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Original Research

Hypertension-Associated Genes in the Mesenteric Artery of Three Spontaneously Hypertensive Rat Substrains Identified Using a DNA Array Method

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Abstract

Background: Although the mesenteric artery plays a key role in regulating peripheral blood pressure, the molecular mechanisms that underlie the development of essential hypertension are not yet fully understood. **Materials and Methods**: We explored candidate genes for hypertension using three related strains of spontaneously hypertensive rats (SHRs) that mimic human essential hypertension. In this study we used DNA microarrays, a powerful tool for studying genetic diseases, to compare gene expression in the mesenteric artery of three SHR substrains: SHR, stroke-prone SHR (SHRSP), and malignant SHRSP (M-SHRSP). **Results**: Compared to normotensive 6-week old Wistar Kyoto rats (WKY), higher blood pressure correlated with overexpression of 31 genes and with down regulation of 24 genes. *Adam23*, which negatively regulates potassium current, and the potassium channel genes, *Kcnc2* and *Kcnq5*, were associated with the onset of hypertension. In addition, *Spock2* and *Agtrap* were identified as strengtheners of hypertension by analyzing up and down regulated genes at 9-weeks of age. **Conclusions**: *Adam23*, *Kcnc2* and *Kcnq5* appear to be factors for the onset of hypertension, while *Spock2* and *Agtrap* are as factors that strengthen hypertension. These findings contribute to our understanding of the pathophysiology of hypertension and to the development of treatment for this condition.

Keywords: hypertension; stroke; DNA microarray; gene expression analyses; genomics; mesenteric artery; spontaneously hypertensive rat (SHR); stroke-prone spontaneously hypertensive rat (SHRSP); malignant stroke-prone spontaneously hypertensive rat (M-SHRSP); potassium voltage-gated chanell subfamilies

1. Introduction

Hypertension is a serious disorder that shows no symptoms even when blood pressure (BP) is elevated. If left untreated, it can lead to life-threatening diseases with atherosclerosis in humans. In rats, arteriosclerosis rather than atherosclerosis is the predominant change associated with hypertension and leads to myocardial infarction, renal failure and stroke [1–3]. Even in Japan where there is sufficient general medical care to treat hypertension, more than 300,000 patients die each year from diseases related to this condition [4,5]. To confront this life-threatening problem, multidisciplinary knowledge of hypertension is required. Together with more effective treatments to avoid serious consequences, elucidation of the genetic background of hypertension is urgently required. Although a number of methods are available for investigating the genetic nature of hypertension, the polygenic nature of human hypertension makes it difficult to identify the responsible genes via meta-analysis of genome-wide studies [6]. The aim of this study was therefore to identify genes related to hypertension by studying rat models of human essential hypertension, namely spontaneously hypertensive rats (SHR) [7], stroke-prone SHR (SHRSP) [8] and malignant SHRSP (M-SHRSP) [9]. The two latter substrains develop stroke at high rates of 77% in SHRSP [8] and more than 96% in M-SHRSP [9].

DNA microarrays are potentially powerful tool for studying the genetics of diseases as they allow simultaneous measurement of the expression level of thousands of genes in experimental studies [10–12]. In this study, we compared gene expression in the mesenteric artery of the three SHR substrains mentioned above [7–9]. SHR was developed as an animal model for research into essential hypertension as part of a breeding program based solely on the selection of elevated BP in normotensive Wistar Kyoto (WKY) rats [7]. SHRSP were derived from SHR following selective inbreeding for susceptibility to stroke [8]. M-SHRSP were selected and established through brother–sister mating of

selected SHRSP animals that showed higher BP and higher incidence rates of stroke [9]. An inbred strain of M-SHRSP has a BP of 250 mm Hg or higher before 14-weeks of age, causing brain stroke in almost all animals [9]. We previously bred three SHR substrains and families in our facility. Using these SHR substrains, we found several blood pressure-regulating genes such as *Rgs2*, *Gja1*, *Uts2*, *Ephx2* in the adrenal glands [13], and *Mapk14*, *Ephx2*, *Kcnc3* in the kidneys that could be related to hypertension [14]. The *Kcnq1*, *Kcnh1*, *Ache* and *Chrm2* genes were identified following study of the mesenteric artery of SHRs, SHRSPs and renovascular hypertensive 2K1C (two kidneys and one clip induced hyper-renin) and 1K1C (one kidney and one clip induced hypervolemic) rats, but not M-SHRSP [15]. Other genes related to hypertension have also been reported [16], and the identify of true candidate genes for hypertension remains controversial.

The mRNA expression profile of different tissues depends on their biochemical and physiological roles and on the states of disease. Hence, it is very important to select mRNA samples from tissues that are responsive to hypertension. Resistance arteries such as the mesenteric and femoral arteries in the rats appear to be the most relevant tissues when analyzing the cause of hypertension [17]. In the present study we therefore chose the mesenteric artery to analyze mRNA in rat hereditary hypertensive models. Genes associated with hypertension were identified in the mesenteric artery of three related substrains of SHR using DNA microarray technology.

2. Materials and Methods

2.1 Animals

The experiments were performed using 6- and 9-week old rats. Wistar Kyoto (WKY)/Izm was used as the control strain, and SHR/Kpo, SHRSP/Kpo and M-SHRSP/Kpo as the hypertensive models [7–9]. Male rats with high blood pressure and frequent strokes as compared to female rats were used. Based on the analyses for genetic polymorphisms, the three rat substrains used here had identical genetic backgrounds (personal communication with Prof. Ota, Department of Pathology, Kindai University Nara Hospital; National Bioresource Project 'Rat' in Kyoto University (http://www.anim.med.kyoto-u.ac.jp/nbr/phylo_jp.aspx#SHR)). In total, three rats from each experimental group were used for the DNA microarray experiments. WKY/Izm rats were purchased from SLC Co. (Shizuoka, Japan), while the three substrains were obtained from the Animal Center, Kindai University School of Medicine. All animals used in this study were handled with due care according to the guidelines established by the Japanese Association for Laboratory Animal Science, which complied with international rules and policies. This study was performed following approval (KAME-19-078 on April 1, 2007) from the Animal Care and Use Committee of the Kindai University. Handling during experiments was performed to minimize the pain and discomfort of the rats.

