Transgenesis and Gene Targeting in the Mouse
Tools for Studying Genetic Determinants of Hypertension
David E. Stec, Robin L. Davisson, and Curt D. Sigmund*

As more effort is made to identify genes responsible for hypertension in human populations and genetically hypertensive animal models, the need for experimental systems in which the functional significance of genes, gene variants, and quantitative trait loci (QTL) can be determined is becoming increasingly important. Over the past five years, transgenic and gene-targeting technology has been utilized to study the cardiovascular effects of over-expression or ablation of genes which have been considered candidates in the genetic basis of hypertension. This review focuses on the most recent major advances in this area, and how this technology aids in our understanding of the molecular mechanisms by which newly discovered genes or gene variants affect blood pressure in the whole organism. We also discuss the potential use of transgenic models in refining the location of a QTL, and discuss some of the limitations and potential pitfalls in the application of these tools to the field of hypertension research. The coupling of genetic manipulations afforded by transgenesis and gene targeting, along with advances in our ability to assess the cardiovascular phenotype in the mouse, provides us with a powerful system for examining the genes responsible for causing essential hypertension. (Trends Cardiovasc Med 1998;8:256–264) © 1998, Elsevier Science Inc.

- Over-expression of Candidate Genes in Transgenic Mice

One of the first uses of transgenic techniques in hypertension research was in examining the expression of candidate genes, which encode proteins that are known to participate in the normal regulation of blood pressure or which differ in their level of expression in cardiovascular tissues of hypertensive and normotensive animal strains. A logical first approach in understanding the possible role of candidate genes in hypertension was to determine the effects of their over-expression in animal models, and numerous such models have now been reported (Table 1).

The Renin-angiotensin System

One of the most extensively studied candidate gene systems is the renin-angiotensin system (RAS). The initial choice of studying the RAS, and in particular the renin gene, was based on: (a) decades of physiological and pharmacologic evidence that demonstrated its importance in the regulation of blood pressure and electrolyte balance; (b) pharmacologic blockade of the RAS remains an effective antihypertensive treatment; (c) the regulation of RAS mRNAs and proteins in cardiovascular tissues have been shown to differ between normotensive and hypertensive rodent models (Yongue et al. 1991); (d) molecular genetic studies report cosegregation between polymorphisms in genes of the RAS and elevated arterial pressure (Jacob et al. 1991, Rapp et al. 1989); and (e) the reported linkage between the angiotensinogen gene and hypertension in humans (Jeunemaitre et al. 1992). Based on these observations, it is not surprising that numerous transgenic models in which the RAS genes are manipulated have now been reported (Table 1). The results of these experiments consistently demonstrate that over-expression of the RAS results in systemic hypertension. For example, we and others have constructed transgenic mice which express both the human renin (HREN) and human angiotensinogen (HAGT) genes in the form of genomic constructs, each under the control of their own endogenous promoters. Since there is a strict species-specificity in the enzymatic reaction between renin and angiotensinogen (AGT), HREN is unable to cleave mouse AGT and vice versa (Hatae et al. 1994). Therefore, the presence of both human transgenes is necessary for a functional human RAS in transgenic rodent models. It is now clear that mice containing both human genes, along with their endogenous mouse counterparts, have elevated plasma angiotensin-II (Ang-II) levels and develop chronic systemic hypertension (Merrill et al. 1996, Fukamizu et al. 1993).

In an interesting variation of this double transgenic mouse model, Takimoto et al. (1996) reported that when HREN transgenic males were mated to HAGT transgenic females, the females exhibited increased blood pressure in late pregnancy (days 16–20). It was proposed that this was due to the secretion of active HREN from the placenta of fetuses (transgenic for HREN) and its interaction with HAGT circulating in the systemic circulation of the mother. Indeed, the model has some features in common with the condition of pre-
eclampsia defined by hypertension during late pregnancy associated with marked proteinuria. While further studies are necessary to determine if the model is a relevant system for examining the role of the RAS in preeclamptic patients, this example serves to demonstrate how a model can be constructed to examine the effect of vasoactive factors on blood pressure during pregnancy.

