Original Article

Myocardial Gene Expression Associated with Genetic Cardiac Hypertrophy in the Absence of Hypertension

Jeremy P. DWYER[1)], Matthew E. RITCHIE[2)], Gordon K. SMYTH[2)], Stephen B. HARRAP[1)], Lea M.D. DELBRIDGE[1)], Andrea A. DOMENIGHETTI[3)], and Robert DI NICOLANTONIO[1)]

The hypertrophic heart rat (HHR) was derived from the spontaneously hypertensive rat of the Okamoto strain and develops cardiac hypertrophy in the absence of hypertension. The genetic basis of this hypertrophy is unknown. Therefore, we compared gene expression profiles in the left ventricular myocardium of young (8–10 weeks of age) and old (38–50 weeks) HHR with rats from an age-matched control strain, the normal heart rat (NHR). cDNA microarrays (National Institute of Aging [NIA], 15,247 clones) were used to evaluate gene expression in cardiac-derived Cy3 and Cy5 labeled cDNA. *M* values ($\log_2$[Cy5/Cy3]) were obtained and significant differential expression was identified using an empirical Bayesian approach with specific results verified using real-time PCR. Compared with NHR, HHR cardiac weight index (heart weight/body weight) was significantly elevated at both ages (young: 5.5±0.5 *vs.* 3.9±0.2; old: 4.2±0.3 *vs.* 3.4±0.2 mg/g; $p<0.05$) with no difference in body weight or in tail-cuff blood pressure detected between the strains at either age. Differential expression was observed in 65 and 390 clones in young and old HHR, respectively, with more genes exhibiting down-regulation than up-regulation in both instances (young: down 44 *vs.* up 21; old: down 292 *vs.* up 98). Our data suggest a role for the Ras/mitogen-activated protein kinase (MAPK) signaling pathway and the tumor necrosis factor (TNF) receptor–mediated activation of nuclear factor-κB (NF-κB) in the etiology of cardiac enlargement in the HHR. These findings support the candidature of previously identified cardiotrophic agents in contributing to the cardiac enlargement in the normotensive HHR, and also identify novel genetic factors which may be involved in the genesis of primary cardiac hypertrophy. (*Hypertens Res* 2008; 31: 941–955)

***Key Words*: hypertrophic heart rat, cardiac hypertrophy, microarray, gene expression, remodeling**

Introduction

Left ventricular hypertrophy (LVH) is a major independent risk factor predictive of cardiovascular mortality and morbidity. In the Framingham study, over 50% of individuals with electrocardiographic evidence of LVH were dead within 8 years as a result of significantly increased rates of cardiac failure, sudden cardiac death and arrhythmia (*1*). Interestingly, not all subjects with hypertension develop LVH and in other instances the increase in LV mass for a given hemodynamic load is inappropriately high (*2–4*). Similarly, in a classic survey of rat strains, Tanase *et al*. showed that although high blood pressure (>150 mmHg) was associated with large

From the [1)]Department of Physiology, University of Melbourne, Melbourne, Australia; [2)]Walter and Eliza Hall Institute of Medical Research, Parkville, Australia; and [3)]Department of Medicine, The University of California (San Diego), La Jolla, USA.

This work was supported by the National Health and Medical Research Council of Australia.

Address for Reprints: Robert Di Nicolantonio, Ph.D., Department of Physiology, University of Melbourne, Victoria 3010, Australia. E-mail: robertdn@unimelb.edu.au

Received April 10, 2007; Accepted in revised form November 27, 2007.

hearts, there was marked variation in heart size among normotensive animals (*5*). In order to provide a model of spontaneous, normotensive cardiac hypertrophy, we derived the hypertrophic heart rat (HHR) strain by the selective inbreeding of offspring that had both large hearts and normal blood pressures. These offspring had been derived, in turn, from a cross between spontaneously hypertensive rats (SHR) of the Okamoto strain and normotensive Fisher 344 animals. As expected, the inbred HHR strain derived through this process exhibits spontaneous cardiac hypertrophy in the absence of hypertension (*6*). While the inherent determinants of the pressure-independent cardiac hypertrophy of HHR have not been identified, they are presumably largely genetic.

A considerable amount is known about the molecular physiology of cardiac hypertrophy, including the identity of a number of genetic pathways that might mediate cardiac enlargement in the HHR. For example, the activation of proto-oncogenes (*e.g.*, *Egr-1*, *c-fos*, *c-jun*, *c-myc*) that encode for peptide transcription factors is believed to induce the transcription of other hypertrophy-related genes (*7*). This so-called "early response" by the proto-oncogenes induces a "fetal gene program" characterized by the re-expression of several genes normally expressed predominantly in the embryonic heart. These genes include those encoding atrial natriuretic factor (ANF) and fetal isoforms of contractile proteins such as β-myosin heavy chain, α-skeletal actin and α-smooth muscle actin (*7*). Concurrent with this re-expression of fetal genes is the down-regulation of genes normally expressed at higher levels in the adult heart, such as *α-myosin heavy chain* and the sarcoplasmic reticulum calcium pump, *SERCA2a* (*7*). These changes in gene expression are believed to be indicative of cardiomyocyte hypertrophy and its associated increase in the production and assembly of contractile proteins into sarcomeric units (*8*).

Of the 15,000 or more genes estimated to comprise the mammalian genome, it has been shown that over two-thirds are expressed in cardiovascular tissues (*9*). Therefore, a case-by-case examination of candidate genes playing a role in the cardiac hypertrophy of the HHR is currently impracticable. High-throughput DNA microarray technology offers a more effective means of uncovering genetic pathways involved in cardiac growth. DNA microarrays consist of large numbers of cDNA or oligonucleotide probes spotted onto a glass slide using a precise robotic system (*10*). Such arrays are used to simultaneously identify genes that are differentially expressed between different cell states or tissue types. At present, DNA microarray studies investigating gene expression in cardiac hypertrophy have suggested that hypertrophy-regulated genes are largely specific to the hypertrophy-inducing stimulus (*11*) and furthermore can be grouped into clusters or "regulons" representing families of genes involved in distinct pathways mediating cardiomyocyte growth (*12*). Genes specifically altered during the development of hypertrophy include those encoding secreted growth factors, receptors, intracellular signaling molecules, proteins involved in intermediary metabolism, structural proteins, transcription factors and protein synthetic genes (*12*). However, determining which expression changes are causative and which are secondary cellular responses is a considerable challenge.

In the present study, we have used cDNA microarrays to compare the gene expression profiles in left ventricular cardiac tissue from the HHR and its genetic control strain, the normal heart rat (NHR). Gene expression of HHR and NHR was compared at 8–10 weeks of age (*i.e.*, in young rats), which was chosen to represent the stage of cardiac hypertrophy in the HHR that coincided with the period of body and heart growth (*6*). Gene expression was also compared between strains at 38–50 weeks (*i.e.*, in old rats), when the growth rate of the animal has largely plateaued. In this way we hoped to determine which genes play a developmental, as opposed to a maintenance role in the cardiac hypertrophy of the HHR. Given that the HHR is normotensive, we also sought to identify primary (causal) hypertrophy genes, rather than those that are induced secondarily to an elevated blood pressure. In doing so we also hoped to identify those SHR genes captured in the HHR and which contribute to the component of the SHR's cardiac hypertrophy that is blood pressure-independent.

Methods

Animals

Male HHR and NHR animals were obtained from the Biological Research Facility (University of Melbourne, Victoria, Australia). Animals were housed under standard conditions with a 12-h light/dark cycle and room temperature maintained at 18–21°C. Animals received standard laboratory fodder (Clarke King, Pakenham, Australia) and tap water ad libitum. All experiments received prior approval by the Animal Experimentation Ethics Committee of the University of Melbourne. Investigations complied with both the Code of Conduct for the Care and Use of Animals as specified by the National Health and Medical Research Council of Australia, as well as the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Blood Pressure Measurements and Tissue Processing

Approximately 1 week before euthanasia, systolic blood pressure measurements were recorded for all HHR and NHR using tail-cuff plethysmography (*13*). Systolic blood pressure was recorded 5 times on two consecutive days, the first day being used solely to adapt the animals to the tail-cuff procedure. On the second day the first of the 5 measures was discarded and the average of the subsequent 4 measures was used as the representative blood pressure for each animal. Because the blood pressure values were not normally distrib-

uted, they were expressed as the median and interquartile ranges, and comparisons were made between groups using the non-parametric Kolmogorov-Smirnov test. The body mass of male HHR and NHR was recorded at 8–10 weeks of age or at 38–50 weeks of age. After induction of surgical anaesthesia with sodium pentobarbitone (60 mg/kg, i.p., Nembutal; Boehringer Manheim, North Ryde, Australia), hearts were excised and then rinsed in phosphate-buffered saline (Sigma, St. Louis, USA). Non-cardiac tissue was trimmed away from the heart, following which it was blotted dry and weighed. The left ventricle was then dissected and immediately frozen in liquid nitrogen. All tissues were stored at −70°C until later use. Total RNA was extracted using Trizol Reagent (GibcoBRL LifeTechnologies, Gaithersburg, USA) according to the manufacturer's recommended protocol. The RNA was then resuspended in 50 μL ultra pure H_2O and stored at −70°C.

