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Original article

Identification of potential target genes of cardioprotection against ischemia–reperfusion injury by express sequence tags analysis in rat hearts

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ABSTRACT

Background: Ischemic preconditioning (IPC) is a powerful mechanism for limiting myocardial infarction during or after ischemia–reperfusion (IR) injury. However, effective target genes and proteins for IPC are unknown. We characterized global changes in gene expression in the heart during IR, and identified effective target genes for IPC.
Methods: Hearts were isolated from Sprague-Dawley rats under control, IR, and IPC conditions. We generated expressed-sequence-tags (ESTs) for each group and investigated their functions and the major biological processes in which they are involved using the eukaryotic clusters of orthologous groups (KOG) database and bioinformatics analysis tools.
Results: IR modified the expression of 126 genes. Of these, 62 were upregulated, 64 were downregulated, and 77 were found to be effective target genes for IPC. In KOG analysis, most of the genes whose expression was modified were involved in energy production and conversion and the cytoskeleton. A gene-to-gene interaction map revealed that IR modified the expression of genes in four major functional modules: electron transport chain/oxidative phosphorylation; tricarboxylic acid cycle/glucose metabolism/amino acid metabolism; cellular structure and contraction; and gene transcription, translation, and protein folding. At the individual gene level, the genes encoding mitochondrial cytochrome c oxidase subunits 2 and 3 were downregulated, and those encoding the major cytoskeleton components tropomyosin, myosin light chain, myomesin 2, and myosin regulatory light chain 2, as well as the gene encoding the iron-storage protein ferritin, were upregulated, and thus were identified as potential target genes. Real time PCR evaluated expression patterns of three mitochondrial IPC effective genes. Two-dimensional electrophoresis proteomic analyses revealed altered expression of 14 target proteins. The expression patterns of six proteins matched the corresponding EST expression patterns.
Conclusion: The global profiling of cardiac ischemia-related genes provides the possible mechanisms of IR and IPC and ways of treating IR injury.

Introduction

Cardiac ischemia and ischemia–reperfusion (IR) injury are major contributors to morbidity and mortality worldwide [1]. Clinically, discovery of early diagnosis markers of ischemic heart disease and myocardial infarction, and pharmacological post-ischemic treatments therapies (e.g. statins or ATP treatment) were widely investigated for reducing infarction size and mortality after IR injury [2–5]. Impairment of calcium balance and lipid homeostasis, accelerated generation of reactive oxygen species, loss of

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high-energy phosphates, and the accumulation of harmful catabolites are believed to play crucial roles in the pathogenesis of IR injury [6,7].

Ischemic preconditioning (IPC) is defined as a short period of myocardial IR that reduces subsequent lethal IR injury [8]; it is the most powerful mechanism known for limiting cardiac infarction [9]. During the past 20 years, much information about the signaling pathways involved in preconditioning has been discovered and has increased our understanding of the underlying mechanism [8]. In spite of the identification of these signaling pathways, the precise mechanism by which these pathways reduce cell death is only beginning to be understood [10]. Recently, genetic profiling of heart and heart disease models was performed to develop strategies for enhancing beneficial gene expression changes, and inhibiting potentially harmful ones, during cardiac development [11], atrial fibrillation [12], oxidative stress-induced cardiac myocyte apoptosis [13], and ischemic preconditions [14]. Furthermore, large-scale, specific, disease-targeted gene expression profiling analyses have provided a large amount of information as like a whole cDNA library with expressed sequence tags (ESTs) in heart and its specific state [15,16]. For example, Hwang et al. [15] suggested a new strategy for developing cardiovascular medicine based on the generation of 43,285 ESTs from human heart cDNA libraries related to hypertrophy genes, while Megy et al. [16] identified 35 heart-specific genes from 4303 ESTs through the analysis of human EST start libraries. These studies demonstrated rapid and effective EST-based approaches for identifying genes expressed in the cardiovascular system. Although the rat has been widely used to study cardiovascular physiology [17,18], a comprehensive set of rat cDNAs and ESTs for analyzing genes related to ischemic heart disease is not yet available.

In the present study, we integrated EST and proteomic approaches to analyze the expression of genes and proteins in rat hearts exposed to control conditions (Con), ischemia preconditioning (IPC), and ischemia/reperfusion (IR) to better understand how IPC treatment regulates gene and protein expression in ways that may protect the heart from serious IR injury.

Methods

Animal cardiac ischemic model

Eight-week-old Sprague-Dawley rats were anesthetized with sodium pentobarbital (1 mg/kg) and heparin (300 IU/mL/kg). Twelve isolated rat hearts were divided into three groups (Con, IR, and IPC groups; four per group), and perfused using a modified Langendorff model with normal Tyrode's (NT) solution and ischemic solution as previously described (see Supplementary Materials for full details of the procedure) [19]. After treatment, two hearts per group were subjected to EST analysis, and the other two were used in proteomic analysis.

cDNA library construction

Rat hearts from the three different groups were homogenized in liquid nitrogen. Total RNA was isolated with TRIzol. Then mRNA was purified from the total RNA using an mRNA purification kit. A cDNA library was generated using a ZAP Express cDNA Synthesis Kit (pBK-CMV vector; Stratagene, La Jolla, CA, USA). Briefly, cDNA was ligated into a TriplEx2 vector and packaged using the Gigapack Gold I packaging system (Stratagene). The library contained 1.6×10^5 plaque-forming units (pfu). A total of 40 random plaques were chosen from each library for polymerase chain reaction (PCR) amplification and digested with the restriction enzyme *Sfi*I. The average insert size was determined through comparison with a standard 100-bp ladder after electrophoresis at 180 V on a 0.8% agarose gel.

DNA sequencing

The lambda TripleEx2 vector (linear form) was converted into the pTriplEx2 vector (circular form) with *Escherichia coli*, which utilizes the Cre-loxP recombination system. Plasmid DNA isolated by the standard alkaline lysis method was amplified by the dye-terminator cycling method using a BigDye® Terminator v3.1 Cycle Sequencing Kit and 5′ universal primers in the vector (Stratagene), and was sequenced using an ABI3730 XL capillary sequencer.

Sequence analysis

The chromatogram files obtained from the sequencer were initially submitted to Phred [20,21] for base calling and quality assignment. The trace files were trimmed with trim-alt 0.05 and sequences shorter than 100 bp were removed. In addition, vector trimming was conducted with cross_match software (http://www.phrap.org), and poly-A sequences were removed using the trimest function in the Emboss package [22]. The prepared MultiFASTA format data were clustered and assembled using TIGR gene indices clustering tools [23]. Finally, the contigs and singletons were analyzed using NCBI local BLAST [24]. Sequences were searched against the NCBI nr database. Matches with *E*-values of 1e−10 for BLASTx were considered putative hits.

Functional annotation and associated network analysis

Identified ESTs were primarily categorized and annotated using the eukaryotic clusters of orthologous groups (KOG) algorithm for complete genomes (http://www.ncbi.nlm.nih.gov/COG/grace/shokog.cgi). Gene interactions and functional associations between identified genes were analyzed using the online search tool for the retrieval of interacting genes/proteins (STRING) 8.0 database (http://string.embl.de) and were rearranged using a network analysis and visualization program (cytoscape 2.6.2) [25]. Protein subcellular localization and functional annotation were analyzed using the proteome analyst specialized subcellular localization server v.2.5 [26] and the protein analysis through evolutionary relationships (PANTHER) classification system [27].

