

# Divergent transcriptional responses to independent genetic causes of cardiac hypertrophy

BRUCE J. ARONOW,<sup>1</sup> TSUYOSHI TOYOKAWA,<sup>2</sup> AMY CANNING,<sup>2</sup> KOBRA HAGHIGHI,<sup>3</sup>  
ULRIKE DELLING,<sup>4</sup> EVANGELIA KRANIAS,<sup>3</sup> JEFFERY D. MOLKENTIN,<sup>4</sup>  
AND GERALD W. DORN II<sup>2,3</sup>

*Departments of <sup>1</sup>Developmental Biology and <sup>4</sup>Molecular Cardiovascular Biology, Children's Hospital Research Center, Cincinnati 45229; and the <sup>2</sup>Division of Cardiology and <sup>3</sup>Department of Pharmacology and Cell Therapeutics, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0542*

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**Aronow, Bruce J., Tsuyoshi Toyokawa, Amy Canning, Kobra Haghighi, Ulrike Delling, Evangelia Kranias, Jeffery D. Molkentin, and Gerald W. Dorn II.** Divergent transcriptional responses to independent genetic causes of cardiac hypertrophy. *Physiol Genomics* 6: 19–28, 2001.—To define molecular mechanisms of cardiac hypertrophy, genes whose expression was perturbed by any of four different transgenic mouse hypertrophy models [protein kinase C- $\epsilon$  activation peptide ( $\Psi\epsilon$ RACK), calsequestrin (CSQ), calcineurin (CN), and  $G\alpha_q$ ] were compared by DNA microarray analyses using the ~8,800 genes present on the Incyte mouse GEM1. The total numbers of regulated genes (tens to hundreds) correlated with phenotypic severity of the model ( $G\alpha_q > CN > CSQ > \Psi\epsilon$ RACK), but demonstrated that no single gene was consistently upregulated. Of the three models exhibiting pathological hypertrophy, only atrial natriuretic peptide was consistently upregulated, suggesting that transcriptional alterations are highly specific to individual genetic causes of hypertrophy. However, hierarchical-tree and K-means clustering analyses revealed that subsets of the upregulated genes did exhibit coordinate regulatory patterns that were unique or overlapping across the different hypertrophy models. One striking set consisted of apoptotic genes uniquely regulated in the apoptosis-prone  $G\alpha_q$  model. Thus, rather than identifying a single common hypertrophic cardiomyopathy gene program, these data suggest that extensive groups of genes may be useful for the prediction of specific underlying genetic determinants and condition-specific therapeutic approaches.

cardiac hypertrophy; transgenic mouse; gene expression; DNA microarray

HYPERTROPHY is the universal adaptive response to increased cardiac workload, stress, or injury. Since myocardial hypertrophy is a common response to dozens of different stimuli, conventional wisdom holds that it is likely to be the result of a defined set of transcriptional events, including embryonic cardiac mRNAs normally

expressed in a “fetal gene program” (3). Consistent with this paradigm, pressure overload, volume overload, ischemic damage, and other naturally occurring events that stimulate cardiac hypertrophy are associated with increased expression of embryonic cardiac genes such as atrial natriuretic peptide (ANP),  $\beta$ -myosin heavy chain ( $\beta$ MHC), and  $\alpha$ -skeletal actin (2, 6, 10). Increased levels of these mRNAs have also been reported in some artificial forms of cardiac hypertrophy and heart failure created by manipulation of the mouse genome (4, 11, 18). Based on the apparent ubiquity with which these genes are upregulated in hypertrophied and failing ventricles, it has been hypothesized that the products of these genes could contribute mechanistically to these cardiac syndromes, and pathological roles for each have been proposed (15, 16, 25). The logical foundation for assigning pathological significance to hypertrophy-associated gene products relies on the existence of a de facto hypertrophy gene program, i.e., a distinct group of genes that constitute a molecular program encoding the developmental process and/or ultimate features of myocardial hypertrophy. However, progress toward elucidating such a unified molecular program is challenged by recently described genetically modified mouse models of hypertrophy in which reexpression of embryonic cardiac genes can be dissociated from hypertrophy (4, 17, 18, 21, 23). In these genetically induced models, cardiac hypertrophy is transduced through multiple parallel and/or redundant signaling pathways, which suggests that hypertrophy could be encoded through multiple genetic pathways. These phenotypically similar, but pathophysiologically dissimilar genetic hypertrophy models therefore constitute a set of biological reagents uniquely suited for evaluating molecular determinants of myocardial hypertrophy through comparison of differentially expressed genes representing diverse functional groups.

In the current studies, DNA microarrays containing ~8,800 DNA sequences were used to compare gene expression changes in four transgenic mouse models of cardiac hypertrophy as a means of detecting the downstream consequences of a spectrum of hypertrophy

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Address for reprint requests and other correspondence: G. W. Dorn II, Division of Cardiology, Univ. of Cincinnati Medical Center, 231 Albert B. Sabin Way, ML 0542, Cincinnati, OH 45267-0542 (E-mail: [dorngw@ucmail.uc.edu](mailto:dorngw@ucmail.uc.edu)).

signaling events by modified or overexpressed signaling proteins. Rather than defining a single gene program that encodes hypertrophy, the results suggest that hypertrophy-regulated genes are largely specific to the hypertrophy-inducing process. Thus, in contrast to the monolithic hypothesis, the uniqueness of model-specific gene expression profiles suggests that expression profiling can give insight into individual pathophysiological characteristics of each hypertrophy model. In particular, we have identified a novel molecular program for cardiomyocyte apoptosis, as well as a small group of genes that do correlate with disease severity. Moreover, since the cardio-disruptive effects are largely unique to each transgene product, rather than marking a general transgenic overexpression stress response, further cross-comparisons of additional models may allow us to gain predictive capability into the genetic or etiologic origins of hypertrophic cardiomyopathy.

## MATERIALS AND METHODS

**Transgenic models.** The  $G\alpha_q$ -, calcineurin (CN)-, and caldesmon (CSQ)-overexpressing mice and the protein kinase C- $\epsilon$  activation peptide ( $\Psi\epsilon$ RACK) transgenic mouse have each been previously described (4, 17, 18, 21, 23). In each of these mice, the full-length mouse  $\alpha$ -MHC promoter (22) was used to drive cardiomyocyte-specific transgene expression on the FVB/N, or in CN mice B6C3F1, backgrounds. All mice used in the current studies (transgenic and nontransgenic littermates) were 8-wk-old males, except CN, which were 18 days old [because older CN mice progress to heart failure (18)]. Thus, in each case mice were used at an age where hypertrophy had developed without associated heart failure, i.e., in a state of nonfailing or “compensated” myocardial hypertrophy (defined as increased cardiac mass without pulmonary congestion). The mechanism for myocardial hypertrophy has been reported to be cardiomyocyte hypertrophy in  $G\alpha_q$ , CN, and CSQ and to be cardiomyocyte hyperplasia in  $\Psi\epsilon$ RACK (4, 17, 18, 20, 21). Left ventricular mRNA was prepared using Trizol (GIBCO BRL; Life Technologies, Grand Island, NY) and the Oligotex mRNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Total RNA was passed twice through oligo-d(T) columns, and mRNA was stored as ethanolic precipitates at  $-70^\circ\text{C}$ . To minimize variations attributable to individual mice and maximize differences attributable to their genotype, each experiment was performed with RNA pooled from 8–10 ventricles.

