2010

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RESEARCH PAPER

Channels 4:2, 101-107; March/April 2010; © 2010 Landes Bioscience

Molecular remodeling of ion channels, exchangers and pumps in atrial and ventricular myocytes in ischemic cardiomyopathy

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Key words: ischemic cardiomyopathy, molecular profiling, atrial myocytes, ventricular myocytes, ion channels, phospholemman, Na^+,K^+-ATPase, CLIC

Abbreviations: ICM, ischemic cardiomyopathy; CE&P, channels, exchangers and pumps

Existing molecular knowledge base of cardiovascular diseases is rudimentary because of lack of specific attribution to cell type and function. The aim of this study was to investigate cell-specific molecular remodeling in human atrial and ventricular myocytes associated with ischemic cardiomyopathy. Our strategy combines two technological innovations, laser-capture microdissection of identified cardiac cells in selected anatomical regions of the heart and splice microarray of a narrow catalog of the functionally most important genes regulating ion homeostasis. We focused on expression of a principal family of genes coding for ion channels, exchangers and pumps (CE&P genes) that are involved in electrical, mechanical and signaling functions of the heart and constitute the most utilized drug targets. We found that (1) CE&P genes remodel in a cell-specific manner: ischemic cardiomyopathy affected 63 CE&P genes in ventricular myocytes and 12 essentially different genes in atrial myocytes. (2) Only few of the identified CE&P genes were previously linked to human cardiac disfunctions. (3) The ischemia-affected CE&P genes include nuclear chloride channels, adrenoceptors, cyclic nucleotide-gated channels, auxiliary subunits of Na^+, K^+ and Ca^{2+} channels, and cell-surface CE&Ps. (4) In both atrial and ventricular myocytes ischemic cardiomyopathy reduced expression of *CACNG7* and induced overexpression of *FXYD1*, the gene crucial for Na^+ and K^+ homeostasis. Thus, our cell-specific molecular profiling defined new landmarks for correct molecular modeling of ischemic cardiomyopathy and development of underlying targeted therapies.

Introduction

Cardiovascular disease is the leading cause of human morbidity and mortality. Ischemic cardiomyopathy[1] (ICM) affects approximately one out of 100 people in the United States, most often middle-aged to elderly men. ICM is the most common form of cardiomyopathy leading to dilation of the cardiac chambers and congestive heart failure. In spite of great efforts, molecular markers and targets of ICM are not currently well understood.[2] The tissue-specific pathogenesis pathways in the human heart reportedly include approximately 20 genes[3] coding for structural proteins and those associated with Ca^{2+} homeostasis and energy metabolism. However, some of the implicated genes may be secondary to ICM because intrinsic noise of investigative platforms precludes from detecting low-signal cell-type-specific critical targets at the whole-tissue level. Different cell types remodel in the development and disease following their unique program. Moreover, this remodeling is anatomically heterogeneous. To overcome these problems and characterize ICM-induced molecular remodeling in a cell-type and anatomical region-specific fashion, we combined two technological innovations, laser-capture microdissection of cells of interest and microarray optimized for detection of alternative RNA splicing events in a narrow catalog of genes coding for ion channels, exchangers and pumps (CE&P genes), which comprise the most acclaimed target classes for top-selling prescription drugs. Because CE&P molecules are involved in electrical, signaling and mechanical functions of the heart, their remodeling in ICM may result in decreased cardiac contractile functions.[4] It is likely that pathophysiological conditions of ICM affect regulation of expression of CE&P splice variants.[5] Thus,

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Submitted: 12/15/09; Revised: 12/17/09; Accepted: 12/18/09
Previously published online: www.landesbioscience.com/journals/channels/article/10975

molecular profiling of disease-induced CE&P remodeling may narrow the search for crucial players and enable better understanding of underlying disease mechanisms and effective ICM prevention and treatment. We focused our research on CE&P molecular remodeling in atrial and ventricular cardiomyocytes because of their ultimate role in cardiac contraction targeted by ICM. This research strategy addressed the complexity of cardiovascular system and tissue heterogeneity complicated by differential gene expression and splice variation. Our findings associate ICM with altered expression of *CACNG7* and *FXYD1* in atrial and ventricular myocytes and show that these cells remodel in ICM following their unique programs involving different subsets of CE&P genes.