Two-dimensional electrophoresis-based proteomic analysis and protein identification

Differences in protein expression between rat hearts exposed to Con conditions, IPC, and IR were investigated by two-dimensional electrophoresis (2DE). Proteins of interest were identified by matrix-associated laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis. These methods have been described previously [19] (see Supplementary Materials for full details of the methods).

Real time PCR

For verifying our EST results, we randomly selected 3 differentially expressed genes for analysis of quantitative expression changes using real time PCR. For real time PCR of cytochrome b (MT-CYB, AC_000022; forward 5′-cgaaaatctcacccctatt-3′ and reverse 5′- agtttacgtctcggcagatg-3′), NADH dehydrogenase subunit 4 (MT-ND4L; AC_000022.2; forward 5′-aggcccttccatattttgac-3′ and reverse 5′-tccctttcttcaaacatcca-3′), and cytochrome c oxidase subunit III (MT-CO3, AC_000022.2; forward 5′-acaggagccctatcagctct-3′ and reverse 5′-aaaatccggcaaagaagaat-3′) mRNA, total RNA was

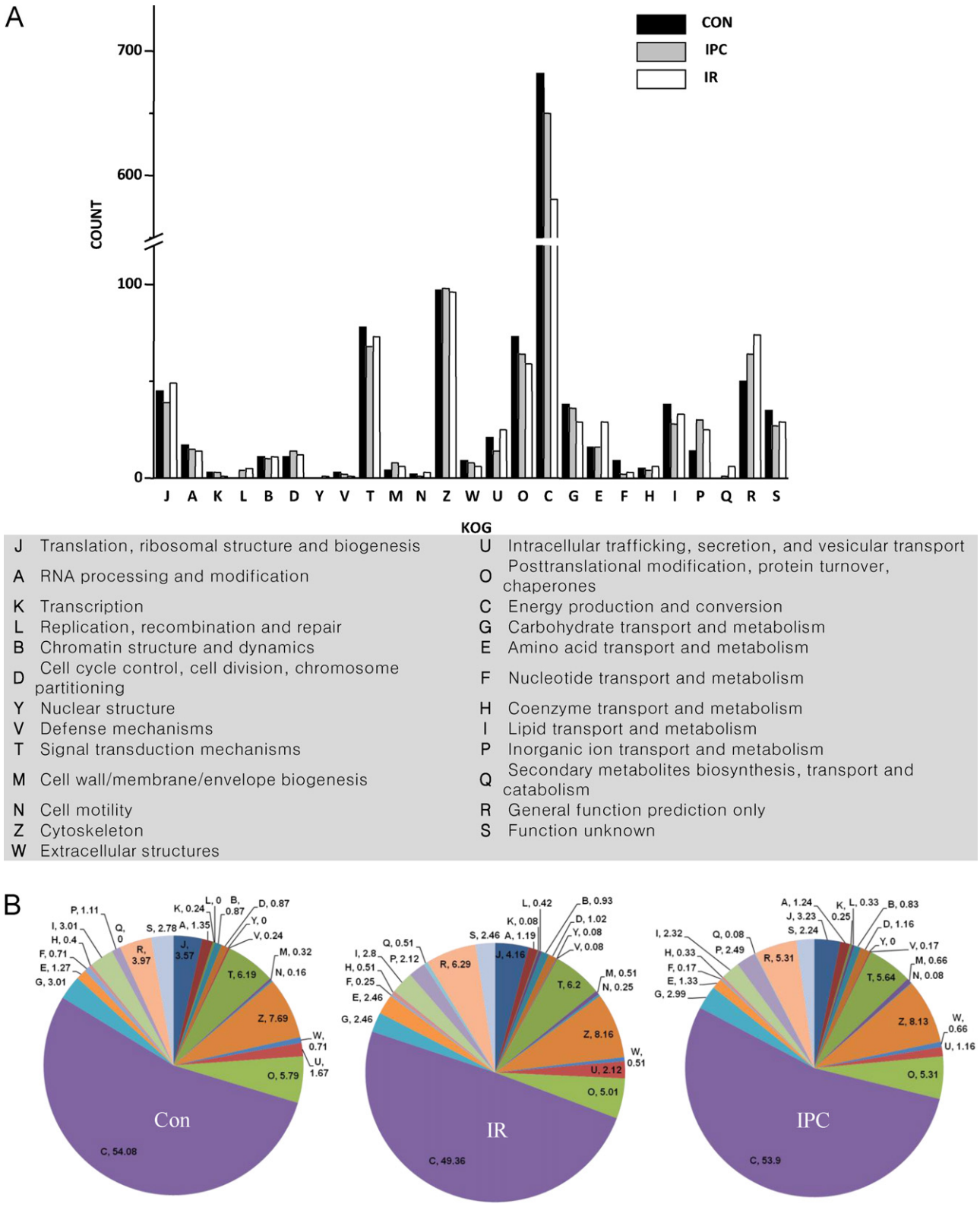

Fig. 1. Results of eukaryotic cluster of orthologous groups (KOG) analysis of rat heart ischemia EST sequences. (A) KOG results for intergroup comparisons between hearts exposed to control conditions (Con), ischemic preconditioning (IPC), and ischemia–reperfusion (IR). (B) Distribution of KOG changes for each group.

Table 1
List of cardiac IR downregulated genes and effectiveness of IPC treatment.

