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Thioredoxin1 Upregulates Mitochondrial Proteins Related to Oxidative Phosphorylation and TCA Cycle in the Heart

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ABSTRACT

Thioredoxin1 (Trx1) inhibits hypertrophy and exhibits protective functions in the heart. To elucidate further the cardiac functions of Trx1, we used a DNA microarray analysis, with hearts from transgenic mice with cardiac-specific overexpression of Trx1 (Tg-Trx1, $n = 4$) and nontransgenic controls ($n = 4$). Expression of a large number of genes is regulated in Tg-Trx1, with a greater number of genes downregulated, versus upregulated, at high-fold changes. The peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) gene was among the top 50 significantly upregulated genes. By pathway analyses, we found that genes involved in both mitochondrial oxidative phosphorylation and the TCA cycle were upregulated in Tg-Trx1. We confirmed upregulation of cytochrome *c* oxidase (COX) components and mitochondrial transcription factor A in Tg-Trx1. The activity of citrate synthase and COX and the cardiac ATP content were significantly higher in Tg-Trx1. A transcription factor binding-site analysis showed that upregulated genes frequently contained binding sites for nuclear respiratory factor 1 (NRF1). Expression of NRF1 and PGC-1α was upregulated in Tg-Trx1, and Trx1 stimulated the transcriptional activity of NRF1 and NRF2 in cardiac myocytes. These results suggest that, in cardiac myocytes, Trx1 upregulates mitochondrial proteins and enhances mitochondrial functions, possibly through PGC-1α and NRFs. *Antioxid. Redox Signal.* 8, 1635–1649.

INTRODUCTION

The cellular reduction–oxidation (REDOX) state is a crucial modifier of intracellular signaling and transcriptional processes (22). Protein thiols with cysteine residues are key mediators in redox sensing and regulation (9). Thioredoxin (Trx) is small and ubiquitously expressed thiol-disulfide oxidoreductase, has two cysteine residues in the catalytic center, and functions as an important redox regulator in cells from *Escherichia coli* to mammals (12, 22). The reduction of the catalytic center in Trx is catalyzed by Trx reductase, using electrons from NADPH. Mammalian cells have two distinct types of Trx; Trx1 and Trx2. Trx1 is normally localized in the cytosol and translocates to the nucleus upon stimulation (29), whereas Trx2 is targeted to the mitochondria (23). Both Trx1 and Trx2 function as scavengers for reactive oxygen species (*i.e.* antioxidants) at the cellular level. In addition, Trx1 interacts with various proteins in a redox-dependent manner, and modulates intracellular signaling pathways and transcription factor activity (27, 37). Accordingly, Trx1 regulates a wide variety of cellular functions, including cell growth, differentiation, apoptosis, immune responses, and gene expression.

We previously showed, *in vivo*, that Trx1 is an essential antioxidant in the mouse heart (36). Trx1 is a stress-inducible protein, which counteracts with oxidative stress and plays a

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cardioprotective role in the heart in response to pathologic insults, such as ischemia–reperfusion (32). Furthermore, inhibition of endogenous Trx1 in the heart causes cardiac hypertrophy, whereas cardiac-specific overexpression of Trx1 attenuates cardiac hypertrophy induced by thoracic aortic banding (36). To elucidate the molecular mechanisms mediating cardiac effects of Trx1, we used DNA microarray analyses, with cardiac mRNA samples prepared from transgenic mice with cardiac-specific overexpression of Trx1 (Tg-Trx1) and nontransgenic mice (NTg). We identified genes whose expression was significantly altered by Trx1 overexpression in the heart *in vivo*. Using pathway analyses, we identified several gene ontology groups whose expression was coordinately upregulated by Trx1. Finally, using a transcription factor binding site (TFBS) analysis, we identified several transcription factor binding sites that are frequently present in the promoter region of the genes altered by Trx1.

MATERIALS AND METHODS

Animal studies

Tg-Trx1 were generated on an FVB background, using the α-myosin heavy chain promoter (courtesy of J. Robbins, University of Cincinnati, Cincinnati, OH), to achieve cardiac-specific expression, as previously described (36). For technical convenience in genotyping, a *tyrosinase* coat-color gene was injected along with the human Trx1 gene into fertile eggs. The basal characterization of Tg-Trx1 and a method of echocardiography have been described (36). All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey.

Gene expression study by DNA microarray

Using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), total RNA was isolated, from the left ventricle, from 2- to 3-month-old Tg-Trx1 and NTg. Expression profiles were generated using Affymetrix GeneChip Mouse Genome 430 2.0. Generation of biotinylated cRNA hybridization and processing of Affymetrix arrays were performed according to Affymetrix's standard protocol. Four independent biologic replicates from each animal group were processed and analyzed.

Microarray data analysis

Intensity values of all probe sets (45,101 total), from each array, were first normalized to the 75th-percentile value of the array, which was arbitrarily set at 100. Probe sets whose fluorescent signals were not detectable ("Absent" by the absent/present call) in more than two of the four samples, in both transgenic and wild-type samples, were discarded. This step resulted in 21,465 probe sets for further analysis. Selected probe sets were then analyzed by the Significance Analysis of Microarrays (SAM) program (33). A false discovery rate (FDR) of ~5% and mean and median changes greater than 1.2-fold (either upregulated or downregulated) were used to select probe sets whose intensity was considered significantly different between the two groups. Selected probe sets were then subjected to cluster analysis, using the CLUSTER program (8) and Pearson Correlation as a metric. Clusters and the heatmap were presented by the TreeView program (8).

Gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses

Selected probe sets were mapped to entries in the NCBI Gene database using Affymetrix online information (NetAffx: https://www.affymetrix.com/analysis/netaffx/index.affx). GO annotations for genes were obtained from the Gene database of NCBI (25). For each GO term, a statistical test based on the hypergeometric distribution was used, as previously described (31). The p values obtained from the tests were adjusted by the Bonferroni method (3). GO terms with a p value of <0.05, after adjustment, were selected. KEGG pathways were analyzed by a similar approach. The mapping between genes and KEGG ids were provided by KEGG (15).

Transcription factor binding site (TFBS) analysis

The transcriptional start sites (TSSs) for mouse genes were mapped by aligning RefSeq sequences from NCBI to UCSC genome sequences using BLAT (18). We retrieved genomic sequences surrounding the TSSs, either from –700 nt to +300 nt or –3,500 nt to +1,500 nt, with the TSS position set at 0. The former is called a 1K set, and the latter is a 5K set. TFBSs were identified in the 1K and 5K sets using the Match tool (16) and the TRANFAC database (version 9.2) (34). TFBSs that were significantly associated with upregulated and downregulated genes were identified by a statistical test based on the hypergeometric distribution, as described earlier. The p values were adjusted, at 0.05, by the Benjamin and Hochberg method (2) to control for the overall false-discovery rate. Sequence logos for TFBSs were generated by position-specific scoring matrices in the TRANSFAC database and the weblogo tool (7).

Preparation of neonatal rat ventricular cardiac myocytes

Primary cultures of cardiac myocytes from 1-day-old Crl:(WI)BR-Wistar rats (Charles River Laboratories Inc., Wilmington, MA) were prepared from a cardiac myocyte–rich fraction, obtained by centrifugation through a discontinuous Percoll gradient, as previously described (1).

Transfection and luciferase assays

Cardiac myocytes were cultured in six-well plates and transfected with the following plasmids (1 μg total): 0.5 μg of pTransLucent reporter vector containing multimeric NRF1- or NRF2-specific binding elements and a firefly luciferase gene (Panomics, Fremont, CA), and 0.5 μg of expression plasmids consisting of pcDNA3.1 alone (Invitrogen Corp.) or pcDNA3.1 harboring rat Trx1. Thirty-six hours after transfection, cells were lysed with Passive Lysis Buffer, and the transcriptional activity was determined using the lu-

ciferase assay system (Promega, Madison, WI), as previously described (1).

Mitochondrial fractionation and enzymatic activity

A mitochondrial fraction was prepared from mouse hearts, as previously described (4). In brief, isolated mouse hearts were homogenized in 10 volumes of ice-cold Buffer A (200 m*M* mannitol, 50 m*M* sucrose, 10 m*M* KCl, 1 m*M* EDTA, 10 m*M* Hepes-KOH (pH 7.4), 0.1% bovine serum albumin, and a mixture of protease inhibitors). Homogenates were centrifuged at 600 *g* for 5 min at 4°C. Supernatants were then centrifuged at 3,500 *g* for 15 min at 4°C. The pellets were resuspended in Buffer A, and centrifuged at 1,500 *g* for 5 min. The supernatants were centrifuged at 5,500 *g* for 10 min at 4°C, and then the pellets were suspended as the mitochondrial fraction in 100 μl of CelLytic M Lysis Reagent (Sigma-Aldrich, St. Louis, MO). Lysates containing equal amounts of proteins were assessed for citrate synthase (citrate synthase assay kit, Sigma-Aldrich) and cytochrome *c* oxidase (cytochrome *c* oxidase assay kit, Sigma-Aldrich) activity.

ATP measurements

Mouse hearts were homogenized in CelLytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma-Aldrich) using polytron (PowerGen 125, Fisher Scientific, Pittsburgh, PA). After centrifugation, lysates (equivalent to 300 μg protein) were used for ATP measurements by Adenosine 5′-triphosphate Bioluminescent Assay Kit (Sigma-Aldrich).