DNA Microarrays

The microarray slides were produced at the Australian Genome Research Facility in Melbourne *via* robotic printing (ChipWriter; Virtek, Waterloo, Canada). PCR-amplified cDNA clones were arrayed at high density onto Corning CMT-GAPS aminosilane-coated glass slides (Corning, New York, USA). The specific clone set printed onto the slides was the NIA 15K mouse cDNA clone set (National Institute of Aging [NIA], National Institute of Health [NIH]; http://lgsun.grc.nia.nih.gov/). This clone set was derived from 11 embryo cDNA libraries and one newborn ovary cDNA library and represents 15,247 (15K) unique genes of which approximately 50% are novel (expressed sequence tags [ESTs]; segments of genes that have been sequenced but have no known function) and the remainder are known genes (*14*). The arrays included a selection of control spots including housekeeping genes and positive, negative and calibration controls. Each probe was printed on slides in duplicate and side-by-side in rows resulting in a total of 32,448 spots in a 12 × 4 grid layout. The microarrays slides were stored in a desiccated, dust- and light-free environment

Labeling of cDNA Probes

The CyScribe cDNA Post Labeling Kit (Amersham Pharmacia, Buckinghamshire, UK) was used to prepare the Cy3- and Cy5-labeled cDNA used in the microarray hybridizations. Five 8–10 week-old HHR rats were age-matched with five NHR animals and a dye-swap pair of microarrays was hybridized with labeled cDNA from each matched pair. Four 38–50 week-old HHR were age-matched with four NHR rats and again a dye-swap pair of microarrays was hybridized from each pair. This produced 10 microarrays comparing young NHR with young HHR and eight arrays comparing old NHR with old HHR. A total of 100 μg total RNA per dye labeling reaction was used for each HHR *vs*. NHR comparison, giving a total of 200 μg total RNA per array (2 labeling reactions per array). Microcon filters (Microcon) were utilized for the purification of the cDNA, and QIAquick columns (Qiagen, Hilden, Germany) were used for the purification of CyDye-labeled cDNA, both in accordance with the manufacturer's recommended protocol. All reactions following CyDye-labeling were carried out in 1.5 mL amber eppendorf tubes to minimize the effect of UV-light on the CyDyes.

Microarray Hybridization

Prior to hybridization, the cDNA microarrays were incubated in a pre-hybridization buffer (10 mg/mL BSA-fraction V [Sigma], 25% Formamide [Sigma], 5 × SSC [Sigma], 0.1% SDS [Sigma]) in a clean slide mailer for 1 h at 42°C. Following incubation, the slides were washed under distilled water and, after drying with a compressed air gun, immediately used for hybridizations. The following blocking agents were added to the purified CyDye-labeled cDNA: 25 μL 1 mg/μL Mouse Cot 1DNA (Gibco BRL), 3.8 μL 10 mg/mL Poly A (Amersham Pharmacia), and 5.0 μL 10 mg/mL Salmon Sperm DNA (Gibco BRL). The hybridization reaction was spun down in a rotary evaporator until a volume of 30 μL was achieved. Thirty microliters 2× hybridization buffer (50% formamide (Sigma), 10× SSC (Sigma), and 0.2% SDS) was added to the 30 μL hybridization reaction, heated at 100°C for 2 min and pipetted using capillary action underneath a 60 × 25 mm cover-slip (Grale Scientific, Ringwood, Australia) placed over the array region on the pre-hybridized microarray slide. The microarray slide was then placed in a hybridization chamber (Corning) and incubated for 16–20 h in a pre-heated water bath at 42°C. Prior to scanning, the microarray slide was gently washed with 1 × SCC (Sigma), 0.2% SDS (Sigma) buffer solution for 5 min, 0.1 × SSC, 0.2% SDS buffer solution for 5 min, and twice with 0.1 × SSC buffer solution for 2 min at room temperature. The microarray slide was spun dry in a plate centrifuge at 500 × *g* for 12 min.

Data Analysis

The hybridized slides were scanned using a GenePix 4000B Scanner (Molecular Devices, Sunnyvale, USA) to produce single-image 16-bit TIFF files. Intensity information was extracted for each spot using the image analysis package Spot (*15*). Background correction used the "morph" morphological background measure (*16*). The data was normalized using print-tip loess normalization (*17*). Log-fold changes were estimated by fitting a gene-wise linear model to the log-ratios from all 18 arrays with coefficients (*18*). The coefficients from the model measured the log-ratio of expression in the direct comparisons of "young" HHR *vs*. NHR animals and "old" HHR *vs*. NHR animals, and their contrast provided an indirect comparison of old *vs*. young animals. Since each probe was printed in duplicate on each array, the linear models were fitted using the generalized least squares method

Table 1. Tail-Cuff Blood Pressure and Cardiac Tissue Measurements in Young (8–10 Weeks) and Old (38–50 Weeks) HHR and NHR Animals

	Young		Old	
	NHR	HHR	NHR	HHR
Sample size	5	6	4	4
Age (weeks)	9.2±0.1	9.1±0.2	41.0±5.2	41.9±2.5
Systolic blood pressure (mmHg)	131 (7.5)	138 (6.6)	123 (16.6)	118 (10.4)
Body weight (g)	261±10	238±14	470±19	463±14
Heart weight (g)	1.02±0.1	1.30±0.1*	1.59±0.1	1.96±0.2*
Heart weight/body weight (mg/g)	3.89±0.2	5.53±0.5*	3.39±0.2	4.20±0.3*

Blood pressures are expressed as median with the interquartile range in parentheses and other variables as mean±SEM. *$p<0.05$ by Student's *t*-test when compared to NHR in the same age group. HHR, hypertrophic heart rat; NHR, normal heart rat.

Table 2. Genes Differentially Expressed in Young HHR (8–10 Weeks) Left Ventricular Tissue When Compared with Age-Matched NHR

Putative gene ID	Accession No./clone ID*	Detected in heart?	Fold change	*B*-stat
Energy/metabolism				
sterol carrier protein 2, liver (Scp2)	BG086181	Yes	1.35	2.84
1-acylglycerol-3-phosphate O-acyltransferase 3 (Agpat3)	BG076329	Yes	1.23	2.49
ATP synthase, H^+ transporting mitochondrial F1 complex, β subunit (Atp5b)	BG076756	Yes	−1.30	0.51
Signal transduction				
DnaJ (Hsp40) homolog, subfamily A, member 3 (Dnaja3)	BG082303	Yes	1.34	8.11
Unc-51–like kinase 1 (*C. elegans*) (Ulk1)	BG079418	Yes	−1.20	3.58
aryl hydrocarbon receptor nuclear translocator-like (Arntl)	BG072020	Yes	−1.33	2.62
transforming growth factor, β induced, 68 kDa (Tgfbi)	BG072750	Yes	−1.51	0.78
Matrix/structural proteins				
actin, β, cytoplasmic (Actb)	BG063870	Yes	−1.32	0.90
procollagen, type V, α 1 (Col5a1)	BG067011	Yes	−1.39	0.68
ribophorin II (Rpn2)	BG078996	Yes	−1.36	0.64
cartilage associated protein (Crtap)	BG066603	Yes	−1.24	0.27
Transcription/chromatin				
SRY-box containing gene 4 (Sox4)	H3089F12	Yes	−1.48	3.93
CCCTC-binding factor (Ctcf)	BG065301	Yes	−1.24	0.09
Protein synthesis/translational control				
similar to 60S ribosomal protein L30 isolog	BG083125	Yes	−1.34	1.34
ribosomal protein L15 (Rpl15) – (2 entries)	Multiple	Yes	−1.34	1.04
proteasome (prosome, macropain) 26S subunit, ATPase 3 (Psmc3)	BG063070	Yes	−1.18	0.06
Cell defence/growth related proteins				
lipocalin 2 (Lcn2)	BG070106		1.35	3.04
histocompatibility 2, class II antigen E β (H2-Eb1)	BG077017	Yes	−1.34	1.29
proteasome (prosome, macropain) 28 subunit, α (Psme1)	BG066650	Yes	−1.41	0.24
Apoptosis				
endonuclease G, mitochondrial precursor (Endog)	BG064577	Yes	−1.67	16.86
15 unknown genes/ESTs†				

HHR, hypertrophic heart rat; NHR, normal heart rat; ESTs, expressed sequence tags. *Multiple accession numbers referring to the same gene are summarised as one entry. Full results are available for download at http://www.physiology.unimelb.edu.au/ †Unknown genes/ESTs are listed separately and available for download at http://www.physiology.unimelb.edu.au/

with an estimated value for the correlation between duplicate spots. The correlation estimate was 0.88. This had the effect of increasing the degrees of freedom available to estimate the gene-wise variances compared with simply averaging the duplicate log-ratios (*19*).

