Physiological Genomics: Implications In Hypertension Research
Susan B. Glueck, Victor J. Dzau

Abstract—In this article we delineate the directions in which the study of physiology will take as it becomes integrated with genomics. We also provide specific examples of the ways in which physiological genomics may be applied to study the complex genetics of hypertension and cardiovascular disease. (Hypertension. 2002;39[part 2]:310-315.)

Key Words: genetics ■ blood pressure ■ hypertension, essential ■ hypertension, genetic

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hysiology is the study of regulatory and homeostatic processes. It involves investigation at the systemic (integrated), organ, and cellular levels. An understanding of normal physiology provides the basis for elucidating the pathophysiology of disease, such as hypertension. Hypertension research has benefited enormously from physiological studies. As we enter this millennium, physiology research has yielded a rich understanding of the processes that control blood pressure and of how the dysregulation of these processes may contribute to the pathophysiology of hypertension. However, the fundamental causes of essential hypertension are still largely unknown. To date, efforts in genetic studies of human hypertension have yielded important but limited information. With the near completion of the sequencing of the human genome, hypertension researchers will possess an unprecedented opportunity to obtain an in-depth, global understanding of all genes regulating blood pressure and their potential contribution to the etiology of essential hypertension. To illustrate this potential, the human genome consists of up to 50,000 genes; yet to date, hypertension researchers have only focused their investigations on 1,000 of them. Thus, many genes that are important in blood pressure regulation and in the cause of hypertension are yet to be discovered and studied.

In this article we delineate the directions that the study of physiology will take as it becomes integrated with genomics. We also provide specific examples of the ways in which physiological genomics may be applied to study the complex genetics of hypertension and cardiovascular disease.

Forward and Reverse Genetics

Modern molecular biology and genomics has transformed physiology from the study of systemic and local homeostatic processes to the study of the genetic interactions that produce the cellular and tissue-level events responsible for those processes. This transformation parallels that of genetics, which itself has changed both in scope and direction. Traditionally, biomedical researchers have relied on “forward genetics” approaches to reveal the genetic underpinnings of specific diseases. Investigators first characterize the physiological defects of a given condition. With an understanding of pathophysiology, it becomes possible to identify the genes whose products mediate or regulate normal biological processes and whose abnormality may result in disease. The roles of these candidate genes may then be verified using knockouts, mutant lines of model organisms, pharmacological agents or antisense technology to block their function. Once a link between a particular candidate gene and disease has been established, researchers may use reporter gene assays in vitro and in vivo; yeast two-hybrid screens; or sequencing of polymorphisms in an allelic series to identify the particular genetic mutations responsible for altered function and subsequent disease state. Such explorations may uncover biochemical or cellular interactions that are crucial to maintaining a normal physiology, which in turn offer further opportunities for study.

Under this paradigm, researchers move from macro to micro, acting as reductionists in search of specific targets that can be demonstrated to contribute to diseases with a known genetic basis. Technologies developed over the last two decades, however, make it possible for scientists to employ “reverse genetics.” In this case, though, rather than testing candidate genes to determine whether alteration of their expression or products results in disease, the entire genome is scanned to identify novel, uncharacterized genes that are associated with disease. This entails assessment of disease-associated phenotypes, which are subsequently mapped by polymerase chain reaction (PCR)-based linkage analysis of evenly spaced repeat sequences throughout the genome (Quantitative Trait Loci, or QTL, mapping). This approach has resulted in the discovery of not only known genes but also novel, uncharacterized genes linked to specific diseases. Despite the different starting point, this method, as with forward genetics, ultimately connects genes to disease. Together, forward and reverse genetic techniques exploit scien-
The Scope of Physiological Genomics

Just as physiology encompasses a wide array of research methodologies to study homeostatic processes, so does physiological genomics encompass various levels of investigation and technology. The integrative aspect of physiological genomics extends from the current scientific capability to study the role of individual proteins from cell to organism. Accordingly, one might first identify genes differentially regulated between a normal and a diseased condition. A novel EST (Expressed Sequence Tag, a short sequence amplified from a pool of mRNA from a particular tissue) or transcript that is abnormally expressed in a pathologic condition could lead one to hypothesize its involvement in that condition. Here, physiological genomics will involve a dissection of the role a particular protein plays in cell-cell interactions, DNA binding affecting transcriptional regulation, or in signaling in a particular pathway. Further research could then ascend to the level of the tissue and thence the organ using more classical physiological techniques allied with genetics, such as the use of genetic strains or surgical or pharmacological interventions, to determine the action of the gene product in question. Thus the contribution of genes to disease might be traced backward in a reverse genetics approach, in contrast to a more classical approach in which a gene of known function was explored to determine its role in physiological function. Classical physiology research also benefits from an expanded ability to study larger numbers of variables simultaneously. Large-scale screening of mutagenized animals holds the potential to speed up the pace of discovery by identifying large numbers of mutants with different phenotypes. Thus, the greatest promise of physiological genomics is its expanded scope, which can enlighten and inform researchers across the spectrum of the disciplines of physiology and medicine.