Results

Laser capture microdissection is an established method for isolation with confidence of histochemically identified cells from heterogeneous cell populations allowing the rational design of comprehensive splice variant profiling of CE&P remodeling in cardiomyocytes. Results of microarray analysis are presented in **Table 3** and summarized in **Table 4**. We found that ICM caused remodeling of 12 genes in atrial myocytes. Increased expression in relation to healthy hearts was found for the *FXYD1* gene coding for protein regulator of Na^+,K^+-ATPase phospholemman (2.3-fold) and intracellular chloride channels *CLIC1* and *CLIC4* (both 1.6-fold). The other nine identified genes were significantly downregulated including *FXYD7*, the inward rectifier $K_{ir}2.4$ channel, the acetylcholine receptor α10 subunit, the Ca^{2+} channel $Ca_V\gamma$ subunits, the AMPA-selective glutamate receptor 3, the $K_V4.3$ K^+ channel involved in initial phase of repolarization and setting the plateau voltage of the action potential, the polyunsaturated fatty acids-activated and mechano-sensitive $K_{2P}4.1$ channel, the Na^+ channel $Na_V\beta1$ subunit. In ventricular myocytes we identified 63 genes affected by ICM. An increase (2.5-fold) was found only for *FXYD1*. The other 62 CE&P genes were downregulated 1.5–3.5 fold. Some of them belong to the same functional groups that are affected by ICM in atrial myocytes (**Table 4**) including *FXYD3*, three A-type K^+ channels, two inward rectifiers, three tandem-pore-domain K^+ channels, three $Ca_V\gamma$ subunits, six acetylcholine receptor subunits, and the AMPA1 glutamate receptor. In addition, we identified three delayed rectifiers, the Ca^{2+}-activated $K_{Ca}3.1$ channel, seven modifiers and β-subunits of K^+ channels, six voltage-gated Ca^{2+} channels, two ENaC and trp channels, cyclic nucleotide-gated channels HCN2 and CNGA3, three connexins, NMDA and glycine receptors, GABA receptor subunits and three adrenoceptors.

To find out whether the splice microarray results reported in **Table 3** are disease-type specific, we supplemented the same splice microarray of ICM ventricular myocytes with one additional sample prepared from left ventricle of a 41-years old *dilated* cardiomyopathy male donor. The top-list ANOVA gene score annotation for combined altered CE&P genes in ventricular myocytes (**Table 3**) was reduced from 63 to just 4 genes (*FXYD1* (G_{fold} = +2.4), *HCN2* (-1.5), *GLRA1* (-1.8) and *GJC1* (-2.3)). This result suggests that dilated and ischemic cardiomyopathy may affect different CE&P subsets and only four indicated genes may be common among the ones affected by these diseases, overexpression of *FXYD1* being the most obvious characteristic feature.

Discussion

Our study is based on the precept that to characterize the ICM-induced molecular remodeling it is essential to investigate it at the level of cells and molecules responsible for altered cardiac function. To achieve this goal, we developed the investigative approach combining laser capture microdissection of identified cardiac cells from tissue biopsies with splice microarray of custom-narrowed sets of CE&P genes that play ultimate role in cardiac contraction targeted by ICM. Our approach for the first time enabled direct comparison of altered gene expression caused by ICM in atrial and ventricular myocytes and identified new essential players.

First of all, we found that atrial and ventricular myocytes remodel following their unique programs where ICM affects different CE&P genes. Although some of these genes were already reported to be expressed in the heart[4,79] (see also refs. in **Tables 3 and 4**), only few of them were previously linked to known cardiac pathologies.[21,39,64,80-84] These include (1) α_{1B}, α_{2C} and β_1-adrenoceptors whose downregulation in cardiomyopathy was linked to pathological remodeling in failing ventricular myocardium.[42,51,85] (2) *KCND2*. In diabetic ventricle, a switch from $K_V4.2$ to $K_V1.4$ may underlie the slower kinetics of the I_{to} K^+ current.[40] (3) *KCND3*. Downregulation of cardiac $K_V4.3$ and minK channels may be associated with arrhythmia and atrial fibrillation.[14,61,62,86] It was reported that congestive heart failure and hypertrophy decrease $K_V4.3$ expression in terminally failing human hearts.[87] In line with this finding, *KCND3* gene transfer abrogates the hypertrophic response to aortic stenosis.[88] (4) *HCN2*. Transfer of this gene was also tested as gene therapy for cardiac arrhythmias in experimental animals with positive results.[89] However, the existing knowledge base remains rudimentary in the absence of attribution to certain cardiac cell types and functions.