Differential expression was assessed using a parametric empirical Bayes model which moderates the standard errors for the test statistics by borrowing information across genes (*18*). This results in far more stable inference when the number of microarrays in the experiment is not large. Genes were considered to be differentially expressed if their posterior log-odds (*B*-statistic) were positive, *i.e.*, if the estimated odds of being differentially expressed was greater than 50–50. All analysis was carried out using the "Limma" software module (*20*).

Annotated genes were classified by their function (*21*) with reference to the Gene Ontology database (http://www.geneontology.org/) (*22*). The SOURCE genomic database (http://source.stanford.edu/) (*23*) was used to determine whether the annotated genes had been previously shown to be expressed in the mouse heart.

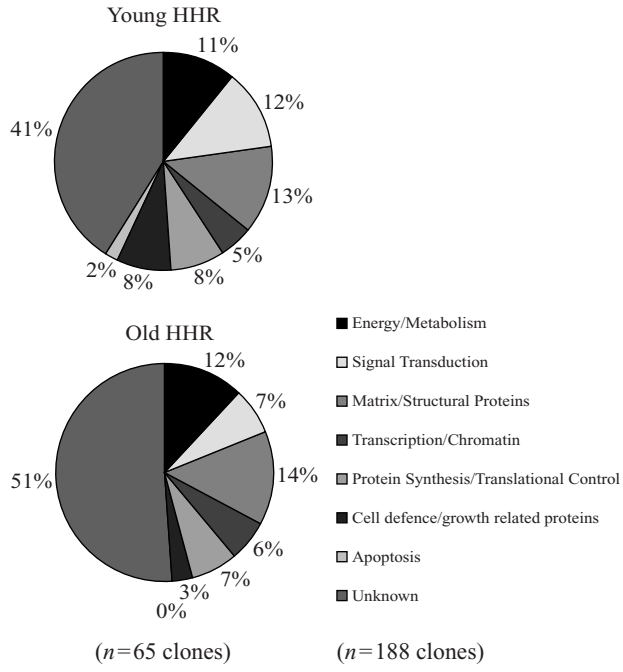

Fig 1. *Functional classification of clones differentially expressed in both young (8–10 weeks) and old (38–50 weeks) HHR. Data are expressed as a percentage of the total differentially expressed clones either over- or under-expressed relative to age-matched NHR.*

Microarray Verification by Quantitative Real-Time PCR

Real-time PCR (RT-PCR) was used to corroborate the fold-differences in RNA levels detected by the microarrays as existing between species. Total RNA from "old" HHR and NHR tissues was extracted using Trizol reagent (Invitrogen Life Technologies, Melbourne, Australia) according to the manufacturer's recommended protocol. Reverse transcription using 5 μg of RNA was performed using the ReactionReady First Strand cDNA Synthesis Kit (SuperArray Bioscience, Frederick, USA). RT^2 Real-Time Gene Expression Assay Kits (SuperArray Bioscience) were used according to the manufacturer's instructions. Four genes were targeted in this approach: *natriuretic peptide precursor type B* (*Nppb*; rat NM_031545), *mitogen activated protein kinase 1* (*Mapk1*; rat NM_053842), *tropomyosin 1* (*Tpm1*; rat NM_019131) and *TRAF family member–associated NF-κB activator* (*Tank*; rat NM_145788). The "housekeeper gene" *ribosomal protein L13a* (*Rpl13a*; rat NM_173340) was used as a control and showed no significant differential expression in our microarray results (fold change=0.94, $B=-4.9$). SYBR green I (Molecular Probes, Eugene, USA) was included in the RT-PCR reactions, which contained the following: 1 μL cDNA, 2.5 μL 5× SYBR Green I solution, 1 μL gene specific primer mix, 12.5 μL 2× PCR cocktail and ultra-pure H_2O to a final volume of 25 μL. PCR reactions were carried out on a Rotor Gene 3000 thermal cycler (Corbett Research, Sydney, Australia). Cycling conditions included an initial incubation at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. At the end of the PCR cycle, a dissociation curve was generated to ensure the amplification of a single product. A cycle threshold (C_T) was calculated for each gene and defined as the number of PCR cycles required for a PCR reaction to reach a fluorescence value within the linear amplification range. The relative fold-difference between strains for a given target gene was calculated using the so-called $2^{-\Delta C_T}$ method (*24*). C_T values for each gene were normalized against the value for the "housekeeper gene" (*Rpl13a*) prior to calculation of fold-differences between strains. Each reported fold change for a gene represents the average of two independent RT-PCR runs, which were each performed in triplicate.

Results

Blood Pressure and Cardiac Measurements

There were no significant differences in systolic blood pressure between the HHR and NHR at either the young or old ages (Table 1). There was also no significant difference in body weight between the HHR and NHR groups at either 8–10 weeks or 38–50 weeks, although the NHR showed a tendency to increase in body weight. As expected, a significant increase in heart weight and the heart weight–to–body weight ratio was seen in the HHR group compared with the NHR group ($p<0.05$) at both 8–10 weeks and 38–50 weeks of age. In both the HHR and NHR, the heart weight–to–body weight ratio was decreased in older animals, as expected.

Table 3. Genes Differentially Expressed in Old HHR (38–50 Weeks) Left Ventricular Tissue When Compared with Age-Matched NHR

Putative gene ID	Accession No./clone ID*	Detected in heart?	Fold change	*B*-stat
Energy/metabolism				
diazepam binding inhibitor (Dbi)	BG073601	Yes	1.44	14.30
cytochrome c oxidase, subunit VIIc (Cox7c) – (2 entries)	Multiple	Yes	1.21	3.94
acid phosphatase 6, lysophosphatidic (Acp6)	BG065427	Yes	1.24	3.84
ATP synthase, H^+ transporting, mitochondrial F0 complex subunit F (Atp5j)	BG072808	Yes	1.24	3.15
succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (Sdha)	BG086026	Yes	1.34	3.07
N-acylsphingosine amidohydrolase 1 (Asah1)	BG071742	Yes	1.24	2.93
ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit f, isoform 2 (Atp5j2)	BG073062	Yes	1.31	2.04
glucan (1,4-α-), branching enzyme 1 (Gbe1)	BG081291	Yes	1.48	1.55
aldehyde reductase-like 6 (Aldrl6)	BG080253		1.20	0.75
thioredoxin 1 (Txn1)	BG086400	Yes	1.26	0.39
domesticus mitochondrial DNA – (16 entries)	Multiple		−2.36	20.71
carbonic anhydrase 8 (Car8)	BG072807	Yes	−2.05	6.11
methylthioadenosine phosphorylase (Mtap)	H3143G03	Yes	−1.31	4.59
EGL nine homolog 1 (*C. elegans*) (Egln1)	BG075252	Yes	−1.34	3.85
glyceraldehyde-3-phosphate dehydrogenase (Gapd) – (5 entries)	Multiple		−1.54	3.84
glutathione S-transferase, π 2 (Gstp2)	BG076872	Yes	−1.19	3.64
phosphoribosylglycinamide formyltransferase (Gart)	BG074918	Yes	−1.39	1.84
arylsulfatase A (Arsa)	BG087046	Yes	−1.29	1.39
ATPase, H^+ transporting, V1 subunit A, isoform 1 (Atp6v1a1)	BG081377	Yes	−1.50	1.16
NADH dehydrogenase (ubiquinone) Fe-S protein 8 (Ndufs8)	BG077969	Yes	−1.39	0.92
ATPase, H^+ transporting, lysosomal 50/57 kDa, V1 subunit H (Atp6v1h)	BG086960	Yes	−1.33	0.36
cytochrome c oxidase, subunit IVa (Cox4a)	BG084085	Yes	−1.48	0.34
Signal transduction				
similar to guanine nucleotide binding protein (G protein), γ 5 (Gng5)	BG079210		1.27	4.87
similar to pyruvate dehydrogenase kinase 1 (*Rattus norvegicus*)	BG078533	Yes	1.30	2.74
channel-interacting PDZ domain protein (Cipp)	BG071152	Yes	1.30	2.41
tripartite motif protein 23 (Trim23)	H3099E03	Yes	1.28	2.29
growth arrest specific 1 (Gas1)	BG087671	Yes	1.52	2.10
RAB12, member RAS oncogene family (Rab12)	H3143E11	Yes	1.27	0.36
mitogen activated protein kinase 1 (Mapk1)	BG069285	Yes	1.33	0.16
neural precursor cell expressed, developmentally down-regulated gene 4 (Nedd4)	BG071875	Yes	1.28	0.14
latrophilin 2 (Lphn2)	BG064852	Yes	−1.58	10.91
CDC-like kinase (Clk) – (2 entries)	Multiple	Yes	−1.88	8.14
TRAF family member–associated Nf-κ B activator (Tank)	BG080604	Yes	−1.82	6.25
interleukin-1 receptor–associated kinase 1 (Irak1)	BG086759	Yes	−1.42	5.90
protein phosphatase 3, catalytic subunit, α isoform (Ppp3ca)	BG072475	Yes	−1.67	5.00
CDC-like kinase 3 (Clk3)	BG088524	Yes	−1.26	3.82
Fyn proto-oncogene (Fyn), mRNA	BG074018	Yes	−1.21	3.38
syntrophin, acidic 1 (Snta1)	BG078505	Yes	−1.93	2.25
protein kinase, AMP-activated, β 1 non-catalytic subunit (Prkab1)	BG071336	Yes	−1.21	1.10
NIMA (never in mitosis gene a)–related expressed kinase 2 (Nek2)	BG065826	Yes	−1.32	0.55
phospholipase C, δ (Plcd)	BG066587	Yes	−1.36	0.31
Traf and Tnf receptor associated protein (Ttrap)	BG070243		−1.39	0.27
ADP-ribosylation factor GTPase activating protein 1 (Arfgap1)	BG086579	Yes	−1.43	0.08
Matrix/structural proteins				
amyloid β (A4) precursor protein (App)	BG086292	Yes	1.22	2.99
growth hormone inducible transmembrane protein (Ghitm)	BG087192	Yes	1.22	2.71
upregulated during skeletal muscle growth 5 (Usmg5)	BG086421	Yes	1.33	2.58