Perhaps the most studied transgenic model in hypertension has been the transgenic rat containing the mouse Ren-2 gene (Mullins et al. 1990). This model has been extensively reviewed and will not be discussed in detail (Ganten et al. 1991). Importantly, however, these rats exhibit severe hypertension reportedly due to the activation of intrinsic tissue RAS (Lee et al. 1996, Sander et al. 1992). Tissue RAS have been proposed to exist in tissues which have the capacity for local generation and action of Ang-II, and have been implicated in mediating some forms of hypertension. In the HREN/HAGT transgenic model just described, one can reasonably hypothesize that the increase in plasma Ang-II in these mice is likely involved in both initiation and maintenance of the hypertension. However, recent evidence from our laboratory suggests that activation of local RAS pathways within the brain are also partially responsible for the maintenance of elevated blood pressure in the HREN/HAGT model (Davison et al. 1997a). Moreover, the concept that tissue RAS exist in the kidney, heart, brain, and vasculature is now being tested directly on transgenic models. In fact, we have recently shown that renal-specific expression of the human RAS can lead to hypertension even in the absence of elevated plasma renin or Ang-II (Ding et al. 1997a and b).

Candidate Genes Encoding Vasodilators or Natriuretic Factors

In addition to genes which are thought to cause an elevation of blood pressure, a number of candidate genes which encode either vasodilators or natriuretic factors have also been over-expressed in transgenic mice. The general hypothesis underlying this approach is that inactivating mutations in such depressor genes could cause hypertension. Examples of these genes include those for atrial natriuretic peptide (ANP), tissue kallikrein, bradykinin B₂ receptor, and the various isoforms of nitric oxide synthase (NOS). Although transgenic models over-expressing most of these genes have now been generated (Table 1) and have resulted in lowered blood pressure, the exact mechanism causing the reduction in blood pressure in some of these models remains unclear. For example, transgenic mice expressing ANP under the liver-specific transthyretin promoter exhibit a 10- to 20-fold increase in plasma ANP levels (Steinhelper et al. 1990). Because of the known effects of ANP on renal function, a logical mechanism causing the reduced blood pressure could be excess sodium excretion and a reduction of intravascular volume. However, changes in sodium intake have no effect on sodium excretion or blood pressure in TTR-ANP mice (Veress et al. 1995), and exaggerated natriuresis does not occur unless the mice are challenged with acute volume expansion (Field et al. 1991). These results suggest that the hypotension observed in these mice may be independent of the direct natriuretic effects of ANF on the kidney (Sommer and et al. 1994). In fact, there is considerable evidence of widespread effects of ANF in extra-renal cardiovascular tissues such as the vasculature and heart.

In general, we must cautiously interpret data from over-expression studies, because the phenotype observed in whole animals may not be directly related to the effects of the transgene product on the anticipated target organ, the kidney in the case of ANF, and the vasculature in the case of Ang-II, but to other important but unpredicted targets and/or compensatory mechanisms. Moreover, the observation that over-expression of a candidate gene results in hypertension does not provide unequivocal proof that the gene causes human hypertension. Instead, these studies should serve as a guide to identify the physiologically relevant genes which may be altered in hypertension and then to examine whether and how these alterations cause hypertension.

• Genetic Alteration of Candidate Genes by Gene Targeting

Simple Gene Targeting

One of the newest applications of genetics to the field of hypertension research has been the use of gene-targeting strategies to functionally ablate (knock out) or duplicate candidate genes of interest. This technique has been used extensively in other fields and has been applied to hypertension research in the past 5 years (Krege 1996). Since genetic manipulations involved in gene targeting are performed in cell culture, the technique affords numerous advantages over classical transgenic approaches. Gene targeting can be used to generate null mutants, point mutations, whole gene duplications, and, as discussed in a later section, deliver whole genes or

