (Paper II-IV)

**Correlation between hyaluronan concentration and gene transcription**

Linear correlation between HA concentration in the left ventricle and expression levels of significantly differentially expressed genes was tested to identify a set of genes whose expression changes are associated with changes in HA concentration. Correlation between HA concentration and gene expression levels were calculated with SPSS (version 16.0, SPSS Inc.). The Pearson correlation coefficient was used and statistical significance was set to \( P<0.05 \).

Factor analysis was performed to examine the change in correlation over time for the genes with expression levels found to correlate with HA concentration. Principal components method was used to analyze correlation matrix and 2 factors were extracted. Since the differentially expressed genes in growing hearts are regulated according to a fetal gene program\(^{17}\), activated by the increased afterload the sham operated animals could not be included in the correlation analysis. MetaCore\textsuperscript{™} (GeneGo Inc.) was used to generate a network between the correlating genes with transcription factors to elucidate common transcriptional relationships.

To further increase the knowledge of the environment in which HA is active, genes coding for proteins associated to HA and the ECM were investigated. Genes coding for proteins such as structural ECM molecules, cell membrane receptors that bind to ECM structures, molecules that interact between cell surface and ECM, growth factors that interact with the ECM and enzymes that regulate the turnover and remodeling of ECM molecules were filtered from the lists of significantly differentially expressed genes at the three time points. These genes were also used to generate a network with transcription factors. (Paper II)

**Preparation of microvesicles**

Cell media were centrifuged to remove cell debris, 3,000 x g for 20 min at 4°C, repeated three times, followed by 10,000 x g for 20 min at 4°C,
repeated three times. The acquired supernatants were ultracentrifuged at 130,000 x g (49,000 rpm) for 2 h at 4ºC in an MLS-50 rotor and a Beckman Optima™ MAX-E Ultracentrifuge (Beckman Coulter) to separate the microvesicle pellet from the supernatant containing soluble molecules. Pellet was dissolved in PBS. A similar ultracentrifugation of cell culture medium alone was carried out to rule out the presence of any microvesicles in the culture medium.

**Cross-talk between cardiomyocytes and fibroblasts**

Measurements of HA concentrations in cell media and gene expression analysis were used to elucidate the existence of signalling crosstalk between cardiomyocytes and fibroblasts.

Three general ways of mediating signalling crosstalk between cells were considered. Direct cell to cell contact, extracellular signalling transferred by microvesicles released into media or extracellular signaling transferred through exocytosis of soluble molecules into media.

After 24 h incubation, media from cardiomyocytes, fibroblasts and cocultured cardiomyocytes and fibroblasts were transferred to both cardiomyocyte and fibroblast cultures. After additional 24 h incubation, media were collected and the cells were harvested and placed in RNAlater. Concentrations of HA in media from cardiomyocytes and fibroblasts were measured as described above.

To distinguish between extracellular signaling through microvesicles and exocytosis of soluble molecules, media that induced increased HA synthesis in cells was centrifuged to separate pellet and media supernatant containing soluble molecules (described above). Cells were then incubated with pellet dissolved in new media or with media supernatant. The concentration of HA in media was measured and compared to controls incubated with fresh media. Gene expression in cells (n=3, except fibroblasts incubated with media supernatant, where n=2) was analyzed with Illumina Beadstation (Illumina).

**Hyaluronan stimulation of cardiomyocytes**

To investigate the presence of HA interaction with cardiomyocytes, native-HA, 10 µg/mL, 800-1200 kDa (Select-HA™ 1000) or oligo-HA, 50 µg/mL, 6-mer (HYA-OLIGO6EF-1) (Hyalose, L.L.C.) was added to cardiomyocytes in Claycomb media, followed by 24 h incubation. The cells were harvested and placed in RNAlater.
Differential gene expression, analyzed with Illumina Beadstation, was used to detect intracellular signaling resulting in changed transcription, compared to control cells (n=2 in each group). (Paper III)

