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Genetic Dissection of a Blood Pressure Quantitative Trait Locus on Rat Chromosome 1 and Gene Expression Analysis Identifies *SPON1* as a Novel Candidate Hypertension Gene

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Abstract

A region with a major effect on blood pressure is located on rat chromosome 1. We have previously isolated this region in reciprocal congenic strains (WKY.SHR-Sa and SHR.WKY-Sa) derived from a cross of the spontaneously hypertensive rat (SHR) with the Wistar-Kyoto rat (WKY) and shown that there are two distinct BP quantitative trait loci (QTLs), BP1 and BP2, in this region. Sisa1, a congenic sub-strain from the SHR.WKY-Sa animals carrying an introgressed segment of 4.3Mb, contains BP1. Here, we report further dissection of BP1 by the creation of two new mutually exclusive congenic sub-strains (Sisa1a and Sisa1b) and interrogation of candidate genes by expression profiling and targeted transcript sequencing. Only one of the sub-strains (Sisa1a) continued to demonstrate a BP difference but with a reduced introgressed segment of 3Mb. Exonic sequencing of the twenty genes located in the Sisa1a region did not identify any major differences between SHR and WKY. However, microarray expression profiling of whole kidney samples and subsequent quantitative RT-PCR identified a single gene, *Spon1* that exhibited significant differential expression between the WKY and SHR genotypes at both 6 and 24 weeks of age. Western blot analysis confirmed an increased level of the *Spon1* gene product in SHR kidneys. *Spon1* belongs to a family of genes with anti-angiogenic properties. These findings justify further investigation of this novel positional candidate gene in BP control in hypertensive rat models and humans.

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INTRODUCTION

Blood pressure (BP) and hypertension exhibit a significant degree of genetic heritability1. In the past 15 years, many genomic locations contributing to hypertension in rodent models have been mapped.2 Most of these regions, known as quantitative trait loci (QTLs), are quite large, typically 10 to >80 million base pairs (Mb). This has hampered the ability to identify the gene(s) responsible for the blood pressure (BP) effects underlying these QTLs. Constructing congenic strains has proved pivotal in reducing the genomic size of QTLs: the strategy creates animal strains where the native genomic background (e.g. from a normotensive rat strain) remains unchanged except for an introgressed (replaced) specific genomic region of interest which is transferred from a contrasting strain (e.g. from a hypertensive rat strain).2 The integration of genetic strategies with expression profiling and targeted sequencing3-6 has also proved a successful approach in accelerating the search for genes underlying various phenotypes related to cardiovascular disease. This integration strategy is based on the assumption that genes that are both differentially expressed or show structural variation and map to a disease-related QTL are likely to be involved in the pathophysiology of that disease.

In a previous study, we mapped a BP QTL region to rat chromosome 1 in F_2 rats derived from a cross of the spontaneously hypertensive rat (SHR) with the Wistar-Kyoto rat (WKY). This QTL region was then isolated in reciprocal congenic strains WKY.SHR-Sa (WConSa) and SHR.WKY-Sa (SConSa) derived from these animals.7 In more recent work8, we have dissected this region further by developing congenic sub-strains and demonstrated that at least two distinct QTLs (BP1 and BP2) affecting BP reside within this region of rat chromosome 1. The sub-strain Sisa1 (WKY.SHR-D1Rat420 / D1Got161) contained BP1, the smallest introgressed segment (4.3Mb) that still demonstrated a BP effect, with BP2 mapping to a separate portion of the introgressed region captured within WConSa.8 In other work utilising our congenic strains, we have shown by cross-transplantation studies that the BP effect of these loci on rat chromosome 1 is mediated through the kidney;9 WKY recipients of a WConSa kidney were found to have significantly higher BP at all time points when compared to recipients of a WKY kidney. The purpose of the present study was to carry out further genetic dissection to provide better physical resolution of the BP QTL in Sisa1 and to undertake both targeted transcript sequencing and microarray expression analyses to identify positional candidate genes.

METHODS

Construction and Genotyping of Novel Congenic Sub-strains of Sisa1

The breeding strategy followed to produce the novel congenic sub-strains was similar to the method used to produce previous congenic strains8 and further details are given in the Online Data Supplement. To determine the size of the introgressed segments in each new strain, animals were subsequently genotyped for all the available polymorphic markers mapped to the original Sisa1 region8, as well as additional microsatellite markers identified from the latest Rat genome assembly (v3.4) using the simple tandem repeat track (located by the Tandem Repeat Finder program10) within the UCSC Genome browser (http://genome.ucsc.edu/). These markers are described in Supplementary Table 1. All genotyping was carried out as described previously.8

Phenotyping

BP was measured in male animals using the same tail plethysmography method we employed previously in the mapping of the QTLs on rat chromosome 1 and analysis of the initial congenic strains.7,8 Further details are given in the Online Data Supplement. All procedures were carried out in accordance with our institutional guidelines.

Transcript Sequencing

For each gene, the longest transcript model according to the Ensembl database (http://www.ensembl.org/index.html) was chosen for targeted exon sequencing. All Ensembl genes were cross checked with the respective RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/) gene model and where differences were found these were only in the lengths of the 5' and 3' UTR sequences and not in the coding sequences. Primers were designed to span the exons. For genes expressed in the kidney sequencing was carried out from cDNAs. Further details are given in the Online Data Supplement. For genes not expressed in the kidney putative exons were amplified directly from genomic DNA and sequenced. All sequences were obtained from a minimum of two SHR and two WKY animals. Sequences were aligned and analysed using Sequencher software (http://www.genecodes.com/).

Gene Expression Profiling and Integration with Genetic Mapping

Affymetrix GeneChip RAE230 expression analysis was used to look for differentially expressed probe sets between SHR, WKY, WConSa, SConSa and Sisa1. Total RNAs extracted from whole kidneys of 5 separate rats from each strain at both 6 and 24 weeks of age were examined. Further details are given in the Online Data Supplement. We identified all probe sets from the RAE230 chip set that mapped within the boundaries of the Chromosome 1 QTLs of interest from Affymetrix GeneChip NetAffx Analysis Centre database (http://www.affymetrix.com/analysis/index.affx) and Ensembl. Genes from the Sisa1 region that were not represented on the RAE230 microarray chip were investigated using semi-quantitative RT-PCR.