Immunoblot analyses

Heart homogenates were prepared in a lysis buffer containing 50 m*M* Tris (pH 7.5), 150 m*M* NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 10 m*M* $Na_4P_2O_7$, 5 m*M* EDTA, 0.1 m*M* Na_3VO_4, 1 m*M* NaF, 0.5 m*M* 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Equal amounts of proteins (10–30 μg) were subjected to 10–15% SDS/PAGE. After proteins were transferred to a PVDF membrane, immunoblots were probed with the following antibodies: anti-cytochrome *c* (1:1,000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-cytochrome *c* oxidase subunit IV (COX IV) (1:1,000 dilution, Invitrogen, Corp.), anti-cytochrome *c* oxidase subunit Vb (COX Vb) (1:750 dilution, Invitrogen, Corp.), anti-peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) (1:500 dilution, EMD Biosciences, Inc., San Diego, CA), Trx1 (1:500) (36) and anti-actin (1:1,000, Sigma-Aldrich). The protein abundance was analyzed densitometrically and standardized with the level of actin.

RT-PCR and quantitative PCR

Total RNA was prepared using TRIzol (Invitrogen Corp.), and first-strand cDNA was synthesized by the ThermoScript RT-PCR system (Invitrogen Corp.). Quantitative PCR was carried out using the DNA Engine Opticon 2 system (Bio-Rad Laboratories, Hercules, CA) and the iQ SYBR Green Supermix (Bio-Rad Laboratories). The specific oligonucleotide primers used in this study are as follows:

NRF1, forward 5′-CAGCAACTCCACCAGTCTCA-3′,
reverse 5′-GGCCAGAGTCAGAGTCGAAC-3′;
NRF2, forward 5′-ACTCATCGATCCCCTCACTG-3′,
reverse 5′-CTAATGGCAGCAGAGGAAGG-3′;
TFAM, forward 5′-ACCAAAAAGACCTCGGTCAG-3′,
reverse 5′-ATGAGATCACTTCGCCCAAC-3′;
GAPDH, forward 5′-TGAACGGGAAGCTCACTGG-3′,
reverse 5′-TCCACCACCCTGTTGCTGTA-3′.
Expression values are standardized by those of GAPDH.

Mitochondrial DNA copy number analysis in mouse hearts

Genomic DNA preparation from mouse hearts (about 5 mg), and subsequent PCR, were performed using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich). For mitochondrial DNA (mtDNA) copy-number analysis, the mitochondrial cytochrome *c* oxidase subunit I gene (COX I), and the 5S ribosomal DNA (rDNA) gene, were examined by PCR using the following primer sets (13):

5S rDNA, forward 5′-ACCGTCTAGCCGTCCTCCTTT-3′,
reverse 5′-CCCACTGTGGATGGATACATG-3′;
COX I, forward 5′-CCCAGATATAGCATTCCCACGA-3′,
reverse 5′-AGTAAGCTCGTGTGTCTACATC-3′.

The cycling conditions were 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 27 cycles. After resolving on a 1.2% agarose gel, and visualizing with ethidium bromide staining, the PCR bands of COX I and 5S rDNA were densitometrically quantitated using NIH image. The mtDNA copy numbers were presented as a density ratio of COX I to 5S rDNA.

Statistics

Values are reported as mean ± SEM. Statistical analyses between the two groups were done by an unpaired *t* test. A p value of <0.05 was considered significant.

RESULTS

Sample preparation

We used 2-month-old male Tg-Trx1 ($n = 4$) and NTg ($n = 4$). Tg-Trx1 do not exhibit an obvious cardiac phenotype at this age, which is consistent with our previous report (36). Echocardiographic analyses indicated that neither LV function nor LV chamber size was significantly different between Tg-Trx1 and NTg (Table 1). Organ weights were also similar between the two groups (Table 1).

Identification of genes whose expression is regulated by Trx1 using microarray analysis

Using the SAM program (33), we identified 4,537 probe sets whose hybridization intensities were significantly different (FDR <5%) between the two mouse groups (Fig. 1A) and that corresponded to 3,803 known genes in the NCBI Gene database. These results suggest that Trx1 overexpression can systematically perturb gene expression in the heart. Interestingly, whereas the overall numbers of upregulated and down-

Table 1. Organ Weight and Echocardiographic Data of NTg and Tg-Trx1

	NTg (n = 4)	*Tg-Trx1 (n = 4)*
Age (days)	76	63
LVW/TL (mg/mm)	4.52 ± 0.18	4.47 ± 0.05
Lung W/TL	8.10 ± 0.35	8.33 ± 0.13
DSEP Wt (mm)	0.77 ± 0.01	0.74 ± 0.02
LVEDD (mm)	3.88 ± 0.15	3.70 ± 0.08
DP wt (mm)	0.72 ± 0.03	0.72 ± 0.01
SSEP wt (mm)	1.21 ± 0.04	1.07 ± 0.01
LVESD (mm)	2.60 ± 0.09	2.44 ± 0.06
SP wt (mm)	1.06 ± 0.06	1.00 ± 0.03
EF (%)	70.0 ± 1.00	71.4 ± 0.80
%FS	33.0 ± 0.51	34.1 ± 0.65
HR (beats/min)	456.3 ± 22.26	411.6 ± 1.5

Data are expressed as mean ± SEM.

BW, body weight; LVW, LV weight; TL, tibia length; DSEP wt, diastolic septal wall thickness; LVEDD, LV end-diastolic dimension; DP wt, diastolic posterior wall thickness; SSEP wt, systolic septal wall thickness; LVESD, LV end-systolic dimension; SP wt, systolic posterior wall thickness; EF, LV ejection fraction; FS, fractional shortening; HR, heart rate.

regulated genes are similar (1,933 and 1,870, respectively), more genes are upregulated in the 1.2- to 1.4-fold change range and, in contrast, more genes are downregulated in the greater than 1.4-fold change range (Table 2), suggesting that the magnitude of upregulation is less than that of downregulation. Clustering analysis, using regulated genes, showed that the Tg-Trx1 samples can be readily separated from the NTg samples, and genes can be largely grouped into two sets (Tg-Trx1 upregulated or downregulated), suggesting low noise in the microarray data (Fig. 1B). The top 50 genes, whose expression was found to be greatly altered, are listed in Table 3. As expected, mouse tyrosinase, exogenously injected with human Trx1, was found to be the prominently upregulated gene (Table 3).

Table 2. Probe Sets and Genes with Different Fold Changes

	Upregulated		*Downregulated*	
Fold change	*Probe sets*	*Genes*	*Probe sets*	*Genes*
1.2–1.4	1,884	1,560	897	745
1.4–1.6	309	278	715	605
1.6–1.8	59	55	340	276
1.8–2.0	25	22	145	123
> 2.0	18	18	145	121
Total	2,295	1,933	2,242	1,870

The numbers of probe sets and genes in different fold change ranges are listed. In total, 4,537 probe sets were evaluated using the SAM analysis and shown to have p value <5% and have mean and median fold changes of >1.2. Upregulated and downregulated probe sets are listed separately and include the number of unique genes annotated by the NCBI Gene database. Of the 3,803 genes selected, 3,682 are unique, as some genes have both probe sets supporting upregulation and downregulation.

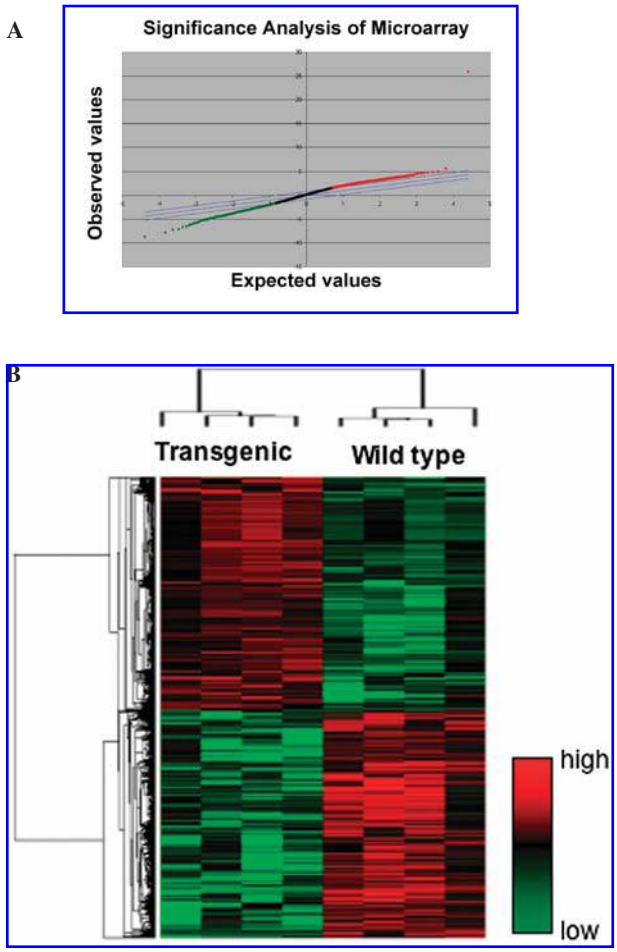

FIG. 1. Significance analysis of gene expression. (**A**) After filtering out probe sets that were considered with no signals, 21,465 probe sets were analyzed by SAM (see Materials and Methods for details). A FDR of ~5% and a fold change >1.2 (either up- or downregulated) were used to select probe sets. Red dots represent upregulated probe sets in the transgenic group, and green dots are upregulated probe sets in the wild-type group. All probe sets were selected. **(B) Cluster analysis.** In total, 4,537 probe sets selected by SAM (supplemental Fig. 1) were subjected to clustering analysis by Pearson Correlation. Values for each probe set across experiments (Rows) are median-centered. Red lines are values above the median, and green lines are values below the median. Two groups of genes can be discerned (*i.e.,* genes whose expression is higher in the transgenic group, and genes whose expression is higher in the wild-type group). Samples from Tg-Trx1 and NTg are also grouped by the cluster analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