2.2 Measurements of Systolic Blood Pressure

Systolic BP (SBP) was measured every week in 6 rats including three rats per group for the DNA microarray experiments, using the tail-cuff method with a UR-5000 instrument (Ueda, Tokyo, Japan). Briefly, three consecutive SBP readings were taken weekly between 09:00 AM and 11:00 AM after warming the body to 35 °C for 5 min in a heater box. SBP values are expressed as the mean ± SEM.

2.3 Tissue Processing and RNA Isolation

The mesenteric arteries, 2nd to 3rd branches because of existences of almost all receptors, ion channels and vascular smooth muscle regulating the blood flow, were harvested under sodium pentobarbital anesthesia (50 mg/kg i.p.), cut with scissors and homogenized twice at a pitch speed of 22 strokes/s for 2 min in a 2 mL plastic tube with 5 mm diameter glass beads using a Qiagen Tissue Lyser (Retsch GmbH & Co., Haan, Germany). Total RNA was extracted with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA quality was evaluated with RNA Nano Chips (Agilent Technologies, Waldbornn, Germany) using an Agilent 2100 Bioanalyzer prior to use in the microarray experiments. Tissues obtained from 3 rats per group of hypertensive and normotensive strains were used for microarray analysis.

2.4 Analysis of Gene Expression Profiles with Oligonucleotide Arrays

To examine the gene expression profiles of rat mesenteric arteries, cRNA labeled with cyanine 3-CTP (PerkinElmer, Boston, MA, USA) was synthesized from 1 μg of DNase I-treated total RNA with a Low RNA Input Amplification kit (Agilent Technologies). This was hybridized by incubating with a Whole Rat Genome Microarray (4x44K formatted) (Agilent Technologies) in a rotor oven (Agilent Technologies) for 17 h at 65 °C, followed by washing. The hybridized slides were scanned with an Agilent GenPix Scanner 4000 (Agilent Technologies), the data was extracted, and the overall raw signal intensities of each array were normalized to the median value of all rat probes with the BRB-Array Tool ver. 3.7.0. (Biometric Research Branch, NIH, Bethesda, Maryland, USA) [18]. A significance level of $p < 0.01$ was set for each probe using a univariate Student's t-test.

2.5 Annotation of Up or Down Regulated Genes in SHR substrains Compared with WKY

A BLASTN search of the NCBI RefSeq database using corresponding 60-nucleotide probes (NCBI, GEO accession: GPL7294) was performed to identify homologous genes with functional annotations [19]. After running a BLASTN search (ratus norvegicus), clones showing a gene expression score that was at least 4-fold higher or 4-fold

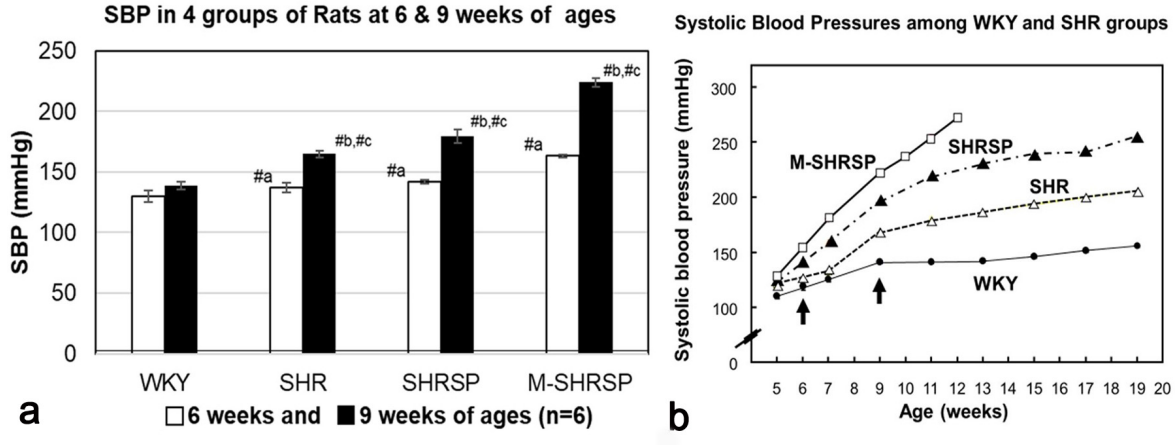


Fig. 1. Systolic blood pressure (SBP) was measured using the tail-cuff method. Three consecutive SBP readings were taken in the morning after warming the body to 35 °C for 5 min in a heater box. SBP values are expressed as the mean ± SEM. (a) The SBP of each male rat (6 rats per group) was measured at 6- (open rectangles) or 9-weeks (closed rectangles) of age. Comparison of the means in each group was performed using ANOVA and Scheffe's multiple comparison tests. Differences were considered to be statistically significant at $p < 0.05$. #**a** indicates statistical significance compared to the SBP of WKY at 6-weeks of age, #**b** indicates statistical significance compared to the SBP of WKY at 9-weeks of age, and #**c** indicates statistical significance compared to the same group of rats at 6-weeks of age. (b) SBP values in 4 strains of rats (6–12 rats each) measured at 5–20 weeks of age (except at 5–12 weeks in M-SHRSP with a high mortality after 12 weeks of age) were obtained in a separate experiment. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone SHR; M-SHRSP, malignant SHRSP.

lower (0.25-fold) than that of WKY were defined as annotated clones. The remaining clones were defined as non-annotated clones (refer to the Results and Discussion sections). The annotated gene and protein symbols are shown in italics and regular font, respectively. The logical basis for establishing this threshold was as follows. When only the genes up regulated more than 4-fold were sought, we identified 566, 669 and 813 genes at 6-weeks of age, and 36, 131 and 9 at 9-weeks of age in SHR, SHRSP and M-SHRSP rats, respectively. For down regulated genes at a threshold of 0.5-fold, we identified 1053, 1401 and 1846 genes at 6-weeks of age, and 121, 591 and 27 at 9-weeks of age in SHR, SHRSP and M-SHRSP rats, respectively. Using a threshold of 0.25-fold down regulation compared to WKY rats, 320, 387 and 576 genes were identified at 6-weeks of age, and 34, 7 and 11 genes at 9-weeks of age in SHR, SHRSP and M-SHRSP rats, respectively.

2.6 Strategies to Identify Candidate Genes Related to Hypertension

The following analyses were carried out to identify candidate genes related to or causing hypertension, as described above. First, data from the comparison of the SHR substrains to WKY was used to survey candidate genes among the SHR, SHRSP and M-SHRSP substrains in ascending order of blood pressure. Second, data from the comparison between the 6- and 9-week old rats from each SHR substrain at similar blood pressures was used to survey the candidate genes in the order of up or down regulated expression.