Gene ID	Con	IPC	IR	Annotation	IPC effect
Energy production and conversion					
AP_004898.1	103.0	101.8	68.6	Cytochrome c oxidase subunit III	E
YP_665632.1	42.0	54.0	26.0	Cytochrome c oxidase subunit II	E
ABG11762.1	24.0	18.7	15.6	Cytochrome b	E
ABG11769.1	25.0	13.5	12.5	NADH dehydrogenase subunit 4	E
AAH59124.1	7.0	2.1	4.2	Malate dehydrogenase 1, NAD (soluble)	NE
CAD21559.1	4.0	5.2	2.1	NADH dehydrogenase subunit 1	E
NP_955417.1	5.0	3.1	3.1	Dihydrolipoamide dehydrogenase	NE
AP_004900.1	5.0	4.2	2.1	NADH dehydrogenase subunit 4L	E
NP_112413.2	5.0	5.2	1.0	Malate dehydrogenase, mitochondrial	E
NP_031834.1	6.0	2.1	2.1	Cytochrome c, somatic	NE
NP_036727.1	4.0	3.1	1.0	L-Lactate dehydrogenase B	E
P10817	4.0	2.1	1.0	Cytochrome c oxidase subunit 6A2, mitochondrial	E
NP_001007621.1	3.0	2.1	1.0	Pyruvate dehydrogenase (lipoamide) beta	E
NP_058721.1	2.0	2.1	1.0	L-Lactate dehydrogenase A	E
AAS75815.1	4.0	1.0	0.0	Mitochondrial aldehyde dehydrogenase precursor	E
ABG11820.1	3.0	1.0	0.0	NADH dehydrogenase subunit 3	E
XP_001071017.1	1.0	2.1	0.0	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit f, isoform 2	NE
NP_075581.1	2.0	0.0	1.0	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	NE
AAH19156.1	2.0	0.0	1.0	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 26	NE
NP_001094009	2.0	1.0	0.0	Succinate dehydrogenase Ip subunit	E
ABG11772.1	4.0	0.0	1.0	NADH dehydrogenase subunit 6	NE
NP_001004250.1	2.0	2.1	0.0	Ubiquinol–cytochrome c reductase core protein I	E
NP_001011907.1	9.0	1.0	4.2	NADH dehydrogenase (ubiquinone) Fe-S protein 2	NE
Cytoskeleton					
P68035	21.0	15.5	7.2	Actin, alpha cardiac muscle 1	E
NP_058935.2	5.0	5.2	2.1	Myosin, heavy polypeptide 6, cardiac muscle, alpha	E
NP_001099960	2.0	2.1	1.0	Myosin-binding protein C, cardiac-type (Cardiac MyBP-C) (C-protein, cardiac muscle isoform)	E
NP_001004080.1	3.0	0.0	1.0	Gelsolin	NE
NP_001102452	3.0	1.0	0.0	Cofilin-2 (Cofilin, muscle isoform)	E
NP_034989.1	3.0	1.0	0.0	Myosin, light polypeptide 3	E
NP_006073.2	1.0	2.1	0.0	Tubulin, alpha, ubiquitous isoform 21	NE
Signal transduction mechanisms					
NP_113865.1	2.0	6.2	0.0	Four and a half LIM domains 2	NE
NP_033419.1	3.0	4.2	1.0	Troponin C, cardiac/slow skeletal	E
NP_001005908.1	2.0	1.0	1.0	Growth hormone inducible transmembrane protein	NE
NP_861434.1	3.0	0.0	1.0	TNNI3 interacting kinase, Serine/threonine-protein kinase	NE
NP_476485.1	2.0	0.0	1.0	Cysteine and glycine-rich protein 3	NE
NP_062244.1	2.0	0.0	1.0	Hypoxia-inducible factor prolyl hydroxylase 3-MITOCHONDRIAL	NE
XP_216433.4	2.0	0.0	1.0	RIKEN cDNA 3110001D03	NE
Lipid transport and metabolism					
NP_446126.1	5.0	7.3	3.1	Phytanoyl-CoA 2-hydroxylase	NE
NP_569117.1	5.0	2.1	3.1	Acetyl-coenzyme A acyltransferase 2	NE
AAH87036.1	2.0	1.0	1.0	Acyl-coenzyme A dehydrogenase, very long chain	NE
NP_077076.1	3.0	1.0	0.0	Fatty acid binding protein 3, muscle and heart	E
NP_001011969.1	2.0	0.0	1.0	Serine/threonine kinase receptor associated protein	NE
AAH31435.1	3.0	0.0	0.0	Chpt1 protein	NE
General function prediction only					
NP_036730.1	5.0	4.2	3.1	Lipoprotein lipase	E
XP_922484.2	1.0	4.2	0.0	Thrombospondin 1	NE
NP_071968.1	1.0	2.1	0.0	CD151 molecule (Raph blood group)	NE
NP_036644.1	2.0	0.0	1.0	Beta-2-microglobulin	NE
NP_001009686.1	3.0	0.0	0.0	WD repeat domain 23	NE
Posttranslational modification, protein turnover, chaperones					
P25236	2.0	2.1	1.0	Selenoprotein P	E
NP_037118.1	2.0	3.1	0.0	E3 ubiquitin-protein ligase NEDD4	E
AAH98673.1	2.0	1.0	1.0	LOC361985 protein	NE
XP_343671.3	3.0	0.0	0.0	UBX domain containing 1	NE
Function unknown					
NP_001007009.1	2.0	1.0	1.0	Coiled-coil-helix-coiled-coil-helix domain containing 10	NE
XP_215468.3	1.0	2.1	0.0	Mitochondrial ribosomal protein S36	NE
Inorganic ion transport and metabolism					
AAH61780.1	4.0	3.1	2.1	Voltage-dependent anion channel 3	E
NP_080665.1	1.0	4.2	0.0	Polymerase delta interacting protein 38	NE
Carbohydrate transport and metabolism					
NP_058704.1	13.0	12.5	5.2	Glyceraldehyde-3-phosphate dehydrogenase	E
NP_997475.1	2.0	2.1	1.0	Glucose phosphate isomerase	E
Extracellular structures					
XP_215375.4	1.0	2.1	0.0	Collagen alpha-1(VI) chain precursor	NE
Intracellular trafficking, secretion, and vesicular transport					
AAC06290.1	2.0	0.0	1.0	Lipocortin V	NE

Table 1 (*Continued*)

Gene ID	Con	IPC	IR	Annotation	IPC effect
Coenzyme transport and metabolism					
AAH07576.2	2.0	0.0	1.0	Putative adenosylhomocysteinase 2	NE
Nucleotide transport and metabolism					
NP_036715.1	2.0	1.0	0.0	Hypoxanthine phosphoribosyltransferase 1	E
Amino acid transport and metabolism					
AAL90860.1	2.0	1.0	1.0	Csr1	NE
Cell cycle control, cell division, chromosome partitioning					
AAH61872.1	2.0	0.0	1.0	Desmin	NE
Chromatin structure and dynamics					
NP_114004.1	3.0	1.0	0.0	Histone-binding protein	E

IR, ischemia–reperfusion; IPC, ischemic preconditioning; E, effective; NE, non-effective.

extracted from rat heart tissue using the RNeasy plus Mini kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. RT reactions were performed with the iScriptTM cDNA Synthesis Kit for RT-PCR (Fermentas, Burlington, Ontario, Canada) using 2 μg of fractionated cellular RNA, which was purified as described above, as a template. Real-time PCR was carried out using SYBR Premix Ex Taq (Takara, Japan). Reactions were prepared following the manufacturer's protocol. All reactions were carried out in triplicate (Bio-RAD, Hercules, CA, USA). Specific primers were used to detect the presence of each of the mRNAs. Standard thermal cycling conditions included a hot start of 2 min at 50 °C and 10 min at 95 °C. The DNA was amplified through 50 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C for cytochrome b, NDUFS2, actin, and tropomyosin alpha-1 chain gene respectively. Data analysis was carried out using Bio-rad software (iCycler iQ^{TM}) and Microsoft Excel. Expression values are presented relative to the measurements for beta-tubulin values in the corresponding samples.

Statistical tests

Real-time PCR and proteomics experiments were done in triplicate, and the results were expressed as mean ± SEM and analyzed using Student's *t*-test. Statistical significance was set at $^{*}p < 0.05$ vs. Con, $\#p < 0.05$ vs. IPC.

Results

Characterization of ESTs

Three cDNA libraries were successfully generated from six rat heart tissue samples. A total of 1714, 1694, and 1715 sequences were acquired from the Con, IPC, and IR groups, respectively. BLAST annotation of the ESTs showed that 4109 ESTs (1402, 1356, and 1351 in the Con, IPC, and IR groups, respectively) had significant hits to the 591, 559, and 615 NCBI non-redundant gene databases, respectively (E-value $\leq e^{-10}$) (Supplementary Table 1).

Functional cluster annotation of ESTs

Functional annotation of ESTs was performed through BLAST searches against the KOG sequence database. Of a total of 4109 ESTs, 1261 (89.9%), 1206 (88.9%), and 1177 (87.1%) ESTs in the Con, IPC, and IR groups, respectively, were successfully annotated into 25 functional KOG categories. The largest population of genes identified from each group was included in the category "energy production and conversion" (coded as C). This population contained 682 (54.08% of total), 650 (53.9%), and 581 (49.36%) genes from the Con, IPC, and IR groups, respectively. The category "cytoskeleton" (coded as Z) contained the second largest number of genes (97, 98, and 96 genes from the Con, IPC, and IR groups, respectively). Additional major categories for each group are shown in Fig. 1A and B.