Table 3. Continued

Putative gene ID	Accession No./clone ID*	Detected in heart?	Fold change	*B*-stat
ubiquitin-conjugating enzyme E2A, RAD6 homolog (Ube2a) (*S. cerevisiae*)	BG068496	Yes	1.21	1.42
solute carrier family 6 (neurotransmitter transporter, taurine), member 6 (Slc6a6)	BG066820		1.22	0.68
zinc finger, DHHC domain containing 3 (Zdhhc3)	BG070950	Yes	1.18	0.49
KRIT1 (Krit1)	H3132F12	Yes	1.28	0.37
actin related protein 2/3 complex, subunit 2 (34 kDa) (Arpc2)	BG063604	Yes	1.32	0.19
formin binding protein 4 (Fnbp4)	BG071493	Yes	−1.41	5.59
tissue inhibitor of metalloproteinase 4 (Timp4)	BG084374	Yes	−1.43	4.72
hemoglobin α, adult chain 1 (Hba-a1) – (15 entries)	Multiple	Yes	−1.56	4.29
37 kDa leucine-rich repeat (LRR) protein (P37NB)	BG085573	Yes	−1.35	4.02
pinin (Pnn)	BG068140	Yes	−1.28	3.65
hydroxysteroid 17-β dehydrogenase 4 (Hsd17b4)	BG072994	Yes	−1.50	2.38
hemoglobin, β adult major chain (Hbb-b1) – (2 entries)	Multiple	Yes	−1.78	1.72
blocked early in transport 1 homolog (*S. cerevisiae*) (Bet1)	BG087731	Yes	−1.33	1.69
vimentin (Vim)	BG073184	Yes	−1.23	1.61
phosphatidic acid phosphatase type 2B (Ppap2b)	BG080374	Yes	−1.50	1.57
lysosomal-associated protein transmembrane 4A (Laptm4a)	BG081646	Yes	−1.73	1.55
caveolin, caveolae protein (Cav)	BG083456	Yes	−1.27	1.24
gap junction membrane channel protein β 3 (Gjb3)	BG063176		−1.22	1.19
drebrin 1 (Dbn1)	BG086358	Yes	−1.37	1.10
cystatin C (Cst3)	BG078926	Yes	−1.73	1.07
cask-interacting protein 2 (Caskin2)	H3016G08	Yes	−1.17	1.10
Mpv17 transgene, kidney disease mutant (Mpv17)	BG066244	Yes	−1.34	0.83
adducin 3 (γ) (Add3)	BG073394	Yes	−1.58	0.83
RUN and FYVE domain containing 1 (Rufy1)	BG088525		−1.56	0.78
Moesin homolog (Msn) [mice, teratocarcinoma F9 cells]	BG066632	Yes	−1.46	0.77
hemoglobin Y, β-like embryonic chain (Hbb-y)	BG073042	Yes	−1.45	0.54
thymosin, β 10 (Tmsb10)	BG063081	Yes	−1.18	0.46
FAT tumor suppressor homolog (Drosophila) (Fath)	BG081126	Yes	−1.21	0.25
cadherin 5 (Cdh5)	BG077522	Yes	−1.40	0.24
tropomyosin 1, α (Tpm1)	BG079039	Yes	−2.74	0.03
Transcription/chromatin				
polymerase (DNA-directed), epsilon 4 (p12 subunit) (Pole4)	H3121D01	Yes	1.37	4.42
RNA polymerase II transcriptional coactivator (Rpo2tc1)	BG074743	Yes	1.38	3.48
ankyrin repeat, family A (RFXANK-like), 2 (Ankra2)	BG081823	Yes	1.24	1.47
WW domain binding protein 5 (Wbp5)	BG086567	Yes	1.43	1.29
similar to general transcription factor IIA, 2 (Gtf2a2)	BG088284	Yes	1.25	0.84
similar to G-rich sequence factor-1 (GRSF-1)	BG079939	Yes	1.24	0.37
Mus musculus bisphosphate 3′-nucleotidase (Bpnt1)	BG063789		1.19	0.35
Max dimerization protein 5 (Mad5)	BG087998	Yes	−2.10	6.41
zinc finger protein 91 (Zfp91)	BG068821	Yes	−1.64	4.74
myeloid/lymphoid leukemia 5 (Mll5)	BG084166	Yes	−1.76	3.82
LIM domains containing 1 (Limd1)	BG069785	Yes	−1.69	3.32
putative homeodomain transcription factor (Phtf)	BG070102		−1.84	3.31
similar to zinc finger protein 40 (LOC224598)	H3065a10		−1.42	3.27
special AT-rich sequence binding protein 1 (Satb1)	BG065579		−1.38	2.35
Kruppel-like factor 9 (Klf9)	BG078359	Yes	−1.43	2.06
homeo box B3 (Hoxb3)	BG086115		−1.69	1.58
suppressor of K^+ transport defect 3 (Skd3)	BG065131	Yes	−1.44	1.54
zinc finger protein 162 (Zfp162)	BG077386	Yes	−1.25	1.42
H3 histone, family 3B (H3f3b) – (2 entries)	Multiple	Yes	−1.19	1.31

