Genome-Wide Identification of Allelic Expression in Hypertensive Rats

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Background—Identification of genes involved in complex cardiovascular disease traits has proven challenging. Inbred animal models can facilitate genetic studies of disease traits. The spontaneously hypertensive rat (SHR) is an inbred model of hypertension that exists in several closely related but genetically distinct lines.

Methods and Results—We used renal gene-expression profiling across 3 distinct SHR lines to identify genes that show different expression in SHR than in the genetically related normotensive control strain, Wistar-Kyoto. To ensure robust discovery of genes showing SHR-specific expression differences, we considered only those genes in which differential expression is replicated in multiple animals of each of multiple hypertensive rat lines at multiple time points during the ontogeny of hypertension. Mutation analysis was performed on the identified genes to uncover allelic variation. We identified those genes in which all SHR lines share a single allele of the gene when normotensive controls (Wistar-Kyoto) have fixed the alternative allele. We then identified which of the differentially expressed genes show expression that is controlled by the allelic variation present in and around the gene. Allelic expression was demonstrated by observing the effect on gene expression of alleles inherited in the freely segregating F2 progeny of a cross between SHR and Wistar-Kyoto animals.

Conclusions—The result of these studies is the identification of several genes (Ptprj, Ela1, Dapk-2, and Gst2) in which each of 4 SHR lines examined have fixed the same allele and in which each of 2 Wistar-Kyoto lines have a contrasting allele for which the inherited allele influences the level of gene expression. We further show that alleles of these genes lie in extensive haplotype blocks that have been inherited identical by descent in the hypertensive lines. (Circ Cardiovasc Genet. 2009;2:106-115.)

Key Words: genetic hypertension ■ spontaneously hypertensive rat ■ gene expression ■ kidney ■ candidate genes ■ gene array ■ haplotype

There is growing evidence that the evolution of organismal diversity arises from progressively more elaborate regulation of gene expression. Similarly, within a species, heritable effects on gene expression are an important element of phenotypic diversity. Such heritable effects can arise from sequence variation within a gene and its adjacent regulatory sequences, a condition termed allelic expression. In the present project, we sought to identify renal genes possessing allelic expression that have been fixed during the selective inbreeding of rats sharing an important cardiovascular phenotype arising from the kidney: arterial hypertension. The spontaneously hypertensive rat (SHR) was generated from the outbred Wistar strain via selection to fix the trait of elevated arterial blood pressure. This was followed by brother-sister mating through multiple generations to achieve homozygosity.1 Fixation of alleles affecting gene expression in SHR may have occurred by chance, because the alleles lie in linkage disequilibrium with a gene subject to selection for the trait of interest (in which case they are positional markers of such genes) or because they contribute to the trait under selection.

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In the studies reported here, we have used genome-wide gene expression analysis in 3 distinct lines of SHR and the related normotensive line, Wistar-Kyoto (WKY) to identify genes that share differential expression in SHR lines compared with WKY. We have then examined these genes for sequence variation across SHR and WKY and determined which genes possess alleles shared by all the SHR lines, but not by WKY. We have then determined which SHR alleles affect gene expression in cis. This was performed in an F2 intercross by examining the relationship between inheritance of SHR alleles and gene expression. Finally, we have examined which of these genes lie in genomic blocks that have been inherited identical by descent (IBD) by all SHR lines in which the WKY region arise from a contrasting ancestral block. This work identifies chromosomal regions that are IBD in multiple lines of SHR that are not shared by the normotensive control line WKY and may therefore include hyper-
tension loci. In addition, it identifies individual genes lying within these IBD regions that can give rise to phenotypes via allelic expression.

Methods
An outline of the experimental approach and hypotheses tested in these studies is provided in a flow chart included in the Data Supplement (supplemental File 1).

Animals and Measurements
Studies were performed on 4-, 8-, 12-, and 18-week-old male animals. We used Wistar-Kyoto (WKY-Heid) and spontaneously hypertensive-A3 rats (SHR-A3, SHRSP, and SHR-stroke prone) that have been maintained in our facility for 8 years. We also used SHR-B2 and SHR-C animals bred from stocks provided to our laboratory in 2002 by T. Suzuki (Kinki University School of Medicine) that are descended from the original SHR lines reported by Okamoto et al. All animals used in the studies were produced in our breeding program and housed under controlled conditions in an American Association for the Advancement of Laboratory Animal Care–approved animal facility. Animals were provided a standard rodent chow diet and drinking water ad libitum. We also obtained genomic DNA samples from SHR/Ncrl and WKY/Ncrl animals purchased from a commercial supplier (Charles River Laboratories, Wilmington, Mass). We have represented the genealogical relationships between these SHR lines in a recent article. Two F2 intercrosses were generated. One was derived from a cross between SHR-A3 male and WKY-Heid female animals. The other was produced by intercrossing SHR-A3 males with SHR-B2 females. Only male progeny were studied.

Blood pressure was measured by radiotelemetry (Data Sciences, St Paul, Minn) in adult animals 16 to 19 weeks of age from SHR-A3 and SHR-B2 as well as the F1 and F2 progeny of an intercross of these lines. Telemetry catheters were implanted in the abdominal aorta in animals under isoflurane anesthesia. Recordings were begun 7 days after implantation and continued for 5 to 7 days.

For tissue collection, animals were anesthetized by isoflurane inhalation and kidneys were rapidly dissected via ventral laparotomy. Renal gene-expression analysis was performed by microarray using total RNA preparations from axial renal segments including the aorta in animals under isoflurane anesthesia. Recordings were begun 7 days after implantation and continued for 5 to 7 days.

For tissue collection, animals were anesthetized by isoflurane inhalation and kidneys were rapidly dissected via ventral laparotomy. Renal gene-expression analysis was performed by microarray using total RNA preparations from axial renal segments including the aorta in animals under isoflurane anesthesia. Recordings were begun 7 days after implantation and continued for 5 to 7 days.