We found that ICM does not alter expression of $Ca_V1.2$ and Na_V5 channel isoforms and Na^+,K^+-ATPase but rather affects some of their accessory subunits. The most profound change in both atrial and ventricular myocytes was overexpression of phospholemman. Binding of phospholemman to Na^+,K^+-ATPase induces a decrease in the affinity of α1-β1 and α2-β1 isozymes to external K^+ and approximately 2-fold decrease in the affinity to internal Na^+.[90] Inhibition of Na^+/Ca^{2+} exchanger by phospholemman[91] may add to the disbalance of Na^+, K^+ and Ca^{2+} gradients across the plasma membrane and contribute to hypertrophy of ICM muscle cells due to overexpression of *FXYD1*. Another unexpected finding that may have profound functional consequences is underexpression of $Na_V\beta1$. Although the functional role of this single-membrane-spanning-repeat protein in the heart remains uncertain, it co-localizes with the $Na_V1.5$ pore-forming α1 subunit in the T-tubule system and intercalating discs levels in cardiomyocytes[17] and modulates $Na_V1.5$ channels in the heart by increasing the Na^+ current density.[92] Confirming its crucial role in the heart, *SCN1B* knock-out caused prolongation of QT and RR intervals[93]

and development of cardiomyopathy.[94] Post-transcriptional gene silencing of $Na_V\beta1$ reduced mRNA and protein levels of $Na_V1.5$, KChIP2 mRNA and $K_V4.3$ resulting in markedly decreased Na^+ and I_{to} currents.[95] Thus, underexpression of $Na_V\beta1$ may lead to a suppression of I_{to}, action potential prolongation and increased susceptibility of the heart to ventricular arrhythmia.

Other new potential targets for drug discovery are *CLIC*s.[8,35] Members of the p64 family, *CLIC* proteins localize to the cell nucleus and exhibit both nuclear and plasma-membrane chloride channel activity, but their functions are not well defined. *CLIC2*, which shares sequence similarity with *CLIC1*, modulates cardiac ryanodine receptors and inhibits Ca^{2+} release from the sarcoplasmic reticulum.[96] Thus, ICM-induced remodeling of *CLIC*s in cardiomyocytes may affect membrane potential, intracellular pH and cell volume.

The four identified $Ca_V\gamma$ calcium channel subunits downregulated in ICM show a broad spectrum of modulating activities that may have a role in cardiac myocytes. The γ7, which is homologous to γ5, regulates stability of certain mRNAs[97] and, along with γ2 and γ8, controls trafficking and gating of AMPA receptors.[98,99] It remains to be studied whether remodeling of AMPA receptors in ICM is associated with $Ca_V\gamma$s. Acetylcholine,[72] AMPA and NMDA receptors,[73] also downregulated in ICM, are present in cardiac neuromuscular junctions and intercalating disk, but little is known about their non-neuronal expression and roles in the heart.

Our study is the first step in molecular characterization of ICM with organ- and cell-specific annotation of altered expression of CE&P genes. Our study does not determine whether upregulation or downregulation of the identified CE&P genes are the primary drivers of ICM or reflect pathophysiological response to the disease. However, it provides an objective context within which it would be easier to find therapeutic targets among the elucidated markers of ICM. Future extension of this study may clarify links between CE&P genes expression and drug therapy, duration of disease, age, gender and race as potential factors in ICM and define key aspects of the principal gene network in relation to the development of ICM at the cellular and specific intercellular levels. In conjunction with protein and immunohistochemical analyses this may yield a more robust approach to better understanding mechanisms and pathophysiology of ICM—a critical need in the clinical utilization of this field.

