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Isolation and analysis of genes mainly expressed in adult mouse heart using subtractive hybridization cDNA library

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Abstract Subtractive hybridization cDNA library (SHL) is one of the powerful approaches for isolating differentially expressed genes. Using this technique between mouse heart and skeletal muscle (skm) tissues, we aimed to construct a cDNA-library that was specific to heart tissue and to identify the potential candidate genes that might be responsible for the development of cardiac diseases or related pathophysiological conditions. In the first step of the study, we created a cDNA-library between mouse heart and skm tissues. The homologies of the randomly selected 215 clones were analyzed and then classified by function. A total of 146 genes were analyzed for their expression profiles in the heart and skm tissues in published mouse microarray dataset. In the second step, we analyzed the expression patterns of the selected genes by Northern blot and RNA in situ hybridization (RISH). In Northern blot analyses, the expression levels of *Myl3*, *Myl2*, *Mfn2*, *Dcn*, *Pdlim4*, *mt-Co3*, *mt-Co1*, *Atpase6* and *Tsc22d1* genes were higher in heart than skm. For first time with this study, expression patterns of *Pdlim4* and *Tsc22d1* genes in mouse heart and skm were shown by RISH. In the last step, 43 genes in this library were identified to have relationships mostly with cardiac diseases and/or related phenotypes. This is the first study reporting differentially expressed genes in healthy mouse heart using SHL technique. This study confirms our hypothesis that tissue-specific genes are most likely to have a disease association, if they possess mutations.

Keywords Heart · Subtractive hybridization cDNA library · Differential expression · Candidate genes · Cardiac diseases

Introduction

The developmental and pathophysiological conditions in several tissues cause qualitative and quantitative differences in gene expression. There are several polymerase chain reaction (PCR)-based techniques used in comparative gene expression analysis. These techniques involve many different approaches regarding the source of genetic material (DNA or mRNA) and the subtraction techniques (solid substrates, magnetic beads, chromatography, polyacrilamide gel systems etc.) [1–6]. Subtractive hybridization cDNA library (SHL) technique is one of the powerful approaches for identifying and isolating genes that are differentially expressed in defined tissues or cell lines. This method has been previously used to distinguish the genes that are differentially expressed in various tissues [1–3, 7–11]. Recent technological developments in large-scale expression analysis have made it possible to rapidly assess many genes for their transcriptional responses in biological and pathological events [12]. The applications of these techniques have various strengths and weaknesses. As a result, the most appropriate method should be determined according to the existing facilities and the experimental design of the planned study.

In a previous article reviewed by Nanni et al. [13] it was pointed out that many studies using microarray technologies have focused on the transcriptional differences on

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human tissue samples, and also cellular and animal models in pathological events related with several genetic and multifactorial cardiac disorders. However, the differentially expressed genes in normal heart have not been fully identified yet. Heart as a whole, with its cellular heterogeneity, is a complex organ. Therefore, the global transcriptional changes of heart in human are not possible to investigate. However, it becomes possible to identify human homologues of the novel heart-specific genes identified and isolated using gene expression analysis techniques such as SHL on rodent models. In this study, we aimed to construct a cDNA-library that was specific to heart tissue by using SHL technique between heart and skeletal muscle (skm) of mouse (BALB/c). Thus, novel and rare genes that are expressed in the heart could be isolated and determined as remarkable candidates for cardiac diseases or pathophysiological conditions.

Materials and methods

Animals and tissue preparation

The animal application procedures were approved by the Local Ethics Committee for Experimental Animals, Institute for Experimental Medicine, Istanbul University. Tissues were obtained from adult healthy male BALB/c mice with a minimum age of 8-week. Heart and skm were dissected, rinsed briefly twice in ice cold RNase free phosphate buffered saline (PBS) and then immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. For SHL and Northern Blot analysis, total RNA was extracted from heart and skm. For in situ hybridization study, heart and skm were fixed with 4 % paraformaldehyde in PBS for 2 h, washed with PBS for 30 min and equilibrated with 30 % sucrose in PBS at +4 °C for overnight, then embedded in OCT medium onto the cryostat holder.

RNA isolation

Total RNAs were extracted from heart and skm tissues using the ToTALLY RNA™ Total RNA Isolation Kit (Ambion® CA, USA). The quantity and quality of each sample were determined spectrophotometrically by A_{260} and $A_{260/280}$ ratio, and evaluated by visualizing the 28S and 18S ribosomal bands with ethidium bromide following electrophoretic separation on formaldehyde denaturing 1.2 % agarose gel.