We then addressed whether regulated genes fell into common pathways. To this end, we conducted statistical analysis of gene pathways using the GO (25) and KEGG (15) databases. As shown in Tables 4 and 5, a number of KEGG pathways and GO terms were found to be significantly regulated. In both cases, a greater number of upregulated versus down-

Table 3. Top 50 Upregulated and Top 50 Downregulated Genes

Rank	*Gene ID*	*Probe Set ID*	*Tg-Trx1*	*NTg*	*FC*	*q Value*	*Annotation*
Upregulated genes							
1	22173	1417717_a_at	2762.18	25.18	109.68	0.00E+00	Tyr, tyrosinase
2	107939	1420004_s_at	56.55	12.85	4.4	1.07E-02	Pom121, nuclear pore membrane protein 121
3	69823	1451325_at	16.92	4	4.23	1.38E-02	Fyttd1, forty-two-three domain containing 1
4	13435	1423066_at	112.33	31.71	3.54	1.74E-02	Dnmt3a, DNA methyltransferase 3A
5	117149	1418685_at	32.95	11.67	2.82	2.75E-02	Tirap, toll-interleukin 1 receptor (TIR) domain-containing adaptor protein
6	68032	1438369_x_at	42.84	15.47	2.77	2.05E-02	2610318K02Rik, RIKEN cDNA 2610318K02 gene
7	74196	1452665_at	16.66	6.2	2.69	1.74E-02	2610511O17Rik, RIKEN cDNA 2610511O17 gene
8	320799	1441454_at	37.76	14.71	2.57	1.38E-02	Zhx3, zinc fingers and homeoboxes 3
9	216991	1425638_at	28.05	11.01	2.55	2.05E-02	Centa2, centaurin, alpha 2
10	22042	1422966_a_at	398.51	157.81	2.53	3.66E-02	Tfrc, transferring receptor
11	73828	1424777_at	63.61	27.41	2.32	2.75E-02	Wdr21,WD repeat domain 21
12	19696	1420710_at	16.46	7.28	2.26	3.66E-02	Rel, reticuloendotheliosis oncogene
13	69576	1432107_at	282.11	126.67	2.23	2.05E-02	2310010M20Rik, RIKEN cDNA 2310010M20 gene
14	214384	1425808_a_at	71.19	32.98	2.16	1.74E-02	Myocd,myocardin
15	14615	1447787_x_at	61.77	28.66	2.16	1.38E-02	Gja7, gap junction membrane channel protein alpha 7
16	109229	1454236_a_at	57.94	27.62	2.1	1.07E-02	C030004A17Rik, RIKEN cDNA C030004A17 gene
17	11910	1449363_at	143.26	70.97	2.02	1.74E-02	Atf3, activating transcription factor 3
18	17763	1449897_a_at	132.49	65.76	2.01	1.74E-02	Mtcp1, mature T-cell proliferation 1
19	329650	1452864_at	88.63	44.42	2	2.05E-02	Med12l, mediator of RNA polymerase II transcription, subunit 12 homologue (yeast)-like
20	15983	1447393_at	75.28	37.79	1.99	3.66E-02	Ifrd2, interferon-related developmental regulator 2
21	72147	1429168_at	96.12	48.54	1.98	1.38E-02	Btbd4, BTB (POZ) domain containing 4
22	15370	1416505_at	895.9	458.74	1.95	1.74E-02	Nr4a1, nuclear receptor subfamily 4, group A, member 1
23	67655	1452697_at	68.26	35.03	1.95	1.38E-02	Ctdp1, CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1
24	106846	1440221_at	13.84	7.15	1.93	3.66E-02	AA408650, expressed sequence AA408650
25	14651	1424171_a_at	179.74	93.16	1.93	1.74E-02	Hagh, hydroxyacyl glutathione hydrolase
26	15374	1438988_x_at	247.34	129.17	1.91	1.74E-02	Hn1,hematological and neurological expressed sequence 1
27	67561	1443836_x_at	185.04	97.76	1.89	2.05E-02	Wdr48,WD repeat domain 48
28	19017	1460336_at	1320	697.82	1.89	2.05E-02	Ppargc1a, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
29	269037	1436047_at	46.81	24.77	1.89	3.66E-02	Gm672, gene model 672, (NCBI)
30	212114	1456026_at	52.19	27.82	1.88	1.74E-02	8030451K01Rik, RIKEN cDNA 8030451K01 gene
31	52535	1452699_at	239.63	128.03	1.87	1.74E-02	D14Ertd209e, DNA segment, Chr 14, ERATO Doi 209, expressed
32	277360	1434069_at	58.31	31.26	1.87	1.74E-02	BC067047, cDNA sequence BC067047
33	27410	1451731_at	51.99	27.93	1.86	2.05E-02	Abca3, ATP-binding cassette, sub-family A (ABC1), member 3
34	12577	1417649_at	126.93	68.8	1.84	1.38E-02	Cdkn1c, cyclin-dependent kinase inhibitor 1C (P57)
35	69000	1435527_at	318.16	174.4	1.82	1.74E-02	1500041O16Rik, RIKEN cDNA 1500041O16 gene
36	17122	1434379_at	203.85	111.94	1.82	1.74E-02	Mxd4, Max dimerization protein 4
37	282619	1459897_a_at	32.77	18.08	1.81	2.05E-02	MGI:2446326, suprabasin
38	14284	1437247_at	252.08	139.18	1.81	1.38E-02	Fosl2, fos-like antigen 2

continued

TABLE 3. CONTINUED

Rank	*Gene ID*	*Probe Set ID*	*Tg-Trx1*	*NTg*	*FC*	*q Value*	*Annotation*
39	72461	1452191_at	28.1	15.56	1.81	3.66E-02	Prcp, prolylcarboxypeptidase (angiotensinase C)
40	20439	1448171_at	75.6	41.94	1.8	2.05E-02	Siah2, seven in absentia 2
41	140570	1416683_at	289.79	162.49	1.78	1.74E-02	Plxnb2, plexin B2
42	55983	1443335_at	55.9	31.41	1.78	2.75E-02	Pdzrn3, PDZ domain containing RING finger 3
43	20856	1449484_at	104.92	59.19	1.77	2.05E-02	Stc2, stanniocalcin 2
44	18595	1421917_at	292.49	165.36	1.77	2.05E-02	Pdgfra, platelet-derived growth factor receptor, alpha polypeptide
45	54158	1427123_s_at	52.9	30.08	1.76	2.75E-02	Copg2as2, coatomer protein complex, subunit gamma 2, antisense 2
46	19126	1419700_a_at	26.73	15.25	1.75	1.74E-02	Prom1, prominin 1
47	12825	1427884_at	166.69	95.91	1.74	2.05E-02	Col3a1, procollagen, type III, alpha 1
48	26414	1437195_x_at	153.63	88.6	1.73	1.74E-02	Mapk10, mitogen activated protein kinase 10
49	100213	1426791_at	125.35	72.54	1.73	1.74E-02	Rusc2, RUN and SH3 domain containing 2
50	73848	1439477_at	188.13	110.15	1.71	1.74E-02	5430406J06Rik, RIKEN cDNA 5430406J06 gene
Downregulated genes							
1	19943	1459436_at	9.52	41.35	4.34	1.07E-02	Rpl28, ribosomal protein L28
2	11669	1434987_at	92.52	381.32	4.12	3.66E-02	Aldh2, aldehyde dehydrogenase 2, mitochondrial
3	74102	1419970_at	28.3	110.08	3.89	2.05E-02	Slc35a5, solute carrier family 35, member A5
4	229841	1439040_at	1.32	4.62	3.5	1.74E-02	Cenpe, centromere protein E
5	22333	1437452_x_at	8.59	29.97	3.49	2.05E-02	Vdac1, voltage-dependent anion channel 1
6	19216	1445445_s_at	5.57	18.73	3.36	8.39E-03	Ptger1, prostaglandin E receptor 1 (subtype EP1)
7	14802	1440891_at	2.43	8.07	3.32	1.38E-02	Gria4, glutamate receptor, ionotropic, AMPA4 (alpha 4)
8	99996	1419831_at	10.65	33.88	3.18	8.39E-03	AA416453, expressed sequence AA416453
9	207165	1427311_at	3.52	11.05	3.14	1.74E-02	Falz, fetal Alzheimer antigen
10	19113	1441873_at	11.58	35.28	3.05	4.90E-03	Prlpe, prolactin-like protein E
11	12561	1449422_at	9.08	26.82	2.95	4.90E-03	Cdh4, cadherin 4
12	22178	1415862_at	10.82	31	2.86	2.05E-02	Tyrp1, tyrosinase-related protein 1
13	69564	1453898_at	80.53	230.63	2.86	2.75E-02	Itgb1bp3, integrin beta 1 binding protein 3
14	407805	1451856_at	58.62	165.07	2.82	8.39E-03	BC006835, cDNA sequence BC006835
15	12326	1438960_at	4.38	11.99	2.73	8.39E-03	Camk4, calcium/calmodulin-dependent protein kinase IV
16	77391	1443648_at	5.1	13.92	2.73	1.38E-02	9530003O04Rik, RIKEN cDNA 9530003O04 gene
17	70632	1453210_at	7.39	18.82	2.54	1.74E-02	5730507C01Rik, RIKEN cDNA 5730507C01 gene
18	70164	1454639_x_at	14.71	37.04	2.52	3.66E-02	2210411K19Rik, RIKEN cDNA 2210411K19 gene
19	14376	1437812_x_at	21.42	53.85	2.51	0.00E+00	Ganab, alpha glucosidase 2 alpha neutral subunit
20	223332	1455707_at	11.04	27.71	2.51	1.38E-02	C130037N17Rik, RIKEN cDNA C130037N17 gene
21	74399	1430931_at	7.35	18.42	2.5	0.00E+00	4933403O03Rik, RIKEN cDNA 4933403O03 gene
22	69434	1447993_a_at	23.57	58.59	2.49	3.66E-02	1700026B20Rik, RIKEN cDNA 1700026B20 gene
23	97091	1442976_at	18.82	46.29	2.46	0.00E+00	C81072, expressed sequence C81072
24	74211	1447063_at	18.4	45.13	2.45	1.38E-02	1700017B05Rik, RIKEN cDNA 1700017B05 gene
25	52677	1459491_at	15.65	38.38	2.45	1.38E-02	D12Ertd673e, DNA segment, Chr 12, ERATO Doi 673, expressed
26	20912	1435058_x_at	20.39	49.98	2.45	8.39E-03	Stxbp3, syntaxin binding protein 3
27	12912	1421583_at	28.83	70.36	2.44	1.38E-02	Creb1, cAMP responsive element binding protein 1
28	55987	1460248_at	201.09	489.11	2.43	0.00E+00	Cpxm2, carboxypeptidase X 2 (M14 family)
29	272031	1446557_at	12.43	30.21	2.43	8.39E-03	E130309F12Rik, RIKEN cDNA E130309F12 gene

