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## Gene expression profiling of the left ventricles in a rat model of intrinsic aerobic running capacity

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Lee, Soon Jin, Justin A. Ways, John C. Barbato, David Essig, Krista Pettee, Sarah J. DeRaedt, Siming Yang, David A. Weaver, Lauren G. Koch, and George T. Cicila. Gene expression profiling of the left ventricles in a rat model of intrinsic aerobic running capacity. *Physiol Genomics* 23: 62–71, 2005. First published July 20, 2005; 10.1152/physiolgenomics.00251.2004.—Our previous work found DA rats superior for intrinsic aerobic running capacity (ARC) and several cardiac function indexes compared with Copenhagen (COP) rats, and identified ARC quantitative trait loci (QTLs) on rat chromosomes 16 (RNO16) and 3 (RNO3). The purpose of this study was to use these inbred rat strains as a genetic substrate for differential cardiac gene expression to identify candidate genes for the observed ARC QTLs. RNA expression was examined globally in left ventricles of 15-wk-old DA, F<sub>1</sub>(COP × DA), and COP rats using microarrays to identify candidate genes for ARC QTLs. We identified 199 differentially expressed probe sets and determined their chromosomal locations. Six differentially expressed genes and expressed sequence tags (ESTs) mapped near ARC QTL regions, including PDZ and LIM domain 3 (*Pdlim3*). Differential expression of these genes/ESTs was confirmed by quantitative RT-PCR. The Ingenuity Pathways program identified 13 biological networks containing 50 (of the 199) differentially expressed probe sets and 85 additional genes. Four of these eighty-five genes mapped near ARC QTL-containing regions, including insulin receptor substrate 2 (*Irs2*) and acyl-CoA synthetase long-chain family member 1 (*Acs1l*). Most (148/199) differentially expressed probe sets showed left ventricular expression patterns consistent with the alleles exerting additive effects, i.e., F<sub>1</sub>(COP × DA) rat RNA expression was intermediate between DA and COP rats. This study identified several potential ARC QTL candidate genes and molecular networks, one of them related to energy expenditure involving *Pik3r1* mRNA expression that may, in part, explain the observed strain differences in ARC and cardiac performance.

quantitative RT-PCR; PDZ and LIM domain 3 (*Pdlim3*); insulin receptor substrate 2 (*Irs2*); phosphatidylinositol 3-kinase, regulatory subunit 1 (*Pik3r1*); acyl-CoA synthetase long-chain family member 1 (*Acs1l*); endurance running; heart

AEROBIC RUNNING CAPACITY (ARC) tests are commonly used to assess cardiorespiratory function and as a general test of overall physical health status in humans. Genetic components for this complex trait in humans have been suggested by association studies of both twins and athletes (e.g., Ref. 40). Recently, genome-wide linkage analyses have identified several chromosomal regions associated with exercise capacity,

cardiac output, and stroke volume in humans (39, 42). Studies with humans and animal models suggest that, not only is the aerobic capacity trait heritable, it is also a strong predictor of disease risk (34, 49). ARC can be divided, conceptually and functionally, into two major heritable components, intrinsic and adaptational (e.g., responses to training), with this study focusing on the intrinsic component.

Animal models have been used to study the genetic components of ARC (3, 20, 24, 25, 29, 30, 47, 49). Our previous studies established the inbred Copenhagen (COP; a low-performing strain) and DA (a high-performing strain) as phenotypically divergent rat strains useful in identifying the gene(s) responsible for heritable differences in intrinsic ARC (3). A strong, positive association of ARC with isolated heart performance ( $r = 0.86$ ) was also observed in 11 rat strains, again with DA having the highest isolated cardiac output and COP having a low isolated cardiac output (3). These two strains were also divergent for a number of phenotypes related to intrinsic cardiac performance (6, 25, 46), implicating functional differences in the heart as a likely contributor to ARC. A genome scan using a segregating intercross population bred from these strains identified at least three distinct ARC quantitative trait loci (QTLs) on rat chromosomes 3 and 16 (RNO3 and RNO16, respectively; Ref. 47).

Knowledge of model organism genome sequences (e.g., Ref. 9) and the ability to study gene expression globally using microarrays (32) now allows searching for candidate genes responsible, in part, for strain differences in complex traits such as ARC, using functional genomics approaches, where genotype and phenotype are studied simultaneously (reviewed by Refs. 27 and 38). Such an approach, expression profiling a congenic strain in concert with the cognate parental inbred strain, identified *cd36* as a candidate gene for an insulin resistance QTL in the spontaneously hypertensive (SHR) rat model (1).

We used a functional genomics approach to identify candidate genes that may be responsible, in part, for the heritable differences in ARC observed in the DA and COP strains. We hypothesize that gene(s) underlying the strain differences in ARC and cardiac performance may be 1) differentially expressed in key organs/tissues, or present in a molecular network or pathway containing differentially expressed genes, and 2) map to an ARC QTL-containing region. Such genes make superior candidates to explain, at least in part, the strain differences in intrinsic ARC and cardiac performance observed between inbred DA and COP rats. Identifying the genes underlying genetically determined differences in ARC and cardiac performance may define new risk factors for cardiovas-

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cular disorders and lead to novel ways of assessing cardiac fitness.

## MATERIALS AND METHODS

### Animals

Male inbred DA and COP rats were initially purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in a breeding colony at the Medical University of Ohio under specific pathogen-free conditions. Rats were provided with pelleted rat chow (diet 5001; Purina Mills, Richmond, IN) and water ad libitum. Rats were maintained on a 12:12-h light-dark cycle, with the light cycle occurring during daytime. F<sub>1</sub>(COP × DA) rats were bred from the same colony. All procedures were carried out with the approval of our Institutional Animal Care and Use Committee and were conducted in accordance with the National Research Council's guidelines.

### Phenotyping and RNA Preparation

ARC was estimated in male DA ( $n = 4$ ), COP ( $n = 4$ ), and F<sub>1</sub>(COP × DA) ( $n = 4$ ) rats by treadmill run to exhaustion tests, conducted essentially as described (3). The first week consisted of introducing 10-wk-old rats to the treadmill (model Exer-4; Columbus Instruments, Columbus, OH) for a gradually increasing duration each day, so that the rats received sufficient treadmill education to run for 5 min at a speed of 10 m/min on a 15° slope. The following week, each 11-wk-old rat was evaluated for maximal endurance running capacity on 5 consecutive days. Daily endurance trials were performed at about the same time (between 10 AM and noon) using a constant slope of 15° and a 10-m/min starting velocity, with velocity increasing by 1 m/min every 2 min. Each rat was run until exhaustion, operationally defined as the third time a rat willingly slid onto the shock grid and sustain 2 s of shock rather than run. At this point, current to the grid was stopped and the rat removed from the treadmill.

Rats were killed by pentobarbital overdose at 15 wk of age, and their body and heart weights were measured. Left ventricles were collected by cutting the atria and major blood vessels from the ventricles, along with the top portion of the left ventricle and septum to avoid contamination. The right ventricle was then bisected and cut away proximal to the left ventricle and septum.

