SUMMARY

Traditional therapeutic approaches for the treatment and control of hypertension are effective in normalizing blood pressure (BP) in less than a third of patients with hypertension. These pharmacological approaches may have reached a plateau in their effectiveness and newer strategies need to be investigated to not only increase the number of patients achieving BP control, but to find ways to cure the disease instead of just manage it. Since completion of the Human Genome Project and the continuous advancement of gene delivery systems, it is now possible to investigate genetic means for the treatment and possible cure for hypertension. In this review, we discuss potential genetic targeting for treatment of hypertension. There are two generalized gene transfer approaches that have been used successfully for hypertension. One is an induction approach where genes that lower blood pressure are overexpressed. A second method is a reduction approach where products of genes that are known to increase blood pressure are decreased. There are a variety of methods that have been utilized to meet these objectives, such as “knockout” and “knock-in” animal models, and the
use of sense and antisense (AS) technology. This review will focus on the sense and antisense applications, and how this technique is becoming more refined and precise through the targeting of specific tissues, the regulation and induction of components of the system, and use of other newer technologies, such as short interfering RNA (siRNA). Our lab has generally focused on the reduction approach, specifically in the genetic manipulation of components of the renin–angiotensin system (RAS). This system not only modulates BP, but has also been implicated in cardiac hypertrophy and morphology and in insulin resistance, which is highly correlated with hypertension. We will also discuss how new genes can be identified and subsequently serve as targets for the treatment of human hypertension.

**Key Words:** Gene therapy; viral vectors; gene delivery; animal models; hypertension; renin–angiotensin system; gene arrays.

### INTRODUCTION

Hypertension is a chronic, debilitating disease that affects more than 50 million Americans (1,2). Although hypertension is defined as a condition of high blood pressure (BP) (3) (systolic BP ≥140 mmHg and diastolic BP ≥90 mmHg), it is a much more complex disorder, with a majority of hypertensive patients being insulin-resistant and/or salt sensitive (3–6). High BP is estimated to account for 6% of all deaths worldwide (7) and is the most common treatable risk factor for cardiovascular disease (CVD). A sustained elevation in BP contributes to serious health complications, such as myocardial ischemia and infarction, renal failure, stroke, and retinal damage (8–10). Vascular and cardiac remodeling occurs in hypertension, which has both hemodynamic and nonhemodynamic effects (11,12). This disorder has such a negative impact on health care that the most recent Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure report has suggested a new category of prehypertension in adults that have a systolic BP of 120–139 mmHg and a diastolic BP of 80–90 mmHg (13). The report states that individuals with this range of BP should be considered pre-hypertensive, and suggests health-promoting lifestyle changes to prevent CVDs.

Hypertension is an asymptomatic, chronic disease that is characterized as either primary or secondary. The latter is indicative of having a separate primary cause, whereas the primary (or essential) form of hypertension, which constitutes more than 90% of the cases, is of unknown etiology. This form of the disease is chronic, multifactorial, and multigenetic in origin, and is difficult to manage and currently not preventable or curable. Thus, the major goal of current therapies has been to control BP and prevent the complications (end-organ damage) associated with the disease, regardless of its origins. Despite the large arsenal of antihypertensive agents currently available, successful control of BP (≤140/90 mmHg) is only observed in a small percentage of patients. Although only 29% of hypertensive patients in the United States have their BP controlled to the nationally recommended level of less than or equal to 140/90 mmHg with pharmacological intervention (13), the control rates are much worse in other countries around the world (14–16). Therefore proper treatment and management of hypertension is of critical importance to society and should be a matter of extreme urgency both in the United States and abroad.

### ADVANTAGE OF GENE THERAPY OVER CONVENTIONAL PHARMACOTHERAPY

Despite a large arsenal of therapeutic agents, conventional pharmacological therapy has not cured hypertension and, in fact, does not control the disease in the majority of
patients. Possible reasons for poor control of BP include incorrect targets of therapy, and poor education, economics, availability of health care, follow-up care, drug availability, and compliance. Even if the “ideal drug” was available for each patient, it is more likely that compliance would be the major factor in determining patient outcome. Reasons for a lack of compliance in hypertensive patients include convenience and side effects of the drugs utilized. Convenience can refer to the drug dosing regiment, which does not always conform to a patient’s everyday activities. Most pharmacological agents are short-acting (≤24 h) and require multiple dosing per day. Another reason a patient may not stay on his/her medication is that symptoms related to the side effects of the drugs may be manifested in this otherwise asymptomatic disease. Patients can “feel better” when they are not on their medication, and they therefore either reduce their own dosage, or stop taking the medication altogether. Thus, we are at a stage in the treatment of hypertension where more focus must be placed on other treatment paradigms rather than further drug developments. Gene therapy offers the possibility of producing long-term effects with specificity based on the particular genetic target. Side effects may no longer be an issue, compliance would be nearly eliminated, as therapy can be directed at the source of the hypertension, and this therapy could be administered a minimal number of times over the patient’s lifetime.

PHILOSOPHIES OF GENE THERAPY

Gene therapy promises to be the next frontier for treatment of and possibly a cure for complex diseases like hypertension. A variety of studies over the past few years have looked at different gene targets and gene delivery vectors as strategies. Currently, therapeutic agents are chosen by targeting the genes that are important in BP regulation. Whereas this technique should yield promising antihypertensive results, ideal gene targets would be those that have not only been implicated in hypertension, but also have been implicated in some of the target organ damage that is associated with hypertensive disease, such as the heart, kidney, vessels, etc. Over the last several years, our lab has mainly focused on the genetically manipulated renin–angiotensin system (RAS). This system has long been associated with hypertension. The RAS is essential in both the normal regulation of BP, as well as in the pathophysiology of hypertension. This system is also involved in, but not limited to, the alterations of nitric oxide metabolism (17), the impact of oxidative stress on endothelial function (18), vascular smooth muscle and cardiac hypertrophy (19,20), and insulin resistance (21,22). Additionally, there are numerous genetic link studies with components of the RAS and hypertension (23–30), as well as the successful use of both angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blocking agents in the treatment of hypertension and some of its related complications.

There are two contrasting philosophies for gene therapy in hypertension—the reduction approach and the “induction” approach. In the “reduction” approach, genes that have been implicated in the elevation of BP are targeted, with the hope of reducing translation (in the case of a signaling molecule) or attenuating their effects by reducing their receptor’s population. With the induction approach, genes that have a BP-lowering effect are introduced or upregulated with selective gene and/or promoter delivery. That is, recombinant DNA is introduced in vivo to express mRNA and increase the concentration of the targeted protein. For each of these approaches there are a variety of methodologies utilized to produce the desired effects.
There are two major contrasting paradigms that can be used to produce these genetic alterations. One is the use of transgenic animals that knock out a specific gene or overexpress a particular gene. The other approach is to alter the genetic makeup of the animal after development with induction (sense) or reduction (antisense [AS]) approaches. A potential drawback to the knockout models is that the animal may undergo some altered physiology to compensate for the removal of a particular gene. This would be especially important if the gene that is removed has a major role in the developmental process. Although knockout models have made significant contributions, any compensatory changes that the animal undergoes may reduce the power of this type of strategy. Both strategies have been successful in reducing BP or in preventing the rise in BP in experimental animals; however, we are of the opinion that the genetic alterations should occur after the developmental process to avoid any compensatory physiological adaptations that may influence the interpretation of the significance of the particular gene of interest. Therefore, this review will focus more on the postdevelopment genetic manipulations in experimental hypertension.