continued

TABLE 3. CONTINUED

Rank	*Gene ID*	*Probe Set ID*	*Tg-Trx1*	*NTg*	*FC*	*q Value*	*Annotation*
30	75169	1454488_at	11.89	28.76	2.42	8.39E-03	4930534I15Rik, RIKEN cDNA 4930534I15 gene
31	14683	1453413_at	13.02	31.39	2.41	2.05E-02	Gnas, GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus
32	73086	1437625_at	5.17	12.45	2.41	1.07E-02	Rps6ka5, ribosomal protein S6 kinase, polypeptide 5
33	18751	1438981_at	4.29	10.32	2.41	8.39E-03	Prkcb1, protein kinase C, beta 1
34	17427	1419402_at	6.4	15.35	2.4	1.38E-02	Mns1, meiosis-specific nuclear structural protein 1
35	71069	1441953_at	19.05	45.48	2.39	1.74E-02	Stox2, storkhead box 2
36	15560	1435513_at	9.27	22.11	2.38	4.90E-03	Htr2c, 5-hydroxytryptamine (serotonin) receptor 2C
37	71990	1447882_x_at	16.31	38.8	2.38	2.75E-02	Ddx54, DEAD (Asp-Glu-Ala-Asp) box polypeptide 54
38	23984	1439618_at	9.84	23.41	2.38	2.05E-02	Pde10a, phosphodiesterase 10A
39	67125	1455853_x_at	23.31	55.19	2.37	4.90E-03	Sas, sarcoma amplified sequence
40	19289	1444981_at	36.04	85.26	2.37	0.00E+00	Punc, putative neuronal cell adhesion molecule
41	64335	1417993_at	13.28	31.2	2.35	4.90E-03	Svs3, seminal vesicle secretion 3
42	70383	1459977_x_at	20.78	48.8	2.35	0.00E+00	Cox10, COX10 homologue, cytochrome *c* oxidase assembly protein, heme A: farnesyltransferase (yeast)
43	16011	1422313_a_at	123.41	289.26	2.34	1.74E-02	Igfbp5, insulin-like growth factor binding protein 5
44	234683	1434489_at	10.36	24.12	2.33	1.74E-02	Elmo3, engulfment and cell motility 3, ced-12 homolog (*C. elegans*)
45	100782	1444675_at	12.37	28.8	2.33	1.07E-02	AL023051, expressed sequence AL023051
46	12287	1447511_at	14.48	33.61	2.32	4.90E-03	Cacna1b, calcium channel, voltage-dependent, N type, alpha 1B subunit
47	99890	1440336_at	15.34	35.4	2.31	8.39E-03	Hrmt1l6, HMT1 hnRNP methyltransferase-like 6 (*S. cerevisiae*)
48	74662	1433344_at	35.09	80.91	2.31	4.90E-03	4930448K20Rik, RIKEN cDNA 4930448K20 gene
49	19046	1456462_x_at	253.41	579.68	2.29	0.00E+00	Ppp1cb, protein phosphatase 1, catalytic subunit, beta isoform
50	50791	1440807_at	3.31	7.53	2.28	3.66E-02	Magi2, membrane associated guanylate kinase, WW and PDZ domain containing 2

Gene ID, ID from the NCBI Gene database; Probe Set ID, Affymetrix probe set ID; Tg-Trx1, the median-intensity value of Tg-Trx1 group; NTg, the median-intensity value of NTg group; FC, fold change of the median-intensity (Tg-Trx1 vs. NTg); q value, q values from SAM analysis; Annotation, gene annotation from the NCBI Gene database.

TABLE 4. SIGNIFICANT KEGG PATHWAYS

Pathway ID	*Gene*	*On-chip*	*Up*	*Down*	*p Value*	*KEGG name*
Upregulated pathway						
3010	84	79	63	6	1.15E-23	Ribosome
190	115	99	70	3	5.04E-21	Oxidative phosphorylation
20	24	21	15	2	1.11E-03	Citrate cycle (TCA cycle)
710	20	14	10	—	4.20E-02	Carbon fixation
Downregulated pathway						
4060	240	96	7	24	3.62E-02	Cytokine-cytokine receptor interaction
4080	278	70	3	20	1.72E-02	Neuroactive ligand-receptor interaction

Pathways with p value <0.05, after Bonferroni correction, were selected.

Pathway ID, identification number in the KEGG database; Gene, number of genes linked to the KEGG pathway; On-chip, number of genes measured by microarray and linked to a particular KEGG pathway; Up, number of genes significantly upregulated in the Tg-Trx1 group; Down, number of significantly downregulated genes in the Tg-Trx1 group; p value, p value derived from statistical tests based on the hypergeometric distribution and adjusted by the Bonferroni method.

regulated entries were identified. Interestingly, entries related to mitochondria were selected in all of the GO categories (*i.e.,* Biological Process, Molecular Function, and Cellular Component). More significantly, both analyses identified genes associated with oxidative phosphorylation and the TCA cycle to be coordinately upregulated (Tables 4 and 5). For example, in complex III and IV of oxidative phosphorylation, upregulation was observed for most genes examined in this study (Fig. 2). In terms of the TCA cycle, many genes are upregulated (Fig. 3). Consistent with these findings, we identified PGC-1α, a coactivator of the nuclear respiratory factor (NRF) and an inducer of mitochondrial proteins (17), as one of the top 50 upregulated genes in Tg-Trx1 (Table 3).

Expression of genes involved in the mitochondrial oxidative phosphorylation and TFAM is increased in Tg-Trx1 hearts

We examined whether components of oxidative phosphorylation are really upregulated in Tg-Trx1 hearts, using immunoblot analyses. Protein expression of cytochrome *c*, COX IV, and COX Vb, components of the mitochondrial oxidative phosphorylation pathway and the genes found to be upregulated in the present microarray analysis (Fig. 2), was significantly greater in Tg-Trx1 than in NTg (Fig. 4A). Furthermore, a qPCR analysis demonstrated that mRNA levels of TFAM, a mitochondrial transcription/replication factor and a known NRF1 target (17), were significantly increased in Tg-Trx1 (Fig. 4B). This observation raises a possibility that the copy number of mitochondrial DNA (mtDNA) is increased in Tg-Trx1 hearts. A ratio of mtDNA to genomic DNA was significantly increased in Tg-Trx1 hearts (Fig. 4C).

Mitochondrial enzymatic activity and ATP content are increased in Tg-Trx1 hearts

We measured the activity of two mitochondrial enzymes, citrate synthase and cytochrome *c* oxidase, using mitochondrial fractions prepared from Tg-Trx1 and NTg hearts. Consistent with the result of the pathway analysis, the enzymatic activity of these enzymes was significantly enhanced in Tg-Trx1 hearts (Fig. 5A). The total ATP content in the heart was significantly greater in Tg-Trx1 than in NTg (Fig. 5B).