Left ventricle samples were processed immediately for total RNA isolation as described (28). Briefly, left ventricles were homogenized in a guanidine thiocyanate-phenol solution (Ultraspec; Biotex Laboratories, Houston, TX), extracted with chloroform, and isolated using RNA Tack Resin (Biotex Laboratories). Total RNA was further purified by ethanol precipitation and absorption to a column (RNeasy; Qiagen, Valencia, CA). Left ventricular RNA integrity was assessed by 1) electrophoretic size fractionation on 1% agarose gels under denaturing conditions and 2) hybridization of newly made cRNA probes to TestChips (Affymetrix).

### Gene Expression Profiling

Oligonucleotide microarray analysis was performed using the Affymetrix Rat Genome U34 array set [GeneChips U34A, U34B, and U34C: a total of 26,379 probe sets, ~7,000 known genes, and 1,000 expressed sequence tag (EST) clusters for U34A, and 8,000 ESTs each for -B and -C chips] according to the manufacturer's protocol. Double-stranded cDNA was synthesized from 15 µg of total RNA from each rat (4 rats/strain) using RT (SuperScript II; Invitrogen, Carlsbad, CA) and T7(dT)<sub>24</sub> as the oligonucleotide primer. Biotinylated cRNA was synthesized using a kit (Enzo Bioarray High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY), and cRNAs were purified on columns (RNeasy mini kit, Qiagen). Each cRNA was fragmented according to the protocol in the Affymetrix GeneChip Expression Analysis manual, with quality assessed by hybridization of a 5-µg aliquot to

a test chip (TestChip3, Affymetrix). Fragmented cRNA probe (5 µg) was then hybridized to each of a set of three rat GeneChips (U34A, -B, and -C; Affymetrix). Hybridization, washing, staining with streptavidin-phycoerythrin, and scanning were performed at the Medical University of Ohio Genomics Core Facility according to the manufacturer's (Affymetrix) instructions. Complete transcription and hybridization were validated using bacterial sequences as an external control, as well as several "housekeeping" genes as internal controls.

### Analysis of Microarray Data

**Identification of differentially expressed probe sets.** Absolute and comparative analyses of data were conducted using the default settings of the Affymetrix software (Microarray Suite, MAS-5.0), which uses multiple statistical algorithms (31). Microarray images were scaled to an average hybridization intensity of 150 to normalize signals between individual chips. This data was deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/projects/geo/>) as series GSE1795. Data from four different DA rats were compared with data from four separate COP rats (i.e., a 4 × 4 matrix comparison) using Data Mining Tool (DMT 3.0, Affymetrix) software. Student's *t*-test, with  $P < 0.05$  as the criterion for significance, was used to initially identify probe sets showing significant strain differences in expression where absolute expression values (signals) from four biological replicates/strains were compared between two strains. Probe sets with signal log ratios greater than 0.38 or less than -0.38 (i.e., a 1.3-fold change) were then selected. We did not include probe sets whose hybridization signals were below background levels, i.e., called "absent" for three of four rats in both strains. Reproducibility was assured by choosing probe sets 1) differentially expressed in >50% of the 4 × 4 matrix comparisons, selecting for consistency of differential calls, and 2) whose signals among the four rats/strains had a standard deviation ≤25%.

**Cluster analysis.** Rat GeneChip sets were also hybridized with cRNA prepared from the left ventricles of four F<sub>1</sub>(COP × DA) rats and analyzed as described above. Probe sets having similar expression patterns in the left ventricles of DA, COP, and F<sub>1</sub>(COP × DA) rats were identified using a correlation coefficient algorithm (Data Mining Tool, Affymetrix) which utilized a nearest neighbor approach to identify probe sets with similar expression patterns, with the correlation coefficient threshold set at 0.98 for seed pattern and 0.90 for cluster, and 1,000 as the maximum number of probe sets included in the seeding set.

**Network analysis of differentially expressed genes/ESTs.** Ingenuity Pathways Analysis (Ingenuity Systems, Mountain View, CA) was used to identify relevant biological networks (<http://www.ingenuity.com>). This application was used to query a proprietary database for interactions between our set of differentially expressed left ventricle genes/ESTs and all other genes stored in the knowledge base to generate a set of networks having a network size of 35 genes/proteins. Ingenuity Pathways Analysis software computed a score for each network according to the fit of the set of supplied focus genes (here, differentially expressed). These scores, derived from *P* values, indicate the likelihood of focus genes found together in a network by chance. A score >2 indicates a ≥99% confidence that a focus gene network was not generated by chance alone.

### Chromosomal Location of Genes

Chromosomal locations of probe sets were determined primarily by comparing their sequences with the rat genomic sequence database (build 2, version 1) at the National Center for Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov>) using the Basic Local Alignment Search Tool (BLAST) program. The BLAST program was used to identify the orthologous location in the mouse genome (mouse genomic sequence database; build 30, NCBI) for

probe sets not mapping in the rat genome. Comparative mapping information for orthologous regions of the mouse and rat genomes was also obtained from the Ensembl (<http://www.ensembl.org>), Rat Genome Database (<http://rgd.mcw.edu/>), and Mouse Genome Informatics (<http://www.informatics.jax.org/>) sites.

Radiation hybrid (RH) mapping of two ESTs and two microsatellite markers was performed using specific primers (Supplemental Table S1; available at the *Physiological Genomics* web site)<sup>1</sup> as described (28). Each primer set was used to amplify DNA from a rat-hamster hybrid cell line panel (Research Genetics; Huntsville, AL) in duplicate, with additional PCR performed to resolve conflicting results. Retention patterns for each marker in the panel were submitted to the RH map server at the Rat Genome Database (<http://rgd.mcw.edu/RHMAPSERVER/>), with logarithm of the odds (LOD) = 14 as the threshold for linkage.

#### Quantitative Real-Time RT-PCR

First-strand cDNAs were synthesized with total RNA (5 µg/reaction) using RT (SuperScript II, Invitrogen) and oligo(dT)<sub>20</sub> primers. Gene/EST-specific primers were designed (Supplemental Table S1) using Primer Express software (version 1.5; Applied Biosystems, Foster City, CA) to amplify 75- to 100-bp PCR products, with design based on two criteria. One, primer pairs were designed from sequences unique to each gene/EST as determined by BLAST search of the nonredundant GenBank database. Two, primer pairs were designed from gene/EST regions containing the Rat GeneChip probe set sequences (Affymetrix). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a gene whose expression in DA and COP rats did not significantly differ in the microarray experiments, was used to normalize data. Quantitative real-time RT-PCR (qRT-PCR) was performed essentially according to the protocol of Johnson et al. (23) using an ABI Prism 5700 thermal cycler and Sequence Detection System (using version 1.6 software), with SYBER Green PCR Master Mix (Applied Biosystems).