**Flow cytometry analysis**

Fluorescence-activated cell sorter (FACS) was used to detect proteins on microvesicle surfaces. Isolated microvesicles were stained with 250 ng mouse anti-annexin-II, mouse anti-clathrin heavy chain, mouse anti-flotillin-1 and mouse anti-caveolin-3 (BD Biosciences) for 20 min, in the dark on ice. After an additional ultracentrifugation to wash the pellet, it was resuspended in 100 µL PBS and 1 µL rat anti-mouse IgG PE and incubated for 20 min, in the dark on ice. The ultracentrifugation was repeated and the pellet resuspended in PBS. Microvesicles were analyzed on FACSCalibur (Becton Dickinson). (Paper IV)

**Electron microscopy**

For electron microscopy, the microvesicles were fixed in a solution containing 3% glutaraldehyde in 75 mM sodium cacodylate buffer (pH 7.4) with 4% polyvinylpyrrolidone and 2 mM CaCl₂, for 6 h. They were subsequently rinsed in the same buffer for one hour, and then post fixed in 1% osmium tetroxide over night at 4ºC. After another rinse in buffer the sample was dehydrated in a graded series of acetone and then embedded in an epoxy resin.

Ultrathin sections (70 nm) were cut, and collected on formvar coated copper grids and then contrasted with uranyl acetate and lead citrate for electron microscopy performed with a JEOL 1200-EX (Jeol Ltd.). (Paper IV)

**Identification of microvesicular contents**

Biotinylated cRNA was converted from microvesicle DNA and RNA (described above). The labeled cRNA samples were hybridized to MouseRef-8 Expression Beadchip (Illumina), incubated with streptavidin-Cy3 and scanned on the Illumina Beadstation GX

Identified mRNAs have to be detected both in cardiomyocytes and microvesicles to be considered as positively detected. Since cardiomyocytes reasonably are the source of microvesicle mRNA, they should themselves contain the same mRNA.

Detected DNA sequences correspond to the 50 nucleotide long probe sequences on the gene expression chip. It does not necessarily mean that the microvesicle DNA is derived from that gene. That 50 nucleotide long sequence could possible also exist in non-coding parts of the genome. (Paper IV)
Microvesicular DNA transfer into target fibroblasts

Microvesicles were stained with 20 µmol/L acridin orange (AO) (Invitrogen) for 90 min, in the dark at room temperature. The sample was diluted to 4 mL and ultracentrifuged at 130,000 x g (49,000 rpm) for 2 h at 4ºC. The supernatant was removed to eliminate contamination of unincorporated AO. The AO-stained microvesicles suspended in 1 mL PBS were then put in a dialysis bag with a 3,500 MWCO (molecular weight cut off) dialysis membrane (Spectra/Por) and dialysed against 300 mL PBS for 24 h with one change of dialysis buffer after 5 h. The sample was ultracentrifuged and the pellet dissolved in DMEM and incubated for 3 h with fibroblasts, grown for 24 h on a cell culture microscope slide (Falcon). The slide was subsequently mounted with 4’-6-Diamidino-2-phenylindole (DAPI) to stain fibroblast nuclei and studied in a Nikon Eclipse E800 confocal microscope. Light microscope was used to add a layer in images to visualize cell borders. (Paper IV)

Microvesicular induced effects on target cells

Fibroblasts, grown on 6-well plates, were incubated for 48 h Claycomb medium, previously incubated for 24 h with cardiomyocytes (n=3).

A part of the same Claycomb medium was ultracentrifuged, (described above) and the supernatant was also incubated for 48 h with fibroblasts (n=2).

Isolated microvesicles from cardiomyocytes incubated for 48 h in Claycomb media were dissolved in DMEM and incubated with fibroblasts for 48 h (n=2).

The stimulated fibroblasts were compared to control fibroblasts incubated in fresh Claycomb medium (n=3) and DMEM (n=2), respectively.

RNA was prepared from fibroblasts, labelled and hybridized to MouseRef-8 Expression Beadchip (Illumina), as described above.

