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Temporal correlation between transcriptional changes and increased synthesis of hyaluronan in experimental cardiac hypertrophy

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ABSTRACT

The role of hyaluronan in cardiac growth has become evident, previously shown by increased myocardial levels of hyaluronan in a rat model of cardiac hypertrophy.
To further investigate the role of hyaluronan and regulation of its synthesis in cardiac hypertrophy, 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. A subset of differentially expressed genes was identified with a transcriptional regulation correlating to the increased synthesis of hyaluronan, suggesting a common regulatory pathway.
Four transcription factors, *Myc*, *Fos*, *Junb* and *Egr1*, were also up-regulated. Furthermore, the *Ace* gene was up-regulated, representing increase of angiotensin II, an inducer of these transcription factors and fetal genes in cardiac hypertrophy. This demonstrates a coordinated synthesis of hyaluronan and pro-hypertrophic gene expression, regulated by immediate early genes, with angiotensin II as a possible mediator.

Introduction

In physiological or pathological cardiac growth, all tissue constituents, including extracellular matrix (ECM), has to expand in a coordinated manner in order to maintain optimal function [1]. The ECM macromolecular structure provides scaffold for myocytes, fibroblasts, endothelial cells and the vasculature to align [2]. The main ECM components include structural proteins such as collagens and elastin, adhesive and anti-adhesive proteins, proteoglycans and glycosaminoglycans (GAG), as well as interstitial cells like fibroblasts and macrophages.

Cells have the ability to bind macromolecular ECM components through transmembrane receptors enabling intracellular signaling between the ECM and the cell machinery. Thus, the ECM not only provides structure but is also crucial for cell function and metabolism. This has been demonstrated in a seminal experiment by reseeding decellularized hearts with cardiac-derived and endothelial cells, which respond to the cardiac ECM environment and forms a functional contracting heart [3].

Variation of ECM physico-chemical properties such as elasticity profoundly affects the maturation of naive mesenchymal stem cells into different lineages [4,5].

The transcriptional changes during cardiac remodeling have been studied in several different models using gene expression microarrays. In these studies the patterns of gene expression have been related to sex, acute and late aortic constriction-induced hypertrophies, myocardial infarction, heart chambers, hypertension, different mutations and stretch vs. pharmacologically stimulated cardiomyocytes [6–15]. Correlations between the gene expression levels and quantitative measurements of cardiac growth have also been studied [12,16,17]. All these studies have provided insights into the underlying genetics of cardiac remodeling and the distinct changes in the expression of genes coding for ECM proteins.

One ECM component widely distributed is the GAG hyaluronan (HA), which forms a highly hygroscopic network around cells and vessels, regulating tissue hydration and osmotic balance. It mediates intracellular signaling, mainly through the transmembrane receptor CD44. Apart from these functions it has been shown to be involved in many processes in the body, including wound healing, tumour growth and inflammation [18–20]. Animal studies have provided evidence that HA affects cancer initiation as well as progression and increased synthesis of HA accelerates tumor growth [21,22]. Absence of HA in mice heart causes abnormalities in heart and blood vessel

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development, resulting in an embryonic lethal phenotype, showing the importance of HA in the cardiovascular morphogenesis [23].

We have previously described the dynamic regulation of HA over time in an experimental rat model of cardiac hypertrophy using abdominal aortic ligation. This model showed increased concentration of HA in hearts from ligated compared to sham-operated animals [24].

The aim of this study was to investigate the role of HA and the regulation of its synthesis. Factor analysis was used to study the correlation between HA concentration and gene expression over time, to associate HA synthesis with transcriptional pathways in order to better understand the hypertrophic process. This is the first study to describe the correlation between levels of HA in cardiac tissue with transcription of growth related genes and signaling pathways.

Materials and methods

Preparation of the animals

A total of 122 male Wistar rats, weighing approximately 200 g, were included in this study. The animals were anesthetized with 0.2 ml pentobarbital intra-abdominally and after abdominal incision, a titanium clip of 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 but without ligating the aorta. The rats were sacrificed at 1, 6 and 42 days postoperatively. To determine if cardiac hypertrophy occurred in the ligated aorta hearts the heart-to-body weight (HBW) ratio was calculated. The six hearts with the highest HBW ratio from each ligated aorta group were used for analysis.

Handling and procedures of laboratory animals were performed according to the French regulation.

Sample preparation

The hearts were excised after pentobarbital anaesthesia and immediately washed in NaCl 0.9%, weighed and placed in liquid nitrogen or RNAlater (Qiagen, Hilden, Germany). For each group, 6 aortic ligated rats and 6 sham operated rats, total RNA was isolated from heart tissue from the lower part of the left ventricular wall using the RNeasy Fibrous Tissue Kit (Qiagen, Stanford, CA). RNA concentration was measured in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc, Wilmington, USA) and the integrity of the RNA was analyzed in a 2100 Bioanalyser (Agilent Technologies Inc, Palo Alto, CA).