Methods

Human cardiac tissue samples. Healthy (**Table 1**) and ICM hearts (**Table 2**) of anonymous donors were obtained from the Cardiac Transplantation Center at the Washington University in St. Louis, MO. Healthy hearts were excluded from transplantation after exceeding 6 h-limit of allograft ischemic time. Showing no evidence of hemorrhagic stroke, they retained normal structure and function evidenced by bimodal biophotonic imaging and optical mapping of the atrioventricular junction.[100] There was no delay in collecting donor's heart tissue. Decision on suitability for transplantation was made prior to harvest of organs, our team members were notified in advance (at least 2 h before cross clamping) and were present during organ harvest. Heart was cardioplegically arrested, harvested and transferred to us immediately after removal from the chest. Tissue samples (0.5–2 g) were dissected from the area outside of scar burden (if any) of left ventricle and left atrial appendages under cold cardioplegic arrest conditions within approximately 30–40 min after removal of the heart from donor's chest. Tissue was washed in 4°C saline, immersed in Tissue-Tek OCT Compound (Electron Microscopy Sciences, Hatfield, PA), flash frozen in liquid nitrogen and stored at -80°C until use. Although availability of donors suitable for this study was limited, the number of selected human hearts (**Tables 1 and 2**) is in full compliance with the NCBI-recommended MIAME guidelines to microarray[101] and enabled us to analyze all individual donor's samples, grouped by cell type, simultaneously on one microarray slide, thus excluding possible slide-to-slide variations.

Table 1. Characteristics of the healthy heart donors

Patient #	Age	Sex	Cause of death	Cardiac tissue studied
H1	58	F	Intracerebral hemorrhage	LV
H2	70	M	Intracerebral hemorrhage	LV, LAA
H3	40	M	Brain tumor	LV
H4	72	F	Intracerebral hemorrhage	LAA
H5	58	M	Intracerebral hemorrhage	LAA

LV, left ventricle; LAA, left atrium appendage.

Laser-capture microdissection and isolation of mRNA. Serial cryostat sections (7–8 μm thick) were cut from the frozen tissue samples using Minotome Plus microtome cryostat. Before laser-capture microdissection, cardiomyocytes in the sections were quickly (10 s) stained with Eosin Y (Sigma-Aldrich, St. Louis, MO) according to standard procedure. Given the notably large size of cardiac muscle cells relative to other cells in the tissue, this method of identification is sufficient to distinguish stained cardiomyocytes from other cells under microscope. Laser-capture microdissection was performed with PixCell II system (Arcturus, Mountain View, CA) using a 7.5-μm laser spot as described earlier.[5] The excised cardiomyocytes were picked up from the slide

Table 2. Characteristics of the ICM heart patients

Patient #	Age	Sex	Complications and prior treatment	PR (ms)	HR (bpm)	QRS (ms)	EF (%)
D1	54	M	CABG, VT, ICD	184	100	202	ND
D2	46	M	VF, ICD	216	91	106	19
D3	50	M	ICD	128	128	144	25

CABG, coronary artery bypass graft; ICD, implantable cardioverter defibrillator; QRS, a waveform on EKG which represents excitation of the ventricles; EF, ejection fraction; ND, not determined.

Table 3. Top-list ANOVA gene-score annotation for CE&P genes altered by ICM in atrial and ventricular myocytes as compared to healthy subjects