Subtractive cDNA hybridization and cloning

For the subtraction of the heart specific transcripts, we used a modified technique reported by Hara et al. [1] which is an efficient method for subtractive cDNA cloning using oligo(dT)30-Latex and PCR. The subtraction was performed by hybridization of cDNA-oligo(dT)30-Latex of skm and the sense strand DNA synthesized from cDNA-oligo(dT)30-Latex of H. In this present study, we designed degenerative primers (EcoRI$(dG)_{14}$N) to be used in an asymmetric PCR for the syntheses of the sense strand DNA. Subsequently, subtractive cDNAs were amplified by twenty rounds of PCR using primers (EcoRI$(dG)_{14}$N and XhoI$(dT)_{19}$N) that were specific for the adapter sequences. All PCR products were inserted into pGEM-T Easy plasmid vector and transformed into the competent cells (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Subtraction efficiency was assessed by comparing of beta-actin (*Actb*, accession number: M12481) and alpha-cardiac myosin heavy chain (*MHC*, accession number: M76601) PCR-products in the subtracted and unsubtracted cDNA aliquots after every four rounds of hybridizations. The forward (5′-GACCCAGATCATGTTTGAGACC-3′) and reverse (5′-TGGTGGTGAAGCTAGCC-3′) *Actb* primers and the forward (5′-GGAAGAGTGAGCGGCGC ATCAAGG-3′) and reverse (5′-CTGCTGGAGAGGTTAT TCCTCG-3′) alpha-cardiac *MHC* primers were used.

Screening of subtracted clones and sequencing

A total of 250 insert positive clones were randomly selected from this cDNA library and analyzed for their insert sizes. Plasmid isolation was performed using the Qiagen Plasmid Isolation Kits (Qiagen, CA, USA). Plasmid DNAs were purified and confirmed by PCR and agarose gel electrophoresis. Successfully cloned cDNA fragments were sequenced with universal primers (Sp6 and T7) by QIAGEN sequencing service (Germany).

Data analysis

The sequences of 224 insert cDNAs were determined, homology searches were performed using the BLAST program, and the functional classifications of the genes were done using Gene database (http://www.ncbi.nlm.nih.gov/gene) of the National Center of Biotechnology Information (GenBank®). The experimental set of Gene Expression Omnibus (GEO) data (http://www.ncbi.nlm.nih.gov/sites/geo) in GenBank was used for the evaluation of the expression levels of the genes in normal mouse heart and skm tissues. The numbers of the used dataset and platform were GDS3052 and GPL1261 (Affymetrix Mouse Genome 430 2.0 Array) respectively. The information of the GEO association IDs and described reporters in this dataset is given in Supplementary Table 1 (Online Resource 1). Furthermore, the relationships of the known genes with

diseases were analyzed using Human Gene Mutation Database (HGMD) of Institute of Medical Genetics in Cardiff University (http://www.hgmd.cf.ac.uk).

Northern blot analysis

Selected cDNAs were analyzed for their tissue expression patterns using Northern blot analysis. cDNA fragments were labeled with PCR using Sp6/T7 primers and Dig-11-dUTP (Roche Applied Sciences, Germany). The detailed information of the labeled probes is shown in Supplementary Table 2 (Online Resource 2). 10 μg of total RNA was separated on Northern gel. Preparation of Northern gels and electrophoresis were performed using the Ambion NorthernMax Kit (Ambion, CA, USA). After the RNAs were blotted overnight on a nitrocellulose filter and ultraviolet cross-linked, hybridization was performed with specific probes overnight at 42–44 °C. After hybridization, filters were washed with 2× and 0.5× SSC containing 0.1 % SDS. Detection was carried with CSPD by using DIG Nucleic Acid Detection Kit (Roche Applied Sciences, Germany). Then, the filters were exposed to X-ray film for autoradiography.

