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ORIGINAL ARTICLE

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Global gene expression in human myocardium—oligonucleotide microarray analysis of regional diversity and transcriptional regulation in heart failure

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Abstract To obtain region- and disease-specific transcription profiles of human myocardial tissue, we explored mRNA expression from all four chambers of eight explanted failing [idiopathic dilated cardiomyopathy (DCM), *n*=5; ischemic cardiomyopathy (ICM), *n*=3], and five non-failing hearts using high-density oligonucleotide arrays (Affymetrix U95Av2). We performed pair-wise comparisons of gene expression in the categories (1) atria versus ventricles, (2) disease-regulated genes in atria and (3) disease-regulated genes in ventricles. In the 51 heart samples examined, 549 genes showed divergent distribution between atria and ventricles (272 genes with higher expression in atria, 277 genes with higher expression in ventricles). Two hundred and eighty-eight

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genes were differentially expressed in failing myocardium compared to non-failing hearts (19 genes regulated in atria and ventricles, 172 regulated in atria only, 97 genes regulated in ventricles only). For disease-regulated genes, down-regulation was 4.5-times more common than up-regulation. Functional classification according to Gene Ontology identified specific biological patterns for differentially expressed genes. Eleven genes were validated by RT-PCR showing a good correlation with the microarray data. Our goal was to determine a gene expression fingerprint of the heart, accounting for region- and disease-specific aspects. Recognizing common gene expression patterns in heart failure will significantly contribute to the understanding of heart failure and may eventually lead to the development of pathway-specific therapies.

Keywords Microarray · Cardiomyopathy · Gene expression · Myocardium · Ion channels · Signal transduction

Abbreviations *CMP:* Cardiomyopathy · *DCM:* Idiopathic dilated cardiomyopathy · *ICM:* Ischemic cardiomyopathy · *LA:* Left atrium · *LV:* Left ventricle · *NF:* Non-failing · *RA:* Right atrium · *RV:* Right ventricle

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Introduction

Transcript profiling has emerged as a powerful tool for delineating complex patterns of tissue- and disease-specific gene expression. In cardiac physiology, this method has been employed to generate hypotheses about the molecular mechanisms underlying observed phenotypes (e.g. myocardial infarction [1], dilated or hypertrophic cardiomyopathy [2, 3, 4, 5, 6, 7], congenital heart disease [8]) and to identify new therapeutic targets. We explored cardiac mRNA expression from left and right atria and ventricles of failing and non-failing hearts using the Affymetrix U95Av2 GeneChip, representing a total of 12,625 probe sets. Our goal was twofold. First, we sought to perform a large-scale screening of the expression profile of atrial and ventricular tissue. Although knowledge of differences in the atrioventricular gene expression profile in human myocardium exists for selected genes, such as ion channels, structural muscle proteins and signal transduction molecules [9, 10, 11, 12, 13, 14, 15], microarray analysis might offer a broader approach to identify sets of new genes exclusively or predominantly expressed in atrium or ventricle. These genes might ultimately become important when regionally specific drug therapy is considered, e.g. targeting exclusively the atrial myocardium in atrial fibrillation. Second, we sought to gain new insights into the pathophysiological mechanism leading to or accompanying cardiomyopathy by comparing gene expression profiles of non-failing and failing atrial and ventricular myocardium. Heart failure is characterized by a remodeling process at the cellular level, which involves many changes in e.g. signaling cascades, cytoskeletal proteins and calcium handling. By examining thousands of genes simultaneously, microarrays offer a unique possibility of unraveling complex regulatory networks. Although a few studies have examined global gene expression changes in heart failure previously [2, 3, 4, 5, 6, 7, 8], novel information is expected from our experiments, as this study presents the first results of myocardial transcript profiling analysis using high-density oligonucleotide arrays accounting for both, region- and disease-specific gene expression patterns in cardiomyopathic hearts.

Materials and methods

Patients

Tissue was prepared from eight hearts of patients with end-stage heart failure (DCM, *n*=5; ICM, *n*=3) and from five non-failing donor hearts that were not transplanted due to palpable coronary calcifications. However, none of the patients in the latter group were known to have a history of heart disease or to take any medication for cardiovascular diseases. Patient characteristics are listed in Table 1. Only one female DCM patient had a positive

Table 1 Patient demographics of non-failing and failing hearts (*ICH* intracerebral hemorrhage, *SAH* subarachnoidal hemorrhage, *EF* left ventricular ejection fraction, as determined by left ventriculography, *A* ACE-inhibitor, *B* beta-blocker, *C* calcium-antagonist, *D* digitalis, *L* loop-diuretic, *S* spironolactone, *T* thiazide-diuretic, *St* statin, *So* sotalol, *Am* amiodarone)