“Induction” Approach

Several investigators have been successful in lowering BP using the induction paradigm. Overexpressing agents that are vasodilators, such as atrial natriuretic peptide (31–34), adrenomedullin (35–38) endothelial nitric oxide synthetase (eNOS) (39–42), human tissue kallikrein (43–55), superoxide dismutase (56–58), and the AT2 receptor (59), have been successful in reducing BP. These studies have examined different models of hypertension, and the gene transfer approach has been systemic or localized in specific tissue(s), depending on the gene target. Systemic delivery of the these transgenes in plasmid DNA or adenovirus by intravenous, intraperitoneal, or intraportal vein injections results in a reduction of BP in a variety of animal models (see Table 1). Innovative delivery systems have been examined by a few research groups. Chen et al. (60) transfected Chinese hamster ovary (CHO) cells with atrial natriuretic peptide (ANP) cDNA, and then the CHO cells were encapsulated in nonantigenic biocompatible polycaprolactone capsules, prior to their implantation into the peritoneal cavity of rats. Li et al. (61) used encapsulated genetically engineered fibroblasts expressing ANP. In both cases, BP was significantly reduced in hypertensive Dahl and spontaneously hypertensive rats (SHR), respectively. In most examples of the induction gene transfer approach, transgenes were administered to adult animals and BP was reduced within a week and remained below controls for 4–6 wk. The extent of the reduction in BP was dependent on the dose of DNA injected, but averaged between 20 and 40 mmHg. Some of the differences observed regarding the extent and duration of BP effects may be related to the choice of promoter directing the expression of the transgene, the route of injection, and the vehicle in which the transgene is delivered. The “induction” gene transfer approach is not restricted to systemic administration. Gene transfer of eNOS, via an adenovirus vector, into the nucleus tractus solitarii of SHR also decreased BP (39,42). In all the studies undertaken, control viral injections were without effect, further demonstrating it was the transgene, and not the virus, that mediated the observed decreases in BP.

BP is not the only parameter assessed in these studies. Investigators have looked at cardiac and renal complications, which are summarized in Table 1. In addition, other parameters also have been evaluated. For instance, overexpression of human tissue kallikrein in a fructose-induced model of hypertension (43) not only lowered BP, but the
insulin-resistant state and the number of angiotensin type I receptors \((AT_1R)\) were also reduced. This gene transfer was without effect on control animals, which was the case in all the other gene transfer experiments described in Table 1. Other investigators \((41,57)\) have also demonstrated that the impaired endothelial function observed in the SHR was significantly improved after gene transfer of eNOS and superoxide dismutase. The specificity of the responses to the transgene has been verified using agents that block or bind to the receptor that the upregulated protein would bind to \((50,53)\).

Reduction Approach

Similar success in reducing BP and cardiovascular pathologies in experimental animals has been realized with the reduction gene transfer approach, which underexpresses agents that vasoconstrict vessels. The most successful agents used in this approach have targeted various components of the RAS. Table 2 summarizes the studies that have been utilized to reduce expression of various genes. These studies produce similar findings as described for the induction approach, as far as its range of effects on reducing basal BP or preventing the rise in BP in several different models of experimental hypertension. In many of these studies, administration of the AS transgene occurs during the neonatal stage and results in prevention of high BP in a variety of hypertensive models. Administration of AS to the \(AT_1R\) in 5-d-old animals completely prevented the development of hypertension in the SHR \((62–65)\). Reverse-transcription polymerase chain reaction (RT-PCR) and receptor-binding studies confirmed a 40–60% knockdown of the endogenous \(AT_1R\), thereby decreasing the gain of the RAS, but still leaving the system intact at a basal level \((64,65)\). Responsiveness to angiotensin II (Ang II) is reduced in \(AT_1R\) antisense-treated rats; however, BP is only reduced in hypertensive and not in normotensive animals. This would suggest that it is an overactivity of the RAS (as opposed to altered function) that is important in hypertension. As was found with the induction gene transfer studies, not only is BP reduced but so are many of the cardiac, renal, and vascular pathophysiology normally associated with the hypertensive state. For instance, cardiac hypertrophy, fibrosis, perivascular necrosis in cardiac tissues, and endothelial dysfunction were prevented in SHRs treated with \(AT_1R\)-AS delivered via a retroviral vector \((62–65)\). Similar results were observed when AS to ACE was used as the transgene in a retroviral vector \((66)\). Like the induction approach, there is also reversal of insulin-resistance in the fructose-model of hypertension with AS to the \(AT_1R\) \((67)\). Recent reports have suggested that the RAS is important in insulin resistance \((22,68,69)\). This implicates an association of the RAS with insulin resistance and Syndrome X. Recent clinical and experimental evidence have demonstrated that antagonism of the RAS reduces the incidence and severity of diabetes and insulin resistance \((70–72)\), providing further rationale for the use of components of the RAS as potential transgenes in gene therapy approaches to other chronic metabolic disorders.

Generally speaking, this reduction approach appears to produce effects on BP that are more long-lasting than the induction approach; however, this may be related more to the delivery system and its resulting integration of the AS into the genome.

When retroviral vector \(AT_1R\)-AS was administered to adult SHR, we observed a transient but significant decrease in BP \((73)\). This transient effect may be a result of the limited infection of nondividing cells by the retrovirus. Other investigators \((74–78)\), using a viral delivery system that can infect nondividing cells, demonstrated that underexpressing genes of the RAS is effective in reducing BP in adult hypertensive rats.
| Transgene        | Animal model                     | Delivery    | Route | Effect on blood pressure | Comments                                                                 | References |
|------------------|----------------------------------|-------------|-------|--------------------------|--------------------------------------------------------------------------|------------|
| Kallikrein       | Fructose-induced SD SHR          | Plasmid DNA | iv    | Decreased               | Decreased AT$_1$R, decreased endothelin, normalized insulin               | 43         |
|                  |                                  | Adenovirus  | im    | No effect                | Increased capillary density in ischemic muscle                           | 44         |
|                  | 5/6 reduction in renal mass      | Adenovirus  | iv    | Decreased               | Left-ventricular hypertrophy attenuated, protects against renal injury and cardiac remodeling | 45         |
|                  | Adult and newborn SHR Dahl salt-sensitive | Adenovirus  | im    | Decreased               |                                                                           | 46         |
|                  | 5-wk-old Goldblatt—2 Dahl salt-sensitive | Adenovirus  | iv    | Decreased               | Reversed cardiac hypertrophy and fibrosis and renal damage               | 47         |
|                  | Kidney/1 Clip                    | Adenovirus  | iv    | Delay of onset          | Reduced left ventricular mass, protection from renal dysfunction         | 48         |
|                  | SHR                               | Adenovirus  | iv    | Decreased               | Decreased left ventricular hypertrophy, attenuated renal injury          | 49         |
|                  | Adult and newborn SHR SHR        | Adenovirus  | iv    | Delay of onset          | Gender differences                                                       | 50         |
|                  |                                   | Oligonucleotide | sc  | Delay of onset          |                                                                           | 51         |
|                  | SHR                               | Adenovirus  | iv    | Decreased               | Decreased BP in adult but not in young SHR                               | 52         |
|                  |                                  | Oligonucleotide | iv  | Decreased               |                                                                           | 53         |
|                  | SHR                               | Plasmid DNA | im   | Decreased               | BP reduction attenuated with bradykinin antagonist                      | 5          |
|                  |                                  | Plasmid DNA  | iv   | Decreased               | Hypotensive effect reversed by kallikrein inhibitor                      | 55         |
| Adrenomedullin   | 5-wk-old Goldblatt—2 Kidney/1 Clip | Adenovirus  | iv    | Delay of onset          | Decreased left ventricular mass, decreased myocyte diameter, decreased myocardial fibrosis, decreased renal | 35         |

(continued)
| Nitric oxide synthase | SHR | Plasmid DNA | iv | Decreased | 2nd injection further reduced BP |
|-----------------------|-----|-------------|----|-----------|---------------------------------|
| SHR                   | Adenovirus | icv (NTS) | Decreased | Depressor response in WKY, but greater in SHR |
| Stroke-prone SHR      | Adenovirus | iv | No effect | Improved endothelial function |
| SHR                   | Plasmid DNA | iv | Decreased | 2nd injection extended therapeutic time limit |
| SHR                   | Adenovirus | icv (NTS) | Decreased | Decrease in HR and BP reversed by microinjection of soluble guanylate cyclase inhibitor |
| Atrial natriuretic peptide | SHR | Plasmid DNA | iv | Decreased | Increased urine volume and sodium excretion |
| Dahl salt-sensitive    | Adenovirus | iv | Decreased | Decrease in cerebral infarction, reduced thickness of arterial wall |
| Dahl salt-sensitive    | Adenovirus | iv | Decreased | Reduction in cardiac myocyte size, attenuation of glomerular sclerotic lesions |
| 4-wk-old SHR           | Plasmid DNA | iv | Decreased | No effect on 12-wk-old SHR |
| Superoxide dismutase   | SHR | Adenovirus | iv | Decreased | Effect greater in anesthetized compared with awake rats; improved endothelial function |
| Stroke-prone SHR       | Adenovirus | iv | No effect | Improved endothelial function |
| Heme oxygenase         | 5-d-old SHR | Retrovirus | ic | Delay of onset | |