RNA in situ hybridization (RISH)

The plasmid DNAs containing the *Tpm1*, *Myl2*, *Tsc22d1*, or *Pdlim4* cDNA fragments were linearized either with SpeI or SacII restriction enzymes. DIG-labeled sense or antisense riboprobes (Online Resource 2) were obtained by in vitro transcription with DIG RNA Sp6/T7 Labeling Kit (Roche Applied Sciences, Germany). Frozen 10-μm tissue sections on RNase-free TESPA subbed glass slides were incubated in PBS containing 1 μg/ml of proteinase K for 15 min and then treated with hybridization buffer (50 % deionized formamide, 1.3× SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2 % Tween 20, 0.5 % CHAPS, 100 μg/ml heparin; pH 5.0 with citric acid) at 65 °C for 2–4 h. The hybridization reaction was carried out at 65 °C for overnight with 50 μl of hybridization mix (with 50 ng/100 μl riboprobe) on each section. After hybridization, slides were washed and incubated with anti-DIG antibody diluted 1:2,000 in a blocking buffer (Roche Applied Sciences, Germany) for 4 h. Following the washes in PBS containing 0.2 % Tween 20, sections were counterstained with NBT/BCIP (Roche Applied Sciences, Germany) at 4 °C for overnight. The analyses of the tissue sections for each probe were done at least in triple. Slides were observed under a light microscope (Leica DM4000B, Leica Microsystems, Germany) and the images were captured using a digital camera (Leica DC160, Leica Microsystems, Germany).

Results

Classification of SHL clones revealed relation to energy metabolism and myocyte structure

A total of 250 clones were randomly selected from this SHL and analyzed for their insert sizes. The average size of cDNA fragments was found to be 500 base pairs. In the next step, 224 out of 250 cDNA fragments were selected according to their sequencing results because 26 of those had insignificant smaller inserts. The homology screening was performed using the BLAST program in GenBank. We found that nine clones had no homology to any sequence in GenBank. The homology results of the remaining 215 clones are given in Table 1. Furthermore, 142 of the clones showed homologies to 117 different known genes and ESTs and next, they were classified according to functional information given in Gene database of GenBank. Although these genes and ESTs appeared to have relationship with many metabolisms and cellular components, most of them were related to energy metabolism and structural compartments of the myocyte (Table 1). Thirty-six of the clones showed homology to 29 different known genes or ESTs, however, their functions were unknown. Moreover, 37 of the clones showed homology to 28 different undetermined ESTs. Additionally, the occurrence numbers of cDNA fragments in the library are also indicated in Table 1.

Almost 50 % of the known genes were expressed in heart tissue

In the following step, we analyzed the known genes ($n = 146$) for their expression profiles in the heart and skm tissues in the published mouse microarray dataset of GEO database (Online Resource 1). It was not surprising that genes had higher expression levels in heart tissue (47.3 %, $n = 69$). In this dataset, we determined that 40.4 % of genes ($n = 59$) had higher expression levels in skm than heart whereas 7.5 % of genes ($n = 11$) had equal expression levels in both tissues. In addition, there were no indicated expression values for some genes (4.8 %, $n = 7$). However, there were dubious (not detected or up to one different values) expression values in this dataset and these values ($n = 22$) are labeled in Supplementary Table 1 (Online Resource 1).

Northern blot analysis confirmed higher expression in heart tissue for most of the analyzed genes

In the next step, a total of 18 known genes or ESTs selected cDNA fragments were analyzed for their tissue expression patterns using Northern blot analysis. Fifty percent were

Table 1 Homology results and functional classifications of the clones isolated using SHL technique between mouse heart and skm