Patient identifier	Diagnosis	EF[%]	Age	Sex	Treatment
Non-failing hearts					
1	ICH		62	female	
2	SAH		60	female	
3	SAH		51	female	
4	ICH		57	male	
5	SAH		54	male	
Failing hearts					
6	DCM	22	37	female	A, B, L
7	DCM	10	54	female	A, C, L, D, Am
8	DCM	25	22	female	A, S, L
9	DCM	25	53	male	A, L, D, So
10	DCM	17	56	male	A, C, D, L, T,
11	ICM	15	60	male	A, B, C, D, L, S, Am
12	ICM	20	60	male	A, B, D, L, S, St
13	ICM	29	64	male	A; D, L

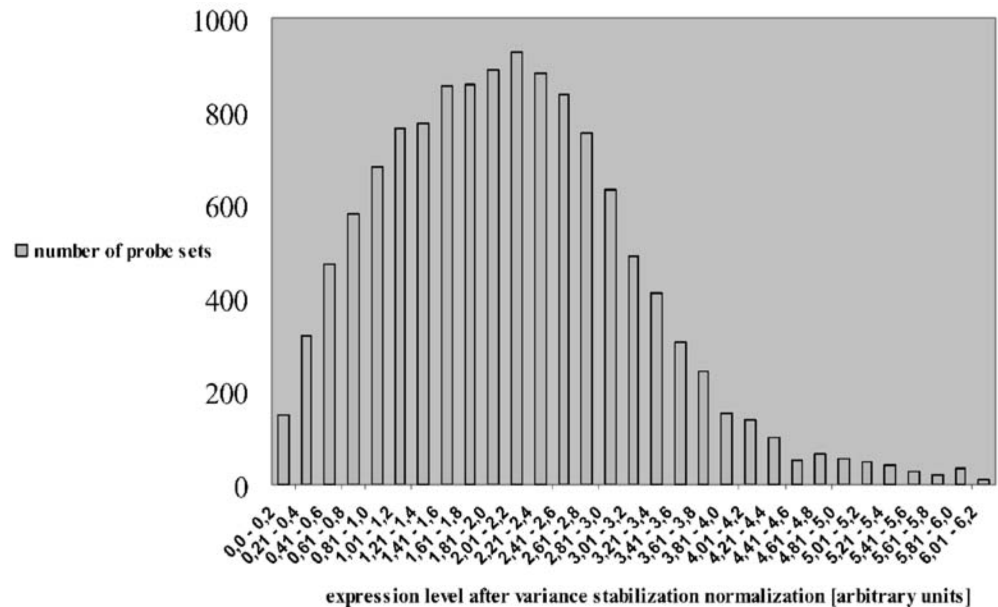

Fig. 1 Distribution of the level of expression of the probe sets represented on the U95Av2 chip after normalization by variance stabilization. The number of probe sets (*y-axis*) is plotted against the expression level (*x-axis*; arbitrary units)

family history suggesting that she was a carrier of Duchenne's disease, as a son had died at the age of 18 (patient identifier no. 7; Table 1). However, screening for disease mutations was not performed for DCM patients. Informed consent was obtained before organ explantation. The Ethical Review Board of the University of Munich approved the protocol.

Tissue selection, mRNA preparation and hybridization to oligonucleotide microarrays

Tissue was collected from all four heart chambers from all but one patient, for whom a left atrial sample could not be obtained due to technical reasons. In total, 51 tissue samples were collected from 13 patients. For ventricular tissue, a 1×1 cm segment of myocardium, approximately midway between the apex and the base of the anterior free wall was used, while in the atria the appendages as well as the sinus node region in the right atrium and the ostia of the pulmonary veins in the left atrium were avoided. Tissue was frozen in liquid nitrogen immediately after explantation.

Total RNA was purified from homogenized deep-frozen tissue samples following the TRIZOL standard protocol as described by the manufacturer (GibcoBRL, Eggenstein, Germany). Total RNA was quantified by spectrophotometry and 150 ng total RNA was used for quality control using the RNA 6,000 Nano LabChip kit and Agilent Bioanalyzer 2,100 (Agilent Technologies, Palo Alto, Calif., USA).