Table 3. Continued

Putative gene ID	Accession No./clone ID*	Detected in heart?	Fold change	*B*-stat
RNA-binding region (RNP1, RRM) containing 2 (Rnpc2)	BG065252	Yes	−1.51	1.06
fragile X mental retardation syndrome 1 homolog (Fmr1)	BG072991	Yes	−1.29	1.06
nuclear receptor subfamily 2, group F, member 2 (Nr2f2)	BG086354	Yes	−1.44	0.93
Kruppel-like factor 4 (gut) (Klf4)	BG069413	Yes	−1.71	0.34
Protein synthesis/translational control				
mitochondrial ribosomal protein L32 (Mrpl32)	BG077318	Yes	1.34	5.13
mitochondrial ribosomal protein L50 (Mrpl50)	BG076629	Yes	1.22	3.28
ribosomal protein S13 (Rps13)	BG085522	Yes	1.31	2.69
ribosomal protein L41 (Rpl41) – (3 entries)	Multiple	Yes	1.26	1.90
ribosomal protein S27-like (Rps27l)	BG076628	Yes	1.38	1.46
eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked (Eif2s3x)	BG083267	Yes	1.29	0.98
ribosomal protein L9 (Rpl9)	BG072542	Yes	1.21	0.82
ribosomal protein S17 (Rps17)	BG073105	Yes	1.39	0.52
similar to eukaryotic translation initiation factor 1A (Eif1a)	H3033G07	Yes	1.34	0.45
ribosomal protein S24 (Rps24)	BG075284	Yes	1.27	0.09
similar to hypothetical protein 2 (rRNA external transcribed spacer) (LOC224783)	BG069712		−1.85	6.24
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 (Ddx6) – (2 entries)	Multiple		−1.81	2.20
ribosomal protein L21 (Rpl21)	BG085871	Yes	−1.39	1.94
general control of amino acid synthesis-like 1 (yeast) (Gcn5l1)	H3096E01	Yes	−1.41	1.86
splicing factor, arginine/serine-rich 3 (SRp20) (Sfrs3)	BG078157	Yes	−1.35	1.80
heterogeneous nuclear ribonucleoprotein H2 (Hnrph2)	BG085988	Yes	−1.32	1.25
deleted in azoospermia-like (Dazl)	BG086966		−1.50	0.62
splicing factor, arginine/serine-rich 3 (SRp20)	BG064952	Yes	−1.47	0.33
ribosomal protein L15 (Rpl15)	BG078436	Yes	−1.33	0.20
Similar to DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 17	BG063903	Yes	−1.23	0.17
ribosomal protein S14 (Rps14)	BG086818	Yes	−1.32	0.06
eukaryotic translation initiation factor 4, γ 1 (Eif4g1)	BG075088	Yes	−1.45	0.01
Cell defence/growth related proteins				
metallothionein II (Mt2) gene	BG063925		1.57	13.91
N-myc downstream regulated 4 (Ndr4)	BG069624	Yes	1.37	1.44
similar to cell cycle checkpoint protein CHFR (*Homo sapiens*)	BG086922	Yes	1.22	0.72
ADP-ribosyltransferase (NAD^+; poly (ADP-ribose) polymerase) 1 (Adprt1)	H3111C07	Yes	1.22	0.60
G1 to phase transition 1 (Gspt1)	BG072757	Yes	1.19	0.37
FK506 binding protein 4 (59 kDa) (Fkbp4) – (2 entries)	Multiple	Yes	−1.51	4.87
anaphase-promoting complex subunit 7 (Apc7)	BG064663	Yes	−1.55	2.57
pleiotrophin (Ptn)	BG086278	Yes	−1.66	0.97
EGF-like-domain, multiple 5 (Egfl5)	BG087063		−1.34	0.40
Apoptosis				
TIA1 cytotoxic granule–associated RNA binding protein (Tia1)	BG086002	Yes	−1.76	2.14
Unknown genes/ESTs				
ethanol induced 6 (Etohi6)	H3014B02	Yes	1.21	3.05
brain protein 44-like protein (Brp44l)	BG086955	Yes	1.26	1.81
tangerine	BG073936	Yes	−1.66	2.27
And 186 unknown genes/ESTs†				

HHR, hypertrophic heart rat; NHR, normal heart rat; ESTs, expressed sequence tags. *Multiple accession numbers referring to the same gene are summarised as one entry. Full results are available for download at http://www.physiology.unimelb.edu.au/ †Unknown genes/ESTs are listed separately and available for download at http://www.physiology.unimelb.edu.au/

Table 4. Genes Differentially Expressed in Both Young (8–10 Weeks) and Old (38–50 Weeks) HHR Left Ventricular Tissue When Compared with Age-Matched NHR

Putative gene ID	Accession No./clone ID	Detected in heart?	Fold change	*B*-stat "young"	Fold change	*B*-stat "old"
Energy/metabolism						
polypeptide GalNAc transferase-T2 (Galnt2)	BG064057	Yes	1.23	4.44	1.25	3.52
translocase of inner mitochondrial membrane 8 homolog b (yeast) (Timm8b)	BG080049	Yes	1.20	1.15	1.28	3.36
paraplegin (Spg7)	BG072876	Yes	1.41	1.37	1.42	0.20
serine (or cysteine) proteinase inhibitor, clade B, member 6c (Serpinb6c)	BG078882		−1.43	2.59	−1.56	4.04
Signal transduction						
retinoblastoma binding protein 4 (Rbbp4)	BG084231	Yes	1.23	7.75	1.18	2.47
adenylate kinase 2 (Ak2)	BG067269	Yes	−1.66	7.09	−1.85	8.92
Homo sapiens jumonji homolog (mouse) (Jmj)	BG079848	Yes	−1.44	5.06	−1.48	4.62
midkine (Mdk)	BG072761	Yes	−1.29	0.30	−1.63	8.21
Matrix/structural proteins						
microtubule-associated protein 1 light chain 3 (Map1lc3)	BG084816	Yes	1.28	3.37	1.38	6.15
endomucin (Emcn)	BG065756	Yes	−1.21	1.56	−1.55	15.38
chloride channel 3 (Clcn3)	BG077600	Yes	−1.26	3.11	−1.52	12.07
phosphatidylinositol glycan, class B (Pigb)	BG067091		−1.37	2.50	−1.52	5.35
ARP2 actin–related protein 2 homolog (yeast) (Actr2)	H3002A08	Yes	−1.13	0.34	−1.19	3.20
Transcription/chromatin						
stromal membrane-associated protein (Smap1)	BG063419	Yes	1.15	2.86	1.16	1.88
Protein synthesis/translational control						
poly(A) binding protein, nuclear 1 (Pabpn1)	BG080210	Yes	−1.44	0.08	−2.09	9.33
Cell defence/growth related proteins						
natriuretic peptide precursor type B (Nppb)	BG063995	Yes	1.40	1.62	1.92	11.44
WAP four-disulfide core domain 1 (Wfdc1)	BG074893	Yes	1.68	6.61	1.81	7.09
12 unknown genes/ESTs[†]						

HHR, hypertrophic heart rat; NHR, normal heart rat; ESTs, expressed sequence tags. [†]Unknown genes/ESTs are listed separately and available for download at http://www.physiology.unimelb.edu.au/

Differentially Expressed Genes in Young HHR

A number of genes were found to be differentially expressed only between HHR and NHR at 8–10 weeks of age (Table 2). With reference to the available literature, genes were grouped into eight broad functional classifications; energy/metabolism, signal transduction, matrix/structural proteins, transcription/chromatin, protein synthesis/translational control, cell defense/growth–related proteins, apoptosis and unknown. Genes are ranked in the tables by the *B*-statistic with over-expressed genes (positive log-ratio) followed by under-expressed genes (negative log change). The log ratios are base 2, and thus a log ratio equal to one corresponds to a two-fold change.

In young HHR, 65 of the 15,247 arrayed clones (0.43%) showed statistically significant differential expression (21 clones over-expressed, 44 clones under-expressed). Twenty-nine of these 65 clones were found to be the same as those differentially expressed in old HHR (see below). A functional annotation was ascribable to 37 (59%) of the differentially expressed clones in young HHR (Fig. 1).

Differentially Expressed Genes in Old HHR

Genes differentially expressed in 38–50 week-old HHR and NHR, but not in young animals (8–10 weeks old), are listed in Table 3. Relative to NHR, older HHR showed a greater proportion of differential expression than at the younger age, such that 390 clones (2.6%) showed statistically significant differential expression (98 clones over-expressed, 292 clones under-expressed). A physiological function was ascribable to 188 (49%) of these clones in old HHR (Fig. 1).

Differentially Expressed Genes Common to Both Young and Old HHR

Of the 426 differentially expressed clones in the young and old HHR there were 29 clones (7%) that were common to both (Table 4). A function was ascribable for 17 (59%) of these clones. The number of differentially expressed clones common to both the young and old animals is shown in Fig. 2.

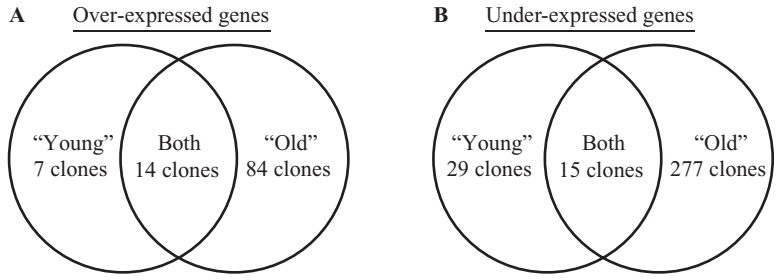

Fig. 2. *Age-specific gene expression. A: The Venn diagram shows the relative number of over-expressed clones in the left ventricular tissue of young HHR (8–10 weeks) compared with that of old HHR (38–50 weeks). B: The relative number of under-expressed clones in young HHR (8–10 weeks) compared with old HHR (38–50 weeks) left ventricular tissue.*

Table 5. Comparison of Fold Changes as Detected by Microarray *vs.* Real-Time PCR for Data Comparing "Old" (38–50 Weeks) HHR and NHR

Target gene	Gene name	Accession number	Microarray fold change	Real-time PCR fold change
Natriuretic peptide precursor type B	*Nppb*	BG063995	1.92	1.15
Mitogen activated protein kinase 1	*Mapk1*	BG069285	1.33	1.06
Tropomyosin 1	*Tpm1*	BG079039	−2.74	−1.34
TRAF family member–associated Nf-κB activator	*Tank*	BG080604	−1.82	−1.85

HHR, hypertrophic heart rat; NHR, normal heart rat.