Gene-Array Analysis
Transcript abundance was determined using the Affymetrix rat gene-expression array data between WKY and SHR lines at each age. Statistical Analysis
A nominal probability value of 0.05 (Mann-Whitney test) was considered statistically significant for the purposes of comparison of gene expression array data between WKY and SHR lines at each age. Genes were selected for further study if this level of significance was reached when comparing each SHR line with WKY at all 4 ages tested. Thus, each gene emerging from the overall analysis did so after achieving statistically significant expression differences across all 4 time points. The resulting high level of statistical significance required for follow-up study (0.05) may have had the effect of eliminating genes that do in fact have allelic effects on expression. However, such genes are clearly subject to effects on expression from other regulatory mechanisms that diminish or make less persistent

Polymorphism Detection
We used denaturing HPLC (dhHPLC, Transgenomic, Wave System, San Jose, Calif) to survey the proximal 5' regulatory region (~1 kb), entire exons and adjacent intron segments of persistently differentially expressed genes and EST sequences for sequence variation. PCR primers were designed to amplify genomic sequences of ~700 bp. A single amplification reaction was performed with each primer pair using a mixture of equal amounts of genomic DNA from SHR-A3 and WKY-Heid animals, and the resulting amplified DNA was analyzed directly for evidence of heteroduplex formation. Polymorphisms that could be detected using this screening approach included insertion and deletion polymorphisms and simple sequence length polymorphisms. The formation of heteroduplexes was indicated in dhHPLC by presence of more than 1 DNA duplex peak with the main peak eluting at the time expected for its fully hybridized strands and with one or more earlier peaks presumed to represent reduced HPLC retention due to partial denaturation around DNA duplex mismatches resulting from genetic dimorphism. Amplicons indicating heteroduplex formation were noted, and the corresponding amplicons from SHR-A3 and WKY were amplified individually and submitted for resequencing to identify the dimorphic base residues.

Genotyping
We determined the allelic state of each polymorphic gene by genotyping genomic DNA from WKY-Heid WKY/Ncrl, SHR-A3, SHR-B2, SHR-C, and SHR/Ncrl animals. Genotyping was also performed on F2 animals from a SHR-A3×WKY-Heid cross. We used the Sequenom (Mass Array, Sequenom, San Diego, Calif) implementation of genotyping by mass spectrometric analysis of oligonucleotides extended across single nucleotide polymorphisms in PCR amplified genomic DNA.

Bioinformatics
We have exploited existing microsatellite (SSLP) and single nucleotide polymorphism (SNP) allelic data to extend the interpretation of our findings. This data are derived from the Allele Characterization Project, a component of the Rat Genome Project and is available through the Rat Genome Database and from Ensembl via the European Union Rat SNP project (http://www.snp-star.de/). Genomic SSLP data were available for 2 SHR lines (SHR/OlaHsd and SHRSP/Riv) and 1 WKY line (WKY/OlaHsd). These lines are closely related to, and may be identical to, the SHR/Ncrl, SHR-A3, and WKY/Ncrl lines, respectively, used in our studies. We used this data to assess the degree of allelic identity in these SSLPs among and across SHR and WKY lines. We further sought evidence that regions identified as IBD in our SHR lines that have nonidentical counterparts in WKY could be further supported as regions of exclusive SHR IBD using SSLP data. We examined recently available genome-wide SNP data in the same manner, seeking evidence of extended haplotypes across additional WKY (WKY, WKY/Izm, and WKY/Nmna) and SHR lines (SHR, SHR/Izm, SHR/Kyo, SHR/NHsd, SHRSP, SHRSP/Ezo, SHRSP/Izm, and SHRSP/Ngsk) in regions harboring allelic expression specific to SHR lines. This comparison was made using the SNPplorer tool (http://www.snpplorer.mcs.anl.gov) and provides higher marker density and strain representation than the SSLP data.

Statistical Analysis
A nominal probability value of 0.05 (Mann-Whitney test) was considered statistically significant for the purposes of comparison of gene expression array data between WKY and SHR lines at each age. Genes were selected for further study if this level of significance was reached when comparing each SHR line with WKY at all 4 ages tested. Thus, each gene emerging from the overall analysis did so after achieving statistically significant expression differences across all 4 time points. The resulting high level of statistical significance required for follow-up study (0.05) may have had the effect of eliminating genes that do in fact have allelic effects on expression. However, such genes are clearly subject to effects on expression from other regulatory mechanisms that diminish or make less persistent
the allelic effect on expression. Such genes may not have the magnitude or persistence of effect to contribute to the strongly contrasting phenotype of blood pressure by which all SHRs are distinct from WKY lines. Thus, the risk from erroneously excluding such genes may not be large.

Results
Several published reports have indicated that systolic blood pressure is not a trait that segregates among the F2 progeny of intercrosses among SHR lines. This has been reported for crosses between SHR-A3 and SHR/N and between SHR-A3 and SHR-C.9–11 In each case, blood pressure was measured by tail cuff, a method no longer recommended in the present context9 that can contribute to relatively large within group variances that reduce statistical power and can lead to type II error. In the present studies using radiotelemetry measurements, we observed that SHR-A3 have higher blood pressure than the F2 progeny of an SHR-A3×SHR-B2 cross (Table 1). However, no difference was found in all other intergroup comparisons. The distribution of blood pressure across these groups and crosses, supports the expectation from genealogy that hypertension arises from a set of alleles largely shared by SHR lines, and our results here support and extend prior evidence of hypertension allele sharing across these lines.

In SHR, hypertension is a phenotype that can be transmitted by renal transplantation, indicating that genes expressed in kidney are essential to the phenotype. Gene expression studies comparing renal gene expression in WKY-Heid with that in 3 SHR lines (SHR-A3, -B2, and -C) at 4 different ages (4, 8, 12, and 18 weeks) using Affymetrix RG-U34A arrays revealed genes that are persistently differentially expressed at (4, 8, 12, and 18 weeks) using Affymetrix RG-U34A arrays that in 3 SHR lines (SHR-A3, -B2, and -C) at 4 different ages.