Double-stranded cDNA was synthesized from 10 μg total RNA using the Superscript double stranded cDNA synthesis kit (Invitrogen, Karlsruhe, Germany) with an HPLC-purified oligo(dT) primer containing a T7 RNA polymerase promoter (GENSET, La Jolla, Calif., USA) following the manufacturer's protocol. Biotinylated cRNA probes were synthesized by in vitro transcription using the ENZO BioArray RNA transcript labeling kit (ENZO Diagnostics, Farmingdale, N.Y., USA).

For gene expression profiling, the Human Genome GeneChip U95Av2 System (Affymetrix, Santa Clara, Calif., USA) representing 12,625 probe sets was used. The sequences are derived from sequence clusters in Build 95 of the UniGene database (sequences in UniGene Build 95 are from GenBank 113 and dbEST/10-02-99). Further information about the Gene Chip System can be obtained at http://www.affymetrix.com [16].

Approximately 7% of genes are represented by multiple probe sets on the U95Av2 array; therefore, the total number of genes is estimated to be ~11,500. One GeneChip was used for each human heart sample. Fragmentation of 10 μg biotinylated cRNA, as well as the subsequent steps of hybridization, washing, and staining followed the instructions provided by Affymetrix.

Data extraction and statistical analysis

The 51 U95Av2 GeneChips passed quality control to eliminate scans with abnormal characteristics, i.e. abnormal low or high dynamic range, high perfect match saturation, high pixel noise, grid misalignment problems, and low mean signal to noise ratio. Statistical analysis was done using R 1.7.0 (http://www.r-project.org) [17]. Reading-in and normalization of the probe level data (.cel files) was done using the affy and vsn packages from Bioconductor (http://www.bioconductor.org) [18, 19]. "Mas" was chosen as background correction, probe specific correction and summary method to convert probe-level data to expression values. In order to enable comparison between different microarray experiments, normalization by variance stabilization was performed [20]. A plot of all signal intensities above background after performing the variance stabilization procedure is given in Fig. 1. Data was further analyzed by two-way ANOVA. Differences were considered statistically significant if a nominal significance of $P<0.05$ was achieved after adjusting the cutoff P value for multiple comparisons by the Bonferroni method and subsequently ranked according to their F value. Clustering was performed using an average linkage algorithm contained in the Genesis Software package (http://genome.tugraz.at) [23].

Functional classification was based on the "biological process" of Gene Ontology [24] using the "GO tool" incorporated in the Netaffx software (http://www.affymetrix.com). Biological processes were annotated for 68% of atrial and 73% of ventricular genes. Relationships between two different gene lists (i.e. region- and disease-specific genes) were checked by means of the "MatchMiner" software tool (http://discover.nci.nih.gov/gd/deploy/MatchMiner/html/index.jsp) [25]. Equally, "MatchMiner" was used to eliminate duplicate probe sets within one gene list.

Validation by real-time quantitative reverse transcription PCR

Expression patterns of seven genes were confirmed by real-time PCR using the SYBR-Green method [26]. Four additional genes were examined using TaqMan probes. Special emphasis was laid on genes displaying expression changes at the atrioventricular level

and in diseased hearts (six of 35 genes in this group were randomly chosen). A complete list of the genes examined with RT-PCR, including the design of the primers and probes is listed in the Electronic Supplementary Material (Table S1).

Before PCR amplification, contaminating genomic DNA was removed from the isolated RNA using the DNA free kit from Ambion (Austin, Tex., USA). Total RNA content was then quantified by spectrophotometry. For in vitro reverse transcription, 200 ng total RNA was preincubated at 70°C for 10 min with random hexamer primer. Then, 1 μl RNAse-inhibitor (1.5 U/μl; RNAsin; from Promega, Heidelberg, Germany), 1 μl dNTP-mix (containing each deoxyribonucleotide at a concentration of 10 mM), 2 μl 0.1 M dithiothreitol, 4 μl 5× reaction buffer (250 mM Tris-HCl pH 8.4, 375 mM KCl, 15 mM $MgCl_2$; from Invitrogen, Karlsruhe, Germany) and 1 μl Superscript II RNAse H reverse transcriptase were added to obtain a total volume of 20 μl and subsequently incubated at 42°C for 60 min. Finally, the enzyme was inactivated at 70°C for 15 min.