Differentially Expressed Unknown Genes/ESTs in HHR

Many clones encoding hypothetical proteins or ESTs with no homology to known proteins exhibited notable over-expression or under-expression in the HHR. These clones are listed in the supplemental materials and are available for download at http://www.physiology.unimelb.edu.au/ftp/SupplementaryDataforMicroarrayManuscript.pdf

RT-PCR Results

The results of the RT-PCR reactions and their concordance with those obtained by microarray are shown in Table 5. Changes in gene expression are shown as mean fold changes (Table 5). The PCR results for the four genes confirm the microarray results (correlation=0.95, $p<0.05$).

Discussion

In the present study, we applied cDNA microarray expression profiling to the study of pressure-independent cardiac hypertrophy with the aim of identifying differentially expressed genes in the HHR that might be associated with its cardiac hypertrophy. To this end, the relative expression of 15,427 cDNA sequences was compared in left ventricular cardiac tissue from both young HHR and NHR at 8–10 weeks, and old HHR and NHR at 38–50 weeks. In young HHR, 65 of the arrayed clones showed differential expression compared with 390 differentially expressed clones in old HHR. The fact that there were a greater number of differentially expressed genes in old HHR may represent an increase in genetic "noise" due to the cumulative effect of time and environment on the gene expression. Alternatively, it is possible that the greater differential gene expression observed in old HHR was the result of pathological changes arising from the cardiac hypertrophy in this model.

A number of genes were found to be expressed only in young or old animals, while some were differentially expressed at both ages. It is difficult to judge which set of genes warrants further investigation. While one could argue that genes differentially expressed only at a young age might be causative of cardiac hypertrophy in the HHR, it is also possible that causal genes are over- or under-expressed throughout life. If this were the case, other factors might be acting to limit cardiac growth at older ages. It might be argued, however, that genes differentially expressed only at the older age are less likely to have been involved in the development of cardiac hypertrophy. Alternatively, a causal gene might not be differentially expressed at all and could exert its action *via* an alteration of protein function rather than transcript level. This latter possibility remains an inherent limitation of the microarray approach.

Genes showing differential expression only in young HHR (Table 2) are involved in several cellular processes. The DnaJ (Hsp40) homolog (*Dnaja3*) was shown to be over-expressed

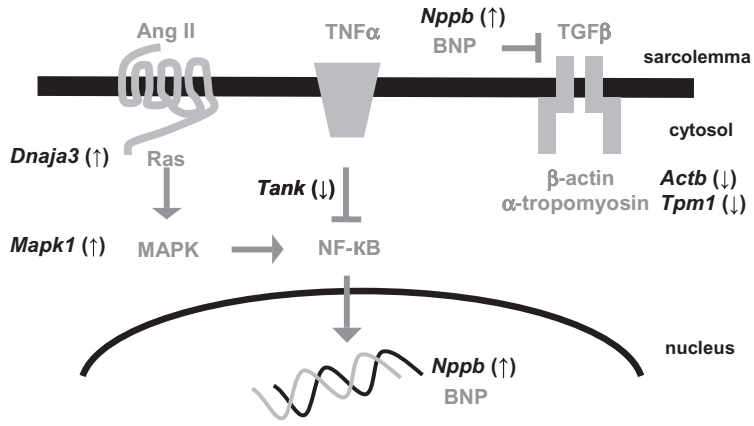

Fig. 3. *Gene dysregulation in the HHR is associated with activation of a hypertrophic signaling pathway involving Ras/MAPK (Dnaja3, Mapk1) and TNF-receptor associated (Tank) proteins which could contribute to increased activation of the nuclear factor NF-κB. The increased expression level of the hypertrophic marker BNP (Nppb), which is known to suppress expression of sarcomeric and matrix proteins, is consistent with the lack of fibrosis observed in the HHR. Gene names are indicated in bold italics, with arrows in brackets denoting over- or under-expression in HHR vs. NHR. Blunt "T" ended pointers indicate an inhibitory effect on the target. Refer to the text for details.*

in young HHR. Genetic and biochemical studies have implicated DnaJ and Hsp70 proteins as important components of intracellular signaling pathways linked to cell survival and growth regulation (*25*). *Dnaja3* (alias *Tid-1*) has been shown to define a novel Ras GTPase–activating protein (RasGAP) binding protein (*26*). Recent evidence suggests a central role for Ras, a small G-protein, in transmitting hypertrophic signals from G-protein coupled receptors, growth factor receptors and cytokine receptors to the nucleus *via* the Ras/mitogen activated protein kinase (MAPK) pathway and other cytosolic effectors (*25*). The over-expression in old HHR of the guanine nucleotide binding protein gene (*Gng5*), *Rab12* (a member of the Ras oncogene family) and mitogen activated protein kinase 1 (*Mapk1*) further suggests a role for the Ras/MAPK signaling pathway in the etiology of cardiac enlargement in the HHR. Evidence that MAPK1 (*27–29*), MAPK14 (*30*, *31*) and MAP2K4 (*32*) are involved in normal and abnormal tissue growth further strengthens the possibility that these are key enzymes in the cardiotrophic process in the HHR. However, the cause and effect relationship between the activity of these enzymes and the elevated cardiac size of the HHR remains to be determined.

It was of interest that transforming growth factor-β (TGF-β) was under-expressed in young HHR. A recent study has shown that human B-type natriuretic peptide (BNP) inhibits TGF-β–induced effects on primary human cardiac fibroblasts, including down-regulating TGF-β–regulated genes related to fibrosis, myofibroblast conversion, proliferation, and inflammation (*33*). The mouse homologue to the polypeptide hormone BNP, natriuretic peptide precursor type B (Nppb), was over-expressed in both young and old HHR, and several matrix/structural protein–related genes (*β-actin*, *Actb*; *procollagen-5*, *Col5a1*; *cartilage associated protein*, *Crtap*) were under-expressed in young HHR. Therefore, one possibility is that the over-expression of the cardiac growth factor Nppb in the cardiac tissue of the HHR inhibits TGF-β expression and the expression of several fibrosis-related genes. Supporting this proposal is the fact that the HHR exhibits increased cardiomyocyte mass with no difference in the proportion of fibrous tissue (*6*). Further studies are required to determine the role of Nppb and its gene in the HHR. The hypothesized molecular pathways by which the genes and proteins mentioned above give rise to the cardiac hypertrophy of the HHR are summarized in Fig. 3.

Among the genes differentially expressed in old HHR, several were assigned an energy/metabolism functional annotation. Of particular interest were the 16 under-expressed mitochondrial DNA clones. Mitochondria are abundant in energy-demanding cardiac tissue, and mitochondrial biogenesis is increased during cardiac hypertrophy (*34*). However, increased levels of point mutations in cardiac mitochondrial DNA and reduced levels of mitochondrial enzymatic activities may occur with aging due to increased production of reactive oxygen species (*35*). Furthermore, a reduction in cardiac mitochondrial DNA levels and respiratory enzymes has been reported in patients with isolated hypertrophic cardiomyopathy and is associated with clinical cardiac dysfunction (*34*). The gene encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (*Gapd*), which was shown to be under-expressed in the old HHR in the present study, has also been shown to be impaired during cardiac oxidative stress (*36*). Thus, the under-expression of mitochondrial DNA and respiratory enzymes in the HHR may reflect an altered oxidative state and cardiac dysfunction in old animals. Perhaps an adaptive response by old HHR to this state of oxidative stress might be the over-expression of the gene encod-

ing the metal-binding protein metallothionein II (MT-II). Metallothionein-overexpressing transgenic mouse models have produced evidence of an antioxidant and protective function of metallothionein from oxidative injury in the heart (*37*).

The tumor necrosis factor (TNF) receptor–associated factor (TRAF) family of proteins is involved in transducing signals from members of the TNF receptor family (*38*). Genes encoding for the TRAF family member–associated nuclear factor-κB (NF-κB) activator (TANK/I-TRAF) and TRAF and TNF receptor associated protein (TTRAP) were under-expressed in old HHR. Studies have shown that over-expression of both TANK/I-TRAF and TTRAP inhibits TRAF-mediated activation of the nuclear transcription factor NF-κB (*38*, *39*). This is of interest because activation of NF-κB has been shown to be required for hypertrophic growth of rat neonatal ventricular cardiomyocytes (*40*). Furthermore, oncogenic Ras enhances NF-κB transcriptional activity through mitogen-activated protein kinase signaling pathways (*41*) and angiotensin II. Angiotensin II is a potent stimulus to hypertrophy of isolated cardiomyocytes in the absence of hemodynamic load (*42*), and induces gene transcription through cell-type-dependent effects on NF-κB (*43*). It has also been shown that metallothionein, over-expressed in old HHR, activates NF-κB, which suggests a potential role for NF-κB in mediating the protective effects of metallothionein (*44*). Thus, it is possible that the under-expression of inhibitors and over-expression of activators for NF-κB may be accentuating its activity, which in turn might contribute to the cardiac hypertrophy in the HHR.

