

Positional identification of variants of *Adamts16* linked to inherited hypertension

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A previously reported blood pressure (BP) quantitative trait locus on rat Chromosome 1 was isolated in a short congenic segment spanning 804.6 kb. The 804.6 kb region contained only two genes, LOC306664 and LOC306665. LOC306664 is predicted to translate into A Disintegrin-like and Metalloproteinase with Thrombospondin Motifs-16 (*Adamts16*). LOC306665 is a novel gene. All predicted exons of both LOC306664 and LOC306665 were sequenced. Non-synonymous variants were identified in only one of these genes, LOC306664. These variants were naturally existing polymorphisms among inbred, outbred and wild rats. The full-length rat transcript of *Adamts16* was detected in multiple tissues. Similar to *ADAMTS16* in humans, expression of *Adamts16* was prominent in the kidney. Renal transcriptome analysis suggested that a network of genes related to BP was differential between congenic and S rats. These genes were also differentially expressed between kidney cell lines with or without knock-down of *Adamts16*. *Adamts16* is conserved between rats and humans. It is a candidate gene within the homologous region on human Chromosome 5, which is linked to systolic and diastolic BP in the Quebec Family Study. Multiple variants, including an Ala to Pro variant in codon 90 (rs2086310) of human *ADAMTS16*, were associated with human resting systolic BP (SBP). Replication study in GenNet confirmed the association of two variants of *ADAMTS16* with SBP, including rs2086310. Overall, our report represents a high resolution positional cloning and translational study for *Adamts16* as a candidate gene controlling BP.

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INTRODUCTION

Essential hypertension (EH), is a very common condition characterized by sustained high blood pressure (BP) with no identifiable cause (1). Genetic susceptibility factors account for ~30% of an individual's risk of developing EH (2). Mapping experiments in humans have successfully identified genetic determinants of rare, monogenic forms of hypertension (3–11), but few have successfully defined the major susceptibility factors for EH, which represents 95% of all forms of hypertension in humans. This void has a bearing on current clinical management of EH, which is limited to symptomatic treatment with select anti-hypertensive agents.

A genome-wide association study by The Wellcome Trust Case Control Consortium (WTCCC) using 2000 cases of EH and 3000 controls represents the single largest human association study for hypertension conducted to date, wherein six single-nucleotide polymorphisms (SNPs) were reported as being associated with hypertension (12). Comparative mapping revealed that the homologous locations of four out of these six human SNPs map within regions of the rat genome identified as BP quantitative trait loci (QTL) in our laboratory (Supplementary Material, Table S1). Notably, among the many strain comparisons that were used for mapping BP QTLs, all four regions are reported as BP QTL containing regions originally identified from a single linkage analysis between the hypertensive Dahl Salt-sensitive (S) rat and the Lewis (LEW) rat (13). Further, by combining transcriptional profiling with substitution mapping of S and LEW rats, we prioritized *Nr2f2* as a positional candidate locus for BP control (14), an observation that was corroborated recently in humans through a haplotypic analysis of the WTCCC data (15). These observations support positional identification of additional rat BP loci previously detected through the genetic analysis of S and LEW rats.

In this report, we focus on the fine-mapping of one such BP QTL (BP QTL2 on rat Chromosome 1) detected using S and LEW rats (16). This BP QTL in rats is homologous to a BP QTL detected on human Chromosome 5 in the Quebec Family Study (QFS) (17).

Deriving and testing minimal congenic strains is a time-consuming process. Several studies have inferred differential segments between two congenic strains with opposite effects as QTL containing regions (18–20). Further, multiple candidate genes with non-synonymous variants and/or differential expression patterns are observed within QTL intervals (21–23), which makes it difficult to pin-point the actual genetic determinant. In contrast to these scenarios, BP QTL2 region was isolated independently in a congenic strain. The congenic interval spanned 804.6 kb and contained only two expressed transcripts. Of these, DNA sequencing coupled with transcript structure and expression analysis identified *Adamts16* as a candidate gene harboring non-synonymous variants. *ADAMTS16* is a member of a family of 19 secreted metalloproteinases, whose function, in contrast to several other family members, is unknown (24–26). There has been very little prior characterization of *ADAMTS16*. It is highly expressed in the kidney compared with other tissues (24), but previous studies reported to date on *ADAMTS16* have

not dealt with the kidney (24,27). Here we report that systolic (SBP) and diastolic BP (DBP) were associated with SNPs of *ADAMTS16* in the QFS and in the GenNet study. This report therefore provides the basis for further investigation of *ADAMTS16* as a novel gene involved in BP regulation.

RESULTS

Fine-mapping the BP QTL

The BP QTL described in this report, also called BP QTL region 2 was previously fine-mapped to a 2.73 Mb region flanked by the microsatellite markers D1Rat211 and D1Rat12 (16). Figure 1 is a comprehensive representation of markers genotyped for linkage analysis and for the construction and characterization of congenic strains. The detailed maps of the congenic substrains S.LEW (D1Mco4x1x3B), S.LEW (D1Mco4x1x3Bx1) and S.LEW (D1Mco4x1x3Bx2) are shown in Figure 2. The measured BP of these congenic strains was significantly lower (19, 18 and 14 mmHg, respectively) than that of the hypertensive S rat as measured by the tail-cuff method (Table 1). The BP of one of these strains, S.LEW (D1Mco4x1x3Bx1) was additionally tested and corroborated by the telemetry method (Fig. 3). The BP of the congenic strain S.LEW (D1Mco4x1x3Bx1) was lower than that of the S at all times, with the maximum difference between the two groups observed during the first 4 h of the resting phase. The BP of the S at this phase was 216 ± 8.6 mmHg compared with that of the congenic strain 192 ± 5.6 mmHg (BP effect of 24 mmHg, $P = 0.03$). Therefore, the smallest congenic interval shared by the congenic strains, S.LEW (D1Mco4x1x3B), S.LEW (D1Mco4x1x3Bx1) and S.LEW (D1Mco4x1x3Bx2), i.e. a fragment of 804597 bp flanked by D1MUO1 and D1MUO28, was inferred as adequate to account for the effect of BP QTL2 (Fig. 1). Note that the entire 804597 bp critical region is represented as congenic intervals of two strains S.LEW (D1Mco4x1x3Bx1) and S.LEW (D1Mco4x1x3Bx2). The data shown in Figure 1 compare the results of substitution mapping with those obtained by genetic linkage analysis of the same region. The location of the QTL determined by congenic strain analysis is closer to one end of the LOD peak from linkage analysis.

Clarifying the location of the BP QTL on Chromosome 1

As we were in the process of mapping this BP QTL, the physical map location of the genomic segment from D1Rat211-D1Rat12 was reported in the rat genome assembly as located on rat Chromosome 17 (http://rgd.mcw.edu/tools/genes/genes_view.cgi?id=1310046; www.ensembl.org). As can be seen in Table 2, the physical locations of the relevant markers in and around the 2.73 Mb BP QTL2 regions are distributed between rat Chromosomes 1 and 17. Genotyping data from our laboratory shows that this fragment was incorrectly placed on the rat genome assembly. It is currently placed towards the p-terminus of the physical map of rat Chromosome 17, whereas, the correct location of the critical 2.73 Mb BP QTL2 flanked by

