ORIGINAL ARTICLE

Refined mapping of blood pressure quantitative trait loci using congenic strains developed from two genetically hypertensive rat models

Sivarajan Kumarasamy¹, Kathirvel Gopalakrishnan¹, Edward J Toland¹, Shane Yerga-Woolwine¹, Phyllis Farms¹, Eric E Morgan² and Bina Joe¹

Previously linkage and substitution mapping were conducted between the Dahl Salt-sensitive (S) rat and the Spontaneously Hypertensive Rat (SHR) to address the hypothesis that genetic contributions to blood pressure (BP) in two genetically hypertensive rat strains are different. Among the BP quantitative trait loci (QTLs) detected, two are located on chromosome 9 within large genomic segments. The goal of the current study was to develop new iterations of congenic substrains, to further resolve both of these BP QTLs on chromosome 9 as independent congenic segments. A total of 10 new congenic substrains were developed and characterized. The newly developed congenic substrains S.SHR(9)x8Ax11A and S.SHR(9)x10Ax1, with introgressed segments of 2.05 and 6.14 Mb, represented the shortest genomic segments. Both of these congenic substrains, S.SHR(9)x8Ax11A and S.SHR(9)x10Ax1 lowered BP of the S rat by 56 mm Hg ($P < 0.001$) and 15 mm Hg ($P < 0.039$), respectively. The BP measurements were corroborated by radiotelemetry. Urinary protein excretion was significantly lowered by SHR alleles within S.SHR(9)x10Ax1 but not by S.SHR(9)x8Ax11A. The shorter of the two congenic segments, 2.05 Mb was further characterized and found to contain a single differentially expressed protein-coding gene, Tomoregulin-2 (*Tmeff2*). The protein expression of *Tmeff2* was higher in the S rat compared with S.SHR(9)x8Ax11A, which also had lower cardiac hypertrophy as measured by echocardiography. *Tmeff2* is known to be upregulated in patients from multiple cohorts with cardiac hypertrophy. Taken together, *Tmeff2* can be prioritized as a candidate gene for hypertension and associated cardiac hypertrophy in both rats and in humans.

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INTRODUCTION

Blood Pressure (BP) is a complex polygenic trait well studied in both model organisms and in humans. Genome-wide association studies in humans have identified a few regions and candidate genes underlying BP regulation.^{1–11} However, the extent of genetic heritability accounted by association studies alone does not represent the total heritability.¹² Therefore, identifying the key determinants of BP control remains to be a major challenge.

Classic linkage analyses and substitution mapping studies using inbred rat models of hypertension have identified BP quantitative trait loci (QTLs) on nearly every rat chromosome^{13–19} (<http://www.rgd.mcm.edu/>). In our lab, we have resolved three of these QTLs and identified short genomic segments of 42.5, 177 and 804.6 kb containing genes coding for Rififylin, 11 β -hydroxylase and Adamts16, respectively, as novel risk factors for hypertension.^{20–23}

These studies were conducted using a hypertensive rat as one of the parental strains and a normotensive rat as the other parental strain.

Two of the most widely used rat models of hypertension are the Dahl Salt-sensitive (S) rat and the Spontaneously Hypertensive Rat (SHR).²⁴ The S and SHR strains are both hypertensive models, but they exhibit differential development of hypertension. The BP of the S rat, but not the SHR is exacerbated by a high-salt diet.^{25,26} In addition, SHR rats are resistant to the kidney damage that is seen in S rats.^{25–27} These phenotypic differences observed between S and SHR suggest that there are different genetic elements governing BP control in S and SHR rats. Previously, we identified two such genetic elements as BP QTLs on rat chromosome 9 (RNO9), in which SHR alleles on the S genetic background lower the BP of S rats on a 2% NaCl diet.²⁸ These two BP QTLs covered long genomic segments of 22.5 and 13.1 Mb and contained 222 and 119 annotations, respectively. The purpose of the

¹Physiological Genomics Laboratory, University of Toledo College of Medicine and Life Sciences, Toledo, OH, USA and ²Center for Diabetes and Endocrine Research, Department of Physiology and Pharmacology, University of Toledo College of Medicine and Life Sciences, Toledo, OH, USA

Correspondence: Dr B Joe, Physiological Genomics Laboratory, Department of Physiology and Pharmacology, University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614-2598, USA.