To determine differentially expressed genes microarray data were analyzed using gene expression module in Beadstudio software, version 3.3.7. (described above). (Paper IV)
Results

Paper I and II:

General characteristics

The heart weight increased in the aorta ligated group compared to the sham group. At day 1 after operation, the heart weight of the aorta ligated animals was 15% higher than in the sham operated group. At day 42 the weight had increased by 43% compared to sham operated animals. The operation initially caused all rats, in particular the aorta ligated group, to lose weight which affected the HBW (heart-to-body weight) ratio calculations. Genes traditionally used as markers for cardiac hypertrophic growth and the fetal gene program (Nppa, Nppb, Acta1, Myh6 and Myh7) showed an increased expression as early as 1 day after surgery in the aorta ligated rat hearts. As expected, expressional changes in early genes was observed only in the acute phase.

Quantitative RT-PCR

Hyaluronan synthases and CD44.

HAS1 expression had increased six-fold in the aorta banded rats at day 1 and 6 and then dropped to three-fold after 42 days.

HAS2 had a similar expression as HAS1 at day 1 but dropped earlier to three-fold by day 6 and was expressed at basal level after 42 days.

HAS3 did not show any detectable cardiac expression at all either in aorta ligated or sham operated rats.

CD44 expression was up-regulated eight-fold at day 1 in the aorta banded group, its expression progressively decreased, to reach control values at day 42.

Fibroblast growth factor-2 and fibroblast growth factor receptor-1.

The expression of FGF-2 had increased almost five-fold at day 1 and was further up-regulated at day 6. After 42 days the level decreased but was still significantly up-regulated two-fold as compared with sham-operated animals.

The expression of FGFR-1 was transiently up-regulated two-fold only at day 1.

Quantification of hyaluronan in heart tissue

The average HA concentration in the hearts of the sham operated animals correlated well to earlier studies. The average HA concentration in the hearts at day 1 was 473±262 μg/g dry weight in the aorta ligated animals and
the concentration was significantly increased compared to sham operated animals ($P=0.037$). The concentrations at day 6 and day 42 were not significantly increased compared to sham operated animals.

**Histochemical analysis of hyaluronan and CD44**

HA was detected in the myocardium and in the adventitia of cardiac arteries in all rats in both aorta ligated and sham operated animals. No difference in staining intensity was detected between the groups.

With one exception, CD44-staining was detected only in the hearts of the aorta ligated animals. CD44 staining was mainly found in and around the adventitia, in some arteries also in the media-intima, and in right ventricular trabeculi.

**Microarray gene expression**

The number of genes that were significantly up- or down-regulated on the chip at day 1, 6 and 42 was 742, 216 and 371 respectively. Out of these genes there were 106 (14%), 4 (2%) and 31 (8%) that correlated with the concentration of HA in the left ventricle in the hearts. Of the 106 genes, 81 were identified and have a known function. The MetaCore™ (GeneGo Inc.) bioinformatics software was used to generate a network between the correlating genes and transcription factors. This analysis revealed an enrichment of genes regulated by 6 transcription factors, JUNB, FOS, MYC, TP53, SP1 and EGR1. Four of these transcription factors, JunB, Fos, Myc and Egr1 were up-regulated at day 1.

The change in correlation with HA concentration over time with the 106 genes at day 1 and the 31 genes at day 42, were analyzed with factor analysis. The 106 genes from day 1 formed two tight clusters, representing positive correlation to HA concentration and negative correlation. The same genes in animals from day 6 showed clustering but less tight. At day 42 the correlation was lost. The 31 genes correlating to HA concentration at day 42 showed an inverse development over time. On day 42 a tight correlation was evident but at day 1 and 6 no cluster was seen. In the sham operated animals no cluster of correlating genes was observed.

Genes with association to HA specifically and the ECM in general were filtered from the lists of significantly differentially expressed genes at the three time points. These 37 genes were also used to generate a network with transcription factors. Most of the genes were also bioinformatically found to be transcriptionally regulated by TP53, SP1, AP-1 and MYC. Four of these genes, CD44, biglycan (Bgn), latent transforming growth factor binding protein 2 (Ltbp2) and fibrillin 1 (Fbn1), correlated significantly with HA concentration levels.
Paper III:

Quantitative analysis of hyaluronan in cell media

The only growth factor that caused cardiomyocytes to synthesize HA was PDGF-BB at 100 ng/mL in the media.