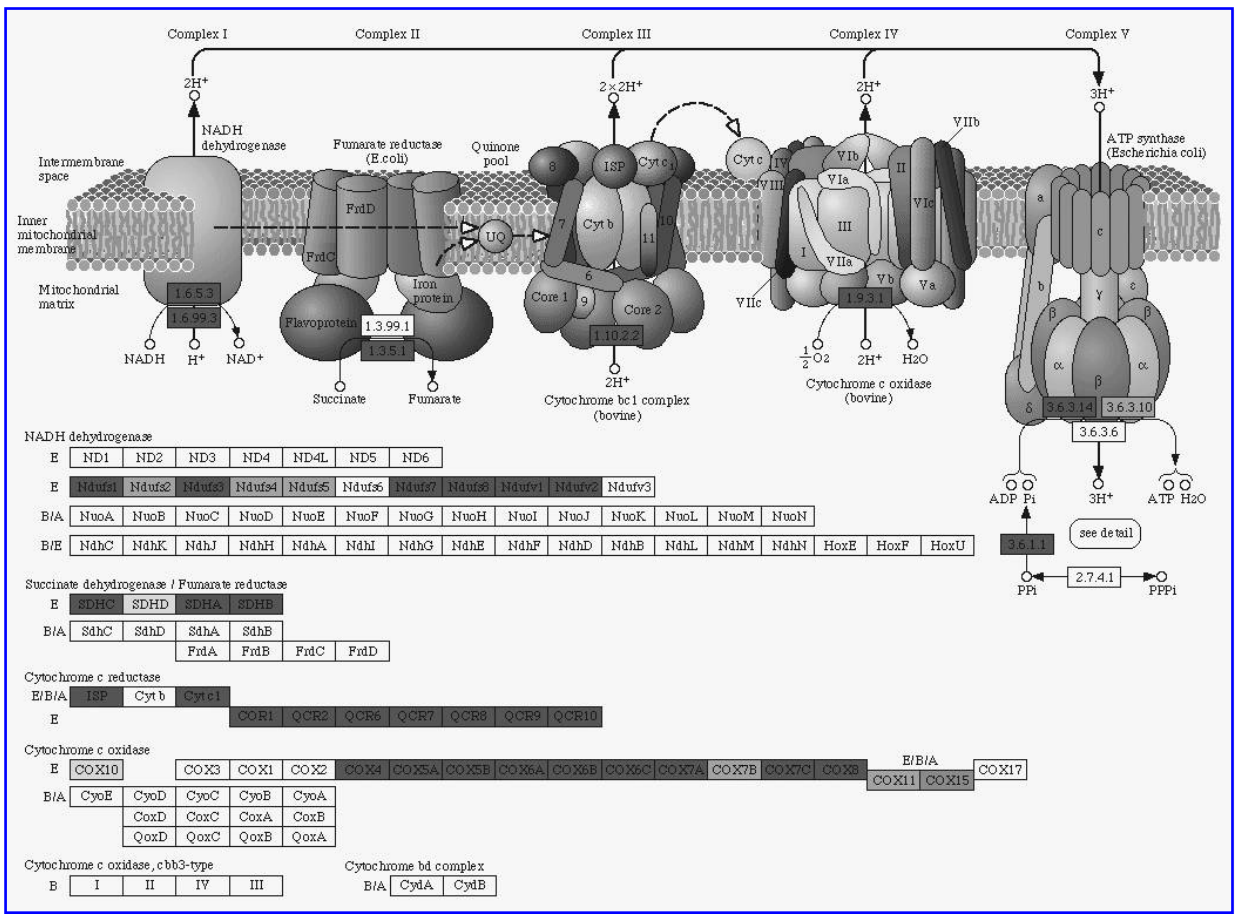

FIG. 2. Genes regulated in oxidative phosphorylation. Genes whose products are involved in the oxidative phosphorylation pathway (annotated by KEGG) are presented in color to reflect their regulation of expression. Red, genes significantly upregulated (p value <5 % and fold change >1.2) in the Tg-Trx1 group; green, genes significantly downregulated in the Tg-Trx1 group; yellow, genes with multiple probe sets in which some are upregulated and some are downregulated in the Tg-Trx1 group; gray, genes that are not significantly regulated in expression; white, genes that have no probes on the microarray. The following numbers are used in the graph to represent gene products: EC:1.6.5.3 and EC:1.6.99.3, NADH dehydrogenase; EC:1.3.5.1, succinate dehydrogenase; EC:1.10.2.2, ubiquinol–cytochrome *c* reductase; EC:1.9.3.1, cytochrome *c* oxidase; E3.6.3.14, H^+-transporting two-sector ATPase; EC:3.6.1.1, inorganic diphosphatase.

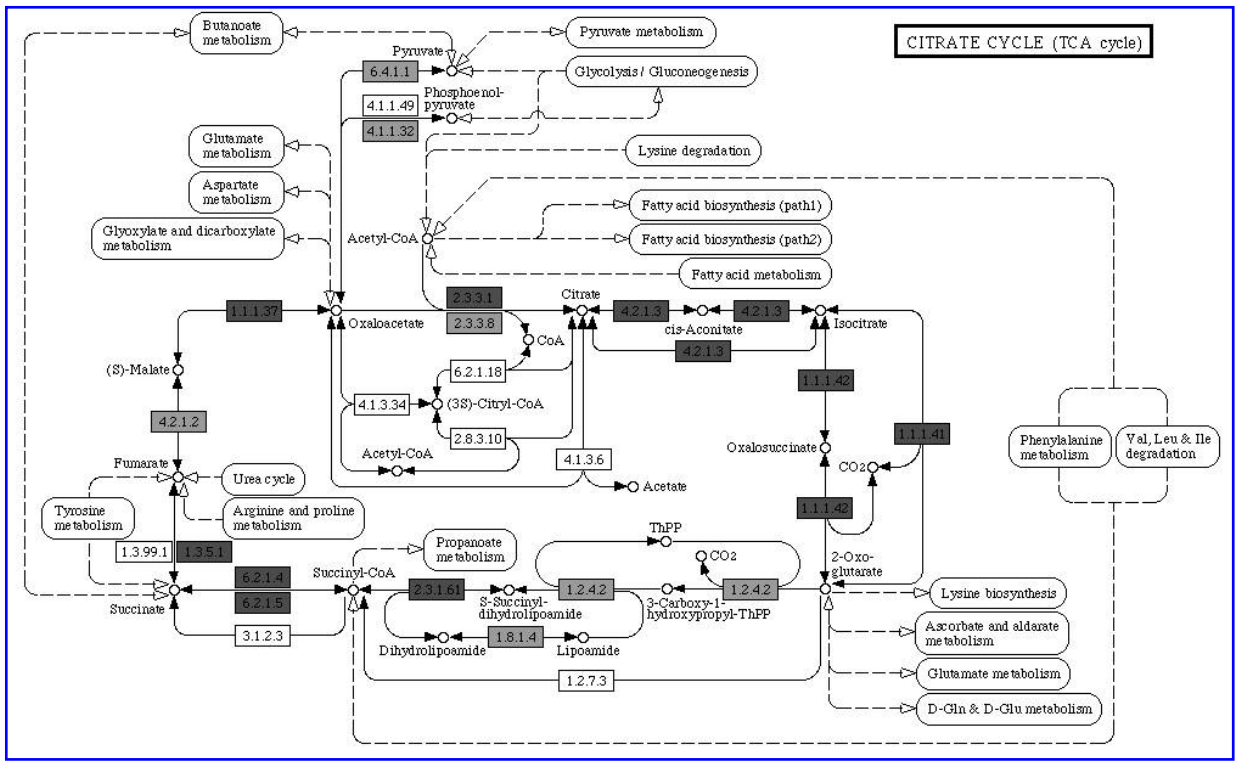

FIG. 3. Genes regulated in the TCA cycle. Genes whose products are involved in the TCA cycle (annotated by KEGG) are presented in color to reflect their regulation of expression. Colors are used in the same way as in supplemental Fig. 2. E2.3.1.61, succinyl transferase; EC:6.2.1.4, succinyl-CoA ligase (GDP forming); EC:6.2.1.5, succinyl-CoA ligase (ADP forming); EC:1.1.1.37, malate dehydrogenase; EC:1.1.1.24, quinate dehydrogenase; EC:1.1.1.41, isocitrate dehydrogenase (NAD^+); EC:1.3.5.1, succinate dehydrogenase (ubiquinone); EC:4.2.1.3, aconitate hydratase.

Identification of transcription factors possibly regulated by Trx1, using TFBS analysis

We further extended our bioinformatic analysis and examined the promoter region of genes whose expression was regulated in Tg-Trx1. We focused on the near promoter region (−700/+300 nt) and the far promoter region (−3,500/+1,500 nt), which both surround the transcriptional start site. Using the TRANFAC database (version 9), which contains a collection of TFBSs, we searched these regions for candidate TFBSs, proposing that genes that are either upregulated or downregulated by a transcription factor would have the corresponding TFBSs enriched in their promoter regions. Statistical analysis showed that a number of TFBSs are significantly associated with genes upregulated or downregulated in Tg-Trx1. Of these, the NRF1 binding site was identified in both the −700/+300 nt (Table 6) and the −3,500/+1,500 nt region (Table 7), highlighting its significance. In addition, the analysis demonstrated that the binding sites of several stress-inducible factors, such as CREB and HIF-1, were associated with upregulated genes, whereas NFκB and AP-1, cardiac hypertrophy–related factors, were not in the list of possibly upregulated transcription factors (Tables 6 and 7).

Trx1 enhances NRF transcriptional activity

To verify the results from TFBS analysis, we examined expression levels and transcriptional activity of NRFs. qPCR analysis showed that NRF1 had higher mRNA expression levels than NRF2 in mouse hearts (50–100-fold). mRNA expression of NRF1 was significantly increased in Tg-Trx1 hearts compared with NTg. Expression of NRF2 was not significantly different between Tg-Trx1 and NTg (Fig. 6A). Immunoblot analyses showed that PGC-1α was significantly upregulated in Tg-Trx1 hearts (Fig. 6B). We examined the transcriptional activity of NRFs using luciferase assays in cultured cardiac myocytes *in vitro*. Consistent with the result of the TFBS analysis, the transcriptional activity of NRF1 and NRF2 was significantly enhanced by Trx1 overexpression (Fig. 6C).