Validation of Microarray Gene Expression Data

To validate differences in mRNA expression detected on the microarray we assessed mRNA abundance by quantitative RT-PCR. PCR was performed by TaqMan assay using a 7900HT (Applied Biosystems). All reactions were performed in duplicate alongside negative controls. Gene expression was measured relative to the endogenous control beta-2-microglobulin. Taqman assays were also used to quantify and compare *Spon1* mRNA levels in aorta and hearts of SHR WKY and Sisa1 rats.

Western blotting

Proteins were extracted from 6 week and 24 week kidneys of WKY, SHR and Sisa1 animals with RIPA buffer in the presence of protease inhibitors. Equal amounts of protein (15μg) were electrophoresed on 8% polyacrylamide gels under denaturing conditions and transferred onto nitrocellulose membranes. Western blots were incubated with an anti-SPON1 polyclonal antibody (Abcam), followed by a horseradish peroxidase- (HRP-) conjugated anti-chicken secondary antibody. As a loading control, membranes were stripped and re-probed with an anti-beta-actin monoclonal antibody (Abcam), followed by a horseradish peroxidase- (HRP-) conjugated anti-mouse secondary antibody. An enhanced chemiluminescence reaction resulted in visualisation of the protein bands.

DNA Sequencing of *Spon 1* gene promoter and exon-intron junctions

Genomic DNA extracted from two WKY and two SHR rats was amplified by PCR using primers designed from rat genome sequence in the Ensembl database. The primers used for

the promoter region mapped to the first 5 kbp of genomic sequence upstream of the rat *Spon1* gene (Ensembl ID = ENSRNOG00000034303). The primers used for the exon-intron junctions of the *Spon1* gene mapped to the flanking introns of each exon. Further details of these primers (Supplementary Table 3) and the PCR and sequencing protocols are given in the Online Data Supplement. Sequences were aligned and analysed using Sequencher software (http://www.genecodes.com/) and *Spon1* data from Ensembl as a reference sequence. All polymorphisms were confirmed with DNAs from additional animals.

Statistical Analysis

To compare the mean BP of each strain, a one-way ANOVA and Bonferroni multiple comparison test was performed. For microarray expression analysis, the MAS 5.0 statistical algorithm (Affymetrix) was utilised to obtain an absolute expression level metric for each probe set. All subsequent data analysis was undertaken using Genespring (v7.2, Silicon Genetics). Data were normalised per chip using the distributions of all genes. In addition we applied a per gene normalisation using the median of each gene. Statistical significance of expression changes across the strains was computed within GeneSpring using a 1-way ANOVA test ($P<0.01$) on an expressed gene list. This expressed gene list was derived by filtering for probe sets that were called “Present” in 3 of the 25 samples (5 replicates per strain) from the same age group. The parameter tested being the 5 strains with a parametric test (assume variances equal) and the Benjamini and Hochberg procedure to control false discovery rates. The Student-Newman-Keuls Post Hoc test was utilised to indicate the strain pairs where statistical differences occur. For the quantitative RT-PCR experiments, ΔC_T values were averaged across biological replicates. The $\Delta\Delta C_T$ values for each strain were then expressed as fold change using the $2^{-\Delta\Delta C_T}$ method. Statistical analysis of quantitative RT-PCR and *Spon1* protein analysis was undertaken using the Students *t* test with a significance level of $P = 0.05$.

Microarray data

This data set has been deposited in the ArrayExpress microarray data public repository (http://www.ebi.ac.uk/arrayexpress/) with the accession number E-TABM-45 (release date January 1, 2006).

RESULTS

Dissection of the Sisa1 congenic region

Two mutually exclusive sub-strains SHR.WKY-D1Got158 / D1Got161 (Sisa1a) and SHR.WKY-D1Rat420 / D1Rat278 (Sisa1b) were produced that had inherited different segments of the Sisa1 congenic region (Figure 1). The original Sisa1 congenic region was effectively dissected into two regions: Sisa1a (3 Mb in length) containing 20 genes and Sisa1b (1.1 Mb) containing 12 genes (Supplementary Table 2). All the genes from the Sisa1 region have been captured in these two sub-strains apart from a novel gene [ENSRNOG00000026767] which is positioned in a gap between the Sisa1a and Sisa1b regions. Furthermore, with additional markers (Supplementary Table 1), uncertainty as to the position of the external boundaries of the congenic regions (Figure 1) has been reduced, to a minimal size (< 60kb) that excludes the residence of any genes in these regions.

BP in congenic sub-strains

The mean BP measurements of Sisa1a and Sisa1b animals at 16 and 20 weeks are shown in Figure 2 as well as the BPs of Sisa1 and SHR animals studied at the same time. As expected from previous results, Sisa1 animals had a significantly lower BP compared to SHR animals with differences of ~11mmHg and 9mmHg at 16 and 20 weeks respectively ($P < 0.001$).

The Sisa1a sub-strain animals also had a significantly lower BP compared to SHR ($P < 0.001$ with differences of ~8mmHg and ~6.5mmHg respectively at the two ages) and exhibited a BP phenotype that was not significantly different to that of Sisa1. However, the Sisa1b animals showed the converse, with an average BP similar to that of SHR but significantly different in comparison to Sisa1 animals ($P < 0.001$).

Exon sequencing of genes located in Sisa1a region

The 20 genes in the Sisa1a region include 17 genes with functional annotation and 3 other (novel) genes (Table 1). Of the known genes, 16 could be amplified from WKY and SHR kidney RNA and cDNA sequencing was used to compare WKY and SHR sequences. The parathyroid hormone precursor was not expressed in the kidney and was not sequenced. No detectable transcripts were observed from the three novel genes in the Sisa1a region either, but the putative exons of these genes were amplified and sequenced from genomic DNA. In total, almost 30kb of exonic sequence was compared between the strains.