The Tools of Physiological Genomics Research

Physiological genomics employs tools of classical physiology as well as those of contemporary molecular genetics. These methodologies make possible an exploration of the connection between genes and disease from both directions. What mainly differentiates physiological genomics from classical physiology is the capability for high-throughput examination of multiple genes, transcripts, or proteins at once—representing a real opportunity to mine the wealth of genomic information. The standard techniques of molecular genetics, classical whole-animal physiological models of disease, and transgenic technology are augmented by the use of expression profiling via microarray analysis and RDA (representational difference analysis) and SAGE (serial analysis of gene expression). Developing proteomics technologies will allow researchers to test multiple protein-protein interactions. The parallel efforts in genomics research in human, rat, and mouse model systems inform each other and facilitate comparative approaches.

The tools of physiological genomics (Table 1) include quantifying expression levels of multiple genes, carrying out both small-scale and large-scale mutagenesis, exploring correlations between genotype and phenotype in humans and model organisms, studying the interactions of nucleic acids and proteins, and experimentally manipulating single genes or entire chromosomes. Technologies including cross-species transgenics, retroviral-mediated gene transfer, and quantitative trait locus analysis are a few examples of the tools available. Also, techniques long in use in the Drosophila model system are now being applied to rats and mice, such as...
large-scale N-ethyl N-nitrosourea (ENU) mutagenesis followed by screening for morphological, physiological, endocrinological, and reproductive effects, or the use of reporter gene constructs to track the expression of genetic splice variants in particular tissues. These tools are described below.

The polymerase chain reaction (PCR) and subsequent PCR-derived technologies make it possible to characterize the prevalence and form of a variety of transcripts at once. With SAGE, sequences derived from different tissues are compared simultaneously, and differentially expressed genes may be identified. A short sequence tag of 10 to 14 base pairs is generated from mRNA by reverse transcription into cDNA, followed by restriction enzyme digestion. Numerous sequence tags are concatenated; the resulting strings of representative transcripts can be cloned and sequenced to identify the RNA population of a given tissue sample. Representational difference analysis combines subtractive hybridization with PCR to differentially amplify transcripts from two different samples—only samples that are expressed at higher levels in one population versus another are amplified, providing a snapshot of differentially expressed mRNAs under particular experimental conditions. Other technologies combine computers and lasers with fluorescently-labeled nucleic acids to carry out thousands of hybridizations at once. These high-tech versions of Southern and Northern blots take the form of cDNA and oligonucleotide microarrays.

Studying Gene Expression as an Approach to Examining Physiology or Function

Microarrays consist of either full-length cDNAs, amplified from the RNA pools of certain tissues, or of synthetic oligonucleotides, synthesized from lists of extant genes characterized by genome projects, spotted onto glass or nylon. These probes are hybridized to differentially fluorescently labeled pools of RNA from a desired tissue, then scanned with a laser. The resulting differences in fluorescent intensity may be statistically analyzed to group genes by expression level in time and in space.

Custom-made cDNA arrays generated from the RNA pools of specific tissues can take advantage of previous genomics research carried out by a particular laboratory group. Such arrays can be a valuable resource for workers with highly specific needs, such as, for example, expression profiles of zebrafish hearts at various stages of development. A disadvantage of cDNA microarrays is that they can only accommodate about 10,000 genes per slide. However, they offer the potential to discover unknown sequences whose expression levels are observed to change between given time or treatment points in such a way as to suggest their involvement in a particular disease pathway.

By contrast, synthetic oligonucleotide microarrays, such as the GeneChips™ which are the proprietary product of Affymetrix, contain duplicate spots that represent every known gene in a particular genome. A given gene is represented by more than one oligonucleotide on the chip. This provides a number of repeat experiments within a single chip to ensure the validity of hybridizations between probe and transcript, providing more precision. These arrays, with up to 40,000 genes per chip, are much higher density than custom glass slides and do not require previous genomic research by the investigators. However, while Affymetrix oligo arrays make possible an encyclopedic survey of the entire annotated genome, they do not allow for the discovery of novel sequences.

