12 Current and Future Novel Targets of Gene Therapy for Hypertension

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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

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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) *(1)* (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, multifactoral, 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 ($\leq$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 kallikrien 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 pathophysiologies 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.

Table 1
Increased Gene Expression

Transgene	*Animal model*	*Delivery*	*Route*	*Effect on blood pressure*	*Comments*	*References*
Kallikrein	Fructose-induced SD	Plasmid DNA	iv	Decreased	Decreased AT_1R, decreased endothelin, normalized insulin	*43*
	SHR	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	Adenovirus	im	Decreased		*46*
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Reversed cardiac hypertrophy and fibrosis and renal damage	*47*
	5-wk-old Goldblatt—2 Kidney/1 Clip	Adenovirus	iv	Delay of onset	Reduced left ventricular mass, protection from renal dysfunction	*48*
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Decreased left ventricular hypertrophy, attenuated renal injury	*49*
	SHR	Adenovirus	iv	Delay of onset		*50*
	Adult and newborn SHR	Oligonucleotide	sc	Delay of onset	Gender differences	*51*
	SHR	Adenovirus	iv	Decreased		*52*
	SHR	Oligonucleotide	iv	Decreased	Decreased BP in adult but not in young SHR	*53*
	SHR	Plasmid DNA	im	Decreased	BP reduction attenuated with bradykinin antagonist	*5*
	SHR	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)

	SHR	Plasmid DNA	iv	Decreased	2nd injection further reduced BP	*38*
Nitric oxide synthase	SHR	Adenovirus	icv (NTS)	Decreased	Depressor response in WKY, but greater in SHR	*39*
	Stroke-prone SHR	Adenovirus	iv	No effect	Improved endothelial function	*40*
	SHR	Plasmid DNA	iv	Decreased	2nd injection extended therapeutic time limit	*41*
	SHR	Adenovirus	icv (NTS)	Decreased	Decrease in HR and BP reversed by microinjection of soluble guanylate cyclase inhibitor	*42*
Atrial natriuretic peptide	SHR	Plasmid DNA	iv	Decreased	Increased urine volume and sodium excretion	*31*
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Decrease in cerebral infarction, reduced thickness of arterial wall	*32*
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Reduction in cardiac myocyte size, attenuation of glomerular sclerotic lesions	*33*
	4-wk-old SHR	Plasmid DNA	iv	Decreased	No effect on 12-wk-old SHR	*34*
Superoxide dismutase	SHR	Adenovirus	iv	Decreased	Effect greater in anesthetized compared with awake rats; improved endothelial function	*56*
	Stroke-prone SHR	Adenovirus	iv	No effect	Improved endothelial function	*57*
Heme oxygenase	5-d-old SHR	Retrovirus	ic	Delay of onset		*58*

SHR, spontaneously hypertensive rats; iv, intravenous; im, intramuscular; ic, intracardiac; sc, subcutaneous; icv, intracerebroventricular; WKY, Wistar–Kyoto; BP, blood pressure; SD, Sprague-Dawley rats.

Table 2
Decreased Gene Expression

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_1R in PVN, decreased angiotensin II in brainstem	*83*
	SHR	Oligonucleotide	icv	Decrease	Decreased angiotensin II in brainstem	*84*
	SHR	AAV*	ic	Decrease, delay of onset	Decreased left ventricular hypertrophy, decreased angiotensinogen	*96*
	SHR	Asialo-glycoprotein	iv	Decrease	Reduced hepatic angiotensinogen, reduced cardiac AT_1R, decreased left-ventricular hypertrophy	*97*
	SHR	AAV	iv	Decrease	Greater effect of AAV-plasmid vector with liposome	*100*
	Cold-induced SD Goldblatt—2 Kidney/1 Clip	Liposome	iv	Decrease	Decreased spontaneous drinking response	*101*
		Oligonucleotide	icv	Decrease	Decreased the elevated hypothalamus angiotensin II levels	*105*
Angiotensin converting enzyme	SHR	Retrovirus	ic	Decrease	No effect on WKY	*66*
AT_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	*67*
	SHR	Retrovirus	ic	Transient Decrease	Repeated daily injections for 6 d	*73*
	SHR	AAV*	icv (Lat. Vent)	Decrease	Intracardiac injection in 3-wk-old SHR also reduced BP	*74*
	SHR	Oligonucleotide	icv	Decrease		*84*
	L-NAME SD	Retrovirus*	ic	Decrease	Decreased left-ventricular hypertrophy, endothelial dysfunction unchanged	*91*
	Renin transgenic	Retrovirus*	ic	Decrease	Prevented cardiac hypertrophy	*93*
	Angiotensin-induced SD	Retrovirus*	ic	Decrease	Protected against angiotensin II-induced increases in BP and cardiac hypertrophy	*99*
	Cold-induced SD	Oligonucleotide	ic and/or icv	Decrease	Spontaneous drinking response to cold reduced	*101*
	Goldblatt - 2 Kidney / 1 Clip	Oligonucleotide	i.v.	Decrease		*102*
	SHR	Oligonucleotide	intracister-nally	Decrease	Decreased cerebral infarct after middle cerebral artery occlusion	*104*
AT_2 receptors	Uni-neprectomized	Oligonucleotide	interstitial pump	Increase	Increased pressor response to angiotensin II	*79*
	SD	Retrovirus*	ic	Increase	Increased pressor response to angiotensin II	*80*
Epidermal growth factor receptor	SHR	Liposome	iv	Decrease	Weekly injections for 2 mo; Decreased left-ventricular hypertrophy, but only effective in 5-wk, not 13-wk-old SHR	*92*
	Angiotensin-induced SD	Oligonucleotide	iv	Decrease	Normalized left-ventricular hypertrophy	*108*
β-adrenergic receptor	SHR	Liposome	iv	Decrease	No effect on heart rate	*90*
	SHR	Liposome	iv	Decrease	Decreased beta 1, but not beta 2, receptors, decreased plasma renin activity and angiotensin II	*95*
CPY-like kininase	DOCA salt SD	Oligonucleotide	iv	Decrease	Increased urine volume and sodium excretion	*107*
Tyrosine hydroxylase	SHR	Oligonucleotide	iv	Decrease	Decreased epinephrine and norepinephrine, decreased TH activity in adrenal medula	*94*
CYP4A1	SHR	Liposome	iv	Decrease	Decreased sensitivity to constrictor action of phenylephrine	*103*
Thyrotropin	SHR	Oligonucleotide	it	Decrease		*106*