The findings from the transcript sequencing experiments are summarised in Table 1. There was no detectable sequence variation between the SHR and WKY transcripts in the majority of the genes. Synonymous single nucleotide polymorphisms (SNPs) were found in K+ channel protein, *R-RAS2* and one of the novel genes ENSRNOG00000025393. A synonymous and non-synonymous SNP was discovered in the RSB-11-77 gene, with the latter resulting in a methionine to valine substitution in the SHR. The cytoskeleton associated-2 gene also had a non-synonymous SNP, causing an arginine to histidine change at residue 142 in the SHR. Interestingly, for the CASL gene both the WKY and SHR strains produced an identical truncated open reading frame when compared to the reference (Brown-Norway) sequence in the Ensembl database. There was also a polymorphism in the 3'UTR region of this gene. For the *Spon1* gene we sequenced the entire known 5' and 3' UTR sequences. *Spon1* had 3 synonymous SNPs and a polymorphism in the 3'UTR region.

Gene Expression Analysis

To identify strong positional candidates from the microarray expression profiling we employed a strict test regime – the genes had to show significant and consistent differential expression between the SHR and WKY genotypes across all 5 strains and at both ages. Sixteen of the Sisa1a genes were represented on the microarray chip and for those genes not represented on the chip, semi-quantitative RT-PCR was used to look for expression differences. Where renal expression was observed in these RT-PCR experiments, no inter-strain differences were observed.

The only gene from the Sisa1a region to pass all the microarray tests for significance and maintained differential expression across all the strains and at both ages was *Spon1* (Ensembl identifier ENSRNOG00000034303), otherwise known as *F-Spondin*. The expression profile of *Spon1* across the strains was validated by quantitative RT-PCR (Figure 3a). At both 6 and 24 weeks of age, relative *Spon1* mRNA levels in the kidney of the WKY rate were significantly lower compared to the SHR rat. *Spon1* mRNA levels were also significantly lower in Sisa1 animals compared to SHR; at 6 weeks of age, *Spon1* mRNA level in Sisa1 animals were intermediate between SHR and WKY but at 24 weeks the Sisa1 *Spon1* mRNA level was similar to that of WKY.

During the microarray analysis, data relating to BP2 (the second BP QTL captured within the introgressed segment of WConSa but distinct from the QTL region of Sisa1)8 was also examined. In total, 853 of the 1,273 genes within the WConSa region were represented on the microarray chip and of these, 3 further genes in addition to *Spon1* were identified as being differentially expressed using the criteria described above; *Homer-2* (Ensembl

identifier ENSRNOG00000019297), *XP_215009* (Ensembl identifier ENSRNOG00000018042) and *Thumpd1* (Ensembl identifier ENSRNOG00000014946). Of these, *Thumpd1* maps to the second BP QTL, BP2, mapped to this region. Differential expression of these genes was also confirmed by quantitative RT-PCR (data not shown).

Expression of *Spon1* in kidneys of Sisa1a and Sisalb sub-congenic strains

In additional experiments, we analysed *Spon1* mRNA levels in kidneys of Sisa1a and Sisa1b sub-congenic strains. Contemperarous SHR, WKY and Sisa1 animals were also studied. The data for 6 week animals are shown in Figure 3b. Sisa1a which still carries the WKY *Spon1* allele showed an expression level similar to WKY and Sisa1 while in Sisa1b the level had increased back to the SHR level. Similar results were obtained at 24 weeks (data not shown).

Expression of *Spon1* in vascular tissues

We also examined *Spon1* expression in the heart and aorta (Figure 4). Both tissues expressed *Spon1*. Comparing Ct and Delta Ct values from the quantitative PCR across the strains and in different tissues indicated that *Spon1* mRNA expression levels are similar in kidney and heart, and approximately 4-fold lower in aorta. Importantly, both vascular tissues showed the same inter-strain profile of *Spon1* expression as the kidney, with a lower level in Sisa1 compared with the SHR supporting the genetic regulation of *Spon1* expression.

Analysis of *F-Spondin* protein levels

F-Spondin is proteolytically cleaved in vivo to yield a 60kDa fragment containing the reelin and spondin domains, and a 50kDa fragment containing the six C-terminal thrombospondin-type 1 repeat (TSR) domains. The anti-*F-Spondin* antibody used in these experiments specifically recognises the TSR domains and thus detected a 50kDa protein in rat kidney lysates (Figure 5). When measured relative to beta-actin, *F-Spondin* protein levels were significantly higher (1.7 fold) in 6 week SHR than in 6 week old WKY animals, but were not significantly different between WKY and Sisa1 kidney samples. A similar trend was observed in 24-week kidney samples, where *F-Spondin* expression was 3.6-fold higher in SHR than in WKY. However, *F-Spondin* levels were 1.8-fold higher in Sisa1 than in WKY although significantly lower (1.8-fold) when compared to SHR animals at 24 weeks.

Comparison of *Spon1* promoter and exon-intron junctions of SHR and WKY

Three single nucleotide polymorphisms (SNPs) were identified between WKY and SHR in the first 5 kbp of sequence upstream of *Spon1*.: a G/A polymorphism (WKY/SHR) at position -2009 relative to the translation start site, a G/A polymorphism at position -4183, and a T/C polymorphism at position -4639. Using the VISTA Genome browser, the 5 kbp of rat sequence upstream of *Spon1* was aligned with orthologous regions in human and mouse. This analysis identified several conserved regions (70% sequence conservation over a 100bp window), one of which contains the SNP at position -4183 (Supplementary Figure 1). A comparative sequence analysis, of all exon-intron junctions for the 19 exons of the Spon1 gene showed only two intron polymorphisms (Supplementary Table 3) between SHR and WKY that were 30 and 58 bases from their respective intron-exon junctions. Neither of these created an alternative splice site according to the known mammalian consensus sequences at the 5’ and 3’ splice sites.