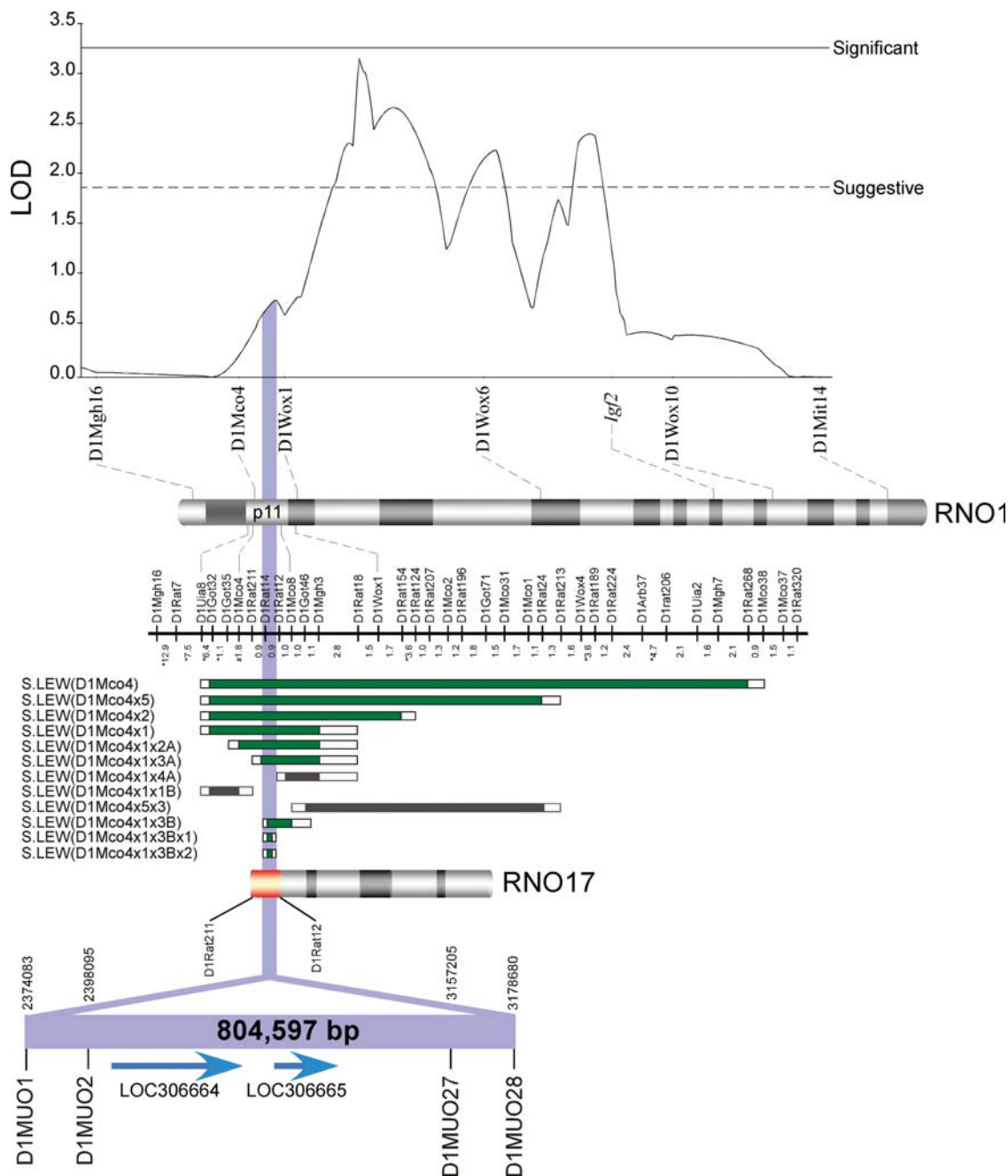


Figure 1. Comprehensive representation of mapping BP QTL2. LOD plot for BP using the F2 (S x LEW) population (13) is shown at the top followed by the congenic strains constructed to map the QTL to a 2.73 Mb region shown in red. Linkage map of Chromosome 1 is drawn to scale except for distances marked with an asterisk. The current location of this region on the physical map of the rat genome is between the markers shown on the cytogenetic map of rat chromosome 17 (Both Ensembl and NCBI databases). Based on our data, it should lie within p11 of Chromosome 1, which is also shown in the diagram (Corroborated by the Celera data on NCBI). All iterations of substitution mapping (16,32,47) are shown by congenic strains with BP lowering effect shown in green and the ones without the BP lowering effect shown in gray. White boxes around the congenic strain schematics represent regions containing the recombination breakpoint (crossover). The region highlighted in purple is the current fine-mapped interval of 804.6 kb. The only two predicted genes within the critical interval are indicated by blue arrows.

D1Rat211 and D1Rat12 should be between the markers D1Mco4 and D1Mco8 on rat Chromosome 1 (Fig. 1 and Table 2). In agreement with our results, the Celera assembly of the rat genome also indicated the location of the fragment from D1Rat211-D1Rat12 as being on Chromosome 1 and not on Chromosome 17.

Genomic composition of the 804.6 kb BP QTL containing critical region

As a logical step after mapping to a 804.6 kb region, the genomic content of this critical region of interest was examined and found to contain only two predicted genes,

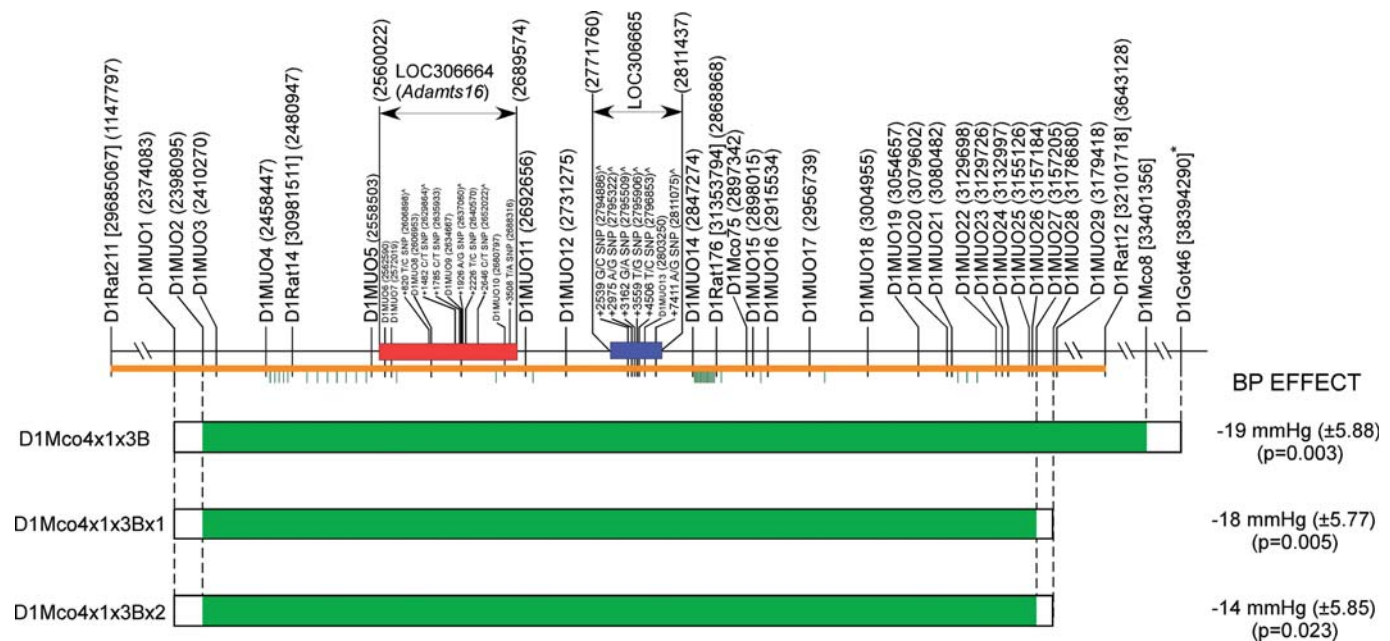


Figure 2. Congenic substrains used for locating the BP QTL. The relevant section of the physical map of RNO1 is shown on top of the Figure. Values in brackets next to the marker names indicate their physical locations in base pairs on RNO17 (NCBI). Values in parenthesis next to the marker names indicate their physical locations in base pairs on RNO17 (NCBI). Information on the new polymorphic markers are available at our website: <http://hsc.utledo.edu/depts/physpharm/rat/marker.html>. The previously mapped BP RNO1 QTL2 region is shown as the orange bar. Congenic strains are shown as solid colored bars flanked by open bars. Solid green bars illustrate the LEW segment introgressed onto the background of S. The open bars at the end of each introgressed segment represent the region of recombination. The BP effect is the BP of the congenic strain minus the BP of the control S. Independent *t*-tests were used to compare the means ($P < 0.05$). The red rectangle represents the limits of *Adamts16*, whereas the blue rectangle represents the limits of LOC306665. Green tabs represent the chromosomal location of SNPs polymorphic between S and LEW deposited in the Ensembl database (www.ensembl.org). ^ Novel polymorphic S and LEW SNPs identified by our study. *Marker location reported by NCBI.