Specifically, qRT-PCR was performed on five replicates of each rat cDNA sample in a 25-µl reaction volume containing varying dilutions of template cDNA, gene-specific primers (0.2–0.4 µM, optimized for each primer pair; Supplemental Table S1), and SYBER Green PCR Master Mix. PCR product was amplified using the following program: 95°C for 3 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. Melt-curve analysis was performed immediately following amplification by increasing the temperature in 0.5°C increments, starting at 55°C for 80 cycles of 10 s each, to confirm amplification of a single PCR product. Standard curves for each PCR primer set were constructed using serial dilutions of cDNA samples, producing median PCR product amounts in preliminary experiments. “No-template” controls were included to ensure amplification specificity. Left ventricular RNA expression levels were calculated for each gene/EST using standard curves constructed for each PCR primer pair and then normalized for GAPDH expression levels. RNA expression levels are presented as the fold change in expression in the left ventricles of COP and F<sub>1</sub>(COP × DA) compared with DA rats.

#### Statistical Analysis

Significance was evaluated using one-way ANOVA followed by the Fisher protected least significant difference modification of the *t*-test as the post hoc test (StatView 5.0.1; Abacus Concepts, Berkeley, CA). The threshold for significance was set at *P* < 0.05.

<sup>1</sup> The Supplemental Material for this article (Supplemental Fig. S1 and Supplemental Tables S1–S3) is available online at <http://physiolgenomics.physiology.org/cgi/content/full/00251.2004/DC1>.

## RESULTS

### Validating the Phenotypes of Rats Used As Source of Left Ventricle RNA

Male DA, COP, and F<sub>1</sub>(COP × DA) rats to be used as a source of left ventricular RNA for microarray experiments were first phenotyped for ARC (measured as best distance run to exhaustion), heart weight, body weight, and relative heart weight (heart wt-to-body wt ratio) (Table 1). As previously reported (3, 25, 47), DA rats had significantly longer best distance run to exhaustion as well as increased heart weight and relative heart weight, but not body weight, values compared with COP rats. F<sub>1</sub>(COP × DA) rat ARC values were intermediate to those of DA and COP rats, as previously reported (47). Heart weight and relative heart weight values for F<sub>1</sub>(COP × DA) rats were also intermediate compared with those of DA and COP rats. Significant differences in heart weight and relative heart weight were also observed between DA and COP rats, consistent with previous results (25).

### Gene Expression Profiling of Left Ventricles

RNA expression was examined globally in the left ventricles of DA, F<sub>1</sub>(COP × DA), and COP rats using an oligonucleotide microarray assay. Data were analyzed initially using the MAS-5.0 (Affymetrix) program, and sixteen pairwise comparisons (4 × 4 matrix comparison) were performed using Data Mining Tool version 3.0 (Affymetrix).

### Overall Gene Detection and Data Validation

The percentage of probe sets corresponding to expressed transcripts on average was 31.1% for DA rats, 32.7% for COP rats, and 36.2% for the F<sub>1</sub>(COP × DA) rats. These differences were not statistically significant. The low variability seen in chip data comparisons validated the array analysis, with the average co-variation (CV) between replicates <20% for the majority of genes/ESTs identified as differentially expressed.

### Identification of Differentially Expressed Probe Sets in Left Ventricles of DA (High-ARC Strain) Compared with COP (Low-ARC Strain)

Differentially expressed probe sets were identified using sixteen pairwise comparisons (4 × 4 matrix comparison of left ventricular expression data from 4 DA rats with data from 4 COP rats). We used stringent selection criteria, as described in MATERIALS AND METHODS, to ensure that differential gene expression identified would be reproducible in four biological replicates for both inbred strains.

We identified 199 differentially expressed probe sets with the microarray analysis, with 104 probe sets showing higher expression levels and 95 probe sets showing lower expression levels in COP rats compared with DA rats (Supplemental Table S2). After elimination of 28 loci represented by multiple probe sets (25 in duplicate and 3 in triplicate), microarray analysis identified 168 genes/ESTs differentially expressed in the left ventricles of DA compared with COP rats (Supplemental Table S2).

Table 1. Comparison of aerobic running capacity, body wt, and heart wt

| Strain                            | COP          | F <sub>1</sub> (COP × DA) | DA           | P Value |
|-----------------------------------|--------------|---------------------------|--------------|---------|
| <i>n</i>                          | 4            | 4                         | 4            |         |
| Best distance run, m              | 392.0 ± 55.2 | 539.0 ± 25.5              | 758.3 ± 34.6 | 0.0004  |
| COP vs. F <sub>1</sub> (COP × DA) |              |                           |              | 0.030   |
| COP vs. DA                        |              |                           |              | 0.0001  |
| F <sub>1</sub> (COP × DA) vs. DA  |              |                           |              | 0.004   |
| Body wt, g                        | 260.5 ± 9.2  | 282.8 ± 10.6              | 270.1 ± 2.8  | NS      |
| Heart wt, mg                      | 566.8 ± 30.8 | 673.0 ± 31.6              | 718.8 ± 8.0  | 0.007   |
| COP vs. F <sub>1</sub> (COP × DA) |              |                           |              | 0.018   |
| COP vs. DA                        |              |                           |              | 0.002   |
| F <sub>1</sub> (COP × DA) vs. DA  |              |                           |              | NS      |
| Heart wt-to-body wt ratio, mg/g   | 2.17 ± 0.04  | 2.38 ± 0.04               | 2.66 ± 0.05  | <0.0001 |
| COP vs. F <sub>1</sub> (COP × DA) |              |                           |              | 0.008   |
| COP vs. DA                        |              |                           |              | <0.0001 |
| F <sub>1</sub> (COP × DA) vs. DA  |              |                           |              | 0.001   |

Comparison of aerobic running capacity (ARC), body wt, and heart wt for Copenhagen (COP), DA, and F<sub>1</sub>(COP × DA) rats. ARC (measured as best distance run to exhaustion, as described in MATERIALS AND METHODS), body wt, heart wt, and relative heart wt (heart wt ÷ body wt) values are presented for the concomitantly raised male COP, DA, and F<sub>1</sub>(COP × DA) rats used in this study. ARC was measured in 11-wk-old rats. Body wt and heart wt were measured in these same rats at 15 wk of age.

### Identifying the Chromosomal Locations of Probe Sets Differentially Expressed in the Left Ventricles of DA and COP Rats

Chromosomal locations of differentially expressed probe sets were determined by comparison of the DNA sequences used to design the probe sets with the Rat Genome Database (build 2, version 1) at the NCBI site using the BLAST program. Due to imprecision of QTL localization (11, 21), probe sets mapping to RNO16, where most of the chromosome was covered by LOD plots above the “suggestive” threshold, or the proximal portion of RNO3 (p-terminus to *D3Rat31*) were considered in ARC QTL-containing intervals. Eight differentially expressed probe sets had sequences mapping to ARC QTL-containing intervals.