DISCUSSION

We have used a combination of creating congenic strains with higher resolution introgressed segments, strong prior evidence from transplant experiments that the QTL effect resides in

the kidney,9 and targeted transcript sequencing and microarray gene expression analysis, to identify putative hypertension candidate genes underlying a BP QTL on rat chromosome 1.

There are several notable findings from the work. The fact that the BP effect seen in Sisa1 could be dissected out with retention of the QTL in the Sisa1a strain and reversion of the phenotype in the Sisa1b strain to the SHR phenotype provides strong evidence that the BP effect is directly due to genomic alterations in the Sisa1a region. We investigated the possibility that the effect may be due to a structural variation in one of the proteins coded by the genes located in Sisa1a. However, this search identified only two non-synonymous changes: one in RSB-11-77 that caused a methionine to valine change at position 186 in the SHR and the other in the Cytoskeleton associated-2 gene leading to an arginine to histidine substitution at position 142. Both types of changes have been reported to alter function in other proteins.11,12 However the respective changes do not occur in any known structural or functional motifs in these genes.

Our most interesting findings came from the microarray gene expression analysis. For this, we set strict criteria whereby the candidate genes not only had to show consistent genotype-related expression differences across several strains but also exhibit differential expression at two chronological points of 6 and 24 weeks of age. This was to identify changes that were likely to be genetically determined and to exclude changes occurring only at the later age, which could be secondary to blood pressure differences. The focus on renal expression was as a result of our previous work, where we observed that the major proportion of the BP effect from the loci on rat chromosome 1 is mediated through the kidney9. The microarray studies highlighted *Spon1* as a gene of particular interest from the Sisa1 region; it was the only gene that showed genotype-related differences in expression at both ages. Thus, compared with WKY, mRNA levels of *Spon1* were higher not only in SHR but also in WConSa, which exhibits a SHR genotype for the chromosomal region in which *Spon1* resides. In contrast, *Spon1* expression levels in the SConSa and Sisa1 congenic strains, which contain the WKY genotype for *Spon1* in an SHR background, were similar to the WKY strain. Furthermore, the Sisa1 sub-congenic strains (Sisa1a and Sisa1b) showed differences in expression corresponding to the *Spon1* allele they carried. Moreover, the *Spon1*-encoded *F-Spondin* protein was expressed at a significantly higher level in the kidney of SHR than WKY and Sisa1 rats at both 6 and 24 weeks of age. The findings as a whole provide persuasive evidence that *Spon1* expression is genetically regulated and tracks with BP.

Spon1 has direct orthologues in many species including mouse and human, indicating that it is a highly conserved gene in evolutionary terms. The *Spon1* orthologue in human has been termed ‘vascular smooth muscle cell growth-promoting factor’ and shown to inhibit angiogenesis via the blockade of integrin receptors on human vascular endothelial cells.13 This is consistent with a large body of research demonstrating that the thrombospondins (proteins with the thrombospondin domain), to which *F-spondin* belongs, have an anti-angiogenic activity.14 Interestingly, it has been demonstrated that the SHR strain has a decreased angiogenic potential compared to the WKY rats and this observation, together with other evidence, has led to the hypothesis that hypertension in the SHR could be caused by an impaired capacity for vascular growth from an early developmental stage.15 In this context, our finding of differential expression of *Spon1* in vascular tissue which appears to be genetically regulated is notable. Indeed, it has been shown that the narrowed lumen of distal afferent arterioles in the SHR kidney at 7 weeks is related to later development of increased blood pressure.16 Therefore, it is possible that *Spon1* over-expression (leading to increased *F-Spondin* protein levels) could affect blood pressure in the SHR by influencing blood vessel formation in the kidney of the developing animal.

The genetic mechanism underlying the differential expression of *Spon1* between SHR and WKY genotypes remains to be elucidated. Our extensive sequencing of the promoter region of Spon1 has identified three single nucleotide polymorphisms (SNPs) between the strains. These provide targets for further analysis as does the SNP identified in the 3'UTR region (Table 1). Also, in order to elucidate whether there were *Spon1* differential splice variants between the SHR and WKY strains, we sequenced the exon-intron junctions of all 19 exons and did not detect any splice site variants (supplementary table 3).

Although not a primary aim of this study, the microarray data also allowed us to look for genes that could underlie the other BP QTL, BP2, in this region of chromosome 1.8 *Thumpd1*, located within this region, showed a consistent genotype-related pattern of expression in the various parental and congenic strains that would make it a putative candidate gene for the second BP QTL. Interestingly, another study6 has recently also confirmed expression differences in *Thumpd1* between hypertensive and normotensive strains and suggested that this is a candidate gene for hypertension. Functional annotation on this gene is sparse. The encoded protein contains an ATP/GTP-binding site motif as well as a THUMP domain that is involved in RNA-binding. We also identified two other genes that are located in the larger WConSa congenic region that showed expression differences between the strains; the first, *Homer-2*, encodes a protein that binds to the cytoplasmic regions of metabotropic glutamate receptors mGluR1 and mGluR5 and appears to aid the coupling of surface receptors to cause intracellular calcium release in neurons.17 The second gene, *XP_215009*, has little functional information attached to it. However, neither maps to one of the narrowed BP QTL regions. In addition, we observed the differential expression of the *Sa* gene, which has previously received considerable attention as a hypertension candidate gene18 between the SHR and WKY genotypes of the 5 strains examined. However, recent research19 utilising gene targeting has shown that the *Sa* gene is unlikely to play a role in blood pressure regulation.

Although our approach has successfully identified *Spon1* as a strong positional candidate gene for one of the BP QTLs on rat chromosome 1, further work is clearly required to confirm its candidacy, including examining the effect on BP and other phenotypes of altering its level of expression through gene modification techniques. In assessing the present findings, limitations of our interrogation of the Sisa1a region need to be borne in mind. Specifically, the microarray chips utilised in this study, and previous studies like this, are not capable of picking up differential expression of alternative spliceforms. Therefore, currently we cannot exclude the possibility that an undetected difference in level of a specific splice variant in one of the Sisa1a genes apart from *Spon1* is responsible for the BP effect. Second, we have focused on the genes located in the region. Increasing evidence is emerging that intergenic regions may influence phenotypic traits. If further interrogation of *Spon1* does not explain the BP QTL effect, then these possibilities will need to be considered.