Table 1. Phenotypic effect of the QTL

Congenic strain	Blood pressure (mmHg)		Effect	<i>P</i> -value	Heart weight (mg)		Effect	<i>P</i> -value
	S rat	Congenic			S rat	Congenic		
S.LEW (D1Mco4x1x3B)	207 (4.7)	188 (3.5)	-19	0.003	1321 (12.54)	1241 (11.86)	-80	0.0001
S.LEW (D1Mco4x1x3Bx1)	190 (5.4)	172 (1.9)	-18	0.005	1276 (17.25)	1201 (9.37)	-75	0.0001
S.LEW (D1Mco4x1x3Bx2)	190 (5.4)	176 (2.2)	-14	0.023	1276 (17.25)	1232 (17.05)	-44	0.078

SBP data reported is from tail cuff determinations. Effect=Congenic value - S value; Negative values indicate a lower value in the congenic strain compared with the S rat. Standard error of the means are in brackets; Number of rats in each group ranged from 18 to 30; all rats were males; In the S.LEW (D1Mco4x1x3B) versus S comparison, rats were fed a 2% NaCl diet for 24 days starting at 40-42 days of age. In the other experiments shown in the last two lines of the table, rats were maintained on a 0.3% NaCl diet and the same group of S rats were used as controls.

LOC306664, a gene annotated as A disintegrin-like metalloproteinase with thrombospondin motif 16 (*Adamts16*) (24) and LOC306665 (Figs 1 and 2). *Adamts16* was predicted to have 33 exons in earlier versions of the annotation of the rat genome at NCBI, but only 23 exons are retained in the later versions of the annotation. Nevertheless, we sequenced all 33 predicted exons of *Adamts16* from S and LEW and detected 7 polymorphisms as shown in Figure 4 (labeled as transcript locations 820, 1482, 1785, 1926, 2226, 2646 and 3508). Out of these, only two were non-synonymous variants that resulted in amino acid substitutions in the predicted mature polypeptide chain of *Adamts16* from Pro274 and Thr1170 in LEW rats to Ser274 and Ser1170 in S rats (Fig. 4). LOC306665 was also sequenced and no exonic non synonymous polymorphisms were detected.

Assessment of *Adamts16* polymorphisms in inbred, outbred and wild rats

Based on the analysis for all seven variants of *Adamts16* detected in this study, inbred strains can be classified into four different haplotypic groups (Supplementary Material, Table S2). Out of the 21 inbred strains tested, the non-synonymous SNPs (at +820 and +3508) of 8 strains were similar to the S and 10 strains had the LEW variants (Table 3). The remaining three strains had a combination of the S and LEW variants at +820 and +3508. The non-synonymous SNPs detected between S and LEW (at +820 and +3508) were additionally screened in other outbred and wild rats. Outbred Sprague-Dawley rats represent the progenitor group of rats from which the inbred S strain was

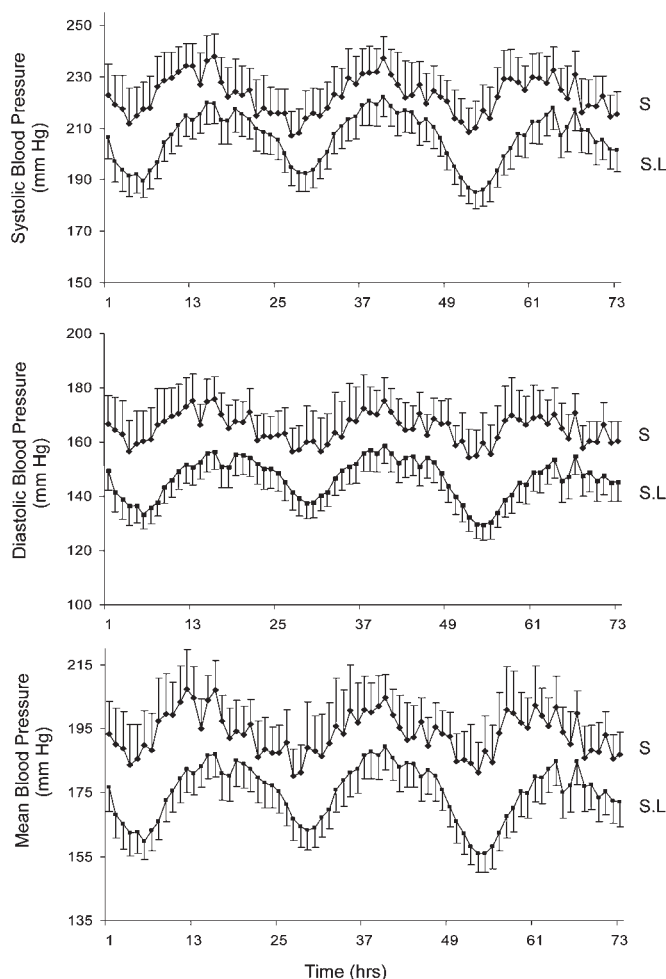


Figure 3. BP effect of the QTL region detected by radiotelemetry. Nine S rats (diamonds) and nine S.LEW (D1Mco4x1x3Bx1) congenic rats (squares) were surgically implanted with the large C40 telemetry transmitters, allowed to recover for a week and BP was recorded over a period of 4 days. The data plotted is obtained by telemetry recording once every 5 min continuously and averaged for 1 h intervals.

derived. Heterozygosity of non-synonymous variants was observed in both the outbred Sprague–Dawley and wild rats tested (data not shown).

In addition to the S versus LEW combination, there are three other strain combinations for which BP QTLs are reported through linkage analysis in the same region (Fig. 5). The three other strain comparisons, i.e. S versus BN, MNS versus MHS and SHR versus F344, all represent contrasting non-synonymous variants (at +820 and +3508) similar to the S versus LEW comparison. Thus, it is possible that S versus LEW is not the only strain combination with variants of *Adamts16* representing candidates for the quantitative trait of BP.

Predicted transcript sequence, tissue distribution and differential expression analysis of *Adamts16*

While attempting to translate the variants of *Adamts16* from the predicted transcript sequence from the NCBI website, we noticed that the Ensembl website predicted a longer transcript, which would potentially alter our inference on protein

Table 2. Physical map locations of microsatellite markers that are linked to RNO1 by genetic linkage analysis of F2 (S × LEW)

Microsatellite marker	Genomic location RNO17 (bp)	RNO1 (bp)
D1Rat7		15999443
D1Rat153		21229309
D1Rat121		21288065
D1Got32		26956572
D1Got33		27326922
D1Got35		28864486
D1Mco4		29841429
D1Rat211	1147797	
D1Rat239	1910351	
D1MUO1	2374083*	
D1MUO2	2398095*	
D1Rat14	2480947	
D1MUO24	3132997*	
D1MUO27	3157205*	
D1MUO28	3178680*	
D1Arb5	3472897	
D1Rat12	3643128	
D1Mco8		33401356
D1Arb4		34480420
D1Rat11		34504757
D1Got40		35839844
D1Got46		38394290
D1Rat18		43747375

Microsatellite markers are those that are placed in order on the genetic linkage map of F2 (S × LEW) (32). *Newly developed markers. Details of these new markers are available at our website: (<http://hsc.utoledo.edu/depts/physiology/research/rat/marker.html>). Additional markers on RNO1 are given in Figure 2. Start of the forward primer sequence on the physical map locations on Chromosome 1 and 17 are given. These were obtained from the rat genome sequence assembly 3.4 at the Ensembl website (www.ensembl.org).

translation. To test the validity of the transcript sequence prediction of *Adamts16* by the NCBI website, we sought to amplify the expressed rat transcript of *Adamts16* using several sets of primers encompassing each of the transcript sequence predictions (Fig. 6). We used these to amplify transcripts from mixed pools of reverse-transcribed RNA from S and LEW rats. Only one of the six primer combinations tested resulted in a transcribed product of a size between 3000 and 4000 bp (Fig. 6). The PCR-amplified samples from both S and LEW rats were cloned and sequence confirmed that the transcript of *Adamts16* as predicted by NCBI, consisted of 3666 bp (XM.225142). In concordance with expression data of *ADAMTS16* in humans (24), expression of *Adamts16* was prominent in the rat kidney, an organ most relevant to the physiology of BP. *Adamts16* was also detected in other tissues including the thymus, lung, heart, liver and spleen (Fig. 7). Real-time RT–PCR of kidney and heart samples revealed that *Adamts16* was not significantly differentially expressed in these organs between S and S.LEW (D1Mco4x1x3Bx1) (data not shown). Expression of the second candidate gene LOC306665 as determined by real-time RT–PCR was also not different between kidney and heart samples of S and S.LEW (D1Mco4x1x3Bx1) (data not shown).

