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High resolution substitution mapping for genetic elements controlling blood pressure located on rat chromosomes 5 and 10

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A Dissertation

High resolution substitution mapping for genetic elements controlling blood pressure located on rat chromosomes 5 and 10.

By

RESMI PILLAI

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

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APRIL 2015
An Abstract of

High resolution substitution mapping for genetic elements controlling blood pressure located on rat chromosomes 5 and 10.

By

Resmi Muralidharan Pillai

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Degree in Doctor of Philosophy

The University of Toledo

APRIL 2015

Changes in blood pressure from high to low are genetically and environmentally controlled. In humans, linkage analysis and GWAS has pointed out to various regions on human chromosome 17 that are linked to blood pressure regulation. Linkage studies in particular, also document that the inheritance of complex traits is not always as a result of mutation/s within several or single genes but may also be due to interactions within genetic determinants. To study this complex aspect of blood pressure regulation, rat models are used. Linkage analysis in rats has led to identification of large segments of chromosome 5 and 10 containing large number of candidates. Hence substitution mapping is used to generate and test progressively shorter genomic segments within congenic strains and thereby facilitate the examination of smaller number of candidates.

Substitution mapping study on rat chromosome 5 shows that epistasis plays a major role in blood pressure regulation and missing this aspect in GWAS study can lead to missing of those determinant in human studies that do not play a direct role in regulation of blood pressure.
Additionally, mapping studies on rat chromosome 10 which shows homology to human chromosome 17, has been used to demonstrate that there could be novel regions on human chromosome 17 currently annotated as gene deserts but have not yet been identified as containing determinants of blood pressure regulation.

The two studies are examples that animal models of hypertension are tools to help find variations on the human genome that are relevant in regulation of complex traits like blood pressure.
To my Parents

Who believed in me at every stage of life

To my son

Who taught me to have fun in whatever I do, however hard it maybe and
To enjoy and cherish the little moments in life for it will be gone too soon
Acknowledgements

I would like to gratefully and sincerely thank Dr. Bina Joe for her guidance, understanding and patience during my graduate studies at University of Toledo. Her mentorship and encouragement helped me grow as an independent thinker and scientist. For everything you’ve done for me, Dr. Joe, I thank you. I would also like to thank all of the members of the Joe research team- Harshal, Xi, Ying, Eric, Blair, Kris and Siva for providing for the help during my time in the lab. I also want to thank the DLAR staff, Department of Physiology and Pharmacology and graduate school for all the support.

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List of Abbreviations

BP ..............................Blood Pressure
SBP ..............................Systolic Blood Pressure
DBP ..............................Diastolic Blood Pressure
PP ..............................Pulse Pressure
MAP ..............................Mean Arterial Pressure
mm Hg ...........................milliliter of mercury
HR ..............................Heart Rate
QTL ..............................Quantitative trait loci
QTN ..............................Quantitative Trait Nucleotide
QTG ..............................Quantitative trait Gene
LEW ..............................Lewis rat strain (normotensive)
S ................................Dahl salt sensitive S rat
MNS ..............................Milan Normotensive Strain
SHR ..............................Spontaneously Hypertensive Rats
SHRSP ............................Spontaneously Hypertensive Rats Stroke Prone
WKY ..............................Wistar Kyoto Rats
HSA ..............................Homo sapiens (Human chromosome nomenclature)
MMU ..............................Mus musculus (mouse)
kb ..............................kilo bases
bp ..............................Base pair
Mb ..............................Mega bases
cM ..............................centi Morgan
UPE ..............................Urine Protein Excretion
FBW ..............................Final Body Weight
RHW ..............................Relative Heart Weight
TKW ..............................Total Kidney Weight
ACE ..............................Angiotensin-converting enzyme
ARB ..............................Angiotensin II Receptor Blockers
CCB ..............................Calcium Channel Blockers
SNP ..............................Single-Nucleotide Polymorphisms
INDELS ..........................Insertions and DELetions
CNV ..............................Copy Number Variations
LOD ..............................Logarithm Of Odds
GWAS ............................Genome Wide Association Studies
WTCC ...........................Wellcome Trust case consortium
HapMAP ..........................Haplotype MAP
List of Symbols

~ Approximately

β Beta

> Greater than

< Less than

= Equal to
Chapter 1

Literature review

1.1 Blood Pressure

Blood pressure or arterial blood pressure is measured by the force or pressure exerted by blood on the walls of the arteries and is expressed as millimeters of mercury (mmHg). It is calculated as the product of cardiac output (volume of blood pumped by each ventricle per minute) and total peripheral resistance (the overall resistance to blood flow through the systemic blood vessels). At the basic level, BP is controlled by only two variables following Ohm's law: peripheral vascular resistance (influenced by factors like arterial wall modifications due to ageing, medial hypertrophy due to hyperinsulinaemia or obesity) and blood flow (modified by, e.g. increased fluid volume due to increased salt intake) [1]. Changes in blood pressure are physiologically done to direct appropriate amounts of oxygen and nutrients to specific parts of the body based on specific needs. There are two mechanisms for regulating BP:

1) Short term mechanism which involves activation/inactivation of baroreceptors in the large arteries like carotid sinus and aortic arch in response to an increase/decrease of BP. The baroreceptors signals the brain to decrease/increase
sympathetic activity and increase/decrease parasympathetic activity causing the heart rate to decrease/increase and arterial diameter to increase/decrease leading to lowering/rise in BP. Short term lowering of osmolarity by dehydration signals the thirst centers in the brain to increase the intake of water thus restoring osmolarity and rehydrate and maintain blood volume thus restoring BP.

2) Long term mechanism- This is done by regulating blood volume in the body after blood loss through hemorrhage, accidents or blood donation leading to lower BP. The juxtaglomerular cells in the kidney recognize the failing BP and releases renin into the bloodstream. Renin causes angiotensin II to be released from angiotensin I and angiotensin II causes release of aldosterone from the adrenal glands located above the kidney. Angiotensin II is also a powerful vasoconstrictor and helps to increase BP by itself. Aldosterone leads to increased sodium and water reabsorption in the renal tubules which inturn leads to increased BP. Incase of excessive dehydration, an increase in blood osmolarity and decrease in BP, the hypothalamus signals the posterior pituitary to release antidiuretic hormone (ADH) that increases reabsorption of water in the kidney by increasing the number of water channels in the distal convoluted tubules and collection duct. This leads to an increase in blood volume and hence an increase in BP. Fluctuations in blood pressure are normal and the body functions to handle it effectively. But when there is persistently low or high blood pressure, it is called as hypotension or hypertension respectively. Hypotension is when the blood pressure readings are lower than 90 mm Hg systolic or 60 mm Hg diastolic or normal systolic
but low diastolic. It can cause dizziness, weakness and a risk of injury from falls. Severe forms of hypotension can cause the brain to receive an inadequate supply of blood and oxygen to carry out its normal functions, leading to damage to your heart and brain. Since hypertension is more common and dangerous it is discussed in detail below.
1.2 Hypertension

In as many as 95% of reported high blood pressure cases in the U.S., the underlying cause cannot be determined. High BP in which secondary causes like renal failure, renovascular disease, aldosteronism or other causes are not present is called primary, idiopathic or essential hypertension. In adults, it is diagnosed when the average of two or more diastolic BP measurements on at least two subsequent visits is >90 mmHg or when the average systolic BP of two or more subsequent visits is >140 mmHg. Isolated systolic hypertension is defined as systolic BP of >140 mmHg and diastolic BP of <90 mmHg. This definition is given in the sixth report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High BP (JNC IV) [2].

There is a strong correlation between high blood pressure and cardiovascular disorders like stroke, myocardial infarction, renal disease and death. In America, 67 million adults (31%) have high blood pressure but only 47% of this population have it under control and management of high blood pressure costs the nation $47.5 billion each year. The factors that influence high BP are [3]:

1. Age and Gender- In an age group of individuals < 45 years old, hypertension is more prevalent in males than females. For people 65 years old or older, high blood pressure affects more women than men.

2. Race- The incidence of high BP is highest among African Americans (43% in males and 45.7% in females) and lowest among the white population (33.9% in males and 31.3% in females).
3. Lifestyle and socioeconomic status- Lack of physical exercise, tobacco and alcohol consumption, unhealthy diet and obesity.

4. Geographic patterns- Hypertension is more prevalent in the southeastern parts of the United States [2].

**Different forms of Hypertension:**

There are two major types of hypertension and four less frequent ones:

1. Primary or essential hypertension- It is diagnosed in the majority of the patients and there is no known cause. It may tend to develop gradually over the years.

2. Secondary hypertension- It is often caused due to various conditions which may include: Kidney diseases, adrenal gland tumors, thyroid imbalances, congenital defects of the blood vessels, certain prescription drugs and birth control pills, alcohol and/or drug abuse and sleep apnea.

3. Malignant hypertension- It is severe and progressive and may lead to organ damage unless properly treated.

4. Isolated systolic hypertension- in this case the BP is consistently above 160 mm Hg, and the diastolic below 90 mm Hg. It usually occurs in older people, and results from the age-related stiffening of the arteries mostly due to arteriosclerosis.

5. White coat hypertension- Also known as anxiety-induced hypertension. The high BP develops only when tested by a health professional and may not need any treatment.
6. Resistant Hypertension- If BP is not reduced to below 140/90 mmHg despite a triple-drug regime, resistant hypertension is considered.

**Essential hypertension: what is known?**

Essential hypertension has its roots in two important factors: Genetic factors that determine predisposition to an increase in blood pressure and extrinsic factors like high salt intake, alcohol intake, obesity, stress, low calcium and potassium in diet, insulin resistance, age and sedentary lifestyle. So, if an individual inherits normal BP and adds one or more of the extrinsic factors, the BP rises but stays within normal range. If an individual has inherited high BP but within the normal range and adds extrinsic factors, he may increase his BP to the hypertensive stage. In addition increasing the number of extrinsic factors to a hypertensive genetic background may increase the persons BP to a severely hypertensive range. Knowing the contributing extrinsic factors can help control or correct the BP increase. Hence, in a constant or ideal environment, determination of the genetic factors must be done in order to control the BP increase in a patient to prevent contributing risk factors [2, 4].
1.3 Current treatment of hypertension

Changes in lifestyle- Lowering sodium and alcohol intake, keeping weight in the ideal range, engaging in regular aerobic exercise, and ending smoking can help some people to control high blood pressure. Reduction in sodium intake (less than 2.3 grams per day), can also work effectively as therapy along with one blood pressure-lowering drug [5-9]

The current medications include [10-13]:

Diuretics- Causes the kidneys to excrete more sodium and water, reducing fluid volume throughout the body and dilates blood vessels. The common diuretic used is thiazides and in some cases, a potassium supplement or a potassium-sparing diuretic is given in combination with a thiazide diuretic to counter the potassium deficiency caused due to its increased excretion in the urine [14-17].

ACE inhibitors- Angiotensin II causes the blood vessels to constrict and BP to increase. Angiotensin-converting enzyme (ACE) inhibitors block production of the hormone, angiotensin II and allow blood vessels to dilate and helps to lowers blood pressure [18-21].

Angiotensin II receptor blockers- The angiotensin II receptor blockers (ARBs) block the effects of angiotensin II on cells in the heart and blood vessels. This helps in dilating blood vessels, lower blood pressure, and improve heart output [20, 21].

Calcium channel blockers- CCBs block the calcium channels and limit the amount of calcium that enters the smooth muscle in blood vessel walls and heart muscle. They reduce contraction of vascular smooth muscle and reduce contraction causing vasodilation. In the
myocardium they reduce force of contraction and also slow the conduction of electrical activity within the heart causing slowing down of heart beat. By blocking the calcium signal on adrenal cortex cells, they directly reduce aldosterone production, and hence lower blood pressure [18, 19, 21].

**Beta blockers**- Beta blockers block some of the effects of the sympathetic nervous system—endogenous catecholamine epinephrine (adrenaline) and norepinephrine (noradrenalin), which helps in increasing the heart rate and raising blood pressure during a fight or flight response. This is done by decreasing the rate and force at which the heart pumps blood [22-24].

**Alpha blockers** — Alpha blockers relax or reduce the tone of involuntary vascular smooth muscle in the walls of blood causing vasodilation, thereby lowering blood pressure [25].

**Direct vasodilators** — Direct vasodilators relax or reduce the tone of blood vessels but are used only in case of severe or resistant high blood pressure [26] [27].

Since hypertension is a multifactorial disease, treating all hypertensive patients using the same drugs or similar cocktail of drugs may not often result in the control of BP. This is because the reason for development of hypertension is different in different individuals as is the genetic variants responsible for this change in BP. This idea of personalized medicine is not very new and has been published in a JNC report in 1977[28]. Personalized medicine refers to the use of diagnostic and screening methods that use the knowledge of the patient’s unique molecular and risk profile to improve the management of the patient’s disease or
predisposition towards it. The ability and availability of the tools to characterize the individual differences at the molecular level has made this concept more realistic. [28-31]
1.4 Genetics of hypertension

Although much is known about BP regulation and the environmental factors regulating it, the intrinsic origin of essential hypertension remains obscure [32]. The genetic influence on a trait like BP is expressed in terms of heritability and is measured as the fraction of the total interindividual variability attributed to the genes that is inherited [33]. Previous epidemiological and familial studies and history of affected individuals along with disease inheritance rate in twins has established that there is a complex interaction between both genetic and environmental factors that determine susceptibility to hypertension [34-36]. Hypertension is about twice as common in subjects who have one or two hypertensive parents [37]. The heritability of hypertension is often cited in the range of 30% - 60%, with multiple contributory genes. This of course should not be taken to imply that the remainder is environmental, as gene–environment interactions are essential yet irresolvable part of the variability in the development of hypertension. Other factors like ethnicity and genetic heterogeneity add to the variability in clinical presentation and drug response in hypertension making the genetic study of this disease challenging [36, 38, 39].