In summary, through a combination of creating novel congenic strains with higher resolution introgressed segments, targeted transcript sequencing and microarray gene expression profiling, we have identified *Spon1* as a novel strong candidate gene underlying a major BP QTL on rat chromosome 1. If further studies confirm its role in BP regulation, then examining its role in BP homeostasis in humans and risk of hypertension will be of great interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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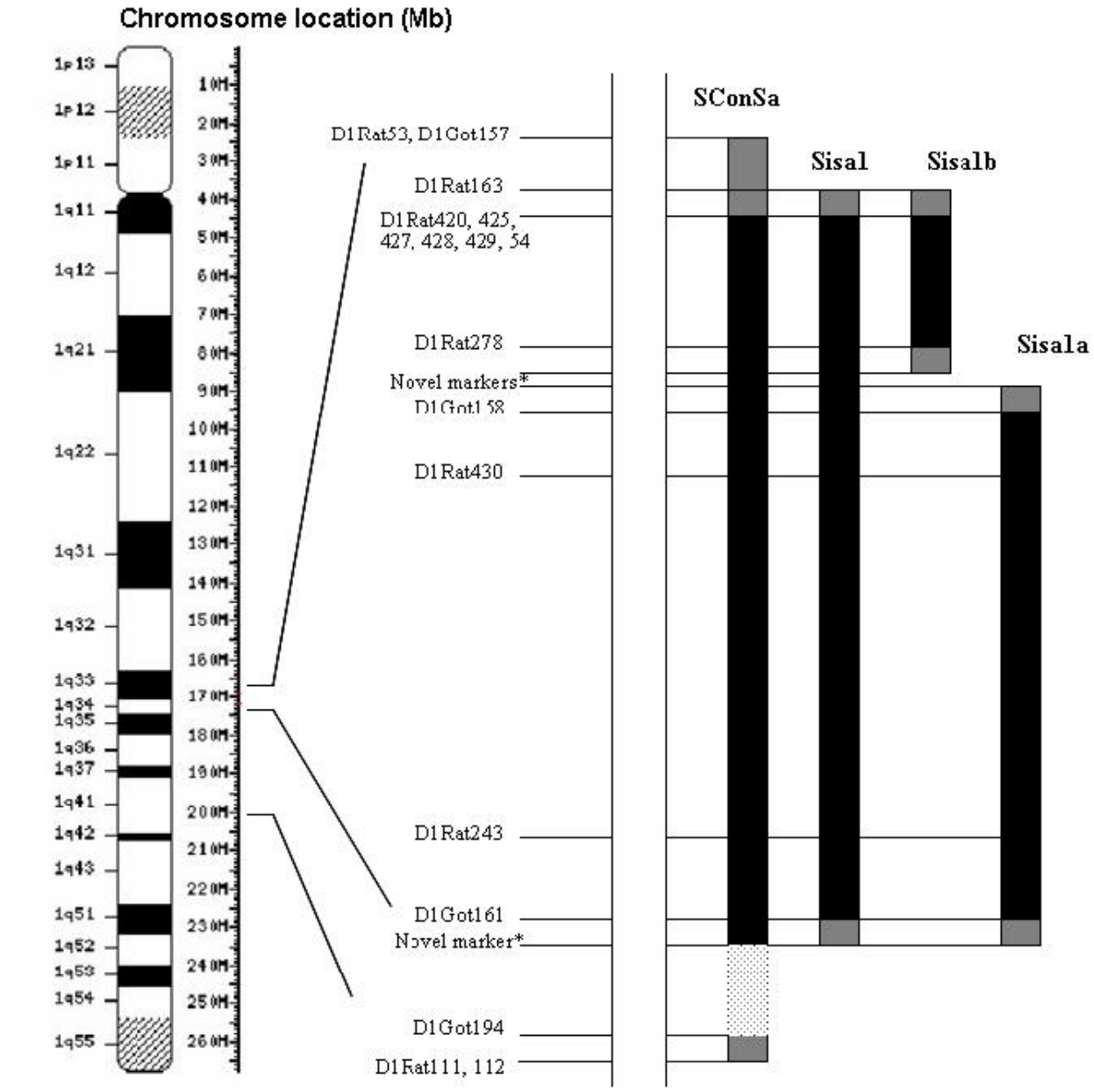

Figure 1.

Chromosomal locations of the congenic regions in the studied strains

An ideogram of rat chromosome 1 is shown on the left side of the figure. The approximate locations of some of the polymorphic genetic markers used to delineate the congenic regions are shown in the middle of the diagram. For the congenic strains and sub-strains, the black (WKY genotype) and shaded areas represent the minimal and potentially maximal introgressed segments, respectively, based on genotypic analysis of the strains. The dotted area within the SConSa region represents the compression of this region to enable clarity of the diagram. The details of all the markers used to define the Sisa1 region are listed in supplementary table 1.