### Table 1. Transgenic models overexpressing candidate genes and their resultant blood pressure phenotypes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Promoter</th>
<th>Blood pressure phenotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>ANF</td>
<td>Transthyretin</td>
<td>Hypotensive</td>
<td>Steinhelper et al. (1990)</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>Endogenous</td>
<td>Normotensive</td>
<td>Hocher et al. (1997)</td>
</tr>
<tr>
<td>Bradykinin B₂ receptor</td>
<td>RSV-3' LTR</td>
<td>Hypotensive</td>
<td>Wang et al. (1997)</td>
</tr>
<tr>
<td>Tissue kallikrein</td>
<td>Endogenous</td>
<td>Hypotensive</td>
<td>Chao and Chao (1996)</td>
</tr>
<tr>
<td>HREN/HAGT</td>
<td>Endogenous</td>
<td>Hypertensive</td>
<td>Fukamizu et al. (1993)</td>
</tr>
<tr>
<td>rREN/rAGT</td>
<td>MT-1</td>
<td>Hypertensive</td>
<td>Merrill et al. (1996)</td>
</tr>
<tr>
<td>rAGT</td>
<td>Endogenous</td>
<td>Hypertensive</td>
<td>Ohkubo et al. (1990)</td>
</tr>
<tr>
<td>NHE-1</td>
<td>hEF-1α</td>
<td>Salt-sensitive hypertension</td>
<td>Kimura et al. (1992)</td>
</tr>
<tr>
<td>ANF</td>
<td>TsRF</td>
<td>Hypotensive</td>
<td>Kuro-o et al. (1995)</td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic factor; RSV, Rous sarcoma virus; LTR, long terminal repeat; HREN, human renin; HAGT, human angiotensinogen; rREN, rat renin; rAGT, rat angiotensinogen; NHE, Na-H exchanger; MT-1, metallothionein; hEF, human elongation factor.
gene variants to specific chromosomal locations. Moreover, gene targeting can also eliminate some problems characteristic of classical transgenic approaches such as ectopic expression of transgenes, expression of non-physiologic levels of transgene products, and copy number artifacts and mutations caused by the random integration of transgene DNA in the genome. The technical aspects of gene targeting have been reviewed elsewhere (Bronson and Smithies 1994). A partial list of the candidate genes that have been targeted for deletion or duplication and their resulting effects on blood pressure are summarized in Table 2.

Each of the genes encoding the RAS proteins have been the subject of gene targeting in mice. The first of the RAS genes to be targeted was AGT. Kim et al. (1995) generated mice with different copy numbers of AGT (one to four copies) caused by targeted gene duplication. They reported that both plasma AGT and blood pressure varied according to the copy number of AGT, suggesting that gradual increases in plasma AGT can cause a significant increase in blood pressure in the mouse. These data were used to argue that similar to what is observed in human patients carrying a molecular variant of HAGT (M235T), small increases in plasma AGT could cause hypertension (Jeunemaitre et al. 1992). However, the data may simply reflect the fact that the concentration of AGT (not renin) in the mouse is rate limiting for the production of Ang-II. Whereas the plasma concentration of AGT in humans is close to the $K_m$ of renin, the plasma renin concentration in the mouse far exceeds that of rats and humans (Campbell et al. 1996).

Although it may have been expected that the renin gene would be the first target of genetic ablation, as it was for overexpression, its complete ablation proved to be more complicated than the other RAS genes and has yet to be accomplished. Since the most commonly used embryonic stem cell lines are derived from a mouse strain called 129Sv, which contains two renin genes (Ren-1 and Ren-2) closely linked on chromosome 1, generating a true renin null mouse would require either individually targeting each allele in succession in ES cells or creating a large deletion removing both genes. Mice carrying individual renin gene deletions have been recently reported, but further studies are needed before a true null is developed. Targeted disruption of the Ren-2 gene has no effect on blood pressure in either heterozygous or homozygous mice, and, surprisingly, the plasma renin concentration in these mice is elevated, suggesting perhaps a defect in feedback regulation (Sharp et al. 1996). In contrast, loss of the Ren-1 allele results in a sexually dimorphic phenotype with hypotension in homozygous female mice (Clark et al. 1997). Although there was no change in plasma renin concentration, plasma prorenin levels were increased in those mice.