It is known that genetic variants play an important role in the origin of essential hypertension reflecting the characteristics of the complex genetic trait that BP is known for. The variations in the genome can be single-nucleotide polymorphisms (SNPs) and consists of two or rarely more different possible nucleotide bases (alleles) at the same genetic position [40]. Other types of variation of the human genome are insertions and deletions (INDELS), structural variation (Copy Number Variations CNV), or epigenetic
modification [41-44]. The variations can be tested by genotyping for the variations and sequencing.

**Hypertension as a monogenic trait**

Certain studies have shown that hypertension maybe inherited as a simple Mendelian trait. Such inheritance patterns may indicate the presence of monogenic forms of hypertension that occurs in rare families are due to rare variants with a large effect [45]. Early onset at a relatively young age or early adolescence may indicate inheritance of a dominant condition while consanguinity (inheritance in first cousins) may indicate recessive inheritance. All monogenic forms of hypertension are marked by specific phenotypic features, such as electrolyte and hormonal abnormalities like suppression of the renin–angiotensin system (low plasma renin level or activity) due to expansion in plasma volume. In addition to hyporeninemia, hypouricemia (decreased uric acid in blood serum by reducing its proximal tubular reabsorption) and hypokalemia are also present [33, 46-48]. Table 1.1 suggests the molecular basis for known monogenic hypertension syndromes [49-63]. Hence, the lesson from monogenic hypertension research is that the 12 genes are members of only two groups of pathways: renal sodium handling and steroid hormone metabolism, including mineralocorticoid receptor activity and most of the genetic abnormalities are present in the proteins related to the kidney and steroid hormone activity. But these rare variants near genes implicated in monogenic hyper- and hypotension and are very low in frequency in the general population and therefore unlikely to explain much of the BP variation in the general population and therefore hypertension. Our findings in
Mendelian forms of hypertension genes do, therefore, not appear to significantly help in the understanding of the pathogenesis of essential hypertension [45].
Table 1.1- Mendelian forms of hypertension - Different known heritable forms of arterial hypertension

<table>
<thead>
<tr>
<th>Monogenic syndrome</th>
<th>Mode of inheritance</th>
<th>Gene mutations</th>
<th>Location in chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liddle’s syndrome (pseudoaldosteronism)</td>
<td>Dominant</td>
<td>SCNN1B or SCNN1G</td>
<td>16p</td>
</tr>
<tr>
<td>Apparent mineralocorticoid excess (AME)</td>
<td>Recessive</td>
<td>11β-HSD-2</td>
<td>16q</td>
</tr>
<tr>
<td>Congenital adrenal hyperplasia- 11-β and 17-α hydroxylases type</td>
<td>Recessive</td>
<td>Nonfunctional hydroxylases CYP11B1 and CYP17A</td>
<td>8q and 10q</td>
</tr>
<tr>
<td>Glucocorticoid remediable aldosteronism (GRA)</td>
<td>Recessive/Dominant</td>
<td>CYP11B1 (11β-hydroxylase)/CYP11B2 (aldosterone synthase)</td>
<td>8q/8p</td>
</tr>
<tr>
<td>Gordon’s syndrome (Pseudohypoaldosteronism type II)</td>
<td>Dominant</td>
<td>WNK1 / WNK4</td>
<td>12p / 17q</td>
</tr>
<tr>
<td>Familial hyperkalemic hypertension</td>
<td></td>
<td>KLHL3 / CUL3</td>
<td>5q / 2q</td>
</tr>
<tr>
<td>Early-onset autosomal dominant HTN with exacerbation in pregnancy</td>
<td>Dominant</td>
<td>NR3C2</td>
<td>4q</td>
</tr>
<tr>
<td>Familial hyperaldosteronism type III</td>
<td>Dominant</td>
<td>KCNJ5</td>
<td>11q</td>
</tr>
</tbody>
</table>
Hypertension as a polygenic trait

The nature of hypertension has been debated by Platt and Pickering where Platt thought that hypertension was a simple disease caused by a single genetic defect that was autosomal dominant and inherited. He thought that the distribution was bimodal indicating that hypertensives were a distinct subpopulation in humans [64]. Some familial forms of hypertension are inherited as single-gene disorders, but they are a small proportion of the total cases. In contrast, Pickering's thought that blood pressures varied constantly and shows continuous and unimodal distribution. This model of multiple susceptibility genes along with environmental interaction is one that can be applied to other common diseases including asthma, diabetes and ischaemic heart diseases. Therefore Pickering’s view of polygenic basis for BP in the general population is widely accepted and is the basis of current understanding and treatment policies [33, 65, 66].

Since BP is a continuous variable [67] and the effect of a single factors like one gene may be small, the current task is to identify variations in genetic elements like protein coding genes, protein noncoding elements, regulatory elements that may independently or in combination modulate BP.
1.5 Human genetic studies of hypertension

The nature of essential hypertension has always been elusive since it was first described in 1877. Historically in human, the genetics of hypertension presents a stark contrast between the high heritability of blood pressure and hypertensive traits and the reality that no clearly reproducible genetic variant for essential hypertension could be found [68]. The methods used to find the genetic elements responsible for changing blood pressure or hypertension have been 1) Candidate gene approach, 2) Genome wide linkage studies and 3) Genome wide association studies (GWAS)

**Candidate Gene Approach** - Several efforts have been made to study the genes linked to hypertension and the candidate gene approach is one such method. The method uses polymorphisms in a subset of genes that have previously been studied to play a role in blood pressure regulation. The limited number of polymorphisms leads to detection of small effects using a moderate sample size. The drawback of this method is that it ignores epistasis (for example, Pascoe et al [69] provided evidence of intragenic interactions in the aldosterone synthase gene where subjects with two missense mutations show elevated serum ratio of 18-hydroxycorticosterone to aldosterone while those with only one mutation were asymptomatic), gene environment interaction (for example significant BP interactions were found in a study where physically active subjects who had SNPs in MR, SCNN1B, APLNR, GNB3 and BDKRB2 showed a reduction of upto 8mm Hg in systolic BP and 5mm Hg in diastolic BP compared to inactive individuals who carry the same variants [69-75] and rare variant effects [76, 77]. This may lead to inconsistent findings and associations that are not well replicated across studies [78-80]. Another disadvantage
of the method is that the proportion of the genes that are associated with a particular trait is small and hence the list of candidate genes is limited [81].

**Genome Wide Linkage studies** - On the human genome, two loci are linked because of their physical connection on the piece of DNA and if they are close to each other, the alleles tend to cosegregate within families. Such cosegregating haplotypes are broken up by recombination. If two loci are close to each other, the probability of a recombination between two loci becomes low, conversely, recombination occurs more frequently between loci that are far apart. Thus, recombination is a function of the distance between the two loci, although not a linear relationship. The search for genes related to a disease trait begins with linkage analysis. The aim of the method is to determine the approximate location of a gene using DNA sequences called genetic markers that show polymorphism and whose position is already known. This is illustrated in Figure 1-1.

The principle of linkage analysis is that in the case of a diseased gene, one of the two alleles (either from the mother or father) will contain the disease allele. The alternate allele will be normal. During meiosis when recombination occurs either the paternal and maternal alleles will mix up due to the large distance between them (see A and C in Figure1-1) or they can recombine and stay together (see A and B in the Figure1-1). Hence the diseased genes can be mapped by measuring recombination against a series of different genetic markers spread over the genome. If the marker and the diseased gene lie in proximity to each other, they will not recombine and hence is said to be linked to it. Ideally, those genetic markers are identified that flank the disease gene and hence define a candidate region of the genome [82].
Figure 1-1 Chromosomal recombination between the maternal (red) and paternal (blue) during meiosis. The three points A, B, C and a, b, c are DNA markers on the paternal and maternal alleles respectively. The recombined chromosome is then passed on to the offsprings. If A is the diseased gene and B and C are the DNA markers, then recombination is most likely to occur between A and C rather than A and B. Hence we can use the two markers B and C to map the relative position of A.

Adapted with permission from http://genome.wellcome.ac.uk
Genetic linkage analysis is a statistical method that is used to associate functionality of genes to their location on chromosomes. Linkage is expressed as a statistic, LOD score (Z); a “logarithm of odds” [83].

\[
\text{LOD score} = \log_{10} \frac{\text{Likelihood of Linkage of loci}}{\text{Likelihood that loci are unlinked}}
\]

The LOD scores are calculated at many selected points in an interval between markers and plotted versus the map position. The term Quantitative Trait Loci is used to describe a broad region on the chromosome that may contain one or more loci controlling or participating in controlling the quantitative trait like BP. The QTL effect enters the likelihood equations through the observed BP data and the QTL map position.

Positive values of Z suggest that two loci are linked and negative values suggest that two loci are not linked. By convention, a LOD score of +3 or greater is considered evidence that two loci are linked. A LOD score below -2 excludes linkage [84] The LOD scores can then be used to obtain the LOD plot. The evidence of association between an allele and a phenotype can be due to: 1) The allele can directly affect the phenotype because it is functional 2) the given allele is correlated or is in linkage disequilibrium with the neighboring causative allele or 3) The association with the trait could be an artifact or coincidence.

Association studies are usually performed in a case control cohort format ensuring that the controls are true controls and dividing the population into subgroups play a
major role [85-87]. The British Genetics of Hypertension (BRIGHT), one of the largest genome wide linkage scans reported a QTL on the end of chromosome 6 [88] and on chromosome 5 [89] in sibling pairs of white British ancestry. The metabolic syndrome factor scores were derived in 4 ethnic groups recruited in 3 Networks of the Family Blood Pressure Program (FBPP): GENOA (blacks, Hispanics, and whites), HyperGEN (blacks and whites), SAPHIRE (Asians). Results from HyperGEN reported a locus on chromosome 2 in the African American sibpairs [90] and GenNet reported a Significant QTL on chromosome 1 which was later reproduced by GENOA and HyperGEN [91-94].

Multiple linkage studies have reported QTLs linked to BP on human chromosome 17. In 1997, Cécile Julier et. al studied a panel of 357 French and UK families containing 384 sibships with one or more hypertensive offsprings and a total of 518 affected sib-pairs and found a strong support for linkage at marker D17S183 [95]. In 1999 Xu et.al. did a blood-pressure screening of >200,000 Chinese adults, sib pairs and found a BP associated QTL with a LOD score of 2.16 near the marker D17S1303 (GATA64B04) [96]. In 2000, Rice et al conducted the Que ´bec Family study consisting of 206 families including 335 sibpairs and found nominal probability values on human chromosome 17 in the region of 17q21.33-17q21.2 [97]. Similarly, using a 10-cM density genome-wide scan in participants from the Framingham Heart Study, Levy et.al found significant evidence of linkage of longitudinal systolic BP to chromosome 17q12–21, at markers GATA25A04 (D17S1299) and ATC6A06 [D18S481] with a LOD score of 4.7 [98, 99]. Further, Rutherford et.al in a study using 177 affected adult Caucasian sibpairs of British descent living in Australia, reported a BP QTL on
chromosome 17 between markers D17S949 (68465676) and D17S799 (13,170,963) [100]. A BP QTL was located on chromosome 17q23.1 with a peak located at the marker GATA49C09 (D17S1290) in the hispanic population in the GENOA study. HyperGEN shows a lower LOD scores for linkage to BP around the same region in the Chinese and Japanese as well as the white population. A1-lod score interval around the linkage peak for candidate genes indicates the Angiotensin I Converting Enzyme (ACE) gene as the probable candidate [101, 102]. To study the genetic factors and to localize sex-specific BP quantitative trait loci (QTL), Franceshini et.al conducted a genome scan using American Indian participants of the Strong Heart Family Study. Systolic blood pressure of both genders were collected separately and combined for the study. A suggestive linkage was observed in a QTL influencing systolic blood pressure on chromosome 17 at 129 cM between markers D17S784 (77802182) and D17S928 (80253028). When accounting for QTL-specific genotype-by-sex interaction, women showed and improved the LOD score of 3.3 for systolic blood pressure on chromosome 17 at 136 cM [103].

All these studies provide meaningful data in linking locations on the genome with a phenotype but the disadvantage of such a method is that the region between the two markers showing linkage is very large and it is assumed that every nucleotide in that region influences BP which may not be true. Another disadvantage is that the study linking a particular phenotype is very specific to the population it was studied on and is difficult to be replicated in another population.

The 10th birthday of the human genome project was celebrated in April 2010 [104, 105]. Technologic advances now permit the genotyping of hundreds of thousands to
more than a million SNPs on a single microarray at a reasonable cost that help to interrogate large proportions of the genome \[106, 107\]. Association of these polymorphisms to GWAS a disease trait like hypertension can help to obtain unbiased data of the genetic causes and hence can be considered a direct application of the human genome project (candidate gene analysis, linkage analysis) and HapMAP project which eventually leads to new methods of preventing, diagnosing, and treating diseases \[105, 108-110\].