DISCUSSION

Our study demonstrated that, when a 1.2-fold change was used as a cutoff, a greater number of genes are upregulated, as opposed to downregulated, whereas an opposite trend was observed when a 1.4-fold changed was used in Tg-Trx1 (Table 2). Trx1 generally activates DNA binding of a number of transcription factors, including NF-κB and AP-1 (22). Thus, it is surprising to see that upregulation of genes predominates only at the level of modest changes (20–40%). We have previously shown that Trx1 has antihypertrophic actions in cardiac myocytes (36). Whether or not the genes with significant changes that we identified are involved in the antihypertrophic action of Trx1 remains to be elucidated. Because Trx1 exhibits growth-promoting effects in some cell types, including cancer cells (5), the effect of Trx1 on cell-growth responses

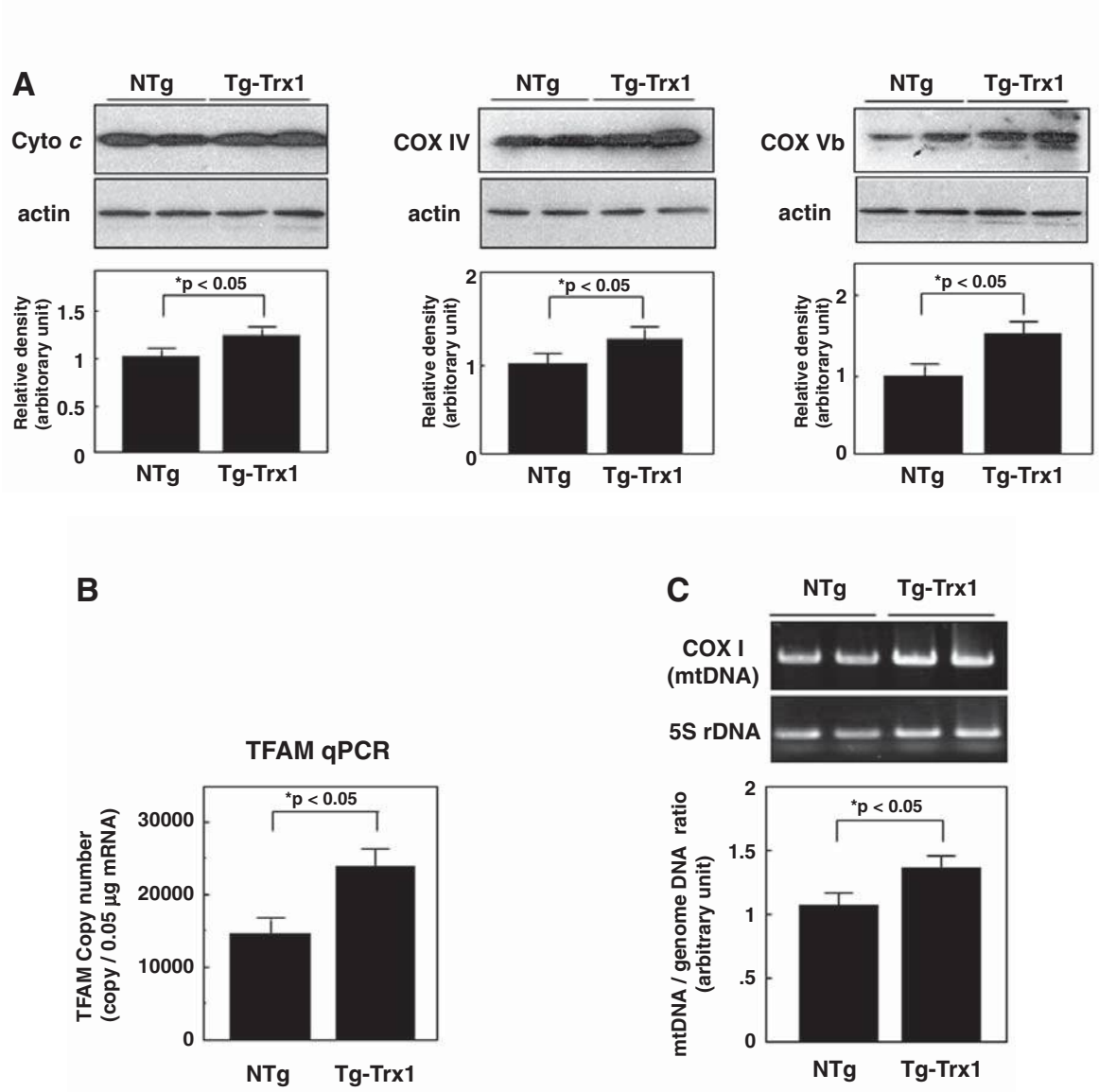

FIG. 4. Expression of genes involved in the mitochondrial oxidative phosphorylation and TFAM. **(A)** Immunoblot analyses of cytochrome *c*, COX IV, and COX Vb. Homogenates were prepared from mouse hearts and equal amounts of protein (10 µg for cytochrome *c*, COX IV and actin, and 30 µg for COX Vb) were loaded onto SDS/PAGE. The protein abundance was analyzed densitometrically and standardized with the level of actin (cytochrome *c* and COX IV, $n = 10$; COX Vb, $n = 6$). Representative immunoblots are shown. (**B**) Quantitative analysis of TFAM mRNA. qPCR was done using 0.05 µg total RNA (RT-products) in each sample ($n = 6$). Values are standardized by those of GAPDH. (**C**) Genomic DNA was prepared from mouse hearts. Mitochondrial COX I and 5S ribosomal DNA genes (internal control) were amplified by PCR. The products were loaded on 1.2% agarose gels and visualized with ethidium bromide. Top: Representative data are shown. Bottom: After each band was densitometrically measured, a ratio of COX I to 5S rDNA was calculated ($n = 6$).

seems to be cell-type specific (38). Therefore, we infer that the suppressive effects of Trx1 on the gene-expression profile could also be cell-type specific.

Although more genes are downregulated than upregulated at high-fold changes in Tg-Trx1, the pathway analyses indicated that expression of genes involved in oxidative phosphorylation and the TCA cycle, which are critical components for ATP synthesis, is coordinately upregulated in Tg-Trx1. Upregulation of these genes was generally modest but statistically significant. Consistent with the result of the pathway analyses, protein expression of the components of oxidative phosphorylation and TFAM was upregulated in Tg-Trx1. Enhanced activity of mitochondrial enzymes and ATP content in Tg-Trx1 hearts also suggest that Trx1 systematically upregulates the mitochondrial proteins involved in ATP synthesis in the mouse heart.

Expression of mitochondrial proteins involved in oxidative phosphorylation and the TCA cycle is downregulated in the mouse heart in response to angiotensin II stimulation (20). Ischemia–reperfusion and other stresses increase oxidative stress, thereby causing mitochondrial dysfunction (14, 28). Because oxidative stress and mitochondrial dysfunction stimulate one

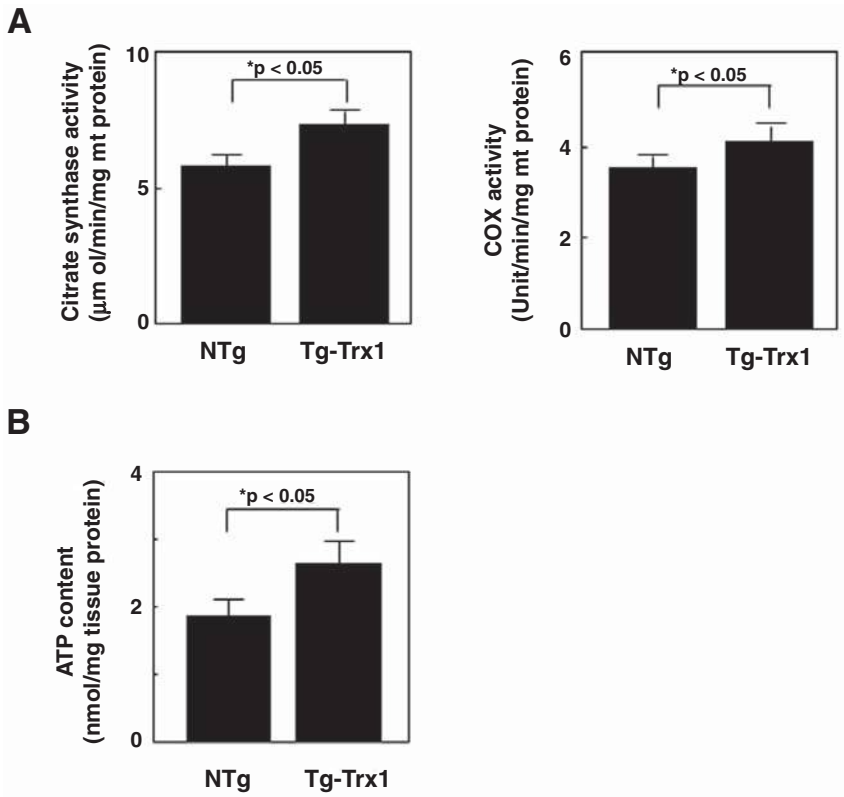

FIG. 5. Mitochondrial enzymatic activity and ATP content in mouse hearts. (A) Mitochondrial fractions were prepared from mouse hearts and lysed with CelLytic M Lysis Reagent. Lysates containing equal amounts of protein were used for measurement of the citrate synthase and cytochrome *c* oxidase activity ($n = 8$). **(B)** ATP content in mouse hearts. Homogenates prepared from mouse hearts equivalent to 300 µg protein were used for ATP measurement ($n = 8$).