Gene-specific primers and probes were designed using Primer 3 software (Applied Biosystems, Foster City, Calif., USA) in order to amplify fragments of 70–150 base pairs in length close to the 3′-end of the transcript. Real-time PCR oligonucleotide hybridization primers were purchased from Thermo Hybaid (Ulm, Germany). Gel electrophoresis and the configuration of the dissociation curves were used to assess the specificity of the amplicon. Real-time PCR was performed in triplicate for each sample with 10 μl aliquots of diluted cDNA (1:3) in the Mx4,000 Detection System using the Brilliant SYBR Green QPCR Master Mix (containing SureStart *Taq*, dNTPs with dUTPs, optimized buffer and passive reference dye) according to the recommendations of Stratagene (La Jolla, Calif., USA). For the genes examined with TaqMan probes, a 2× Universal PCR Master-Mix from Perkin Elmer (containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTPs, passive reference dye and optimized buffer including $MgCl_2$), 900 nM primer and 200 nM probe were used. 18S RNA served as an internal control in all RT-PCR experiments.

PCR amplification of cDNA started with a "hot start" activation of the SureStart *Taq* polymerase at 95°C for 10 min, followed by 40 cycles of 15 s denaturation at 95°C, annealing for 60 s at 58°C, and 10 s elongation at 72°C. All experimental results for the samples with a coefficient of variation >10% were retested. To evaluate differences in gene expression, a relative quantification method based on the REST program developed by Pfaffl and colleagues was used [27].

Results

Regional gene expression

Five hundred and forty-nine genes were identified to be differentially expressed between atria and ventricles (Electronic Supplementary Material, Table S2). Two hundred and seventy-two and 277 genes showed higher expression values in atria and ventricles, respectively. According to Gene Ontology, distinct functional classes could be identified for specific biological processes (Fig. 2). Compared to the ventricles and to all genes present on the U95Av2 chip, genes involved in cell proliferation, development, cell communication and signal transduction showed relatively higher expression in the atria, while expression of genes encoding for metabolic processes was more abundant in the ventricles compared to the atria (Fig. 2).

Of note, we were able to reproducibly find different expression values for the two isoforms of the myosin alkali light chain, known to be differentially expressed between atria and ventricles [12], thus confirming the regional specificity of our dataset [*MYL3* and *MYL4* with higher expression in ventricles and atria, respectively (Table S2)].

Figure S1 (Electronic Supplementary Material) shows an unsupervised clustering of the 549 genes with different atrioventricular localizations. As expected, atrial and ventricular samples formed different clusters. However, atrial as well as ventricular non-failing samples clustered separately from DCM/ICM samples, suggesting that for some genes, the atrioventricular gradient of expression is altered by heart failure.

Disease-specific gene expression in the ventricles

Atria and ventricles have different developmental, structural, hemodynamic and physiological properties. Addi-

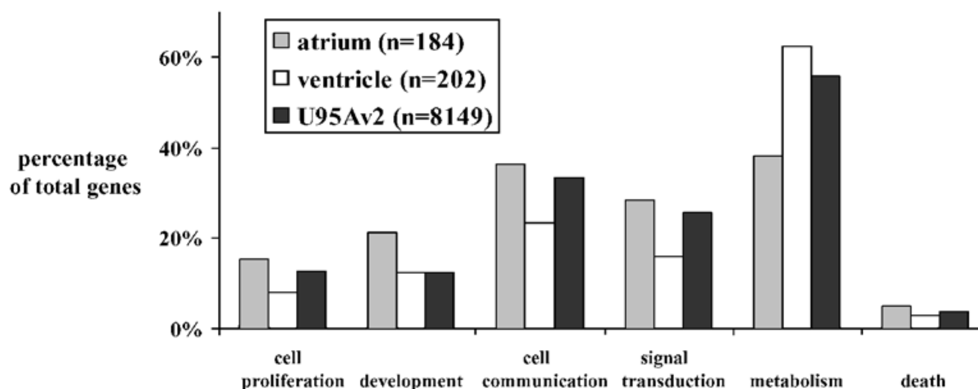

Fig. 2 Functional classification for genes displaying an atrioventricular gradient in mRNA expression. Six major categories of biological processes are shown as percentage of total genes for the respective functional class. For 272 atrial-specific genes, 184 functional annotations were available (*gray columns*), while 202 annotations were available for 277 genes with a predominant ventricular localization (*white columns*). All genes represented on the U95Av2 chip are indicated by the *black column*. Elements of cell proliferation, development, cell communication, signal transduction are more abundant in the atria, while metabolic genes appear more prominently regulated in ventricles