E-mail: bina.joe@utoledo.edu

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current study was to utilize the S.SHR congenic strains constructed in the previous study as tools to generate panels of congenic substrains and further map each of the BP QTLs to shorter genomic segments. Data are presented that support further resolution of two distinct BP QTLs to short genomic segments of 2.05 and 6.14 Mb. In addition, methodical characterization of the shorter of the two segments (2.05 Mb) lending support to suggest that the expression of a novel gene, *Tmeff2* is linked to the development of hypertension in the S rat, is presented.

METHODS

Animals

All animal procedures and protocols used in this report were approved by the University of Toledo Health Science Campus Institutional Animal Care and Use Committee. The Spontaneously Hypertensive Rat (SHR/Hsd), was originally obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA) and maintained in our colony. The Dahl salt-sensitive rat (SS/jr) from our colony was used in all experiments and is referred to as S. The panel of congenic substrains reported in this study was generated from S.SHR (9) x8A and S.SHR (9) x10A²⁸ as previously described.²¹

Genotyping

PCR genotyping using microsatellite markers was done as previously described.¹⁹

Tail-cuff BP measurement

The experimental design for BP measurement by the tail-cuff method was as described previously.²⁸ Briefly, at 30 days of age rats were weaned onto a low salt (0.3% NaCl) Harlan Teklad 7034 diet. One or two congenic strains along with S rats were housed two to a cage such that two different strains were in each cage. At 40–42 days of age the rats were switched to a 2% NaCl diet (Harlan Teklad, TD 94217) and maintained on this diet for the duration of the experiment. During days 25–28 on the 2% NaCl diet, each rat had its systolic BP measured by two blinded operators. During BP measurements, rats were restrained and warmed to 28 °C. The operators' readings for each rat were averaged together and recorded as that animal's systolic BP. The following new congenic substrains were concomitantly raised and tested together for BP along with S rats: S.SHR(9)x8Ax6 and S.SHR(9)x8Ax12; S.SHR(9)x10Ax1 and S.SHR(9)x10Ax2; and S.SHR(9)x10Ax3 and S.SHR(9)x10Ax4.

Telemetry

BP was also collected using a telemetry system (Data Sciences International, St. Paul, MN, USA) as explained in detail previously.²⁹ Briefly, 4 days after the BP measurements by the tail-cuff method, rats were surgically implanted with telemetry probes through their femoral arteries into the lower abdominal aortae. Rats were allowed to recover from surgery for 4 days before the transmitters were turned on for recording BP. All statistical analyses were as previously conducted.²⁹

Urinary protein excretion (UPE)

UPE determination was done as previously described.^{26,30} Briefly, within 5 days following BP measurements, each rat was housed individually in a metabolic cage and its urine was collected over a 24-h period. The pyrogallol based QuanTtest Red Total Protein Assay from Quantimetrix (Redondo Beach, CA, USA) was used to determine protein concentrations of the urine samples. A VERSAmax microplate reader from Molecular Devices (Sunnyvale, CA, USA) was used to determine absorbance at 600 nm. Protein concentrations were determined by reading against the absorbance of the QuanTtest human protein standards (25–200 mg dl⁻¹). UPE data are presented as milligrams over a 24-h period (mg per 24 h).

Echocardiography

Left-ventricular function of S (*n*=6) and congenic rats (*n*=7) were evaluated by echocardiography as described previously.^{31,32}

Survival study

S (*n*=8) and S.SHR.(9)x8Ax11A (*n*=8) rats were raised and administered 2% NaCl containing diet as described under the BP measurements section. These rats were continued on the 2% NaCl diet until their natural death.

Gene sequencing

DNA samples were obtained by tail biopsies as previously described¹⁹ from S.SHR(9)x8Ax11A, SHR and S. Primers were designed for the exons of *Tmeff2* and *LOC685674* using ExonPrimer (<http://ihg.gsf.de/ihg/ExonPrimer.html>). M13 primer sequences were added during the commercial synthesis of these custom exon primers by Integrated DNA Technologies (IDT, Coralville, IA, USA). PCR reactions were carried out using Platinum Taq High Fidelity DNA