Chromosomal locations of probe sets not mapped using the Rat Genome Database were inferred by comparison of their DNA sequences with the mouse genome database (build 30, NCBI) using the BLAST program. One EST, AI072658, mapped to a mouse chromosome 2 region orthologous to the RNO3 p-terminus, with radiation hybrid mapping confirming this placement (Supplemental Fig. S1), proximal to microsatellite markers defining the location of the RNO3 ARC QTL (47). Thus nine differentially expressed probe set sequences mapped to ARC QTL-containing intervals (Table 2). However, sequences used to design two probe sets (S69874 and AI231572) had high similarity to sequences in multiple genomic locations.

### Confirming the Differential Expression of Genes Using qRT-PCR

qRT-PCR, using GAPDH expression levels as an internal control, was used to confirm the differential expression for these nine genes observed in the microarray analysis (Table 3). Values for expression in the left ventricles of F<sub>1</sub>(COP × DA) rats, whose ARC are intermediate to those of the parental, DA, and COP strains, are also presented for comparison. Differential expression was confirmed for all but one EST, AA924103, in the left ventricles of COP compared with DA rats, with six genes/ESTs showing significant differences ( $P < 0.05$ ; Table 3A). Left ventricular expression for four of the remaining eight

genes/ESTs (U68544, AI231572, AI072166, and AA818129) was intermediate in F<sub>1</sub>(COP × DA) rats compared with that observed in the inbred, DA, and COP strains from which they were bred. DA and F<sub>1</sub>(COP × DA) rats showed similar levels of expression for the AA800318 and AI072166 ESTs, while COP and F<sub>1</sub>(COP × DA) rats showed similar expression levels for the AI072238 and AI072658 ESTs. EST AA924103 did not follow any of the aforementioned patterns.

qRT-PCR was also used to validate differential left ventricular expression for seven genes/ESTs, AA849518, AI008865, U50412, AA059931, AI176584, AA891242, and AI234849, that showed large differences in expression in the microarray experiments but did not map near ARC QTLs (Table 3B). qRT-PCR confirmed differential expression for five of these seven genes/ESTs.

### Identification of Gene Expression Cluster Patterns

The 199 differentially expressed probe sets were analyzed for clustering pattern among DA, COP, and F<sub>1</sub>(COP × DA) rats based on their left ventricular expression levels. Three major gene expression patterns were identified using the correlation coefficient algorithm (Data Mining Tool, Affymetrix) with *clusters 1* [DA > F<sub>1</sub>(COP × DA) > COP] and *2* [COP > F<sub>1</sub>(COP × DA) > DA] containing the most probe sets (148 of 199; Supplemental Table S2). The 199 differentially expressed probe sets listed in Supplemental Table S2 are divided into four sections (*clusters 1, 2, and 3* and “other clusters,” which contains the remaining probe sets) reflecting the results of the correlation coefficient clustering using the parameter described in MATERIALS AND METHODS.

### Identification of Additional Candidate Genes for ARC QTL Using Network Analysis

The Ingenuity Pathways Analysis program (Ingenuity Systems) identified 13 biological networks (Supplemental Table S3) containing 50 differentially expressed probe sets, with the 3 largest networks collectively containing 40 differentially expressed genes/ESTs (Supplemental Table S3). Table 4 describes the probable functions/pathways associated with the three largest networks identified by the Ingenuity Pathways

Table 2. *Genes/ESTs identified as ARC candidates*

| Probe Set ID   | Chromosome | Cluster | Current Annotation   |
|----------------|------------|---------|--|
| A.             |            |         |  |
| rc_AA924103_at | 16         |         | EST (Rn.7966), similar to elastin microfibril interfacier 3, EMILIN-like protein EndoGlyx-1  |
| rc_AA818129_at | 16q12.4    | 2       | LOC290833 (Rn.1289), similar to <i>M. musculus</i> RIKEN cDNA 2410018G3; deduced amino acid sequence similar to <i>H. sapiens</i> BBP-like protein 1 |
| rc_AI072166_at | 16q11      |         | PDZ and LIM domain 3 (actinin alpha 2 associated LIM protein), <i>Pdlim3</i>   |
| rc_AI231572_at | 16         | 1       | HesB protein, <i>Hbld2</i>   |
| U68544_at      | 16p16      | 1       | peptidylprolyl isomerase F (cyclophilin F), <i>Ppif</i>  |
| rc_AI072238_at | 3p10       | 2       | LOC296558 (Rn.20099), anaphase-promoting complex subunit 2 - predicted, <i>Anapc2</i>  |
| S69874_s_at    | 3q12       | 1       | fatty acid-binding protein, epidermal, <i>Fabp5</i>  |
| rc_AA800318_at | 3q24       | 2       | LOC295703 (Rn.100285), serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary), <i>Serping1</i>         |
| rc_AI072658_at | 3          | 2       | EST, similar to (Q9D301) <i>M. musculus</i> RIKEN cDNA 9130003M10  |
| Symbol         | Chromosome | Network | Current Annotation   |
| B.             |            |         |  |
| <i>Irs2</i>    | 16q12      | 1       | insulin-receptor substrate 2   |
| <i>Acs11</i>   | 16q11      | 1       | acyl-CoA synthetase long-chain family member 1   |
| <i>Nmi</i>     | 3q12       | 2,3     | <i>N-myc</i> (and STAT) interactor   |
| <i>Snpc4</i>   | 3p13       | 13      | small nuclear RNA activating complex, polypeptide 4  |

A: probe sets differentially expressed in left ventricles of 15-wk-old COP rats, compared with DA rats, whose sequences map to ARC quantitative trait locus (QTL)-containing regions. Chromosomal locations of the 199 differentially expressed probe sets were determined primarily by comparing the probe set sequence (NetAffyx) with the rat genome database [build 2, version 1; National Center for Biotechnology Information (NCBI)] using the Basic Local Alignment Search Tool (BLAST) program. The chromosomal location of AI072658 was determined by both comparative mapping in the mouse genome and radiation hybrid mapping (Supplemental Fig. S1). Probe set annotation was obtained from the NCBI site, with the exception of AI072658, which was not present. AI07268 annotation was obtained from the TIGR Rat Gene Index. Sequences on multiple rat chromosomes showed similarity to those from which the S69874 and AI231572 probe sets were derived. A listing of all differentially expressed probe sets is provided in Supplemental Table S2. B: genes identified by molecular network analysis that map to ARC QTL-containing regions. Chromosomal locations of the 85 additional genes present in the 13 networks identified by the Ingenuity Pathways program (Supplemental Table S3) using the Homologene and Gene databases at the NCBI website. Symbol, location, network, and current annotation are shown for the 4 genes in the networks that mapped to ARC QTL-containing regions. A listing of all genes in these pathways is provided in Supplemental Table S3.

program, as well as the genes in each network associated with the function/pathway.

Chromosomal locations were determined for the 85 genes belonging to these networks that were not among the 199 differentially expressed probe sets. Four genes, insulin receptor substrate 2 (*Irs2*), acyl-CoA synthetase long-chain family member 1 (*Acs11*), small nuclear RNA activating complex polypeptide 4 (*Snpc4*) and *Nmyc* (and STAT) interactor (*Nmi*) mapped to ARC QTL-containing regions on RNO3 and RNO16 and thus are potential ARC QTL candidate genes (Table 2B).

