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Author
O'Hearn, James David

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Unraveling the Genetic Basis of Heart Failure via Systems Genetics

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

James David O’Hearn

2017
ABSTRACT OF THE DISSERTATION

Unraveling the Genetic Basis of Heart Failure via Systems Genetics

by

James David O’Hearn

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology
University of California, Los Angeles, 2017

Professor Yibin Wang, Chair

Heart failure (HF) is the leading cause of death worldwide. It is a complex disease involving multiple aspects of pathology affected by genetic pre-disposition, the aging process, and environmental stressors. Defining the genetic basis for HF can provide keys to the underlying mechanisms and lead to a better personalized diagnosis and therapy. Recent progress has improved our understanding and management of diseases of the heart, yet its incidence, prevalence, and economic costs are steadily adding an increased burden to an already stressed health care system. Although life expectancy increases with the plethora of mechanical and chemical treatments, as well as lifestyle changes, overall life expectancy after diagnosis still has a poor prognosis.
Genome Wide Association Studies (GWAS) and Genome Module Association Studies (GMAS) in humans have had very limited successes in heart failure, in part, because of a lack of a quantifiable matrix for the disease and difficulties in tissue access for molecular studies. A systems genetics approach was developed to perform functional characterization of 107 mouse strains from a Hybrid Mouse Diversity Panel (HMDP). This approach takes full advantage of the genetic diversity offered by the HMDP combined with quantitative characterization of cardiac phenotypes. For the first time, we are able to systematically unravel the genetic basis of common forms of HF.

Three categories were designated, sensitive, intermediate, and resistant, after β-adrenergic stimulation using isoproterenol (ISO), a β-adrenergic receptor agonist. Extremely resistant and sensitive strains were selected for angiotensin II (AngII), a α1-adrenergic agonist, and transverse aortic constriction (TAC) treatment, a pressure overload model, to study the contribution of genetic background on the pathogenesis of HF. ISO, AngII, and TAC share the hypertrophic and fibrotic responses in a strain and stressor specific manner. The contractility phenotype is common for sensitive and resistant strains. Additionally, in cell culture, ISO, AngII, and phenylephrine increased cell size. Itga1, the alpha subunit of integrin receptors was found to have significant association with left ventricular hypertrophy (LVH). Together, these results suggest that genetic variants significantly contribute to the development of heart failure and the genes implicated in the specific pathological features of heart failure can be revealed via systems genetics.
We have identified a number of cardiac phenotypes implicated in diastolic dysfunction based on initial echocardiographic, blood pressure, tissue weight, and nitric oxide content data. More extensive studies using highly sensitive and resistant strains from the HMDP have shown differential responses. BXD75/RwwJ, C58/J, and AXB15/PgnJ revealed a clear conclusion for Heart Failure with preserved Ejection Fraction (HFpEF).

These results provide validation for our approach to unraveling the effects of genetic background associated with multiple cardiac phenotypic manifestations to pathological stressors and continue to generate hypotheses and analytical understanding in heart failure.
The dissertation of James David O’Hearn is approved.

James N. Weiss

Thomas M. Vondriska

Jau-Nian Chen

Yibin Wang, Committee Chair

University of California, Los Angeles

2017
Dedication

For everyone that has suffered from the illnesses associated with heart failure.
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VITA

2006
John Spielman Award in Chemistry
California State University
Los Angeles, CA

2008-2009
Undergraduate Student Researcher
Biochemist
Dr. Jamil Momand, Ph.D.
Department of Chemistry and Biochemistry
California State University
Los Angeles, CA

2009
B.S., Biochemistry
Department of Chemistry and Biochemistry
California State University
Los Angeles, CA

2009-2010
Research Scientist
Cell Signaling
University Auxiliary Services
Los Angeles, CA

2009-2010
Graduate Student Researcher
Breast Cancer Biologist
Dr. Jamil Momand, Ph.D.
Department of Chemistry and Biochemistry
California State University
Los Angeles, CA

2010
Graduate Student Researcher
Lung Cancer Biochemist
ACCESS
Dr. Steve Dubinett, M.D.
University of California
Los Angeles, CA

2010
Graduate Student Researcher
Lipid Metabolism Biochemist
ACCESS
Dr. Steve Young, M.D.
University of California
Los Angeles, CA
2011  Graduate Student Researcher  
Cancer Metastasis Geneticist  
ACCESS  
Dr. Lily Wu, M.D.  
University of California  
Los Angeles, CA

2011  Graduate Student Researcher  
Aging and Cancer Biochemist  
ACCESS  
Dr. Jing Huang, M.D.  
University of California  
Los Angeles, CA

2011-2017  Graduate Student Researcher  
Cardiovascular Biologist  
Molecular, Cellular, and Integrative Physiology  
Dr. Yibin Wang, Ph.D.  
Department of Anesthesiology  
Division of Molecular Medicine  
David Geffen School of Medicine  
University of California  
Los Angeles, CA

2011-2012  Teaching Assistant  
Molecular Biology  
Department of Life Sciences  
University of California  
Los Angeles, CA

2013-2016  National Institutes of Health  
Ruth L. Kirschstein  
National Research Service Award  
T32HL69766  
Vascular Biology Training Grant

2016  Judge  
Los Angeles County Science Fair  
Pasadena, CA
Chapter 1

Introduction
Disease Background

Heart failure (HF) is the leading cause of death worldwide. Recent progress has improved our understanding and management of diseases of the heart, yet its incidence, prevalence, and economic costs are steadily adding an increased burden to an already stressed health care system. Although life expectancy increases with the plethora of mechanical and chemical treatments, as well as lifestyle changes, overall life expectancy after diagnosis still has a very poor prognosis. Therefore, studying the etiologies of cardiovascular diseases and their associated pathologies have never been more pressing.

According to the American Heart Association, HF is a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood. A wide range of cardiac conditions, hereditary defects, and systemic diseases can result in HF. Of the estimated 38 million people living with HF more than half of all cases can be attributed to four underlying conditions: ischemic heart disease, chronic obstructive pulmonary disease (COPD), hypertensive heart disease, and rheumatic heart disease. Ischemia is a condition in which blood flow, and thus oxygen, to the heart is restricted or reduced by atherosclerosis, often leading to heart attack and sudden death. In COPD environmental factors play a strong role and lead to low oxygen levels which when present for a prolonged period can result in narrowing of the arteries. Hypertensive heart disease is one of the complications of high blood pressure which is present if the resting blood pressure is above 140/90 mmHg. Rheumatic heart disease, a major challenge in developing areas of the world, is the result of heart valve damage caused by an abnormal
immune response resulting in atrial fibrillation and HF\textsuperscript{5}. Considering several similarities with respect to symptoms it is easy to understand there is a complexity attributed to HF and many difficulties for diagnosis and treatment.

Over the past 50 years there have been many challenges for diagnoses and treatments including pharmacological and non-pharmacological approaches. For example, for atrial fibrillation, rate and rhythm control has used sotalol, disopyramide, and amiodarone, for cardio-embolic prevention there are oral anticoagulants, and electric cardioversion or AF ablation. For ventricular arrhythmias amiodarone and nadalol have been used to reduce ventricular tachycardia as well as implantable cardioverter defibrillators. β-blockers and ranolazine are customary for diastolic dysfunction and microvascular ischemia. For patients presenting with HF symptoms ACE inhibitors, β-blockers, mineralocorticoid receptor antagonists, diuretics, cardiac resynchronization therapy, ventricular assist devices, and heart transplant have been used\textsuperscript{6}.

In contrast to HF with reduced ejection fraction (HFrEF), HF with preserved Ejection Fraction (HFpEF) has many unique challenges in the research field because no treatments have improved outcomes\textsuperscript{7}. Imaging studies have demonstrated normal LV geometry in 30-45\% of patients, traditional noninvasive measures of diastolic function are normal in approximately one third of HFpEF patients, and left ventricular diastolic dysfunction (LVDD) is frequently detected in older persons without HF\textsuperscript{8}. HFpEF accounts for ~50\% of HF\textsuperscript{9}. This suggests that the heterogeneity of phenotypic manifestations may be much greater than in HFrEF. Understanding this increased heterogeneity may allow
more targeted clinical trials\textsuperscript{10}. A comprehensive examination of cardiac dysfunction and its association with adverse end stages may overcome deficiencies in the areas of diagnosis and, ultimately, treatment\textsuperscript{11}. There is great need for a method to classify HFP EF in a manner that defines a more homogeneous group of similar individuals, effectively leading to treatment of HFP EF patients for abnormalities overlooked by HFr EF investigations. HFP EF has historically been referred to as 'diastolic heart failure'. However, rather than being solely caused by diastolic dysfunction, HFP EF is caused by many impairments in ventricular diastolic and systolic reserve function, atrial dysfunction, stiffening of the ventricles and vasculature, and impaired vasodilatation\textsuperscript{12}.

Differences in the extent of cardiovascular injury lead to dynamic outcomes with inherited and environmental factors contributing to the risk of HF\textsuperscript{13}. Environmental variables add to the phenotypic pleiotropy seen in single-gene disorders and are believed to have early influences on cardiac biology and injury response\textsuperscript{14}. Although more than 40 single-gene disorders have been identified, researchers obtaining the ability to effectively explain the genetic component in the remainder of HF disorders will have less complicated obstacles for investigating the environmental etiologies\textsuperscript{15}.

Precision medicine is a health-care model in its infancy that facilitates accurate identification of the optimal course of care for an individual patient that integrates as much information that is currently available about not just the condition of the patient of interest but also the sum of all patients’ histories, such as systems biology, clinical research, molecular diagnostics, imaging, and health records\textsuperscript{16}. For example, the efforts to classify
patients according to renin, sodium, and volume profiling are a simple form of physiological and biochemical phenotyping that give precision medicine a basis for development\textsuperscript{17}. The efficacy of this approach currently is unproven and will require defining and understanding phenotypic and genotypic patterns before the targeting by a specific, customized, and individual cure can be realized.

Activation of neurohormonal systems is one of the most important mechanisms contributing to cardiac homeostasis in the short term, however, chronic stimulation leads to the progression of HF. Drug therapies against neurohormonal systems, including the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone systems (RAAS), has become widely used for HF\textsuperscript{18}. Patients with HF have increased chemosensitivity which correlates with increased neurohormonal activation, worsened arterial function, and decreased survival\textsuperscript{19}. The deleterious effects of sustained SNS and RAAS activation on the heart are counteracted by β-adrenergic blocking agents and angiotensin converting enzyme (ACE) inhibitors and attempt to correct the imbalance between endogenous vasoconstrictors, such as AngII, and vasodilators, such as natriuretic peptides, that results from sustained neurohormonal activation.

**Heart Failure Induction**

Inducing HF in animal models is one of the most efficient and cost effective ways to investigate the diseases it is associated with. There are several methods in the field of cardiovascular research to induce HF. Some methods use chemical pathological stressors, such as angiotensin II (AngII) or isoproterenol (ISO) with an osmotic pump. There are also
physiologically invasive methods including transverse aortic constriction (TAC), a pressure overload model, and ischemia/reperfusion (I/R) and coronary artery ligation, which produce myocardial infarction, the most common cause of subsequent HF in humans. Several common and well proven methods will be discussed below.

**Pathogenesis of Heart Failure**

There is a large body of evidence showing that AngII, through the renin-angiotensin aldosterone system (RAAS), is involved in cardiac hypertrophy, fibrosis, and elevated blood pressure20-23. Angiotensinogen, from the liver, is the substrate for renin, found in the kidney, which produces angiotensin I (AngI). The angiotensin converting enzyme (ACE) converts AngI to AngII. The mechanism of AngII is to stimulate one of the α1-adrenergic receptors, a G-protein coupled receptor (GPCR) known as AngII type-1 receptor (AT1). AT1 interacts with multiple heterotrimeric G-proteins producing second messengers, such as inositol trisphosphate (IP3), diacylglycerol (DAG), and reactive oxygen species (ROS). It also activates various intracellular protein kinases, the mitogen-activated protein kinase (MAPK) family, c-Jun N terminal kinase (JNK), protein kinase B (PKB), as well as many others22. There is no question as to why ACE has become a target of pharmaceutical inhibitors. There are a myriad of experimental models using AngII in animals and cell culture. In rodents there has been an effort to use subpressor doses, those which do not elevate blood pressure, in order to examine the effects of AngII on other pathologies, such as cardiac hypertrophy or fibrosis, without the confounding of hypertensive etiologies. These subpressor doses are most commonly in the range of 100-500 ng/kg/min but are still
able to produce ~20% increases in the heart weight to body weight ratio (HW/BW) and several-fold changes in fibrosis. The range of pressor doses, those which do stimulate high blood pressure, are usually from 500-1000 ng/kg/min, but may be higher\textsuperscript{24-34}.

Chronic activation of the β-adrenergic receptor (βAR) has also been shown to add to the development of adverse cardiac remodeling, cardiac myocyte death, and replacement fibrosis\textsuperscript{35}. ISO, a βAR agonist, was a clinical drug for the treatment of bradycardia but is now used in research for stimulation of cardiac hypertrophy and fibrosis\textsuperscript{36}. ISO increases heart rate by altering the conduction speed of the atrioventricular node and also increases the strength of contraction. ISO, as well as AngII, has a multitude of animal and cell models. Doses are often administered using osmotic pumps (10-60 mg/kg/day), by injection (60 mg/kg), intravenously (0.1–0.4 ug/kg/min), or added to culture media (10-100 uM)\textsuperscript{35, 37-40}.

**Surgical Methods**

Myocardial infarction (MI) is one of the leading causes of mortality and the most frequent proximal cause of HF in humans\textsuperscript{41, 42}. MI can be induced by ligation of the proximal left anterior descending coronary artery (LAD)\textsuperscript{43}. Rats often develop congestive HF after 3 weeks with reduced cardiac output and have little capacity to respond to pre- and after-load stress\textsuperscript{43}. Infarct size, the fraction of the total circumference of the left ventricle, thinning of the scar measured at the thinnest point, area of the left ventricular chamber, and the area of the left ventricular myocardium are all measured to determine the severity of this disease state\textsuperscript{44}. Reperfusion injury is caused by the restoration of circulation
and results in inflammation and oxidative stress instead of normal function and, therefore, the ischemia/reperfusion (I/R) model is an important line of inquiry in cardiovascular research. In this model the ligature is removed, usually within 1 hr, to allow reperfusion and subsequent injury which, depending on experimental design, may last from 1-24 hrs\textsuperscript{45}.

Transverse aortic constriction (TAC) employs partial ligation of the aorta between the innominate and left carotid arteries. In this procedure a silk suture is tied tightly around the artery and a needle (16-30 gauge) and then the needle is removed\textsuperscript{46-48}. This allows a 60-80\% reduction in internal diameter of the aorta with the severity increasing with higher gauge. This action reduces blood flow through the artery, significantly inhibiting flow into the left carotid artery and downstream aorta while increasing flow into the right carotid artery. Consequentially, due to pressure overload in the left ventricle, TAC surgery induces hypertension, left ventricular hypertrophy, and cardiac failure\textsuperscript{49}. The secondary dysfunctional effects of aortic banding are increased collagen content, stroke work and congestion of the lungs and liver, among others\textsuperscript{50}. When compared to LAD coronary artery occlusion, TAC provides a more reproducible model of cardiac hypertrophy and a more gradual time course in the development of heart failure\textsuperscript{51}.

**Candidate Gene Identification: Purposes, Methods, and Application**

**Purposes**

Although humans share significant genomic homology amongst one another, millions of differences still exist between individuals. There are several variations in the
individual, including single nucleotide polymorphisms (SNPs), copy number variants (CNVs), insertions, and deletions, the most abundant being SNPs\textsuperscript{52}. All of these may affect susceptibility to disease, physical attributes such as height, or do nothing at all. Linkage analysis has been the primary investigative tool in which family inheritance was closely monitored. This is a successful approach for Mendelian disorders; however, for polygenic diseases the results of these studies have been modest at best, leaving questions of complex diseases unanswered\textsuperscript{53}. Linkage studies are performed when you have a pedigree of related individuals and a phenotype, such as cardiomyopathy, that is present in some but not all of the family members. For each locus, cases are tabulated where parents and offspring both are positive or negative for the phenotype and also have the same allele. Linkage analysis is a very powerful approach when studying highly penetrant phenotypes, in which case, if the allele is present there is a strong probability of exhibiting the phenotype.

With the reduced costs of genotyping, methods such as Genome Wide Association Studies (GWAS) have become a reliable alternative to linkage analysis. In these cases the statistical test is a logistic regression or a related test for trends. They work when the phenotype has much lower penetrance and are, in fact, more powerful than linkage analysis, provided you have enough informative cases and matched controls. Association studies are how you find common, low penetrance alleles. The principle of an association study is straightforward, gather some individuals with a disease and some without and use statistical modeling to see if a certain genotype, usually a SNP or group of SNPs, is present more often in the cases than the controls. If the allele has a role in causing the disease, or is correlated with a causal allele, it will have a higher frequency in the case population than
the control population. It’s been 20 years since the statistical power of association studies have been postulated as being superior at finding weak genetic effects that go unnoticed in linkage studies\textsuperscript{54}. Over the last decade, human GWASs have dissected common complex diseases, such as cancer, type II diabetes, and various cardiovascular etiologies, to provide unbiased views of their genetic background\textsuperscript{55}. After genotyping millions of genetic variants this study type asks if there is bias in allele distributions between a diseased cohort compared to its non-diseased counterpart\textsuperscript{56}. Over 16,000 common genetic variants associated with disease have been cataloged and are available for download from the National Human Genome Research Institute\textsuperscript{56}. Several other factors have contributed to the advancement of GWAS, such as GWAS Central, which has no individual level genotypes or phenotypes, only group level aggregated data\textsuperscript{57}.