*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_1R 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

Table 3
Major Pros and Cons of Delivery Methods

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 intregration
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, arge payload, infects dividing and nondividing cells	Random integration

AAV, adeno-associated virus.

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 nondividing 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

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_1R in hypertensive rat models. Use of rAAV containing AT_1R-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_2R 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_2R and cardiac hypertrophy, and suggest that the AT_2R do antagonize effects mediated by the AT_1R.

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 AT_1R-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. Kantachuvesiri 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 AT_1R *(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 gene-specific 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 AT_1R) 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 AT_1R 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 α/β or α/γ heterodimers, and is involved in intracellular protein trafficking, Ca^{2+} mobilization, and the phosphorylation state of certain kinases *(208)*. Genetic variations in α-adducin have been associated with primary human hypertension and the BP phenotype in Milan hypertensive rats *(25,209–211)*. Polymorphisms of the β and γ subunits may contribute to BP variation, especially when associated with α-adducin polymorphisms, consistent with the notion that biological activity of adducin is dependent on α/β or α/γ heterodimers *(25,210,211)*.

A role for adducin in BP regulation has been further supported by the demonstration of higher BP in β-adducin deficient mice than in wild-type mice *(212)*. We have identified a 22% decrease in γ-adducin (*Add3*) expression in SHR neurons compared with WKY neuronal cultures using gene expression profiling. Real-time RT-PCR confirmed the decrease in γ-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 γ-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 γ-adducin transcript and protein levels, indicating a regulation of expression by Ang II. These observations demonstrate a decrease in γ-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 γ-adducin inhibition on the firing rate of neuronal cultures. Using intracellular delivery of γ-adducin-specific antibodies to inhibit γ-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 γ-adducin; however, in contrast with the genetic models of hypertension, there was no change in brainstem γ-adducin. Neuronal cultures from the WKY strain of rats, when incubated with Ang II, resulted in a 60% decrease in the neuronal γ-adducin *(213)*. Decreased γ-adducin may therefore contribute to augmented basal neuronal firing rate in cardiovascular-regulatory brain areas of hypertensive animals. Because central γ-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 AT_1R *(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/AT_1R-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 AT_1R 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 AT_1R 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 AT_1R 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 AT_2R *(229,230)*. This receptor, like the AT_1R, is a G protein-coupled receptor with seven transmembrane regions *(229–233)*. Although similar in size (363 amino acids for AT_2R and 359 amino acids for AT_1R), there is only 34% homology between the two receptor subtypes. The AT_2R 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 AT_2Rs oppose those of the AT_1R *(229–233)*. AT_2R knockout models