The predicted protein sequence described in our study is translated from the validated transcript of *Adamts16*. The nascent polypeptide chain coding for *Adamts16* contains

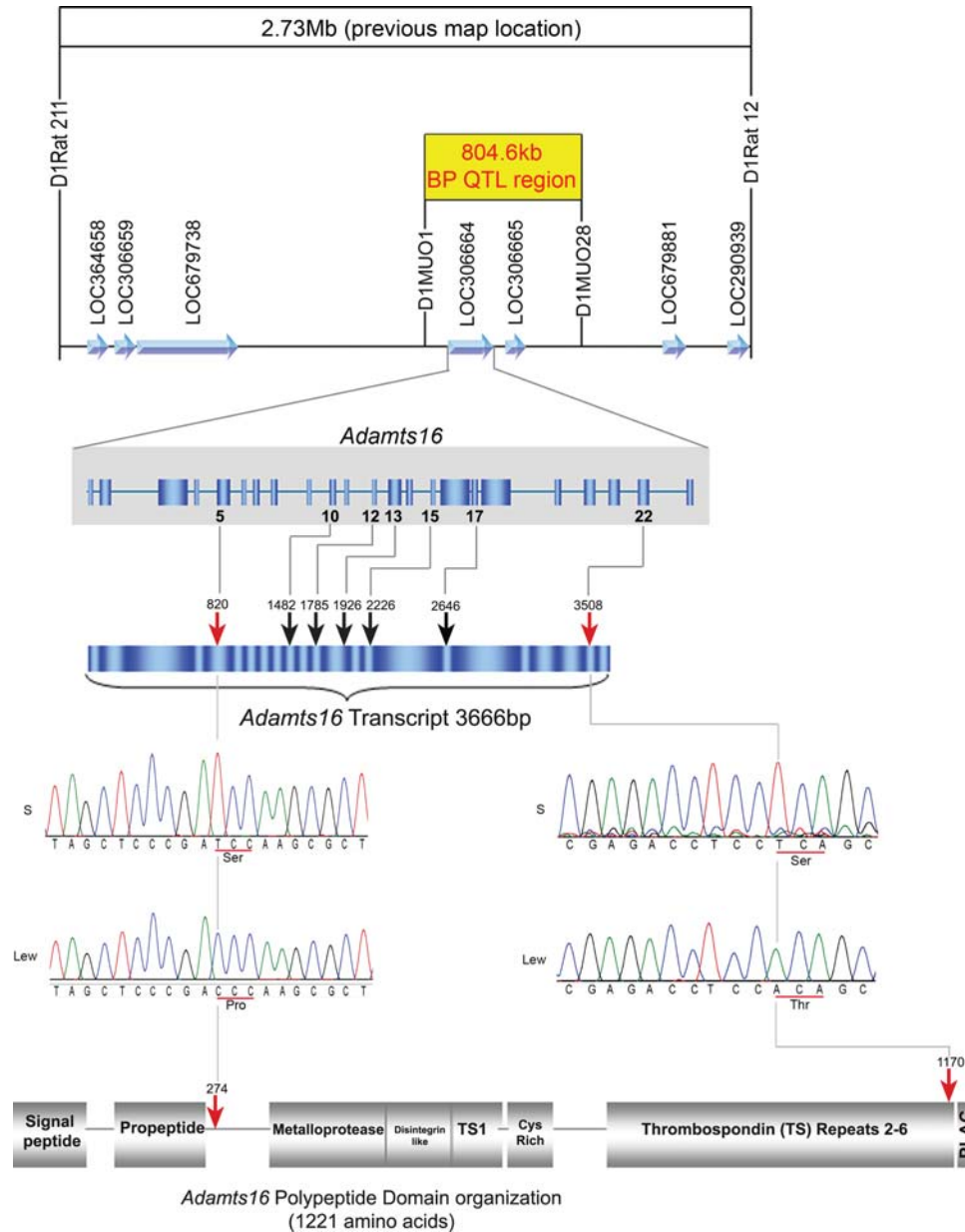


Figure 4. Detection of variants of *Adamts16* within the BP QTL region. Predicted candidate genes for the 2.73 Mb QTL interval are shown in the top panel of the Figure as blue arrows within the region flanked by the markers D1Rat211 and D1Rat12. This information was extracted from <http://www.ncbi.nlm.nih.gov/genome/guide/rat/index.html>, Build 4.1. Arrows indicate the orientation of the gene sequence on the chromosome. The exons and introns of LOC306664, which codes for *Adamts16*, are shown above the corresponding transcript sequence. Locations of transcript variants detected between S and LEW are numbered on the transcript sequence. These transcript variations in S versus LEW are as follows: (i) T/C820, (ii) C/T1482, (iii) C/T1785, (iv) A/G1926, (v) T/C2226, (vi) C/T2646, (vii) T/A3508. Resultant amino acid changes and their locations on the schematic diagram of the predicted polypeptide domain of *Adamts16* are shown at the bottom of the Figure. PLAC, protease and lacunin domain.

1221 amino acids in rats (present study) in comparison to 1224 amino acids reported for the human ortholog of *Adamts16* (www.ncbi.nlm.nih.gov). Closer inspection of the primary amino acid sequence indicated that the variant at location 274 in rat and the corresponding location of 277 in human are within putative furin cleavage sites (Arg-Xaa-Arg/Lys-Arg). ADAMTS proteases are synthesized as precursor proteins (zymogens) that are proteolytically processed at such consensus sites by furin to excise their propeptides. Pro-

peptide excision typically converts the zymogen to an active protease. Therefore, the efficiency of cleavage of the variants within this furin cleavage site of S and LEW was tested in a protein construct containing the propeptide and catalytic domains (*Adamts16* Pro-Cat). However, there were no detectable differences in the efficiency of cleavage by furin (Supplementary Material, Fig. S1). Next, we tested the possibility that *Adamts16* may function as a QTL gene that regulates the expression of downstream 'effector genes', leading to a

Table 3. Predicted amino acids of the polypeptide chain of *Adamts16* in 21 rat inbred strains

AA	AA	Number of inbred strains	Inbred strains
274	1170		
Ser	Ser	8	S, R, SHR, MHS, WKY, LH, LN, LL
Pro	Thr	10	LEW, BN, MNS, F344, SBH, SBN, AS, LOU, MWF, SJH
Pro	Ser	3	ACI, DA, COP

AA, Amino acid; ACI, August × Copenhagen Irish; AS, Albino Surgery; BN, Brown Norway; COP, Copenhagen; DA, Dark Agouti; F344, Fisher; LEW, Lewis; LH, Lyon hypertensive; LL, Lyon hypotensive; LN, Lyon normotensive; LOU, Louvain; MHS, Milan hypertensive strain; MNS, Milan normotensive; MWF, Munich Wistar Fromter; R, Dahl salt-resistant; S, Dahl salt-sensitive; SBH, Sabra hypertensive; SBN, Sabra normotensive; SHR, spontaneously hypertensive rat; SJH, San Juan hypertensive; WKY, Wistar Kyoto.

physiological response related to BP control (28). To explore the downstream genes and/or networks affected by *Adamts16* polymorphisms, the renal transcriptomes of S and S.LEW (D1Mco4x1x3Bx1) were analyzed. A total of 1294 differentially expressed genes were identified (Supplementary Material, Table S3). Of these genes, 256 were found to be differentially expressed upon siRNA-mediated knock-down of *Adamts16* in rat kidney NRK-52E cells (Supplementary Material, Table S4). This subset of 256 overlapping genes represents potential 'effector genes' regulated by *Adamts16* (Supplementary Material, Fig. S2). Successful knock-down of *Adamts16* was confirmed by quantitative RT-PCR, where *Adamts16* mRNA levels in cells transfected with targeting siRNAs were $32 \pm 4\%$ of control cells transfected with non-targeting siRNA ($n = 6$, data not shown). Analysis of the 'effector genes' subset by Expression Analysis Systematic Explorer (EASE) identified a number of gene ontologies of potential interest in kidney function including regulation of transcription, metal ion binding, extracellular space and organ development (Supplementary Material, Fig. S2). Interestingly and particularly relevant in this study was the identification of gene networks regulated by *Adamts16* involved in renal and urological disease which impacts on BP control (Supplementary Material, Fig. S3) and in organ development (Supplementary Material, Fig. S4).