Functional classification	Gene/EST number ($n = 174$)	Total clone number ($n = 215$)	Gene symbols/ESTs (clone number)
Actin binding	2	2	Tpm3, Tmod2
Calcium ion binding	4	4	Sparcl1, S100a16, Epdr2, Cltb
Catalytic activity	2	2	Ppme1, 9630033F20Rik
Chemokine activity	1	2	Cxcl10 (2)
GTP binding and GTPase activity	5	7	Rheb, Gpsm1, **Mfn2**, Gnb2 (3), Rhoa
Guanyl-nucleotide exchange factor activity	1	1	Arhgef9
Hydrolase activity	6	9	Mmp15, Ctsz, Cdc25a (4), Dedd, Atp6v1f, Atp5b
Ion channel activity	6	6	Fxyd1, Kctd12, Kcnk7, Trpv4, Pllp, Pln
Kinase activity	4	5	Psmc5 (2), Acvrl1, Pim1, Hspb8
Metal ion binding	2	2	Nptx2, Rfwd3
Motor activity	5	9	**Myl3**, **Myl2** (4),Myl6, Plec1, Dynll1 (2)
Nucleic acid binding	7	8	Arid5b, **Tspyl1**, Zmat2, Ptbp1, Safb2, Kars (2), Rpl8
Other enzymatic activity	6	6	Ywhag, Steap3, Lipe, Cdc26, Ndufb7, Za20d1
Oxidoreductase and hydrogen ion transporter activity	12	13	Aldh2, Cox7a2, Ndufb5 (2), Sdha, Ndufa11, **Rpl4**, **mt-Co1**, **mt-Co2**, **mt-Co3**, Nd1, **Atpase6**, Atp5k
Proapoptotic activity	1	1	Ppp2r5b
Protein binding	8	9	Sgca, Dpt, **Pdlim4** (2), Tor1aip2, Neurl1a, Atrnl1, Snx17, **Dcn**
Receptor activity	5	11	Spryd1 (5), Gabarapl1 (2), Adora3 (2), Il10r2, Frs2
Structural constituent of cytoskeleton	5	6	**Actb**, Arpc3, **Tpm1** (2), Myom2, Tpm2
Structural constituent of ribosome	3	3	mt-Rnr2, Rps13, Rpl31
Structural constituent	2	2	Cryab, Lama4
Structural molecule activity	4	4	Acta2, Nf2, Col4a1, Actc1
Sugar binding	2	2	Lgals1, Reg3b
Syntaxin binding	1	1	Stxbp2
Transcription	8	8	Gata6, Jun, Lass5, Ahctf1, Smyd1, **Tsc22d1**, Taf8, Ptrf
Transferase activity	8	11	Nek7, **St3gal6** (3), St3gal2, Ddost, Gys1, Lfng, Pdk4, Csnk1a1 (2)
Translation	2	3	Eef2, Eif1 (2)
Transporter and transporter activity	5	5	Mb, Serinc2, Kpnb1, Apod, Slc25a4
No functional information	29	36	Cnnm3, Ei24, Stom, Deb1, Blcap, Vapb, **Chchd1**, **2810428I15Rik**, 2610528E23Rik, Tmem107, 2900010M23Rik (2), Ociad1, **1810027O10Rik** (4), Oaf (2), Cuedc1, Flcn, Ubxn1, Tmem150a, Fam55c (2), 3425401B19Rik, Emd, Midn, 0610031J06Rik, Spsb2 (2), Psap, Vps33a, Mreg, Erh, Serf2
Unknown	28	37	AC153899 (5), CT025556 (2), AK039595 (2), AC158582 (2), CT025708, AC110537, AC098934, AC122458, AC131800, AK075891, AC108944, AC127690, AK155211, AC118216, AC124401 (2), AC153820, AK080494, AC153993, AY940477, AC113486, AK053465, V00711, AC126441, AC125460, AE014183, AE014174, AL596025, AJ289605 (2)

Selected genes for Nothern Blot analysis are indicated in bold. Numbers in parenthesis indicates the occurrence numbers of the regarding cDNA fragments in the SHL

confirmed to have increased expression in heart, however, 28 % had higher expression in skm, and 22 % had equal expression in both tissues. Northern blot results showed that expression levels of *Myl3*, *Myl2*, *Mfn2*, *Dcn*, *Pdlim4*, *mt-Co3*, *mt-Co1*, *Atpase6* and *Tsc22d1* genes were relatively higher in heart than skm (Fig. 1a, b). We observed different band patterns for *mt-Co3*, *mt-Co1*, *Atpase6* and *Tsc22d1* in Northern blot results (Fig. 1b). With Northern

hybridization analysis, it was not clearly confirmed that there existed two *Tsc22d1* transcripts for heart tissue (approximately 1.8 kb, *Tsc22d1*-isoform 2) and for skm tissue (approximately 4.8 kb, *Tsc22d1*-isoform 1). Furthermore, the expression levels of *mt-Co2*, *Rpl4*, *1810027O10Rik* and *Tspyl1* genes were similar in both tissues (Fig. 1c). However, *Chchd1*, *Tpm1*, *St3gal6*, *2810428I15Rik* and *Actb* showed higher expression in skm than the heart tissue (Fig. 1d). These Northern blot results were compared with the results of mouse microarray dataset except for the mitochondrial genes (*mt-Co3*, *mt-Co1*, *Atpase6* and *mt-Co3*). In the result of this comparison, the expression profiles of ten genes were consistent whereas results of *Pdlim4* and *Rpl4* were relatively different.