Figure 1 Schematic representation of the first set of newly developed congenic substrains. Congenic substrains were developed from S.SHR(9)x8A as described under Methods. The physical map of rat chromosome 9 is shown on the left with locations of markers in base pairs shown within parenthesis. Newly developed polymorphic microsatellite markers are given D9Mco numbers. Markers that begin with RNO9 are single-nucleotide polymorphic markers. RNO9 (SDPR-Exon 2) is a C/T single-nucleotide polymorphism between S and SHR. *The S.SHR(9)x8A congenic strain was previously reported and is shown here for reference. The solid bars represent SHR introgressed segments of the congenic strains. Open boxes flanking the solid bars are where recombinations occurred. The blood pressure (BP) data obtained from these congenic substrains are provided in Table 1. Black bars indicate congenic strains with BP significantly lower than that of the S. Gray bars indicate congenic strains with no BP effect.

Table 1 Blood pressure effects of S.SHR(9) congenic substrains

Congenic substrain	Blood pressure (mm Hg)				Body Weight (g)			
	S	Congenic	Effect	P	S	Congenic	Effect	P
S.SHR(9)x8Ax1	194 [7.36]	183 [7.72]	−11 [10.73]	0.289	nr	nr	—	—
S.SHR(9)x8Ax6	197 [3.53]	188 [3.67]	−9 [5.61]	0.293	307 [4.93]	302 [2.88]	−5 [5.74]	0.689
S.SHR(9)x8Ax7	197 [3.85]	188 [3.21]	−9 [5.01]	0.068	312 [2.80]	307 [2.84]	−5 [3.99]	0.217
S.SHR(9)x8Ax9	214 [5.26]	181 [3.91]	−33 [6.66]	<0.001	334 [5.60]	324 [2.30]	−10 [6.05]	0.132
S.SHR(9)x8Ax11A	263 [5.60]	207 [3.50]	−56 [6.61]	<0.001	296 [14.72]	328 [6.89]	−32 [14.60]	0.041
S.SHR(9)x8Ax11A	222 [5.01]	183 [4.15]	−39 [6.50]	<0.001	nr	nr	—	—
S.SHR(9)x8Ax12	197 [3.53]	176 [5.24]	−21 [5.87]	0.003	307 [4.93]	300 [4.41]	−7 [6.01]	0.547
S.SHR(9)x10Ax1	200 [4.22]	185 [4.84]	−15 [6.22]	0.039	353 [6.24]	339 [3.37]	−14 [6.86]	0.095
S.SHR(9)x10Ax2	200 [4.22]	183 [4.14]	−17 [6.13]	0.014	353 [6.24]	347 [4.08]	−6 [6.76]	0.622
S.SHR(9)x10Ax3	213 [5.14]	208 [5.78]	−5 [7.73]	0.912	367 [6.23]	367 [3.77]	0 [6.85]	1.00
S.SHR(9)x10Ax4	213 [5.14]	203 [6.11]	−10 [7.73]	0.559	367 [6.23]	365 [3.84]	−2 [6.85]	0.995
Congenic substrain	Heart Weight (g)				Relative Heart Weight			
	S	Congenic	Effect	P	S	Congenic	Effect	P
S.SHR(9)x8Ax1	nr	nr	—	—	nr	nr	—	—
S.SHR(9)x8Ax6	1.28 [0.018]	1.20 [0.015]	−0.08 [0.026]	0.017	4.19 [0.081]	3.99 [0.039]	−0.20 [0.091]	0.085
S.SHR(9)x8Ax7	1.27 [0.020]	1.23 [0.014]	−0.04 [0.000]	0.088	4.09 [0.079]	4.01 [0.031]	−0.08 [0.085]	0.348
S.SHR(9)x8Ax9	1.38 [0.016]	1.21 [0.018]	−0.17 [0.024]	<0.001	4.16 [0.065]	3.75 [0.063]	−0.41 [0.091]	<0.001
S.SHR(9)x8Ax11A	1.68 [0.058]	1.40 [0.038]	−0.28 [0.066]	<0.001	5.82 [0.423]	4.30 [0.156]	−1.52 [0.391]	0.001
S.SHR(9)x8Ax11A	nr	nr	—	—	nr	nr	—	—
S.SHR(9)x8Ax12	1.28 [0.018]	1.15 [0.025]	−0.13 [0.028]	<0.001	4.19 [0.081]	3.84 [0.070]	−0.35 [0.095]	0.001
S.SHR(9)x10Ax1	1.53 [0.019]	1.38 [0.021]	−0.15 [0.031]	<0.001	4.36 [0.073]	4.09 [0.067]	−0.27 [0.100]	0.024
S.SHR(9)x10Ax2	1.53 [0.019]	1.43 [0.025]	−0.10 [0.031]	0.004	4.36 [0.073]	4.13 [0.070]	−0.23 [0.098]	0.060
S.SHR(9)x10Ax3	1.55 [0.019]	1.52 [0.013]	−0.02 [0.029]	0.860	4.24 [0.098]	4.16 [0.059]	−0.08 [0.113]	0.893
S.SHR(9)x10Ax4	1.55 [0.019]	1.50 [0.019]	−0.04 [0.029]	0.393	4.24 [0.098]	4.12 [0.072]	−0.12 [0.113]	0.725