## DISCUSSION

Microarray analysis was used to examine gene expression patterns globally in the left ventricles of DA, COP, and F<sub>1</sub>(COP × DA) rats. Sequences of 9 differentially expressed probe sets mapped to RNO16 and the proximal portion of RNO3 (Table 2), where our previous studies identified ARC QTLs (47). While such genes/ESTs have potential as candidates, based on their 1) differential expression in a biologically relevant tissue and at a biologically relevant time point and 2) location near known ARC QTLs, additional evidence is required.

Only a small fraction of probe sets, 199 of ~27,000 interrogated, were differentially expressed in the left ventricles of 15-wk-old male DA and COP rats. A relatively low threshold (1.3-fold change) was used for determining differential expression due to the expectation that many QTLs exert small effects; however, the reproducibility of these modest changes in expression were ensured by the stringent criteria used. Differen-

tial left ventricular RNA expression was confirmed for 13 of 16 genes/ESTs examined (Table 3), reflecting well on data reproducibility.

Most differentially expressed probe sets grouped into two clusters, based on their expression patterns in DA, F<sub>1</sub>(COP × DA), and COP rat left ventricles (Supplemental Table S2), with F<sub>1</sub>(COP × DA) rats showing intermediate expression levels between the two parental strains. This suggests that the effects of the alleles on ARC are additive. Most of the 9 differentially expressed probe sets mapping to ARC QTL-containing chromosomal regions belong to these two clusters. Two genes/ESTs (U68544 and AI231572) were in *cluster 1*, where DA rat left ventricular expression was greater than that of COP rats, and four genes/ESTs (AA800318, AA818129, AI072238, and AI072658) were in *cluster 2*, where COP rat left ventricular expression was greater compared with DA rat.

### Eliminating Genes/ESTs from Consideration as ARC Candidate Genes

S69874 (*Fabp5*) was initially considered a candidate based on its differential expression (Table 3A) in a key tissue (left ventricle), having *Fabp5*-like sequences near the RNO3 ARC QTL, and its biological relevance. However, radiation hybrid and comparative mapping, as well as the lack of EST sequences corresponding to the predicted coding regions in the RNO3 site, eliminated *Fabp5* as a candidate gene (see Supplemental Material). Similarly, AI231572 (*Hbld2*) is an unlikely candidate, as the RNO16 sequence (with the highest BLAST score) was intron-less, unlike the intron-containing sequence on RNO17 orthologous to the

Table 3. Validation of differential left ventricular gene expression by quantitative real-time RT-PCR

| EST ID       | Strain                           | Relative Ratio $\pm$ SD |                  | R Value | P Value |
|--------------|----------------------------------|-------------------------|------------------|---------|---------|
|              |                                  | Microarray              | qRT-PCR          |         |         |
| <b>A.</b>    |                                  |                         |                  |         |         |
| U68544       | DA                               | 1.00 $\pm$ 0.11         | 1.00 $\pm$ 0.13  | 0.998   | 0.003   |
| <i>Ppif</i>  | F <sub>1</sub> (COP $\times$ DA) | 0.89 $\pm$ 0.09         | 0.88 $\pm$ 0.14  |         |         |
|              | COP                              | 0.69 $\pm$ 0.08         | 0.65 $\pm$ 0.09  |         |         |
|              | DA                               | 1.00 $\pm$ 0.14         | 1.00 $\pm$ 0.04  | 0.994   | 0.0007  |
| S69874       | DA                               | 1.00 $\pm$ 0.14         | 1.00 $\pm$ 0.04  |         |         |
| <i>Fabp5</i> | F <sub>1</sub> (COP $\times$ DA) | 0.85 $\pm$ 0.14         | 0.80 $\pm$ 0.07  |         |         |
|              | COP                              | 0.75 $\pm$ 0.08         | 0.67 $\pm$ 0.14  |         |         |
|              | DA                               | NA                      | 1.00 $\pm$ 0.14  | 0.993   | 0.004   |
| *S69874 (A)  | F <sub>1</sub> (COP $\times$ DA) |                         | 0.78 $\pm$ 0.18  |         |         |
|              | COP                              |                         | 0.62 $\pm$ 0.07  |         |         |
|              | DA                               | 1.00 $\pm$ 0.10         | 1.00 $\pm$ 0.18  |         |         |
| AA924103     | F <sub>1</sub> (COP $\times$ DA) | 1.09 $\pm$ 0.10         | 0.83 $\pm$ 0.18  | 0.965   | NS      |
|              | COP                              | 0.79 $\pm$ 0.09         | 1.07 $\pm$ 0.18  |         |         |
|              | DA                               | 1.00 $\pm$ 0.12         | 1.00 $\pm$ 0.17  | 0.994   | <0.0001 |
| AI231572     | F <sub>1</sub> (COP $\times$ DA) | 0.64 $\pm$ 0.07         | 0.50 $\pm$ 0.01  |         |         |
|              | COP                              | 0.36 $\pm$ 0.04         | 0.25 $\pm$ 0.03  |         |         |
|              | DA                               | 1.00 $\pm$ 0.12         | 1.00 $\pm$ 0.20  | 0.998   | 0.009   |
| AI072166     | F <sub>1</sub> (COP $\times$ DA) | 1.94 $\pm$ 0.57         | 1.29 $\pm$ 0.18  |         |         |
|              | COP                              | 1.93 $\pm$ 0.20         | 1.41 $\pm$ 0.13  |         |         |
|              | DA                               | 1.00 $\pm$ 0.15         | 1.00 $\pm$ 0.22  | 0.997   | NS      |
| AA800318     | F <sub>1</sub> (COP $\times$ DA) | 1.43 $\pm$ 0.14         | 1.01 $\pm$ 0.13  |         |         |
|              | COP                              | 1.58 $\pm$ 0.27         | 1.19 $\pm$ 0.28  |         |         |
|              | DA                               | 1.00 $\pm$ 0.08         | 1.00 $\pm$ 0.25  | 0.977   | NS      |
| AA818129     | F <sub>1</sub> (COP $\times$ DA) | 1.49 $\pm$ 0.11         | 1.56 $\pm$ 0.20  |         |         |
|              | COP                              | 1.78 $\pm$ 0.19         | 1.86 $\pm$ 0.80  |         |         |
|              | DA                               | 1.00 $\pm$ 0.22         | 1.00 $\pm$ 0.04  | 0.994   | NS      |
| AI072238     | F <sub>1</sub> (COP $\times$ DA) | 1.45 $\pm$ 0.28         | 1.39 $\pm$ 0.23  |         |         |
|              | COP                              | 1.65 $\pm$ 0.23         | 1.40 $\pm$ 0.37  |         |         |
|              | DA                               | 1.00 $\pm$ 0.09         | 1.00 $\pm$ 0.43  | 0.976   | 0.046   |
| AI072658     | F <sub>1</sub> (COP $\times$ DA) | 1.34 $\pm$ 0.21         | 1.27 $\pm$ 0.72  |         |         |
|              | COP                              | 1.46 $\pm$ 0.20         | 1.78 $\pm$ 0.40  |         |         |
|              | <b>B.</b>                        |                         |                  |         |         |
| AI008865     | DA                               | 1.00 $\pm$ 0.17         | 1.00 $\pm$ 0.02  | 0.999   | 0.001   |
|              | F <sub>1</sub> (COP $\times$ DA) | 0.46 $\pm$ 0.03         | 0.84 $\pm$ 0.18  |         |         |
|              | COP                              | 0.35 $\pm$ 0.07         | 0.62 $\pm$ 0.10  |         |         |
| U50412       | DA                               | 1.00 $\pm$ 0.18         | 1.00 $\pm$ 0.05  | 0.998   | <0.0001 |
|              | F <sub>1</sub> (COP $\times$ DA) | 0.38 $\pm$ 0.22         | 0.53 $\pm$ 0.10  |         |         |
|              | COP                              | 0.10 $\pm$ 0.02         | 0.07 $\pm$ 0.01  |         |         |
| AA849518     | DA                               | 1.00 $\pm$ 0.18         | 1.00 $\pm$ 0.14  | 0.993   | <0.0001 |
|              | F <sub>1</sub> (COP $\times$ DA) | 0.65 $\pm$ 0.09         | 0.66 $\pm$ 0.15  |         |         |
|              | COP                              | 0.02 $\pm$ 0.01         | 0.36 $\pm$ 0.07  |         |         |
| AA059931     | DA                               | 1.00 $\pm$ 0.08         | 1.00 $\pm$ 0.15  | 0.998   | NS      |
|              | F <sub>1</sub> (COP $\times$ DA) | 0.74 $\pm$ 0.04         | 1.06 $\pm$ 0.27  |         |         |
|              | COP                              | 0.47 $\pm$ 0.09         | 1.05 $\pm$ 0.18  |         |         |
| AI176584     | DA                               | 1.00 $\pm$ 0.18         | 1.00 $\pm$ 0.13  | 0.996   | 0.003   |
|              | F <sub>1</sub> (COP $\times$ DA) | 1.11 $\pm$ 0.10         | 1.18 $\pm$ 1.15  |         |         |
|              | COP                              | 2.38 $\pm$ 0.50         | 1.56 $\pm$ 0.29  |         |         |
| AA891242     | DA                               | 1.00 $\pm$ 0.15         | 1.01 $\pm$ 0.43  | 0.996   | <0.0001 |
|              | F <sub>1</sub> (COP $\times$ DA) | 1.19 $\pm$ 0.26         | 4.30 $\pm$ 1.40  |         |         |
|              | COP                              | 2.73 $\pm$ 0.38         | 14.83 $\pm$ 2.80 |         |         |
| AI234849     | DA                               | 1.00 $\pm$ 0.10         | 1.00 $\pm$ 0.07  | 0.998   | NS      |
|              | F <sub>1</sub> (COP $\times$ DA) | 4.97 $\pm$ 1.02         | 0.93 $\pm$ 0.09  |         |         |
|              | COP                              | 6.59 $\pm$ 1.60         | 0.93 $\pm$ 0.08  |         |         |