Table 1. Blood Pressure Levels Measure by Radiotelemetry in SHR Parental Lines SHR-A3 and SHR-B2 and in the F2 Progeny of a Cross Between These Lines

<table>
<thead>
<tr>
<th>Line/Cross</th>
<th>n</th>
<th>Systolic</th>
<th>SEM</th>
<th>Mean</th>
<th>SEM</th>
<th>Diastolic</th>
<th>SEM</th>
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</thead>
<tbody>
<tr>
<td>SHR-A3</td>
<td>16</td>
<td>198.6</td>
<td>3.9</td>
<td>171.7</td>
<td>3.2</td>
<td>144.6</td>
<td>2.8</td>
</tr>
<tr>
<td>SHR-B2</td>
<td>20</td>
<td>186.7</td>
<td>2.5</td>
<td>158.8</td>
<td>3.3</td>
<td>136.3</td>
<td>2.6</td>
</tr>
<tr>
<td>F1 A3×B2</td>
<td>13</td>
<td>195.0</td>
<td>4.6</td>
<td>160.1</td>
<td>1.7</td>
<td>133.8</td>
<td>2.0</td>
</tr>
<tr>
<td>F2 A3×B2</td>
<td>121</td>
<td>184.0</td>
<td>1.3</td>
<td>158.1</td>
<td>1.4</td>
<td>132.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The only statistically significant differences when comparing across the 4 groups of animals was between SHR-A3 and the F2 A3 X B2 (P<0.05). Statistical analysis in multiple group comparisons was performed by ANOVA followed by Tukey honestly significant difference test.

We measured the by polymorphism in the gene that we detected. Resequencing Sod1 in the 2 rat strains across the RGU34A hybridization probes revealed an insertion-deletion polymorphism in the transcribed sequence from which differential array hybridization signals arose.

We screened all genes in Table 2 by dHPLC for evidence of sequence variation between SHR-A3 and WKY. Among the 36 genes and ESTs surveyed, 21 have been found to be polymorphic between SHR-A3 and WKY. Genotyping assays were developed and used to determine which of these 21 genes share the same allele in all SHR lines (SHR-A3, -B2, -C, and SHR/N), but contrast with 2 WKY lines (WKY-Heid and WKY/N). We found that 11 genes showed this consistent distribution of alleles. These genes identify chromosomal regions that have been inherited IBD by all SHR lines from the 2 animals that were founders of the SHR lines.2 Normotensive WKY lines have inherited contrasting DNA sequences in these chromosomal regions. Genes contributing to hypertension in SHR lie within such regions that are shared IBD by all hypertensive lines, but which are not IBD to SHR in related normotensive lines. Table 3 indicates the identity of these autozygous genes and their chromosomal locations. Expression of these genes across the 4 ages studies and 4 lines examined is provided in supplemental File 2.

Genes that are differentially expressed in all SHR lines and that share the same allele in these lines may contribute to the hypertensive trait. However, some genes may have these properties (differential expression, IBD, and contrast with WKY alleles) because differential expression is due to trans-acting mechanisms arising from other genomic loci. We have assessed whether gene expression differences between SHR and WKY lines arise in cis or in trans. We measured the level of gene expression in the freely segregating F2 progeny of an SHR-A3×WKY cross to examine the relationship between the number of SHR alleles of a gene that are inherited in F2 individuals and the level of gene expression in those individuals. This test was performed with kidney RNA from a group of 10 F2 animals evaluated with the E230A array and using regression analysis to assess the relationship between SHR alleles inherited and level of gene expression. Evidence of cis-mediated effects on expression was obtained for 5 of the 11 genes in which all SHR lines share the same allele (Table 3, supplemental File 3). The concordance of these 5 genes with known rat quantitative trait loci was investigated using data summarized at the Rat Genome Database (http://rgd.mcw.edu). Of the 5 genes identified, 4 corresponded to rat blood pressure quantitative trait loci that
had been identified in multiple crosses involving several rat models with divergent blood pressures, suggesting that these 4 genes lie within regions that affect blood pressure in several distinct rat strains, including SHR (supplemental File 4).

We used microsatellite data from the Allele Characterization Project of the Rat Genome Project to estimate the genetic relationship between SHR and WKY. We calculated that 19% of the genomes of the 4 SHR lines are expected to be shared IBD and to not be shared with WKY (supplemental File 5).

One target of our study has been the identification of genes possessing alleles common to multiple SHR lines, differing from WKY alleles of these genes and creating allelic expression as a means by which renal phenotypes can arise. We have identified 5 genes (Table 3) that manifest allelic expression in all 4 SHR lines. We sought to further test the robustness of our conclusion that these genes exist as alleles identical in SHR, but dissimilar in WKY. Using microsatellite (simple sequence length polymorphism, SSLP) genotype data from the Allele Characterization Project of the Rat Genome Database (http://rgd.mcw.edu), we examined the geno-

### Table 2. Reevaluation of 36 Genes and ESTs Showing Persistent Differential Gene Expression Between WKY and All SHR lines