Quantitative analysis of HA

The wet weight of the tissue samples from the lower part of the left ventricular wall was measured before being dried in a rotary vacuum pump for 3 hours. Dry weights were measured, and then the samples were homogenised. Sample weight was from 3 to 33 mg of homogenised tissue. The samples were suspended in a solution of the protease pronase, 5 Units/ml (Pronase from *Streptomyces griseus*, BioChemica, Fluka, Buchs, Switzerland) in tris-buffer (0.05 M Tris–HCl, 0.01 M $CaCl_2$). Thereafter, 0.1 unit protease per mg tissue was added to each tissue sample before they were incubated in a water bath at 55 °C for 16 h. The temperature was then raised to 100 °C, and samples were boiled for 10 min. 50–100 µl phenylmethanesulfonyl fluoride (0.1 M in ethanol, BioChemika, Fluka, Buchs, Switzerland) was added, and samples were centrifuged for 15 minutes at 15000 G. The supernatant was diluted 20–100 times in PBS and analysed for HA using an enzyme-linked binding protein assay (Corgenix, Westminister, USA) according to the manufacturer's instructions. Absorbance was read at 450 nm with correction at 650 nm on a spectrophotometer (Multiscan Ascent, Thermo Labsystems, Helsinki, Finland).

Microarray gene expression

Aliquots of total RNA were converted to biotinylated double-stranded cRNA according to the specifications of the Illumina Totalprep RNA Amplification Kit (Ambion, Austin, TX, USA). The labelled cRNA samples were hybridized to Sentrix RatRef-12 Expression Beadchip (Illumina, San Diego, CA, USA), incubated with streptavidin-Cy3 and scanned on the Illumina Beadstation GX (Illumina, San Diego, CA, USA).

Data analysis

To determine differentially expressed genes microarray data were analyzed using Illumina Beadstudio software (version 3.3). Intensity data were normalized using Beadstudios cubic spline algorithm. Microarray data and information is available through NCBIs Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE21600. 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) [25,26]. The gene expression fold change was calculated as the average signal value of aorta ligated animals relative to the average signal value of the sham operated animals. 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 upregulated genes the signal intensity was set at >50 in the ligated aorta group, 2.5 times the highest background signal. For downregulated genes the signal intensity was set at >50 in the sham group. Linear correlation between HA concentration in 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., Chicago, Ill, USA). 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 [27], activated by the increased afterload the sham operated animals could not be included in the correlation analysis. MetaCore™ (GeneGo Inc. USA) was used to generate a network between the correlating genes with transcription factors to elucidate common transcriptional relation between them.

To further increase the knowledge of the environment where 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.

Confirmation of the microarray data with quantitative RT-PCR

Relative quantification of mRNA expression of four genes differentially expressed was performed to confirm the validity of the microarray expression data, using an Applied Biosystems Prism 7900HT Sequence Detection System, according to the manufacturer's specifications (Foster City, CA). Omniscript RT Kit (Qiagen, Hilden, Germany) was used to synthesise cDNA from an aliquot of the remaining total RNA. Rat cDNA-specific TaqMan Gene Expression

Table 1
General characteristics and quantification of HA in the hearts.

Parameter	1 day		6 days		42 days	
	Sham	A.I.	Sham	A.I.	Sham	A.I.
	(N=6)	(N=6)	(N=6)	(N=5)	(N=6)	(N=6)
Heart weight (g)	0.77±0.02	0.89±0.06	0.78±0.12	0.80±0.06	1.15±0.20	1.65±0.08
Body weight (g)	206.7±13.2	210.7±14.4	259.1±11.3	215.2±33.5	403.3±49.2	425.0±39.6
Weight change (g)	−8.0±6.6	−24.2±4.5	29.9±20.1	-25.4±24.1	171.5±82.9	250.2±35.4
HBW ratio (mg/100 g)	374.0±18.7	423.2±17.0	298.7±39.1	395.1±36.5	282.6±17.0	391.2±27.2
HA conc. (μg/g d.w.)	141±13	473±262	208±76	251±54	150±34	324±172
P-value	0.037		0.201		0.055	
HA conc. (μg/g w.w.)	67±6	178±137	139±56	167±26	65±22	90±54
P-value	0.055		0.234		0.423	
Amount of water (%)	52±3	65±15	34±8	32±9	57±9	72±9
P-value	0.332		0.46		0.01	

Sham, sham operated animals; A.I., aorta ligated animals; HBW, heart body weight; d.w., dry weight; w.w., wet weight; HA conc., the average HA concentration in the hearts in the three groups and significance of increase in aorta ligated animals compared to sham operated.

Assays for *Vcan, Itgb1, Ccn2/Ctgf,* and *Tgfb2* from Applied Biosystems were used in the study. The rat *Gapdh* gene was used as an endogeneous control (part number 4352338E; Applied Biosystems). All samples were run in triplicates and amplification was analyzed using Applied Biosystems Prism Sequence Detection Software (version 2.3). Relative quantification was calculated according to the comparative C_T method (Applied Biosystems Inc, Foster City, CA) 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 $2^{-\Delta\Delta C}{}_T$. [28]. The gene expression fold change of the animals with ligated aorta is the average $2^{-\Delta\Delta C}{}_T$ value relative to the average $2^{-\Delta\Delta C}{}_T$ value for the sham operated animals. All analyses on real-time RT-PCR data were performed with SPSS statistical analysis package (version 16.0, SPSS Inc., Chicago, IL, USA). Differences between two groups were compared using Mann–Whitney *U* test and bivariate correlation was calculated.