A: differentially expressed genes/ESTs with sequences mapping to ARC QTL-containing regions. B: 7 highly differentially expressed genes present in major molecular networks that did not map to ARC QTL-containing regions. Expression levels in the left ventricles of COP, F<sub>1</sub>(COP  $\times$  DA), and DA rats are presented for the 9 differentially expressed probe sets. Expression levels were determined by both oligonucleotide microarray analysis and quantitative RT-PCR (qRT-PCR) using normalized values for 4 rats per strain, as described in MATERIALS AND METHODS. Gene expression values are presented as relative ratios, with the mean DA rat value set to 1.00. Gene symbols are shown below the expressed sequence tags (ESTs), where known. R, correlation coefficient for the standard curve for a given gene/EST, obtained by qRT-PCR and calculated as described in MATERIALS AND METHODS; P, probability obtained in the comparison of DA and COP qRT-PCR expression values determined using a 1-way ANOVA, using all 3 groups with a Fisher's protected least significant difference post hoc test; NS, not significant; NA, not applicable. \*Level of gene expression that was measured using specific primers designed to distinguish *Fabp5* cDNA (S69874) sequence from *Fabp5*-like sequences (see Supplemental Materials, Supplemental Table S1).

Table 4. Probable functions for 3 networks

| Network Function                                     | Genes in Network   |
|--|--|
| <i>NW1</i>   |  |
| Lipid metabolism                                     | <i>Acs11</i> , <i>Casp8</i> , <i>Elovl6</i> , <i>Fabp5</i> (↓), <i>Fads2</i> , <i>Hnf4a</i> , <i>Irs2</i> , <i>Pik3ca</i> , <i>Pparg</i> , <i>Sc5dl</i> , <i>Scap</i> , <i>Scd</i> , <i>Srebf1</i> , <i>Slc3a2</i> (↓)   |
| Endocrine system disorders                           | <i>Cblb</i> (↑), <i>E2f1</i> , <i>Hnf4a</i> , <i>Irs2</i> , <i>Pik3r1</i> * (↓), <i>Pparg</i> , <i>Rela</i> , <i>Srebf1</i>  |
| Metabolic disease                                    | <i>Cblb</i> (↑), <i>E2f1</i> , <i>Hnf4a</i> , <i>Irs2</i> , <i>Pik3r1</i> * (↓), <i>Pparg</i> , <i>Srebf1</i>  |
| <i>NW2</i>   |  |
| Cell cycle   | <i>Akap12</i> (↑), <i>Fyn</i> , <i>RT1-Ba</i> (↑), <i>Dbi</i> (↓), <i>Id1</i> (↑), <i>Id2</i> (↑), <i>Inhba</i> , <i>Mycn</i> , <i>Npm1</i> , <i>Myc</i> , <i>Myod1</i> , <i>Nfkb1a</i> (↑), <i>Ptn</i> (↑), <i>Tp53</i>   |
| Hematological system development and function        | <i>Fyn</i> , <i>Gstp1</i> , <i>Id1</i> (↑), <i>Id2</i> (↑), <i>Il2rb</i> (↑), <i>Inhba</i> <i>Myc</i> , <i>Nfkb1a</i> (↑), <i>Npm1</i> , <i>Tp53</i> , <i>Cd74</i> , <i>Myod1</i> , <i>RT1-Da</i> (↑)  |
| Immune and lymphatic system development and function | <i>Fyn</i> , <i>Gstp1</i> , <i>Id1</i> (↑), <i>Id2</i> (↑), <i>Il2rb</i> (↑), <i>Inhba</i> <i>Myc</i> , <i>Nfkb1a</i> (↑), <i>Npm1</i> , <i>Tp53</i> , <i>Cd74</i> , <i>Myod1</i>  |
| <i>NW3</i>   |  |
| Cell death   | <i>Cd9</i> (↑), <i>Cebpb</i> (↑), <i>Dcn</i> , <i>Fn1</i> * (↑), <i>Fos</i> , <i>Fos11</i> , <i>Gata4</i> , <i>Ifng</i> , <i>Igfbp5</i> (↑), <i>Inha</i> (↓), <i>Nid</i> (↑), <i>Nppa</i> , <i>Odc1</i> , <i>Srf</i> , <i>Stat3</i> * (↓), <i>Tcf1</i>   |
| Cellular development                                 | <i>Cebpb</i> (↑), <i>Eno1</i> * (↑), <i>Fn1</i> * (↑), <i>Fos</i> , <i>Fos1</i> , <i>Gata4</i> , <i>Hop</i> * (↓), <i>Ifng</i> , <i>Nkx2-5</i> , <i>Nmi</i> , <i>Srf</i> , <i>Stat3</i> * (↓), <i>Tcf1</i>   |
| Gene expression                                      | <i>Cd9</i> (↑), <i>Cdc2</i> , <i>Cebpb</i> (↑), <i>Crp</i> , <i>Dcn</i> , <i>Eno1</i> * (↑), <i>Fn1</i> * (↑), <i>Fos</i> , <i>Gata4</i> , <i>Ifng</i> , <i>Igfbp5</i> (↑), <i>Inha</i> (↓), <i>Lgals3bp</i> , <i>Mmp11</i> , <i>Nkx2-5</i> , <i>Nppa</i> , <i>Odc1</i> , <i>Sat</i> (↑), <i>Serpine2</i> , <i>Stat3</i> * (↓), <i>Tcf1</i> , <i>Tgfb1</i> |