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>SHR-A3</th>
<th>SD</th>
<th>WKY</th>
<th>SD</th>
<th>P</th>
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<tbody>
<tr>
<td>Ahm</td>
<td>Afamin</td>
<td>739</td>
<td>72</td>
<td>1393</td>
<td>135</td>
<td>0.014</td>
</tr>
<tr>
<td>Bhmt</td>
<td>Betaine-homocysteine methyltransferase</td>
<td>330</td>
<td>47</td>
<td>373</td>
<td>30</td>
<td>0.271</td>
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<tr>
<td>Dtt</td>
<td>o-dopachrome tautomerase</td>
<td>4041</td>
<td>509</td>
<td>4720</td>
<td>337</td>
<td>0.043</td>
</tr>
<tr>
<td>Dpep1</td>
<td>Dipeptidase 1</td>
<td>714</td>
<td>83</td>
<td>1373</td>
<td>73</td>
<td>0.014</td>
</tr>
<tr>
<td>Ela1</td>
<td>Elastase 1</td>
<td>251</td>
<td>53</td>
<td>2398</td>
<td>137</td>
<td>0.014</td>
</tr>
<tr>
<td>Ephx2</td>
<td>Epoxide hydrolase 2, cytoplasmic</td>
<td>247</td>
<td>98</td>
<td>1629</td>
<td>152</td>
<td>0.014</td>
</tr>
<tr>
<td>Gsto1</td>
<td>Glutathione S-transferase omega 1</td>
<td>5236</td>
<td>624</td>
<td>7526</td>
<td>655</td>
<td>0.014</td>
</tr>
<tr>
<td>Gstt2</td>
<td>Glutathione S-transferase, theta 2</td>
<td>530</td>
<td>18</td>
<td>783</td>
<td>62</td>
<td>0.014</td>
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<tr>
<td>Hmox2</td>
<td>Heme oxygenase-2</td>
<td>796</td>
<td>48</td>
<td>885</td>
<td>49</td>
<td>0.157</td>
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<td>Homer2</td>
<td>Homer, neuronal immediate early gene, 2</td>
<td>197</td>
<td>40</td>
<td>276</td>
<td>42</td>
<td>0.086</td>
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<tr>
<td>Lgal1</td>
<td>Lectin, galactose binding, soluble 1</td>
<td>412</td>
<td>37</td>
<td>652</td>
<td>62</td>
<td>0.014</td>
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<tr>
<td>Mat2a</td>
<td>Methionine adenosyltransferase II, α</td>
<td>1505</td>
<td>248</td>
<td>1971</td>
<td>370</td>
<td>0.043</td>
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<tr>
<td>Mep1b</td>
<td>Meprin 1 β</td>
<td>1630</td>
<td>193</td>
<td>3005</td>
<td>385</td>
<td>0.014</td>
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<tr>
<td>Mgl2</td>
<td>Monoglyceride lipase 1</td>
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<td>181</td>
<td>2355</td>
<td>82</td>
<td>1</td>
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<td>Ptpj</td>
<td>Protein tyrosine phosphatase, type J</td>
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<td>47</td>
<td>543</td>
<td>20</td>
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<td>RoDHII</td>
<td>Retinol dehydrogenase type II (RODH II)</td>
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<td>291</td>
<td>476</td>
<td>102</td>
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<td>Rpl30</td>
<td>Ribosomal protein L30</td>
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<td>Solute carrier family 17A1</td>
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<td>Slipr</td>
<td>Scaffolding protein SLIPR</td>
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<td>445</td>
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<tr>
<td>Sod1</td>
<td>Superoxide dismutase 1</td>
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<td>1065</td>
<td>12506</td>
<td>618</td>
<td>0.586</td>
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<tr>
<td>Sparc</td>
<td>Secreted acidic cysteine rich glycoprotein</td>
<td>6201</td>
<td>248</td>
<td>4378</td>
<td>318</td>
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<tr>
<td>Timm17a</td>
<td>Translocator of inner mitochondrial memb1r 17a</td>
<td>1169</td>
<td>54</td>
<td>1255</td>
<td>88</td>
<td>0.086</td>
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<tr>
<td>Ugt1a6</td>
<td>UDP glycosyltransferase 1, A6</td>
<td>2166</td>
<td>350</td>
<td>5126</td>
<td>5151</td>
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<tr>
<td>AA799440</td>
<td>EST</td>
<td>1002</td>
<td>122</td>
<td>1017</td>
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<tr>
<td>AA799567</td>
<td>EST</td>
<td>148</td>
<td>25</td>
<td>245</td>
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<tr>
<td>AA800735</td>
<td>Similar to supervillin</td>
<td>423</td>
<td>27</td>
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<tr>
<td>AA866432</td>
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<td>363</td>
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<td>515</td>
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<td>AA892189</td>
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<td>1136</td>
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<td>40</td>
<td>467</td>
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<td>AA892762</td>
<td>EST, Pleckstrin domain containing, H1</td>
<td>566</td>
<td>93</td>
<td>106</td>
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<tr>
<td>AA892986</td>
<td>EST</td>
<td>656</td>
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<td>1566</td>
<td>54</td>
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<td>AA893147</td>
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<td>507</td>
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<tr>
<td>AA893984</td>
<td>Similar to p53 inducible protein</td>
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<td>937</td>
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<td>Al638939</td>
<td>Similar to transcription factor BBX</td>
<td>384</td>
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<td>406</td>
<td>36</td>
<td>0.586</td>
</tr>
<tr>
<td>Al638974</td>
<td>EST, FMS-like tyrosine kinase 1, FIt</td>
<td>289</td>
<td>32</td>
<td>242</td>
<td>11</td>
<td>0.014</td>
</tr>
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</table>

The reproducibility of gene expression differences observed with the RG-U34A array was reexamined using the E230A array. All except Homer2 and AA638939 were found to have similar directional changes in expression. Among the other genes, Sod1 was found to have an indel polymorphism across hybridization probes used in the RGPU34A array, but not in the E230A array, whereas 6 genes were found not to reach statistical significance in this comparison.

The table shows the reproducibility of gene expression differences observed with the RG-U34A array was reexamined using the E230A array. All except Homer2 and Al638939 were found to have similar directional changes in expression. Among the other genes, Sod1 was found to have an indel polymorphism across hybridization probes used in the RGPU34A array, but not in the E230A array, whereas 6 genes were found not to reach statistical significance in this comparison.
progeny of such crosses,9–11 and this conclusion is generally supported by the blood pressure telemetry measurements made in the novel cross reported in this study. This evidence supports the expectation from genealogy2 that a common set of gene variants creates hypertension across all SHR lines.