Expression analysis of individual genes

After functional cluster analysis, we compared the expression level of each identified gene based on the number of counts for each treatment. A total of 126 genes were selected base on a fold-change of >1.5 after IR treatment. Of these, 62 were upregulated and 64 were downregulated. The KOG functional category for each gene was annotated (Tables 1 and 2). In the upregulated gene group, the categories containing the largest numbers of genes were C (16.4%) and Z (16.4%) (Fig. 2A). The categories in the downregulated gene group containing the largest numbers of genes were C (34.3%), Z (10%), and "lipid transport and metabolism" (8.6%, coded as I) (Fig. 2B).

Subsequently, up- and downregulated genes were re-categorized into "IPC-effective" and "IPC-ineffective groups" to identify potential target genes for IPC treatment that achieve cardioprotection during IR. Genes whose expression was >1.5-fold different between the IR and Con groups and for which the IR-induced change in expression was reduced by IPC treatment were considered IPC-effective genes. For example, the most frequently detected gene in the upregulated group was myosin light chain 3, with a total of 37 counts (9, 12, and 16 for the Con, IPC, and IR groups, respectively). Expression of the myosin light chain 3 gene was 1.77-fold higher in the IR group and only 1.33-fold higher in the IPC group. In the downregulated group, the most frequently detected gene was cytochrome c oxidase (COX) subunit 3, with a total of 267 counts (103, 98, and 66 in the Con, IPC, and IR groups, respectively). There was a 1.56-fold change in the expression of COX subunit 3 in the IR group, but only a 1.05 fold-change in the IPC group. In total, we identified 32 and 29 IPC-effective genes in the down- and upregulated groups, respectively (Tables 1 and 2).

Subcellular localization analysis of encoded proteins revealed that the mitochondrion is a major target organelle in IR injury (Fig. 3A) and IPC treatment (Fig. 3B). Moreover, functional classification of IPC-effective mitochondrion protein-coding genes revealed that the respiratory electron transport chain in mitochondria is the major target of the IPC-induced cardioprotective mechanism (Table 3).

Functional associations of ESTs

A total of 183 non-redundantly selected genes (those detected more than three times in all groups), comprising 61 upregulated genes, 70 downregulated genes, and 50 constantly expressed genes, were queried to analyze functional network clusters. A functional association network with 512 interactions between 120 genes was constructed. A total of 26 genes had no direct interactions with other queried genes (Fig. 4A). In the network, genes implicated in the same function were located at short distances from each other and formed four major functional modules: electron transport chain/oxidative phosphorylation (23 members: 14 downregulated, 7 upregulated, and 2 constantly expressed); tricarboxylic acid (TCA)

Table 2
List of cardiac IR upregulated genes and effectiveness of IPC treatment.

Gene ID	Con	IPC	IR	Annotation	IPC effect
Energy production and conversion					
NP_077374.2	6.0	6.2	9.3	Aconitase 2, mitochondrial	E
NP_001005550.1	2.0	3.1	4.2	NADH dehydrogenase (ubiquinone) Fe–S protein 1, 75 kDa	E
NP_569112.1	2.0	1.0	3.1	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	E
NP_059007.1	0.0	2.1	2.1	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit c, isoform 1	NE
NP_942037.2	0.0	3.1	1.0	Electron-transferring-flavoprotein dehydrogenase	NE
NP_112287.1	0.0	1.0	2.1	Dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	E
AP_004896.1	0.0	2.1	1.0	ATP synthase F0 subunit 8	NE
NP_001178661	0.0	3.1	0.0	Cytochrome c-type heme lyase (CCHL) (holocytochrome c-type synthase)	NE
NP_001099830	0.0	2.1	1.0	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	NE
NP_001020317.1	0.0	0.0	3.1	NADH dehydrogenase (ubiquinone) Fe–S protein 4	E
Cytoskeleton					
P04692	6.0	20.8	11.5	Tropomyosin alpha-1 chain	NE
NP_036738.1	9.0	12.0	16.0	Myosin, light chain 3, alkali; ventricular, skeletal, slow	E
AAA42294.1	8.0	9.3	12.4	Troponin I	E
NP_035782.3	2.0	1.0	3.1	Titin isoform N2-A	E
NP_001162612	1.0	2.1	2.1	Myomesin 2	NE
NP_036810.1	0.0	2.1	2.1	Tropomyosin 4	NE
NP_631977.1	0.0	3.1	1.0	Nexilin (F actin binding protein) isoform b	NE
NP_074058.2	0.0	1.0	2.1	Microtubule-associated protein 1 light chain 3 beta	E
XP_848839.1	0.0	1.0	2.1	Myosin regulatory light chain 2, nonsarcomeric (Myosin RLC) isoform 1	E
General function prediction only					
XP_001081394.1	0.0	4.2	2.1	Telethonin (Titin cap protein)	NE
NP_001100565.1	0.0	3.1	2.1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	NE
Q8R560	1.0	2.1	2.1	Ankyrin repeat domain-containing protein 1	NE
NP_001099190.2	0.0	3.1	1.0	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha [*Rattus norvegicus*]	NE
AAH60583.1	1.0	1.0	2.1	Cd81 protein	E
NP_476484.1	1.0	0.0	2.1	Parkinson disease protein 7	E
Posttranslational modification, protein turnover, chaperones					
NP_037067.1	4.0	7.3	8.3	Crystallin, alpha B	E
EDL75730.1	1.0	2.1	5.2	Complement component 7 precursor	E
NP_001034090.1	2.0	1.0	3.1	TNF receptor-associated protein 1, HSP75	E
NP_001030167	1.0	2.1	2.1	RING finger protein 14 (androgen receptor-associated protein 54) (Triad2 protein)	E
AAH06722.1	0.0	1.0	2.1	Heat shock protein 8	E
NP_037215.1	0.0	2.1	1.0	Heat shock protein 5	NE
Signal transduction mechanisms					
NP_001121052	3.0	1.0	5.2	3-Oxoacid CoA transferase 1	E
NP_113749.2	0.0	1.0	4.2	Cd36 antigen	E
NP_071946.1	0.0	2.1	3.1	Cysteine-rich protein 2	E
NP_001013949.1	2.0	0.0	3.1	Phospholipase B-like 1 precursor	NE
NP_001013072.1	0.0	2.1	1.0	ADP-ribosylhydrolase like 1	NE
Lipid transport and metabolism					
NP_598302.1	3.0	1.0	6.2	Mitochondrial trifunctional protein, beta subunit	E
NP_445872.1	0.0	2.1	3.1	BCL2/adenovirus E1B 19 kDa-interacting protein 3	E
NP_058682	1.0	1.0	3.1	Medium-chain acyl-CoA dehydrogenase	E
NP_001006967.1	0.0	0.0	3.1	Peroxisomal D3,D2-enoyl-CoA isomerase	E
Q62651	1.0	0.0	2.1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial; Flags: Precursor	E
Chromatin structure and dynamics					
NP_001100058.1	0.0	0.0	3.1	Coiled-coil-helix-coiled-coil-helix domain containing 3	E
AAH49523.1	0.0	1.0	2.1	H2afvl protein	E
XP_001071565	1.0	0.0	2.1	Histone H1.2 (H1 VAR.1) (H1c)	E
NP_002097	1.0	0.0	2.1	Histone h2a.z	E
Translation, ribosomal structure and biogenesis					
CAA33199.1	4.0	5.2	11.4	60S acidic ribosomal protein P0	E
AAH29688.1	0.0	0.0	3.1	Csde1 protein	E
NP_001162617	0.0	3.1	0.0	40S ribosomal protein S16	NE
Carbohydrate transport and metabolism					
NP_075211.2	1.0	4.2	5.2	Triosephosphate isomerase 1	E
NP_036770.1	2.0	0.0	3.1	Glycogen phosphorylase, muscle form (Myophosphorylase)	NE
NP_445743.2	1.0	0.0	2.1	Phosphoglycerate kinase 1	E
Intracellular trafficking, secretion, and vesicular transport					
NP_444180.2	0.0	3.1	6.2	Clusterin	E
NP_001011918.1	1.0	0.0	2.1	Annexin A11	E