Probable functions for 3 networks (NWs) identified using the Ingenuity Pathways Analysis and Knowledge Base. The Ingenuity Pathway Analysis tool (winter version, 2004) was used to analyze a focus set of 199 genes differentially expressed in the left ventricles of COP compared with DA rats. Thirteen molecular NWs were identified that contained a total of 135 genes (50 differentially expressed). These networks and the genes contained in them are described in detail in Supplemental Table S3. This computer program was used to determine the most probable functions for the 3 molecular networks containing the largest numbers of differentially expressed (focus) genes. Genes associated with each of these probable functions are shown. Genes showing decreased (↓) and increased (↑) RNA expression in the left ventricles of COP compared with DA rats are indicated by arrows. Genes mapping to ARC QTL-containing intervals are indicated by italics. \*Multiple probe sets have the same gene annotation.

mouse and human *Hbld2* genes. AA924103 was also eliminated as a candidate, as qRT-PCR did not confirm its differential expression. Thus six genes/ESTs were differentially expressed in a key tissue, the heart, and map to ARC QTL-containing chromosomal intervals.

#### Using Network Analysis to Identify ARC Candidate Genes

Genes interacting with the 199 probe sets differentially expressed in the left ventricles of DA, compared with COP rats (Supplemental Table S3) were also used to identify potential candidate genes for ARC QTLs. We reasoned that while allelic differences in some gene(s) affecting ARC would not alter RNA expression and thus be undetected by microarray assay, such mutations could affect the expression of downstream genes. Thus candidate genes for ARC

QTL might also be found among genes interacting with differentially expressed genes/ESTs. Molecular networks containing the differentially expressed genes/ESTs were identified using the Ingenuity Pathways program, and the chromosomal locations of additional nondifferentially expressed genes belonging to these gene networks were determined, with four genes/ESTs mapping to ARC QTL-containing regions (Table 2B).

#### Biological Relevance of Putative Candidate Genes/ESTs to ARC

*Genes differentially expressed and mapping to ARC QTL-containing intervals.* PDZ and LIM domain 3 (*Pdlim3*) is a member of the PDZ-LIM protein family. PDZ-LIM proteins, through binding of their PDZ domains to  $\alpha$ 2-actinin, localize to Z lines of muscle. However, while *Pdlim3* knockout mice have normal skeletal muscle development and structure, they become cardiomyopathic (36), demonstrating the importance of *Pdlim3* in cardiac development and function. Peptidyl-prolyl-*cis-trans*-isomerase (*Ppif*) is a component of the mitochondrial permeability transition pore (5) whose activation is believed important in inducing death by both apoptosis and necrosis (17, 26). Anaphase promoting complex 2 (*Anapc2*) encodes one of two subunits responsible for the ubiquitin-ligase activity of the anaphase promoting complex and thus the ubiquitin-mediated destruction of cell cycle regulatory proteins, such as securin and cyclins, shortly before anaphase (51). Targeted deletion of *Anapc2* in mouse livers led to unscheduled re-entry of normally quiescent hepatocytes into the cell cycle (48). *Serp1g1* encodes C1 inhibitor protein (C1INH), a member of the serine proteinase inhibitor gene family that regulates all three pathways of complement activation (i.e., the classical, alternative, and contact). C1INH protected ischemic myocardium from reperfusion injury in animal models and in patients with acute myocardial infarctions (12). Because the remaining differentially expressed probe sets mapping to ARC QTL-containing chromosomal intervals (AI072658 and AA818129) are ESTs, their function and relevance to ARC remain unknown.

*Genes identified in molecular networks and mapping to ARC QTL-containing intervals.* Acyl-CoA synthetase long-chain family member 1 (*Acs11*) gene encodes one of several isoforms catalyzing ligation of long-chain fatty acids to CoA, the first step for fatty acid utilization in mammals. The resulting acyl-CoA products are primary substrates for energy production by  $\beta$ -oxidation in the heart and other tissues and the synthesis of cholesteryl esters, as well as functioning as signaling molecules (reviewed by Refs. 8, 13). The Nmi protein has been shown to potentiate STAT-dependent transcription and augment coactivator protein recruitment to at least some members of a group of sequence-specific transcription factors (52). *Snape4* (small nuclear RNA activating complex, polypeptide 4) encodes the 190-kDa subunit of SNAPc involved in the nucleation of both RNA polymerase II and III transcription initiation complexes (33).

A fourth gene, insulin receptor substrate 2 (*Irs2*), maps below the more distal RNO16 ARC QTL and is of particular interest. In the present study, 15-fold higher phosphatidylinositol 3-kinase, regulatory subunit 1 (*Pik3r1*) RNA expression

was observed in the left ventricles of DA compared with COP rats (Table 3B). Previous studies indicated that phosphorylated Irs2 protein interacts with Pik3r1 protein (35, 45). Zabolotny et al. (50) found reduced Irs2 protein phosphorylation in muscle following intramuscular injection of insulin in leukocyte antigen-related transgenic mice, a model of insulin resistance, causing decreased *Pik3r1* kinase activity and, ultimately, decreased glucose uptake. Higher *Pik3r1* mRNA levels observed in the left ventricles of DA rats suggest a more efficient cardiac glucose uptake, which could lead to incrementally better ARC and/or cardiac performance in this strain, particularly at later stages of the ramped endurance test. Recently, *IRS2* was proposed as a candidate gene for a baseline maximal power output QTL on human chromosome 13q33 (42) near the *IRS2* locus, orthologous to the more distal rat RNO16 ARC QTL identified in our previous study (47).