**DNA microarray hybridization and analysis.** DNA microarray hybridizations were performed using Incyte mouse gene expression microarray (GEM, version 1.12; Genome Systems, St. Louis, MO). For each model, a microarray was run using poly(A)<sup>+</sup> mRNA from transgenic hearts vs. its corresponding nontransgenic control. Cy3 and Cy5 derivatized cDNA was prepared using random primers and reverse transcriptase. Fluorescent cDNAs were competitively hybridized to the DNA chips. Primary data were examined using Incyte Gemtools software and Silicon Genetics GeneSpring software. Defective cDNA spots (signal/noise ratio  $<2.5$ , irregular geometry, or  $<40\%$  spot area compared with average) were eliminated from the data set of 8,799 sequence tags; 7,808 fit the criteria for inclusion in all four comparative experiments. Data sets were subjected to normalization first for each microarray by multiplication of the Cy5 channel

with a balance coefficient that set its median gene signal value equal to that for the Cy3 channel. The balance coefficient values were 1.07 for  $\Psi\epsilon$ RACK, 0.77 for  $G\alpha_q$ , 0.52 for CSQ, and 1.50 for CN. Genes whose expression increased or decreased in any one of the four transgenic models were pooled into a group of dynamically regulated genes. The selection criterion was gene expression greater than 1.7-fold induced or repressed from the mean of genes within that region of the scattergram, equaling 2 standard deviations removed from the mean. The high correlation value for the bulk of the unchanged genes allowed for relatively fine differences to be detected. Expression pattern clusters were defined by subjecting the  $\log_2$  transformed relative differential expression data set to K-means and hierarchical tree clustering algorithms as implemented in the GeneSpring program (Silicon Genetics). The hierarchical tree analysis was performed using a minimum distance value of 0.001, separation ratio of 0.5, and the standard correlation distance definition. This hierarchical tree structure was used to suggest the optimal group number for K-means clusters, and it was empirically confirmed that the major patterns were detected. The minimal cluster number to accommodate all major patterns was 10, although several clusters were quite similar (example, sets D and E).

**Northern analysis.** Northern blot analysis was performed using standard methodology and formaldehyde/agarose gels in which 3  $\mu\text{g}$  of poly(A)<sup>+</sup> mRNA was loaded per lane. Each lane represented RNA from four to six different hearts. Blots were probed with  $^{32}\text{P}$ -labeled (random priming) cDNAs obtained from Incyte which correspond exactly to the DNA targets on the GEM microarrays. RNA dot blots were performed as previously described (4, 14) using 2  $\mu\text{g}$  total RNA per dot. Blots were probed with  $^{32}\text{P}$ -labeled antisense oligonucleotides specific for the indicated genes (14).

## RESULTS

Cardiac poly(A)<sup>+</sup> RNA from each transgenic model (8–10 ventricles) along with that from paired nontransgenic siblings was simultaneously submitted to Genome Systems for GEM microarray hybridization. The four models studied represent a continuum of hypertrophy phenotypes.  $\Psi\epsilon$ RACK mice, in which a transgenically expressed synthetic octapeptide selectively activates protein kinase C- $\epsilon$  (7), has increased myocardial mass, normal cardiomyocyte size, and normal ventricular function, i.e., “physiological hypertrophy” (17). CSQ mice, in which the calcium binding protein CSQ is overexpressed, have mild ventricular hypertrophy and contractile depression (21), whereas CN and  $G\alpha_q$  overexpressors have more pronounced ventricular hypertrophy and contractile depression (4, 18). Contractile depression in the latter three models classifies them as “pathological hypertrophy” (although in the current studies no mice were studied in a state of heart failure). A standard screening of cardiac gene expression by RNA dot blot analyses demonstrated a range of molecular abnormalities in the four models (Fig. 1A), corresponding to those noted in previously published descriptions (4, 17, 18, 21). Individual DNA microarray results for each of the four transgenic models are shown in Fig. 1B, with hybridization intensity for the transgenic mice plotted vertically, vs. that for the nontransgenic siblings from each model on the horizontal axis. Increased gene expression in the

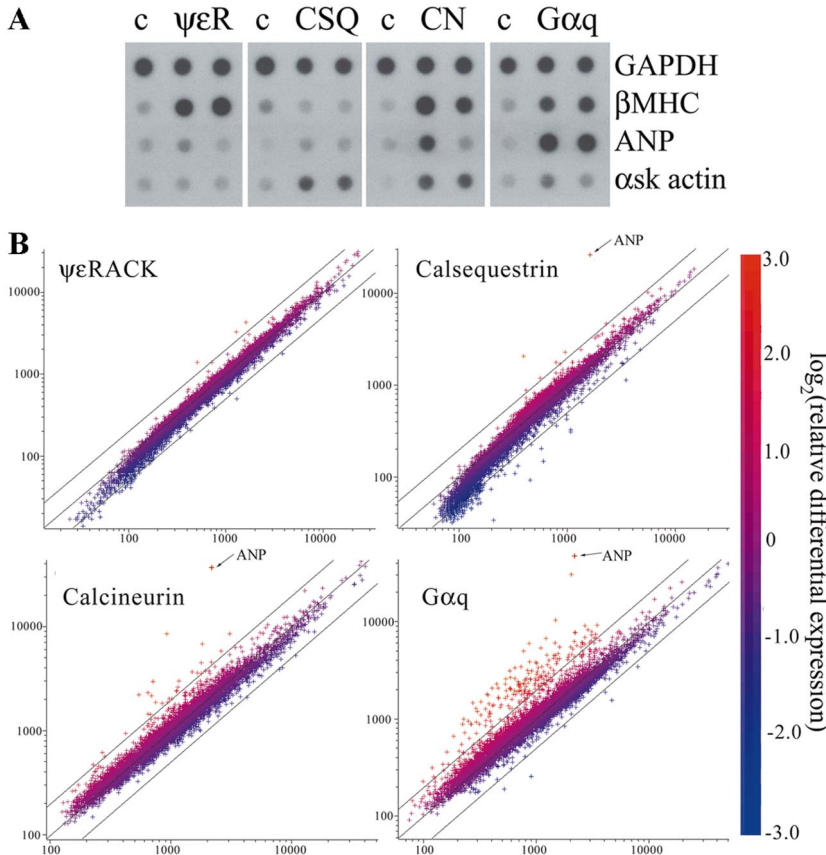


Fig. 1. *A*: gene expression profiles of four transgenic mouse hypertrophy models. RNA dot blot analysis of four transgenic mouse hypertrophy models; c, control;  $\Psi\epsilon R$ ,  $\Psi\epsilon RACK$  (protein kinase C- $\epsilon$  activator); CSQ, calsequestrin; CN, calcineurin; ANP, atrial natriuretic peptide;  $\beta MHC$ ,  $\beta$ -myosin heavy chain;  $\alpha sk$  actin,  $\alpha$ -skeletal actin. *B*: microarray analysis of the four hypertrophy models. For each mouse, hybridization signal intensity is plotted logarithmically on the vertical axis for transgenic and on the horizontal axis for nontransgenic. Relative differential expression (transgenic signal/nontransgenic signal) is indicated by red-to-blue shading and displacement from the line of unity.

transgenic model is indicated by deviation above the line of unity slope and a red shift, whereas a decrease in gene expression in the transgenic is indicated by downward deviation and a blue shift. Optimal hybridization for each of the four experiments is indicated by gene expression data that consistently fit along the line of slope unity. A high degree of reproducibility between experiments was shown by comparative analysis of the 88 internal standards included in each microarray, the results for which were highly correlated between all four differential hybridizations (data not shown).