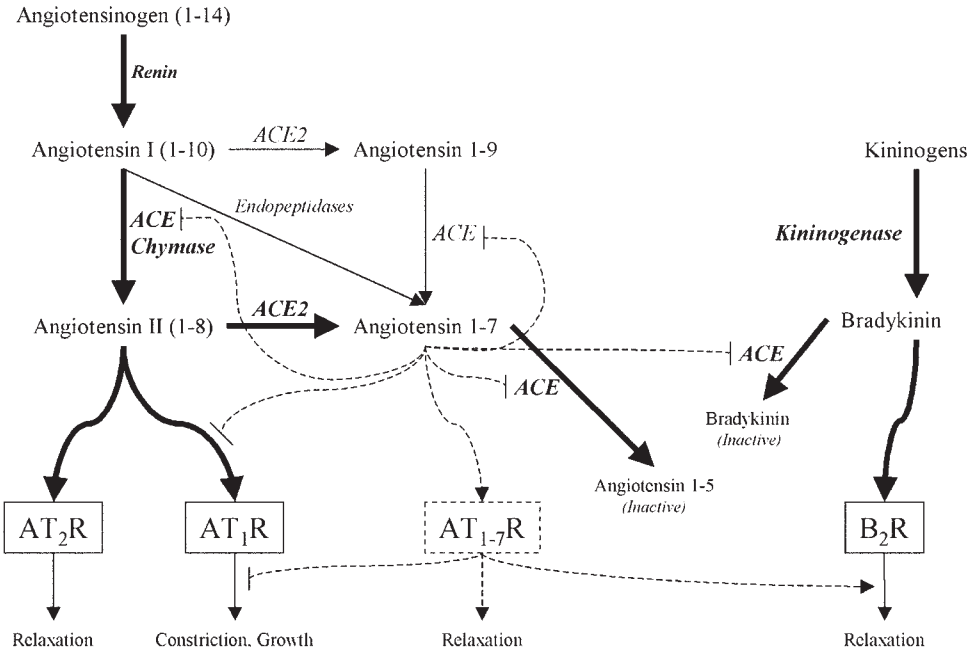

Fig. 1 The renin–angiotensin system. ACE, angiotensin-coverting enzyme; AT_1R, angiotensin type I receptors; AT_2R, angiotension type II receptors; B_2R, bradykinin type II receptors.

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_2R inhibits cardiac hypertrophy in the SHR *(59)* and that underexpression of the AT_2R can elevate BP in Sprague-Dawley animals *(80)*. The recent finding that the AT_2R 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_2R *(59)* or underexpression of the AT_1R *(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.

REFERENCES

1. Deshmukh R, Smith A, Lilly LS. Hypertension. In: Pathophysiology of heart disease. (Lilly LS, ed.). 1998; Williams & Wilkins, Baltimore, MD: 267–288.
2. American Heart Association Heart Disease and Stroke Statistics, 2004. Update Dallas, TX; American Heart Association, 2003. www.americanheart.org/statistics/index.html
3. Sowers, JR. Is hypertension an insulin-resistant state? Metabolic changes associated with hypertension and antihypertensive therapy. Am Heart J 1991;122:932–935.
4. Ferrannini E, Natali A. Essential hypertension, metabolic disorders, and insulin resistance. Am Heart J 1991;4:1274–1282.
5. Reaven GL. Insulin resistance, hyperinsulinemia, and hypertriglyceridemia in the etiology and clinical course of hypertension. Am J Med 1991;90(suppl 2A):7S–12S.
6. Cusi D, Barlassina C, Taglietti MV. Genetics of human arterial hypertension. J Nephrol 2003;16:609–615.
7. Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet 1997;349:1436–1442.
8. Stamler J, Stamler R, Neaton JD. Blood pressure, systolic and diastolic, and cardiovascular risks. Arch Intern Med 1993;153:598–615.
9. Whelton PK. Epidemiology of hypertension. Lancet 1994;334:101–106.
10. Devereux RB, Pickering TG, Harshfield GA, et al. Left ventricular hypertrophy in patients with hypertension: importance of blood pressure response to regularly recurring stress. Circulation 1983;68:470–476.
11. Wang X, Ren B, Liu S, Sentex E, Tappia PS, Dhalla NS. Characterization of cardiac hypertrophy and heart failure due to volume overload in the rat. J Appl Physiol 2003;94:752–763.
12. de Simone G, Pasanisi F, Contaldo F. Link of nonhemodynamic factors to hemodynamic determinants of left ventricular hypertrophy. Hypertension 2001;38:13–18.
13. Chobanian AV, Bakris GL, Black HR, et al., and the national high blood pressure educational program coordinating committee. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure. Hypertension 2003;42:1206–1252.
14. Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure: The sixth report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure. Arch Intern Med 1997;157:2413–2446.
15. Joffres MR, Ghadirian P, Fodor JG, Petrasovits A, Cockalingam A, Hamet P. Awareness, treatment, and control of hypertension in Canada. Am J Hypertens 1997;10:1097–1102.
16. Wolf-Maier K, Cooper RS, Kramer H, et al. Hypertension treatment and control in five European countries, Canada, and the United States. Hypertension 2004;43:10–17.