**Genome wide association studies (GWAS)** - One of the first traits to be studied using GWAS is hypertension. The first large collaborative study for hypertension, the Wellcome Trust case consortium (WTCC) reported the first GWAS results in 2007 \[111\]. The study consisted of 2000 cases and 3000 shared controls for 7 complex human diseases including hypertension. However none of the SNPs that was associated with BP were present on human chromosome 17 \[111\]. In a study done by Sabatti et. al., no genetic variants were found to be associated to blood pressure \[112\]. In 2009 Christopher Newton-Cheh et. al, formed the Global Blood Pressure Genetics (Global BPgen) consortium and conducted meta-analysis of GWAS in 34,433 individuals of European ancestry with SBP and DBP measurements followed by genotyping and in-silico analysis. The results of the joint analysis in up to 134,258 individuals of European ancestry along with reports obtained from the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) consortium indicate genome wide significant associations for SBP at SNP id rs12946454 on chromosome 17-17q21 with the genes *PLCD3* (Phospholipase C, Delta), *ACBD4* (Acyl-CoA binding domain containing 4), *HEXIM, HEXIM2* (encoding Hexamethylene bis-
acetamide inducible proteins 1 and 2) and FMNL1 (Formin-Like Protein 1) nearby, the SNP is in the intron of PLCD3 3 (Phospholipase C-delta isoform). The SNP, rs16948048 associated with DBP at 17q21 is upstream of ZNF652 (zinc finger protein 652) and PHB (prohibitin) [113-115]. Neither gene was previously implicated in hypertension or other cardiovascular phenotypes. A follow up study of 133,661 additional individuals of European descent added GOSR2 (Golgi SNAP Receptor Complex Member 2) (SNP id rs17608766) as being associated with SBP. The SNP rs16960228 located in the gene encoding PRKCA (Protein kinase C, alpha), in chromosome 17q24.3 is found to be significantly associated with diastolic BP response to hydrochlorothiazide treatment in the [116]PEAR (Pharmacogenomic Evaluation of Antihypertensive Responses Study) study of African Americans [117]. In a study published in 2014 by Franceschini et.al, three genes with multiple SNPS were marginally associated with SBP [TBC1D16 (TBC1 domain family, member 16), HRNBP3 (RAN Binding Protein 3) and AZI1 (5-azacytidine induced gene 1)] with AZI1 showing most prominence [118]. In a study published in 2014 by Simino et. al. using data from CHARGE, GBPgen, and ICBP from 99,241 individuals of European ancestry, they found two SNPs in the intron of NXN (Nucleoredoxin) gene (rs747685 and rs747687) that were associated with BP [119]. Recently Huan et al, conducted an association study of global gene expression levels in whole blood with BP traits (SBP, DBP, and hypertension) in six independent studies [116]. The study samples included: 3679 from the Framingham Heart Study (FHS), 972 from the Estonian Biobank (EGCUT), 604 from the Rotterdam Study (RS) [Division of Preventive Medicine, Brigham and Women's Hospital, 900 Commonwealth Avenue East, Boston MA 02215, USA], 597 from the InCHIANTI Study, 565 from the Cooperative Health Research in the Region of
Augsburg (KORA F4) Study [Harvard Medical School, Boston, MA, USA], and 600 from the Study of Health in Pomerania (SHIP-TREND) [Icelandic Heart Association, Kopavogur, Iceland] totaling 7017 individuals. The results speculate that KCNJ2 (Potassium Inwardly-Rectifying Channel) on HSA 17 may be important for BP regulation [116].

The findings of GWAS is promising in the field of blood pressure genetics and paves the way for subsequent investigations. Larger sample size can lead to greater power and help to find more variants involved in BP regulation. One major drawback of GWAS is that most of the published SNPs associated with diseases are not located in or near genes. They are located in regions where there are no annotated genes referred to as ‘gene deserts’. These regions previously referred to as ‘junk DNA’ are now known to be reservoirs for novel genes and regulators of distant genes. Another major drawback of GWAS is that the proportion of heritability explained by known variants indirectly assume that traits do not involve genetic interactions or epistasis and one can argue that high estimates of heritability have been misinterpreted. Such studies also do not give a clear answer to whether the variants are causal or only associated with the trait. The complexity of the complex traits is apparent from the range of factors that need to be considered as potentially contributing to the ‘missing heritability’. These can be rare variants whose significance is not yet recognized, less uncommon variants of small effect, or common variants of very small effect (very weakly penetrant) [120-125].

**Epistasis and Missing heritability** - Complex traits or diseases can involve one or multiple genetic elements that work independently, additively or interactively with one another to
contribute to the trait. Such genetic interactions are known as epistasis. Epistasis can be defined biologically as well as statistically. Biologically, epistasis refers to gene-gene interaction where the action of a gene is modified by one or many other genetic elements. Statistically, epistasis can be defined as interaction between variants at multiple loci and the total effect that contributes to the trait is the combination of variants at the different loci and this effect may differ considerably from the effect of a linear combination of individual loci. This can help to indicate genetic loci that have a biological interaction [126-128]. One example of genetic interaction is the genetic heritability of obesity in family studies which in human familial studies is \( \sim 40\% \), but in twin studies the number is higher (\( \sim 65\% \)) [129, 130]. This difference suggests that almost one-third of the heritable variance may be due to nonadditive genetic variance, including allelic and nonallelic gene interactions. In a study done by Dong et.al. the percentage fat mass analysis based on a two-locus epistatic model yielded significant evidence for interaction between chromosome 20q and the chromosome 10 centromere whose LOD score = 1.74; \( P=.024 \), compared with a two-locus additive model whose LOD = 0.90. The results indicate that epistatic interactions between loci in these regions play a role in obesity [129].

Candidate gene, Genome wide Linkage Studies, and GWAS are methods that have significantly increased our understanding of the genetic components underlying BP regulation. To complete the puzzle, focused studies considering epistasis, gene-environment interactions, and rare variants in systematic and biologically ways are required and are underway. Existing candidate gene and pathway studies can test epistasis and gene environment interactions using all available genotype and phenotype information even if no prior evidence linking them is available [131-135].
1.6 Animal models of hypertension and why the rat is a model of choice

The genetic analysis of complex human traits like hypertension and blood pressure has been less successful than the genetic dissection of mendelian traits. Even the results obtained by linkage and association studies cannot be replicated consistently [136, 137]. This is usually due to effects of environment variability, genetic heterogeneity and incomplete penetrance of the disease [138]. Analytical approaches using animal models have the potential to overcome some of the genetic and environmental complexities of human studies. Researchers in the 1960’s and 1970’s developed genetic models of hypertension by selectively breeding rats and mice [139-154]. The first animal model of hypertension was made when Harry Goldblatt clipped the renal artery of a dog [155]. Rat and mouse have been used as models to study the genetics associated with disease traits but the rat has been the model of choice for many researchers because not many inbred mouse models of hypertension have yet been developed. Mouse is an animal of choice for gene knockout and overexpression transgenic studies and experimental manipulations of physiological systems are very informative on a physiological level because they provide information on how quantitative variation in candidate genes influences BP in the context of the whole animal with all its regulatory and compensatory systems intact [145]. Inspite of this the mouse does not make a good model for the discovery of naturally recombining genes that regulate blood pressure. Mice are also not very useful in finding novel genetic variants or pathways that can lead to hypertension. The rat genome has approximately 2.75 billion base pairs and the mouse has 2.6 billion base pairs compared to the human genome that contains 2.9 billion base pairs and the rat genome also contains almost all the corresponding genes known to be associated with diseases in human and are highly
conserved through evolution confirming that rat models are excellent for medical research [156, 157]. The larger size helps with ease of invasive measurements and mechanistic studies and better characterized physiology in rats also make it a suited model to study the trait of hypertension. Besides spontaneous hypertension is hard to occur in mouse hence the candidate genes for hypertension seem to be relevant more in rats and since the study on rats started early, various genetic models have been used to study the etiology of hypertension, most of them being rat models [145, 158-160].

More than 50 years ago, Dahl et. al. selectively bred rats for sensitivity (S rats) and resistance (R rats) to study hypertensive effect of a high salt (NaCl) diet [161]. This further helped to develop inbred strains of S and R rats from Dahl’s selectively bred lines and were the first rat models for studying salt-induced hypertension [162]. Other inbred strains used are the normotensive Lewis (LEW) rat, the spontaneously hypertensive rat (SHR), DOCA-salt rats, Sabra hypertensive-prone rats, Milan, Lyon, fawn-hooded and Prague hypertensive rats [144, 163, 164]. These models provide a rapid and affordable way to identify and validate those novel candidate genes that play a role in the complex pathways that determine arterial pressure as predicted from human studies. Recently rat transgenic technology has been well established and assemblies that link genotype to phenotype is available. All this information not only helps in the initial ascertainment of QTLs, but also in narrowing down the QTL regions of interest. Rats provide a rapid and affordable way to identify and validate novel candidate genes that are predicted from human studies to have a role in the complex pathways that determine arterial pressure [144, 145, 159, 165-170]. One example of a success story is the identification of a BP QTL on rat chromosome 10 which is homologous to region on human chromosome 17 containing both a human QTL.
[95], and the locus for Gordon's syndrome [33]. Variation in 11 \( \beta \)-hydroxylase gene in Dahl rats and similarly in human is also an example of quantitative variation in a steroid biosynthetic pathway causing an increase in BP [171-174].

Rodent studies have also shown that inspite of similar levels of increased BP, the susceptibility to end organ damage varies highly among different rodent strains. This implies that the phenotype of end organ damage is also genetically determined and identifying the causative genes can better our approach to risk assessment in human hypertension [175].

**Congenic substitution mapping**

Linkage analysis provides evidence for large chromosomal segments containing multiple genes and other regulatory determinants that influence BP. These segments are too large for positional cloning. As we now know, BP alteration may not be due to a single genetic determinant but could also be due to interactions and hence the region directly under the peak of the LOD plot may not always be a precise indication of the location [176, 177]. Hence to shorten and resolve the large segment into smaller segments by natural recombination, congeneric strains and substrains must be constructed [139, 178].

Figure1-2 outlines the basic concept of making congeneric models. It involves moving of genetic segments along with the polymorphic marker gene from one inbred (donor) strain (indicated in blue) to another inbred (recipient) strain (indicated in red). The two strains are crossed, and the F1 heterozygotes are obtained. The F1 is then backcrossed to the recipient strain. The offspring will segregate to give a combination of homozygous and heterozygous strains in the ratio of 1 donor homozygous: 2 heterozygous:
A homozgyous recipient. Heterozygotes are selected and backcrossed again to the recipient strain and the procedure is repeated for at least eight cycles of backcrossing and now the two offspring homozygous are bred to fix the donor allele on the recipient background. During each backcross, 50% of the genetic background becomes progressively enriched for the recipient strain genes until the background is 99% recipient genes after the eighth backcross. But the donor genes linked to locus we are trying to introgress will be pulled along and the resulting congenic strain will have only the locus of interest flanked by donor chromosomal segment [145, 179].

The construction of congenic strains and backcrossing requires about 2.5–3 years of breeding to dilute out the unwanted donor genome outside the desired congenic region. The elimination of the donor genome can be expedited by selecting against donor strain alleles at markers strategically placed throughout the genome, at the same time one selects for donor alleles. This approach is also known as Marker Assisted Selection Protocols (MASPs) or speed congenic and utilizes genetic markers to facilitate the controlled introgression of genes within strains [180].
Figure 1-2 Diagram of the chromosomes as they appear in congenic strains constructed using the genomes of the normotensive LEW rat (blue) as a donor and a hypertensive S rat (red) as a recipient. The congenic genetic segment in the congenic strain is shown in blue. This strain will have short segments of the LEW rat genome introgressed onto the genetic background of the S genome. (Picture courtesy Dr. Bina Joe)
Defining quantitative trait loci for blood pressure

The genetic basis for variation in BP between different rat strains is hereditary and is passed down through generations but unlike Mendelian traits, the genotype based on change in BP does not yield any unique information about the genotype at any given single locus, because BP is the net effect across many loci. The existence of a QTL within a chromosomal region was initially detected using the candidate gene approach in 1972 using the biochemical genetic marker for steroid 11β- hydroxylase and later by restriction fragment length polymorphisms [181, 182]. Garrett et al did a study using F2 population derived from the Dahl salt sensitive S rat and normotensive LEW rats raised on 8% NaCl diet for 9 weeks and doing a systematic genome scan for BP QTL using MAPMAKER/QTL software. They found that congenic strains S.LEW(5) and S.LEW(10) made on chromosome 5 and 10 respectively yielded significant LOD scores (>3.5) [84, 183]. This data is represented in Figure 1-3.

The BP QTL on rat chromosome 10 is also reported on other F2 populations like F2(SxMNS) [184] and F2(SHRxWKY) [167, 185]. On RNO5, the BP QTL was previously noted by Deng et al. [186] and a stroke QTL SHRSP and WKY is described in the similar location by Jeffs et al [187].
Figure 1-3 LOD plots for linkage to BP on chromosomes 5, 8, and 10 are shown. The thick bars on the plots of chromosome 5 and 10 represent the area of LEW chromosome introgressed into S rats in the construction of congenic strains. Only the representative markers are shown. The broken vertical line is the LOD threshold for suggestive linkage and the solid line represents significant linkage as defined by Lander and Kruglyak.
The software MAPMAKER/QTL can also be used to determine the existence of epistatic interactions making it especially beneficial for linkage analysis. An example is the detection of interacting loci in a double congenic strain on rat chromosomes 2 and 10 [188].