Gene	Genbank #	Variant accession #	p-value* (ICM/H)	G_{fold} change* (ICM/H)
Atrial myocytes				
FXYD1[6,7]	NM_021902	H75358	0.02859	2.3
CLIC1[8]	NM_001288	BU173816	0.04412	1.6
CLIC4[8]	NM_013943	BG436443	0.04047	1.6
KCNJ14[9,10]	NM_013348	na	0.01289	-1.6†
FXYD7	NM_022006	CQ722304	0.01907	-1.6
CHRNA10[11]	NM_020402	CR744383	0.02780	-1.6
CACNG7[12]	NM_031896	H19702	0.01039	-1.6
GRIA3[13]	NM_000828	DA531074	0.01945	-1.7
KCND3[14]	NM_004980	NM_172198	0.00837	-1.7
KCNK4[9,15]	NM_016611	BE900958	0.01063	-1.8
SCN1B[16-18]	NM_001037	DA062026	0.03059	-1.8
CACNG2	NM_006078	na	0.02046	-4.7
Ventricular myocytes				
FXYD1[6,7]	NM_021902	H75358	0.00511	2.5
KCNN4	NM_002250	AL552182	0.00551	-1.5
CLCN2	NM_004366	BM789394	0.00391	-1.5
CACNG7	NM_031896	H19702	0.00069	-1.5
CACNG8	NM_031895	CQ718803	0.00092	-1.5
CACNA1A	NM_000068	BF529475	0.01245	-1.5
KCNH6	NM_173092	CQ730511	0.00758	-1.5
CACNG5	NM_145811	AX101266	0.03112	-1.5
GRIN2A	NM_000833	BG718790	0.01562	-1.5
SCNN1D	NM_002978	AK093372	0.00046	-1.6†
GABRA3	NM_000808	DA801686	0.00407	-1.6
CACNA1E[19,20]	NM_000721	L27745	0.00148	-1.6
KCNH1[9,21,22]	NM_172362	DB021985	0.00092	-1.6
KCNV2[9,23]	NM_133497	CQ724488	0.00935	-1.6
CHRNA6	NM_004198	DA415543	0.00817	-1.6
KCNK6[9,24]	NM_004823	AW883970	0.02392	-1.6
CFTR[25]	NM_000492	BG386556	0.00212	-1.6
GLRA3	NM_006529	BG186165	0.00657	-1.6
TRPC4[26,27]	NM_016179	AF421361	0.02904	-1.6
FXYD3	NM_021910	DR006067	0.01264	-1.6†
GRIN2D	NM_000836	AB209292	0.00249	-1.6†,‡
CACNA1D[9,28,29]	NM_000720	CQ731466	0.00347	-1.7
HCN2[9,30]	NM_001194	BX281160	0.00039	-1.7
KCNS1[9]	NM_002251	DA231979	0.02663	-1.7
KCNK18[31]	NM_181840	AX319992	0.00469	-1.7
VMD2[32]	NM_004183	AA205892	0.03238	-1.7
TRPM1[33]	NM_002420	na	0.02334	-1.7
CHRND[34]	NM_000751	BF306695	0.01945	-1.7†
CLIC3[35]	NM_004669	BE902424	0.01425	-1.7†
CACNA1H[36,37]	NM_021098	DB100395	0.00183	-1.7
CACNA1G[29]	NM_198396	BM451648	0.00045	-1.7
KCND2[38-40]	NM_012281	DA125095	0.01268	-1.7
CNGA3	NM_001298	AK131300	0.02203	-1.7
GABRG2	NM_198904	BI819259	0.02867	-1.8
KCNA4[9,28,39-41]	NM_002233	CQ741592	0.02019	-1.8
ADRA1B[42]	NM_000679	na	0.02796	-1.8†
CHRNA4	NM_000744	BC096291¶	0.00353	-1.8
CACNA1I	NM_021096	AX068892	0.00270	-1.8
GRIA1	NM_000827	DA749477	0.01665	-1.8
KCNJ1[9,43]	NM_153767	NM_000220	0.00360	-1.8
KCNC3[9,28]	NM_004977	BM474777	0.00913	-1.8
KCNQ4[9,44]	NM_004700	AK074957	0.02336	-1.9
KCNMB2[45]	NM_181361	BG185231	0.01402	-1.9
GABRG1	NM_173536	CQ714573	0.02843	-1.9
KCNG1[46]	NM_172318	DA497732	0.02389	-1.9
KCNE1[14,47,48]	NM_000219	AY789480	0.01744	-1.9
GLRA1	NM_000171	BP208426	0.01999	-2.0
CHRNA1	NM_000079	CD013888	0.02807	-2.0†
NOX1[49]	NM_013954	NM_013955	0.00356	-2.0
GRID2	NM_001510	DB052812	0.00299	-2.0
CHRNA9	NM_017581	BF513332¶	0.00625	-2.0
ADRA2C[50-53]	NM_000683	T39448	0.00030	-2.1†
GJB1	NM_000166	BF571436	0.01233	-2.1
GABRR1	NM_002042	CB959800	0.01415	-2.1
SCNN1G[9]	NM_001039	CQ721445	0.00501	-2.2
CNGB3	NM_019098	BX104558	0.00254	-2.5
CHRNB3	NM_000749	DA127065	0.00064	-2.5
GJC1[54]	NM_152219	na	0.00582	-2.5
ADRB1[52,55,56]	NM_000684	na	0.00674	-2.5
KCNG4	NM_172347	CQ728641	0.01073	-2.8
KCTD11	NM_00100291	na	0.00300	-3.1†
KCNA2[9,16,28,57]	NM_004974	BI907383	0.00795	-3.1
GJB6	NM_006783	AY789474	0.00040	-3.5