Use of comprehensive gene expression analysis as a tool to refine mapped hypertension loci has been reported.15–20 The present approach combines a genetic with a genome-wide expression approach across multiple SHR lines and investigates heritability of gene expression in association with the hypertension trait. We have identified 5 genes that are consistently differentially expressed between SHR lines and WKY that exist as allelic variants with one form common to 4 SHR lines and another to 2 WKY lines, in which the expression effect associated with the gene is inherited from the locus of the gene affected. The result is identification of a compact set of genes containing allelic variation affecting the expression of the genes that are IBD in multiple SHR lines and that include potential hypertension candidate genes.

There are 3 main explanations why a gene harboring allelic expression could be present in one allelic form in multiple SHR lines whereas the alternate allele is present in WKY. First, random assortment of alleles has produced this pattern by chance. Second, the SHR allele of the gene contributes to hypertension and was subject to selection during breeding to fix the hypertension trait. Third, the allele demonstrating allelic expression has been retained by SHR lines because it is in linkage disequilibrium with one or more nearby alleles that have been subject to selection because they influence the trait. Further work will be required to determine whether the genes identified in this study are hypertension genes or whether they contribute to other phenotypic differences shared by SHRs.

The Sa gene illustrates one of the strengths of our design. The Sa gene has been very thoroughly studied after it was initially identified and shown to be highly differentially expressed using differential hybridization in SHR kidney.21 Our analysis finds that 3 of 4 of SHR lines examined possess the high expressing allele originally identified in SHR. In contrast, WKY possesses a different allele, providing further support that these genes lie in genomic regions shared IBD in all SHR lines. Each gene of the 5 genes was found to lie in haplotype blocks of allelic identity among SHR lines across which WKY possesses a different allele (Table 4). Thus, the candidacy of these SHR alleles for involvement in the hypertension trait through their expression phenotype is promoted by being integrated within haplotype blocks common to SHR lines.

This IBD haplotype approach can now be further supported by the use of recently obtained high density SNP data available via Ensembl for a broad range of rat strains including several SHR and WKY lines. We have used this SNP data to increase the number of SHR and WKY lines in which IBD can be assessed across the 5 genes of interest. The resolution of these blocks is ~10-fold higher overall as a result of the increased marker density available using SNP genotypes compared with microsatellites. Figure 1 indicates the distribution of SNP alleles in a 3 Mbase region around each of the 5 genes. Here again, in each case, there was strong evidence of haplotype block-sharing among all SHR lines and contrasting haplotypes in WKY lines.

**Discussion**

The SHR model, like other inbred rodent strains, offers an important opportunity for trait gene identification that arises from the multiple lines that were created during the development of the model. The genealogy of these lines is well recorded2–14 and lines have remained separated until present. The fact that all SHR lines experience hypertension because of a shared set of gene variants has been tested and supported by the creation of crosses among SHR lines. It has been reported that blood pressure does not segregate among the F2 progeny of such crosses,9–11 and this conclusion is generally supported by the blood pressure telemetry measurements made in the novel cross reported in this study. This evidence supports the expectation from genealogy2 that a common set of gene variants creates hypertension across all SHR lines.

**Table 3.** Differentially Expressed Genes That Are Polymorphic in SHR and WKY in Which All SHR Lines (SHR-A3, SHR-B2, SHR-C, and SHR/N) Share the Same Allele and Contrast With Both WKY-Heid and WKY/N

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene</th>
<th>Band</th>
<th>Position</th>
<th>Expression Inheritance in cis (P Value)</th>
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<td>Gsto1</td>
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<tr>
<td>Mat2a</td>
<td>Methionine adenosyltransferase II, α</td>
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<td>chr4:105 984 423-105 989 953</td>
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<tr>
<td>Pptra</td>
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<tr>
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<tr>
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</table>

* Genes whose differential expression is determined in cis. The P value of the regression analysis relating inheritance of SHR alleles of these genes with level of gene expression in 10 F2 animals is also indicated.
Table 4. Genes That Possess SHR-Specific Alleles That Manifest Allelic Expression Are Contained Within Haplotype Blocks Shared by SHR Lines and Distinct From WKY Lines

<table>
<thead>
<tr>
<th>RGD Name</th>
<th>Chromosome</th>
<th>Position</th>
<th>SHRSP/Riv</th>
<th>SHR/OlaHsd</th>
<th>WKY/OlaHsd</th>
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<td>70 039 735</td>
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</table>

(Continued)
a well-substantiated SHR blood pressure locus,22–26 is expressed predominantly in the kidney, is polymorphic between SHR and WKY, 27,28 and there is evidence from human studies suggesting involvement of Sa in blood pressure determination.29–31 Recent rat congenic studies have not found support for the involvement of Sa in blood pressure regulation, but rather suggest that one or more genes in an adjacent locus is responsible for elevation of blood pressure.32,33 The criteria arising from the rationale of our studies require that all SHR lines share the same allele in SHR for a gene to be considered further as a hypertension candidate. Sa gene fails this test using the approach we have applied.

The assignment of the remaining genes that have been identified in this study to the 3 categories outlined earlier is less certain. However, because the product of our analysis is specific genes (rather than extensive chromosomal loci containing many genes) that are shown here to contain functional polymorphism, the path forward from these studies can be much more narrowly focused than follow-up studies of trait mapping. Such follow-up studies are made more compelling because of the functional attributes of many of the genes we have identified that indicate that they are potential hypertension genes. We have discussed the potential functional implications of the genes we have identified in the pathogenetic mechanisms of hypertension in SHR. This discussion is included in the Data Supplement (supplemental File 6).