SHR, spontaneously hypertensive rats; iv, intravenous; im, intramuscular; ic, intracardiac; sc, subcutaneous; icv, intracerebroventricular; WKY, Wistar-Kyoto; BP, blood pressure; SD, Sprague-Dawley rats.
| Transgene                      | Animal model | Delivery | Route | Effect on blood pressure | Comments                                                                 | References |
|-------------------------------|--------------|----------|-------|--------------------------|--------------------------------------------------------------------------|------------|
| Fibroblast growth factor      | SHR          | Liposome | iv    | Acute decrease           | Augments number of endothelial cells, ameliorated endothelial dependent response to vasoconstrictors | 98         |
| Angiotensinogen               | SHR          | Oligonucleotide | iv    | Decrease                | Plasma angiotensinogen and angiotensin II levels reduced                | 81         |
|                               | SHR          | Oligonucleotide | iv    | Decrease                | Angiotensinogen and angiotensin II levels reduced                      | 82         |
|                               | SHR          | Oligonucleotide | icv   | Decrease                | Decreased AT\textsubscript{1}R in PVN, decreased angiotensin II in brainstem | 83         |
|                               | SHR          | Oligonucleotide | icv   | Decrease, delay of onset| Decreased angiotensin II in brainstem                                  | 84         |
|                               | SHR          | Axial- glycoprotein | iv    | Decrease                | Decreased left ventricular hypertrophy, decreased angiotensinogen      | 96         |
|                               | SHR          | AAV\*     | iv    | Decrease                | Greater effect of AAV-plasmid vector with liposome                     | 100        |
| Cold-induced SD               | SHR          | Retrovirus | ic    | Decrease                | Decreased spontaneous drinking response                                 | 101        |
| Goldblatt—2 Kidney/1 Clip     | SHR          | Oligonucleotide | icv   | Decrease                | Decreased the elevated hypothalamic angiotensin II levels              | 105        |
| Angiotensin converting enzyme | SHR          | Retrovirus | ic    | Decrease                | No effect on WKY                                                       | 66         |
| AT\textsubscript{1} receptors  | SHR          | Retrovirus* | ic    | Decrease                | Decreased cardiac hypertrophy and fibrosis, similar effects seen in progeny | 62         |
|                               | SHR          | Retrovirus* | ic    | Decrease                | Prevented left ventricular hypertrophy and myocardial perivascular fibrosis | 63         |
|                               | SHR          | Retrovirus* | ic    | Decrease                | Decreased angiotensin II mediated responses in WKY and SHR but only decreased basal pressure in SHR | 64         |
|                               | SHR          | Retrovirus* | ic    | Decrease                | Angiotensin II-induced BP and dipsogenic responses attenuated           | 65         |
| Fructose-Induced SD | Retrovirus* | ic | Decrease | Prevented glucose intolerance |
|---------------------|-------------|----|----------|-----------------------------|
| SHR                 | Retrovirus  | ic | Transient| Repeated daily injections for 6 d |
| SHR                 | AAV*        | icv (Lat. Vent) | Decrease | Intracardiac injection in 3-wk-old SHR |
| SHR                 | Oligonucleotide | icv | Decrease | also reduced BP |
| L-NAME SD           | Retrovirus* | ic | Decrease | Decreased left-ventricular hypertrophy, endothelial dysfunction unchanged |
| Renin transgenic Angiotensin-induced SD | Retrovirus* ic | Decrease | Prevented cardiac hypertrophy |
| Cold-induced SD     | Oligonucleotide ic and/or icv | Decrease | Protected against angiotensin II-induced increases in BP and cardiac hypertrophy |
| Goldblatt - 2 Kidney / 1 Clip | Oligonucleotide i.v. intracysterinally | Decrease | Spontaneous drinking response to cold reduced |
| SHR                 | Oligonucleotide | Decrease | Decreased cerebral infarct after middle cerebral artery occlusion |
| AT_2 receptors      | Uni-neprectomized SD | Oligonucleotide | interstitial pump | Increased pressor response to angiotensin II |
| Epidermal growth factor receptor | SHR | Liposome | iv | Decrease | Increased pressor response to angiotensin II |
| Angiotensin-induced SD | Oligonucleotide | iv | Decrease | Normalized left-ventricular hypertrophy |
| SHR                 | Liposome    | iv | Decrease | No effect on heart rate |
| SHR                 | Liposome    | iv | Decrease | Decreased beta 1, but not beta 2, receptors, decreased plasma renin activity and angiotensin II |
| CPY-like kinase     | DOCA salt SD | Oligonucleotide | iv | Decrease | Increased urine volume and sodium excretion |
| Tyrosine hydroxylase | SHR | Oligonucleotide | iv | Decrease | Decreased epinephrine and norepinephrine, decreased TH activity in adrenal medulla |
| CYP4A1              | SHR | Liposome | iv | Decrease | Decreased sensitivity to constrictor action of phenylephrine |
| Thyrotropin         | SHR | Oligonucleotide | it | Decrease | |

*Injection performed at 5 d of age. SHR, spontaneously hypertensive rats; AAV, adeno-associated virus; WKY, Wistar-Kyoto; it, intrathecal; iv, intravenous; icv, intracerebroventricular; ic, intracardiac.
Further, there have been some recent studies (79,80) that have used gene therapy approaches to lower expression of the AT$_2$ receptor, which results in the increase in basal BP. Collectively, these studies indicate that both induction and reduction of components of the RAS are promising for the treatment of hypertension.

### DELIVERY METHODS FOR GENE THERAPY

In hypertension, whether using an AS approach to express reduction or AS mRNA to inhibit an overexpressed protein, or using an induction or sense approach to increase the synthesis of proteins critical to the pathogenesis of the disease, one generally has to transduce somatic cells of the body. Therefore, methodologies for efficient gene transfer are paramount for successful gene therapy. Other essential components of successful gene therapy are the ability to appropriately express the gene, to have long-term survival of the transduced cell(s), and to be able to control expression of the particular gene. There have been several vehicles explored for somatic gene delivery for hypertension, such as naked DNA, liposomes, receptor-mediated delivery, and viral delivery. This last method can be further divided by the type of virus utilized: retroviral, adenoviral, adeno-associated, or lentivirus. Each system has certain advantages and disadvantages, which influence their selection. The duration of the effect of the target gene is dependent on the delivery system utilized. The following is a brief summary of some of the more common gene delivery vehicles.

#### Nonviral Vectors

Nonviral vectors possess some advantages over viral vectors for gene therapy, in that these vectors are usually devoid of the safety issues related to insertional mutagenesis and immunogenecity. Early attempts to modify genes to lower BP used oligodeoxynucleotides (ODN). In particular, AS-ODN was directed to the AT$_1$R and angiotensinogen mRNA (81–84). ODN are single-stranded, short sequences of nucleotides that are made of DNA (or modified DNA) designed to interact specifically with its target mRNA by Watson–Crick basepairing. Once bound to its target mRNA, the AS-ODN inhibits protein synthesis by directly blocking translation (85,86). ODNs may further work to block expression by stimulating RNase H, which sterically inhibits the mRNA from translating its message. This reduces the total number of mRNA copies and frees the AS-ODNs to hybridize again. The ODN approach has some specific disadvantages (77,80–87). Its effects are long-lasting in comparison to available pharmaceutical agents, but usually the effects are still limited a week after administration. No adverse or toxic effects have been observed, they can be produced in larger quantities relatively inexpensively, and they do not cross the blood–brain barrier when given peripherally (80). There have been numerous studies conducted, mostly in the SHR, with BP generally reduced anywhere from 16 to 40 mmHg for 3–7 d (see Tables 1 and 2). Naked AS-ODN is effective, but cationic liposomal carriers (77,88–90) can increase the effectiveness.

Naked DNA can be delivered directly to the cells, in a plasmid (circular, naked DNA) or with liposomes (lipid bilayers that carry the DNA into cells). These methods are relatively safe and easy. Plasmids are effective vectors, but administer relatively short transgene duration because they do not allow for integration into the genome. Human kallikrein and NOS have been manipulated using these techniques to transiently lower BP in hypertensive rats (see Table 1). However, these methods are not very efficient, may be cytotoxic, and only produce a transient effect (109).
Modifications to nonviral vectors can increase the efficiency of gene transfer to cardiovascular tissues. Liposomes developed with cationic lipids can allow for a higher transfection efficiency of plasmid DNA. Another method to deliver DNA is a receptor-mediated method using hemagglutinating virus of Japan liposome. In this method, DNA is combined with a ligand to be internalized by a cell in a specific receptor-mediated mechanism (110). Although this method is an improvement over the basic liposomal method and is highly specific, it is more difficult to produce the modified liposomes.