*A two-way ANOVA model was used to compare ICM vs. healthy (H) cardiac samples as described in splice microarray data analysis. p-values, geometric mean (G_{mean}), and the respective G_{fold} Change values were calculated. †Partial (tiling) human cardiac cDNA clones: AI082799 (*KCNJ14*); DA560890 (*SCNN1D*); AI138642, AI144215 and AI183491 (*FXYD3*); AA455065 (*GRIN2D*); AA716738 and AI367583 (*CHRND*); W69541, AI146716 and W69457 (*CLIC3*); AA010313 (*ADRA1B*); W81677 (*CHRNA1*); AAB31164 (*ADRA2C*); AJ709249 and AJ711264 (*KCTD11*). ‡CK845061.1 cDNA from rat ventricle (*GRIN2D*). ¶This sequence may represent a bonafide polyA tail.

surface and captured on LCM Caps. To confirm the quality of cell isolation, the sectional images taken before and after micro-dissection were thoroughly inspected to exclude contamination with non-muscle cells, and only then the captured samples from serial sections were joined together. High-quality cellular RNA was recovered from the collected cells using PicoPure™ RNA isolation kit (Arcturus) and treated with RNase-free DNase (Qiagen, Valencia, CA). Quality of RNA was tested right before the labeling for microarray by measuring the OD and 28S/18S

Table 4. CE&P genes altered by ICM in atrial and ventricular myocytes

CE&P class protein	Atrial myocytes	Ventricular myocytes
Cl^- channel	*CLIC1*[8], *CLIC4*[8]	*CLIC3*[35], *CLCN2*[*], *CFTR*[25,58,59], *VMD2*[32]
Transient outward (I_{to}) K^+ channel, A-type	K_V4.3 (*KCND3*)[*14]	K_V1.4 (*KCNA4*)[*9,28,39-41], K_V3.3 (*KCNC3*)[9,28], K_V4.2 (*KCND2*)[*38-40]
Delayed rectifier K^+ channel		K_V1.2 (*KCNA2*)[*9,16,28,57], K_V7.4 (*KCNQ4*)[*9,44], K_V10.1 (*KCNH1*)[*9,21,22]
K^+ channel modifiers and β subunits		K_V6.1 (*KCNG1*)[46], K_V6.3 (*KCNG4*), K_V8.2 (*KCNV2*)[*9,23], K_V9.1 (*KCNS1*)[9], minK (*KCNE1*)[*14,47,48,60-62], tetramerization domain containing 11 (*KCTD11*)
Inward rectifier K^+ channel	K_{ir}2.4 (*KCNJ14*)[*9,10]	K_{ir}1.1 (*KCNJ1*)[*9,43], K_V11.2 or HERG2 (*KCNH6*)
Tandem pore domain K^+ channel	K_{2P}4.1 (*KCNK4*)[9,15]	K_{2P}6.1 (*KCNK6*)[*9,24], K_{2P}18.1 (*KCNK18*)[31]
Ca^{2+}-activated K^+ channel		K_{Ca}3.1 (*KCNN4*)[*], β2 subunit of maxiK (*KCNMB2*)[45]
Voltage-gated Ca^{2+} channel α_1 subunit[63,64]		$Ca_V1.3\alpha_{1D}$ (*CACNA1D*)[*9,28,29], $Ca_V2.1\alpha_{1A}$ (*CACNA1A*)[*], $Ca_V2.3\alpha_{1E}$ (*CACNA1E*)[*19,20], $Ca_V3.1\alpha_{1G}$ (*CACNA1G*)[*65,66], $Ca_V3.1\alpha_{1H}$ (*CACNA1H*)[*36,37,67], $Ca_V3.1\alpha_{1I}$ (*CACNA1I*)
Ca^{2+} channel γ subunit	$Ca_V\gamma_2$ (*CACNG2*), $Ca_V\gamma_7$ (*CACNG7*)[12]	$Ca_V\gamma_5$ (*CACNG5*), $Ca_V\gamma_7$ (*CACNG7*)[12], $Ca_V\gamma_8$ (*CACNG8*)
Na^+ channel subunits	$Na_V\beta_1$ (*SCN1B*)[*17,18]	ENaC-γ (*SCNN1G*)[*9], ENaC-δ (*SCNN1D*)
TRP channel		*TRPC4*[26,27,68], melastatin-1 (*TRPM1*)[33]
Cyclic nucleotide-gated channel[69]		*HCN2*[*9,30], *CNGA3*
Connexin		Cx32 (*GJB1*), Cx30 (*GJB6*), Cx31.9 (*GJC1*)[54,70,71]
Acetylcholine receptor[72]	α10 (*CHRNA10*)[11]	α1 (*CHRNA1*), α4 (*CHRNA4*), α6 (*CHRNA6*), α9 (*CHRNA9*), β3 (*CHRNB3*), δ (*CHRND*)[34]
Glycine receptor		α1 (*GLRA1*), α3 (*GLRA3*)
GABA receptor		α3 (*GABRA3*)[*], γ1 (*GABRG1*), γ2 (*GABRG2*), rho (*GABRR1*)
NMDA receptor receptor[73,74]		2A (*GRIN2A*), 2D (*GRIN2D*)
Glutamate receptor receptor[73,74]	AMPA3 (*GRIA3*)[*13]	AMPA1 (*GRIA1*), δ2 (*GRID2*)
Adrenergic receptor[75]		α_{1B} (*ADRA1B*)[42,76-78], α_{2C} (*ADRA2C*)[50,52,53], β_1 (*ADRB1*)[*52,56]
H^+ channel		NADPH oxidase (*NOX1*)[49]
Na^+,K^+-ATPase regulator	*FXYD1*[*7], *FXYD7*	*FXYD1*[*7], *FXYD3*