2.7 Analyses of Genes Expressed in Biochemical Pathways

Analyses of the roles of genes expressed in biochemical pathways was performed using Skypainter from REACTOME, a free and open-source database (http://www.reactome.org/) available at the Cold Spring Harbor Laboratory website, The European Bioinformatics Institute and The Gene Ontology Consortium. Gene biochemical information was mainly obtained from the GenBank database (the NIH genetic sequence database, https://ncbi.nlm.nih.gov). The possibility of the epigenetic modification of candidate genes was explored using the Genome Browser Gateway (University of California Santa Cruz Genomic Institute, http://genome-asia.ucsc.edu/cgi-bin/).

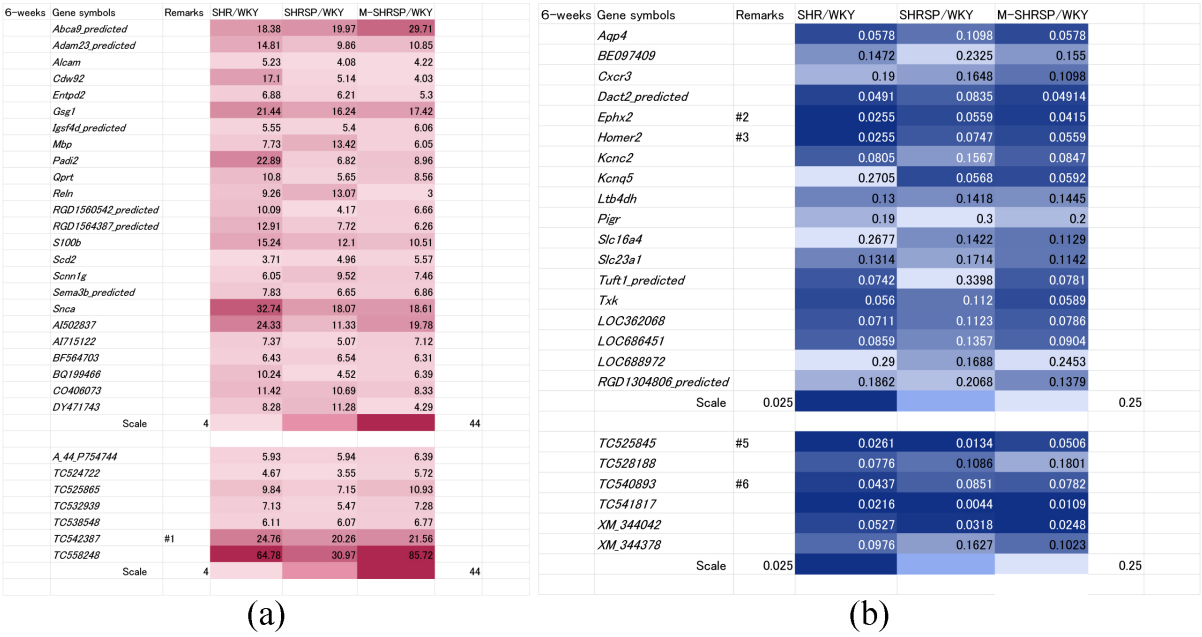
2.8 Statistical Analyses

Comparisons between the means of the data in each group were performed using one-way analysis of variance (ANOVA) and Scheffe's multiple comparison tests. Differences were considered significant at $p < 0.05$ for BP measurements, and $p < 0.01$ and FDR <0.05 for DNA array measurements.

3. Results

3.1 Systolic Blood Pressure at 6- and 9-Weeks of Age in Four Rat Strains

Systolic blood pressure (SBP) was measured for the WKY, SHR, SHRSP and M-SHRSP strains was measured every week between 6- to 9-weeks of age (Fig. 1a). SBP levels were significantly higher (or tended higher) at both



6-weeks	Gene symbols	Remarks	SHR/WKY	SHRSP/WKY	M-SHRSP/WKY
	Abca9_predicted		18.38	19.97	29.71
	Adam23_predicted		14.81	9.86	10.85
	Alcam		5.23	4.08	4.22
	Cdw92		17.1	5.14	4.03
	Entpd2		6.88	6.21	5.3
	Gsg1		21.44	16.24	17.42
	Igsf4d_predicted		5.55	5.4	6.06
	Mbp		7.73	13.42	6.05
	Padi2		22.89	6.82	8.96
	Qprt		10.8	5.65	8.56
	Reln		9.26	13.07	3
	RGD1560542_predicted		10.09	4.17	6.66
	RGD1564387_predicted		12.91	7.72	6.26
	S100b		15.24	12.1	10.51
	Scd2		3.71	4.96	5.57
	Scnn1g		6.05	9.52	7.46
	Sema3b_predicted		7.83	6.65	6.86
	Snca		32.74	18.07	18.61
	AI502837		24.33	11.33	19.78
	AI715122		7.37	5.07	7.12
	BF564703		6.43	6.54	6.31
	BQ199466		10.24	4.52	6.39
	CO406073		11.42	10.69	8.33
	DY471743		8.28	11.28	4.29
	Scale	4			44
	A_44_P754744		5.93	5.94	6.39
	TC524722		4.67	3.55	5.72
	TC525865		9.84	7.15	10.93
	TC532939		7.13	5.47	7.28
	TC538548		6.11	6.07	6.77
	TC542387	#1	24.76	20.26	21.56
	TC558248		64.78	30.97	85.72
	Scale	4			44

(a)

6-weeks	Gene symbols	Remarks	SHR/WKY	SHRSP/WKY	M-SHRSP/WKY
	Aqp4		0.0578	0.1098	0.0578
	BE097409		0.1472	0.2325	0.155
	Cxcr3		0.19	0.1648	0.1098
	Dact2_predicted		0.0491	0.0835	0.04914
	Ephx2	#2	0.0255	0.0559	0.0415
	Homer2	#3	0.0255	0.0747	0.0559
	Kcnc2		0.0805	0.1567	0.0847
	Kcnq5		0.2705	0.0568	0.0592
	Ltb4dh		0.13	0.1418	0.1445
	Pigr		0.19	0.3	0.2
	Slc16a4		0.2677	0.1422	0.1129
	Slc23a1		0.1314	0.1714	0.1142
	Tuft1_predicted		0.0742	0.3398	0.0781
	Txk		0.056	0.112	0.0589
	LOC362068		0.0711	0.1123	0.0786
	LOC686451		0.0859	0.1357	0.0904
	LOC688972		0.29	0.1688	0.2453
	RGD1304806_predicted		0.1862	0.2068	0.1379
	Scale	0.025			0.25
	TC525845	#5	0.0261	0.0134	0.0506
	TC528188		0.0776	0.1086	0.1801
	TC540893	#6	0.0437	0.0851	0.0782
	TC541817		0.0216	0.0044	0.0109
	XM_344042		0.0527	0.0318	0.0248
	XM_344378		0.0976	0.1627	0.1023
	Scale	0.025			0.25