Fibroblasts on the other hand synthesize HA with or without any addition of growth factors. The addition of growth factors showed that FGF2 and PDGF-BB at higher concentrations (10 and 100 ng/mL) induced a lower concentration of HA than at lower concentrations (5 and 50 ng/mL). TGFB2 induced the highest concentration of HA but no difference was seen in relation to its concentration.

Co-culturing cardiomyocytes and fibroblasts resulted in increased HA concentration compared to cells cultured separately, especially when taking into account the lower amount of fibroblasts in the co-culture.

In contrast, FGF2 and PDGF-BB stimulated co-cultured cells resulted in lower HA concentration in the media compared to cells cultured alone. Media from co-cultured cells stimulated with TGFB2 contained twice as much HA as in TGFB2 stimulated fibroblasts.

Real-time polymerase chain reaction analysis of hyaluronan synthases

The endogenous control Gapdh, used in real-time PCR analysis to normalize for differences in the amount of total RNA added, were in some cell/growth factor combinations not at a consistent level between samples and controls. Therefore, the β-actin and 36B4 genes were also tested as endogenous controls but with the same result. For this reason the real-time PCR results have only been used as a detection of expression of the different Has’s, without the possibility to calculate foldchange and significance of changes in expression.

Dynamic Light Scattering Size analysis of hyaluronan

The size of HA synthesized by fibroblasts, cardiomyocytes or co-culture of the two cell types was estimated by DLS.

Cardiomyocytes produced HA with a diameter of 135 ±15 nm when stimulated with a high concentration of PDGF-BB. Under other conditions cardiomyocytes did not produce any detectable levels of HA.

Non-stimulated fibroblasts produced HA with a hydrodynamic diameter of 175 ±25 nm.

Upon stimulation by low levels of FGF2, fibroblasts released HA with a diameter of 140 ±20 nm, whereas fibroblasts cultured with high levels of FGF2 produced HA with a diameter of 60 ±15 nm.

Fibroblasts stimulated by the low dose of PDGF-BB produced HA with a diameter of 100 ±15 nm. When the fibroblasts were stimulated by a high
dose of PDGF-BB the concentration of HA became too low to ensure reliable DLS measurements.

The different concentrations of TGFB2 showed little effect on the size of produced HA, with a hydrodynamic diameter of 110 ±15 nm and 100 ±5 nm for the low and high concentration of this growth factor, respectively.

When cardiomyocytes and fibroblasts were co-cultured without any growth factors they secreted HA with a diameter of 160 ±20 nm.

The addition of low levels of FGF2 induced HA of 115 ±45 nm, whereas the high level of FGF2 yielded no detectable HA.

Low levels of PDGF-BB did not induce a HA concentration high enough for reliable DLS measurements, whereas a high dose of PDGF-BB yielded HA with a size of 220 ±80 nm.

When a low dose of TGFB2 was added to co-cultured cells, HA with a size of 170 ±35 nm was produced, whereas the high concentration of TGFB2 resulted in HA with a diameter of 85 ±15 nm.

**Crosstalk between cardiomyocytes and fibroblasts**

Synthesis of HA by fibroblasts increased after incubation with Claycomb media from either co-cultured cells or cardiomyocytes alone, compared to both fibroblasts incubated in DMEM and fibroblasts incubated in fresh, unused Claycomb media. A lower HA concentration was obtained after incubation of fibroblasts with fresh, unused Claycomb media.

Fibroblasts incubated with pellet from ultracentrifugation of Claycomb media from cardiomyocytes, dissolved in DMEM showed similar HA concentration to fibroblast controls while fibroblasts incubated in the supernatant of ultracentrifuged Claycomb media from cardiomyocytes demonstrated a two-fold increase in HA concentration. Cardiomyocytes showed no synthesis of HA irrespective of media added.