Evidence for association of SNPs of *ADAMTS16* in human BP

To test whether *ADAMTS16* is associated with human BP, we sought populations in which evidence for linkage to BP or EH was reported in the homologous region. There is one such report in a non-obese subset of the QFS in which a homologous QTL for SBP was found (17). Consequently, twelve known human *ADAMTS16* SNPs were genotyped in 945 subjects of this study. The LD plot for the associated SNPs is shown in the Supplementary Material, Figure S5. Three SNPs including an Ala to Pro variant in codon 90 (rs2086310) of human *ADAMTS16* were associated with human resting BP (Table 4). The association of the non-synonymous variant rs2086310 within *ADAMTS16* to SBP in the QFS was confirmed in the GenNet study with a total

sample size of 3205 (Table 4). The association of rs2086310 was observed in Whites but not in Blacks or Hispanics (Table 4). Another SNP, rs2964433, was associated with SBP in Whites and Hispanics and with DBP in Hispanics only. Overall human variants were associated with lower SBP and DBP.

DISCUSSION

The data presented is the result of sustained applications of substitution mapping for over a decade and constitutes the first report of a BP QTL within a very short congenic segment, containing variants within a single rat gene, *Adamts16*. Similar associations of variants within human *ADAMTS16* that were prevalent in two independent populations provide the rationale for further functional analysis of *ADAMTS16* in the context of BP regulation and EH. Additional replications would be required to confirm these associations in humans.

Previously we inferred that QTL2 was localized to a 2.73 Mb region on the basis of the differential segment between two congenic strains, one with a BP effect and the other without (16). By capturing the QTL in its entirety in two congenic substrains, which can be considered as replicated experiments, this QTL was shown to exert a phenotypic effect independently, i.e. without genetic interaction from introgressed LEW alleles of the previously reported strain with the BP effect, S.LEW (D1Mco4x1x3A) (16).

Data presented in Figure 1 allowed us to compare the results of substitution mapping to the evidence obtained by genetic linkage analysis of the same region. It is interesting to note that the location of the QTL gene under study here is at some distance from the confidence intervals of LOD peaks for linkage to BP in the F2 (S × LEW) population. This phenomenon is known from theoretical work on simulations of linkage analysis involving two QTLs on the same chromosome and the creation of a 'ghost' LOD peak between them (29–31). This artifact occurs when two QTLs are ~40 cM apart and when the plus alleles at both QTLs are on the same chromosome, and the contrasting minus alleles are on the other chromosome. In the present case, the two QTLs on rat Chromosome 1 [QTL2 under consideration here and another adjacent QTL called QTL1 (32)] are configured such that the LOD peak is generated between them, rather than directly over either of them. Other segregating populations, in which QTL1 is presumably not polymorphic, show excellent alignment of QTL2 with the LOD peak (Fig. 5). The above situation reiterates the difficulty of mapping QTL genes solely based on linkage analysis as was often done in humans and exemplifies the power of substitution mapping in animal models to resolve QTLs by isolating them as 'monogenic' QTL effectors. The advantage of this approach is shown by the data presented here, because the fine-mapped BP QTL did not contain any gene with previously known function related to BP.

The accuracy of mapping QTLs and detecting underlying genetic determinants depends heavily on the quality of assembly of genomes and gene predictions. Early impediments to the positional cloning effort described in this report included

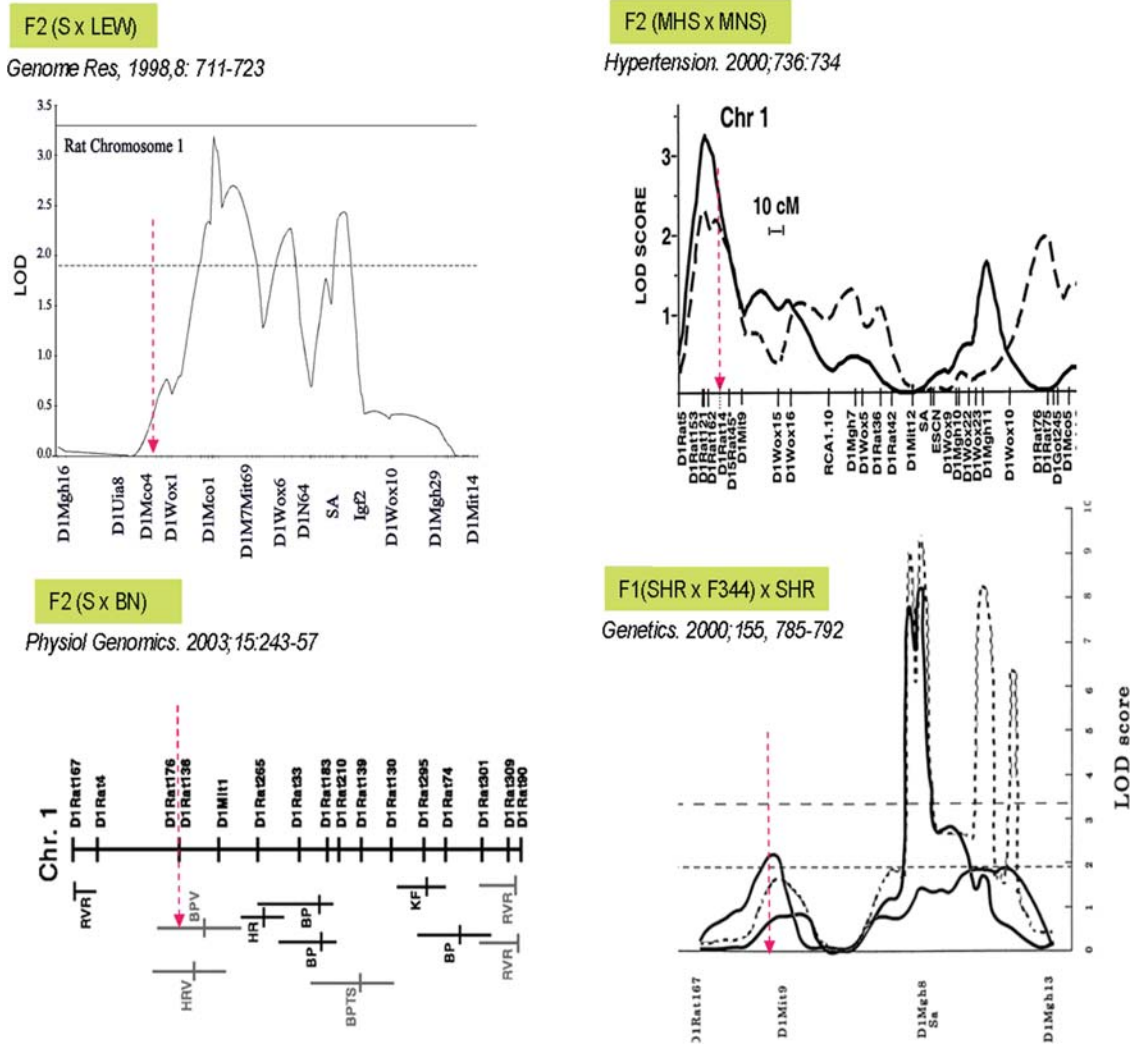


Figure 5. Comparisons of linkage maps of Chromosome 1. Individual Figures of linkage data for BP extracted from the published data of crosses shown in Green. Colored arrows show the relative location of *Adamts16*.

the incorrect assignment of the QTL region to rat Chromosome 17 and multiple, discordant gene predictions of *Adamts16*. The data presented in Figure 1 and Table 2 resolved the error in sequence assembly by correctly placing the region spanned by markers D1Rat211 and D1Rat12 on Chromosome 1. Similarly, the biological data that we have obtained allowed for solving the discrepancies among the available predictions for gene and transcript sequences of rat *Adamts16*. The most recent version of the predicted *Adamts16* gene at the NCBI rat genome resources website (Build 4.1) concurs with the evidence that we have provided in the present study.