TABLE 5. SIGNIFICANT GENE ONTOLOGY TERMS

GO ID	*Gene*	*On-chip*	*Up*	*Down*	*p Value*	*GO name*
				Biological process		
				Upregulated GO		
GO:0006091	427	289	115	34	2.48E-14	Generation of precursor metabolites and energy
GO:0006412	491	374	131	42	2.27E-11	Protein biosynthesis
GO:0006092	84	63	36	7	3.20E-08	Main pathways of carbohydrate metabolism
GO:0046356	23	23	19	2	1.91E-07	Acetyl-CoA catabolism
GO:0009059	544	409	139	44	5.14E-11	Macromolecule biosynthesis
GO:0006099	22	22	18	2	8.63E-07	Tricarboxylic acid cycle
GO:0009060	22	22	18	2	8.63E-07	Aerobic respiration
GO:0044249	826	602	181	73	2.18E-09	Cellular biosynthesis
GO:0006118	286	182	64	25	2.18E-04	Electron transport
GO:0007582	10264	5420	1114	743	4.22E-08	Physiological process
GO:0045333	25	25	19	2	2.61E-06	Cellular respiration
GO:0042773	11	10	10	-	1.97E-04	ATP synthesis -coupled electron transport
GO:0009058	928	678	193	84	8.81E-08	Biosynthesis
GO:0006084	33	32	22	2	2.67E-06	Acetyl-CoA metabolism
GO:0006119	40	37	24	-	2.75E-06	Oxidative phosphorylation
GO:0050875	7726	5014	1052	670	7.53E-10	Cellular physiological process
GO:0007028	107	90	39	8	1.74E-04	Cytoplasm organization and biogenesis
GO:0008152	5582	3758	809	483	2.29E-07	Metabolism
GO:0019538	2327	1605	368	203	1.37E-03	Protein metabolism
GO:0042254	96	83	34	7	6.29E-03	Ribosome biogenesis and assembly
GO:0006120	8	7	7	-	3.17E-02	mitochondrial electron transport, NADH to Ubiquinone
GO:0006810	2129	1314	307	170	3.05E-03	Transport
GO:0007046	87	77	32	7	8.78E-03	Ribosome biogenesis
GO:0044267	2312	1594	365	203	1.82E-03	Cellular protein metabolism
				Downregulated GO		
GO:0050877	1537	134	9	38	4.24E-02	Neurophysiological process

continued

TABLE 5. CONTINUED

GO ID	Gene	On-chip	Up	Down	p Value	GO name
				Molecular function		
				Upregulated GO		
GO:0003735	196	176	92	17	7.28E-21	Structural constituent of ribosome
GO:0005198	548	349	140	41	1.85E-18	Structural molecule activity
GO:0015077	132	107	65	1	9.38E-19	Monovalent inorganic cation transporter activity
GO:0015078	127	105	63	1	1.08E-17	Hydrogen ion transporter activity
GO:0005386	382	236	95	24	9.14E-12	Carrier activity
GO:0008137	37	35	27	-	2.85E-10	NADH dehydrogenase (ubiquinone) activity
GO:0003954	37	35	27	-	2.85E-10	NADH dehydrogenase activity
GO:0015075	607	305	107	33	7.38E-09	Ion transporter activity
GO:0008324	457	254	91	28	9.81E-08	Cation transporter activity
GO:0005215	1263	699	191	91	5.05E-06	Transporter activity
GO:0016651	57	53	32	1	6.18E-08	oxidoreductase activity, acting on NADH or NADPH
GO:0005489	130	100	43	10	4.67E-05	Electron transporter activity
GO:0016491	623	390	121	46	2.62E-06	Oxidoreductase activity
GO:0004129	18	15	13	-	8.83E-05	Cytochrome- *c* oxidase activity
GO:0008121	8	8	8	-	5.84E-03	Ubiquinol-cytochrome -*c* reductase activity
GO:0046873	71	63	32	2	2.58E-05	Metal ion transporter activity
				Downregulated GO		
GO:0004871	3281	892	112	178	5.83E-04	Signal transducer activity
GO:0004888	1971	273	25	68	3.40E-03	Transmembrane receptor activity
GO:0004930	1668	131	6	38	2.36E-02	G- protein –coupled receptor activity
				Cellular component		
				Upregulated GO		
GO:0005739	737	588	239	57	2.33E-35	Mitochondrion
GO:0005840	167	153	84	17	9.89E-21	Ribosome
GO:0005830	48	44	34	4	1.33E-13	Cytosolic ribosome (sensu Eukaryota)
GO:0005737	2922	2178	609	230	3.18E-34	Cytoplasm
GO:0005746	26	22	19	1	4.01E-08	Mitochondrial electron transport chain
GO:0019866	133	111	56	7	7.30E-11	Inner membrane
GO:0015935	31	28	21	3	4.78E-07	Small ribosomal subunit
GO:0005740	142	114	56	8	3.18E-10	Mitochondrial membrane
GO:0005843	14	12	11	2	3.62E-04	Cytosolic small ribosomal subunit (sensu Eukaryota)
GO:0030529	363	295	109	30	8.59E-11	Ribonucleoprotein complex
GO:0043234	1706	1159	318	141	1.37E-12	Protein complex
GO:0005743	110	89	43	6	5.12E-07	Mitochondrial inner membrane
GO:0005622	6077	4232	944	527	1.33E-16	Intracellular
GO:0030016	56	38	21	5	1.73E-03	Myofibril
GO:0005623	10601	5763	1163	786	3.17E-06	Cell
GO:0030017	52	34	20	4	8.14E-04	Sarcomere
GO:0045259	19	17	12	-	1.63E-02	Proton-transporting ATP synthase complex
GO:0043231	4562	3268	701	403	5.89E-05	Intracellular membrane-bound organelle
GO:0043292	59	41	21	5	9.13E-03	Contractile fiber
GO:0005829	261	214	72	16	2.57E-04	Cytosol

GO entries were selected by statistical tests based on the hypergeometric distribution. Entries with p values <0.05, after Bonferroni correction (either upregulated or downregulated) were selected. Of the 12,311 genes measured by the microarray, 8,393 of them have GO entry annotations according to the NCBI LocusLink database. There are 1,933 genes significantly upregulated and 1,870 genes significantly downregulated. See Table 4 for descriptions of Gene, On-chip, Up, Down, and p Value.

another, and eventually lead to heart failure, a modality to preserve the mitochondrial function may inhibit the progression of heart failure by disrupting this vicious cycle. Trx1 plays a protective role against ischemia–reperfusion injury (30, 32). Ischemic preconditioning induces expression of Trx1, which may in turn mediate upregulation of mitochondrial proteins. In a murine model of adriamycin-induced cardiotoxicity, overexpression of Trx1 attenuates morphologic changes in the heart, including swelling of mitochondria, by inhibiting the formation of hydroxy radical and protein oxidation (26). Trx1 is upregulated in human heart failure, where the extent of Trx1 upregulation is proportional to the severity of heart failure (19). Because the function of mitochondria is decreased in failing hearts (14), Trx1 may be upregulated to compensate for the mitochondrial dysfunction. Taken together, Trx1 not only acts as an antioxidant but also enhances the mitochondrial function by inducing expression of mitochondrial proteins under pathologic insults.

What is the mechanism mediating the upregulation of mitochondrial genes by Trx1? Our results suggest that protein expression of NRF1 and PGC-1α, major inducers of mitochon-