A similar interactive BP QTL was detected on RNO5 when the larger QTL obtained during linkage studies was dissected further by construction of a new iteration containing nine congenic substrains [189] demonstrating the presence of two closely linked BP QTLs. One of the QTLs was 6.3cM and another, 4.6 cM with a 1 cM distance between them. These substrains are shown in Figure 1-4a and b. The data implies that both the QTLs are closely linked and that neither one of the indicated QTLs when present alone gave a significant BP effect but when present adjoining each other cause a significant BP lowering effect [189]. A hunt for the heritable transcriptional network regulated by the two interacting BP QTLs led researcher Lee et al to hypothesize that allelic interactions and transcriptional crosstalk between QTL 1 harboring gene Dmrrta2 and QTL2 containing the gene Nfia are important for the BP lowering effect [190].
Figure 1-4a Congenic strains for rat chromosome 5 with LEW as the donor strain on the S-rat genetic background. The linkage map is at the right, and the numbers denote map distances in centiorgans (cM). The solid bars to the left of the linkage map indicate the extent of the donor regions homozygous for the LEW alleles for each congenic strain. The open bars on the ends of these congenic segments indicate the interval in which recombination occurred. The effect on blood pressure of each strain compared with S rats is shown by the bar graph at the bottom. (n=20 per group); the standard error of this difference is indicated by the thin line at the end of the blood pressure bars. Significance levels by a t-test for the blood pressure effect are shown below each bar. A negative blood pressure deviation means that the congenic strain had a lower blood pressure than concomitantly studied S rats. The 2-LOD interval from our previous genome scan (10) is shown at the right. The LOD score peak is indicated by the solid diamond symbol and was 4.5. The deduced position of the blood pressure quantitative trait locus (QTL) based on the data from the congenic strains is indicated at the left, labeled “QTL 1.” *A linkage distance between markers D5Rat108 and D5Rjr1 could not be determined from the linkage panel because these markers are very close together.
Figure 1-4b Further derivation of congenic strains for rat chromosome 5. The format is the same as Fig. 1. Strains S.LEW(5)×6 and S.LEW(5)×5 are from Fig. 1. The other four strains were derived from S.LEW(5)×6 and are labeled S.LEW(5)×6×6, S.LEW(5)×6×9, S.LEW(5)×6×10, and S.LEW(5)×6×11. The data indicate the presence of two QTL (labeled “QTL 1” and “QTL 2”), both of which are required to retain the LEW allele in a given congenic strain in order for a significant blood pressure effect to be observed; the double-headed arrow between QTL 1 and QTL 2 is meant to imply this interaction. QTL 1 here is the same as QTL 1 in Fig. 1. See legend to Fig. 1-4a for description of the asterisk.
Chapter 2

Materials and methods

2.1 Rat strains

Dahl Salt-sensitive (SS/Jr) inbred rats, designated as S, were from our colony maintained at the University of Toledo Health Science Campus. LEW/NCrI BR (LEW rats) were originally obtained from the Charles River Laboratories (Wilmington, MA) and maintained in our colony. All the animals were maintained and studied as per the institutional review committee’s approved protocol and IACUC protocols [191].

2.2 Rat Diets

The animals were maintained on a low salt (0.3% NaCl) diet from Harlan Teklad diet 7034 (Madison, WI). The rats that were used for experiments on high salt (2% NaCl) were given the Harlan Teklad diet TD94217.

2.3 Genotyping

Genomic DNA was extracted from rat tail biopsies using the Promega Wizard SV-96, genomic DNA purification system. Primers were designed to amplify microsatellite
markers and SNP’s from genomic DNA sequences in the region of interest obtained from the rat genome data available and the Ensembl web site (www.ensembl.org). Polymorphic microsatellite markers were identified by PCR of genomic DNA and used for genotyping the congenic strains [192]. To narrow down the region of recombination, primers were designed around the SNPs and the genomic segment of S, LEW and congenic substrains were amplified using PCR. The PCR products were sequenced using the standard read sequencing service (http://www.operon.com/).

2.4 **Systolic Blood Pressure (SBP) measurement by the tail-cuff method**

Fifteen-20 male rats of each congenic strain were used for this study. Each congenic rat strain was bred, housed and studied concomitantly along with the control S rats. The animals were weaned at 30 days of age rats onto a low salt diet. At 42 days of age the high salt group were switched to a 2% NaCl diet and the low salt group was maintained on the low salt diet for the duration of the experiment. Each cage contained one congenic and one S rat. After 24-25 days on the respective diets, each rat had its systolic BP measured by two blinded operators. During BP measurements, rats were restrained and warmed to 28°C. The operators’ readings for each rat were averaged together and recorded as that animal’s systolic BP [193-195].

2.5 **Corroboration of tail-cuff BP measurements by telemetry**

Rats from the tail-cuff study were continued on a 2% NaCl diet and surgically implanted with C40 radiotelemetry transmitters as described previously [191]. Telemetry was also done independent of the tail cuff method on younger rats (42do) by using the C10
transmitters and equipment from Data Sciences International [196]. The radio-telemetry transmitters (C-40 and C10) is implanted in anesthetized animals using 2% isoflurane. The cannula of the transmitter is inserted through the femoral artery and advanced to the lower abdominal aorta. The transmitter is inserted into the abdominal cavity. Once the procedure is completed, the animal is allowed to recover for a period of one week and continued on the 2% NaCl diet for the duration of the experiment. The blood pressure is then recorded continuously for 3 days.

2.6 Urinary protein excretion

Urinary protein excretion (UPE) was done by measurement of total protein in the urine over a 24 hour period after BP measurement was completed. The rats were caged individually in metabolic cages (Lab Products, Seaford, DE) with only water but no food. Urine was collected in measuring cylinders in the presence of 0.01% sodium azide and the total urine volume was recorded after 24 hours. The total protein level was determined by ready-to-use colorimetric method using Pyrogallol Red Molybdate dye binding mechanism (Quan Ttest red total protein assay system- Quantimetrix Corporation, Redondo Beach, CA).

2.7 Genomic sequencing and analysis

Primers are designed around the region to be sequenced (Integrated DNA Technologies Inc. (Coralville, IA). If large segments are involved, 1000kb overlapping regions are chosen. The PCR samples were sent to Eurofins MWG Operon (Huntsville, AL) for sequencing and the results were analyzed using the software Sequencher 4.9 (Gene
Codes Corporation). Once the RGD sequence variation data was publically available, the SNPs and INDELS were compared.

2.8 Bioinformatics analysis

List of websites used:


http://www.ensembl.org

https://genome.ucsc.edu/

http://rgd.mcw.edu

http://regrna2.mbc.nctu.edu.tw

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

http://ihg.gsf.de/ihg/ExonPrimer.html

http://www.genecards.org/

http://www.omim.org/

http://www.cbs.dtu.dk/services/Promoter

https://cm.jefferson.edu/rna22/Interactive

http://rnasoft-new.cs.ubc.ca/cgi-bin/RNAsoft/PairFold/pairfold.pl

http://www.gwascentral.org

2.9 RNA isolation and Real-time PCR

Animals (S and congenic) were euthanized after a 2% NaCl diet after six weeks of age. The total RNA from kidney and heart were then prepared using RNeasy Mini Kit (Qiagen) and TURBO DNA-free™ Kit (Invitrogen) was used to remove the traces of
genomic DNA. Reverse transcription PCR (RT-PCR) was done using up to 5 ug of total RNA and reverse transcribed using Oligo-dT primers by using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Life Technologies, Grand Island, NY) to obtain cDNA. The cDNA concentration was measured by NanoDrop 2000 (Thermo Scientific). Real time PCR using Power SYBER® Green Mater Mix (Applied Biosystems, Foster City, CA) in the Step One Plus Real-time PCR System (Applied Biosystems) was performed using gene specific primers and 18sRNA was used as control.

**Promoter Assay**

Promoter function was determined by means of a luciferase reporter gene assay system (Promega Corp).

**Plasmid construction** - The genomic region that was predicted by the software (Promoter 2.0 prediction server) to contain promoter activity was PCR amplified using primers that flanked it. The total size of the construct was between 700-1000bp and constructs obtained from both, the S and LEW rats. The primers were designed such that it includes maximum number of polymorphisms in that region between S and LEW and contained the restriction sites for Nhe1 and Xho1. The fragment containing the predicted promoter segment was digested with Xho I and Nhe1 restriction enzymes and was then sub-cloned into the multiple cloning sites (Xho I and Nhe1) of pGL3-basic vector that lack eukaryotic promoter and enhancer sequences (Promega). The pGL-3 vector contains the cDNA encoded for firefly luciferase which when fused with a promoter, can be used to analyze the inserted promoter activity once transfected into mammalian HEK293T (Human Embryonic Kidney) cells. All clones were verified by sequencing. Transfection was done using lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA).
The vector encoded for the Renilla luciferase transcribed by a HSV-TK promoter was used as internal controls to normalize firefly luciferase expression. Forty-eight hours after transfection, the cells were lysed in passive lysis buffer (Promega). Cell lysate was added to the luciferase substrate (dual luciferase reporter system, Promega) and firefly and Renilla luciferase activity was measured with a luminometer (GloMax® 96 Microplate Luminometer w/Dual Injector).
Chapter 3

Genetic determinants of blood pressure on rat chromosome 5

Introduction

Many blood pressure (BP) quantitative trait loci (QTLs) have been successfully located and fine mapped to short genomic segments spanning a few megabases or kilobases of the rat genome [176, 191, 196-205], (http://rgd.mcw.edu/). While some of the fine-mapped QTLs act independently of each other, few BP QTLs have been identified as interacting or epistatic loci [188, 189, 206-218]. The work described here involves the study of two such QTLs on rat chromosome 5 (RNO5) that are closely linked and interact with each other to influence BP. The interacting QTLs, QTL1 and QTL2 linked to BP, were previously inferred based on differential segments between congenic strains constructed from the parental congenic strain S.LEW(5). These introgressed Lewis rat (LEW) segments were 6.3 and 4.6 cM on the genetic background of the Dahl salt sensitive (S) rat. Both these QTLs were captured in a single congenic strain S.LEW(5)x6x9 that was used as a parental strain for the current study [189, 190]. Isolating QTL1 and QTL2 as individual introgressed segments in two different monocongenic strains and reconstituting
them in a single bicongenic strain would provide direct evidence that LEW alleles within these two BP QTLs interact with each other to cause a BP lowering effect in the S rat. To obtain this important evidence, we generated a series of four different groups of congenic strains. Each group consisted of the following:

1) One bicongenic strain containing introgressed LEW alleles on RNO5 at QTL1 and QTL2 separated by an S genomic segment,
2) The two corresponding monocongenic strains, and
3) The parental S strain.

Each of these bicongenic strains were unique because they were developed by combining novel lines of monocongenic substrains containing introgressed LEW segments potentially different in their regions of recombination with the S genome. Data obtained from these new groups of congenic strains that is presented includes

1) Definitive evidence for the presence of epistasis between the two closely linked QTLs,
2) Capture of the two proposed epistatic QTL segments in independent congenic strains within better resolved intervals,
3) Sequencing of the QTL regions, and
4) Enlistment of potential candidate genetic elements.
Construction of the panel of monocongenic and bicongenic strains

The bicongenic strains used for this study were derived from the parental strain S.LEW(5)x6x9 developed in our laboratory [190]. The parental strain S.LEW(5)x6x9 was bred with the S rat to generate an F1 population. The F1 rats were intercrossed to generate an F2 population. F2 rats that had recombinations on RNO5 between the two microsatellite markers D5Mco39 and D5Mco47 were selected and backcrossed to the parental S strain to fix the recombination to homozygosity. Additional markers were used to determine the extent of the introgressed LEW alleles containing segments retained within each of the monocongenic strains. To develop bicongenic strains, male and female pairs of the monocongenic strains, each representing one of the two QTLs were randomly selected and intercrossed to develop F1 strains that were heterozygous at the two QTL regions. The animals containing the heterozygous alleles within each of the two QTLs were intercrossed to generate the bicongenic strains that were homozygous LEW at QTL1 and 2.