Since the Incyte GEM microarrays contain numerous genes that are not significantly expressed in cardiac tissue, the gene expression profiles were initially

refined by deleting genes with absolute expression levels in the lower quartile of both nontransgenic and transgenic mice for all four models. This low-signal region of the microarray experiment is also more susceptible to minor distortion effects that prevent linear normalization (see, for example, the falling tails of CSQ and  $\Psi\epsilon RACK$ ; Fig. 2*B*). Several genes tested from this region failed to show signal in poly(A)<sup>+</sup> Northern analyses (data not shown). Thus 887 sequence tags, or ~10% of total number of sequences (representing those in the *bottom left* quadrant of the expression profiles shown in Fig. 1), were excluded from subsequent comparative analysis. It is apparent from examination of the differential expression data in Fig. 1 that the gene

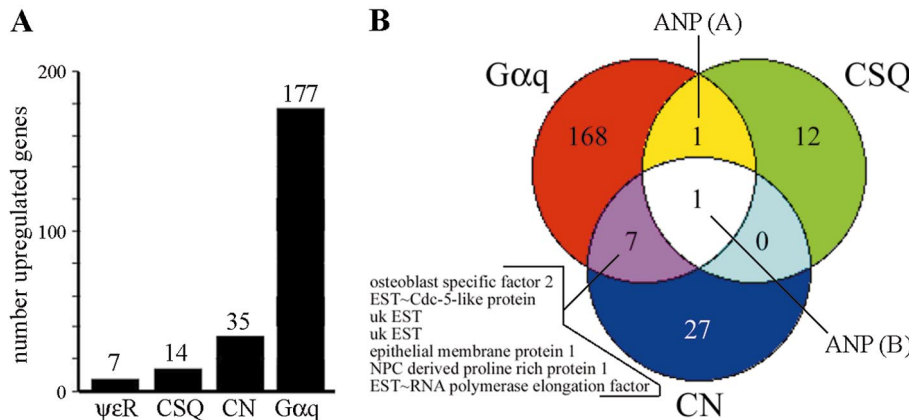


Fig. 2. Dynamically regulated genes in mouse cardiac hypertrophy. *A*: the absolute number of upregulated genes in each model corresponds to severity of phenotype ( $G\alpha_q > CN > CSQ > \Psi\epsilon RACK$ ). *B*: comparison of dynamically upregulated sequences in the three pathological hypertrophy models identifies few coregulated genes. EST, expressed sequence tag; uk, unknown; NPC, nasopharyngeal carcinoma.

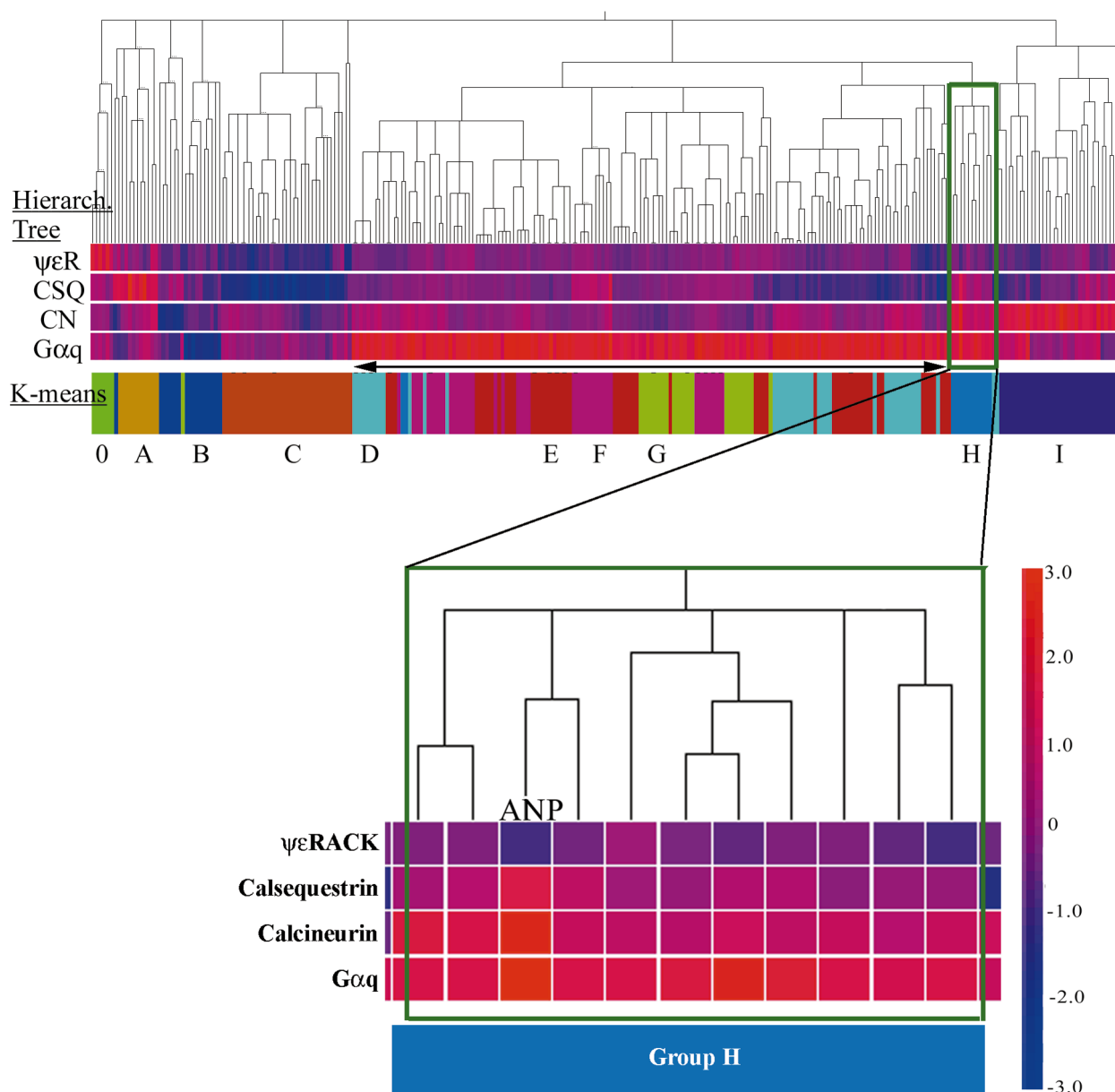


Fig. 3. Hierarchical analysis of gene expression patterns in mouse hypertrophy models: grouping of expressed sequences into gene clusters based on expression trends between models. The hierarchical tree is depicted leading to individual gene expression values (red/blue) for the four models. Gene clusters are color coded and assigned alphabetical designation A through I, except for the seven genes upregulated only in  $\Psi\epsilon$ RACK (group 0). Groups D–G form a superfamily of genes highly expressed in  $G\alpha_q$ . Group H, consisting of genes expressed in direct proportion to phenotypic severity, is magnified below for clarity. The red/blue color scale for differential expression is identical to that in Fig. 1. ANP is indicated.