Gene expression analysis of fibroblasts incubated for 24 h with Claycomb media transferred from cardiomyocytes, resulted in 333 differentially expressed genes compared to fibroblasts incubated with unused Claycomb media. The same analysis of fibroblasts incubated for 24 h with ultracentrifuged Claycomb media supernatant transferred from cardiomyocytes, resulted in 96 differentially expressed genes. In both cases the Tgfb2 gene was up-regulated 3-fold.

**Hyaluronan stimulation of cardiomyocytes**

The addition of native-HA and oligo-HA to Claycomb media before incubation for 24h, affected cardiomyocytes gene expression. When native-HA was added to Claycomb media 35 differentially expressed genes were detected and 63 genes were detected with oligo-HA added.
Paper IV:

Preparation and characteristics of microvesicles
Transmission electron microscopy of microvesicles secreted from cardiomyocytes revealed small, closed exosome-like vesicles (40-300 nm) surrounded by a bilayered membrane. Since the microvesicles are cardiomyocyte-derived they are called cardiosomes to denote their origin. Some of them had a distinct electron dense appearance while others displayed an electron lucent interior. Furthermore, approximately 80% of the cardiosome population was positive for flotillin-1 and 30% for caveolin-3 in the flow cytometry analysis, indicative of a certain degree of heterogeneity.

Identification of cardiosomal contents
The cardiosomes contained 1595 detected mRNAs of which 1520 also were detected in cardiomyocytes. It was possible to connect 423 of these genes/proteins in a network by using the MetaCore™ (GeneGo Inc.) bioinformatics software. Furthermore, mRNA from 35 genes coding for proteins in the small and large ribosomal subunit and 8 additional genes were detected and could be connected in a network. Finally 33 genes coding for proteins in mitochondria could be detected.

Analysis of cardiosomal DNA converted to biotinylated cRNA detected signals from gene probes corresponding to 343 different chromosomal DNA sequences.

Cardiosomal DNA transfer into target fibroblasts
Examination by confocal microscopy of fibroblasts incubated with AO-stained cardiosomes revealed intracellular AO-stained spots, localized in cytoplasm and inside the nuclear membrane.

Cardiosome induced effects on fibroblasts
Incubation of fibroblasts with cardiosomes resuspended in fresh DMEM medium induced 161 differently expressed genes compared to incubation with control medium.
Discussion

This study has focused on investigating the role of HA in the development of cardiac hypertrophy. Both an experimental rat model of cardiac hypertrophy and cultured cardiomyocytes and fibroblasts have been used.

An early and pronounced increase in the expression of Has1 and Has2 in the hearts of the aorta ligated animals was observed, paralleled by a similar increase in the expression of CD44 and Fgf2. This was accompanied by an increase in HA concentration in the rat hearts. The induced expressions of Has1 and Fgf2 were an early (day 1) and sustained response (day 6 and 42). This pattern contrasted with that of Has2 and CD44, the expressions of which were transient, the levels decreasing as soon as after day 6 to reach control levels by day 42.

This indicates that HA derived from HAS1 might be synthesized for a different purpose in the late phase of the hypertrophic development of the heart and therefore regulated by other factors than Has2 is regulated by.

Assuming that genes involved in the same biological chain of events have similar temporal expression patterns, then HA, synthesised by HAS2, is linked to the expression of CD44. HA concentration and CD44 expression levels correlated day 1. The CD44 receptor was mostly localised around the arteries in the hearts from aorta ligated animals. It is known that CD44 has a role during angiogenesis\(^{53, 118}\) and the HA synthesised by HAS2 may be correlated to a vascular response to either hypertension and/or adaptation to a growing heart.

In contrast, in cultured cardiomyocytes and fibroblasts, Has2, but not Has1 was detected both in controls and all growth factor treated combinations. One reason for Has1 not being expressed, as it was in heart tissue, could be the absence of vascular cells in the cell cultures. This would mean that HA synthesized by HAS1 is associated with vessels which is contradictory to the assumption made from the results from cardiac tissue where Has2 is associated to the vasculature.

Obviously the regulation of the HASs is very complex.