Statistical significance was set to $P<0.05$.

Results

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 the sham operated group. At day 42 the weight had increased by 43% (Table 1). The operation initially caused all rats, in particular the aorta ligated group, to lose weight which affected the HBW 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 changes in early genes was observed only at the acute phase (Table 2).

Table 2
Differentially expressed genes associated with cardiac hypertrophy, in rats at three time points after aortic ligation.

	1 day		6 days		42 days	
	Foldchange	*P*-value	Foldchange	*P*-value	Foldchange	*P*-value
Nppa	2.6	0.031	7.4	0.0007	7.0	0.044
Nppb	1.39	0.24	1.7	0.10	2.4	0,019
Acta1	1.6	0.073	1.9	0.19	2.5	<0.00001
*Myh6**	0.6	0.010	1.1	0.34	0.9	0.33
*Myh7**	3.9	0.004	2.4	0.054	3.0	0.045
Egr1	2.1	0.032	2.0	1.0	2.0	0.0016
Myc	4.0	0.0012	3.0	1.0	1.2	1.0
Fos	4.1	0.0017	1.9	1.0	0.4	1.0
Junb	2.6	0.014	3.9	0.75	1.4	0.29
Ace	3,1	0,002	4,6	0,187	0,7	0,793
Ace2	1,2	1,000	0,6	1,000	1,9	0,002

Nppa, Natriuretic peptide precursor type A; Nppb, Natriuretic peptide precursor type B; Acta1, skeletal actin alpha 1; Myh6, myosin heavy chain, cardiac muscle, alpha; Myh7, myosin heavy chain, cardiac muscle, beta; Egr1, Early growth response 1; Myc, myelocytomatosis viral oncogene homolog; Fos, FBJ murine osteosarcoma viral oncogene homolog; Junb, jun B proto-oncogene; Ace and Ace2, angiotensin-converting enzyme 1 and 2. *Myh6 and Myh7 analysed with real-time PCR in earlier study.

Quantification of HA

The average HA concentration in the hearts of the sham operated animals correlated well to earlier studies [29]. The average HA concentration in the hearts at day 1 was increased threefold [30] in the aorta ligated animals and the concentration was significantly increased compared to sham operated animals ($P=0.037$). The concentration at day 6 was not significantly increased compared to sham operated animals (Table 1).

Microarray gene expression

The microarray data was filtered by controlling FDR, setting statistical significance to $P<0.05$, foldchange to ≥ 1.5 and ≤ 0.67 and minimum signal intensity to ≥ 50. The number of genes that were significantly up- or down-regulated on the chip at day 1, 6 and 42 was

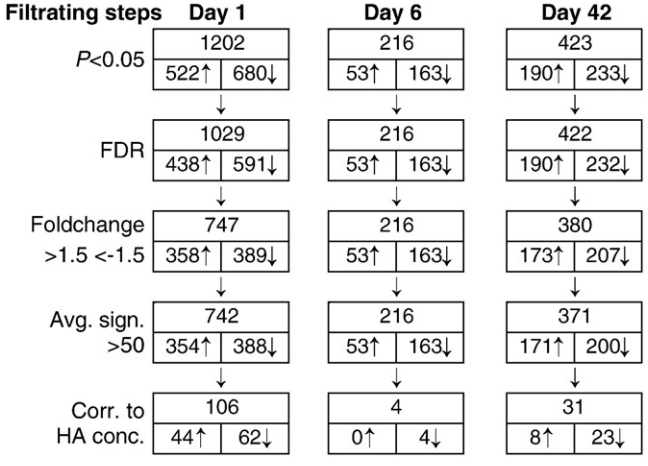

Fig. 1. Number of differentially expressed genes after different filtering steps. Statistical significance was set to $P<0.05$. Multiple testing corrections using False Discovery Rate (FDR) was applied. A significant up-regulation was defined as a foldchange ≥ 1.5 and a significant down-regulation was defined as foldchange ≤ 0.67. A minimum signal intensity value of 50 was utilized. Linear correlation between HA concentration and expression levels of differentially expressed genes was tested. Avg. sign., average signal. Corr., correlates. Conc., concentration.

742, 216 and 371, respectively (Fig. 1). 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. USA) bioinformatics software was used to generate a network between the correlating genes with transcription factors (Fig. 2). The three networks with the best *P*-values and centered on transcription factors expressed in the rat hearts were merged into one network, including other significantly differentially expressed transcription factors at day 1. This revealed an enrichment of genes regulated by 5 transcription factors, tumour protein p53 (*Tp53*), specificity protein 1 (*Sp1*), myelocytomatosis viral oncogene homolog (*Myc*), FBJ murine osteosarcoma viral oncogene homolog (*Fos*) and jun B proto-oncogene (*Junb*). *Fos*, *Junb* and *Myc* were up-regulated at day 1 (Table 2), the first two are likely to form a complex, activator protein-1 (AP-1).