Microarray analysis enables researchers to elucidate and identify pathways of genetic regulation; genes mediating physiological function; and genes mediating disease. Microarrays can be used in expression profiling, in which the expression levels of large numbers of genes at a given time point is presented. There is the potential to elucidate the interconnected nature of preexisting pathways, which currently requires many sequential experiments. Expression profiling thus has great predictive and diagnostic capabilities. The challenge is to separate signal from noise as well as to make valid assessments of the relative importance of differentially expressed transcripts. Furthermore, the variability both between and within laboratories should sound a cautionary note: it is essential to follow up expression profiling experiments with RT- (real-time-) PCRs and Northern blots for individual transcripts. Not every differentially expressed mRNA found in a particular tissue will be relevant to a particular pathophysiology, just as certain physiological symptoms of disease are byproducts of, rather than cause for, that condition. It is incumbent on researchers to select worthwhile genes for further study through reasoned application of clustering algorithms with stringent limits of statistical significance.

Mutagenesis to Study Physiology or Function

Large-scale mutagenesis has long been used by geneticists studying yeast, Escherichia coli, Caenorhabditis elegans, Drosophila and other well-established model organisms. It has been only recently that scientists have been able to apply such large schemes to vertebrates. ENU, or N-ethyl N-nitrosourea, induces point mutations and small deletions in premeiotic spermatogonia, via DNA ethylation. ENU may be used either to mutagenize mouse embryonic stem cells (ES cells) to generate germline chimeras; or to mutagenize males whose affected progeny may then be characterized by screening back-crossed individuals for physiological and morphological abnormalities. Large-scale ENU mutagenesis allows researchers to perturb a number of important pathways at once, experimentally (Table 2). Such screens, when combined with established mapping techniques, can lead to more rapid identification of genes in which mutations bring about physiological change.

Other modes of mutagenesis include classical methodologies, such as X-irradiation, and vector-based methods including insertional mutagenesis and “gene trapping.” Although X-irradiation induces mutations at a rate up to 100-fold greater than the spontaneous frequency, the mutations result in large deletions, translocations, or similar chromosomal aberrations. In insertional mutagenesis, target vectors are recombined into the genome at specific locations in order to disrupt open reading frames. With insertional mutagenesis, very precise results can be obtained; additionally, disrupted genes can be tagged so that alterations can be followed at the cellular and tissue level. Gene trapping in mice is a variant of
the widely used “enhancer traps” of Drosophila research. LacZ reporter vectors are integrated into the genome of mouse embryonic stem cells, where they frequently disrupt open reading frames, thus simultaneously serving as insertional mutagens and flagging their location through the expression of LacZ in a pattern that mimics that of the endogenous gene. As with ENU mutagenesis, gene trapping is being used in large-scale screens. Libraries of gene trap sequence tags representing insertional mutations representative of most of the mouse genome are being generated and will represent a considerable resource to researchers interested in genes with particular regions of expression.4

Genetic Manipulation In Vivo to Study Physiological Genomics

A host of methods for genetic manipulations are available for use in mice or rats (Table 3). It is possible to eliminate the protein products of desired genes, to alter those proteins such that they are expressed abnormally or fail to interact with their usual targets, or to alter the expression levels of the proteins in order to recapitulate a particular disease state. Such experiments entail the use of vectors to transfer genes or reporter constructs into a given animal strain, as well as breeding to introgress particular chromosomal segments into different genetic backgrounds. Knockouts may be generated through mutagenesis, vector-mediated recombination in embryonic stem cells, or gene targeting. Through genetic manipulations such as these, researchers can confirm the function of genes in vivo whose activity has previously been assessed in vitro. Additionally, the degree to which particular genes contribute to multigenic inherited disorders can be assessed by altering their expression. “Unknown” genes, identified through expression profiling or PCR-based assays, can be knocked out or overexpressed in an effort to determine their function. These genetic tools can also help researchers ascertain novel functions of previously characterized genes, because the products of many genes in fact play multiple physiological roles. Furthermore, genetic manipulations can generate new strains of research animals which provide new models of complex disease.5 Lastly, physiological genomics may be employed to test specific hypotheses of the onset and progression of disease. Indeed, this approach has been employed in hypertension research via experimental alteration of the expression of renin-angiotensin, nitric oxide synthase, and kallikrein-kinin, among others.