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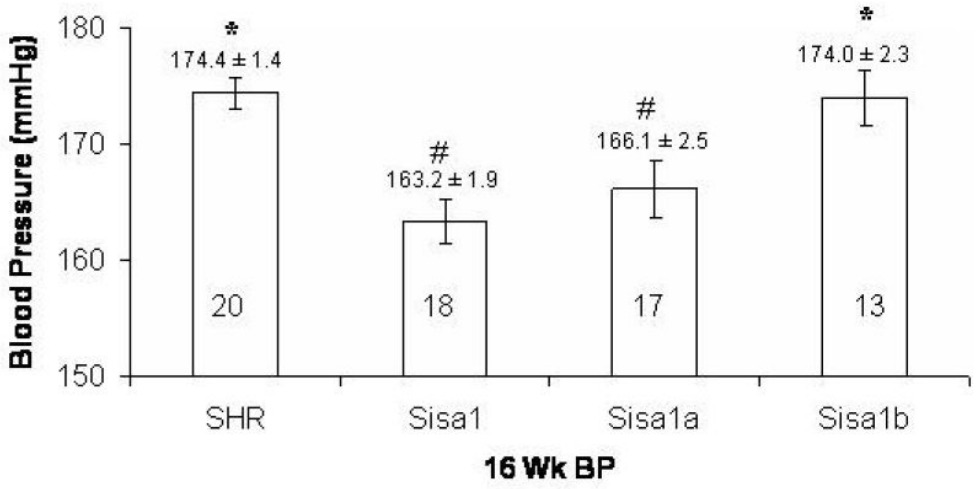
A



B

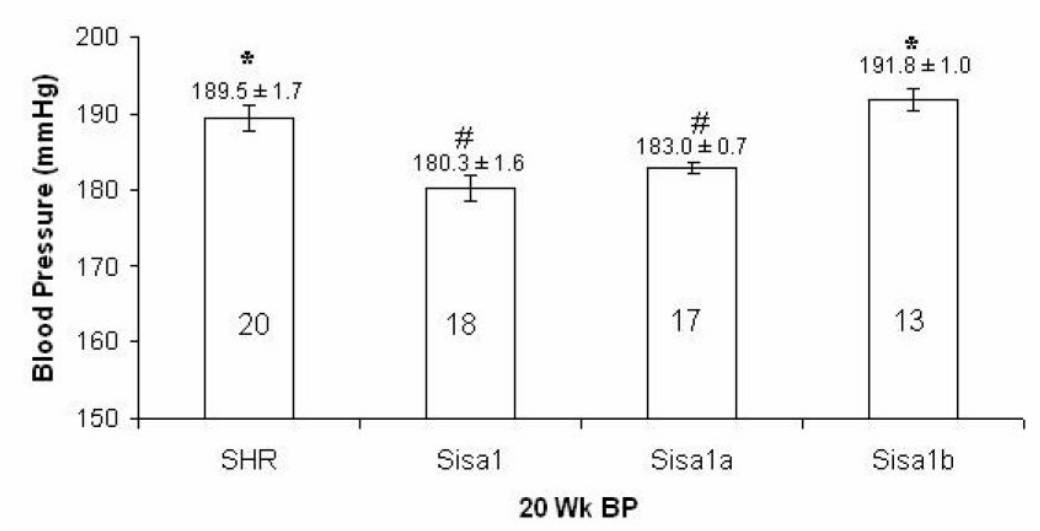


Figure 2.

Mean blood pressure values of the strains

The mean BP values of the Sisa1a and Sisa1b strains in comparison to the SHR and Sisa1 strains at A) 16 weeks and B) 20 weeks of age. The figures within the column bars represent the number of animals used. BP values are ± standard deviation. * $P < 0.001$ vs. Sisa1 $^{\#}P < 0.001$ vs. SHR.

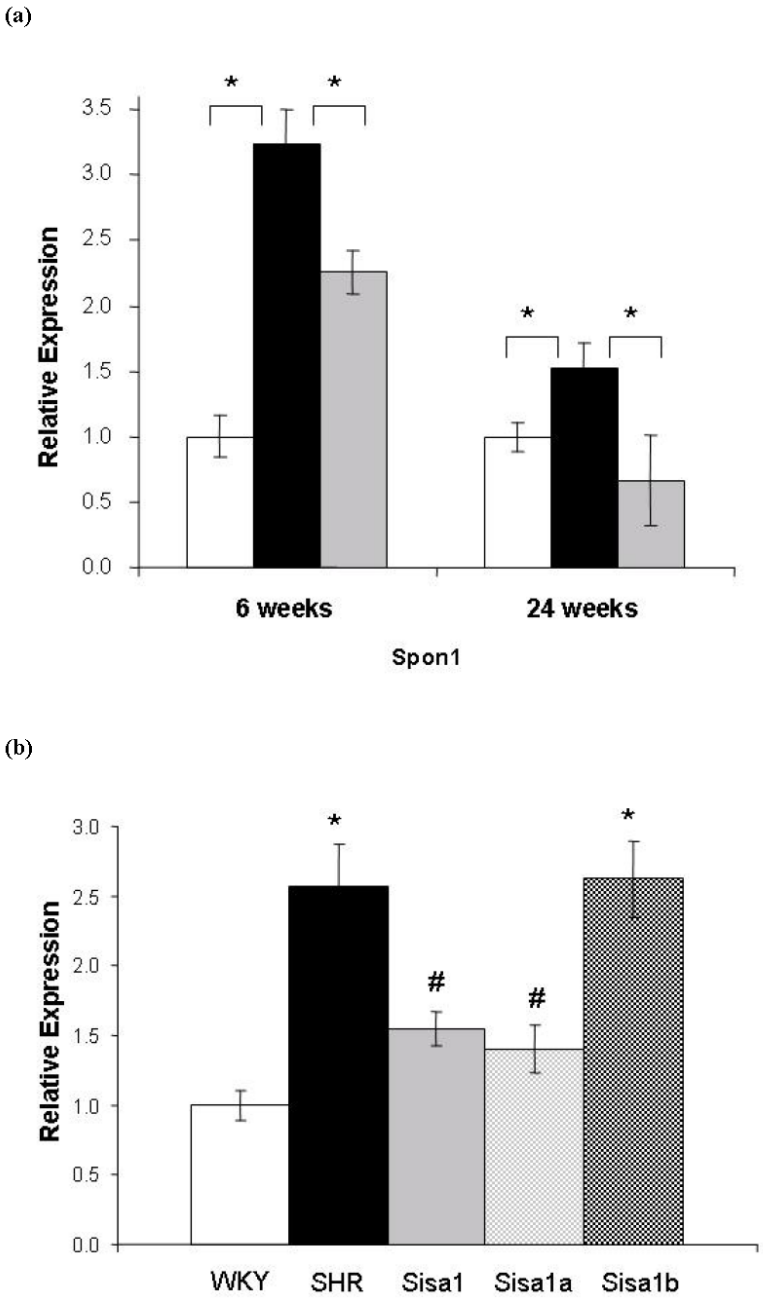


Figure 3.