Abbreviations: S, dahl salt-sensitive; SHR, spontaneously hypertensive rat.

Values are given as the mean with the standard error of the mean in brackets. The effect is defined as the Congenic mean minus the S mean. Effects were considered significant if *P* value is ≤0.05. Relative heart weight is defined as HW divided by BW times 1000. Body weight and heart weight were not recorded (nr) for S.SHR(9)x8Ax1.

Polymerase from Invitrogen (Carlsbad, CA, USA). The amplified products were resolved on 2% agarose gels, purified using Qiaquick purification kits (Qiagen, Valencia, CA, USA), and sequenced commercially (MWG—<http://www.operon.com>). DNA sequences were analyzed using Sequencher (Genecodes Corporation, Version 4.9, Ann Arbor, MI, USA).

RNA Isolation and RT-PCR analysis

After the BP measurements by the tail-cuff method, heart, kidney and brain samples were obtained from S (*n*=6) and congenic S.SHR(9)X8Ax11A (*n*=6) rats. Total RNA was extracted using TRI reagent (Invitrogen). One microgram of total RNA was used to detect gene expression by Real-Time PCR (BioRad, Hercules, CA, USA) and expression levels relative to *Gapdh* were calculated by the $2^{-\Delta\Delta CT}$ method.¹⁸

5' RACE transcript analysis

First-strand cDNA was synthesized from total RNA using a gene-specific primer (GSP1) and superscriptTM II reverse (Invitrogen). The remaining RNA was degraded by Rnase H treatment after the completion of cDNA synthesis. cDNA was purified to remove unincorporated dNTP's, GSP1 and proteins using an SNAP column. A homopolymeric tail was then added to the 3' end of the purified cDNA using TdT and dCTP. Then the tailed cDNA was used as template for subsequent PCR reaction using GSP2 that anneals to a site located within the cDNA molecule and a novel deoxyinosine containing anchor primer (provided by the manufacturer). Following amplification the PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide and the bands were visualized under UV-light.

Immunoblot analysis

Antibodies were from the following sources: goat polyclonal anti- *Tmeff2* (N-15) (SC-47504), rabbit monoclonal anti-*Gapdh* (Cell Signaling, Danvers, MA, USA), bovine anti-rabbit IgG-HRP conjugate (SC-2370) and donkey anti-goat IgG-HRP conjugate (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) (SC-2020). Samples were homogenized in ice-cold RIPA lysis buffer with protease inhibitor cocktail (Pierce, Rockford, IL, USA). Forty microgram of proteins were boiled with Laemmli loading buffer for 5 minutes at 95 °C. Protein samples were resolved using 10% NuPAGE Bis-Tris-gel (Invitrogen) at room temperature, and transferred on to PVDF membrane (Millipore, Temecula, CA, USA). The membrane was blocked with 3% BSA, incubated with primary and secondary antibodies before visualization by the chemiluminescence method (Pierce). Image J was used to quantify the protein expression level.

Statistical analysis

All BP statistical analyses were done using the SPSS software (SPSS, Chicago, IL, USA). Data were analyzed by the Student's *t*-test unless more than two strains were tested concomitantly, in which case, a one-way analysis of variance was used to assess statistical significance. Data are presented as the mean ± standard error. A *P*-value of ≤0.05 was used as a threshold for statistical significance. For BP and UPE experiments and subsequent heart weight and body weight measurements the number of rats in each group ranged from 10 to 20. Survival analysis was done using the Kaplan—Meier analysis.