The use of recombinant inbred rat strains derived from a cross between SHR and Brown Norway rats to perform genome-wide mapping of the genetic control of gene expression has recently been reported.15 This study identified 7 genes for which both regulation of expression in cis was identified and for which gene sequence variation in SHR was identified. When the allelic distribution of these genes was compared across SHR and WKY lines, only 1 gene, Pik3c3, was identified as possessing SHR-specific alleles. We have confirmed that all SHR lines in our study share the same Pik3c3 allele and differ from both the WKY strains. However, when comparing gene expression across multiple time points and SHR lines, consistently different expression was not observed. Furthermore, we found no evidence of allelic expression of Pik3c3 in the F2 cross between SHR-A3 and WKY-Heid (data not shown).

The utility of SHR as a model to uncover genes of relevance to human cardiovascular disease is well illustrated by other studies performed with this model. Among SHR lines, we have shown that the Ephx2 gene exists as 2 distinct alleles with profound expression phenotypes.4 The function of Ephx2 in metabolism of eicosanoids34 involved in vascular inflammation35,36 and distribution of these contrasting expression alleles among SHR lines suggested that Ephx2 variation might contribute to susceptibility to vascular injury in the SHR-A3 line. Subsequently, we have investigated the association between human polymorphism of Ephx2 and the haplotypes these polymorphisms create and have uncovered important modification of risk for atherosclerosis and ischemic stroke associated with Ephx2 alleles in humans.37–39 This precedent underscores the value of elucidating the role of the

<table>
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<th>RGD Name</th>
<th>Chromosome</th>
<th>Position</th>
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This is demonstrated by examining the distribution of microsatellite marker alleles from the Allele Characterization Project of the Rat Genome Project. The table indicates the microsatellite allele sizes in each locus surrounding the 5 genes (‡) that we have identified as possessing SHR allele–specific expression. In each case, the allelic expression genes are outlined in a block (*) in which both microsatellite alleles and alleles of the genes identified in this study share the same pattern of distribution between SHR and WKY. Each block is accompanied by the contiguous 3 markers up and downstream of the block (†) and is defined by a shift in the immediately adjacent marker pattern so that the pattern of allele sharing among SHR, but not by WKY, is broken.
genes identified in this study in the causation of hypertension in both SHR and in humans.

Our approach to hypertension gene mapping that combines identification of allelic expression with IBD in multiple SHR lines is new and relies on technical resources that are continuing to evolve (rat genome sequence and gene array content). The approach faces limitations that arise from: the fact the genes involved in hypertension may have their disease-causing actions as a result of coding, not regulatory sequence differences; the possibility that genes involved and functioning as hypertension genes through effects on expression are not represented on the array; the possibility that allelic variation exists, but is not discovered by the polymorphism screening approach used; and as well as other possible confounding effects. However, it draws support from emerging evidence of the important role played by allelic expres-

Figure 1. SNP haplotype blocks in SHR and WKY lines around genes showing allelic expression shared by SHR lines. The haplotype is the linear sequence of SNPs that is read as a vertical column for each line. WKY is used as the reference strain with its SNP genotype indicated as a black box. Contrasting SNP genotypes are indicated in gray. Occasional heterozygous alleles are observed (light gray) and occasional SNP genotypes are missing (white). The Ensembl database SNP identification numbers are indicated in the leftmost column, followed by the chromosome and base position in the rat 3.4 genome assembly.
sion that result in phenotypic diversity, including disease susceptibility and including susceptibility to hypertension in SHR. Thus, this study illustrates clearly a potential path by which heritable effects on gene expression can be identified, resolved at the level of sequence diversity in the differentially expressed gene, and tested for association with blood pressure. An important element present in this approach which is lacking in purely positional mapping approaches is that it yields outputs in the form of specific genes, rather than broad chromosomal regions containing large numbers of genes. This provides an important opportunity to specifically test the hypothesis of involvement of any gene by reverse genetic approaches (eg, transgenic rats expressing the alternative allele) or by pharmacological approaches designed to alter expression of a target gene or change the activity of its protein product.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Blood pressure is influenced by both environmental and heritable factors. Identification of genes carrying susceptibility alleles for hypertension in human populations has been a difficult challenge, probably because of the interaction of multiple susceptibility genes within both individuals and populations. Animal models that share the polygenic nature of human hypertension offer advantages for hypertension gene identification studies. However, mapping studies in rodent models of hypertension have lacked resolution and as a result the genomic regions linked to hypertension are large and contain many genes. We have combined mapping for both hypertension and related gene expression traits to identify allelic variation in the spontaneously hypertensive rat that regulates gene expression across multiple spontaneously hypertensive rat lines but not in normotensive controls. This was accomplished by examining genes expressed in the kidney to identify those that are differentially expressed in hypertensive rats before, during, and after the elevation of blood pressure in spontaneously hypertensive rats. We then identified which of these genes contain allelic variation and which among such genes show expression levels linked to this variation. These genes may be relevant to the pathogenesis of hypertension or other traits in spontaneously hypertensive rats and potentially also in humans.
Genome-Wide Identification of Allelic Expression in Hypertensive Rats
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Genome-wide identification of allelic expression in hypertensive rats

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SUPPLEMENTAL MATERIAL
Flow Chart and Hypothesis testing

Step 1.

Procedure: Determine gene expression in 1 WKY line and 3 SHR lines at 4 weeks of age.

Test hypothesis that genes can be identified that are differentially expressed in all SHR lines compared with WKY and that the directional difference of expression compared with WKY is the same in all SHR lines tested.

Step 2.

Procedure: Repeat gene expression analysis in same animals, but at age 8wks, age 12wks and age 18wks.

Test same hypothesis as Step 1, but for each new age added.

Step 3.

Procedure: Examine expression data obtained in Steps 1 and 2.

Test hypothesis that a set of genes can be identified in which differential expression identified in Steps 1 and 2 is present at all ages tested for that gene.

Step 4.

Procedure: Amplify genomic DNA from genes identified in Step 3 that show different expression in all SHR lines compared to WKY and screen PCR amplified DNA from WKY and SHR by dHPLC to determine whether variation between WKY and SHR can be identified. When variation is identified, resequence the amplified DNA to identify the explicit sequence change obtained through screening. Genotype 2 distinct WKY lines and 4 distinct SHR lines to identify the alleles present in the differentially expressed genes.