Recent advances in nonviral delivery technology have infused renewed vigor into the field. These newer advances include ultrasound-mediated enhancement of gene delivery and peptide-targeted liposomal formulations to target enhancement. With regard to cardiovascular physiology, ultrasound has recently been shown to enhance transduction into vascular tissue by increasing permeability of the cell membrane. Lawrie et al. (111) has reported a 300-fold increase in gene transfer in smooth muscle cells compared with naked DNA alone using an ultrasound approach. Use of a lipid integrin DNA (LID) nonviral vector system, which is a modification of the liposomal-mediated gene delivery, consists of a standard liposomal/DNA component that is complexed with an integrin-targeting peptide. This LID vector complex can be used to attach to cell receptors on vascular tissues. This targeting has been used effectively in vitro for both vascular smooth muscles and endothelial cells (112). This technology can be a versatile one in which substitution of other peptides that can mediate selective uptake into specific cell types can be developed to enhance targeted gene delivery. If more prolonged effects are desired, the delivery of the transgene requires a viral vector. There are numerous viral vectors used for gene therapy, each having its specific advantages and disadvantages (Table 3).

**Viral Vectors for Gene Therapy Approach**

For hypertension, where a long-term (or permanent) control of pathophysiology is the goal, integration of the transgene into the host cell’s genome is necessary. Whereas naked DNA and liposomes are incapable of this task, viruses require such actions for their life cycle. Various viral vectors have been modified and used to deliver and incorporate transgenes of interest into cells. There are four major classes of viral vectors currently being used for both in vivo and in vitro applications in hypertension (Table 3). However, the “ideal viral vector” has yet to be found. An ideal vector should be safe (have a low toxicity when delivered), should not elicit an immune response, should integrate in a predictable, safe region of the genome, must be efficiently taken up by the target tissue, and must infect the target tissue with a high enough efficiency to have a physiological effect. For practical purposes, the vector also has to be packaged with high efficiency into the viral envelope, be easily produced in high concentrations, and have enough space to accommodate the required transgene and its promoter. Each of the known viral vectors has some of these characteristics, but not all of them.

**Adenovirus**

Adenovirus is a double-stranded DNA virus that can infect both dividing and nondividing cells with high efficiency as it targets mammalian cells with specific membrane receptors. Viruses then enter the cells by receptor-mediated endocytosis and translocate into the nucleus. Most of the adenoviruses are episomal, and thus do not integrate into the host DNA. Because they are not integrated, the episomal DNA eventually becomes inactive or degraded, and the transgene will no longer be expressed. As the adenoviral genome
expresses numerous viral proteins, the adenovirus stimulates the immune system and can cause vascular inflammation (113, 114). Utilization of this vector system has been shown to delay the development of hypertension in genetic and nongenetic models of hypertension for approx 1 mo (see Tables 1 and 2). Because of the transient expression patterns and induction of the immune response, the conventional forms of the vector have serious limitations and will likely not be used for human gene therapy for chronic diseases. With advances in virology, however, use of this vector may prove beneficial in the future.

Recent advances in engineering retroviral vectors have increased transduction of cardiovascular cells. Two methods shown to improve gene delivery into vascular beds are serotype switching and the use of antibodies. The adenovirus family is very diverse and there have been over 50 different serotypes identified. Pseudotyping technology can exploit certain serotypes to target gene delivery to specific target tissues. For CVDs, utilization of this approach has been successful in improving gene delivery. For example, serotypes 4 and 11 possess higher affinity binding and infectivity for vascular endothelial cells (115). Harari et al. (116) reported that they were able to target activated endothelium in vitro by using an antibody-mediated adenovirus directed against e-selectin. This concept may allow for strategies to target specific vascular beds in vivo.

### Adeno-Associated Virus

Adeno-associated virus (AAV) is a stable, nonpathogenic vector that can infect non-dividing cells. The AAV is a parvovirus, and its replication is dependent on the presence of a helper virus. The wild-type AAV integrates in a specific region of chromosome 19. Although its site of integration is known, the recombinant AAV (rAAV) loses this property and randomly integrates. There are some limitations with this vector system because it is difficult to produce large quantities of virus and it has a limited transgene carrying capacity (117–119). Although the carrying capacity is limited, this virus is amply suited

| **Vehicle** | **Pros** | **Cons** |
|-------------|----------|----------|
| Oligonucleotide | Easy to produce, safe to handle | Lack of target specificity, Transient expression patterns |
| Liposome | Easy to produce, safe to handle | Lack of target specificity, Transient expression patterns |
| Viruses | | |
| Adenovirus | Infects dividing and nondividing cells | Immunogenic, transient expression, random integration |
| AAV | Nonpathogenic, specific integration site (wild-type), infects nondividing cells | Very small payload |
| Retrovirus | Stable long-term expression, large payload | Random integration, only infects dividing cells |
| Lentivirus | Stable long-term expression, large payload, infects dividing and nondividing cells | Random integration |

AAV, adeno-associated virus.
for the delivery of small genes and AS cDNA, which only needs to code for a small fragment of the target gene to be effective. This virus is also fairly safe for human use. AAV is not pathogenic and is not associated with any known disease state.

To produce rAAV a helper virus (adenovirus) is required. In producing the rAAV, viral coding sequences are removed so that the source of immune reactions to viral gene expression is removed and no inflammation response is evoked in vivo. AAV infects all mammalian cells, and its expression in target tissue is long-lasting (118). With no helper virus present, AAV infection remains latent indefinitely. Upon infection of the cell with helper virus, the AAV genome is excised, replicated, packaged, and finally released by the cell.

The AAV is a good candidate for gene therapy because it is safe and has a very broad host range. AAV has been successfully used in phase 1 clinical trials for the treatment of cystic fibrosis (120). It has also been used to deliver AS to the AT,R in hypertensive rat models. Use of rAAV containing AT,R-AS has been administered systemically as well as centrally in the SHR (74), and resulted in a decrease in BP and a reduction in left-ventricular hypertrophy.

**Retroviral Vectors**

Retroviral vectors, which have a large transgene payload capacity, can be produced with high efficiency and titer. The viral genome is a single-stranded RNA that is converted to DNA in the host cell by reverse transcription mediated by viral reverse transcriptase. The DNA is then integrated into the genome and can result in long-term expression of the transgene. This virus type is highly efficient at delivering genes to dividing cells; however, this vector is not efficient at infecting nondividing cells. The other major disadvantage of retroviruses lies in their random genome integration pattern (121), which raises concerns about their safety for practical use in vivo. Despite this disadvantage, we and others have been very successful in using the retrovirus to prevent hypertension, cardiac hypertrophy, and restenosis in several experimental models (62–67,91,93,99,122).

**Lentivirus**

The lentiviral vectors are a subfamily of retrovirus and are the most recent addition of vectors to the rapidly developing field. The lentiviral vectors are derived from the human immunodeficiency virus type 1 (HIV-1). These highly “humanized” vectors combine the advantages of retroviral and adenoviral vectors, and they are emerging as the vectors of choice for long-term, stable in vitro and in vivo gene transfer. These vectors are attractive because they can efficiently carry large transgene cassettes (up to 18 kb in size) and they are capable of transducing both dividing and quiescent cells. Lentiviruses can allow for long-term expression of the transgene with little immune response. Lentiviruses can infect noncycling and postmitotic cells and have the potential to generate transgenic mice by infecting stem cells (123). This vector has been successfully used to transduce genes into selected tissues of adult animals with a long-term expression potential (124). However, its use in vivo has been limited by production. We have recently developed an efficient method for packaging and concentrating lentiviral vectors that consistently yields high-titer virus on a scale suitable for in vivo applications (125). We demonstrated that lentiviral vector delivered systemically can transduce several cardiovascular-relevant tissues, including the brain, and the transgene exhibited long-term (120 d, duration
of experiment) expression (126). Recent findings from our lab using this lentivirus to overexpress the AT$_2$R in SHR demonstrated that our transgene effectively transduced cardiac myocytes and significantly reduced the cardiac hypertrophy in these animals without effecting BP (59). These findings provide significant information with respect to the AT$_2$R and cardiac hypertrophy, and suggest that the AT$_2$R do antagonize effects mediated by the AT$_1$R.