RESULTS

Mapping a BP QTL within 2.05 Mb

Using a congenic strain reported in our previous study,²⁸ S.SHR (9)x8A, a new panel of congenic substrains of the S rat containing

Figure 2 Radiotelemetry measures on (a) systolic BP, (b) diastolic BP (c) heart rate S and S.SHR(9)x8Ax11A. Rats ($n=6$ per group) were surgically implanted with C40 blood pressure radiotelemetry transmitters as described under Methods. Data points are 4-hour moving averages $\pm$ s.e.m. *** $P<0.001$; ns, not significant.

introgressed segments of chromosome 9 from the SHR were generated. A schematic diagram of these new congenic substrains is shown in Figure 1. The BP of the congenic substrains shown in gray in Figure 1, S.SHR(9)x8Ax1, S.SHR(9)x8Ax7 and S.SHR(9)x8Ax6 were not significantly different from that of the S (Table 1), whereas the BP of the three other strains shown in black in Figure 1, S.SHR(9)x8Ax9, S.SHR(9)x8Ax12 and S.SHR(9)x8Ax11A, were significantly lower than that of S by 33, 21 and 56 mm Hg, respectively (Table 1). The three congenic substrains with BP lowering effects also had significantly lower relative heart weights compared with that of S rats (Table 1). S.SHR(9)x8Ax11A was the congenic substrain with the shortest introgressed segment of 2054387 bp, which demonstrated a BP lowering effect (Figure 1). The BP study of this strain was repeated by the tail-cuff method. S.SHR(9)x8Ax11A had 39 mm Hg lower BP com-

Table 2 Urinary protein excretion of S.SHR(9) congenic substrains

Congenic substrain	Urinary protein excretion (mg per 24 h)			
	S	Congenic	Effect	P
S.SHR(9)x8Ax1	78 [13.86]	72 [5.442]	-6 [16.16]	0.716
S.SHR(9)x8Ax6	102 [6.81]	86 [5.72]	-16 [9.39]	0.212
S.SHR(9)x8Ax7	127 [10.32]	87 [8.11]	-40 [13.12]	0.004
S.SHR(9)x8Ax9	94 [7.54]	69 [4.55]	-25 [9.01]	0.013
S.SHR(9)x8Ax11A	253 [20.37]	212 [14.66]	-41 [25.10]	0.109
S.SHR(9)x8Ax12	102 [6.81]	72 [8.09]	-30 [9.86]	0.012
S.SHR(9)x10Ax1	160 [6.78]	135 [7.39]	-25 [9.66]	0.036
S.SHR(9)x10Ax2	160 [6.78]	140 [5.78]	-20 [8.96]	0.080
S.SHR(9)x10Ax3	nr	nr	nr	nr
S.SHR(9)x10Ax4	nr	nr	nr	nr

Urinary protein excretion data are presented as milligrams over a 24 h period (mg per 24 h). Values are given as the mean with the standard error of the mean in brackets. The effect is defined as the Congenic mean minus the S mean. Effects were considered significant if P value is $P\leq 0.05$. Urinary protein excretion were not recorded (nr) for S.SHR(9)x10Ax3 and S.SHR(9)x10Ax4.

pared with S ($P<0.001$, $n=20$ per group). Additional confirmation of the BP effect was sought by the radiotelemetry method (Figure 2). Both systolic and diastolic BP of S.SHR(9)x8Ax11A were significantly lower than that of the S (Figure 2a and b). Heart rates of S.SHR(9)x8Ax11A were, however, not different from that of the S (Figure 2c). These data suggest that a major BP QTL is located within the 2.05 Mb congenic segment of S.SHR(9)x8Ax11A.

The profiles of the panel of congenic substrains in Figure 1 compared with S were not the same for BP and UPE effects. Among the strains that lowered BP of the S, S.SHR(9)x8Ax9 and S.SHR(9)x8Ax12 had similar significant effects on UPE, reducing protein excretion by 25 (± 9.01 , $P=0.013$) and 30 (± 9.86 , $P=0.012$) mg per 24h, respectively (Figure 1, Table 2). S.SHR(9)x8Ax11A did not affect UPE of the S rat. The congenic substrain S.SHR(9)x8Ax7, which did not affect BP, reduced the UPE of the S rat by 40 (± 13.12 , $P=0.004$) mg per 24h. The only two strains that did not show either a BP effect or a UPE effect were S.SHR(9)x8Ax1 and S.SHR(9)x8Ax6 (Figure 1, Table 2).