expression profile was most “abnormal” in the  $G\alpha_q$  model, with abnormal being defined as genes with a relative differential expression value greater than +1.7 or less than -1.7 (corresponding to >2 standard deviations from the mean). This threshold level of differential gene expression was applied across all four experiments. An initial profiling of genes in this manner showed that the number of differentially regulated genes was greater in  $G\alpha_q$  than CN or CSQ models and that expression of relatively few genes was altered in  $\Psi\epsilon$ RACK (Fig. 2A). Interestingly, when the gene expression data for each model were compared as shown

in Fig. 2B, no genes were found to be regulated in common among the four hypertrophy models. Furthermore, only one gene, ANP, was coregulated in the three pathological hypertrophy models. Thus a conserved multigene hypertrophy expression program was not detected among the genes represented on the GEM microarrays.

An advantage of comparing gene expression in genetic forms of cardiac hypertrophy, where variously perturbed signaling events cause hypertrophy via different signaling mechanisms, is the potential to identify model-specific molecular events, i.e., unique molec-

ular features specific for each form of hypertrophy. Therefore, despite the absence of an obvious “hypertrophy gene program,” we sought to test the hypothesis that model-specific gene expression changes could provide insight into the pathophysiology of their respective phenotypes and perhaps of myocardial hypertrophy in general. To detect such patterns, hierarchical tree and K-means clustering algorithms were applied to the expression profiles of the pool of 276 genes that exhibited dynamic regulation among any of the four models. As depicted in Fig. 3, patterns of gene expression existed which could be grouped into clusters reflecting both shared and individual genetic profiles across the four models. Nine of the ten expression profiles are shown in Fig. 4 (not shown is the cluster of seven uniquely upregulated genes in  $\Psi\epsilon$ RACK, *group O*; see Table 1). The approximate biophysical correlates to these mathematical subgroups are as follows: *group A*, genes expressed greatly more in CSQ than other models; *group B*, genes downregulated in CN and/or

$G\alpha_q$ ; *group C*, genes downregulated in CSQ; *groups D–G*, a superfamily of genes upregulated in  $G\alpha_q$ , with subgroups based on patterned expression in other models; *group H*, genes whose expression increases in direct proportion to phenotypic severity of the four models; and *group I*, genes expressed more in CN than other models. Gene annotations for each hierarchical cluster are in Table 1, and Northern blot analyses of select genes are shown in Fig. 5, confirming the results of microarray experiments. As expected (and serving as an important internal control), the microarrays detected mRNAs corresponding to the transgenes for each model; CSQ was in *group A*, CN in *group I*, and  $G\alpha_{11}$ , which is closely related to  $G\alpha_q$ , was in *group E* ( $G\alpha_q$  is not represented on these GEM microarrays.)

Simple comparative analyses of the genes in the four hypertrophy models upregulated by more than  $\times 1.7$  failed to identify any commonly expressed genes (see Fig. 2B). However, application of the K-means clustering algorithm allowed detection of a group (*group H*) of

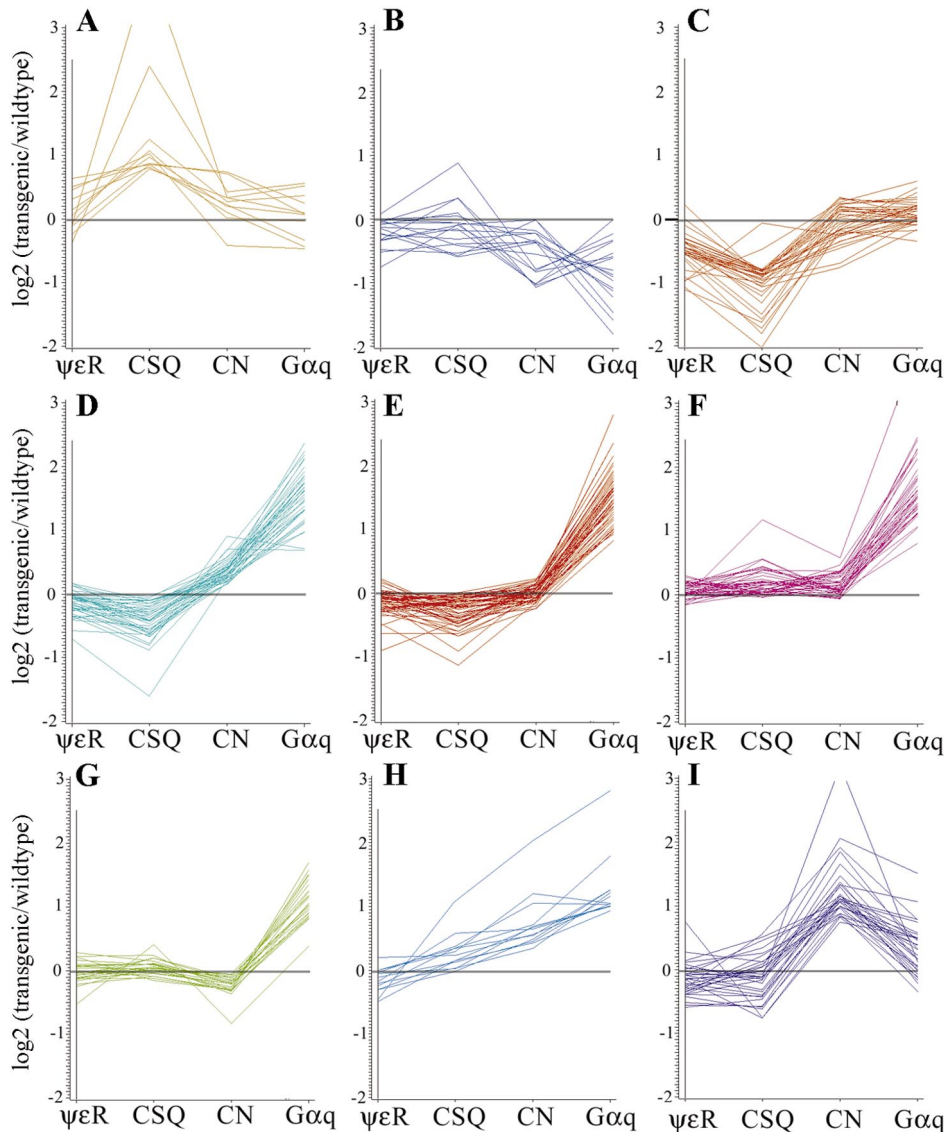


Fig. 4. Expression of genes from various hierarchical clusters in mouse hypertrophy models. The color coding and alphabetical designations are same as Fig. 3. Individual gene differential expression is depicted for each model. Vertical scale is  $\log_2$  of relative differential expression. Gene designations for each cluster are in Table 1.