The lentivirus preparations we used were administered to 5-d-old animals. Future studies will determine the effectiveness of utilizing this vector delivery method in adults. Because the lentivirus can infect nondividing cells, it should be effective in adult animals and is most likely more applicable in a clinical setting over the retrovirus, because it could reverse the disease state in contrast to preventing the development of the disease.

CELL TYPE-SPECIFIC PROMOTERS

Numerous technological advances have afforded us the ability to underexpress (reduction approach) or overexpress (induction approach) transgenes of interest in various animal models. However, ubiquitous expression of a transgene may lead to undesirable effects. Local or targeted gene delivery has the advantage of concentrating the gene therapy in a relevant tissue, and reduces the risk of losing the vector to sites where its under- or overexpression may have deleterious consequences. One way to obviate this potential problem is to utilize tissue- or cell-selective promoters to limit transgene expression to a subset of cells or tissues. Such systems have been developed to selectively alter gene expression in the heart and vascular smooth muscle (127,128). For example, tissue-specific gene expression can be enhanced by the use of specific cardiac myocytes promoters, such as mlc-v (ventricular-specific myosin light chain-2), or cardiac troponin T gene promoter (129,130). Others have used a rat neuron-specific enolase promoter (Ad-NSE) to specifically target neuronal cells (131). Thus, one can selectively target gene delivery to specific tissue types, provided one has utilized the appropriate tissue-specific promoter. Utilization with these specific promoters will limit expression of the transgene to specific tissue sites that are relevant for the particular disease state.

Targeting specific tissues/cells with a transgene early in development can have detrimental or even lethal effects. For example, transgenic mice with endothelially expressed genes have been reported to harbor severe developmental abnormalities (132). Thus, the more optimal approach to gene therapy is the development of system(s) that allow for externally regulated control of transgene expression.

REGULATABLE PROMOTERS

Constitutive expression of a transgene may produce undesirable effects if nonphysiological levels of proteins are produced. Although gene therapy approaches have extraordinary promise, uncontrolled transgene expression may lead to deleterious consequences. Therefore, the ability to modulate transcription of a transgene or vector would be desirable for effective and safe gene therapy in humans. One way of overcoming this problem is to utilize a regulated gene expression system that uses exogenous ligands to control transgene expression. Some of these previously developed systems use inducible promoters, which are modulated by exogenous ligands, such as mifepristone (133), rapamycin (134), ecdysone (135), or tetracycline (136), to drive the expression of the transgene. These systems can allow for the activation of a transgene when needed, thereby
allowing researchers and clinicians to “turn off” of the transgene expression if complications arise, or to “turn on” the transgene when it is needed. In general, these systems use an inducer or repressor that can reversibly bind the endogenous ligand, and its chimeric state then acts as a transcriptional factor for its particular promoter. Ideally, such an exogenous inducer would allow a wide therapeutic window and few, if any, side effects when compared to conventional pharmacological therapy.

Pioneering work by Bujard and Gossen (137) established the tetracycline transactivator system as a reliable tool for regulating transgene expression. This tetracycline (tet)-inducible system consists of three major components: the tetracycline-binding-transcriptional modulator protein, the corresponding tetracycline-responsive promoter element and the tetracycline-class pharmacological agent. Doxycycline (Dox) is the most commonly used tetracycline drug used with the tet system because it has a high affinity for the tet transcriptional modulator protein, has a low toxicity, and favorable pharmacokinetic properties for use in vivo (138,139). This system has been used for transcriptional control of transgenes for over 10 yr, and a continuous line of modifications to the system have made it much more effective in tightly regulating gene expression (140).

The original tet system was a “tet-off” version, in which application of a tetracycline caused attenuated expression of the transgene. Subsequent development of the tet system by Gossen et al. (136) resulted in the generation of a “tet-on” version, where application of a tetracycline causes increased transgene expression. Despite recent improvements in the tet-on system, including the addition of a tet-silencer protein (141), this inducible system has an inherent leak in basal transgene expression and causes only mild increases in transgene expression (when compared with the range of expression of the tet-off system) (142). Certain other problems with the tet-on system are currently being worked out, such as cellular toxicity, insensitivity to Dox in certain tissues, and unstable transcripts (143,144). Some groups (141,145), however, have suggested that differences in regulation may exist among various mouse strains.

A possible drawback with this system is that it requires two vectors, and these vectors both have to infect the same cell to achieve a properly regulated system. Despite this issue, our lab constructed dual cassette retrovirus vectors that together encoded a modified enhanced green fluorescent protein (d2EGFP) under the control tet-on system. Viral particles were then used to infect rat aortic vascular smooth muscle cells and pulmonary endothelial cells. Cells transduced with both vectors were examined by fluorescence microscopy for inducible expression of d2EGFP. Incubation of dual-vector infected cells with Dox caused a robust expression of d2EGFP within 48 h, whereas removal of Dox caused a disappearance of green fluorescence within 24 h. (146). These results demonstrate tightly regulated gene expression using a retroviral vector system. We subsequently used this system to effectively induce AT1R-AS, resulting in a lowering of BP in the SHR (147) when the animals were maintained on Dox.

However, with newer technologies, these regulatable systems will have even greater advantages. For instance, Teng et al. (148) have recently combined the components of the regulated system with tissue-specific properties to direct the controlled induction of an exogenous transgene to the vascular endothelium of an adult mouse by utilizing an endothelial cell-specific promoter through the tet-on system.

Recently some tet-regulated transgenic mice have been developed. This reduces the payload requirement of a gene therapy vector in these animals, because the animals already harbor half of the gene-switch system ubiquitously. Corbel and Rossi (140) recently
summarized work on new tissue-specific tetracycline (activation and repression) transgenic mice, as well as the recent methods utilized to deliver tetracycline-regulated genes in vitro and in vivo. Ju et al. (149) recently generated transgenic mice with the use of an arterial smooth muscle cell (SMC)-restricted (SM22α promoter-driven), tetracycline-controlled transactivator (tTA) to effect conditional expression of a tTA-dependent transgene encoding rat vascular chymase (RVCH). The recombinant RVCH converts Ang I to Ang II in vitro. In their study, hypertension was completely reversed and the medial thickening of mesenteric arteries from tTA+/RVCH+ mice was prevented by doxycycline. Therefore, by utilizing such combined technologies, more specific and controlled targeting of selected transgenes can be employed more effectively as therapeutic tools.

More recently, use of endogenous instead of exogenous regulatable systems have been used effectively in vivo, further demonstrating the importance and versatility of effectively controlling gene expression and its application to hypertensive therapy. Kantachuvessiri et al. (150) have used a rat CPY1a1 enhancer/promoter sequence to control mouse Ren2 cDNA expression in rats to create an inducible model of hypertension. BP and the RAS returned to normal in the absence of the inducer. Other in vivo work has used adenovirally mediated transfer of ecdysone-inducible constructs to the heart and carotid bodies of rats (151). Thus, these regulated systems offer significant advantages in utilizing gene therapy approaches to better understand disease processes and provide potential therapy for a variety of disease states.

Another example of an endogenous state-specific promoter is the hypoxia response element (HRE), which responds to hypoxic conditions within the cell. Under hypoxia, the transcription of numerous genes can be activated, including erythropoietin (152,153), the β-adrenergic receptor (154), glycolytic enzymes (155,156), and vascular endothelial growth factor (157,158), among others. Thus, this promoter could be used in various cardiovascular or metabolic disorders to regulate expression of genes that have been proven to have beneficial effects in conditions of ischemia, such as AS to the AT1R (159), the β-adrenergic receptor (160,161), superoxide dismutase (162,163), angiotensin-converting enzyme (164,165), and vascular endothelial growth factor (166).

Vigna et al. (167) were the first to report the generation of lentiviral vectors capable of delivering tet-regulated gene delivery to human hematopoietic progenitor cells ex vivo. The combination of lentiviral delivery with improved transcriptional activators of the tet system resulted in the regulation of an inducible gene that was maintained for over 20 wk after infection of these engineered cells. The lentivirus, with its much larger cassette site, may allow for both the tetracycline transactivator and the tetracycline response element to be housed in a single vector. We have used such a system for in vitro manipulation and are currently attempting to develop such a vector for subsequent in vivo gene therapy for hypertension in order to better control expression of our transgene of interest. Thus, these regulated systems offer significant advantages for the utilization of gene therapy approaches to better understand disease processes and to provide effective therapy for a variety of disease states.