Fine-mapping a second closely linked BP QTL within 6.14 Mb

In our previous report,²⁸ a second BP QTL distal to the region fine-mapped above was described. To fine-map this distal QTL, a second panel of congenic substrains were generated from the progenitor strain S.SHR(9)x10A shown in Figure 3. The congenic substrains S.SHR(9)x10Ax2 and S.SHR(9)x10Ax1 significantly lowered BP of the S rat by 17 (± 6.13 , $P=0.014$) and 15 mm Hg (± 6.22 , $P=0.039$, Table 1). Two other substrains with shorter introgressed segments, S.SHR(9)x10Ax4 and S.SHR(9)x10Ax3 did not lower BP of the S rat (Table 2). The congenic substrain S.SHR(9)x10Ax1, which had the shortest introgressed segment and demonstrated a BP-lowering effect by the tail-cuff method, was further tested by the radiotelemetry approach. Systolic and diastolic BP of S.SHR(9)x10Ax1 was significantly lower than that of the S (Figure 4a and b). Further, heart rate of this strain was higher than that of the S at all time points tested (Figure 4c). These data suggest that BP QTL2 is located within the congenic limits of S.SHR(9)x10Ax1, which is 6.14 Mb. UPE of S.SHR(9)x10Ax1 was lower than that of the S by 25 ± 9.66 mg per 24h, $P<0.036$ (Table 2), suggesting that unlike the BP QTL located within 2.05 Mb, this BP QTL is associated with UPE. For further characterization, we focused on the shorter of the two mapped QTLs, that is, the BP QTL within 2.05 Mb.

Further physiological genomic characterization of the BP QTL within 2.05 Mb

To assess the impact of the BP lowering effect on heart function, S and congenic S.SHR(9)x8Ax11A animals were studied by echocardiography. The left-ventricular end-diastolic area, end-systolic area, posterior wall thickness and antero-septal wall thickness were significantly lower in the S.SHR(9)x8Ax11A compared with S (Table 3). These data corroborate the relative heart weight data of S.SHR(9)x8Ax11A (Table 1) and are reminiscent of cardiac hypertrophic effects driven by the observed differences in BP. Further, the Kaplan–Meier plot shown in Figure 5 suggests that the congenic strain S.SHR(9)x8Ax11A survived significantly longer than that of the S. The median survival of the S was 108 days, while that of the congenic strain S.SHR(9)x8Ax11A was 146 days ($P < 0.02$ by Kaplan–Meier analysis).

Sequencing of positional candidate genes

The 2.05 Mb congenic interval of S.SHR(9)x8Ax11A contained one protein coding gene, *Tmeff2* – transmembrane protein with EGF-like and two follistatin-like domains. The other predicted annotations

included *LOC685674*, one exon of the gene *Sdpr* coding for serum-deprivation response protein, a pseudogene with no known protein product, two unknown predicted genes and five genes for small nuclear RNAs. The exons for *Sdpr*, *Tmeff2*, *LOC685674* and a neighboring gene (*Ofc2a*) were sequenced, revealing no coding sequence variations. Note that the neighboring gene sequence of *Ofc2a* was completed before the detection of the SNP in the 3'-UTR of *Sdpr*, which delimits the top of the congenic segment of S.SHR(9)x8Ax11A (Figure 1). Detection of this SNP ruled out *Ofc2a* as a positional candidate within S.SHR(9)x8Ax11A.

Characterization of the rat *Tmeff2* transcripts

We next focused on differential expression of *Tmeff2*, the only known complete predicted protein-coding gene within the 2.05 Mb critical

Figure 3 Schematic representation of the second set of newly developed congenic substrains. Congenic substrains were developed from S.SHR(9)x10A as described under Methods. The physical map of rat chromosome 9 is shown on the left with locations of microsatellite markers in base pairs shown within parenthesis. *The S.SHR(9)x10A congenic strain was previously reported and is shown here for reference. The solid bars represent SHR introgressed segments of the congenic strains. Open boxes flanking the solid bars are where recombination's occurred. The blood pressure (BP) data obtained from these congenic substrains are provided in Table 1. Black bars indicate congenic strains with BP significantly lower than that of the S. Gray bars indicate congenic strains with no BP effect.