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 (Table 3). 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 (Fig. 3). Four of these genes, *CD44, Biglycan* (*Bgn*), Latent transforming growth factor beta binding protein *(Ltbp2)* and *fibrillin1* (*Fbn1*), correlated significantly with HA concentration levels.

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 and illustrated with component plots in rotated space (Fig. 4a and b). The 106 genes from day 1 formed two tight clusters, positive correlation to HA concentration in the upper right corner and negative correlation in the lower left corner. The same genes in animals from day 6 showed clustering but less tight. At day 42 the

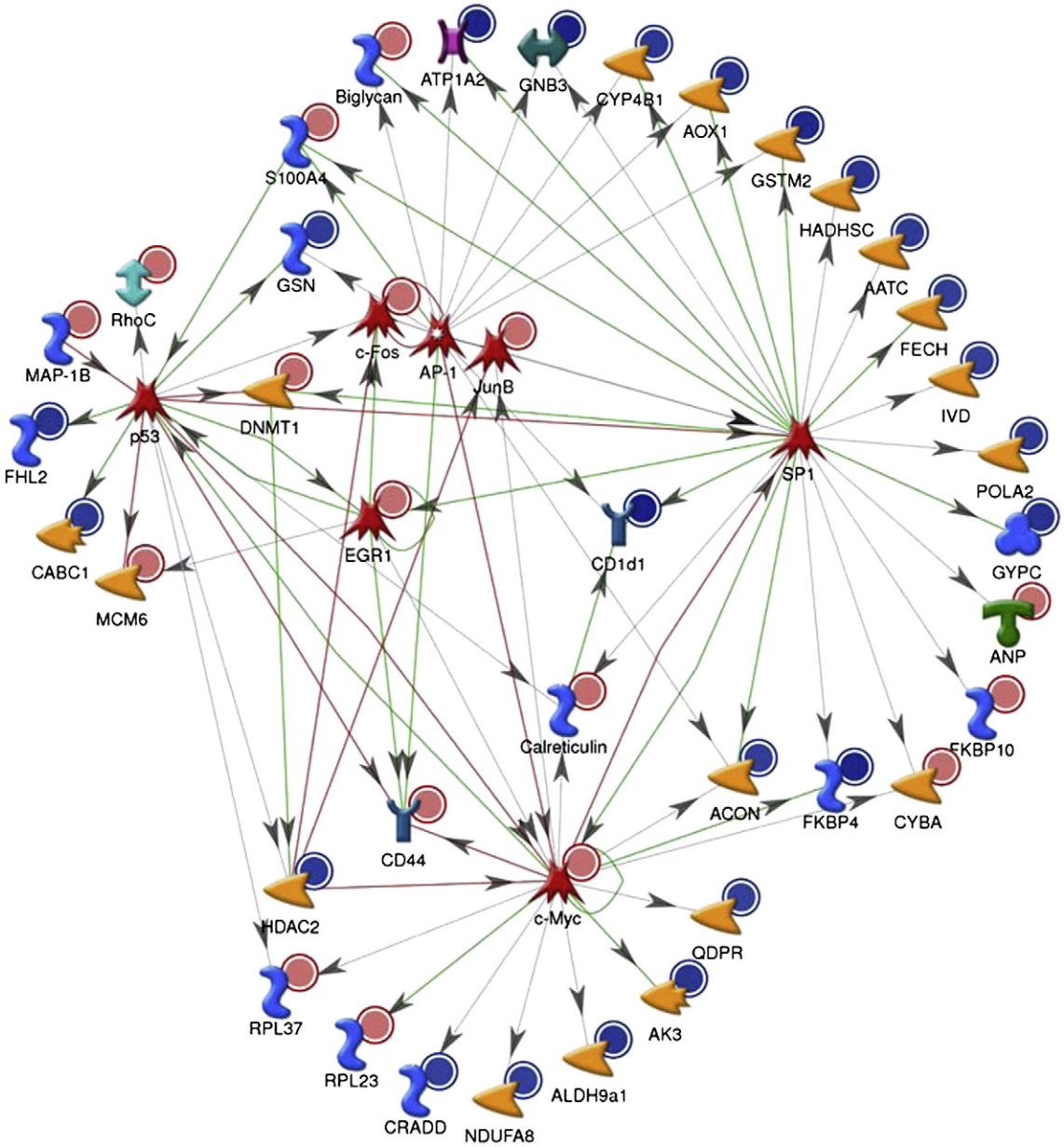

Fig. 2. Network generated by the MetaCore™ (GeneGo Inc. USA) bioinformatics software. The differentially expressed genes correlating with HA concentration are centered on transcription factors. The expression of transcription factors do not correlate to HA concentration but *Egr1*, *Fos*, *Junb* and *Myc* are up-regulated. Blue circles indicate down-regulation and red circles up-regulation. Green arrows indicate activation and red arrows inhibition. The symbols are as follows: enzyme; kinase; protease; protein; generic binding protein; transcription factor; GTPase; G-protein adaptor; receptor; receptor ligand; channel.

