Online Supplement

Female-specific hypertension loci on rat chromosome 13

Matthew J. Hoffman[1,3*], Michael J. Flister[1,3*], Lizbeth Nunez[1,3], Bing Xiao[1,3], Andrew S. Greene[2,3], Howard J. Jacob[1,3,4], Carol Moreno[1,3]

[1]Human and Molecular Genetics Center; [2]Biotechnology and Bioengineering Center; Departments of [3]Physiology and [4]Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin

*M.J. Hoffman and M.J. Flister contributed equally to this manuscript.

Expanded Materials and Methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (MCW). The 3.7 Mb congenic line 9 [SS.BN-(D13Hmgc41-D13Rat101)/Mcwi)] was derived from the SS-13BN/Mcwi consomic by marker-assisted breeding with SS/JrHsD/Mcwi, as described previously.[1] Line 9 has also previously been referred to as Ren1-BN[2] and line 13D.[3] To generate the smaller congenics, line 9 was backcrossed to the parental SS/JrHsD/Mcwi to generate the line 9C [SS.BN(D13Rat124-D13Rat101)/Mcwi], as reported elsewhere.[4] Line 9C has also been previously referred to as line $13D_C$.[3] The line 9C was subsequently backcrossed to the parental SS/JrHsD/Mcwi to generate the line 9E [SS.BN(D13Rat25-D13rs106935835)/Mcwi] and line 9F [SS.BN(D13Rat25-D13rs198199323)/Mcwi] congenic strains. Followed each backcross, the F1 progeny and F2 generations were intercrossed to capture different portions of different regions of the line 9 congenic interval by marker-assisted selection.

Blood Pressure Measurement

Experiments were performed on conscious 9-week old male and female rats. Rats were anesthetized with isoflurane and a gel-filled catheter attached to a blood pressure transmitter (TA11PA-C40, Data Sciences International, St. Paul, MN) was implanted into the femoral artery for continuous BP measurement. After 5 days of recovery, mean arterial pressure (MAP) was measured from 9am-1pm for 3 consecutive days, at 500Hz for 10-second intervals, every 2 minutes, and averaged. The rats were then switched to a high salt diet (8% NaCl, AIN-76, Dyets) and blood pressure was measured again after 21 days of 8% NaCl diet.

Measurement of Albumin Excretion

After 16 days of 8% NaCl diet, rats were acclimated in metabolic cages (40615, Lab Products) for 24 hours, followed by a 24-hour urine collection. Urine samples were cleared of insoluble particulate by centrifugation at 2,800xG and total albumin was assessed using an Albumin Blue 580 assay (Molecular Probes, Eugene, OR).

RT-qPCR

RNA was extracted by Trizol (Life Technologies, Carlsbad, CA) from the renal cortex and medulla of 9-week old male and female SS and $SS\text{-}13^{BN}$ congenic rats fed 0.4% NaCl (low salt) or 8% NaCl (high salt) diets for 7 days (n=4-6 per group). cDNA was synthesized from 4µg of RNA using a RevertAid First Strand cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada). Primers for RT-qPCR were designed against rat CDS found in the NCBI database. Primer sequences were designed using the OligoPerfect Designer website (http://tools.invitrogen.com/content.cfm?pageid=9716) and validated for specificity and primer efficiency. All primers (listed in Table S1) were purchased as annealed oligos from Life Technologies (Carlsbad, CA). RT-qPCR was performed using GoTaq qPCR Master Mix (Promega, Madison, WI) and an ABI HT7900 Real-Time machine (Applied BioSystems, Foster City, CA). Data were normalized to GAPDH and relative mRNA expression was determined using the ΔΔCt method as described previously.[5]

Inflammation and Fibrosis RT-qPCR Array

Four micrograms of medullary RNA was pooled within each strain (line 9E, line 9F, and SS) on low salt diet (0.4% NaCl) and high salt diet (8% NaCl) (n = 4-6 rats per strain) was synthesized using a RevertAid First Strand cDNA synthesis kit, according to the manufacturer's protocol (Fermentas, Burlington, Ontario, Canada). Inflammatory and fibrotic gene expression was examined using a rat inflammatory cytokines and receptors RT2 Profiler PCR Array, according to the manufacturer's protocol (PARN-120ZE-4, SABiosciences, Fredrick, MD). Target gene expression was normalized to a combination of 5 housekeeping genes: *Rplp1, Hprt1, Rpl13a, Ldha, and Actb*. Relative changes in mRNA expression in line 9E, line 9F, and SS on high salt diet were compared to the low salt values from each respective group. Fold changes were determined using the ΔΔCt, method as described previously.[5]