17. Liu R, Persson AE. Angiotensin II stimulates calcium and nitric oxide release from Macula densa cells through AT1 receptors. Hypertension 2004;43:649–653.
18. Zhou MS, Jaimes EA, Raij L. Inhibition of oxidative stress and improvement of endothelial function by amlodipine in angiotensin II-infused rats. Am J Hypertens 2004;17:167–171.
19. Higashi M, Shimokawa H, Hattori T, et al. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. Circ Res 2003;93:767–775.
20. Yamakawa T, Tanaka S, Kamei J, Kadonosono K, Okuda K. Phosphatidylinositol 3-kinase in angiotensin II-induced hypertrophy of vascular smooth muscle cells. Eur J Pharmacol 2003;478:39–46.
21. Yavuz D, Koc M, Toprak A, et al. Effects of ACE inhibition and AT1-receptor antagonism on endothelial function and insulin sensitivity in essential hypertensive patients. J Renin Angiotensin Aldosterone Syst 2003;4:197–203.
22. Henriksen EJ, Jacob S. Angiotensin converting enzyme inhibitors and modulation of skeletal muscle insulin resistance. Diabetes Obes Metab 2003;5:214–222.
23. Levesque S, Moutquin JM, Lindsay C, Roy M-C, Rousseau. Implications of an AGT halotype in multigene association study with pregnancy hypertension. Hypertension 2004;43:71–78.
24. Sethi AA, Nordestgaard BG, Agerholm-Larsen B, Frandsen E, Jensen G, Tybjaerg-Hansen A. Angiotensinogen polymorphisms and elevated blood pressure in the general population. Hypertension 2001;37:875–881.
25. Clark CJ, Davies E, Anderson NH, et al. α-adducin and angiotensin I-converting enzyme polymorphisms in essential hypertension. Hypertension 2000;36:990–994.
26. Tsai C-T, Fallin D, Chiang F-T, et al. Angiotensinogen gene haloytpe and hypertension. Interactions with ACE gene I Allele. Hypertension 2003;41:9–15.
27. Ueda S, Elliot HL, Morton JJ, Connell JMC. Enhanced pressor response to angiotensin I in normotensive men with the deletion genotype (DD) for angiotensin converting enzyme. Hypertension 1995;25:1266–1269.
28. Jeunemaitre X, Soubrier F, Kotelevtsev YV, et al. Molecular basis of human hypertension: role of angiotensinogen. Cell 1992;71:169–180.
29. Zee RY, Lou YK, Griffiths LR, Morris BJ. Association of a polymorphism of the angiotensin 1-converting enzyme gene with essential hypertension. Biochem Biophys Res Commun 1992;184:9–15.
30. Bonnardeaux A, Davies E, Jeunemaitre X, et al. Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. Hypertension 1994;24:63–69.
31. Qin YJ, Zhang JF, Wei YJ, Ding JF, Chen KH, Tang J. Gene suture—a novel method for intramuscular gene transfer and its application in hypertension therapy. Life Sci 1999;65:2193–203.
32. Lin KF, Chao J, Chao L. Atrial natriuretic peptide gene delivery reduces stroke-induced mortality rate in Dahl salt-sensitive rats. Hypertension 1999;33(1 Pt 2):219–224.
33. Lin KF, Chao J, Chao L. Atrial natriuretic peptide gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in salt-sensitive rats. Hum Gene Ther 1998;9:1429–1438.
34. Lin KF, Chao J, Chao L. Human atrial natriuretic peptide gene delivery reduces blood pressure in hypertensive rats. Hypertension 1995;26(6 Pt 1):847–853.
35. Wang C, Dobrzynski E, Chao J, Chao L. Adrenomedullin gene delivery attenuates renal damage and cardiac hypertrophy in Goldblatt hypertensive rats. Am J Physiol Renal Physiol 2001;280:F964–F971.
36. Zhang JJ, Yoshida H, Chao L, Chao J. Human adrenomedullin gene delivery protects against cardiac hypertrophy, fibrosis, and renal damage in hypertensive Dahl salt-sensitive rats. Hum Gene Ther 2000;11:1817–1827.
37. Dobrzynski E, Wang C, Chao J, Chao L. Adrenomedullin gene delivery attenuates hypertension, cardiac remodeling, and renal injury in deoxycorticosterone acetate-salt hypertensive rats. Hypertension 2000;36:995–1001.
38. Chao J, Jin L, Lin KF, Chao L. Adrenomedullin gene delivery reduces blood pressure in spontaneously hypertensive rats. Hypertens Res 1997;20:269–277.
39. Hirooka Y, Sakai K, Kishi T, Ito K, Shimokawa H, Takeshita A. Enhanced depressor response to endothelial nitric oxide synthase gene transfer into the nucleus tractus solitarii of spontaneously hypertensive rats. Hypertens Res 2003;26:325–331.
40. Alexander MY, Brosnan MJ, Hamilton CA, et al. Gene transfer of endothelial nitric oxide synthase but not Cu/Zn superoxide dismutase restores nitric oxide availability in the SHRSP. Cardiovascular Res 2000;47:609–617.
41. Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. Hypertension 1997;30(3 Pt 1):307–313.