Table 1. *Relative expressions of groups A–I*

Gene	Relative Expression (transgenic/control)				
	Accession No.	$\psi$ eRACK	CSQ	CN	G $\alpha_q$
<i>Group O</i>					
Cytochrome P450, 2f2	AA220582.1	2.55	1.24	1.10	1.28
Metallothionein 1	AI595466.1	1.93	1.20	1.12	1.15
And 5 ESTs					
<i>Group A</i>					
Calsequestrin 2	AA033488.1	0.93	16.12	1.26	1.07
Four and a half LIM domains 1	AA047966.1	0.77	5.26	1.35	1.47
HS1 binding system	AA472437.1	1.04	2.11	1.01	0.98
Peripheral myelin protein, 22 kDa	AA416246.1	1.25	1.76	1.17	0.80
And 7 ESTs					
<i>Group B</i>					
CCAAT/enhancer binding protein (C/EBP), alpha	AA271223.1	1.00	0.96	0.49	0.99
EST, highly similar to human carboxylesterase 3 (CES3)	AA509566.1	0.70	0.76	0.78	0.29
Ferritin heavy chain	AA124396.1	1.00	1.03	0.78	0.55
Four and a half LIM domains 2	AA023645.1	0.84	0.80	0.86	0.33
Nuclear factor of activated T-cells, cytoplasmic 2	AA451395.1	0.96	0.96	0.88	0.51
Peroxisomal/mitochondrial dienyol-CoA isomerase	W29607.1	0.90	0.67	0.99	0.43
Smoothelin	W11792.1	0.79	0.94	0.57	0.85
And 10 ESTs					
<i>Group C</i>					
Early growth response 1	AA465846.1	0.73	0.54	1.17	1.23
Hemoglobin alpha, adult chain 1	AA109900.1	0.70	0.51	0.61	1.00
Histocompatibility 2, K region	W14540.1	0.85	0.56	1.09	1.02
Lectin, galactose binding, soluble 9	AA250039.1	0.93	0.57	0.82	1.09
<i>Mus musculus</i> BAF57 (baf 57) gene	AA125197.1	0.78	0.49	1.11	1.00
<i>Mus musculus</i> mel (MEL 91) mRNA	W18484.1	0.72	0.55	1.08	1.08
<i>Mus musculus</i> mRNA for <i>N</i> -acetylglucosamine-6- <i>O</i> -sulfrtransferase	AA538322.1	0.69	0.55	0.77	1.04
Myeloid/lymphoid or mixed-lineage leukemia	AA174521.1	0.69	0.35	1.04	1.00
Peroxisome biogenesis factor 16	AA064183.1	0.74	0.48	0.59	0.89
And 26 ESTs					
<i>Group D</i>					
Adducin 3 (gamma)	AA066202.1	0.95	0.93	1.24	3.20
BCL2/adenovirus E1B 19 kDa-interacting protein 3	AA240743.1	1.01	0.67	1.19	3.63
Calcium channel, P/Q type, alpha 1A	AA492651.1	0.83	0.94	1.18	2.20
Caspase 1	AI326615.1	0.95	0.84	1.31	3.97
DNA segment, Chr 6, Wayne State University 16, expressed	AA241132.1	0.96	0.88	1.22	2.50
FK506 binding protein 7 (23 kDa)	AA242149.1	0.84	0.87	1.46	3.06
Glutathione <i>S</i> -transferase, theta 2	AA230348.1	0.92	0.66	1.20	2.95
Integral membrane protein 2	AA066921.1	0.96	0.66	1.60	4.53
Lymphocyte protein tyrosine kinase	AA098002.1	0.75	0.71	1.21	4.38
<i>Mus musculus</i> mRNA for cysteine and histidine-rich protein	AA537095.1	0.81	0.57	1.87	1.64
Protein kinase, DNA activated, catalytic polypeptide	AI386256.1	0.97	0.93	1.14	2.16
Purine-nucleoside phosphorylase	AA172867.1	1.09	0.80	1.32	2.77
Transforming growth factor beta regulated gene 4	AA240480.1	0.67	0.65	1.31	3.57
And 28 ESTs					
<i>Group E</i>					
A kinase anchor protein 4	AA106031.1	0.87	0.77	1.01	2.89
Alkaline phosphatase 2, liver	AI552105.1	0.85	0.92	0.95	1.99
Aryl-hydrocarbon receptor-interacting protein	AA087650.1	0.54	0.74	0.87	3.04
B-cell translocation gene 2, anti-proliferative	AA154848.1	0.84	0.62	0.96	1.93
Cardiac morphogenesis	AA023480.1	0.71	0.94	1.09	1.90
CD97 antigen	AA080529.1	0.94	0.96	1.01	2.70
Chromodomain protein, Y chromosome-like	AA080272.1	0.91	0.87	1.02	2.93
Cysteine rich protein 61	AA423149.1	0.94	0.88	1.05	2.25
Esterase 1	AA403730.1	0.82	0.70	0.95	2.77
Fas-associated factor 1	AA072606.1	0.93	0.98	1.05	3.13
Forkhead box M1	AA066741.1	0.94	0.98	0.95	3.05
Guanine nucleotide binding protein, alpha 11	AA451269.1	0.98	0.94	1.06	4.11
Heparan sulfate (glucosamine) 3- <i>O</i> -sulfotransferase 3B	AA272859.1	0.93	0.63	0.85	1.77
<i>Mus musculus</i> mRNA for GTP-binding protein (drg2 gene)	AA071987.1	0.97	0.85	1.05	2.48
<i>Mus musculus</i> putative endo/exonuclease MmMre11b	AI326588.1	0.95	0.78	0.99	3.11
Nuclear receptor coactivator 1	AI326579.1	0.97	0.73	1.06	2.35
Renin 1 structural	AA207872.1	1.02	0.80	0.93	6.94
Small inducible cytokine A3	AI326603.1	0.08	0.60	0.98	1.93
<i>Trans</i> -acting transcription factor 1	AA212645.1	1.14	0.76	1.06	2.55
And 37 ESTs					