Sequence Analysis

Genomic DNA sequence of BN (rn4 assembly) and SS/JrHsD/Mcwi were accessed from the RGD website (Full details of gDNA library preparation, sequencing, and analysis were described elsewhere).[6] Putative microRNA target sites in the 3'-untranslated regions of known genes were identified by TargetScan[7] (http://www.targetscan.org/). The consequences of sequence variants were analyzed by variant effect predictor[8] (www.ensembl.org/info/docs/variation/vep/) and Polyphen2[9] (http://genetics.bwh.harvard.edu/pph2/). Consensus transcription factor binding sites were predicted using TRANSFAC (http://www.gene-regulation.com/pub/databases.html) and MatInspector (http://www.genomatix.de/).

References

1. Moreno C, Kaldunski ML, Wang T, Roman RJ, Greene AS, Lazar J, Jacob HJ, Cowley AW, Jr. Multiple Blood Pressure Loci on Rat Chromosome 13 Attenuate Development of Hypertension in the Dahl S Hypertensive Rat. *Physiol Genomics*. 2007;31:228-235.
2. Durand MJ, Moreno C, Greene AS, Lombard JH. Impaired Relaxation of Cerebral Arteries in the Absence of Elevated Salt Intake in Normotensive Congenic Rats Carrying the Dahl Salt-Sensitive Renin Gene. *Am J Physiol Heart Circ Physiol*. 2010;299:H1865-1874.
3. Stodola TJ, de Resende MM, Sarkis AB, Didier DN, Jacob HJ, Huebner N, Hummel O, Saar K, Moreno C, Greene AS. Characterization of the Genomic Structure and Function of Regions Influencing Renin and Angiogenesis in the Ss Rat. *Physiol Genomics*. 2011;43:808-817.
4. Flister MJ, Hoffman MJ, Reddy P, Jacob HJ, Moreno C. Congenic Mapping and Sequence Analysis of the Renin Locus. *Hypertension*. 2013;61:850-856.
5. Schmittgen TD, Livak KJ. Analyzing Real-Time Pcr Data by the Comparative C(T) Method. *Nature protocols*. 2008;3:1101-1108.
6. Flister MJ, Prisco SZ, Sarkis AB, O'Meara CC, Hoffman M, Wendt-Andrae J, Moreno C, Lazar J, Jacob HJ. Identification of Hypertension Susceptibility Loci on Rat Chromosome 12. *Hypertension*. 2012;60:942-948.
7. Friedman RC, Farh KK, Burge CB, Bartel DP. Most Mammalian Mrnas Are Conserved Targets of Microrna*s*. *Genome Res*. 2009;19:92-105.
8. McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the Consequences of Genomic Variants with the Ensembl Api and Snp Effect Predictor. *Bioinformatics*. 2010;26:2069-2070.
9. Ramensky V, Bork P, Sunyaev S. Human Non-Synonymous Snps: Server and Survey. *Nucleic acids research*. 2002;30:3894-3900.

Table S1. RT-qPCR primer sequences.

Name	Primer Sequences
Fmod	
Sense	5'-ACGTCTACACCGTCCCTGAC-3'
Antisense	5'-GTGCAGAAACTGCTGATGGA-3'
Optc	
Sense	5'-GCAACAGAGGAGTGCTCCAG-3'
Antisense	5'-GTAGCATTCCCGTGGACAGT-3'
Prelp	
Sense	5'-CAGCTTCCAGGGAGACAAAG-3'
Antisense	5'-CAGCAGGACACGACAAAGAA-3'
Btg2	
Sense	5'-TTAAATCTGTTCCTCACTGCCCG-3'
Antisense	5'-TGCTCAACAACAGTCCAGCTCTGTG-3'
Renin	
Sense	5'-GGTGCCCTCCACCAAGTGT-3'
Antisense	5'-GCTAGAGGATTCCGAGGAGTC-3'

Table S2. Predicted transcription factor binding sites 5 Kb upstream of *Optc* , *Prelp* , and *Fmod*