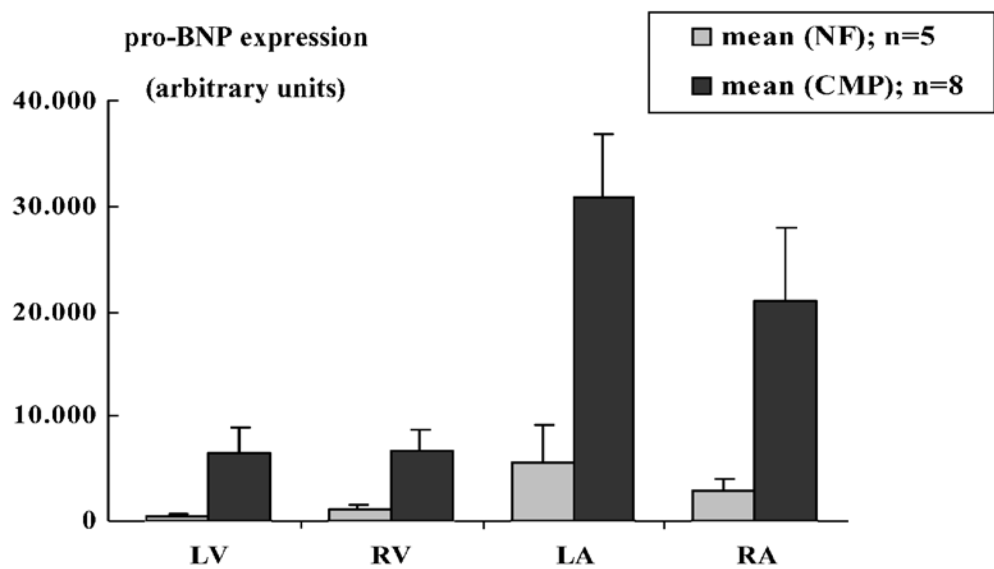

Fig. 3 Region- and disease-specific expression of pro-BNP. Expression values (arbitrary units) from RT-PCR experiments are given for non-failing (*NF*, *gray columns*) and failing myocardium (*CMP*, *black columns*). It is evident that pro-BNP transcripts are more abundant in the atria compared to the ventricles. In heart failure up-regulation of pro-BNP occurs in ventricles and atria

tionally, a differential response to pathologic stress of atria and ventricles is suggested as only 19 disease-regulated genes (less than 10% of all disease-regulated genes identified) were found to be regulated by heart failure concomitantly in ventricles and atria (Electronic Supplementary Material, Tables S3a and S3b). Moreover, pro-BNP, an established marker for heart failure, was differentially expressed between atria and ventricles, highlighting the importance of accounting for atrioventricular differences in gene expression when examining disease-regulated genes (Fig. 3).

In the ventricles, a total of 116 genes was regulated by disease, with 13 and 103 genes found to be up- and down-regulated, respectively (Electronic Supplementary Material, Table S3a). Thirteen genes displaying an atrioventricular expression gradient with higher expression in the ventricle also displayed expression changes in response to heart failure in the ventricle. Interestingly, five genes with a modest but statistically significant atrial predominance were found to be regulated by disease in ventricles only (Electronic Supplementary Material, Table S3a).

Disease-specific gene expression in the atria

A total of 191 genes were regulated by disease in atria (Electronic Supplementary Material, Table S3b). Similar to genes regulated in ventricles, down-regulation was more common than up-regulation (148 versus 43 genes, respectively).

Fourteen genes displaying an atrioventricular expression gradient with higher expression in the atria also displayed atrial expression changes in response to heart failure. Six additional genes with a predominant ventricular localization were found to be regulated by disease only in the atria (Electronic Supplementary Material, Table S3b).

Among the genes up-regulated in failing hearts, many elements of signal transduction pathways and transcription factors were found [transcription factor AP-2 beta; transcription factor AR1; Spi-B transcription factor (Spi-1/PU.1 related); POU domain, class 2, transcription factor 2; hepatic transcription factor 2; variant hepatic nuclear factor (LF-B3)].

Validation by real-time quantitative PCR

The changes in expression levels revealed by the microarray experiments were validated for selected genes using semiquantitative real-time PCR assays. From the genes regulated differentially between atria and ventricles, nine were randomly selected with confirmation of the expression changes for all of these genes (Table 2). For disease-regulated genes, expression changes were confirmed for all but one gene (*AMD1*). As the variance stabilization transformation introduced a non-linear (logarithmic) correction, only qualitative differences were available for microarray data, instead of the traditional "-fold changes" [20].

Discussion

Using an unbiased approach of microarray gene expression analysis, we were able to identify genes expressed in a unique region- and disease-specific pattern. To our knowledge, this is the first study to account for region- and disease-specific human myocardial gene expression using Affymetrix oligonucleotide technology. The reliability of these findings is supported by confirmation of the results by semiquantitative RT-PCR and recognition of known atrial and ventricular genes.