Mice homozygous for gene targeted deletions of AGT or ACE develop severe renal abnormalities characterized by cortical thinning, focal fibrosis, tubular atrophy, chronic lymphocytic inflammation, and occlusive renal vascular hypertrophy (Krege et al. 1995, Niimura et al. 1995, Smithies and Kim 1994). These findings suggested an essential role for the RAS in the normal development of the kidney. Interestingly, initial reports indicated that mice deficient in AT1A, AT1B, or AT2 receptors exhibit a phenotypically normal renal morphology, suggesting the possibility that the actions of Ang-II during development of the kidney may act through an unidentified Ang-II receptor, or that the presence of other receptor subtypes in any single receptor-deficient genetic background may compensate for the loss of an individual subtype (Chen et al. 1997, Hein et al. 1995, Ichiki et al. 1995, Ito et al. 1995). Since the AT1A and AT1B genes are localized on different chromosomes, hypotheses concerning the interaction of these receptor subtypes can be tested simply by generating double knockouts by breeding. Indeed, mice lacking both receptor subtypes exhibit phenotypes similar to

<table>
<thead>
<tr>
<th>Gene</th>
<th>Blood pressure phenotype</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td>$\alpha_{1b}$ Adrenergic Receptor</td>
<td>None, decreased response to phenylephrine</td>
<td>Cavalli et al. (1997)</td>
</tr>
<tr>
<td>$\alpha_{2b}$ Adrenergic Receptor</td>
<td>None</td>
<td>Link et al. (1996)</td>
</tr>
<tr>
<td>$\alpha_2$ Adrenergic Receptor</td>
<td>None</td>
<td>Link et al. (1995 and 1996)</td>
</tr>
<tr>
<td>Adenosine A2a Receptor</td>
<td>Hypertensive</td>
<td>Ledent et al. (1997)</td>
</tr>
<tr>
<td>ACE</td>
<td>Hypotensive, lethal</td>
<td>Esther, Jr. et al. (1996), Krege et al. (1995)</td>
</tr>
<tr>
<td>AT1A Receptor</td>
<td>Hypotensive</td>
<td>Ito et al. (1995), Sugaya et al. (1995)</td>
</tr>
<tr>
<td>AT1B Receptor</td>
<td>None</td>
<td>Chen et al. (1997)</td>
</tr>
<tr>
<td>AT2 Receptor</td>
<td>None, hypertensive</td>
<td>Hein et al. (1995), Ichiki et al. (1990)</td>
</tr>
<tr>
<td>ANP</td>
<td>Salt-sensitive hypertension</td>
<td>John et al. (1995)</td>
</tr>
<tr>
<td>$\beta_1$ Adrenergic Receptor</td>
<td>Hypotensive, lethal</td>
<td>Rohrer et al. (1996)</td>
</tr>
<tr>
<td>$D_2A$ Dopamine Receptor</td>
<td>Hypertensive</td>
<td>Albrecht et al. (1996)</td>
</tr>
<tr>
<td>Endothelin-I</td>
<td>Hypertensive, lethal</td>
<td>Kurihara et al. (1994)</td>
</tr>
<tr>
<td>eNOS</td>
<td>Hypertensive</td>
<td>Huang et al. (1995), Shesely et al. (1996)</td>
</tr>
<tr>
<td>GC-A Receptor</td>
<td>Hypertensive</td>
<td>Lopez et al. (1995)</td>
</tr>
<tr>
<td>iNOS</td>
<td>Not determined</td>
<td>Laubach et al. (1995)</td>
</tr>
<tr>
<td>nNOS</td>
<td>None</td>
<td>Huang et al. (1993)</td>
</tr>
<tr>
<td>Ren-1$^d$</td>
<td>Hypotensive (females)</td>
<td>Clark et al. (1997)</td>
</tr>
<tr>
<td>Ren-2</td>
<td>None</td>
<td>Sharp et al. (1996)</td>
</tr>
</tbody>
</table>