Physiological Genomics to Study Hypertension and Cardiovascular Disease

The expression of genes involved in cardiovascular disease is influenced by hemodynamics, humoral factors, and environmental factors such as diet, stress, and exercise. The response to the above is influenced further by individual genetic variation (Figure 2). Physiological genomics has the potential to elucidate genetic pathways of hypertension and to assess the expression profile of transcripts in affected target organs. Following are a number of examples of research into the effect of single genes or numerous genes on hypertension.

Testing Hypotheses and Elucidating Function at the Level of Single Genes

There are numerous ways to assess the effect of single genes: to study their function, to test specific hypotheses, and to examine their roles in diseases such as hypertension. For example, the paradigm of the tissue renin-angiotensin system (RAS) has been examined recently by altering expression levels via transgensics, knockouts, and gene targeting. Indeed, Esther et al6 demonstrated that tissue ACE (angiotensin-converting enzyme) plays a critical role in blood pressure regulation. By generating a transgenic protein that lacked C-terminal sequences which serve to anchor somatic ACE to the plasma membrane of cells, the authors demonstrated that animals lacking tissue-bound ACE had significantly lower systolic blood pressure. The importance of local angiotensin in regulating tissue structure and function has also been studied using physiological genomics approaches. For example, overexpression of the AT1 receptor in mouse myocardium results in cardiac enlargement and failure.7,8 Finally, the

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**TABLE 2. ENU Mutagenesis**

<table>
<thead>
<tr>
<th>Strategy for ENU Mutagenesis</th>
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<tr>
<td>● ENU induces point mutations and small deletions via DNA ethylation, in premeiotic spermatogonia</td>
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<tr>
<td>● Either mutagenize ES cells to generate germ-line chimeras or mutagenize males</td>
</tr>
<tr>
<td>● Matings and back-crosses followed by panel of screening:</td>
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<tr>
<td>● Subsequent mapping, identification of mutations</td>
</tr>
</tbody>
</table>

ENU indicates N-ethyl N-nitrosourea; ES, embryonic stem. Modified from Rathkolb et al.3

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**TABLE 3. Genetic Manipulation In Vivo to Study Physiology or Function**

| ● Reporter gene constructs |
| ● Transgenic animals |
| ● Knockouts |
| ● Congenics and consomics |
| ● Gene transfer for functional studies or therapy |

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![Figure 2. Physiological genomics of the cardiovascular system. Gene expression that regulates the function and structure of the heart and blood vessels is influenced by hemodynamics and humoral factors. The qualitative and quantitative response of individual gene expression is determined by genetic variation and influenced by environmental factors.](Image 305x631 to 540x718)
role of tissue RAS in hypertension was confirmed by tissue-specific overexpression in transgenic mice. Expression of human angiotensinogen (AGT) in the mouse brain can raise blood pressure, as demonstrated by Morimoto et al.11,12 mice expressing human AGT in glial cells (driven by a brain-specific promoter) had higher blood pressure because of an increase in angiotensin II. A tissue-specific intrarenal transgenic renin-angiotensin system, when activated, can induce hypertension in mice.13 These innovative studies, taken together, provide important support for the existence of a functional tissue angiotensin system and are excellent examples of the application of physiological genomics to test specific hypotheses.

One classic example of the use of genetic manipulation to study suspected agents of hypertension is the work of Kim et al.,11 which demonstrated a direct causal relationship between Agt genotype and blood pressure in mice. Using gene targeting in embryonic stem cells to introduce extra copies of angiotensinogen into mice, these researchers demonstrated that the greater the number of copies of Agt, the higher the blood pressure in the transgenic animals. Physiological genomic tools have also been used to clarify the role of genes of unknown function in disease, as in studies of the AT2 receptor. Several years ago, the functions of the AT2 receptor were not known. Using gene disruption techniques, the AT2 receptor gene was knocked out in the mouse.12 The homozygous mutant, as compared with wild-type mice, had lower blood pressure, attenuated pressor response to angiotensin infusion, impaired drinking behavior after water deprivation, and subtle locomotor and circadian defects. Furthermore, the AT2 receptor knockout mice exhibited a marked increase in the size of the neointimal lesions of injured femoral arteries as compared with the wild-type mice,13 confirming the in vitro observation that this receptor mediates growth inhibition and apoptosis. The above studies are examples of the use of a physiological genomics approach to test specific hypotheses or to elucidate the function of specific genes.

Physiological genomics takes advantage of comparative studies in mice, rats, and humans that complement each other. Some diseases are more tractable in particular experimental systems; findings from such systems may be translated into testable hypotheses or proposed treatments in other models. One such example, of cross-species transgenics, is the work of Mullins, Peters, and Ganten,5 in which the mouse Ren-2 gene was inserted into rats. The result was fulminant hypertension whose specific genetic basis was known.