Table 2 Validation of expression changes observed in microarray experiments with RT-PCR. Mean values for -fold changes were compared between non-failing and failing hearts for all 51 tissue samples. Please note that comparison of the fold changes for the microarray data was limited by a non-linear correction introduced by the variance stabilization transformation; therefore, only qualitative differences are given for the microarray results (*A* atria, *V* ventricles, *CMP* cardiomyopathy, *NF* non-failing)

Gene	Group	Microarray	Fold-change	Validation status
		U95Av2	RT-PCR	
ABCC9	atria vs. ventricles	V >A	0.6	Yes
ABCC9	NF vs. CMP (atria)	NF >CMP	1.8	Yes
TIMP3	atria vs. ventricles	A >V	1.2	Yes
TIMP3	NF vs. CMP (ventricles)	NF >CMP	1.4	Yes
TUBA2	atria vs. ventricles	V >A	0.6	Yes
TUBA2	NF vs. CMP (ventricles)	NF >CMP	1.2	Yes
AMD1	atria vs. ventricles	V >A	0.4	Yes
AMD1	NF vs. CMP (atria + ventricles)	CMP >NF	0.7	No
KIAA0592	atria vs. ventricles	A >V	1.5	Yes
KIAA0592	NF vs. CMP (atria)	NF >CMP	1.3	Yes
APOA1	atria vs. ventricles	V >A	0.5	Yes
APOA1	NF vs. CMP (ventricles)	CMP >NF	0.5	Yes
MYL4	atria vs. ventricles	A >V	14.0	Yes
STAT3	NF vs. CMP (atria + ventricles)	NF >CMP	1.4	Yes
SERPINA3	NF vs. CMP (atria + ventricles)	NF >CMP	7.1	Yes
NPPB (pro-BNP)	atria vs. ventricles (NF)	A >V	7.3	Yes
NPPB (pro-BNP)	NF vs. CMP (ventricles)	NF >CMP	8.3	Yes
NPPA (pro-ANP)	atria vs. ventricles (NF)	A >V	138	Yes
NPPA (pro-ANP)	NF vs. CMP (ventricles)	NF >CMP	13.9	Yes

Regional gene expression

We identified a whole set of genes with divergent atrioventricular distribution (Electronic Supplementary Material, Table S2), including several genes known to show differential distribution (*KCNA5* [9], *AGTR1* [10], *TBX5* [11], *MYL3* and *MYL4* [12], *KCNJ3* [13], *KCNK1* and *KCNK3* [14], *ADM* [15]). *MYL3* and *MYL4* are two transcripts of the myosin alkali light chain showing 78% sequence homology at the level of the coding sequence [12]. The Affymetrix probe sets 40062_s_at (*MLY4*, atrial isoform) and 41730_at (*MYL3*, ventricular isoform) can accurately discriminate between both highly similar transcripts in atria and ventricles, thus confirming the regional specificity of our data set.

Differential expression of genes between atria and ventricles may contribute to the functional and structural differences of these chambers. In agreement with a recent study examining atrioventricular differences in gene expression [8], metabolic genes belonged to a functional class with higher expression levels in ventricles than in atria (Fig. 2). For instance, the gene for lipoprotein lipase was found among the genes with a dominant ventricular distribution pattern. As fatty acids released by the catalytic activity of lipoprotein lipase from triacyl-glycerol are used by cardiomyocytes as oxidative fuel, the higher expression levels of *LPL* observed in ventricles might reflect the higher metabolic needs of ventricular myocytes due to greater force generation compared to atrial myocardium [28].

Moreover, 28 ion channels and their associated subunits were found to be differentially expressed between atria and ventricles (Electronic Supplementary Material, Table S2), reflecting their electrophysiological differences. For example, *KCNJ3* encodes for a subunit of the atrial-specific acetylcholine-activated K^+ channel which transmits vagal input to the myocardium and thereby causes a decrease in heart rate and myocardial contractility [13]. Similarly, *KCNA5*, another ion channel gene with atrial predominance, has been identified as the molecular substrate for the ultrarapid delayed rectifier current, found in human atrial myocytes [9]. *KCNK1* and *KCNK3* encode for two-pore domain K^+ channels, giving rise to background currents, also found predominantly in the atria [14].

Understanding the physiological distribution of genes and their regulation by pathophysiological states might significantly contribute to the targeted development of therapeutic interventions. For instance, drug therapy for atrial arrhythmias is often limited by proarrhythmia at the ventricular level. Therefore, determining unique electrical properties of atrial cardiomyocytes might give rise to new therapeutic strategies targeting atrial-specific ion channels like KCNA5 [29].