Continued

Table 1.—Continued

Gene	Relative Expression (transgenic/control)				
	Accession No.	ψεRACK	CSQ	CN	Gα <sub>q</sub>
<i>Group F</i>					
Cytochrome P450, 4a10	AA097980.1	1.02	1.12	1.08	3.51
Ganglioside-induced differentiation-associated-protein 2	AA068478.1	1.00	1.16	1.07	5.52
Glutathione peroxidase 3	AA080443.1	1.07	1.16	0.97	2.84
<i>Mus musculus</i> anti-HIV-1 reverse transcriptase	AA098196.1	0.95	1.04	1.02	2.63
Natriuretic peptide precursor type A	W14325.1	0.97	2.25	1.49	14.91
Phospholipase A2 group VII	AI324436.1	1.03	1.15	1.05	2.36
Proteasome (prosome, marcopain) subunit, alpha type 7 And 34 ESTs	AA214752.1	1.09	1.13	1.23	2.70
<i>Group G</i>					
Aquaporin 1	AA241281.1	1.16	0.90	0.81	1.76
Kidney androgen regulated protein	AI550046.1	0.91	1.15	0.90	2.04
Leukemia inhibitory factor receptor	AA207338.1	1.21	1.11	0.86	2.54
<i>Mus musculus</i> AKAP95 mRNA for A kinase anchor protein 95, Proteasome (prosome, macropain) subunit, alpha type 7 Syndecan 2 And 18 ESTs	AA067731.1	0.98	1.04	0.93	1.88
	AA098608.1	0.92	0.92	0.81	2.96
	AA106952.1	0.86	0.94	0.80	2.35
<i>Group H</i>					
Epithelial membrane protein 1	W64798.1	1.00	1.22	2.30	2.07
ESTs, highly similar to α-actinin, smooth muscle isoform	AA403949.1	0.96	1.01	1.61	2.01
ESTs, highly similar to down syndrome candidate region 1	AA200984.1	0.84	1.28	1.64	3.45
Fibroblast inducible secreted protein	W36541.1	0.81	1.11	1.35	1.90
Natriuretic peptide precursor type B	AA030805.1	0.72	2.11	4.09	7.04
NPC derived proline rich protein 1	AA217593.1	0.98	1.30	2.07	2.05
NPC derived proline rich protein 1	AA444365.1	0.89	1.50	1.56	2.01
Procollagen, type 1, alpha 1 And 5 ESTs	AA073604.1	0.71	1.11	1.59	2.00
<i>Group I</i>					
Bloom syndrome homolog (human)	AA030433.1	0.90	0.98	2.08	1.03
Brain lipid binding protein	AI894027.1	0.85	1.04	1.83	0.79
Chloride intracellular channel 4 (mitochondrial)	AA537234.1	0.91	0.78	2.22	1.50
Follistatin-like	AA242611.1	0.79	1.17	1.98	1.39
Glucose phosphate isomerase 1 complex	W29397.1	0.77	1.06	2.45	0.86
Glucose phosphate isomerase 1 complex	AA276216.1	0.84	0.92	2.77	1.12
Insulin-like growth factor 2	AA030546.1	1.01	1.27	2.23	0.92
Mini chromosome maintenance deficient 5 ( <i>S. cerevisiae</i> )	AA031056.1	0.91	0.93	2.17	1.17
<i>Mus musculus</i> mRNA for sid23p,	AI390104.1	0.66	0.72	2.15	0.96
<i>Mus musculus</i> osf-2 mRNA for osteoblast specific factor 2	W81878.1	0.82	1.47	3.77	1.72
<i>Mus musculus</i> protein phosphatase X (Ppx) mRNA	AA05069.1	0.98	0.91	2.58	0.99
Phosphomannomutase 1	AA387369.1	0.70	0.67	1.80	1.05
Procollagen, type V, alpha 2	AA034564.1	0.81	1.10	1.77	1.41
Protein phosphatase 3, catalytic subunit, alpha isoform	AA245461.1	0.96	0.93	9.19	1.09
Recombining binding protein suppressor of hairless And 17 ESTs	W97158.1	1.68	0.59	1.77	0.93

ψεRACK, protein kinase c-ε activation peptide; CSQ, calsequestrin; CN, calcineurin; EST, expressed sequence tag

defined genes with a continuum of expression that paralleled the phenotype, i.e., genes whose expression correlated with severity of pathological hypertrophy (Table 1). ANP, as expected, is in this grouping, along with procollagen type I α1 and expressed sequence tags (ESTs) highly similar to smooth muscle α-actinin and the Down syndrome candidate gene recently described as a putative negative regulator of CN (19). Upregulated genes specific to the four hypertrophy models included, in addition to the transgenes themselves (and a large number of unidentified sequence tags), several unique functional gene groups with likely pathophysiological relevance. Most striking was the segregation of upregulated apoptosis genes into subgroups *D*, *E*, and *F* of the superset of genes upregulated in Gα<sub>q</sub>. These apopto-

sis genes include caspase 1 (*group D*), Fas-associated factor 1 and a Bcl-2/E1B interacting protein (*group E*), and apoptosis inhibitor 1 (*group F*) (see Fig. 5). Importantly, of the four transgenic models studied herein, only the Gα<sub>q</sub> overexpressor is predisposed to cardiomyocyte apoptosis (1). In contrast, several upregulated protein phosphatases segregated into *group I*, and included, in addition to CN, the α-catalytic subunit of protein phosphatase 3 and protein phosphatase X, suggesting a role for regulated phosphatases in the CN model. Several individual genes also showed intriguing model-specific expression patterns; the renin 1 gene in *group E*, the A kinase anchor proteins AKAP-4 and -95 genes in *groups E* and *G*, and the osteoblast-specific factor 2 (OSF-2) gene in *group I*.

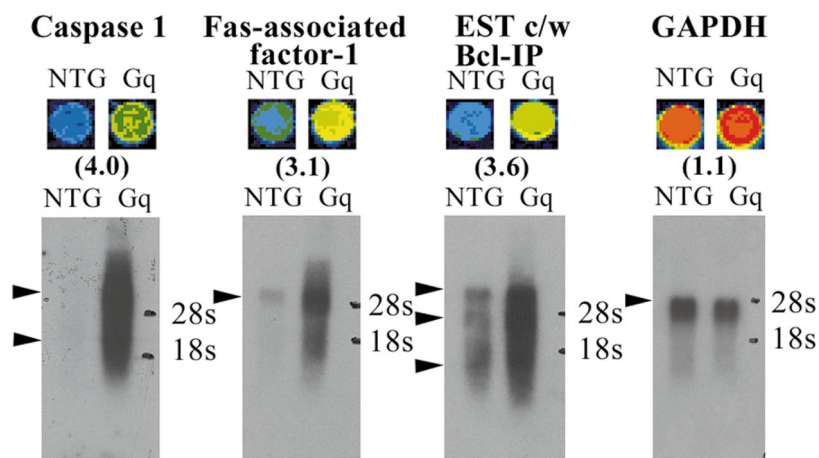


Fig. 5. Northern analysis of significant upregulated genes in mouse cardiac hypertrophy: upregulation of apoptosis genes in  $G\alpha_q$ . Images of array hybridization spots are shown above their corresponding lanes of Northern blots and the corresponding numerical value. EST, expressed sequence tag; NTG, nontransgenic; C/W, consistent with.