RISH analyses revealed the tissue expression patterns of selected genes

In this study, *Pdlim4* and *Tsc22d1* genes were selected for the determination of the heart and skm expression patterns in tissue sections to ensure integrity with the results of previous studies. As shown in Fig. 2, the tissue expression patterns of *Pdlim4*, *Tsc22d1*, *Tpm1* and *Myl2* genes in heart and skm were analyzed with RISH technique (Fig. 2). The Tpm1-antisense probe for both tissues and Myl2-antisense probe for heart were used as positive controls. Sense probes for four genes and Myl2-antisense probe for skm were used as negative controls, produced no distinct signals in all slides. *Pdlim4* antisense transcript was localized in heart and skm and the localization patterns appeared to be uniform. In heart, *Tsc22d1* antisense transcript was mainly localized in atrium in comparison to the ventricular site. Additionally, as shown in Fig. 2, *Tsc22d1* isoform 2 in atrial myocytes and *Tsc22d1* isoform 1 in skm had more dense expressions.

The relationships of known genes with diseases and/or cardiac phenotypes

In the scope of this study, we also analyzed the relationships of known genes detected in this SHL with cardiac diseases and/or phenotypes in human. Overall, mutations of the 43 genes were determined to cause several diseases and/or cardiac phenotypes depending on the data of HGMD and other published references (Table 2). However, there is no published information about the relationships of other genes in this library with cardiac diseases or pathological situations yet.

Discussion

The present study provides novel information on differentially expressed genes in healthy heart tissue and also data for remarkable candidate genes for cardiac diseases. Of the 215 clones analyzed within this study, we determined several novel ESTs using SHL technique between heart and skm of mouse. Not surprisingly, the functional classification showed that 117 different known transcripts from 142 clones were mostly related to energy metabolism and structural compartments of the myocyte. In addition, we showed that the 43 known genes in present cDNA library were mostly associated with cardiac diseases and/or related phenotypes.

The SSH technique combines equalization of abundantly expressed cDNAs and enrichment of (over 1,000-fold) rare sequences [2]. In most of the previous studies, higher success rates (84–94 %) were reported [3, 8, 9]. However, a high false positive rate has been reported, resulting in the identification of <20 % of clones and validated differential expression of approximately 2 % of the candidates [30]. In another study, using SSH, 56 candidates of 4,000 clones (1.4 %) have been identified [7]. In our study, differential expressions of 146 different genes were analyzed in published microarray dataset and 47.3 % of these genes were found to have higher expression levels in the mouse hearts when compared to skm. On the other hand, nine of the randomly selected 18 genes, namely *Myl3*, *Myl2*, *Mfn2*, *Dcn*, *Pdlim4*, *mt-Co3*, *mt-Co1*, *Atpase6* and *Tsc22d1*, were confirmed to have differentiated expression in heart by Northern blot analysis (50 % in random).

The similarity of the gene expression pattern of the other genes in both tissues and analyses of the randomly selected small number of clones may explain the low success rate of our study; however, this rate does not reflect the overall success rate. The limitation of the present study is that the expression levels of all transcripts in this library have not been experimentally confirmed yet. The valid success rate of SHL techniques may be determined with next generation sequencing approaches in the future.

Similar to our Northern blot results, recent studies reported prominent expression of *Pdlim4* and *Tsc22d1*-isoform 2 (short transcript) in heart [31, 32]. The role of PDZ and LIM domain protein 4 (*Pdlim4*; also known as *ril*), a member of PDZ–LIM protein family, in cardiac disease is unknown. However, another member of this family, *PDLIM3* (also known as *ALP*) gene has been previously associated with dilated cardiomyopathy [33, 34]. On the other hand, *Tsc22d1* (TGFβ-stimulated clone 22; also known as *TSC-22*) was reported to significantly enhance CNP (C-type natriuretic peptide) promoter activity in human aortic endothelial cells by the stimulation of transforming growth factor β [31]. *Pdlim4* and *Tsc22d1* mRNAs have been described to have roles as transcriptional regulator or interaction with other proteins in important mechanisms. In this study, we investigated the