The addition of growth factors in cell media of cultured cardiomyocytes and fibroblasts consistently affected the HA size, demonstrating that growth factors not only influence the amount of synthesized HA but the processes initiated by growth factors also induced a change in HA size. In most cases, the cells produced HA in smaller size when stimulated with growth factors. The predominant synthase expressed was Has2, suggesting that HA size was not controlled by shift in type of HAS expressed.
Which type of cells that contributes to HA synthesis and expression of HASs in the cardiac tissue in the aorta ligated rats is unknown. Cell cultures were used to investigate the capability of cardiomyocytes to synthesize HA.

The addition of PDGF-BB, at the higher concentration (100 ng/ml), to cultured cardiomyocytes, activated synthesis of HA in the cells. Thus cardiomyocytes must express HAS and indeed, real-time PCR showed both Has2 and Has3 transcription.

Adding HA to cardiomyocyte media showed that both oligo-HA and native HA influenced transcription of cardiomyocyte genes. This suggests that cardiomyocytes express HA binding receptors on the surface, capable of triggering gene transcription through intracellular signalling. Hence, cardiomyocytes can produce, detect and be affected by HA in their environment.

An interesting finding was the distinctly affected HA concentration levels found both in untreated and TGFB2 treated co-cultured cells compared to cardiomyocytes and fibroblasts cultured alone. This was explained by the observation that cardiomyocytes secrete through exocytosis soluble molecule(s), a still unknown factor which after transfer to fibroblasts up-regulated the Tgfb2 gene and increased the synthesis of HA. HA seems to be requested by the growing and proliferating cardiomyocytes, which is in concordance with the observations of many other cell types needing increased HA synthesis for growth.\textsuperscript{79, 80}

The lower concentration of PDGF-BB induced no synthesis of HA. This may indicate a threshold that must be exceeded before synthesis is initiated in cardiomyocytes. It is therefore tempting to speculate that fibroblasts are the primary source of HA synthesis in the myocardium and cardiomyocytes are a secondary producer of HA that start their synthesis when PDGF-BB rises to a certain level. The range in sizes of HA synthesized in cardiomyocytes overlapped sizes of HA seen in fibroblasts and co-cultured cell controls. Thus, cardiomyocytes might produce HA in similar size to fibroblasts when an acute need for high amounts of HA arises to support the HA synthesis derived from fibroblasts.

Cardiomyocytes are also capable of intercellular signalling via exosomes, denoted cardiosomes. The cardiosome population observed was not homogenous in size, expressed surface proteins or electron density observed in electron microscopy. This suggests that cardiomyocytes secrete cardiosomes with different purpose and perhaps intended for different targets cells.

They contained both mRNA and DNA, which could be transferred to fibroblasts with subsequent change in gene transcription. This internalization of cardiosomes, even into the nucleus, was visualized by confocal microscopy.
Cardiosomal mRNA coding for both small and large ribosomal subunits as well as proteins involved in mitochondrial energy generation were identified. This implies that cardiosomes are carrying means to support protein production and energy generation in the target cells for the transcription of their delivered genetic material.

To further investigate the changes in HA synthesis in this animal model, correlation between HA synthesis and gene expression changes was investigated.

Subsets of the differentially expressed genes that correlated with HA concentrations at day 1, 6 and 42 were identified. Thus, HA synthesis and such genes are likely to be governed by a common regulatory pathway.

With factor analysis it was possible to demonstrate that genes, which expression levels correlated with HA concentration at day 1 in aorta ligated animals, also correlated in a similar way after 6 days. No correlation was found between these genes in the sham operated animals at any time point.

One explanation for the temporal differences seen in gene correlation could be the regulation and expression of different HA synthases.

Also the genes coding for angiotensin-converting enzyme (ACE) and ACE2 showed distinct temporal patterns of expression with acute up-regulation of Ace at day 1 and a late response of Ace2 at day 42.

The renin angiotensin system (RAS) is a known regulator of cardiac growth. Angiotensin II (Ang II) is a prohypertrophic effector peptide, while angiotensin 1-7 has the opposite effect, activated by angiotensin-converting enzyme (ACE) and ACE2 respectively.