Validation of microarray gene expression data for *Spon1* by quantitative RT-PCR and analysis of *Spon1* mRNA levels in sub-congenic strains.

(a) *Spon1* mRNA levels in kidneys of 6 and 24 week old rats. *Spon1* expression in WKY (white), SHR (black) and Sisa1 (grey) was measured relative to the endogenous control beta-2-microglobulin (n=5 for each strain). Relative expression values are ± s.e.m * $P < 0.03$

(b) *Spon1* mRNA levels in kidneys of 6 weeks old WKY, SHR, Sisa1, Sisa1a and Sisa1b animals (n = 5 per strain). Relative expression values are ± s.e.m * $P < 0.01$ when

comparing to WKY expression level and # $P < 0.05$ when comparing to SHR expression level.

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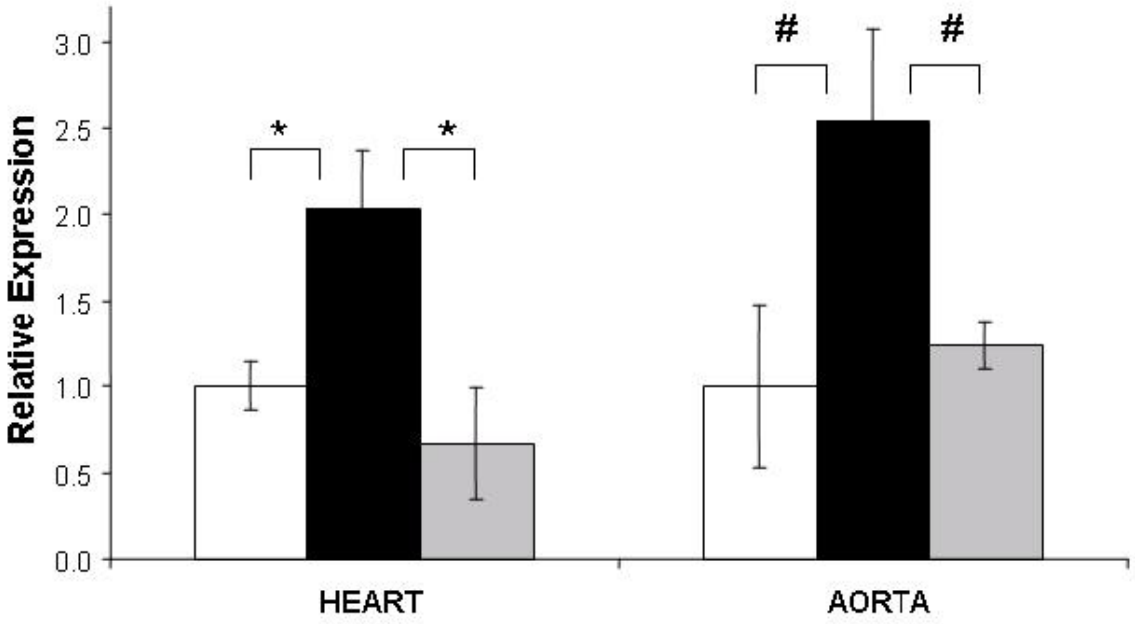

Figure 4.

Spon1 mRNA Expression in Heart and Aorta of 6 weeks old animals

Spon1 expression in WKY (white), SHR (black) and Sisa1 (grey) measured relative to the endogenous control beta-2-microglobulin (n=5 for each strain). Relative expression values are ± s.e.m * $P < 0.01$ and # $P < 0.05$

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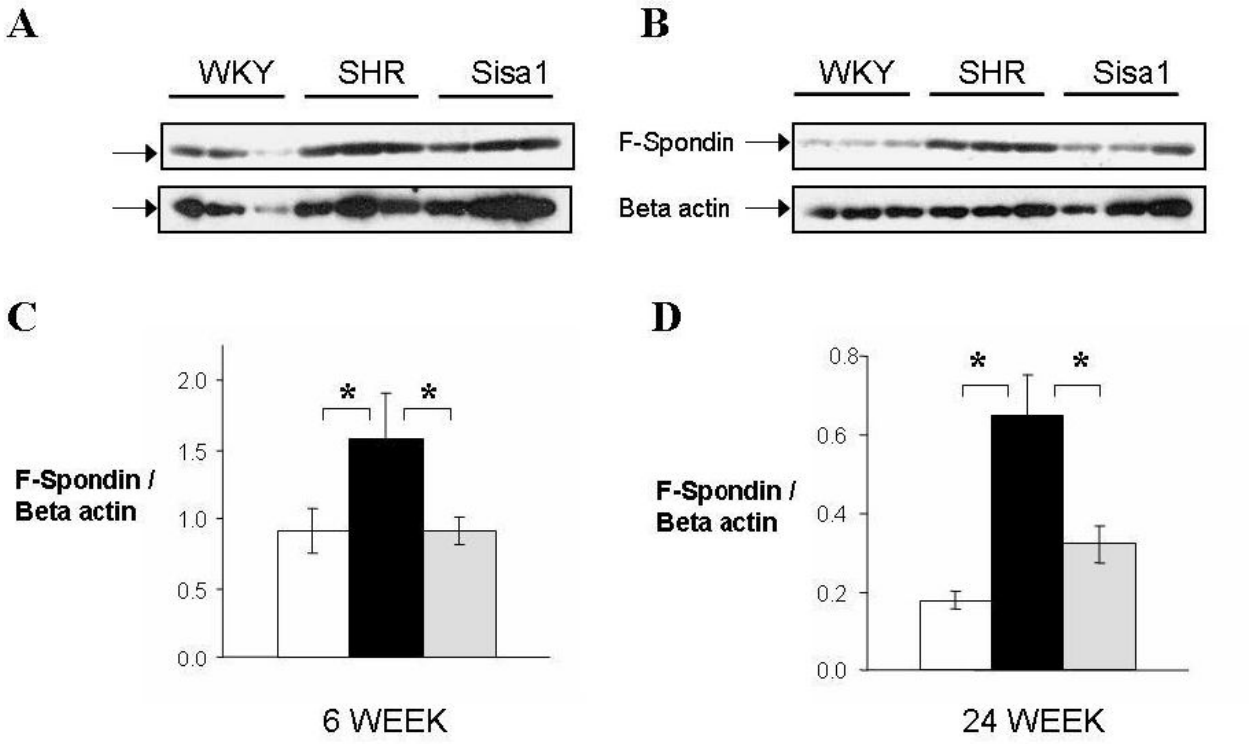