Table 3
ECM related gene expression of aorta ligated vs. sham-operated animals.

Gene	1 day	6 days	42 days
Structural ECM genes			
Vcan	↑	=	=
Cspg4	(↑)	(↑)	↑
Cspg5	=	↓	=
Fbn1	↑	↑	=
Fbn2	↑	=	=
Fbln1	↓	=	=
Fbln2	=	↑	(↑)
Fn1	(↑)	↑	=
Selp	↑	=	=
Lamc1	↑	=	=
Lama5	(↑)	↑	=
Col6a3	↑	=	=
Col8a1	↑	(↑)	↑
Col15a1	↓	=	=
Col16a1	↑	(↑)	=
Col18a1	↑	↑	=
Bgn	↑	(↑)	↑
ECM associated genes			
Itgb1	↑	↑	=
Itga5	↑	=	=
CD38	↓	=	↓
CD44*	↑	↑	=
Ccn1/Cyr61	↑	=	=
Ccn2/Ctgf	↑	↑	(↑)
Ccn3/Nov	↓	↑	=
Ccn5/Wisp2	(↑)	(↑)	=
Tgfb1	(↑)	=	=
Tgfb2	↑	↑	↑
Ltbp1	↓	=	(↓)
Ltbp2	↑	↑	↑
Thbs4	(↑)	(↑)	↑
Timp1	↑	(↑)	↑
Timp2	↓	=	(↓)
Mmp23	↑	↑	=
Ctsc	↓	=	(↓)
Ctsk	(↑)	↑	=
Adamts1	↑	=	=
Adamts5	=	=	↓
Ugdh	↑	=	=

Vcan, versican; Cspg, chondroitin sulphate proteoglycan; Fbn, Fibrillin; Fbln, Fibulin; Fn1, Fibronectin1; Selp, P-selectin; Lamc1, Laminin c1; Lama5, Laminin a5; Col, Collagen; Bgn, Biglycan; Itgb1, Integrin beta1; Itga5, Integrin alpha5; Ctgf, Connective tissue growth factor; Cyr, Cysteine rich protein; Nov, Nephroblastoma overexpressed; Wisp, Wnt-induced secreted proteins; Tgfb, Transforming growth factor beta; Ltbp, Latent transforming growth factor beta binding protein; Thbs, Thrombospondin; Timp, Tissue inhibitor of metalloproteinase; Mmp, Matrix metallopeptidase; Ctsc, Cathepsin C; Ctsk, Cathepsin K; Adamts, A disintegrin-like and metallopeptidase with thrombospondin type 1 motif; Ugdh, UDP-glucose dehydrogenase.
↑ and ↓ indicates change in expression in aorta ligated animals compared to sham operated. = indicates no significant change. Arrows in brackets indicate non-significant changes with a *P*-value between 0.05 and 0.3. *CD44 analysed with real-time PCR in earlier study.

correlation was lost. The 31 genes correlating to HA concentration at day 42 showed an inverse development over time. Day 42 a tight correlation was evident but at days 1 and 6 no cluster was seen. In the sham animals no cluster of correlating genes were observed.

Quantitative RT-PCR

The levels of *Vcan*, *Itgb1*, *Ccn2*/*Ctgf*, and *Tgfb2* mRNA were measured with real-time PCR and in all cases the foldchange was greater than with the Illumina microarray beadchip (Supplemental Table 2). The *P*-values showed higher significance in all cases except Ccn2, where the *P*-value was slightly higher but still significant. Differences between the two methods can be explained by the several more steps of handling the RNA before hybridizing it to the chip compared to the single RT-PCR step before the real-time PCR. This is likely to cause more sample variation in the microarray analysis and therefore higher *P*-values compared to using real-time PCR. Correlation between the results was significant expect in the case of Itgb1. Despite some differences between real-time PCR and microarray the results confirm the expression patterns seen on the microarrays.

Discussion

Up-regulation of HA synthases and an increased HA concentration as well as up-regulation of CD44 occurs, have previously been shown in growing hearts of aorta ligated rats [24]. To further investigate the changes in HA synthesis in this animal model, correlation between HA synthesis and gene expression changes was investigated. Correlation between gene expression levels and quantitative measurements of cardiac growth has been proposed to be more informative than traditional comparison between two conditions. By using this method it was possible to associate an increased synthesis of HA to an activation of the fetal gene program in the heart, indicating a possible mechanism for the involvement of HA in the cardiac growth process.

A stepwise bioinformatic approach was used to analyse gene expression data. After filtering the differentially expressed genes by applying FDR, limiting foldchange and signal levels, the expression levels of the remaining genes were ascertained for correlation with HA concentration in the hearts.