Genomic sequencing and analysis

Genomes of the SS/Jr and LEW/NCrlBR rat strains were sequenced on the next generation sequencing platform Illumina HiSeq2000 using paired end sequencing technology with 100 bp read length and 350bp insert size. Sequence reads were mapped to the reference BN genome RGSC-3.4 [219] using short read aligner BWA-0.5.8c [220]. Genomic variants in SS/Jr and LEW/NCrlBR, relative to the BN reference genome, were identified using Genome Analysis Toolkit (GATK version 1.0.6001) [221] after rigorous pre-processing of
mapped reads including removal of clonal reads, indel realignment and base call recalibration. Potential false positives were filtered using variant quality score recalibration function of GATK which employs the Bayes Gaussian mixture model (GMM). Genomic variants (SNPs and indels) polymorphic between SS/Jr and LEW/NcrI BR in the two congenic intervals were then extracted using Perl script. Functional consequences of the SNPs and indels were estimated using Variant Effect Predictor (VEP) version 2.4 [222] on the Ensembl v66 rat gene set. All nonsynonymous variations in QTL 1 and 2 were independently validated with genomic DNA of S and S.LEW(5)x6Bx9x5 using primers designed to detect the variants by sequencing as described elsewhere[177] The list of primers used is provided in Table 3.1.
Table 3.1 List of primers for independent validation of nonsynonymous variations in protein-coding genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>LEFT PRIMER</th>
<th>RIGHT PRIMER</th>
<th>PRODUCT SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zcchc11</td>
<td>AGAATTCTCCCCAACCGTCT</td>
<td>AGTCACCCAACCTTTCCACA</td>
<td>900</td>
</tr>
<tr>
<td>Osbp19</td>
<td>GCCACCATGCCTCATCTACT</td>
<td>AAGAATGTGATGTGAGCCCTTGA</td>
<td>601</td>
</tr>
<tr>
<td>Raver2</td>
<td>CCCCTATGGGGGAAAGTAGA</td>
<td>AAAGCCGATTGCATAAGGAC</td>
<td>840</td>
</tr>
<tr>
<td>Zyg11a</td>
<td>TCCCCCAGGACTGAGTTACA</td>
<td>GAAAAGCTTTCATGTGTGCTACAA</td>
<td>780</td>
</tr>
<tr>
<td>Zyg11b</td>
<td>GGAACCTAAAAAGAAAAACCA AC</td>
<td>CCTTTTGCCACCACAAGCTA</td>
<td>780</td>
</tr>
<tr>
<td>Faf1</td>
<td>TGCCCATGCCATGTAGATTA</td>
<td>CCTCCTACACGTAGGCATCC</td>
<td>960</td>
</tr>
</tbody>
</table>
Results

A panel of 12 congenic substrains including monocongenic and bicongenic strains were developed and assigned into four groups as shown in Figure 3-1. Each group had a unique combination of LEW alleles within the two QTLs, QTL1 and QTL2 (Figure 3-1). Each group of animals was concomitantly raised along with the control S rats and their BP was measured using the tail cuff method (n=15-20 per group). None of the strains in groups 1, 2 or 3 differed in their BP compared to the S rat (Figure 3-1, Table 3.2). However, within group 4, the SBP of the two monocongenic strains S.LEW(5)x6Bx9x5a and S.LEW(5)x6Bx9x5b were comparable to that of the S, but the bicongenic strain, S.LEW(5)x6Bx9x5 had a systolic BP of 157± 4.3 mmHg, which was 39 mmHg lower than the SBP of the S rat (SBP =196 ± 6.8 mmHg) (p < 0.001, Table 3.2). Statistical analysis of the data obtained with the 3 strains in group 4 along with the S using two-way ANOVA showed significant interaction (p=0.041, Table 3.3). These data point to an interactive effect (labeled as ‘C’ in Figure 3-2). The interactive effect was observed between the two introgressed segments within the monocongenic strains labeled as S.LEW(5)x6Bx9x5a and S.LEW(5)x6Bx9x5b in Figure 3-1. Further, because this interaction was not observed with any of the other groups of congenic rats, the introgressed segment contained within the strains S.LEW(5)x6Bx9x2a, S.LEW(5)x6Bx9x3a and S.LEW(5)x6Bx9x4a could be eliminated as BP QTL containing segments. Therefore the most likely location of one of the interacting BP QTLs was interpreted to be within the 7.77 Mb orange-colored segment labeled as QTL1 in Figure 3-1. The second orange-colored segment labeled as QTL2 in Figure 3-1 represents the BP
Figure 3-1 **Schematic representation of the monocongenic and bicongenic strains developed**. Physical map of rat chromosome 5 (RNO5) along with the microsatellite markers, genes and their locations according to the Ensembl database (www.ensembl.com, RGSC 3.4) are shown in the left. The vertical bars alongside the physical map represent schematics of congenic strains with black boxes representing introgressed Lewis segment and white boxes indicating S allele. The blood pressure effect is shown as black bars at the bottom of the congenic strains as measured by tail cuff method. BP data were analyzed by one-way ANOVA for each group. The blood pressure QTLs are indicated as orange bars at the right side.
**Table 3.2** Blood Pressure effect of the four groups of monocongenic and their respective bicongenic strains by the tail-cuff method.

<table>
<thead>
<tr>
<th>Congenic substrain</th>
<th>SBP (mmHg) ± SEM</th>
<th>BP Effect(CONGENIC-S)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.LEW(5)x6Bx9x2</td>
<td>205 ± 5.1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x2A</td>
<td>199 ± 4.2</td>
<td>-6</td>
<td>0.8</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x2B</td>
<td>204 ± 5.4</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>S</td>
<td>205 ± 3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x3</td>
<td>226 ± 5.5</td>
<td>6</td>
<td>0.9</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x3A</td>
<td>221 ± 6.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x3B</td>
<td>231 ± 4.3</td>
<td>12</td>
<td>0.2</td>
</tr>
<tr>
<td>S</td>
<td>220 ± 5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x4</td>
<td>181 ± 2.5</td>
<td>-10</td>
<td>0.2</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x4A</td>
<td>184 ± 4.3</td>
<td>-7</td>
<td>0.5</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x4B</td>
<td>179 ± 3.2</td>
<td>-12</td>
<td>0.06</td>
</tr>
<tr>
<td>S</td>
<td>191 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x5</td>
<td>157 ± 4.3</td>
<td>-39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x5A</td>
<td>190 ± 5.6</td>
<td>-6</td>
<td>0.9</td>
</tr>
<tr>
<td>S.LEW(5)x6Bx9x5B</td>
<td>187 ± 5.2</td>
<td>-9</td>
<td>0.6</td>
</tr>
<tr>
<td>S</td>
<td>196 ± 6.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3 Report of Statistical Analyses for Interactive Effects for Systolic BP measured by the Tail-cuff method by two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>7624.999^a</td>
<td>3</td>
<td>2541.666</td>
<td>9.718</td>
<td>.000</td>
</tr>
<tr>
<td>Intercept</td>
<td>1037823.409</td>
<td>1</td>
<td>1037823.409</td>
<td>3967.973</td>
<td>.000</td>
</tr>
<tr>
<td>QTL1</td>
<td>3584.586</td>
<td>1</td>
<td>3584.586</td>
<td>13.705</td>
<td>.001</td>
</tr>
<tr>
<td>QTL2</td>
<td>2555.919</td>
<td>1</td>
<td>2555.919</td>
<td>9.772</td>
<td>.004</td>
</tr>
<tr>
<td>QTL1 * QTL2</td>
<td>1199.786</td>
<td>1</td>
<td>1199.786</td>
<td>4.587</td>
<td>.041</td>
</tr>
<tr>
<td>Error</td>
<td>7323.401</td>
<td>28</td>
<td>261.550</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1082464.590</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>14948.400</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. R Squared = .510 (Adjusted R Squared = .458)
Figure 3-2 Interactive Systolic BP effect. Results from group 4 are presented. The BP differences of the monocongenic strains compared to S rat are represented by intervals A and B. The additive BP effect expected in the bicongenic strain S.LEW(5)x6Bx9x5 is represented by the interval labeled A+B. The difference between the expected and the observed BP of the congeneric strain S.LEW(5)x6Bx9x5 is labeled as ‘interactive effect’ represented by the interval C. A two-way ANOVA showed that this interaction was significant, p=0.041
QTL that interacts with QTL1. BP QTL2 spans 4.18 Mb, which is the introgressed LEW segment of S.LEW(5)x6Bx9x5b.

To corroborate the result obtained by the tail-cuff method, the BP of S.LEW(5)x6Bx9x5a, S.LEW(5)x6Bx9x5b, S.LEW(5)x6Bx9x5 and S were additionally tested by the telemetry method (n=6/group for all strains except S.LEW(5)x6Bx9x5a, n=5). Similar to the data obtained by the tail-cuff method, the SBP of the two monocongenic strains S.LEW(5)x6Bx9x5a and S.LEW(5)x6Bx9x5b were not different from that of the S. However, the SBP of the bicongenic strain S.LEW(5)x6Bx9x5 was significantly lower than that of the S (p=0.041, Figure 3-3). Similarly, the diastolic blood pressure (DBP), mean arterial pressure (MAP) and pulse pressure (PP) of the bicongenic strain S.LEW(5)x6Bx9x5, but not either one of the monocongenic strains, were significantly lower than that of the S (DBP, p=0.036; MAP, p=0.009; PP, p=0.049; Figure 3-3). A two-way ANOVA of the SBP, DBP, MAP, PP data obtained by the telemetry method further confirmed that the observed interaction was statistically significant in the bicongenic strain S.LEW(5)x6Bx9x5 (Table 3.4).

**Sequence variants within two identified QTL regions**

The location of the two QTL regions were queried by Blast searches of the sequences delimiting the two QTLs labeled as QTL1 and 2 in Figure 3-1. The genomic sizes of these QTLs differed between the rat genome assembly versions RGSC 3.4 and RGSC 5.0 (Table 3.5). The interpretations of genomic content of the two QTLs in the current report were based on RGSC 3.4 because this assembly was used to determine the sequences of the two QTL regions. As per RGSC 3.4, QTL1 was located within 7.77 Mb
containing 85 annotations (Table 3.5) and QTL2 within 4.18 Mb containing 34 annotations. Genomic DNA sequencing of the QTL regions 1 and 2 identified 7360 and 2753 variants respectively, between S and LEW. These variant counts include single nucleotide polymorphisms and insertion and deletion polymorphisms. The majority of the variants in both the QTLs were outside of protein-coding genes. However, within QTL1, variants were confirmed in three protein-coding candidate genes (Table 3.6). These genes are Fas (TNFRSF6) associated factor 1 (Faf1) whose function is to potentiate FAS-induced apoptosis [223], zyg-11 homolog A (Zyg11a) that probably acts as target recruitment subunit in an E3 ubiquitin ligase complex ZYGA-CUL2-elongin BC (By similarity) and zyg-11 homolog B (Zyg11b) is predicted as a target recruitment subunit in the E3 ubiquitin ligase complex ZYG11B-CUL2-Elongin BC [224] (Table 3.6). Similarly, within QTL2, one nonsynonymous variation and one splice-site variation was confirmed in the gene ribonucleoprotein, PTB-binding 2 (Raver2) that may bind single stranded nucleic acids (Potential) [225]. SIFT/PROVEAN (Protein Variation Effect Analyzer) software tool was used to analyze those variations in amino acids in genes that were confirmed to have non synonymous polymorphisms (http://sift.jcvi.org/). The results indicate a neutral effect due to the change in amino acid on the biological function of the protein coded by genes Zyg11b and Raver2. Given that the focus of the current study was to obtain definitive evidence for genetic interactions and localization of the two QTLs, constructing hypotheses around these candidate variants as the only potentially causative variants for the observed interactive effect is premature. The congenic strains reported will serve as genetic tools for further fine-mapping and positional cloning of the two interacting loci.
Figure 3-3 BP effect of the QTL region detected by radiotelemetry - Radiotelemetry measures of systolic blood pressure, diastolic blood pressure, mean arterial pressure and pulse pressure of S and congenic strains (n=5-6). Rats were surgically implanted with large C40 radiotelemetry transmitters, allowed to recover for a week and BP was recorded over a period of 3 days. The data plotted is obtained by telemetry recording once every 5 min continuously and averaged for 4 hour intervals and is represented as Mean ± SEM
Green: S.LEW(5)X6BX9X5; Black: S.LEW(5)X6BX9X5a; Blue: S.LEW(5)X6BX9X5b; Red: S
Table 3.4 Report of Statistical Analyses by two-way ANOVA for Systolic, Diastolic and Mean arterial blood pressure measured by the telemetry method

(a) Dependent Variable: Systolic BP

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>5036.072&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>1678.691</td>
<td>4.912</td>
<td>.011</td>
</tr>
<tr>
<td>Intercept</td>
<td>869142.857</td>
<td>1</td>
<td>869142.857</td>
<td>2543.049</td>
<td>.000</td>
</tr>
<tr>
<td>QTL1</td>
<td>892.857</td>
<td>1</td>
<td>892.857</td>
<td>2.612</td>
<td>.123</td>
</tr>
<tr>
<td>QTL2</td>
<td>845.873</td>
<td>1</td>
<td>845.873</td>
<td>2.475</td>
<td>.132</td>
</tr>
<tr>
<td>QTL1 * QTL2</td>
<td>3200.635</td>
<td>1</td>
<td>3200.635</td>
<td>9.365</td>
<td>.006</td>
</tr>
<tr>
<td>Error</td>
<td>6493.667</td>
<td>19</td>
<td>341.772</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>881431.000</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>11529.739</td>
<td>22</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a. R Squared = .437 (Adjusted R Squared = .348)

(b) Dependent Variable: Diastolic BP

<table>
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<tr>
<th>Source</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
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<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>10313.070&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>3437.690</td>
<td>14.477</td>
<td>.000</td>
</tr>
<tr>
<td>Intercept</td>
<td>479136.311</td>
<td>1</td>
<td>479136.311</td>
<td>2017.729</td>
<td>.000</td>
</tr>
<tr>
<td>QTL1</td>
<td>88.406</td>
<td>1</td>
<td>88.406</td>
<td>.372</td>
<td>.549</td>
</tr>
<tr>
<td>QTL2</td>
<td>3552.406</td>
<td>1</td>
<td>3552.406</td>
<td>14.960</td>
<td>.001</td>
</tr>
<tr>
<td>QTL1 * QTL2</td>
<td>6185.200</td>
<td>1</td>
<td>6185.200</td>
<td>26.047</td>
<td>.000</td>
</tr>
<tr>
<td>Error</td>
<td>4511.800</td>
<td>19</td>
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<td></td>
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<tr>
<td>Total</td>
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<tr>
<td>Corrected Total</td>
<td>14824.870</td>
<td>22</td>
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</table>
Table 3.4 Continued…