## DISCUSSION

The stated purpose of this study was to identify a defined set of genes that are commonly regulated among four genetic models of cardiac hypertrophy, anticipating that these genes would encompass those molecular events necessary for, or critical to, the hypertrophy process. Therefore, the relative expression of ~8,800 expressed sequences was compared in multiple genetically manipulated mouse models exhibiting increased myocardial mass and including both cardiomyocyte hypertrophy and hyperplasia. Surprisingly, not a single assayed gene was coregulated among all four models, and for the three models of pathological hypertrophy, only ANP was coordinately upregulated. A reciprocal observational approach of defining genes that were uniquely regulated in one model or another proved to be more illuminating than identifying conserved genes, especially after cluster analysis was used to identify trends of gene expression across the hypertrophy phenotypic spectrum. Thus an important outcome of these comparative experiments may be a renewed focus on model-specific genetic events as determinants of unique phenotypic features.

The notion of deemphasizing common or highly conserved genetic events in hypertrophy is further supported when the present results are compared with other recently reported gene expression profiles of cardiac hypertrophy. Hwang et al. (12) identified transcript profiles of hypertrophied human hearts by comparative analysis of cDNA libraries, and their 10 strongest candidates for differential expression included ANP, brain natriuretic peptide (BNP), and  $\alpha$ -skeletal actin. These three genes were also in the top 10 list of genes reported by Friddle et al. (9) as being upregulated during angiotensin II and isoproterenol induction of cardiac hypertrophy in mice. In the current studies, these genes were all members of the *group H* gene cluster which correlated closely with the pathological severity of the cardiac phenotype. In fact, these genes have long been recognized as molecular markers of cardiomyocyte hypertrophy both in vivo and in vitro when assayed by Northern or RNA dot blot analysis (2–4, 6, 10, 11, 17, 18, 21, 23; see Fig. 1A).

Thus the genes that tend to be expressed in common among various different forms of hypertrophy are not, in this case, molecular keys that unlock the hypertrophic process, but rather appear to be nonspecific transcriptional adaptations to myocardial contractile dysfunction or cardiac injury (24).

Taking the opposite approach, that of identifying transcriptional features that distinguished the four models, was more revealing. Perhaps predictably, the absolute number of genes regulated in the hypertrophy models corresponded approximately with the severity of the observed pathology. Thus, in  $\Psi$ eRACK where it is thought that postnatal cardiomyocyte hyperplasia results in normally functioning, but enlarged heart (17), only seven genes fell outside the established limits of “normality.” In contrast, in the  $G\alpha_q$  model of cardiomyocyte hypertrophy, which develops contractile dysfunction and a predisposition to apoptosis (4, 7, 20), a large number of genes were strongly regulated. It is worthwhile noting that it is impossible to determine from these data which genes are regulated as direct consequence of  $G\alpha_q$  signaling vs. those genes that are regulated indirectly as a consequence of contractile depression. It is interesting, however, that in previous reports of physiological “rescue” of the  $G\alpha_q$  phenotype by superimposed overexpression of  $\beta_2$ -adrenergic receptors (8) or  $\Psi$ eRACK (26), ANP and  $\alpha$ -skeletal actin expression were not normalized and  $\beta$ MHC gene expression remained very high. This suggests a direct effect of  $G\alpha_q$  signaling on these genetic markers.

Perhaps the most striking and potentially physiologically meaningful observation from these studies is the delineation of a previously undescribed apoptosis gene program in  $G\alpha_q$  overexpressors.  $G\alpha_q$  mice are uniquely susceptible to apoptotic cardiomyocyte death, with resulting progression from nonfailing hypertrophy to dilated cardiomyopathy (1). This apoptotic degeneration has been described in vivo in  $G\alpha_q$  overexpressors with high levels of transgene expression (compound heterozygous  $G\alpha_q$  mice), in a peripartum cardiomyopathy (1), and after aortic banding (5, 20). Furthermore, adenoviral transfection of mutationally activated  $G\alpha_q$  into cultured neonatal rat

cardiomyocytes causes apoptosis (1). The increased expression of a caspase, a fas effector and a Bcl-2 interacting protein described herein for  $G\alpha_q$  mice, is likely to contribute to the characteristic apoptotic proclivity of this model. In this regard, it is worthwhile noting that the mice used for these genetic analyses were nonfailing, nonapoptotic  $G\alpha_q$  overexpressors in which apoptosis [assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) labeling] is no greater than nontransgenic controls (1, 5). It is also intriguing that phosphatase genes were uniquely upregulated in the CN model. Since CN is itself a phosphatase, increased expression of protein phosphatases X and 3 suggests a certain degree of coordinated regulation between these three phosphatases. In contrast to the current studies, a previous transcript profiling of CN and pressure overload hypertrophy mice did not report upregulation of phosphatases (13). Furthermore, whereas we observed significant upregulation of OSF-2, this and other genes were not identified in CN mice, even though they were detected in pressure overload hypertrophy in this prior study (13). These differences may reflect our selection of 18-day-old CN mice, i.e., the early hypertrophic phase, as opposed to older CN mice in which progression to heart failure is already advanced (18). Another interesting observation in the current study is the upregulation of mouse skeletal LIM protein and another gene exhibiting homology to titin, specifically in CSQ. Since both of those are sarcomeric proteins, their increased expression may provide important cytoskeletal support for this model, such that hypertrophy does not progress to failure, even over the long term (L. Kranias, unpublished observations).

Although the power of gene expression profiling by the use of DNA microarrays is great, the application of this technology is still in its infancy, and there are technical, intellectual, and economic limitations to these types of studies. First, annotation of the expressed sequence library at this date is such that only approximately half of the sequences have been related to specific genes, although the database is constantly being updated. Second, the number of sequences assayed (~8,800), although substantial, represents only a fraction of the total number of genes expressed in the heart. Furthermore, many of the putative cDNAs on the microarrays no doubt represent genes not expressed to any significant extent in the normal or perturbed heart. For this study, which really represents a first pass without microarray replicates, to increase data trust, we excluded from analysis those genes whose absolute expression levels in both the nontransgenic and transgenic arrays from all four models was in the lower quartile. Clearly, however, much work needs to be done before the entire cardiac "transcriptome" can be assayed using these techniques. In addition, we believe that microarray data should be reviewed using multiple quality measures of the hybridized array, and all potentially significant

findings should be confirmed by additional quantitative approaches such as Northern blots, nuclease protection, or internally controlled RT-PCR.