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. It is important to point out that using the same strategy, no correlation was found between these genes in the sham operated animals at any time point. This makes it less likely that the observed changes seen in gene expression are caused by stress due to operation trauma.

One explanation to the temporal differences seen in gene correlation could be the expression of different HA synthases. Previous studies in the same rat model, showed both *Has1* and *2* up-regulation at day 1 and 6 [24]. *Has2* returned to control levels at day 42 while *Has1* was still up-regulated. This indicates that HA derived from HAS1 is synthesized for a different purpose in the late phase of the hypertrophic development of the heart and therefore regulated by other factors.

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 [31,32]. These corresponding genes also showed distinct temporal patterns of expression with acute up-regulation of *Ace* at day 1 and a late response of *Ace2* at day 42 (Table 2). 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*.

To test the hypothesis that there is a common signaling pathway, regulated by the same transcription factors, the bioinformatic software MetaCore™ (GeneGo Inc. USA) was used. The differentially expressed genes correlating with HA concentration at day 1 as well as genes with association to HA specifically and the ECM in general, were investigated. This analysis revealed an enrichment of genes regulated by 6 transcription factors, JUNB, FOS, MYC, TP53, SP1 and EGR1, the former two possibly assembling AP-1 complexes [33–36] (Figs. 2 and 3). Four of these transcription factors, *Junb*, *Fos*, *Myc* and *Egr1* were up-regulated at day 1, indicating strong association to the transcriptional changes in the aorta ligated animals. *Junb*, *Fos*, *Myc* and *Egr1* are immediate early genes (IEG), activated in response to stimuli

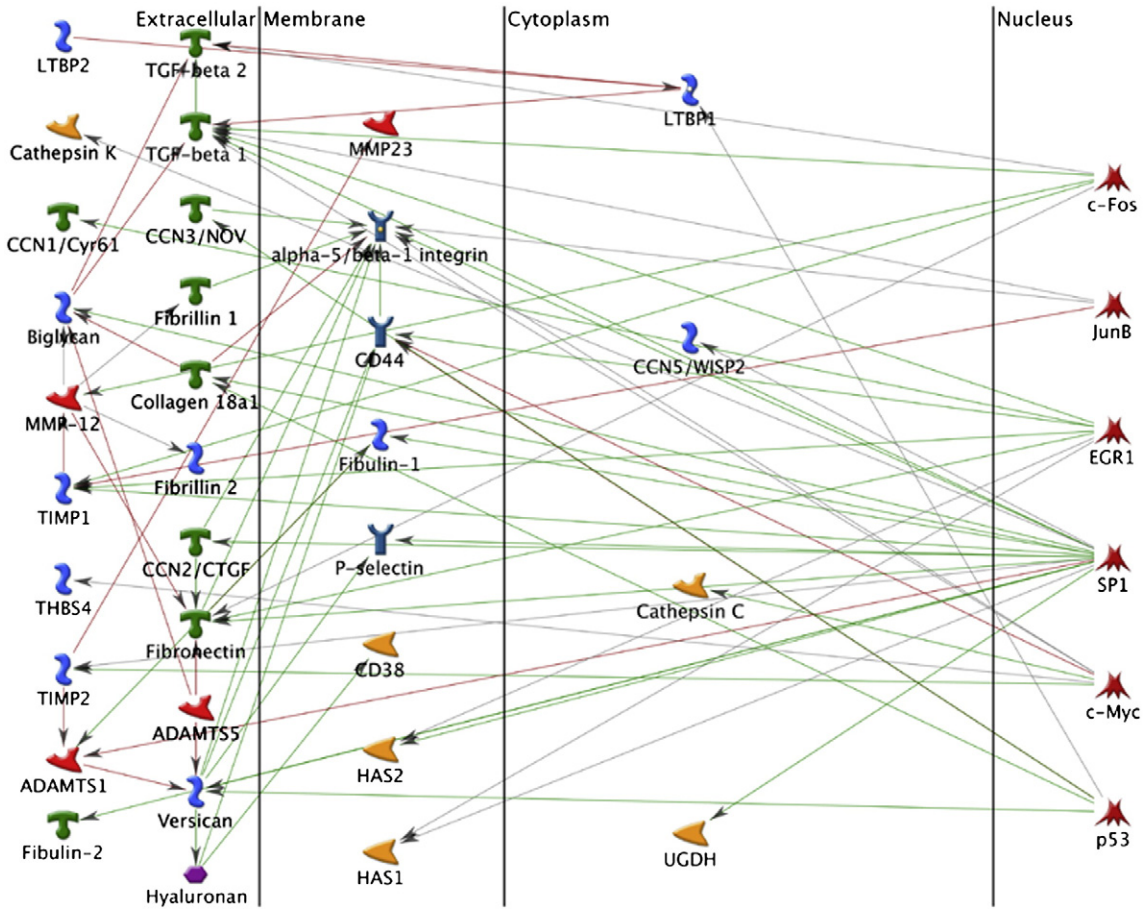

Fig. 3. Network generated by the MetaCore™ (GeneGo Inc. USA) bioinformatics software. Genes coding for proteins such as structural ECM molecules, cell membrane receptors that bind to ECM structures, molecules that interact between cell surface and ECM, growth factors that interact with the ECM and enzymes that regulate the turnover and remodeling of ECM molecules were filtered from the lists of significantly differentially expressed genes at all three time points. Gene and protein network was generated by the MetaCore™ (GeneGo Inc. USA) bioinformatics software. Green arrows indicate activation and red arrows inhibition. The symbols are as follows: enzyme; kinase; protease; protein; generic binding protein; transcription factor; GTPase; G-protein adaptor; receptor; receptor ligand.