Test the hypothesis that some of the genes that demonstrate persistently differential expression between WKY and SHR lines exist as allelic variants and to identify which genes possess allelic variants for which SHR lines have all fixed one variant while WKY lines have all fixed the alternative variant.

Step 5.

Procedure: Examine expression of differentially expressed genes in which all WKY and all SHR lines have fixed contrasting alleles in the F2 progeny of a WKY x SHR cross in which these alleles freely segregate.

Test the hypothesis that among the differentially expressed genes in which all SHR and all WKY lines have fixed alternate alleles, that the expression of the gene in the F2 progeny will be correlated with the number of SHR alleles inherited.

Step 6.
Further procedures: Bioinformatic analysis to determine whether those genes, in which SHR and WKY lines have fixed alternative alleles for which the expression level of the gene is determined by the allele inherited, lie in genomic blocks that have been inherited identical by descent in all SHR lines for which microsatellite and SNP genotypes are available.
Expression of SHR differentially expressed genes in individual SHR lines at 4 ages, compared with WKY.

**Ela1**

**Gstt2**
Dapk-2

Fluorescence intensity

age (wks)

W
S-A3
S-B2
S-C
Relationship between expression levels (normalized fluorescence intensity) measured using Affymetrix E230A arrays and the allele inherited for each gene showing allelic expression specific to SHR lines. Determined using 12 F2 animals that were the progeny of a cross between SHR-A3 and WKY.
Rat BP QTL’s overlapping genes with SHR expression alleles.

QTL’s are numbered using the identifiers provided by the Rat Genome Database (http://rgd.mcw.edu).

QTL’s marked with asterisk were identified in crosses using SHR progenitors.

**Ptprj**

Physical position: Chr3:74,693,135  
Rat BP QTL 207, 208, Cluster 4*, 264, 251, 152

**Ela1**

Physical position: Chr7:139,515,742  
Rat BP QTL 265, 266, 183

**AA866432**

Physical position: Chr8:11,151,250  
Rat BP QTL 35*, 217

**Dapk-2**

Physical position: Chr8:70,581,319  
Rat BP QTL 35*, 262, 253, 62*, 39,

**Gstt2**

Physical position: Chr20:13,221,934  
Rat BP QTL None
Estimated Identity By Descent across SHR and WKY lines

Data on genome-wide SSLP allele size from the Allele Characterization Project (ACP) of the Rat Genome Project (http://rgd.mcw.edu/ACPHAPLOTYPER/) indicates that SSLP genotypes for WKY/OlaHsd, SHR/OlasHsd and SHRSP/Riv are available for all 3 strains at 3236 loci. All three strains share the same allele at 50.4% of these loci, reflecting the high level of relatedness among the progenitor animals that gave rise to these strains. Since all SHR lines are the progeny of a single pair of animals and were inbred through 8 generations before separation of isolated lines, the expected degree of allele sharing among any two SHR lines should be greater than between WKY and any SHR line.

Allelic identity was observed at 79.0% of loci comparing SHR/OlaHsd with SHRSP/Riv, 51.5% of loci comparing WKY/OlaHsd with SHR/OlaHsd and 50.4% of loci comparing WKY/OlaHsd with SHRSP/Riv. Loci which are shared by both SHR lines, but differ from the WKY line are potential hypertension loci and account for 34.6% of loci for which the ACP has genotype information for all three strains.

Based on strain origin information in the ACP database, the SHRSP/Riv line is presumed to be the same lineage as our SHR-A3 line, while the SHR/OlaHsd line is distinct from SHRSP/Riv and is likely from the same lineage as the SHR/NCrl line used in our studies. The SHR-B2 and SHR-C lines that we have investigated in the present study are known to be independent lines from the two SHR lines in the ACP database and thus are likely to have inherited similar alleles at any loci at the same frequency (0.79) as SHR/OlaHsd and SHRSP/Riv. Thus we can estimate that the likelihood that any locus contains alleles identical by descent in SHR/OlaHsd, SHRSP/Riv, SHR-B2 and SHR-C is (0.79)^4 or 0.39. Given that differences in the frequency of allele sharing between any SHR line and any WKY line arise from the fact that these strains (i.e., SHR and WKY) arose from different founders, we can similarly estimate that the degree of
allelic identity between SHR-B2 and SHR-C with WKY will be similar to that between SHR/OlaHsd and SHRSP/Riv with WKY, i.e. approximately 50%. Thus, it is possible to estimate the likelihood at the genome-wide level that any single locus will share identity in all 4 SHR lines (SHR-A3, SHR-B2, SHR-C and SHR/N) and differ at the same locus with WKY as \((0.79)^4 \times 0.5 = 0.19\).
Notes on differentially expressed SHR genes.

Ptprj

This gene encodes a receptor-type protein tyrosine phosphatase. The protein consists of a large extracellular domain incorporating multiple fibronectin-like repeats. A single transmembrane pass connects this extracellular domain to an intracellular protein phosphatase domain. Two mouse knockout models of Ptprj have been reported. One knockout was generated by replacing exons 3-5 with a beta-galactose-neomycin phosphotransferase cassette and produced no observed phenotype. Another knockout model replaced the intracellular catalytic domain with enhanced green fluorescent protein. This resulted in an embryonic lethal phenotype with failure of vascularization, disorganized vascular structures and cardiac defects. This phenotype might be explained by actions arising from the untargeted extracellular portion of the Ptprj molecule. Administration of antibody to the extracellular portion of Ptprj results in clustering of the ectodomain and inhibits vascular endothelial cell growth and blocks angiogenesis. The antibody blocks phosphorylation of ERK1/2 kinases in the same way that activated Ptprj does. Evidence of a role of Ptprj in vascular development also comes from studies of the effects of morpholinos targeting the two Zebrafish isoforms of Ptprj (Dep1a and Dep1b) that revealed reduced arterial marker specification and increased venous marker specification.