In conclusion, this comparative analysis of gene expression profile of four genetic models of hypertrophy has failed to define a common hypertrophy gene expression program, but rather identifies specific mRNA profile changes likely to represent either mediators or adaptive responses by the heart to specific individual genetic perturbations. Regarding a widely conserved "hypertrophy gene program," it is likely that such a genetic program does not exist and that myocardial hypertrophy, as a universal response to numerous different hemodynamic, toxic, injurious, or genetic stimuli, exhibits molecular characteristics that reflect its original stimulus. Our studies suggest that unique genetic characteristics of hypertrophy, such as upregulated apoptosis gene expression in  $G\alpha_q$  overexpressors, can be important phenotypic determinants and are therefore attractive targets for experimental manipulation and therapeutic modification. As additional types of multi-gene comparisons are applied to other disease models, we expect that expression clusters will suggest both etiologic classification and the selection and optimization of therapeutic strategies.

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## REFERENCES

1. Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB, Chien KR, Brown JH, and Dorn GW II. Enhanced  $G\alpha_q$  signaling: a common pathway mediates cardiac hypertrophy and heart failure. *Proc Natl Acad Sci USA* 95: 10140–10145, 1998.
2. Brown LA, Nunez DJ, and Wilkins MR. Differential regulation of natriuretic peptide receptor messenger RNAs during the development of cardiac hypertrophy in the rat. *J Clin Invest* 92: 2702–2712, 1993.
3. Chien KR, Knowlton KU, Zhu H, and Chien S. Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. *FASEB J* 5: 3037–3046, 1991.
4. D'Angelo DD, Sakata Y, Lorenz JN, Boivin G, Walsh RA, Liggett SB, and Dorn GW II. Transgenic  $G\alpha_q$  overexpression induces cardiac contractile failure in mice. *Proc Natl Acad Sci USA* 94: 8121–8126, 1997.
5. De Windt LJ, Lim HW, Taigen T, Wencker D, Condorelli G, Dorn GW II, Kitsis RN, and Molkentin JD. Calcineurin mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: an apoptosis independent model of dilated heart failure. *Circ Res* 86: 255–263, 2000.
6. Dorn GW II, Robbins J, Ball N, and Walsh RA. Myosin heavy chain regulation and myocyte contractile depression after left ventricular hypertrophy in aortic banded mice. *Am J Physiol Heart Circ Physiol* 267: H400–H405, 1994.
7. Dorn GW II, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN, and Mochly-Rosen D. Sustained in vivo cardiac protection by a rationally designed peptide that causes  $\epsilon$  protein kinase C translocation. *Proc Natl Acad Sci USA* 96: 12798–12803, 1999.
8. Dorn GW II, Tepe NM, Lorenz JN, Koch WJ, and Liggett SB. Low- and high-level transgenic expression of  $\beta_2$ -adrenergic receptors differentially affects cardiac hypertrophy and function in  $G\alpha_q$  overexpressing mice. *Proc Natl Acad Sci USA* 96: 6400–6405, 1999.

9. **Friddle CJ, Koga T, Rubin EM, and Bristow J.** Expression profiling reveals distinct sets of genes altered during induction and regression of cardiac hypertrophy. *Proc Natl Acad Sci USA* 97: 6745–6750, 2000.
10. **Hama N, Itoh H, Shirakami G, Nakagawa O, Suga S, Ogawa Y, Masuda I, Nakanishi K, Yoshimasa T, Hashimoto Y, Yamaguchi M, Hori R, Yasue H, and Nakao K.** Rapid ventricular induction of brain natriuretic peptide gene expression in experimental acute myocardial infarction. *Circulation* 92: 1558–1564, 1995.
11. **Hunter JJ, Tanaka N, Rockman HA, Ross J Jr, and Chien KR.** Ventricular expression of a MLC-2v-ras fusion gene induces cardiac hypertrophy and selective diastolic dysfunction in transgenic mice. *J Biol Chem* 270: 23173–23178, 1995.
12. **Hwang DM, Dempsey AA, Lee CY, and Liew CC.** Identification of differentially expressed genes in cardiac hypertrophy by analysis of expressed sequence tags. *Genomics* 66: 1–14, 2000.
13. **Johnatty SE, Dyck JR, Michael LH, Olson EN, and Abdelatif M.** Identification of genes regulated during mechanical load-induced cardiac hypertrophy. *J Mol Cell Cardiol* 32: 805–815, 2000.
14. **Jones WK, Grupp IL, Doetschman T, Grupp G, Osinski H, Hewett TE, Boivin G, Gulick J, Ng WA, and Robbins J.** Ablation of the murine alpha myosin heavy chain gene leads to dosage effects and functional deficits in the heart. *J Clin Invest* 98: 1906–1917, 1996.
15. **Kumar A, Crawford K, Close L, Madison M, Lorenz J, Doetschman T, Pawlowski S, Duffy J, Neumann J, Robbins J, Boivin GP, O'Toole BA, and Lessard JL.** Rescue of cardiac alpha-actin-deficient mice by enteric smooth muscle gamma-actin. *Proc Natl Acad Sci USA* 94: 4406–4411, 1997.
16. **Miyata S, Minobe W, Bristow MR, and Leinwand LA.** Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circ Res* 86: 386–390, 2000.
17. **Mochly-Rosen D, Wu G, Hahn HS, Osinska H, Liron T, Lorenz JN, Yatani A, Robbins J, and Dorn GW II.** Cardiogenic effects of  $\epsilon$  protein kinase C: analysis by in vivo modulation of  $\epsilon$ PKC translocation. *Circ Res* 86: 1173–1179, 2000.
18. **Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, and Olson EN.** A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 93: 215–228, 1998.
19. **Rothermel B, Vega RB, Yang J, Wu H, Bassel-Duby R, and Williams RS.** A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. *J Biol Chem* 275: 8719–8725, 2000.
20. **Sakata Y, Lorenz JN, Hoit BD, Liggett SB, Walsh RA, and Dorn GW II.** Decompensation of pressure overload hypertrophy in  $G\alpha_q$  overexpressing mice. *Circulation* 97: 1488–1495, 1998.
21. **Sato Y, Ferguson DG, Sako H, Dorn GW II, Kadambi VJ, Yatani A, Hoit BD, Walsh RA, and Kranias EG.** Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *J Biol Chem* 273: 28470–28477, 1998.
22. **Subramaniam A, Gulick J, Neumann J, Knotts S, and Robbins J.** Transgenic analysis of the thyroid-responsive elements in the alpha-cardiac myosin heavy chain gene promoter. *J Biol Chem* 268: 4331–4336, 1993.
23. **Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, and Walsh RA.** Transgenic overexpression of constitutively active protein kinase C epsilon causes concentric cardiac hypertrophy. *Circ Res* 86: 1218–1223, 2000.
24. **Taylor LA, Carthy CM, Yang D, Saad K, Wong D, Schreiner G, Stanton LW, and McManus BM.** Host gene regulation during coxsackievirus B3 infection in mice, assessment by microarrays. *Circ Res* 87: 328–334, 2000.
25. **Wu CF, Bishopric NH, and Pratt RE.** Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes. *J Biol Chem* 272: 14860–14866, 1997.
26. **Wu G, Toyokawa T, Hahn H, and Dorn GW II.** Epsilon protein kinase C in pathological myocardial hypertrophy: analysis by combined transgenic expression of translocation modifiers and  $G\alpha_q$ . *J Biol Chem* 275: 29927–29930, 2000.