mediated via Ang II and/or mechanical factors [37–40]. They are well known as early regulators of cell growth and to precede the expression of cardiac hypertrophy markers, e.g. skeletal α-actin and ANP. The correlation of HA synthesis to the expression of ANP, which is regulated by the ACE and IEGs, opens the possibility that HA also is part of the fetal gene program activated in cardiac hypertrophy.

A hallmark of cardiac hypertrophy is ECM remodeling. The extensive involvement of the ECM in cardiac hypertrophy, including the increased HA synthesis, is illustrated by the structural ECM genes with changed expression shown in Table 3a. HA binds directly to collagen VI α3 and VCAN [41]. The proteoglycan VCAN forms huge aggregates with HA, around which an extensive matrix is organised [42,43]. Both HA and VCAN levels have been correlated to progression of tumor growth [21,22,44,45]. Fibronectin1 (FN1) and FBN1 also bind to VCAN and the expression of *Fbn1* correlated with HA concentration. Expression of *Bgn*, another proteoglycan, also correlated with HA concentration. BGN binds to FN1, collagen VI and TGFB. TGFB is a positive regulator of *Bgn* and TGFB/BGN have been proposed to have a role in pathological remodeling of the heart [46]. Furthermore two ECM receptors, associated to tissue and tumour growth, *CD44* and *integrin α5β1* (*Itga5*/*Itgb1*), were both up-regulated. The main receptor of HA, *CD44*, correlated to HA concentration, indicating a close co-regulation. The up-regulated *Itga5*/*Itgb1*, the isoform being more common in the fetal heart, suggests that the myocardium is expressing receptors capable of recognizing an ECM similar to that of a fetal heart. Integrins transmit mechanical force across the cell membrane, triggering intracellular signaling pathways including the hypertrophic gene program [47,48]. ITGA5/ITGB1 is also a receptor for VCAN, FN and CCN2/CTGF. Recently it was shown that CCN2/CTGF induces cardiac hypertrophy and is up-regulated during myocardial ischemia [49,50]. One of the most potent inducers of *Ccn2*/*Ctgf* is TGFB and the involvement of TGFB in cardiac hypertrophy and angiogenesis is well recognized [51,52]. CCN2/CTGF can also bind directly to TGFB and FN. Interestingly, both TGFB1 and TGFB2 induce synthesis of HA and expression of VCAN [53,54]. TGFB is secreted and stored in the ECM as a complex between a latent TGFB dimer and LTBP, which binds to both FBN and FN. The up-regulation of *Tgfb2* and *Ltbp2* together with *Fbn1* and *Fn1* in this model suggests an ECM serving as a repository for TGFB as a part of the induced hypertrophic remodeling. Both *Ltbp2* and *Fbn1* expression levels correlated to HA concentration. 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.

Assuming that cells need a certain ECM composition for growth, the observed changes in transcription may represent a demand for a remodeling of the ECM. Subsequently when cell receptors respond to the remodeled ECM, the growth can progress. This is in accordance with a recently published review by Richard Hynes who postulated that the ECM, together with growth factors, play “chords” on several receptors simultaneously to transduce intracellular signaling [55]. An example of ECM influence over cells, is the reseeding of decellularized hearts with cardiac-derived and endothelial cells, where the cells respond to the cardiac ECM environment and form a functional contracting heart [3].