AGT, angiotensinogen; ACE, angiotensin converting enzyme; AT1A, Ang-II type 1A receptor; AT1B, Ang-II type 1B receptor; ANP, atrial natriuretic peptide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; GC-A, guanylyl cyclase A receptor.
those of mice lacking AGT (Tsuchida et al. 1997). Complicating this however are more recent observations of increased mortality and incidence of renal morphological abnormalities in congenic AT1A-deficient mice successively backcross bred onto pure genetic backgrounds (Oliverio et al. 1997). These mice differ from the original AT1A-deficient strains by having a genome which is >95% C57BL/6 or >95% 129 while the original mice lacking severe lesions were an F2 generation of C57BL/6 × 129 and therefore contained a random mix of genetic information from these strains. Similarly, in the case of the AGT ablation experiments, differences in the incidence of postnatal lethality and renal abnormalities were reported by different groups simultaneously developing the models (Smithies and Kim 1994, Tanimoto et al. 1994). Although this could potentially be attributed to differences in the extent of the deletion breakpoints or in some aspect of animal husbandry, it is more likely due to genetic differences unlinked to AGT. Indeed, the genetic background of the ES cells used as the host for gene targeting differed in these experiments (129/Sv vs. C57BL/6 × CBA) potentially explaining the phenotypic differences (Smithies and Kim 1994, Tanimoto et al. 1994). These examples serve to illustrate the importance of the genetic background on phenotypic outcomes in gene targeted mice, and imply that a specific combination of genes or alleles of a gene unlinked to the locus of interest may strongly influence the phenotype observed in the knockout mice. Consequently, it must be recognized that as other candidate genes are evaluated in this way, phenotypes may only become manifested when placed onto a permissive genetic background or when subjected to some environmental or physiological stress.

Another aspect to emerge from the recent gene-targeting studies in mice has been the increased appreciation for the redundancy in the mechanisms responsible for the control of blood pressure and organ function. For example, ANP-deficient mice exhibit blood pressure changes that are sensitive to changes in dietary sodium, but without changes in renal function or sodium balance even when challenged with high dietary salt (8% NaCl) (John et al. 1995). This is most likely due to the effects of other hormonal factors which are able to compensate for the lack of ANP and maintain normal sodium and water homeostasis in these mice. One possible candidate is B-type natriuretic peptide (BNP) which shares the same receptor (GC-A) as ANP and binds with comparable affinity (Suga et al. 1992). Consistent with this is the observation of salt-independent hypertension in mice lacking the GC-A receptor (Lopez et al. 1995). Another example of redundancy is evident when one considers endothelial-dependent vasodilatory responses in endothelial nitric oxide synthase (eNOS)- and neuronal nitric oxide synthase (nNOS)-deficient mice. It was reported that acetylcholine still elicited NO-dependent dilatation of the pial arteriole isolated from mice deficient in either eNOS or nNOS (Meng et al. 1996). Further examination using inhibitors of NOS revealed that these responses were due to compensation by the remaining NOS isoform (eNOS in an nNOS-deficient mouse and vice versa). These studies illustrate how potentially diverse regulatory circuits can interact to maintain normal organ function, even in the absence of important modulators.

It is interesting to note that while blood pressure alterations in many transgenic experiments are dramatic (30–50 mm Hg increases), the changes in blood pressure in many knockouts are modest (10–30 mm Hg) in comparison. This should not come as a surprise given the presence of compensatory mechanisms (alluded to above), and the fact that hypertension is a polygenic trait, which may only manifest itself with a particular combination of genes and alleles. It will be of significant interest, therefore, to combine individual gene knockout models to assess how combinations of gene deficiencies affect blood pressure regulation. Moreover, such studies have the potential to identify important gene–gene interactions controlling blood pressure.

**Tissue-Specific Gene Targeting**

One of the major problems associated with creating knockouts is that the targeted deletion of the gene of interest could lead to a lethal phenotype, as in the case of AGT and endothelin-1 (Kuriharra et al. 1994, Smithies and Kim 1994). Clearly, a lethal phenotype cannot be predicted a priori with any accuracy. Furthermore, when a lethal phenotype occurs in the homozygous deletion in which 50% of gene function remains. Another equally important limitation of conventional knockout strategies is the global loss of gene function throughout the entire organism from the earliest point in development. This is particularly troubling for genes which exhibit a wide cell-specific expression profile and whose gene products are integral members of multiple paracrine systems. For example, AGT mRNA is expressed in liver, kidney, heart, brain, and adipose tissue (Yang et al. 1994). In addition to supplying hepatic-derived circulating AGT contributing to the systemic or endocrine RAS, extra-hepatic AGT may be important for local generation of Ang-II in a number of tissues including kidney. Abnormalities in the latter may be the primary cause of lethality in the homozygous knockout mice. Therefore, a system in which the ablation of gene function could be controlled in a developmental and tissue-specific fashion would provide an ideal tool for understanding the importance of tissue- and cell-specific gene expression and may provide an opportunity to study both chronic and acute effects of the loss of function caused by the knockout.