Insulin resistance, and thus abnormal glucose homeostasis, is associated with cardiovascular disease (15, 41). Studies of vasculature of obese rat (22) and human skeletal muscle biopsy samples from obese nondiabetic individuals demonstrated that insulin stimulation of the phosphoinositide 3-kinase (PI3K) pathway was dramatically reduced (10). A similar mechanism may operate in our rat model, i.e., the reduced activity of PI3K pathway, as is suggested by the 15-fold lower levels of *Pik3r1* mRNA in COP rat left ventricles, may contribute to the lower ARC performance of COP compared with DA rats.

*Highly differentially expressed genes and the ARC phenotype.* Although PI3K is most commonly associated with insulin signaling and glucose uptake, it is also a component of the Janus kinase (Jak)/STAT (and other) signaling pathways (19, 43). *Stat3* (signal transducer and activator of transcription 3) is another differentially expressed gene present in a molecular network (Tables 3 and 4, Supplemental Table S3). Both *Stat3* and *Pik3r1* have elevated RNA expression levels in DA rat left ventricles (Table 3, Supplemental Table S3) and interact with Jak proteins (2, 19, 43) including *Jak3* (Janus kinase 3), which is located on RNO16 near an ARC QTL. The Jak/STAT pathway has been associated with cardiac hypertrophy, apoptosis, angiotensin signaling, ischemia-reperfusion injury, and preconditioning (44). Thus differences in the Jak/STAT signaling pathway could lead to altered cardiac function, which may explain, in part, strain differences in ARC observed between DA and COP rats. While not part of the networks identified by the Ingenuity Pathways program (Supplemental Table S3), *Tsc1* (tuberous sclerosis complex 1) maps to the proximal portion of RNO3 and is a downstream participant in insulin/PI3K signaling. The tuberous sclerosis complex regulates insulin/PI3K signaling by inhibiting S6K protein action on insulin receptor substrate proteins (18) and is implicated in processes involved in regulating tissue size and mass (reviewed by Ref. 37).

While the rat ortholog of human myosin light chain 2a gene (*MLC2a*) does not map to an ARC QTL-containing interval, its differential expression in DA compared with COP rats may explain, in part, the observed strain differences in ARC and contractility (3, 6, 25, 47). Indeed, decreased myosin heavy chain  $\alpha/\beta$ -isoform expression ratio (for both RNA and protein) was associated with decreased cardiac contractility in Buffalo rats, another low-performing inbred strain, compared with DA rats (4).

*Metabolic pathways/molecular networks that may be responsible for strain differences in ARC between COP and DA rats.* The Ingenuity Pathways program was used to identify gene networks (NW) that contained 168 genes and ESTs differentially expressed in DA and COP rat left ventricles. Potential molecular mechanisms operating in this model that are responsible for the strain differences in ARC were sought based on 1) examination of networks containing candidate genes that map to ARC QTL-containing intervals (see above) and 2) the most probable functions assigned to the three largest networks, containing 104 different genes, by the Ingenuity Pathways program (Table 4). These functional groups, and the candidate genes contained within, can be classified into those involving 1) energy expenditure, especially lipid and glucose metabolism, and 2) regulation of cell/tissue growth and development.

Lipid metabolism and endocrine system/metabolic disorders are the top functions listed for *NW1* (Table 4). Two members of this network, *Acs1l* and *Irs2*, are involved in lipid metabolism and map to ARC QTL-containing intervals. *Irs2*, along with *Nmi*, *Tsc1*, and *Jak3* (the latter 2 map to ARC QTL-containing intervals, but not in the Ingenuity Pathways networks), encodes downstream components of the insulin receptor/PI3K signaling pathway that are key in regulating glucose and fat metabolism, and thus energy expenditure, which can affect ARC. *Pik3r1* and *Stat3*, while not mapping to ARC QTL-containing regions, are differentially expressed in this model and key members of this pathway.

The most probable functions of *NW2* are 1) immune, lymphatic, and hematological system development and function and 2) cell cycle regulation. The most probable functions of *NW3* are the regulation of 1) cellular death and development and 2) gene expression (Table 4). We should consider these two networks, as well as their most probable functions, together for two reasons. *Nmi*, which maps to an ARC QTL-containing interval, is present in both networks. The majority of the differentially expressed genes in both networks (12/13 in *NW2*, and 8/13 in *NW3*) were expressed at higher levels in the left ventricles of COP rats compared with DA rats (Table 4). Indeed, most of the genes in *NW2* and *NW3* expressed at higher levels in COP rats are in *cluster 2* (Supplemental Tables S2 and S3), suggesting that these genes may be coordinately regulated. These genes are involved in the regulation of cell growth, cell death, and development and may be responsible for the greater heart weight observed for DA rats compared with COP rats shown in this study (Table 1) and our previous studies (3, 25, 47). Indeed, these strain differences in cardiac mass are observed in male rats at 7 wk and 10 mo of age (unpublished observations). Several other genes that map to ARC QTL-containing regions may also be involved in the processes of gene expression regulation (*Snapc4*), cell cycle regulation (*Anapc2*), cell growth (*Tsc1*), and cell death (*Csp3* and *Ppif1*). Aside from differences in size, DA and COP rat hearts were shown to differ for a number of measures of cardiac performance (3, 6, 25, 46, 47), with the DA rats uniformly showing significantly greater values. *Pdlim3* (which maps to an ARC QTL-containing interval) and *Mlc2a* are both highly differentially expressed in this model and both key cardiac muscle components that may influence cardiac contractility and performance, and thus ARC.

### Limits to Using Functional Genomic Approaches to Identify Candidate Genes

Proving that any of the above-described candidate genes are causative, in part, of an ARC QTL will require identification of strain-specific allelic differences and altered protein expression in a key organ, such as the left ventricle. Eventually, demonstration that replacement of the variant nucleotide results in phenotypic alteration will be required to prove a candidate gene causative, in part, for the complex trait (16).

We acknowledge limitations in using microarray data to identify candidate genes for QTL, as elegantly reviewed by Pravenec et al. (38). We studied only one time point and only one tissue, which may or may not be when, or where, critical ARC molecular events occur. However, this is an appropriate age for studying expression differences, as this was when final physical measurements of F<sub>2</sub>(COP × DA) rats used to identify the ARC QTLs were taken (47), and it is intermediate to when ARC and intrinsic cardiac performance were initially measured in DA and COP rats (3). However, other genes, expressed at different time points, may exert important influences on ARC. Indeed, several studies of blood pressure and related traits showed temporal differences in QTL identification (e.g., Refs. 7, 14).

ARC is a complex trait, dependent on the interplay of numerous tissues and organs, and the expression differences observed in this study may not arise solely from differences in myocardial mRNA expression. Finally, potential ARC candidate genes may be missed for the following technical reasons: 1) expression of all rat genes could not be interrogated because they are not all present on the oligonucleotide microarray chips, and 2) the functions of most genes/ESTs remain poorly understood or unknown and are not present in available molecular network/pathway databases.

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