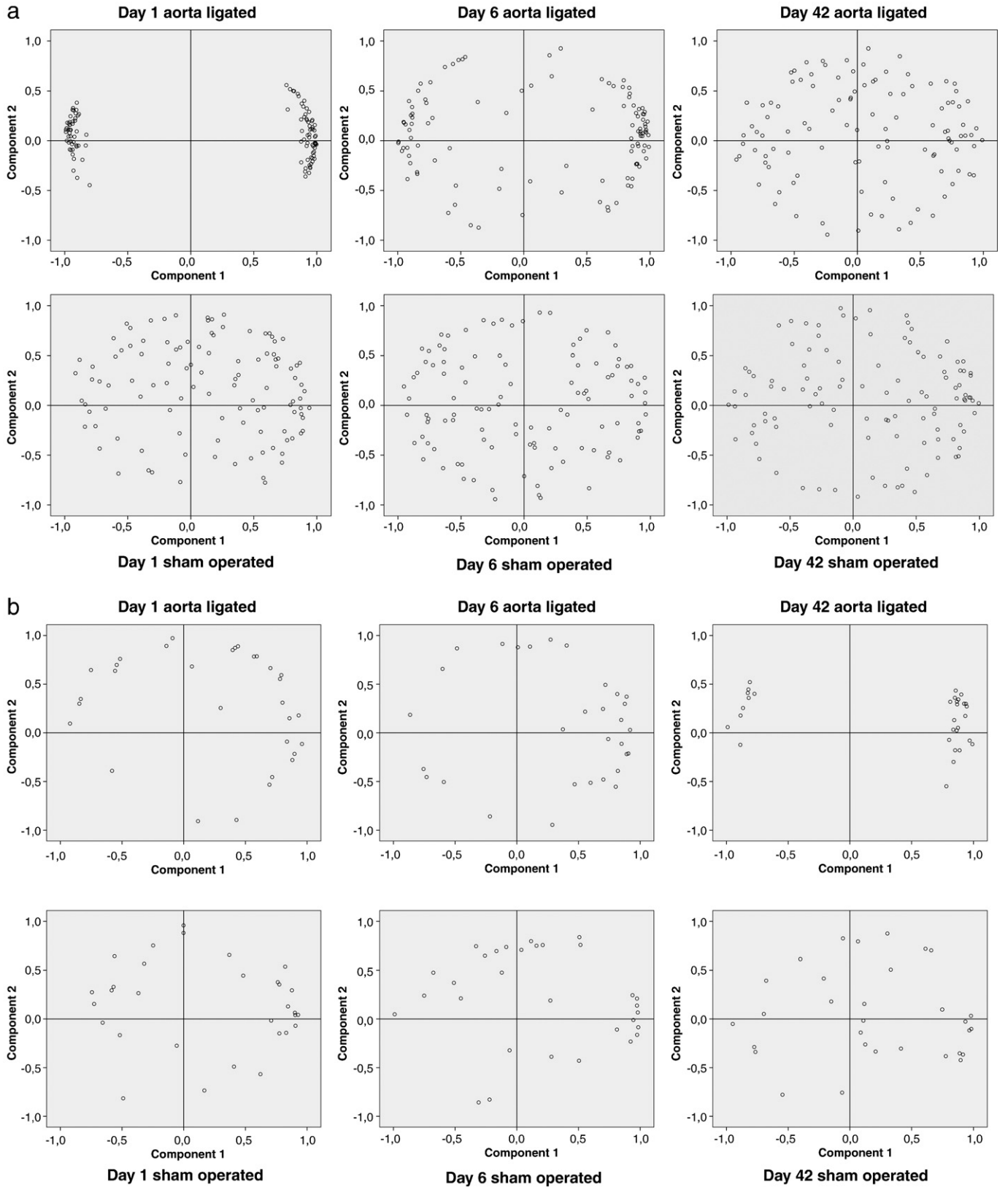

Fig. 4. (a) Factor analysis of genes correlation with HA concentration at day 1. There were 742 differentially expressed genes at day 1 in aorta banded animals, of which mRNA levels of 106 genes correlated with the concentration of HA. Factor analysis was performed to examine the change in correlation over time between these 106 genes, illustrated by component plots. The plots show positive correlation to HA concentration to the left side and negative correlation on the right side of the plot. Clustering of genes in the plot indicate that they have similar expression regulation. The 106 genes formed two tight clusters on day 1. The same genes also showed clustering at day 6 but less tight. At day 42 the correlation between the genes was lost. No correlation was found between these genes in the sham operated animals at any time point. (b) Factor analysis of genes correlation with HA concentration at day 42. There were 371 differentially expressed genes at day 42 in aorta banded animals, of which mRNA levels of 31 genes correlated with the concentration of HA, forming a tight cluster in the component plot (top right). At days 1 and 6 no cluster was seen, nor was any correlation found between these genes in the sham operated animals at any time point.

A limitation of this study is that the cardiac hypertrophy induced by aortic ligation is an extreme procedure and causes acute responses that are hardly found in human diseases like aortic stenosis and hypertrophic cardiomyopathy. However, the controlled timing of the cardiac growth and high influence on gene transcription makes it a suitable model, where relatively few animals are needed for cardiac growth studies.

In conclusion we found a co-variation between HA and specific genes indicating a common regulatory pathway and transcription factor(s). The importance of HA in cardiac remodeling is also reflected by the differential expression of several genes coding both for structural and regulatory molecules interacting within the ECM and the expression of immediate early transcription factors. These transcription factors, MYC, FOS, JUNB and EGR1, are known to regulate several of the genes that correlated to the HA concentration, indicating the involvement of immediate early genes in the synthesis of HA. The up-regulation of the *Ace* gene induces the expression of these transcription factors and also the re-expression of fetal genes (e.g. skeletal α-actin and ANP) in cardiac hypertrophy. The coordinated synthesis of HA and expression of pro-hypertrophic genes, regulated by immediate early genes, suggests that HA is a part of the fetal gene program.

Conflict of interest statement

The authors have no conflicting financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2010.04.003.

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