Table 2 (*Continued*)

Gene ID	Con	IPC	IR	Annotation	IPC effect
Function unknown					
NP_001099939	3.0	2.1	8.3	Myozenin 2	E
NP_001101793.1	0.0	0.0	3.1	Ser/Thr-rich protein T10 in DGCR region	E
Amino acid transport and metabolism					
NP_112319.2	1.0	4.2	4.2	Aldehyde dehydrogenase family 6, subfamily A1	E
P13221.3	0.0	1.0	2.1	Aspartate transaminase (EC 2.6.1.1) (clone 8C7) – human	E
Extracellular structures					
NP_036788.1	1.0	2.1	3.1	Secreted protein, acidic, cysteine-rich (osteonectin)	E
Inorganic ion transport and metabolism					
AAH78892.1	4.0	13.5	14.6	Ferritin – Fth1 protein	E
Cell motility					
P62329	1.0	0.0	2.1	Thymosin beta-4 precursor – rat (fragment)	E
Cell cycle control, cell division, chromosome partitioning					
NP_037238.1	1.0	2.1	2.1	Guanine nucleotide binding protein (G protein), alpha inhibiting 3	E
RNA processing and modification					
EAW62676	1.0	0.0	2.1	Heterogenous nuclear ribonucleoprotein K isoform 10	E

Con, control; IR, ischemia–reperfusion; IPC, ischemic preconditioning; E, effective; NE, non-effective.

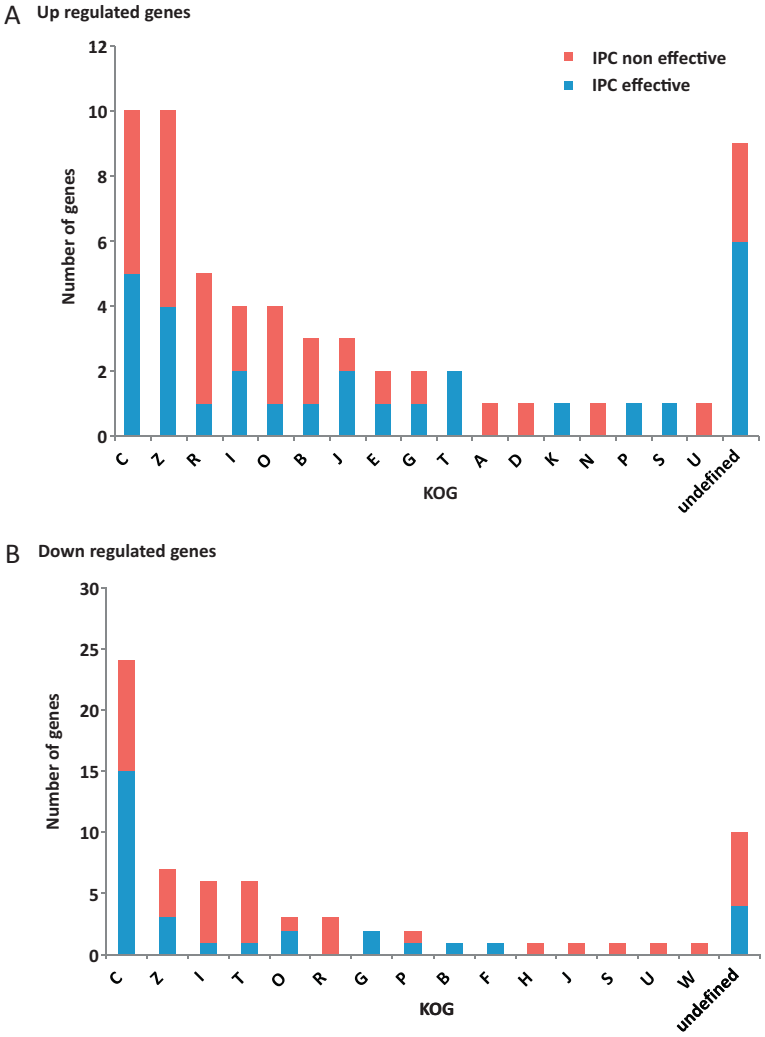

Fig. 2. Eukaryotic cluster of orthologous groups (KOG) annotation of cardiac genes whose expression was altered by ischemia–reperfusion (IR). Numbers of genes that were upregulated (A) and downregulated (B) by IR treatment in each KOG category are shown. The effectiveness of ischemic preconditioning (IPC) was determined by comparing gene expression in the control (Con), IR, and IPC groups. Groups for which the difference in gene expression between the Con and IR groups was larger than that between the Con and IPC groups were considered to be IPC-effective.

cycle/glucose metabolism/amino acid metabolism (17 members: 10 downregulated, 4 upregulated, and 3 constantly expressed); cellular structure and contraction (23 members: 7 downregulated, 11 upregulated, and 5 constantly expressed); and gene transcription, translation, and protein folding (11 members: 3 downregulated, 6 upregulat ed, and 2 constantly expressed). Biological processes for each modified gene were identified using the PANTHER program and were used to construct a gene-to-biological process

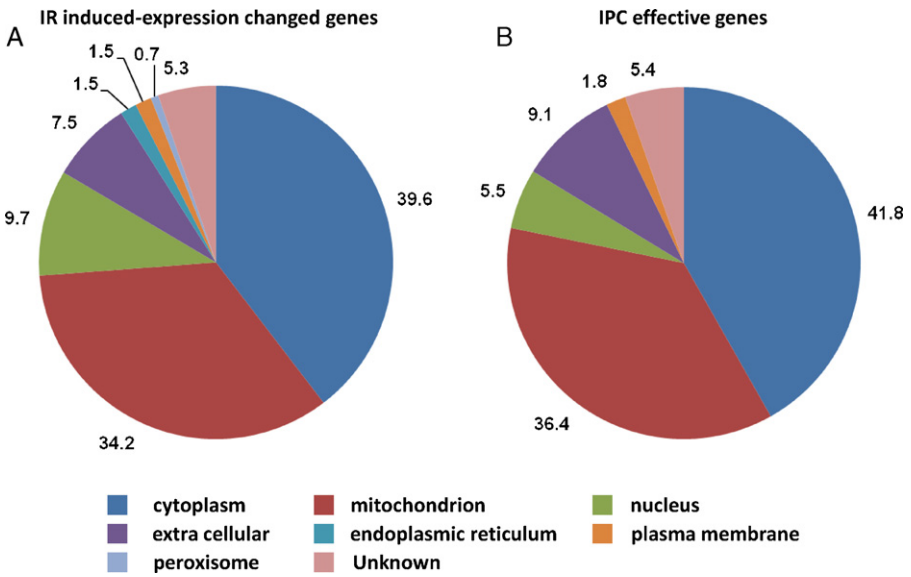

Fig. 3. Subcellular localization of the proteins encoded by identified genes. (A) Subcellular localization of proteins encoded by genes whose expression was altered by ischemia–reperfusion (IR). (B) Subcellular localization of proteins encoded by ischemic preconditioning (IPC)-effective genes.

network (Supplementary Fig. 1). Finally, we constructed a gene-to-biological process-to-gene network, which included information on changes in the expression of cardiac genes following IR treatment, biological processes in which the genes are implicated, and gene-to-gene, gene-to-function, and function-to-function relationships (Fig. 4B).