(a) Dependent Variable: Mean arterial pressure

<table>
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<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>4070.713(^a)</td>
<td>3</td>
<td>1356.904</td>
<td>5.159</td>
<td>.009</td>
</tr>
<tr>
<td>Intercept</td>
<td>624046.959</td>
<td>1</td>
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<td>2372.707</td>
<td>.000</td>
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<tr>
<td>QTL1</td>
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<td>751.340</td>
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<tr>
<td>QTL2</td>
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<td>2.890</td>
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</tr>
<tr>
<td>QTL1 * QTL2</td>
<td>2472.229</td>
<td>1</td>
<td>2472.229</td>
<td>9.400</td>
<td>.006</td>
</tr>
<tr>
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<td>4997.200</td>
<td>19</td>
<td>263.011</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
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<td>22</td>
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</tbody>
</table>

\(^{a}\) R Squared = .449 (Adjusted R Squared = .362)
<table>
<thead>
<tr>
<th>QTL</th>
<th>RGSC 3.4 (base pairs)</th>
<th>Size of the QTLs (base pairs)</th>
<th>RGSC 5.0 (base pairs)</th>
<th>Size of the QTLs (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QTL1</td>
<td>124,085,611-131,853,815</td>
<td>7,768,204</td>
<td>126,545,004-134,002,747</td>
<td>7,457,743</td>
</tr>
<tr>
<td>QTL2</td>
<td>117,894,038-122,070,175</td>
<td>4,176,137</td>
<td>124,137,683-124,137,683</td>
<td>4,043,618</td>
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Table 3.6 List of single nucleotide polymorphisms within protein-coding genes

<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>POSITION</th>
<th>SINGLE NUCLEOTIDE POLYMORPHISM (SNP)</th>
<th>S</th>
<th>LEW</th>
<th>BN</th>
<th>LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faf1</td>
<td>131357775</td>
<td>SNP- SPLICE SITE, INTRONIC</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>QTL1</td>
</tr>
<tr>
<td>Zyg11a</td>
<td>129293730</td>
<td>SNP- SPLICE SITE, INTRONIC</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>QTL1</td>
</tr>
<tr>
<td>Zyg11b</td>
<td>129367632</td>
<td>SNP- NONSYNONYMOUS</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>QTL1</td>
</tr>
<tr>
<td>Raver2</td>
<td>121784282</td>
<td>SNP- SPLICE SITE, INTRONIC</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>QTL2</td>
</tr>
<tr>
<td>Raver2</td>
<td>121786520</td>
<td>SNP- NONSYNONYMOUS</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>QTL2</td>
</tr>
</tbody>
</table>
DISCUSSION

The locations of the interacting BP QTLs reported in this study were previously interpreted based on collective data obtained from several S.LEW congenic strains containing differential introgressed segments on RNO5 [189, 190]. Such interpretations, however logical, could be misleading without definitive evidence provided through the isolation and reconstitution of each of the two interacting BP QTLs [226]. Isolating interacting loci as introgressed segments within monocongenic strains and reconstituting these introgressed segments within bicongenic strains is relatively easier when the two interacting loci are on two different chromosomes. For example, two BP QTLs on chromosomes 2 and 10 are demonstrated to interact by the comparisons between two monocongenic strains with introgressed segments of chromosomes 2 and 10 and a bicongenic strain with both of the introgressed segments on chromosomes 2 and 10 [188]. Compared to this example, the present study was challenging because (1) the two epistatic loci were within a few megabases on the same chromosome and (2) the parental strain that was used for isolating the two interacting QTLs contains a contiguous LEW introgressed segment spanning the entire genomic segment from QTL1 through QTL2. The study therefore required the replacement of the LEW segment between the locations of the two interacting loci with S alleles. This was achieved first by the construction of several monocongenic strains, followed by grouping these strains such that there were two monocongenic strains, one containing QTL1 and other containing QTL2. The two monocongenic strains within a group were used as progenitor strains for reconstitution into a bicongenic strain within the same group. The data collected with four such groups of
monocongenic and bi-congenic strains compared with the S clearly demonstrated that the two interacting BP QTLs were resolved as single introgressed segments within congenic strains that were 2.02 Mb apart.

The sizes of the two QTL regions are amenable for further dissection using the congenic approach. QTL1 is homologous to two regions on human chromosome 1 (HSA 1: 50513686 - 59012474 and 67390578 - 67600639bp). QTL2 is homologous to chromosome 1 in humans (HSA1: 61330931 - 65697828 bp). Within these two regions there are no reported direct associations to blood pressure (http://www.genome.gov/gwastudies/). Genome-wide association studies (GWAS) do not typically account for gene-gene interactions, whereby data on epistasis could be easily missed [123]. If similar epistasis, as described in our studies using rat models, does exist between the human homologous segments of QTLs 1 and 2, one would not expect current genome-wide association studies to demonstrate associations with either one of the homologous segments per se. Indeed, there are no known associations to blood pressure within QTL2 on human chromosome 1. The current data thus points to epistasis, as described in our report, as perhaps contributing towards BP regulation by genetic elements within the homologous regions of QTLs 1 and 2 in humans. This, of course, remains to be determined.

Genome-wide association studies of hypertension accounts for only about 1% of the inheritance of blood pressure. This observation is leading to recent literature pointing to epistasis as one of the factors contributing to ‘missing heritability’ of quantitative traits such as BP [220, 227]. The present study attests to epistasis as a possible factor contributing to ‘missing heritability’ in human hypertension GWAS because inheritance of a protective effect of LEW alleles at either QTL1 or QTL2 by itself did not result in an observable
change in BP. Therefore, the genomic segments could have been easily dismissed as ones that do not contain any genetic elements that causally alter BP. However, reconstitution of LEW alleles within QTL1 and QTL2 demonstrate that the ‘missing’ heritability is indeed accounted for by the presence of LEW alleles at both loci. Thus, results of rodent studies such as this, which are inherently more powerful in terms of their ability to dissect polygenic traits because of their ability for selective breeding schemata, points to epistatic effects in the molecular basis of hypertension in rodents. This may also contribute to the molecular basis of hypertension in humans and study designs that will detect such effects with the possibility that these will explain more of the heritability of human hypertension than has been possible with human hypertension GWAS to date.
Chapter 4

Genetic determinants of blood pressure on rat chromosome 10

4.1 Introduction

The presence of blood pressure QTLs on rat chromosome 10 (RNO10) has been demonstrated previously by linkage analysis from our laboratory as well as other laboratories [167, 183-185, 206, 228-237]. Linkage mapping resulted in the identification of large genomic segments as being linked to BP and was located between markers D10Mit10 and D10Mco6 (~79-Mb). To dissect and refine this larger segment into smaller genomic segments, substitution mapping was applied in our laboratory. Accordingly congenic strains that encompass large regions of RNO10 were constructed by replacing the genomic suspect region of the hypertensive S rat with that of the normotensive LEW rats (S.LEW) [233]. One of the congenic strains used to study chromosome 10 is S.LEW(10) that contains the above mentioned ~79-Mb genetic segment that showed a significant lowering of BP by ~ 43 mm Hg compared with that of S [183, 233]. Using this congenic as a parental strain, further iterations of congenic substrains were constructed and the critical genomic segment was further resolved. Along with other congenic substrains in the iteration, S.LEW(10)x12 showed a significant lowering of BP of 25 mm Hg compared to S. A similar study had been previously done on a 32-cM segment from MNS rats
introgressed into the S genetic background which also showed a BP-lowering effect of 34 mm Hg compared with S [230]. In humans, a sib pair analysis by Julier et al found significant linkage of BP to markers in the region of HSA17 that was homologous to the BP QTL region of RNO10 obtained by linkage analysis [95] which was later confirmed by Baima et al [238].

Fine mapping of the congenic segment on rat strain S.LEW(10)x12 was important to locate Quantitative trait nucleotides (QTNs) or Quantitative trait genes (QTGs) that are functional in regulating BP. After making a new iteration of congenic substrains and based on differential congenic segmentation, the QTL on RNO10 was further resolved and mapped to a 1.17Mb (shown by the orange bars labelled as S.LEW BP QTL in Figure 4.1) on substrain S.LEW(10)x12x2x3 which had a 2.35 Mb LEW segment. This region contains 18 genes and homology mapping indicates that the 1.17-Mb BP QTL in S.LEW(10)x12x2x3 is not within any of the BP regulating regions mapped in human showing significant linkage. However, the region is also a candidate region for human familial hyperkalemia and hypertension (pseudohypoaldosteronism type II, Gordon’s syndrome) [239]. This may suggest that there are perhaps many genetic factors influencing BP that are yet to be discovered in humans [191]. The alternate possibility is that rats and humans do not share this location as BP QTLs.

During phenotyping of any congenic strain, the net effect of alleles is what is measured but the underlying contributing factors to the observed net effect remains unknown. Hence mapping using congenic intervals as QTL is more appropriate than mapping by differential segments as QTL intervals. Differential segmentation is effective in hypothesis generation but requires further validation using congenic strains. Hence to
locate the genetic determinants of BP within congenic strains with shorter introgressed segments, a new iteration of substrains was made from the parental S.LEW(10)x12x2x3 congenic that had an introgressed LEW segment of 2.35 Mb encompassing the entire 1.17 Mb BP QTL [192]. However this genetic segment is not directly under the apex of the LOD plot suggesting that we may miss the underlying genetic determinants of BP if we fixate on those genes that are close to the point of the highest statistical evidence in a linkage study. One of the congenic substrains obtained from the parental S.LEWx12x2x3 is S.LEW(10)x12x2x3x8 (1.3Mb LEW segment) showed a significant BP lowering effect. Another substrain S.LEW(10)x12x2x3x5 (375kb LEW segment) showed a significant increasing BP effect compared to S rat. This helped to divide the 1.3Mb segment on S.LEW(10)x12x2x3x8 into multiple regions- region1 (401kb), region2 (104kb) and region3 (409kb). This is represented in Figure 4-1.

In an attempt to study the strain S.LEWx12x2x3x5 containing the 104kb LEW segment (region 2) that led to a further increase in BP of the S rat, SNP genotyping was conducted to resolve the region of recombination and the QTL was resolved to a <42.5kb between the base pairs 71028112 and 71070581 and this critical (<42.5kb) region contained a single predicted gene, Rififylin (Rffl) with no exonic variants [196, 240]. This gene was prioritized as a candidate gene in BP regulation.

The aim of the current study is to fine map these three regions and resolve them to the highest possible extent. The strain S.LEW(10)x12x2x3x8 spanning a 1.34Mb LEW introgressed segment containing all the three regions, region 1 (401 kb), region 2 (<42.5 kb) and region 3 (409 kb) was chosen as a parental strain to make future congenic substrains and conduct fine mapping studies to refine the three regions.
Figure 4-1 Congenic substraains and their phenotypic effects. The relevant section of the physical map of RNO10 is shown to the left of the Figure. Values in parenthesis next to the marker names indicate their physical locations in base pairs. The previously mapped BP QTL1 region is shown as the orange bar flanked by 2 open arrows. Congenic strains are shown as solid colored bars flanked by open bars. Solid color bars illustrate the LEW segment introgressed onto the background of S. The open bars at the end of each introgressed segment represent the region of recombination. Green colored bars represent the LEW introgressed segment in congenic substraains with a BP effect, whereas the dark gray colored bars represent the congenic substraains without a BP effect. The locations of the newly determined BP Regions 1, 2, and 3 are presented at the right of the illustration as orange, peach, and blue bars. The bottom portion of the Figure illustrates the BP and HW effect observed for each congenic substrain compared with that of the S rat BP: Green bars represent a significant BP effect, black bars represent a BP effect with a probability value <0.05, hatched bars represent a significant HW effect, and open bars represent a HW effect with a probability value <0.05.
4.2 HYPOTHESIS

Genetic determinants within the three regions (region 1, region 2, and region 3) regulate blood pressure.
4.3 Construction of congenic strains- The original congenic strains S.LEW(10)X12X2X3X8 and S.LEW(10)X12X2X3X5 (shown in Figure 4.1) and congenic substrains were constructed in our laboratory and maintained in the animal facility. These two strains were used as the progenitors and were backcrossed with S rats to obtain an F1 generation and this F1 generation was intercrossed to produce a F2 population. The rats with recombinations within the congenic segment were crossed with S to duplicate the recombinant chromosome and then selectively bred to fix the recombinant chromosome to homozygosity on the S background [233].
4.4 Results