We were able to identify a whole set of genes with a previously unknown region-specific distribution. The advent of gene therapy, which necessitates a very localized transfection of selected regions of the myocardium (e.g. modulation of AV-conduction in atrial fibrillation [30] or creation of a biological pacemaker [31]), emphasizes the need for identifying region-specific gene expression in order to make use of region-specific promoters.

Disease-specific gene expression

Given the profound structural and functional differences between atria and ventricles, we have attempted a genomic comparison of the transcriptional profile of 51 myocardial samples from cardiomyopathic and non-failing atria and ventricles. To our knowledge, this is the first study to address regional differences in gene expression in patients with dilated and ischemic cardiomyopathy. The importance of accounting for regional differences when examining disease-regulated genes is highlighted by the fact that only 19 out of 288 genes regulated in cardio-

myopathies, i.e. less than 10%, were found to be co-regulated in atria and ventricles. Consequently, we were able to show for the first time a different genomic response of atrial and ventricular genes to heart failure, with nearly twice as many genes regulated in the atria in response to heart failure compared to the ventricles (172 versus 97, respectively). Presently, the reason for this is unclear. Still, it is interesting to note that in a recent microarray study of murine heart failure, Zhao and colleagues found that atrial-predominant genes as a class were more responsive to adrenergic stimuli [32]. In their study, seven of 14 atrial-predominant genes showed >1.8-fold up-regulation in the atria of isoproterenol-treated mice, while only one of the seven genes examined with neutral or ventricular predominance was up-regulated. Using a comparative bioinformatic approach to search for evolutionarily conserved noncoding sequences, they found that atrial-expressed genes have more predicted myocyte enhancer factor-2 (MEF2) binding sites than ventricle-predominant genes. The higher sensitivity of atrial genes to hypertrophic stimuli, like isoproterenol, might in part be conferred through an evolutionary conserved transcription factor binding site for MEF2 which responds to isoproterenol stimulation, leading to the regulation of many atrial-predominant genes [32]. Additionally, a "transcriptional field" defined by a chamber-specific mix of transcription factors, that are enriched or more active in the atrium has been suggested to explain the regulation of genes independent of MEF2 [32]. This seems to be supported by our finding of signal transduction elements being more abundant in atria compared to ventricles (Fig. 2). Although this might serve as a first hypothesis for the differences in the genomic response observed between atria and ventricles, further studies are definitively needed to elucidate region-specific differences in gene expression in response to heart failure.

Despite chamber-specific differences in heart disease, several genes were found to be regulated in both, the atria and the ventricles. For instance, a lower expression of *STAT3* (signal transducer and activators of transcription, member 3) was observed in all failing tissue samples examined. Next to glycoprotein 130, *STAT3* is one of the main downstream effectors of cardiotrophin-1, a member of the interleukin-6 family with potent hypertrophic but also cardioprotective properties. It has been proposed that preserved or even enhanced glycoprotein130/*STAT3* signaling might delay the onset of cardiac failure [33]. In an animal model of doxorubicin-induced cardiomyopathy, cardiac-specific overexpression of *STAT3* provided protection against heart failure and resulted in improved survival [34]. It is therefore tempting to speculate that the lower expression of *STAT3* in failing myocardium attenuates cardioprotective pathways and favors the development of heart failure.

Moreover, down-regulation of the proteinase inhibitor alpha1-antichymotrypsin (*SERPINA3*), previously shown for failing ventricular myocardium only [2], was observed in atria and ventricles (Electronic Supplementary Material, Tables S3a and S3b) and might therefore reflect a general shift towards increased myocardial degradation in heart failure. However, additional chamber-specific differences in tissue degradation and remodeling may be operative, since *SERPINB1*, another member of this proteinase inhibitor family, *TIMP3* (tissue inhibitor of metalloproteinase, member 3), and *ADAM23* (a disintegrin and metalloproteinase domain, member 23) were found to be down-regulated only in the ventricles, while *MMP15* (matrix metalloproteinase, member 15) was up-regulated in the atria only (Electronic Supplementary Material, Tables S3a and S3b). A reduction in the levels of *TIMP3*, corresponding with adverse extracellular matrix remodeling in a cardiomyopathic hamster model and in the failing human heart was observed in a recent study, suggesting a possible role of *TIMP3* in heart disease [35]. Extracellular matrix remodeling with increased fibrosis and myocardial scarring is also thought to contribute to electrical instability of the failing heart by providing a substrate for reentrant ventricular tachycardias. The propensity to ventricular arrhythmias is a characteristic feature of the failing heart, accounting for up to 50% of deaths [36, 37]. Amongst other things, such as abnormal intracellular calcium handling and stretch-induced mechanisms due to altered ventricular loading conditions, substantial evidence from recent years indicates a crucial role of altered expression of ion channels for the electrical instability of failing myocardium [38]. In this respect, *KCNJ8*, part of the mitochondrial ATP-gated potassium channel mediating ischemic preconditioning, was found to undergo transcriptional changes in ventricular myocardium in heart failure (Electronic Supplementary Material, Table S3a). Differences in the behavior of the mitochondrial I_{KATP} in failing hearts may have profound implications for susceptibility to arrhythmias induced by myocardial ischemia.