**CRE/Lox System**

Another type of regulated system is an older CRE (“causes recombination”)/Lox system. This technique allows for the modification of a specific gene with locus of crossover (loxP) sites flanking the specific region of interest though the use of standard
gene targeting vectors in embryonic stem cells. These targeted alleles are said to be “floxed”, or flanked by the loxP, and thus may not be fully functional. CRE recombinase directs recombination between loxP sites. Application of this technique in mice allows for deletion of selected material at specific times. This offers advantages over knockout models in that it can avoid the complications of not only embryonic lethality, but also the developmental compensatory changes that occur when there is a genetic knockout. Because this technology relies on homologous recombination in embryonic stem cells, its in vivo use is currently restricted to mice. Mice that are derived from these targeted embryonic stem cells are then bred to homozygosity for the targeted floxed allele and can then be crossed with other mice transgenic for CRE recombinants under the control of specific promoters to allow for a tissue-specific deletion of the floxed segment. Thus, the activity of a particular gene can be modified in a limited number of cells or tissues while the genetic content of the rest of the animal is essentially unaltered. Many tissue-specific CRE transgenic mice strains are becoming available and have provided insights into endocrine physiology in recent years. Most of these tissue-specific CRE/loxP-mediated gene deletions have been studied in endocrine or other target tissues, and have been nicely summarized by Ryding et al. (168). A database cataloging these mouse models is available at http://www.mshri.on.ca/nagy/cre.htm.

Unfortunately, most CRE/loxP mouse experiments have several potential pitfalls. First, expression of CRE within a particular tissue or cell is rarely uniform, which can lead to mosaic recombination. A second potential pitfall is that of transcriptional interference by vector-derived sequences. Like strain differences observed with the tet system (141,169), one might also anticipate that the efficiency of CRE recombination may also be strain-dependent, although there has yet to be any formal investigation of this possibility. Despite these potential drawbacks, the CRE/loxP technology is a well established genetic tool for the study of conditional deletion of genes in mice.

**Vigilant Vector Approach**

Recently, some groups have begun work on developing cell-state-specific promoter systems for transgenes. A popular example of this methodology is referred to as the “vigilant vector,” which senses hypoxic conditions within the cell and responds by increasing transgene transcription. This approach has been used to signal dormant transgenes to become active and to protect specific tissues with high amplification of the specific transgene. In order for this system to be effective, four components are required (170). The first component is stable vector that is safe and can be administered by systemic injection and in which the transgene is expressed in a particular organ or tissue. The second component is a reversible gene switch that acts as a biosensor and can detect certain physiological signals. The third component is a tissue-specific promoter, and the fourth component is an amplification system. This type of system can have broad applications. One can switch tissue-specific promoters or switch out different transgenes and/or protective genes in order to apply this vigilant approach in a variety of disease states. Tang et al. (171) recently characterized a hypoxic inducible double plasmid system for myocardial ischemia. This hypoxia-sensitive promoter could be used to drive transcription of hypoxia-protective genes, such as superoxide dismutase (162), the β-adrenergic receptor (160), and components of the RAS (159,164), thus conferring some immunity to hypoxia upon these cells.
**siRNA Technology**

Selective downregulation (knockdown) of a specific gene can be a useful method for therapeutic intervention, provided the targeted gene is important in the pathogenesis of the disease. One way to facilitate this genetic “knockdown” is the use of AS gene technology. The major disadvantage of this method is that the transgene product (the AS mRNA) is destroyed along with the target mRNA. A recent advancement to the AS approach is a conserved posttranscriptional gene silencing (PTGS) mechanism mediated by double-stranded RNA (dsRNA). Silencing of gene expression using dsRNA, known as RNA interference (RNAi) or short interfering RNAs (siRNAs), provides a powerful tool for analyzing gene function. Although not all of the mechanisms by which this system operates are completely known, this RNAi method is more efficient than AS in that it is able to cleave the target mRNA and mark future copies of the target mRNA to be destroyed, while the interfering mRNA itself is not destroyed along with the target RNA (172). It can therefore cleave and destroy multiple pieces of target RNA. An RNase III-like enzyme called a “Dicer” cuts long dsRNA, or hairpin RNAs, into double-stranded siRNAs (173). These are typically 20–25 nucleotides (nt) long, and can trigger formation of an RNA silencing complex (RISC). The siRNA–RISC complex seeks out the mRNA with the targeted sequence and degrades it, effectively silencing that gene. Short dsRNAs can be synthesized in vitro and introduced into mammalian cells. Using these short siRNAs does not trigger antiviral mechanisms within the cell and can mediate genespecific suppression in mammalian cells. As a result, a few copies of the interfering dsRNA can cause total degradation of cognate transcripts in a cell. This technology has been used successfully as a tool to analyze gene function in plants, insects, and nematodes (174–176); however, use of long dsRNA in mammalian somatic cells results in activation of antiviral defense systems that can result in nonspecific degradation of RNA transcripts and a general loss of host cell protein synthesis, thus investigators soon discovered that introduction of smaller siRNA, less than 30 nt, can lead to gene specific silencing (177).

Although there is significant potential for the use of this new AS technology to target specific genes associated with hypertension, important issues remain unresolved. Because there is no set pattern or rule yet discovered to determine the optimal sequence to target within a gene, investigators are encouraged to select several siRNA sequences in different locations along the gene of interest to test in order to find the most efficient site that will be important in silencing the gene of interest. A single-point mutation in certain locations of the paired region of the siRNA duplex can abolish target mRNA degradation (177). siRNAs are extraordinarily effective at lowering the amounts of targeted RNA—and, by extension, proteins—frequently to undetectable levels. However, any polymorphism in the gene of interest can complicate the process. There are several web-based tools to assist in design of siRNAs (examples include those provided by Ambion [http://www.ambion.com] and Dharmacon [http://www.dharmacon.com]). Once a sequence is identified, the siRNA transgene must be chemically synthesized and transfected. Recent evidence suggests that the effectiveness of siRNAs may depend greatly on the method of transfection (178). There is no uptake of siRNAs into cells, and the delivery to selected sites of therapy remains problematic (179). Handling of the RNAi is laborious and complex because of ubiquitous RNAses. However, with advancing technologies, this may be a very effective means to knock down targeted disease-producing genes. Various viral vectors are being evaluated to increase infection for in vivo administration of siRNA.
Lentiviral and other retroviral systems have been shown to be effective in many settings and adenovirally based vectors are also being evaluated (180,181). The ability to efficiently and stably produce and deliver sufficient amounts of siRNA to the proper target tissues still requires refinement before this new technology can be tried clinically. Xia et al. (246) recently described a virally mediated delivery mechanism that results in specific silencing of targeted genes through expression of siRNA. These investigators demonstrated expression of exogenous and endogenous genes in vitro and in vivo in brain and liver, and further applied this strategy to a model system of a major class of neurodegenerative disorders, finally suggesting that this virally mediated strategy could be successfully used to reduce expression of target genes to provide therapy for human diseases. Lewis et al. (182) recently described a method for efficient in vivo delivery of siRNAs to organs of postnatal mice. Lentivirus-based vectors may be the most promising siRNA expression system to drive gene inhibition in stem cells, because the transgenes that are expressed from the lentiviruses are not silenced during the developmental process and can thus be used to generate transgenic animals through infection of embryos (180,183). Cheng et al. (184) recently reviewed this field, and provide an overview of its potential applications in the treatment of human disease.

MIcroARRAY TECHNOLOGY AND ITS IMPLICATIONS IN GENE THERAPY

Recent advances in genomic techniques and their integration into physiology have resulted in the genesis of the “functional genomics” era. Functional genomics is a multidisciplinary approach to studying genes, their products, and interactions among them that are responsible for mediating physiological responses. Also included in functional genomics is the detection and characterization of aberrations of genetic processes that may result in diseases. Recent advances in molecular biology and technology have made it possible to monitor the expression level of numerous genes simultaneously. Cardiovascular research has focused mainly on only a small fraction of known genes; however, information gleaned from genome sequencing and the development of microarray technologies has the potential to provide for genome-wide analysis of genes that can mediate BP regulation and their potential contribution to the pathogenesis of hypertension. Protein microarrays also have been developed for protein expression analysis and interaction analysis, which may include protein–protein, ligand–receptor, enzyme–substrate, and nucleic acid–protein interactions. However, the use of high-throughput arrays (both RNA and protein) has yet to realize its full potential in cardiovascular research. Issues concerning reproducibility and preanalytic variables can make the discovery process from these methods tedious (185,186).