4.4.1 High resolution mapping of BP QTL on S.LEW(10)x12x2x3x8 (region 1 and 3)

The F2 rats obtained from the parental S.LEW(10)x12x2x3x8xS (Figure 4-1) population were screened for polymorphism using the microsatellite markers D10Mco83 and D10Mco62 (Table A.1). Two recombinant progeny obtained were backcrossed to the S to fix for homozygosity. The resultant congenics were named as S.LEW(10)x12x2x3x8x1 (C1) and S.LEW(10)x12x2x3x8x2 (C2) and contained a 893.76kb and 435.5kb respectively of introgressed LEW alleles (Figure 4-2). Systolic BP (SBP) of male rats on a high salt diet for 21 days belonging to the two strains along with concomitantly raised S rats were measured by the tail cuff method (Figure 4-3) and survival, final body weight (FBW), relative heart weight (RHW) and total kidney weight (TKW) was also recorded (Table 4.1). As seen in Figure 4-3, C1 showed a significant BP decreasing effect of -27 ± 8.6 mmHg (p=0.0041) whereas C2 showed no BP effect (-4 ± 10 mmHg, p=0.7) compared to the S rat. As seen in Table 4.1, RHW of only C1 was significantly lower than that of S (0.2 ± 0.05gms, p=0.0013) but this effect was not seen in case of C2 (0.1 ± 0.06gms, p=0.06). The final body weight effect of C1 and C2 were not significantly different from that of S (11.5 ± 6 gm, p= 0.07 and 12.7 ± 9 gm, p=0.2 respectively), and total kidney weight of congenic 1 (0.09 ± 0.1 gm, p= 0.4) and C2 (0.10 ± 0.1 gm, p= 0.3) strains were also not significantly different from that of the S. The survival of these animals as measured by the Kaplan Meier plot shows that the lifespan of the C1 is significantly higher than the S rat (p<0.001) on high salt but C2 showed no such
effect (p=0.54) (Figure 4-3). Urinary protein excretion of these two strains was not measured.
**Figure 4-2 New iteration of congenic substrains.** The black bar on the left side shows the relevant section of the physical map of RNO10. The left side of this bar has the names of the polymorphic end markers and the SNPs in the form of (S/LEW) and the right side of the bar has the physical location of these markers and SNPs in base pairs. The bars in orange on left side are the previously mapped QTLs and the current QTLs are given at the right end. Congenic strains are shown as solid colored bars flanked by open bars. Solid color bars illustrate the LEW segment introgressed onto the background of S. The open bars at the end of each introgressed segment represent the region of recombination. Green colored bars represent the LEW introgressed segment in congenic substrains with a BP effect, whereas the black colored bars represent the LEW introgressed segment in congenic substrains without a BP effect. At the bottom is the BP effect of the corresponding strains above.
Table 4.1 Phenotypic characterization of congenic substrains - Urinary protein excretion (UPE), relative heart weight (RHW), total kidney weight (TKW - left + right kidney weight) and final body weight (FBW) was recorded at the end of the BP study. The significant values (p<0.05) are indicated by *.

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Figure 4-3 Systolic blood pressure and the Kaplan-Meier survival plots for C1 and C2.

The black bar indicates SPB (mmHg) of the S, Grey bar indicates the SBP of strain C1 and white bar represents SBP of C2
Figure 4-3 Systolic blood pressure and the Kaplan-Meier survival plots for C1 and C2.

Top - Systolic blood pressure of male rats on 2% NaCl diet was measured using tail cuff method. Error bars (SEM) are indicated on top of each bar and * indicates significant difference. (n=15-20 per group) the SBP values are S= 213.4±7.1 mmHg, C1= 186.6±5.2 mmHg, C2= 217.5±6.4 mmHg. The y axis represents the SBP in mmHg and the x axis represents the strains. The black bar is S, grey bar is C1 and white bar is C2

Bottom - The survival study for S1 (n=14) was done along with C1 (n=15) and S2 (n=14) was done along with C2 (n=16). These animals were continued on high salt (2% NaCl) after the BP experiment until their natural death. S1 versus C1, p=0.0009 and S2 versus C2, p=0.51
The two strains, congenic 1 and 2 (C1 and C2) were then used as parental strains to further narrow region 1, 2 and 3 by making a new iteration of congenic substrains. The four new congenic substrains obtained are - S.LEW(10)x12x2x3x8x1x1 (C3), S.LEW(10)x12x2x3x8x1x2 (C4), S.LEW(10)x12x2x3x8x2x3 (C5), and S.LEW(10)x12x2x3x8x2x4 (C6) (Figure 4-2). BP by telemetry method was measured and urinary protein excretion was studied as a unit of renal function. RHW, TKW and FBW was noted after the experiment was completed.

The LEW introgressed segment for C3 is 752.4kb (71169252bp to 71921657bp) and C4 is 517.08kb (71545930bp to 72063014bp). The region of recombination was genotyped using SNP data for S and LEW rat genome on [http://rgd.mcw.edu/rgdweb/front/select.html](http://rgd.mcw.edu/rgdweb/front/select.html) and hence SNP locations along with microsatellite marker locations have been indicated in the figure (Figure 4-2). C3 did not show a significant SBP effect at any time point during the three day course of BP measurement (Figure 4-3a) but C4 had significantly lower SBP at every time point compared to the S rat during the three days of BP measurement with the effect ranging from -13 to -20 mmHg (Figure 4-4b). The UPE effect of C3 (p=0.4) or C4 (p=0.6) did not differ significantly compared to the S rats. (Table 4.1).

C5 has an introgressed LEW segment of 49.4kb and spans across the region 70752452bp to 70801867bp. (Figure 4-2). The strain shows no significant change in SBP or DBP compared to the S rat at any time point during the three days that the telemetry recordings were monitored but MAP showed significant lowering at all time points. (Figure4-4c) There was also no significant change in the UPE, RHW, FBW or TKW as compared to the S rat (Table 4.1).
C6 has an introgressed LEW segment between 70878449bp and 71170090bp (291.64kb). The alleles in the LEW segment causes a significant increase in BP of the S rat (Figure 4-4d). The RHW (p=0.04) and TKW (p=0.04) is significantly higher that the S rat strain but there is no change in the UPE (p=0.27) and FBW (p=0.1) measured on high salt diet compared to S (Table 4.1).
Figure 4-4 Radiotelemetry data for C3, C4, C5 and C6. Data plotted shows the recording obtained once every 5 minutes over a period of three days and the data points plotted are averaged for 4 hour intervals. The p values are obtained by 1-way ANOVA followed by Tukey- HSD test. Error bars represent the standard error of the mean. The values obtained from the S rat are plotted in red and the values from the congenic are plotted in green. The black bars on the x axis indicate the night cycles.

Figure 4-4a BP data for S (n=11) and C3 (n=10). The data points are not significantly different at any time point (p>0.05).
Figure 4-4b BP data for S (n=10) and C4 (n=11). The data points that are not significant (p>0.05 is indicated in #) and if the data points are not significant except at few points they are marked by *.
Figure 4c BP data for S (n=6) and C5 (n=8). The data is not significantly different at any time point except for mean arterial pressure where p<0.05 at all points.
Figure 4-4d BP data for S (n=11) and C4 (n=10). The data points are not significant except at few points that are marked by *. 
Discussion

The three regions (region 1, 2 and 3) mapped previously on the parental strain S.LEW(10)x12x2x3x8 containing a LEW segment of 1.34Mb is now divided between the two substrains S.LEW(10)x12x2x3x8x1 containing a LEW segment of 902.26kb and S.LEW(10)x12x2x3x8x2 containing a 435.5kb LEW segment.

The substrain C1 shows a significant BP lowering effect and significant reduction in relative heart weight. This could indicate that the LEW alleles responsible for lowering of BP of the S rat could also be involved in protection of the heart against hypertrophy [145, 241-243]. The LEW introgressed within the strain spans across the genomic location 71160976bp to 72063232bp and contains the entire region 1 (71662574bp to 72063232bp) and part of region 3 (71160976bp to 71476900bp).

The substrain C2 (LEW segment lies within genomic location 70725437bp to 71160976bp), showed no BP effect even though it contains the previously reported strain S.LEW(10)x12x2x3x5 (<320.6 kb) in its entirety which exhibited a significant BP increasing effect (Figure 4-2). The substrain contains all of region 2 (70996834bp to 71100513bp) and part of region 3 (71067496bp to 71160976bp). This may be evidence that C2 contains genetic elements within the QTL that are interacting with each other to mask out or cancel the BP increasing effect when additional LEW alleles in the location 71067496bp to 71160976bp are present along with the LEW segment in the S.LEW(10)x12x2x3x5 strain.

This helps to strengthen the hypothesis that the previously prioritized gene Rf/fl [196, 240] is a genetic factor involved in BP regulation.
Strain C3 contains part of region 1 (71662574bp-71921657bp) and region 3 (71169252bp-71476900bp). This strain showed no BP effect and no UPE, RHW or FBW effect compared to S rat strain indicating that the LEW alleles in the integrated segment may not independently regulate BP. Strain C4 has a significantly lower SBP, DBP and MAP compared to S strain. Measurement of heart beat per minute shows significantly higher heart rate during the active night cycles in this strain which may indicate a better heart function compared to the S rats. The LEW introgressed segment in this congenic strain contains the entire region1. By differential segmentation using C3 and C4, the region 1 (401 kb) can be fine mapped to a 141kb BP QTL that contains BP regulating elements. This shorter region 1 contains three protein coding genes- Heat repeat containing protein 6 (Heatr6) which has been implicated in breast cancer in human [244] studies, Extracellular proteinase inhibitor (Expi), and LOC360228 each of which are potential candidates for BP regulation. Previously it was reported that Expi did not show any variations between S and LEW [191] but the gene showed a significant change in expression in the kidney samples (2.71 times higher in S). LOC360228 showed significant change in expression in heart samples (3.6 times higher in S compared to parental S.LEWx12x2x3x8) [192].

Strain C5 did not show any BP effect or changes in the other measured phenotypes measured whereas strain C6 containing part of region3(71070581bp-71170090bp) and the entire region 2 showed a BP increasing effect further strengthening the hypothesis that Rffl may be an important factor in BP regulation. With the help of C3 and C6, region 3 has also been refined by congenic differential segmentation to a smaller 103 kb region containing 4 protein coding genes- DNA repair protein RAD51 homolog 4 (Rad51l3), Fibronectin
Type III Domain Containing 8 (Fndc8), Notchless Homolog 1 (Nle1), Unc-45 Homolog B (Unc45b).
4.4.2 High resolution mapping of BP QTL on S.LEW(10)x12x2x3x5 (region 2)

The strain S.LEW(10)x12x2x3x5 containing region 2 was previously reported to show a BP increasing effect compared to the hypertensive S rat and the gene *Rffl* was prioritized as a candidate in BP regulation [196, 240]. To confirm the role of *Rffl* gene, we attempted to make substrains containing shorter congenic segments that have only the *Rffl* gene. Before I started my dissertation work in the laboratory, several rounds of breeding, genotyping to screen for recombinants was carried out on approximately 2000 animals but the parental strain S.LEW(10)x12x2x3x5 did not produce any sub-strains that recombined to include the LEW alleles at region 2 containing the *Rffl* gene. But the process of natural recombination yielded a congenic substrain S.LEW(10)x12x2x3x5x1 (C7) (Figure 4-2). Hence we phenotyped this strain to help us fine map the LEW segment on the parental S.LEW(10)x12x2x3x5.

C7 spans the genomic region 70752452bp to 70868736bp (116.3kb). Using the tailcuff method, SBP of male rats on high salt was measured. The C7 strain has significantly lower SBP (183.4 ± 4 mmHg) compared to the S rat (211.5 ± 6 mmHg) with a SBP effect of -28 mmHg, p<0.001 (Figure 4-5). To determine if the alleles responsible for the BP lowering effect is salt sensitive, SBP of male rats was again measured using the tailcuff method on a high (2% NaCl) and low (0.3% NaCl) salt diet. The results demonstrate that the BP lowering effect was observed only when the animals were on a high salt diet (SBP effect= -28 mmHg, p=0.001) and not on a low salt diet (SBP effect= ...
-6 mmHg, p=0.2) (Figure 4-5). Further a similar experiment was done on female rats to determine if the BP lowering effect was gender specific. The results demonstrated that SBP of female C7 rats also showed a significant BP lowering effect of -19 mmHg, p=0.001 (C7 = 167.3 ± 3 mmHg and S = 186.1 ± 4 mmHg) on a high salt diet but no BP effect was noted on a low salt diet (C7 was 171.7 ± 3 mmHg and S was 172.5 ± 3 mmHg, p=0.8) (Figure 4-5).
Figure 4-5 SBP data obtained by tail cuff method for C7. Error bars represent the standard error of the mean. The BP effect that are significant (p<0.05) is indicated in *.

Top- BP data for S (red) and C7 (green) males. (On 2% NaCl (S=18 and C7 =20) and 0.3% NaCl (S= 13 and C7 =13).

Bottom- BP data for S (red) and C7 (green) females. (On 2% NaCl S= 13, C7=13) and 0.3% NaCl S= 16 and C7=18).
As seen in Figure 4-6, the urinary protein excretion of both male and female C7 rats were significantly lower compared to the S strain following a high salt diet. The UPE effect of the male C7 strain was -65.41/kg bwt/24hrs (p=0.006) (male C7=249.0 ± 20/kg bwt/24hrs and S rat was 183.6 ± 7/kg bwt/24hrs) and the female C7 showed a UPE lowering effect of -46.48 ± 21/kg bwt/24hrs (p=0.04) (C7female= 225.3 ± 10/kg bwt/24hrs and S female rat (271.7 ± 19/kg bwt/24hrs) on a high salt diet. A similar UPE lowering effect was not observed on a low salt diet. Figure 4-6 also shows that the survival of the male C7 animals on high salt as represented by the Kaplan Meier plot is also significantly higher (p=0.0023) than the S rats with a median survival of 145.5 days for C7 and 110 days for the S rats.