This approach is now feasible with use of the Cre/loxP system from bacteriophage P1. Cre-recombinase binds 34-base pair sequences, termed loxP sites, and mediates a homologous recombination event between the sites thus resulting in the deletion of any stretch of DNA lying between them (Figure 1). Genes can be appropriately modified to contain loxP sequences by gene targeting in embryonic stem (ES) cells. By placing the sites into introns, the gene will be functional in the absence of cre because the loxP sites will be spliced out along with the introns during normal mRNA processing. However, in the presence of cre, the gene will be rendered nonfunctional owing to deletion of intervening genetic material at the DNA level. Selectively expressing cre-recombinase under the control of a developmentally-regulated and/or cell-specific promoter provides temporal and/or spatial control over the function of the gene. The selectivity is limited by the availability of promoters which exhibit the desirable temporal and spa-
tial expression characteristics. For instance, if deletion of AGT from the kidney is responsible for the post-natal renal abnormalities in AGT-deficient mice, promoters which either target cre-recombinase to extra-renal sites of AGT expression (spatial control) or target widespread cre-recombinase expression after the critical time period for renal development (temporal control) may allow the animals to escape the lethal phenotype.

The first reported use of this system to tissue-specifically inactivate a gene in vivo was the DNA polymerase β gene (polβ), specifically in T cells. This was accomplished by driving cre-recombinase expression with the T-cell-specific lck promoter (Gu et al. 1994). Further studies by this same group demonstrated that deletion of the polβ gene could be temporally controlled by placing cre-recombinase under the control of the mouse interferon responsive Mx1 promoter (Kuhn et al. 1995). Following interferon induction, near total deletion of the polβ gene was observed in interferon responsive tissues such as the liver and spleen, while lower levels of deletion were detected in non-interferon responsive tissues such as the brain. More recent studies have employed the cre-loxP system to specifically target cells of the CA1 hippocampal region of the brain and thereby functionally delete the N-methyl-D-aspartate (NMDA) receptors (Tsien et al. 1996).

It should be pointed out that this system is not without its drawbacks. Technical limitations of the cre-loxP recombinase system center largely on problems of efficiency. That is, it may be difficult to target every cell in a tissue which expresses the gene of interest. Although many promoters can target transgenes to very specific and specialized cell types, their expression is often variegated, or limited to a subset of expressing cells within a tissue. The reason why variegated expression of transgenes is observed remains unclear, but may reflect the absence of important regulatory sequences in the cloned promoter which normally controls cell-specific activity. One potential solution will be to take advantage of knock-in strategies, placing cre-recombinase directly under the control of the desired promoter at its normal site in the genome.

An alternative to tissue-specific knockout is tissue-specific complementation, or the restoration of gene expression in a specific tissue of a knockout animal. This can be achieved by the use of stan-

Figure 1. The cre-loxP recombinase system. This schematic shows that a typical mammalian gene can be altered to contain loxP (arrowheads) sites within introns (thin lines) and surrounding a central exon (green, blue, and red boxes). In the absence of cre recombinase (−Cre) normal processing of the mRNA results in the production of a normal mRNA. In the presence of cre-recombinase (+Cre), an intra-molecular recombination takes place between the two loxP sites resolving (yellow line) into a gene containing only one loxP site and lacking the central exon. Transcription of that gene will result in a defective mRNA missing a portion of the protein coding region.
dard transgenic techniques to target the gene of interest to a specific tissue, followed by two generations of backcross breeding of the transgenics to knockout mice lacking that gene. We recently reported the complementation of a lethal phenotype in AGT-deficient mice by breeding the knockout mice with transgenic mice containing the human renin and human angiotensinogen genes (Davission et al. 1997b). Finally, the recent development of a number of ligand-inducible promoters can provide additional flexibility to temporally regulate the expression of a gene in a tissue-specific manner. Ligand-inducible promoters based on tetracycline (Furth et al. 1994) and ecdysone-receptor (No et al. 1996) have been previously used in transgenic mice to provide “on/off” switches for gene expression.