### Linking Genes to Function at the Scale of Numerous Genes

The capability for high-throughput testing makes possible the identification of novel genetic interactions and novel drug targets. As detailed by Hoit and Nadeau14 (Table 4), a strategy combining large-scale chemical mutagenesis of mice or rats with high-throughput screening for cardiovascular phenotypes has the potential to uncover a variety of genes suitable for further testing. A major requirement is a large number of genetic variants which can be produced using ENU. They describe a 3-tiered series of screens for cardiovascular phenotypes consisting of a high-throughput, large scale assessment of cardiac function (eg, echocardiography and measurement of blood pressure), followed by more invasive procedures on the variant mice identified in the first screen. Once secondary assays, such as evaluating the physiological response to particular pharmacological agents, have been carried out, tertiary assays such as force frequency analysis may be employed to pinpoint the subtleties of the identified genetic variations. Mapping techniques used in QTL analysis are employed to localize the induced point mutations to particular genetic regions.

Methodologies similar to those described above were employed to screen ENU-mutagenized zebrafish for cardiovascular mutations.15 This task was made easier by the fact that zebrafish embryos are transparent, making possible ready identification of cardiac abnormalities. A suite of mutations were identified that affect heart morphology, vascular integrity, and cardiac function. Since there is widespread synteny homology between vertebrate genomes, the ease of studies in tractable model organisms such as the zebrafish has the potential to inform human studies.

### Physiological Genomics Strategy to Study Target Organ Response and Damage

Expression profiling provides a sophisticated way to compare mouse models and human conditions of target organ damage in hypertension. One important target organ complication is cardiac hypertrophy and failure. The key question is which genes are critical in mediating this pathological cardiac remodeling. Since many genes are presumably differentially regulated between the disease and normal state, the challenge is to determine the physiological significance of differences in expression. Our strategy is summarized in Figure 3. In brief, RNA derived from diseased human heart tissue or from experimental mouse models of cardiac hypertrophy/failure is hybridized to oligonucleotide microarrays, then analyzed to provide a profile of transcripts that are up- or downregulated relative to wild type. Genes from mouse and human samples may then be compared with identify those common transcripts between the two species whose expression levels are significantly altered. This set of candidate heart failure genes can then be compared with cardiomyocyte-specific transcripts identified from cell culture. The resulting cluster of cardiomyocyte-specific genes whose expression level is different between normal and heart failure forms the basis of the validation process. In a cell-based high throughput screen, antisense oligonucleotides (loss of function) or novel genes

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**TABLE 4. Strategy for High-Throughput Cardiovascular Screening of ENU-Induced Mutations**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>Large-scale chemical mutagenesis</td>
</tr>
<tr>
<td>2.</td>
<td>Three-tiered phenotypic screening</td>
</tr>
<tr>
<td>A.</td>
<td>Primary assays, suitable for high throughput</td>
</tr>
<tr>
<td>B.</td>
<td>More invasive secondary assays</td>
</tr>
<tr>
<td>C.</td>
<td>Subtle and specific tertiary assays</td>
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</table>

**Electrophysiologic tests, force-frequency analysis**
(gain of function) can be transduced into cultured cells to assess the cellular response. A series of screening assays may then be carried out to look for particular phenotypes, such as apoptosis, proliferation, or markers of inflammation. With further validation using more specific and detailed confirmatory assays, the pool of candidate genes may be narrowed down to a manageable number suitable for testing in vivo in mice. At this stage transgenics or targeted knockouts may be utilized to verify the individual contribution of certain cardiomyocyte-specific genes to heart failure. This strategy would provide a rapid and thorough method to analyze a large number of candidates with a minimum of effort, aided by bioinformatics technology.

Conclusions

The greatest extent to which physiology researchers stand to benefit from physiological genomics is that their ability to test hypotheses will be expanded greatly. This is the promise of the wealth of genomic data and the strength of high-throughput technology. It is certainly not yet possible to test the pairwise interactions of each protein encoded by every open reading frame in the genome, as is being done in yeast. However, possession of the complete sequence of the human and mouse genomes facilitates the rapid identification and characterization of novel disease genes, as well as genes which can be shown to play a role in preexisting pathways. The application of physiological-genomics approaches should have a significant impact on hypertension research. It promises the identification of genes responsible for hypertension, the elucidation of genetic and molecular interactions mediating target organ pathology, and novel therapeutic strategies that may lead to a cure.

Acknowledgments

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References


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