Position	BN	SS	Downstream Gene	Predicted transcription fractor binding
46,858,389	A	G	Optc	
46,859,135	C	G	Optc	CP2
46,860,161	A	G	Optc	Gata1
46,860,237	A	G	Optc	
46,860,931	T	A	Optc	Cdxa,Oct-1
46,861,406	A	C	Optc	N-Myc, Gata X, Usf
46,862,340	T	C	Optc	
46,863,030	T	C	Optc	
46,875,266	G	C	Prelp	Gata1
46,875,597	A	G	Prelp	
46,877,106	G	A	Prelp	Brn-2
46,983,892	G	T	Fmod	
46,983,893	A	C	Fmod	
46,984,626	A	G	Fmod	
46,984,719	C	T	Fmod	
46,984,884	G	T	Fmod	Gata1
46,984,886	T	A	Fmod	Gata1
46,984,966	A	G	Fmod	
46,984,967	T	C	Fmod	
46,985,551	A	G	Fmod	
46,986,451	A	G	Fmod	CdxA and TATA box
46,987,190	A	G	Fmod	
46,987,233	T	C	Fmod	Ik-2
46,987,580	T	C	Fmod	

Table S3. Predicted transcription factor binding sites for the *Btg2* region

Position	BN	SS	VEP Prediction	Predicted transcription fractor binding
47,010,821	C	T	Intergenic	
47,011,033	T	G	Intergenic	
47,011,253	A	G	Intergenic	Mzf1
47,013,668	A	G	Intergenic	Mzf2
47,014,824	C	G	Intergenic	
47,015,944	C	T	Intergenic	
47,016,463	C	T	Intergenic	Gata-3
47,019,947	G	T	Intergenic	CdxA
47,020,509	G	A	Intergenic	Gata-1, Gata-2
47,020,558	A	C	Intergenic	CdxA, Nkx-2
47,023,437	A	G	Downstream	
47,025,793	G	T	Downstream	Aml1a, ERRα
47,026,156	C	T	Downstream	vMyb
47,027,822	A	G	UTR3	
47,028,011	G	A	UTR3	
47,029,505	T	C	Intronic	
47,030,784	A	G	upstream	
47,031,810	T	C	upstream	Sry

Table S4. Renin expression in the renal cortex.

Gene Groups	LS	7 days HS
Line 9F (male)	1.0 ± 0.1	0.3 ± 0.1†
Line 9E (male)	0.7 ± 0.1*	0.2 ± 0.1†
Line 9F(female)	1.1 ± 0.3	0.3 ± 0.1†
Line 9E (female)	1.1 ± 0.2	0.4 ± 0.1†