Genetic association studies and linkage analysis are both important in terms of their application. In Mendelian disorders, genetic linkage analysis is useful and informative. Whereas in polygenic or complex disorders, both environment and genetics play roles and, therefore, GWAS are superior for identifying loci with weak effects. The main differences between the two methods is that linkage analysis in based on pedigrees with a particular segregating phenotype with poorer genetic resolution in the centimorgan range. Association studies are based on larger populations that detect linkage disequilibrium with fine genetic resolution in the kilobase range. There are several advantages to association studies, such as not needing pedigrees, but instead a well-defined phenotype. Because linkage analysis does work on such a broad genetic scale, it often identifies many genes, most of which have little or no effect. On the other hand, GWAS examines the region more
closely. If there is SNP association it may be within the gene responsible or, possibly, another associated gene that is causal is in strong disequilibrium with your SNP.

With linkage studies, how do you identify what is going to be your cut off range for segregating two populations? How are the very mild versions of these diseases assessed when affected members of the pedigree have the gene of interest but maybe not all the required variants? Excluding presumably healthy family who have never been checked before they die and considering them healthy in your pedigree will lead to misinterpretation. With GWAS, you are not looking for a specific inheritance pattern, free of any hypothesis, and will identify variants that could be the cause, or have some responsibility for the severity, for the disease.

Methods

A case/control study is a common approach to GWAS. This results in unbiased allele frequencies underlying the distribution in the entire population. The individuals comprising each group are genotyped for known SNPs. Typically, there are approximately 500,000 to 1 million SNPs, or more, on a chip-based microarray which will capture >80% of commonly occurring SNPs. The case and control group allele frequency is compared for each SNP to determine if they are significantly different. In these studies, the fundamental unit for calculating effect sizes is the odds ratio for having an associated allele vs. not having the allele. Additionally, a chi-squared test may be used to determine genome wide significance (typically $p < 5 \times 10^{-8}$ in human studies). Less commonly used for GWAS are fully quantitative traits such as biomarker concentration, body mass index, or gene
expression, despite these GWASs having several advantages over simple case/control studies.

Gene expression may be studied in greater detail than other traits since the measured mRNA is the product of a single gene and, therefore, has a specific chromosomal location. The results of a GWAS on gene expression are termed expression quantitative trait loci (eQTL) and can be further classified as either cis, meaning the locus lies near to the gene in question, or trans, if the gene and locus are separated on the chromosome or are on different chromosomes. The Howard Hughes Medical Center was the first organization to publish a GWAS using eQTL as a trait. Many eQTL studies followed in plants and animals, including humans. Still, there are other methods for studying control of mRNA expression, such as RNA sequencing.

Imputation is the prediction of genotypes that have not already been genotyped in a sample of individuals. The purpose is to increase power for discovering rare variants, fine mapping, and in meta-analyses in which studies with different arrays may be combined with a reference panel of haplotypes. Large scale resequencing of reference panels are available from the International HapMap Project and 1000 Genomes Project. Several advanced software packages, such as IMPUTE2 and MaCH, are readily available for imputation.

It is common to take into account any variables that could potentially confound the results. Sex, age, and geographical origin are common examples of these variables. Because of these associations, for example, studies must take into account the geographic
and ethnic background of participants by controlling for population stratification. Using Efficient Mixed Model Association (EMMA), bioinformaticians can statistically test for model organism association mapping, essentially solving the problem of confounding from population structure and genetic relatedness.\(^{65}\)

The creation of correlation networks has become a valuable tool for geneticists and bioinformaticians for uncovering groups of genes, or modules, which affect a particular phenotype. Weighted Gene Co-expression Network Analysis (WGCNA) has been successful at creating modules which have high associations with cardiovascular disease, DNA methylation, and analysis of liver data.\(^{66}\) However, this software package only allows a gene to exist in a single module. Because genes have a vast array of important functions, and in order to account for non-linear interactions in the data, Maximal Information Component Analysis (MICA) combines the Maximal Information-based Nonparametric Exploration (MINE) with the Interaction Component Modeling for Genes (ICMg).\(^{67-69}\) This enables a gene to belong to multiple modules, an advanced biological representation over hierarchical clustering based algorithms such as WGCNA.

**Applications**

**Successes of Population Based Studies for Genetic Basis of Human Diseases**

Recently, GWAS-derived hypothesis generation for polygenic obesity traits in humans have uncovered common gene variants with subtle effects. These discoveries have generated new research into poorly understood signaling pathways.\(^{70}\) For example, whole
Exome sequencing has helped determine the role of leptin in energy homeostasis using extremely obese individuals from four consanguineous families. The study found two nonsense mutations (p.C186AfsX27 and p.H160LfsX9), leaving leptin lacking its necessary binding domain for signaling.

Another GWAS using electrocardiogram data as a measure of left ventricular hypertrophy (ECG-LVH), in which the community-based Korean Association REsource (KARE) was analyzed by microarray, showed the GWAS results were validated in a hospital-based cohort. Fourteen SNPs in 8 genetic loci were associated with ECG-LVH. Out of these 14, 12 were genotyped in the hospital sample with consistent association with the region which contains the RYR1 gene (ryanodine receptor 1), which encodes a major Ca²⁺ channel in skeletal muscle. From these, 3 SNPs in RYR1 were replicated (p-values < 2.7 × 10⁻² and 3.6 × 10⁻²) in the hospital cohort. However, the combined analysis of the KARE/replication sample demonstrated that the association signals of all three SNPs passed the threshold of significance for genome-wide associations (p < 7.2 × 10⁻⁸). The most significant SNP in the region (rs10500279) had genome-wide significance in the combined cohort (odds ratio = 1.58, p = 1.0 × 10⁻⁸). Additionally, mutations in RYR1 have also been reported to correlate with cardiovascular diseases.

A GWAS on a population consisting of BP extremes identified a SNP (rs13333226) in the 5′ region of the uromodulin (UMOD) gene which was associated with hypertension and later validated. A follow up study provided functional evidence that UMOD is involved in BP regulation by altering sodium excretion. In this study, there were clear BP
differences between UMOD KO mice and WT animals in that they displayed lower baseline BP and were insensitive to salt-induced changes in BP. In addition, they showed that this may be because of an inverse association between the expression levels of UMOD and the Na\(^+\)-K\(^+\)-Cl\(^2-\) cotransporter\(^{75}\).

**Limitations of Current Approaches for Complex Human Traits**

As an important advancement in discovering genetic variants that influence disease, there are still limitations to the GWAS approach. This includes a lack of well-defined case and control groups, controlling for multiple testing and population stratification, and insufficient sample size, all which can lead to false-positive and -negative results\(^{76}\). In many cases these can be minimized through quality control and study setup.

In particular, in 2010 Sebastiani et al. identified several SNPs associated with longevity\(^{77}\). However, in 2011 MacArthur discovered there was a flaw in the genotyping arrays between the case and control groups, leading to some of these SNPs being incorrectly associated with longevity\(^{78}\). Although the study was later retracted and subsequently corrected, Sebastiani had accurately differentiated between centenarians and controls\(^{79}\).

As another example, in the KARE study, only Minnesota ECG criteria, a code used mainly for population research and clinical trials and not for hospital care, were used to diagnose ECG-LVH cases. However, it has been suggested that voltage-only criteria are more strongly predictive of mortality\(^{80}\). The sample size of the replication study also was
quite small with many suggestive loci which are known risk factors being overlooked. In order to confirm these suggestive loci they must be examined in a larger population.

This begs the question, have past studies been stringent enough? Although present methods appear to be correcting past issues, inevitably, there will be new criticisms. There has also been a phenomenological dispute over the idea that common genetic variation plays a significant role in heritable variation. Furthermore, the results yielded by GWAS have only managed to explain 10% of heritability and; therefore, the funding may be better utilized in other ways. Fortunately, biotechnological advances continue to reduce the costs of array-based and complete genome sequencing methods.

**Candidate Gene Validation**

It is necessary and interesting to learn whether or not rare variants in the coding regions of candidate genes actually contribute to HF. Therefore, these candidate genes must be tested in order to validate their SNP-association with HF. Genetic factors play an important role in the development of HF. Up to now, >6,000 HF loci have been documented in [Online Mendelian Inheritance in Man](https://www.omim.org) (OMIM). However, the exact genes in a significant portion of HF loci remain unknown. Recently, Rau et al. identified 7 loci associated with cardiac hypertrophy, fibrosis, and surrogate traits related to HF in a panel of mice. In this study they identified Abcc6 \((p = 7.1 \times 10^{-7})\) as a novel gene which markedly and quickly promoted βAR-induced cardiac fibrosis.
In order to develop reliable techniques to investigate genotype-phenotype associations, there have been several breakthroughs in recent decades in genetic engineering. Engineered nucleases which induce site-specific double strand breaks (DSB) have enabled efficient gene modifications by homology directed repair (HDR) and non-homologous end joining (NHEJ). Cre-Lox recombination is also commonly used for deletions or insertions at specific sites. These modifications allow targeting to a specific cell type or triggering by a conditional stimulus.

Transcription activator-like effector nucleases (TALENs) changed the standard for genomic engineering methods. TALENs contain a nuclease domain fused to a TALE (transcription activator-like effector) DNA binding domain. TALEs contain 33–35 amino acid repeat domains that recognize a single base. TALE specificity is determined by repeat-variable di-residues (RVDs) made up of two hyper variable amino acids. The TALE repeats use four RVD domains, one for each specific nucleotide. The design of DNA segments encoding TALE repeats requires special cloning techniques and customized TALENs are commercially available.

Another gene editing breakthrough is CRISPR-Cas9. The clustered regularly interspaced palindromic repeat (CRISPR) has been shown to be a dual-RNA–guided DNA that uses CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) for DNA recognition. Cas9 has two putative nuclease domains, HNH and RuvC-like. It uses its HNH domain to cleave the DNA strand that is complementary to the 20-nucleotide sequence of the crRNA and the RuvC-like domain for the other strand. DNA target
recognition requires pairing to the crRNA sequence and the presence of a protospacer adjacent motif (PAM), a short sequence just downstream of the crRNA-targeted sequence on the target DNA\textsuperscript{85,86}. The dual crRNA:tracrRNA has also been engineered as a single guide RNA (sgRNA) that maintains two necessary features, the 20-nucleotide sequence at the 5′ end of the sgRNA and the double-stranded structure at the 3′ side of the guide sequence that binds to Cas9. This has created a simple two-component system in which changes to the guide sequence (20 nucleotides in the native RNA) of the sgRNA can be used to program CRISPR-Cas9 to target any DNA sequence\textsuperscript{84}.

In contrast to TALENs, which require substantial protein engineering for each DNA target site to be modified, the CRISPR-Cas9 system simply needs a change in the guide RNA sequence. For this reason, the CRISPR-Cas9 system has been adopted to target the genomes of a myriad of research models. Some analyses have shown that CRISPR-Cas9–mediated editing efficiencies can reach >80%, as high as observed using TALENs. The use of CRISPR-Cas9 for genome-wide studies will enable large-scale screening for drug targets, transcriptional regulatory domains, and many other phenotypes, expanding their utility in these models. There is overwhelming potential for use in human gene therapy as well\textsuperscript{87}.

The creation of conditional and tissue-specific recombination systems in transgenic mice has enabled monogenic trait analysis. For example, these transgenic mice may contain a tamoxifen-inducible Cre recombinase protein flanked by two mutant estrogen-receptor ligand-binding domains (MerCreMer). Placed under the control of the α-myosin heavy
chain promoter, these mice are powerful tools for inducing and investigating heart failure phenotypes. Sohal et al. crossed these mice with ROSA26 lacZ-flox–targeted mice and Cre recombinase activity was examined. Their studies showed no Cre-mediated recombination in the embryonic, neonatal, or adult heart without tamoxifen. However, with tamoxifen >80% recombination was achieved after four injections. Expression of this MerCreMer fusion protein did not affect cardiac fractional shortening, cellular morphology, or expression of the HF marker genes ANF, BNP, and aMHC	extsuperscript{88}.

In summary, these model systems are essential tools for temporally regulated inactivation of any gene of interest within the developing and/or challenged heart or for temporally recombinating and expressing an inactivated cardiac transgene	extsuperscript{88}.

Conclusion

Once a gene of interest has been properly manipulated, standard molecular biology and biochemical techniques may be used to interrogate its biological role. For example, it is important to understand if a candidate gene is cell, tissue, or organ specific, whether its protein product is circulating, and if it is extracellular to name a few possibilities. Additionally, determining at what developmental stage it has primary activity as well as its functional role (i.e. structural, signaling, transport, etc.). Most importantly, finding the mechanism of action is of utmost importance when attempting to elucidate the causality of candidate gene effects in cardiovascular genetics. This information will provide new methods for treatment, such as inhibition, reversal, and prevention of disease progression, using gene therapy, drug discovery, surgical techniques, and all other methods that have
proven efficacy. Importantly, reducing costs and providing easier access to healthcare is a challenge as great as overcoming the obstacles in solving the detriments suffered under heart failure.
Figure 1. Heart failure is the leading cause of death worldwide. Bridging the gap from phenotypic manifestation, medical prognosis, gene discovery, candidate gene validation, and translational medicine to improving quality of life and disease prevention is a multifactorial process. A process that is often shunted in the validation phase back to the phenotypic manifestation phase. Many treatments have been tried, many with proven efficacy, longer survival rates, and a slowed rate of disease progression, but far too often the side effects of these treatments result in altered HF phenotypes.
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Chapter 2

Comparative Characterization of Genetic Contribution to Multiple Pathological Stressors
Introduction

This chapter contains a copy of a manuscript currently in submission regarding the major findings of the comparative characterization of the role of genetic contribution, following treatment from multiple pathological stressors, with left ventricular hypertrophy, contractility, and fibrosis. The results showed significant genetic contribution to contractile dysfunctions are by ISO and TAC while to cardiac hypertrophy by AngII and TAC. Surprisingly, there is a minimally shared genetic contribution to cardiac fibrosis induced by all three stressors. Together, these results imply that distinct sets of genetic variants contribute to the specific features of cardiac remodeling and heart failure in a stressor specific manner.
Comparative Characterization of Genetic Contribution to Multiple Pathological Stressors

Jim O’Hearn1-3, Christoph Rau1,2, Jessica Wang2,4, Rozeta Avetisyan1,2, Aldons J. Lusis2-4, Yibin Wang1,4

1Department of Medicine/Division of Molecular Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA

2Departments of Anesthesiology, Physiology and Medicine, Cardiovascular Research Laboratory, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA

3Department of Molecular, Cellular, and Integrative Physiology, David Geffen School of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA

4Department of Physiology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA

5Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA
ABSTRACT

Heart failure (HF) is a complex disease contributed by both genetic diversity and multiple pathological stressors. Although multiple stressors often converge to similar pathological manifestations in hypertrophy, cardiac fibrosis, and contractile dysfunction, it is not clear whether different pathological stressors have shared or distinct genetic pathways, thus modulated by the same or different genetic background. We attempted to address this question using a cohort of the Hybrid Mouse Diversity Panel (HMDP). In a previous study, resistant and sensitive strains were identified in response to chronic β-adrenergic stimulation using isoproterenol (ISO). Selected strains were further characterized for cardiac phenotypes following angiotensin II (AngII), an α1-adrenergic agonist, and transverse aortic constriction (TAC). The results showed significant genetic contribution to contractile dysfunctions are by ISO and TAC while to cardiac hypertrophy by AngII and TAC. Surprisingly, there is minimal shared genetic contribution to cardiac fibrosis induced by all three stressors. Together, these results imply that distinct sets of genetic variants contribute to the specific features of cardiac remodeling and heart failure in a stressor specific manner.

Keywords: isoproterenol, angiotensin II, transverse aortic constriction, echocardiography, hypertrophy, fibrosis, contractility, ejection fraction, Hybrid Mouse Diversity Panel, Genome Wide Association Study

INTRODUCTION
Heart failure (HF) is the leading cause of death worldwide\(^1\). It is a complex disease involving multiple aspects of pathology affected by genetic pre-disposition, the ageing process, and environmental stressors. Defining the genetic basis for HF can provide keys to the underlying mechanisms and lead to a better personalized diagnosis and therapy. GWAS in humans have had very limited success in heart failure, in part, because of a lack of a quantifiable matrix for the disease and difficulties in tissue access for molecular studies\(^2\). We developed a systems genetics approach to perform functional characterization of >100 mouse strains from a cohort of thoroughly genotyped mice, the Hybrid Mouse Diversity Panel (HMDP)\(^3\). This approach takes full advantage of the genetic diversity offered by the HMDP combined with quantitative characterization of cardiac phenotypes. For the first time, we are able to systematically unravel the genetic basis of common forms of HF. However, heart failure is a complex disease involving diverse etiologies involving different pathological conditions. It is a pressing question for the field whether different pathological stressors confer cardiac remodeling through common or distinct genetic pathways. Utilization of the HMDP in response to different pathological stressors offers a unique opportunity to address this question.

In humans, a major pathological condition associated with HF is chronic hypertension which leads to increased mechanical overload to the left ventricle (LV) and triggers LV hypertrophy, remodeling, and eventual dysfunction. In mice, this condition can be partially simulated by transverse aortic constriction (TAC), which employs partial ligation of the aorta between the innominate and left carotid arteries. In this procedure, a silk suture is tied tightly around the artery and a needle (16-30 gauge) and then the needle
is removed\textsuperscript{4,6}. This allows a 60-80\% reduction in internal diameter of the aorta with the severity increasing with higher gauge. This action reduces blood flow through the artery, significantly inhibiting flow to the downstream aorta. Consequentially, due to pressure overload in the left ventricle, TAC surgery induces LV hypertrophy and heart failure\textsuperscript{7}. The secondary dysfunctional effects of aortic banding are increased collagen content, stroke work, and congestion of the lungs and liver, among others\textsuperscript{8,9}. Pressure-overload induced LV hypertrophy and HF are clinically relevant as disease models for humans and involves both \( \alpha \) - and \( \beta \) -AR overdrive.