Verification of EST results by using real time-PCR

The expression patterns of three IPC effective mitochondrial genes were verified by real-time PCR. The expression of MT-ND4L, MT-CYB, and MT-CO3 were downregulated by IR and effectively attenuated by IPC in both real-time PCR (Fig. 5) and EST result (Table 3).

Proteomic analysis

To complement the EST-based genomic analysis, 2DE-based proteomic analysis was performed to analyze the wide and dynamic IR and IPC mechanisms. We identified 14 proteins whose expression was different in the IR and Con groups (Fig. 6 and Supplementary Table 2). Notably, cytoskeletal proteins including myosin, myosin regulatory light chain, tropomyosin, alpha-actin, myomesin-1, myomesin 2, and myosin-binding protein C were upregulated in the IR group, paralleling similar changes detected in the EST analysis (Fig. 6).

Discussion

In the present study, we used ESTs, 2DE proteomics, and systemic analysis tools to identify IPC target genes and proteins. Comparative analysis of ESTs provided not only general profiling of changes in the expression of genes in rat hearts exposed to IR or IPC, but also IPC effect-associated genes that could be effective targets for cardioprotective therapies.

Robust regulation of energy production and consumption and structural integrity are essential for maintaining the function of the heart, which continuously provides blood to the whole body. Half of all cardiac genes function in energy production and conversion, with the second largest proportion playing a role in the cytoskeleton (Fig. 1). Any serious transcriptomic/proteomic perturbation of these two major categories leads to critical heart failure [28,29]. Our results demonstrate that IR damage causes extensive transcriptomic perturbation in both categories. The large proportion of IPC-effective genes in the C and Z categories further suggests that the cardioprotective effect of IPC is mediated via these two functional categories (Fig. 2A and B). In the energy metabolism category, the mitochondrion is the major target organelle for IR and IPC (Fig. 3). About 40% of IR- and IPC-implicated genes encode mitochondrial proteins. Notably, the predominant IR and IPC target genes were the genes encoding components of mitochondrial complex IV in oxidative phosphorylation complexes. COX complex or complex IV is a large transmembrane protein complex consisting of 13 protein subunits (10 of nuclear origin, and 3 synthesized in the mitochondria) [30]. IR injury reduces COX activity and IPC maintains COX activity [31]. In this process, loss of cytochrome c and cardiolipin leads to a decrease in COX activity [32]. Our results further suggest that expression of at least two mitochondrial COX subunit-encoding genes, COX II (YP_665632.1) and COX III (AP_004898.1), was downregulated by IR and preserved by IPC.

We observed reduced expression of genes encoding components of the cytoskeleton (e.g. actin, myosin, myosin-binding protein, and alpha tubulin) and increased expression of genes encoding cytoskeleton regulatory proteins (e.g. troponin I, tropomyosin, myosin light chain 3, and myosin regulatory light chain 2). Similar perturbations in cytoskeletal protein expression were detected in muscle in aged rats, in which upregulation of cytoskeletal regulatory proteins compensated for aging-induced disorganization of sarcomeric myofibrillar proteins [33]. Troponin I, tropomyosin, and myosin light chain 3 are important regulators of muscle contraction and calcium sensitivity. Recovery of these proteins during reperfusion is essential for restoring cardiac contraction.

The results of the proteomic analysis support the conclusion that IR induced changes in the expression of cytoskeletal and structural proteins in the heart. Increased expression of cytoskeletal proteins including myosin light chain, myosin regulatory light chain 2, tropomyosin, and myomesin 2 in the IR group paralleled the changes detected in the EST analysis.

Table 3
Ischemic preconditioning effective mitochondria protein coding genes.

NCBI-GI	Gene name	Expression	GO biological process
38512108	Voltage-dependent anion-selective channel protein 3	Down	Anion transport
209915614	Succinate dehydrogenase [ubiquinone] iron–sulfur subunit, mitochondrial	Down	Tricarboxylic acid cycle oxidative phosphorylation respiratory electron transport chain carbohydrate metabolic process
117884	Cytochrome b	Down	Oxidative phosphorylation respiratory electron transport chain
110189669	Cytochrome c oxidase subunit 3	Down	Oxidative phosphorylation respiratory electron transport chain
110189718	Cytochrome c oxidase subunit 2	Down	Oxidative phosphorylation respiratory electron transport chain
53850628	NADH–ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Up	Respiratory electron transport chain
8392833	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	Up	Respiratory electron transport chain acyl-CoA metabolic process nitrogen compound metabolic process lipid metabolic process
128726	NADH–ubiquinone oxidoreductase chain 3	Down	Oxidative phosphorylation respiratory electron transport chain
68341995	NADH dehydrogenase [ubiquinone] iron–sulfur protein 4, mitochondrial	Up	Oxidative phosphorylation respiratory electron transport chain
110189671	NADH–ubiquinone oxidoreductase chain 4L	Down	Oxidative phosphorylation respiratory electron transport chain
51948476	Cytochrome b–c1 complex subunit 1, mitochondrial	Down	Respiratory electron transport chain protein metabolic process
16758160	Bnip3:BCL2/adenovirus E1B 19 kDa-interacting protein 3	Up	Unknown
45737868	Aldehyde dehydrogenase, mitochondrial	Down	Carbohydrate metabolic process nucleobase, nucleoside, nucleotide and nucleic acid metabolic process cellular amino acid and derivative metabolic process
128764	NADH–ubiquinone oxidoreductase chain 4	Down	Oxidative phosphorylation respiratory electron transport chain
56090293	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Down	Vitamin biosynthetic process carbohydrate metabolic process cellular amino acid and derivative metabolic process lipid metabolic process
119364626	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	Up	Coenzyme metabolic process carbohydrate metabolic process
42476181	Malate dehydrogenase, mitochondrial	Down	Tricarboxylic acid cycle carbohydrate metabolic process
1352170	Cytochrome c oxidase polypeptide 6A2, mitochondrial	Down	Oxidative phosphorylation respiratory electron transport chain