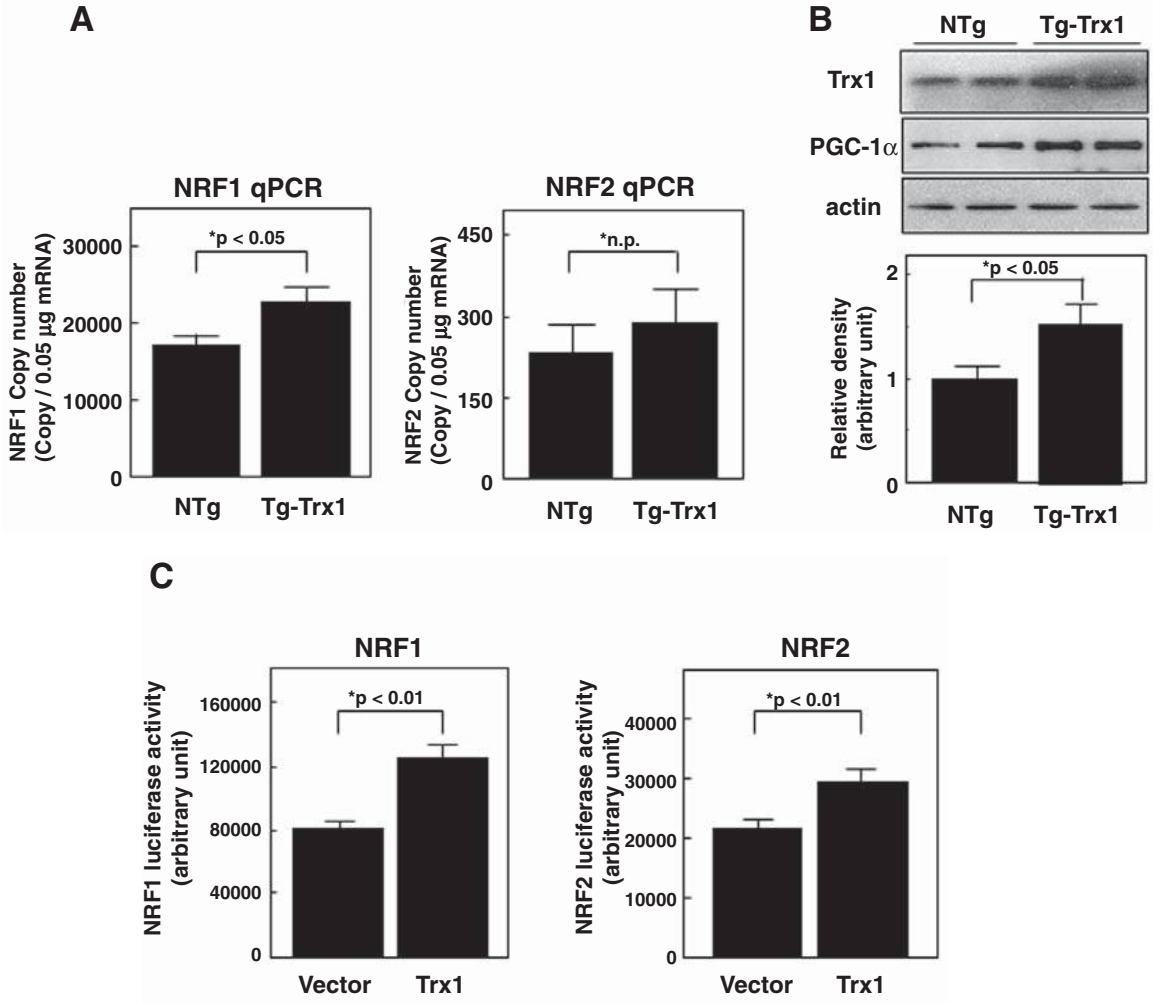

FIG. 6. Expression and transcriptional activity of NRF1, NRF2, and PGC-1α **(A)** Quantitative analysis of NRF1 and NRF2 mRNAs. Total RNA was prepared from mouse hearts, and qPCR was done using RT-products with 0.05 μg total RNA in each sample ($n = 6$). Values are standardized by those of GAPDH. **(B)** Immunoblot analysis of PGC-1α. Homogenates were prepared from mouse hearts and equal amounts of protein (10 μg for Trx1 and actin, and 30 μg for PGC-1α) were loaded onto SDS/PAGE. The protein abundance was densitometrically analyzed and standardized with the level of actin ($n = 8$). Representative immunoblots are shown. **(C)** NRF luciferase activity. Cardiac myocytes were cultured in six-well plates and co-transfected with the NRF1- or NRF2-luciferase vector and either pcDNA3.1 empty vector or pcDNA3.1-Trx1. Thirty-six hours after transfection, cells were lysed, and luciferase assays were done (NRF1, $n = 15$; NRF2, $n = 12$).

drial proteins, is increased in Tg-Trx1, and that the transcriptional activity of NRF1 and NRF2 is upregulated in cardiac myocytes under Trx1 overexpression. Although protein expression of NRF2 was not significantly upregulated in Tg-Trx1, its transcriptional activity was likely to be enhanced due to increased expression of PGC-1α, because NRFs and PGC-1α function cooperatively to induce expression of mitochondrial proteins (17, 35). At the present time, the involvement of other transcription factors, such as HIF-1, Krox, and E2F-1, in upregulation of mitochondrial proteins in Tg-Trx1, remains to be elucidated.

The mechanism by which Trx1 upregulates PGC-1α and NRFs expression levels has yet to be clarified. The TFBS analysis suggested that CREB, a key transcription factor mediating transcription of PGC-1α (11), is upregulated in Tg-Trx1. It is reported that Trx1 enhances CREB activity in the heart (6). Thus, CREB may mediate upregulation of PGC-1α in Tg-Trx1 (11). Furthermore, PGC-1α is a potent inducer of NRF1 and NRF2 (35). Nuclear translocation of NRF1 is promoted through phosphorylation of NRF1 by the PI3K/Akt pathway (24). Because Trx1 binds directly to, and inhibits PTEN, a lipid phosphatase attenuating PI3K activation (21), nuclear translocation of NRF1 in Tg-Trx1 may possibly be enhanced through this mechanism. In addition, DNA binding activity of NRFs is enhanced by Trx1 (10). Thus, Trx1 may increase the transcriptional activity of NRFs by both transcriptional upregulation and posttranslational modification.

Overexpression of Trx1 in the mouse heart alters the intracellular redox state, which secondarily affects expression of other antioxidant molecules (36). Thus, the observed changes in gene expression in the Tg-Trx1 heart may also be mediated indirectly by other antioxidant molecules, such as glutathione. Further studies are required to elucidate how Trx1

Table 6. Transcription Factor Binding Sites in the −700 to +300nt Region That Are Significantly Associated with Regulated Genes

Matrix	*Gene*	*On-chip*	*Up*	*Down*	*p Value*	*TFBS*
			Associated with upregulated genes			
V$ATF1_Q6	1169	772	172	100	1.04E-02	ATF-1
V$E2F1_Q3_01	**5871**	**4198**	**796**	**560**	**1.09E-02**	**E2F-1**
V$USF_Q6	9225	5706	1050	794	1.26E-02	USF
V$MAZR_01	5977	3909	743	551	1.28E-02	MAZR
V$CREB_Q2_01	4606	3159	615	420	1.47E-02	CREB
V$E2F1_Q4	**6959**	**4809**	**891**	**664**	**3.40E-02**	**E2F-1**
V$HIF1_Q5	**1678**	**1150**	**237**	**165**	**3.56E-02**	**HIF-1**
V$E2F_Q3_01	5720	4000	747	556	3.77E-02	E2F
V$CREB_Q4_01	4485	3073	585	399	3.93E-02	CREB
V$WHN_B	**3422**	**2369**	**460**	**319**	**4.00E-02**	**Whn**
V$NFMUE1_Q6	1491	1003	208	131	4.14E-02	NF-muE1
V$LXR_DR4_Q3	94	45	17	5	4.20E-02	LXR direct repeat 4
V$CREBATF_Q6	3569	2471	476	315	4.25E-02	CREBATF
V$E2F1_Q4_01	**3760**	**2638**	**505**	**378**	**4.27E-02**	**E2F-1**
V$CREB_Q4	1369	991	205	129	4.41E-02	CREB
V$NRF1_Q6	**904**	**734**	**156**	**112**	**4.81E-02**	**Nrf-1**
V$ATF4_Q2	3428	2189	423	300	4.87E-02	ATF4
V$MAZ_Q6	11658	7263	1298	1022	4.90E-02	MAZ
			Associated with downregulated genes			
V$SOX9_B1	**5496**	**2541**	**415**	**429**	**9.05E-03**	**SOX-9**
V$TAL1_Q6	4345	2256	373	380	1.06E-02	TAL1
V$SOX5_01	**4446**	**2031**	**325**	**348**	**1.12E-02**	**Sox-5**
V$CDX2_Q5	9177	4559	762	721	1.24E-02	Cdx-2
V$FOXJ2_02	2893	1182	174	209	2.49E-02	FOXJ2
V$POU1F1_Q6	**3904**	**1657**	**263**	**284**	**2.64E-02**	**POU1F1**
V$NRSF_01	23	4	-	4	2.50E-02	NRSF

Entries in bold are also found using the –3,500/+1,500nt promoter region. Some TFBSs, such as several CREB-related sites, are similar to one another because of similar matrices.

Matrix, the TFBS matrix name from the TRANSFAC database; Gene, number of genes that have the TFBS in their promoter regions (−700/+300nt); On-chip, number of genes in the gene group measured by microarray; Up, number of significantly upregulated genes in the On-chip group; Down, number of significantly downregulated genes in the On-chip group; *p* value, *p* values from the hypergeometric tests adjusted by the Benjamin and Hochberg method; TFBS, name of transcription factor binding site.

Table 7. Transcription Factor Binding Sites in the −3,500 to +1,500 nt Region that are Significantly Associated with Regulated Genes

Matrix	*Gene*	*On-chip*	*Up*	*Down*	*p Value*	*TFBS*
			Associated with upregulated genes			
V$PAX5_01	4417	2694	539	374	2.41E-03	BSAP
V$WHN_B	**7137**	**4543**	**858**	**631**	**6.85E-03**	**Whn**
V$PAX9_B	8766	5126	952	735	1.37E-02	Pax-9
V$KROX_Q6	10985	7100	1284	955	1.48E-02	KROX
V$E2F1_Q4	**11171**	**7117**	**1284**	**1001**	**1.81E-02**	**E2F-1**
V$NRF1_Q6	**1303**	**981**	**208**	**144**	**2.77E-02**	**Nrf-1**
V$HIF1_Q5	**4335**	**2747**	**527**	**393**	**4.63E-02**	**HIF-1**
V$SREBP_Q3	11633	6500	1176	947	4.66E-02	SREBP
			Associated with downregulated genes			
V$POU1F1_Q6	**14249**	**7838**	**1321**	**1178**	**1.91E-02**	**POU1F1**
V$CHOP_01	11785	6388	1054	979	2.33E-02	CHOP
V$SOX9_B1	**15448**	**8579**	**1474**	**1269**	**2.59E-02**	**SOX-9**
V$TEF_Q6	13315	7349	1231	1106	2.64E-02	TEF
V$GATA2_02	13006	7097	1188	1071	2.73E-02	GATA-2
V$SOX5_01	**14485**	**8023**	**1372**	**1197**	**2.82E-02**	**Sox-5**
V$HSF1_Q6	2242	1209	201	214	2.86E-02	HSF1

Entries in bold are also found by using the −700/+300 nt promoter region.

See Table 6 for descriptions of Matrix, Gene, On-chip, Up, Down, *p* Value, and TFBS.

changes the activity of transcription factors, thereby causing upregulation of mitochondrial genes in the mouse heart.

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ABBREVIATIONS

COX, cytochrome *c* oxidase; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes, mtDNA, mitochondrial DNA; NRF, nuclear respiratory factor; NTg, nontransgenic mice; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1; qPCR, quantitative PCR; SAM, significance analysis of microarrays; TFAM, mitochondrial transcription factor A; TFBS, transcription factor binding site; TSS, transcriptional start site; Tg-Trx1, transgenic mice with cardiac-specific overexpression of Trx1; Trx1, thioredoxin1.

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