It is interesting to note that in heart failure more genes were down-regulated than up-regulated in both, the atria and the ventricles. This differs from the majority of studies so far [4, 5, 6, 7], even though down-regulation has also been reported for DCM in one study [3].

To address these divergent findings, several aspects need to be considered: First, the choice of tissue for "non-failing controls" is a critical point. Given the extremely limited availability of human non-failing tissue, we used specimens from donor hearts which could not be transplanted due to palpable coronary calcifications. No left ventricular hypertrophy or scars indicative of prior myocardial infarctions were evident. At the molecular level, pro-BNP was uniformly low in the non-failing LV samples compared to the LV from cardiomyopathic hearts, supporting the fact that the prominent down-regulation observed was not due to inadequate control tissue (Fig. 3).

The second possibility might be that the "down-regulation" observed is related to a change in cellular composition of the myocardium in heart disease, such as replacement of myocytes by non-cellular connective tissue. As laser microdissection has not been performed, we cannot ultimately exclude this possibility. However, the expression of several genes, generally assumed to be re-

stricted to cardiomyocytes, such as cardiac myosin heavy polypeptide 7 (*MYH7*), cardiac myosin light polypeptide 2 (*MYL2*), cardiac troponin T2 (*TNNT2*) and calsequestrin (*CASQ2*) [6, 21] was not significantly different between failing and non-failing tissue in the 51 samples examined (data not shown). Therefore, it is unlikely that the down-regulation observed is due to a quantitative change of cardiomyocytes.

Third, normalization has a profound influence on the statistical analysis [39]. Therefore, great care was taken to ensure that the small number of genes found to be up-regulated was not caused by normalization artifacts: performing a different method of normalization (global scaling) confirmed the same overall result of down-regulation being more common than up-regulation (data not shown).

Fourth, gender-specific differences were corrected for by performing a neighborhood analysis [22] to eliminate genes regulated by sex but not by heart failure, as it has been shown that some heart-failure responsive genes demonstrated significant changes in expression as a function of sex [21]. However, only one gene was identified in our regional- and disease-specific results and excluded from subsequent analysis (*RPSY4*; ribosomal protein S4, Y-linked, probe set 41214_at).

Still, one has to consider that a number of pathophysiologically relevant genes were probably missed by our combined analysis of left and right myocardial samples, as a differential regulation between right and left chambers may occur. It is well known that congestive heart failure can present clinically as predominant left, right or biventricular failure. Thus, depending on the clinical presentation, specific heart chambers might be affected to a different degree. A separate analysis of left and right ventricular samples to account for left or right ventricular involvement might have overcome this limitation, however, would have reduced sample sizes, resulting in inadequate statistical power. Hence, this approach was discarded at the expense of potentially missing some regulated genes.

Limitations of microarray analysis

While the technique of microarray data analysis has reached widespread use, reproducibility of microarray data continues to be a critical issue. Analysis of human myocardial tissue is complicated by biological variability, which is expected to be greater than in well-controlled animal models of heart failure or in cell-based systems. Different etiologies of heart failure, medication, age, sex, individual course of the disease and clinical conditions at the time of transplantation might contribute considerably to the variability of microarray data in different studies. Moreover, comparison between microarray studies is complicated by different study designs (cDNA versus oligonucleotide technology, different statistical methods, pooled tissue samples versus samples from single patients, endomyocardial biopsies versus transmural tissue slices) which might also explain the conflicting results of different studies.

Moreover, transcriptome data may not accurately reflect protein levels, since translational control and post-translation processing occur. Still, identification of candidate genes on the basis of mRNA quantification and correlation of these results with possible disease genes will help to develop the "genes-to-mechanisms" paradigm further. Understanding the region- and disease-specific regulation of myocardial genes might ultimately help to develop new rationales for therapeutic interventions.

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