It is interesting to note that in addition to other tissues, *Adamts16* is expressed in the kidney, which is a major organ involved in BP homeostasis. The function of *Adamts16* is unknown (http://rgd.mcw.edu/tools/genes/genes_view.cgi?id=1310046). Based on the roles of the ADAMTS family members and based on the presence of a metalloproteinase domain and intact active site sequence HESGH+HD within *Adamts16*, it is likely that *Adamts16* functions as a metallo-

proteinase *in vivo*. The non-synonymous SNPs reported in this study do not reside within the metalloproteinase domain of *Adamts16*. However, they are located in potentially critical regions of the protease, since furin cleavage is known to relieve enzyme latency of other members of the Adamts family of proteins (33–38), and the thrombospondin repeat domain is known to influence substrate binding and proteolysis. As the SNPs within the nucleotides coding for the furin cleavage site did not account for changes in efficiency of cleavage by furin, it is possible that the full-length protein may be required for eliciting a differential functionality of *Adamts16* variants. For example, a signal peptide mutation in ADAMTS10 causing Weill–Marchesani syndrome was found to have different effects on ADAMTS10 Pro-Cat and full-length ADAMTS10 (39). Alternately, the ADAMTS16 thrombospondin-motifs, one of which is affected in the second non-synonymous variant (+3508), reported in our study could have key biological activities. Since a substrate is not known for *Adamts16*, these possibilities are currently unexplored and beyond the scope of the present work. Regard-

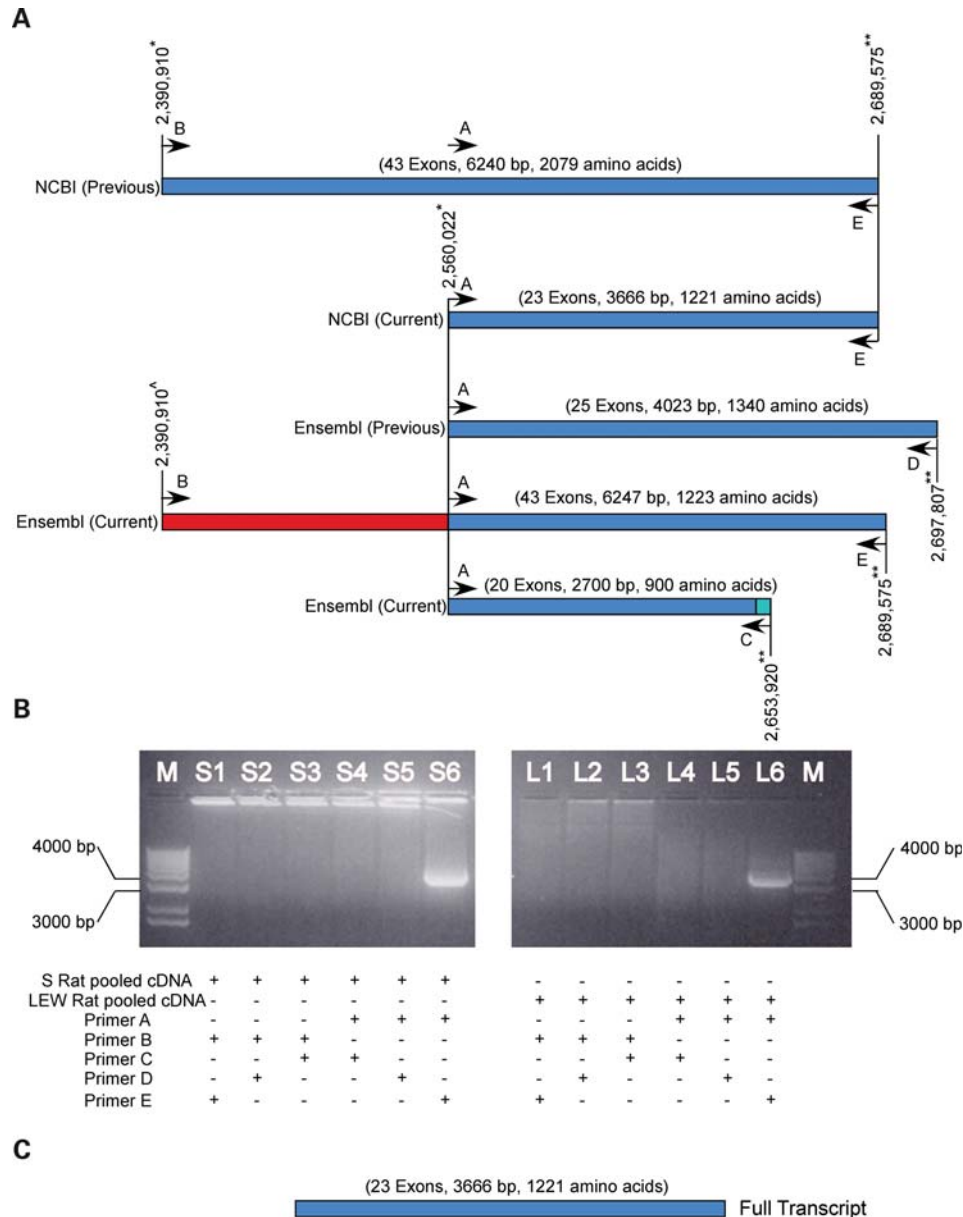


Figure 6. *Adamts16* predicted transcript analysis. (A) Schematic representations of previous and current NCBI and Ensembl annotations for *Adamts16* (LOC306664). Primers were designed at the locations specified as (A) through (E) in the directions depicted by the arrows. All primers are 5'-3': Primer A = ATGGAACCCCGCGTTGC, Primer B = ATGGGAGCCAATGCAAATGTATAC, Primer C = TGAAGATATTTCCAAATGTCCTCC, Primer D = CTCACAGTTGGACTTGGAAC, Primer E = CTA CTTTAGAACTCGGTGGTTG. (B) Ethidium bromide stained agarose gel of PCR products obtained from a mixed pool of either S or LEW cDNA and different combinations of primer pairs. The combinations of primers are as follows (S, S rat mixed cDNA pool; L, LEW rat mixed cDNA pool). Only the primer A + E combination gave a product with an approximate size of 3700 bp. (C) Schematic representation of the cloned *Adamts16* transcript (both the S and LEW RT-PCR reactions yielded a gene with 3666 bp, coding for 23 exons that translate into 1221 amino acids). *Location of the initiation codon; **location of the termination codon; ^location of start of transcription; M, molecular weight marker.

less of the functional alterations, we hypothesized that alterations of the renal transcriptome as a consequence of altered functions of *Adamts16* could be important in understanding the potential link between the mechanism of action of this gene product and the physiology of BP regulation. Because the renal transcriptome of the congenic strain may not be the exclusive representation of the downstream effectors regulated by *Adamts16*, but may rather represent the net effect of the introgressed congenic segment, we compared the results

of our study to the 'biological signature' obtained by knock-down of *Adamts16*. Overlapping, multiple networks related to BP and organogenesis were perturbed in both cases. The precise mechanism through which such changes in expression lead to the alteration in BP remains to be determined in this model. The results suggested interesting gene targets for BP regulation such as histone deacetylases *Hdac5* and *Hdac10*, genes involved in epigenetic regulation. Taken together, the results are highly supportive of the kidney as one of the

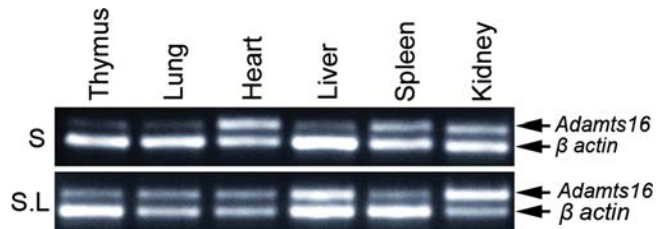


Figure 7. Tissue distribution of *Adamts16*. Various tissues from one S and one S.LEW (D1Mco4x1x3Bx1) congenic rat (labeled as S.L) were collected after euthanization of the rats by CO₂ inhalation. Total RNA was extracted from the tissues using the Trizol reagent (Invitrogen). Three microgram of RNA from each tissue obtained from either an S or S.LEW (D1Mco4x1x3Bx1) (3 months old) maintained on 0.3% NaCl containing diet was reverse transcribed using Superscript III (Invitrogen). Resultant cDNA was amplified by multiplexing PCR for both *Adamts16* and beta-actin using the following four primers in the same reaction: Primers for *Adamts16*: (i) 5'-TTGCTTTGCGCTCTGGGT-3'; (ii) 5'-AGAAAGGGCCACAGAAGAA-3' and primers for beta-actin; (iii) 5'-GTGTGATGGTGGGTATGG-3' and (iv) 5'-CGCGTAACCTCATAGAT-3'. PCR products were resolved on a 3% agarose gel.

sites of action through which variants of *Adamts16* participate in the regulation of BP.