Figure 4 Radiotelemetry measures on (a) systolic BP, (b) diastolic BP (c) heart rate S and S.SHR(9)x10Ax1. Rats ($n = 6$ per group) were surgically implanted with C40 blood pressure radiotelemetry transmitters as described under Methods. Data points are 4-hour moving averages $\pm$ s.e.m. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, ns, not significant.

Table 3 Echocardiography of S and S.SHR(9)X8AX11A Congenic rats

Strain	N	EDA, cm ²	ESA, cm ²	PWT, cm	SWT, cm	RWT
S	6	0.62 ± 0.11	0.25 ± 0.07	0.29 ± 0.03	0.26 ± 0.02	1.07 ± 0.34
S.SHR(9)x8Ax11A	7	0.88 ± 0.06 [†]	0.44 ± 0.05 [†]	0.24 ± 0.01 [†]	0.20 ± 0.01 [†]	0.62 ± 0.03

Abbreviations: EDA, end-diastolic area; ESA, end-systolic area; LV, left ventricular; N, Number of samples; PWT, posterior wall thickness; RWT, relative wall thickness; SWT, antero-septal wall thickness.

Data are presented as the mean ± s.e.m. [†]*P* < 0.05 vs. S rats.

Figure 5 Comparison of survival times of S vs. S.SHR(9)x8Ax11A. Rats (*n*=8 per group) were studied for survival as described under Methods. **P* < 0.02 by the Kaplan–Meier analysis.

interval. The Ensembl database (www.ensembl.org) predicted three transcripts of *Tmeff2* (Figure 6a). Primers were designed to amplify segments that are unique to each of these transcripts (Figure 6a). PCR reactions with cDNA isolated from the heart resulted in products for only two out of these three predicted transcripts (Figure 6b). Similar data were obtained from kidney samples also (data not shown). The 139bp and 118bp PCR products (Figure 6b) were confirmed to belong to *Tmeff2* by cDNA sequencing. To quantitate these transcripts, RNA samples from S and S.SHR(9)x8Ax11A were assessed by real-time PCR. The mRNA levels of the full-length transcript ENSRNOT00000066231 were highly expressed in the brain, but were not differential between S and S.SHR(9)x8Ax11A. However, both in the hearts and kidneys, the *Tmeff2* transcript, ENSRNOT00000066231, was significantly higher in the S compared with S.SHR(9)x8Ax11A (Figure 6c). To ascertain the 5′-end prediction of ENSRNOT00000066231, a 5′-RACE experiment was conducted. Amplification and sequencing of the 585bp 5′-RACE PCR product shown in Figure 6d confirmed that the start-site of this transcript was correct as predicted on the Ensembl website (www.ensembl.org). Further, the protein product of the *Tmeff2* transcript ENSRNOT00000066231 was significantly higher only in the hearts of S compared with S.SHR(9)x8Ax11A (Figures 6e and f).

DISCUSSION

Using congenic strains introgressing SHR alleles onto the S genetic background we previously identified two BP QTLs on RNO9.²⁸ Mapping studies where differential congenic segments were used to determine the location of BP QTLs are increasingly recognized as not being ideal.^{13,33} Therefore, to further resolve the two BP QTLs, we developed two new iterations of congenic substrains for each of the BP QTLs and interpreted the location of the QTLs as the introgressed regions within the shortest congenic strains with BP effects. Accordingly, two distinct short genomic segments of 2.05 and 6.14 Mb on

RNO9 were resolved as BP QTL containing congenic segments. The 6.14Mb segment contained 39 annotations and requires further genetic dissection. Therefore, the current study was further focused on the shorter of the two segments, that is, 2.05 Mb.

The single known and complete predicted protein-coding gene retained within the congenic segment was experimentally verified as a rat gene coding for at least two alternate transcripts of Tomoregulin-2, also known as a transmembrane glycoprotein with EGF-like and two Follistatin-like domains 2 (*Tmeff2*). *Tmeff2* is predominantly expressed in the brain, but the levels of *Tmeff2* expression were not different between the brains of S and the congenic strain S.SHR(9)x8Ax11A, which has SHR alleles at *Tmeff2*. *Tmeff2* was also expressed in other tissues relevant to BP control such as the kidney and heart. The mRNA of the longest transcript of *Tmeff2* was upregulated in hearts of the S rat, which demonstrated increased cardiac hypertrophy compared with the congenic strain carrying SHR alleles of *Tmeff2*. The protein product of this long transcript of *Tmeff2* was also higher only in the heart samples of S compared with the congenic strain. These results suggest that decreasing the expression of *Tmeff2* protein in the S rat by introgression of SHR alleles of a genomic segment containing *Tmeff2* is associated with lowering of BP of the S.