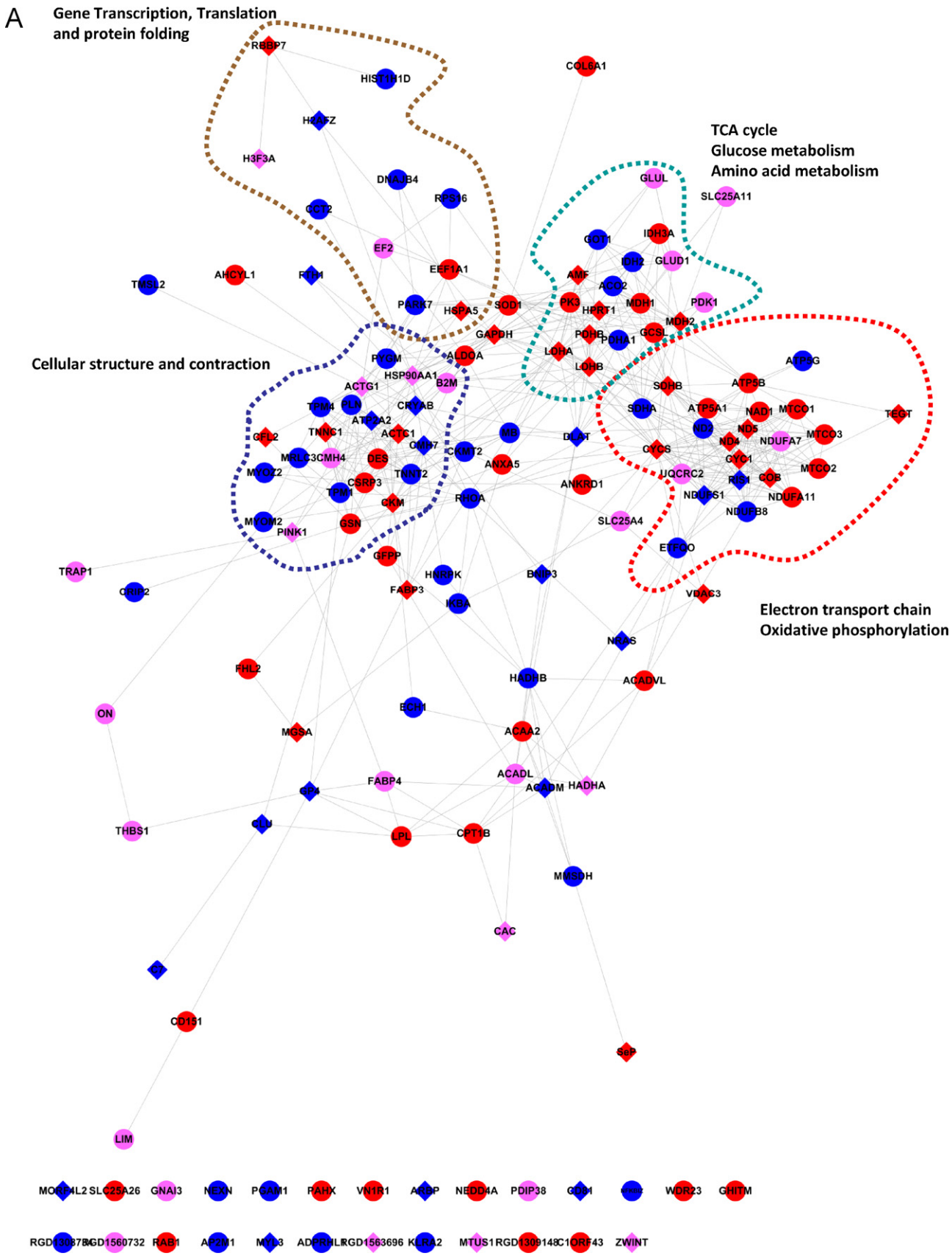

Fig. 4. Interaction map showing genes identified from expressed-sequence-tags of hearts exposed to control conditions (Con), ischemic preconditioning (IPC), or ischemia–reperfusion (IR). (A) Map showing known interactions between identified genes, IR-induced changes in heart expression for each gene, and effectiveness of IPC for attenuating IR-induced gene expression changes in the heart. (B) Map showing known interactions between identified genes and their biological functions. The size of each biological function node indicates the number of implicated genes. Key: color: (●) expression downregulated by IR (compared to Con); (●) expression upregulated; (●) expression unchanged; (●) biological function; shape: (◊) IPC-effective gene (genes for which IPC reduced the IR-induced change in expression); (○) IPC-ineffective gene.

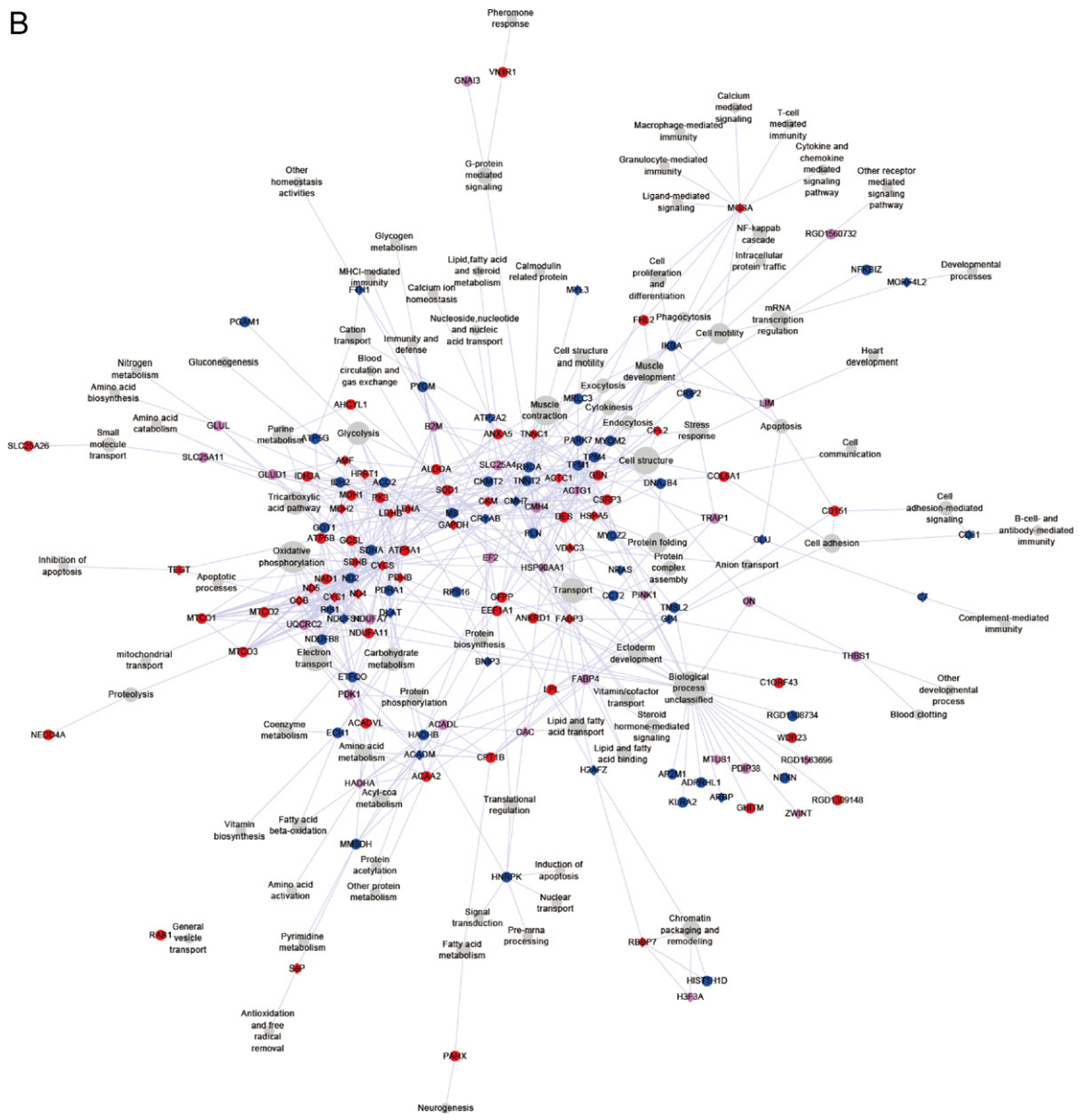

Fig. 4. (*Continued*).