There was a significant lowering of relative heart weight of C7 male rats on high salt diet by -1.222 ± 0.2 g (p= < 0.001) compared to that of the S rat (Table 4.1). This effect was not observed in the total kidney weight (p= 0.73).

The SBP results obtained by tail cuff method was corroborated using the telemetry method at 68 days of age and the results are shown in Figure 4-7. The results obtained from a three day BP period recording indicate that at every data point plotted, there is a significant decrease of SBP and DBP of the C7 compared to the S rat ranging from -26 mmHg to -38 mmHg. DBP lowering effect was also significant and ranged from -18 to -27 mmHg.

To determine the age at which the protection from high BP on high salt starts, BP measurements were monitored on 42 days old rats using by C10 radiotelemetry probes. Even at young age of 42 days, SBP of the C7 rats were significantly lower than the S rat (Figure 4-7).
Figure 4-6 Urinary Protein Excretion and Kaplan Meier plot for C7.
Top- UPE of male and female congenic substrain C7 (green) was compared to the S rat strain (red). SEM is plotted on the bar graphs and * indicates significant effect where p<0.05. n =15-17.
Bottom- Kaplan- Meier curves- Animals from both groups, C7 and S (n=10-12) were fed with 0.3% dietary salt for 6 weeks and then fed with 2% NaCl until their natural death. (P= 0.0023).
Figure 4-7 Radiotelemetry data for C7.
Figure 4-7 Radiotelemetry data for C7.
Top panel- C40 probes were used on 63 day old S rats (red) and C7 (green) animals and the data plotted is obtained by taking a 4 hour moving average of telemetry recording obtained every 5 minutes continuously over a period of 3 days. N= 12 per group. The black rectangles indicate the night cycle in a 24 hour time frame.
Bottom panel- Early time point assessment of BP by telemetry method was done in younger animals (42do) S-red and C7 (green) by implanting them with the C10 probes. Data is collected every 5 minutes for one day and plotted using the 4 hour moving average.
To find which SNPs/INDELS may be candidates for the change in BP, the genomic sequencing of the introgressed LEW segment of congenic 7 strain was completed using the standard read sequencing services by eurofinsgenomics.com before the genomic sequence of the SS/JR (ICL) and LEW/NCrl (ICL) was published [245]. Data obtained from RGD (http://rgd.mcw.edu) indicated that the LEW segment between positions 70752452 to 70868736 has a total of 174 SNPs. According to the genomic sequencing done by our lab the LEW segment has a total of 224 SNPs and 50 INDELS (Table 4.2).
<table>
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<th>Position (RGSC 3.4)</th>
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<td>Entire LEW segment</td>
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</tr>
<tr>
<td>Region 4</td>
<td>10: 70801867 to 70868736</td>
</tr>
</tbody>
</table>

Table 4.2 **Number of variations between S and LEW** within the introgressed LEW segment and within the QTL (region4)
In an attempt to prioritize candidates for BP regulation in the LEW segment of C7, a detailed bioinformatics analysis generated no known annotation on rat chromosome 10 (RNO10: 70752452bp to 7086736bp) whereas in homologous region on mouse chromosome 11 (MMU11) there was a novel long non-coding RNA LnC RNA - GM11426 at position 11:82633353-82636309 and in human chromosome 17 (HSA 17) there was NOVEL LnC RNA (CODING GENE) - RP11-642M2.1 at position 17:33164890-33186065 (Table A.2). Characterization or functional studies of either of these novel Lnc RNA has not been done as of now. Hypothesizing that a similar LnC RNA is present in the LEW segment in C7, we designed several primers but inspite of several modifications in PCR conditions and using various different sets of primers, we could not isolate any mRNA expressed from S, LEW or C7 strains (A.3). Further, to check if the expression of the adjacent genes was altered due to the presence of a regulator in this region, we did expression analysis for the genes Tmem132e and Cct6b genes (A.4). The results show that there was no significant differential expression in both of the adjacent genes, Tmem132e (heart p=0.96 and kidney p=0.29), Cct6b (heart p=0.17 and kidney p=0.87) compared to the heart and kidney samples of S rats on a high salt diet. Since we were incapable of obtaining evidence of presence of a regulator we hypothesized this genomic segment may be involved in RNA protein interaction (Table A.5). Real time experiments to observe for change in expression of the three genes that was given a high score by the prediction software (RPI seq) did not show any significant difference between the C7 and S (data not shown). This led to further hypothesize that the segment may be involved in promoter activity. Then we looked at promoter predictions in the genomic segment using the Promoter 2.0 prediction server and found four predicted segments. Luciferase assay was
conducted on these prediction and the results indicate that there is no promoter activity noted in these four predicted regions (A.6).
DISCUSSION

The goal of this study was to make congenic substrains from parental strains S.LEW(10)x12x2x3x5 that contains the region 2 and to confirm the role of Rffl in regulation of BP [196, 240]. Even though this goal was not attained, a fourth region has been added along with the BP QTLs (region 1, 2 and 3). This region 4 may contain genetic elements that once independent from parental strain S.LEW(10)x12x2x3x5 promotes BP lowering effect as seen in strain C7.

One of the reasons for the lack of substrains developed around the Rffl gene could be due to the presence of other important genes like Lig3 and Rad51 that play an important role in double strand break and repair mechanism. Hence lack of viable offsprings could be due to the presence of chromosomal segments called ‘recombination deserts’ or recombinationally suppressed regions of DNA where genetic recombination may lead to mutation, fixation or extinction of all allelic variants within an important haplotype that play a major role in survival. [246-253]

Overall the study resulted in the following findings:

1. The LEW segment in the parental strain S.LEW(10)X12X2X3X5 (10:70752452 to 71070581) shows a BP increasing effect compared to the hypertensive S rat indicating that the LEW genetic segment at this location are prohypertensive and causes the S rat BP to increase.

2. The LEW segment in C7 (10:70752452 to 70868736) when isolated from the parental S.LEW(10)X12X2X3X5 showed a BP decreasing effect compared to the
hypertensive S rat strain. The C7 also showed a rescue from high UPE indicating improved kidney function and increased lifespan. This could indicate that this LEW segment that currently contains no known annotations contains a functional variant/s that could be switched on or off relative to the neighboring genes or other variants [254].

3. Epistasis has been implicated as one of the major players in regulation of complex traits like essential hypertension, cancer, neurological disorders and cardiovascular disorders [194, 255-263]. Strong evidence of epistasis was observed between genetic elements on rat chromosome 10. To explain better, if we divide the LEW segment on the parental strain into three segments (Figure 4-8) - orange, blue and yellow. When the red color segment is present in the strain S.LEW(10)X12X2X3X5, there is a BP increasing effect, loss of the light blue and green segment in strain C7 causes the BP to be significantly lower than S. The presence of green segment or loss of red segment in S.LEW(10)X12X2X3X1 causes the BP effect to be lost. The black segment in C5 may be ruled out not involved in BP regulation. Hence the light blue and yellow segments may contain genetic elements regulating BP but the effect of yellow segment is lost or negated when present in S.LEW(10)X12X2X3X5. Hence if there is a BP regulating elements in either the light blue, green and yellow segments, interactions between them can lead to loss of function, gain of function or negating effect.

4. The missing or phantom heritability is due, at least in part, to epistasis or gene–gene interactions, which is yet to be explored in human and murine models. This study in the rat model is an example of one such interaction between genetic
elements from gene desert region with other genetic elements. The results are not
only an example of the prevalence of epistasis in BP regulation but also strengthen
the fact that SNPs within gene deserts as in C7 are important regulators of disease
development and prevention [124, 264-267].
Figure 4-8 Epistasis Model for BP regulation.

The black bar on the left side shows the relevant section of the physical map of RNO10. The left side of this bar has the names of the SNPs in the form of (S/LEW) and the right side of the bar has the physical location of these SNPs in base pairs. Strains C5, C7, S.LEW(10)X12X2X3X5 and S.LEW(10)X12X2X3X1 are shown. The LEW segment of S.LEW(10)X12X2X3X5 is red and light blue, that of S.LEW(10)X12X2X3 is black and green, C7 is dark blue and yellow and C5 is all black. The BP effects of respective strains are shown below.
Future Direction

Spatial organization of chromatin structures plays an important role in transcription of genes. Enhancers and insulators that lie distally influence formation of protein mediated loops [268-272]. Many of these regulators may lie in gene deserts and variations in the epigenome associated with SNPs in gene deserts help define complex phenotypes and diseases [123, 124, 264, 273, 274]. Techniques like Chromosome Conformation Capture and Hi-C is being currently used to study the architecture of genomes [268, 275-277]. Any of these methods will be used in future to fine map the gene desert on C7 strain to assign a functional role in BP regulation.
Chapter 5

Perspective

High blood pressure (hypertension) is the most common risk factor for other diseases which in turn accounts for more morbidity and mortality than any other category of diseases. Within the near future, it is conceivable that advances in medical technology and basic science will allow for therapy predicated on an individual’s genetic profile and variants.

Personalized medicine is based on the notion that all individuals are uniquely defined by their genome and the difference between health and disease is determined by their genetic makeup along with a blend of environmental factors. Hence understanding the mechanisms and roles of genetic elements and screening for variations within these elements helps to understand person’s predisposition to a disease and the susceptibility to environmental factors. This can change a person’s response to a given drug. But for common conditions like hypertension, the cause may be heterogeneous, with multiple genes involved and interacting with several environmental factors. Sorting out which characteristics in an individual patients profile can serve as a useful biomarkers in the detection, evaluation, treatment, and prevention of hypertension remains an arduous process. Although uniquely personalized medicine for each patient may not yet be
practical, progress can certainly be made towards an intermediate goal of identifying subgroups within the patient population with more homogeneous pathophysiology and greater likelihood of favorable responses to particular therapeutic and preventative interventions.
Appendix A

A.1 Primers for amplification of microsatellite markers and SNPs

<table>
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<th>Primers</th>
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A.2 Bioinformatics analysis of S.LEW(10)x12x3x5x1 (C7).

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A. mRNA expression analysis in C7 heart and kidney samples.

The top green line is the genomic region homologous in rat. The strains SLEW(10)x12x3x5x1 and S were used in the experiments. The strains used in the experiments were SLEW(10)x12x3x5x1 and S. The shorter lines are the regions where primers were designed. The gel picture is of the bands obtained from cDNA amplification and these bands were excised and sequenced. The gel picture is of the bands obtained from cDNA amplification and these bands were excised and sequenced. The gel picture is of the bands obtained from cDNA amplification and these bands were excised and sequenced.
A.4 Expression of *Tmem132e* and *Cct6b* transcripts from heart and kidney tissues of 54 days old animals of C7 were detected by RT-PCR. (Male rats, n=3 on 2% NaCl diet) the data shown is relative to the S rat assuming that S transcript level = 1
A.5 List of genes that are predicted for RNA-protein interaction on strain C7. The list is arranged not by the prediction numbers but the gene names.

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**total=46 genes**
A.6- **Luciferase activity** (for predicted promoters) was expressed as relative values to those generated by empty vector PGL3. HRK-293 cell lines were cotransfected with the PGL3 vector containing the inserted predicted promoter region. After 24 hours of transfection, the cells were harvested and luciferase activity was determined. The values are normalized to Renilla expression.
References


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95. Julier, C., et al., Genetic susceptibility for human familial essential hypertension in a
region of homology with blood pressure linkage on rat chromosome 10. Hum Mol Genet,
96. Xu, X., et al., An extreme-sib-pair genome scan for genes regulating blood pressure. Am J
97. Rice, T., et al., Genome-wide linkage analysis of systolic and diastolic blood pressure: the
Genome scan linkage results for longitudinal blood pressure phenotypes in subjects from
100. Rutherford, S., et al., Chromosome 17 and the inducible nitric oxide synthase gene
101. Kraja, A.T., et al., Two major QTLs and several others relate to factors of metabolic
102. Knight, J., et al., Human chromosome 17 in essential hypertension. Ann Hum Genet,
103. Franceschini, N., et al., A quantitative trait loci-specific gene-by-sex interaction on
systolic blood pressure among American Indians: the Strong Heart Family Study.
105. Dominiczak, A.F. and P.B. Munroe, Genome-wide association studies will unlock the
106. Fan, J.B., et al., Parallel genotyping of human SNPs using generic high-density
107. Oliphant, A., et al., BeadArray technology: enabling an accurate, cost-effective approach
p. 789-96.
110. International HapMap, C., et al., A second generation human haplotype map of over 3.1
111. Wellcome Trust Case Control, C., Genome-wide association study of 14,000 cases of
112. Sabatti, C., et al., Genome-wide association analysis of metabolic traits in a birth cohort
113. Dey, A., S.H. Chao, and D.P. Lane, HEXIM1 and the control of transcription elongation:
from cancer and inflammation to AIDS and cardiac hypertrophy. Cell Cycle, 2007. 6(15):
p. 1856-63.
114. Newton-Cheh, C., et al., Genome-wide association study identifies eight loci associated
115. International Consortium for Blood Pressure Genome-Wide Association, S., et al.,
Genetic variants in novel pathways influence blood pressure and cardiovascular disease