Tmeff2 has additional aliases, *TENB2*, *HPPI1*, *TR*, *TPEF* and *CT120.2*, and is a known epigenetic factor modulating cancer-related phenotypes.^{34–36} It is also recognized as a trophic factor for dopaminergic mesencephalic neurons.³⁷ Human TMEFF2 contributes to cell proliferation in an ADAM17-dependent autocrine fashion in cells expressing this protein.³⁸ Perhaps related to this observation, a recent genome-wide association study indicates that intronic single-nucleotide polymorphisms of *TMEFF2* are associated with body mass index in humans.³⁹ However, beyond the demonstrated alterations in expression of the transcript of *Tmeff2* in tissues other than the brain, information regarding the function of *Tmeff2* is limited. TMEFF2 contains two follistatin-like domains that directly interact with TGF-β and regulate related growth factor signaling;³⁶ it is possible that the increased TMEFF2 levels may allow for increased interaction with TGF-β leading to alterations in fibrosis. Lack of a proteinuria effect in the congenic strain, S.SHR(9)x8Ax11A, containing SHR alleles at *Tmeff2* suggests that the functional effect of *Tmeff2* related to BP regulation may be elicited from tissues other than the kidney. Significant decreases in left-ventricular diastolic and systolic area were observed in the S rats with higher levels of both mRNA and protein expression of *Tmeff2* compared with the congenic with SHR alleles of *Tmeff2*. Therefore, the heart could be further investigated as one of the potential sites from where the functional effects of *Tmeff2* contribute to BP regulation. The translational significance of our work is that it is similar to the observation that the left ventricles of patients from multiple cohorts with hypertrophic cardiomyopathy had a 2.05 fold increase in mRNA of *TMEFF2* compared with healthy individuals (<http://www.ncbi.nlm.nih.gov/geo/>; Geo Accession Number: GSE1145). The causal genetic element responsible for the differential expression of *Tmeff2* remains to be explored in both rats and in

Figure 6 Transcript and protein expression analysis of *Tmeff2* (Tomoregulin-2) in S and S.SHR(9)x8Ax11A. The schematics of the three predicted transcripts of *Tmeff2* from the Ensembl database (www.ensembl.org) are shown in Panel (a). Open white boxes are 5' and 3' untranslated regions. Thick vertical bars are exons. ENS numbers are transcript numbers assigned by the Ensembl database. GSP, location of gene-specific primer used for 5'RACE experiment; RT-F1 and RT-R1, locations of primers designed to specifically amplify the full-length predicted transcript ENSRNOT00000066231. Expected product size=118bp (b). RT-F2 and RT-R2, locations of primers designed to amplify the second predicted transcript ENSRNOT00000035114. No product amplified (b); RT-F3 and RT-R3, locations of primers designed to amplify the third predicted transcript ENSRNOT00000022312. Expected product size=139bp (b). (c) is the real-time PCR data from S vs. S.SHR(9)x8Ax11A congenic substrain. mRNA obtained from heart and kidney samples were converted to cDNA and amplified using primers RT-F1 and RT-R1 shown in panel (a). Values are fold change in congenic relative to S ± s.e.m. ***P<0.001; (d) 5' RACE analysis. The GSP primer represented in panel (a) was used in the 5' RACE experiment detailed under Methods. Expected product size of 585bp was obtained with the ENSRNOT00000066231 transcript of *Tmeff2*. Expected product size of 247bp was obtained with GAPDH used as the positive control. (e) Western blot analysis of heart samples from S and S.SHR(9)x8Ax11A. S1 and S2 are two different S rats. C1 and C2 are two different congenic rats. (f) Quantitation of the blots shown in panel (e); values are fold change in congenic relative to S ± s.e.m. **P<0.01.

humans. These elements could be allelic variants within regulatory regions including non-coding RNA/s within the congenic interval. Nevertheless, the observation that differential expression of *Tmeff2* in the heart is linked to hypertension and cardiac hypertrophy is novel.

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