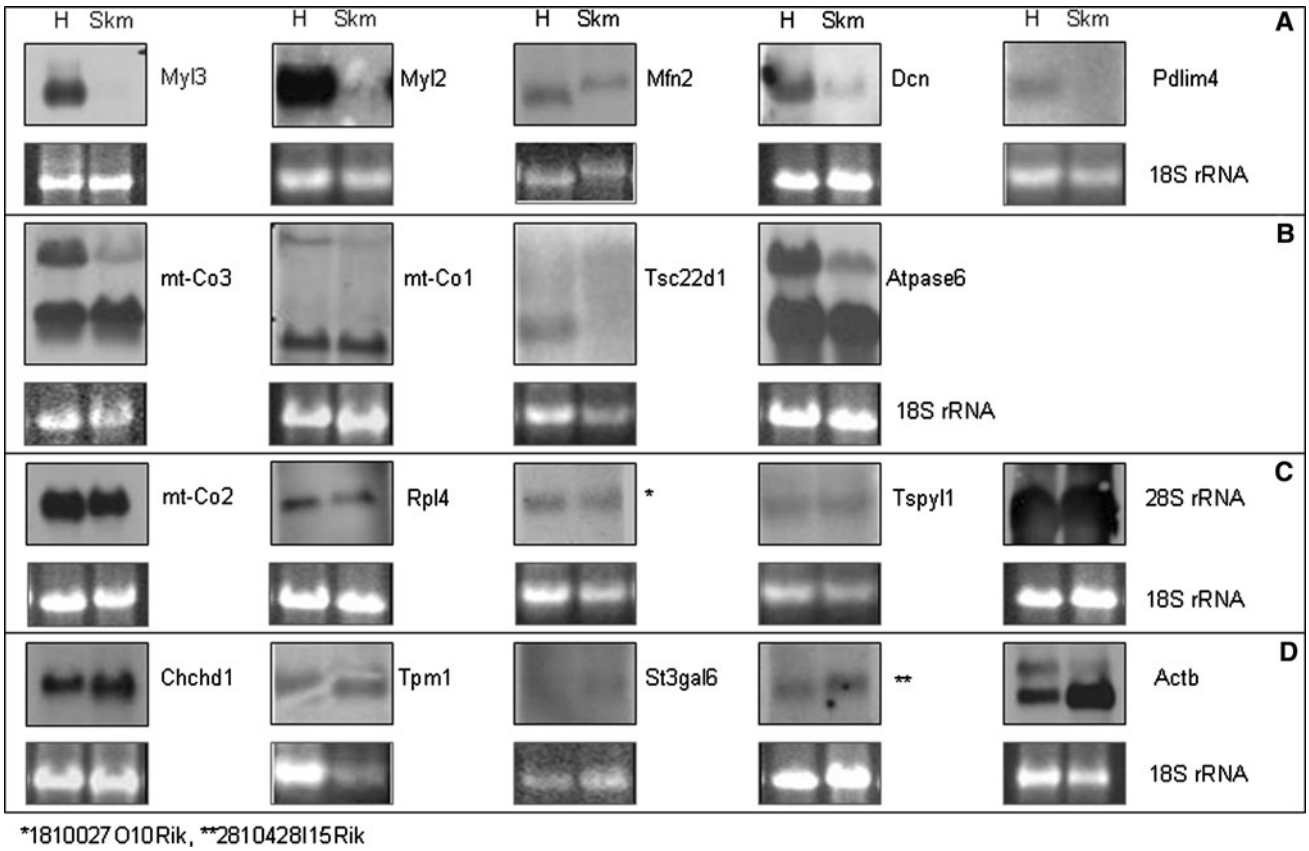

Fig. 1 Northern blot analysis of the selected mRNAs in mouse adult heart and skm. The blots were visualized by autoradiography (*upper panels*). Each lane contained 10 μg of total RNA from the heart (*H*) and skeletal muscle (*skm*). The *lower panels* display the ethidium bromide staining of the 18S rRNA as control. **a** higher expression levels in heart, **b** different band patterns in heart, **c** similar expression levels in both tissue, and **d** higher expression levels in skm