One of the genes showing the greatest under-expression in old HHR was that encoding for α-tropomyosin (*Tpm1*), a component of the cardiac sarcomere. Mutations in the gene encoding α-tropomyosin (*Tpm1*) have been described in hypertrophic cardiomyopathy (HCM) (*45*). Moreover, it has been suggested that mutations in sarcomeric proteins may have some effect on proto-oncogenes, and indeed, the expression of nuclear proto-oncogenes *Ras* and *c-myc* has been shown to be up-regulated in HCM patients (*46*). It was also of interest that the gene for the cell growth inhibitor Max dimerization protein 5 (*Mad5*) was under-expressed in old HHR. Transcription repression is mediated by Max heterodimer formation with members of the Mad family such that over-expression of Max dimerization protein 5 can inhibit cell growth and cellular transformation by Myc and Ras (*47*). Thus, it stands to reason that under-expression of *Mad5* may increase the positive effects of Myc and Ras on cellular growth and gene expression in cardiac tissue of the HHR.

Genes differentially expressed at both the developmental and established hypertrophic stages in the HHR were expected to represent the common genetic events in pressure-independent cardiac hypertrophy. As mentioned previously, Nppb was over-expressed in both young and old HHR. Expression of its gene (*Nppb*), predominantly from the cardiac ventricles (*48*), increases dramatically in response to hypertrophic stimuli (*49*). The gene encoding the chloride channel subunit Clcn3 was under-expressed at both young and old ages in the HHR. An increased chloride current develops in hypertrophied rat ventricular myocytes (*50*), and changes in Clcn3 mRNA levels have been shown in cardiac hypertrophy induced by abdominal aortic banding (*51*). Perhaps the reduced expression of *Clcn3* in the HHR is an adaptive response to a hypertrophy-induced increase in chloride current.

The genes in the HHR contributing to their cardiac hypertrophy are either hypertrophic genes transmitted from the SHR progenitors or "hypotrophic" genes not received from the F344 strain. As such, it should be possible to identify those genes differentially expressed in our study and which have also previously been reported to have an altered expression in the SHR. We would argue that such genes would be more likely to be those that cause altered heart size in the SHR and act independently of the elevated blood pressure in this strain. For example, we found differential expression of the *NF-κB* gene in HHR in our study, a finding which has previously been reported in SHR (*52*). The suggestion by Gupta *et al*. that this particular gene causes cardiac hypertrophy independently of blood pressure is thus supported by our finding that the normotensive HHR has altered expression of this gene (*52*). Other genes that we report as differentially expressed between HHR and NHR, and which have also been reported as having altered expression in the SHR include TGF-β1 (*53*, *54*), *Nppb* (brain natriuretic peptide) (*55–57*) and *Mapk1* (*58*). In contrast, given that we found altered expression of the chloride channel subunit gene *Clcn3* in HHR, our results do not agree with the suggestion that chloride channels do not play a role in cardiac hypertrophy (*59*). These genes differentially expressed in both HHR and SHR, we believe, are particularly worthy of further investigation in either strain.

Our study describes for the first time the gene expression changes in hypertrophic cardiac tissue from the HHR. Moreover, this study investigated age-specific expression changes representative of pressure-independent cardiac hypertrophy in younger and older animals. Despite the analytical power of gene expression profiling through the use of DNA microarrays, there are some limitations to studies such as those reported here. First, in the present study we hybridized rat total RNA to a mouse cDNA clone set. This may explain the low fold changes reported, especially in the HHR *vs*. NHR comparison at 8–10 weeks of age. However, an independent assessment of rat-mouse sequence homology in the NIA 15K clone library has suggested that inter-species use of this clone library is valid (*60*). Future gene expression studies of this model will incorporate rat microarray analyses when these are more readily available. The relatively low fold changes reported may also be due to the genetic similarity of the HHR and the NHR, which were inbred from the same parental strains, the spontaneously hypertensive and Fischer 344 rats (*6*). It is unlikely that large differences in gene expression will

be found between similar tissues, as previous studies of gene expression in cardiac growth have reported fold-change values of 1.5-fold as significant (*61*). However, we are confident that most of the putative differentially expressed genes represent real differences in gene expression given the biological and technical replication involved in the experiment and the statistical analysis of significance, which analyzed variability as well as observed fold changes. The co-identification of known hypertrophic genes adds further confidence to our results. Our data suggest candidate genetic factors that contribute to the establishment and maintenance of the cardiac hypertrophy of the HHR. Further exploration and verification of the precise role of identified genes in this process is merited.

Acknowledgements

The valuable technical assistance of Ms. Anna Caldwell, Ms. Catherine Jensen and Ms. Melanie O'Keefe is gratefully acknowledged.

References

1. Kannel WB, Dannenberg AL, Levy D: Population implications of electrocardiographic left ventricular hypertrophy. *Am J Cardiol* 1987; **60**: 85I–93I.
2. Lauer MS, Anderson KM, Kannel WB, Levy D: The impact of obesity on left ventricular mass and geometry. The Framingham Heart Study. *JAMA* 1991; **266**: 231–236.
3. Palmieri V, de Simone G, Roman MJ, Schwartz JE, Pickering TG, Devereux RB: Ambulatory blood pressure and metabolic abnormalities in hypertensive subjects with inappropriately high left ventricular mass. *Hypertension* 1999; **34**: 1032–1040.
4. Tingleff J, Munch M, Jakobsen TJ, *et al*: Prevalence of left ventricular hypertrophy in a hypertensive population. *Eur Heart J* 1996; **17**: 143–149.
5. Tanase H, Yamori Y, Hansen CT, Lovenberg W: Heart size in inbred strains of rats. Part 1. Genetic determination of the development of cardiovascular enlargement in rats. *Hypertension* 1982; **4**: 864–872.
6. Harrap SB, Danes VR, Ellis JA, Griffiths CD, Jones EF, Delbridge LM: The hypertrophic heart rat: a new normotensive model of genetic cardiac and cardiomyocyte hypertrophy. *Physiol Genomics* 2002; **9**: 43–48.
7. MacLellan WR, Schneider MD: Genetic dissection of cardiac growth control pathways. *Annu Rev Physiol* 2000; **62**: 289–319.
8. Lee HR, Henderson SA, Reynolds R, Dunnmon P, Yuan D, Chien KR: Alpha 1−adrenergic stimulation of cardiac gene transcription in neonatal rat myocardial cells. Effects on myosin light chain-2 gene expression. *J Biol Chem* 1988; **263**: 7352–7358.
9. Dempsey AA, Dzau VJ, Liew CC: Cardiovascular genomics: estimating the total number of genes expressed in the human cardiovascular system. *J Mol Cell Cardiol* 2001; **33**: 1879–1886.
10. Snijders AM, Meijer GA, Brakenhoff RH, van den Brule AJ, van Diest PJ: Microarray techniques in pathology: tool or toy? *Mol Pathol* 2000; **53**: 289–294.
11. Aronow BJ, Toyokawa T, Canning A, *et al*: Divergent transcriptional responses to independent genetic causes of cardiac hypertrophy. *Physiol Genomics* 2001; **6**: 19–28.
12. Friddle CJ, Koga T, Rubin EM, Bristow J: Expression profiling reveals distinct sets of genes altered during induction and regression of cardiac hypertrophy. *Proc Natl Acad Sci U S A* 2000; **97**: 6745–6750.
13. Lovenberg W: Animal models for hypertension research. *Prog Clin Biol Res* 1987; **229**: 225–240.
14. Tanaka TS, Jaradat SA, Lim MK, *et al*: Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proc Natl Acad Sci U S A* 2000; **97**: 9127–9132.
15. Buckley MJ: Spot User's Giuide. Sydney, CSIRO Mathematical and Information Sciences, 2000.
16. Yang YH, Buckley MJ, Dudoit S, Speed TP: Comparison of methods for image analysis on cDNA microarray data. *J Comput Graph Stat* 2002; **11**: 108–136.
17. Smyth GK, Speed TP: Normalization for cDNA microarray data. *Methods* 2003; **31**: 265–273.
18. Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article3.
19. Smyth GK, Michaud J, Scott HS: Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 2005; **21**: 2067–2075.
20. Smyth GK, Yang YH, Speed T: Statistical issues in cDNA microarray data analysis. *Methods Mol Biol* 2003; **224**: 111–136.
21. Kargul GJ, Dudekula DB, Qian Y, *et al*: Verification and initial annotation of the NIA mouse 15K cDNA clone set. *Nat Genet* 2001; **28**: 17–18.
22. Ashburner M, Ball CA, Blake JA, *et al*: Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; **25**: 25–29.
23. Diehn M, Sherlock G, Binkley G, *et al*: SOURCE: a unified genomic resource of functional annotations, ontologies, and gene expression data. *Nucleic Acids Res* 2003; **31**: 219–223.
24. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–408.
25. Hefti MA, Harder BA, Eppenberger HM, Schaub MC: Signaling pathways in cardiac myocyte hypertrophy. *J Mol Cell Cardiol* 1997; **29**: 2873–2892.
26. Trentin GA, Yin X, Tahir S, *et al*: A mouse homologue of the Drosophila tumor suppressor l(2)tid gene defines a novel Ras GTPase-activating protein (RasGAP)-binding protein. *J Biol Chem* 2001; **276**: 13087–13095.
27. Hayes GM, Carrigan PE, Miller LJ: Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas. *Cancer Res* 2007; **67**: 2072–2080.
28. McWhinnie R, Pechkovsky DV, Zhou D, *et al*: Endothelin-1 induces hypertrophy and inhibits apoptosis in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**: L278–L286.
29. Stockand JD, Meszaros JG: Aldosterone stimulates prolifer-