Most likely the observed co-regulation of genes with HA concentrations at day 1 and 6 reflects the acute hypertrophic process while the genes being active at day 42 shows a shift to a steady state program mirrored by the expression levels seen in Has1 and Has2 as well as Ace and Ace2.

Differentially expressed genes with expression levels correlating with HA concentration are likely to be regulated by the same transcription factors. These genes were analyzed in the bioinformatic software MetaCore™ (GeneGo Inc. USA) and an enrichment of genes regulated by 6 transcription factors, JUNB, FOS, MYC, TP53, SP1 and EGR1 were identified. Four of these transcription factors, JunB, Fos, Myc and Egr1 were up-regulated at day 1, indicating strong association with the transcriptional changes in the aorta ligated animals.

JunB, Fos Myc and Egr1 are immediate early genes (IEG), activated in response to stimuli mediated via Ang II and/or mechanical factors.

They are well known as early regulators of cell growth and to precede the expression of cardiac hypertrophy markers, e. g. ACTA1 and ANP. The correlation of HA synthesis to the expression of ANP, which is regulated by
the ACE and IEG’s, opens the possibility that HA also is part of the fetal gene program activated in cardiac hypertrophy.

When this bioinformatic analysis was repeated on differently expressed genes associated to HA specifically and to the ECM in general, the same six transcription factors, JUNB, FOS, MYC, TP53, SP1 and EGR1 were found to regulate these genes. The physical proximity of these ECM proteins and receptors to HA and correlation to HA synthesis reveals a collective change in response to aortic ligation and cardiac hypertrophy.
Conclusions

The aim of this thesis was to investigate HA and its role in cardiac hypertrophy, using both an experimental rat model of cardiac hypertrophy as well as cultured mouse cardiomyocytes and fibroblasts.

Increased concentration of HA in hypertrophied cardiac tissue was observed together with an up-regulation of two Has genes. The different expression patterns of the Has1 and Has2 genes in both the cardiac hypertrophy rat model and cultured cardiomyocytes and fibroblasts, may indicate that HA is needed for two different purposes in the hearts exposed to aortic ligation, one for an acute and transient response and one for a sustained response. One could be for cardiomyocyte growth, one for vascular growth.

In cultured fibroblasts it was shown that different growth factors induced different concentrations and sizes of HA, even though the main synthase expressed was Has2. FGF2 and PDGF-BB induced HA synthesis in fibroblasts and co-cultivated cells in a reverse dose dependent response, the highest dose induced least HA production and smallest size.

This indicates that different growth factors and their concentrations regulate HA synthesis and size in other ways than just switching between different HASs.

Future research needs to investigate the localisation of HAS1 and HAS2 in the cardiac tissue from aorta ligated animals, as well as the growth factor regulation of HA size.

Four transcription factors, Myc, Fos, JunB and Egr1 were up-regulated in the aorta ligated animals. These immediate early genes are known to be early regulators of the re-expression of fetal genes (e.g. Acta1 and Nppa) in cardiac hypertrophy. They are also known to regulate both several of the differently expressed genes coding for structural and regulatory molecules interacting within the ECM and several of a subset of differently expressed genes which expression levels correlated to HA concentration, in cardiac tissue of the aorta ligated animals.

The coordinated synthesis of HA and expression of genes regulated by immediate early genes, suggests the involvement of immediate early genes in the regulation of synthesis of HA and that HA is a part of the fetal gene program. Functional studies should be performed to elucidate the involvement of immediate early genes in the regulation of HAS expression and HA synthesis.

Cell cultures revealed the ability of cardiomyocytes to both synthesize and detect HA in their environment with subsequent altered gene expression.
Conclusions

Cardiomyocytes also secreted a factor into culture media that after transfer to fibroblasts increased synthesis of HA. This ability of cardiomyocytes to send out a request to fibroblasts to synthesize HA indicates a need for HA of the cardiomyocytes. When fibroblasts deliver the request, receptors on the cardiomyocytes surface can detect the HA signal and after signal transduction, a changed gene transcription in the cardiomyocytes is achieved.