*Statisticallly significant between strains

†Stistically significant within strains

Table S5. Fold-change in gene expression after 7 days of HS diet

		Male			Female		
Gene Symbol	**Gene Name**	**SS**	**Line 9E**	**Line 9F**	**SS**	**Line 9E**	**Line 9F**
Acta2	Smooth muscle alpha-actin	2.7	3.6	4.3	7.7	2.0	1.2
Agt	Angiotensinogen	-1.6	-1.1	-3.0	-1.1	1.2	-7.7
Akt1	V-akt murine thymoma viral oncogene homolog 1	-1.3	-1.3	-1.1	1.8	1.7	1.2
Bcl2	B-cell CLL/lymphoma 2	-1.3	1.3	-1.3	-1.4	1.1	-2.8
Bmp7	Bone morphogenetic protein 7	-1.3	-2.4	-2.5	-1.2	1.1	-2.3
Cav1	Caveolin 1, caveolae protein	-1.0	-1.3	1.0	-1.1	1.3	-1.4
Ccl11	Chemokine (C-C motif) ligand 11	1.8	-1.5	-2.4	-1.1	1.1	-9.8
Ccl12	Chemokine (C-C motif) ligand 12	-1.5	-1.0	1.1	2.9	1.7	-1.7
Ccl3	Chemokine (C-C motif) ligand 3	1.2	-1.8	-1.2	1.5	1.2	-1.1
Ccr2	Chemokine (C-C motif) receptor 2	1.5	3.1	1.7	1.5	1.1	18.6
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	1.0	1.4	1.2	1.2	1.4	-2.6
Col1a2	Collagen, type I, alpha 2	1.6	1.0	3.3	2.0	1.2	1.5
Col3a1	Collagen, type III, alpha 1	1.2	1.4	1.9	2.6	1.2	-1.3
Ctgf	Connective tissue growth factor	-1.1	-1.6	1.2	1.2	1.5	1.0
Cxcr4	Chemokine (C-X-C motif) receptor 4	1.4	2.6	1.6	1.1	1.1	1.1
Dcn	Decorin	-1.2	1.3	-1.1	1.1	1.2	-1.5
Edn1	Endothelin 1	1.0	-1.6	-1.3	1.3	1.3	-1.8
Egf	Epidermal growth factor	-2.1	-2.3	-2.0	-2.4	1.1	-1.7
Eng	Endoglin	-1.5	-1.9	-1.6	-1.1	1.2	-2.4
Faslg	Fas ligand (TNF superfamily, member 6)	-1.2	1.1	-1.0	-1.5	2.0	-2.8
Grem1	Gremlin 1	1.1	1.4	-1.3	1.1	2.4	-1.0
Hgf	Hepatocyte growth factor	-1.2	2.6	1.0	-1.0	-1.3	-1.4
Hprt1	Hypoxanthine phosphoribosyltransferase 1	-1.0	1.1	1.3	-1.0	1.3	5.6
Ifng	Interferon gamma	1.3	-1.0	-2.4	-1.5	2.2	-9.7
Il10	Interleukin 10	2.0	1.1	-1.5	1.4	3.1	-2.8
Il13	Interleukin 13	1.8	1.2	-2.3	-1.1	1.0	-10.2
Il13ra2	Interleukin 13 receptor, alpha 2	1.3	-1.3	-4.8	-2.1	3.8	-21.9
Il1a	Interleukin 1 alpha	1.2	-1.6	-1.6	1.3	N/A[†]	-25.4
Il1b	Interleukin 1 beta	-1.0	1.7	-1.1	1.3	1.4	-1.4
Il4	Interleukin 4	1.2	-1.7	-2.8	-3.3	3.4	-18.6
Il5	Interleukin 5	1.3	-1.7	-3.9	-1.4	-1.2	-18.8
Ilk	Integrin-linked kinase	-1.3	-1.4	-1.3	-1.2	-1.0	-1.3
Inhbe	Inhibin beta E	1.5	-1.0	-4.2	-1.3	3.4	-14.9
Itga1	Integrin, alpha 1	-1.3	-1.0	-1.0	-1.1	1.2	-1.3
Itga2	Integrin, alpha 2	1.1	1.1	-1.3	1.0	1.1	-2.0
Itga3	Integrin, alpha 3	-1.2	1.1	1.0	1.0	1.3	-1.2
Itgav	Integrin, alpha V	-1.2	-1.7	-1.2	-1.3	1.2	-2.3
Itgb1	Integrin, beta 1	-1.2	1.2	1.1	1.1	1.1	1.6
Itgb3	Integrin, beta 3	1.3	-1.1	1.1	-1.0	1.4	-1.6
Itgb5	Integrin, beta 5	-1.4	-1.4	-1.3	-1.6	1.1	-1.2
Itgb6	Integrin, beta 6	-1.3	-1.7	-1.6	-1.4	1.2	-1.6
Itgb8	Integrin, beta 8	-1.1	1.8	-1.0	-1.4	1.3	-2.1
Jun	Jun oncogene	-1.0	1.4	1.2	-1.5	1.8	-1.6
Ldha	Lactate dehydrogenase A	1.1	1.1	-1.2	-1.0	1.3	-1.1
Lox	Lysyl oxidase	1.4	2.1	2.0	1.9	1.4	1.2
Ltbp1	Latent transforming growth factor beta binding protein 1	1.1	-1.0	-1.2	1.2	-1.1	1.3
Mmp13	Matrix metallopeptidase 13	1.7	-1.3	-6.3	-1.7	4.8	-21.7
Mmp14	Matrix metallopeptidase 14	1.7	-1.0	1.1	1.0	1.0	-1.6
Mmp1a	Matrix metallopeptidase 1a	1.2	1.1	-13.3	-7.6	4.4	-61.5
Mmp2	Matrix metallopeptidase 2	1.0	1.6	1.1	1.3	1.2	1.1
Mmp3	Matrix metallopeptidase 3	-1.2	-1.6	-5.2	-1.4	2.5	-24.1
Mmp8	Matrix metallopeptidase 8	1.1	-1.1	-4.0	-3.1	3.0	-21.5