• Characterization of Gene Variants Using Transgenic Mice

With the advent of gene mapping techniques, it is now possible to genetically dissect the genes responsible for hypertension in genetically homogeneous strains of hypertensive rats and mice, and in diverse human populations. Lifton et al. have identified specific mutations causing rare Mendelian forms of hypertension such as glucocorticoid-remediable aldosteronism (GRA) (Lifton et al. 1992) and Liddle’s syndrome (Shimkets et al. 1994), and both transgenic and knockout models of the latter are currently being investigated in the hope of understanding how the disease-causing genes (subunits of the epithelial sodium channel) may participate in the regulation of blood pressure.

Progress in identifying genes causing essential hypertension has been hampered by the multifactorial and polygenic nature of the disorder; its mixed etiology, and the complicated nature of the populations accessible for study. Despite these difficulties, segregation analysis of molecular variants of several candidate genes has been performed in humans. Of these, the AGT gene remains the only gene reported to be either linked or associated with increased blood pressure, preeclampsia, or one of its intermediate phenotypes in humans (Jeunemaitre et al. 1992). Within the AGT gene, a variant at position 235 replacing a methionine (M235) with a threonine (T235) has been found to be associated with elevated plasma AGT and was more prevalent in hypertensive patients than in normal patients (Jeunemaitre et al. 1992, Ward et al. 1993). However, other population studies failed to find any association between these variants and hypertension (Caulfield et al. 1994, Fornage et al. 1995, Ukkola et al. 1995), although an association between a microsatellite polymorphism downstream of AGT and hypertension was found in some of these patients (Caulfield et al. 1994). The complexity of this story was fully recognized when the T235 variant was: (a) found to be the prevalent form in some populations; (b) the primary form of the gene present in non-human primates; and (c) present in strong linkage disequilibrium with a nucleotide substitution in the promoter (position -6) that effects transcriptional activity of the angiotensinogen promoter (Inoue et al. 1997).

Clearly, these data leave unresolved the significance, if any, of molecular variants of AGT in causing genetic hypertension in humans. Moreover, it is likely that similarly complicated scenarios will emerge when other genes causing essential hypertension or other equally complex polygenic diseases are mapped. This will be particularly true when the variants are subtle amino acid substitution mutations and not null alleles, and when the arguments for their importance are based solely on a statistical analysis of their prevalence in affected and unaffected individuals. Therefore, reliable experimental systems are necessary to determine the importance of a gene and its variants in causing a phenotype or participating in a quantitative trait. The methods described later, as exemplified by AGT, can be used to test the importance of a candidate gene allele (or variant) observed more frequently in a hypertensive than an unaffected population.

In the case of AGT, a model in which each of the variants is placed on an isogenic genetic background would be ideal. Although it is possible to create transgenic mice in inbred strains of mice, traditional transgenic techniques rely on the integration of DNA at random sites in the genome and in multiple copy numbers. This would be problematic in a system in which the effects of single gene variants are being tested, since transgene expression can be affected both by its position in a chromosome and its copy number. In fact, we previously demonstrated that expression of HAGT is proportional to its copy number in transgenic mice (Yang and Sigmund 1998). This feature of transgenesis makes comparison between different transgenic lines, and therefore different variants or haplotypes of a gene, impossible.

Gene targeting offers several solutions to this problem by providing a tool for targeting single copies of a gene to a specific and chosen site in the genome. In the simplest case, where the human gene variant of interest lies in a region of the gene which is highly conserved among species, the variant mutation can be made directly in the mouse homologue. Methods for introducing subtle point mutations into the genome have been described (Bronson and Smithies 1994). This has the advantage of causing only the desired alteration to the genome, without changing the position or copy number of the gene relative to its normal location. In more complicated cases where the variant of interest is in a region of the gene which is poorly conserved or in a gene with low sequence homology across species (as is the case for AGT which is less than 70% identical in mRNA and protein between mouse and human) the entire mouse gene can be physically replaced with the human gene. While this would allow a retention of the “normal” chromosomal location of the gene, it can be technically difficult to achieve. Generating a HAGT model would be further complicated by the need for species-homologous renin.