Numerous investigators are using gene expression profiles in hypertensive models in hopes of identifying genes relevant to hypertension. Candidate genes can be identified from transcript profiling, and different strategies can be utilized to evaluate their potential relevance in the pathogenesis of hypertension. Clinical studies have demonstrated that genetic variation accounts for up to half of the phenotypic variation in BP (187). Numerous studies have demonstrated links between genes of the RAS (i.e., angiotensinogen, renin, ACE, and AT1R) and hypertension in various populations (188,189). The use of inbred rat models of hypertension and congenic strains also has identified large chromosomal regions containing quantitative trait loci that account for genetic variation in BP.
(190–193). The construction of minimal congenic strains has been used to further narrow down the particular loci involved (194). However, consistent associations have been difficult to demonstrate, and little progress has been made toward identifying the specific genetic variants that contribute to a particular phenotype. Therefore, to better understand a polygenic disease such as hypertension, we need to identify which groups of genes account for the hypertensive phenotype, and how these gene clusters are regulated.

Gene expression profiling has been utilized to gain insight into disease mechanisms. Assessment of the expression of a large number of genes have been realized by the use of high-throughput gene profiling technology, such as cDNA and oligonucleotide microarrays, as well as serial analysis of gene expression (SAGE). In addition, for genomes that are not yet fully sequenced, transcript profiling of expressed sequence tags (EST) provides expression information on novel genes. The technical limitations and issues relating to analysis of microarray data have been reviewed recently (195–198). Gene expression profiles most often represent complex phenotypes. Cluster analysis of expression data may distinguish particular groups of genes that are regulated in a similar manner. Profiling has been used to identify genes involved in hypertension, and usually compares differentially expressed genes in inbred genetic rat models of hypertension with their normotensive controls. Differential gene expression between these two groups, however, may not necessarily reflect causative mechanisms in hypertension. The differences may reflect a genetic difference between the strains that are unrelated to hypertension, or they may be expressed as a result of the high BP and not be related to any cause for the increase in BP. In addition, like any method, there are some technical drawbacks for the sole use of microarrays in identifying candidate genes. Some limitations and issues relating to analysis of microarray data have been reviewed recently (196,197,199,200). Therefore, it is essential that other techniques, such as QTL mapping, complementation testing, or loss/gain of function experiments, be combined with the gene profiling to determine if differential gene expression reflects a causative role in hypertension (201–203).

Transcript profiling of inbred genetic models of hypertension, such as the SHR, have proven useful in identifying potential genetic mechanisms of hypertension. Microarray profiling from kidneys of SHR and Wistar–Kyoto (WKY) animals has identified a number of differentially expressed genes in SHR (203,204). However, even when comparing different strains of SHR, there is a large variability in genes that are differentially expressed, which underscores the genetic heterogeneity of the different strains (203). These observations suggest that there can be both common and distinct genetic mechanisms in hypertension in different SHR strains.

The most appropriate tissue type for gene profiling is debatable. Most transcript profiling studies in hypertensive rats have used RNA derived from kidneys (202,203) to identify genes that may contribute to hypertension, as the kidney has a well-established role in long-term regulation of BP. We are currently utilizing gene expression profiling in different areas of the brain, such as the hypothalamus and brainstem, in order to identify target candidate genes that may be regulated by the brain RAS in the SHR (205,206). To eliminate any genetic alteration resulting secondarily from an elevation in BP, we have also assessed gene expression profiles in primary neuronal cultures derived from the hypothalamus and brainstem areas of neonatal (prehypertensive) SHR and WKY rats (205). We found that neonatal SHR neurons in culture exhibit increased AT1R transcript expression and functional receptors as compared to WKY cultures. These results indicate that the hyperactivity of the brain RAS observed in the adult SHR is also present in the
neuronal cultures (207). We identified genes that are regulated by Ang II by comparing transcript profiles of neuronal cultures treated with vehicle to those treated with Ang II. Utilizing gene filtering and statistical analysis, we identified differential expression of 299 genes and 109 EST between strains (205). Interestingly, SHR neurons exhibit an altered Ang II-induced pattern of gene expression as most differentially expressed genes were upregulated, whereas their expression was downregulated in WKY neurons (a complete list is available online at www.med.ufl.edu/phys/raizada/veerasingham.doc). This effort, like most of the gene expression profiling, has been of a correlational or descriptive nature. Further experiments are required to evaluate the physiological relevance of the altered gene profile of Ang II-responsive genes in SHR neurons, as well as that of genes that are differentially expressed between the two strains.

One potential therapeutic target gene in the brain that was differentially expressed in SHR compared to WKY neuronal cultures was adducin. Adducin is a ubiquitously expressed tetrameric cytoskeletal protein composed with either $\alpha/\beta$ or $\alpha/\gamma$ heterodimers, and is involved in intracellular protein trafficking, $Ca^{2+}$ mobilization, and the phosphorylation state of certain kinases (208). Genetic variations in $\alpha$-adducin have been associated with primary human hypertension and the BP phenotype in Milan hypertensive rats (25,209–211). Polymorphisms of the $\beta$ and $\gamma$ subunits may contribute to BP variation, especially when associated with $\alpha$-adducin polymorphisms, consistent with the notion that biological activity of adducin is dependent on $\alpha/\beta$ or $\alpha/\gamma$ heterodimers (25,210,211).

A role for adducin in BP regulation has been further supported by the demonstration of higher BP in $\beta$-adducin deficient mice than in wild-type mice (212). We have identified a 22% decrease in $\gamma$-adducin (Add3) expression in SHR neurons compared with WKY neuronal cultures using gene expression profiling. Real-time RT-PCR confirmed the decrease in $\gamma$-adducin transcript, and Western blot analysis indicated a more dramatic decrease (approx 60%) in protein levels in SHR compared with WKY neurons in culture (206). This decrease in $\gamma$-adducin expression was maintained in the hypothalamus and brainstem of adult SHR and was also observed in mRen2 rats, suggesting that it is common to hypertensive models that exhibit an overactive brain RAS (206). Furthermore, Ang II treatment of either WKY or SHR neuronal cultures resulted in a decrease of $\gamma$-adducin transcript and protein levels, indicating a regulation of expression by Ang II. These observations demonstrate a decrease in $\gamma$-adducin expression in hypothalamic and medullary areas of hypertensive rats, and support the concept that an overactive brain RAS may be responsible for the decreased expression. We also evaluated the effect of $\gamma$-adducin inhibition on the firing rate of neuronal cultures. Using intracellular delivery of $\gamma$-adducin-specific antibodies to inhibit $\gamma$-adducin resulted in an increased neuronal firing rate, which was similar to that observed with Ang II. This effect was not additive, suggesting that a common mechanism resulted in the increased firing rate (206). We subsequently investigated two nongenetic models of hypertension, the chronic Ang II model and the desoxycorticosterone acetate model (213). In both models, the increase in BP was associated with a 70% decrease in hypothalamic $\gamma$-adducin; however, in contrast with the genetic models of hypertension, there was no change in brainstem $\gamma$-adducin. Neuronal cultures from the WKY strain of rats, when incubated with Ang II, resulted in a 60% decrease in the neuronal $\gamma$-adducin (213). Decreased $\gamma$-adducin may therefore contribute to augmented basal neuronal firing rate in cardiovascular-regulatory brain areas of hypertensive animals. Because central $\gamma$-adducin expression is decreased in SHR, we would predict that a gene therapy approach to overexpress this gene in the brain of the SHR may
decrease BP and reverse hypertension, whereas deleting or reducing its expression in WKY rats would induce hypertension.

Yu et al. (244) demonstrated an elevation in the expression of soluble epoxide hydrolase (sEH) from the kidney of the SHR when compared with the WKY. In addition, this group showed that blocking sEH lowered the BP in the SHR. Furthermore, Sinal et al. (214) reported that BP is reduced in a knockout mouse model for sEH, although this change in BP was observed only in male rats. However, when the kidneys from multiple strains of SHR and WKY rats were utilized in subsequent gene profiling studies no consistent changes in this gene were observed and there was no clear correlation between expression of the gene and hypertension (203,245). This led us to propose a novel hypothesis about the role of this gene in hypertension. Our hypothesis is that the expression of brain sEH may be key in the regulation of BP, and its altered expression of dysregulation could be better linked to hypertension. This hypothesis was tested and subsequently validated by demonstrating an upregulation of this enzyme in the hypothalamus and brain stem areas of the SHR by expression profiling analysis. In spite of the excellent physiological link between dysregulated expression of brain sEH and hypertension, direct gene transfer studies must be carried out to conclusively demonstrate the role of this enzyme in hypertension. It is from studies like these that gene profiling experiments that initially correlate changes in gene expression can move beyond to seek out mechanisms of functional significance.