Considering the difficulty of defining what constitutes a good standard for identifying a gene underlying a QTL, Glazier *et al.* have suggested some criteria (40). In a recent review on the genetic dissection of hypertension (41), Cowley indicated that the gold standard for validation of a potential candidate gene for hypertension is that the rescue of the phenotype must be done in the same species and with the same genetic background that was used to identify the QTL. Our study almost meets this 'gold standard' because (i) The QTL is fine-mapped to a small region solely based on natural recombination events, thus closely mimicking the performance of a natural allele in the rat as opposed to artificially genetically manipulated systems such as transgenics and knock-ins; and (ii) we are able to map the QTL to a gene-sparse region as confirmed by comparative mapping in rats, mice and humans (16), thus allowing for testing of the two expressed candidate transcripts, only one of which had coding sequence variants. One could argue that in addition to the variants within *Adamts16*, there may be other variants within inter-genic or intra-genic regions of the critical QTL interval that contribute to the QTL effect. Like in any positional cloning study, regardless of any criteria, such possibilities cannot be ruled out. However, with the view of improving the clinical management strategies for high BP through the identification of novel and effective drug targets, the current study in rats as model organisms with follow-up analyses in independent association studies in humans from two unrelated populations, provide the rationale for prioritization of functional studies of *ADAMTS16* as a candidate for the BP QTL on human Chromosome 5.

MATERIALS AND METHODS

Animals

All animal experiments were conducted as per pre-approved protocols by the Institutional animal care and use committee

(IACUC) of the University of Toledo College of Medicine (UTCOM). Dahl salt-sensitive (SS/Jr) inbred rats were from our colony maintained at UTCOM. LEW/NCrIBR (LEW rat) was originally obtained from the Charles River Laboratories (Wilmington, MA) and maintained in our colony. The congenic substrain S.LEW (D1MCO4X1X3B) was derived from S.LEW (D1MCO4X1) (16) as the parental strain contributing to the introgressed LEW alleles. Congenic substrains S.LEW (D1MCO4X1X3BX1) and S.LEW (D1MCO4X1X3BX2) were derived from S.LEW (D1MCO4X1X3B) as the progenitor with LEW alleles at the QTL region. The congenic substrains were constructed by crossing the progenitor congenic strain to the S rat to obtain a heterozygous F1 population. An F1 × F1 intercross then yielded an F2 population. The F2 animals were selected for recombinants with varying sizes of the LEW RNO1 introgressed region. A new recombinant animal was backcrossed to S rats to duplicate the new recombinant chromosome. The resulting progeny were genotyped to identify heterozygotes. Heterozygotes were then intercrossed to produce progeny, which were screened for animals homozygous for the new recombinant chromosome. Finally, the homozygous animals were bred to generate a congenic strain containing the desired LEW chromosomal segment in the homozygous form on the S genetic background.

Microsatellite marker development and tracking recombination events in congenic strains

Microsatellite repeats in the region of interest were identified on the rat genomic sequence (version 3.1) at the NCBI website (www.ncbi.nlm.nih.gov). In order to amplify these microsatellite repeats, primers were designed using the Primer3 software available at the website: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi and synthesized by integrated DNA technologies (IDT) (www.idtdna.com). DNA was isolated with the QIAamp Tissue Kit (Qiagen Inc.) from S, LEW and congenic substrains. PCR reactions were set up using the primers designed to amplify microsatellite repeats from S and LEW rats. Polymorphic repeats were used as markers to genotype DNA from tail biopsies of the relevant rat populations.

Predicted genomic and transcript analysis

Predicted gene sequences within the QTL region were downloaded from the rat genome sequence at the NCBI website (www.ncbi.nlm.nih.gov). Primers to amplify exons were designed using the ExonPrimer software (<http://ihg.gsf.de/ihg/ExonPrimer.html>). Primers tagged with M-13 sequences were synthesized by IDT. All primer sequences mentioned in our work are available at our website (<http://hsc.utoledo.edu/depts/physiology/research/rat/>). Genomic DNA was amplified using these primers and sequenced by MWG Biotech Inc. Predicted transcript sequences were extracted from the NCBI database and/or Ensembl (www.ensembl.org). Primers were designed encompassing the start and stop codons of each predicted transcript using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or Oligonucleotide properties calculator ([http://](http://www.ensembl.org)

Table 4. Human Adams16 variants with associations to SBP and DBP in the QFS and GenNet study

Study	Variant	Genotype	<i>n</i>	SBP	P1	P2	DBP	P1	P2
QFS	rs1871468	C/C	654	115.7 ± 0.6	ns	0.0024	71.5 ± 0.4	0.0002	0.0019
		C/G	165	114.1 ± 1.1			69.4 ± 0.7		
		G/G	7	106.4 ± 2.2			68.9 ± 1.3		
QFS	rs2086310	G/G	587	116.0 ± 0.6	0.009	0.0025	71.6 ± 0.5	0.006	0.0008
		C/G	210	114.2 ± 0.9			70.1 ± 0.5		
		C/C	14	106.5 ± 2.1			67.1 ± 1.5		
QFS	rs10512769	C/C	596	115.8 ± 0.6	0.06	0.0004	71.6 ± 0.4	0.0005	0.0027
		C/T	205	114.3 ± 1.2			69.6 ± 0.7		
		T/T	27	108.4 ± 2.3			69.8 ± 1.4		
GenNet (Whites)	rs2086310	G/G	975	123.7 ± 0.6	0.01	0.02	76.8 ± 0.4	ns	ns
		C/G	434	120.9 ± 0.8			75.7 ± 0.5		
		C/C	56	124.5 ± 2.9			76.1 ± 1.44		
GenNet (Whites)	rs2964433	G/G	1062	123.2 ± 0.5	0.01	0.009	76.6 ± 0.3	ns	ns
		A/G	357	123.0 ± 0.9			76.6 ± 0.6		
		A/A	30	112.5 ± 1.7			72.2 ± 1.7		
GenNet (Blacks)	rs2086310	G/G	409	122.8 ± 0.9	ns	ns	76.3 ± 0.7	ns	ns
		C/G	434	122.6 ± 0.9			75.6 ± 0.7		
		C/C	105	124.1 ± 1.8			75.7 ± 1.4		
GenNet (Blacks)	rs2964433	G/G	776	122.5 ± 0.7	ns	ns	75.6 ± 0.5	0.07	0.08
		A/G	159	123.6 ± 1.4			77.6 ± 1.1		
		A/A	9	127.1 ± 6.6			79.2 ± 2.7		
GenNet (Hispanics)	rs2086310	G/G	610	112.6 ± 0.8	ns	ns	69.5 ± 0.5	ns	ns
		C/G	169	113.7 ± 1.4			69.8 ± 0.8		
		C/C	8	110.2 ± 3.7			64.5 ± 2.6		
GenNet (Hispanics)	rs2964433	G/G	633	113.1 ± 0.8	<0.0001	<0.0001	69.6 ± 0.5	<0.0001	<0.0001
		A/G	90	108.4 ± 1.7			67.5 ± 1.1		
		A/A	2	104.2 ± 0.4			66.2 ± 0.3		

Values for SBP and DBP are Means ± SEM after adjustment for age, sex, sodium intake and waist circumference. DBP, diastolic blood pressure; SBP, systolic blood pressure; P1, *P*-values for age- and sex-adjusted data; P2, *P*-values for data adjusted for age, sex, sodium intake and waist circumference. ns, non significant. QFS, Quebec family study; GenNet, one of four multicenter networks investigating blood pressure genetics.

www.basic.northwestern.edu/biotools/oligocalc.html). Messenger RNA from various tissues of S and LEW were extracted using TRIzol Reagent (Life Technologies). cDNA of the predicted transcripts within the QTL region was obtained by reverse transcription using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Standard polymerase chain reaction, using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) and gene-specific primers, was used to amplify the cDNA. S and LEW gene amplicons were cloned into the Topo-TA-Cloning System (Invitrogen) as per the manufacture's recommended procedures. Plasmid DNA was purified using the QIAprep-miniprep (Qiagen). The full-length S and LEW alleles for each gene within the QTL region were sequenced by MWG (High Point, NC), using vector-based and gene-specific primers. All sequence data were analyzed using DNA Star (DNASTar Inc.) and/or Sequencher (Genecodes Corp.).