(b)

Fig. 2. Genes significantly up or down regulated in all three SHR substrains at 6-weeks of age vs age-matched WKY controls. Genes either up regulated more than 4-fold (a) or down regulated less than 0.25-fold (b) in all three SHR substrains (SHR, SHRSP or M-SHRSP) compared to age-matched WKY are expressed in heat maps. The color grading corresponds to fold-changes in gene expression, as illustrated by the scale shown in the final row. Numbers shown in the remarks column refer to the duplication of gene data from other figures. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone SHR; M-SHRSP, malignant SHRSP.

6- and 9-weeks in all SHR substrains compared with the age-matched WKY controls. The SBP of control rats was 110 to 140 mm Hg, while in hypertensive rats including SHR the SBP was considered to be hypertensive at 150 mm Hg or more. The SBP of SHRSPs increased from 142 mm Hg at 6-weeks to 179 mm Hg at 9-weeks. In the same period, the SBP of M-SHRSPs increased from 163 to 224 mm Hg. Moreover, the SBP values of SHRSPs and M-SHRSPs at 12-weeks of age increased further to 235 and 265 mm Hg, respectively (Fig. 1b). The SBP of 9-week old SHRs (165 mm Hg) was similar to that of M-SHRSP (163 mm Hg) at 6-weeks of age. These results show that the SBP of SHR and M-SHRSP at 9- and 6-weeks, respectively, was almost the same, and at 9-weeks it was significantly higher in both SHRSPs and M-SHRSPs. In light of these observations, we hypothesized that genes associated with the onset of hypertension are preferentially expressed in the former comparisons (SHR vs M-SHRSP) at 6-weeks of age, while those involved in the maintenance/strengthening of hypertension are predominant in the latter comparison (SHRSP vs M-SHRSP). This hypothesis was evaluated further by additional analysis of the data.

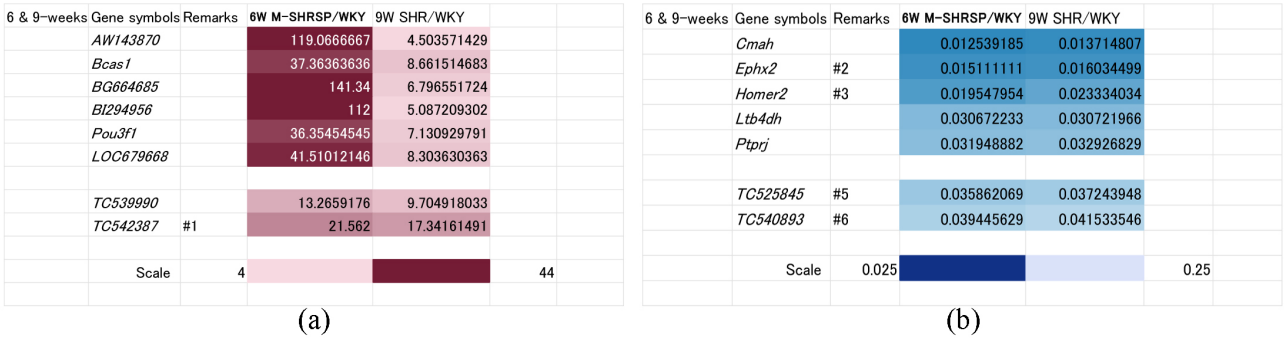
3.2 Genes Up or Down Regulated in All Three SHR Substrains Compared with WKY

Genes whose expressions were up regulated more than 4-fold in all three SHR substrains compared to WKY rats at 6-weeks of age are shown in Fig. 2a. In total, 31 genes were identified, of which 24 were known and 7 unknown.

The use of heat maps allows the comprehensive comparison of gene expression between different SHR strains and WKY. Expression of 13 of the 31 genes was significantly elevated compared with WKY, indicating a strong correlation between higher gene expression and high blood pressure at 6-weeks of age. The known genes were *Abca9*_predicted, *Adam23_predicted*, *Gsg1*, *Padi2, Qprt, S100b*, *Snca*, *AI502837*, *AI715122*, and *CO406073*, while the unknown genes were *TC525865*, *TC542387* and *TC558248*. Conversely, the expression of 24 genes (18 known and 6 unknown) was down regulated more than 0.25-fold in all 3 substrains compared to WKY (Fig. 2b). Amongst these, 9 showed a strong correlation between low expression levels and higher blood pressure in the SHR substrains (known genes: *Aqp4*, *Cxcr3*, *Ephx2*, *Homer2*, *Kcnc2*, *Kcnq5*, *Ltb4dh*; unknown genes: *XM_344042* and *XM_344378*).

3.3 Genes Significantly Up or Down Regulated in 9-Week old SHR and in 6-Week Old M-SHRSP Compared with Age-Matched WKY

As shown above, the SBP of 9-week old SHRs was comparable to that of 6-week old M-SHRSPs (Fig. 1), suggesting the involved genes are associated with the onset and/or strengthening of hypertension. Comparison using heat maps identified 8 up regulated genes (known: *AW143870*, *Bcas1*, *BG664685*, *BI292956*, *Pou3f1*, and *LOC679668*; unknown: *TC539990*, and *TC542387*)



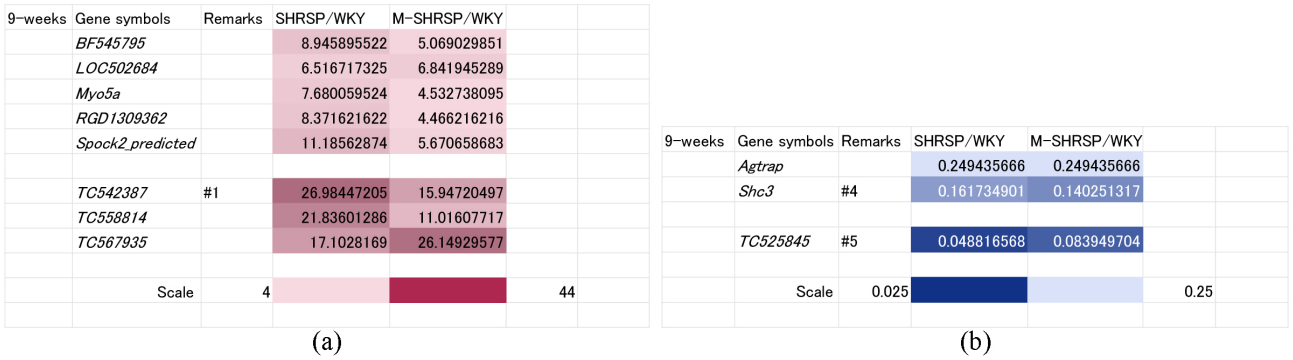
6 & 9–weeks	Gene symbols	Remarks	6W M–SHRSP/WKY	9W SHR/WKY
	AW143870		119.0666667	4.503571429
	Bcas1		37.36363636	8.661514683
	BG664685		141.34	6.796551724
	BI294956		112	5.087209302
	Pou3f1		36.35454545	7.130929791
	LOC679668		41.51012146	8.303630363
	TC539990		13.2659176	9.704918033
	TC542387	#1	21.562	17.34161491
	Scale	4		44

(a)

6 & 9–weeks	Gene symbols	Remarks	6W M–SHRSP/WKY	9W SHR/WKY
	Cmah		0.012539185	0.013714807
	Ephx2	#2	0.015111111	0.016034499
	Homer2	#3	0.019547954	0.023334034
	Ltb4dh		0.030672233	0.030721966
	Ptprj		0.031948882	0.032926829
	TC525845	#5	0.035862069	0.037243948
	TC540893	#6	0.039445629	0.041533546
	Scale	0.025		0.25

(b)

Fig. 3. Genes significantly up or down regulated both in 9-week old SHRs and in 6-week old M-SHRSPs compared with age-matched WKYs. Heat maps of genes up regulated more than 4-fold (a), or down regulated more than 0.25-fold (b), in both 9-week old SHRs and in 6-week old M-SHRSPs vs age-matched WKY. Numbers shown in the remarks column refer to the duplication of gene data from other figures. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; M-SHRSP, malignant SHRSP.



9–weeks	Gene symbols	Remarks	SHRSP/WKY	M–SHRSP/WKY
	BF545795		8.945895522	5.069029851
	LOC502684		6.516717325	6.841945289
	Myo5a		7.680059524	4.532738095
	RGD1309362		8.371621622	4.466216216
	Spock2_predicted		11.18562874	5.670658683
	TC542387	#1	26.98447205	15.94720497
	TC558814		21.83601286	11.01607717
	TC567935		17.1028169	26.14929577
	Scale	4		44

(a)

9–weeks	Gene symbols	Remarks	SHRSP/WKY	M–SHRSP/WKY
	Agtrap		0.249435666	0.249435666
	Shc3	#4	0.161734901	0.140251317
	TC525845	#5	0.048816568	0.083949704
	Scale	0.025		0.25

(b)

Fig. 4. Genes significantly up or down regulated both in SHRSPs and in M-SHRSPs at 9-weeks of age compared with age-matched WKY. Heat maps show genes either up regulated more than 4-fold (a) or down regulated more than 0.25-fold (b) in both 9-week old SHRSPs and in M-SHRSPs vs age-matched WKY. Numbers shown in the remarks column refer to the duplication of gene data from other figures. WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; M-SHRSP, malignant SHRSP.

(Fig. 3a). Conversely, 7 genes were down regulated in this period (Fig. 3b), including *Cmah*, *Ephx2*, *Homer2*, *Ltb4dh*, and *Ptprj* (known genes), and *TC525845* and *TC540893* (unknown genes).

3.4 Genes Significantly Up or Down Regulated Both in SHRSPs and in M-SHRSPs Compared with WKY at 9-Weeks of Age

As shown in Fig. 1, SBP was already significantly elevated at 9-weeks of age in both SHRSPs and M-SHRSPs, suggesting the likely dominance of genes involved in the maintenance and/or strengthening of hypertension rather than its onset. The SBP of SHRSPs and M-SHRSPs was 179 and 224 mm Hg, respectively, compared to 164 mm Hg for age-matched SHRs. Accordingly, 8 genes were up regulated (known: *BF545795*, *LOC502684*, *Myo5a*, *RGD1309362*, and *Spock2*_predicted; unknown: *TC542387*, *TC558814*, and *TC567935*; Fig. 4a) and three were down regulated (known: *Agtrap* and *Shc3*; unknown: *TC525845*; Fig. 4b).

4. Discussion

In this study, we identified hypertensive-related genes that were either up or down regulated in SHR, SHRSP and/or M-SHRSP rats compared to WKY rats. These genes were classified into four categories according to their postulated association with hypertension: (1) genes primarily pathogenic for the onset of hypertension (Group-1); (2) genes whose expression does not appear to be related to the onset of hypertension, but which may strengthen hypertensive conditions (Group-2); (3) genes whose involvement in hypertension is presently unknown, but which could in future be linked to the onset or the strengthening of hypertension after further investigation (Group-3), and (4) genes whose involvement in hypertension is less likely, but which may be associated with physiological characteristics of SHRs other than hypertension (Group-4).

Through careful measurement, we found that the increase in SBP of SHRs at 9-weeks of age was similar to that of M-SHRSPs as young as 6-weeks old (Fig. 1a). Furthermore, the SBP was significantly higher in 9-week old SHRSP and M-SHRSP, even when compared with that

Table 1. Common up and down regulated genes in SHR/WKY, SHRSP/WKY, and M-SHRSP/WKY rats at 6-weeks of age, as well as their postulated hypertension group (Group-1 to Group-4).

Gene symbols	Remarks	Protein names	Postulated associated with hypertension
Abca9_predicted		ATP binding cassette subfamily A member 9	Group-4
Adam23_predicted		ADAM metallopeptidase domain 23	Group-1
Gsg1		germ cell associated 1	Group-3
Padi2		peptidyl arginine deiminase 2	Group-3
Qprt		quinolinate phosphoribosyltransferase	Group-3
S100b		S100 calcium binding protein B	Group-4
Snca		synuclein alpha	Group-3
AI502837		cDNA clone UI-R-BT0-pl-e-03-0-UI 3'	Group-3
AI715122		cDNA clone UI-R-Y0-abl-d-08-0-UI 3'	Group-3
CO406073		cDNA clone IMAGE:7319013 5'	Group-3
TC525865		Unknown gene	Group-3
TC542387	#1	Unknown gene	Group-3
TC558248		Unknown gene	Group-3
Aqp4		aquaporin 4	Group-4
Cxcr3		C-X-C motif chemokine receptor 3	Group-3
Ephx2	#2	epoxide hydrolase 2	Group-4
Homer2	#3	homer scaffold protein 2	Group-3
Kcnc2		potassium voltage-gated channel subfamily C member 2	Group-1
Kcnq5		potassium voltage-gated channel subfamily Q member 5	Group-1
Ltb4dh	#4	leukotriene B4 protein	Group-4
XM_344042		Unknown gene	Group-3
XM_344378		Unknown gene	Group-3

Based on physiological and biochemical information, each gene was classified into one of four groups as follows: Group-1: gene primarily pathogenic for the onset of hypertension; Group-2: gene whose expression does not appear to be related to the onset of hypertension, but which might strengthen hypertensive conditions; Group-3: gene whose involvement in hypertension is presently unknown, but which may in future be associated with the onset or strengthening of hypertension after further investigation; Group-4: gene whose involvement in hypertension is less likely but which might be associated with physiological characteristics of SHRs other than hypertension.
Numbers shown in the remarks column refer to the duplication of gene data from other figures.
WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; M-SHRSP, malignant SHRSP.

of 20-week old SHR from other experiments (Fig. 1b). Based on these observations, we hypothesize that most upstream genetic alterations relevant to the onset of hypertension occur in the early period, while the expressions of genes in the latter period may instead act to maintain or strengthen hypertensive conditions. Moreover, we postulate that the expression level of candidate genes would be altered in different substrains of SHRs, but not in WKY. To investigate this we employed DNA microarray technology as described previously by our group [15]. We identified several genes that may be associated with spontaneous hypertension in SHRs. At the age of 6-weeks, 13 mostly up regulated genes were selected from 31 genes listed in the upper part of Table 1. These genes were associated with increases in the SBP of SHR substrains: *Abca9*_predicted, *Adam23_predicted*, *Gsg1*, *Padi2, Qprt, S100b*, *Snca*, *AI502837, AI715122, CO406073, TC525865*, *TC542387* (#1) and *TC558248*.

Various hypertension-related genes identified in previous reports will now be considered one by one. The functional involvement of *Abca9*_predicted gene [20] in hypertension has so far been uncertain, but it may be causal for hypermetabolism in SHRs through ABC-mediated proteins transport of various molecules across extra- and intracellular membranes. We, therefore, categorized *Abca9*_predicted as Group-4. Adam23 was originally reported to be involved in cancer and neuronal differentiation [21,22]. More recently, Adam23 was reported to be a negative regulator of $K_{v1.1}/K_{v1.4}$ potassium currents [23]. Hence, the up regulation of *Adam23*_predicted observed in the present study is quite suggestive. It is well known that the opening of K^+ permeable channels will hyperpolarize the plasma membrane potential (E_m). The excitation of vascular smooth muscle cells (VSMC) followed by the depolarization of the E_m via the influx of Ca^{2+} and Na^+ or the efflux of Cl^- leads to the opening of voltage-dependent Ca^{2+} channels (VDCCs). In VSMC, the opening of VDCCs causes Ca^{2+} influx, leading to a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent activation of the contractile machinery. Depolarization also leads to the voltage-dependent opening of K_v channels, giving rise to a compensatory hyperpolarizing current that reduces the like-

lihood of VDCCs and inhibits vasoconstriction [24]. Although the role of Adam23 in VSMC is still unclear, its marked up-regulation may negatively regulate vasodilatation via functional suppression of vasodilative potassium currents, leading finally to increased vasocontraction. The overexpression of *Adam23*_predicted was therefore categorized as Group-1. The selective expression of *Gsg1* in testis may cause redistribution of PAPOLB from the cytosol to the endoplasmic reticulum. This prompted us to categorize *Gsg1* as Group-3, since the mechanism of action is not known. It has been reported that *Padi2* mediates arginine citrullination and modulates transcription in cancer [25] as well as the progression of neurodegenerative disorders. Since the role of citrullination in hypertension remains largely unknown, we classified the overexpression of *Padi2* into Group-3. There is some information regarding *Qprt* and elevated quinolinate levels in the brain linked to the pathogenesis of neurodegenerative disorders. However, to our knowledge there is no information in relation to SHRs. *Qprt* was therefore classified as Group-3. *S100b* [26] is expressed in the cytoplasm and/or nucleus of a wide range of cells and is a calcium binding protein involved in the regulation of various processes such as cell cycle progression and differentiation. This gene could thus be linked to hypermetabolic states in SHRs, and was therefore categorized as Group-4. According to a previous reports, *Snca* is preferentially expressed on neuronal cells [27], where it may serve to integrate presynaptic signaling and membrane trafficking. However, its role in arteries is not known and therefore it was categorized in Group-3. Almost no information is available for *AI502837, AI715122, CO406073, TC525865*, *TC542387* (#1, duplication gene also in other comparisons) and *TC558248.* These were classified as Group-3 given they have no known role in the biochemical mechanism of hypertension.

Nine of the 24 genes that were down regulated by more than 0.25-fold in all three SHR substrains at 6-weeks of age are shown in the lower part of Table 1. Known genes were *Aqp4*, *Cxcr3*, *Ephx2* (#2), *Homer2* (#3), *Kcnc2*, *Kcnq5*, and *Ltb4dh* (#4), while the unknown genes were *XM_344042* and *XM_344378*. *Aqp4* (*aquaporin 4*) is expressed by astrocytes in the CNS (central nervous system) [28], and is involved in water movement, cell migration and neuroexcitation [29]. It was classified into Group-4, since down regulation of this gene may cause hydrocephaly in SHRs. Previously, it was reported that mice lacking the Cxcr3 chemokine receptor suffer from hypertension [30]. Mice deficient in *Cxcr3* exhibit up regulation of angiotensin II type I receptor (ATIR) in VSMC, suggesting the importance of this gene in vascular contractility and hypertension through up regulation of *AT1R* expression. However, since the mechanistic relationship between down regulation of *Cxcr3* and up regulation of *AT1R* is not yet clear, this gene was classified as Group-3. Due to the potent vasodilatory effects of epoxyeicosatrienoic acids (EETs) on VSMC, the metabolizer of EETs on the overproduction of Ephx2 leads to reduced vasorelaxation and hence to subsequent hypertension [31]. However, down regulation of *Ephx2* (#2) in the mesenteric artery does not appear to be actively involved in the pathogenesis of hypertension. Based on our observations, the down regulation of *Ephx2* may be compensatory for increased blood pressure and was therefore classified as Group-4. *Homer2* is widely expressed in many tissue types including muscles (NCBI Gene Databank, Gene ID: 29547). Although the long form Homer 1c plays a role in synaptic changes during long-term potentiation, the biological role of *Homer 2* in the circulatory system is not yet clear. Thus, *Homer2* was categorized as Group-3. The *Kcnc2* and *Kcnq5* genes are likely to be involved in the development of hypertension. Bearing in mind the role of K^+ channels in vasodilatation, down regulation of *Kcnc2* and *Kcnq5* causes spontaneous hypertension. Furthermore, Yoshimura Y previously reported that potassium-induced contraction of isolated aortic strips at concentrations of 10–100 mM were significantly greater in SHRSP than in WKY at 1.5, 3, 6 and 12 months of age [32]. This finding could indicate increased susceptibility of the vascular contractile machinery of SHR to K^+. Therefore, the *Kcnc2* and *Kcnq5* genes were classified as Group-1 in the present study. *Ltb4dh* protein catalyzes leukotriene B4 (LTB4) to increase its activity [33]. LTB4 activates human pulmonary artery adventitial fibroblasts in pulmonary hypertension, causing increased vascular stiffness of the pulmonary artery [34]. The down regulation of *Ltb4dh* observed in the present study might result in prolonged activation of LTB4, thereby causing perivascular fibrosis in the mesenteric artery and increasing the vascular tone. Therefore, *Ltb4dh* was classified into Group-4. The biological role of the two unknown genes (XM_344042 and XM_344378) observed here is uncertain according to the NCBI 'Gene' information resources and were therefore classified as Group-3.

Particular attention was also paid in this study to genes that were up or down regulated in M-SHRSP and SHR at 6- and 9-weeks of age, respectively. As shown in Fig. 3, 8 genes were up regulated and 7 genes were down regulated. The known up regulated genes were *AW143870*, *Bcas1*, *BG664685*, *BI292956*, *Pou3f1*, and *LOC679668*, while the unknown genes were *TC539990*, and *TC542387* (upper part in Table 2).

Since there is little information on *AW143870,* this gene was categorized as Group-3. *Bcas1* is known to reside in a region of 20q13 that is amplified in several tumor types [35]. However, there is no information with regard to hypertension and hence this gene was categorized as Group-3. There is also little biochemical information available for *BG664685* and *BI294956. Pou3f1* is thought to be involved in early embryogenesis and neurogenesis [36]. *LOC679668* encodes a leucine-rich repeat transmembrane protein that interacts with neurexins and neuroligins to modulate synap-

Table 2. Common up and down regulated genes in 6-week old M-SHRSP/WKY and in 9-week old SHR/WKY together with their postulated hypertension group as shown in upper, and in 9-week old SHRSP/WKY and M-SHRSP/WKY together with their postulated hypertension group as shown in below (Group-1 to Group-4).

Gene symbols	Remarks	Protein names	Postulated association with hypertension
(Up and down regulated genes in 6-week old M-SHRSP/WKY and in 9-week old SHR/WKY)			
AW143870		cDNA clone RGICB87 5' end	Group-3
Bcas1		brain enriched myelin associated protein 1	Group-3
BG664685		cDNA clone DRABHF03 5'	Group-3
BI294956		cDNA clone UI-R-DK0-cee-a-12-0-UI 3'	Group-3
Pou3f1		POU class 3 homeobox 1	Group-3
LOC679668		LRRTM1:leucine rich repeat transmembrane neuronal 1	Group-3
TC539990		unknown gene	Group-3
TC542387	#1	unknown gene	Group-3
Cmah		cytidine monophospho-N-acetylneuraminic acid hydroxylase	Group-3
Ephx2	#2	epoxide hydrolase 2	Group-4
Homer2	#3	homer scaffold protein 2	Group-3
Ltb4dh	#4	leukotriene B4 protein	Group-4
Ptprj		protein tyrosine phosphatase receptor type J	Group-4
TC525845	#5	unknown gene	Group-3
TC540893		unknown gene	Group-3
(Up and down regulated genes in 9-week of old SHRSP/WKY and M-SHRSP/WKY)			
BF545795		cDNA clone UI-R-BT0-qc-d-07-0-UI 5'	Group-3
LOC502684		hypothetical protein LOC502684	Group-3
Myo5a		myosin VA	Group-3
RGD1309362		similar to interferon-inducible GTPase	Group-3
Spock2_predicted		osteonectin, cwcv and kazal like domains proteoglycan 2	Group-2
TC542387	#1	unknown gene	Group-3
TC558814		unknown gene	Group-3
TC567935		unknown gene	Group-3
Agtrap		angiotensin II receptor associated protein	Group-2
Shc3		SHC adaptor protein 3	Group-4
TC525845	#5	unknown gene	Group-3

Based on physiological and biochemical information, each gene was classified into one of the four groups described in Table 1 above. Numbers shown in the remarks column refer to the duplication of gene data from other figures. WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; M-SHRSP, malignant SHRSP.

tic cell adhesion. *TC539990* and *TC542387* are unknown genes. Hence, these 6 genes were also classified as Group-3.

Cmah is expressed in the brain and neuronal cells and produces N-glycolylneuraminic acid [37]. However, there is no information available in relation to hypertension and therefore it was categorized as Group-3. *Ephx2*, *Homer2* and *Ltb4dh* were categorized as Group-4, Group-3 and Group-4, respectively, as mentioned above. *Ptprj* is a member of the protein tyrosine phosphatase family and regulates a variety of cellular processes including cell growth. Since the down regulation of *Ptprj* might act as compensatory factor for hypermetabolism in SHRs, this gene was classified as Group-4. *TC525845* and *TC540893* are unknown genes and were therefore classified as Group-3.

Lastly, we focused on genes that were up regulated in both SHRSPs and M-SHRSPs at 9-weeks of age (Fig. 4). These were categorized as shown in below of Table 2. Since there is little information on *BF545795*, *LOC502684* and *RGD1309362* in relation to hypertension [38], these genes were classified as Group-3. *Myo5a* belongs to the Myosin V heavy-chain class of genes [39] that code for actin-based motor protein involved in cytoplasmic vesicle transport and mRNA translocation. Since this could relate to hyperactivation in hypertension, *Myo5a* was classified as Group-4 because there is still little information on the circulatory system. *Spock2* is an extracellular heparan/chondroitin sulfate proteoglycan [40]. Vascular stiffness is an important factor for vascular resistance and is regulated by ECM [41]. Increased vascular stiffness in SHRs caused by the overproduction of *Spock2* may strengthen hypertension, hence this gene was categorized as Group 2. There is little information on the biochemical pathways for *TC542387* (#1), *TC558814*, and *TC567935* and hence these were classified as Group-3.

Agtrap, *Shc3* and *TC525845* were markedly down reg-

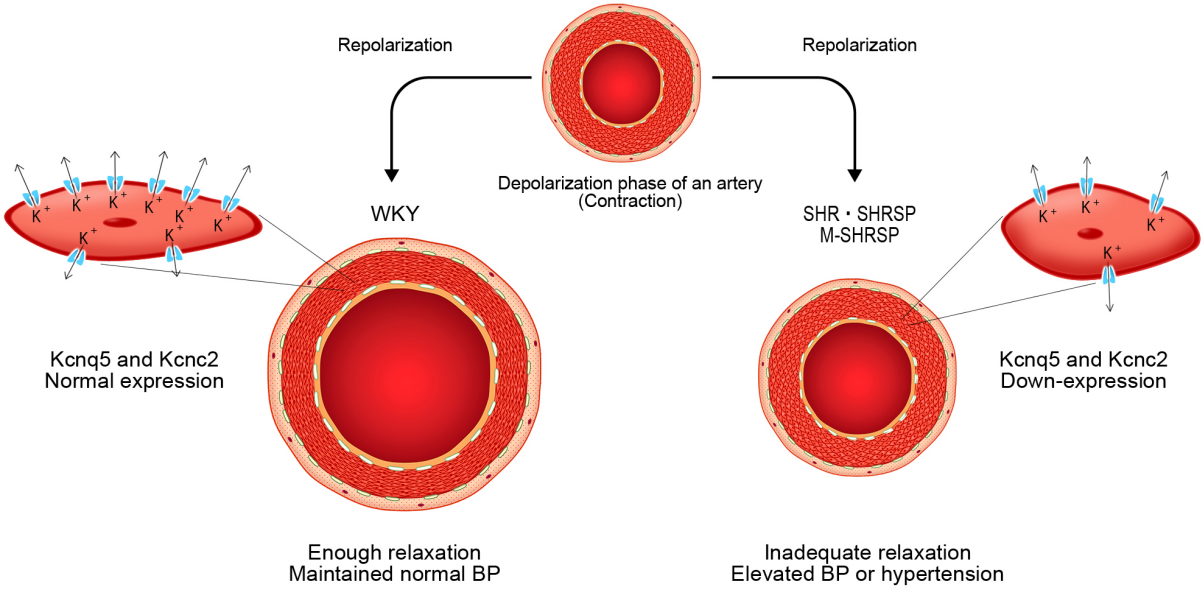


Fig. 5. Schematic representation of the initial changes that occur during the onset of spontaneous hypertension. In WKY, the mesenteric-resistant artery recovers from contraction following the depolarization of the E_m for VSMC via the repolarization processes, in which the efflux of K^+ occurs through K^+ channels, i.e., Kcnq5 and Kcnc2. In 6-week old SHRs, SHRSPs and M-SHRSPs at the age of 6 weeks, both K^+ channels are constitutively downregulated, resulting in inadequate vasorelaxation and consequently an increase in blood pressure (BP). WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; M-SHRSP, malignant SHRSP.

ulated at 9-weeks of age. *Agtrap* interacts with AT1R to promote its constitutive internalization, thus inhibiting the hyperactivation of downstream signaling and augmenting the effects of angiotensin II (Ang II) [42]. Using an *ex vivo* system, Zhang *et al*. [43] recently reported that treatment of the mesenteric artery with Ang II led to the attenuation of acetylcholine (Ach)-induced vascular relaxation, thus demonstrating a direct effects of Ang II on the mesenteric artery. *Agtrap* is likely to counteract vasoconstriction of the mesenteric artery via the constitutive internalization of AT1R, thus inhibiting the activation of Ang II signaling. Down regulation of *Agtrap* in SHRs may therefore lead to increased activation of Ang II signaling in the mesenteric artery. This is at least partly causative of hypertension and *Agtrap* was therefore classified as Group-2. *Shc3* is predicted to be involved in glutamatergic synaptic transmission and epidermal growth factor/neuregulin signaling in the tyrosine kinase-signaling pathway. Thus, *Shc3* reduces hypertension and was classified as Group-4. No information was available for the down regulated gene *TC525845* (#5, unknown gene) and therefore it was categorized as Group-3.

5. Conclusions

To summarize the above findings, the genes thought to be involved in the onset of hypertension (Group-1) or to promote, maintain or strengthen hypertension (Group-2) were identified as follows.

Group-1: *Adam23*_predicted, *Kcnc2*, and *Kcnq5*

Group-2: *Spock*_predicted, and *Agtrap*

The down regulation of K^+ channel genes (*Kcnq5* and *Kcnc2*) and the functional suppression of potassium currents via overexpression of *Aadm23* may impair the capacity for vasodilatation and subsequently enhance vasoconstriction. These hypertensive conditions may be increased by genes belonging to Group 2 including reduced expression of *Agtrap* and overexpression of *Spock2*_predicted. This may lead to increased activation of Ang II signaling, and to increased vascular stiffness via the deposition of ECM and vascular fibrosis. Although further validation is required, the current findings provide a framework to understand the pathophysiological basis of hypertension.

Two genes thought to be directly involved in the onset of hypertension were *Kcnc2*, and *Kcnq5*. Fig. 5 shows the proposed action of these genes in more detail.

Author Contributions

Conceptualization—YA and HH; methodology—MSA and HH; software—TH and KK; validation—TH and KK; investigation—MSA and HH; data curation—YA and HH; writing—YA and HH; and supervision—HH.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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