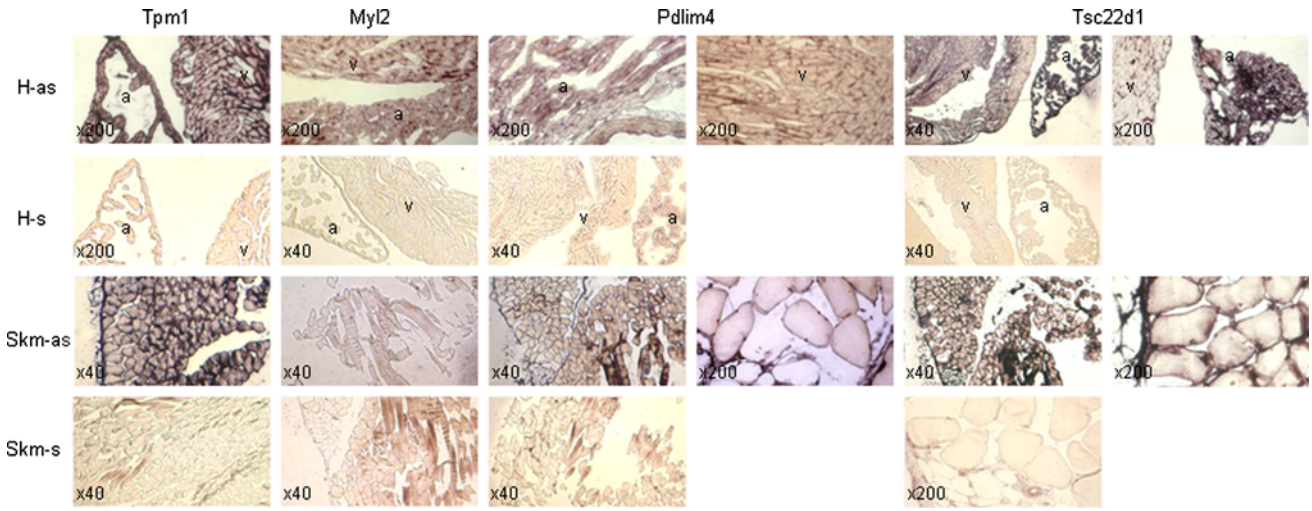

Fig. 2 Sections in situ localization of *tpm1*, *myl2*, *pdlim4* and *tsc22d1* mRNAs in heart and skm. In situ hybridization of *tpm1* (control for both tissues), *myl2* (control for heart), *pdlim4* and *tsc22d1* mRNAs was performed with tissue cryosections (10 μm) of adult BALB/c mice. *H* heart, *skm* skeletal muscle, *a* atrium, *v* ventricle, *as* antisense probe, and *s* sense probe. NBT/BCIP staining was performed for overnight. Magnifications: ×40 and ×200

Table 2 The relationships of the known genes in subtractive cDNA library with several diseases and/or cardiac phenotypes in human

Gene symbols	Disease/phenotype in human	References
TPM3	Congenital fibre-type disproportion myopathy, nemaline myopathy	HGMD
MFN2	Charcot–Marie–Tooth disease 2a	HGMD
	Essential hypertension, association	[14]
ARHGEF9	Hyperekplexia and epilepsy	HGMD
TRPV4	Scapuloperoneal spinal muscular atrophy, spondyloepiphyseal dysplasia, metatrophic dysplasia, Charcot–Marie–Tooth disease 2c, autosomal dominant brachyolmia	HGMD
PLN	Dilated cardiomyopathy type 1P, Hypertrophic cardiomyopathy	HGMD
ACVRL1	Haemorrhagic telangiectasia 2, primary pulmonary hypertension, pulmonary arterial hypertension, pituitary cancer	HGMD
HSPB8	Distal hereditary motor type II neuropathy, Charcot–Marie–Tooth disease 2L	HGMD
MYL3	Hypertrophic cardiomyopathy	HGMD
MYL2	Hypertrophic cardiomyopathy	HGMD
PLEC	Muscular dystrophy with epidermolysis bullosa	HGMD
	Dilated cardiomyopathy	[15]
TSPYL1	Anomalies of testicular function/development	HGMD
ALDH2	Acetaldehyde dehydrogenase deficiency, association	HGMD
SDHA	Leigh syndrome, optic atrophy ataxia myopathy, mitochondrial complex II deficiency	HGMD
	Familial neonatal isolated cardiomyopathy	[16]
MT-CO1	Mitochondrial complex IV deficiency	HGMD
	Mitochondrial cardiomyopathies	[17]
MT-CO2	Mitochondrial complex IV deficiency, MELAS	HGMD
MT-CO3	Leber hereditary optic neuropathy (LHON)	HGMD
MT-ND1	LHON	HGMD
	Maternally inherited type 2 diabetes mellitus and hypertrophic cardiomyopathy, left ventricular noncompaction	[18–21]
MT-ATP6	Leigh syndrome (LS), LHON	HGMD
	Essential hypertension, association	[22]
SGCA	Limb girdle muscular dystrophy type 2D	HGMD
DCN	Congenital stromal dystrophy	HGMD
ADORA3	Ischaemic cardiomyopathy, association with infarct size	[23]
ACTB	Dystonia juvenile-onset, sensory hearing loss and dystonia developmental malformations, neutrophil dysfunction and recurrent infection	HGMD
TPM1	Dilated cardiomyopathy, hypertrophic cardiomyopathy	HGMD
TPM2	Nemaline myopathy type 4, dystal arthrogryposis type 1, muscle weakness and distal limb deformity	HGMD
CRYAB	Cataract, desmin related myopathy, myofibrillar myopathy, dilated cardiomyopathy	HGMD
LAMA4	Dilated cardiomyopathy	HGMD
ACTA2	Thoracic aortic aneurysms and dissections	HGMD
NF2	Neurofibromatosis 2	HGMD
COL4A1	Porencephaly type 1, hereditary angiopathy with nephropathy, aneurysms, and muscle cramps (HANAC), intracerebral haemorrhage, small vessel disease	HGMD
	Myocardial infarction, association	[24]
ACTC1	Hypertrophic cardiomyopathy type 11, dilated cardiomyopathy type 1R, atrial septal defect type 5	HGMD
STXBP2	Haemophagocytic lymphohistiocytosis type 5	HGMD
GATA6	Atrioventricular septum defects, Fallot tetralogy, persistent truncus arteriosus	HGMD
JUN	Cardiac hypertrophy	[25]
PTRF	Generalized lipodystrophy with mild metabolic derangements, myopathy, cardiac arrhythmias, atlantoaxial instability, and pyloric stenosis	[26]
GYS1	Glycogen storage disease, insulin resistance, association	HGMD
LFNG	Spondylocostal dysostosis	HGMD
APOD	Plasma lipid levels, association	[27]
SLC25A4	Progressive external ophthalmoplegia, mitochondrial myopathy and hypertrophic cardiomyopathy	HGMD