In response to hemodynamic overload, chronic activation of sympathetic and humoral systems is also a hallmark of HF\textsuperscript{10}. There is a large body of evidence showing that AngII, through the renin-angiotensin aldosterone system (RAAS), is involved in cardiac hypertrophy, fibrosis, and elevated blood pressure\textsuperscript{10-13}. Angiotensinogen, from the liver, is the substrate for renin, found in the kidney, which produces angiotensin I (AngI). The angiotensin converting enzyme (ACE) converts AngI to AngII. The mechanism of AngII is to stimulate one of the \( \alpha_1 \) -adrenergic receptors, a G-protein coupled receptor (GPCR) known as AngII type-1 receptor (AT\textsubscript{1}). AT\textsubscript{1} interacts with multiple heterotrimeric G-proteins producing second messengers, such as inositol trisphosphate (IP3), diacylglycerol (DAG), and reactive oxygen species (ROS). It also activates various intracellular protein kinases, the mitogen-activated protein kinase (MAPK) family, c-Jun N terminal kinase (JNK), protein kinase B (PKB), as well as many others\textsuperscript{12}. There is no question as to why ACE has become a target of pharmaceutical inhibitors. There are a myriad of experimental models using AngII in animals and cell culture. In rodents there has been an effort to use
subpressor doses, those which do not elevate blood pressure, in order to examine the effects of AngII on other pathologies, such as cardiac hypertrophy or fibrosis, without the confounding of hypertensive etiologies. These subpressor doses are most commonly in the range of 100-500 ng/kg/min but are still able to produce ~20% increases in the heart weight to body weight ratio (HW/BW) and several-fold changes in fibrosis. The range of pressor doses, those which do stimulate high blood pressure, are usually from 500-1000 ng/kg/min, but may be higher

In addition to AngII, catecholamine is also chronically induced during heart failure. Originally viewed as a compensatory response in order to augment cardiac contractility and output, as well as peripheral vessel constriction to improve cardiac return, chronic catecholamine over-activation is detrimental by inducing cardiac hypertrophy, remodeling, and dysfunction. AngII is a main driver of cardiac hypertrophy via α-AR, PKC, and MAPK signaling and contributes to fibrosis via direct impact on fibroblasts and ECM.

Isoproterenol (ISO), a β-AR agonist, is a main driver of not only contractile dysfunction via PKA signaling, but also cardiac hypertrophy and fibrosis due to calcium overload, CaM kinase activity, and cell death. Whereas α-adrenergic stimulation promotes myocyte protein synthesis predominantly through activation of the Gq signaling pathway, β-AR stimulation promotes hypertrophy through Gs- and Gi-mediated activation of the MAPK cascades. In addition, β-agonists can also enhance Akt and IL-6 synthesis in cardiomyocytes and fibroblasts. Ornithine decarboxylase and calcineurin have also been implicated in hypertrophy in mice. The physiologic effect of ISO is positive cardiac inotropy and chronotropy and, therefore, is administered in cases of bradycardia, heart
block, or pulmonary emergencies. In mice, sustained activation of the adrenoceptors by chronic administration of ISO is a classical and well characterized model of LVH independent of blood pressure. Earlier literature suggests ISO-induced LVH is modulated by strain-specific factors. For example, phenotypic characterization showed that A/J mice developed greater changes in morphology than C57BL/6J and other evidence from FVB and C57BL6/SV129 mice showed that strain-specific genetic variants are also likely to modulate the chronotropic action of ISO on heart rate.

In our initial study, chronic stimulation of isoproterenol (ISO) was implemented in the HMDP. HMDP mice were subjected to ISO treatment for 21 days using an osmotic pump implanted in the peritoneum. Due to the ability of ISO to activate βAR, it was expected to produce a LV hypertrophy (LVH) phenotype. Detailed echocardiogram and pathological analyses were performed and the phenotypic diversity in cardiac LVH, contractility, and fibrotic remodeling in the HMDP strains demonstrates the significant contribution of genetic background on the pathogenesis of HF. From this study, a cohort of resistant and sensitive strains were identified regarding their pathological responses to ISO stimulation as measured from hypertrophy, interstitial fibrosis, and contractile dysfunction. These insights allowed us to investigate the effects of genetic variants on cardiac pathology under different stressors including AngII and TAC.

**MATERIALS/METHODS**

**Mouse surgery.** Strains (4 resistant, 2 sensitive) were selected based on the most resistant/sensitive to ISO for the formation of increased left ventricular (LV) weight,
fibrotic tissue in the LV, and LV ejection fraction. A/J, BALB/cJ, C3H/HeJ, BTBRT<+>tf/J are sensitive and C57BL/6J and AXB8/PgnJ are resistant. Mice received micro-osmotic pumps (Alzet, model 1004) for ISO (20 mg/kg/day), AngII (1 ug/kg/min), or transverse aortic constriction (TAC), all for 21 days.

**Echocardiography.** Mice were anesthetized with 1.5% isoflurane. Using a Vevo 2100 with an MS250 (13-24 MHz) transducer (VisualSonics), LV long and short axes were taken in M- and B-mode, E/A ratio was taken with color and tissue doppler. Aortic arch and abdominal velocity and wall thickness were measured with color doppler and M-mode, respectively, as well. Procedures were conducted for baseline before treatment and for day 7, 14, and 21 after treatment.

**Mouse tissue.** Mice were sacrificed on day 21 using isoflurane and cervical dislocation. Plasma, heart, adrenal glands, liver, lung, and kidney weights were measured. The heart was subdivided by chamber and individual weights were measured. Tissue was flash frozen in liquid nitrogen and stored at -80° Celsius and/or placed in formalin. The tibia was also removed and length was measured.

**Histology.** LV tissue, after being stored in formalin, was placed in paraffin wax blocks, sections were cut, and placed on microscope slides. Using Masson trichrome staining, sections were stained for collagen fibers.

**Cell Culture.** Neonatal rat ventricular myocytes (NRVM) were plated in 6 well plates (250,000 cells/well) on day 0 using DMEM with FBS and P/S. After 24 hrs, wells were
washed twice with PBS, given DMEM with ITS. At 48 hrs, either ISO (20 µM), AngII (20 µM), or phenylephrine (50 nM) was administered. On day 3 cells were imaged and cross-sectional area was measured.

**Microscopy.** An Eclipse TE-2000U inverted microscope with NIS Elements (Nikon) imaging software was used to measure cross-sectional area of NRVMs and to quantify collagen fibers in Masson trichrome stained LV tissue sections.

**Candidate gene identification.** GWAS was performed using Factored Spectrally Transformed Linear Mixed Models (FaST-LMM).

**RESULTS**

In previous studies, HMDP mice were subjected to ISO treatment for 21 days using an osmotic pump implanted in the peritoneum. Among the 105 mouse strains (Fig. 1a), the response was highly variable in terms of cardiac hypertrophy (LV weight/tibia length), contractile function (ejection fraction), or interstitial fibrosis (Masson trichrome staining of LV) due to genetic diversity. These results suggest that the underlying genetic background plays an important role in the heterogeneity of multiple cardiac phenotypes. Accordingly, the HMDP cohort was ranked based on being either sensitive or resistant strains to ISO treatment\(^{29}\) (Fig. 1b, c).

We then wanted to determine if strains that had higher levels of ISO induced LVH also manifest more fibrotic remodeling and/or higher degrees of contractile dysfunction. Using the criteria of >25% LV weight gain (Fig. 1a), >5% LV fibrosis (Fig. 1b), and >10%
reduction in ejection fraction (Fig. 1c), we identified C3H/HeJ and BTBRT+ as developing higher levels of all three phenotypic features, namely LVH, fibrosis, and contractile dysfunction. In contrast, AXB8/PgnJ was resistant to all three pathological features (<25% LVH, <5% fibrosis, and <10% reduction in EF). This observation suggests that a certain genetic background can confer disease vulnerability in all three features. This led us to question if the genetic factors responsible for resistance or vulnerability to ISO-induced cardiac remodeling and dysfunction would be shared with other pathological stresses. To address this notion, 6 strains were selected for the degrees of ISO induced cardiac pathology, 4 were highly sensitive (A/J, BALB/cJ, C3H/HeJ, and BTBRT+) and 2 were resistant (C57BL/6J and AXB8/PgnJ). We then subjected them to two different pathological stressors: one is chronic stimulation by angiotensin II (AngII, 1 ug/kg/min) and another is transverse aortic constriction (TAC, 27 gauge needle) for 21 days.

Among the two ISO-resistant strains, there were relatively low levels of LVH (Fig. 3 AngII: C57BL/6J +11%, p=NS; AXB8/PgnJ +3%, p=NS and TAC: AXB8/PgnJ +20%, p=0.006), fibrosis (Fig. 4 AngII: C57BL/6J 3.8-fold, p=NS; AXB8/PgnJ 3.6-fold, p=0.001 and TAC: C57BL/6J 1.9-fold, p=NS; AXB8/PgnJ 1.4-fold, p=NS), and loss of EF (Fig. 5 AngII: C57BL/6J -6%, p=NS; AXB8/PgnJ no change, p=NS and TAC: C57BL/6J +2%, p=0.004; AXB8/PgnJ -4%, p=NS) in response to AngII or TAC, except C57BL/6J displaying +65% (Fig. 3 p=9.7x10^{-7}) increase in LV post-TAC. These results suggest that resistance to pathological hypertrophy, fibrotic remodeling, and contractile dysfunction can be conferred potentially by a common genetic background shared by the resistant strains.
Among the strains that had a sensitive response to ISO treatment, A/J mice, which showed significant LV enlargement following ISO (+35%, p=0.004), did not develop LV hypertrophy in response to AngII (-2%, p=NS) and had modest hypertrophy after TAC (+13%, p=NS). In contrast, BALB/cJ mice developed significant hypertrophy in response to all 3 stressors (ISO +36%, p=2.5x10^-5; AngII +13%, p=0.038; and TAC +48%, p=3.6x10^-8). Furthermore, mice from the other two sensitive strains C3H/HeJ and BTBRT+ showed no significant changes in LV weight after AngII treatment (C3H/HeJ +3%, p=NS and BTBRT+ +4%, p=NS); however, they developed significant LV hypertrophy post-TAC (C3H/HeJ +32%, p=0.003 and BTBRT+ +76%, p=4.4x10^-6) in a similar pattern as observed after ISO treatment. Therefore, the results in Fig.3 show the ISO-sensitive strains have highly heterogeneous hypertrophic responses to AngII but relatively more uniformed chamber enlargement after TAC, indicating that βAR stimulation and pressure overload appear to share a common genetic pathway leading to hypertrophy which is likely more distinct from AngII induced hypertrophy.

For ventricular fibrosis, the induction patterns to different stressors among the sensitive strains were dramatically different from LV hypertrophy. While A/J was not significantly affected by any stressor (ISO: 3.3-fold, p=NS; AngII: 7.2-fold, p=NS; and TAC: 1.9-fold, p=NS), BALB/cJ mice showed significant induction of fibrosis only after ISO (7.2-fold, p=7.5x10^-7), but not after AngII (3.3-fold, p=NS) or TAC (no change, p=NS). In contrast, C3H/HeJ and BTBRT+ mice showed significant induction of fibrosis after both ISO (C3H/HeJ: 11.3-fold, p=0.001 and BTBRT+: 6.2-fold, p=2.8x10^-7) and AngII (C3H/HeJ: 2.4-fold, p=0.001 and BTBRT+: 6.3-fold, p=3.8x10^-5) but not after TAC.
The heterogeneity shown in fibrosis induction among the different sensitive strains indicates complex genetic factors leading to cardiac fibrosis (Fig. 4).

When focusing on cardiac contractility based on percent loss of EF, it was A/J that was most sensitive to all three stressors (ISO: -22%, p=0.011; AngII: -6%, p=0.010; and TAC: -15%, p=0.003), which was surprising considering it had a modest, at best, increase in LVH and fibrosis after AngII and TAC treatment. BALB/cJ was only sensitive to AngII (-9%, p=0.042) and TAC (-22%, p=3.9x10^-4). Interestingly, C3H/HeJ and BTBRT+ were sensitive to ISO (C3H/HeJ: -16%, p=0.034 and BTBRT+: -6%, p=0.046) and TAC (C3H/HeJ: -15%, p=0.003 and BTBRT+: -15%, p=0.006), but not AngII (C3H/HeJ: +8%, p=NS and BTBRT+: +2%, p=NS), identical to their LVH responses. The continued heterogeneity shown in loss of EF among the different sensitive strains also indicates complex genetic factors leading to cardiac fibrosis (Fig. 5).

These studies illustrate complex genetic contributions to specific features of cardiac pathology in response to different pathological stressors. To qualitatively analyze the genetic landscape associated with stressor-dependent manifestation of cardiac hypertrophy, fibrosis, and contractile dysfunction, we took advantage of the well-established genotypic information for the HMDP cohort and conducted a Genome Wide Association Study (GWAS) using FaST-LMM to identify associations of genetic variants and their commonality with each of the pathological traits30.
Figure 3A shows that cardiac hypertrophy induced by TAC and AngII shared 29,225 common single nucleotide polymorphisms (SNP). Surprisingly, cardiac hypertrophy induced by ISO and AngII only had 8 SNPs in common while none was found to be shared between ISO and TAC treatments. For cardiac fibrosis as illustrated in Figure 3B, however, there were no common SNPs detected among any of the three AngII, ISO and TAC treatments. Remarkably, for impact on contractility, 13,282 SNPs were shared between ISO and TAC treatment, while none were detected to be shared with AngII. The 8 SNPs that are common for ISO and AngII revealed association of rs6410794 (ISO, $p=4.22\times10^{-5}$ and TAC, $p=5.15\times10^{-6}$) with LVH and is located in the gene body of integrin, alpha 1 (Itga1) which encodes the alpha subunit of integrin receptors and is a member of the focal adhesion pathway.

**DISCUSSION**

LVH is a compensatory mechanism in response to an increased hemodynamic load on the heart\textsuperscript{31}. The heart, in particular the LV, must contract with more force to pump blood through a degraded vascular complex. Eventually, cardiomyocytes suffer from a chronic lack of energy and they lose their ability to maintain homeostasis, which, when coupled with an increase in fiber disarray, replacement fibrosis, and perivascular fibrosis, myocardial contraction is further degraded\textsuperscript{32}. AngII-induced hypertension may also result in interstitial fibrosis, both of which may contribute to an increase in left ventricular stiffness, resulting in diastolic dysfunction\textsuperscript{33}. Because LVH is a compensatory response to pressure or volume stress, which can subsequently lead to fibrosis, why is there a lack of
fibrosis in some sensitive strains? For example, these results show that post-TAC mice have a loss of contractility with increased LV weight; however, they are without fibrotic deposits. In contrast, the majority of sensitive post-ISO mice showed a loss of contractility, increased LVH, and elevated fibrosis. Surprisingly, the fibrosis response post-AngII for C3H/HeJ and BTBRT+ was not accompanied by a loss of contractility or increased LVH. These results support the idea that fibrosis is not only the result of myocardial dysfunction, but also may provide stability to an AngII-induced hypertensive heart.

Although no particular etiology predicts a specific cardiac pathology, there are 3 variables that we have uncovered with multiple similarities: strain, stressor, and phenotype. These results have raised several issues: why is a strain, such as A/J, able to resist increases in fibrosis and LVH while suffering such significant decreases in contractility? Moreover, C3H/HeJ and BTBRT+ showed signs of all 3 cardiac phenotypes post-ISO but not after AngII and TAC. Additionally, TAC, had a magnificent effect on LVH and loss of EF, but adds almost no fibrotic tissue. Furthermore, loss of contractility was present in all sensitive but not resistant strains. These studies have identified specific strains of HMDP mice with dramatically different manifestations of cardiac pathology in response to ISO. Using different stressors, such as pressure-overload (TAC) and AngII, we found the strains with different phenotypic responses to ISO also have similar but not identical patterns of responses to other stressors. This indicates that the genetic contribution revealed by ISO-induced HF may also have significant implications in other HF etiologies and can, therefore, be potentially translated to HF in humans. Additionally, these results suggest
the phenotypic heterogeneity of different pathological stressors are not mutually exclusive and may share a significant level of overlap.

In this analysis, there was either massive amounts of overlap or absolutely none. Most interestingly, though, the 8 SNPs that are common for ISO and AngII revealed association of rs6410794 (ISO, $p=4.22 \times 10^{-5}$ and TAC, $p=5.15 \times 10^{-6}$) with LVH. This SNP was found in the gene body of integrin, alpha 1 (Itga1) which encodes the alpha subunit of integrin receptors and is a member of the focal adhesion pathway. Together with the beta subunit, they dimerize to form a cell-surface receptor for collagen and laminin which has been implicated in inflammation and fibrosis\textsuperscript{34}. Human ITGA1 is downregulated in atherosclerotic plaques\textsuperscript{35}. Additionally, chicken Itga1 is highly expressed and normally associated with reduced H3K27me3 differentially marked regions in virus-resistant birds after infection with Marek’s disease virus (MDV); however, virus-sensitive birds showed basal expression with increased H3K27me3 and H3K4me3 marks after MDV infection\textsuperscript{36}. Furthermore, in two separate GWASs, ITGA1 was found to have significant correlation with immune function and apoptosis in cardiac manifestations of neonatal lupus (rs2432143, $p=4.54 \times 10^{-5}$) in the first study and in the second it was found to have associations with inflammation and immunity in liver disease (rs4074793, $p=3.7 \times 10^{-10}$)\textsuperscript{34,37}. Interestingly, although there was a small sample size for sufficient power, FaST-LMM was able to reveal an Itga1 association with LVH. Because Itga1 has been implicated by multiple biochemical and GWA studies as having a role in cardiac pathologies,
including inflammation, fibrosis, and atherosclerosis, we believe this provides additional evidence that genetic variants significantly contribute to the development of heart failure and the genes implicated in the specific pathological features of heart failure can be revealed via systems genetics.

**Conflicts of Interest:** None to declare.
Figure 1. Four sensitive and two resistant HMDP strains were subjected to ISO, AngII, and TAC stress for 21 days. Before surgery all mice were subjected to echocardiography and weighed. After surgery at day 7, 14, and 21 mice were subjected to echocardiography and on day 21 were weighed, sacrificed, and tissues was weighed. Subsequent experiments included Masson trichrome staining of the left ventricle (LV) for collagen fiber, qPCR for LV hypertrophy marker expression, neonatal rat ventricular myocyte cell culture, and a genome wide association study.

Figure 2. Left ventricular weight vs. body weight (a), fibrosis (b), and LV ejection fraction (EF) in control and ISO-treated HMDP mice. The scales are ordered clockwise by increasing weight, fibroed tissue, or EF in controls (blue) with the corresponding ISO-treated (yellow) strains. The scales are in mg (a), fold change (b), and percentage (c).

Figure 3. LVH after ISO (A), AngII (B), and TAC (C) treatment was quantified as the ratio of left ventricular weight to tibia length for control (black) and treated (colored) HMDP mice. Grey bars in (C) are for sham mice that received surgery without banding.

Figure 4. Fibrosis after ISO (A), AngII (B), and TAC (C) treatment was quantified as the ratio of fibroed area to total area for control (black) and treated (colored) HMDP mice. Grey bars in (C) are for sham mice that received surgery without banding.

Figure 5. LV ejection fraction in control (A) and after ISO (B), AngII (C), and TAC/Sham (D) treatment for day 0 (black) and day 21 (colored) HMDP mice.
Figure 6. Overlap of genetic variation due to each stressor within the LV hypertrophy (A), fibrosis (B), and contractility (C) phenotypes. The numeric values indicate the amount of single nucleotide polymorphisms (SNP) for each stressor and the overlapping areas indicate the number of SNPs common to multiple stressors.

Supplemental Figure 1. Fibrosis after ISO (A), AngII (B), and TAC (C) treatment was quantified as the ratio of fibrosed area to total area for control (black) and treated (colored) HMDP mice.

Supplemental Figure 2. LV ejection fraction after ISO (A), AngII (B), and TAC (C) treatment for day 0 (black) and day 21 (colored) HMDP mice.

Supplemental Figure 3. Cross-sectional surface area of neonatal rat ventricular myocytes in control and ISO, AngII, and PE treatment.
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References


Chapter 3

Gene Expression and Candidate Gene Validation

in

Isoproterenol Treated Mice
Abstract

Human GWASs have dissected common complex diseases, such as cancer, type II diabetes, and various cardiovascular etiologies, to provide unbiased views of their genetic background. Using an isoproterenol-induced hybrid mouse diversity panel (HMDP), GWAS identified Motile sperm domain containing 3 (Mospd3) and 2610028H24Rik (H24) as having significant association with left ventricular hypertrophy. Expression analysis using quantitative PCR (qPCR) on select strains of ISO-sensitive and -resistant HMDP strains shows strain and stressor specific dynamic regulation. Small interfering RNA (siRNA) in neonatal rat ventricular myocyte (NRVM) cell culture was used against Mospd3 and H24 implicate Mospd3 as a regulator of basal cell size and H24 as a driver of hypertrophy. Overexpression of Mospd3 and H24 in NRVM and Madin Darby canine kidney (MDCK) cultures showed cytosolic localization.

Introduction

Although humans share significant genomic homology amongst one another, millions of differences still exist between individuals. These variations include single nucleotide polymorphisms (SNPs), copy number variants (CNVs), insertions, and deletions, with the most abundant being SNPs. All of these may affect susceptibility to disease, physical attributes such as height, or do nothing at all. Linkage analysis has been the primary investigative tool in which family inheritance was closely monitored. This is a successful approach for Mendelian disorders; however, for polygenic diseases the results of these studies have been modest at best, leaving questions of complex diseases
unanswered\textsuperscript{39}. With the reduced costs of genotyping, methods such as GenomeWide Association Studies (GWAS) have become a reliable alternative to linkage analysis. It’s been 20 years since the statistical power of association studies have been postulated as being superior at finding weak genetic effects that go unnoticed in linkage studies\textsuperscript{40}. Over the last decade, human GWASs have dissected common complex diseases, such as cancer, type II diabetes, and various cardiovascular etiologies, to provide unbiased views of their genetic background\textsuperscript{41}. After genotyping millions of genetic variants this study type asks if there is bias in allele distributions between a diseased cohort compared to its non-diseased counterpart\textsuperscript{42}. Over 16,000 common genetic variants associated with disease have been cataloged and are available for download from the National Human Genome Research Institute\textsuperscript{42}. Several other factors have contributed to the advancement of GWAS, such as GWAS Central, which has no individual level genotypes or phenotypes, only group level aggregated data\textsuperscript{43}.

It is necessary and interesting to learn whether or not rare variants in the coding regions of candidate genes actually contribute to HF. Therefore, these candidate genes must be tested in order to validate their SNP-association with HF. Genetic factors play an important role in the development of HF. Up to now, >6,000 HF loci have been documented in Online Mendelian Inheritance in Man (OMIM). However, the exact genes in a significant portion of HF loci remain unknown. Recently, Rau et al. identified 7 loci associated with cardiac hypertrophy, fibrosis, and surrogate traits related to HF in a panel of mice. In this study they identified Abcc6 ($p = 7.1 \times 10^{-7}$) as a novel gene which markedly and quickly promoted βAR-induced cardiac fibrosis\textsuperscript{29}.
Methods

Mouse surgery. Strains (4 resistant, 2 sensitive) were selected based on the most resistant/sensitive to ISO for the formation of increased left ventricular (LV) weight, fibrotic tissue in the LV, and LV ejection fraction. A/J, BALB/cJ, C3H/HeJ, BTBRT<+>tf/J are sensitive and C57BL/6J and AXB8/PgnJ are resistant. Mice received micro-osmotic pumps (Alzet, model 1004) for ISO (20 mg/kg/day), AngII (1 ug/kg/min), or transverse aortic constriction (TAC), all for 21 days.

Mouse tissue. Mice were sacrificed on day 21 using isoflurane and cervical dislocation. Plasma, heart, adrenal glands, liver, lung, and kidney weights were measured. The heart was subdivided by chamber and individual weights were measured. Tissue was flash frozen in liquid nitrogen and stored at -80° Celsius and/or placed in formalin. The tibia was also removed and length was measured.

Candidate gene identification. GWAS was performed using Efficient Mixed Model Association (EMMA).

Gene expression. Mouse tissue and NRVMs were homogenized using the phenyl/chloroform method (Trizol) to obtain total RNA. RNA was reverse transcribed to generate cDNA with SuperScript II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) employed the LightCycler® 480 II (Roche) with iTaq Universal SYBR Green Supermix (BioRad). The Livak Method was used for quantification (\(-\Delta\Delta C_t\)).
**Cell Culture and Transfection.** Neonatal rat ventricular myocytes (NRVM) were plated in 6 well plates (250,000 cells/well) on day 0 using DMEM with FBS and P/S. After 24 hrs, wells were washed twice with PBS, and given DMEM with ITS. Lipofectamine 2000 was used for siRNA (30 nM) transfection. At 48 hrs, either ISO (20 µM), AngII (20 µM), or phenylephrine (50 nM) was administered. On day 3 cells were imaged and cross-sectional area was measured. Madin-Darby canine kidney (MDCK) epithelial cells were plated in 6 well plates (50,000 cells/well) on day 0 using MEM with FBS and P/S. After 24 hrs, wells were washed twice with PBS, and given MEM with ITS. Lipofectamine 3000 was used for plasmid (1 µg) transfection. At 48 hrs, wells were washed twice with PBS, and given MEM with ITS. At 72 hrs, cells were imaged.

**Immunohistochemistry.** MDCK and NRVM plated cover slips were fixed with 4% sucrose in formalin for 15 min at room temperature. They were then washed in phosphate buffered saline (PBS) 4x for 5 min and permeabilized with 0.25% Triton X-100 in PBS for 5 min. Samples were then washed in PBS 4x for 5 min and blocked in 10% BSA/PBS for 30 min at 37 C. They were washed again in PBS 4x for 5 min and then incubated with anti-FLAG M2 (1:500) in 3% BSA/PBS overnight at 4 C. Samples were then washed in PBS 4x for 5 min and incubated with Alexa 488 goat anti-mouse (1:200) in 3% BSA/PBS with anti-mouse for 90 min at room temperature. Next, they were washed in PBS 4x for 5 min, counterstained with wheat germ agglutinin (WGA), and transferred to slides with Prolong Gold with DAPI (Invitrogen).
Microscopy. An Eclipse TE-2000U inverted microscope with NIS Elements (Nikon) imaging software was used to measure cross-sectional area of NRVMs. An Eclipse Ti inverted confocal microscope with NIS Elements (Nikon) imaging software was used to detect the presence of Mospd3 and H24 in MDCK and NRVM fixed cells.

Results

Mospd3 and H24 were identified using Efficient Mixed Model Association and both were strongly associated with LV hypertrophy (Mospd3 p = , H24 p = ). Mospd3 has been shown to regulate right ventricular chamber size and wall thickness and has 50% neonatal mortality in a knockout mouse model. H24 is a Riken gene of unknown function and previously has only been predicted to have a protein product with no existing data to support this assertion. Because these two genes were the most highly correlated with LV hypertrophy it was important to understand whether or not there was dynamic regulation of expression levels in sensitive and resistant strains. Considering the differential response of hypertrophy from earlier studies (Chapter 2), which showed that increased LV mass presented with a strain and stressor specific pattern, it was logical and customary to test LV tissue expression from that segment of the investigations with these candidate genes as the focus.

On one hand, it may be hypothesized that two signal transduction pathways that are involved in generating similar cardiac phenotypes, such as a- and B-adrenergic receptors in LVH, will have similar effects on the expression level of genes that are closely associated with LVH. On the other hand, it may be postulated that the expression levels may be very dissimilar simply because the two pathways are inherently different with alternative
intermediate and downstream signaling. Therefore, assaying the mRNA levels of Mospd3 and H24 was conducted using PCR for quantification. Mospd3 exhibited various levels of expression in all 6 strains with no defined pattern. Interestingly, in C57BL/6J mice Mospd3 was shown to be upregulated post-AngII and downregulated post-TAC. In AXB8/PgnJ, Mospd3 was significantly upregulated post-ISO, however, AngII and TAC had no effect on Mospd3 levels (Figure 1A). What’s more interesting is that H24 exhibited as much as 40-fold higher expression after ISO and AngII treatment in sensitive and resistant strains but was significantly downregulated after TAC (C3H/HeJ, BTBRT+, C57BL/6J, and AXB8/PgnJ). In contrast, H24 expression was upregulated after TAC in the other two sensitive strains (A/J and BALB/cJ) (Figure 1B). Hypertrophy marker expression of natriuretic peptide type A and B (Nppa and Nppb) had indications of increased expression in these strains after TAC surgery but were mostly unchanged with either ISO or AngII (Figures 1C and D). These results indicate genetic background plays a key role in the phenotypic manifestation of LVH and Mospd3 and H24 are dynamically regulated by each stressor, regardless of their ability to resist any particular stressor.

In order to determine the morphological effects of Mospd3 and H24 at the cellular level, each gene was inhibited with siRNA, expression levels were recorded, and cross sectional surface area was measured. The dynamic regulation seen in mice tissue was not apparent in neonatal rat ventricular myocytes (NRVM) after ISO, AngII, and phenylephrine (PE). However, siRNA managed to knockdown these genes quite efficiently compared to untreated and uninhibited controls (Figure 2A and B). As expected, ISO, AngII, and PE increased cell size by nearly 50% in control and scramble cells (Figure 3).
Strikingly, siRNA against H24 prevented the hypertrophic effects of each stressor. In contrast, Mospd3 inhibition had an additive effect in which surface area increased over that of treatment alone in control and scrambled cells (Figure 3). These results implicate H24 as a driver of hypertrophy and Mospd3 as a basal regulator of cell size.

Curiosity abound as to cellular localization of the protein products of these two genes. In order to select a cell line for overexpression it was important to understand which types of tissue these two genes were more highly expressed in. Relative expression of Mospd3 was ubiquitous in several types of tissue but strongest in liver, lung, and kidney (Figure 4A). H24 was also detected in many tissue types but was an order of magnitude higher in kidney (Figure 4B). Therefore, Madin-Darby canine kidney (MDCK) epithelial cells were selected for overexpression experiments because of their robust survivability and ease of transfection.

In order to determine the localization of Mospd3 and H24, overexpression vectors were transfected into MDCK epithelial cell cultures. These vectors were either empty or contained the gene of interest within the multiple cloning site (MCS) (Figure 5). The MCS was flanked by a 5’ cytomegalovirus (CMV) promoter and Kozak consensus sequence (gccRccAUGG) and a 3’ Myc-DDK tag for detection by a fluorescent microscope. MDCK epithelial cells were cultured alone, with an empty vector, or a Mospd3 or H24 overexpression vector. Cell membranes were visualized with wheat germ agglutinin (WGA), Mospd3 and H24 with anti-FLAG/Alexa 488, and nuclear DNA with DAPI. As expected, there were no FLAG antibodies in control and empty vector samples. However, Mospd3 and H24 both appear to be localized in the cytosol (Figure 6). Likewise, when
NRVM cells were cultured by the same method as MDCK cells, there were no FLAG antibodies in control and empty vector samples. Again, Mospd3 and H24 both appear to be localized in the cytosol. However, H24 also appears to be highly punctate (Figure 7).

Discussion

An extensive and comprehensive list of candidate genes were identified from the ISO-induced HMDP as having high correlation with a multitude of cardiac phenotypes. These current studies have attempted to validate those findings by providing preliminary data that serves to form a basis for future mechanistic studies that will eventually define the detailed role of Mospd3 and H24 in LVH. Thus far, Mospd3 appears to be a cytosolic protein that regulates basal cell size. Mospd3 has been previously identified in another study as having a role in RV dysfunction and remodeling accompanied by 50% neonatal lethality in a mouse model. H24 was virtually unknown and was only predicted to have a protein product based on its DNA sequence. We now believe it also is a cytosolic protein but, in contrast to Mospd3, is a driver of cell hypertrophy in NRVM cultures. The fact that H24 appears highly punctate in NRVM cells may suggest several possibilities, such as there being vesicle trafficking for signal transduction or, alternatively, degradation and recycling for other cell functions. Co-immunoprecipitation and mass spectrometry may prove to be a viable approach for discovering binding partners for both candidates and, therefore, a signaling pathway for a future assessment of a functional role.
Figure 1. Relative fold change for mouse LV mRNA expression of Mospd3, H24, and LVH markers natriuretic peptide A and B (Nppa, Nppb) using qRT-PCR. Con (black), ISO (yellow), AngII (red), and TAC (green); p-value compared to control of respective strain or as indicated by bracket.
Figure 2. Relative fold change of Mospd3 (A) and H24 (B) after siRNA inhibition. Con (black), ISO (yellow), AngII (red), and PE (green); p-value compared to untreated, uninhibited control. Gene inhibition appears to be sufficient for knockdown.
Figure 3. Fold change of cross sectional surface area of NRVM 48 after siRNA inhibition and 24 hrs after treatment. Con (black), ISO (yellow), AngII (red), and PE (green); p-value compared to control of respective treatment or as indicated by brackets.

Figure 4. Relative expression Mospd3 (A) and H24 (B) in various types of tissues (left atrium, right atrium, left ventricle, right ventricle, liver, lung, kidney, and skeletal muscle) after 3 weeks of AngII treatment (control - black, treated – white). Mospd3 was expressed highest in liver, lung, and kidney and H24 was an order of magnitude higher in kidney.
Figure 5. Genetic map for plasmid (pCMV6-Entry, Origene) used in overexpression experiments. Either Mospd3 or H24 was inserted into the multiple cloning site which was flanked by a 5’ CMV promoter and Kozak sequence and 3’ Myc-DDK fluorescent tag.
Figure 6. Madin-Darby canine kidney (MDCK) epithelial cells cultured alone, with an empty vector, or with either a Mospd3 or H24 overexpression vector. Cell membranes were visualized with wheat germ agglutinin (WGA), Mospd3 and H24 with anti-FLAG/Alexa 488, and nuclear DNA with Hoechst. As expected, there were no FLAG antibodies in control and empty vector samples. However, Mospd3 and H24 both appear to be localized in the cytosol.
Figure 7. Neonatal rat ventricular myocytes (NRVM) cells cultured alone, with an empty vector or with either a Mospd3 or H24 overexpression vector. Cell membranes were visualized with wheat germ agglutinin (WGA), Mospd3 and H24 with anti-FLAG/Alexa 488, and nuclear DNA with Hoechst. As expected, there were no FLAG antibodies in control and empty vector samples. However, Mospd3 and H24 both appear to be localized in the cytosol. H24 also appears highly punctate.
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Chapter 4

An Angiotensin II-Induced Hybrid-Mouse Diversity Panel

Reveals Genetic Basis of Diastolic Dysfunction:

Implication in HFpEF
Abstract

Heart failure is a complex disease with heterogeneous contributing factors, affecting both systolic and diastolic functions. Due to this challenge, the genetic basis for stress induced left ventricular diastolic dysfunction (LVDD) remains to be poorly defined based on genetic analysis of the human population and the genetic contribution to clinical manifestation of heart failure remains enigmatic. In this study, we employed a Hybrid Mouse Diversity Panel (HMDP), which contains well over 100 strains of classic inbred and recombinant inbred mice, and systems genetic approaches to uncover the genetic basis of LVDD. Using an implanted mini-pump as the delivery vehicle, we treated 37 strains of mice of the HMDP with AngII (500 ng/kg/min) for three weeks. Many of the HMDP strains showed sub-hypertensive blood pressure increase, significant cardiac hypertrophy, and molecular changes in the left ventricle following AngII treatment.

For proof of concept and to investigate LVDD, we employed three strains, AXB15/PgnJ (resistant), C58/J (delayed sensitivity), and BXD75/RwwJ (rapid sensitivity), from the HMDP and used nitric oxide bioavailability, the pressure/volume relationship, and differential gene expression in the LV to investigate the genetic basis of LVDD in heart failure with preserved ejection fraction (HFpEF). The 2 sensitive strains both showed sub-hypertensive blood pressure increase, significant cardiac remodeling, reduced NO bioavailability, impaired relaxation, diastolic dysfunction, and specific differential gene expression-derived GO terms following AngII treatment. While left ventricular ejection fraction was preserved, a large diversity of left atrial weight was observed among these
strains, indicative of strain specific LVDD in response to AngII treatment. Echocardiogram and invasive hemodynamic analysis further support the state of LVDD and HFP EF associated with LA enlargement. These data support the idea that there is a significant genetic contribution to AngII-induced diastolic dysfunction in heart failure that can be revealed from systematic analysis of the HMDP strains. Further studies of the HMDP strains will allow better dissection of the genetic basis of LVDD and the sensitive strains identified in this study may represent a valuable model to investigate HFP EF.

**Introduction**

Heart failure is the leading cause of death worldwide and costs continue to surge, creating a two-fold strain on quality of life: pain and suffering and depleted financial resources\(^1\). Several factors contribute to HF manifestation, such as age, family history, environment, diet, and exercise to name a few, all of which add to the complexity of these diseases. Despite past efforts, recent advances in technology and the evolution of bioinformatic methods to uncover the genetic basis of HF, the future remains bleak as the genetic basis of HF remains poorly understood. Understanding the genetic component of HF will help unravel mechanisms for early diagnosis, targeted therapy, and, hopefully, treatments for reversing intermediate and advanced disease states. Genome wide association studies (GWAS) in humans have identified a multitude of genetic loci that correlate with cardiac phenotypes, however, a specific single nucleotide polymorphism (SNP) within the locus is often flanked by other SNPs that, in statistical terms, are just as likely to be the SNP of interest due to linkage disequilibrium\(^2\). We have previously
developed several systems genetics approaches to perform functional characterization of >100 mouse strains from the HMDP\(^3\). In the current study our approach, again, takes full advantage of the genetic diversity offered by the HMDP. Combining this with quantitative characterization of cardiac phenotypes, systematically unraveling the genetic basis of common forms of HF has become an effective research tool for bioinformaticians, geneticists, and cardiologists.

Importantly, one must understand the many ways that HF can manifest in order to select an induction method suitable for answering questions raised by a hypothesis. Whether HF is chemically or surgically induced in an animal model or studied via other methods are important considerations. Other considerations include whether the model selected will yield viable results, the size is large enough to inform statistical relevance while not being wasteful or causing unnecessary pain and suffering, and that it is hypothesis driven.

Chronic activation of sympathetic systems is a hallmark of HF\(^4\). They are induced in response to an increased mechanical load to the heart. In an earlier study chronic stimulation of isoproterenol (ISO) was implemented in the HMDP. Due to the array of cardiac phenotypes, dynamic levels of heart function, and previous molecular studies from ISO-infused HMDP strains, we wanted to learn the effects of other stressors. Therefore, we employed the use of angiotensin II (AngII) in another mouse panel that, with the experience of our collaborators and advances in computing power, we expect an
improvement in analytical methods and precision in determination of the role of genetic background in HF and its related diseases.

**HMDP**

Because linkage analysis has low mapping resolution and has failed to identify loci with subtle or modest effects, the Hybrid Mouse Diversity Panel (HMDP), which contains well over 100 strains of classic inbred and recombinant inbred mice, was created as an alternative for association mapping using a systems approach. Together, these mouse strains have manifested several intermediate and advanced cardiac phenotypes. In previous studies the HMDP has been shown to have sufficient power and resolution to map genes to less than a megabase. There are several advantages to the HMDP, such as the panel has been completely genotyped, they are commercially available and renewable, and useful for studying gene-by-environment interactions and systems biology. For example, Abcc6, which when inhibited is the cause of progressive tissue calcification, was identified and subsequently validated in the development of isoproterenol-induced cardiac fibrosis. Additionally, Asxl2 was identified in a bone mineral density (BMD) study and confirmed to be involved in myeloid cell differentiation. Due to these proven methodologies with and without HMDP genetic perturbation, the focus of this project is the adrenergic receptor for angiotensin II (AngII).

**Angiotensin II**
There is a large body of evidence showing that angiotensin II (AngII), through the renin-angiotensin-aldosterone system (RAAS), is involved in cardiac hypertrophy, fibrosis, and elevated blood pressure\textsuperscript{4,9-11}. Angiotensinogen, from the liver, is the substrate for renin, found in the kidney, which produces angiotensin I (AngI). The angiotensin converting enzyme (ACE) converts AngI to AngII. The mechanism of angiotensin II is to stimulate one of the $\alpha_1$-adrenergic receptors, a G-protein coupled receptor (GPCR) known as AngII type-1 receptor (AT$_1$). AT$_1$ interacts with multiple heterotrimeric G-proteins producing second messengers, such as inositol trisphosphate (IP3), diacylglycerol (DAG), and reactive oxygen species (ROS). It also activates various intracellular protein kinases such as the mitogen-activated protein kinase (MAPK) family, c-Jun N terminal kinase (JNK), protein kinase B (PKB), as well as many others\textsuperscript{10}. There is no question as to why ACE has become a target of pharmaceutical inhibitors.

There are a myriad of experimental models using AngII in animals and cell culture. In rodents there has been an effort to use subpressor doses, those which do not elevate blood pressure, in order to examine the effects of AngII on other pathologies, such as cardiac hypertrophy or fibrosis, without the confounding of hypertensive etiologies. These subpressor doses are most commonly in the range of 100-500 ng/kg/min but are still able to produce ~20% increases in the heart weight to body weight ratio (HW/BW) and several-fold changes in fibrosis. The range of pressor doses, those which do stimulate high blood pressure, are usually from 500-1000 ng/kg/min, but may be higher\textsuperscript{12-22}. Therefore, AngII serves as an excellent molecule for generating an array of cardiac phenotypes in mouse models.
Heart Failure with Preserved Ejection Fraction

Heart failure is the result of heterogeneous factors that ultimately lead to abnormal cardiac structure and subsequent reduced function in the form of cardiac output and/or filling pressures. Despite this heterogeneity, HF with reduced ejection fraction (HFrEF) has several drugs and devices that have been shown to improve outcomes. In contrast, HF with preserved Ejection Fraction (HFpEF) has no treatments that have improved outcomes\textsuperscript{23}. Imaging studies have demonstrated normal LV geometry in 30-45\% of patients, traditional noninvasive measures of diastolic function are normal in approximately one third of HFpEF patients, and LVDD is frequently detected in older persons without HF\textsuperscript{24}. HFpEF accounts for ~50\% of HF\textsuperscript{25}. This suggests that the heterogeneity of phenotypic manifestations may be much greater than in HFrEF. Understanding this increased heterogeneity may allow more targeted clinical trials\textsuperscript{26}. A comprehensive examination of cardiac dysfunction and its association with adverse end stages may overcome deficiencies in the areas of diagnosis and, ultimately, treatment\textsuperscript{27}. There is great need for a method to classify HFpEF in a manner that defines a more homogeneous group of similar individuals, effectively leading to treatment of HFpEF patients for abnormalities overlooked by HFrEF investigations.

Diastolic Dysfunction and Impaired Relaxation

The heartbeat consists of two phases, systole, when the ventricles contract, and diastole, when the ventricles relax. As blood collects in resting atria during systole, the sinoatrial node signals the atria to contract, forcing blood to pump through the tricuspid
and mitral valves into the resting ventricles during diastole. In the second phase, the electrical signals cause the ventricles to contract and push blood through the pulmonary and aortic valves. DD is the presence of abnormal pumping of one or both ventricles during the diastolic phase. In these hearts, the ventricle does not relax properly during diastole. When the ventricle experiences impaired relaxation, the internal pressure will increase as blood from the next heartbeat tries to enter, subsequently leading to pulmonary and/or systemic congestion. DD has been positively correlated with cardiac mortality. However, a diagnostic method has not been fully established and is often difficult.

Patients suffering with HFpEF are more likely to have LVH, LVDD, and LA enlargement. For hypertensive hearts, physiological changes of the LA often increase atrial arrhythmias, heart failure, and cardiovascular death, which may remain undetected due to its occurrence before LVH and DD. In hypertrophic cardiomyopathy (HCM), LA enlargement has been shown to be associated with LVH, DD, higher filling pressures, worse functional class, and higher risk of atrial arrhythmias. LV filling is modulated by the LA, which increases LV filling pressure. Hypertension and LVH may also have roles in LVDD, elevated filling pressure, and enlarged LA. LA enlargement has been strongly associated with LVDD severity, stroke, atrial fibrillation, duration and severity of increased LA pressure, poor exercise capacity, and death. Clinicians need a measure of LVDD that is easy to obtain and can be done so in a heterogeneously, phenotypically diverse population. Despite the fact that LA volume has been suggested to fit this need, the use of LA enlargement as a predictor of HFpEF is uncertain.
Nitric Oxide Bioavailability

Nitric oxide (NO) is the key humoral factor implicated in the pathogenesis of hypertension. It is synthesized in the vascular endothelium by NO synthase (eNOS). NO stimulates guanylate cyclase to produce cyclic guanosine monophosphate (cGMP), which subsequently inhibits Ca\(^{2+}\) influx into the cytosol by mediating protein kinase G, eventually leading to vasodilation\(^35\). During excitation-contraction (EC) coupling in the myocyte, the sarcoplasmic reticulum (SR) sequesters Ca\(^{2+}\), allowing Ca\(^{2+}\) to leave its troponin-C binding sites, followed by release of actin from myosin. If this mechanism is burdened then the rate and extent of relaxation are decreased which, with time, will reduce the rate of preload during the rapid filling phase. Therefore, lack of NO bioavailability affects the cardiovascular system by adversely reducing perfusion, promoting apoptosis, and unbalancing vasoconstriction/dilation\(^36\).

AngII is a pleiotropic hormone that regulates many organ systems. In the cardiovascular system it is a potent vasoconstrictor that elevates arterial pressure\(^37\). There is increasing evidence that locally produced vasoconstrictor peptides contribute to blood vessel homeostasis, as well as the development of vascular pathologies\(^38\). In the vascular wall, smooth muscle growth is dependent on the net balance between intracellularly generated ROS in smooth muscle cells, diffusible ROS produced in adjacent cells in the endothelium and adventitia, and endothelium-derived NO. These actions include counteracting the tonic inhibition on smooth muscle cells by decreasing bioavailable endothelium-derived NO\(^39\). Because the normal function of NO to promote vasodilation is
functionally opposed to AngII, measuring NO bioavailability in the remodeled arterial vasculature can be used to monitor AngII activity.

Increases in cardiac myocyte stiffness in HFpEF is related to cGMP deficiency and nitroso-oxidative stress. The loss of NO bioavailability may have a prominent role in the pathophysiology of LVDD and HFpEF. Obesity and metabolic disease can increase nitroso-oxidative stress and limit NO bioavailability in the heart and vasculature. Activation of RAAS exacerbates atrial remodeling, especially in a condition with decreased NO bioavailability and it has been shown that AngII infusion causes left atrial enlargement, atrial interstitial fibrosis, perivascular fibrosis, cardiomyocyte hypertrophy, and atrial superoxide production.

HFpEF induces a systemic inflammatory state in which endothelial adhesion molecules, such as vascular cell adhesion molecule (VCAM), were shown to be highly expressed. Leukocytes begin migrating and activation of nicotinamide adenine dinucleotide phosphate oxidases (NOX) and proinflammatory cytokines produce reactive oxygen species (ROS). Low NO bioavailability in HFpEF myocardium is believed to be from conversion of NO to peroxynitrite by superoxide anion. It has also been shown that infusion of NO donors acutely lowered diastolic LV stiffness in human controls, in aortic stenosis, and in dilated cardiomyopathy. Chronic inhibition of NOS in rats induced leftward displacement of the diastolic LV pressure-volume loop and lowered stroke volume. Taken together, these studies indicate the need for assaying NO content when monitoring LVDD and impaired relaxation in HFpEF research models.
Pressure Volume Relationship

LVDD is characterized by an altered diastolic pressure-volume relationship (PVR). It is manifested by the inability of the LV to relax and completely fill during diastole. Unless LA pressure increases to compensate, small increases in volume manifest disproportionate amounts of LV compliance and, therefore, LV filling is delayed or incomplete\textsuperscript{45}. The PVR is influenced primarily by relaxation and passive filling properties. Mitral inflow patterns inform on these dynamics and are commonly imaged with echocardiography\textsuperscript{46}. Changes in LV compliance affect cardiac performance and adaptations, thus, diastolic mechanisms are important in assessing dysfunction in general\textsuperscript{47}. A series of LV pressure-volume loops can be obtained using variable loads in order to assess LV performance\textsuperscript{48}. With increased inotropy, the pressure volume loop reflects a smaller end-systolic volume (ESV) and a higher end-systolic pressure (ESP), so that the slope of the PVR moves upward and to the left. PVR has been the most reliable analysis method for measuring LV contractility. In order to do so, a conductance catheter is used to measure pressure-volume loops\textsuperscript{49}.

Preload, or ventricular filling, occurs when the LV fills with blood after the mitral valve opens. Because function of the LV depends on preload and contractility, or compliance, stroke volume (SV) is adversely affected when either is impaired. EDV depends upon return of blood and compliance of the LV during diastole. Because, a reduction in LV compliance, as occurs in LVH, will result in decreased EDV and a greater EDP, SV and stroke work will decrease as well. These concepts suggest the usefulness of
assessing the PVR for defining a more detailed role for LVDD and impaired relaxation in HFpEF.

Materials/Methods

Mouse surgery. Of 321 total control and treated mice, 190 received micro-osmotic pumps (Alzet, model 1004) for [Val⁵] Angiotensin II acetate salt hydrate (100 - 2000 ng/kg/min) for 21 days implanted either intraperitoneally or subcutaneously on the back. Vehicle solution was either water or an acetic acid-NaCl solution (final concentration 0.008% acetic acid, 120 mM NaCl).

Echocardiography. Mice were anesthetized with 1.5% isoflurane. Using a Vevo 2100 with an MS250 (13-24 MHz) transducer (VisualSonics), LV long and short axes were taken in M- and B-mode, E/A ratio was taken with color and tissue doppler. Aortic arch and abdominal velocity and wall thickness were measured with color doppler and M-mode, respectively, as well. Procedures were conducted for baseline before treatment and for day 7, 14, and 21 after treatment.

Blood Pressure. Using a Visitech Systems BP2000 Blood Pressure Analysis System, mice were placed into the apparatus and monitored for 30 min weekly for 3 weeks preceding surgery and sacrifice, for a total of 4 sessions, with a tail cuff and transmission photoplethysmographe. Mice were not anesthetized before pressure readings. Within the 30 min session, the first 10 min was for training, then the next 20 min there was 1 measurement per minute lasting 10 seconds for a total of 20 reading per mouse per session.
Mouse tissue. Mice were sacrificed on day 21 using isoflurane and cervical dislocation. Plasma, heart, adrenal glands, liver, lung, and kidney weights were measured. The heart was subdivided by chamber and individual weights were measured. Tissue was flash frozen in liquid nitrogen and stored at -80° Celsius and/or placed in formalin. The tibia was also removed and length was measured.

Pressure Volume Relationship. Using a Transonic ADVantage Pressure-Volume System (ADV500), a 3.5 mm catheter was inserted through the apex into the LV. Measurements were taken 3 weeks post-AngII just before sacrifice. P/V loop, including pressure, volume, phase, and magnitude, were collected for 10 min after baseline and analyzed using AD Instruments LabChart software.

Electron Spin Resonance Detection of Aortic Nitric Oxide Production. Freshly isolated aortic rings were incubated with freshly prepared nitric oxide specific spin trap Fe$^{2+}$(DETC)$_2$ (0.5 mmol/L) in modified Krebs/HEPES buffer at 37°C for 60 min, in the presence of calcium ionophore A23187 (10 µmol/L). After the incubation, the aorta was snap frozen in liquid nitrogen and loaded into a finger Dewar for analysis with ESR spectrophotometer (eScan, Bruker). The instrument settings were as the followings: bio-field, 3,280; field sweep, 77.54 G (1 G=0.1 mT); microwave frequency, 9.78 GHz; microwave power 40 mW (4 dB); modulation amplitude, 10 G; 4,096 points of resolution; and receiver gain 900.$^{50}$

Differential Gene Expression. Left ventricles were flash frozen in liquid nitrogen then crushed with a pestle inside a microcentrifuge tube to extract total-RNA using TRIzol
reagent. Isolated total-RNA was bioanalyzed with minimum RIN >7 before cDNA library preparation. RNA sequencing was performed using mRNA on an Illumina Nextseq 500 platform with a coverage of 1x75 bp and depth of 20-30 million reads. Tophat NGS tools were used for transcriptome assembly and differential expression analysis including Bowtie, Cufflinks, Cuffmerge, and Cuffdiff functions51.

Molecular and Genetic Basis of AngII Induced HFpEF

In order to establish the effects of AngII on HMDP mice an osmotic micro-pump was implanted intraperitoneally in one ISO-resistant (FVB/nJ) and one ISO-sensitive (BALB/cByJ) strain6. The purpose was to determine a subpressor dose using a response gradient (1.0, 1.33, 1.67, and 2.0 µg/kg/min) that would effectively allow a focus on AngII perturbation of genetic background, avoiding the confounding of hypertension. These values were selected based on a literature search in which the majority of publications indicated a dose of 1.0 ug/kg/min in order to obtain an LV hypertrophic phenotype15, 16, 19, 20, 52-54. After 3 weeks of treatment both FVB/nJ and BALB/cByJ strains experienced some amount of hypertrophy, however, the ISO-sensitive BALB/cByJ strained reached significance with the lower dose of 1.0 and 1.33 µg/kg/min (Figure 1). In the ISO-resistant FVB/nJ strain all doses increased systolic BP except for the highest dose (p-value = NS for all doses) and BALB/cByJ systolic BP was unchanged (Figure 1).

To be more certain of obtaining LV hypertrophy and other cardiac related phenotypes 2.0 µg/kg/min was selected as the dose for the HMDP panel. After 23 strains there were 14 that had an increase in LV weight. Surprisingly, only 2 had statistical
significance (p-value<0.05) and just 1 strain had an increase more than 25% (p-value = NS, Figure 2). LA enlargement, a phenomarker of diastolic dysfunction, was present in 13 strains (9>50%, 7>75%, 4>2-fold, 3>2.25-fold, and 1>2.5-fold, 6 with p<0.05, Figure 2). A subpressor dose was apparent in which systolic BP was increased in 11 strains (2 with p<0.05), however, the panel as a whole was not suffering from hypertension (Figure 3).

Because of the poor response for LV hypertrophy the dose and method of delivery were once again investigated. Osmotic pumps with water as the vehicle had been implanted intraperitoneally thus far. A response with two different vehicles, water or acetic acid-NaCl, was prepared with a range of doses from 100-2000 ng/kg/min and were implanted in C57BL/6J mice, a widely used strain in several disease models, including cardiovascular disease. The LV response was still less than +25%, however, the LA response was very robust, as high as 2-fold with 2.0 µg/kg/min IP and 500 ng/kg/min subcutaneous in acid or water (p<0.05, Figure 4), duplicating previous results from the first 23 strains. More importantly, systolic BP was elevated to similar levels with the original 2.0 µg/kg/min IP and 500 ng/kg/min subcutaneous doses after weeks 1 and 2, but only the subcutaneous pump with acid was effective throughout the entire 3 week treatment (Figure 4). Thus far, because there were many similarities between the 2.0 µg/kg/min IP with water and 500 ng/kg/min subcutaneous with acid doses, while the 500 ng/kg/min subcutaneous with water dose was also effective, the pump location seemed to be the determining factor for effectiveness. Therefore, the subcutaneously implanted pump with acetic acid-NaCl as the vehicle was now the method of interest since it was somewhat more robust than IP or subcutaneous with water (Figure 4).
Now that a preliminary dose and method of delivery was established, dose response 3 (DR3) was tested using strains from DR1 (100, 200, 500, and 1000 ng/kg/min). The resistant FVB/nJ strain had similar LV, LA, and systolic BP responses in DR3 as in DR1, however, the sensitive BALB/cByJ had a more severe response with LA and LV weight (p<0.05 with various doses) while systolic BP was unaffected. These results instilled confidence in the 500 ng/kg/min subcutaneous acid pump as robust and superior for inducing subpressor cardiac phenotypes compared to the previous 4-fold higher dose using water as a vehicle and the peritoneum for location. Therefore, the panel was continued with the new method.

With both doses several other cardiac phenotypes besides heart weight and systolic BP were also measured and recorded. Tissue weights (total heart, left atrium, right atrium, left ventricle, right ventricle, adrenal glands liver, lung, and kidney), tibia length, blood pressure (systolic, diastolic, mean arterial pressure, and heart rate), echocardiogram parameters (blood flow through the mitral valve, aortic arch, and abdominal aorta, mitral valve contractility, ejection fraction, left ventricular internal diameter, posterior wall thickness, and relative wall thickness, and aortic arch and abdominal aorta wall thickness) were measured and recorded for each mouse. The controls and treated mice values were averaged separately and then compared. Due to the fact that after comparing the two different doses, one from each cohort, when combined they were quite similar in regards to the phenotypes of interest. Several strains were repeated and some had received both doses. Because of the inter-cohort and intra-strain similarities between the two cohorts and
repeated strains, respectively, an analysis of all 37 strains as one large panel was conducted (Figure 6).

Of 37 strains, 26 showed an increase in total heart weight (4 with p<0.05) and 3 were reduced (Figure 6A), 24 had LA enlargement (13 with p<0.05) and 3 were reduced (Figure 6B), 19 had RA enlargement (2 with p<0.05) and 3 were reduced (Figure 6C), 26 had LV hypertrophy (5 with p<0.05) and 3 were reduced (Figure 6D), and 9 had increased RV weight (all NS) while 14 were reduced (5 with p<0.05, Figure 6E).

Other tissues showed much more variation. For example, the adrenal glands had 17 strains with increased weight (5 with p<0.05) and 13 were reduced (5 with p<0.05, Figure 6F), 12 had increased liver weight (1 with p<0.05) and 10 were reduced (3 with p<0.05, Figure 6G), 19 had increased lung weight (5 with p<0.05) and 5 were reduced (Figure 6H), and 10 had increased kidney weight while 12 were reduced (1 with p<0.05, Figure 6I).

At this point in the study LA enlargement was clearly the most interesting phenotype. More specifically, of the 24 strains that had enlargement 13 were >25% (10 with p<0.05) and 8 were >50% (6 with p<0.05), one experiencing a fold-change as high as 2.55 (+/- 0.28 S.E.M., p=0.025, BXD5/TyJ, Figure 6B). This was more evidence implicating diastolic dysfunction as an emerging disease state in this panel.

Because AngII has been used in multiple animal models to stimulate LV hypertrophy and culture models to stimulate cell growth, a much more robust response was expected. However, of the 26 stains that did, indeed, have hypertrophy not one was above
the 25% threshold and, surprisingly, only a handful of individual mice were above this level (Figure 6D).

AngII stimulates increased blood pressure, and therefore hypertension, therefore, blood pressure was monitored with a tail cuff for 30 min, 10 min of training and 20 min of 1 measurement per minute for 10 seconds for a total of 20 measurements per mouse. Measurements were taken weekly starting before surgery and continued through week 3 before the mice were sacrificed for a total of 4 data sets each. Control and treated mice were averaged separately and compared (Figure 7). Of the 37 strains, 18 had increased systolic BP (12 with p<0.05) and no strain was reduced (Figure 7A), 10 had increased diastolic BP (1 with p<0.05) and 2 were reduced (1 with p<0.05, Figure 7B), 35 had increased MAP (4 with p<0.05) and no strain was reduced (Figure 7C), and 3 had increased heart rate (2 with p<0.05) and 6 were reduced (Figure 7D). Considering the degree of systolic BP increase, taken together, these results indicate that the panel is receiving a subpressor dose of AngII and, therefore, is not suffering from hypertension. Even though several models have used subpressor doses of AngII to stimulate LV hypertrophy, these results may provide evidence to suggest why these mice did not manifest more severe remodeling.

Echocardiography is used as a standard for monitoring heart function. A VisualSonics Vevo 2100 was used to image the panel (1.5% isoflurane) using color and tissue Doppler, M-mode, and B-mode with an MS400 transducer (18-38 MHz). More specifically, LV function is monitored by ejection fraction (EF = stroke volume/end
diastolic volume x 100), a measure of cardiac output which is reduced in hypertrophy, left ventricular internal diameter (LVID) which is also reduced in hypertrophic cardiomyopathy (HCM) and increases during dilated cardiomyopathy (DCM), left ventricular posterior wall thickness (LVPWT) is expected to increase during hypertrophy, and left ventricular relative wall thickness (LVRWT), which is calculated from LVID and LVPWT (LVRWT = (2 x LVPWT)/LVID), is also expected to increase with hypertrophy.

MV function is monitored by measuring blood flow through the MV into the LV with color Doppler and contractility of MV with tissue Doppler imaging techniques. E/A ratio measures the early (E) flow when the MV opens and blood flows into the LV and atrial (A) flow is when the LA contracts pumping its remaining contents of the LA into the LV, which is expected to increase with systolic dysfunction and DCM. Similarly, both early’ (E’) and atrial’ (A’) measure contractility of the mitral annulus/septum interface and are expected to decrease with impaired relaxation. E/E’ is a measure of LV filling pressure and is expected to increase in diastolic dysfunction, effectively, blood flow through the MV remains steady but the LV has reduced contractility and does not reach complete relaxation at end diastole.

Of the many effects AngII has on the body, one is vasoconstriction. Therefore, aortic blood flow was monitored in the arch and abdomen along with wall thickness, internal diameter, and relative wall thickness. The idea was that flow would increase due to a narrowed vasculature. Peak velocities were imaged in color Doppler mode and dimensions were imaged in M-mode.
These mice were monitored on a weekly basis starting before surgery and continued through week 3 before the mice were sacrificed for a total of 4 data sets each. Control and treated mice were averaged separately and compared (Figure 8). Of the 37 strains, 7 had increased EF (2 with p<0.05) and 4 were reduced (1 with p<0.05) (Figure 8A), 8 had increased LVID (0 with p<0.05) and 7 were reduced (4 with p<0.05, Figure 8B), 10 had increased LVRWT (5 with p<0.05) and no strain was reduced (Figure 8C), and 10 had increased LVPWT (5 with p<0.05) and no strain was reduced (Figure 8D). Given that rEF, reduced LVID, increased LVRWT, and increased LVPWT are expected in AngII-induced LV hypertrophy, in contrast, these results clearly indicate that this mouse panel has pEF.

MV function was altered in several strains. E/A ratio was elevated in 5 strains (3 with p<0.05) and 5 were reduced (Figure 9A) indicating normal blood flow into the LV, 12 had increased E/E’ ratio (7 with p<0.05) and 5 were decreased (2 with p<0.05, Figure 9B) indicating increased LV filling pressure, 10 had increased E’ (3 with p<0.05) and 8 were reduced (6 with p<0.05, Figure 9C) indicating loss of LV compliance, and 6 had increased A’ (2 with p<0.05) and 10 were reduced (5 with p<0.05, Figure 9D) indicating impaired relaxation.

Aortic function was largely unchanged. AV peak velocity was modestly increased in 9 strains (6 with p<0.05) and was reduced in 4 (3 with p<0.05, Figure 10A), 12 had wider internal diameter (5 with p<0.05) and none were more narrow (Figure 10B), wall thickness in 12 strains and reduced in 2 (Figure 10C), and relative wall thickness was increased in 6 (only 1 with p<0.05) and reduced in 8 (Figure 10D). Wall thickness and
relative wall thickness, at 0.08 – 0.3 mm, are just below the lower limit of the capability of the transducer (0.3 mm), therefore, significant differences are not detectable with any level of confidence. These results suggest conserved vascular dimension and function, indicating a lack of hypertension.

**Extreme Strains**

Because LV hypertrophy was not as robust as expected and LA enlargement was the first phenotype that manifested with such severity and interest in the AngII-induced HMDP, as high as 2-3 fold in several strains, more attention was focused on this phenomenon. First, the strains were ordered by the degree of LA enlargement. Then this pattern was used for other phenotypes of interest, such as LV hypertrophy, systolic BP, LV function, and MV function (Figure 10). Eleven strains had significant increases in LA and LV size (p<0.05). Ten also had pEF, while the eleventh actually had an increase in EF. Six of these had a combination of phenotypes consistent with diastolic dysfunction, including normal E/A ratio, increased LV filling pressure, and reduced mitral valve contractility. Based on these analyses, AngII-induced stress in this panel may be stimulating HFpEF. Therefore, 2 strains were selected for more in depth studies, one that was sensitive (BXD75/RwwJ) and one that was resistant (C58/J).

In order to determine at what point these mice began experiencing diastolic dysfunction, the research strategy included a time course in which mice were sacrificed at the end of each week for a total of 4 subgroups: 1 week, 2 weeks, and 3 weeks and the fourth group consisted of controls from each week. This effort was made not only to
confirm previous findings, but also to explore the possibility that this model is truly experiencing HFpEF. Therefore, the pressure/volume loop was invasively measured using an intracardiac catheter. In addition, nitric oxide bioavailability was determined using the aorta, from the arch to the abdomen, to add biochemical evidence to the previous physiology that was observed as possibly impaired relaxation.

BXD75/RwwJ experienced LA enlargement as early as the first week and LV hypertrophy at week 2; however, C58/J took 3 weeks for LA enlargement and LV hypertrophy to manifest (Figure 11). BXD75/RwwJ experienced rapidly increased systolic BP, during the first week, and C58/J started at 2 weeks. Diastolic BP was unaffected at the 3 week time point for both strains. Mean arterial pressure was unchanged for the sensitive BXD75/RwwJ but was increased for the resistant C58/J at 2 and 3 weeks. Interestingly, BXD75/RwwJ had a significant increase in heart rate from week 1 to 3, possibly as a compensatory mechanism for impaired contractility (Figure 12). Looking closer at LV wall dimensions, both strains had a slight decrease in internal diameter and increase in posterior wall thickness but, apparently, there was no change at 3 weeks and they both continued to have pEF (Figure 13). MV function as a physiological marker of diastolic dysfunction showed no change in E/A ratio, increased LV filling pressure (E/E’), and abnormal tissue Doppler contractility (reduced E’ and A’) (Figure 14). Using nitric oxide as a biomarker for impaired relaxation, both strains exhibited reduced NO content post-AngII.

In these studies, after increasing the quantity of mice, these results revealed that C58/J was actually a sensitive strain with delayed onset. Therefore, we sought to use
AXB15/PgnJ as a sensitive strain. In the main panel, AXB15/PgnJ appeared to have no phenotypes associated with diastolic dysfunction, including LA enlargement, LV hypertrophy, increased LV filling pressure, and reduced MV contractility. This held true in this 3 strain subpanel. AXB15/PgnJ LA and LV weight and EF and MV function were unaffected (Figures 11-14). NO bioavailability was reduced, similar to the sensitive strains (Figure 15), however, this indicates this model is useful for resistant, delayed sensitivity, and rapid sensitivity phenotypes. The fact that the availability of NO was reduced in sensitive and resistant strains but there was adverse physiology only in sensitive strains indicated that a reduction in NO content may be useful as a co-marker for DD but is not sufficient to predict DD in this smaller cohort.

**Differentially Expressed Genes**

We employed mRNA-sequencing to determine the state of differentially expressed genes (DEGs) after AngII stimulation in C58/J (sensitive) and AXB15/PgnJ (resistant) mice using control and 3 weeks post-AngII LV tissue. Twelve samples had total RNA extracted and cDNA libraries prepared for RNAseq with a read depth of 75 bp and reference coverage of 30 million paired sequence reads per sample. Each sample produced 4 data files that were concatenated into one. Each of these 12 files were subjected to standard quality control measures FASTQ Groomer and Fastqc QC\(^55\). Reads and differentially expressed genes (DEGs) were quantified using Tophat next generation sequencing (NGS) tools including Bowtie, Cufflinks, Cuffmerge, and Cuffdiff\(^56-64\).
Principal component analysis (PCA) was conducted on 44,340 unique transcripts across all samples. Principal component 2 (PC2) efficiently segregated sensitive C58/J from resistant AXB15/PgnJ mice (Figure 7A). Using the 33,000 transcripts with FPKM>1 showed PC1 segregated the two strains (Figure 7B). These results indicate genetic background plays a key role, as opposed to AngII, in the expression of these varying transcripts. Clustered Image Maps (CIMs) were used to help understand the role of AngII in these two strains. Using only the 931 post-AngII differentially expressed genes with a fold change <0.5 or >2 (FDR corrected p<0.05), several but not all of the sample mice clustered together based on control/AngII status (Figure 8). For example, controls (AXB15/PgnJ 2 and C58/J 1, 8, and 22) clustered separately from AngII (AXB15/PgnJ 6 and 7 and C58/J 30, 32, and 33). When using CIMs on each strain alone, AngII was a determining factor for clustering in the resistant AXB15/PgnJ mice (Figure 9). Considering the pre-selection of AngII-induced differentially expressed genes, these results are no surprise and suggest that AngII plays a major role in clustering for resistant mice. Surprisingly, however, for the sensitive C58/J mice the clustering was random (Figure 10). These results suggest that post-AngII stress is a subtle, perturbing factor rather than the major contributor that genetic background exerts in sensitive mice, which also offers an explanation as to why the two strains together did not completely cluster separately based on control/AngII status. In other words, AngII has the ability to perturb the genetic background of sensitive mice enough to cross a threshold that leads to molecular dynamics that exhibit multiple cardiac dysfunction phenotypes that are a result of the genetic background itself, whereas the genetic background of resistant mice remained unperturbed,
short of crossing that threshold, and maintained normal function and, thus, implicated AngII as having the main effect on clustering. This result agrees with the PCA results in which AXB15/PgnJ control/AngII mice segregated efficiently but C58/J were not clustered (Figure 7B).

Of the 30 million paired sequence reads per sample compiled after control measures were qualified, there were 931 unique transcripts that had significant differential expression (FDR corrected p<0.05). Of these 931, there were 739 and 288 expressed in C58/J and AXB15/PgnJ, respectively, with 96 genes that were duplicated in both strains. These unique transcripts were then filtered by eliminating transcripts between fold-change cutoff values of >0.5-fold and <2-fold, leaving only the most extreme DEGs. There were 308 and 135 expressed in C58/J and AXB15/PgnJ, respectively, with 42 being duplicates. Therefore, there were 266 transcripts unique to C58/J that, individually, may have negatively or positively affected HF function and progression, which together ultimately led to LA enlargement, diastolic dysfunction, and HFpEF. In contrast, there were 93 transcripts in AXB15/PgnJ that may additively serve to protect against these phenotypes (data available in supplemental material).

Within these smaller groups there was a wide range of expressional fold-change. For example, transcripts that were lowly expressed in C58/J AngII-sensitive mice included Rnf225 (0.006-fold, FDR corrected p<0.024), a novel ring finger protein, and Ppp1r10 (0.05-fold, FDR corrected p<0.002) which, when inhibited, has been shown to ameliorate LV diastolic function and prevent adverse left ventricular remodeling in muscle LIM

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protein deficient mice, a model of dilated cardiomyopathy and hypertrophy\textsuperscript{66, 67}. In contrast, highly expressed transcripts in C58/J included Cox5b (100-fold, FDR corrected p<0.002), which has been shown to increase ATP synthesis in failing cardiomyocytes, and Gng4 (16-fold, FDR corrected p<0.05), the gamma subunit of G-protein coupled receptors, which is essential to the stress responses of the heart\textsuperscript{68, 69}.

Interestingly, transcripts that were lowly expressed in AXB15/PgnJ AngII-resistant mice included Celsr3 (0.016-fold, FDR corrected p<0.002), which also has GPCR activity, and Rcan3 (also known as Mcip1, 0.09-fold, FDR corrected p<0.002) which has been shown to blunt cardiac hypertrophy in response to pressure overload or chronic adrenergic stimulation in Mcip1\textsuperscript{−/−} mice\textsuperscript{69, 70}. In contrast, highly expressed transcripts in AXB15/PgnJ included Scgb1a1 (13-fold, FDR corrected p<0.02), which has been shown to inhibit phospholipase A2 and may counteract RAAS-induced inflammatory effects\textsuperscript{71}. In addition, when Top2a (15-fold, FDR corrected p<0.002), a type II topoisomerase that simplifies DNA topology by separating entangled daughter strands, is inhibited it is unable to repair DNA damage-induced apoptosis, suggesting the high expression seen here either allows normal function or corrects abnormal function\textsuperscript{72}.

Next, in order to preliminarily define the structural, molecular, and cellular biological functions of these DEGs, Gene Ontology (GO) terms were compiled and analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.8)\textsuperscript{73, 74}. Of 7,074 terms associated with the 931 DEGs from both strains combined there were 946 GO terms, 42 with Benjamini-Hochberg corrected p<0.05. Of
the 192 statistically significant DEGs from AXB15/PgnJ there were 410 GO terms, 29 with BH corrected p<0.05, and 1,474 for C58/J, 37 with BH corrected p<0.05. In addition there were 313 GO terms that were associated with each strain individually and when grouped, 58 with BH corrected p<0.05 (Supplemental Material). Interestingly, there were GO terms that were associated with only one group. For example, there were 12 GO terms that were associated with both strains as a group but not with either one individually. More importantly, there were 8 GO terms associated only with AXB15/PgnJ (4 with BH corrected p<0.05) and 47 GO terms associated only with C58/J (18 with BH corrected p<0.05) (Table 2, 3A, and 3B).

Among the significant GO terms for AXB15/PgnJ were interferon-inducible GTPase, guanylate-binding protein C-terminal, guanylate-binding protein N-terminal, and guanine nucleotide-binding protein 1 (Gbp1) (Table 1). Within these terms were a concise list of genes including several isoforms of Gbp (3, 4, 5, 6, 8, 9, and 10) among other well annotated members such as Irgm1, Igtp, Irgm2, and Iigp1, and several novel genes such as Bc023105, Gm12250, Gm4841, and Gm4951 (Table 2A). These results suggest that these genes may have significant activity to help the underlying genetic background counteract or mitigate the adverse effects of AngII-mediated pathological stress. In contrast, there was a much longer and more comprehensive list of GO terms associated with the sensitive C58/J strain (Table 1). These terms included S-100 protein, extracellular matrix, calcium binding, S100/CaBP-9k-type, MhcI, EF-Hand type, glycosaminoglycan binding, heparin-binding, insulin-like growth factor-binding protein (Igfbp), thrombospondin type 1 repeat, and apoptosis (genes are listed in Table 3A and 3B). These results suggest that these genes
may drive the expression of inductors of multiple cardiac failure phenotypes or, if they act to maintain normal function in untreated mice, are unable to provide the protection conferred by basal expression.

**Discussion**

HFpEF is a disease state with a group of heterogeneous phenotypes that, thus far, has manifested without a specific pathological pattern, leaving its diagnosis undetectable and outcome unpredictable. The genetic landscape has remained elusive as well. It is necessary to explore and identify additional phenotypes that may serve as a database for finding phenotypic diversity that is homogeneous enough to be useful for focusing on the genetic basis of HFpEF. Our approach, using inbred mouse strains supports this idea by providing an infinite number of essentially genetically identical intra-strain mice that provide power for identifying these phenotypes, while still balancing the necessary diversity of inter-strain genetic differences required for systems analyses across the entire panel.

The panel as a whole had moderately increased LV weight, severely elevated LA enlargement, varied levels of non-heart tissue weight, pEF, normal LV dimensions, sub-hypertension BP, and unchanged aortic blood flow. Interestingly, MV function was altered in which there was normal blood flow into the LV but compliance and relaxation was impaired. When combining this phenomenon with LA enlargement there is a suggestion for LVDD.
Our main goal is to continue the study and complete the entire panel. Thus far, the results suggest LVDD and HFpEF after 3 weeks of AngII treatment. If these trends continue, we expect to generate a mouse model primed for hypothesis generation and gene discovery for a field of cardiovascular disease that has a substantial proportion of the leading cause of hospitalization and mortality, not to mention it being poorly understood, diagnostically elusive, and largely untreatable.

Replicable methodology and subsequent data are imperative for the fulfillment of future studies. For interesting hypotheses to be formed the basis for their ideas must be true. Therefore, the overall purpose of the 3-strain cohort was to use select AngII-sensitive and -resistant strains from the HMDP to further investigate the pathologies observed in that panel and determine whether they are informative for suggestive hypotheses.

In this sub-study, the sensitive mice had already shown several indications of impaired relaxation, LVDD, and HFpEF in the main panel and in this 3-strain cohort there was increased LA and LV weight, normal measures of EF, LVID, LVPWT, and LVRWT, and normal E/A ratio combined with increased LV filling pressure (E/E’ ratio), and reduced MV function (E’ and A’). In addition to the main panel, which did not receive invasive catheters or have NO bioavailability assayed, the magnitude of the minimum derivative of pressure (dP/dT min) in AngII-induced BXD75/RwwJ mice is lower compared to controls, indicating reduced compliance, and all 3 strains exhibited lowered NO content, suggesting that AngII is not the driving factor for HF but, instead, the underlying genetic background is.
BXD75/RwwJ and AXB15/PgnJ were in agreement with the main panel with respect to data replication. In contrast, C58/J, which was believed to be resistant to AngII-induced pathologies was re-categorized as sensitive with a delayed onset. This conclusion is owed to the increase in mice used in this follow-up study. In the main panel C58/J had 3 controls and 3 treated mice, whereas, in this study there were 14 controls and 6, 7, and 8 treated mice for the groups of 1, 2, and 3 weeks, respectively. These increased values effectively separated the strain into two groups, one of unaffected controls and one of AngII-sensitivity, as indicated by delayed onset overlooked by the smaller groups.

RNAseq analysis has served to provide additional evidence that AngII does have a contributory role but has subtle effects when compared to that of genetic background as evidenced by PCA and CIM. Furthermore, the most extreme dynamic expression levels of select genes (Ppp1r10, Cox5b, Gng4, Celsr3, Rcan3, Scgb1a, and Top2a) agree with published literature\textsuperscript{66-72}. Only 4 GO terms, comprised of 15 genes, were significantly associated with AXB15/PgnJ, 3 of which have identical genes. In contrast, C58/J had 16 GO terms with 96 genes. This suggests that resistant mice have mechanisms to resist changes in nucleotide structure and function. On the other hand, sensitive mice undergo a much larger change in the number of genes with dramatically altered expression such that they are unable to resist or mitigate stressor-induced adversity that results in LA enlargement, LVDD, HFpEF, and impaired relaxation.

The immediate goal is to continue to prove the usefulness of a systems approach for unraveling the genetic basis of HF and the present model may serve as a first for
investigating HFpEF in a large mouse panel. Consequently, the discovery of previously unknown gene candidates that are differentially expressed with temporal and strain specific variation may provide new opportunities for the longer term goals of in-depth analyses of impaired relaxation, LVDD, and HFpEF.
Figure 1. Two sensitive and one resistant HMDP strains were subjected to AngII stress via an osmotic pump for 21 days. Before surgery all mice were subjected to echocardiography, tail-cuff blood pressure, and weighed. After surgery at day 7, 14, or 21 mice were subjected to echocardiography, sacrificed, and body/tissues were weighed. Subsequent experiments included NO bioavailability assay, PV loop analysis, and RNAseq.
Figure 2. HMDP tissue weight and physiology ordered by degree of LA enlargement. HMDP tissue weight and physiology ordered by LA size. Sensitive (red) and resistant (green) strains segregate efficiently when based on the degree of LA enlargement. Seven of eleven strains with significant LA enlargement ($p<0.05$) have preserved ejection fraction and diastolic dysfunction, including elevated LV filling pressure or abnormal MV tissue Doppler contractility.
Table 1. Sensitive strains exhibited phenotypes associated with DD and HFpEF. In contrast, AXB15/PgnJ was resistant to AngII-induced cardiac pathology.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Increased LA</th>
<th>Increased LV</th>
<th>Abnormal tissue doppler</th>
<th>Subpressor BP</th>
<th>pEF</th>
<th>Reduced NO content</th>
<th>Normal E/A ratio</th>
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<td>+</td>
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Figure 3. Left atria, left ventricle, systolic blood pressure, and diastolic blood pressure vs. control mice (black-control, color-3 weeks, red-sensitive, green-resistant) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ experienced LA enlargement as early as the first week, C58/J took 3 weeks for LA enlargement and LV hypertrophy to manifest, and AXB15/PgnJ was unaffected (Figures S1 and S2). All 3 strains experienced modestly increased LV weight and systolic BP but were all sub-hypertensive. This indicates this model is useful for sensitive, resistant, and intermediate phenotypes.
Figure 4. LV Function. Treated vs. control mice (black-control, yellow-1 week, red-2 weeks, green-3 weeks) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ and C58/J internal diameters were slightly reduced at week 1 but were unchanged after 3 weeks, BXD75 also showed a thicker posterior wall at 1 week but no change overall, C58/J had increased relative wall thickness at the 3 week mark. However, all 3 strains experienced preserved ejection fraction.
Figure 5. MV Function. Treated vs. control mice (black-control, yellow-1 week, red-2 weeks, green-3 weeks) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ E/A ratio was slightly reduced at week 1 but was unchanged after 3 weeks, C58/J did have a slightly increased E/A ratio at 3 weeks, and AXB15/PgnJ was unchanged overall. Increased filling pressure, as indicated by E/E’, was present in the sensitive strains. Likewise, sensitive strains had abnormal tissue Doppler, as indicated by E’ and A’. Normal E/A ratio combined with abnormal tissue Doppler and increased filling pressure indicate impaired relaxation and diastolic dysfunction in sensitive strains while the resistant strain did not manifest these challenges.
Figure 6. NO content is a biomarker of impaired relaxation and diastolic dysfunction. Treated vs. control mice after 1, 2, and 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ NO content was reduced at week 2 and 3, C58/J was reduced at 1-3 weeks, and AXB15/PgnJ was also reduced at 3 weeks. These results indicate that, biochemically, the sensitive and resistant mice are showing signs of impaired relaxation but, interestingly, AXB15/PgnJ is not physiologically presenting this impairment as indicated in figure 8.
Table 1. Sensitive strains exhibited phenotypes associated with DD and HFpEF. In contrast, AXB15/PgnJ was resistant to AngII-induced cardiac pathology.

<table>
<thead>
<tr>
<th></th>
<th>BXD75/RwwJ</th>
<th>C58/J</th>
<th>AXB15/PgnJ</th>
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<td>Increased LA</td>
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<tr>
<td>Increased LV</td>
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<td>Abnormal tissue doppler</td>
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<td>pEF</td>
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<td>Reduced NO content</td>
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<tr>
<td>Normal E/A ratio</td>
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</table>
Figure 7. Principal component analysis (PCA) of AXB15/PgnJ and C58/J mice using RNAseq data. Principal component 2 (PC2) efficiently segregated C58/J (sensitive) from AXB15/PgnJ (resistant) in 44,340 unique transcripts across all samples (A). Using the 33,000 transcripts with FPKM>1 showed PC1 segregated the two strains and AXB15/PgnJ treated from control mice (B).
Figure 8. Clustered Image Maps (CIMs) were used to help understand the role of AngII in these two strains. Using only the 931 post-AngII differentially expressed genes with a fold change <0.5 or >2 (FDR corrected p<0.05), several but not all of the sample mice clustered together based on control/AngII status.
Figure 9. Clustered Image Maps (CIMs) of AXB15/PgnJ. Using only the 192 post-AngII differentially expressed genes with a fold change <0.5 or >2 (FDR corrected p<0.05), AngII was a determining factor for clustering in the resistant AXB15/PgnJ mice. Considering the pre-selection of AngII-induced differentially expressed genes, these results are no surprise and suggest that AngII plays a major role in clustering for resistant mice.
Figure 10. Clustered Image Maps (CIMs) of C58/J. Using only the 643 post-AngII differentially expressed genes with a fold change <0.5 or >2 (FDR corrected p<0.05), AngII was not a determining factor for the random clustering in the sensitive C58/J mice. These results suggests that post-AngII stress is a subtle, perturbing factor rather than the major contributor that genetic background exerts in sensitive mice, which also offers an explanation as to why the two strains together did not completely cluster separately based on control/AngII status.
Table 2. Gene Ontology (GO) terms that were associated with either both strains together or only one strain. For example, the first GO term in the list is brown fat cell differentiation which was associated with both strains together but not either strain alone. Likewise, the first GO term for AXB15/PgnJ, interferon-inducible GTPase, had no association with C58/J or both strains combined, making it a unique GO term for the resistant strain. Bolden entries have Benjamini-Hochberg (BH) corrected $p<0.05$ which was considered significant.
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Table 3A. Genes that correspond to the GO terms in Table 2. Bolden entries have Benjamini-Hochberg (BH) correction methods in which a p-value<0.05 was considered significant. The list is continued in Table 3B.
Table 3B. Genes that correspond to the GO terms in Table 2. The list is continued from Table 3A.
Figure S1. Dose response 1. AngII in one ISO-resistant (FVB/nJ) and -sensitive (BALB/cByJ) strain. (top) Ratio of treated vs. control mice after 3 weeks of AngII induction administered by osmotic pump (1.0 - grey, 1.33 - green, 1.67 - yellow, and 2.0 - red ug/kg/min). (bottom) Systolic BP at baseline through week 3 (p-value = not significant).
Figure S2. Tissue weight after 23 strains. Ratio of treated vs. control mice after 3 weeks of AngII induction administered by osmotic pump (2000 ng/kg/min).
Figure S3. Systolic BP after 23 strains. Control and AngII-treated strains after 3 weeks of AngII induction administered by osmotic pump (2000 ng/kg/min).
Figure S4. Dose response 2. Ratio of treated vs. control mice after 3 weeks of AngII induction administered by osmotic pump. Pumps were implanted intraperitoneally (IP) or subcutaneously (sub) (IP = 100, 200, 500, or 1000 ng/kg/min with water vehicle, sub = 500 ng/kg/min with acetic acid-NaCl vehicle or water vehicle). (bottom) Systolic BP at baseline through week 3.
**Figure S5.** Dose response 3. AngII in one ISO-resistant (FVB/nJ) and -sensitive (BALB/cByJ) strain. (top) Ratio of treated vs. control mice after 3 weeks of AngII induction administered by osmotic pump (100 - grey, 200 - green, 500 - yellow, and 1000 - red ng/kg/min). (bottom) Systolic BP at baseline through week 3 (p-value = not significant).
Figure S6. Tissue weight vs. tibia length. Ratio of treated vs. control mice after 3 weeks of AngII induction administered by osmotic pump (500 or 2000 ng/kg/min). A. total heart, B. left atria, C. right atria, D. left ventricle, E. right ventricle, F. adrenal gland, G. liver, H. lung, and I. kidney.
Figure S7. HMDP tissue weight and physiology ordered by degree of LA enlargement. Sensitive (red) and resistant (green) strains segregate efficiently when based on the degree of LA enlargement. Seven of eleven strains with significant LA enlargement ($p<0.05$) have preserved ejection fraction and diastolic dysfunction, including elevated LV filling pressure or abnormal MV tissue Doppler contractility.
Figure S8. HMDP tissue weight and physiology ordered by degree of LA enlargement. HMDP tissue weight and physiology ordered by LA size. Sensitive (red) and resistant (green) strains segregate efficiently when based on the degree of LA enlargement. Seven of eleven strains with significant LA enlargement (p<0.05) have preserved ejection fraction and diastolic dysfunction, including elevated LV filling pressure or abnormal MV tissue Doppler contractility.
Figure S9. HMDP tissue weight and physiology ordered by degree of LA enlargement. HMDP tissue weight and physiology ordered by LA size. Sensitive (red) and resistant (green) strains segregate efficiently when based on the degree of LA enlargement. Seven of eleven strains with significant LA enlargement (p<0.05) have preserved ejection fraction and diastolic dysfunction, including elevated LV filling pressure or abnormal MV tissue Doppler contractility.
**Figure S10.** Heart weight. Treated vs. control mice (black-control, yellow-1 week, red-2 weeks, green-3 weeks) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ experienced LA enlargement as early as the first week, C58/J took 3 weeks for LA enlargement and LV hypertrophy to manifest, and AXB15/PgnJ was unaffected. This indicates this model is useful for sensitive, resistant, and intermediate phenotypes.
Figure S11. Blood Pressure. Treated vs. control mice (black-control, yellow-1 week, red-2 weeks, green-3 weeks) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ experienced increased systolic BP as early as the first week, C58/J took 2 weeks for systolic BP to manifest, and AXB15/PgnJ initially had a drop in BP before increasing at week 2. It is important to note that all 3 strains have suppressor levels of increased BP. Interestingly, BXD75/RwwJ showed increased heart rate during the 3 week period, possibly as a compensatory mechanism for impaired function.
**Figure S12.** LV Function. Treated vs. control mice (black-control, yellow-1 week, red-2 weeks, green-3 weeks) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ and C58/J internal diameters were slightly reduced at week 1 but were unchanged after 3 weeks, BXD75 also showed a thicker posterior wall at 1 week but no change overall, C58/J had increased relative wall thickness at the 3 week mark. However, all 3 strains experienced preserved ejection fraction.
Figure S13. MV Function. Treated vs. control mice (black-control, yellow-1 week, red-2 weeks, green-3 weeks) after 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ E/A ratio was slightly reduced at week 1 but was unchanged after 3 weeks, C58/J did have a slightly increased E/A ratio at 3 weeks, and AXB15/PgnJ was unchanged overall. Increased filling pressure, as indicated by E/E’, was present in the sensitive strains. Likewise, sensitive strains had abnormal tissue Doppler, as indicated by E’ and A’. Normal E/A ratio combined with abnormal tissue Doppler and increased filling pressure indicate impaired relaxation and diastolic dysfunction in sensitive strains while the resistant strain did not manifest these challenges.
Figure S14. NO content is a biomarker of impaired relaxation and diastolic dysfunction. Treated vs. control mice after 1, 2, and 3 weeks of AngII induction administered by osmotic pump (500 ng/kg/min). BXD75/RwwJ NO content was reduced at week 2 and 3, C58/J was reduced at 1-3 weeks, and AXB15/PgnJ was also reduced at 3 weeks. These results indicate that, biochemically, the sensitive and resistant mice are showing signs of impaired relaxation but, interestingly, AXB15/PgnJ is not physiologically presenting this impairment as indicated in figure 8.
RNAseq data processing. RNAseq on AXB15/PgnJ and C58/J mice generated 4 files per mouse which were first concatenated into one file. Quality control steps were taken to validate the data, Tophat was used for transcript alignment, and then Cufflinks was used to generate RPKM (reads per kilobase per million reads) and differential expression values.
References


43. Paulus WJ. The Role of Nitric Oxide in the Failing Heart. *Heart Fail Rev*. 2001;6:105-118.


Chapter 5

Conclusions

and

Implications for Future Studies
Heart failure (HF) is the leading cause of death worldwide. Recent progress has improved our understanding and management of diseases of the heart, yet its incidence, prevalence, and economic costs are steadily adding an increased burden to an already stressed health care system. Although life expectancy increases with the plethora of mechanical and chemical treatments, as well as lifestyle changes, overall life expectancy after diagnosis still has a very poor prognosis. Therefore, studying the etiologies of cardiovascular diseases and their associated pathologies have never been more pressing.

Importantly, one must understand the many ways that HF can manifest in order to select an induction method suitable for answering questions raised by a hypothesis. Whether HF is chemically or surgically induced in an animal model or studied via other methods are important considerations. Other considerations include whether the model selected will yield viable results, the size is large enough to inform statistical relevance while not being wasteful or causing unnecessary pain and suffering, and that it is hypothesis driven.

GWA studies have discovered implicative and causal loci in a vast array of human cohorts and animal models. However, GWAS has only explained a fraction of the role of genetic background in disease. Gene identification and subsequent validation require extensive studies to prove the usefulness of systems approaches, otherwise, it is a wasteful endeavor that diverts resources away from methodology that would have otherwise been actually useful. There have been several gene manipulation techniques, such as CRISPR and MerCreMer, which have eased the validation process. Along with this ease are
complicated and multifactorial experiments with obstacles at every step, including troubleshooting, optimization, and properly conducted analyses, to name a few.

Notwithstanding, the many successes of systems biology are accompanied by limitations, such as inadequate study designs that produce sub-genome wide significance or, more importantly, candidate genes with genome wide significance uncovered by these investigations being published but then never having follow ups conducted. GWAS, in and of itself, has proven its usefulness in the researchers tool collection. Supporting these findings by driving the research past gene discovery compliments the gargantuan efforts of a systems approach. For example, it is important to understand if a candidate gene is cell, tissue, or organ specific, whether its protein product is circulating, and whether it is extracellular to name a few possibilities. Additionally, determining at what developmental stage it has primary activity as well as its functional role (i.e. structural, signaling, transport, etc.). Most importantly, finding the mechanism of action is of utmost importance when attempting to elucidate the causality of candidate gene effects in cardiovascular genetics. This information may provide new methods for treatment, such as inhibition, reversal, or prevention of disease progression, using gene therapy, drug discovery, surgical techniques, and all other methods that have proven efficacy. Importantly, reducing costs and providing easier access to healthcare for all people is a challenge as great as overcoming the obstacles in solving the detriments suffered under HF.
Multiple Stressors

LVH is a compensatory mechanism in response to an increased hemodynamic load on the heart\(^1\). Eventually, cardiomyocytes suffer from a chronic lack of energy and they lose their ability to maintain homeostasis, which, when coupled with an increase in fiber disarray, replacement fibrosis, and perivascular fibrosis, myocardial contraction is further degraded\(^2\). AngII contributes to an increase in left ventricular stiffness, resulting in diastolic dysfunction\(^3\). LVH is a response to pressure or volume stress, which can subsequently lead to fibrosis. Some strains showed a loss of contractility, increased LVH, and elevated fibrosis. There must be a compensatory mechanism that precludes other strains that have a loss of contractility and increased LV weight from building these fibrotic deposits.

Although no particular etiology predicts a specific cardiac pathology, there are 3 variables that we have uncovered with multiple similarities: strain, stressor, and phenotype. The results of the multiple stressors study in Chapter 2 have begged the question why are some strains of mice sensitive to some cardiac failure related phenotypes and not others? Dogma within the cardiovascular research community shows that HF is a complicated, multifactorial condition resulting from a heterogeneous mixture of adverse physical, functional, and biochemical conditions. These studies have identified specific strains of HMDP mice with dramatically different manifestations of cardiac pathology in response to ISO, TAC, and AngII, and we found, in several cases, the strains with different phenotypic responses to ISO also have similar patterns of responses to other stressors. This
indicates that the genetic contribution revealed by multiple stressor-induced HF may also have significant implications in other HF etiologies and can, therefore, be potentially translated to HF in humans.

In addition, because Itga1 has been implicated by multiple biochemical and GWA studies as having a role in cardiac pathologies, including inflammation, fibrosis, and atherosclerosis, we believe genetic variants significantly contribute to the development of HF and the genes implicated in the specific pathological features of HF can be revealed via systems genetics.

To assess the replicability of this study there should be an effort to repeat one resistant and one sensitive strain to confirm previous findings. This will not only provide physiological data, but also new tissue which has been extinguished by previous studies. For example, A/J mice only had 4 controls and 3 ISO-treated mice. Combining this with the fact that of the 4 sensitive strains, A/J mice had the least amount of LVH and fibrosis but the largest drop in EF, raising the possibility that this small data set within the larger study is not sufficient to explain the loss of LV function. With additional mice there may be larger changes in LVH and fibrosis or smaller changes in EF, leading to statistically significant differences that were not observable with this small group. Additionally, other ISO-sensitive and –resistant strains becoming part of this study will provide larger, more physiologically detailed datasets than already obtained.

With LV weight, fibrosis, and contractility as the 3 main phenotypes of our focus, several other contributory factors have been overlooked. For example, echocardiographic
strain analysis provides direct information on intrinsic myocardial function. This method divides the left ventricle into 6 sections and uses speckle tracking echocardiography to determine the amount of tissue movement in each section, which enables the researcher to visually and numerically assess whether or not the sectioned LV is contracting with a wave-like rhythmic motion. Strain and strain rate measurements, the amount of contraction and the time to maximum contraction, are newer quantitative indices of intrinsic cardiac deformation in real time. Having these additional phenotypes will offer another avenue of approach for categorizing the differential responses of these animals and serve as supplemental support for the conclusions already observed. It will be easier to categorize these mice into sensitive or resistant categories with more than LV weight, fibrotic content, and contractility as the only variables since assessment of other phenotypes will aid in inclusion or exclusion from the respective category.

**Candidate Gene Validation**

An extensive and comprehensive list of candidate genes were identified from the ISO-induced HMDP as having high correlation with a multitude of cardiac phenotypes. The investigations in Chapter 3 have attempted to validate those findings by providing preliminary data that serves to form a basis for future mechanistic studies that will eventually define the detailed role of Mospd3 and H24 in LVH. Thus far, Mospd3 appears to be a cytosolic protein that regulates basal cell size. Mospd3 has been previously identified in another study as having a role in RV dysfunction and remodeling accompanied by 50% neonatal lethality in a mouse model. H24 was virtually unknown and was only
predicted to have a protein product based on its DNA sequence. We now believe it also is a cytosolic protein but, in contrast to Mospd3, is a driver of cell size in NRVM cultures. The fact that H24 appears highly punctate in NRVM cells may suggest several possibilities, such as there being vesicle trafficking for signal transduction or, alternatively, degradation and recycling for other cell functions. Co-immunoprecipitation and mass spectrometry may prove to be a viable approach for discovering binding partners for either gene product and, therefore, a signaling pathway for future assessment of a functional role.

Much of the ISO-treated tissue was used before this study began and, as a result, some strains have not had expression levels established. Therefore, additional ISO-treated tissue should be collected from new mice. This will pin down a truer, more accurate view of Mospd3, H24, and several other hypertrophy markers in addition to Nppa and Nppb. Do cells that have lower levels of hypertrophy have higher hypertrophy marker expression, suggesting that the GOI has a role downstream of hypertrophy signaling?

The overexpression of Mospd3 and H24 was able to show cytosolic localization. Unfortunately, overexpression in NRVM appeared to have low efficiency. We plan to optimize by adjusting the existing transfection protocol or switching from plasmid to adenoviral transfection. The mRNA and protein levels have not been verified for overexpressed Mospd3 and H24 in NRVM and MDCK. FLAG antibodies will suffice for exogenous expression, however, ascertaining endogenous protein levels will require antibody generation. A non-physiological abundance of protein product may enable a dual role for a protein of interest, or for an alternative activity altogether. Exogenous and
endogenous co-IP should allow us to examine states of basal and high activity. Mass spectrometry of binding partners captured by co-IP will also help us learn the specific endogenous and exogenous signal transduction occurring in diseased and control environments. Transgenic and knock-out mice, either as tissue specific or global, will be necessary for our long term goals of understanding activity in the heart, vasculature, and other systems.

**Angiotensin II Induced HMDP**

HFpEF is a disease state with a group of heterogeneous phenotypes that, thus far, has manifested without a specific pathological pattern, leaving its diagnosis undetectable and outcome unpredictable. The genetic landscape has remained elusive as well. It is necessary to explore and identify additional phenotypes that may serve as a database for finding phenotypic diversity that is homogeneous enough to be useful for focusing on the genetic basis of HFpEF. Our approach, using inbred mouse strains supports this idea by providing an infinite number of essentially genetically identical intra-strain mice that provide power for identifying these phenotypes, while still balancing the necessary diversity of inter-strain genetic differences required for systems analyses across the entire panel.

The panel as a whole had moderately increased LV weight, severely elevated LA enlargement, varied levels of non-heart tissue weight, pEF, normal LV dimensions, sub-hypertension BP, and unchanged aortic blood flow. Interestingly, MV function was altered in which there was normal blood flow into the LV but compliance and relaxation was
impaired. When combining this phenomenon with LA enlargement there is a suggestion for DD.

Our main goal is to continue the study and complete the entire panel. Thus far, the results suggest DD and HFpEF after 3 weeks of AngII treatment. If these trends continue, we expect to this HMDP will be primed for hypothesis generation and gene discovery for a field of cardiovascular disease that covers a substantial proportion of the leading cause of hospitalization and mortality, not to mention it being poorly understood, diagnostically elusive, and largely untreatable.

As explained earlier, echocardiographic strain analysis, also known as speckle tracking echocardiography (STE), provides direct information on intrinsic myocardial function. In fact, the ratio of early mitral flow to peak early diastolic strain rate (E/SRE) ratio can be used to predict LVEDP and peak SR during the isovolumic relaxation period (SRIVR) has been used diagnostically for LV relaxation in patients with HCM. These are additional tools that can be used on existing echocardiographic data to help confirm DD in the AngII-induced HMDP. In the long term, it will help identify mouse strains, which have not yet been treated, as experiencing DD and impaired relaxation that could have been overlooked by small sample numbers or by other echocardiographic parameters already in use.

The long term goals of this study, after completing the panel, are to perform GWAS to identify gene loci that correlate with phenotypes of interest, including quantitative trait loci expression (eQTL), echocardiographic parameters, changes in tissue weight,
hemodynamic changes, and differentially expressed genes. Gene modules can be constructed via network analysis, as well.

Multiple studies have shown that it can take as much as 4 weeks for mice to develop HF by AngII administration\textsuperscript{7-17}. It is possible that resistant strains simply need more time to develop impaired relaxation, DD, and HFpEF. Therefore, it would be interesting to select 3 strains of mice that appear to be resistant and treat them for extended periods of time. For example, AXB15/PgnJ mice could be treated for 4, 6, and 8 weeks and then assess whether or not they have remained resistant. This could potentially create a model that searches for reasons as to how a particular genetic background maintains complete resistance, experiences delayed onset, or has mild or acute sensitivity.

Another way to systematically investigate HFpEF is to data mine other studies that were not focused on HFpEF and, therefore, overlooked or ignored the possibility of detecting its existence. For example, the ISO-induced HMDP has echocardiographic data available. Considering the fact that approximately half of human HF has pEF, there is a strong possibility that other studies will have large datasets with valuable HFpEF phenotypic data. A meta-analysis of echocardiographic data from several studies may provide enough power to uncover subtle effects hidden by smaller studies.

**Impaired Relaxation, Diastolic Dysfunction, and Preserved Ejection Fraction**

Replicable methodology and meaningful data are imperative for the fulfillment of future studies. For interesting and useful hypothesis generation the basis for their ideas
must be true. Therefore, the purpose of the 3-strain cohort studies was to use select AngII-sensitive and -resistant strains from the AngII-HMDP to further investigate the pathologies observed in that panel and determine whether they are informative for suggestive hypotheses.

In this sub-study, the sensitive mice showed several indications of impaired relaxation, DD, and HFpEF in the main panel and in this 3-strain cohort including increased LA and LV weight, normal measures of EF, LVID, LVPWT, and LVRWT, and normal E/A ratio combined with increased LV filling pressure (E/E’ ratio) and reduced MV function (E’ and A’). In addition to the main panel, which did not receive invasive catheters or have NO bioavailability assayed, the magnitude of the minimum derivative of pressure (dP/dT min) in AngII-induced BXD75/RwwJ mice is lower compared to controls. All 3 strains exhibited lowered NO content, more evidence that suggests genetic background as the underlying cause of the phenotypic manifestations rather than AngII.

BXD75/RwwJ and AXB15/PgnJ were in agreement with the main panel with respect to data replication. In contrast, C58/J, which was believed to be resistant to AngII-induced pathologies was re-categorized as sensitive because of a delayed onset. This conclusion is owed to the increase in mice used in this follow-up study. In the main panel C58/J had 3 controls and 3 treated mice, whereas, in this study there were 14 controls and 6, 7, and 8 treated mice for the groups of 1, 2, and 3 weeks, respectively. These increased values effectively separated the strain into two groups, one of unaffected controls and one of AngII-sensitivity, as indicated by delayed onset overlooked by the smaller groups. The
immediate goal is to continue to prove the usefulness of a systems approach for unraveling the genetic basis of HF and the present model may serve as a first for investigating HFpEF in a large mouse panel. Consequently, the discovery of previously unknown gene candidates that are differentially expressed with temporal and strain specific variation may provide new opportunities for the longer term goals of in-depth analyses of impaired relaxation, DD, and HFpEF.

Similar to the future studies proposed for the main panel, this study should continue along those lines using STE for strain analysis for detection of impaired relaxation, look at differentially expressed genes throughout the entire longitudinal study rather than just week 3, and perform P/V loop analysis with occlusion in order to adjust the load of load-dependent hemodynamics.

Because the 3 strains for this study were selected close to this panel’s inception there are several other strains with higher levels of LA enlargement and functional markers of impaired relaxation, LVDD, and HFpEF. Therefore, selecting 2 of these extremely sensitive strains and another that is mostly resistant/modestly sensitive will provide a model containing 4 sensitive strains and 2 that are resistant. Combining this proposal with the idea that one of the sensitive strains has delayed onset and one of the resistant strains is not completely resistant, this approach has the potential to exhibit a gradient effect on levels of AngII-induced HFpEF within the HMDP strains.

With regards to NO bioavailability, is lower NO due to impaired activity of eNOS or lack of BH4, the eNOS co-factor for generating NO? Assaying eNOS, BH4, and other
signal transduction members for their respective activities will help inform as to whether or not DD is necessarily a result of impaired eNOS function.

**Concluding Remarks**

Heart failure, a heterogeneous group of adverse cardiovascular phenotypes, is the leading cause of death. In my investigations several interesting phenomenon have developed. First, it has been shown that genetic variants significantly contribute to the development of heart failure and the genes implicated in the specific pathological features of heart failure can be revealed using a systems genetics approach. Also, the molecular basis for the ISO-induced genetic contribution of candidate genes and gene modules can be understood for additional perturbations, such as AngII and TAC, and their outcomes. Cardiac hypertrophy, contractility, and fibrosis induced by different pathological stressors are subsequently influenced by different genetic factors.

Hypertrophy and fibrosis do not have a strain-specific phenotypic manifestation due to other stressors. For contractility, ISO and TAC share strain-specific manifestations. Therefore, genetic background plays a significant role in the manifestation of cardiac phenotypes which is also dependent on the stressor. Strains sensitive to a particular stressor may not be sensitive to another stressor for any of the phenotypes tested. However, strains susceptible to a particular genetic perturbation that leads to multiple cardiac phenotypes appear to be susceptible to other perturbations, although it may present with a different pattern of phenotypic manifestations.
With respect to Mospd3 and H24 candidate genes, there is a significant genetic contribution to H24 expression that is stressor dependent in which it is dynamically regulated by pathological stressors with a specific pattern under different genetic backgrounds. In NRVMs, inhibition of Mospd3 added to this increase in cell size. Inhibition of H24 enabled cells to resist these hypertrophic effects. We now believe H24 produces a protein product and the localization of both Mospd3 and H24 appears to be non-nuclear.

The AngII-HMDP has indicated impaired relaxation, diastolic dysfunction, and heart failure with preserved ejection fraction based on left atrial enlargement, left ventricular hypertrophy, subpressor systolic blood pressure, and reduced mitral valve function. The 3 extreme strains have provided replicable data to support what was obtained from the main panel. BXD75/RwwJ showed results indicating acute, C58/J had a delayed onset, taking 2-3 weeks, and AXB15/PgnJ continued to show resistance.

Progress has been made. Many questions have been asked. Some have been answered. However, with all the unanswered questions and remaining progress to be achieved, decades of work still remain. Which differentially expressed genes are associated with dysfunction? Are these genes leading to multiple phenotypic abnormalities? At what developmental point do these changes occur? Can the genes or proteins be targeted by drugs or have its DNA edited? Although the challenge often seems insurmountable, with each experiment we come one step closer to understanding the underlying genetic
background of heart failure and, eventually, we will find ways to slow or reverse the progression of disease in general, preferably preventing it from happening in the first place.
References