Mmp9	Matrix metallopeptidase 9	1.7	1.4	-2.1	1.7	1.5	-3.2
Myc	Myelocytomatosis oncogene	1.6	1.9	3.3	1.6	1.3	-1.4
Nfkb1	Nuclear factor of kappa B1	-1.2	1.2	-1.0	-1.1	1.3	-1.2
Pdgfa	Platelet-derived growth factor alpha polypeptide	-1.1	-1.2	-1.3	-2.2	1.1	1.0
Pdgfb	Platelet-derived growth factor beta polypeptide	-1.6	1.1	-1.6	-1.4	-1.1	-1.6
Plat	Plasminogen activator, tissue	-1.1	-1.2	1.0	-1.3	1.3	-1.6
Plau	Plasminogen activator, urokinase	-1.2	-1.3	-1.1	-1.4	1.1	-1.1
Plg	Plasminogen	1.1	1.4	-2.3	-1.1	5.6	-12.1
Rpl13a	Ribosomal protein L13A	-1.1	1.2	-1.2	-1.2	1.0	-3.2
Rplp1	Ribosomal protein, large, P1	-1.1	-1.1	1.1	1.1	-1.7	1.7
Serpina1	Serpin peptidase inhibitor A1	2.0	-1.1	-2.9	-1.1	1.7	-5.3
Serpine1	Serpin peptidase inhibitor E1	1.5	1.4	1.9	1.6	1.9	-1.1
Serpinh1	Serine (or cysteine) peptidase inhibitor H1	-1.0	-1.2	1.2	1.2	1.4	-1.0
Smad2	SMAD family member 2	-1.2	1.0	-1.1	-1.1	1.1	-1.3
Smad3	SMAD family member 3	-1.2	-1.1	-1.2	-1.2	1.2	-1.2
Smad4	SMAD family member 4	-1.4	-1.4	-1.2	-1.4	-1.1	-1.6
Smad6	SMAD family member 6	-1.3	-1.1	-1.6	-1.3	-1.0	-1.3
Smad7	SMAD family member 7	-1.1	-1.2	-1.4	-1.9	1.0	-1.9
Snai1	Snail homolog 1 (Drosophila)	-1.6	1.4	-1.2	-1.0	1.4	-1.6
Sp1	Sp1 transcription factor	1.1	-1.5	-1.2	-1.7	1.1	-1.5
Stat1	Signal transducer and activator of transcription 1	1.1	-1.4	-1.1	-1.4	1.2	-1.3
Stat6	Signal transducer and activator of transcription 6	-1.1	1.2	-1.1	-1.1	1.3	-1.7
Tgfb1	Transforming growth factor, beta 1	-1.1	1.2	1.1	1.0	1.4	-1.8
Tgfb2	Transforming growth factor, beta 2	-1.1	1.4	1.2	1.1	1.0	-1.0
Tgfb3	Transforming growth factor, beta 3	1.2	-1.1	1.4	1.8	3.1	-1.5
Tgfbr1	Transforming growth factor, beta receptor 1	-1.2	1.4	1.1	-1.2	1.2	-2.3
Tgfbr2	Transforming growth factor, beta receptor II	-1.3	-1.1	-1.3	-1.2	1.5	-1.6
Tgif1	TGFB-induced factor homeobox 1	1.2	1.3	1.4	1.0	1.6	-1.3
Thbs1	Thrombospondin 1	-1.2	-1.5	1.0	1.3	1.3	-1.7
Thbs2	Thrombospondin 2	1.1	-1.4	1.1	1.1	1.3	-1.6
Timp1	TIMP metallopeptidase inhibitor 1	1.9	1.0	2.4	1.8	1.4	-1.1
Timp2	TIMP metallopeptidase inhibitor 2	-1.1	-1.2	1.1	1.0	1.6	-1.6
Timp3	TIMP metallopeptidase inhibitor 3	-1.5	-1.7	-1.9	-1.7	1.0	-1.8
Timp4	Tissue inhibitor of metalloproteinase 4	-1.0	-1.5	-1.3	-1.8	1.2	-2.6
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	-1.1	1.1	-1.5	-1.4	-1.1	-2.3
Vegfa	Vascular endothelial growth factor A	-1.2	-1.2	-1.6	-1.6	1.2	-2.1

Data is presented as fold expression after 7 days high salt diet (8% NaCl) compared with low salt diet (0.4% NaCl).

[†]Gene expression was not detected.

Figure S1. Time course of the development of hypertension in the SS, line 9, line 9E, and line 9F rats fed low salt (0.4% NaCl) or high salt (8% NaCl) diets for 21 days (group sizes are given in Figure 1). Mean arterial pressure (MAP) was recorded by radiotelemetry in male (**A**) and female (**B**) rats, as described in the methods. MAP at 21 day of high salt diet is also reported in Figure 1. Data are presented as MAP ± SEM. *P*-values are determined by a 1-way ANOVA followed by a Holm-Sidak post hoc test. For (**B**), **P*<0.05, line 9 vs. SS; †*P*<0.05, line 9 and line 9F vs. line 9E and SS; ‡*P*<0.001, line 9 and line 9F vs. line 9E and SS.