Figure 5.

F-Spondin protein is differentially expressed in the kidney of WKY, SHR and Sisa1 rats at 6 and 24 weeks of age.

Western blot analysis was performed on kidney lysates from 6 week (A) and 24 week (B) animals (n = 3 for each strain) using anti-F-Spondin and anti-beta-actin antibodies. A 50kDa F-Spondin protein was normalised to the loading control, beta-actin, and quantified by densitometry for 6 week (C) and 24 week (D) animals. WKY (white), SHR (black) and Sisa1 (grey). Relative expression values are ± s.e.m * $P < 0.05$ for 6 week data, $P < 0.02$ for 24 week data.

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Table 1

A summary of the exon sequencing of genes located in the Sisa1a region.

Ensembl Gene ID	Gene Description	Expression status in kidney	Transcript size sequenced (bp)	Open reading frame size (amino acid residues)	Polymorphisms detected between SHR and WKY	Putative effect of polymorphism
ENSRNOG00000017021	GALNAC-9	Expressed	2143	622	NONE	
ENSRNOG00000033668	Novel *	Not expressed	207	69	NONE	
ENSRNOG00000026754	Hydrolase	Expressed	4701	1364	NONE	
ENSRNOG00000016343	Dkk3	Expressed	1047	348	NONE	
ENSRNOG00000016244	CASL	Expressed	2886	961	1025bp C>T in SHR	No effect on ORF - The WKY and SHR cDNA sequences both produce an ORF which begins in the same Frame as the reference protein sequence, but both result in a truncated termination at taa (750 bp), which is before the SNP (1,025bp).
ENSRNOG00000016210	RSB-11-77 Protein	Expressed	1766	687	332bp G>A in SHR, 630bp A>G in SHR, 671bp A>A in SHR, 576bp A>G in SHR	All synonymous changes except the 576bp A>G mutation in SHR that causes a M to V amino acid change in SHR - both of these amino acids are small and hydrophobic and are not in a protein structural domain.
ENSRNOG00000015713	PARVA	Expressed	1122	373	NONE	
ENSRNOG00000015488	TEAD	Expressed	1281	426	NONE	
ENSRNOG00000014847	PAM-monooxygenase	Expressed	1714	Pseudogene - not translated	NONE	
ENSRNOG00000036773	Cytoskeleton associated-2	Expressed	435	145	425bp G>A in SHR	R to H amino acid change in SHR - both polar and positive charged. Not in a structural domain.
ENSRNOG00000014448	ARNTL	Expressed	2389	626	NONE	
ENSRNOG00000014341	K+ channel protein	Expressed	1428	475	756bp C>T in SHR	Synonymous change
ENSRNOG00000014318	Parathyroid hormone precursor *	Not expressed	704			
ENSRNOG00000033924	Novel *	Not expressed	315	106	NONE	
ENSRNOG00000013176	Male Sterility protein	Expressed	2481	517	NONE	
ENSRNOG00000025393	Novel *	Not expressed	1164	388	2bp A>G in SHR	Synonymous change
ENSRNOG00000034303	F-SPONDIN	Expressed	4029#	807	2079bp T>C in SHR, 2153bp T>C in SHR, 2241bp T>C in SHR, 3800bp T>C in SHR	3 × Synonymous changes and one polymorphism (3800bp T>C) in 3′ UTR region

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Ensembl Gene ID	Gene Description	Expression status in kidney	Transcript size sequenced (bp)	Open reading frame size (amino acid residues)	Polymorphisms detected between SHR and WKY	Putative effect of polymorphism
ENSRNOG00000029854	Putative RNA Binding Protein	Expressed	519	173	NONE	
ENSRNOG00000012258	R-RAS2	Expressed	615	204	268bp T>C in SHR	Synonymous change
ENSRNOG00000025122	Ribosomal protein S11	Expressed	483	143	NONE	

*Those genes with no known function or previously ascribed description from an orthologous gene are labelled as novel. The transcript that was used to design primers for cDNA sequencing was the longest transcript model found for that gene from Ensembl.

#For Spon1 the entire 5' and 3' UTR as defined by NCBI RefSeq database was sequenced. **Galnac-9** - Putative polypeptide N-acetylgalactosaminyltransferase-like protein. **Hydrolase** - Ubiquitin carboxyl-terminal hydrolase. **Dkk3** - Dickkopf-related protein 3 precursor. **CasL** - similar to flavoprotein oxidoreductase. **Parva** - Alpha-parvin (Calponin-like integrin-linked kinase binding protein). **Tead** - Transcriptional enhancer factor TEF-1. **PAM-monoxygenase** - similar to peptidylglycine alpha-amidating monooxygenase COOH-terminal interactor; peptidylglycine alpha-amidating monooxygenase COOH-terminal interactor protein-1. **Arntl** - Aryl hydrocarbon receptor nuclear translocator-like protein 1. **Male sterility protein** - male sterility domain containing. **Spon1** - Spondin-1 precursor (F-spondin) (Vascular smooth muscle cell growth- promoting factor). **R-Ras2** - related RAS viral (r-ras) oncogene homolog 2.