IR and IPC also altered gene expression of major transcription factors such as nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFkBIZ) and alpha (IKBA) and Parkinson disease protein 7 (PARK7). Regulation of transcription factors, NFkB and IKB, are considered as an important therapeutic target for cardiac protection [34]. Activation of IkB alpha and blockade of following NFkB signaling is known to be a protective mechanism of IPC induced-cardiac protection [35,36]. In agreement with those, we found the upregulation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFkBIZ) and alpha (IKBA) by IPC treatment. PARK7 is a positive regulator of androgen receptor-dependent transcription and it protects neurons against oxidative stress, cell death, and stroke-induced damage [37]. PARK7 is also suggested as a plasma marker for stroke [38]. However, its expressional alteration in the IR- or IPC-treated heart is unknown. Because the major cause of stroke is brain ischemia, in this consideration, increased level of PARK7 in the IR heart may suggest that PARK7 is implicated in ischemic injury.

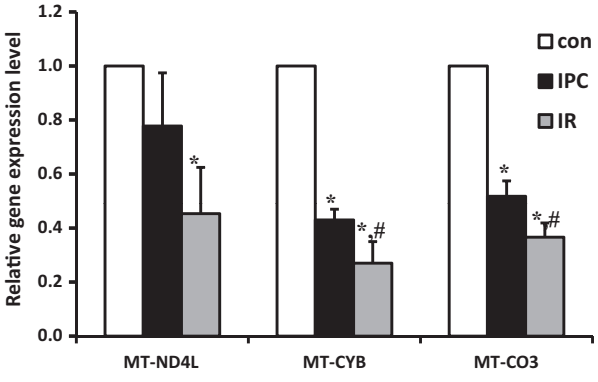

Fig. 5. Verification of expressed-sequence-tags (EST) result using real time polymerase chain reaction. Expression patterns of randomly selected 3 genes including NADH dehydrogenase subunit 4 (MT-ND4L), cytochrome b (MT-CYB), and cytochrome c oxidase subunit III (MT-CO3) were matched with EST results. Ischemic preconditioning (IPC) treatment attenuated the decrease of gene expression in ischemia–reperfusion (IR) model [$n = 3$ each group, $^{*}p < 0.05$ vs. control (con), $^{\#}p < 0.05$ vs. IPC].

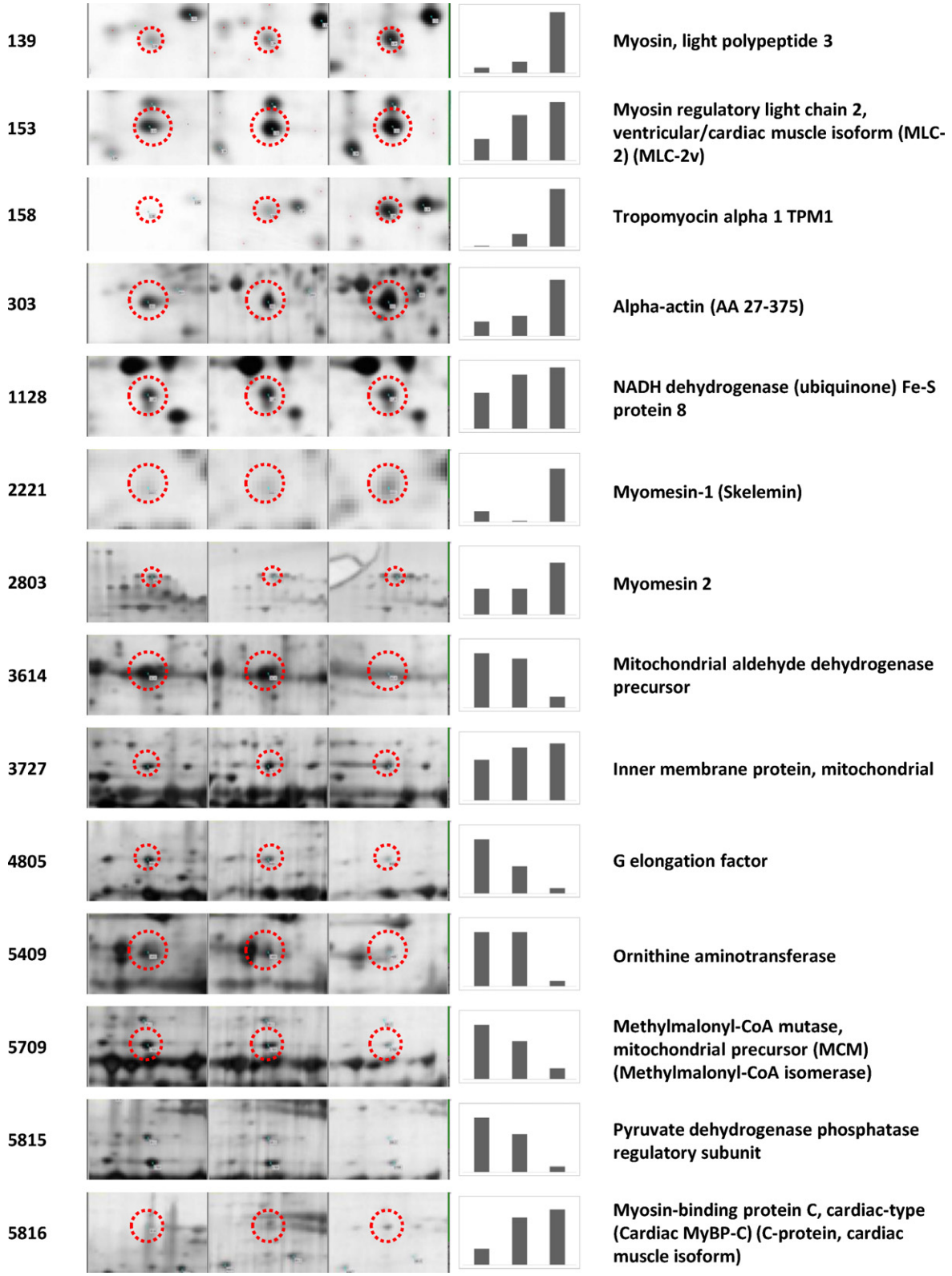

Fig. 6. Relative expression changes for 14 identified proteins. Representative images of protein spots for control (left), ischemic preconditioning (middle), and ischemia–reperfusion samples (right), and expression histograms.

Besides the well-known cardiac IR-related genes, we also identified two interesting IPC-effective genes, myozenin 2 and ferritin, whose expression increased. Myozenin 2 is a novel gene in human hypertrophic cardiomyopathy [39], and high blood and tissue ferritin levels are a risk factor for atherosclerosis and ischemic heart disease [40]. Their role in cardioprotection against IR injury remains unclear.

In conclusion, we evaluated various IPC-effective cardiac genes in rat hearts exposed to IR and IPC using ESTs and gene/protein systemic analysis tools. Our well-matched gene expression and

proteomics results suggest that EST-based studies may be an effective tool for primary screening and validation of complex biological processes. Further studies are required to functionally validate the IPC-effective genes identified herein. We identified major functional pathways that were modified by IR and that were effectively protected against modification by IPC. Genes or proteins that we have identified will be used in the development of IPC-mediated cardioprotective therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jjcc.2012.02.004.

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