42. Tai MH, Hsiao M, Chan JY, et al. Gene delivery of endothelial nitric oxide synthase into nucleus tractus solitarii induces biphasic response in cardiovascular functions of hypertensive rats. Am J Hypertens 2004;17:63–70.
43. Zhao C, Wang P, Xiao X, et al. Gene therapy with human tissue kallikrein reduces hypertension and hyperinsulinemia in fructose-induced hypertensive rats. Hypertension 2003;42:1026–1033.
44. Emanueli C, Salis MB, Stacca T, et al. Rescue of impaired angiogenesis in spontaneously hypertensive rats by intramuscular human tissue kallikrein gene transfer. Hypertension 2001;38:136–141.
45. Wolf WC, Yoshida H, Agata J, Chao L, Chao J. Human tissue kallikrein gene delivery attenuates hypertension, renal injury, and cardiac remodeling in chronic renal failure. Kidney Int 2000;58:730–739.
46. Zhang JJ, Wang C, Lin KF, Chao L, Chao J. Human tissue kallikrein attenuates hypertension and secretes into circulation and urine after intramuscular gene delivery in hypertensive rats. Clin Exp Hypertens 1999;21:1145–1160.
47. Chao J, Zhang JJ, Lin KF, Chao L. Adenovirus-mediated kallikrein gene delivery reverses salt-induced renal injury in Dahl salt-sensitive rats. Kidney Int 1998;54:1250–1260.
48. Yayama K, Wang C, Chao L, Chao J. Kallikrein gene delivery attenuates hypertension and cardiac hypertrophy and enhances renal function in Goldblatt hypertensive rats. Hypertension 1998;31:1104–1110.
49. Chao J, Zhang JJ, Lin KF, Chao L. Human kallikrein gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in Dahl salt-sensitive rats. Hum Gene Ther 1998;9:21–31.
50. Jin L, Zhang JJ, Chao L, Chao J. Gene therapy in hypertension: adenovirus-mediated kallikrein gene delivery in hypertensive rats. Hum Gene Ther 1997;8:1753–1761.
51. Chao J, Yang Z, Jin L, Lin KF, Chao L. Kallikrein gene therapy in newborn and adult hypertensive rats. Can J Physiol Pharmacol 1997;75:750–756.
52. Chen LM, Chao L, Chao J. Adenovirus-mediated delivery of human kallistatin gene reduces blood pressure of spontaneously hypertensive rats. Hum Gene Ther 1997;8:341–347.
53. Chao J, Jin L, Chen LM, Chen VC, Chao L. Systemic and portal vein delivery of human kallikrein gene reduces blood pressure in hypertensive rats. Hum Gene Ther 1996;7:901–911.
54. Xiong W, ChaoJ, Chao L. Muscle delivery of human kallikrein gene reduced blood pressure in hypertensive rats. Hypertension 1995;25(4 Pt 2):715–719.
55. Wang C, Chao L, Chao J. Muscle delivery of human kallikrein gene reduces blood pressure in hypertensive rats. Hypertension 1995;25(4 Pt 2):715–719.
56. Chu Y, Iida S, Lund DD, et al. Gene transfer of extracellular superoxide dismutase reduces arterial pressure in spontaneously hypertensive rats: role of heparin-binding domain. Circ Res 2003;92:461–468.
57. Fennell JP, Brosnan MJ, Frater AJ, et al. Adenovirus-mediated overexpression of extracellular superoxide dismutase improves endothelial dysfunction in a rat model of hypertension. Gene Ther 2002;9:110–117.
58. Sabaawy HE, Zhang F, Nguyen X, et al. Human heme oxygenase-1 gene transfer lowers blood pressure and promotes growth in spontaneously hypertensive rats. Hypertens 2001;38:210–215.
59. Metcalfe BL, Huentelman MJ, Parilak LD, et al. Prevention of cardiac hypertrophy by angiotensin II type 2 receptor gene transfer. Hypertension 2004;43:1233–1238.
60. Chen LG, Qu Y, Peng WZ, Wang YQ, Xiao J, Wang ZR. Encapsulated ANP cDNA transfection cells attenuate hypertension in hypertensive rats. Space Med Eng (Beijing) 2003;16:77–78.
61. Li T, Liang H, Lu G, Shi R, Lu S. Hypotensive effect of encapsulated genetically engineered fibroblasts expressing mutant atrial natriuretic peptide in hypertensive rats. Zhonghua Yi Xue Za Zhi 2002;82:1086–1089.
62. Reaves PY, Gelband CH, Wang H, et al. Permanent cardiovascular protection from hypertension by the AT(1) receptor antisense gene therapy in hypertensive rat offspring. Circ Res 1999;85:e44–e50.
63. Martens JR, Reaves PY, Lu D, et al. Prevention of renovascular and cardiac pathophysiological changes in hypertension by angiotensin II type 1 receptor antisense gene therapy. Proc Natl Acad Sci USA 1998;3;95:2664–2669.
64. Lu D, Raizada MK, Iyer S, Reaves P, Yang H, Katovich MJ. Losartan versus gene therapy: chronic control of high blood pressure in spontaneously hypertensive rats. Hypertension 1997;30(3 Pt 1):363–370.
65. Iyer SN, Lu D, Katovich MJ, Raizada MK. Chronic control of high blood pressure in the spontaneously hypertensive rat by delivery of angiotensin type 1 receptor antisense. Proc Natl Acad Sci USA 1996;93:9960–9965.
66. Wang H, Katovich MJ, Gelband CH, Reaves PY, Phillips MI, Raizada MK. Sustained inhibition of angiotensin I-converting enzyme (ACE) expression and long-term antihypertensive action by virally mediated delivery of ACE antisense cDNA. Circ Res 1999;85:614–622.

67. Katovich MJ, Reaves PY, Francis SC, Pachori AS, Wang HW, Raizada MK. Gene therapy attenuates the elevated blood pressure and glucose intolerance in an insulin-resistant model of hypertension. J Hypertens 2001;19:1553–1558.
68. Henriksen EJ, Jacob S. Angiotensin converting enzyme inhibitors and modulation of skeletal muscle insulin resistance. Diabetes Obes Metab 2003;5:214–222.
69. Katovich MJ, Pachori A. Effects of inhibition of the renin-angiotensin system on the cardiovascular actions of insulin. Diabetes Obes Metab 2000;2:3–14.
70. Dahlof B, Devereux RB, Kjeldsen SE, et al., LIFE Study Group. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002;359:995–1003.
71. Lindholm LH, Ibsen H, Dahlof B, et al., LIFE Study Group. Cardiovascular morbidity and mortality in patients with diabetes in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002;359:1004–1010.
72. Lindholm LH, Ibsen H, Borch-Johnsen K, et al., for the LIFE study group. Risk of new onset diabetes in the Losartan Intervention For Endpoint reduction in hypertension study. J Hypertens 2002;20:1879–1886.
73. Katovich MJ, Gelband CH, Reaves P, Wang HW, Raizada MK. Reversal of hypertension by angiotensin II type 1 receptor antisense gene therapy in the adult SHR. Am J Physiol 1999;277(3 Pt 2):H1260–H1264.
74. Phillips MI, Mohuczy-Dominiak D, Coffey M, et al. Prolonged reduction of high blood pressure with an in vivo, nonpathogenic, adeno-associated viral vector delivery of AT1-R mRNA antisense. Hypertension 1997;29(1 Pt 2):374–380.
75. Phillips MI. Antisense inhibition and adeno-associated viral vector delivery for reducing hypertension. Hypertension 1997;29(1 Pt 2):177–187.
76. Phillips MI. Gene therapy for hypertension: sense and antisense strategies. Expert Opin Biol Ther 2001;1:655–662.
78. Phillips MI. Gene therapy for hypertension: the preclinical data. Hypertension 2001;38(3 Pt 2):543–548.
79. Moore AF, Heiderstadt NT, Huang E, et al. Selective inhibition of the renal angiotensin type 2 receptor increases blood pressure in conscious rats. Hypertension 2001;37:1285–1281.
80. Wang H-W, Gallinat S, Li H-W, Sumners C, Raizada MK, Katovich MJ. Elevated blood pressure in normotensive rats produced by "knockdown" of the angiotensin type 2 receptor. Exp Physiol 2004;89:313–322.
81. Tomita N, Morishita R, Higaki J, et al. Transient decrease in high blood pressure by in vivo transfer of antisense oligodeoxynucleotides against rat angiotensinogen. Hypertension 1995;26:131–136.
82. Wielbo D, Simon A, Phillips MI, Toffolo S. Inhibition of hypertension by peripheral administration of antisense oligodeoxynucleotides. Hypertension 1996;28:147–151.
83. Phillips MI, Wielbo D, Gyurko R. Antisense inhibition of hypertension: a new strategy for renin-angiotensin candidate genes. Kid Int 1994;46:1554–1556.
84. Gurko R, Wielbo D, Phillips MI. Antisense inhibition of AT1 receptor mRNA and angiotensinogen m RNA in the brain of spontaneously hypertensive rats reduces hypertension of neurogenic origin. Regul Pept 1993;49:167–174.
85. Galderisi U, Cascino A, Giordano A. Antisense oligonucleotides as therapeutic agents. J Cell Physiol 1999;181:251–257
85a. Lavrovshy Y, Chen S, Roy AK. Therapeutic potential and mechanism of action of oligonucleotides and ribozymes. Biochem Mol Med 1997;62:11–22.
86. Neckers L, Whitesell L, Rosolen A, Geselowitz DA. Antisense inhibition of oncogene expression. Crit Rev Oncogen 1992;3:175–231.
87. Kagiyama S, Kagiyama T, Phillips MI. Antisense oligonucleotides strategy in the treatment of hypertension. Curr Opin Mol Ther 2001;3:258–264.
88. Phillips MI, Galli SM, Mehta JL. The potential role of antisense oligodeoxynucleotide therapy for cardiovascular disease. Drugs 2000;60:239–248.
89. Phillips MI. Somatic gene therapy for hypertension. Braz J Med Biol Res 2000;33:715–721.
90. Zhang YC, Bui JD, Shen L, Phillips MI. Antisense inhibition of beta(1)-adrenergic receptor mRNA in a single dose produces a profound and prolonged reduction in high blood pressure in spontaneously hypertensive rats. Circulation 2000;15;101:682–688.
91. Reaves PY, Beck CR, Wang HW, Raizada MK, Katovich MJ. Endothelial-independent prevention of high blood pressure in L-NAME-treated rats by angiotensin II type I receptor antisense gene therapy. Exp Physiol 2003;88:467–473.

92. Kagiyama S, Qian K, Kagiyama T, Phillips MI. Antisense to epidermal growth factor receptor prevents the development of left ventricular hypertrophy. Hypertension 2003;41(3 Pt 2):824–829.
93. Pachori AS, Numan MT, Ferrario CM, Diz DM, Raizada MK, Katovich MJ. Blood pressure-independent attenuation of cardiac hypertrophy by AT(1)R-AS gene therapy. Hypertension 2002;39:969–975.
94. Kumai T, Tateishi T, Tanaka M, Watanabe M, Shimizu H, Kobayashi S. Tyrosine hydroxylase antisense gene therapy causes hypotensive effects in the spontaneously hypertensive rats. J Hypertens 2001;19:1769–1773.
95. Clare Zhang Y, Kimura B, Shen L, Phillips MI. New beta-blocker: prolonged reduction in high blood pressure with beta(1) antisense oligodeoxynucleotides. Hypertension 2000;35(1 Pt 2):219–224.
96. Kimura B, Mohuczy D, Tang X, Phillips MI. Attenuation of hypertension and heart hypertrophy by adeno-associated virus delivering angiotensinogen antisense. Hypertension 2001;37(2 Part 2):376–380.
97. Makino N, Sugano M, Ohtsuka S, Sawada S, Hata T. Chronic antisense therapy for angiotensinogen on cardiac hypertrophy in spontaneously hypertensive rats. Cardiovasc Res 1999;44:543–548.
98. Cuevas P, Garcia-Calvo M, Carceller F, et al. Correction of hypertension by normalization of endothelial levels of fibroblast growth factor and nitric oxide synthase in spontaneously hypertensive rats. Proc Natl Acad Sci USA 1996;93:11,996–12,001.
99. Pachori AS, Wang H, Gelband CH, Ferrario CM, Katovich MJ, Raizada MK. Inability to induce hypertension in normotensive rat expressing AT(1) receptor antisense. Circ Res. 2000;86:1167–1172.
100. Tang X, Mohuczy D, Zhang YC, Kimura B, Galli SM, Phillips MI. Intravenous angiotensinogen antisense in AAV-based vector decreases hypertension. Am J Physiol. 1999;277(6 Pt 2):H2392–H2399.
101. Peng JF, Kimura B, Fregly MJ, Phillips MI. Reduction of cold-induced hypertension by antisense oligodeoxynucleotides to angiotensinogen mRNA and AT1 receptor mRNA in brain and blood. Hypertension 1998;31:1317–1323.
102. Galli SM, Phillips MI. Angiotensin II AT(1A) receptor antisense lowers blood pressure in acute 2-kidney, 1-clip hypertension. Hypertension 2001;38:674–678.
103. Wang MH, Zhang F, Marji J, Zand BA, Nasjletti A, Laniado-Schwartzman M. CYP4A1 antisense oligonucleotide reduces mesenteric vascular reactivity and blood pressure in SHR. Am J Physiol Regul Integr Comp Physiol 2001;280:R255–R261.
104. Yamakawa H, Phillips MI, Saavedra JM. Intracisternal administration of angiotensin II AT1 receptor antisense oligodeoxynucleotides protects against cerebral ischemia in spontaneously hypertensive rats. Regul Pept 2003;111:117–122.
105. Kagiyama S, Varela A, Phillips MI, Galli SM. Antisense inhibition of brain renin angiotensin system decreases blood pressure in chronic 2-kidney, 1-clip hypertensive rats. Hypertension 2001;37:371–375.
106. Suzuki S, Pilowsky P, Minson J, et al J. Antisense to thyrotropin releasing hormone receptor reduces arterial blood pressure in spontaneously hypertensive rats. Circ Res 1995;77:679–683.
107. Hayashi I, Majima M, Fujita T, et al. In vivo transfer of antisense oligonucleotide against urinary kininase blunts deoxycorticosterone acetate-salt hypertension in rats. Br J Pharmacol 2000;131:820–806.
108. Kagiyama S, Eguchi S, Frank GD, Inagami T, Zhang YC, Phillips MI. Angiotensin II-induced cardiac hypertrophy and hypertension are attenuated by epidermal growth factor receptor antisense. Circulation 2002;106:909–912.
109. Wivel NA, Wilson JM. Methods of gene delivery. Hematol Oncol Clin North Am 1998;12:483–501
110. Mann MJ, Morishita R, Gibbons GH, vonderLeyen HE, Dzau VJ. DNA transfer into vascular smooth muscle using fusigenic Sendai virus (HJV) liposomes. Mol Cell Biochem 1997;172:2–12.
111. Lawrie A, Brisken AF, Francis SE, et al. Ultrasound enhances reporter gene expression after transfection of vascular cells in vitro. Circulation 1999;99:2617–2620.
112. Parkes R, Meng QH, Siapati KE, McEwan JR, Hart SL. High efficiency transfection of porcine vascular cells in vitro with a synthetic vector system. J Gene Med 2002;4:292–299.
113. Kovesdi I, Brough DE, Bruder JT, Wickham TJ. Adenoviral vectors for gene transfer. Curr Opin Biotechnol 1997;8:583–589.
114. Lu D, Yang H, Raizada, MK. Attenuation of ANG II actions by adenovirus delivery of AT1 receptor antisense in neurons and SMC. Am J Physiol 1998;274:H719–H727.
115. Zhang LQ, Mei YF, Wadell G. Human adenovirus serotypes 4 and 11 show higher binding affinity and infectivity for endothelial and carcinoma cell lines than serotype 5. J Gen Virol 2003;84(Pt 3):687–695.
116. Harari OA, Wickham TJ, Stocker CJ, et al. Targeting an adenoviral gene vector to cytokine-activated vascular endothelium via E-selectin. Gene Ther 1999;6:801–807.
117. Flotte TR, Carter BJ. Adeno-associated virus vectors for gene therapy. Gene Ther 1995;2:357–362.
118. Hallek, M. Wendtner CM. Recombinant adeno-associated virus (rAAV) vectors for somatic gene therapy: recent advances and potential clinical applications. Cytokines Mol. Ther. 1996;2:69–79.

119. Langer JC, Klotman ME, Hanss B, et al. Adeno-associated virus gene transfer into renal cells: potential for in vivo gene delivery. Exp Nephrol 1998;6:189–194.
120. Flotte T, Carter B, Conrad C, et al. Phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. Hum Gene Ther 1996;10;7:1145–1159.
121. Guntaka RV, Swamynathan SK. Retroviral vectors for gene therapy. Indian J Exp Biol 1998;36:359–345.
122. Gardon M, Raizada MK, Katovich MJ, Berecek KH, Gelband CH. Gene therapy for hypertension and restenosis. J Renin Angiotensin Aldosterone Syst 2000;1:211–216.
123. Rubinson DA, Dillon CP, Kwiatkowski AV, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 2003;33:401–406.
124. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci USA 1996;93:11,382–11,388.
125. Huentelman MJ, Reaves PY, Katovich MJ, Raizada MK. Large-scale production of retroviral vectors for systemic gene delivery. Methods Enzymol 2002;346:562–573.
126. Coleman JE, Huentelman MJ, Kasparov S, et al. Efficient large-scale production and concentration of HIV-1-based lentiviral vectors for use in vivo. Physiol Genomics 2002;12:221–228.
127. Ribault S, Neuville P, Mechine-Neuville A, et al. Chimeric smooth muscle-specific enhancer/promoters: valuable tools for adenovirus-mediated cardiovascular gene therapy. Circ Res 2001;88:468–475.
128. Hoggatt AM, Simon GM, Herring BP. Cell-specific regulatory modules control expression of genes in vascular and visceral smooth muscle tissues. Circ Res 2002;91:1151–1159.
129. Franz WM, Rothmann T, Frey N, Katus HA. Analysis of tissue-specific gene delivery by recombinant adenoviruses containing cardiac-specific promoters. Cardiovasc Res 1997;35:560–566.
130. Wang Q, Sigmund CD, Lin JJ. Identification of cis elements in the cardiac troponin T gene conferring specific expression in cardiac muscle of transgenic mice. Circ Res 2000;86:478–484.
131. Navarro V, Millecamps S, Geoffroy MC, et al. Efficient gene transfer and long-term expression in neurons using a recombinant adenovirus with a neuron-specific promoter. Gene Ther 1999;6:1884–1892.
132. Carmeliet P, Lampugnani MG, Moons L, et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 1999;98:147–157.
133. Wang Y, O'Malley BW Jr, Tsai SY, O'Malley BW. A regulatory system for use in gene transfer. Proc Natl Acad Sci USA 1994;91:8180–8184.
134. Pollock R, Issner R, Zoller K, Natesan S, Rivera VM, Clackson T. Delivery of a stringent dimerizer-regulated gene expression system in a single retroviral vector. Proc Natl Acad Sci USA 2000;97:13,221–13,226.
135. No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc Natl Acad Sci USA 1996;16;93:3346–3351.
136. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995;268:1766–1769.
137. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992;89:5547–5551.
138. Gossen M, Bujard H. Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. Nucleic Acids Res 1993;21:4411–4412.
139. Efrat S, Fusco-DeMane D, Lemberg H, Emran OL, Wang X. Conditional transformation of a pancreatic β-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. PNAS 1995;92:3576–3580.
140. Corbel SY, Rossi FMV. Latest developments and in vivo use of the Tet system: ex vivo and in vivo delivery of tetracycline-regulated genes. Curr Opin Biotechnol 2002;13:448–452.
141. Zhu Z. Ma B, Homer RJ, Zheng T, Elias JA. Use of the tetracycline controlled transcriptional silencer (tTS) to eliminate transgene leak in inducible overexpression transgenic mice. J Biol Chem 2001;276:25,222–25,229.
142. Imhof MO, Chatellard P, Mermod N. A regulatory network for the efficient control of transgene expression. J Genetic Med 2000;2:107–116.
143. Baron U, Gossen M, Bujard H. Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential Nucleic Acids Res 1997;25:2723–2729.
144. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. PNAS 2000;97:7963–7968.

145. Robertson A, Perea J, Tolmachova T, Thomas PK, Huxley C. Effects of mouse strain, position of integration and tetracycline analogue on the tetracycline conditional system in transgenic mice. Gene 2002;282:65–74.
146. Raizada MK, Francis SC, Wang H, Gelband CH, Reaves PY, Katovich MJ. Targeting of the renin-angiotensin system by antisense gene therapy: a possible strategy for the long-term control of hypertension. J Hypertens 2000;18:353–362.
147. Pachori AS, Huentelman MJ, Francis SC, Gelband GH, Katovich MJ, Raizada MK. The future of hypertension therapy: sense, antisense or nonsense? Hypertension 2001;37:357–364.
148. Teng PI, Dichiara MR, Komuves LG, Abe K, Quertermous T, Topper, JN. Inducible and selective transgene expression in murine vascular endothelium. Physiol. Genomics 2002;11:99–107.
149. Ju H, Gros R, You X, Tsang S, Husain M, Rabinovitch M. Conditional and targeted overexpression of vascular chymase causes hypertension in transgenic mice. Proc Natl Acad Sci USA 2001;98:7469–7474.
150. Kantachuvesiri S, Fleming S, Peters J, et al. Controlled hypertension: a transgenic toggle switch reveals differential mechanisms underlying vascular disease. J Biol Chem 2001;276:36,727–36,733.
151. Hoppe UC, Marban E, Johns DC, Adenovirus-mediated inducible gene expression *in vivo* by a hybrid ecdysone receptor. Mol Ther 2000;1:159–164.
152. Madan A, Curtin PT. A 24-base-pair sequence 3' to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. Proc Natl Acad Sci USA 1993;90:3928–3932.
153. Beck I, Ramirez S, Weinmann R, Caro J. Enhancer element at the 3'-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. J Biol Chem. 1991;266:15,563–15,566.
154. