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Identification of blood pressure genes in the Dahl salt-sensitive hypertension model

par
Julie Dutil

Département de Biologie Moléculaire
Faculté des études supérieures

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présentée par

Julie Dutil

A été évaluée par un jury composé des personnes suivantes:

Dr Richard Bertrand
président-rapporteur

Dr Alan Deng
directeur de recherche

Dr Christian Deschepper
membre du jury

Dr Joseph Dent
examinateur externe

Dr Pierre Moreau
représentant du doyen de la FES

Summary in English

Hypertension afflicts 21% of the Canadian population and is a leading cause of death as it is associated with an increased risk of cardiovascular diseases. Blood pressure (BP) regulation is a complex trait controlled by multiple genes and influenced by environment. Little is known about the genes responsible for BP regulation. Inbred rats genetically predisposed to hypertension have been developed to facilitate the study of the genetic basis to this disease. Preliminary linkage and congenic experiments have shown the presence of at least one BP quantitative trait loci (QTL) in a 80cM region of rat chromosome (chr) 2. The objective of the present thesis was to identify and characterize the rat chr 2 regions involved in the genetic control of hypertension. We are using congenic strains in which various segments from the Dahl salt-sensitive (DSS) rat chr 2 are replaced by the homologous segments coming from the Milan normotensive rat (MNS). This model has allowed to narrow down the rat chr 2 BP QTL to three non-overlapping regions each containing at least one distinct BP QTL: C2QTL1 (5.7cM), C2QTL2 (3.5cM) and C2QTL3 (1.5cM). These closely linked BP QTL showed epsitatic and additive interactions. We also identified a QTL for vascular smooth muscle cell (VSMC) number acting independently of BP. The gene coding for the angiotensin receptor 1b (*Agtr1b*) is located in the region containing the QTL for SMCN. *Agtr1b* is a candidate for the SMCN QTL because 1) two non-synonymous mutations were found in the coding region between DSS and MNS rats, and 2) contractile responses to Ang II are reduced in rats harbouring the MNS allele of the gene compared with DSS rats. The current work provide new insights about the genetic determination of hypertension and of vascular remodelling disorders. In the future, these knowledge may be useful in the development of new therapeutic targets, genetic diagnostic tools and pharmacogenomics.

Keywords: genetics of hypertension, Dahl salt-sensitive rat, quantitative trait loci, aortic hyperplasia, vasoreactivity.

Résumé en français

L'hypertension affecte 21% de la population canadienne et est un facteur de mortalité important car elle est associée à un risque accru de maladies cardiovasculaire. La régulation de la pression artérielle (PA) est un trait complexe impliquant plusieurs gènes et une composante environnementale. Les gènes responsables de la régulation de la PA sont peu connus. Les modèles de rats consanguins ont été développé pour faciliter l'étude des bases génétiques de l'hypertension. Des études préliminaires de liaison génétique et de lignées congéniques ont démontré la présence d'au moins un locus pour trait quantitatif (QTL) pour la PA dans une région de 80 cM sur le chromosome (chr) 2 du rat. L'objectif de cette thèse était d'identifier, de définir et de caractériser les régions du chr 2 du rat impliquées dans la détermination génétique de l'hypertension. Pour atteindre cet objectif, nous utilisons des lignées congéniques dans lesquelles divers segments du chr 2 du rat Dahl salt-sensitive (DSS) ont été remplacé par la région homologue provenant du rat Milan normotendu (MNS). Ce modèle a permis de réduire la région d'intérêt du QTL du chr 2 du rat à 3 régions non-chevauchantes contenant chacune un QTL pour la PA distinct: C2QTL1 (5.7 cM), C2QTL2 (3.5 cM) and C2QTL3 (1.5 cM). Ces loci ont démontré des interactions additives et épistatiques. Nous avons aussi identifié un QTL pour le nombre de cellules musculaire lisses (SMCN) vasculaires agissant indépendamment de la PA. Le gène codant pour le récepteur 1b de l'angiotensine (*Agtr1b*) est situé dans la région contenant le SMCN QTL. Agtr1b est un gène candidat pour le SMCN QTL puisque 2 mutations non-synonymes ont été identifiées dans la région codante entre les séquences du rat DSS et du MNS, et 2) la réponse contractile à l'angiotensine II est diminuée dans les rats portant les allèles MNS du gène comparativement aux rats DSS. Ce travail apporte de nouvelles précisions sur les déterminants génétiques de l'hypertension et des maladies de remodelage vasculaire. Ces connaissances pourraient être utiles au développement de nouvelles cibles thérapeutiques, d'outils de diagnostique génétique et à l'avancement de la pharmacogénomique.

Mots clé: génétique de l'hypertension, rat Dahl salt-sensitive, loci pour trait quantitatif, hyperplasie aortique, vasoréactivité.

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List of abbreviations

AA	Arachidonic acid
ACE	Angiotensin converting enzyme
Ach	Acetylcholine
ACTH	Adrenocorticotropic-hormone
AGT	Angiotensinogen
AME	Apparent mireralocorticoid excess
Ang I	Angiotensin I
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
AT1	Ang II receptor 1
AT2	Ang II receptor 2
BC1	Backcross generation
BNP	Brain natriuretic peptide
BP	Blood pressure
Ca2+	Calcium
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CHF	Congestive heart failure
CHHS	Canadian heart health survey
chr.	Chromosome
cM	centiMorgans (cM)
CNP	C-type natriuretic peptide
CNS	Central nervous system
CO_2	Carbon dioxide
CO	Cardiac output
COX	Cyclooxygenase
cR	CentiRays
DBP	Diastolic blood pressure
DG	Diacylglycerol
DSR	Dahl salt-resistant
DSS	Dahl salt-sensitive
EET	Epoxyeicosatrienoic acids
ENaC	Amiloride-sensitive epithelial sodium channel
eNOS	Endothelial NOS
ET-1	Endothelin 1
EST	Expressed sequence tag
FAK	Focal adhesion kinase
F1	First filial generation
GFR	Glomerular filtration rates
GH	the New Zealand
GPK	GPR protein kinase
GPR	G protein-coupled receptor
GRA	Glucocorticoid remediable aldosteronism

GTP	Guanosine triphosphate
HR	Heart rate
iNOS	Inducible NOS
IP3	Inositol triphosphate
JAK/STAT	Janus Kinase /Signals transducers and activators transcription
LDL	Low density lipoproteins
Lew	Lewis
LH	Lyon hypertensive
LT	Leukotrienes
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinases
MHS	Milan hypertensive
MME	Metalloendopeptidase
mmHg	Milimeter of mercury
MNS	Milan normotensive
NaCl	Sodium chloride
NCCT	Na-Cl co-transporter
NCX	Sodium/Calcium Na+/Ca2+ exchanger
Nedd4	neuronal precursor cells expressed, developmentally downregulated
NO	Nitric oxide (NO)
NOS	NO synthase (NOS)
NPR	Natriuretic peptide receptor
NTS	Nucleus tractus solitarius
O2	Oxygen
PDE	Phophodiesterase
PGs	Prostaglandins
PHAII	Pseuhypodoaldosteronism type II
PIP2	4,5 biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PNS	Peripheral nervous system
PP	Pulse pressure
QTL	Quantitative trait locus
RAS	Renin angiotensin system
RFLP	Restriction fragment lenght polymorphism
RH	Radiation hybrid
RIHP	Renal interstitial hydrostatic pressure
ROMK	Renal outer medullary potassium channel
ROS	Reactive oxygen species
S-GC	Soluble guanylyl cyclase
SNP	Single nucleotide polymorphism
STS	Sequence tag site

RVLN	Rostral ventrolateral nucleus
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SHR-SP	SHR stroke prone
SMC	Smooth muscle cells
TPR	Total peripheral resistance
TXs	Thromboxanes
UTR	Untranslated region
WNK	With no lysine
WKY	Wistar Kyoto
11ßHSD	11 ß-hydroxysteroid dehydrogenase
20-HETE	20-hydroxyeicosatetraenoic acid
5,6-EET	5,6-epoxyeicosatrienoic acid

CHAPTER 1

INTRODUCTION

1.1 PHYSIOLOGY OF BLOOD PRESSURE

The cardiovascular system is responsible for the distribution of blood to all parts of the organism. It is composed of a pump, the heart and of a network of conduits, the blood vessels. The heart provides the force necessary for the blood to circulate through the arteries and veins. The blood is the vehicle ensuring the transport of several substances. A proper blood flow allows the distribution of heat, ensures that the nutriments and oxygen (O_2) reach the tissues and that carbon dioxide (CO_2) and waste are removed from the tissues. Blood also ensures the maintenance of a stable pH in the organism with the buffer substances it contains.

1.1.1 Definition

Blood pressure (BP) is defined as the force exerted by the blood per unit of vessel wall area. Circulation of the blood in the body follows a pulsatile pattern reaching its highest peak during ventricular contractions of the heart and its lowest level between cardiac contractions. The force necessary for the propulsion of the blood in the entire organism relies on the strength of heart beats and on the pressure gradient present in the cardiovascular system. In effect, there is a progressive lowering of the blood pressure as it travels from large arteries such as aorta to arterioles until it reaches heart atria through vena cava. Systolic blood pressure (SBP) corresponds to the blood pressure during ventricular contraction whereas diastolic blood pressure (DBP) is defined as the blood pressure between each ventricular contraction. Mean arterial pressure (MAP) is the average between diastolic and systolic blood pressures and reflects the pressure to which small vessels are continuously submitted. Pulse pressure (PP) is calculated by subtracting DAP to SAP and is an indication of the compliance of large arteries. BP is measured in millimetres of mercury (mmHg). [1]

1.1.2 Hemodynamics

From a hemodynamic point of view, MAP is function of two major variables: i) cardiac output (CO), which is the quantity of blood ejected by the left ventricle during systole; and ii) total peripheral resistance (TPR) exerted by small arteries and arterioles. Thereby:

$$MAP = CO \times TVR$$

Where CO is influenced by systolic precharge and heart rate, and total vascular resistance is determined by the rayon of small arteries and the physical properties of the blood which include viscosity and concentration of fibrinogen. [1]

1.1.3 Regulation

A complex system of regulation is responsible blood pressure control to ensure constant blood flow to tissues and adjustment to meet specific demands of the organism. Regulation is achieved through the control of blood volume and of resistance vessels. It involves the participation of the endocrine system, of the central and peripheral nervous systems and of vasoactive substances acting locally.

1.1.3.1 Nervous system

The nervous system is a major player in the BP homeostasis as it is involved in short-term and long-term regulation. The effects of the nervous system on the cardiovascular system require the integration of afferent input from visceral organs and various brain regions by the central nervous system (CNS) and the transduction of the input in the appropriate cardiovascular adaptation via the parasympathetic and sympathetic divisions of the autonomic peripheral nervous system (PNS). The autonomic nervous system is responsible for controlling vital functions including heart rate and BP. Its actions are mostly involuntary. The autonomic nervous system is divided into the parasympathetic nervous system (PSNS) and the sympathetic nervous system (SNS) on the basis of structural and functional differences. Peripheral nervous system projects to target organs where it regulates cardiovascular function. The influence of the two divisions is opposed. [2]

1.1.3.1.1 Afferents inputs

The hypothalamus, the baroreceptors and the chemoreceptors ensure a constant monitoring of the cardiovascular system. The hypothalamus is the brain region responsible for the integration of environmental and behavioural input. It is the link between the endocrine and the neural systems to maintain homeostasis. Arterial baroreceptors are located mainly in the carotid sinus and in the wall of the aortic arch. They are involved in adaptation to rapid and important changes in blood pressure, like it is the case during postural changes. Cardiac baroreceptors are positioned in the walls of atria and ventricles. Baroreceptors respond to an increase in pressure or stretching. Chemoreceptor are found proximity to baroreceptors in carotid sinus and aortic arch. They are specialized in detecting changes in O_2, CO_2 and H^+ levels. [2,3]

1.1.3.1.2 Integration of afferent inputs

The nucleus tractus solitarius (NTS) of the medulla oblongata is the principal site of termination of afferent fibres arising from cardiovascular receptors, hypothalamus and cerebral cortex. Signal from different types of cardiovascular afferent fibres have different sites of termination in the NTS. The NTS integrates information from afferent fibres and adjust the cardiovascular system accordingly. [2,3] The NTS controls the parasympathetic nerves of the peripheral nervous system directly and projects to the rostral ventrolateral nucleus (RVLN) in the medulla. The RVLN is responsible for controlling the output of the sympathetic preganglionic neurons. The signalling of the NTS to RVLN is inhibitory, which means that excitatory signalling to NTS are translated by an increase in inhibition of RVLN and sympathetic output. [3,4]

1.1.3.1.3 Efferent outputs

Both division of the peripheral nervous system require two neurons to reach the target organs: a preganglionic neuron to conduct influx from the central nervous system to the ganglion and a postganglionic neuron which transmit the signal form the ganglion to the target organ. Preganglionic neurons of the PSNS and SNS signal by releasing the neurotransmitter acetylcholine (Ach) which binds to nicotinic receptors on postganglionic cells. [5] All major organs are innervated by both, the PSNS and the SNS,

with the exception of the adrenal medulla which is under the control of the SNS exclusively. [2] The PSNS inhibitory tone predominates at rest whereas SNS stimulation is triggered in a stress or exercise situation. [5]

1.1.3.1.3.1 PSNS

The parasympathetic system exerts its effect via the vagal postganglionic nerves using acetylcholine as the main neurotransmitter. Parasympathetic postganglionic neurons are very short since the ganglion is located at organ. These neurons are cholinergic as they release Ach which binds muscarinic receptors at target organs. Effects of PSNS signalling include a decreased rate and force of contraction of the heart and vessel dilation. [5]

1.1.3.1.3.2 SNS

The sympathetic neurons are longer since they run from the ganglion in the spinal cord to the target organ. They are adrenergic as they release the neurotransmitter norepinephrine which can bind to several subtypes of the adrenergic receptors. Sympathetic stimulation increases heart rate and cardiac output through β1 adrenergic receptors, provokes constriction of arteries and veins through α1 adrenergic receptors, and lead to dilation of vessels and renin secretion through β2 adrenergic receptors. All the effects of sympathetic activation contribute to the 'fight or flight' response aiming at preparing the body to respond to danger. More specifically, SNS actions allow to redistribute the blood away from skin and viscera to concentrate it to skeletal muscles, brain and heart, it stimulates heart beat, raises BP and promotes the release of adrenaline in the bloodstream by the adrenal medulla. This latter action of the SNS is necessary for ensuring that all the body is reached by SNS effects. [5,6]

1.1.3.2 Endocrine system

1.1.3.2.1 Renin angiotensin system (RAS)

The circulating RAS is a major system responsible for maintenance of body fluid volume homeostasis and regulation of BP. It consists of a proteolytic cascade that

results in the production of a biologically active peptide, angiotensin II (Ang II), which exerts its effects via a signal transduction system.

1.1.3.2.1.1 Angiotensin II proteolytic cascade

Generation of Ang II begins with the molecule angiotensinogen (AGT), expressed constitutively from the liver and secreted in the circulation. Under normal conditions, AGT levels remain constant at a concentration near its K_m for cleavage by renin [7]. AGT is cleaved by the aspartic protease renin to yield angiotensin I (Ang I). In the kidney, pre-prorenin (406aa) is first synthesized and is converted to prorenin by the removal of 23 amino acids as it enters the rough ER. Through a secretory pathway, prorenin is processed to renin by the cleavage of 43 amino acids. In response to low perfusion pressures, low sodium delivery to the distal tubules or β adrenergic stimulation, renin is secreted in the circulation. The opposite situation inhibits renin secretion from the kidneys. Inhibition of renin secretion by the product of the cascade itself, Ang II allows negative feedback regulation. [2] As the biologically inactive peptide Ang I reaches pulmonary circulation, it is converted to active Ang II by the Angiotensin converting enzyme (ACE). ACE is a zinc metallopeptidase that exists in 3 isoforms: somatic ACE, soluble ACE and testicular ACE. Somatic ACE is mainly responsible for the conversion of Ang I to Ang II by cleavage of a dipeptide from its C-terminal end. ACE has a broad substrate specificity. In addition to cleaving Ang I, it can also inactivate bradykinin by removal of a C-terminal dipeptide. [8]

1.1.3.2.1.2 Angiotensin receptors and signalling

Ang II exerts its effect by binding to two receptors, the Ang II receptor 1 (AT1) and the Ang II receptor 2 (AT2).

1.1.3.2.1.2.1 AT1

Most known effect of Ang II are mediated through coupling with the AT1 receptor. The AT1 receptor is a member of the G-protein-coupled 7-transmembrane receptor family. The tertiary structure of the 4 extracellular loops is stabilized by disulfide bonds and contains 3 glycosylation sites. The cytoplasmic tail is rich in serine and threonine

residues, which are potential phosphorylation sites. AT1 receptors exhibit a wide tissue distribution in adult including spleen, liver, kidney, heart, vascular smooth muscle cells (SMC), blood vessels, adrenal gland, liver and brain. Molecular cloning studies have distinguished 2 receptor subtypes, AT1a and AT1b differing in their distribution and regulation. At the molecular level, AT1a and AT1b show differences in the carboxy domain of the protein and in the 3' and 5' untranslated region (UTR). The 2 receptor subtypes are present in the mouse and in the rat. In human, only one AT1 subtype is found. [8,9]

Upon activation by Ang II, the AT1 receptor couples to G proteins of the G_i/G_q family which in turn activate a wide range of signalling cascades including the phospholipase C (PLC), phospholipase D (PLD), and phospholipase A2 (PLA_2) pathways. In addition, Ang II stimulation of the AT1 receptor has an inhibitory effect on adenylate cyclase. [10-12]

Activation of PLC leads to the production of inositol triphosphate (IP_3) and diacylglycerol (DG) from the hydrolysis phosphatidylinositol 4,5 biphosphate (PIP2). In parallel, PLD generates choline and phosphatidic acid (PA) from phosphatidylcholine (PC). PA is subsequently converted to DG. IP_3 production is involved in the mobilization of intracellular calcium (Ca^{2+}) resulting in contraction of smooth muscle cells (SMC) and vasoconstriction. DG and intracellular Ca^{2+} mobilization both contribute to the activation of protein kinase C (PKC) which mediates the proliferative effects of Ang II via the phosphorylation and activation of the mitogen-activated protein kinases (MAPK). PC is also hydrolyzed by phospholipase A_2 upon Ang II binding to AT1R. This cascade generates arachidonic acid (AA), a precursor for the production of eicosanoid vasoactive substances. [8,9,11]

The consequence of the inhibition of adenylate cyclase is a decrease in cyclic AMP (cAMP) levels and as a result a reduction in protein kinase A (PKA) activity. In renal proximal tubules, the net effect is a reduction in Na^{+}/H^{+} exchange inhibition leading to an augmented sodium reabsorption. In the adrenal cortex, a decrease in adenylate

cyclase activity results in a reduction in inhibition of adrenocorticotropic-hormone (ACTH)-stimulated aldosterone secretion. [8,9,11]

Effects of AT1R on stimulation of growth and proliferation involves additional signalling mechanisms including the Janus kinase signal transducers and activators transcription (JAK/STAT) system and ras/raf activation of MAPK. [12]

1.1.3.2.1.2.2 AT2

The structure of the AT2 receptor resembles that of the G-protein-coupled 7-transmembrane receptor family. Despite the similarities to the family, a G protein coupling AT2 to its signalling pathways has yet to be identified. The AT2 receptor shares 34% sequence homology with the AT1 receptor. [10] Contrarily to AT1 which is expressed mainly in adult tissue, AT2 expression is stronger in foetuses and declines after birth. Weak AT2 expression remains in adult endothelium, myocardium, brain, adrenal gland, ovaries and uterus [13].

Description of signalling from AT2 receptor remains incomplete. Its effects are thought to balance the effects of AT1 on cellular proliferation. In effect, AT2 is believed to have effects in cell differentiation and anti-proliferation. These effects are thought to be mediated by the activation of phosphotyrosine phosphatase which in turn inactivates mitogen-activated protein (MAP) kinase. [10] AT2 is also involved in vasodilatation and natriuresis via a local increase in nitric oxide and bradykinin. [10]

1.1.3.2.1.3 Local RAS

In addition to the well described endocrine RAS, evidence for the local generation of Ang II within tissues accumulates. In addition to their presence in the circulation, the components of the RAS are also found in several tissues including brain, kidney, adrenals, testis and arterial wall. [14] It is believed that the circulating RAS mediates the acute effects of Ang II on vasoconstriction, salt and water homeostasis and cardiac rhythm. In contrast, tissue RAS seems to be involved in the establishment of the long term effect on Ang II including cellular proliferation and differentiation. [13]

1.1.3.2.1.4 Biological actions of Ang II

In the vasculature, Ang II increases total peripheral resistance by directly causing vasoconstriction and indirectly by facilitating SNS transmission. Ang II is a mitogenic factor for vascular SMCs and myocardium. In addition, Ang II interacts with other endocrine factors as it is one of the main stimulus for the synthesis of aldosterone by the adrenal cortex and the release ACTH and vasopressin by the pituitary. ACTH stimulates the adrenals to release aldosterone. Aldosterone acts on distal tubules where it stimulates sodium/potassium exchange leading to increased sodium reabsorption. Vasopressin triggers the insertion of water pores in the distal tubules and the collecting duct of the kidney, thereby promoting water reabsorption. Ang II also regulates sodium and fluid reabsorption from proximal tubules of the kidney by affecting locally the constriction state of the renal vasculature. From its action on the CNS, Ang II induces thirst and salt appetite. Inhibition of renin secretion in the kidney provides a negative feedback loop mechanism for the effects of Ang II. [15]

1.1.3.2.2 Natriuretic peptides

The natriuretic peptide family includes the atrial natriuretic peptide (ANP), the brain natriuretic peptide (BNP) and the C-type natriuretic peptide (CNP). ANP is synthesized and released mainly by the atria and BNP is synthesized and released predominantly by the ventricular myocytes. [16] These peptides exert their function as circulating hormones. [17] Even though ANP and BNP are constitutively released from the heart, mechanical stimuli such as atrial stretch or endocrine stimuli may affect their rate of synthesis and secretion [16]. CNP is a paracrine factor produced by the cerebral cortex and endothelial cells of the vessels. [18]

1.1.3.2.2.1 Receptors and signalling

Natriuretic peptides exert their function by binding to the natriuretic peptide receptors (NPR) A and B. NPR-A and NPR-B have a guanylyl cyclase intracellular domain that catalyses the formation of cyclic guanosine monophosphate (cGMP) second messenger from guanosine triphosphate (GTP). Targets of cGMP include cGMP-dependent protein

kinases and cGMP-gated ion channels. [16] ANP and BNP have higher affinity for NPR-A whereas CNP associates preferentially with NPR-B [17].

1.1.3.2.2.2 Biological actions

Natriuretic peptides have an important influence on the cardiovascular homeostasis. More specifically, ANP and BNP antagonize the RAS by inhibiting the synthesis and release of renin and aldosterone. They also increase natriuresis by constriction of efferent arterioles and dilation of afferent arterioles in the glomeruli which results in an increased glomerular filtration rate (GFR), they inhibit the effects of endothelin, vascular SMC proliferation, and SNS. In a general way, the effects of ANP and BNP oppose those of the RAS. [18] In contrast to ANP and BNP, CNP has only minimal natriuretic effects and doesn't inhibit the RAS. It is an venous and arterial vasodilator which can inhibit endothelin and SMC proliferation. [17,18]

1.1.3.2.2.2 Metabolism

Metabolism of natriuretic peptides include enzymatic degradation by neutral metalloendopeptidase (MME) and receptor-mediated clearance via NPR-C. In effect, NPR-C is similar to NPR-A and NPR-B, but lacks the guanylate cyclase activity due to a truncated intracellular domain. [16]

1.1.3.3 Vasoactive substances

Regulation of blood flow is also achieved by locally acting vasoactive compounds that control the contractile state in a paracrine or autocrine fashion. Some of the vasoactive substances have a constrictor action (endothelins, Ang II, eicosanoids) and some of them exert a dilator effect (nitric oxide, kinins, eicosanoids). The balance of vasoconstrictors and vasodilators will determine the contraction state of the vessel. A detailed description of the effects of Ang II on the vasculature can be found in section 1.1.3.2.1.

1.1.3.3.1 Endothelin

The endothelium responds to mechanical stress and to humoral stimulation by producing endothelin 1 (ET-1). Humoral factors such as Ang II, vasopressin and catecholamines

can also trigger the release of ET-1 by endothelial cells. The endothelial cells first produce a precursor, preproendothelin, which is processed to a 21 amino acid biologically active peptide by a series of cleavage reactions occurring inside or outside the endothelial cells. Upon release by endothelial cells, ET-1 binds to ET1A and ET1B receptors at the surface of underlying SMC. Endothelin receptors are members of the 7 transmembrane domain family of receptors coupled to G proteins and signal through PLC, PLD, PLA_2, and intracellular calcium modulation. Activation of the MAPK is involved in mediating the effect of ET-1 on cellular growth. [19]

ET-1 induces vasoconstriction and stimulates SMC proliferation in the vasculature. The vasculature of kidneys is especially sensitive to the vasoconstrictor effects of ET-1, which results is a decrease in sodium excretion and water retention. ET1 can also indirectly induce vasodilatation through ET1B receptor via the release of NO. In the heart, ET1 increases contractility and stimulates ANP production and release. It has growth promoting effect on myocytes and cardiac fibroblasts, mediates collagen production leading to fibrosis and impaired myocardial relaxation. [20]

1.1.3.3.2 Nitric oxide

Nitric oxide is a small lipophilic molecule that exerts its function by diffusing to nearby cells. [21] It is produced from the precursor L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). [22] NOS exists in 3 different isoforms that are differentially expressed throughout the cardiovascular system and the kidney. [22] The constitutive forms of NOS (eNOS and nNOS) are always expressed and activated upon binding of calcium and calmodulin. In contrast, iNOS is expressed upon stimulation by proinflammatory signals and act in a calcium-independent fashion. [23] In the cell, iNOS and nNOS isoforms are soluble whereas eNOS is membrane bound. [23] Table I summarizes the sites of expression, the regulation of expression, the subcellular localization and the requirements for the activation of the different isoforms of NOS.

Table I Properties of the different isoforms of NOS

Isoform	Sites of expression	Regulation of expression	Subcellular localization	Activation
eNOS	Vascular endothelium, cardiomyocytes [22]	Constitutive	Membrane bound	Ca2+ dependent
iNOS	Endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells [23]	Induced by proinflammatory signals	Soluble	Ca2+ independent
nNOS	Nerve terminals, vascular smooth muscle cells, cardiomyocytes, macula densa cells and collecting duct. [22]	Constitutive	Soluble	Ca2+ dependent

NO has a wide spectrum of biological actions throughout the body. In the cardiovascular system, NO is involved in the maintenance of vascular tone, in cardiac contractility, and in vascular remodeling. [22] By diffusing to nearby platelets in the lumen of the blood vessels, NO inhibits platelet aggregation and adhesion [21] In the kidney, NO contributes to water and sodium homeostasis, hemodynamics and renin secretion. [24] NO also acts as a neurotransmitter in the central and peripheral nervous systems, and participates in immune responses. [25]

NO exerts its functions via cGMP-dependent and cGMP-independent pathways. [25] It acts as a paracrine signal and an intracellular messenger. [26] The short half life of NO limits its capacity to spread and restricts its actions to cells in close proximity. [21] Activation of eNOS and nNOS by transient intracellular calcium increases promotes the formation of NO at relatively low concentrations. [25] Most of the effects of eNOS and nNOS derived NO are mediated through binding to the soluble guanylyl cyclase (S-GC).[27] Binding of NO to S-CG leads to an increase in cyclic guanosine monophosphate (cGMP) levels. [27] Subsequently, cGMP activates downstream effectors including the activation of cGMP-gated ion channels, cGMP-dependent protein kinases (PKG), and cGMP- regulated phosphodiesterase (PDE). [27] In contrast, induction of iNOS in the context of a immune response leads to the production of high levels of NO over a long period of time [25]. In this situation, NO exerts pro-apoptotic effects by releasing the cytochrome c from the mitochondria, by increasing p53 expression, and by regulating the expression of apoptosis associated proteins. [25]

It is interesting to note that nNOS and eNOS are both involved in the regulation of the vascular tone. In knock out mice for the gene encoding nNOS, no changes in vascular tone was observed. [22] In contrast, in the knock out mice lacking the eNOS gene, the BP increase was accompanied by an increase in peripheral resistance. [22] From these data, it was proposed that eNOS might play a role in the regulation of vascular tone under basal conditions, whereas nNOS would be involved in adjusting the vessel contractility in response to specific modifications of its environment. [22] It was shown that nNOS activity is regulated at the translational level in response to hypoxia leading to changes in vessel contractility. [26]

1.1.3.3.1 Kinins

Kinin peptides are formed from the precursor kinininogen. The glycoprotein enzyme kallikrein is responsible for the formation of bradykinin and kallidin in plasma and tissue, respectively. Kinins exert their effects through the kinin receptors B1 and B2. The B2 receptor, a G-protein coupled receptor mediates most of the known biological actions of kinins. Kinins are converted to metabolically inactive peptides by the action of kininases. [28]

Kinins are known to produce vasodilatation in vessels of the heart, kidney, intestines, skeletal muscles and liver. [28] Effect of kinins on the renal vasculature is believed to promote natriuresis and diuresis. [29] Other functions of kinins include inflammatory process and stimulation of pain receptors. [28]

1.1.3.3.2 Eicosanoids

Eicosanoids are 20-carbon unsaturated fatty acids synthesized in response to a mechanical trauma, cytokines, growth factors or bradykinin. [30] Because eicosanoids are rapidly metabolized, they act locally on neighbouring cells. Biosynthesis of eicosanoids is initiated with the release of arachidonic acid (AA) from membrane phospholipids by PLA_2 or from DG by PLC pathway. AA is further oxidized by 1) the cyclooxygenase (COX) pathway to produce prostaglandins (PGs) and thromboxanes (TXs), 2) the lipoxygenase pathway producing leukotrienes (LT) and 3) the P-450 epoxygenase

pathway resulting in the formation of epoxyeicosatrienoic acids (EETs). Eicosanoids exert their effects by binding G-protein linked receptors in an autocrine or paracrine fashion. [31]

Eicosanoids are involved in a large array of biological functions including immunity, vascular and renal functions. Products of the COX pathway can lead either to vasodilatation of resistance vessels and diminution of SNS activity by a decrease in release of norepinephrine from sympathetic nerves (PGI_2, PGE_2) or to stimulation of VSM cells contraction and facilitation of sympathetic activity (TXA_2, $PGF_{2\alpha}$). [32] Through their effects on renal vasculature, proastaglandin and thromboxanes also affect salt and water excretion. [32] PGI_2, PGE_2 also increase renin release in the kidney and inhibit platelet aggregation. In contrast TXA_2 is a potent platelet aggregator. [31] Leukotrienes are synthesized mostly by mast cells, eosinophils, neutrophils and macrophages. [33] The effects of LTC_4, LTD_4, LTE_4 leukotrienes include vasoconstriction and increased vascular permeability and LTB_4 act as a chemo attractant [33]. Major cytochrome P450-derived eicosanoids include the vasodilator 5,6-epoxyeicosatrienoic acid (5,6-EET) and the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE). In the preglomerular microvasculature, vasoconstriction by 20-HETE decreases GFR and consequently promotes salt and water reabsorption. [32]

1.2 HYPERTENSION

1.2.1 Definition and classifications

A disruption in the homeostasis of the systems regulating blood pressure results in a condition known as hypertension. According to the recommendations established by the World Health Organization, blood pressure (SBP/DBP in mmHg) can be classified in the following categories: optimal (<120/80 mmHg), normal (<130/85 mmHg), normal-high (130-139/85-89mmHg), hypertension grade 1 (140-159/90/99 mmHg), hypertension grade 2 (160-179/100-109 mmHg) and hypertension grade 3 (≥ 180/110 mmHg) [34].

For 5% of the hypertensive patients, high blood pressure is explained by an underlying renal or endocrine abnormality. This type of hypertension for which the physiological cause is known is called secondary hypertension. The remaining 95% of hypertensive patients suffer from primary or essential hypertension. The pathological process leading to the development of this type of hypertension remains unknown. [35]

1.2.2 Sequelae

Hypertension is often referred to as the 'silent killer' because it is asymptomatic and painless until complications develop. However, with time, hypertension is associated with an increased risk of cardiovascular or renal complication (Table II). Oxidative stress and endothelial dysfunction, atherosclerosis and remodeling of the heart and vessels are at the basis of a sequelae to hypertension including coronary artery diseases, renal damage, angina pectoris, myocardial infarction, stroke and congestive heart failure (Figure 1).

Table II Risk of cardiovascular or renal event in hypertensive subjects

Cardiovascular or renal disease	Relative risk	
	Men	Women
Coronary artery disease*	2.0	2.2
Stroke*	3.8	2.6
Cardiac failure*	4.0	3.0
End stage renal disease**	3.1	-

The relative risk refers to the ratio of incidence rate of cardiovascular events/renal disease among hypertensive subjects to the incidence rate cardiovascular events/renal disease among normotensives subjects. Adjusted for age. * Adapted from Kannel, W.B. [36]; ** Adapted from Klag, M.J. [37] (hypertension stage 1 patients only).

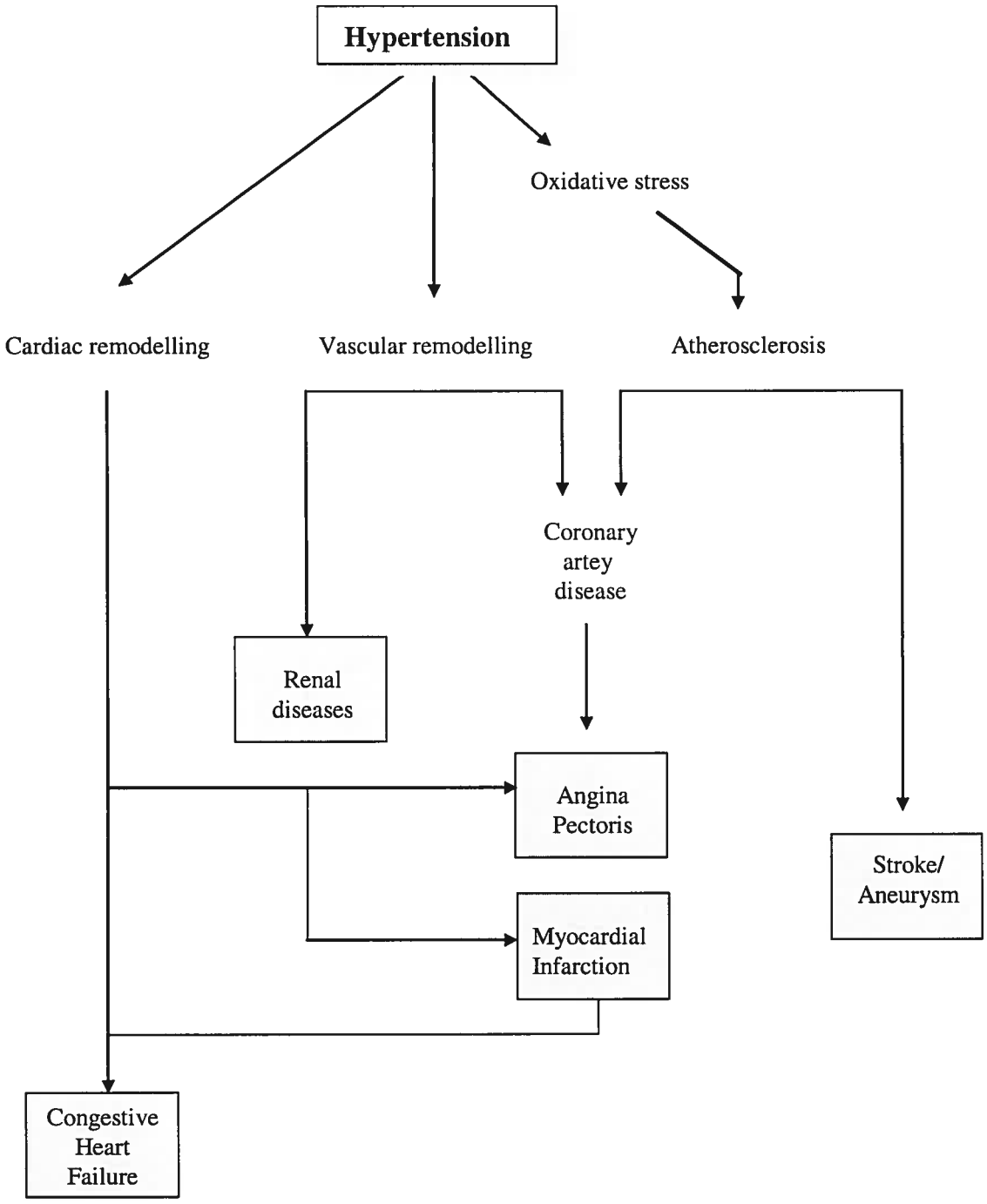


Figure 1- The sequelae of hypertenion. See text for detailed explanation.

1.2.2.1 Oxidative stress and endothelial dysfunction

Reactive oxygen species (ROS) are a family of molecules including free radicals (molecules that possess unpaired electrons) and other molecules that have an oxidizing effect. [38] They are part of normal cellular processes such as metabolic events, or growth factors and cytokines signaling. [39] Under normal physiological conditions, scavenging mechanisms protect against the toxic effects of excess ROS. [39] The rate of oxidant formation is balanced by the rate of their elimination. [40] Oxidative stress refers to the situation where there is an overproduction of ROS that cannot be buffered by scavenging mechanisms.

Several evidences are in favor of a role of oxidative stress in the development of hypertension. Correction of oxidative stress lowers BP whereas creation of oxidative stress raises BP in most animal models of hypertension. [41] Xanthine oxidase, NADH/NADPH oxidase and uncoupled eNOS has been extensively studied as sources of ROS in pathological situations. [38] It is noteworthy that Ang II induced hypertension in rats is accompanied by an increase in expression and activity of NADH/NADPH oxidase. [41] Mechanical stress on the vascular wall can also trigger increases in NADH/NADPH oxidase. [38] As a result of increased oxidative stress, the endothelium-dependent vascular relaxation is impaired, a condition known as endothelial dysfunction. [40] It the consequence, at least in part, of a diminished bioavailability of NO. [42] The reduction in NO-mediated relaxation could be explained by a decreased synthesis, increased breakdown or interaction and inactivation of NO by other endothelium derived substances. [43] For example, ROS react with NO which results in the production of peroxinitrite, a cytotoxic oxidant that has poor relaxing properties on the vessels. [42]

1.2.2.2 Atherosclerosis

Atherosclerosis is a chronic immune-inflammatory disease leading to the progressive accumulation of plaques within the arterial wall. [44] Several epidemiological studies have revealed that hypertension is associated with an increased frequency of complications due to atherosclerosis. [45]

According to the response-to-injury hypothesis, elevated blood pressure would result in sustained endothelial damage which would be responsible for initiating the formation of a plaque. [45] Regions of curvature and in close proximity to branch sites in the large arteries are especially susceptible to plaque formation. [46] In early stages of atherosclerosis, several elements are required for the plaque to form: 1) increased permeability of the endothelium facilitates the migration of oxidized low density lipoproteins (LDL) through the endothelium into the underlying extracellular matrix, 2) oxidized LDL stimulate blood monocytes to migrate towards the endothelium, 3) following their adhesion to the activated endothelium, the monocytes migrate across the endothelium to reach the intima. [44] In the intima, the majority of the monocytes differentiates in macrophages, and starts internalizing lipids to become foam cells. [44] As the plaque progresses, the foam cells aggregate and a core of lipids and necrotic debris is formed. [44] A fibrous cap composed of extracellular matrix components and recruited SMC cover the necrotic core. [46]

Atherosclerotic plaques built up over the years and as they grow in size, they can interfere with blood flow partially or completely. In this advanced state of the disease, calcification and increased degradation of the extracellular matrix by matrix metalloproteinases predispose to plaque rupture. [47] Once the plaque is released from the arterial wall, it produces a thrombus that can travel in the circulation and eventually block blood flow in an organ different from the site of plaque formation.

1.2.2.3 Vascular remodeling

Essential hypertension has been associated with an increase in peripheral resistance in animal models and human. [40] The resistance vessels responsible for this increased peripheral resistance are the small arteries and arterioles. [48] The vascular resistance of a blood vessel is function of its structural properties (media: lumen ratio and media thickness) and its active properties (force per cross sectional area). [48] The increased peripheral resistance observed in hypertension is explained by: 1) vascular remodeling

of the vessels, which affects their structural properties, and 2) modifications of the active properties of the vessel and hence, of the vascular tone. [40]

During the development of hypertension, inward hypertrophic remodeling and inward eutrophic remodeling have been observed in small arteries. [49] Inward hypertrophy is characterized by a decrease in the lumen diameter accompanied by an increase in the cross sectional area of the vessel.[48] In eutrophic remodeling the lumen diameter also decreases but there is no change in the cross sectional area. [48] Therefore, eutrophic and hypertrophic remodeling lead to a reduced lumen diameter. However, the increase in wall thickness is only present in hypertrophic remodeling. In contrast, eutrophic remodeling is a rearrangement of the same material around a smaller lumen. In hypertension, a process called rarefaction also contributes to the increase in vascular resistance. Rarefaction refers to a decrease in the number of parallel-connected vessels and is observed in arterioles. [50]

Vascular remodeling of resistance vessels is the consequence of humoral factors, oxidative stress and mechanical factors. [40] It involves cell growth, cell death by apoptosis, cell migration and modifications of the extracellular matrix. [51] Vasoactive agents (Ang II, ET-1, catecholamine, vasopressin), growth factors and cytokines mediate vascular remodeling via activation of the MAP kinases. [40] The increase in wall stress, that results from high BP induces the stimulation of integrins, recruitment of the focal adhesion kinase (FAK), and remodeling processes in the vasculature through MAP kinase signaling. [48] Reactive oxygen species also contribute to vascular remodeling by stimulating growth of the vascular smooth muscle cells and accumulation of ECM. [40]

1.2.2.4 Cardiac remodeling

In situations where the work load is increased, the heart responds by a compensatory increase in mass, or hypertrophy. During exercise, a repeated but episodic mechanical stress is imposed to the heart. In this case, the increase in cardiac mass is explained by the homogenous remodeling of cardiomyocytes and non-cardiomycocytes. In contrast to exercise, hypertension exerts a constant mechanical and non-mechanical stress on the

heart. An increase in left ventricular hypertrophy (LVH) is accompanied by proliferation of fibroblasts, accumulation of extracellular matrix (ECM) components and loss of cardiomyocytes by apoptosis or necrosis. Pathologic remodeling of the heart may also lead to dilated cardiomyopathy, a condition characterized by a decrease in wall thickness, increase in volume of the heart chambers and loss of contractility. It is a consequence of increased blood volume in the heart and cardiomyocyte death. In hypertrophic cardiomyopathy, sarcomeres are added in parallel, leading to a thickening of the cardiomyocytes. In contrast, dilated cardiomyopathy presents a lengthening of the cardiomyocytes due to addition of new sarcomeres in series. [52]

Mechanical and non-hemodynamic factors are the initiators of molecular events leading to a complex reorganization of the cardiac tissue. At the surface of the cell membranes, integrins act as mechanoreceptors that can detect changes in the work load of the heart. [53] When submitted to mechanical force, the interaction between ECM components and integrins triggers intracellular signalling via the activation of the focal adhesion kinase (FAK). [53] Among the mechanisms leading to the build-up of fibrosis in the myocardium, a disregulation of the MMP-1 was shown to be involved. [54] MMP1 is the metalloproteinase responsible for degrading collagen type I, one of the predominant ECM component observed in fibrosis. [54] Activation of G-protein coupled receptors by Ang II, ET-1, and activators of the adrenergic receptors, is also known to mediate the different manifestations of pathological hypertrophy. [52]

1.2.2.5 Coronary artery disease

The coronary arteries are the vessels that distribute oxygenated blood to the heart. Myocardial ischemia refers to the lack of oxygenation of the myocardium tissue resulting from a partial or complete obstruction of the coronary arteries. Hypertension can facilitate partial obstruction by an atherosclerotic plaque growing in the coronary arteries combined to an increased metabolic demand from thickening of the ventricular wall in LVH. The combination of plaque formation in the coronary arteries and increased metabolic demand due to LVH results in a blood flow to the heart that is not sufficient to meet the organ requirements, a condition called coronary heart disease

(CHD). Chest pain resulting from partial blockage of the arteries in CHD is known as angina pectoris and is usually triggered by physical exertion or emotional stress. Alternatively, the blood supply to heart can be interrupted completely by a thrombus originating from a plaque rupture. Complete privation of oxygen to heart tissue is known as myocardial infarction or heart attack. In this case, part of heart muscle will die from ischemia. The extend of the damage will depend on the proportion of myocardium that suffers from the lack of oxygen. [55]

1.2.2.6 Congestive heart failure

Congestive heart failure (CHF) refers to a condition in which the heart cannot supply enough blood to meet the metabolic requirements of the body, due to a progressive weakening of the pump function of the organ. [56] LVH or loss of myocytes due to past events CHD or MI are important risk factors for the development of CHF. [56] In addition to an increase in LVH, remodeling of the heart is accompanied fibrosis. One of the consequence of the accumulation of ECM is a decreased compliance of the left ventricle resulting in diastolic dysfunction. [57] To maintain the cardiac output and fill the left ventricle to normal levels, the heart compensates the diastolic dysfunction by increasing the left atrial pressure. Pulmonary oedema can occur if the elevated atrial pressure is transmitted to the pulmonary circulation. [58] In advanced states of CHF, the condition progresses to a state of uncompensated CHF resulting in a lowering of the CO that will eventually lead to pump failure. [58]

1.2.2.7 Stroke and cerebral aneurysms

Stroke is the term used to define the vascular events leading to cerebral ischemia. Several studies came to the conclusion that hypertension is the most important modifiable risk factor for stroke. [45] In addition, active treatment of hypertension can reduce the occurrence of stroke by 25 to 45 %. [45] Damage to the brain linked to hypertension can occur via several mechanisms. In large vessels, cerebral ischemia can result from partial or total blockage of a cerebral artery by an atherosclerotic plaque or from the interruption of the blood flow by a thrombus. Another type of stroke occurs from cerebral hemorrhage. The latter can be the result of an aneurysm, which is a blood

filled sac forming in the wall of an artery due to increased pressure on a thrombus or from bursting of an artery. Because the small diameter penetrating end arteries that supply cerebral tissue arise directly from main arterial trunk, these are especially vulnerable to the effect of high blood pressure. [45]

1.2.2.7 Renal damage

As a result of hypertension, glycoproteins and collagen matrix form a homogeneous deposit on the wall of the renal vessels accompanied by atrophy of smooth muscle cells and irregular thickening of the basement membrane. Preglomerular arterioles also undergo hypertrophic and hyperplasic remodelling of the smooth muscle cells. At the molecular level, these modifications of the renal microvasculature are known to involve changes in cell cycle and activation of genes encoding proteins relating to growth and secretion of matrix. Deposition of fibrin and fibrinogen is also observed. A gradual obstruction of the blood flow to the glomeruli is associated with a decrease in function and subsequent atrophy due to the resulting ischemia. Alternatively, the kidney can suffer direct damage from an increase in blood pressure entering the glomeruli due to the incapacity of preglomerular vessel to adjust diameter in responses to changes in blood pressure. These mechanisms lead to glomerulosclerosis and eventually renal failure. [59]

In larger arteries, lesions attributable to atheromatous plaque obstruction and smooth muscle hyperplasia and fibrosis can lead to renal artery stenosis, which is a complete or partial narrowing of the artery. If occurring unilaterally, renal arterial stenosis will lead to secondary hypertension. [59]

1.2.3 Epidemiology and burden

Statistics indicate that hypertension, obesity, physical inactivity, and tobacco smoking are among the modifiable risk factors of developing a cardiovascular disease. 80.2% of Canadian adults aged between 20 and 59 years have at least one of these risk factors. [60] High blood pressure alone affects 21.1% of the Canadian population according to the 1988-1994 evaluation of the Canadian Heart Health Survey (CHHS). [61]

The biennial publication of the Heart and Stroke Foundation of Canada ranks cardiovascular diseases as the leading cause of death in Canada, responsible for 74 626 deaths in 2002 only. [62]. In addition to the high mortality associated with cardiovascular diseases, Health Canada classifies cardiovascular diseases as the most costly contributor to both direct and indirect health costs, accounting for 11.6 % of total illness costs in Canada. In 1998, the cost rising from cardiovascular disease was evaluated at 18.5 billion dollars, 6.8 billion in direct cost related to hospitalisation, prescription drug consumption and physician care expenditure and 11.7 billion dollars in indirect cost explained by disability and mortality. [6]

1.2.4 Treatment

When it comes to treating hypertension, modification to the lifestyle and pharmacological treatments are often needed to reach therapeutic BP levels. Lifestyle modifications can reduce SBP by 4.6- 11.4 mmHg and the diastolic pressure by 2.5 - 7.5 mmHg. [63] Changes recommended include weight loss, diminution of alcohol and salt consumption and regular exercise. [64] In cases where BP is higher than stage I hypertension, various classes of drugs can be used alone or in combination to reach target values of BP. The major classes of agents available to fight hypertension are the diuretics that act by depleting body sodium, the sympathoplegic agents that act on the SNS, the direct vasodilators that relax smooth muscle cells and the molecules that prevent the actions of the RAS such as the ACE inhibitors and the angiotensin receptor blockers. [65] Reduction of the blood pressure below 140/90 mmHg is achieved in only 13% of the patients suffering from hypertension [61], leaving room for improvement in awareness and efficiency of treatment.

1.3 GENETICS OF HYPERTENSION

Primary hypertension can be sub-divided into monogenic and essential hypertension. Monogenic hypertension represents 5% of all cases of primary hypertension. It is explained by a single gene defect inherited following a Mendelian transmission. The

remaining 95% of hypertension cases are classified as essential hypertension and are under a polygenic influence with an environmental component. The search for genes responsible for Mendelian forms of hypertension has been a successful one, but the genes involved in essential hypertension remain unknown.

1.3.1 Monogenic hypertension

Mendelian forms of hypertension include Liddle's syndrome, glucocorticoid remediable aldosteronism (GRA), apparent mineralocorticoid excess (AME), and pseudohypoaldosteronism type II (PHAII). All these forms of hypertension affect homeostasis of salt and water reabsorption. Even though Mendelian forms of hypertension are more rare and severe than essential hypertension, there was a tremendous effort to better understand their etiology in the hope that it would lead to clues about the pathophysiology of essential hypertension.

1.3.1.1 Glucocorticoid Remediable Aldosteronism

Normally, cortisol is synthesized in the zona fasciculata of adrenal cortex whereas aldosterone is synthesized in the zona glomerulosa. 11βhydroxylase encoded by the gene *CYP11B1* is involved in the synthesis of cortisol and the aldosterone synthase encoded by the gene *CYP11B2* is part of the biosynthetic pathway leading to aldosterone production. *CYP11B1* and *CYP11B2* are both located on human chromosome (chr) 8. Both genes have 9 exons sharing 95% DNA similarity and 8 introns similar to 90%. [66] The major difference between *CYP11B1* and *CYP11B2* resides in the 5'flanking region. *CYP11B1* expression is regulated by adrenocorticotropic hormone (ACTH) and *CYP11B2* is regulated by angiotensin II.

In GRA, an autosomal dominant form of hypertension, there is an increased and ectopic synthesis of aldosterone. [67] It was shown that in families suffering from GRA, there is a chromosomal rearrangement resulting from unequal meiotic cross over between *CYP11B1* and *CYP11B2* leading to an additional copy of a chimeric gene made of the 5'flanking sequence and first few exons of *CYP11B1* and of the 3'exons of *CYP11B2*. [68,69] The result is that the gene aldosterone synthase is placed under the regulatory

control of ACTH. The rate of cortisol to aldosterone secretion is normally 1000:1. [70] Under the control of ACTH aldosterone is produced in excessive quantities therefore leading to salt retention, volume expansion and hypertension.

1.3.1.2 Apparent Mineralocorticoid Excess

Mineralocorticoid receptors have similar affinity for mineralocorticoid aldosterone and glucocorticoid cortisol. It was proposed that specificity of aldosterone its receptor in target tissues is achieved by tissue specific expression of 11 β-hydroxysteroid dehydrogenase (11βHSD) which converts cortisol to cortisone, an analog that has no affinity for the mineralocorticoid receptor. [71] Therefore, in the distal tubule, 11βHSD acts as a protector of the mineralocorticoid receptor by preventing high levels of cortisol to occupy the aldosterone receptor. [72]

AME is an autosomal recessive form of hypertension. It is caused by stimulation of the mineralocorticoid receptor despite low levels of aldosterone. Mutations in the kidney isoform of 11βHSD that affect enzyme activity or pre m-RNA splicing have been identified in AME patients. [73,74] A deficiency of 11βHSD in the kidney impairs the conversion of cortisol to cortisone, thereby leaving the mineralocorticoid receptor unprotected from high levels of cortisol. [72] The activation of the aldosterone receptor by cortisol leads to hypertension through sodium and water retention.

1.3.1.3 Liddle's syndrome

The amiloride-sensitive epithelial sodium channel (ENaC) is expressed in the distal portion of kidney tubules and is regulated by aldosterone. This channel is made up of three subunits: α, β, and γ. Reabsorption of sodium through ENaC is an important determinant of BP homeostasis. [67]

Liddle is characterized by early onset severe hypertension. Early linkage studies have shown association between βENaC locus on human chr. 16 and Liddle's Syndrome. [75] Further analysis of *ENaC* gene have revealed mutations in the β subunit gene [75,76] and in the γ subunit gene [77]. All mutations identified consist in a premature stop codon or

frameshift mutation in the carboxy terminal domain of the protein resulting in the removal of the normal carboxy terminal. [75-77] Subsequently, it was shown that truncation or frameshift of the carboxy terminal domain does not affect the biophysical properties of the channel but causes an increase in the number of receptors present in the plasma membrane. [78] Nedd4 (neuronal precursor cell expressed developmentally downregulated) is a ubiquitin-protein ligase that binds to αβγENaC PY (xPPxY) motif located in the COOH terminus of the channel. [79] Upon ENaC/Nedd4 interaction, the channel is ubiquitinated and targeted for degradation by lysosomes. [79] The loss of Nedd4 binding sites in Lidddle's syndrome may result in impaired degradation of the channel and hence, increased channel activity would result in abnormal renal reabsorption of sodium and water, and explains hypertension.

1.3.1.4 Pseudohypoaldosteronism type II

Pseudohypoaldosteronism type II (PHAII), or Gordon's syndrome, is an autosomal dominant form of hypertension. [80] In PHAII, hypertension is accompanied by hyperkalemia, hyperchloremic metabolic acidosis, and normal to high levels of aldosterone. [72] Genomic wide linkage search of families affected with PHAII have revealed strong linkage on regions of chr. 1, 12 and 17. [72] Subsequently, identification of two of the genes responsible for PHAII lead to the discovery of a novel family of serine/threonine kinases, the WNK. [81]

The WNK (with no lysine kinases) are recognized by the lack of a key lysine residue in the catalytic domain. [82] In the epithelium of the distal nephron, WNK4 is necessary for the coordination of the transcellular and paracellular flux to achieve NaCl and K+ homeostasis. This function is accomplished by: 1) inhibition of the NCCT (NaCl co-transporter) by preventing its insertion into the membrane, 2) induction of clathrin-dependent reduction of renal outer medullary potassium channel (ROMK) expression at cell surface, and 3) regulation of paracellular permeability to Cl- by phosphorylation of claudin proteins in the tight juctions. [80] In the kidney, WNK1 expression is restricted to the cytoplasm of distal epithelial cells.[83] Its function is to prevent WNK4-induced inhibition of NCCT. [80] The mechanism behind the inhibitory effect of WNK1 on

WNK4 is not fully understood, but it may involve a direct interaction between the two kinases.[80]

Mutation in genes encoding WNK1 (on human chr.12) and WNK4 (on human chr.17) are responsible for PHAII. [83] In the gene coding for WNK4, missense mutations clustered to a highly conserved sequence 20-25 amino acids downstream of the coiled coil domains of the enzyme have been identified. [83] The mutated WNK4 protein looses the ability to retain NCCT in the cytoplasm leading to increased expression of NCCT on the cell surface and excessive Na+ reabsorption. [80] In a gain of function fashion, the mutations in *WNK4* gene increase internalization of the ROMK channel and the permeability of tight junctions to Cl- leakage across the epithelium thereby explaining hyperkalemia associated with PHAII. [80] The mutation in the *WNK1* gene are large deletions in the first intron resulting in increased WNK1 expression without altering the protein structure. [83] The presence of higher levels of WNK1 in the kidney increases the inhibitory effect of WKN1 on WNK4, which results in increased amount of NCCT at the cell surface and increased sodium reabsorption. [80]

1.3.2 Essential Hypertension

1.3.2.1 Evidence for a genetic contribution

As described in the previous section, the search for a genetic basis to several forms of monogenic hypertension has been a successful one. However, Mendelian forms of hypertension represent merely 5% of all cases, the remaining being polygenic with an environmental component. The challenge now resides in identifying genes involved in essential hypertension.

It has been known for a long time that hypertension aggregates in certain families. Familial clustering of high BP could be the consequence of genetic inheritance of blood pressure gene or genes, household environment or a combination of both. Familial aggregation studies have shown that clustering occurs early in life since siblings living apart do not become dissimilar and spouses do not become more similar as they share

the same environment. Despite these observations, studies of familial aggregation were not sufficient to estimate the relative contribution of genetic and household environment to hypertension. [84]

In an effort to define more precisely the genetic contribution to BP determination, adoption and twin studies have been carried out. Adoption studies have demonstrated that the correlation between parents and their natural children is two times greater than the correlation between parents and their adoptive children. In addition, natural siblings have correlation coefficients twice those of adopted children. Together, these observations are in favour of a genetic contribution to blood pressure. On the other hand, the correlation coefficient of BP of children within the same household is greater than that of parents and their children, indicating that the household environment, which is more closely shared within than between generations, also plays a part in the blood pressure regulation. Overall, adoption studies have estimated the genetic contribution to be 30-50%. The household environment would be responsible of 11% of the population BP variability. Remaining variability would be explained by environmental non-familial components. [84]

In twin studies, the phenotypic resemblance of monozygotic twins, who share 100% of their genes, is compared to that of dizygotic twins, who share only 50% of their genes. Twin studies assume that the environment is constant. Results of several twin studies have shown that BP correlation is very high in monozygotic twins and much lower in dizygotic twins. However, the heritability was estimated to be higher than with adoption studies. This may be explained by the fact that monozygotic twins share more closely their environment than dizygotic twins. [84]

It is currently accepted that hypertension shows familial aggregation, which is explained by both genetic factors and the household environment. Continuous distribution of blood pressure results from a genetic component involving several genes, each of which interacts with the environment and with other genes. Therefore, essential hypertension is now recognized as a polygenic complex trait. [84]

1.3.2.2 Rat models

1.3.2.2.1 General characteristics

Identification of the genetic bases of essential hypertension is limited by the genetic heterogeneity of populations and environmental factors. To overcome these problems, animal models of hypertension have been developed. Working with animal models allows one to conduct the experiment in a controlled environment following strict protocols. Such rigor would not be possible in a clinical setting. Even though the mouse, the rabbit and the dog have been used as tools to study hypertension, the rat remains the most widely used model for hypertension studies. Compared to the mouse, its size is more suitable for physiological measurements and it retains the advantages of the mouse. The rat has a short life span allowing studies of the progression of the disease on a relatively short period of time, it reaches maturity for breeding at early ages, the gestation period is short and it yields reasonable litter size.

To ensure the use of homogeneous populations of hypertensive rats, several inbred strains of rats have been developed. An inbreeding program consists of two steps: 1) from an outbred stock, animals are selected for the phenotype of interest over several generation to fix the trait, 2) animals are then brother-sister mated for at least 20 generations in order to achieve genetic homogeneity. In the case of several inbred models of hypertension, a normotensive control was developed simultaneously using similar inbreeding techniques.

Most of the genetically inbred models of hypertension have been developed from original stocks of Wistar, Sprague-Dawley and Otago rats. Genetically hypertensive rats can be classified in two categories. In some rat models, hypertension appears spontaneously or without stimulation from the environment. Examples include the Spontaneously Hypertensive Rat (SHR) [85] and the SHR stroke prone (SHR-SP) [86], the New Zealand (GH) Rat [87], the Milan Hypertensive Strain (MHS) [88], and the Lyon Hypertensive (LH) rat [89]. The second type of genetically hypertensive rat was selected for the development of high blood pressure after an environmental stimulus. The Dahl

Salt-Sensitive (DSS) is an example of such a strain [90,91]. Normotensive control strains commonly used are Milan Normotensive (MNS), Wistar Kyoto (WKY), Lewis (Lew), and Dahl Salt-Resistant (DSR).

It is interesting to note that all the rat strains listed above represent polygenic models of hypertension. In effect, the distribution of the blood pressure in a F_2 population originating from a cross between any of these strains and a normotensive control follows a normal distribution rather than a bimodal distribution. Rat models of hypertension are therefore likely to be realistic models of the human disease, since human hypertension is also a polygenic trait. [35]

1.3.2.2.2 The Dahl Salt-Sensitive rat

1.3.2.2.2.1 Development of the strain

The Dahl Salt-Sensitive (DSS) model of hypertension was developed after the observation that consumption of salt resulted in hypertension in some individuals whereas others remained normotensive despite a high salt diet. The objective was to separate, by selective inbreeding, strains that would differ in their sensibility to salt consumption. A population of 55 Sprague-Dawley rats constituted the original breeding stock. Each animal was subjected to a diet containing 7.3% Sodium chloride (NaCl) and L-triiodothyronine, an agent that accelerates the appearance of salt-induced hypertension. The rats with the lowest blood pressure values were selected for the creation of a normotensive strain. In parallel, the rats with the highest blood pressure values were used to generate the hypertensive strain. Selected rats were brother sister-mated for 3 generations leading to the DSS and the Dahl Salt Resistant (DSR) strain. The initial characterisation of these strains showed that after 3 weeks of the combined NaCl-triiodothyronine diet, the DSS rat develops hypertension whereas DSR strain was resistant to salt. [91]

However, in his original work, Dahl had inbred the rats for the first few generations only. The strain was then maintained by introducing foreign stocks while keeping

selecting for high blood pressure at very generation. In 1985, John Rapp described the production of inbred rats from the Dahl colony. He randomly selected 5 breeder pairs from Dahl DSR colony and 7 breeder pairs from DSS stock. Each pair were brother-sister mated for 20 generations several sub-lines for each strain. Only one inbred strain of DSR and of DSS rat were retained. [90]

1.3.2.2.2.2 Blood pressure

In the DSS, a high salt diet starting from weaning at 3 weeks of age will provoke fulminant hypertension reaching 200 mmHg and the death of the animal within 16 weeks. By contrast, the DSR rat submitted to the same conditions will remain normotensive and show a 80% survival rate at the 48th week. If salt feeding is delayed to 3 months of age, hypertension will develop less rapidly and reach 185 mmHg by 16-20 weeks of age. It should be noted that even on regular diet containing 1% NaCl, the DSS rat will eventually develop hypertension. On the low salt diet (0.4% NaCl), DSS BP is 15-20 mmHg higher than that of DSR rat. [92]

1.3.2.2.2.3 Hemodynamics

Hemodynamic modifications generated by an increase in salt consumption in the Dahl rats are dependent on the salt load and on the amount of time salt was consumed. The first 4 weeks on a 8% sodium chloride diet result in an increase in blood volume and cardiac output. [93] After 8 weeks on the same diet, blood volume and cardiac output return to normal values and an increase in peripheral resistance is responsible for sustaining hypertension. [93] The hemodynamic pattern is different with prolonged consumption of 1% NaCl diet for 46 weeks as it is characterised by an increase in total peripheral resistance without the preceding increase in blood volume and cardiac output observed on a 8% salt diet. [93] Studies of the regional blood flow distribution determined that the increase in total peripheral resistance in the DSS under high salt diet was uniformly distributed among most vascular beds except for the kidney where it was more important in comparison to other organs. [94]

1.3.2.2.2.4 Kidney

Initial evidences for a role of the kidney in BP response to salt in DSS and DSR rats were first obtained from kidney transplant experiment. Transplanting a kidney from the DSS rat to the DSR rat is sufficient for the DSR to develop hypertension. In addition, the BP increase in the transplanted rat is more important when 2 kidneys are transplanted than when only one kidney is transplanted. Conversely, the transfer of a kidney from a DSR to a DSS rat reduces blood pressure significantly. The mechanism explaining the different response to salt in DSS kidneys compared to DSR is not known to date. But it is thought to involve a shift in pressure-natriuresis curve since at any renal perfusion pressure, DSR kidney excrete more urine and sodium than DSS. [92] Renal interstitial hydrostatic pressure is a major player in adjusting sodium excretion in responses to changes in sodium load. Despite equivalent BP and GFR, pre-hypertensive DSS rats show significant decreases in proximal tubule and whole kidney natriuretic response to increases in renal interstitial hydrostatic pressure (RIHP) compared with DSR rats [95]. These results suggest that a reduced sensibility of the kidney to changes in RIHP could be one of the defects contributing to salt-sensitivity of DSS rats. [95]

1.3.2.2.2.5 Vasculature: endothelial dysfunction

Measures of contractile responses to acetylcholine in aortas of Dahl rats revealed a depressed endothelium-dependent relaxation of aortas in DSS on a 8%NaCl diet when compared to DSS on low salt diet or DSR. [96] Since endothelin causes vasodilatation through the release of NO, it was hypothesized that a decrease in NO could be responsible for increased peripheral resistance in DSS rats consuming a high salt diet. [96,97] Subsequent studies using a competitive inhibitor of NO synthesis L-NMMA, provided evidence for the role of NO in maintaining a constant BP despite an increase in salt consumption in DSR rats. [97] One of the defects leading to hypertension in DSS would involved reduced NO production, since BP is normalized by an infusion of L-arginine, a precursor of NO synthesis. [97] Measures of the ratio of reduced-to-oxidized glutathione in the whole blood of DSS and DSR rats on high salt diet showed that an increase in the level of oxidative stress may induce endothelial dysfunction in this model. [98]

1.3.2.2.2.6 Vasculature: Na+/Ca2+ exchanger

Vascular contractility is determined mostly by intracellular calcium concentration. The mechanisms regulating intracellular calcium concentration include influx and efflux from the plasma membrane as well as calcium mobilisation from intracellular storage sites. The Na^+/Ca^{2+} exchanger (NCX) is an ATP-dependent pump located at plasma membrane transporting 3 Na^+ ions in exchange for 1 Ca^{2+} ion. Studies of isolated afferent renal arterioles and cultured mesangial cells have shown that the baseline the activity of NCX is upregulated upon PKC activation in DSR but not in DSS rat. [99-101] It was proposed that a failure of the DSS to upregulate the pump could lead to excess accumulation of Ca^{2+} in the cells and contribute to increased vascular resistance, decreased GFR and high blood pressure in DSS rat [99].

Comparison of the kidney cDNA sequence of the gene coding for Na^+/Ca^{2+} in DSS and DSR showed a substitution of an isoleucine for a phenylalanine at position 218 in the carboxy terminal membrane spanning domain. [101] In addition, the region coding for the cytoplasmic domain varies at an alternative splice sites leading to the production of different isoforms in DSS and DSR. [100] Activation of the pump by PKC results in an increase in the protein levels at plasma membrane, and the time frame of the increase was not suggestive of de novo protein synthesis. [100] Since the DSS rat model is know to be associated with increased oxidative stress, a link between oxidative stress and the differential regulation in the DSS and DSR was investigated and showed that in the presence of oxidative stress, cells expressing DSS NCX show significant increase in intracellular calcium levels when compared to cells expressing DSR NCX. [102] A link between sequence differences, protein levels and sensibility to oxidative stress remains to be demonstrated.

1.3.2.2.2.7 Renin angiotensin system

The pathology of DSS implicates the local kidney and cardiac RAS rather than the circulating RAS. The plasma renin activity and the circulating levels of Ang II are low in this animal model. [103] In the kidney, renin mRNA is not suppressed in presence of

dietary sodium [104], and blockade of the AT1R with candesartan has no beneficial effect on the BP but ameliorates glomerular injury. [105,106] The renoprotective effect of candesartan is explained by a decrease in Ang II levels and MAP kinase activities. [106] In the heart, a combination of ACE inhibitor and AT1R blocker improves cardiac remodeling and function without affecting BP. [107] From these evidences, it seems that a derangement of RAS in DSS affects renal injury and cardiac remodeling independently of BP.

1.3.2.3 Genetic tools

1.3.2.3.1 Genetic markers

Before undertaking genetic analyses, it is fundamental to develop tools for genotyping individuals or animals. A genetic marker is defined as a segment of DNA whose physical location on a chromosome is known and whose inheritance can be followed. Eukaryotic DNA contains very short simple sequence repeats spread throughout the genome. [108] Known as microsatellites, these markers are inherited following a codominant pattern and variations in the number of repeats between individuals is frequent, making them extremely useful for genotyping. [109] Microsatellites can be amplified by polymerase chain reaction using primers specific to their unique flanking DNA and typed on polyacrylamide sequencing gels or agarose gel electrophoresis. [109] The informativeness of the microsatellites was shown to be dependent of the length of the repeats for perfect repeats. [110] An interrupted repeat shows lower informativeness than expected on the basis of his length. [110]

Early studies evaluated that in the human genome, there are approximately 100 000 CA blocks whose length averages 25 repeats. [108] Subsequently, hybridization experiments and searches in the Genbank and EMBL sequences revealed that other microsatellites ranging from mononucleotides to tetranucleotides also exist and exhibit length polymorphisms. [111,112] Even though AC is the most frequent repeat with a frequency of one every 30 kb in human, and one every 21 kb in the rat [113], AG, AAAN, and A are example of other repeats that were abundantly represented in the human and

rat genomes [111]. When all types of microsatellites repeats are combined, one is expected to encounter on average one microsatellite every 6 kb in the human genome. [111]

1.3.2.3.2 Mapping of genetic markers

To align the genetic makers along the chromosomes and to determine the distance between them, different mapping techniques are available. These include linkage, radiation hybrid (RH), and physical mapping.

1.3.2.3.2.1 Linkage mapping

Genetic linkage maps are based on the principle that the frequency of recombination between two given loci is dependent on the physical distance that separates them. In a linkage map, the distances are measured in centiMorgan (cM). Hence, 1 cM is equal to a 1% chance that a marker at one genetic locus will be separated from a marker at another locus due to crossing over in a single generation. Although crossovers are random events, they are not uniformly distributed along the mammalian genome. [114] In human, mouse, and rat, the local recombination rate is influenced by the GC content of the sequence, the CpG density, the presence of repetitive elements, and the neutral mutation rate. [114] As a result of the variable recombination rates along the chromosomes, the distances obtained from linkage does not always correlate with the physical distance. In addition, linkage mapping is limited to polymorphic genetic markers, since the detection of recombination events necessitates informative markers.

1.3.2.3.2.2 Radiation hybrid mapping

Genetic distances in cM are influenced by the recombination rate and restricted to polymorphic markers. RH mapping techniques were developed to fulfill the emerging need to map of non-polymorphic markers as well.

The principle behind RH mapping is that the further apart two markers are on a chromosome (chr.), the less chances they have to be separated by a given dose of X-ray irradiation. In the construction of a RH map [115], donor cells (human, rat or mouse) are submitted to x-rays irradiation before being fused to hamster cells by somatic cell

fusion. The generation of somatic cell hybrids results in the recovery of broken chromosomal fragments by insertion or translocation in the hamster chromosomes. Every hybrid cell contains the complete hamster genome, but only a limited number of chr. segments from the donor cells. A panel of several different clones is then screened for the presence or absence of the marker one wishes to test. Finally, linkage determines if the marker of interest is linked to markers of known position on a framework map. On a RH map, the distances between markers are expressed in centiRays (cR). For example, a value of 1 cR_{3000} corresponds to a 1% frequency of breakage between two loci after exposure to 3000 rads of x-rays. A panels of 106 rat/hamster hybrids is available commercially. [116]

The scientific community has greatly beneficed of this methods of mapping since it allows the mapping of non-polymorphic markers. Examples of such markers include expressed sequence tags (ESTs) and sequence tag sites (STS) markers. However, it also presents some limitations. The resolution of a RH map depends the size of the fragments after irradiation. It is determined by 1) the radiation dose used, and 2) the retention frequency of the fragments in the generation of the hybrid cell. It was observed that there is a wide variation in the retention frequency between and across chr. [117] Some of the variation is attributable to an effect of the centromere and the influence of the chr. size. [117] Consequently, the cR distances may not correlate with the actual physical distances.

1.3.2.3.2.3 Physical mapping

The publication of the draft sequences for human [118], mouse [119] and rat [120] genomes has greatly facilitated the mapping of markers. It is now possible to locate the genetic markers simply by blasting their sequence on the human, rat or mouse genomic sequence. The maps obtained provide the physical position of a marker and the distances are measured in base pairs.

Physical maps are not based on pattern of inheritance like the linkage maps. Therefore, they include polymorphic and non-polymorphic markers. Since the distances are given

in base pairs, the physical map provide the most accurate estimate of the relative position of the makers and of the distance separating them.

1.3.2.3.3 Quantitative trait locus definition

The following section details a genetic strategy that allows one to identify the genetic bases to hypertension. It involves the localization of quantitative trait loci (QTLs) on chr. regions. A QTL is defined as a chr. region that genetically determines a blood pressure difference between two contrasting strains of rats or between individuals.

1.3.2.3.4 Co-segregation analyses

Co-segregation analysis are tools frequently used to identify loci related to BP regulation. It is based on the principle that the frequency of recombination between two given loci is dependent on the physical distance that separates them. As illustrated in Figure 2a, since the M marker is very close to the blood pressure gene, the A allele will always be inherited with the high blood pressure allele and the B allele will co-segregate with the low pressure phenotype, since no recombination occurs. On the contrary, Figure 2b shows that if more physical distance separate the M marker from the blood pressure gene, recombination will occur at a higher frequency. In this case, some individuals will inherit the A allele of M marker with the low blood pressure phenotype, and the B allele of M with the high blood pressure genotype. Co-segregation analyses provide a statistical assessment of the probability that a marker is inherited in conjunction with the blood pressure phenotype because the two loci are physically close or by chance only. [121]

Co-segregation analysis can be performed using different samples. In linkage studies, co-inheritance is evaluated in families. For example, a certain allele for a marker associated with a blood pressure gene would be shared between siblings of similar phenotype more frequently than expected by chance. An alternative to linkage is the association study, known in some cases as a case-control study. In this approach, the frequency of alleles at a marker loci is compared between a group of hypertensive individuals (cases) and a group of normotensive individuals (controls). If a marker is

close to a blood pressure gene, the frequency of alleles at this loci should be significantly different between cases and controls. This approach generally uses the general population rather than families or sibpairs.[122]

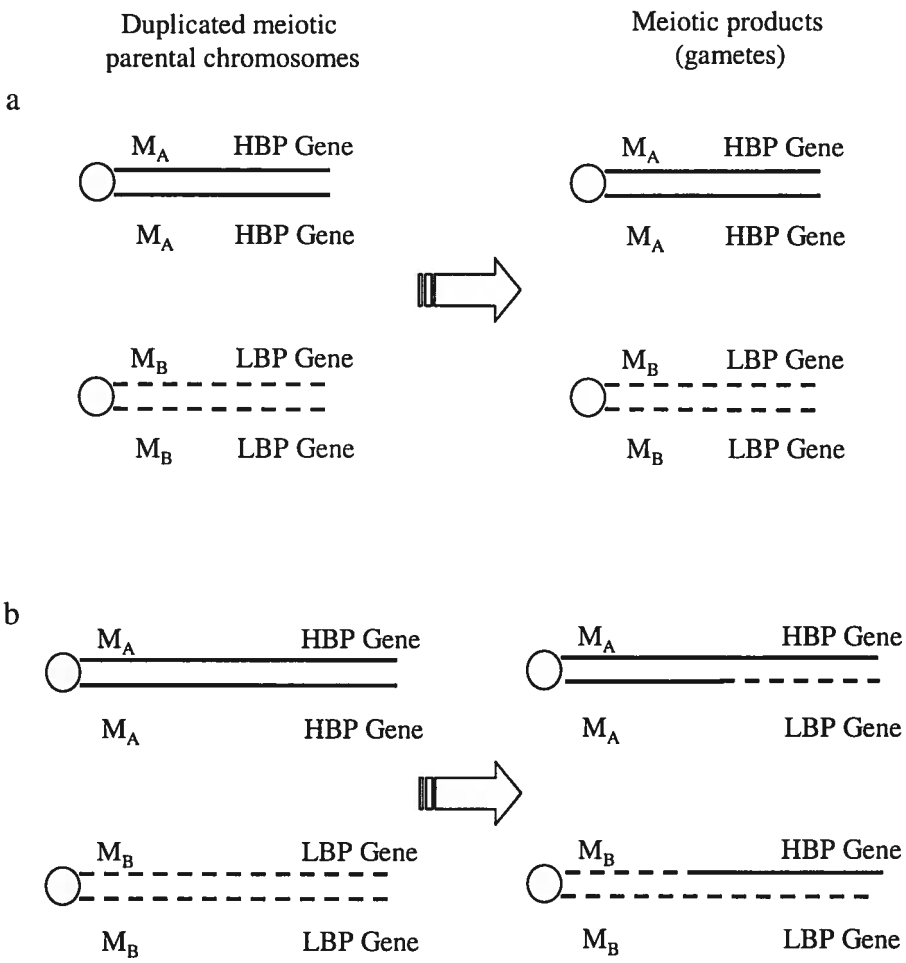


Figure 2 The principle of linkage Adapted from Rapp, J.P. [121] M refers to genetic marker, allele A and B, BP gene refers to the gene involved in blood pressure regulation, L for the BP lowering allele and H for the BP increasing allele. a represents meiotic products of two loci closely linked where no recombination events occur and b illustrates the meiotic products after recombination between distant loci. See text for detailed explanation.

1.3.2.3.4.1 Candidate gene approach

In identifying the genetic basis of essential hypertension, the candidate gene approach is based on the knowledge concerning biochemical and physiological basis of blood pressure regulation. In theory, genetic variants in the sequence of genes coding for proteins involved in the cardiovascular system could explain some of the variation in the blood pressure levels between individuals.

In the candidate gene approach, the gene of interest is positioned on the genome and its DNA sequence identified. Subsequently, the sequence of the gene is searched for the presence of variations between different individuals or between contrasting strains of rats. Variants are tested for co-segregation with the blood pressure phenotype using linkage or association.

Despite the tremendous number of candidate genes involved in the regulation of the cardiovascular, renal and nervous systems, the candidate approach is limited to molecules that are already known to be involved in the regulation of blood pressure (either in normal or pathological conditions).

1.3.2.3.4.2 Total genome scan approach

An alternative to the candidate gene approach is the total genome scan. This strategy presents the advantage of studying a genomic locus for its involvement in blood pressure regulation, without making any assumption on its function. Therefore, it expands the search for genes beyond the known proteins towards new possibilities.

The genome scan allows one to identify quantitative trait loci, or QTL, which can be defined as chromosomal locations that genetically determine blood pressure differences between normotensive and hypertensive individuals or animals. Using polymorphic anonymous genetic markers, such as microsatellites, equally spread in the genome, the genome scan assesses which locations co-segregate with the blood pressure phenotype. Therefore, rather than associating a gene or a variant to the BP phenotype, a genome scan allows one to identify a region of interest.

1.3.2.3.5 Congenic strains

Linkage and association studies provide a statistical evaluation of the probability of a QTL being in a given region of the genome. A more definitive evidence for the presence of a QTL is obtained by the construction of congenic strains. A congenic strain can be made by replacing a region from a hypertensive strain (recipient) by the homologous region coming from a normotensive strain (donor). The blood pressure of the resulting congenic strain is measured and compared to that of the hypertensive parent. If the region replaced contains a blood pressure QTL, a significant lowering of the BP of the congenic strain is expected when compared to the hypertensive parent. In parallel, the inverse strain can be built. This strain consists in a hypertensive region on a normotensive background. In the inverse congenic strain, a significant increase in the BP is expected when compared to that of the normotensive parental strain. [123]

Figure 3 illustrates the breeding steps needed for the construction of a congenic strain, a process requiring 3 to 4 years on average. The construction starts with two parental strain, a hypertensive strain and a normotensive strain. The parental strains are bred to yield the first filial generation (F1). The F1 generation is then backcrossed to the recipient parent, leading the first backcross generation (BC1). Rats that retained an allele from the donor parent in the region of interest are identified by PCR amplification of anonymous genetic markers located in the region of interest. Rats selected are backcrossed again to the recipient parent. The backcross/selection cycle is repeated until the BC8 are obtained. At BC8, more than 99% of the donor genome have been replaced by that of the recipient except for the selected region of study. Finally, brother-sister mating makes the region of interest homozygous for the donor alleles. [123,124]

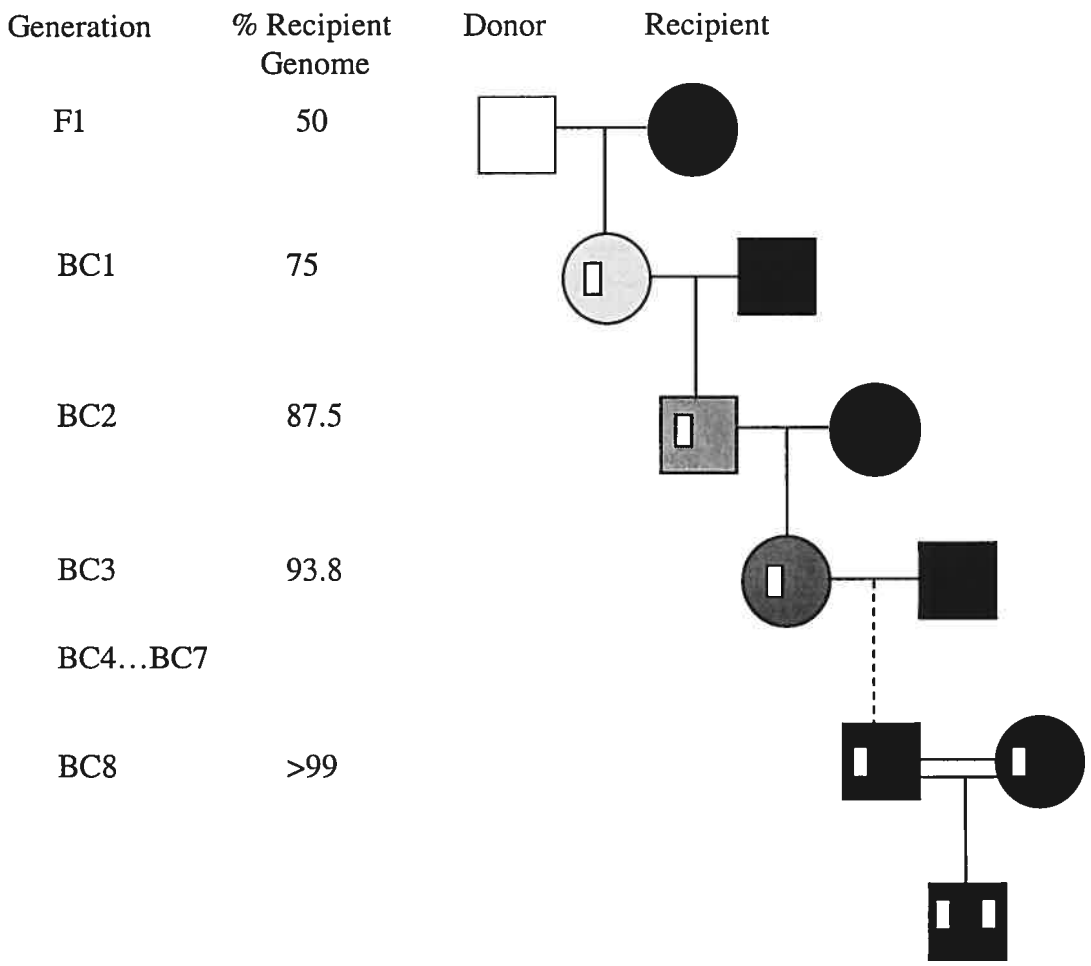


Figure 3 – Construction of a congenic strain. Adapted from Rapp, JP *et al.* [123] Construction of a congenic strain. The white square represents the selection of the donor genotype at the genetic marker targeted. See text for detailed explanation.

What is the mechanism responsible for the replacement of the background genome by the recipient genome? F1 rats have received one allele from each parental strain, these rats are heterozygous at all loci. According to Mendel's laws, the loci other than the region selected have 50% chances of retaining the recipient allele at each backcross. Therefore, the percentage of the genetic background coming from the recipient strain increases following each back-cross: 75% at BC1, 87.5% at BC1, 93.8% at BC3 and so on until it reaches 99% at BC8. [123,124]

In recent years with the constant progress made in the development of new polymorphic microsatellite markers spread all over the genome, the traditional protocol for building congenic strains has evolved towards a more efficient and less time consuming method. In speed congenics, the most suitable breeders are chosen based on the genotyping of polymorphic markers spread throughout the genome. The rats that have maintained recipient alleles at the highest number of loci and that have acquired the donor allele in the region targeted are selected as the best breeders for generating the next BC generation. An initial study in the mouse showed that the use of markers distributed every 25 centiMorgans (cM) combined with the selection of at least 16 best breeders at every generation allows to achieve more than 99% homozygocity in 3 to 4 backcross generations. [125] Using the speed congenic approach, one can establish a congenic strain in 15-18 months. Subsequently, the same strategy has successfully been applied to the rat. [125]

1.3.2.3.6 Congenic substrains

The original congenic strain allows one to confirm the presence of a QTL in a given region of interest. However, the size of the region replaced (usually around 20cM) does not allow the molecular analysis of which gene within the interval may be responsible for the blood pressure effect. Construction of congenic substrains will allow one to narrow down the QTL region until it reaches 1-2cM.

In generating congenic substrains, the original congenic strain is bred with the recipient parental strain. Therefore, the background remains homozygous for alleles coming from

the recipient parents. In the QTL region, there is the possibility for meiotic recombination between the congenic and the recipient parental strain. The rats obtained from this cross are screened at markers flanking the QTL for the presence of a cross-over in the QTL region. Progeny showing recombination are bred to the recipient parents and in the subsequent generation, rats that have retained the QTL region are brother-sister mated to achieve homozygocity. Such recombination events allow to reduce the region of the QTL to gradually smaller regions.

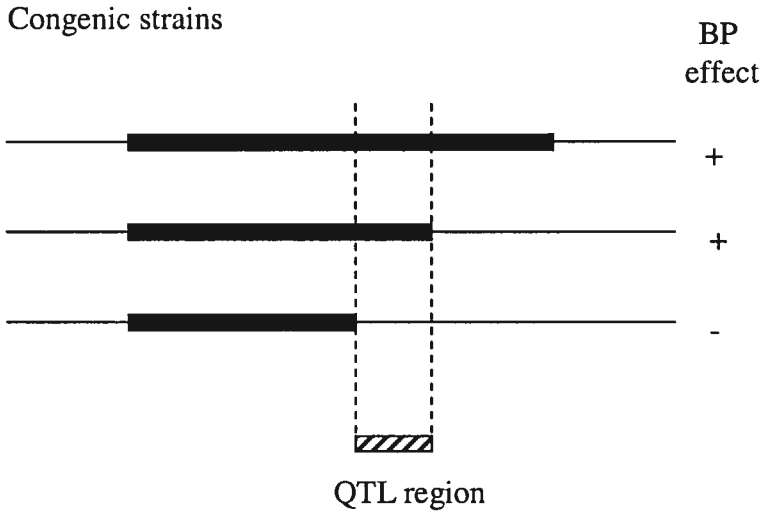


Figure 4- Substitution mapping using congenic strains. Adapted from Rapp *et. al* [123] Black bars represent the regions coming from the donor strain on a recipient background. + sign designates the strains for which a blood pressure effect was observed and – sign designate the strains showing no blood pressure effect. QTL region is designated by a striped line. See text for detailed explanation.

Several substrains can be obtained with this method in relatively few generations. The blood pressure of these strains is measured and substitution mapping (Figure 4) allows to locate the QTL to a narrow chromosomal region. Some of the strains obtained will retain the blood pressure effect observed in the original congenic strain whereas others will show no blood pressure difference with the hypertensive parental strain. The QTL is located in the region retained by the substrains showing a blood BP but not conserved in the substrains that lost the BP effect. Detection of crossovers in regions becoming progressively smaller requires the development of a dense genetic map with polymorphic markers distributed every 1-2 cM. [123]

1.3.2.4 Candidate genes for essential hypertension

In the past, several studies evaluated candidate genes for hypertension using a combination of several approaches in animal models as well as in various human populations. Examples of candidates studied include genes coding for proteins involved in the renin angiotensin system and other molecules whose function might be relevant to the cardiovascular homeostasis.

1.3.2.4.1 Renin angiotensin system

It has been known for a long time that variations in the level of several components of the renin-angiotensin system including renin, angiotensinogen, and angiotensin II correlate with inter-individual variance in blood pressure. [126] Given the well established role of the RAS in blood pressure regulation and sodium handling, the question arise whether some variants in one or more components of the RAS could be responsible for the genetic susceptibility to hypertension in animal models or human.

1.3.2.4.1.1 Angiotensinogen gene

The observation that hypertensive subjects and offspring of hypertensive parents have higher concentration of plasma AGT compared with normotensive controls [127] was the rationale that lead to a tremendous amount of studies assessing the role of *AGT* gene polymorphisms in genetic predisposition to hypertension. Following the original publication of a linkage between *AGT* gene locus and hypertension in hypertensive

sibships from France and United States, and the identification of two variants M235T and T174M that were more prevalent in hypertensive subjects [128], studies yielded conflicting results. Linkage detection was reproduced in African Caribbeans [129], but not in Europeans [130,131] or Australian Anglo-Caucasians [132] families. Furthermore, allelic frequency of M235T polymorphism was associated with high blood pressure in some studies [133-138] but not in others [129,131,132,139-142]. Association of T174M variant with hypertension was not confirmed [131,140]. Association studies cannot rule out the possibility that a locus in close proximity with the disease locus could be responsible for the blood pressure variations observed. A G to A substitution located 6 residues upstream of initiation site for transcription and in linkage disequilibrium with M235T [143,144]. This variation was shown to affect the promoter basal rate of transcription of the gene [144], thereby suggesting that the -6A polymorphism could be the causative mutation explaining the association between M235T and hypertension.

When it comes to polymorphisms of the *AGT* gene, hundreds of studies have published variable results. The different studies have looked for associations in various ethnic groups using a wide range of experimental designs, which makes it difficult to draw general conclusions. A meta-analysis was designed to investigate the association of the M235T polymorphism of the *AGT* gene with angiotensinogen levels, systolic and diastolic BP, hypertension and risk of ischemic heart disease. This meta-analysis reviewed and averaged the data published in 63 studies with a total of 45 267 participants from 3 different ethnic groups (Asian, black and white). [145] The major findings of this study confirm that the M235T polymorphism of *AGT* is associated with increased levels of plasma angiotensinogen in white, accompanied by a higher risk of developing hypertension in white and Asian subjects. The plasma angiotensinogen levels and the risk of hypertension are lower in homozygotes MM and follow a stepwise increase in heterozygotes MT and in homozygotes TT. In black subjects, no association was detected between M235T polymorphism and hypertension. Despite the clarifications brought by this study, one should note that the meta-analysis methodology presents some limitations. These include unmatched cases and controls, lack of adjustment for confounding factors, and heterogeneity of the selection criteria for cases

and controls. [145] Ultimately, the quality of the meta-analysis is dependent on the quality of the studies it combines. [146]

The *AGT* gene has also been studied in animal models. In the rat, there was cosegregation of the *AGT* locus on rat chr 19 with pulse pressure in a F2 (SHRXWKY) population. [147]. Aorta of SHR and WKY showed tissue- and age-specific differences in AGT mRNA. [147] In contrast, in a cross F2 (SHR-SP X WKY), no significant linkage of the *AGT* locus to systolic or diastolic pressure were found. [148]

1.3.2.4.1.2 Renin gene

Conversion of angiotensinogen to Ang I by renin is the rate limiting step for generation of the biologically active peptide of the RAS, AngII. Furthermore, transgenic rats expressing high amount of the mouse renin gene develop fulminant hypertension from 4 weeks of age. [149] For these reasons, the renin gene was a logical candidate for the genetic basis of hypertension.

In the DSS rat, a 1.2Kb deletion in intron 1 of the renin gene was shown to be associated with blood pressure. [150] Various crosses involving the Dahl rat and several contrasting strains (Lew, MNS, WKY, DSR, SHR) revealed the existence of 7 alleles for the renin intron 1 polymorphism. [151] In all populations, the DSS allele had a blood pressure raising effect, though the magnitude of the effect varied, indicating an effect of the genetic background. A restriction fragment length polymorphism (RFLP) in intron 1 of the renin gene also cosegregated with the blood pressure phenotype in the SHR. [152] Since no differences were observed in the coding, 5' regulatory sequence or splice pattern between DSS and DSR rats, it was suggested that the BP effect observed is either explained by a locus linked to the renin gene or to regulatory sequences situated farther from the renin gene. [153]

Human association of renin RFLP polymorphisms [154] and linkage studies of renin haplotypes on affected sib pairs [155] both failed to implicate the renin gene as a genetic determinant of human essential hypertension.

1.3.2.4.1.3 Angiotensin I converting enzyme gene

The angiotensin I converting enzyme is involved in the conversion of Ang I to the active peptide Ang II and in the degradation of bradykinin to inactive fragments. [8] Interest in ACE as a candidate for genetic determination of blood pressure comes from the fact that despite large inter-individual difference in ACE level, healthy families show significant intra-familial resemblances in plasma ACE level. [156]

Interest in ACE as a candidate gene has allowed the identification of an insertion/deletion polymorphism of 250 bp in intron 16 of human ACE gene. [157] Association of the I/D polymorphism with plasma ACE levels has been demonstrated. [157] However, studies investigating the implication of the polymorphim in genetic determination of blood pressure yielded conflicting results. In effect, no association was detected between blood pressure and the I/D genotype in normal subjects [158] or in Dutch hypertensive subjects [159]. In contrast, the I/D genotype was associated with salt sensitivity in hypertensive patients [160] and with hypertension in men in the Framingham Heart Study [161], in the Suita study in a Japanese population [162], and in old hypertensive subjects with severe early onset familial hypertension [163]. I/D polymorphism was not associated with blood pressure in healthy young Caucasian adults with genetic predisposition to high or low blood pressure. [159,164] Positive [165] and negative [165] linkage of genetic markers in linkage disequilibrium with the ACE locus have been reported.

In the rat, a highly significant linkage between a loci closely linked to ACE on chromosome 10 was reported in a F2 (WKY X SHRSP) population. [166] Fine substitution mapping using congenic strains excluded the ACE locus from the QTL. [167]

1.3.2.4.1.4 Angiotensin II receptor gene

The angiotensin type 1 receptor mediates most of the known biological actions of Ang II. The receptor is encoded by a 55kb gene consisting of 5 exons and 4 introns [168] which maps to human chr. 3 [169]. In the rat, the 2 sub-types of AT1R, a and b have been mapped to chr. 17 and 2, respectively [169]. Analysis of the sequence variations in human *AT1R* have revealed that an A to C transversion at position 1166 in 3' untranslated

region was more frequent in a hypertensive than in a normotensive group. [170] Association of the A1166C polymorphism with hypertension was confirmed in other populations. [171,172] Linkage of the *AT1R* locus to blood pressure failed to reach statistical significance in some studies [170] but showed evidence of a suggestive peak in others. [171] In rat models of hypertension, weak linkage was detected near the locus of the b subtype of AT1R. [173]

1.3.2.4.2 Others

1.3.2.4.2.1 SA gene

Based on the information that blood pressure regulation and renal function are linked, the S_A gene was originally identified from a pool of complementary DNA differentially expressed between SHR and WKY rats kidneys. [174] In effect, it was observed that the mRNA for this novel gene was 10 times more abundant in SHR kidneys than in those of WKY, an increase observed at 28 days of age, preceding blood pressure increase. [174] Subsequently, the site of increased expression S_A mRNA in SHR was narrowed to the kidney proximal tubules, a region well known for its involvement in sodium handling. [175] S_A gene showed no significant homology to any known gene [174] and was mapped to rat chr. 1. [169,176]

Following its discovery, the S_A gene has motivated a number of studies assessing its probable role in BP control and in the genetic basis of hypertension. In the rat, a cosegregation study in an F2 (SHR X WKY) population showed a significant effect of the genotype at the S_A locus on blood pressure. [177] These results were reproduced in a F2 (DSS X Lew) population [178], and in a F2 (SHRSP X WKY) population [176], but not in a F2 (DSS X WKY) population [178], indicating that the expression of the S_A phenotype is influenced by the genetic background. Initial congenic strains harbouring a segment of WKY in a SHR background and its reciprocal strain brought further evidence in favour of a role of the S_A gene in the genetic basis of hypertension in the genetically hypertensive rats. [179] Subsequently, refining of the regions introgressed in chr. 1

congenics issued from WKY X SHRSP [180] and from Dahl X Lew crosses [181] ruled out the S_A locus for these specific strains.

Mice and human experiments have yielded conflicting results. A S_A null mice showed no blood pressure reduction under basal conditions, or under salt loading when compared to the wild type counterpart. [182] The human homologue of rat S_A was cloned and mapped to human chromosome 16. [183,184] An association study in a Japanese population was able to associate the rare allele of a *Pst I* RFLP of the S_A gene with a significant increase in BP. [183] These results could not be confirmed by linkage in affected sib-pairs [184] or by association [184,185] in French Caucasians. In young adults predisposed to hypertension (Caucasian from Scotland), the S_A *Pst I* RFLP polymorphism was also excluded as a genetic determinant of blood pressure and physiological renal parameters using the four corner approach. [186] These discrepancies suggest that either S_A is not involved in the genetic determination of BP in the White populations studied, or alternatively, the polymorphism tested is not in complete linkage disequilibrium with the causative variants of hypertension. [185]

1.3.2.4.2.2 Nitric oxide synthase gene

In human and animal models of hypertension, high blood pressure is associated with a decrease in NO levels. [187] Linkage studies looking for association of the endothelial NOS (*NOS3*) with hypertension were not positive. [188,189] In the rat, the blood pressure phenotype did not cosegregate with the *NOS1* locus in the cross tested but cosegregated with the *NOS2* locus in F2 (SXMNS) and F2 (SXWKY) populations. [190] Markers in close proximity to *NOS3* were not linked to BP phenotype in various F2 populations involving the DSS rat. [191] Fine mapping of the region surrounding *NOS2* locus by the use of congenic substrains has ruled out *NOS2* as a gene involved in the genetic predisposition of the DSS rat. [167]

1.3.2.4.2.3 Epithelial Na^+ channel gene

Mutations in the sodium epithelial channel are responsible for the autosomal dominant form of hypertension in Liddle syndrome. [75-77] It was hypothesized that more subtle

changes in *ENaC* coding genes that would affect the enzyme activity without causing a phenotype as severe as the Liddle syndrome might also be involved in polygenic essential hypertension. In human, coding variants have been identified in the gene coding for the beta subunit of ENaC. [192] Even if some of the variants could slightly alter the activity of the corresponding channel, they did not cosegregate with the blood pressure phenotype. [192] The rat homolog to the human epithelial Na^+ channels was mapped to rat chr. 1 (β and γ subunits) and to rat chr. 4 (α subunit). [193] However, despite linkage of the loci of the β and γ subunits to the blood pressure phenotype in a SHR-SP X WKY cross, no biologically meaningful mutations were identified in the coding sequence comparing the two strains and no difference in expression levels was noted in kidneys. [194] These data fail to implicate *ENaC* gene in the genetic determination of essential hypertension.

1.3.2.4.2.4 WNK kinases genes

Most of the Mendelian forms of hypertension occur rarely and are characterized by early onset moderate to severe hypertension. In contrast, the gradual increase in BP described in forms of PHAII involving *WNK1* mutations might be more representative of the slow and progressive development of essential hypertension with aging. [83] For this reason, the WNK kinases are especially appealing as candidates for the genetic control of essential hypertension.

Initially, SNP in intron 10 of *WNK4* was showed to be associated with hypertension in a white American population, but not in an African American population. [195] These results were not reproduced in an Anglo-Australian [196] or in a white European population [197]. In the Japanese population, the *WNK1* SNPs studied did not show significant association with hypertension, whereas the genotype at a SNP in intron 14 of *WNK4* did.[198] A family-based association study of extremely hypertensive white Europeans have showed positive association of a SNP located in close proximity of the promoter region with hypertension. [199] Finally, a population-based have revealed that several common variant of *WNK1* were associated with mean 24-hour SBP and DBP. [197] This study provided extremely convincing data implicating *WNK1* locus in essential

hypertension in the general population. Its impact resides in 1) the use of 24hr ambulatory BP rather than punctual measures, 2) the association of the genetic polymorphisms in samples that have not been selected for the presence or absence of hypertension. In the study of continuous traits such as blood pressure, such an approach should be privileged.

1.3.2.4.3 Limitations of linkage and association studies

In general, the numerous studies looking at association between polymorphisms in candidate genes and hypertension have yielded conflicting results. Some general conclusions about this approach can be drawn. First, one can expect variable results across different population or strains of rats. Second, a particular attention should be paid to analyse intermediate phenotypes rather than BP alone. Third, one should recognize that since BP is not a dichotomous phenotype, studying association across the BP range in population-based association studies rather than in case versus controls might be more appropriate to the continuous distribution of BP in the population. Furthermore, the variations associated with hypertension in severe cases might not be relevant to the majority of the hypertensive population which presents a milder phenotype. Fourth, a given polymorphism can be statistically associated with hypertension for two reasons: it is possible that the variation studied itself contributes to the physiological changes resulting in high BP; alternatively, it may be a marker for another variation in close proximity. Molecular studies investigating the effect of individual variations on the biological function of proteins and on the regulation of protein levels are necessary to discriminate between these possibilities.

1.4 RATIONALE AND GENERAL OBJECTIVE

Attention to a possible role of rat chr. 2 in the genetic determination of hypertension comes from the observation that the locus of the gene coding for the atrial natriuretic peptide receptor A (*Gca*) cosegregates with the blood pressure phenotype in F_2(DSS X MNS) and F_2(DSS X WKY) population. [200] A second study the region surrounding *Gca* gene confirmed the linkage in a region containing several candidate genes including the gene coding for the Na+,K+-ATPase α1 isoform and calmodulin-dependent protein kinase II-delta. In addition, a suggestive peak unique to the F_2(DSS X MNS) cross was detected around the gene coding for the angiotensin receptor 1 b (AT1b). [201] Since statistical evidence is not definitive, congenic strains were built in which a segment of WKY or MNS rat chr. 2 was introgressed within the background of DSS rats. The two congenic strains showed significant lowering of blood pressure when compared to the DSS rat. These data established without a doubt the presence of a BP QTL on rat chr 2. [202] However, the length of the region involved in making the congenic strain was about 80 cM and contained several candidate genes.

The aim of the present work was to study the rat chr. 2 region involved in the genetic control of hypertension. Specific objectives included 1) to determine the number of QTL in the original 80 cM region, 2) to develop a detailed genetic map of the chr 2 region of interest, 3) to reduce the region of interest for the chr 2 BP QTL and 4) to assess the possible implication of candidate genes present on chr 2 in genetic determination of hypertension in the DSS rat model.

CHAPTER 2

METHODS

2.1 GENETIC MAPS

Genetic maps for the chr 2 regions of interest were built by integrating different sources of information: 1) a previously published linkage genetic map of rat chr. 2 [203], 2) data obtained by the use of a radiation hybrid panel 3) data available in rat database websites and genotyping data for existing strains.

Most of the genetic markers used are microsatellites of di-, tri- or tretra-nucleotide repeats. For regions where the density of markers from existing rat databases was insufficient, screening of the rat genome and design of new markers was undertaken as described below. The regions of interest were first identified by blasting informative markers at the website, http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html. The sequence obtained was searched for regions containing microsatellites. From these regions, new markers were designed for genotyping of rats based on PCR. These new markers were designated with D2Chm prefixes, where Chm stands for Centre Hospitalier de l'Université de Montreal (CHUM).

2.2 CONGENIC SUBSTRAINS

Congenic substrains are obtained from meiotic recombination events between an existing congenic strain or substrain and the hypertensive parental line, DSS. The strategy for obtaining of congenic substrains is described in section 1.3.2.3.6 (page 42). Genotyping of the of the genetic makers is verified by amplification by polymerase chain reaction (PCR) amplification of rat tail DNA followed by electrophoretic migration on a 4% agarose gel or on a polyacrylamide gel when agarose resolution is not sufficient. The specific breeding scheme for each of the congenic substrain presented in this work is available in sections in sections 3.3.2 (page 62), 4.3.2 (page 76), and 5.3.2 (page 94).

2.2.1 Nomenclature and genetic boundaries of congenic strains

The designation of a congenic strain includes the following information: the recipient strain (MNS) goes first, followed by a period and the donor strain (S/DSS). After the dash, the two genetic markers represent the region from the recipient strain that was replaced by the homologous region coming from the normotensive strain. [204] The genetic maps used to define the strains the boundaries of the strains are constantly evolving as new genetic markers are developed. As a result, the number of microsatellite markers available for mapping, and the resolution of mapping of the congenic strains are always improving. For these reasons, nomenclature of the congenic strains evolves as well. In chapter 3 to 6, more than one designation/abbreviation may be used for a given congenic strain. In certain cases, different markers are used to describe the same boundary for a given congenic strain. Table III is a guide to the nomenclature of the different congenic strains presented in chapters 3 to 6 and to the genetic markers mentioned to define them.

Table III Nomenclature and genetic markers defining the congenic strains presented in chapters 3-6.

Nomenclature of the congenic strains		Genetic markers			
Full designation	Abbreviation	SS (1)	MM (1)	MM (2)	SS (2)
S.MNS-*Adh*/D2Mit6	S.M	Prlr	D2Mit6	Adh	-
S.MNS-D2Mit6/D2Rat166, S.MNS-D2Mit6/Nep	S.M1, C2S.M1	Prlr	D2Mit6	D2Rat166	Nep, D2Mit9
S.MNS-D2Mit6/D2Rat303, S.MNS-D2Mit6/D2Rat302	S.M2, C2S.M2	Prlr	D2Mit6	D2Rat303, D2Mco9, D2Rat302	D2Rat166
S.MNS-*Nep*/D2Mit14	S.M5	D2Rat166	Nep	D2Mit14	D2Wox37
S.MNS-Nep/Gca, S.MNS- Mme/D2Rat131	S.M6, C2S.M6	D2Rat166	Nep	Gca, D2Rat131	D2Mgh10
DSS.MNS-D2Rat183/D2Chm113	C2S.M7	D2Chm33	D2Rat183	D2Chm113	D2Chm90
DSS.MNS-D2Chm25/D2Mit14	C2S.M8	D2Mco8	D2Chm25	D2Mit14	D2Wox27
DSS.MNS-D2Chm25/D2Rat131	C2S.M9	D2Mco8	D2Chm25	D2Rat131	D2Chm57
DSS.MNS-D2Wox27/Adh	C2S.M10	D2Mit14	D2Wox27	Adh	-
DSS.MNS-D2Chm51/D2Rat341, DSS.MNS-D2Chm51/D2Rat38	C2S.M11	D2Chm90	D2Chm51	D2Rat38, D2Rat341	D2Chm225
DSS.MNS-D2Chm25/Fgg	C2S.M12	D2Mco8	D2Chm25	Fgg	Gca

Table III Genetic markers are the markers at the extremity of the MNS region for each congenic strain. Each strain is defined by two markers homozygous MM (1 and 2) and two markers homozygous SS (1 and 2). Detailed genetic maps for each strain are presented in figure 1 of chapter 3, figure 1 of chapter 4, figure 1 and 2 of chapter 5 and figure 1 of chapter 6. *Adh* Alcohol dehydrogenase, *Prlr* prolactine receptor, *Nep*/*Mme* neutral endopeptidase, *Fgg* fibrinogen gamma.

2.3 PREPARATION OF ANIMALS

Male rats were weaned at 21 days of age, maintained on a low salt diet (0.2% NaCl, Harlan Teklad 7034) and then fed a high salt diet (2% NaCl, Harlan Teklad 94217) starting from 35 days of age until the end of the experiment. Telemetry probes are implanted after 3 weeks of the high salt diet with their body weights between 250-320 grams. After the surgery, the rats are allowed at least 10 days for the postoperative recuperation. The BP measurements lasts until the time of sacrifice. Rats are sacrificed by decapitation 30 days after the commencement of their BP measurements, i.e. at 14 weeks of age. The organs of interest are removed, cleaned carefully from surrounding

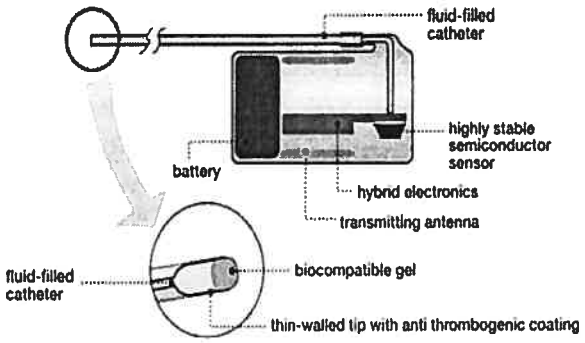
adventitial connective tissues and fat, blotted to remove excess blood, and prepared for subsequent experiments.

2.3.1 Radiotelemetry

The radiotelemetry system from Data Science Inc (St. Paul, MN) was used to measure BP. The telemetry system consist in a probe (surgically implanted in the rat) that sends data to a receiver through radiotransmission. The data are then processed and forwarded to a computer by an exchange matrix. The dataquest software stores, processes and displays the physiological data detected.

The telemetry probe consists in a fluid-filled catheter attached to a body containing the sensor, the battery and the electronic section for radiotransmission. The catheter of a telemetry probe is inserted to the descending aorta via the renal artery and secured by a suture to the blood vessel. The telemetry probe is then fixed to the muscle wall in the abdominal cavity. (Figure 5a). [205]

a



b

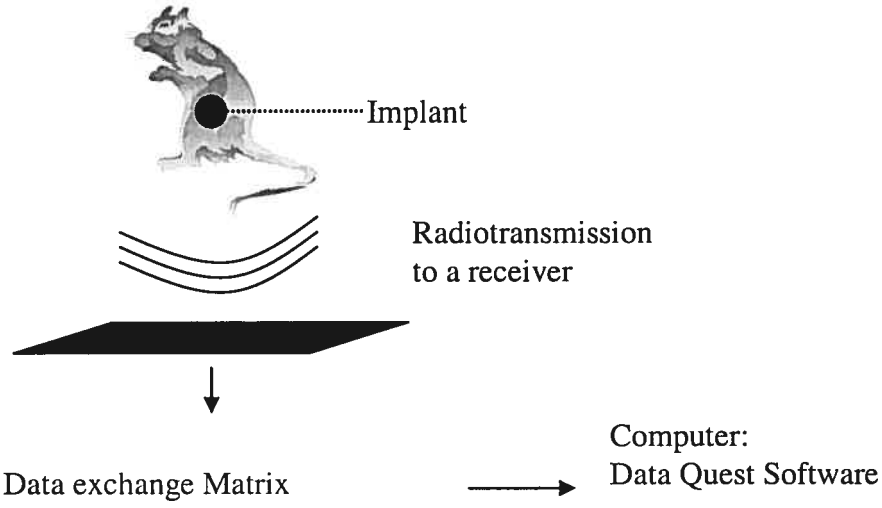


Figure 5- Measure of the blood pressure by radiotelemetry. a- the implant (Mills, P.A. *et al.* [205]) b- transmission of the data from the animal to the computer (Adapted from Data Science International) .

2.4 STATISTICAL ANALYSIS

Congenic strains are compared to the DSS rat for each phenotype studied. The ANOVA is the first step to detect a difference between any of the groups compared. For BP measures, a variation of the traditional ANOVA, the repeated measures ANOVA, allows to take into account that for any given animal, BP is measured at several points in time. In cases where the ANOVA detects no significant difference, it indicates that there is no significant difference between any of the groups tested and no further analysis is required. If the ANOVA detects a significant difference it indicates a significant difference between some of the groups compared. To distinguish which pairs of groups are different, a Dunnett *post hoc* comparison is performed. The Dunnett test allows one to perform multiple pairwise comparisons to determine which congenic strains are different from a reference control, the DSS rat. It adjusts the p values for differences in sample size and multiple comparisons. SYSTAT 9.0 program (SPSS Sci. Chicago, IL) is used for statistical analysis.

CHAPTER 3

Mapping a Blood Pressure Quantitative Trait Locus to a 5.7 cM region in Dahl Salt Sensitive Rats

Julie Dutil, Alan Y. Deng

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Research Centre-CHUM, Hotel Dieu, 3840 rue St. Urbain, Montréal, Quebec, H2W 1T8, Canada

Running head: Blood pressure QTL mapping

Corresponding author: Alan Deng, Ph.D.

Chief of Molecular Genetics and Associate Professor of Medicine

Research Centre

Centre Hospitalier de l'Universite de Montreal (CHUM)

7-132 Pavillon Jeanne Mance

3840, rue St. Urbain

Montreal, Quebec, H2W 1T8

Canada

Phone: 514-843-2611 ex. 5522

Fax: 514-843-2911

[redacted]

3.1 ABSTRACT

A region on rat Chromosome (Chr.) 2 of the Dahl salt-sensitive rat (S) was shown previously to contain a quantitative trait locus (QTL) for blood pressure (BP). This was achieved first by linkage, followed by the use of congenic strains. A congenic strain, designated S.MNS-D2Mit6/*Adh*, contained a segment of Chr. 2 from the Milan Normotensive (MNS) rat in the S genetic background. Since the region containing the QTL was roughly 80 cMs in size, a further reduction is needed towards the positional or candidate gene cloning. Currently, two congenic substrains were made from the original strain S.MNS-D2Mit6/*Adh*. One of these two substrains showed a BP-lowering effect, whereas the other substrain did not. Deducing the segment not shared in the two substrains, the BP QTL has to be present in a chromosome region of roughly 5.7 cMs between the marker D2Rat303 and the locus for the neutroendopeptidase gene (*Nep*). *Nep* is not included in within the segment. This region does not seem to contain any candidate genes well-known for the BP control. Thus the final identification of the QTL will most likely lead to the discovery of a brand new gene for the BP regulation.

3.2 INTRODUCTION

The mapping of quantitative trait loci (QTL) for blood pressure (BP) using animal models has been greatly facilitated by the genetic approach, such as linkage analysis and the use of congenic strains (Rapp and Deng 1995, Deng 1998, Dominiczak et al. 2000). In our previous work, regions on rat Chr.2 were shown to contain QTL, first by linkage analysis (Deng and Rapp 1992, Deng et al. 1994) followed by the use of two congenic strains (Deng et al. 1997a). In one congenic strain, the chromosome region of interest from the Dahl salt-sensitive strain (S) was replaced by that of the Milan normotensive (MNS) rat (Deng et al. 1997a). The length of the region involved in making the congenic strain was about 80 cMs, because the linkage evidence suggested (Deng et al. 1994) that one QTL was near the locus for the Na^+/K^+ATPas subunit α gene (*Atp1a1*) and another near the locus for the angiotensin receptor AT1B gene (*Agtr1b*) in an F_2(S x MNS) population. These two QTL could be separated by more than 40 cMs.

To further narrow down the region containing the QTL, we made congenic substrains from the original strain, S.MNS-D2Mit6/ alcohol dehydrogenase (*Adh*) (Deng et al. 1997a), and studied blood pressure of these substrains.

3.3 METHODS

3.3.1 Animals

The SS/Jr rat used for making congenic substrains was provided by Dr. J. Rapp and will be designated as S in the present report. The genomic DNA for every S rat used for maintaining the strain and BP studies at each generation has been extracted and verified by genotyping. In order to insure that the S strain remains as genuine as the SS/Jr rat directly obtained from Dr. J. Rapp in our facility, a rigorous and strict quality control procedure has been instituted. It consists of two parts, (a) genetic testing; (b) physical distinction. In an approximate genomic scan, 88 markers roughly evenly spaced (on average 10-15 cM) throughout the rat genome were tested. Each DNA sample for every S rat tested was compared with the true S DNA standard that we used in our original work (Deng et al. 1997a). So far, no marker differences have been detected (data not shown). Moreover, all our S rats have, in addition to a skin tag, an ear mark punch hole designed to facilitate their identification and to reduce potential handling errors.

The original congenic strain used to initiate the study is designated as S.MNS-D2Mit6/*Adh*, and is the same as published previously (Deng et al. 1997a). In brief, it was made by 8 consecutive backcrosses, and consequently putting the MNS chromosome region between D2Mit6 and *Adh* markers on the S genetic background. This strain, therefore, is homozygous MNS (i.e. MM) for the region in question and homozygous SS for the rest of the genome. This conclusion has been supported by the genotyping of 57 markers scattered throughout the rat genome other than on Chr.2 (data not shown). Figure 1 contains the Chr. 2 map, additional markers tested for the Chr.2 regions, and the chromosome fragment in question in the congenic strain. This original congenic strain is abbreviated as S.M. The authenticity of the strain has been established by genotyping the markers between D2Mit6 and *Adh* on Chr.2 (Figure 1).

Protocols for handling as well as maintaining animals were approved by our institutional animal committee. All the procedure for the experiment was in accordance with the guidelines of local, provincial and federal regulations.

3.3.2 Breeding scheme for generating substrains

Rats of the original congenic strain, S.M (Figure 1) were first bred with S to produce F_1 rats, which in turn were intercrossed to produce F_2. An F_2 rat with crossovers in the region between D2Mit6 and *Adh* markers was retained, and then backcrossed (BC) to a S rat to duplicate the region of interest. A female and a male BC rats were crossed to finally generate rats homozygous MM for the region of interest, but homozygous SS for the rest of Chr. 2 and rest of the genome. The progeny of each of these crosses constitute a congenic substrain dividing the initial segment involved in S.MNS-D2Mit6/*Adh*. Substrains, S.MNS-D2Mit6/D2Rat166 and S.MNS-D2Mit6/D2Rat303 are abbreviated as, S.M1 and S.M2 respectively (Figure 1). The chromosome region homozygous MM in the substrain and the original S.M strain are shown as solid bars in Figure 1. All the markers in the region were genotyped for each congenic strain in question.

3.3.3 Preparation of rats for BP measurements

The mating pairs for the S strain and congenic strains to be studied were bred simultaneously and in the same facility. Male rats were chosen from two separate litters of the same strain when possible, in order to minimize potential litter effects. The chosen rats were weaned at 21 days of age, maintained on a low salt diet (0.2% NaCl, Harlan Teklad 7034) and then fed a high salt diet (2% NaCl, Harlan Teklad 94217) starting from 35 days of age until the end of the experiment.

Telemetry probes were implanted when rats were 56 days old with their body weights between 250-320 grams. Before the surgery, the rats were anesthetized by the inhalation of isoflurane at a dose of 4% for 3 min. During surgery, the state of anesthesia was maintained by isoflurane at a dose of 1.5-2%. The catheter of a telemetry probe was implanted through the femoral artery until it reached all the way up

to the abdominal aorta, and secured by a suture to the blood vessel. Some investigators place the catheter in the abdominal aorta directly. In our experience and as advised by Data Sciences Inc., the placement in the abdominal aorta could cause more paralysis in the rats than it was through the femoral artery. Both methods are reliable for BP measurements. The telemetry probe was fixed to the muscle wall, and the transmitter was left in the abdominal cavity. After the surgery, the rats were allowed to recuperate for 10-17 days. In the first three days of recuperation, the rats were fed Jello-O containing the analgesic buprenorphine at 0.5% mg/kg. Afterwards, they were given a diet of Ensure milkshake with chocolate in addition to the 2% NaCl regular food. This supplement usually lasted up to 5 days to facilitate their appetite and thus weight gain. In total, the rats are allowed at least 10 days for the postoperative recuperation.

3.3.4 BP measurement

The telemetry system from the Data Sciences Inc. (St. Paul, MN) was used. Each telemetry probe was calibrated before and cleaned after each usage according to manufacturer's instructions. A dedicated technician was thoroughly trained by the Data Sciences Inc. for the procedure of telemetry.

3.3.5 Statistical analysis

Repeated measures' Analysis of variance (ANOVA) followed by Tukey in the SYSTAT 9 program (SPSS Sci. Chicago, IL) was used to compare the significance level for a difference or a lack of it in a BP component between a congenic strain (or substrain) and the S strain. In the analysis, a BP component was compared at each day for the period of measurement among the strains.

3.3.6 DNA extraction and Genotyping

DNA for each rat was extracted by tail biopsy using a Qiagen Genome kit and the genotype of each rat was determined by PCR based on the methods previously published (Deng et al. 1994, 1997a, b).

3.3.7 Radiation Hybrid mapping

A rat/hamster (RH) panel of 96 radiation hybrids was purchased from Research Genetics (Huntville, AL, http://www.resgen.com/). For chromosome mapping, each marker was genotyped using RH by PCR according to a previously published protocol (5-7). To locate a marker of interest onto an existing RH framework map, the results of genotyping were entered into a web site, http://rgd.mcw.edu/RHMAPSERVER.

3.4 RESULTS

3.4.1 Congenic strain monitoring

All the congenic strains in Figure 1 have been tested for 57 markers scattered throughout the chromosomes and turned out to be SS homozygous (data not shown). All the markers for the region of interest on Chr. 2 in each congenic strain are MM homozygous. An effort was made to produce a congenic strain, which is homozygous MM for a Chr.2 region, but does not show a BP-lowering effect. This strain is designated as a negative control (e.g. S.M2 in Figures 1 and 2).

3.4.2 BP Study designs

The basic design of raising animals is similar to our previous congenic work regarding the age and sex, and in terms of the timetable of dietary treatments (Deng et al.1997a). The most apparent difference is that BP measurements in the current study were direct and continuous for a period of 10-20 days. All the BP components were measured including systolic, diastolic and mean arterial pressures.

3.4.3 BP measurements

Readings for each BP parameter were recorded every 10 seconds for each rat. Theoretically, one could obtain 10 x 6 x 60 x 24 = 8640 readings for a rat daily. However, to cover all the rats in one experimental setting, a ten-second reading was done for each rat at a 2- minute interval during a 24-hour period for the duration of the experiments. As a result, 60/2 x 24 = 720 measurements were collected daily for each

rat. For the simplicity of presentation, each point in the graphs in Figure 2 represents averaged 24-hour readings taken from every four hours.

3.4.4 Mapping of a BP QTL by analyzing BP effects associated with chromosome segments

Figure 2 shows the comparison of MAP, DAP and SAP of S rats with those of two congenic rats. The chromosome regions containing MNS substitutions in the congenic rats are shown in Figure 1. A total of 9 S rats were pooled from separate and independent cohorts of measurement for different periods of times, because the differences in S rats among the separate cohorts turned out not to be statistically significant ($P>0.39$). Two independent cohorts for each of S.M1 and S.M2 substrains were pooled also for the same reason ($p>0.86$).

The Mapping of a QTL of interest can be done as follows. Congenic strains were compared to each other in the length of the chromosome replacements and for their effects on BP. The region containing the QTL can be localized to the segment either in common between two strains both having a BP effect; or in the fragment not shared in two strains, one having and the other lacking a BP effect.

3.5 DISCUSSION

Mean arterial (MAP), diastolic (DAP) and systolic (SAP) pressures of the original congenic strain S.M was significantly ($p<0.0001$) lower than that of the S strain (lowest line tracing in each graph in Figure 2), thus confirming the presence of BP QTL in the chromosome segment involved. S.M. differs from the S strain essentially in the Chr.2 regions in question, i.e. homozygous MM (Figure 1), and homozygous SS for the rest of the genome as shown by genotyping 57 markers across the rat genome (data not shown). More importantly, no matter what effects the remaining MNS genome had in the S background in the congenic S.M strain, it definitely did not influence BP because S.M2, which was entirely derived from S.M, showed the same BP as that of S. This fact indicates, without doubt, that the BP-lowering effect shown in S.M is entirely due to the

Chr.2 segment not shared between S.M and S.M2. Therefore, at least one BP QTL has to exist in the Chr.2 segment between D2Mco9 and *Adh* markers.

A region containing a BP QTL can be narrowed further by comparing S.M1 and S.M2. S.M1 has a BP significantly ($p<0.03$) lower than that of S, whereas S.M2 is practically the same ($p>0.87$, Figure 2). Thus, one can conclude that a BP QTL should exist in the non-overlapping region. That is between D2Rat302 and *Nep* markers, which effectively places the QTL in question within a segment of 5.7 cM. The current work provides the first line of evidence for locating a BP QTL to such a small region on Chr 2. A definitive proof will come from making a congenic substrain just for this 5.7 cM section, which would show a comparable BP-lowering effect.

It is noteworthy that BP of the S.M1 congenic strain is significantly ($p<0.03$) lower than that of the S strain, but significantly ($p<0.02$) higher than that of the S.M congenic strain (Figure 2). This observation indicates that the entire BP effect contained in S.M can not be fully accounted for by S.M1. Therefore, there seems to be another QTL located in the region between *Nep* and *Adh* markers.

The work of investigators using other hypertensive models has also shown that there is a QTL in the same, but broad chromosome region (Pravenec et al. 1995, Schork et al. 1995, Samani et al. 1996, Vincent et al. 1997, Jeffs et al. 2000). The QTL of interest appears to be common to most of the hypertensive rat strains. By extrapolation, the QTL might be conserved in other mammals potentially including humans. Although, our experiments put rats on 2% NaCl, this setup was largely designed to hasten the expression of hypertension without killing the animals in a shorter period of time than otherwise would have been without it. The salt sensitive nature of the S rats may restrict the general applicability of other BP QTL discovered from the S strain to different hypertensive strains.

Because the S.M2 substrain does not show a BP effect, the *Agtr1b* gene can not be supported as a candidate for a BP QTL. Combining our previous sequencing analysis

(Deng and Rapp 1994), the *Agtr1b* gene does not appear to be a candidate. The various linkage analyses conducted in different crosses (Deng et al. 1994, Deng and Rapp 1994, Dubay et al. 1993) most likely resulted from statistical artifacts. Once again, our current work illustrates the importance of the congenic approach to either support or refute the results of linkage analyses.

3.6 ACKNOWLEDGEMENTS

We thank Dr. J. Rapp for providing the S strain and for collaborating on making the original congenic strain, S.MNS-*Adh*/D2Mit6. We also thank Dr. J. Tremblay in helping set up the telemetry system and Ms. Marie-Claude Guertin in statistical analyses. This work was supported by grants from American Heart Associate National Center (0140149N) and from Medical Research Council of Canada (MOP36378) to AY. Deng. AY. Deng is an Established Investigator of the American Heart Association.

3.7 REFERENCES

Deng Y, Rapp JP. (1992). Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic peptide receptor genes using Dahl salt-sensitive rats. *Nature Genet* 1:267-272.

Deng AY, Dene H, Rapp JP. (1994). Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. *J. Clin. Invest.* 94:431-436.

Deng AY, Rapp JP. (1994). Evaluation of the angiotensin II receptor AT1B gene as a candidate gene for blood pressure. *J. Hyperten.* 12:1001-1006.

Deng AY, Dene H, Rapp JP. (1997a). Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension* 30:199-202.

Deng AY, Jackson CM, Hoebee B, Rapp JP. (1997b). Mapping of rat chromosome 2 markers generated from chromosome-sorted DNA. *Mamm. Genome* 8:731-735.

Deng AY. (1998). In search of hypertension genes in Dahl salt-sensitive rats. *J. Hypertension* 16:1707-1717.

Dominiczak AF, Negrin DC, Clark JS, Brosnan MJ, McBride MW, Alexander MY. (2000). Genes and Hypertension: from gene mapping in experimental models to vascular gene transfer strategies. *Hypertension* 35:164-172.

Dubay C, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi J, Kotelevtsev Y, Beckmann JS, Soubrier R, Sassard J, Lathrop GM. (1993). Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nature Genet* 3:354-357.

Jeffs B, Negrin CD, Graham D, Clark JS, Anderson NH, Gauguier D, Dominiczak AF. (2000). Applicability of a 'speed' congenic strategy to dissect blood pressure quantitative trait loci on rat Chromosome 2. *Hypertension* 35:179-187.

Pravenec M, Gauguier D, Schott J-J, Buard J, Krén V, Bila V, Szpirer C, Szpirer J, Wang J-M, Huang H, St. Lezin E, Spence MA, Flodman P, Printz M, Lathrop GM, Vergnaud G, Kurtz TW (1995). Mapping of quantitative trait loci for blood pressure and cardiac mass in the rat by genome scanning of recombinant inbred strains. *J Clin Invest* 96:1973-1978.

Rapp JP, Deng AY. (1995). Detection and positional cloning of blood pressure quantitative trait loci: is it possible? *Hypertension* 25:1121-1128.

Samani NJ, Gauguier D, Vincent M, Kaiser MA, Bihoreau M-T, Lodwick D, Wallis R, Parent V, Kimber P, Rattray F, Thompson JR, Sassard J, Lathrop M. (1996). Analysis of quantitative trait loci for blood pressure on rat chromosomes 2 and 13. *Hypertension* 28:1118-1122.

Schork NJ, Krieger JE, Trolliet MR, Franchini KG, Koike G, Kreiger, EM, Lander ES, Dzau VJ, Jacob HJ. (1995). A biometrical genome search in rats reveals the multigenic basis of blood pressure variation. *Genome Res* 5:164-172.

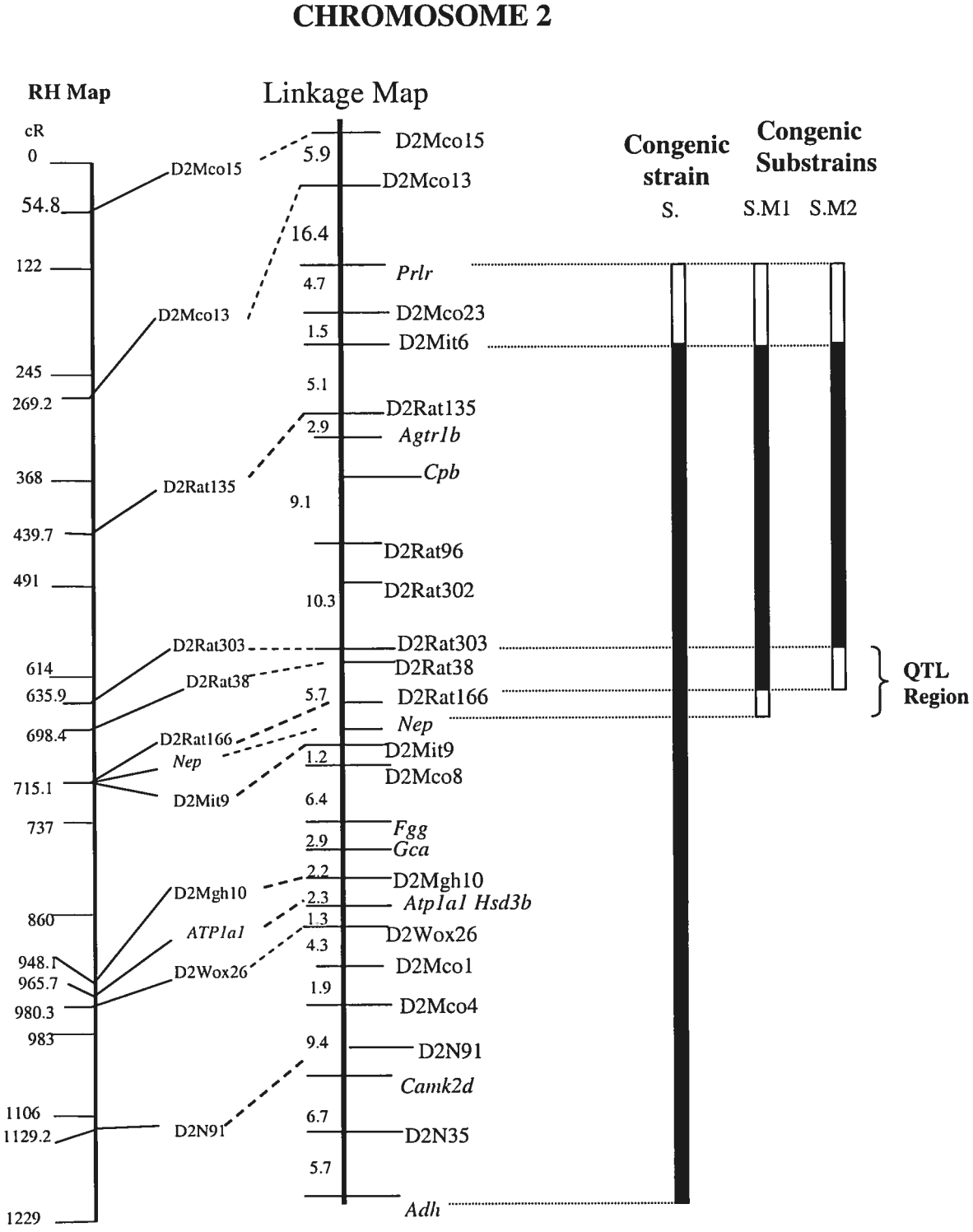
Vincent M, Samani NJ, Gauguier D, Thompson JR, Lathrop G, Sassard J. (1997). A pharmacogenetic approach to blood pressure in Lyon hypertensive rats. *J Clin Invest* 100:2000-2006.

3.8 FIGURE LEGENDS AND FIGURES

Figure 1. **Chr. 2 regions replaced in the original congenic strain and two substrains.** The linkage map is essentially the same as published previously (Deng et al.1997b), which is based on an F_2(S x MNS) population. Numbers to the left of the linkage map are units in cM. The order of most of the loci on the map has been initially determined by linkage using the MAPMAKER program, and then verified by scoring crossovers during the construction of congenic substrains. RH map refers to the map based on rat/hamster radiation hybrids, in which units are in centiRays (cR). S.M represent the original congenic strains S.MNS-*Adh*/D2Mit6 (Deng et al.1997a). S.M1 and S.M2 are substrains that have been derived from S.M and studied. Solid bars under congenic strain and the substrains symbolize the S chromosome fragments that have been replaced by that of the MNS rat. The entire region indicated by solid bars and junctions between the solid and open bars are homozygous MM on the map for all the markers listed in the corresponding positions. Open bars on ends of solid bars indicate the ambiguities of crossover breakpoints between markers. Junctions between solid and open bars as well as ends of chromosome regions of interest in each strain are connected by dotted lines to the marker positions on the map. *Adh*, alcohol dehydrogenase; *Agtr1b*, angiotensin receptor type 1B; *Atp1a1*, Na^+K^+-ATPase α1; *Camk2d*, calmodulin-dependent protein kinase II-delta; *Cpb*, carboxypeptidase B; *Fgg*, fibrinogen gamma; *Gca*, guanylyl cyclase A/atrial natriuretic peptide receptor; *Hsd3b*, 3-hydroxysteroid dehydrogenase/delta 5 isomerase; *Nep*, neutral endopeptidase; *Prlr*, prolactin receptor. The rest of the markers are anonymous (Deng et al. 1997b, http://waldo.wi.mit.edu/rat/public/). S.M1 and S.M2 represent substrains S.MNS-D2Mit6/166 and S.MNS-D2Mit6/D2Rat303 respectively. S, the Dahl salt-sensitive strain.

Figure 2. **Comparison in BP components between the S.M, S.M1 and S.M2 congenic strains, and the S strain**. Mean arterial (MAP, a), diastolic (DAP, b) and systolic (SAP, c) pressures were significantly different ($p<0.0001$) between the S.M (n=5) congenic and the S (n=9), different ($p<0.03$) between the S.M1 (n=8) congenic strain and the S (n=9) strains, and different ($p<0.02$) between S.M1 and S.M. In contrast, the difference in MAP, DAP and SAP between the S.M2 (n=8) and S (n=9) strains is not significant ($p>0.87$). Each time point on the graph represents an average of 24-hour readings. (d) represents a sampling of the diurnal variations in two strains. All the numbers on the vertical axes (or Y) are in mmHg. S.M, S.M1 and S.M2 represent S.MNS-D2Mit6/*Adh*, S.MNS-D2Mit6/*Nep* and S.MNS-D2Mit6/D2Rat302 strains respectively. S, the Dahl salt-sensitive strain.

Figure 1


CHROMOSOME 2
RH Map
Linkage Map
cR
0
54.8
122
245
269.2
368
439.7
491
614
635.9
698.4
715.1
737
860
948.1
965.7
980.3
983
1106
1129.2
1229
D2Mco15
D2Mco13
D2Rat135
D2Rat303
D2Rat38
D2Rat166
Nep
D2Mit9
D2Mgh10
ATP1a1
D2Wox26
D2N91
5.9
16.4
4.7
1.5
5.1
2.9
9.1
10.3
5.7
1.2
6.4
2.9
2.2
2.3
1.3
4.3
1.9
9.4
6.7
5.7
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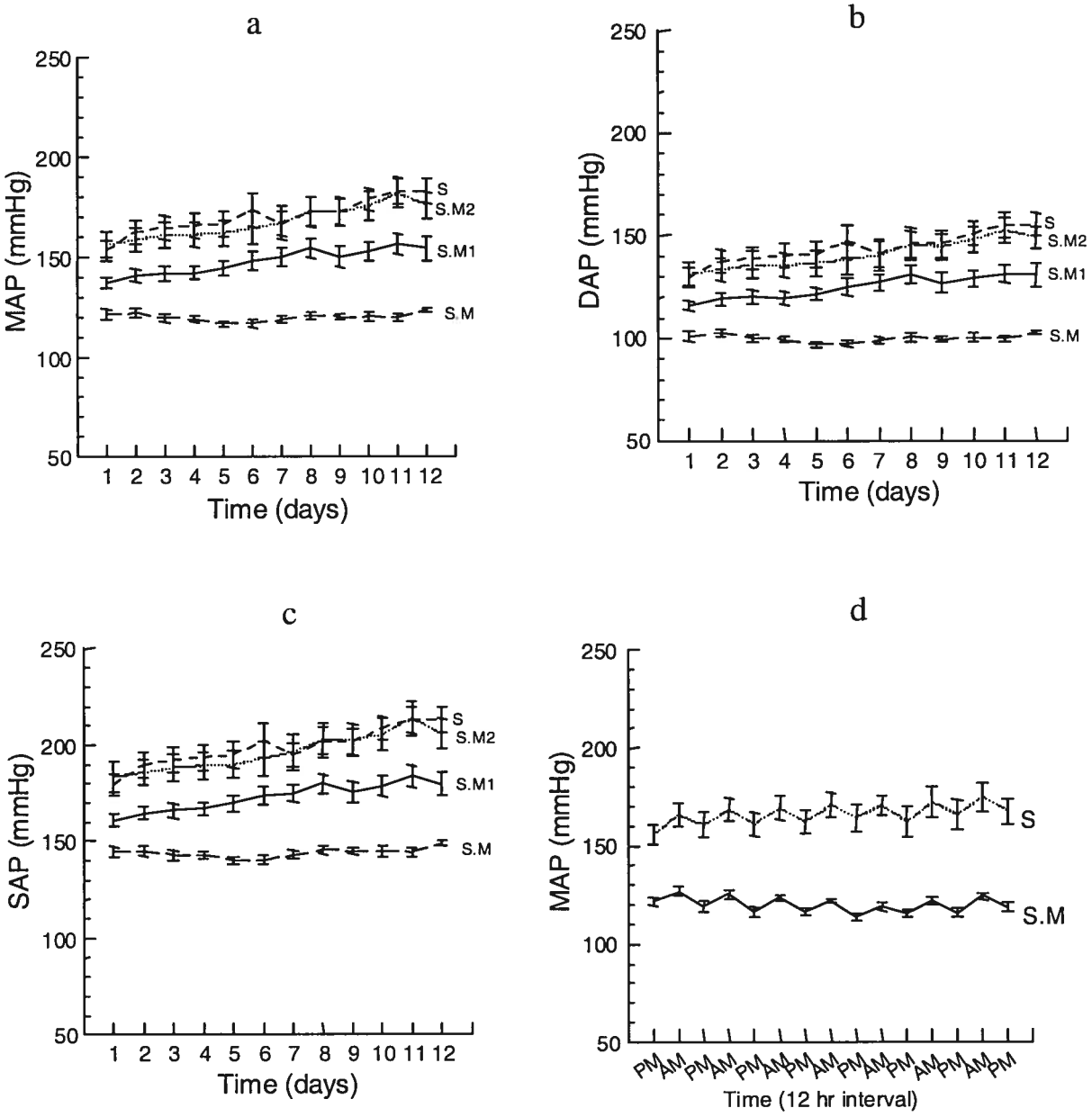
D2Mco13
Prlr
D2Mco23
D2Mit6
D2Rat135
Agtr1b
Cpb
D2Rat96
D2Rat302
D2Rat303
D2Rat38
D2Rat166
Nep
D2Mit9
D2Mco8
Fgg
Gca
D2Mgh10
Atp1a1 Hsd3b
D2Wox26
D2Mco1
D2Mco4
D2N91
Camk2d
D2N35
Adh
Congenic strain
S.
Congenic Substrains
S.M1
S.M2
QTL Region

Figure 2


a
MAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12
Time (days)
S
S.M2
S.M1
S.M
b
DAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12
Time (days)
S
S.M2
S.M1
S.M
c
SAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12
Time (days)
S
S.M2
S.M1
S.M
d
MAP (mmHg)
250
200
150
100
50
PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM
Time (12 hr interval)
S
S.M

CHAPTER 4

Further chromosomal mapping of a blood pressure quantitative trait locus in Dahl rats on Chromosome 2 using congenic strains

Julie Dutil, Alan Y. Deng

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Research Centre-CHUM, Hôtel Dieu, 3840 rue St. Urbain, Montréal, Québec, H2W 1T8, Canada

Running title: Blood pressure QTL mapping

Corresponding author: Alan Deng, Ph.D.

Chief of Molecular Genetics and Associate Professor of Medicine

Research Centre

Centre Hospitalier de l'Université de Montreal (CHUM)

7-132 Pavillon Jeanne Mance

3840, rue St. Urbain

Montreal, Quebec, H2W 1T8

Canada

Phone: 514-843-2611 ex. 5522

Fax: 514-843-2911

[redacted]

4.1 ABSTRACT

Both linkage and use of congenic strains have shown that a region on rat Chromosome (Chr.) 2 of Dahl salt-sensitive rats (S) contained a quantitative trait locus (QTL) for blood pressure (BP). A congenic strain was made by replacing a segment of the S rat by the homologous region of the Milan normotensive rat (MNS). Since the region was roughly 80 centiMorgans (cMs) in size, a further reduction is required towards the final identification of the QTL. Currently, three congenic substrains were made by replacing smaller sections within the 80 cM. Each strain contains a specific region of MNS in the S genetic background. Two of the three congenic strains shared a segment in common and both showed a BP-lowering effect. One of the three congenic strains carried a unique segment and had the same BP as S. Deducing the fragment shared in the two substrains having an effect, the BP QTL has to be present in a region of roughly 15 cMs. In contrast to BP, heart rates of all the congenic rats were the same as that of the S rat. Thus, BP and the heart rate are under the control of independent genetic determinants.

Key words: genetic hypertension, rat Chromosome 2, Milan normotensive rat

4.2 INTRODUCTION

The mapping of quantitative trait loci (QTL) for blood pressure (BP) using animal models has been greatly facilitated by the genetic approach, such as linkage analysis and the use of congenic strains (1, 7, 13). In our previous work, regions on rat Chr.2 were shown to contain a QTL first by linkage analysis (2, 3, 5). The evidence suggested (2) that there might be two QTL separated by more than 40 cM, one near the locus for the Na^+/K^+ATPase subunit α gene (*Atp1a1*) and another near the locus for the angiotensin receptor AT1B gene (*Agtr1b*) in an F_2(S x MNS) population. The presence of a QTL was proved by the use of two congenic strains (3). In one congenic strain, the chromosome region of interest from the Dahl salt-sensitive strain (S) was replaced by that of the Milan normotensive (MNS) rat (3). The length of the region involved in making the congenic strain was about 80 cMs.

To further narrow down the region containing the QTL, we made congenic substrains from the original strain, S.MNS-D2Mit6/ alcohol dehydrogenase (*Adh*) (3), and studied blood pressures of these strains using telemetry.

4.3 METHODS

4.3.1 Animals

The S rat used for making congenic strains was provided by Dr. John Rapp. The original litter of 5 male and 5 female S rats was obtained directly from Dr. John Rapp (January 14, 1998). From these rats, the first independent breeding of them by Alan Deng was done on February 6, 1998, followed by three more generations at the Medical College of Ohio, Toledo, USA. On December 7, 1998, two litters of S rats were shipped to the authors' current location at Montreal, Canada. After a period of quarantine and at the third generation (born on December 10, 1999) bred from the shipped S rats, the current telemetry studies began. Therefore, a total of 6 generations had elapsed from the time the rats originated from Dr. John Rapp to our first telemetry study. The genomic DNA for every S rat used for maintaining the strain and BP studies at each generation has been extracted and verified by genotyping.

In order to insure that the S strain remains as genuine as the SS/Jr rat directly obtained from Dr. J. Rapp in our facility, a rigorous and strict quality control procedure has been instituted. It consists of two parts, (a) genetic testing; (b) physical distinction. In an approximate genomic scan, 88 markers roughly evenly spaced (on average 10-15 cM) throughout the rat genome were tested at every third generation of breeding starting from generation 5. At every generation at least one marker randomly chosen for each chromosome was genotyped, and at every other generation, at least 2-3 markers for each chromosome were genotyped. Each DNA sample for every S rat tested was compared with the true S DNA standard that we used in our original work (3). At the time the current work was done, there were only S, S.M and S.M strain derivatives present in our animal facilities (see below). If any potential genetic contamination of the S strain would happen, it could only come from an accidental breeding between the S and S.M

strains. This possibility was categorically eliminated by genotyping markers (shown in Figure 1) on the rats of the S strain in each generation. Moreover, all our S rats have, in addition to a skin tag, an ear mark punch hole designed to facilitate their identification and to reduce potential handling errors.

The original congenic strain used to initiate the study is designated as S.MNS-D2Mit6/*Adh*, and is the same as published previously (3). This original congenic strain is abbreviated as S.M. In brief, it was made by 8 consecutive backcrosses, and consequently putting the MNS chromosome region between D2Mit6 and *Adh* markers on the S genetic background. This strain, therefore, is homozygous MNS (i.e. MM) for the region in question and homozygous SS for the rest of the genome. This conclusion has been supported by the genotyping of 57 markers scattered throughout the rat genome other than on Chr.2 (data not shown). Figure 1 contains the Chr. 2 map, and the chromosome fragment in question in the congenic strain.

The S.M strain was transferred to Alan Deng from Dr. John Rapp (January 14, 1998) and has been maintained for approximately the same generations as S. Two S.M litters were then shipped along with the S rats to Montreal at the same time as S. The authenticity of the strain has been established by genotyping the markers between D2Mit6 and *Adh* on Chr.2 (Figure 1).

Protocols for handling as well as maintaining animals were approved by our institutional animal committee. All the procedure for the experiment was in accordance with the guidelines of local, provincial and federal regulations.

4.3.2 Breeding scheme for generating substrains

Rats of the original congenic strain, S.M (Figure 1) were first bred with S to produce F_1 rats, which in turn were intercrossed to produce F_2. An F_2 rat with crossovers in the region between D2Mit6 and *Adh* markers was retained, and then backcrossed (BC) to an S rat to duplicate the region of interest. A female and a male BC rats were crossed to finally generate rats homozygous MM for the region of interest, but homozygous SS for

the rest of Chr. 2 and rest of the genome. The progeny of each of these crosses constitute a congenic substrain dividing the initial segment involved in S.MNS-D2Mit6/*Adh.* S.MNS-D2Mit6/D2Rat303 is simplified as S.M2; S.MNS-*Neutral endopeptidase* (*Nep*)/D2Mit14 as S.M5; and S.MNS-*Nep*/atrial natriuretic peptide receptor/guanylyl cyclase A (*Gca*) as S.M6. The chromosome region homozygous MM in each substrain is shown as solid bars in Figure 1.

The first backcross between the S and S.M rats occurred on February 6, 1998, and all the substrains were finally fixed to the MNS homozygosity for each region in question by January 9, 2000. The chromosome region homozygous MM in the substrain and the original S.M strain are shown as solid bars in Figure 1. All the markers in the region were genotyped for each congenic strain in question.

4.3.3 Preparation of rats for BP measurements

The mating pairs of the S and congenic strains to be studied were bred simultaneously and in the same facility. Male rats were chosen from two separate litters of the same strain when possible, in order to minimize potential litter effects. The chosen rats were weaned at 21 days of age, maintained on a low salt diet (0.2% NaCl, Harlan Teklad 7034) and then fed a high salt diet (2% NaCl, Harlan Teklad 94217) starting from 35 days of age until the end of the experiment.

Telemetry probes of PA-D70 implantable device were implanted when rats were 56 days old with their body weights between 250-320 grams. The procedure follows that of Data Sciences Inc (St. Paul, MN, USA). Before the surgery, the rats were anesthetized by the inhalation of isoflurane at a dose of 4% for 3 min. During surgery, the state of anesthesia was maintained by isoflurane at a dose of 1.5-2%. The pressure catheter, which contains the Biocompatible gel at the tip and the noncompressable fluid connecting the tip to the pressure sensor, was implanted through the femoral artery until it reached all the way up to the abdominal aorta. It is secured by sutures to the blood vessel. The telemetry devise body, which includes the pressure sensor, the reusable electronics module and a battery, was fixed to the muscle wall, and was left in the

abdominal cavity. After the surgery, the rats were allowed to recuperate for 10-17 days. In the first three days of recuperation, the rats were fed Jello-O containing the analgesic buprenorphine at 0.5% mg/kg. Afterwards, they were given a diet of Ensure milkshake with chocolate in addition to the 2% NaCl regular food. This supplement usually lasted up to 5 days to facilitate their appetite and thus weight gain. In total, the rats are allowed at least 10 days for the postoperative recovery.

4.3.4 BP measurement

The telemetry system from the Data Sciences Inc. was used. Each telemetry probe was calibrated before and cleaned after each usage according to manufacturer's instructions. A dedicated technician was thoroughly trained by the Data Sciences Inc. for the procedure of telemetry.

4.3.5 Statistical analysis

Repeated measures' Analysis of variance (ANOVA) followed by Tukey in the SYSTAT 9 program (SPSS Sci. Chicago, IL) was used to compare the significance level for a difference or a lack of it in BP between a congenic strain (or substrain) and the S strain. In the analysis, BP was compared at each day for the period of measurement among the strains.

4.3.6 DNA extraction and Genotyping

DNA for each rat was extracted by tail biopsy using a Qiagen Genome kit and the genotype of each rat was determined by PCR based on the methods previously published (2-4).

4.3.7 Radiation Hybrid mapping

A rat/hamster (RH) panel of 96 radiation hybrids was purchased from Research Genetics (Huntville, AL, http://www.resgen.com/). For chromosome mapping, each marker was genotyped using RH by PCR according to a previously published protocol (2-4). To locate a marker of interest onto an existing RH framework map, the results of genotyping were entered into a web site, http://rgd.mcw.edu/RHMAPSERVER (9).

4.4 RESULTS

4.4.1 BP Study designs

The basic design of raising animals is similar to our previous congenic work in terms of age and sex, and for the timetable of dietary treatments (3). The most apparent difference is that BP measurements in the current study were direct and continuous for a period of 10-20 days. All the BP components were measured including mean arterial (MAP), systolic (SAP), and diastolic (DAP) pressures.

4.4.2 BP measurements

Readings for each BP parameter were recorded every two minutes during a 24-hour period for the duration of the experiments. As a result, 60/2 x 24 = 720 measurements were collected daily. For the simplicity of presentation, each point in the graphs in Figure 2 represents averaged 24-hour readings taken from every four hours. Each error bar represents SEM for all the individual data points collected from every four hours for all the rats of that strain. MAPs, SAPs and DAPs for all the strains are given to show their magnitudes and ranges.

4.4.3 Mapping of BP QTL by analyzing BP effects associated with chromosome segments

Figure 2 shows the comparison of MAPs, SAPs and DAPs of S rats with those of congenic rats. The chromosome regions containing MNS substitutions in each congenic strain are shown in Figure 1. A total of 9 S rats were pooled from separate and independent cohorts of BP measurement during different periods of times, because the differences in S rats among the separate cohorts turned out not to be statistically significant ($P>0.39$). The BP data from two independent cohorts for the S.M2 substrain were pools also for the same reason ($p>0.87$).

As shown in Figure 2, MAPs, DAPs and SAPs were significantly lowered ($p<0.001$) in the S.M (n=5), S.M5 (n=4) and S.M6 (n=7) congenic strains in comparison with that of

the S (n=9) strain. But the difference in MAP, DAP and SAP between the S.M2 (n=8) and S (n=9) strains is not significant ($p>0.86$).

Heart rates of the rat strains were measured and are shown in Figure 3. Each graph represents the rat strains compared simultaneously during the same period of time. The heart rates of S rats were significantly different from one cohort to another, and thus could not be pooled. This fluctuation probably reflects mostly the effects of the environment.

4.5 DISCUSSION

BP of the original congenic strain S.M was shown to be significantly lower than that of the S strain (Figure 2), thus confirming the presence of BP QTL in the chromosome segment involved. To narrow down the location of the QTL, the original segment was split into subsections (shown as solid bars for S.M2, S.M5 and S.M6 in Figure 1).

Our congenic strains differ from the S strain essentially in the Chr.2 regions in question, i.e. homozygous MM (Figure 1), and homozygous SS for the rest of the genome as shown by genotyping a number of markers across the rat genome (data not shown). More importantly, no matter what effects the remaining MNS genome had in the S background in the congenic S.M strain, it definitely did not influence BP because S.M2, which was entirely derived from S.M, showed the same BP as that of S. This fact indicates, without doubt, that the BP-lowering effect shown in S.M is entirely due to the Chr.2 segment not shared between S.M and S.M2. Therefore, at least one BP QTL has to exist in the Chr.2 segment between D2Mit9 and *Adh* markers.

A region containing a BP QTL can be narrowed further by examining S.M5 and S.M6. By comparing these substrains, one can reasonably conclude that a region containing a BP QTL should be located in a minimum segment shared by both substrains, i.e. between D2Rat166 and D2Mgh10 (roughly 15 cMs). In this case, the same region was tested independently two times and turned out to have a BP-lowering effect every time.

In contrast to BP, heart rates of the all the congenic strains are the same as that of the S rat ($p>0.49$) when averaged at every 24 hours. This is striking when noticing that MAPs of S.M5 and S are significantly different and MAPs of S.M2 and S are not (Figure 2d), whereas the heart rates of all three strains were not significantly different (Figure 3c). These observations indicate that a QTL present in the chromosome region of interest influences only blood pressure, not heart rate. Thus, blood pressure and heart rate are controlled independently by different genes.

Upon closer examinations, it appears that there could be a sizable difference in heart rates between S and both S.M (Figure 3b) and S.M6 (Figure 3f) within an 24-hour period. It is to be determined, however, if these differences within 24 hours could be genetic because S.M5 had a similar heart rate as S (Figure 3d), whereas S.M6 had a slower heart rate (Figure 3f). Both S.M5 and S.M6 shared a chromosome segment in common (Figure 1). There are reports of genetic determinants for heart rates independent of BP in both humans (16) and the spontaneously hypertensive rats (11).

Although S.M5 contained *Atp1a1* whereas S.M6 did not, but both showed a BP effect with a similar magnitude (Figure 2b and c). This fact would argue against the candidacy of *Atp1a1*. But since the possibility of an epistatic gene-gene interaction among potential multiple QTL in the same region could not be ruled out at this point, *Atp1a1* could not be excluded as a candidate QTL. A definitive proof will come from a congenic strain involving a minimum chromosome segment containing the *Atp1a1* locus.

For a more in-depth study of a BP QTL, especially when one attempts to study a time course relationship, telemetry appears to be more appropriate, because it can provide continuous measures for all components of BP. Moreover, if diastolic and systolic pressures are controlled differently, they might contain separate genetic determinants also. Telemetry is ideal for monitoring each separate component of BP and following it with time. On the other hand, the BP measurements obtained by telemetry have an environmental element not present in tail cuff, that is the stress induced by surgery. It is

reassuring, though, that both tail cuff (3) and telemetry (the present study) showed a BP-lowering effect for S.M.

The work of investigators using other hypertensive models has also shown that there is a QTL in the same broad chromosome region either in linkage analysis (12, 14, 15, 17) or using congenic strains (10). The QTL of interest appears to be common to most of the hypertensive rat strains. By extrapolation, the QTL might be conserved in other mammals potentially including humans. Although, our experiments put rats on 2% NaCl, this setup was largely designed to hasten the expression of hypertension in a shorter period of time than otherwise would have been without it. The salt sensitive nature of the S rats may restrict the general applicability of other BP QTL discovered from the S strain to different hypertensive strains.

Because the S.M2 substrain does not show a BP effect, the *Agtr1b* gene can not be supported as a candidate for a BP QTL. Combining our previous sequencing analysis (6), the *Agtr1b* gene does not appear to be a candidate. The various results of linkage analyses conducted in different crosses (2, 6, 8,) most likely reflected statistical artifacts. Once again, our current work illustrates the importance of the congenic approach to either support or refute the results of linkage analyses.

The region containing the BP QTL in the rat shares a conserved synteny relationship with a segment of mouse Chr.3, and segments of human CHR.1 and 4 (http://www.well.ox.ac.uk/rat_mapping_resources/Comparative_maps/compa_map_chr02.html). A fine mapping of the QTL in question may further refine the exact portion of the human chromosome with which it has a conserved synteny, and eventually help unravel some of the genetic determinants in human hypertension. As the human and mouse genome projects progress, genomic information obtained might also contribute to the identification of the QTL in the rat.

4.6 ACKNOWLEDGMENTSs

We thank Dr. J. Rapp for providing the S strain and for collaborating on making the original congenic strain, S.MNS-*Adh*/D2Mit6. We also thank Dr. J. Tremblay in helping set up the telemetry system and Ms. Marie-Claude Guertin in statistical analyses. This work was supported by grants from American Heart Associate National Center (0140149N) and from Canadian Institutes for Health Research (MOP36378) to AY. Deng. AY. Deng is an Established Investigator of the American Heart Association.

4.7 REFERENCES

1. Deng AY. In search of hypertension genes in Dahl salt-sensitive rats. *J. Hypertension* 16: 1707-1717, 1998.
2. Deng AY, Dene H, Rapp JP. Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. *J. Clin. Invest.* 94: 431-436, 1994.
3. Deng AY, Dene H, Rapp JP. Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension* 30: 199-202, 1997.
4. Deng AY, Jackson CM, Hoebee B, Rapp JP. Mapping of rat chromosome 2 markers generated from chromosome-sorted DNA. *Mamm. Genome* 8: 731-735, 1997.
5. Deng Y, Rapp JP. Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic peptide receptor genes using Dahl salt-sensitive rats. *Nature Genet* 1: 267-272, 1992.
6. Deng AY, Rapp JP. Evaluation of the angiotensin II receptor AT1B gene as a candidate gene for blood pressure. *J. Hyperten.* 12: 1001-1006, 1994.
7. Dominiczak AF, Negrin DC, Clark JS, Brosnan MJ, McBride MW, Alexander MY. Genes and Hypertension: from gene mapping in experimental models to vascular gene transfer strategies. *Hypertension* 35: 64-172, 2000.
8. Dubay C, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi J, Kotelevtsev Y, Beckmann JS, Soubrier R, Sassard J, Lathrop GM. Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nature Genet* 3: 354-357, 1993.

9. Dutil J, Moujahidine M, Lemieu C, Jankowski M, Gutkowska J, Deng AY. Chromosomal and comparative mapping of rat oxytocin, oxytocin receptor and vasopressin genes. *Cytogenet. Cell Genet.* In press.
10. Jeffs B, Negrin CD, Graham D, Clark JS, Anderson NH, Gauguier D, Dominiczak AF. Applicability of a 'speed' congenic strategy to dissect blood pressure quantitative trait loci on rat Chromosome 2. *Hypertension* 35: 179-187, 2000.
11. Kreutz R, Struk B, Stock P, Hubner N, Ganten D, Lindpaintner K. Evidence for primary genetic determination of heart rate regulation. *Circulation* 96:1078-1081.
12. Pravenec M, Gauguier D, Schott J-J, Buard J, Krén V, Bila V, Szpirer C, Szpirer J, Wang J-M, Huang H, St. Lezin E, Spence MA, Flodman P, Printz M, Lathrop GM, Vergnaud G, Kurtz TW. Mapping of quantitative trait loci for blood pressure and cardiac mass in the rat by genome scanning of recombinant inbred strains. *J Clin Invest* 96: 1973-1978, 1995.
13. Rapp JP, Deng AY. Detection and positional cloning of blood pressure quantitative trait loci: is it possible? *Hypertension* 25: 1121-1128, 1995.
14. Samani NJ, Gauguier D, Vincent M, Kaiser MA, Bihoreau M-T, Lodwick D, Wallis R, Parent V, Kimber P, Rattray F, Thompson JR, Sassard J, Lathrop M. Analysis of quantitative trait loci for blood pressure on rat chromosomes 2 and 13. *Hypertension* 28: 1118-1122, 1996.
15. Schork NJ, Krieger JE, Trolliet MR, Franchini KG, Koike G, Kreiger, EM, Lander ES, Dzau VJ, Jacob HJ. A biometrical genome search in rats reveals the multigenic basis of blood pressure variation. *Genome Res* 5: 164-172, 1995.
16. Singh JP, Larson MG, O'Donnell CJ, Tsuji H, Evans JC, Levy D. Heritability of heart rate variability, the framingham heart study. *Circulation* 99:2251-2254, 1999.
17. Vincent M, Samani NJ, Gauguier D, Thompson JR, Lathrop G, Sassard J. A pharmacogenetic approach to blood pressure in Lyon hypertensive rats. *J Clin Invest* 100: 2000-2006, 1997.

4.8 FIGURE LEGENDS AND FIGURES

Figure 1. **Chr. 2 regions replaced in the original congenic strain and substrains, and the QTL region mapped.** The linkage map is essentially the same as published previously (4), which is based on an F2(S x MNS) population. Numbers to the left of the linkage map are units in cM. The order of most of the loci on the map has been initially determined by linkage using the MAPMAKER program, and then verified by scoring crossovers during construction of congenic substrains. RH map refers to the map based on rat/hamster radiation hybrids, in which units are in centiRays (cR). S.M represent the original congenic strains S.MNS-*Adh*/D2Mit6 (3). S.M2, S.M5 and S.M6 are substrains that have been derived from S.M. Solid bars under congenic strain and the substrains symbolize the S chromosome fragments that have been replaced by that of the MNS rat. The entire region indicated by solid bars and junctions between the solid and open bars are homozygous MM on the map for all the markers listed in the corresponding positions. Open bars on ends of solid bars indicate the ambiguities of crossover breakpoints between markers. Junctions between solid and open bars as well as ends of chromosome regions of interest in each strain are connected by dotted lines to the marker positions on the map. *Adh*, alcohol dehydrogenase; *Agtr1b*, angiotensin receptor type 1B; *Atp1a1*, Na^+K^+-ATPase α1; *Camk2d*, calmodulin-dependent protein kinase II-delta; *Cpb*, carboxypeptidase B; *Fgg*, fibrinogen gamma; *Gca*, guanylyl cyclase A/atrial natriuretic peptide receptor; *Hsd3b*, 3-hydroxysteroid dehydrogenase/delta 5 isomerase; *Nep*, neutral endopeptidase; *Prlr*, prolactin receptor. The rest of the markers are anonymous (http://waldo.wi.mit.edu/rat/public/). S.M2, S.M5 and S.M6 are S.MNS-D2Mit6/D2Rat303, S.MNS-*Nep*/D2Mit14, and S.MNS-*Nep*/*Gca* respectively. S, the Dahl salt-sensitive strain.

Figure 2. **Comparisons in mean arterial (MAP), diastolic (DAP) and systolic (SAP) pressures between the S.M, S.M2, S.M5 and S.M6 congenic strains, and the S strain**. Each time point on the graph represents an average of 24-hour readings. S, the Dahl salt-sensitive strain. S.M, S.MNS-D2Mit6/*Adh*, S.M2, S.MNS-D2Mit6/D2Rat303; S.M5, S.MNS-*Nep*/D2Mit14; and S.M6, S.MNS-*Nep*/*Gca* strains respectively. Each point on the 24-hour graph is an average of readings taken at every 1 hour (j, l, and n) or 4 hours (k, m and o). **a, b** and **c**, MAP, DAP and SAP of S and S.M; **d, e** and **f**, MAP, DAP and SAP of S, S.M2 and S.M5; **g, h** and **i**, MAP, DAP and SAP of S and S.M6; **j** and **k** represent MAP readings of S and S.M in a 24-hour period averaged every hour and every four hours respectively; **l** and **m** represent MAP readings of S, S.M2 and S.M5 in a 24-hour period averaged every hour and every four hours respectively; **n** and **o** represent MAP readings of S and S.M6 in a 24-hour period averaged every hour and every four hours respectively. Error bars represent SEM.

Figure 3. **Comparisons in heart rates between the S and congenic strains**. The difference in heart rates among S, S.M, S.M2, S.M5 and S.M6 are compared. Each time point on the graph in **a, c**, and **e** represents an average of 24-hour readings. **a**, comparing S (n=5) with S.M (n=5), $p>0.47$; **c**, comparing S (n=3), S.M2 (n=4), and S.M5 (n=4), $p>0.49$; and **e**, comparing S (n=4) and S.M6 (n=7), $p>0.30$. Along side each graph for the 12-day period of measurements (left, a, c and e) are variations of heart rates in a 24-hour period (right, b, d and f) to show the hemodynamic variance among the strains. Each point on the 24-hour graph is an average of readings taken at every 4 hours. S, the Dahl salt-sensitive strain. For congenic strain designations, see the legend for Figure 2. Error bars represent SEM.

CHROMOSOME 2

Figure 1

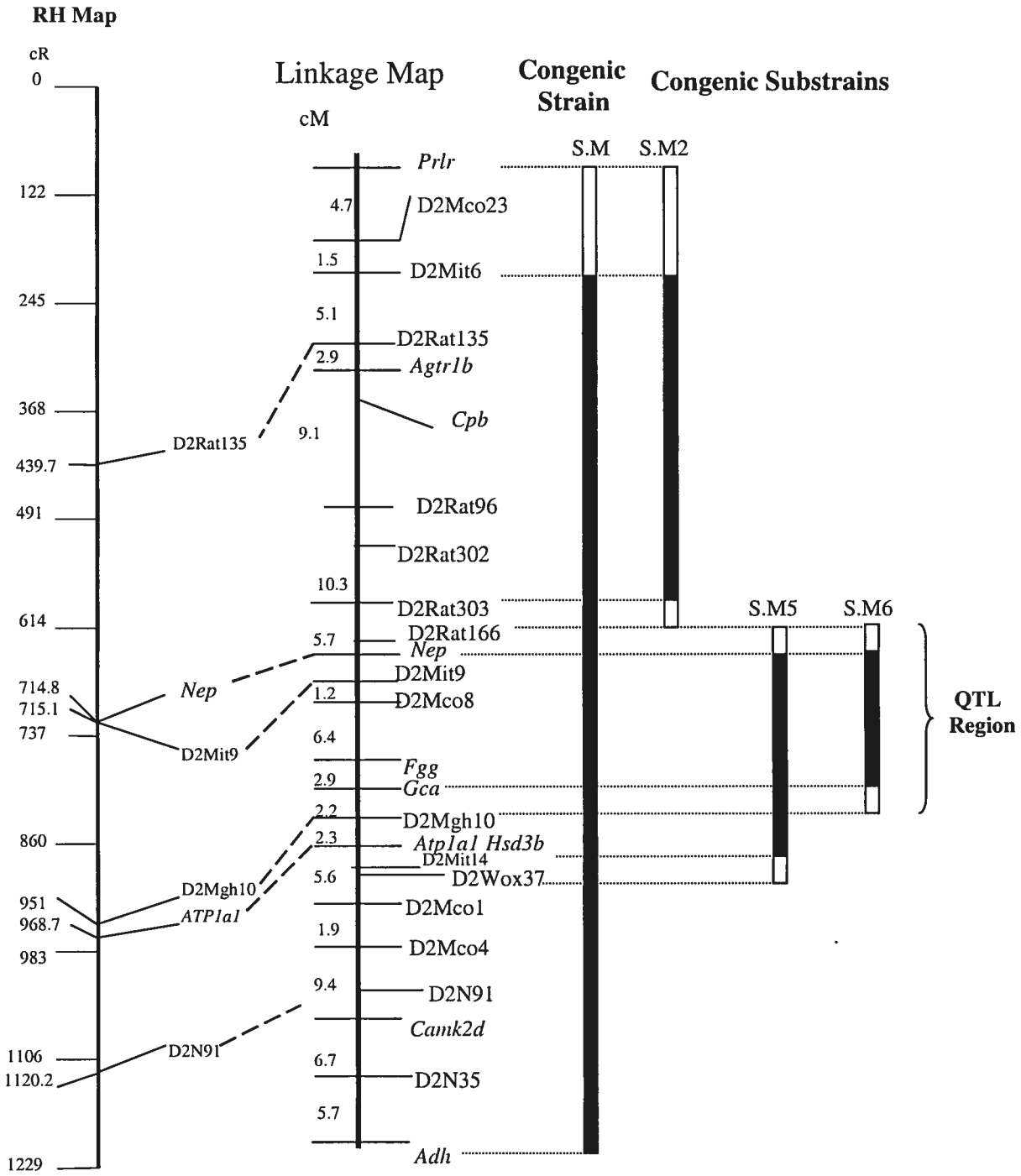


Figure 2

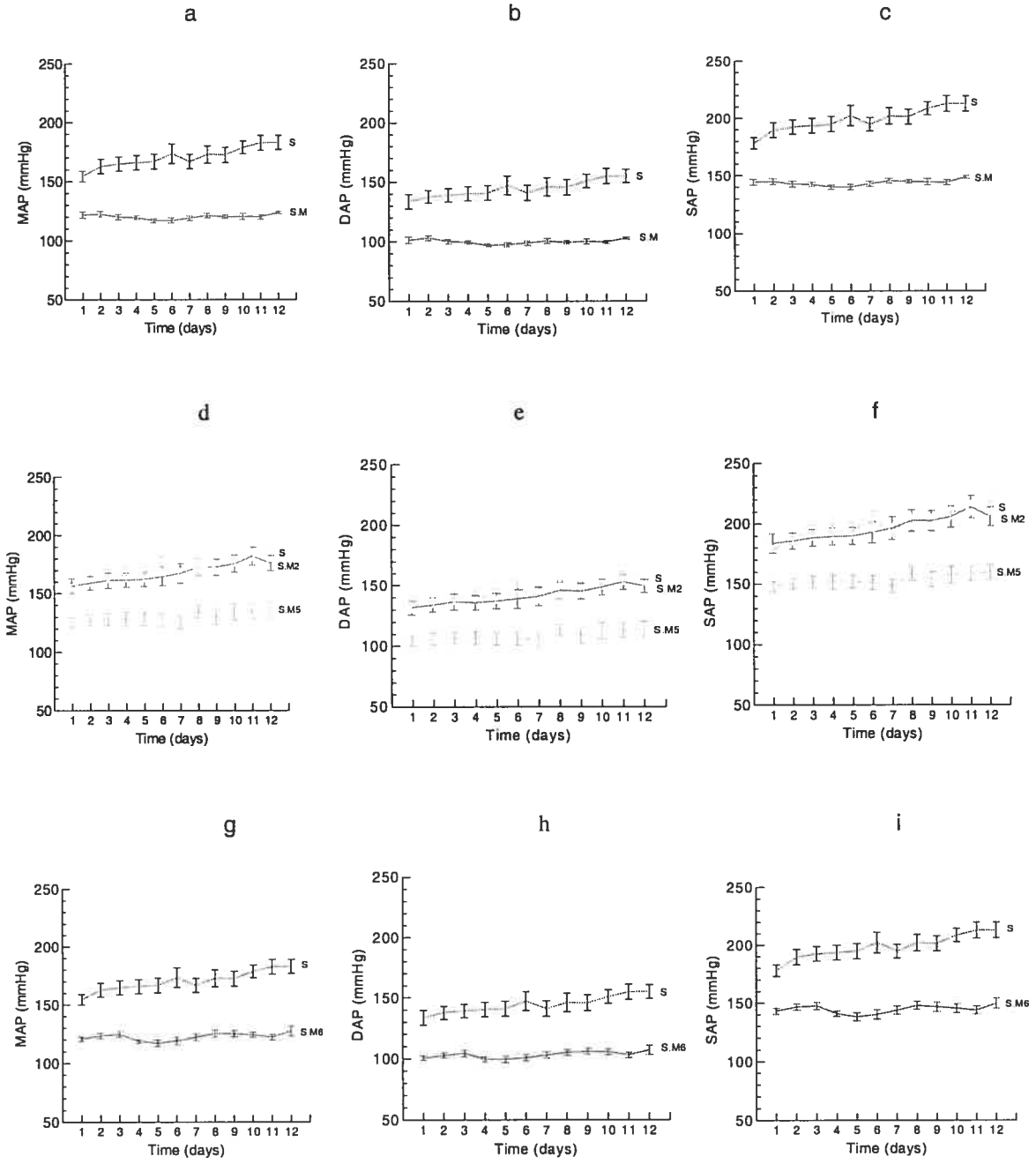

a
b
c
d
e
f
g
h
i
MAP (mmHg)
DAP (mmHg)
SAP (mmHg)
Time (days)
S
S.M
S.M2
S.M5
S.M6
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2 continued

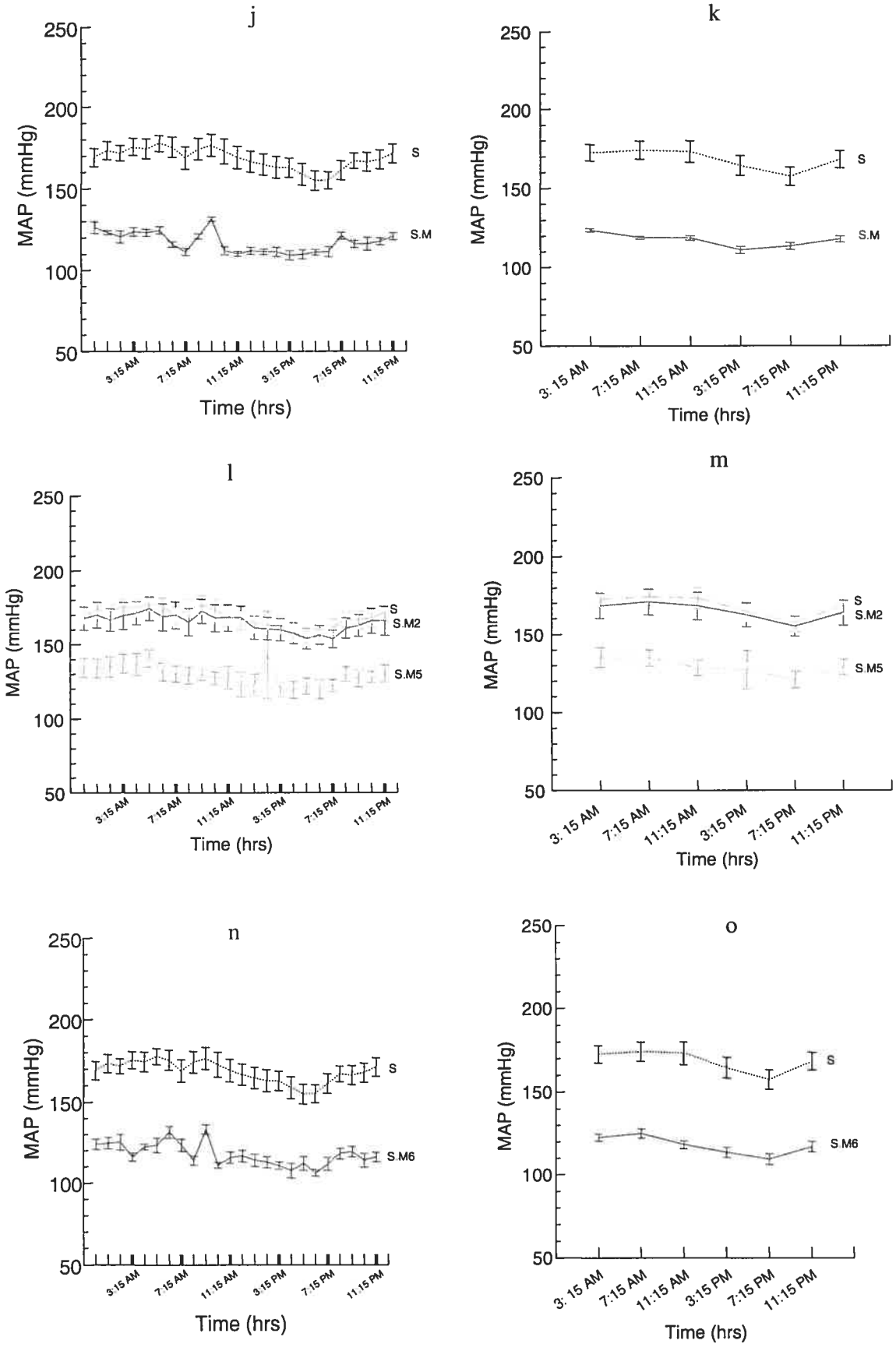
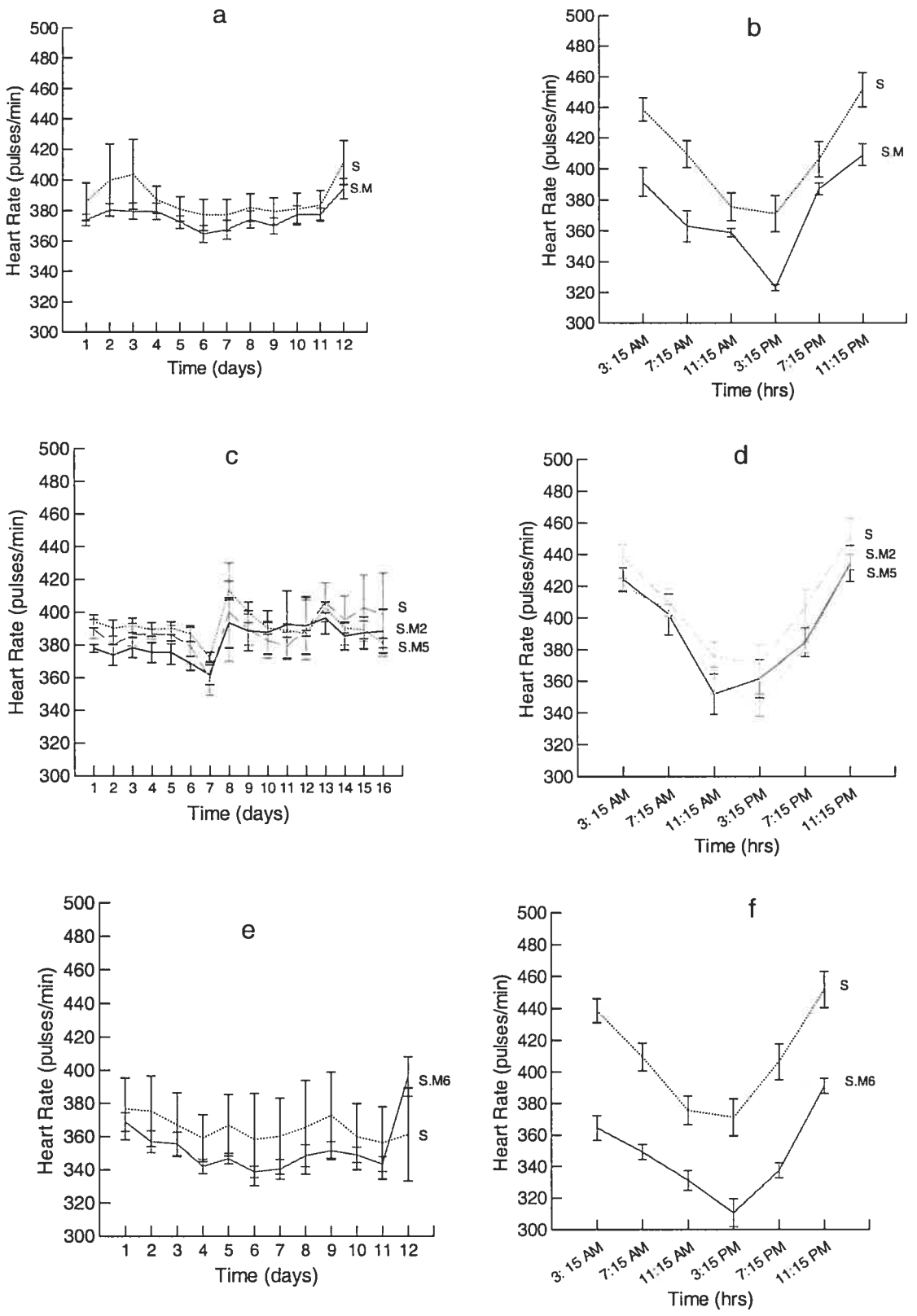


Figure 3


a
Heart Rate (pulses/min)
500
480
460
440
420
400
380
360
340
320
300
1 2 3 4 5 6 7 8 9 10 11 12
Time (days)
S
S.M
b
Heart Rate (pulses/min)
3: 15 AM
7:15 AM
11:15 AM
3:15 PM
7:15 PM
11:15 PM
Time (hrs)
S
S.M
c
Heart Rate (pulses/min)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
S
S.M2
S.M5
d
Heart Rate (pulses/min)
Time (hrs)
S
S.M2
S.M5
e
Heart Rate (pulses/min)
Time (days)
S.M6
S
f
Heart Rate (pulses/min)
Time (hrs)
S
S.M6

CHAPTER 5

Multiple quantitative trait loci for blood pressure interacting epistatically and additively on Dahl Rat Chromosome 2

Julie Dutil, Vasiliki Eliopoulos, Johanne Tremblay, Pavel Hamet, Sophie Charron, Alan Y. Deng

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Research Centre-CHUM, 3840 rue St. Urbain, Montréal, Québec, H2W 1T8, Canada

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Corresponding author: Alan Deng, Ph.D.

Research Centre

Centre Hospitalier de l'Université de Montréal (CHUM)

7-132 Pavillon Jeanne Mance

3840, rue St. Urbain

Montreal, Quebec, H2W 1T8, Canada

Phone: 514-890-8000 ex. 15522

Fax: 514-412-7152

[redacted]

5.1 ABSTRACT

Our previous work demonstrated two quantitative trait loci (QTLs), C2QTL1 and C2QTL2, for blood pressure (BP) located on Chromosome (Chr) 2 of Dahl salt-sensitive (DSS) rats. However, for a lack of markers, the two congenic strains delineating C2QTL1 and C2QTL2 could not be separated. The position of the C2QTL1 was only inferred by comparing two congenic strains, one having and another lacking a BP effect. Furthermore, it was not known how adjacent QTLs would interact with one another on Chr 2. In the current investigation, first, a critical chromosome marker was developed to separate two C2QTLs. Second, a congenic substrain was created to cover a chromosome fragment thought to harbor C2QTL1. Finally, a series of congenic strains were produced to systematically and comprehensively cover the entire Chr 2 segment containing C2QTL2 and other regions previously untested. Consequently, a total of three QTLs were discovered, with C2QTL3 located between C2QTL1 and C2QTL2. C2QTL1, C2QTL2 and C2QTL3 reside in chromosome segments of 5.7 centiMorgan (cM), 3.5 cM and 1.5 cM respectively. C2QTL1 interacted epistatically with either C2QTL2 or C2QTL3; whereas C2QTL2 and C2QTL3 showed additive effects to each other. These results suggest that BP QTLs closely linked in a segment interact epistatically and additively to one another on Chr 2.

Key words: hypertension, epistasis, additivity, comparative congenics, comprehensive congenic coverage

5.2 INTRODUCTION

Ever since the revelation of a quantitative trait locus (QTL) for blood pressure (BP) on Chromosome 2 (Chr 2) of Dahl salt-sensitive (DSS) rats [1;2], Chr 2 seems to play a role in the development of hypertension in several of the hypertensive strains [3-13]. In our initial work, two BP QTLs designated C2QTL1 and C2QTL2 were localized to regions on Chr 2 of the DSS rat [5;6]. C2QTL1 was found between the markers D2Rat303 and D2Rat166 [6] and C2QTL2 was found between the markers D2Rat166 and D2Rat131 [5].

However at the time, the position of C2QTL1 was solely inferred from comparing two overlapping congenic strains, one having and the other lacking a BP effect [6]. It was uncertain whether this deduction was valid in localizing a QTL for a polygenic trait. Another issue was that C2QTL1 defined by S.M1 [6] and C2QTL2 defined by S.M5 and S.M6 [5] shared a chromosome region of ambiguity. As a result, it could not be ruled out that there might be just one QTL instead of two in the Chr 2 region in question.

Subsequent to our original work on Chr 2 QTL localizations [1;2;5;6;14], another group found several QTLs situated adjacent to one another in one Chr 2 segment of DSS rats [7]. However, it was not clear how these QTLs could act with reference to one another.

Based on these observations, two questions were addressed: are there truly multiple BP QTLs in a Chr 2 segment of the DSS rat? If there are, how do they interact with reference to one another in determining BP?

5.3 METHODS

5.3.1 Animals

Congenic strains, S.M, S.M1, S.M2, S.M5 and S.M6 and DSS strain are the same as used previously [5;6] (Figure 1).

5.3.2 Constructions of congenic substrains

S.M, S.M1, S.M5 and S.M6 [5;6] were used to derive congenic substrains. The basic design was to systematically and as completely as possible cover the entire Chr 2 region of interest. The goal is to define each QTL unambiguously.

The procedure was similar to that published previously [15]. In brief, rats of the DSS and S.M (or S.M1 or S.M5 or S.M6) were used to derive congenic substrains (see the online supplement for detail). In the end, the authenticity of each congenic substrain has been established by genotyping the markers for the region of interest, and 57 additional markers scattered throughout the rat genome (data not shown). The chromosome region homozygous MM for each congenic substrain is depicted by a solid bar in Figures 1 and 2. New congenic substrains produced in the present work are: DSS.MNS-(D2Rat183-D2Chm113)/Lt (abbreviated as C2S.M7), DSS.MNS-(D2Chm25-D2Mit14)/Lt (C2S.M8), DSS.MNS-(D2Chm25-D2Rat131)/Lt (C2S.M9), DSS.MNS-(D2Wox27/*Adh*)/Lt (C2S.M10), DSS.MNS-(D2Chm51-D2Rat341)/Lt (C2S.M11) and DSS.MNS-(D2Chm25-*Fgg*)/Lt (C2S.M12).

5.3.3 BP measurements

BP studies on the congenic strains were essentially the same as reported previously [5;6;15-21]. In brief, male rats were weaned at 21 days of age, maintained on a low salt diet (0.2% NaCl, Harlan Teklad 7034) and then fed a high salt diet (2% NaCl, Harlan Teklad 94217) starting from 35 days of age until the end of the experiment. Telemetry probes were implanted when rats were 56 days old (i.e. after 3 weeks of the high salt diet) with their body weights between 250-320 grams. BPs for all the strains were measured at least at two different times to exclude seasonal as well as environmental influences. Thus the BP data were pooled from separately reproducible measurements for each strain

5.3.4 Statistical analysis

Repeated measures' Analysis of variance (ANOVA) followed by Dunnett, (which permits a correction for multiple comparisons and sample sizes), was used to compare a parameter between 2 group as presented previously [5;6;15-21]. During the BP comparison, ANOVA was, first, used to analyze the data to see if there was any difference among the groups. If the difference is significant, then, the Dunnett test was followed to see which group is different and how much significant from the DSS strain.

The 2 x 2 ANOVA determines a QTL-QTL interaction (or a lack of it) by evaluating whether the observed BP effect of a congenic strain combining two separate congenic strains is significantly different from a predicted sum of BP effects from each individual congenic strain.

5.4 RESULTS

5.4.1 A chromosome marker defining separate QTLs

The marker designated as D2Chm90 was instrumental in separating QTLs [Figure 1 and Table 1 (see the online supplement for the detail as to how these markers were generated)]. It came from the same supercontig containing *Mme* and is homozygous MM for S.M, but SS for S.M1, S.M2, and S.M6 (Figure 1). Thus D2Chm90 effectively separated S.M1 and S.M2 from S.M6. Consequently, there are definitively at least 2 different BP QTLs present (Figure 1). Because S.M1 had a BP 22 mmHg lower ($p<0.03$) than that of DSS, whereas S.M2 is different ($p>0.82$) from DSS by 2 mmHg, C2QTL1 should be present between D2Rat303 and D2Chm90 (Figure 1). Since BP of S.M6 was 48 mmHg lower ($p<0.001$) than that of DSS, C2QTL2 should exist between D2Chm90 and D2Mgh10 (Figure 1). BP of S.M (i.e. 120 mmHg) is not different from that of S.M6 (i.e. 122 mmHg) ($p>0.5$).

5.4.2 Constructions of new congenic substrains to fine map multiple BP QTLs

C2S.M7, C2S.M8, C2S.M9, C2S.M10, C2S.M11 and C2S.M12 span around 5.7 cM, 11.5 cM, 8 cM, 28 cM, 1-2 cM and 5.5 cM respectively.

Figure 3 shows the actual tracings of systolic (SAP), diastolic (DAP) and mean arterial (MAP) of DSS and congenic strains by telemetry. For the simplicity of comparisons among the strains, averaged MAPs were shown at the bottom of Figures 1 and 2.

C2S.M7 was produced by targeting the chromosome segment between D2Rat303 and D2Rat166 of S.M1 (Figure 1). C2S.M7 exhibited a BP lower ($p<0.03$) than that of DSS (Figures 2 and 3).

When C2S.M9 (Figure 2) were produced from S.M6 [5], its MAP of around 139 mmHg was 31 mmHg lower ($p<0.02$) than that of 170 mmHg of DSS rats (Figure 2). This lowering in BP could only explain about 65% (i.e. 31/48) of that observed in S.M6 [5] (i.e. 170-122 = 48 mmHg), suggesting that there might be another QTL, C2QTL3, that would have an additive BP effect to C2QTL2. To prove this prediction, a new congenic substrain, C2S.M11 (Figure 2), was constructed specially targeting the sub-segment in S.M6 none-overlapping with C2S.M9 (Figures 1 and 2). Indeed, MAP of C2S.M11 was around 147 mmHg (Figures 2 and 3), which was lower ($p<0.03$) than that of DSS.

From C2S.M9, C2S.M12 was produced (Figure 2) and its BPs were not different ($p>0.3$) from those of DSS (Figures 2 and 3). As a result, the interval for C2QTL2 can be further narrowed to the section non-overlapping between C2S.M9 and C2S.M12 by subtracting the segment shared between them (Figure 2).

The genes for the soluble subunits α (*Gucy1a*) and β (*Gucy1b*) of guanylate cyclase 1 are located in the chromosome fragment in C2S.M12 (Figure 2), which had a BP not different ($p>0.3$) from that of DSS.

5.4.3 Epistatic and additive QTL-QTL interactions

Figure 4 summarizes the relationships among the three C2QTLs, assuming that one QTL was involved in each QTL interval. A 2 x 2 factorial ANOVA [22] indicated that there is an epistatic interaction ($p<0.03$) between C2QTL1 and a combination of [C2QTL2 and C2QTL3] (Figure 4a). This interaction can be viewed in another way. That is: S.M (Figure 1), which harbors three QTLs, C2QTL1, C2QTL2 and C2QTL3, possessed a similar BP (i.e. 120 mmHg) as that of S.M6 (i.e. 122 mmHg), which contained only two QTLs, C2QTL2 and C2QTL3 (Figure 1). The BP effect of C2QTL1 was apparently masked when combined with C2QTL2 and C2QTL3.

C2QTL3 and C2QTL2 appear to act additively to each other, because a 2 x 2 factorial ANOVA demonstrated that there is no epistatic interactions between them (*p* interaction >0.5, Figure 4b). In other words, a combined BP effect of C2QTL2 and C2QTL3 is greater than each of the two QTLs acting alone.

5.5 DISCUSSION

Major findings from the current studies are (a), a comprehensive and systematic congenic coverage unraveled multiple QTLs closely linked in a segment on Dahl rat Chr 2. (b), Certain of these QTLs demonstrated epistatic interactions, while others exhibited additive effects.

5.5.1 Comprehensive congenic coverage divulging multiple BP QTLs in a closely linked region

All the available markers in the rat data base for the region between D2Rat166 and *Mme* were tested, but, none was either homozygous SS or MM for S.M1 and S.M6 (Figure 1). Therefore, potential overlaps could not be ruled out between the lower segment for C2QTL1 [6] and the upper segment for C2QTL2 [5] (Figure 1). Two approaches of proving that C2QTL1 and C2QTL2 are separate genes were that a marker between D2Rat166

and *Mme* should be SS for both S.M1 and S.M6; and/or non-overlapping congenic strains separately covering C2QTL1 and C2QTL2 would each show a BP effect.

Indeed, the new marker, D2Chum90, turned out to be SS for S.M1 and S.M6 (Figure 1). Moreover, both C2S.M8 and C2S.M9 are clearly separate from C2S.M7 (Figure 2), and all three showed BP effects (Figure 2). Combining both sets of evidence, C2QTL1 and C2QTL2 indisputably represent distinctive genetic loci.

In addition to C2S.M7, C2S.M8, and C2S.M9, congenic strain C2S.M11 (Figure 2) also showed a BP significantly lower ($p<0.03$) than that of DSS (Figure 3). In contrast, C2S.M10 and C2S.M12 (Figures 2 and 3) had BPs not statistically different ($p>0.3$) from that of DSS (Figure 3). Since congenic strains C2S.M7, C2S.M9, and C2S.M11 unequivocally do not overlap in the chromosome segments that they cover (Figure 2) and all showed BP effects, consequently, three separate QTLs, C2QTL1, C2QTL2 and C2QTL3, must be located in C2S.M7, C2S.M9, and C2S.M11 respectively. C2QTL1, C2QTL2 and C2QTL3 reside in intervals of 5.7, 3.5 and 1.5 cMs respectively.

Because C2S.M10 and C2S.M12 as 'negative' controls did not manifest significant alterations in BP from DSS, the BP effects of the three QTLs observed could not be due to the genetic background in congenic strains C2S.M7, C2S.M9, and C2S.M11. It is a QTL that is responsible for lowering BP in each congenic strain.

For a lack of congenic strain specifically made for the fragment between D2Chm57 and D2Wox27 (Figure 2), it can not be ruled out that an additional QTL could still exist in that region.

Our results are consistent with those of other investigators [3;7;8], which showed the existence of several BP QTLs in similar regions. Specifically in comparing our current results with those of Garrett and Rapp [7], several features are evident. First, MNS and Wistar Kyoto (WKY) strains may or may not share the same alleles at each QTL on Chr 2. For example, C2QTL1 seems unique to the DSS and MNS contrast. Second,

C2QTL2 and C2QTL3 shared the same chromosome segment with QTL1 and QTL2 in Garrett and Rapp [7], implying that the same QTL alleles could be in common between MNS and WKY. Third, the C2QTL3 region in our current work is now small enough (i.e. 1.5 cM) for positional cloning to identify the gene. Finally, our telemetry affords more accurate measurements to assess a QTL effect and QTL-QTL interactions. In a nutshell, our current work fine mapped the three C2QTLs and presented evidence for both epistatic and additive QTL-QTL interactions among them.

5.5.2 Epistatic and additive QTL interactions among C2QTL1, C2QTL2 and C2QTL3

From Figure 4, it is apparent that C2QTL1 is epistatic to either C2QTL2 or C2QTL3. It is unknown if the interaction was between C2QTL1 and C2QTL2, or between C2QTL1 and C2QTL3. Mechanistically, it is probable that C2QTL1 belongs to the same pathway/cascade as either C2QTL2 or C2QTL3 [23;24]. Increasingly, epistasis in determining BP [2;20;22;25] has been recognized as an important organizational hierarchy among BP QTLs [23;24], not simply a background genetic noise.

It will be worthwhile to ascertain with which of the two QTLs, i.e. C2QTL2 or C2QTL3, C2QTL1 interacts epistatically. One way to accomplish this task is to make 'double' congenic strains between both C2QTL1 and C2QTL2, and C2QTL1 and C2QTL3, much as S.M6 being for C2QTL2 and C2QTL3. As these three QTLs are located in close proximity on the same chromosome (Figure 2), any 'double' congenic strain as such will require a crossover between two closely linked markers. So far, no such crossovers have been achieved (data not shown). Another way to study these epistatic interactions is to construct 'double' congenic strains between a congenic strain from a different chromosome and every one of the single congenic strains harboring the three QTLs on Chr 2. In this case, no chromosome crossovers are required so that a production of such a 'double' congenic strain is practically achievable. This line of work is ongoing.

The phenomenon of additive QTL interactions between C2QTL2 and C2QTL3 is not an isolated instance. Several BP QTLs adjacent to one another acting additively have also been detected on another chromosome using congenic strains [15]. In a mechanistic sense, it is possible that QTLs in an additive relationship belong to different pathways/cascades [23;24].

5.5.3 Perspective

There are three BP QTLs closely linked in a chromosome segment of 19.6 cM of the DSS rat in contrast to the MNS rat. C2QTL1 interacted with either C2QTL2 or C2QTL3 epistatically; whereas C2QTL2 and C2QTL3 seemed to be additive in affecting BP. By extrapolation, the identifications of these QTLs and how they interact with one another could have an impact on the revelation of mechanisms governing certain forms of human essential hypertension. The chromosome segment harboring the three QTLs has a conserved synteny with a fragment of Chr 3 of the mouse and with segments of human CHRs 3 and 13 (C2QTL1), CHRs 1 and 4 (C2QTL2) and CHR 3 (C2QTL3) (http://www.ncbi.nlm.nih.gov/). The newly available rat genome information [26] will no doubt be helpful in positional cloning of these C2QTLs [23;27].

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5.7 REFERENCES

1. Deng AY, Dene H, Rapp JP. Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. *J Clin Invest*. 1994;94:431-436.

2. Deng Y, Rapp JP. Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic peptide receptor genes using Dahl salt-sensitive rats. *Nature Genetics*. 1992;1:267-272.

3. Alemayehu A, Breen L, Krenova D, Printz MP. Reciprocal rat chromosome 2 congenic strains reveal contrasting blood pressure and heart rate QTL. *Physiol Genomics*. 2002;10:199-210.

4. Dubay C, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi JP, Kotelevtsev Y, Beckmann JS, Soubrier F, Sassard J, Lathrop GM. Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nat Genet*. 1993;3:354-357.

5. Dutil J, Deng AY. Further chromosomal mapping of a blood pressure QTL in Dahl rats on chromosome 2 using congenic strains. *Physiological Genomics*. 2001;6:3-9.

6. Dutil J, Deng AY. Mapping a blood pressure quantitative trait locus to a 5.7-cM region in Dahl salt-sensitive rats. *Mammalian Genome*. 2001;12:362-365.

7. Garret MR, Rapp JP. Multiple blood pressure QTL on rat chromosome 2 defined by congenic Dahl rats. *Mammalian Genome*. 2002;13:41-44.

8. Jeffs B, Negrin CD, Graham D, Clark JS, Anderson NH, Gauguier D, Dominiczak AF. Applicability of a "speed" congenic strategy to dissect blood pressure quantitative trait loci on rat chromosome 2. *Hypertension*. 2000;35:179-187.

9. Pravenec M, Gauguier D, Schott JJ, Buard J, Kren V, Bila V, Szpirer C, Szpirer J, Wang JM, Huang H, . Mapping of quantitative trait loci for blood pressure and

cardiac mass in the rat by genome scanning of recombinant inbred strains. *J Clin Invest.* 1995;96:1973-1978.

10. Samani NJ, Gauguier D, Vincent M, Kaiser MA, Bihoreau MT, Lodwick D, Wallis R, Parent V, Kimber P, Rattray F, Thompson JR, Sassard J, Lathrop M. Analysis of quantitative trait loci for blood pressure on rat chromosomes 2 and 13. Age-related differences in effect. *Hypertension.* 1996;28:1118-1122.

11. Schork NJ, Krieger JE, Trolliet MR, Franchini KG, Koike G, Krieger EM, Lander ES, Dzau VJ, Jacob HJ. A biometrical genome search in rats reveals the multigenic basis of blood pressure variation. *Genome Res.* 1995;5:164-172.

12. Stoll M, Cowley AW, Jr., Tonellato PJ, Greene AS, Kaldunski ML, Roman RJ, Dumas P, Schork NJ, Wang Z, Jacob HJ. A genomic-systems biology map for cardiovascular function. *Science.* 2001;294:1723-1726.

13. Vincent M, Samani NJ, Gauguier D, Thompson JR, Lathrop GM, Sassard J. A pharmacogenetic approach to blood pressure in Lyon hypertensive rats. A chromosome 2 locus influences the response to a calcium antagonist. *J Clin Invest.* 1997;100:2000-2006.

14. Deng AY, Dene H, Rapp JP. Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension.* 1997;30:199-202.

15. Palijan A, lambert R, Sivo Z, Dutil J, Deng AY. Comprehensive congenic coverage revealing multiple BP QTLs on Dahl rat Chromosome 10. *Hypertension.* 2003;42:515-522.

16. Ariyarajah A, Palijan A, Dutil J, Prithiviraj K, Deng Y, Deng AY. Dissecting quantitative trait loci into opposite blood pressure effects on Dahl rat Chromosome 8 by congenic strains. *J Hypertens.* 2004;22:1495-1502.

17. Deng AY, Dutil J, Sivo Z. Utilization of marker-assisted congenics to map two blood pressure quantitative trait loci in Dahl rats. *Mamm Genome.* 2001;12:612-616.

18. Moujahidine M, Dutil J, Hamet P, Deng AY. Congenic mapping of a blood pressure QTL on Chromosome 16 of Dahl rats. *Mammalian Genome*. 2002;13:153-156.

19. Moujahidine M, lambert R, Dutil J, Palijan A, Sivo Z, Ariyarajah A, Deng AY. Combining congenic coverage with gene profiling in search of candidates for blood pressure QTLs in Dahl rats. *Hypertension Research*. 2004;27:203-212.

20. Palijan A, Dutil J, Deng AY. Quantitative trait loci with opposing blood pressure effects demonstrating epistasis on Dahl rat chromosome 3. *Physiological Genomics*. 2003;15:1-8.

21. Sivo Z, Malo B, Dutil J, Deng AY. Accelerated congenics for mapping two blood pressure quantitative trait loci on Chromosome 10 of Dahl rats. *J Hypertens*. 2002;20:1-9.

22. Rapp JP, Garret MR, Deng AY. Construction of a double congenic strain to prove an epistatic interaction on blood pressure between rat chromosomes 2 and 10. *J Clin Invest*. 1998;101:1591-1595.

23. Deng AY. Functional genomics of blood pressure determination: dissecting and assembling a polygenic strait by experimental genetics. *Current Hypertension Reviews*. 2005;1:35-50.

24. Deng AY. In search of hypertension genes in Dahl salt-sensitive rats. *J Hypertens*. 1998;16:1707-1717.

25. Monti J, Plehm R, Schulz H, Ganten D, Kreutz R, Hubner N. Interaction between blood pressure quantitative trait loci in rats in which trait variation at chromosome 1 is conditional upon a specific allele at chromosome 10. *Hum Mol Genet*. 2003;15:435-439.

26. Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, Scott G, Steffen D, Worley KC, Burch PE, Okwuonu G, Hines S, Lewis L, DeRamo C, Delgado O, Dugan-Rocha S, Miner G, Morgan M, Hawes A, Gill R,

Celera, Holt RA, Adams MD, Amanatides PG, Baden-Tillson H, Barnstead M, Chin S, Evans CA, Ferriera S, Fosler C, Glodek A, Gu Z, Jennings D, Kraft CL, Nguyen T, Pfannkoch CM, Sitter C, Sutton GG, Venter JC, Woodage T, Smith D, Lee HM, Gustafson E, Cahill P, Kana A, Doucette-Stamm L, Weinstock K, Fechtel K, Weiss RB, Dunn DM, Green ED, Blakesley RW, Bouffard GG, De Jong PJ, Osoegawa K, Zhu B, Marra M, Schein J, Bosdet I, Fjell C, Jones S, Krzywinski M, Mathewson C, Siddiqui A, Wye N, McPherson J, Zhao S, Fraser CM, Shetty J, Shatsman S, Geer K, Chen Y, Abramzon S, Nierman WC, Havlak PH, Chen R, Durbin KJ, Egan A, Ren Y, Song XZ, Li B, Liu Y, Qin X, Cawley S, Worley KC, Cooney AJ, D'Souza LM, Martin K, Wu JQ, Gonzalez-Garay ML, Jackson AR, Kalafus KJ, McLeod MP, Milosavljevic A, Virk D, Volkov A, Wheeler DA, Zhang Z, Bailey JA, Eichler EE, Tuzun E, Birney E, Mongin E, Ureta-Vidal A, Woodwark C, Zdobnov E, Bork P, Suyama M, Torrents D, Alexandersson M, Trask BJ, Young JM, Huang H, Wang H, Xing H, Daniels S, Gietzen D, Schmidt J, Stevens K, Vitt U, Wingrove J, Camara F, Mar AM, Abril JF, Guigo R, Smit A, Dubchak I, Rubin EM, Couronne O, Poliakov A, Hubner N, Ganten D, Goesele C, Hummel O, Kreitler T, Lee YA, Monti J, Schulz H, Zimdahl H, Himmelbauer H, Lehrach H, Jacob HJ, Bromberg S, Gullings-Handley J, Jensen-Seaman MI, Kwitek AE, Lazar J, Pasko D, Tonellato PJ, Twigger S, Ponting CP, Duarte JM, Rice S, Goodstadt L, Beatson SA, Emes RD, Winter EE, Webber C, Brandt P, Nyakatura G, Adetobi M, Chiaromonte F, Elnitski L, Eswara P, Hardison RC, Hou M, Kolbe D, Makova K, Miller W, Nekrutenko A, Riemer C, Schwartz S, Taylor J, Yang S, Zhang Y, Lindpaintner K, Andrews TD, Caccamo M, Clamp M, Clarke L, Curwen V, Durbin R, Eyras E, Searle SM, Cooper GM, Batzoglou S, Brudno M, Sidow A, Stone EA, Venter JC, Payseur BA, Bourque G, Lopez-Otin C, Puente XS, Chakrabarti K, Chatterji S, Dewey C, Pachter L, Bray N, Yap VB, Caspi A, Tesler G, Pevzner PA, Haussler D, Roskin KM, Baertsch R, Clawson H, Furey TS, Hinrichs AS, Karolchik D, Kent WJ, Rosenbloom KR, Trumbower H, Weirauch M, Cooper DN, Stenson PD, Ma B, Brent M, Arumugam M, Shteynberg D, Copley RR, Taylor MS, Riethman H, Mudunuri U, Peterson J, Guyer M, Felsenfeld A, Old S, Mockrin S, Collins F.

Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature*. 2004;428:493-521.

27. Glazier AM, Nadeau JH, Aitman TJ. Finding genes that underlie complex traits. *Science*. 2002;298:2345-2349.

28. Deng AY, Jackson CM, Hoebee B, Rapp JP. Mapping of rat chromosome 2 markers generated from chromosome-sorted DNA. *Mamm Genome*. 1997;8:731-735.

5.7 FIGURE LEGENDS, FIGURES AND TABLES

Table 1: Newly generated polymorphic Chr 2 markers:

Rat Marker	Supercontig	Primer sequences (5'→ 3')	Size (Bp)	Temp (°C)	Condition (P or A)	Polymorphism among rat strains
D2Chum24	NW_047625	TTCGGTTGTTGAGACCCATT GCCAGAGGTCGAGTTCAAAG	245	60	A	DSS<MNS
D2Chum25	NW_047625	GAGAGGGATATAACATGGACAGG CCACTTTCATTGCTTCTTTGG	244	60	A	DSS>MNS
D2Chum33	NW_047625	AGGCAGAGTTGGACGAAGAA TGCAGCTCAACCAGAAAATG	209	60	A	DSS>MNS
D2Chum46	NW_047625	TTCCTCCCTTGCCCTACTCT CACCAGGTGAAACTCCCACT	204	60	A	DSS<MNS
D2Chum51	NW_047625	ACTTCGCAAGCATTGGAAAC CCCCTTTTTCTCTCCCAACA	227	60	A	DSS>MNS
D2Chum57	NW_047627	CTACTGGAAACAGCCCCATC GGGGGTGTTTATTTTTGCAT	225	60	A	DSS<MNS
D2Chum90	NW_047625	TTGGAGTTCATTAAGCAACACAG CCTTCTGGAAAAAGGTAAACCA	242	60	P	DSS>MNS
D2Chum113	NW_047625	TGTGGGTGTGGGTGTGTATC TGGCCCAAATACTTTCCTGA	248	60	A	DSS<MNS
D2Chum122	NW_047625	GTGTCACAGAGCAGCAGGAA GGTGGAAAGGTTGTTGAGGA	197	60	A	DSS<MNS
D2Chum225	NW_047625	ACAAGCAAGAAGGCACCACT GCCTGTGGAGAGAAGTGACC	168	60	A	DSS<MNS

Footnote to Table 1: DSS, the Dahl salt-sensitive strain; MNS, the Milan normotensive strain strain; bp, base pairs; Supercontigs are those obtained from blasting an existing marker on the map to the rat genome database at http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html. Only polymorphic markers used in our present work are listed. Under condition: P represents polyacrylamide and A represents agarose gel electrophoresis.

Figure 1. **Separations of two congenic strains by a critical new chromosome marker.** The linkage map is essentially the same as published previously [1;5;6;28], which is based on an F_2(S x MNS) population. Numbers to the left of the linkage map are units in cM. RH map refers to the map using rat/hamster radiation hybrids, in which units are in centiRays (cR). Numbers on the RH map are arbitrary units descending from the top of the chromosome. Solid bars under congenic strains symbolize the DSS chromosome fragments (i.e. in an open bar) that have been replaced by that of the MNS rat. Hatched bars on ends of solid bars indicate the ambiguities of crossover breakpoints between two markers. The rest of Chr 2 and the rest of the congenic genomes were homozygous SS. *Adh*, alcohol dehydrogenase; *Agtr1b*, angiotensin receptor type 1B; *Atp1a1*, Na^+K^+-ATPase α1; *Camk2d*, calmodulin-dependent protein kinase II-delta; *Fgg*, fibrinogen gamma; *Gca*, guanylyl cyclase A/atrial natriuretic peptide receptor; *Mme*, membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase); *Prlr*, prolactin receptor. D2Chm90 and the rest of the markers are anonymous either from our current (Table 1) and previous [28] work, and from the rat genome data bases (http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html). DSS, the Dahl salt-sensitive strain. Congenic strains were as follows: S.M, DSS.MNS-(D2Mit6-*Adh*)/Lt; S.M1, DSS.MNS-(D2Mit6-D2Rat166)/Lt; S.M2, DSS.MNS-(D2Mit6-D2Rat303)/Lt; S.M5, DSS.MNS-(Mme-D2Mit14/Lt) and S.M6, DSS.MNS-(*Mme*-D2Rat131)/Lt. BPs of these strains were reported previously [5;6]. MAP refers to the averaged mean arterial pressure during the period of measurement for each strain. The localization of C2QTL1 is shown. The QTL region indicated that the interval initially thought to harbor only C2QTL2 actually contains C2QTL2 and C2QTL3 (Figure 2).

Figure 2. **Mapping of multiple BP C2QTLs.** Chr 2 map and depictions of new congenic substrains are essentially the same as that for Figure 1. The regions harboring the QTLs for BP have been enlarged for better presentations. Solid bars under congenic substrains symbolize the DSS chromosome fragments (i.e. an open bar) that have been replaced by that of the MNS rat. Hatched bars at ends of solid bars represent the segments of crossover ambiguity between two markers. The entire region indicated by solid bars and junctions between the solid and open bars are homozygous for MNS, i.e.

MM, on the map for all the markers listed in the corresponding positions. The rest of Chr 2 and the rest of the congenic genomes were SS. DSS, Dahl salt-sensitive strain; DSS.MNS-(D2Rat183-D2Chm113)/Lt (abbreviated as C2S.M7), DSS.MNS-(D2Chm25-D2Mit14)/Lt (C2S.M8), DSS.MNS-(D2Chm25-D2Rat131)/Lt (C2S.M9), DSS.MNS-(D2Wox27/*Adh*)/Lt (C2S.M10), DSS.MNS-(D2Chm51-D2Rat38)/Lt (C2S.M11) and DSS.MNS-(D2Chm25-*Fgg*)/Lt (C2S.M12). C2Chm markers are given in Table 1. For explanations of MAP comparisons and most of the marker designations, see the legend for Figure 1. *Gucy1a* and *Gucy1b* are the soluble subunits α and β of guanylate cyclase 1 genes respectively. They have been excluded as candidate genes for a C2QTL because C2S.M12 did not exhibit a BP effect. The placements of C2QTL1, C2QTL2 and C2QTL3 were indicated by brackets to the right.

Figure 3. **Comparisons of BPs between congenic substrains, and the DSS strain**. a, d, g, j, m: systolic arterial pressures (SAPs), b, e, h, k, n: Diastolic arterial pressures (DAPs), and c, f, i, l, o: mean arterial pressures (MAPs). Error bars represent SEM. n refers to the number of rats. BP response patterns such as diurnal variations among all the congenic strains were not different (data not shown). BPs of each strain were measured, at least, during two different time periods, and then pooled to produce the final BP readings. For the simplicity of presentation and comparison, only a 24-hour average of BP is taken as one data point on the graph for each strain. DSS, Dahl salt-sensitive strain; For designations of the rest of the strains see the legend for Figure 2.

Figure 4. **Epistatic and additive interactions among C2QTL1, C2QTL2 and C2QTL3**: The left three columns in (a) and (b) represent differences in mean arterial pressure (MAP) between a congenic and DSS strains, i.e. BP lowered by a congenic strain. The last column to the right in (a) and (b) indicates a 'predicted' value of MAP lowered by combining different C2QTLs. The BP effect of C2QTL1 is represented by congenic strain S.M1 (Figure 1) or C2S.M7 (Figure 2); the BP effect of C2QTL2 is represented by congenic strain C2S.M9 (Figure 2); the BP effect of C2QTL3 is represented by congenic strain C2S.M11 (Figure 2); the BP effect of [C2QTL2 and C2QTL3] is represent by congenic strain S.M6 (Figure 1); and the BP effect of

[C2QTL1 and C2QTL2 and C2QTL3] is represented by congenic strain S.M (Figure 1). *p* indicates a significant QTL-QTL interaction or no interaction as performed by 2 x 2 factorial analyses [22]. a), The 'observed' BP effect of one congenic strain combining [C2QTL1 and C2QTL2 and C2QTL3] is different (*p* interaction <0.03) from a 'predicted' sum of BP effects from an individual congenic strain containing [C2QTL2 and C2QTL3] plus another individual congenic strain containing only C2QTL1. Thus, there is a QTL-QTL interaction between C2QTL1 and [C2QTL2 and C2QTL3]. b), The 'observed' BP effect of one congenic strain combining C2QTL2 and C2QTL3 is not different (*p* interaction >0.5) from a 'predicted' sum of BP effects of C2QTL2 + C2QTL3. Thus, there is no QTL-QTL interaction between C2QTL2 and C2QTL3.

Figure 1

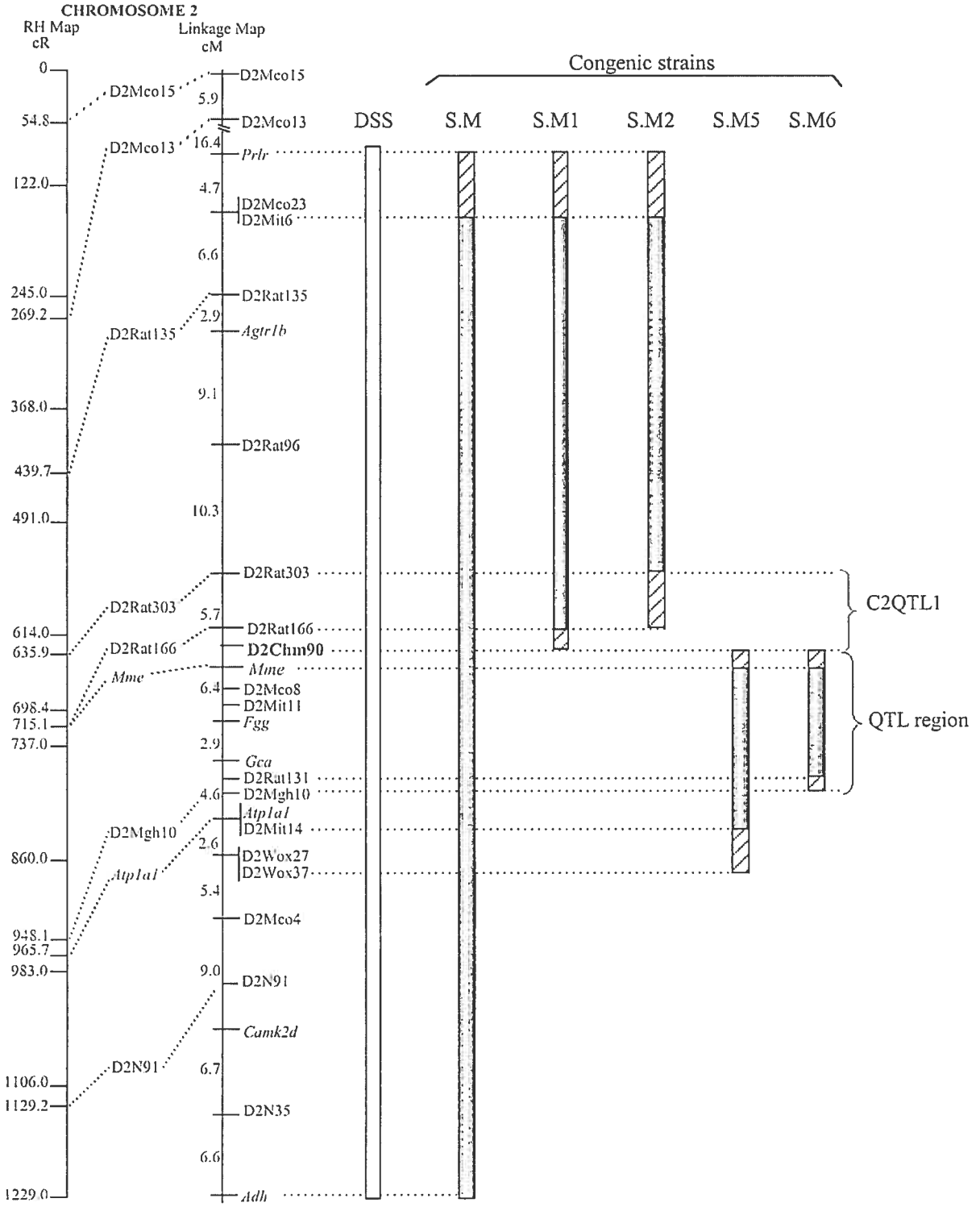
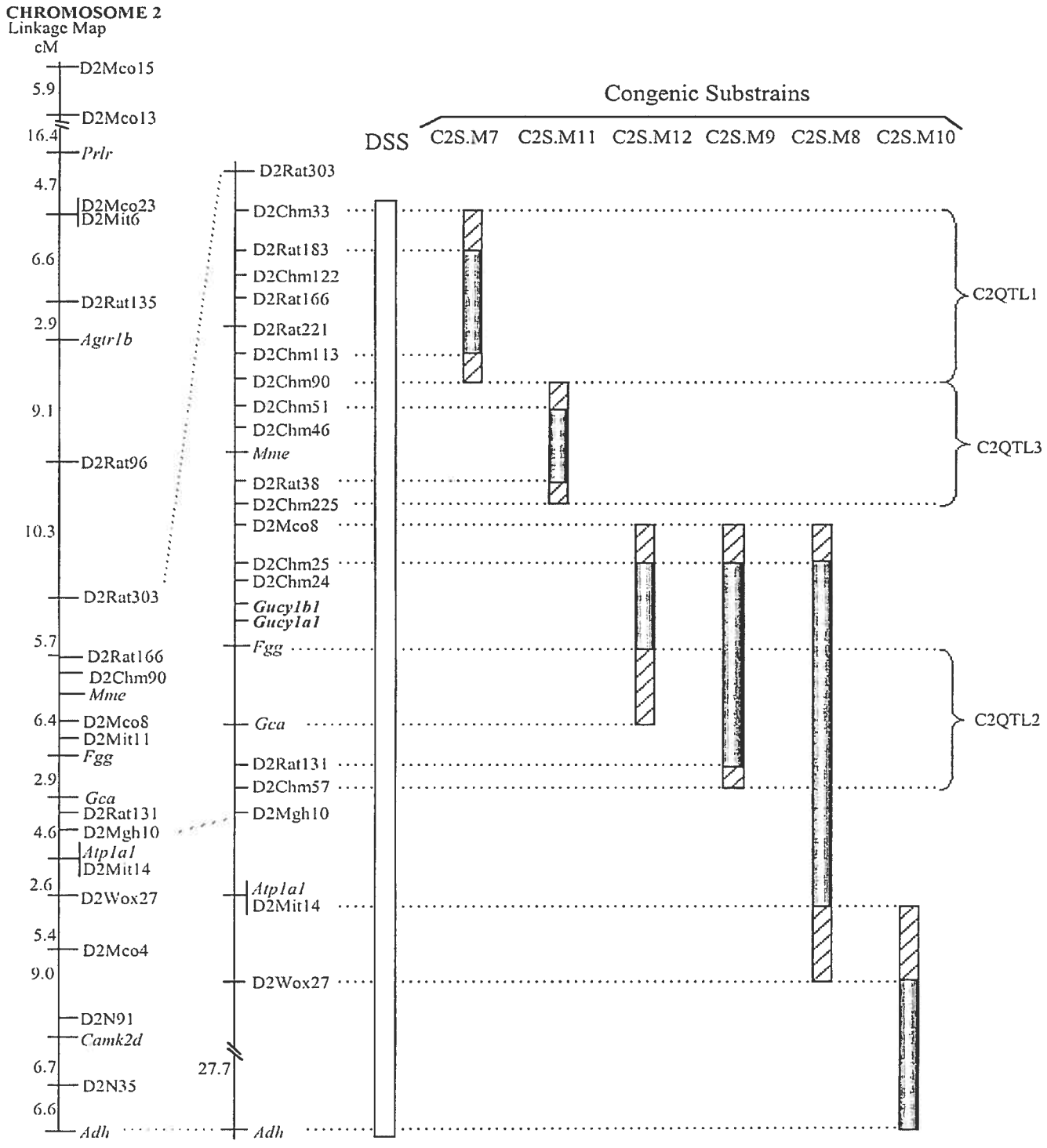

CHROMOSOME 2
RH Map
cR
Linkage Map
cM
Congenic strains
DSS
S.M
S.M1
S.M2
S.M5
S.M6
0
D2Mco15
5.9
54.8
D2Mco13
16.4
122.0
Prlr
4.7
D2Mco23
D2Mit6
6.6
245.0
D2Rat135
269.2
2.9
Agtr1b
9.1
368.0
D2Rat96
439.7
10.3
491.0
D2Rat303
5.7
614.0
D2Rat166
635.9
D2Chm90
Mme
6.4
D2Mco8
698.4
D2Mit11
715.1
Fgg
737.0
2.9
Gca
D2Rat131
4.6
D2Mgh10
Atp1a1
D2Mit14
2.6
860.0
D2Wox27
D2Wox37
5.4
D2Mco4
948.1
965.7
983.0
9.0
D2N91
Camk2d
6.7
1106.0
1129.2
D2N35
6.6
1229.0
Adh
C2QTL1
QTL region
MAP (mmHg)
170±5 (n=10)
120±2 (n=5)
148±4 (n=9)
168±4 (n=9)
130±5 (n=4)
122±2 (n=5)
MAP difference between S and congenics (mmHg)
—
50
22
2
40
48
ANOVA + Dunnet P
—
<0.0001
<0.03
>0.82
<0.001
<0.001

Figure 2



MAP (mmHg)	170±4 (n=18)	145±4 (n=8)	144±2 (n=8)	160±7 (n=9)	139±8 (n=5)	139±5 (n=8)	162±7 (n=5)
MAP difference between S and congenics (mmHg)	—	25	26	10	31	31	8
ANOVA + Dunnett *P*	—	**<0.03**	**<0.03**	>0.3	**<0.02**	**<0.003**	>0.5

Figure 3

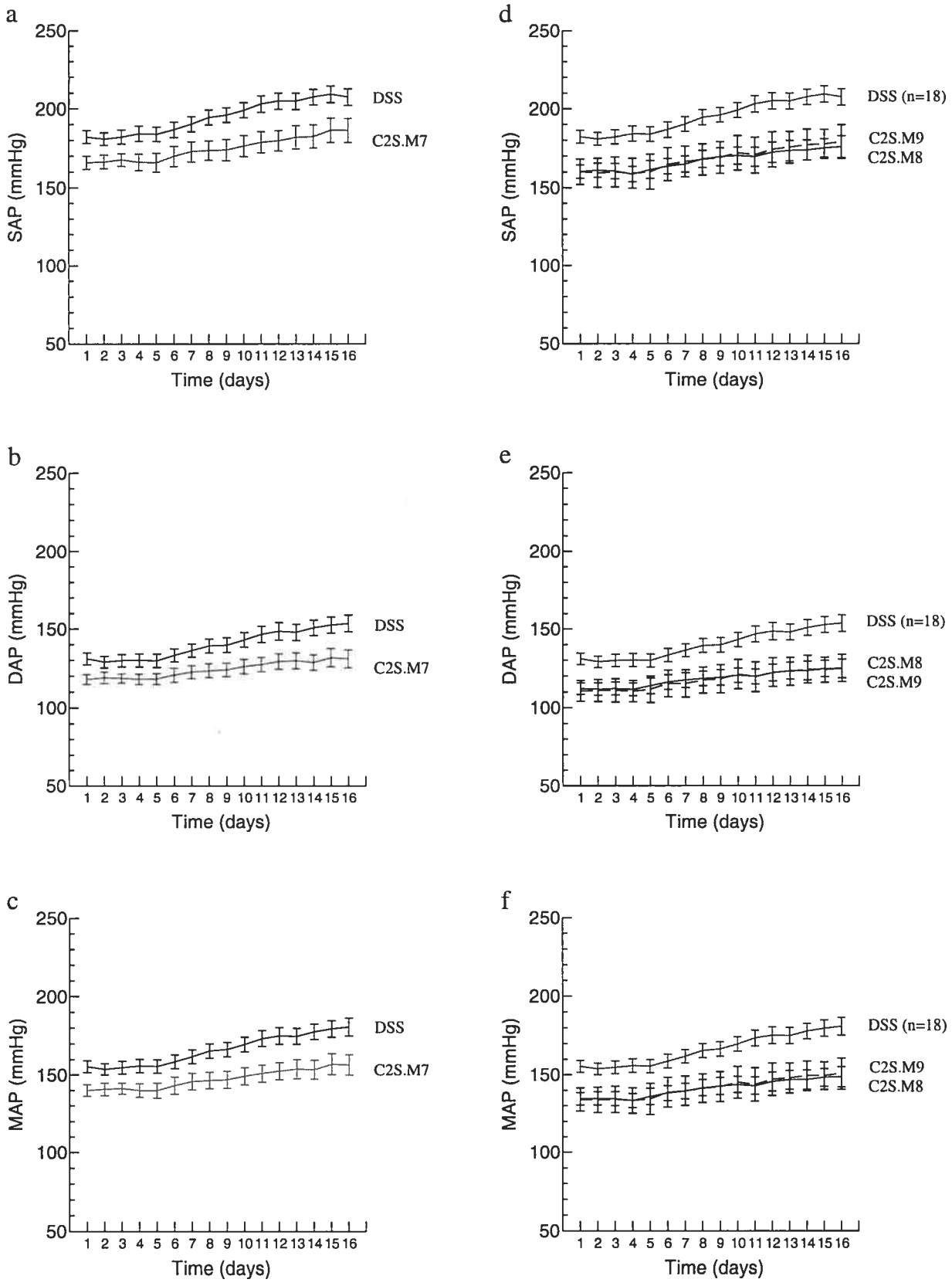

a
SAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M7
d
SAP (mmHg)
Time (days)
DSS (n=18)
C2S.M9
C2S.M8
b
DAP (mmHg)
Time (days)
DSS
C2S.M7
e
DAP (mmHg)
Time (days)
DSS (n=18)
C2S.M8
C2S.M9
c
MAP (mmHg)
Time (days)
DSS
C2S.M7
f
MAP (mmHg)
Time (days)
DSS (n=18)
C2S.M9
C2S.M8

Figure 3

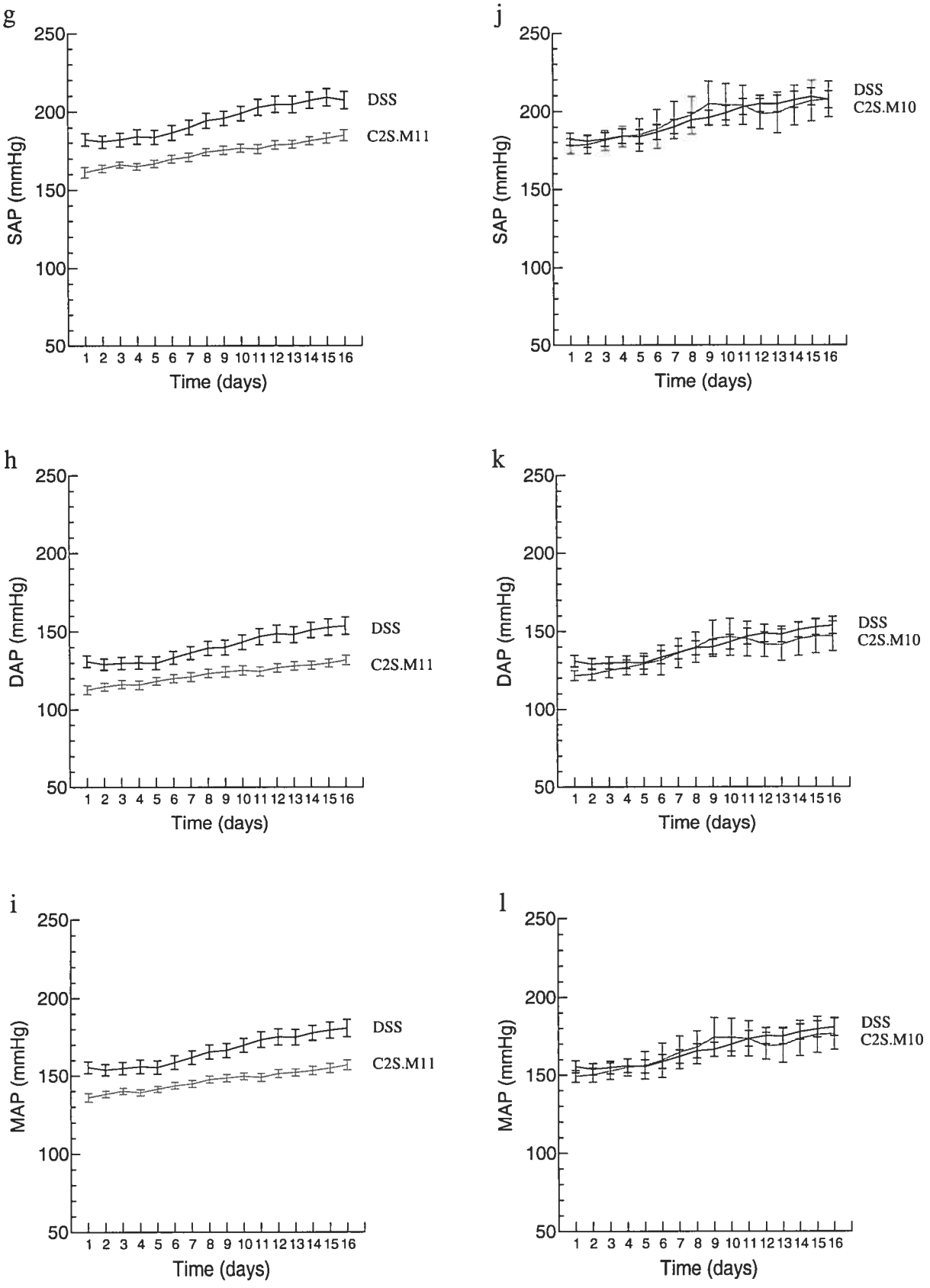

g
SAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M11
j
SAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M10
h
DAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M11
k
DAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M10
i
MAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M11
l
MAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS
C2S.M10

Figure 3

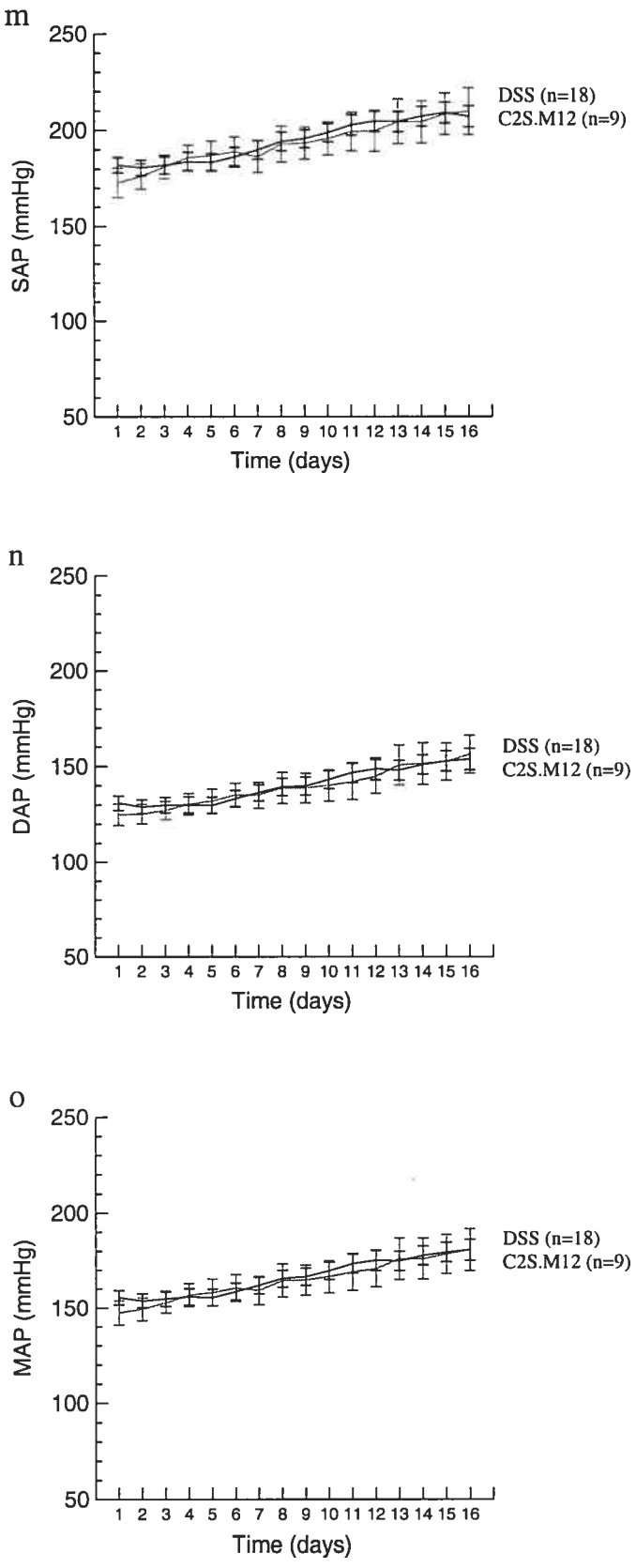
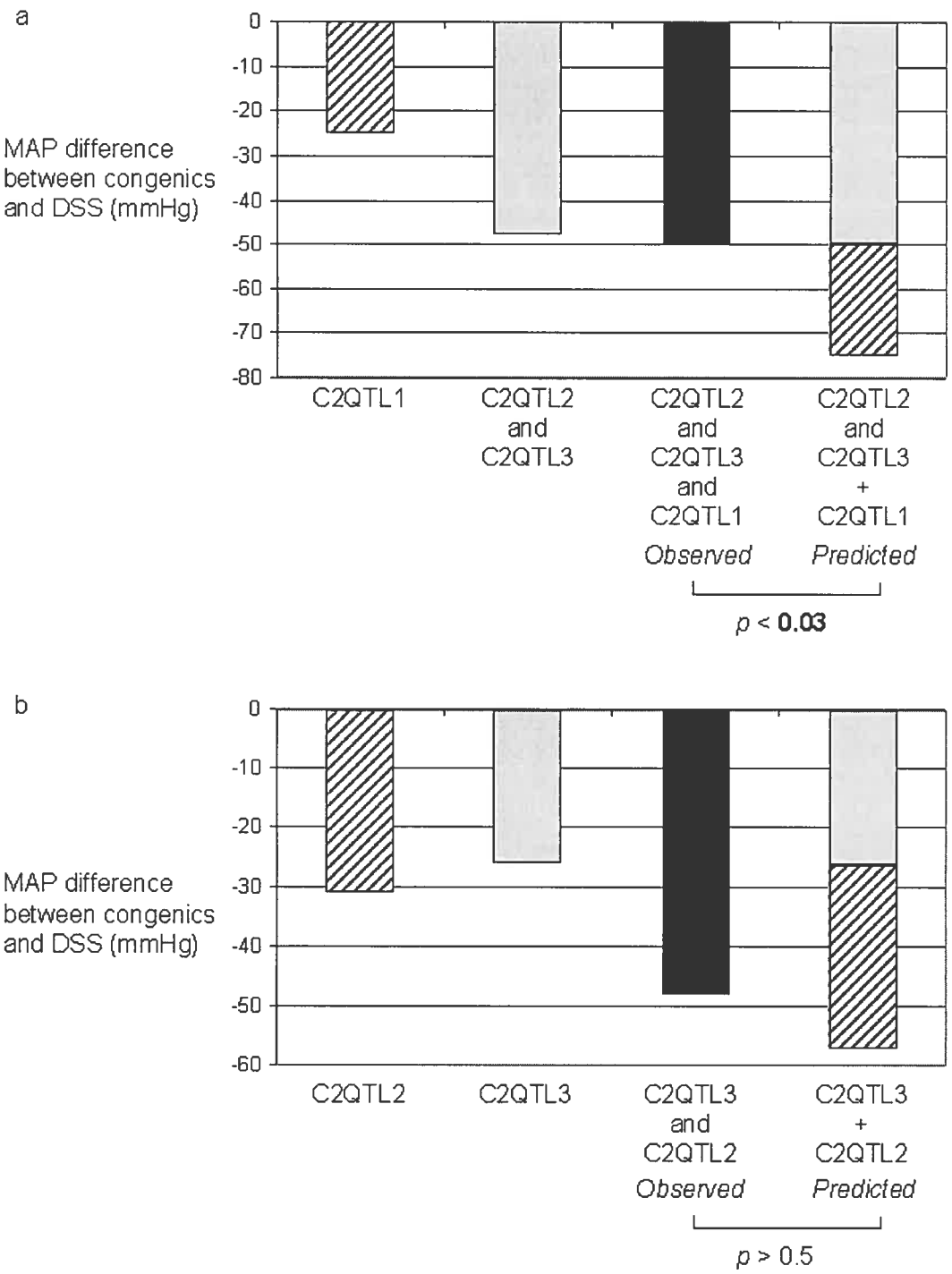

m
SAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS (n=18)
C2S.M12 (n=9)
n
DAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS (n=18)
C2S.M12 (n=9)
o
MAP (mmHg)
250
200
150
100
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (days)
DSS (n=18)
C2S.M12 (n=9)

Figure 4


a
MAP difference between congenics and DSS (mmHg)
0
-10
-20
-30
-40
-50
-60
-70
-80
C2QTL1
C2QTL2 and C2QTL3
C2QTL2 and C2QTL3 and C2QTL1
Observed
C2QTL2 and C2QTL3 + C2QTL1
Predicted
p < 0.03
b
MAP difference between congenics and DSS (mmHg)
0
-10
-20
-30
-40
-50
-60
C2QTL2
C2QTL3
C2QTL3 and C2QTL2
Observed
C2QTL3 + C2QTL2
Predicted
p > 0.5

CHAPTER 6

A quantitative trait locus for aortic smooth muscle cell number acting independently of blood pressure: implicating the angiotensin receptor AT1B gene as a candidate

Julie Dutil, Vasiliki Eliopoulos, Éve-Lyne Marchand, Alison M. Devlin, Johanne Tremblay, Kalyani Prithiviraj, Pavel Hamet, Annik Migneault, Denis deBlois, Alan Y. Deng

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Research Centre-CHUM, Hôtel Dieu, 3840 rue, St. Urbain, Montréal, Québec, H2W 1T8, Canada

Short title: QTL for aortic hyperplasia and angiotensin receptor AT1B

Corresponding author: Alan Deng, Ph.D., F.A.H.A.

Research Centre-Centre Hospitalier de l'Université de Montréal (CHUM)

7-132 Pavillon Jeanne Mance, 3840, rue St. Urbain

Montréal, Québec, H2W 1T8, Canada

Phone: 514-890-8000 ex. 15522

Fax: 514-412-7152

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6.1 ABSTRACT

Vascular hyperplasia may be involved in the remodeling of vasculature. It was unknown if there were genetic determinants for aortic smooth muscle cell number (SMCN) and, if yes, whether they acted independently of those for blood pressure (BP). To unravel this issue, we utilized congenic strains previously constructed for BP studies. These strains were made by replacing various Chromosome 2 segments of the Dahl salt-sensitive (S) rat with those of the Milan normotensive rat (MNS). We measured and compared SMCN in aortic cross sectional areas and BPs of these strains. Consequently, a quantitative trait locus (QTL) for SMCN was localized to a chromosome region not containing a BP QTL, but harboring the locus for the angiotensin II receptor AT1B (*Agtr1b*). *Agtr1b* became a candidate for the SMCN QTL because a) two significant mutations were found in the coding region between S and all congenic strains possessing the MNS alleles and b) contractile responses to angiotensin II were significantly and selectively reduced in congenic rats harboring the MNS alleles of the SMCN QTL as compared to S rats. The current investigation presents the first line of evidence that a QTL for aortic SMCN exists, and it acts independently of QTLs for BP. The relevant congenic strains developed therein potentially provide novel mammalian models for the studies of vascular remodeling disorders.

Key words: functional genomics, Dahl salt-sensitive rats, Milan normotensive rats, aortic hyperplasia, vasoreactivity, congenic strains

6.2 INTRODUCTION

Hypertrophy and/or vascular hyperplasia may contribute to the elasticity/rigidity of the blood vessel, and peripheral vascular resistance. Although, cardiac hypertrophy and hypertension were sometimes associated, there were indications that genes for BP and left ventricular hypertrophy (LVH) could be separated in hypertensive models [1,2]. LVH can be determined by genes from normotensive rats, independently of hypertension [3].

As for a relationship between hypertension and hyperplasia, the initial evidence indicated that the cultured aortic smooth muscle cells (SMC) proliferated faster (i.e. hyperplasia) in the spontaneously hypertensive rats (SHR) than those in the Wistar Kyoto rats (WKY) [4,5]. These findings suggested a link between hypertension and the SMC proliferation. Since then, a large body of evidence has been accumulated on their associations [6-11]. Based on these observations, one would expect that a higher SMC proliferation might correlate with a higher BP. Nevertheless, there was one genetic study reporting that hypertension was not associated with the growth of skin fibroblast in a F2 cross between SHR and WKY [12]. This is not surprising because an association between BP and the SMC growth in these two strains could simply be accidental not causal, one way or the other.

Up to date, it remained unclear whether or not there are QTLs determining vascular SMC number and, if yes, whether or not mechanisms determining hypertension and vascular hyperplasia could be separated. Our current goal is to discover QTLs controlling vascular SMC number regardless of, or unprejudiced by, its association or dissociation with BP.

The power and utility of genetic analyses are evident in finding causal genes for vascular hyperplasia. The beauty of the genetic approach lies in its objectivity, i.e., one is not biased towards predicting an outcome based on a preconceived notion linking two associated phenomena, e.g. hypertension and hyperplasia. Nor are we biased towards the perception that hyperplasic alleles of a QTL, if it existed, had to come from a hypertensive strain, but not from a normotensive strain. If hypertensive alleles of a QTL

for BP could originate from a normotensive strain, and vice versa [13,14], one can expect that SMC-increasing alleles of a QTL could originate from a normotensive strain.

Ever since the revelation of a QTL for BP in Dahl salt-sensitive (S) rats [15,16], rat Chr 2 appears to play an important role in the development of hypertension in several of the hypertensive strains [17-27]. In the process, a number of congenic strains have been developed that cover various regions of Chr 2 of S rats [28,29]. Taking advantage of these resources, some questions could be addressed regarding QTLs for hyperplasia. Specifically (a), is there a QTL for vascular hyperplasia, and particularly for aortic smooth muscle cell number (SMCN) in S rats? If yes, does it act independently of blood pressure? And (b), if there is a QTL specific for SMCN, can any gene be supported as a candidate?

In the present studies, BPs of certain congenic strains were compared along with their respective indices of vascular SMCN, and vascular, cardiac, and renal weights, and the internucleosomal DNA fragmentation, an indicator of apoptosis. Additionally, when a candidate gene was found in the region containing the QTL for SMCN, sequencing would be conducted to detect significant mutations that could have the potential to alter the expression or/and the function of the gene.

6.3 METHODS

6.3.1 Animals

For the sake of easy distinctions for congenic strains specifically made for Chr 2, a prefix of C2 is added in front of congenic designations. Thus the congenic strains previously utilized [28,29], S.M, S.M1, S.M2 and S.M6, are re-designated as C2S.M, C2S.M1, C2S.M2 and C2S.M6 respectively (Figure 1).

6.3.2 Marker development

We examined all the available markers in the rat database for the region between D2Rat166 and *Mme* (Figure 1). But none of them was either homozygous SS or MM for C2S.M1 and C2S.M6. Therefore, potential overlaps could not be ruled out between the

lower segment for BP C2QTL1 [29] and the upper segment for BP C2QTL2 [28]. This ambiguity called into question whether or not BP C2QTL1 and BP C2QTL2 were separate or merely represented the same QTL, but under the influence of a different background.

To prove that BP C2QTL1 and BP C2QTL2 are separate genes, one had to find a marker between D2Rat166 and *Mme* that is SS for both C2S.M1 and C2S.M6. For this purpose, we employed the rat genome data base. Supercontigs located in Chr 2 region between D2Rat166 and *Mme* were first identified by blasting them at the website, http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html. From such a supercontig, regions containing microsatellites were searched, and when found, were used to design markers for genotyping rats based on PCR. These new markers were designated with D2Chm prefixes, which represent the Centre Hospitalier de l'Université de Montreal (CHUM). Consequently, a series of markers were generated. One particular marker designated D2Chm90 was polymorphic and located in the region of interest (Figure 1).

6.3.3 BP studies and tissue extractions

BP studies on the congenic strains were reported in detail previously [28,29]. In brief, male rats were weaned at 21 days of age, maintained on a low salt diet (0.2% NaCl, Harlan Teklad 7034) and then fed a high salt diet (2% NaCl, Harlan Teklad 94217) starting from 35 days of age until the end of the experiment. Telemetry probes were implanted when rats were 56 days old (i.e. after 3 weeks of the high salt diet) with their body weights between 250-320 grams. The BP measurements lasted until the time of sacrifice.

Rats were sacrificed by decapitation 30 days after the commencement of their BP measurements, i.e. at 14 weeks of age. The organs of interest (see the following sections) were removed, cleaned carefully from surrounding adventitial connective tissues and fat, blotted to remove excess blood, and weighed immediately. To minimize any potential inconsistency in the collection, one person was designated for harvesting one particular organ and also for the subsequent dissections into sub-sections of interest.

The whole heart was then dissected into the left ventricle plus the septum, and into right ventricle. The weight of each section was then recorded and corrected for the body weight of the respective animal. The dissection was according to a previously published procedure [30,31].

6.3.4 Measurement of aortic cross sectional areas (CSA)

The following procedure was done essentially according to that previously published [32]. The thoracic aorta was isolated from the diaphragm to above the first intercostal artery and cleaned of adherent adventitial tissue and fat. A 3-mm-long ring of aorta was cut between the third and fourth intercostal arteries. The aortic rings were immerse-fixed in 4% parformaldehyde overnight and processed according to routine histological procedures for morphological examinations in parafin-embedded tissues. The fixation of aortas was done in non-pressure-controlled conditions. The aortic endothelium of the remaining aorta segments was removed by scrubbing the intimal surface with a cotton tip applicator. The aortic media was immediately snap frozen in liquid nitrogen and kept at –80°C until further processing for DNA extraction.

The medial cross-sectional area was evaluated in 5-μm-thick, hematoxylin-eosine-stained sections of aorta. Photomicrographs of the aortic sections were taken at 400x magnification, digitalized, and analyzed using the public domain NIH Image program 1.61 (developed at the National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Two independent measurements of each tissue section were conducted to ensure the reproducibility of the image analysis.

6.3.5 Determination of vascular SMCN

The following was done essentially according to the procedure previously published [33]. Briefly, three consecutive sections (5 μm thickness) were obtained from aortic rings sectioned perpendicularly to the longitudinal axis of the vessel. Tissue sections were stained with hematoxylin, photographed, and printed at a final magnificatlon of 400X. In the top section, an area, a_d, of vessel wall was delineated by two parallel lines approximately perpendicular to the wall edges. A disector was defined as the three-

dimensional probe bounded by a_d and the top surface of the "bottom" section. The disector has volume $v_d = a_d X h_d$, where a_d is the area of the disector and h_d is the height of the disector. The term h_d is obtained by the following equation $h_d = (s- l) X t$, where s is the number of serial sections and t the average section thickness. Within a_d, the number of nucleus profiles, n_t, was determined and in the subsequent sections, each of the n_t nuclei was followed and marked. In the final section, the number of nuclei still present was determined, n_b. The number $n_d = (n_t-n_b)$ is then the number of "downward-pointing" nucleus ends within the disector when counting from top to bottom. Binucleate SMC account for less than 0.5% of the SMC in the thoracic aorta of hypertensive rats [34]. Therefore, on the assumption that each cell contains only one nucleus, an estimate of cell numerical density, N_v, is given by $N_d = n_d / v_d$. The number of cells per unit vessel length then was estimated from $a_l X N_v$, where a_l is the medial cross-sectional area determined with the image analyzer as described above.

6.3.6 DNA content and apoptosis

The remaining aortic media was pulverized in liquid nitrogen. The total tissue DNA was extracted and quantified as described previously [35]. To quantify the degree of internucleosomal DNA fragmentation, a hallmark of apoptosis (programmed cell death), the 3'OH ends in the extracted DNA were radiolabeled using ^{32}P-dNTP and terminal deoxynucleotidyl-transferase. DNA was separated by gel electrophoresis, blotted, and radioactivity incorporated in the small fragments (150 to 1500 bps) was quantified using a PhosphoImager as described previously [36].

6.3.7 Vasoreactivity studies

Vasoactive responses to angiotensin II (AngII) and phenylphrine (PE) were examined as described previously [37] in rings of thoracic aorta isolated from of S and C2S.M2 implanted or not with a telemetry probe. Briefly, rats were killed by exposure to CO_2 and freshly-isolated vessels without adventitial adipose and connective tissue or endothelium were cut into 3-mm-long rings to be placed at 37°C in organ chambers filled with oxygenated Krebs solution containing in mM: dextrose, 11; NaCl, 117.5; $MgSO_4$, 1.18; KH_2PO_4, 1.2; $NaHCO_3$, 25; KCl, 4.7; $CaCl_2$, 2.5. Isometric contractions

were measured with the use of isometric force transducers (Harvard Apparatus Canada) and a digitalized data acquisition system (Model MP100, Biopac System, Harvard). Rings were equilibrated under a passive tension of 1 g and then challenged with 40 mmol/L KCl to document receptor-independent contractility. Tissues were also stimulated with cumulative concentrations of PE (1 – 100 mmol/L) and AngII (300 pmol/L to 300 μmol/L). Separate sets of rings were used to measure contractile responses in the presence or absence of L-NAME (100 μmol/L). The integrity of the endothelium was evaluated on the plateau of PE-induced maximal contraction by measuring the relaxant response to Ach (1 μmol/L). In a subset of tissues, AngII-induced contractile effect was also evaluated in the presence of losartan (75 nmol/L).

6.3.8 Statistical analysis

In BP analyses, repeated measures' analysis of variance (ANOVA) followed by Dunnett, (which permits a correction for multiple comparisons and sample sizes), was used to compare a parameter between 2 group as presented previously [28,29]. During the comparison, ANOVA was, first, used to analyze the data to see if there was any difference among the groups. If the difference is significant, then, the Dunnett test was followed to see which group is different and how much significant from the S strain. If the difference is not, then, no Dunnett test followed. In functional vasoreactivity studies, data from S and C2S.M2 were compared using unpaired Student *t* test using Prism 4.0 (GraphPad Software).

6.4 RESULTS

6.4.1 Localization of a QTL for vascular SMCN independent of BP QTLs

The marker, D2Chm90, is important (Figure1). It came from the same supercontig, NW_043524, containing *Mme* and is homozygous MM for C2S.M, but SS for C2S.M1, C2S.M2, and C2S.M6 (Figure 1). Thus D2Chm90 effectively separated C2S.M1 from C2S.M6.

The most informative strain is C2S.M2 (Figure 1), which had no effect on BP (reference 17 and summarized at the bottom of Figure 1) and had the same cardiac, left ventricular and renal weights as S (Figure 2 a and b), but showed higher SMCN (Table 1). In fact, aortic SMC counts of both C2S.M1 and C2S.M2 were higher than that of the S strain ($p<0.039$). In contrast, SMC counts of the C2S.M6 and C2S.M strains were not different from that of the S strain (Table 1).

Figure 2c shows comparisons of aortic cross sectional areas (CSAs) between a congenic and the S strain. C2S.M6 had smaller CSAs than S ($p<0.006$). CSAs (Figure 2c) showed the same tendency as the SMC counts (Table 1). Both C2S.M1 and C2S.M2 had slightly larger, although not significantly different, CSAs than S.

Figure 2 also shows comparisons of cardiac and renal weights corrected for body weights among the strains. Kidney weights in C2S.M1, C2S.M6 and C2S.M were lower ($p<0.03$) than those of the S rats; whereas these of C2S.M2 were not different from those of the S rats (Figure 2 a and b). Cardiac and left ventricular weights were only significantly lowered in C2S.M and C2S.M6 ($p<0.03$), but not in C2S.M1 strains ($P>0.10$). Table 2 shows assessments of DNA content and DNA fragmentation indices in both cardiac ventricles and aortas.

In terms of the DNA fragmentation indices and DNA contents comparing various strains, a possible relationship between apoptosis (or DNA synthesis) and hypertension or aortic SMC number remains to be investigated further. There was a suggestive correlation between tissue weights and the DNA content in both ventricles (Table 2). Moreover, although the DNA fragmentation suggested a tendency to be inversely correlated with the DNA content in the left ventricle, with a significant decrease in DNA content in the C2S.M and C2S.M6 strains as compared to the S strain. In the right ventricle, the DNA fragmentation was significantly increased in the C2S.M strain only.

6.4.2 The angiotensin-receptor AT1B (*Agtr1b*) gene as a candidate for the SMCN QTL:

In previous pharmacological studies, we observed that AT1 not AT2 receptors were involved in the SMC growth [38,39], implying that AT1 receptors might be important in mediating the SMC proliferation. There are two types of AT1 receptors, AT1A and AT1B, encoded by *Agtr1a* and *Agtr1b* genes respectively. In our congenic strains, the *Agtr1a* gene on Chr 17 [40] was the same as S [28,29,41], but the *Agtr1b* on Chr 2 (Figure 1) was different in that it contains the alleles from the Milan normotensive strain (MNS) (Figure 1). Because it is located in the chromosome region harboring the SMCN QTL (Figure 1), *Agtr1b* could be conceived as a candidate gene for the SMCN QTL. We reasoned that to qualify *Agtr1b* to be the candidate gene, it had to contain significant mutations that could potentially result in differences either in its function or in its level of expression between S and congenic strains trapping the SMCN QTL.

Mutational evidence supporting *Agtr1b* as a candidate gene of the SMCN QTL: Indeed, when *Agtr1b* genes were cloned and sequenced, several nucleotide mutations were detected in its coding region. Two of these mutations were of special interest in that they were found in MNS, C2S.M, C2S.M1, and C2S.M2, but were different from those in C2S.M6 and S, which had the same nucleotides. These two nucleotides resulted in significant changes in amino acids. One of T to C change at nucleotide position 6 caused a change of (aliphatic) isoleucine to (hydroxyl) threonine at amino acid position 2. Another nucleotide of A to G change at nucleotide position 118 resulted in a significant change of (sulfuric) methionine to (aliphatic) valine at amino acid position 20. The nucleotide positions were numbered from the sequence coding for the first amino acid as given previously [42]. These mutations support functional implications of *Agtr1b* as the candidate for the SMCN QTL (Figure 1), since C2S.M, C2S.M1, and C2S.M2 possess MNS alleles at *Agtr1b* (i.e. C at position 6 and G at position 118); whereas C2S.M6 possesses S alleles at *Agtr1b* (i.e. T at position 6 and A at position 118).

Functional evidence supporting *Agtr1b* as a candidate gene of the SMCN QTL: If the mutations found in *Agtr1b* were truly significant, one is expected to observe functional differences between the MNS *Agtr1b* and S *Agtr1b* genes.

For the subsequent functional study, C2S.M2 was chosen because it carries a smaller chromosome interval of MNS than C2S.M1 (Figure 1). In our work, contractile responses to AngII were documented in rings of aorta isolated from C2S.M2 and S rats. The analysis indicated that contractile responses were not different whether a telemetry probe had been implanted (N=6/strain) or not (N=6-7/strain) and the data were therefore pooled for each strain. As shown in Table 3, contractile responses to AngII were significantly reduced in C2S.M2 rats as compared to S rats, without a significant change in apparent receptor affinity. The contractions induced by AngII were AT1 receptor-dependent since they were significantly inhibited (>90%) in the presence of losartan (data not shown). In contrast, there was no inter-strain difference in the contractile responses to phenylephrine ($alpha_1$-adrenergic agonist) or KCl (receptor-independent contractile stimulus) (Table 3). Similar conclusions were reached with tissues challenged in the presence or absence of the NO synthase inhibitor L-NAME (Table 3). Thus, the hyperplasic aorta from C2S.M2 rats exhibited a selective decrease in AngII-induced, AT1 receptor-dependent contractility as compared to S rats.

6.5 DISCUSSION

Major findings from the current studies are (a), there is a QTL(s) determining vascular smooth muscle cell number (SMCN) which acts independently of QTLs for blood pressure. (b), The *Agtr1b* is a candidate for such a QTL based on two lines of evidence, gene mutations and their functional consequences. That is to say that significant nucleotide differences resulted in functional differences in *Agtr1b* between the S strain and the congenic strain carrying *Agtr1b* alleles from MNS. The significant functional correlates were shown in vasoreactivity studies.

6.5.1 A QTL for the vascular SMCN functions independently of QTLs for hypertension

C2S.M1 had a BP lower than S [Figure 1 and reference [29]], but had more aortic SMCN (Table 1). In contrast, C2S.M2 had the same BP as S [Figure 1 and reference [29]], but showed SMCN similar to those of C2S.M1 (Table 1). This fact clearly indicates that these genetic determinants for BP and aortic SMCN act independently of each other. Upon examining the chromosome interval on the map, the QTL for the aortic SMCN should be present in the chromosome fragment shared between C2S.M1 and C2S.M2, which is between *Prlr* and D2Rat303 markers (Figure 1). In contrast, BP C2QTL1 exists in the segment not shared between C2S.M1 and C2S.M2, i.e. between the D2Rat303 and *Mme* markers (Figures 1). It is noted that the QTL for the vascular SMCN acts independently, also, of cardiac, left ventricular, and renal hypertrophy, because C2S.M2 had the same cardiac, left ventricular and renal masses as S (Figure 2a and b).

By the use of two congenic strains, a stringent genetic testing, aortic SMCN and BP have different genetic, and possibly physiological, bases. Although MNS rats *per se* were not directly analyzed for SMCN, C2S.M6 served effectively as a negative control, because it contained a small segment from the MNS strain not including the region delineated by *Prlr* and D2Rat303 markers (Figure 1). Since C2S.M6 has no hyperplastic effect, whereas C2S.M1 and C2S.M2 do (Table 1), the effects to increase SMCN in C2S.M1 and C2S.M2 have to be attributed to the QTL residing in the *Prlr*/D2Rat303 segment, not to any MNS genetic background. The MNS rat would not be a good control, because it differs greatly from the S genome on the rest of the chromosomes [43,44] and because it gives no information on the effects of genome changes during the congenic construction [45].

Because SMCNs were slightly different (Table 1) between C2S.M1 (170 ± 11) vs C2S.M2 (134 ± 21), a possibility can not be ruled out that there could be another QTL for SMCN in the region of BP C2QTL1 (Figure 1). In that case, the same QTL could be responsible for both BP and SMCN.

It is noteworthy that the alleles to increase SMCN come from MNS, which is normotensive. Nonetheless, it is not surprising that MNS QTL alleles cause SMCN to increase, whereas S QTL alleles decreased SMCN. In an independent study, Lewis rats, a normotensive strain, actually carry BP-increasing alleles in comparison with S at a QTL on Chr 3, although the overall BP of the Lewis strain is considerably lower than that of the hypertensive S strain [14]. This fact indicated that the BP-increasing QTL alleles in Lewis were hidden and were only unveiled by isolating the region harboring it from the rest of the Lewis genome by congenic strains. Therefore, our present and other studies further demonstrated the power and necessity of genetic dissections of individual QTLs using congenic strains.

The detection of BP C2QTL1 added a further proof for the power of congenic approach. In linkage analysis, there was no indication that a QTL existed in the region around C2QTL1 [46,47]. It is only after the isolation of a segment by using a congenic strain (C2S.M1) that BP C2QTL1 was found [29]. This phenomenon is due, probably, to epistatic gene-gene interactions [45,48], among other things and indicates that the effect of the genetic background impacted by other QTLs affecting the same phenotype may need to be removed for the manifestation of a QTL for SMCN (Figure 1).

It is also of note that C2S.M strain included the chromosome segment contained in C2S.M1 and C2S.M2 (Figure 1), and yet, C2S.M did not show hyperplasia (Table 1). This phenomenon can be explained by the following. There could be another QTL or a modifying gene present in the segment in C2S.M but lacking in C2S.M1, i.e. between D2Rat166 and *Adh* that would have a hypoplasic effect. When combined, the overall effect would be hypoplasic. There was an example of this epistatic interaction between two QTLs. When BP-lowering alleles at one QTL was experimentally put together with BP raising alleles at another QTL, the combined effect was the same as that of the BP-lowering alleles on Chr 3 [14]. This fact indicates that these QTLs do not act alone, but in a hierarchical relationship with each other in controlling the overall BP. The same principle could be applied to the genetic determinants of SMCN.

As to the mechanisms for increasing SMCN, there are two possibilities. The QTL could either favor the SMC proliferation or reduce the SMC death. The present data showing an inverse relationship between DNA fragmentation and SMCN (Table 2) suggest a possible role for reduced apoptosis. Defining the relative contribution of these cellular mechanisms, however, is worthy of further investigations.

6.5.2. Agtr1b is a candidate gene for a SMCN QTL

It is noted that *Agtr1b* is present in the region containing the QTL for SMCN (Figure 1). Interestingly, we detected two nucleotide differences leading to significant changes in amino acids in congenic strains possessing MNS *Agtr1b* alleles (i.e C2S.M, C2S.M1, and C2S.M2), and that possessing S *Agtr1b* allele (i.e. C2S.M6) (see the result section for detail). The presence of these mutations in *Agtr1b* corresponding to the location of the QTL provides a possible mechanism for increased SMCN, because the pro-growth and anti-apoptotic effects of AT1 receptors on SMC are well known [49].

The mutations found in *Agtr1b* could have functional roles in controlling SMC proliferation. As an initial investigation of this issue, we chose to measure contractile responses to AngII since this is a proximal marker of receptor function. Surprisingly, we observed that responses to AngII were reduced in C2S.M2 vs S rats. We speculate that a prior AT1 receptor activation *in vivo* may have resulted in receptor desensitization in the aortic tissue isolated from C2S.M2 rats. Such a negative effect of *in vivo* receptor activation on the vascular sensitivity *ex vivo* seems reminiscent of hypertensive human and rat arteries, where an enhanced local expression of endothelin resulted in a selective attenuation of contractile responses to endothelin in tissues stimulated *ex vivo* [50]. Alternatively, the altered responses to AngII may be an epiphenomenon. *In vitro* studies with cultured SMCs are needed to better define the functional correlates of the mutations in *Agtr1b* and its significance for the SMC proliferation.

Another way to validate if *Agtr1b* would remain a good candidate gene or not for the SMCN QTL is by fine congenic mapping. If *Agtr1b* is the SMCN QTL, no matter how small the region harboring the QTL is minimized to (e.g. 100-200 kb), *Agtr1b* should

always be included in the same region, and this congenic substrain should also have an effect on SMCN. Alternative, *Agtr1b* could simply be a marker indicating the approximate location of the QTL for SMCN. The inducible form of the nitric oxide synthase (*Nos2*) is a case in point. *Nos2* was located in a broad region containing a QTL for BP initially, but a fine mapping ruled it out as a QTL because a congenic strain harboring it did not show a BP effect [51]. Furthermore, fine congenic mapping will also test the possibility if there are multiple QTLs for SMCN present in the interval between *Prlr* and D2Rat303.

In summary, a major finding in the present work is that there is a QTL(s) for vascular SMCN that acts independently of hypertension. This discovery has laid the foundation for the identification of the QTL and will lead to revelations of its underlying physiological mechanisms regulating of vascular remodeling independently of BP. *Agtr1b* is a candidate gene worthy of further investigations to be the SMCN QTL by fine mapping and *in vitro* functional assays. Utilizing the same strategy of fine congenic mapping and comparative congenic mapping of BP QTLs, the QTL(s) for SMCN can and will be localized to a smaller interval for candidate cloning (i.e. *Agtr1b*), or if not, then positional cloning.

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6.7 REFERENCES

References

1. Clark JS, Jeffs B, Davidson AO, Lee WK, Anderson NH, Bihoreau MT, Brosnan MJ, Devlin AM, Kelman AW, Lindpaintner K, Dominiczak AF. Quantitative trait loci in genetically hypertensive rats. Possible sex specificity. *Hypertension* 1996;28:898-906.

2. Hamet P, Kaiser MA, Sun Y, Page V, Vincent M, Kren V, Pravenec M, Kunes J, Tremblay J, Samani NJ. HSP27 locus cosegregates with left ventricular mass independently of blood pressure. *Hypertension* 1996;28:1112-1117.

3. Masciotra S, Picard S, Deschepper CF. Cosegregation analysis in genetic crosses suggests a protective role for atrial natriuretic factor against ventricular hypertrophy. *Circ Res* 1999;84:1453-1458.

4. Hadrava V, Tremblay J, Hamet P. Abnormalities in growth characteristics of aortic smooth muscle cells in spontaneously hypertensive rats. *Hypertension* 1989;13:589-597.

5. Yamori Y, Igawa T, Kanbe T, Kihara M, Nara Y, Horie R. Mechanisms of structural vascular changes in genetic hypertension: analyses on cultured vascular smooth muscle cells from spontaneously hypertensive rats. *Clin Sci (Lond)* 1981;61 Suppl 7:121s-123s.

6. deBlois D, Tea BS, Than VD, Tremblay J, Hamet P. Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension* 1997;29:340-349.

7. Dominiczak AF, Devlin AM, Lee WK, Anderson NH, Bohr DF, Reid JL. Vascular smooth muscle polyploidy and cardiac hypertrophy in genetic hypertension. *Hypertension* 1996;27:752-759.

8. Hadrava V, Tremblay J, Hamet P. Abnormalities in growth characteristics of aortic smooth muscle cells in spontaneously hypertensive rats. *Hypertension* 1989;13:589-597.

9. Hamet P, Thorin-Trescases N, Moreau P, Dumas P, Tea BS, deBlois D, Kren V, Pravenec M, Kunes J, Sun Y, Tremblay J. Workshop: excess growth and apoptosis: is hypertension a case of accelerated aging of cardiovascular cells? *Hypertension* 2001;37:760-766.

10. Marchand EL, Der SS, Hamet P, deBlois D. Caspase-dependent cell death mediates the early phase of aortic hypertrophy regression in losartan-treated spontaneously hypertensive rats. *Circ Res* 2003;92:777-784.

11. Tea BS, Der SS, Touyz RM, Hamet P, deBlois D. Proapoptotic and growth-inhibitory role of angiotensin II type 2 receptor in vascular smooth muscle cells of spontaneously hypertensive rats in vivo. *Hypertension* 2000;35:1069-1073.

12. Guicheney P, Soussan K, Dausse E, Rota R. Dissociation of hypertension and genetically enhanced cell growth capacity in skin fibroblasts of F2 hybrid spontaneously hypertensive rats/Wistar-Kyoto rats. *Am J Hypertens* 1992;5:556-565.

13. Ariyarajah A, Palijan A, Dutil J, Prithiviraj K, Deng Y, Deng AY. Dissecting quantitative trait loci into opposite blood pressure effects on Dahl rat Chromosome 8 by congenic strains. *J Hypertens* 2004;22:1495-1502.

14. Palijan A, Dutil J, Deng AY. Quantitative trait loci with opposing blood pressure effects demonstrating epistasis on Dahl rat chromosome 3. *Physiological Genomics* 2003;15:1-8.

15. Deng AY, Dene H, Rapp JP. Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. *J Clin Invest* 1994;94:431-436.

16. Deng Y, Rapp JP. Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic peptide receptor genes using Dahl salt-sensitive rats. *Nature Genetics* 1992;1:267-272.

17. Alemayehu A, Breen L, Krenova D, Printz MP. Reciprocal rat chromosome 2 congenic strains reveal contrasting blood pressure and heart rate QTL. *Physiol Genomics* 2002;10:199-210.

18. Deng AY, Jackson CM, Hoebee B, Rapp JP. Mapping of rat chromosome 2 markers generated from chromosome-sorted DNA. *Mamm Genome* 1997;8:731-735.

19. Deng AY, Dene H, Rapp JP. Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension* 1997;30:199-202.

20. Dubay C, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi JP, Kotelevtsev Y, Beckmann JS, Soubrier F, Sassard J, Lathrop GM. Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nat Genet* 1993;3:354-357.

21. Garret MR, Rapp JP. Multiple blood pressure QTL on rat chromosome 2 defined by congenic Dahl rats. *Mammalian Genome* 2002;13:41-44.

22. Jeffs B, Negrin CD, Graham D, Clark JS, Anderson NH, Gauguier D, Dominiczak AF. Applicability of a "speed" congenic strategy to dissect blood pressure quantitative trait loci on rat chromosome 2. *Hypertension* 2000;35:179-187.

23. Pravenec M, Gauguier D, Schott JJ, Buard J, Kren V, Bila V, Szpirer C, Szpirer J, Wang JM, Huang H, . Mapping of quantitative trait loci for blood pressure and cardiac mass in the rat by genome scanning of recombinant inbred strains. *J Clin Invest* 1995;96:1973-1978.

24. Samani NJ, Gauguier D, Vincent M, Kaiser MA, Bihoreau MT, Lodwick D, Wallis R, Parent V, Kimber P, Rattray F, Thompson JR, Sassard J, Lathrop M. Analysis of quantitative trait loci for blood pressure on rat chromosomes 2 and 13. Age-related differences in effect. *Hypertension* 1996;28:1118-1122.

25. Schork NJ, Krieger JE, Trolliet MR, Franchini KG, Koike G, Krieger EM, Lander ES, Dzau VJ, Jacob HJ. A biometrical genome search in rats reveals the multigenic basis of blood pressure variation. *Genome Res* 1995;5:164-172.

26. Tremblay J, Hum DH, Sanchez R, Dumas P, Pravenec M, Krenova D, Kren V, Kunes J, Pausova Z, Gossard F, Hamet P. TA repeat variation, Npr1 expression, and blood pressure: impact of the Ace locus. *Hypertension* 2003;41:16-24.

27. Vincent M, Samani NJ, Gauguier D, Thompson JR, Lathrop GM, Sassard J. A pharmacogenetic approach to blood pressure in Lyon hypertensive rats. A chromosome 2 locus influences the response to a calcium antagonist. *J Clin Invest* 1997;100:2000-2006.

28. Dutil J, Deng AY. Further chromosomal mapping of a blood pressure QTL in Dahl rats on chromosome 2 using congenic strains. *Physiological Genomics* 2001;6:3-9.

29. Dutil J, Deng AY. Mapping a blood pressure quantitative trait locus to a 5.7-cM region in Dahl salt-sensitive rats. *Mammalian Genome* 2001;12:362-365.

30. Moujahidine M, Dutil J, Hamet P, Deng AY. Congenic mapping of a blood pressure QTL on Chromosome 16 of Dahl rats. *Mammalian Genome* 2002;13:153-156.

31. Sivo Z, Malo B, Dutil J, Deng AY. Accelerated congenics for mapping two blood pressure quantitative trait loci on Chromosome 10 of Dahl rats. *J Hypertens* 2002;20:1-9.

32. deBlois D, Tea BS, Than VD, Tremblay J, Hamet P. Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension* 1997;29:340-349.

33. deBlois D, Tea BS, Than VD, Tremblay J, Hamet P. Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension* 1997;29:340-349.

34. Owens GK. Differential effects of antihypertensive drug therapy on vascular smooth muscle cell hypertrophy, hyperploidy, and hyperplasia in the spontaneously hypertensive rat. *Circ Res* 1985;56:525-536.

35. deBlois D, Tea BS, Than VD, Tremblay J, Hamet P. Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension* 1997;29:340-349.

36. deBlois D, Tea BS, Than VD, Tremblay J, Hamet P. Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension* 1997;29:340-349.

37. Lemay J, Hou Y, Tremblay J, deBlois D. Angiotensin I-converting enzyme activity and vascular sensitivity to angiotensin I in rat injured carotid artery. *Eur J Pharmacol* 2000;394:301-309.

38. deBlois D, Tea BS, Than VD, Tremblay J, Hamet P. Smooth muscle apoptosis during vascular regression in spontaneously hypertensive rats. *Hypertension* 1997;29:340-349.

39. Tea BS, Der SS, Touyz RM, Hamet P, deBlois D. Proapoptotic and growth-inhibitory role of angiotensin II type 2 receptor in vascular smooth muscle cells of spontaneously hypertensive rats in vivo. *Hypertension* 2000;35:1069-1073.

40. Deng AY, Dene H, Pravenec M, Rapp JP. Genetic mapping of two new blood pressure quantitative trait loci in the rat by genotyping endothelin system genes. *J Clin Invest* 1994;93:2701-2709.

41. Deng AY, Dene H, Rapp JP. Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension* 1997;30:199-202.

42. Deng AY, Rapp JP. Evaluation of the angiotensin II receptor AT1B gene as a candidate gene for blood pressure. *J Hypertens* 1994;12:1001-1006.

43. Garret MR, Zhang X, Dukhanina OI, Deng AY, Rapp JP. Two linked blood pressure QTL on chromosome 10 defined by Dahl-rat congenic strains. *Hypertension* 2001;38:779-785.

44. Kato N, Hyne G, Bihoreau MT, Gauguier D, Lathrop GM, Rapp JP. Complete genome searches for quantitative trait loci controlling blood pressure and related traits in four segregating populations derived from Dahl hypertensive rats. *Mamm Genome* 1999;10:259-265.

45. Deng AY. In search of hypertension genes in Dahl salt-sensitive rats. *J Hypertens* 1998;16:1707-1717.

46. Deng AY, Dene H, Rapp JP. Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. *J Clin Invest* 1994;94:431-436.

47. Deng AY, Rapp JP. Evaluation of the angiotensin II receptor AT1B gene as a candidate gene for blood pressure. *J Hypertens* 1994;12:1001-1006.

48. Deng AY. Functional genomics of blood pressure determination: dissecting and assembling a polygenic strait by experimental genetics. *Current Hypertension Reviews* 2005;1:35-50.

49. deBlois D, Orlov SN, Hamet P. Apoptosis in cardiovascular remodeling--effect of medication. *Cardiovasc Drugs Ther* 2001;15:539-545.

50. Schiffrin EL. State-of-the-Art lecture. Role of endothelin-1 in hypertension. *Hypertension* 1999;34:876-881.

51. Palijan A, lambert R, Sivo Z, Dutil J, Deng AY. Comprehensive congenic coverage revealing multiple BP QTLs on Dahl rat Chromosome 10. *Hypertension* 2003;42:515-522.

52. Deng AY, Dene H, Rapp JP. Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. *J Clin Invest* 1994;94:431-436.

53. Deng AY, Rapp JP. Evaluation of the angiotensin II receptor AT1B gene as a candidate gene for blood pressure. *J Hypertens* 1994;12:1001-1006.

54. Deng AY, Jackson CM, Hoebee B, Rapp JP. Mapping of rat chromosome 2 markers generated from chromosome-sorted DNA. *Mamm Genome* 1997;8:731-735.

55. Deng AY, Dene H, Rapp JP. Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension* 1997;30:199-202.

6.8 FIGURE LEGENDS, LEGENDS AND TABLES

Figure 1. **Mapping of a QTL for vascular SMCN independently of BP QTLs.** The linkage map is essentially the same as published previously [16,52-55], which is based on an F_2(S x MNS) population. Numbers to the left of the linkage map are units in cM. RH map refers to the map using rat/hamster radiation hybrids, in which units are in centiRays (cR). Numbers on the RH map are arbitrary units descending from the top of the chromosome. Solid bars under congenic strains symbolize the S chromosome fragments that have been replaced by that of the MNS rat. The entire region indicated by solid bars and junctions between the solid and open bars are homozygous for MNS, i.e. MM, on the map for all the markers listed in the corresponding positions. Open bars on ends of solid bars indicate the ambiguities of crossover breakpoints between markers. *Adh*, alcohol dehydrogenase; *Agtr1b*, angiotensin receptor type 1B; *Atp1a1*, Na^+K^+-ATPase α1; *Camk2d*, calmodulin-dependent protein kinase II-delta; *Fgg*, fibrinogen gamma; *Gca*, guanylyl cyclase A/atrial natriuretic peptide receptor; *Mme*, membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase); *Prlr*, prolactin receptor. D2Chm90 is a newly produced marker. Primers used are forward 5'-TTGGAGTTCATTAAGCAACACAG-3', and reverse, 5'-CCTTCTGGAAAAAGGTAAACCA-3'. The rest of the markers are anonymous. S, the Dahl salt-sensitive strain. Congenic strains were as follows: C2S.M, S.MNS-(D2Mit6-*Adh*)/Lt; C2S.M1, S.MNS-(D2Mit6-D2Rat166)/Lt; C2S.M2, S.MNS-(D2Mit6-D2Rat303)/Lt; and C2S.M6, S.MNS-(*Mme*-D2Rat131)/Lt. MAP refers to the averaged mean arterial pressure during the period of measurement for each strain [28,29]. ANOVA with the Dunnett's correction compares MAPs between S and each of the congenic strains. The placement of the QTL specifically for SMCN is indicated by a bracket. So are these for two BP C2QTL1 and BP C2QTL2.

Figure 2. **Comparisons of cardiac and renal parameters and cross sectional areas of aortas between the congenic and the S strains.** The number of rats for each strain in the comparisons is as follows: for (a), C2S.M (n=5), C2S.M1 (n=9), C2S.M2 (n=10), C2S.M6 (n=5), S (n=11); for (b) C2S.M (n=5), C2S.M1 (n=5), C2S.M2 (n=10), C2S.M6 (n=5), S (n=7); and for (c), C2S.M (n=5), C2S.M1 (n=8), C2S.M2 (n=8), C2S.M6 (n=4), S (n=10). HW, heart weight; LVW and RVW, left and right ventricular weight; KW, kidney weight; LKW and RKW, left and right kidney weight; BW, body weight; CSAs, cross sectional areas of aortas; mg, milligram; g, gram. HW, LVW, RVW, KW, LKW and RLW were corrected for BW. For congenic strain designations, see the legend for Figure 1.

Table 1: Comparisons in aortic smooth muscle cell numbers (SMCN) between the congenic and the S strains.

Strains	SMC number (SMCN)		
	n	(cells/μm)	*p*
C2S.M	5	58 ± 9	0.275
C2S.M1	8	170 ± 11	**<0.0001**
C2S.M2	8	134 ± 21	**0.039**
C2S.M6	4	71 ± 4	0.775
S	10	90 ± 4	

Footnotes to Table 1: *p*, ANOVA comparing the S with a congenic strains with Dunnett corrections; SEM, ± standard error of the mean. SMC, smooth muscle cells; n, number of rats. For congenic strain designations, see the legend for Figure 1. Rats for each strain were taken from multiple litters, and any potential litter effect among litters of the same strain were found not to be significant.

Table 2: Comparisons in cardiac and vascular parameters between congenic and S strains.

Strains n	Aortic	Cardiac			
		Left Ventricle		Right Ventricle	
	DNA Frag. Index (arb units/pixel/ µg DNA)	DNA content (µg/g)	DNA Frag Index (arb units/ pixel/µg DNA)	DNA content (µg/g)	DNA Frag Index (arb units/ pixel/µg DNA)
C2S.M 5	36 ± 9	3.0 ±0.2	83 ± 10	1.13 ± 0.05	83 ± 10
p	***0.0015***	***0.005***	*0.075*	*0.114*	***<0.0001***
C2S.M1 4	0.23 ± 0.06	5.0 ± 0.5	6 ± 5	0.50 ± 0.09	3 ± 1
p	*0.291*	*0.833*	*0.075*	*0.114*	*0.878*
C2S.M2 5	6 ± 2	5.0±0.5	8 ± 1	1.2 ± 0.09	7 ± 2
p	*0.784*	*0.331*	*0.075*	*0.114*	*0.999*
C2S.M6 5	6 ± 1	3.7 ± 0.3	249 ± 170	1.1 ± 0.2	4 ± 1
p	*0.711*	***0.045***	*0.075*	*0.114*	*0.909*
S 11	8 ± 5	5.8 ± 0.6	16 ± 7	1.1± 0.1	8± 3

Footnotes to Table 2: *p*, ANOVA comparing the S with a congenic strains with Dunnett corrections, which take into account multiple group comparisons and differences in sample sizes; SEM, ± standard error of the mean; DNA content in the aorta is calculated as microgram (µg) of DNA per millimeter (mm) of aorta corrected for BW (g). DNA content in the ventricle is calculated as µg of DNA corrected for BW. DNA fragmentation (Frag) is calculated in arbitrary units per pixel unit area per µg of DNA. For detail, see the 'Method' section. For congenic strain designations, see the legend for Figure 1. *P* values for the DNA fragmentation index of the left ventricle and for the

DNA content of the right ventricle were given uniformly among the strains because ANOVA was not significant among the strains. Consequently, no further analysis was necessary.

Table 3: Comparisons in contractile responses to KCl, angiotensin II and phenylephrine between congenic strain C2S.M2 and S strain.

	n	KCl	Phenylephrine		Angiotensin II	
		(g)	Emax (%KCl)	log EC_{50}	Emax (%KCl)	log EC_{50}
Control						
S	12	1.59 ± 0.11	104.06 ± 4.70	7.26 ± 0.06	34.85 ± 2.94	8.22 ± 0.26
C2S.M2	13	1.62 ± 0.09	100.16 ± 5.63	6.96 ± 0.17	**19.33 ± 3.67** **	7.86 ± 0.27
L-NAME (100 umol/L)						
S	12	1.39 ± 0.06	141.72 ± 7.73	7.60 ± 0.05	50.61 ± 5.29	7.92 ± 0.36
C2S.M2	13	1.60 ± 0.12	143.03 ± 6.62	7.41 ± 0.16	**26.63 ± 4.00** †	7.20 ± 0.31

Footnotes to Table 3: Paired sets of aortic rings with endothelium were stimulated in the presence or absence of L-NAME. For each strain, data from rats implanted (n=6/strain) or not implanted (n=6-7/strain) with a telemetry probe were not significantly different (data not shown), and were pooled. Results are expressed as mean ± SEM. ** $p<0.01$ vs corresponding S group; † $p<0.001$ vs corresponding S group by unpaired Student *t*-test.

Figure 1

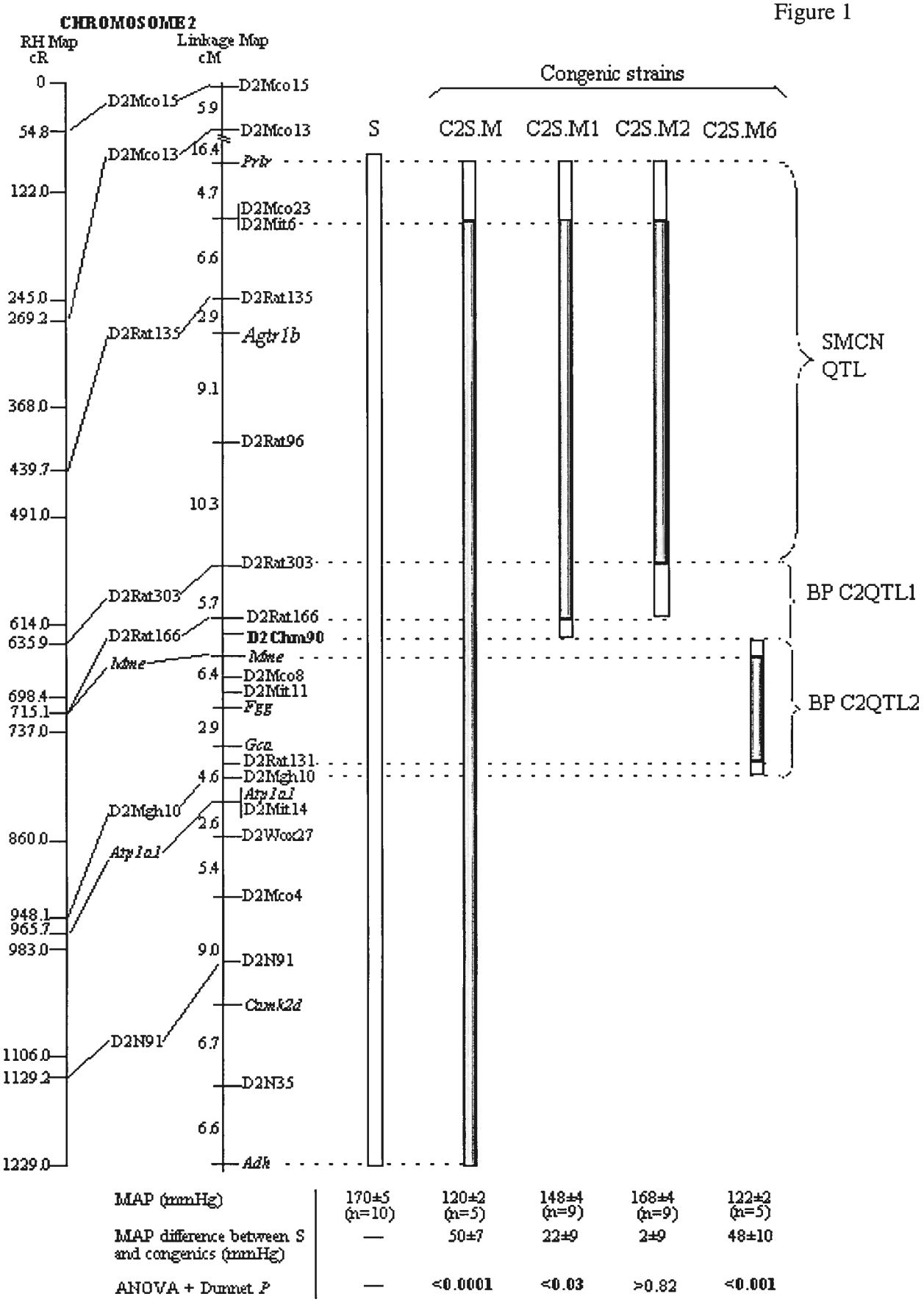
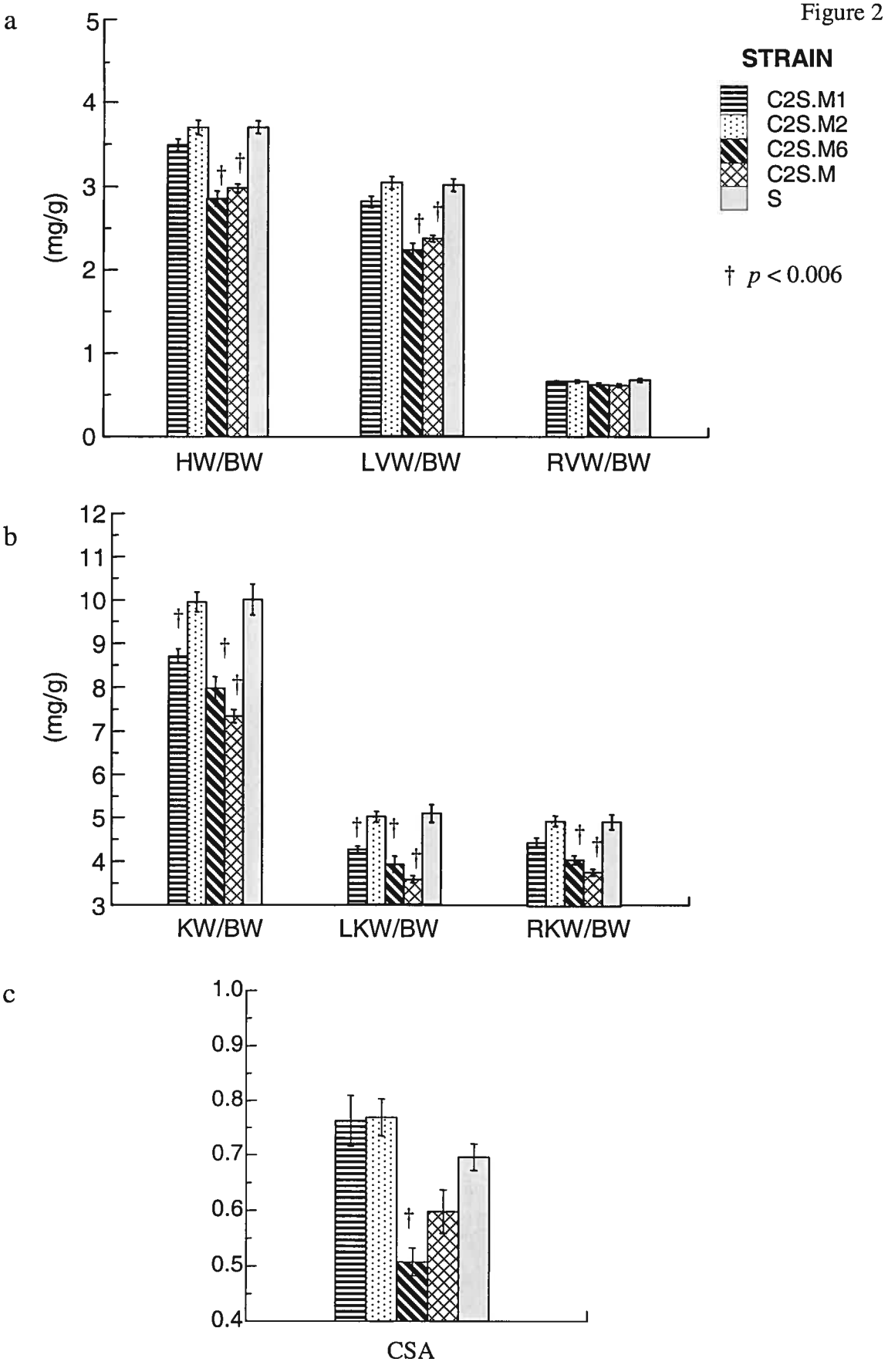

CHROMOSOME 2
RH Map
cR
Linkage Map
cM
Congenic strains
S
C2S.M
C2S.M1
C2S.M2
C2S.M6
SMCN QTL
BP C2QTL1
BP C2QTL2
0
D2Mco15
54.8
122.0
245.0
269.2
368.0
439.7
491.0
614.0
635.9
698.4
715.1
737.0
860.0
948.1
965.7
983.0
1106.0
1129.2
1229.0
D2Mco13
D2Rat135
D2Rat303
D2Rat166
Idme
D2Mgh10
Atp1a1
D2N91
5.9
16.4
4.7
6.6
2.9
9.1
10.3
5.7
6.4
2.9
4.6
2.6
5.4
9.0
6.7
6.6
Prlr
D2Mco23
D2Mit6
Agtr1b
D2Rat96
D2Rat303
D2Rat166
D2Chm90
Idme
D2Mco8
D2Mit11
Fgg
Gca
D2Rat131
D2Mgh10
Atp1a1
D2Mit14
D2Wox27
D2Mco4
D2N91
Chmk2d
D2N35
Adk
MAP (mmHg)
170±5 (n=10)
120±2 (n=5)
148±4 (n=9)
168±4 (n=9)
122±2 (n=5)
MAP difference between S and congenics (mmHg)
—
50±7
22±9
2±9
48±10
ANOVA + Dunnet P
—
<0.0001
<0.03
>0.82
<0.001

Figure 2


a
5
4
3
2
1
0
(mg/g)
HW/BW
LVW/BW
RVW/BW
STRAIN
C2S.M1
C2S.M2
C2S.M6
C2S.M
S
† $p < 0.006$
b
12
11
10
9
8
7
6
5
4
3
(mg/g)
KW/BW
LKW/BW
RKW/BW
c
1.0
0.9
0.8
0.7
0.6
0.5
0.4
CSA

CHAPTER 7

DISCUSSION

7.1 Effect of the chr. 2 congenic strains on heart rate

In chapter 4, we showed that the congenic strains S.M, S.M5 and S.M6, all of which having a BP lowering effect, had no effect on heart rate (HR) when averaged at 24 hours. From these observations, we concluded that blood pressure and heart rate are controlled independently by different genes. However, some issues regarding the effect of these congenic strains on HR remain to be discussed.

7.1.1 Circadian rhythms in HR and BP

It has been well described that mammals are submitted to an internal clock that is responsible for controlling circadian rhythms of behavior, physiology and metabolism, including 24hr variations in BP and HR. [206] In the transgenic rat TGR(mREN2)27, an additional copy of the mouse renin gene leads to overactivity of the renin-angiotensin system.[207] The TGR(mREN2)27 is characterized by hypertension, hypertensive end-organ damage and inverse circadian BP patterns [207]. Consequently, one can wonder if the HR effect in S.M, S.M5 and S.M6 may be masked by displaced circadian rhythms? Upon closer examination of the HR curves within a 24 hour period, we noticed a lowering of the heart rate (HR) in both S.M and S.M6, when compared to the DSS rat. However, the timing circadian pattern did not deviate from what we expected, reaching the highest values during the nighttime.

It has been previously shown that in the DSS rat fed a high salt diet, there is an increase in the amplitude of the circadian BP variations, but no shift in the timing of the BP peak is observed. [208] Similarly, when the TGR(mREN2)27 is submitted to salt loading, the amplitude of the circadian MAP fluctuation is increased, without affecting the circadian timing of the peak MAP. [209] In our congenic strains, despite a normal pattern of circadian variation, we cannot exclude the possibility that the amplitude of the circadian variations may hinder the statistical revelation of a significant difference between the

HR in S.M and S.M6 when compared to DSS. For a more definitive analysis of the HR, it would be interesting to: 1) increase statistical power by measuring more animals, 2) compare the congenic strains to the DSS at specific time points during the days and night rather than averaging data for 24 hours.

7.1.2 Localization of a HR QTL

If S.M and S.M6 congenic strains have in fact a lower HR than DSS, where would the HR QTL map? Both S.M5 and S.M6 are congenic substrains derived from the original congenic strain, namely S.M. From S.M, a first crossover event in the region between D2Rat166 and *Nep* resulted in the creation of a first substrain (data not shown). Subsequently, crossovers between D2Mit14 and D2Wox37 and between *Gca* and D2Mgh10 resulted in S.M5 and S.M6, respectively. From the familial history of the strains, we can conclude that the ambiguous regions in S.M5 and S.M6 are identical. Hence, the MM region in S.M6 is entirely encompassed by the MM region of S.M5. For this reason, the genetic determinants that are responsible for the HR lowering in S.M and S.M6, but not in S.M5, could not be located in the region between D2Rat166 and *Nep*.

Given that the MM region in S.M5 encompasses completely the MM region of S.M6, how could we explain the presence of a HR effect in S.M and S.M6, but not in S.M5? The study of epistatic interactions may provide an explanation for this apparent discrepancy. In chr. 1 and chr. 10 congenic strains carrying SHR alleles on a WKY background, it was showed that only the chr. 10 congenic strain had a significant increase in BP. [210] When taken individually, the SHR chr. 1 congenic strain showed a slight increase in BP that was not sufficient to reach statistical significance. [210] However, in a double congenic strain combing the chr. 1 and chr. 10 SHR regions, the BP effect was higher that the sum of the chr. 1 and chr. 10 QTLs when taken separately. [210] In this case, the full expression of the BP effect was conditional to the presence of SHR alleles on both, chr. 1 and chr. 10. The HR effect observed in S.M6, but not in S.M5, may also be explained by epistatic relationships between QTLs. In effect, it is possible that the combination of genetic factors present in S.M5 is not favorable to the

expression of a HR QTL, whereas the genetic environment is more favorable in S.M6. This issue could be resolved by the construction of congenic substrains in which the MM region is smaller and therefore less likely to contain more than one genetic factor influencing the cardiovascular system.

7.2 QTL-QTL interactions

The use of congenic strains have revealed the presence of BP QTLs on Chr. 1, 3, 5, 7, 8, 9, 10, 12, 13, 15, 16, 17, and 18 of the Dahl rat [211]. Given the number of QTL identified in the Dahl rats, it will be interesting to investigate how these QTLs relate to one another, how they interact. By definition, an additive relationship between two QTL occurs when the sum of the BP effects of each QTL taken individually is equal the BP effect of a congenic strain containing both QTL. In contrast, an epistatic relationship occurs when the individual QTL BP effect are not equal to that of a congenic containing both QTLs. To further describe the relationships between the different BP QTL, an approach was proposed to assemble the QTL without knowing the physiological role that each gene plays. [211] Using this strategy, one can hypothesize that the QTLs demonstrating an additive relationship might be part of different physiological pathways, whereas the QTLs that interact epistatically might be involved in the same physiological pathway or cascade.

In Chapter 5, we have shown that the Dahl rat Chr. 2 contains at least 3 BP QTL, and that C2QTL1 is epistatic to either C2QTL2 or C2QTL3. At this point, we were not able to determine if C2QTL1 was epistatic to C2QTL2 or to C2QTL3. In addition, the C2S.M strain encompassing C2QTL1, C2QTL2 and C2QTL3 also includes regions that were not tested individually for the presence of QTL (region between D2Chm225 and D2Mco8, and the segment between D2Chm57 and D2Wox27). It is possible that these regions hide more BP QTLs.

To resolve these issues, we propose to construct double congenics. As mentioned in chapter 5, the construction of double congenic strains for rat chr. 2 requires a recombination event between two closely linked loci. This concept can be illustrated

with the following example: the production of a double congenic strain for C2QTL1 and C2QTL3. The first step to obtain such a strain is to cross the C2QTL1 congenic (C2S.M7) to the C2QTL3 congenic (C2S.M7). The progeny obtained, F_1(C2S.M7XC2S.M11), will be heterozygous at the C2QTL1 locus and at the C2QTL3 locus. (Figure 6a). Figure 6b illustrates the outcomes of the meiosis of F_1(C2S.M7XC2S.M11) if no crossover event occurs between the C2QTL1 locus and the C2QTL3 locus. In this case, the gametes obtained carry either the MM allele for C2QTL1 with the SS allele for C2QTL3 or the SS allele for at QTL1 with the MM allele at C2QTL3. The progeny obtained from such gametes will bear the MM allele at C2QTL1 or at C2QTL3. In contrast, if a cross over event occurs between C2QTL1 and C2QTL3 (figure 6c) loci, 25% of the gametes obtained will bear the MM alleles at both loci. Brother sister mating of rats obtained from these gametes will result in a rat homozygous MM at C2QTL1 and C2QTL3 loci. The frequency with which recombination occurs between two given loci is dependent on the distance separating those loci, but also on the intrinsic properties of the sequence. [114] So far, we have not been able to obtain crossovers between C2QTL1 and C2QTL3 or between C2QTL3 and C2QTL2.

In this context, one could believe that it would be more practical to compare directly F_1(C2S.M7XC2S.M11), which is heterozygous at C2QTL1 and at C2QTL3 loci, to heterozygotes for C2QTL1 or C2QTL3 only. However, the study of heterozygotes is complicated by the presence of dominant and recessive alleles. For example, the BP of C2S.M7 rats heterozygous at the C2QTL1 locus is not significantly different from that of C2S.M7 rats that are homozygous MM at the C2QTL1 locus (data not shown). This implies that for the C2QTL1 locus, the MNS allele is dominant over the DSS allele. In effect one copy of the MNS allele at C2QTL1 is sufficient for the heterozygote to display the same BP effect as the homozygote. The opposite situation is observed for C2S.M11 heterozygous rats which have the same BP as DSS. For this reason, heterozygous congenic strains may not be suitable for the study of QTL-QTL interactions.

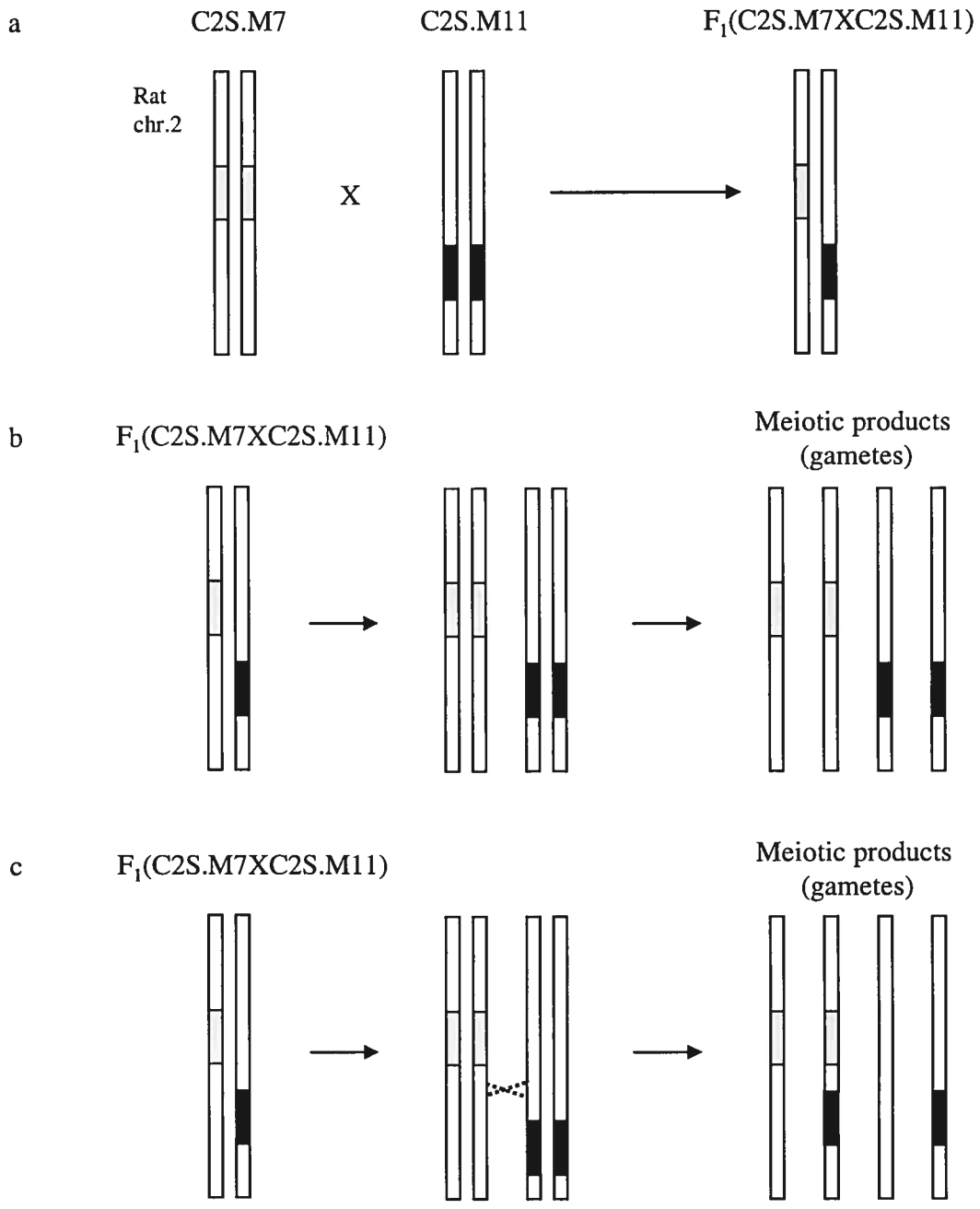


Figure 6 Construction of double congenic strains for rat chr. 2. Grey and black bars indicate the region coming from the MNS strain in the C2QTL1 and C2QTL3 congenic strains, respectively. White bars represent the DSS genetic background. a- Breeding of C2S.M7 and C2S.M11 congenic strains. b- and c- Generation gametes from the meiotic division in F1(C2S.M7 X C2S.M11) rats when no recombination occurs between C2QTL1 and C2QTL3 loci (b) or when a recombination event occurs between C2QTL1 and C2QTL3 loci (c). Refer to text for detailed expalnations.

7.3 QTL for SMC number implicating the AT1B gene as a candidate

7.3.1 Model for regulation of SMC number independently of BP

In chapter 6, we reported that the congenic strain C2S.M2 had an significant increase in SMC number in the thoracic aorta when compared to the DSS rat. This increase in SMC number was accompanied by a slightly higher but non-significant increase in the cross sectional area of the aorta. It is of importance that we didn't observe any BP effect in C2S.M2, despite the increase in SMC number. Several hypotheses can be emitted to explain the absence of a relationship between hyperplasia and BP in the C2S.M2 congenic rat.

First, the increase in peripheral resistance that has been associated with essential hypertension is explained mainly by the remodeling of small arteries and arterioles. [48] At this point, we cannot extrapolate about the structural reorganization of the resistance arteries, if any. It is possible that the hyperplasia is restricted to the large arteries. In this case, no increase in peripheral resistance is expected. It would be interesting to study the structural characteristics of the resistance arteries as well.

Second, in large arteries such as the aorta, remodeling is characterized by outward hypertrophy of SMC that results in a thicker wall without a reduction in the size of the lumen. [49] It is possible that the increase in SMC number is compensated by an increase in the lumen of the artery. The measurement of the SMC number and of the CSA of the artery give information about the wall thickness only. At this time, we have no information about the media:lumen ratio of C2S.M2 and DSS aortas. As hypertension progresses, the outward hypertrophy will be accompanied with an increase in arterial stiffness that is mainly due to an increase in collagen content. [212] From hemodynamic studies, BP is known to be composed of a steady component (MAP) and a pulsatile component, the pulse pressure (PP). Pulse pressure is determined by the stiffness of the large arteries and the wave reflexion, whereas MAP is influenced by the resistance vessels. [213] If the hyperplasia in the aorta of the C2S.M2 strain were accompanied with an increase in the rigidity, we would observe a difference in the PP which is determined

by the response of the vessel to ventricular ejection. Because of its pulsatile nature, PP varies along the arterial tree, which makes its measurement difficult.

Finally, the contraction state of the vessel is not only influenced by its structural characteristics but it also depends on its the contractile properties. It is possible that the structural changes we observe in the aorta of C2S.M2 are compensated for by changes in contractile properties of the SMC.

7.3.2 Agtr1b as a candidate gene for SMC number QTL

In looking at the molecular mechanisms responsible for the increase in SMC number in S.M2, *Agtr1b* was proposed as a candidate gene based on its localisation in the QTL region. This candidate is validated by the presence of two nucleotide substitutions resulting in significant amino acid changes in the MNS sequence when compared to that of DSS. Furthermore, the contractile responses to Ang II were significantly reduced in C2S.M2 when compared to DSS. Given that C2S.M2 had a higher SMC number than DSS, one could expect an increase in contractility in that vessel rather than the decrease we observed.

In chapter 6, we proposed that the decrease in contractility observed in aortic rings from C2S.M2 may be a result of prior receptor desensitization *in vivo*. It is well recognized that G-protein-coupled receptors (GPRs) undergo agonist-induced desensitization as a mechanism to turn off the signal resulting from ligand-receptor interaction. [214] The mechanisms for desensitization of the family of GPRs involve three levels of inactivation: 1) rapid inactivation following agonist-induced phosphorylation due to the uncoupling of the G protein from the receptor, 2) internalization of the receptor and, 3) targeted degradation of the internalized receptor by lysosomes. [214] Even though lysosomal degradation has been demonstrated in some cases, it is more frequent for the receptor to be recycled at the cell surface. [214] GPRs can be phosphorylated by the GPR protein kinases (GPKs) and by the second messenger-activated kinases such as PKC and PKA.[215] In bovine adrenal glomerulosa cells, it was demonstrated that binding of Ang II to $AT1_A$ receptor induces the phosphorylation of the receptor. [215] Specifically, agonist-

induced desensitisation of $AT1_A$ involved a serine/threonine rich segment of the carboxy terminal intracellular region of the protein. [216]

7.3.3 Further implication of *Agtr1b* as a candidate gene for the SMC number QTL

To further implicate Agtr1b in the regulation of SMC number in S.M2, we proposed the congenic strategy as a way to progressively narrow down the region of interest around *Agtr1b*. An alternative genetic approach to validate *Agtr1b* as a candidate gene for the SMC number QTL would be to generate a transgenic strain by inserting the normal copy of *Agtr1b* (from DSS rat) in the C2S.M2 strain. If *Agtr1b* is responsible for the SMC number phenotype, we would expect the transgenic strain to have the same SMC number as DSS.

Molecular approaches are also available for confirming the role of the *Agtr1b* mutations on the receptor function. By comparing cultured SMC from DSS and from C2S.M2, one could study the effect of the mutations on the receptor affinity for Ang II, and on the activation of downstream signal transduction pathways. For example, it would be interesting to compare the activity of PLC, PLD and PLA_2, which are known target of the activation of $AT1_B$ by Ang II. [217] Since the amino substitutions we identified are at position 2 and 20 of the protein, they are located in the extracellular amino terminal domain of the protein. [217] It is also possible that changes in this position affect the interaction between Ang II and the $AT1_B$ receptor.

7.4 The next step: from a region to a gene

The congenic strategy described in the current work has allowed us to narrow down the regions of interest for the chromosome 2 BP QTL to 3 non-overlapping regions: C2QTL1, C2QTL2, and C2QTL3. The next question one can ask is: what are the genes in these regions of interest that are responsible for the blood pressure differences observed between the congenic and the parental strain?

7.4.1 Identification of functional and positional candidates

Identification of the gene responsible for the BP effect of the congenic is the next task to achieve. This step will be facilitated by the bioinformatic tools available at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) which is a gate to access genomic data generated by a worldwide effort to sequence the genomes of human and model organisms. From the list of genes generated, two types of candidate genes are obtained : functional and positional candidates. Functional candidates are genes that are already known to be involved in the cardiovascular system. For example, the QTL2 region includes the gene coding for the atrial natriuretic peptide receptor A (*Gca*) and the QTL3 region includes the gene coding for neutral endopeptidase (*Mme*). Positional candidates are candidates based on their position in the genome, making no assumption on their function.

7.4.2 Identification of sequence polymorphisms

For a given functional or positional candidate gene to be involved in BP regulation, the hypertensive (DSS) and normotensive control strain (MNS) could differ in their coding sequences or in their expression levels. The angiotensin receptor gene *Agtr1b* is a candidate genes for the QTL for vascular SMC number on rat chr 2. Sequencing of the coding region of the gene has revealed non-synonymous mutations between the DSS and the MNS rats. Identification of the genes responsible for the BP effects observed in C2QTL1, C2QTL2 and C2QTL3, will require us to undertake systematic sequence and expression level comparisons between DSS and the MNS rats for all the functional and positional candidate genes located in the QTL region. A variation between those contrasting strains could indicate a gene responsible for the BP variations.

7.4.3 Demonstrating the causative link between a variant and the BP effect

Identification of a functional or positional gene variant is necessary but not sufficient to provide a definitive evidence of the contribution of a gene to the genetic basis of hypertension. During the selection process of genetically hypertensive and normotensive rats, a series of differences unrelated to the blood pressure phenotype were also selected and fixed. The end result is that two contrasting strains of rat might

exhibit polymorphisms at loci involved in BP control and at loci unrelated to BP control. In this case, how can we differentiate between the genetic variations involved in BP regulation and the polymorphisms randomly selected and fixed along the way? The burden of proof is to demonstrate that the variations in the candidate gene are actually the cause of the BP effect.

The preferred approach to solve this issue will depend on the type of variation identified. A polymorphism affecting the expression levels of a gene could be resolved by transgenic rescue experiments. For example, if a candidate gene was found to be downregulated in the hypertensive strain when compared to the normotensive control, inserting additional copies in the hypertensive rat by mean of transgenic technology would be expected to have a BP lowering effect. In cases where the regulatory regions allowing a temporal and regional regulation of the gene expression are not known, bacterial artificial chr (BAC) constructs can be used as a transgene. Due to their large size, they offer the advantage of protecting the gene of interest from positional effect of the region surrounding the insertion site and ensure that the cis regulatory elements are also included in the transgene. Interestingly, a variation of the classical transgenic strategy, where the transgene is overexpressed, can be used to silence a gene of interest. A transgenic rat line expressing an antisense RNA targeted against the 5' region of the angiotensinogen mRNA resulted in an animal deficient in brain angiotensinogen. [218] This approach may be especially beneficial for the rat model since the knock out widely used in the mouse is not yet available in the rat.

7.5 Identification of causative genes for complex trait: is it possible?

There are examples in the literature where positional identification of genes responsible for complex diseases was achieved using the classical positional cloning approach. Rheumatoid arthritis is a complex trait for which linkage analysis had demonstrated the presence of a major QTL on rat chromosome 12. Fine mapping using congenic strains has allowed researchers to reduce the region of the QTL to a 300 Kb interval containing only two genes. Sequence analysis of the two positional candidates revealed several polymorphisms in the coding sequence of the gene *Ncf1*. This gene codes for a

component of the NADPH oxidase complex. Differences in enzyme activity associated with that polymorphism were associated severe destructive arthritis. [219] A susceptibility gene for rat type 1 diabetes mellitus was identified in the Komeda diabetes-prone (KDP) rat using a similar positional cloning approach. In this case, the region was narrowed to a 3.1cM region on rat chr. 11 containing two genes. One of the genes did not show sequence or expression levels variations in the KDP rat. The other gene, *Cblb*, contained a non-sense mutation changing an arginine to a stop codon and leading to a protein truncated by 448 amino acids. Transgenic rescue with a wild type *Cblb* was sufficient to rescue the KDP phenotype.[220] Hence, the use of a traditional positional cloning strategy can lead to the identification of single genes involved in the genetic determination of complex traits.

7.6 Alternative strategies to identify genes for complex traits

7.6.1 DNA Microarrays

The current work has demonstrated how powerful the congenic strategy is to break down the complex traits and refine the position of the regions of interest to regions reaching less than 2 cM. However, the limit of the congenic approach to reduce the region of interest using substrains is the distance between the two closest recombination events. [124] Depending on the gene density of the region of interest, even a very small QTL region may contains an important number of genes. For example, we estimated that BP QTL3, which is less than 1.5 cM, corresponds to 9 Mb, a region containing roughly 80 known and predicted genes according to the national center for biotechnology information (NCBI) (unpublished results). From this observation, one can ask whether the strategy we use is capable of narrowing the candidate to a single gene, or whether we need complementary approaches to achieve this task.

Successful positional cloning of the *Cd36* gene for insulin resistance combined the congenic and the microarrays technologies. The search for the genetic basis to insulin resistance started with the identification of a highly significant linkage peak for glucose and fatty acid metabolism, hypertriglyceridaemia, and hypertension close to the

telomere of rat chr 4 in F2 populations issued from a SHR X WKY cross and from a SHR X BN cross. [221] Subsequently, the QTL was confirmed by congenic strains in which part of the SHR chr. 4 region were replaced by the homologous region from the BN. [222] The molecular identification of the gene responsible for the difference in insulin dependence between the congenic and the SHR rat was achieved with the use of DNA microarrays rather than with a traditional positional cloning approach. Three clones encoding rat *Cd36* showed reduced hybridization signals in the SHR when compared to BN control and mapped to the region of interest on rat chr. 4. [223] The transgenic expression of *Cd36* in the SHR ameliorates insulin resistance and lowers serum fatty acid. [224]

The successful identification of *Cd36* as a candidate gene for insulin resistance lead the way to a new strategy of positional cloning. Since the congenic approach may not be sufficient to reasonably narrow down the number of candidates to study, the microarray technology may be a powerful complement to identify the genes of interest. Some of the pitfalls that have been associated with large scale analysis of expression patterns is the number of candidates obtained and the quantity of data generated. In effect, when comparing cDNA expression levels in the BN and the SHR, it is expected that 1% of the genes tested will be differentially expressed, leading to hundreds of genes to further study. [225] Some of these differentially expressed genes may be responsible for physiological differences between the strains that are not relevant to BP control. A congenic strain is genetically identical to the recipient strain except in the region coming from the donor strain. Combining congenic strain mapping to microarray technology allows one to limit the differentially expressed candidates to those that are located in the region of interest of the congenic. The success of this strategy is however highly dependent on the choice of the organ to study and the point in time at which the comparison is made. [225]

7.6.2 Importance of intermediate phenotypes

The construction of a map for cardiovascular function using more than 239 cardiovascular and renal intermediate phenotypes has highlighted the possibilities of

such an approach to study complex traits. [226] Linkage and association studies would gain statistical power by using intermediate phenotypes rather than looking at the blood pressure only. Since hypertension shows a complex and diverse pathophysiology, it is possible that populations studied are composed of an admixture of several sub-types of hypertension involving different causative genes. In this case, using intermediate phenotyping and classifying the population according to the type of hypertension they have may help to group individuals likely to have the same genetic defect and gain statistical power. The lack of detailed phenotyping may explain the highly contradictory results obtained from the numerous association and linkage studies aiming at assessing candidate genes.

7.7 Clustering of functionally related QTL

In the current work, we have shown that the original region of interest on rat chr. 2 contained at least 3 separate blood pressure QTLs in addition to a QTL for smooth muscle cell number. Using a similar fine mapping strategy, we have shown that the rat chromosome 10 contained at least 3 BP QTLs. [167,227] Therefore, it seems that functionally related QTLs have the tendency to cluster in certain chromosomal regions. The murine systemic lupus erythematosus (SLE) susceptibility locus is an example of functionally related genes cluster. [228] It was demonstrated that the genes *sle1a*, *sle1b* and *sle1c* contained within a 7 cM segment of mouse chr. 1 can each independently cause a loss of tolerance to nuclear antigen all contributing distinctly to the SLE pathogenesis. [228] It was concluded that they are part of a functionally related cluster of genes. [228] A similar phenomenon was described for hypertension. In effect, a genome wide scan of 239 cardiovascular and renal phenotypes in a F2 (BN X DSS) population revealed the presence of aggregates composed of 6 or more QTL on chr 1, 2, 3 and 18 of the rat. [226] From these finding, we can predict that further systemic coverage and fine mapping of the chr. 2 regions of interest may lead to the discovery of additional cardiovascular related QTLs.

The biological significance of the clustering of functionally related traits remain to be investigated. It was proposed that clustering may be the result of functional and

evolutionary pressures since clustered loci tend to belong to the same functional pathway such as it is the case for the loss of tolerance to nuclear antigen for the *Sle1* cluster. [226] The chr. 2 region of interest studied in this thesis presents an additional evidence in favour of this hypothesis since the genes coding for several components of the atrial natriuretic peptide pathway are located in adjacent chr. 2 regions. These functionally related loci include the gene coding for the receptor for the atrial natriuretic peptide (*Gca*), and the gene coding for the metallo-endopeptidase (*Mme*).

7.7.1 Implications of gene clustering and interactions on QTL detection

Even if the biological signification of the clustering of functionally related genes remains hypothetic, this observation has important consequences on experimental design for future genetic studies. Knowing that certain regions of the genome are more concentrated in genes involved in cardiovascular regulation, it is important to proceed to fine mapping of the QTLs. In effect, the current work shows that so far, the original region of interest contained more that one QTL for blood pressure and it is not impossible that further dissection of the C2QTL1, C2QTL2 and C2QTL3 regions lead to identification of additional QTLs. We have demonstrated that the C2QTL1 is epistatic to either C2QTL2 or C2QTL3. Such an epistatic interaction imply that comparing the magnitude of the blood pressure lowering in congenic strains is not reliable for evaluating the number of QTL in a given region. In effect, in this particular case, the BP lowering effect of C2QTL1 was hidden by that of C2QTL2 or C2QTL3. Furthermore, since QTLs involved in the same pathways have been shown to cluster [228] and given that we propose that the mechanistic behind epistatic interaction involves genes in the same biological pathways, it is highly probable that clusters of genes show epistatic interaction. In this regard, it is essential to proceed with the fine mapping work even if the region identified contains interesting candidate genes. Fine mapping should be performed to narrow down the region of interest to the maximum possible given the limitation of the recombination events between two close loci.

7.8 The animal model : background effect

7.8.1.1 Residual background

From a theoretical point of view, after the eight backcrosses required for the construction of a congenic strain, more than 99% of its genetic background is that of the recipient strain. [123] The possibility remains that the blood pressure effect observed in the congenic strains could be explained by residual donor genotype rather than by the region studied. In the original publication of the chr. 2 congenic strains, 2 different strains were obtained from independent breeding : S.WKY-D2N35/Nep and S.MNS-Adh/D2Mit5, both of which showing a significantly lower blood pressure than the DSS rat. [229] Therefore, it is unlikely that the blood pressure lowering the congenic strains discussed in the present work is explained by the presence of residual background since 2 independently derived congenic strain showed the same effect. Because the position of residual background is determined by chance only, it is highly unlikely that the same region would be obtained in 2 independent crosses.

7.8.1.2 Background of the recipient strain

The effect of the genetic background on expression of blood pressure controlling alleles has been well illustrated in a study of the effect of specific renin alleles on BP.[230] Cosegregation of blood pressure with the RFLP in intron 1 of the renin gene was evaluated in different populations issued from DSS X DSR crosses where the amount of genetic background coming from the DSR rat varied. A positive association between the renin alleles and blood pressure was observed in the F2 (DSS X DSR) and in F1 (DSS X DSR) X DSS cross but not in the F1 (DSS X DSR) X DSR population. It was concluded that the relationship of genes to blood pressure was highly dependent on the genetic background since the F1 population backcrossed to DSR did not show the association, thus indicating that having too much background from DSR prevented the blood pressure effects of the renin alleles. [178] The choice of the normotensive contrasting strain also influences the expression of the high blood pressure alleles.

Evaluation of the S_A gene in F2 populations showed a positive correlation with blood pressure in the F2 (Lew X DSS) cross but not in a F2 (WKY X DSS) cross. [178]

7.9 Relevance to other rat models of hypertension

Since the different genetic models of hypertension in the rat were bred separately, we can expect that the pathophysiology of the disease differs in each model. For example, the DSS rat is derived from outbred Sprague-Dawley rats but the genetic basis of this model depends on the alleles present in the few individuals from which the line was established. Therefore, it is likely that the DSS model of hypertension has retained only a proportion of the genes involved in the genetic determination of blood pressure in outbred stocks. Animal models of hypertension therefore present a reductionist experimental approach. From these observations, one can wonder how can the results obtained for the DSS rat be generalized to other rat models of hypertension?

Several groups of investigators have been interested in the mapping of the chromosome 2 QTLs and some groups were using different rat models. The figure 7 compares BP QTLs on rat chr. 2 identified by other groups of investigators to the regions presented in this work. [231] The regions we have identified correlate with those of other studies involving different crosses. [226,232-237] This indicates that our QTLs are not unique to the Dahl rats and that the potential genes responsible for the BP effect in C2QTL1, C2QTL2 and C2QTL3 are likely to be relevant to other genetic rat models of hypertension.

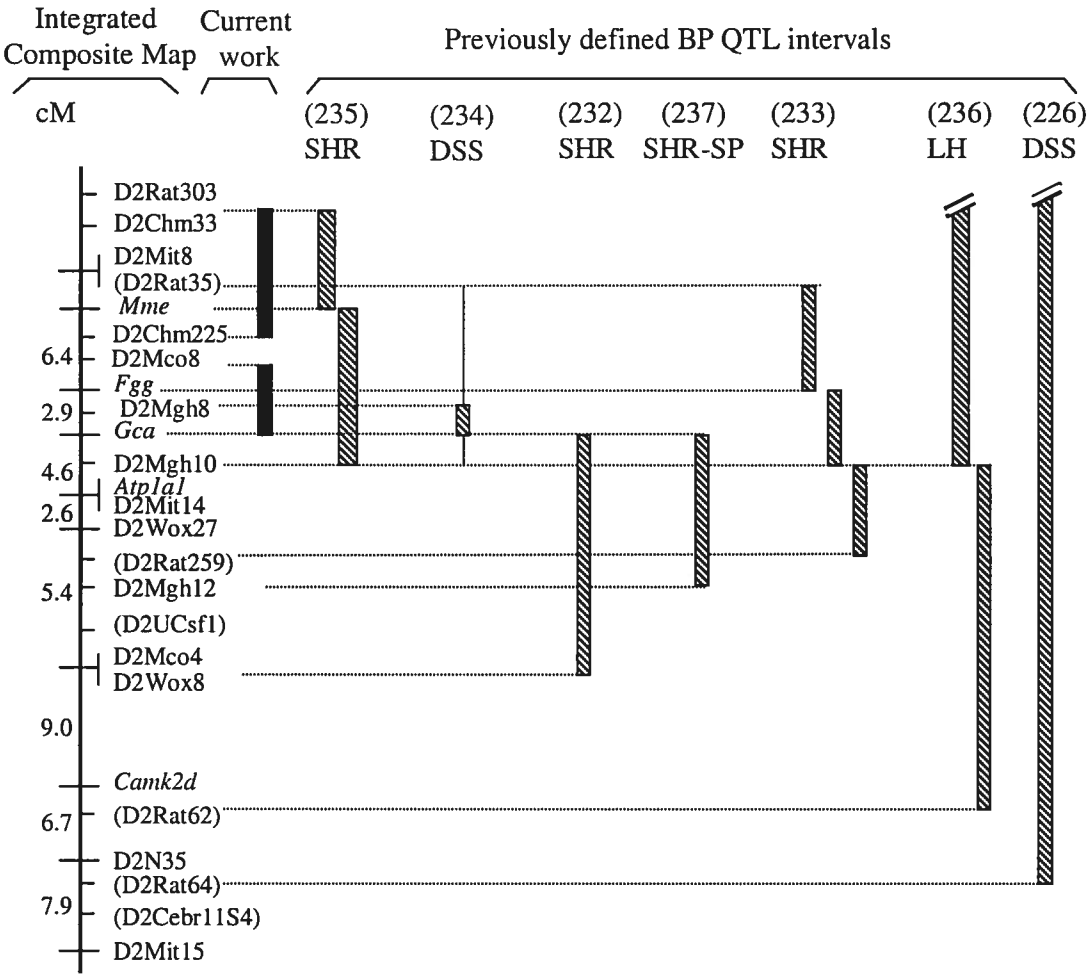


Figure 7- Comparison of the chr 2 BP QTL. Adapted from Eliopoulos *et al.* [231] The current work is represented by the black rectangles. The striped rectangles indicate the positions of QTLs identified by other groups. Numbers in parenthesis refer to the references cited. DSS Dahl salt-sensitive, LH Lyon hypertensive, SHR spontaneously hypertensive, SHR-SP stroke prone spontaneously hypertensive

Even if rat genetic models of hypertension present a simplified form of the human essential hypertension etiology, study of the DSS pathophysiology (1.3.2.2.2) clearly indicates that several systems are altered to contribute to the high BP phenotype. Therefore, simultaneous studies of the several rat models may be the solution to ensure a complete coverage of the genetic components accounting for the more heterogeneous human essential hypertension.

7.10 Relevance human hypertension

Comparative mapping using the information available on the National Center for Biotechnology Information (NCBI) has allowed us to determine that the chromosome 2 regions of interest in the rat are homologous to segments of chr 13, 3 and 4 in human Within each synthenic segment, gene order is conserved between rat and human. In the human genes present in these regions of interest, more than a thousand single nucleotide polymorphisms (SNPs) have been inventoried in the coding and regulatory regions (unpublished observations). These results indicate that the human homologues of the rat genes in the regions of interest show inter-individual variability potentially important for blood regulation.

7.11 Perspectives

7.11.1 Basic sciences

Identification of the genes involved in hypertension is promising from a clinical point of view but also to gain a better understanding of the normal BP regulatory systems and of the pathophysiology of hypertension. Using a global positional approach rather than a candidate approach may lead to the discovery of new pathways involved in the control of blood pressure. The identification of *Ncf1* as a gene responsible for rheumatoid arthritis also led to the discovery that NADPH oxidase complex was involved in the progression of severe autoimmune diseases. [219]

The current work combines positional cloning approach and global genomics approach. The *Agtr1b* gene was identified on the basis of its function. C2QTL2 and C2QTL3 contain potential gene candidates whose function is known to be associated with the

cardiovascular system. Despite the presence of candidate genes in C2QTL2 and C2QTL3, it is not excluded that a yet unknown gene is responsible for the blood pressure effects. To date, C2QTL1 region doesn't include any obvious candidate genes. Identification of the genes at the molecular level may contribute to defining the mechanisms and regulation of known cardiovascular pathways or to discover new cascades and molecules.

7.11.2 Clinical

Hypertension is a major risk factor for the onset of lethal cardiovascular diseases. However, detection and control is achieved in only 13% of hypertensive patients. [61] Identification of genes for hypertension is necessary for the development of genetic diagnostic tools, design of new medications and a personalized treatment based on the genetic profile of a given patient, a field known as pharmacogenomics. Prevention and individualized treatment may translate into a reduction in cost and optimal efficiency of health delivery system.

In hypertension, it has been estimated that several genes are involved in the disease, each of them contributing to 30- 50% of the blood pressure phenotype. [84] Given the number of parameters regulating blood pressure, is it realistic to expect that discoveries of the individuals genes in animal models or human hypertension will translate into novel therapeutic approaches, pharmacogenomics and genetic diagnostic tests?

7.11.2.1 Gene therapy

Gene therapy is most of the time associated with the correction of single gene diseases. At first sight, given the variety of pharmacological treatments available for treating hypertension, gene therapy may not be an appealing solution. Interestingly, it was proposed that gene therapy offers important advantages over traditional drug treatment: 1) it would diminish the side effects and 2) compliance would be achieved more easily since the patient would be required to take a single dose lasting several months rather than a pill every day. As knowledge of hypertension genes progresses, more targets will become available. [238] However, significant technical issues such as inflammatory and

immune responses, and poor efficiency of delivery of the therapeutic gene will have to be resolved before gene therapy becomes available as a treatment for hypertension. [124]

7.11.2.2 Genetic diagnosis and pharmacogenomics

Knowing the genetic variations present in the population that underlie hypertension will allow us to diagnose individuals at risk for hypertension before the onset of cardiovascular complications. Individuals respond differently to antihypertensive therapy suggesting the possibility of personalized treatment. [168] The A1166C polymorphism of AT1R receptor in humans is associated with differences in humoral and renal hemodynamic responses to AT1R blockade with losartan. [239] This example illustrates how the identification of a genetic variation may lead to a pharmacogenomic strategy of treatment.

7.12 Summary and conclusions

Since the publication of the original congenic strain in a 80 cM region of Dahl rat chr 2[229], we have used a systematic mapping strategy to provide a comprehensive coverage and fine analysis of the BP QTL it contains. Consequently, a total of three QTLs were discovered. C2QTL1, C2QTL2 and C2QTL3 reside in chromosome segments of 5.7 centiMorgan (cM), 3.5 cM and 1.5 cM respectively. C2QTL1 interacted epistatically with either C2QTL2 or C2QTL3, whereas C2QTL2 and C2QTL3 showed additive effects to each other. In addition, the current investigation presents the first line of evidence that a QTL for aortic SMCN exists, and it acts independently of QTLs for BP. The SMC number QTL is also located in the segment studied in the original chr. 2 congenic strain. A candidate gene for the SMC number QTL, the gene *Agtr1b* contains two non-synonymous mutations in the coding region between DSS and MNS rats and a reduced contractile responses to Ang II was observed in aortas of rats harbouring the MNS allele of the gene compared with DSS rats. Further work will be necessary to identify the genes responsible for the BP effect in C2QTL1, C2QTL2 and C2QTL3. Identification of genes for complex diseases remains challenging but the new genomic tools available are promising. Genes identified will provide new insights about the BP regulatory systems and the defects leading to hypertension. This knowledge may be useful in the development of new therapeutic targets, genetic diagnostic tools and pharmacogenomics.

REFERENCES

1. Delbarre B, Delbarre G. Mécanismes de régulation de la pression artérielle. In: Delbarre B, Delbarre G, eds. *Hypertension artérielle: Physiopathologie et pharmacologie.* Masson; 1993:7-35.

2. Dampney RAL. Fonctional Organization of Central Pathways Regulating the Cardiovascular System. *Physiological Reviews* 1994;74:323-364.

3. Izzo JL. Sympathetic Nervous System in Acute and Chronic Blood Pressure Elevation. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's The Kidney.* Philadelphia: W.B. Saunders Company; 2000:42-59.

4. De Wardener HE. The Hypothalamus and Hypertension. *Physiological Reviews* 2001;81:1599-1658.

5. Amerena J, Julius S. Role of the Nervous System in Human Hypertension. In: Hollenberg NK, ed. *Hypertension: Mechanisms and Therapy.* Philadelphia: Current Medicine; 1995:2.1-2.23.

6. Policy Research Division Strategic Policy Directorate Population and Public Health Branch. Economic Burden of Illness in Canada, 1998. 2002. Her Majesty the Queen in Right of Canada, represented by the Minister of Public Works and Governement Services Canada (http://www.phac-aspc.gc.ca/publicat/ebic-femc98/).

7. Catanzaro DF. Angiotensinogen: Physiology, Molecular Biology, and Relevance to Hypertension. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's The Kidney.* Philadelphia: W.B. Saunders Company; 2000:77-89.

8. Fabiani ME, Dinh DT, Nassis L, Jonhston CI. Angiotensin-Converting Enzyme: Basic Prperties, Distribution and Functional Role. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's The Kidney.* Philadelphia: W.B. Saunders Company; 2000:90-110.

9. Griendling KK, Lassègue B, Alexander RW. Angiotensin Receptors and their therapeutic implications. *Annual Reviews of Pharmacology and Toxicology* 1996;36:281-306.

10. Carey RM, Wang Z-Q, Siragy HM. Role of the Angiotensin Type 2 Receptor in the Regulation of Blood Pressure and Renal Function. *Hypertension* 2000;35:155-163.

11. Harris RC, Inagami T. Molecular Biology and Pharmacology of Angiotensin Receptor Subtypes. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis, and Management*. New York: Raven Press, Ltd.; 1995:1721-1738.

12. Lucius R, Gallinat S, Busches S, Rosenstiel P, Unger T. Behond blood pressure: new roles for angiotensin II. *Cellular and Molecular Life Sciences* 1999;56:1008-1019.

13. Unger T. Neurohormonal modulation in cardiovascular disease. *American Heart Journal* 2000;139:S2-S8.

14. Campbell DJ. Tissu Renin-Angiotensin System: Sites of Angiotensin Formation. *Journal of Cardiovascular Pharmacology* 1987;10:S1-S8.

15. Zhuo J, Allen AM, Alcorn D, Alderd P, MacGregor DP, Mendelsohn FAO. The Distribution of Angiotensin II Receptors. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis and Management*. New York: Raven Press, Ltd.; 1995:1739-1762.

16. Campese VM, Widerhorn ALM. Natriuretic Peptides. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's the kidney.* Philadelphia: W.B. Saunders Company; 2000:165-176.

17. Burnett JC, Shenker Y. Endogenous Natriuretic Peptides. In: Izzo JL, Black HR, eds. *Hypertension Primer*. Dallas: American Heart Association; 1999:42-43.

18. Tremblay J, Desjardins R, Hum D, Gutkowska J, Hamet P. Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. *Molecular and Cellular Biochemistry* 2002;230:31-47.

19. Schiffrin EL. Endothelin. In: Izzo JL, Black HR, eds. *Hypertension Primer.* Dallas: American Heart Association; 1999:36-37.

20. Burnett JC. Endothelin in Hypertension. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's the Kidney*. Philadelphia: W.B. Saunders Company; 2000:152-158.

21. Ignarro LJ. Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension* 1990;16:477-483.

22. Ortiz PA, Garvin JL. Cardiovascular and renal control in NOS-deficient mouse models. *American Journal of Physiology* 2003;284:R628-R638.

23. Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sciences* 2004;75:639-653.

24. Herrera M, Garvin JL. Recent advances in the regulation of nitric oxide in the kidney. *Hypertension* 2005;45:1062-1067.

25. Choi B-M, Pae H-O, Jang S, Kim Y-M, Chung H-T. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. *Journal of Biochemistry and Molecular Biology* 2002;35:116-126.

26. Schmidt HHHW, Lohmann SM, Walter U. The nitric oxide cGMP signal transduction system: regulation and mechanism of action. *Biochimica and Biophysica Acta* 1993;1178:153-175.

27. Friebe A, Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circulation Research* 2003;93:96-105.

28. Reid IA. Vasoactive Peptides. In: Katzung BG, ed. *Basic and Clinical Pharmacology*. Stamford: Appleton and Lange; 1998:287-303.

29. Margolius HS. Kallikrein-Kinin Systems. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's The Kidney*. Philadelphia: W.B. Saunders Company; 2000:190-201.

30. Funk CD. Prostaglandins and Leukotrienes: Advances in Eicosanoid Biology. *Science* 2001;294:1871-1875.

31. Foegh ML, Ramwell PW. The Eicosanoids: Prostaglandins, Thrmboxanes, Leukotrienes, and Related Compounds. In: Katzung BG, ed. *Basic and Clinical Pharmacology*. Stamford: Appleton and Lange; 1998:304-318.

32. McGiff JC, Nasjletti A. Prostaglandins and P450 Metabolites. In: Izzo JL, Black HR, eds. *Hypertension Primer*. Dallas: American Heart Association; 1999:30-32.

33. Nadler JL. Lipoxygenase Products. In: Izzo JL, Black HR, eds. *Hypertension Primer*. Dallas: American Heart Association; 1999:33-34.

34. Hypertension Artérielle 2002: Guide Thérapeutique de la Société québécoise d'hypertension artérielle. Québec: La Société québécoise d'hypertension artérielle; 2002.

35. Rubattu S, Struk B, Kreutz R, Volpe M, Lindpaintner K. Animal models of genetic hypertension: what can we learn for human hypertension? *Clinical and Experimental Pharmacology and Physiology* 1995;Suppl. 2:S386-S393.

36. Kannel WB. Blood pressure as a cardiovascular risk factor: prevention and treatment. *JAMA* 1996;275:1577-1580.

37. Klag MJ. Renal Risk. In: Izzo JL, Black HR, eds. *Hypertension Primer*. Dallas: American Heart Association; 1999:211-214.

38. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases. The role of oxidant stress. *Circulation Research* 2000;87:840-844.

39. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2005;25:29-38.

40. Touyz RM. Molecular and cellular mechanisms regulating vascular function and structure- Implications in the pathogenesis of hypertension. *Canadian Journal of Cardiology* 2000;16:1137-1146.

41. Wilcox C. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? *American Journal of Physiology* 2005;289:R913-R935.

42. Endemann DH, Schiffrin EL. Endothelial dysfunction. *Journal of the American Society of Nephrology* 2004;15:1983-1992.

43. Panza JA. Nitric Oxide in Hypertension. In: Oparil S, Weber MA, eds. *Hypertension: a companion to Brenner and Rector's The kidney*. Philadelphia: W.B. Saunders Company; 2000:158-165.

44. Bobryshev YV. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron* 2006;37:208-222.

45. Phillips SJ, Whisnant JP. Hypertension and Stroke. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis, and Management*. New York: Raven Press, Ltd.; 1995:465-478.

46. Fornage M, Turner ST, Sing CF, Boerwinkle E. Variation at the M235T locus of the angiotensinogen gene and essential hypertension: a population -based case-control study from Rochester, Minnesota. *Human Genetics* 1995;96:295-300.

47. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* 1995;91:2488-2496.

48. Mulvany MJ. Small artery remodeling in hypertension. *Current Hypertension Reports* 2002;4:49-55.

49. Prewitt RL, Rice DC, Dobrian AD. Adaptation of resistance arteries to increases in pressure. *Microcirculation* 2002;9:295-304.

50. Standley PR. Mechanisms of vascular hypertrophy. In: Izzo JL, Black HR, eds. *Hypertension Primer*. Dallas: American Heart Association; 1999:157-159.

51. Zervoudaki AI, Toutouzas PK. Remodeling of resistance vessels in essential hypertension. *Hellenic Journal of Cardiology* 2003;44:116-124.

52. Wakatsuki T, Schlessinger J, Elson E. The biochemical response of the heart to hypertension and exercise. *TRENDS in Biochemical Sciences* 2004;29:609-617.

53. Corda S, Samuel J-L, Rappaport L. Extracellular matrix and growth factors during heart growth. *Heart Failure Reviews* 2000;5:119-130.

54. Lopez B, Gonzalez D, Diez J. Role of matrix metalloproteinases in hypertension-associated cardiac fibrosis. *Current Opinion in Nephrology and Hypertension* 2004;13:197-204.

55. Francis CK. Pathogenesis of Coronary Artery Disease. In: Izzo JL, Black HR, eds. *Hypertension Primer.* Dallas: American Heart Association; 1999:175-176.

56. Sonnenblick EH, LeJemtel TH. Pathogenesis of Congestive Heart Failure. In: Izzo JL, Black HR, eds. *Hypertension Primer.* Dallas: American Society of Hypertension; 1999:180-182.

57. Susic DS, Frohlich ED. Hypertension and the heart. *Current Hypertension Reports* 2000;2:565-569.

58. Andrew P. Diastolic heart failure demystified. *Chest* 2003;124:744-753.

59. Kashgarian M. Hypertensive Disease and Kidney Structure. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis, and Management.* New York: Raven Press, Ltd.; 1995:433-443.

60. The Growing Burden of Heart Disease and Stroke in Canada 2003. Ottawa: Heart and Stoke Foundation of Canada (http://www.cvdinfobase.ca/cvdbook/); 2003.

61. Joffres MR, Hamet P. Distribution of Blood Pressure and Hypertension in Canada and in the United States. *American Journal of Hypertension* 2001;14:1099-1105.

62. The Growing Burden of Heart Disease and Stroke in Canada 2003. Ottawa: Heart and Stoke Foundation of Canada (http://www.cvdinfobase.ca/cvdbook/); 2003.

63. Hypertension Artérielle 2002: Guide Thérapeutique de la Société québécoise d'hypertension artérielle. Québec: La Société québécoise d'hypertension artérielle; 2002.

64. Hypertension Artérielle 2002: Guide Thérapeutique de la Société québécoise d'hypertension artérielle. Québec: La Société québécoise d'hypertension artérielle; 2002.

65. Benowitz NL. Antihypertensive Agents. In: Katzung BG, ed. *Basic and Clinical Pharmacology*. Stamford: Appleton and Lange; 1998:153-178.

66. Mornet E, Dupont J, Vitek A, White PC. Characterization of Two Genes Encoding Human Steroid 11-beta Hydroxylase (P-450 11 beta). *The Journal of Biological Chemistry* 1989;264:20961-20967.

67. Lifton RP. Molecular Genetics of Human Blood Pressure Hypertension. *Science* 1996;272:676-680.

68. Pascoe L, Curnow K, Slutsker L, Connell JMC, Speiser PW, New MI, White PC. Glucocorticoid-suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between CYP11B1 and CYP11B2. *Proceedings of the National Academy of Science of the United States of America* 1992;89:8327-8331.

69. Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S, Lalouel J-M. A chimeric 11beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature* 1992;355:262-265.

70. Mornet E, Dupont J, Vitek A, White PC. Characterization of Two Genes Encoding Human Steroid 11-beta Hydroxylase (P-450 11 beta). *The Journal of Biological Chemistry* 1989;264:20961-20967.

71. Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid Action: Target Tissue Specificity Is Enzyme, Not Receptor, Mediated. *Science* 1988;242:583-585.

72. Luft FC. Mendenlian forms of human hypertension and mechanisms of disease. *Clinical Medicine and Research* 2003;1:291-300.

73. Wilson RC, Krozowski ZS, Li K, Obeyesekere VR, Razzaghy-Azar M, Harbison MD, Wei JQ, Shackleton CHL, Funder JW, New MI. Mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess. *Journal of Clinical Endocrinology and Metabolism* 1995;80:2263-2266.

74. Mune T, Rogerson FM, Nikkilä H, Agarwal AK, White PC. Human hypertension caused by mutations in the kidney isozyme of 11beta-hydroxysteroid dehydrogenase. *Nature Genetics* 1995;10:394-399.

75. Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR, Ulick S, Milora RV, Findling JW, Canessa CM, Rossier BC, Lifton RP. Liddle's Syndrome: Heritable Human Hypertension Caused by Mutations in the beta Subunit of the Epithelial Sodium Channel. *Cell* 1994;79:407-414.

76. Hansson JH, Schild L, Lu Y, Wilson TA, Gautschi I, Shimkets R, Nelson-Williams C, Rossier BC, Lifton RP. A de novo missense mutation of the beta subunit of the epithelial sodium channel causes hypertension and Liddle syndrome, identifying a proline-rich segment critical for regulation of channel activity. *Proceedings of the National Academy of Science of the United States of America* 1995;92:11495-11499.

77. Hansson JH, Nelson-Williams C, Suzuki H, Schild L, Shimkets R, Lu Y, Canessa C, Iwasaki T, Rossier B, Lifton RP. Hypertension caused by a truncated epithelial sodium channel gamma subunit: genetic heterogeneity of Liddle syndrome. *Nature Genetics* 1995;11:76-82.

78. Snyder PM, Price MP, McDonald FJ, Adams CM, Volk KA, Zeiher BG, Stokes JB, Welsh MJ. Mechanisms by Which Liddle's Syndrome Mutations Increase Activity of a Human Epithelial Na+ Channel. *Cell* 1995;83:969-978.

79. Abriel H, Loffing J, Rebhun JF, Pratt JH, Schild L, Horisberger J-D, Rotin D, Staub O. Defective regulation of the epithelial Na+ channel by Nedd4 in Liddle's syndrome. *Journal of Clinical Investigation* 1999;103:667-673.

80. Gamba G. Role of WNK kinases in regulating tubular salt and potassium transport and in the development of hypertension. *American Journal of Renal Physiology* 2005;288:F245-F252.

81. Rossier BC. Negative regulators of sodium transport in the kidney: Key factors in understanding salt-sensitive hypertension? *Journal of Clinical Investigation* 2003;111:947-950.

82. Delaloy C, Lu J, Houot A-M, Disse-Nicodeme S, Gasc J-M, Corvol P, Jeunemaitre X. Multiple promoters in the WNK1 gene: one controls expression of a kidney-specific kinase-defective isoform. *Molecular and Cellular Biology* 2003;23:9208-9221.

83. Faure S, Delaloy C, Leprivey V, Hadchouel J, Warnock DG, Jeunemaitre X, Achard J-M. WNK kinases, distal tubular handling and hypertension. *Nephrology Dialysis Transplantation* 2003;18:2463-2467.

84. Ward R. Familial Aggregation and Genetic Epidemiology of Blood Pressure. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis and Management*. New York: Raven Press, Ltd.; 1995:67-88.

85. Okamoto K, Aoki K. Development of a Strain of Spontaneously Hypertensive Rats. *Japanese Circulation Journal* 1963;27:282-293.

86. Okamoto K, Yamori Y, Nagaoka K. Establishment of the Stroke-prone Spontaneously Hypertensive Rat (SHR). *Circulation Research* 1974;Suppl. 1:I-143-I-153.

87. Smirk FH, Hall WH. Inherited Hypertension in Rats. *Nature* 1958;182:727-728.

88. Bianchi G, Fox U, Imbasciati E. The Development od a New Strain of Spontaneously Hypertensive Rats. *Life Sciences* 1974;14:339-347.

89. Dupont J, Dupont JC, Froment A, Milon H, Vincent M. Selection of three strains of rats with spontaneously different levels of blood pressure. *Biomedicine* 1973;19:36-41.

90. Rapp JP, Dene H. Development and Characteristics of Inbred Strains of Dahl Salt-Sensitive and Salt-Resistant Rats. *Hypertension* 1985;7:340-349.

91. Dahl LK, Heine M, Tassinari L. Role of genetic factors in susceptibility to experimental hypertension due to chronic salt ingestion. *Nature* 1962;194:480-482.

92. Rapp JP. Dahl Salt-Susceptible and Salt-Resistant Rats. *Hypertension* 1982;4:753-763.

93. Simchon S, Manger WM, Brown TW. Dual Hemodynamics Mechanisms for Salt-Induced Hypertension in Dahl Salt-Sensitive Rats. *Hypertension* 1991;17:1063-1071.

94. Boegehold MA, Huffman LJ, Hedge GA. Peripheral vascular resistance and regional blood flows in hypertensive Dahl rats. *American Journal of Physiology* 1991;261:R934-R938.

95. Kato T, Kassab F, Wilkins FC, Kirchner KA, Granger JP. Decreased Sensitivity to Renal Interstitial Hydrostatic Pressure in Dahl Salt-Sensitive Rats. *Hypertension* 1994;23:1082-1086.

96. Lüscher TF, Raij L, Vanhoutte PM. Endothelium-dependent Vascular Response in Normotensive and Hypertensive Dahl Rats. *Hypertension* 1987;9:163.

97. Chen YP, Sanders P. L-Arginine Abrogates Salt-Sensitive Hypertension in Dahl/Rapp Rats. *Journal of Clinical Investigation* 1991;88:1559-1567.

98. Bayorth MA, Ganafa AA, Socci RR, Silverstrov N, Abukhalaf IK. The Role of Oxidative Stress in Salt-Induced Hypertension. *American Journal of Hypertension* 2004;17:31-36.

99. Nelson LD, Unlap MT, Lewis JL, Bell PD. Renal arteriolar Na+/Ca2+ exchange in salt-sensitive hypertension. *American Journal of Physiology* 1999;276:F567-F573.

100. Mashburn NA, Unlap MT, Runquist J, Alderman A, Jonhson GVW, Bell PD. Altered protein kinase C activation of Na+/Ca2+ exchange in mesangial cells from salt-sensitive rats. *American Journal of Physiology* 1999;276:574-580.

101. Unlap MT, Peti-Peterdi J, Bell PD. Cloning of mesangial cell Na+/Ca2+ exchangers from Dahl/Rapp salt-sensitive/resistant rats. *American Journal of Physiology* 2000;279:F177-F184.

102. Unlap MT, Bates E, Williams C, Komlosi P, Williams I, Kovacs G, Siroky B, Bell PD. Na+/Ca2+ Exchanger. Target for Oxidative Stress in Salt-Sensitive Hypertension. *Hypertension* 2003;42:363-368.

103. Zhao X, White R, Van Huysse J, Leenen FHH. Cardiac hypertrophy and cardiac renin-angiotensin system in Dahl rats on high salt intake. *Journal of Hypertension* 2000;18:1319-1326.

104. Tank JE, Moe OW, Henrich WL. Abnormal regulation of proximal tubule renin mRNA in the Dahl/Rapp salt-sensitive rat. *Kidney International* 1998;54:1608-1616.

105. Otsuka F, Yamauchi T, Kataoka H, Mimura Y, Ogura T, Makino H. Effetcs of chronic inhibition of ACE and AT1 receptors on glomerular injury in Dahl salt-sensitive rats. *American Journal of Physiology* 1998;274:R1797-R1806.

106. Nishiyama A, Yoshizumi M, Rahman M, Kobori H, Seth DM, Miyatake A, Zhang G-X, Yao L, Hitomi H, Shokoji T, Kiyomoto H, Kimura S, Tamaki T, Kohno M, Abe Y. Effects of AT1 receptor blockade on renal injury and mitogen-activated protein activity in Dahl salt-sensitive rats. *Kidney International* 2004;65:972-981.

107. Kim S, Yoshiyame M, Izumi Y, Kawano H, Kimoto M, Zhan Y, Iwao H. Effects of Combination of ACE Inhibitor and Angiotensin Receptor Blocker on Cardiac Remodeling, Cardiac Function, and Survival in Rat Heart Failure. *Circulation* 2001;103:148-154.

108. Hamada H, Kakunaga T. Potential Z-DNA forming sequences are highly dispersed in the human genome. *Nature* 1982;298:396-398.

109. Weber JL, May PE. Abundant Class of Human DNA Polymorphisms Which Can Be Typed Using the Polymerase Chain Reaction. *American Journal of Human Genetics* 1989;44:388-396.

110. Weber JL. Informativeness of Human (dC-dA)n.(dG-dT)n Polymorphisms. *Genomics* 1990;7:524-530.

111. Beckmann JS, Weber JL. Survey of Human and Rat Microsatellites. *Genomics* 1992;12:627-631.

112. Tautz D, Renz M. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acid Research* 1984;12:4127-4138.

113. Stallings RL, Ford AF, Nelson D, Torney DC, Hildebrand CE, Moyzis RK. Evolution and Distribution of (GT)n Repetitive Sequences in Mammalian Genomes. *Genomics* 1991;10:807-815.

114. Jensen-Seaman MI, Furey TS, Payseur BA, Lu Y, Roskin KM, Chen C-F, Thomas MA, Haussler D, Jacob HJ. Comparative recombination rates in the rat, mouse, and human genomes. *Genome Research* 2004;14:528-538.

115. Cox DR, Burmeisterm M, Price ER, Kim S, Myers RM. Radiation Mapping: A Somatic Cell Genetic Method for Constructing High-Resolution Maps of Mammalian Chromosomes. *Science* 1990;250:245-250.

116. Watanabe M, Bihoreau M-T, McCarthy LC, Kiguwa SL, Hishigaki H, Tsuji A, Browne J, Yamasaki Y, Mizogushi-Miyakita A, Oga K, Ono T, Okuno S, Kanemoto N, Takahashi E-I, Tomita K, Hayashi H, Adashi M, Webber C, Davis M, Kiel S, Knights C, Smith A, Critcher R, Miller J, Thangaranja T, Day PJR, Hudson JR, Irie Y, Takagi T, Nakamura Y, Goodfellow PN, Lathrop M, Tanigami A, James MR. A radiation hybrid map of the rat genome containing 5,255 markers. *Nature Genetics* 1999;22:27-36.

117. Gyapay G, Schmitt K, Fizames C, Jones H, Vega-Czarny N, Spillett D, Muselet D, Prud'Homme J-F, Dib C, Auffray C, Morissette J, Weissenbach J, Goodfellow PN. A radiation hybrid map of the human genome. *Human Molecular Genetics* 1996;5:339-346.

118. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di F, V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferriera S,

Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigo R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M. The sequence of the human genome 82. *Science* 2001;291:1304-1351.

119. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, Antonarakis SE, Attwood J, Baertsch R, Bailey J, Barlow K, Beck S, Berry E, Birren B, Bloom T, Bork P, Botcherby M, Bray N, Brent MR, Brown DG, Brown SD, Bult C, Burton J, Butler J, Campbell RD, Carninci P, Cawley S, Chiaromonte F, Chinwalla AT, Church DM, Clamp M, Clee C, Collins FS, Cook LL, Copley RR, Coulson A, Couronne O, Cuff J, Curwen V, Cutts T, Daly M, David R, Davies J, Delehaunty KD, Deri J, Dermitzakis ET, Dewey C, Dickens NJ, Diekhans M, Dodge S, Dubchak I, Dunn DM, Eddy SR, Elnitski L, Emes RD, Eswara P, Eyras E, Felsenfeld A, Fewell GA, Flicek P, Foley K, Frankel WN, Fulton LA, Fulton RS, Furey TS, Gage D, Gibbs RA, Glusman G, Gnerre S, Goldman N, Goodstadt L, Grafham D, Graves TA, Green ED, Gregory S, Guigo R, Guyer M, Hardison RC, Haussler D, Hayashizaki Y, Hillier LW, Hinrichs A, Hlavina W, Holzer T, Hsu F, Hua A, Hubbard T, Hunt A, Jackson I, Jaffe DB, Johnson LS, Jones M, Jones TA, Joy A, Kamal M, Karlsson EK, Karolchik D, Kasprzyk A, Kawai J, Keibler E, Kells C, Kent WJ, Kirby A, Kolbe DL, Korf I, Kucherlapati RS, Kulbokas EJ, Kulp D, Landers T, Leger JP, Leonard S, Letunic I, Levine R, Li J, Li M, Lloyd C, Lucas S, Ma B, Maglott DR, Mardis ER, Matthews L, Mauceli E, Mayer JH, McCarthy M, McCombie WR, McLaren S, McLay K, McPherson JD, Meldrim J, Meredith B, Mesirov JP, Miller W, Miner TL, Mongin E, Montgomery KT, Morgan M, Mott R, Mullikin JC, Muzny DM, Nash WE, Nelson JO, Nhan MN, Nicol R, Ning Z, Nusbaum C, O'Connor MJ, Okazaki Y, Oliver K, Overton-Larty E, Pachter L, Parra G, Pepin KH, Peterson J, Pevzner P, Plumb R, Pohl CS, Poliakov A, Ponce TC, Ponting CP, Potter S, Quail M, Reymond A, Roe BA, Roskin KM, Rubin EM, Rust AG, Santos R, Sapojnikov V, Schultz B, Schultz J, Schwartz MS, Schwartz S, Scott C, Seaman S, Searle S, Sharpe T, Sheridan A, Shownkeen R, Sims S, Singer JB, Slater G,

Smit A, Smith DR, Spencer B, Stabenau A, Stange-Thomann N, Sugnet C, Suyama M, Tesler G, Thompson J, Torrents D, Trevaskis E, Tromp J, Ucla C, Ureta-Vidal A, Vinson JP, Von Niederhausern AC, Wade CM, Wall M, Weber RJ, Weiss RB, Wendl MC, West AP, Wetterstrand K, Wheeler R, Whelan S, Wierzbowski J, Willey D, Williams S, Wilson RK, Winter E, Worley KC, Wyman D, Yang S, Yang SP, Zdobnov EM, Zody MC, Lander ES. Initial sequencing and comparative analysis of the mouse genome73. *Nature* 2002;420:520-562.

120. Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, Scott G, Steffen D, Worley KC, Burch PE, Okwuonu G, Hines S, Lewis L, DeRamo C, Delgado O, Dugan-Rocha S, Miner G, Morgan M, Hawes A, Gill R, Celera, Holt RA, Adams MD, Amanatides PG, Baden-Tillson H, Barnstead M, Chin S, Evans CA, Ferriera S, Fosler C, Glodek A, Gu Z, Jennings D, Kraft CL, Nguyen T, Pfannkoch CM, Sitter C, Sutton GG, Venter JC, Woodage T, Smith D, Lee HM, Gustafson E, Cahill P, Kana A, Doucette-Stamm L, Weinstock K, Fechtel K, Weiss RB, Dunn DM, Green ED, Blakesley RW, Bouffard GG, De Jong PJ, Osoegawa K, Zhu B, Marra M, Schein J, Bosdet I, Fjell C, Jones S, Krzywinski M, Mathewson C, Siddiqui A, Wye N, McPherson J, Zhao S, Fraser CM, Shetty J, Shatsman S, Geer K, Chen Y, Abramzon S, Nierman WC, Havlak PH, Chen R, Durbin KJ, Egan A, Ren Y, Song XZ, Li B, Liu Y, Qin X, Cawley S, Worley KC, Cooney AJ, D'Souza LM, Martin K, Wu JQ, Gonzalez-Garay ML, Jackson AR, Kalafus KJ, McLeod MP, Milosavljevic A, Virk D, Volkov A, Wheeler DA, Zhang Z, Bailey JA, Eichler EE, Tuzun E, Birney E, Mongin E, Ureta-Vidal A, Woodwark C, Zdobnov E, Bork P, Suyama M, Torrents D, Alexandersson M, Trask BJ, Young JM, Huang H, Wang H, Xing H, Daniels S, Gietzen D, Schmidt J, Stevens K, Vitt U, Wingrove J, Camara F, Mar AM, Abril JF, Guigo R, Smit A, Dubchak I, Rubin EM, Couronne O, Poliakov A, Hubner N, Ganten D, Goesele C, Hummel O, Kreitler T, Lee YA, Monti J, Schulz H, Zimdahl H, Himmelbauer H, Lehrach H, Jacob HJ, Bromberg S, Gullings-Handley J, Jensen-Seaman MI, Kwitek AE, Lazar J, Pasko D, Tonellato PJ, Twigger S, Ponting CP, Duarte JM, Rice S, Goodstadt L, Beatson SA, Emes RD, Winter EE, Webber C, Brandt P, Nyakatura G, Adetobi M, Chiaromonte F, Elnitski L, Eswara P, Hardison RC, Hou M, Kolbe D, Makova K, Miller W, Nekrutenko A, Riemer C, Schwartz S, Taylor J, Yang S, Zhang Y, Lindpaintner K, Andrews TD, Caccamo M, Clamp M, Clarke L, Curwen V, Durbin R, Eyras E, Searle SM, Cooper GM, Batzoglou S, Brudno M, Sidow A, Stone EA, Venter JC, Payseur BA, Bourque G, Lopez-Otin C, Puente XS, Chakrabarti K, Chatterji S, Dewey C, Pachter L, Bray N, Yap VB, Caspi A, Tesler G, Pevzner PA, Haussler D, Roskin KM, Baertsch R, Clawson H, Furey TS, Hinrichs AS, Karolchik D, Kent WJ, Rosenbloom KR, Trumbower H, Weirauch M, Cooper DN, Stenson PD, Ma B, Brent M, Arumugam M, Shteynberg D, Copley RR, Taylor MS, Riethman H, Mudunuri U, Peterson J, Guyer M, Felsenfeld A, Old S, Mockrin S, Collins F. Genome sequence of the Brown Norway rat yields insights into mammalian evolution 66. *Nature* 2004;428:493-521.

121. Rapp JP. The Search for the Genetic Basis of Blood Pressure Variation in Rats. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysilogy, Diagnosis and Management*. New York: Raven Press, Ltd.; 1995:1289-1300.

122. Lander ES, Schork NJ. Genetic Dissection of Complex Traits. *Science* 1994;265:2037-2048.

123. Rapp JP, Deng AY. Detection and Positional Cloning of Blood Pressure Quantitative Trait Loci: Is It Possible? Identifying the Genes for Genetic Hypertension. *Hypertension* 1995;25:1121-1128.

124. Dominiczak AF, Negrin DC, Clark JS, Brosnan MJ, McBride MW, Alexander MY. Genes and Hypertension From Gene Mapping in Experimental Models to Vascular Gene Transfer Strategies. *Hypertension* 2000;35:164-172.

125. Markel P, Shu P, Ebeling C, Carlson GA, Nagle DL, Smutko JS, Moore KJ. Theoretical and empirical issues for marker assisted breeding of congenic mouse strains. *Nature Genetics* 1997;17:280-284.

126. Walker WG, Whelton PK, Saito H, Russell RP, Hermann J. Relation Between Blood Pressure and Renin, Renin Substrates, AngiotensinII, Aldosterone and Urinary Sodium and Potassium in 574 Ambulatory Subjets. *Hypertension* 1979;1:287-291.

127. Fasola AF, Martz BL, Helmer OM. Plasma renin activity during supine exercise in offsprings of hypertensive parents. *Journal of Applied Physioogy* 1968;25:410-415.

128. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel J-M, Corvol P. Molecular Basis of Human Hypertension: Role of Angiotensinogen. *Cell* 1992;71:169-180.

129. Caulfield M, Lavender P, Newell-Price J, Farrall M, Kamdar S, Daniel H, Lawson M, De Freitas P, Fogarti P, Clark AJL. Linkage of the Angiotensinogen Gene Locus to Human Essential Hypertension in African Carribeans. *Journal of Clinical Investigation* 1995;96:687-692.

130. Brand E, Chatelain N, Keavney B, Caulfield M, Cittero L, Connell J, Grobbee D, Schmidt S, Schunkert H, Schuster H, Sharma AM, Soubrier F. Evaluation of the Angiotensinogen Locus in Human Essential Hypertension. *Hypertension* 1998;31:725-729.

131. Caulfield M, Lavender P, Farrall M, Munroe P, Lawson M, Turner P, Clark AJL. Linkage of the angiotensinogen gene to essential hypertension. *The New England Journal of Medicine* 1994;330:1629-1633.

132. Wang WYS, Glenn CL, Zhang W, Benjafield AV, Nyholt DR, Morris BJ. Exclusion of Angiotensinogen Gene in Molecular Basis of Human Hypertension: Sibpair Linkage and Association Analyses in Australian Anglo-Caucasians. *American Journal of Medical Genetics* 1999;87:53-60.

133. Iwai N, Shimoike H, Ohmichi N, Kinoshita M. Angiotensinogen Gene and Blood Pressure in the Japanese Population. *Hypertension* 1995;25:688-693.

134. Schorr U, Blaschke K, Beige J, Disler A, Sharma AM. Angiotensinogen M235T variant and salt sensitivity in young normotensive caucasians. *Journal of Hypertension* 1999;17:475-479.

135. Hata A, Namikawa C, Sasaki M, Sato K, Nakamura K, Tamura K, Lalouel J-M. Angiotensinogen as a Risk Factor for Essential Hypertension in Japan. *Journal of Clinical Investigation* 1994;93:1285-1287.

136. Pereira AC, Mota GFA, Cunha RS, Herbenhoff FL, Mill JG, Krieger JE. Angiotensinogen 235T Allele 'Dosage' Is Associated With Blood Pressure Phenotypes. *Hypertension* 2003;41:25-30.

137. Kamitani A, Rakugi H, Higaki J, Yi Z, Mikami H, Miki T, Ogihara T. Association analysis of a polymorphism of the angiotensinogen gene with essential hypertension in Japanese. *Journal of Human Hypertension* 1994;8:521-524.

138. Hunt SC, Geleijnse JM, Wu LL, Witteman JCM, Williams RR, Grobbee DE. Enhanced Blood Pressure Response to Mild Sodium Reduction in Subjects With the 235T Variant of the Angiotensinogen Gene. *American Journal of Hypertension* 1999;12:460-466.

139. Hingorani AD, Sharma P, Jia H, Hopper R, Brown MJ. Blood Pressure and the M235T Polymorphism of the Angiotensinogen Gene. *Hypertension* 1996;28:907-911.

140. Rotimi C, Morrison L, Cooper R, Oyejide C, Effiong E, Ladipo M, Osotemihen B, Ward R. Angiotensinogen Gene in Human Hypertension. *Hypertension* 1994;24:591-594.

141. Fornage M, Turner ST, Sing CF, Boerwinkle E. Variation at the M235T locus of the angiotensinogen gene and essential hypertension: a population -based case-control study from Rochester, Minnesota. *Human Genetics* 1995;96:295-300.

142. Bennett CL, Schrader AP, Morris BJ. Cross-sectional analysis of Met235 -> Thr variant of angiotensinogen gene in severe, familial hypertension. *Biochemical and Biophysical Research Communications* 1993;197:833-839.

143. Jeunemaitre X, Inoue I, Williams C, Charru A, Tichet J, Powers M, Sharma AM, Gimenez-Roqueplo A-P, Hata A, Corvol P, Lalouel J-M. Haplotypes of Angiotensinogen in Essential Hypertension. *American Journal of Human Genetics* 1997;60:1448-1460.

144. Inoue I, Nakajima T, Williams CS, Quackenbush J, Puryear R, Powers M, Cheng T, Ludwig E, Sharma AM, Hata A, Jeunemaitre X, Lalouel J-M. A Nucleotide Substitution in the Promoter of Human Angiotensinogen Is Associated with Essential Hypertension and Affects Basal Transcription In Vitro. *Hypertension* 1997;99:1786-1797.

145. Sethi AA, Nordestgaard BG, Tybjaerg-Hansen A. Angiotensinogen gene polymorphism, plasma angiotensinogen, and risk of hypertension and ischemic heart disease: a meta-analysis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2003;23:1269-1275.

146. Corvol P, Persu A, Gimenez-Roqueplo A-P, Jeunemaitre X. Seven lessons from two candidate genes in human essential hypertension. Angiotensinogen and epithelial sodium channel. *Hypertension* 1999;33:1324-1331.

147. Lodwick D, Kaiser MA, Harris J, Cumin F, Vincent M, Samani NJ. Analysis of the Role of angiotensinogen in Spontaneous Hypertension. *Hypertension* 1995;25:1245-1251.

148. Hubner N, Kreutz R, Takahashi N, Ganten D, Lindpaintner K. Unlike Human Hypertension, Blood Pressure in a Hereditary Hypertensive Rat Strain Shows no Linkage to the Angiotensinogen Locus. *Hypertension* 1994;23:797-801.

149. Mullins JJ, Peters J, Ganten D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 1990;344:541-544.

150. Rapp JP, Wang S-M, Dene H. A Genetic Polymorphism in the Renin Gene of Dahl Rats Cosegregates with Blood Pressure. *Science* 1989;243:542-544.

151. Rapp JP, Dene H, Deng AY. Seven renin alleles in rats and their effects on blood pressure. *Journal of Hypertension* 1994;12:349-355.

152. Kurtz TW, Simonet L, Kabra PM, Wolfe S, Chan L, Hjelle BL. Cosegregation of the Renin Allele of the Spontaneously Hypertensive Rat with an Increase in Blood Pressure. *Journal of Clinical Investigation* 1990;85:1328-1332.

153. Alam KY, Wang Y, Dene H, Rapp JP. Renin gene nucleotide sequence of coding and regulatory regions in Dahl rats. *Clinical and Experimental Hypertension* 1993;15:599-614.

154. Soubrier F, Jeunemaitre X, Rigat B, Houot A-M, Cambien F, Corvol P. Similar Frequencies of Renin Gene Restriction Fragment Lenght Polymorphisms in Hypertensive and Normotensive Subjects. *Hypertension* 1990;16:712-717.

155. Jeunemaitre X, Rigat B, Charru A, Houot A-M, Soubrier F, Corvol P. Sib pair linkage analysis of the renin gene haplotypes in human essential hypertension. *Human Genetics* 1992;88:301-306.

156. Cambien F, Alhenc-Gelas F, Herbeth B, Andre L, Rakotovao R, Gonzales D, Bloch C. Familial resemblance of plasma angiotensin-converting enzyme level: the Nancy Study. *American Journal of Human Genetics* 1988;43:774-780.

157. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An Insertion/Deletion Polymorphism in the Angiotensin I-converting Enzyme Gene Accounting for Half the serum Enzyme Levels. *Journal of Clinical Investigation* 1990;86:1343-1346.

158. Lachurié M-L, Azizi M, Guyene T-T, Alhenc-Gelas F, Ménard J. Angiotensin-Converting Enzyme Gene Polymorphism Has No Influence on the Circulating Renin-Angiotensin-Aldosterone System or Blood Pressure in Normotensive Subjects. *Circulation* 1995;91:2933-2942.

159. Schmidt S, van Hooft IMS, Grobbee D, Ganten D, Ritz E. Polymorphism of the angiotensin I converting enzyme gene is apparently not related to high blood pressure: Dutch Hypertension and Offspring Study. *Journal of Hypertension* 1993;11:345-348.

160. Hiraga H, Oshima T, Watanabe M, Ishida T, Shingu T, Kambe M, Matsuura H, Kajiyama G. Angiotensin I-Converting Enzyme Gene Polymorphism and Salt Sensitivity in Essential Hypertension. *Hypertension* 1996;27:569-572.

161. O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH, Levy DL. Evidence for Association and Genetic Linkage of the Angiotensin-Converting Enzyme Locus With Hypertension and Blood Pressure in Men but not in Women in the Framingham Heart Study. *Circulation* 1998;97:1766-1772.

162. Higaki J, Baba S, Katsuya T, Sato N, Ishikawa K, Mannami T, Ogata J, Ogihara T. Deletion Allele of Angiotensin-Converting Enzyme Gene Increases Risk of Essential Hypertension in Japanese Men. The Suita Study. *Circulation* 2000;101:2060-2065.

163. Morris BJ, Zee RYL, Schrader AP. Different Frequencies of Angiotensin-converting Enzyme Genotypes in Older Hypertensive Individuals. *Journal of Clinical Investigation* 1994;94:1085-1089.

164. Harrap SB, Davidson HR, Connor JM, Soubrier F, Corvol P, Fraser R, Foy CJW, Watt GCM. The Angiotensin I Converting Enzyme Gene and Predisposition to High Blood Pressure. *Hypertension* 1993;21:455-460.

165. Jeunemaitre X, Lifton RP, Hunt SC, Williams RR, Lalouel J-M. Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. *Nature* 1992;1:72-75.

166. Jacob HJ, Lindpaintner K, Lincoln SE, Kusumi K, Bunker RK, Mao Y-P, Ganten D, Dzau VJ, Lander ES. Genetic Mapping od a Gene Causing Hypertension in the Stroke-Prone Spontaneously Hypertensive Rat. *Cell* 1991;67:213-224.

167. Palijan A, Lambert R, Dutil J, Sivo Z, Deng AY. Comprehensive Congenic Coverage Revealing Multiple Blood Pressure Quantitative Trait Loci on Dahl Rat Chromosome 10. *Hypertension* 2003;42:515-522.

168. Baudin B. Angiotensin II receptor polymorphisms in hypertension. Pharmacogenomics considerations. *Pharmacogenomics* 2002;3:65-73.

169. Szpirer C, Rivière M, Szpirer J, Levan G, Guo DF, Iwai N, Inagami T. Chromosomal assignment of human and rat hypertension candidate genes: type 1 angiotensin II receptor genes and the SA gene. *Journal of Hypertension* 1993;11:919-925.

170. Bonnardeaux A, Davies E, Jeunemaitre X, Féry I, Charru A, Clauser E, Tiret L, Cambien F, Corvol P, Soubrier F. Angiotenin II Type I Receptor Gene Polymorphisms in Human Essential Hypertension. *Hypertension* 1994;24:63-69.

171. Kainulainen K, Perola M, Terwilliger J, Kaprio J, Koskenvuo M, Syvanen A-C, Vartiainen E, Peltonen L, Kontula K. Evidence for Involvement of the Type I Angiotensin Receptor Locus in Essential Hypertension. *Hypertension* 1999;33:844-849.

172. Wang WYS, Zee RYL, Morris BJ. Association of angiotensin II type I receptor gene polymorphism with essential hypertension. *Clinical Genetics* 1997;51:31-34.

173. Deng AY, Rapp JP. Evaluation of the angiotensin II receptor AT1B gene as a candidate gene for blood pressure. *Journal of Hypertension* 1994;12:1001-1006.

174. Iwai N, Inagami T. Isolation of Preferentially Expressed Genes in the Kidneys of Hypertensive Rats. *Hypertension* 1991;17:161-169.

175. Patel HR, Thiara AS, West KP, Lodwick D, Samani NJ. Increased expression of the SA gene in the kidney of the spontaneously hypertensive rat is localized to mthe proximal tubule. *Journal of Hypertension* 1994;12:1347-1352.

176. Lindpaintner K, Hilbert P, Ganten D, Nadal-Ginard B, Inagami T, Iwai N. Molecular genetics of the SA-gene: cosegregation with hypertension and mapping to rat chromosome 1. *Journal of Hypertension* 1993;11:19-23.

177. Iwai N, Inagami T. Identification of a candidate gene responsible for the high blood pressure of spontaneously hypertensive rats. *Journal of Hypertension* 1992;10:1157.

178. Harris EL, Dene H, Rapp JP. SA Gene and Blood Pressure Cosegregation Using Dahl Salt-Sensitive Rats. *American Journal of Hypertension* 1993;6:330-334.

179. Frantz F, Clemitson J-R, Bihoreau M-T, Gauguier D, Samani NJ. Genetic Dissection of Region Around the Sa Gene on Rat Chromosome 1. *Hypertension* 2001;38:216-221.

180. Hübner N, Lee Y-A, Lindpaintner K, Ganten D, Kreutz R. Congenic Substitution Mapping Excludes Sa as a Candidate Gene Locus for a Blood Pressure Quantitative Trait Locus on Rat Chromosome 1. *Hypertension* 1999;34:643-648.

181. Saad Y, Garrett MR, Lee SJ, Dene H, Rapp JP. Localization of a blood pressure QTL on rat chromosome 1 using Dahl rat congenic strains. *Physiological Genomics* 1999;1:119-125.

182. Walsh V, Somody L, Farrell A, Zhang B, Brown J, Pritchard P, Vincent M, Samani NJ. Analysis of the Role of the SA Gene in Blood Pressure Regulation by Gene Targeting. *Hypertension* 2003;41:1212-1218.

183. Iwai N, Ohmichi N, Hanai K, Nakamura K, Kinoshita M. Human SA Gene Locus as a Candidate Locus for Essential Hypertension. *Hypertension* 1994;23:375-380.

184. Nabika T, Bonnardeaux A, James M, Julier C, Jeunemaitre X, Corvol P, Lathrop M, Soubrier F. Evaluation of the SA Locus in Human Hypertension. *Hypertension* 1995;25:6-13.

185. Zee RYL, Stephen AL, Iwai N, Morris BJ. Association Analyses of SA Gene Variant in Essential Hypertensives. *American Journal of Hypertension* 1997;10:235-242.

186. Harrap SB, Samani NJ, Lodwick D, Connor JM, Fraser R, Davies DL, Lever AF, Foy CJW, Watt GCM. The SA gene: predisposition to hypertension and renal function in man. *Clinical Science* 1995;88:665-670.

187. Linder L, Kiowski W, Buhler FR. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulationin vivo. Blunted response in essential hypertension. *Circulation* 1990;81:1762-1767.

188. Benjafield AV, Morris BJ. Association Analyses of Endothelial Nitric Oxide Synthase Gene Polymorphisms in Essential Hypertension. *American Journal of Hypertension* 2000;13:994-998.

189. Bonnardeaux A, Nadaud S, Charru A, Jeunemaitre X, Corvol P, Soubrier F. Lack of Evidence for Linkage of the Endothelial Cell Nitric Oxide Synthase Gene to Essential Hypertension. *Circulation* 1995;91:96-102.

190. Deng AY, Rapp JP. Locus for the Inducible, but not a Constitutive, Nitric Oxide Synthase Cosegregates with Blood Pressure in the Dahl Salt-Sensitive Rat. *Journal of Clinical Investigation* 1995;95:2170-2177.

191. Deng AY, Rapp JP. Absence of Linkage for 'Endothelial' Nitric Oxide Synthase Locus to Blood Pressure in Dahl Rats. *Hypertension* 1997;29:49-52.

192. Persu A, Barbry P, Bassilana F, Houot A-M, Mengual R, Lazdunski M, Corvol P, Jeunemaitre X. Genetic Analysis of the Beta Subunit of the Epithell Na+ Channel in Essential Hypertension. *Hypertension* 1998;32:129-137.

193. Kreutz R, Struk B, Rubattu S, Hubner N, Szpirer J, Szpirer C, Ganten D, Lindpaintner K. Role of the alpha, beta, and gamma subunits of epithelial sodium channel in a model of polygenic hypertension. *Hypertension* 1997;29:131-136.

194. Kreutz R, Struk B, Rubattu S, Hubner N, Szpirer J, Szpirer C, Ganten D, Lindpaintner K. Role of the alpha, beta, and gamma Subunits of Epithelial Sodium Channel in a Model of Polygenic Hypertension. *Hypertension* 1997;29:131-136.

195. Erlich PM, Cui J, Chazaro I, Farrer LA, Baldwin CT, Gavras H, DeStephano AL. Genetic variants of WNK4 in Whites and African Americans with hypertension. *Hypertension* 2003;41:1191-1195.

196. Speirs JL, Morris BJ. WNK4 intron 10 polymorphism is not associated with hypertension. *Hypertension* 2004;43:766-768.

197. Tobin MD, Raleigh SM, Newhouse S, Braund P, Bodycote C, Ogleby J, Cross D, Gracey J, Hayes S, Smith T, Ridge C, Caulfied M, Sheehan NA, Munroe P, Burton PR, Samani NJ. Association of WNK1 gene polymorphisms and haplotypes with ambulatory blood pressure in the general population. *Circulation* 2005;112:3423-3429.

198. Kokubo Y, Kamide K, Inamoto N, Tanaka C, Banno M, Takiuchi S, Kawano Y, Tomoike H, Miyata T. Identifiation of 108 SNPs in TSC, WNK1, and WNK4 and their association with hypertension in a Japanese general population. *Journal of Human Genetics* 2004;49:507-515.

199. Newhouse S, Wallace C, Dobson R, Mein C, Pembroke J, Farrall M, Clayton D, Brown M, Samani N, Dominiczak AF, Connell JM, Webster J, Lathrop M, Caulfied M, Munroe P. Haplotypes of the WNK1 gene associate with blood pressure variation in a severely hypertensive population from the British Genetics of Hypertension study. *Human Molecular Genetics* 2005;14:1805-1814.

200. Deng AY, Rapp JP. Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic peptide receptor genes using Dahl salt-sensitive rats. *Nature Genetics* 1992;1:267-272.

201. Deng AY, Dene H, Rapp JP. Mapping of a Quantitative Trait Locus for Blood Pressure on Rat Chromosome 2. *Journal of Clinical Investigation* 1994;94:431-436.

202. Deng AY, Dene H, Rapp JP. Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension* 1997;30:199-202.

203. Deng AY, Jackson CM, Hoebee B, Rapp JP. Mapping of rat Chromosome 2 markers generated from chromosome-sorted DNA. *Mammalian Genome* 1997;8:731-735.

204. Deng AY. Determining quantitative trait loci for blood pressure in thwe rat-implication for human genetic hypertension. In: Dragani TA, ed. *Human Polygenic Diseases : Animal Models*. Harwood Academic Publishers; 1998:81-111.

205. Mills PA, Huetteman DA, Brockway BP, Zwiers LM, Gelsema AJM, Schwartz RS, Kramer K. A new method for measurement of blood pressure, heart rate, and activity in the mouse bt radiotelemetry. *Journal of Applied Physioogy* 2000;88:1537-1544.

206. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature* 2002;418:935-941.

207. Lemmer B, Mattes A, Bohm M, Ganten D. Circadian blood pressure variation in transgenic hypertensive rats. *Hypertension* 1993;22:97-101.

208. Mohri T, Emoto N, Nonaka H, Fukuya H, Yagita K, Okamura H, Yokohama M. Alterations of Circadian Expression of Clock Genes in Dahl Salt-Sensitive Rats Fed a High-Salt Diet. *Hypertension* 2003;42:189-194.

209. Li P, Morris M, Ferrarion CM, Barrett C, Ganten D, Callahan MF. Cardiovascular, endocrine, and body fluid-electrolyte responses to salt loading in mRan-2 transgenic rats. *American Journal of Physiology* 1998;275:H1130-H1137.

210. Monti J, Plehm R, Schulz H, Ganten D, Kreutz R, Hubner N. Interactions between blood pressure quantitative trait loci in rats in which trait variation at chromosome 1 is conditional upon a specific allele at chromosome 10. *Human Molecular Genetics* 2003;12:435-439.

211. Deng AY. Functional Genomics of Blood Pressure Determination: Dissecting and Assembling a Polygenic Trait by Experimental Genetics. *Current Hypertension Reviews* 2005;1:35-50.

212. Safar ME, London GM, Asmar R, Frohlich ED. Recent advances on large arteries in hypertension. *Hypertension* 1998;32:156-161.

213. Safar ME, Boudier HS. Vascular development, pulse pressure, and the mechanisms of hypertension. *Hypertension* 2005;46:205-209.

214. Bunemann M, Lee KB, Pals-Rylaarsdam R, Roseberry AG, Hosey MM. Desensitization of G-protein-coupled receptors in the cardiovascular system. *Annual Reviews of Physiology* 1999;61:169-192.

215. Smith RD, Baukal AJ, Zolyomi A, Gaborik Z, Hunyady L, Sun L, Zhang M. Agonist-induced phosphorylation of the endogenous AT1 angiotensin receptor in bovine adrenal glomerulosa cells. *Molecular Endocrinology* 1998;12:634-644.

216. Smith RD, Hunyady L, Olivares-Reyes JA, Mihalik B, Jayadev S, Catt KJ. Agonist-induced phosphorylation of the angiotensin AT1A receptor is localized to a serine/threonine-rich region of its cytoplasmic tail. *Molecular pharmacology* 1998;54:935-941.

217. Griendling KK, Lassègue B, Alexander RW. Angiotensin receptors and their therapeutic implications. *Annual Reviews of Pharmacology and Toxicology* 1996;36:281-306.

218. Schinke M, Baltatu O, Bohm M, Peters J, Rascher W, Bricca G, Lippoldt A, Ganten D, Bader M. Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proceedings of the National Academy of Science of the United States of America* 1999;96:3975-3980.

219. Olofsson P, Holmberg J, Tordsson J, Lu S, Akerstrom B, Holmdahl R. Positional identification of Ncf1 as a gene that regulates arthritis severity in rats. *Nature Genetics* 2003;33:25-32.

220. Yokoi N, Komeda K, Wang H-Y, Yano H, Kitada K, Saitoh Y, Seino Y, Yasuda K, Serikawa T, Seino S. Cblb is a major susceptibility gene for rat type 1 diabetes mellitus. *Nature Genetics* 2002;31:391-394.

221. Aitman T, Gotoda T, Evans AL, Imrie H, Heath KE, Trembling PM, Truman H, Wallace CA, Rahman M, Doré C, Flint J, Kren V, Zidek V, Kurtz TW, Pravenec M, Scott J. Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nature Genetics* 1997;16:197-201.

222. Aitman T, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St-Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nature Genetics* 1999;21:76-83.

223. Aitman T, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St-Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nature Genetics* 1999;21:76-83.

224. Pravenec M, Landa V, Zidek V, Musilova A, Kren V, Kazdova L, Aitman T, Glazier AM, Ibrahimi A, Abumrad NA, Qi N, Wang JM, St-Lezin E, Kurtz TW. Transgenic rescue of defective Cd36 ameliorates insulin resistance in spontaneously hypertensive rats. *Nature Genetics* 2001;27:156-158.

225. Pravenec M, Wallace C, Aitman T, Kurtz TW. Gene Expression Profiling in Hypertension Research. *Hypertension* 2003;41:3-8.

226. Stoll M, Cowley AW, Tonellato PJ, Greene AS, Kaldunski ML, Roman RJ, Dumas P, Schork NJ, Wang Z, Jacob HJ. A Genomic-System Biology Map for Cardiovascular Function. *Science* 2001;294:1723-1726.

227. Sivo Z, Malo B, Dutil J, Deng AY. Accelerated congenics for mapping of two blood pressure quantitative trait loci on chromosome 10 of Dahl rats. *Journal of Hypertension* 2001;20:45-53.

228. Morel L, Blenman KR, Croker BP, Wakeland EK. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proceedings of the National Academy of Science of the United States of America* 2001;98:1787-1792.

229. Deng AY, Dene H, Rapp JP. Congenic Strains for the Blood Pressure Quantitative Trait Locus on Rat Chromosome 2. *Hypertension* 1997;30:199-202.

230. Rapp JP, Wang S-M, Dene H. Effect of genetic background on cosegregation of renin alleles and blood pressure in Dahl rats. *American Journal of Hypertension* 1990;3:391-396.

231. Eliopoulos V, Dutil J, Deng Y, Grondin M, Deng AY. Severe hypertension caused by alleles from normotensive Lewis for a quantitative trait locus on chromosome 2. *Physiological Genomics* 2005;22:70-75.

232. Alemayehu A, Breen L, Krenova D, Printz MP. Reciprocal rat chromosome 2 congenic strains reveal contrasting blood pressure and heart rate QTL. *Physiological Genomics* 2002;10:199-210.

233. Samani NJ, Gauguier D, Vincent M, Kaiser MA, Bihoreau M-T, Lodwick D, Wallis R, Parent V, Kimber P, Rattray F, Thompson JR, Sassard J, Lathrop M. Analysis of quantitative trait loci for blood pressure on rat chromosome 2 and 13. Age-related differences in effect. *Hypertension* 1996;28:1118-1122.

234. Garrett MR, Rapp JP. Multiple blood pressure QTL on rat chromosome 2 defined by congenic Dahl rats. *Mammalian Genome* 2002;13:41-44.

235. Schork NJ, Krieger JE, Trolliet JE, Franchini KG, Koike G, Krieger EM, Lander ES, Dzau VJ, Jacob HJ. A biometrical genome search in the rats reveals the multigenic basis of blood pressure variation. *Genome Research* 1995;5:164-172.

236. Dubay C, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi JP, Kotelevtsev YV, Beckmann JS, Soubrier F, Sassard J, Lathrop M. Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nature Genetics* 1993;3:354-357.

237. Jeffs B, Negrin DC, Grahams D, Clark JS, Anderson NH, Gauguier D, Dominiczak AF. Applicability of a 'Speed' Congenic Strategy to Dissect Blood Pressure Quantitative Trait Loci on Rat Chromosome 2. *Hypertension* 2000;35:179-187.

238. Phillips MI. Is Gene Therapy for Hypertension Possible? *Hypertension* 1999;33:8-13.

239. Miller JA, Thai K, Scholey JW. Angiotensin type I receptor gene polymorphism predicts response to losartan. *Kidney International* 1999;56:2173-2180.

Annex

Annexe I : Description de la contribution aux articles à auteurs multiples

Julie Dutil
[redacted]

Programme de Biologie Moléculaire
Faculté des Études Supérieures
Pour l'obtention de: Ph.D. Biologie Moléculaire

Chapitre 3
Dutil J, Deng A Y.
Mapping a blood pressure quantitative trait locus to a 5.7 cM region in Dahl salt sentitive rats.
Publié Mammalian Genome 12 : 362-365 (2001).

Chapitre 4
Dutil J, Deng A Y.
Further chromosomal mapping of a blood pressure QTL in Dahl rats on chromosome 2 using congenic strains.
Publié Physiological Genomics 6 :3-9 (2001).

Chapitre 5
Dutil J, Eliopoulos V, Tremblay J, Hamet P, Charron S, Deng A Y.
Multiple quantitative trait loci for blood pressure interacting epistatically and additively on Dahl rat chromosome 2.
Publié Hypertension 45: 557-564 (2005).

À titre d'auteure principale de l'article identifié ci-dessus, ma contribution inclue : la planification des expériences, les manipulations ayant permis d'obtenir les résultats présentés, l'analyse statistique, la préparation des figures, l'interprétation des résultats.

Chapitre 6
Dutil J, Eliopoulos V, Marchand È-L, Devlin A M, Tremblay J, Prithiviraj K, Hamet P, Migneault A, deBlois D, Deng A Y.
A quantitative trait locus for aortic smooth muscle cell number acting independently of blood pressure: implicating the angiotensin receptor AT1B gene as a candidate.
Publié Physiological Genomics 21: 362-369 (2005).

L'article identifié ci-dessus a été produit en collaboration avec l'équipe du Dr Denis deBlois. Ma contribution inclue : la planification des expériences, les manipulations ayant permis d'obtenir les résultats présentés (excluant les études fonctionnelles du récepteur de l'angiotensine 1B), l'analyse statistique (excluant les études fonctionnelles du récepteur de l'angiotensine 1B), la préparation des figures, l'interprétation des résultats.