This demonstrates the close involvement between HA and cardiomyocytes. A more detailed study in the changes in gene expression in cardiomyocytes, when stimulated by HA, could reveal the reason for demanding the fibroblasts to synthesize HA.

It was also demonstrated that cardiomyocytes possess an intercellular way of communication via secreted microvesicles. These cardiomyocyte-derived exosomes, cardiosomes, contained both DNA and mRNA and were shown to be internalized when transferred to fibroblasts with subsequent altered gene expression. Through the transfer of the cardiomyocytes own mRNA to target cells, where they are translated to proteins, the cardiomyocyte can influence behaviour and function of target cells in their neighbourhood. The destinations and purposes of secreted cardiosomes need to be clarified in the future.

This study shows that increased HA synthesis in cardiac tissue during hypertrophic development is a part of the extracellular matrix remodelling.

Assuming that growing cells need a certain ECM composition for growth, the observed changes in transcription may in part represent a demand for a remodelling of the ECM. The new ECM, together with growth factors transduce intracellular signalling through several receptors simultaneously. Subsequently when cell receptors respond to the remodelled ECM, the growth can progress.

It has in many cases been demonstrated that HA enriched extracellular matrices are necessary for cells to be able to grow and proliferate. The discovery that cardiomyocytes under certain circumstances can synthesize HA themselves opens up for interesting speculations. Recently, it has been shown that the myocardium has the ability to regenerate, although very slowly\textsuperscript{125, 126}. HA might be an important factor in this process.
Sammanfattning på svenska


Syftet med denna avhandling var att undersöka hyaluronan och dess roll både i en experimentell hjärthypertrofi modell samt i odlade muskardiomyocyter och fibroblaster.

Hjärthypertrofi inducerades hos råttor genom ligering av bukaorta. Hyaluronankoncentration mättes och uttryck av gener som kodar för hyaluronan syntaser kvantifierades i hjärtväv från vänstra kammarens vägg efter 1, 6 och 42 dagar efter operation. Lokalisation av hyaluronan och CD44-receptorn studerades histokemiskt. Hyaluronansyntesens korrelation till transkription av gener studerades med hjälp av microarray genexpressionsanalys.


Ökad koncentration av hyaluronan i hypertrofisk hjärtväv sågs tillsammans med uppreglering av två hyaluronansyntasgener. Hyaluronan upptäcktes i hjärtmuskeln och i hjärtartärernas adventitia medan färgning av CD44 huvudsakligen fanns i och runt adventitia. Hyaluronansyntes korrelerade till uttrycket av gener som regleras av transkriptionsfaktorer som är kända för att inducera hjärthypertrofi.

innehöll både DNA och RNA. Isolerade mikrovesiklar inkuberades med fibroblaster varefter en internalisering observerades i fibroblaster med konfokalmikroskopi. Förändrat genuttryck observerades i fibroblaster efter stimulering med mikrovesiklar.

Denna studie visar att en ökad hyaluronansyntes i hjärtvävnad under utveckling av hypertrofi är en del av anpassningen av extracellulär matrix. Cellkulturer visade att kardiomyocyter kan både syntetisera hyaluronan och förmedla signaler till fibroblaster, vilket får dem att öka sin hyaluronansyntes. Kardiomyocyter kan också uttrycka receptorer för hyaluronan, vilka förmedlar intracellulära signaler som orsakar de observerade förändringarna i genuttryck hos kardiomyocyter som stimulerats med hyaluronan. Detta påvisar en omfattande medverkan av hyaluronan i den process som leder till hjärthypertrofi.
Acknowledgments
References

38. Stuhlmeier, K.M. & Pollaschek, C. Differential effect of transforming growth factor beta (TGF-beta) on the genes encoding hyaluronan synthases and utilization of the


Spicer, A.P., Joo, A. & Bowling, R.A., Jr. A hyaluronan binding link protein gene family whose members are physically linked adjacent to chondroitin sulfate


104. Williams, O.W. & Savani, R.C. Neonatal CD44 null mice have defects in TGFβ and ERK signaling and are protected from hypoxia-induced pulmonary hypertension and decreased alveolarization. *8th International Conference on Hyaluronan* (2010).