BP measurements

Each set of congenic substrains (*n* = 20 males) and control S rats (*n* = 20 males) were bred, housed and studied concomitantly to minimize environmental effects. Rats were weaned at 30 days of age and given a low salt diet (0.4% NaCl, Harlan Teklad). At 40–42 days of age rats on a high salt dietary study only were fed 2% NaCl diet (Harlan Teklad diet TD94217) for 24 days. Rats on a low salt dietary study were continued on the 0.4% NaCl in their diet. SBP was

measured using the tail-cuff method commencing on the 25th day (42). Briefly, conscious restrained rats were warmed to 28°C. The BP of each rat was measured for four consecutive days by two blinded operators. BP values for each day were the mean of three to four consistent readings. The final BP value used was the mean of the four daily BP values. The day after the last BP measurements, rats were euthanized and heart weights were recorded. BP was also collected using a telemetry system (Data Sciences International, St. Paul, MN) as explained in detail previously (16). Briefly, 4 days after the BP measurements by the tail-cuff method, rats S (*n* = 9) and congenic rats (*n* = 9) on a low salt diet were surgically implanted with a transmitter into the left flank and the probe was inserted through the femoral artery and advanced to the lower abdominal aorta. Rats were allowed to recover from surgery for a week before the transmitters were turned on and BP data collected for 24 h at a time over several weeks. All statistical analyses were performed as previously reported (16), using SPSS software.

Transcriptome and network analyses

Gene expression analysis was carried out on the Rat Genome 230 version 2 GeneChip as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Hybridizations were performed to compare the transcriptome in hypertensive S rat kidney (three independent hybridizations from individual animals) versus S.LEW (D1Mco4x1x3Bx1) congenic rat

kidney (three independent hybridizations), and control non-targeting siRNA versus two different Adamts16-targeting siRNAs transfected into rat kidney NRK-52E cells (three independent transfections for control, two independent transfections for each targeting siRNA). siRNAs were transfected into cells with DharmaFECT reagent (Dharmacon) for 48 h prior to RNA isolation. The control siRNA is a non-sense sequence with no human GenBank database match (sense sequence: 5'-CCTCCAATGTGCTCGGTAAT-3'), and two Adamts16-targeting siRNAs were separately employed to account for potential off-target effects (sense sequences: 5'-AGAAGTACGTCTCCGAAA-3' and 5'-CCTATATTC TGTGCGCAA-3'). Gene expression data files were processed and scaled with the Affymetrix GeneChip Operating Software. Statistical analysis for differential gene expression was accomplished by an analysis of variance with 10% false discovery rate to control for type I error resulting from multiple comparisons. All data are accessible at the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE12869. Gene ontologies, gene networks and canonical pathways were investigated using EASE (43), Ingenuity Pathway Analysis (IPA, www.ingenuity.com, Redwood City, CA), Pathway Studio (www.ariadnegenomics.com), the GO Consortium (www.geneontology.org), GenMAPP (www.genmapp.org) and Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg).

Furin cleavage assay. The S and LEW alleles of the 1.6 kb fragment encoding the furin cleavage site along with the propeptide and catalytic domains (Adamts16 Pro-Cat) was amplified by PCR and subcloned into the *Not1* and *BamH1* restriction endonuclease sites of the pcDNA3.1/myc-HIS vector. Primer sequences for amplification of the S and LEW alleles are 5'-GCT AGC TAT ATA GCG GCC ACC ATG GAA CCC CGC GG-3' and 5'-TAG AAA GCT TGG TAC CGA GCT CGG ATC CAG CTT TTC CGG GTA CTT GTA-3'. Amplified products were sequence verified by bidirectional dideoxy sequencing. Furin cleavage assay was performed as described in the legend to Supplementary Material, Figure S1.

Association studies in humans

Quebec family study. The QFS is composed of French Canadian families from the greater Quebec City area recruited through the media between 1978 and 1981 (Phase 1), 1989 and 1994 (Phase 2) and 1995 and 2001 (Phase 3). The QFS study represents a mixture of random sampling (Phase 1) and ascertainment through and obese proband (Phases 2 and 3), as described previously (44). The present analyses are based on participants from phases 2 and 3 not taking anti-hypertensive drug and include a total of 854 individuals (373 men and 481 women) from 220 families. The Medical Ethics Committee of Laval University approved the protocol and all participants gave their written consent to participate in the study.

All exonic *ADAMTS16* SNPs (five total) as well as seven SNPs in the vicinity of exons with confirmed minor allele frequency of 10% or greater in Caucasians were genotyped in the QFS. Genotyping of the SNPs was done by the primer exten-

sion method with fluorescence polarization detection (FP-TDI; PerkinElmer Inc.) (45). Changes in fluorescence polarization after excitation of the samples by plane-polarized light were measured using a Victor2 Plate Reader (Perkin Elmer Life Sciences). The allele calling was performed with the SNP scorer genotyping software (Perkin Elmer Life Sciences). Details for PCR conditions and primer sequences are available upon request at rankint@pbrc.edu.

GenNet study. The GenNet study comprises 3467 individuals in total, recruited between 1995 and 2004 [1101 African Americans (AA), 839 Hispanic Americans (HA) and 1496 European Americans (EA)] (46). Individuals were recruited at two field centers: EA were recruited from Tecumseh, Michigan, and AA and HA were recruited from Maywood, IL. Recruitment was performed in families starting from a proband with high BP. DNA was available for 3205 individuals (968 AA, 824 HA and 1471 EA). Measured SBP and DBP (average over two manual measurements) was adjusted for age and sex, or age, sex and BMI, by linear regression and the residuals were used in the analysis. The impact of anti-hypertensive therapy on BP levels was empirically corrected for by adding 10 mmHg to SBP and 5 mmHg to DBP if a participant took BP-lowering medications.

SNP genotyping in the GenNet study. SNP genotyping was performed on all DNA samples available and genotypes could be obtained from using a 5'-nuclease-based assay (TaqMan, ABI) analyzed on an ABI Prism 7900 Real Time PCR System according to the manufacturer's instructions. Call rates were 0.989 for rs2086310 and 0.982 for rs2964433. There was no evidence for deviation from Hardy-Weinberg equilibrium within each ethnic group ($P > 0.1$ for all populations in both variants tested). As additional quality control, we produced duplicate genotypes ($n = 102$) that gave identical results and genotypes from seven HapMap samples that were all consistent with the HapMap dataset. Minor allele frequencies were 0.3, 0.188, 0.129 (rs 2086310) and 0.1, 0.134, 0.043 (rs2964433) for AA, EA and HA, respectively.

Analyses: QFS and GenNet study. Association testing for measured SBP and DBP (average over two manual measurements) was performed with a mixed regression model using the SAS PROC MIXED procedure (version 9.1.3; SAS Institute, Cary, NC). The non-independence of the data resulting from the familial structure in GenNet and QFS was accounted for in the analyses by using a sandwich procedure which asymptotically yields the same parameter estimates as an ordinary least squares or regression method but the standard errors and consequently hypothesis tests are adjusted for the dependencies. Two sets of analyses were performed, one on age- and sex-adjusted data and a second one adjusted for age, sex and BMI (for GenNet) and for age, sex, sodium intake and waist circumference (for QFS). In GenNet, the impact of anti-hypertensive therapy on BP levels was empirically corrected for by adding 10 mmHg to SBP and 5 mmHg to DBP if a participant took BP-lowering medications.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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