[*]Mouse Genome Database gene expression data (www.informatics.jax.org) confirming expression of the marked genes in mouse heart.

RNA ratios. On average, the OD ratio at 260/280 nm and 260/230 nm was >1.8 and the ratio of 28S/18S in the RNA samples selected for experiments was ≥1.6. Individual samples of total RNA were optionally amplified without 3'-bias using the TransPlex™ Whole Transcriptome Amplification kit (Rubicon Genomics, Ann Arbor, MI). (The non-bias 3'-amplification was confirmed by the automated RT-PCR with capillary electrophoretic quantification of amplicons executed on a commercial human cardiac total RNA samples (Sigma) with primers to CACNA1C and CACNB2, calcium channel genes characterized by complex alternative splicing). The amplified products were purified using QIAquick PCR purification kit (Qiagen).

cDNA labeling and microarray. The PCR products were fragmented with DNase I, denatured and end-labeled with Cy-3 fluorescent dye. The individual donor's samples, grouped according to cell type, were analyzed simultaneously on Human Ion Channel Splice Arrays 8-pack 4 x 44K slides (ExonHit Therapeutics, Gaithersburg, MD) manufactured on the Ion Channel Splice Array sv1.1 platform representing 287 human CE&P, including 248 alternatively spliced ones in total 1,655 splicing events and supplemented with additional capabilities to recognize connexins and ryanodine receptors.

Microarray data analysis. The statistically significant differential expression patterns between ICM and healthy atrial and ventricular cell samples was analyzed using long- to short-form ratio statistics and expression level statistics to identify genes and splice events affected by ICM. All analyses were performed at ExonHit using Partek Genomics Suite. Principal Component Analysis was carried out to illustrate the level of spread between samples and experimental groups. A two-way ANOVA model was used to perform statistical tests on the probe set level intensities, to compare ICM vs. healthy cells. A Source of Variation plot was generated from this data to find the relative level of difference contributed by each factor. A cutoff level was determined to generate "top hit lists" of probe sets that indicate the most statistically significant differences between the sample groupings. The raw and transformed data sets are submitted to Gene Expression Omnibus, accession # GSE17294 and GSE17530. The method accounted for specific splice variants shown in the results, but did not evaluate the ICM-induced changes in alternative splicing. Low variability between the individual RNA samples in meaningful ($p < 0.01$) probesets, estimated as average of individual probesets standard deviations normalized to the respective mean values (0.08 ± 0.04, mean ± st.dev), suggests that differences in

donor's age and gender in our study have not notably affected the results of microarray. Additional details are presented in Supplementary Methods.

Acknowledgements

The authors thank Kevin Becker and William H. Wood (Gene Expression and Genomics Unit, NIA) for development of splice microarray slides, Heather Jordan and Weiyin Zhou (ExonHit Therapeutics) for help with splice microarray analysis, and Cardiac Transplantation Unit of the Washington University in St. Louis, MO for help with cardiac tissues. This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Institute on Aging (Z01 AG000294-08 to Nikolai M. Soldatov) and by the National Heart, Lung and Blood Institute grant (RO1 HL085369 to Igor R. Efimov).

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/GronichCHAN4-2-Sup.pdf

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