**FUTURE GENE TARGETS OF THE RAS**

The RAS is classically known as a hormonal system that is involved in salt and water regulation and BP control. The RAS is one example of a system in which dysregulated expression and hyperactivity have been associated with the development and maintenance of hypertension. Both the systemic (endocrine) and tissue (paracrine/autocrine) versions of the RAS contribute to hypertension (238,242). In the systemic RAS, a coordinated sequence of events involving various organs work together to generate circulating active hormone, Ang II. In this classical systemic endocrine system, angiotensinogen, which is produced in the liver, is converted to Ang I by the enzyme renin, which is secreted by the kidney. ACE is an enzyme that is found both circulating in the blood and membrane bound in numerous tissues including epithelial and endothelial cells (215,216), converts ANG I to ANG II. Various areas of the brain are also rich in ACE (217). Ang II produced as a result of the endocrine RAS is responsible for many short-term effects of Ang II, such as arterial vasoconstriction, aldosterone release, and sodium and water reabsorption by signaling through the AT1R (223,230,238). This classical understanding of the RAS led to the use of ACE inhibitors (ACEI) and Ang II receptor blockers (ARBs) as therapeutic agents in the treatment of hypertension. Because a hyperactive RAS is a key player in hypertension, and because 40–60% of hypertensive patients respond to ACE-inhibitors/AT1R-antagonists, one could hypothesize that genetic manipulation of this system to inhibit signaling within the RAS may, in principle, be an ideal method to attempt a genetic cure for this disease. We and others (62–67,73,74,84,91,93,99,101,102,104) have used an AS technique to downregulate transcription of the ACE enzyme and/or the AT1R to prevent the development of hypertension in both genetic and nongenetic models of experimental hypertension. However, this classical RAS is more complex, and new discoveries regarding angiotensin degradation fragments provide even more potential targets for gene therapy in hypertension.
Recently, the ACE2 enzyme has been characterized (218,219). This enzyme, which initially was found in the testis, kidney, and heart, has also been identified in a wide variety of tissues and is most likely localized, much like ACE (218–220). It shares 40% homology with ACE, but differs greatly in substrate specificity, and its activity is not altered by ACEI. ACE2 is one of several enzymes that catalyze the formation of degradation fragments angiotensin 1–9 (Ang 1–9) and angiotensin 1–7 (Ang 1–7) from both Ang I and Ang II, respectively (see Fig. 1). In mice lacking the ACE2 gene, Allred et al. (227) observed a decrease in baseline BP and an enhanced pressor response during intravenous infusion of Ang II as compared with normal mice. BP was also decreased in animals that overexpressed ACE2 (228). Therefore, the physiological effect of an imbalance of ACE2 over that of ACE could shift the RAS to increase vasodilator effects and reduce vasoconstrictor effects, thus resulting in a reduced BP (see Fig. 1). It is conceivable that the balance between ACE and ACE2 may be a pivotal mechanism in the regulation of BP and in the tissue pathophysiology (i.e., cardiac hypertrophy, renal disease) that has been associated with the tissue RAS. ACE2 also has the potential to be a better target than ACE because (1) it does not have the confounding interpretation effects that ACE does on bradykinin levels; (2) the gene has both a secreted form and a membrane-bound form that may allow us to differentiate BP effects from end-organ target pathologies; and (3) control of this unique enzyme can shift the emphasis of the entire RAS to one of vasodilation instead of vasoconstriction, and thus regulation of this enzyme may have significant therapeutic effects in the treatment of hypertension and other cardiovascular abnormalities.

Even though the actions of Ang II are best characterized, a role for other Ang peptides, such as Ang III, Ang IV, and Ang 1–7, as well as other Ang receptors, is rapidly emerging (223–225). Because ACE inhibitors and ARBs are effective in treating essential hypertension, it is conceivable that some of the actions of these drugs could be mediated through these nonclassical components of the RAS. Blocking the ACE enzyme, for example, causes bradykinin levels to increase, and this leads to a lowering of BP (226). Ang I levels would increase with ACE inhibition, and Ang II levels would increase with ARB treatment. In both cases, this could lead to an increase in Ang 1–7 via ACE2 and other endopeptidases. Ang 1–7 has been suggested to antagonize Ang II action directly at the AT1R as well as indirectly via other pathways (such as antagonizing ACE) and its concentrations are increased during ACE inhibition (221,222). Although Ang 1–7 is catabolized by ACE, it is also considered a competitive inhibitor of ACE (219). This new knowledge of the RAS suggests several potential genetic targets. Overexpression of ACE2, or increased production of Ang 1–7, are two future possible targets for gene therapy in the treatment of hypertension.

Most studies of the RAS have focused on the AT1R subtype. This subtype is one that is responsible for most of the known actions of Ang II, such as vasoconstriction, enhancement of noradrenergic neurotransmission, and release of hormones from the adrenal gland (229,230). There is an additional receptor subtype that can be activated by Ang II, the AT2R (229,230). This receptor, like the AT1R, is a G protein-coupled receptor with seven transmembrane regions (229–233). Although similar in size (363 amino acids for AT2R and 359 amino acids for AT1R), there is only 34% homology between the two receptor subtypes. The AT2R is more widely distributed in fetal tissues, and it has been suggested that this receptor is involved in developmental processes (229–233). Generally, the actions of AT2Rs oppose those of the AT1R (229–233). AT2R knockout models
exhibit increases in basal BP and/or show enhanced pressor responses to Ang II (234,235). Further, we have shown recently that overexpression of the AT\(_2\)R inhibits cardiac hypertrophy in the SHR (59) and that underexpression of the AT\(_2\)R can elevate BP in Sprague-Dawley animals (80). The recent finding that the AT\(_2\)R can act in a constitutive manner (236) is also intriguing, and may suggest some clinical significance. Its use as a genetic target will continue to be examined.

In contrast to the classical systemic (endocrine) RAS, there is the local-tissue (paracrine/autocrine) RAS. Components of the classical, systemic RAS are found in numerous tissues (237–240). Ang II and its other active degradation fragments can be produced within given tissues, and thus have their physiological effects locally at that (or neighboring) tissues. This paracrine/autocrine system appears to play a major role in long-term cardiovascular regulation, especially in regulating cardiac hypertrophy and remodeling of the arteriole vasculature in other tissues (241–243). We have demonstrated that overexpression of the AT\(_2\)R (59) or underexpression of the AT\(_1\)R (62–64,91,93,99) is effective in preventing cardiac hypertrophy in genetic and nongenetic models of experimental hypertension. Targeting specific tissues with gene therapy approaches may be able to reverse and/or prevent the pathophysiological alterations that occur in hypertensive and other cardiovascular diseases. Figure 1 identifies most of the well-characterized components of the RAS, and thus identifies some current and future targets for gene therapy.

**SUMMARY AND PERSPECTIVES**

Hypertension is a prevalent disease in which current intervention with pharmaceutical agents is effective in only about one-third of patients. Additionally, the current pharmacological approach aims to control BP, and will not lead to prevention or cure of the
disease. Gene therapy has the potential to prevent and/or cure hypertension, as clinical findings strongly support the notion that hypertension is genetically based. Many of these clinical findings involve components of the RAS, although there are many other candidate genes. Although there are numerous technologies available to use for discovery and examination of candidate genes, and for the delivery and manipulation of gene therapies, each has its own strengths and weaknesses. Current knowledge of the pathways that can lead to hypertension can suggest some of these potential genes, but this method will not reveal novel pathways or mechanisms to study and target. Microarray expression profiling studies can be used to identify a number of differentially expressed genes, but the challenge remains to determine the physiological relevance of these findings, and to identify which of these genes are linked to the development and/or maintenance of hypertension. The combination of gene expression profiling and the phenotypic characterization of in vitro and in vivo loss- or gain-of-function experiments has the potential to identify genes involved in the pathogenesis of hypertension, and thereby presents novel targets for therapy. Our primary research task then, still remains, to identify significant candidate genes.

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