ation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling. *Am J Physiol Heart Circ Physiol* 2003; **284**: H176–H184.
30. Bhattacharya S, Macdonald ST, Farthing CR: Molecular mechanisms controlling the coupled development of myocardium and coronary vasculature. *Clin Sci* (*Lond*) 2006; **111**: 35–46.
31. Fang JY, Richardson BC: The MAPK signalling pathways and colorectal cancer. *Lancet Oncol* 2005; **6**: 322–327.
32. Murphy KM, Brune KA, Griffin C, *et al*: Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: deleterious BRCA2 mutations in 17%. *Cancer Res* 2002; **62**: 3789–3793.
33. Kapoun AM, Liang F, O'Young G, *et al*: B-type natriuretic peptide exerts broad functional opposition to transforming growth factor-beta in primary human cardiac fibroblasts: fibrosis, myofibroblast conversion, proliferation, and inflammation. *Circ Res* 2004; **94**: 453–461.
34. Marin-Garcia J, Goldenthal MJ: The mitochondrial organelle and the heart. *Rev Esp Cardiol* 2002; **55**: 1293–1310.
35. Raha S, Robinson BH: Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem Sci* 2000; **25**: 502–508.
36. Eaton P, Wright N, Hearse DJ, Shattock MJ: Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol* 2002; **34**: 1549–1560.
37. Kang YJ, Li Y, Sun X, Sun X: Antiapoptotic effect and inhibition of ischemia/reperfusion-induced myocardial injury in metallothionein-overexpressing transgenic mice. *Am J Pathol* 2003; **163**: 1579–1586.
38. Rothe M, Xiong J, Shu HB, Williamson K, Goddard A, Goeddel DV: I-TRAF is a novel TRAF-interacting protein that regulates TRAF-mediated signal transduction. *Proc Natl Acad Sci U S A* 1996; **93**: 8241–8246.
39. Pype S, Declercq W, Ibrahimi A, *et al*: TTRAP, a novel protein that associates with CD40, tumor necrosis factor (TNF) receptor-75 and TNF receptor–associated factors (TRAFs), and that inhibits nuclear factor-kappa B activation. *J Biol Chem* 2000; **275**: 18586–18593.
40. Purcell NH, Tang G, Yu C, Mercurio F, DiDonato JA, Lin A: Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. *Proc Natl Acad Sci U S A* 2001; **98**: 6668–6673.
41. Norris JL, Baldwin AS Jr: Oncogenic Ras enhances NF-kappaB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. *J Biol Chem* 1999; **274**: 13841–13846.
42. Booz GW, Baker KM: Role of type 1 and type 2 angiotensin receptors in angiotensin II-induced cardiomyocyte hypertrophy. *Hypertension* 1996; **28**: 635–640.
43. Brasier AR, Jamaluddin M, Han Y, Patterson C, Runge MS: Angiotensin II induces gene transcription through cell-type-dependent effects on the nuclear factor-kappaB (NF-kappaB) transcription factor. *Mol Cell Biochem* 2000; **212**: 155–169.
44. Abdel-Mageed AB, Agrawal KC: Activation of nuclear factor kappaB: potential role in metallothionein-mediated mitogenic response. *Cancer Res* 1998; **58**: 2335–2338.
45. Marian AJ, Roberts R: Recent advances in the molecular genetics of hypertrophic cardiomyopathy. *Circulation* 1995; **92**: 1336–1347.
46. Kai H, Muraishi A, Sugiu Y, *et al*: Expression of proto-oncogenes and gene mutation of sarcomeric proteins in patients with hypertrophic cardiomyopathy. *Circ Res* 1998; **83**: 594–601.
47. Ayer DE, Laherty CD, Lawrence QA, Armstrong AP, Eisenman RN: Mad proteins contain a dominant transcription repression domain. *Mol Cell Biol* 1996; **16**: 5772–5781.
48. Ogawa Y, Nakao K, Mukoyama M, *et al*: Natriuretic peptides as cardiac hormones in normotensive and spontaneously hypertensive rats. The ventricle is a major site of synthesis and secretion of brain natriuretic peptide. *Circ Res* 1991; **69**: 491–500.
49. Nakagawa O, Ogawa Y, Itoh H, *et al*: Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. *J Clin Invest* 1995; **96**: 1280–1287.
50. Benitah JP, Gomez AM, Delgado C, Lorente P, Lederer WJ: A chloride current component induced by hypertrophy in rat ventricular myocytes. *Am J Physiol* 1997; **272**: H2500–H2506.
51. Hiramatsu M, Furukawa T, Sawanobori T, Hiraoka M: Ion channel remodeling in cardiac hypertrophy is prevented by blood pressure reduction without affecting heart weight increase in rats with abdominal aortic banding. *J Cardiovasc Pharmacol* 2002; **39**: 866–874.
52. Gupta S, Young D, Sen S: Inhibition of NF-kappaB induces regression of cardiac hypertrophy, independent of blood pressure control, in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 2005; **289**: H20–H29.
53. Jing L, Zhang JZ, Zhao L, Wang YL, Guo FY: High-expression of transforming growth factor beta1 and phosphorylation of extracellular signal–regulated protein kinase in vascular smooth muscle cells from aorta and renal arterioles of spontaneous hypertension rats. *Clin Exp Hypertens* 2007; **29**: 107–117.
54. Satoh C, Fukuda N, Hu WY, Nakayama M, Kishioka H, Kanmatsuse K: Role of endogenous angiotensin II in the increased expression of growth factors in vascular smooth muscle cells from spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 2001; **37**: 108–118.
55. Haugen E, Chen J, Wikstrom J, Gronros J, Gan LM, Fu LX: Parallel gene expressions of IL-6 and BNP during cardiac hypertrophy complicated with diastolic dysfunction in spontaneously hypertensive rats. *Int J Cardiol* 2007; **115**: 24–28.
56. Kuroski de Bold ML: Atrial natriuretic factor and brain natriuretic peptide gene expression in the spontaneous hypertensive rat during postnatal development. *Am J Hypertens* 1998; **11**: 1006–1018.
57. Ye P, West MJ: Cosegregation analysis of natriuretic peptide genes and blood pressure in the spontaneously hypertensive rat. *Clin Exp Pharmacol Physiol* 2003; **30**: 930–936.
58. Kwon S, Fang LH, Kim B, Ha TS, Lee SJ, Ahn HY: p38 Mitogen-activated protein kinase regulates vasoconstriction in spontaneously hypertensive rats. *J Pharmacol Sci* 2004; **95**: 267–272.
59. Scherer CR, Linz W, Busch AE, Steinmeyer K: Gene

expression profiles of CLC chloride channels in animal models with different cardiovascular diseases. *Cell Physiol Biochem* 2001; **11**: 321–330.

60. Boutros PC, Okey AB; Assessment of Rat-Mouse Sequence Homology in the NIA 15K Clone Library: Implications for Gene Expression Studies in Species Widely-Used in Toxicology, Society of Toxicology of Canada 35th Annual Symposium (STC). Montreal, 2002.
61. Sehl PD, Tai JT, Hillan KJ, *et al*: Application of cDNA microarrays in determining molecular phenotype in cardiac growth, development, and response to injury. *Circulation* 2000; **101**: 1990–1999.