Ptprj first came to be understood through its effects on contact-mediated inhibition of cell growth and is alternatively named density-enhanced phosphatase-1 (Dep1). In vascular endothelial cells, the function of Ptprj may depend on its ability to intercept signaling between vascular cadherin (VEC) and the VEGF receptor 2 that is activated by VEC. The signaling induced by this pathway is reduced by Ptprj, which may bind to the activated receptor, reducing its tyrosine phosphorylation and attenuating the activation of MAPK pathways driven by the activated receptor. Since VEGF is known to be involved in formation of blood vessels in both embryogenesis and in proliferative disease, Ptprj may play a critical role in modulating the pathway of vasculogenesis. In the kidney, VEGF has been shown to have functions in maintaining both renal vascular and tubular structure under normal conditions and antagonism of VEGF signaling results in reduced vascular formation and nephrogenesis in the superficial cortex. Thus, Ptprj, through modification of renal VEGF signaling in SHR may have effects on blood pressure in SHR by altering both the vascular and renal tubular epithelium as a result of lower levels of expression than are found in WKY. The effect of reduced of Ptprj in SHR may result in increase VEGF receptor phosphorylation and increased signaling in this pathway.

However, the effects of Ptprj variation in SHR may be broader than the reduced expression we have observed to be associated with the SHR allele of Ptprj. In surveying the gene to detect sequence variation we discovered that SHR lines all possess a single SNP variation that leads to a non-synonymous replacement of alanine for threonine at position 39. Analysis of the predicted effects of this substitution using the InterProScan tool (www.ebi.ac.uk) indicates that this change alters one of the fibronectin type III domains that may have consequences for Ptprj activation. Finally, VEGF has been shown to play an important protective role in hypertension in which increased glomerular expression of VEGF occurs and drives signaling pathways that support glomerular integrity and prevent albuminuria. It is possible that the Ptprj allele expressed in SHR helps to protect these animals from the adverse renal effects of hypertension by maintaining the efficacy of VEGF signaling.
These observations regarding Ptpnj function do not directly tie this gene to blood pressure regulation; however, they do strongly suggest that Ptpnj function in the kidney may have important consequences for renal tubular and vascular development.

**Elastase 1**

Defects in the elastic component of the extracellular matrix of resistance arteries are known to be associated with hypertension. This suggests that the elastic component of resistance vessels is more than a simple reactive element allowing adaptation to fluctuations in arterial pressure, but rather molecular and genetic changes in elastin may contribute to vascular dysfunction and hypertension. Haploinsufficiency of the elastin gene in both mice and humans is associated with hypertension \(^9,10\). In the SHR, altered elastin structure and organization occurs in resistance vessels and this disorganization precedes the elevation of blood pressure \(^11,12\). Furthermore, manipulation of SHR vascular elastin so that it resembles in its organization that of WKY was associated with change in mechanical properties and lower blood pressure \(^13,14\).

Elastase 1 is a proteolytic enzyme with specificity for elastin and cleavage activity that extends to certain other proteins (e.g., hemoglobin, fibrin). Although pancreatic expression of elastase 1 is silenced in the human, the gene appears to be conserved and is expressed in at least one other tissue in humans \(^15\). While little knowledge of elastase function in kidney exists, analysis of expression array data indicates that it is abundantly expressed in the pancreas and kidney in rats and mice (http://genome.ucsc.edu). Thus, one potential mechanism for the involvement of this secreted proteolytic enzyme in the generation of hypertension is via the effect of secretion from the kidney on local renal vessels or perhaps even systemic effects of secreted renal elastase 1.

These observations suggest that elastase 1 may have the capacity to alter vascular function in a manner that would alter blood pressure regulation.

**Dapk-2**

The function of Dapk-2 is not well understood. There is evidence of Dapk-2’s involvement in involvement of immune cell activation, and this might contribute to the degree of inflammation in kidney that is known to be involved in the pathogenesis of hypertension in SHR \(^16\). Death-associated protein kinases are involved in regulation of cell proliferation via apoptotic and autophagic cell death and has been shown to be down-regulated by beta-catenin and Tcf4 in order to induce anoikis of malignant epithelial cells \(^17,18\).

In the kidney, the closely related protein Dapk (Dapk-1) is upregulated in a number of renal injury models and apoptosis of injured cells may require the kinase activity of Dapk \(^19-22\). In renal injury, Dapk appears to increase apoptosis and reduce fibrosis \(^23\). It is unknown at present if renal Dapk-2 is involved in similar processes.

The limited knowledge of renal function of Dapk-2 at present precludes a well-developed hypothesis regarding its potential role as a source of hypertensive disease processes in SHR.
Gstt2

This gene is a member of the glutathione s-transferases that share general functions in detoxification of xenobiotics and reactive endogenous compounds. Little specific knowledge regarding the function of Gstt2 is known. It is expressed predominantly in liver, lung and kidney, though no reports of it distribution within the kidney have been published. Given that hypertension may have a pathogenic element arising from renal oxidative stress and that conjugation of glutathione to reactive oxidants can reduce redox stress, it is conceivable that Gstt2 might play a role in hypertensive mechanisms through this aspect of its function. However, the GST family is large it is can be questioned whether the moderately reduced expression of Gstt2 in SHR lines would be sufficient to produce a generalized shift in renal redox balance sufficient to effect blood pressure regulation.

Thus, any hypothesis regarding functional consequences of inheritance of a SHR allele of Gstt2 that contributes to the genesis of hypertension must await further understanding of its role with specific regions of the kidney and within specific elements of renal function.

AA866432

This expressed sequence tag has been mapped in the rat genome to a location in which there are no known rat genes. Further interpretation of the functional implications of this allelic expression signal will require some rudimentary understanding of which, if any, aspects of genomic function are reflected by this expression signal.

References