Table 2 continued

Gene symbols	Disease/phenotype in human	References
STOM	Dyserythropoiesis	[28]
VAPD	Amyotrophic lateral sclerosis type 8	HGMD
FLCN	Birt–Hogg–Dube syndrome, primary spontaneous pneumothorax, colorectal cancer	HGMD
EMD	Emery–Dreifuss muscular dystrophy type 1, conduction cardiomyopathy	HGMD
	X-linked nonsyndromic sinus node dysfunction and atrial fibrillation	[29]
PSAP	Prosaposin deficiency, metachromatic leukodystrophy, atypical gaucher disease	HGMD

HGMD Human Gene Mutation Database

tissue expression profiles of these genes with RISH technique and showed their expression in all parts of the heart and also skm. Furthermore, we found that *Tsc22d1* was predominantly expressed in atrium. This finding suggests that *Tsc22d1* and the genes regulated by *Tsc22d1* in heart tissue might be potential candidates for atrial fibrillation or other related phenotypes. A previous study reporting 4.0-fold upregulated expression of *Tsc22d1* in porcine atria with pacing-induced fibrillation [35] strongly supports our suggestion.

Genes that are specifically or predominantly expressed in heart are likely to be important for the cardiac function. The subtracted genes can lead to uncover novel physiological functions of the heart in healthy conditions and also in diseases. Furthermore, this data has potential value in the identification of the novel candidate genes for cardiac diseases. Although, SSH was successfully used to identify differentially upregulated myocardial genes in idiopathic dilated cardiomyopathy [36], and ventricular septal defect [8], heart as a whole, with its cellular heterogeneity is a complex organ. Gene expression differences in normal heart tissue comparing with skm have not been studied before. This approach enabled us to compare the transcripts of the two muscle origined tissues. Thus, by using this approach, we identified several differentially expressed candidate genes whose malfunction might be responsible for the development of cardiac diseases or related pathophysiological conditions. These genes are mostly involved in energy metabolism and structural compartments of the cell in heart tissue. In addition, mutations or variants in some of these candidate genes were determined to cause cardiac diseases and/or related phenotypes [HGMB and 14–29]. However, other differentially expressed genes or functionally active genes in heart might be important in the determination of the novel candidate genes for cardiac diseases.

In our study, we utilized mice for creating cDNA library, because to obtain postmortem hearts are rather difficult. Mice and other animal models are being used in several experimental studies to explore mechanisms underlying pathophysiological conditions [13]. After identifying a mouse-specific gene as an effector, one can select the corresponding human counterpart of that particular gene. In fact, the human homologies of 43 genes in our mouse cDNA-library were identified to have relationship mostly with cardiac diseases and/or related phenotypes. For the rest, large-scale gene expression and functional analyses are needed. As a consequence of limitations, differentially gene expression analyses between heart and skm using subtractive hybridization library, Northern Blot and RISH techniques were applied in this study. This only allowed us to analyze a limited number of genes.

Our preliminary findings suggest that unknown genes and ESTs in this SHL are crucial candidates that might have a role in cardiac physiology. Moreover, further studies of heart specific subtracted genes will provide insights into their functional roles in heart, even provide new knowledge for the etiology of the cardiovascular diseases. This is a useful approach for tissue-specific genes related with pathophysiological conditions.

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