Another approach is to introduce a single copy of the transgene at a common, specifically defined, site in the genome. One method, first described by Bronson et al. (1996), introduces transgenes into the hypoxanthine phosphoribosyltransferase (hprt) locus. This method takes advantage of an hprt-deficient ES cell line and a targeting vector, which, after homologous recombination, restores hprt (allowing selection of recombinants on Hypoxanthine Aminopterin Thymidine [HAT] media) and delivers the transgene. Since transgenes are all introduced into the same site in a single copy, direct comparisons can be made between independent transgenic lines carrying different haplotypes of a construct. In the example of HAGT, mice carrying different HAGT haplotypes could be crossed with mice containing HREN, and differences in cardiovascular and endocrine parameters can be
compared among the haplotype-specific mice. Moreover, the model could be extended further by crossing these double transgenic mice with knockout mice which lack endogenous AGT to generate a model in which the effects of the endogenous RAS on blood pressure could not interfere with the effects of the gene variants being examined. We have previously demonstrated that the presence of both the HREN and HAGT genes can rescue the lethal phenotype in angiotensinogen knockout mice (Davisson et al. 1997b).

- Genetic Mapping of QTLs and Conclusion

Finally, as additional QTLs for hypertension are identified, bioassay systems which can either functionally separate multiple QTLs located on the same chromosome or determine the function of a single unknown QTL will become increasingly necessary. Recent developments in transgenic methodology have made it possible to insert large fragments of DNA in the form of bacterial or yeast artificial chromosomes into fertilized eggs [reviewed by Peterson (1997)]. With genetic maps becoming increasingly refined, it will soon be possible to isolate single QTLs in large genomic clones, generate transgens, and then determine the effects of the QTLs on blood pressure. Functional analysis of QTLs using this strategy has the advantage of being significantly quicker than generating congenic strains, however, species-specific differences and effects of genetic background must also be taken into account when interpreting the results of such studies.

With the advances in the molecular genetics of hypertension that have taken place within the last decade, we are rapidly becoming aware of the need for model systems to test identified gene variants and QTLs. New advances in transgenic and gene-targeting technology offer us an opportunity to test the functional significance of variants and newly discovered genes in the context of an experimentally and genetically manipulable animal system. These advances, when coupled with the increasing ability to perform sophisticated cardiovascular physiological studies in the mouse, will provide the tools necessary for moving into the next era of hypertension research:

- Gene\n
sorting out the complex genetic determinants of pathophysiologic regulation of blood pressure. This goal will be most efficiently accomplished by the partnership between molecular biology, molecular genetics, and integrative physiology.


Cx43 Gap Junctions in Cardiac Development

Cecilia W. Lo* and Andy Wessels

Studies utilizing knockout and transgenic mouse models revealed an important role for connexin 43 (Cx43) gap junctions in cardiac development. This may involve a quantitative requirement for gap junctions in modulating the development of cardiac crest cells. In addition, studies in humans and Xenopus indicate that Cx43 gap junctions also may play a role in regulating heart laterality. Together, these findings indicate that the perturbation of Cx43 function could play a significant role in specific congenital heart malformations. (Trends Cardiovasc Med 1988;8:264–269) © 1998, Elsevier Science Inc.

Gap junctions are cell junctions containing hydrophilic membrane channels that mediate the diffusion of ions and low molecular weight metabolites between cells (Bruzzone et al. 1996). They are comprised of a hexameric array of polypeptides encoded by the connexin multi-gene family (Beyer et al. 1990, Kumar and Gilula 1992). Connexin 43 (Cx43) is the predominant connexin isoform expressed in the mammalian heart. In the adult heart, Cx43 containing gap junctions are primarily found in the intercalated disks of the working myocardium and in the myocytes of the fast components of the atrioventricular conduction system (Gourdie et al. 1990 and 1991, Oosthoek et al. 1990, Van Kempen et al. 1991 and 1996). They play a vital role in adult cardiac function by facilitating the synchronization of myocardial contractions [see for example Veenstra and DeHaan (1988)]. Recent studies suggest that perturbation in the expression and distribution of Cx43 gap junctions in the adult myocardium may play a role in reentry and lethal cardiac arrhythmia (Saffitz et al. 1992, Severs et al. 1996, Spach 1997).

* Address correspondence to: Dr. C.W. Lo, University of Pennsylvania, Goodard Laboratory, Department of Biology, Philadelphia, PA 19104-6017. E-mail: <clo@mail.sas.upenn.edu>.

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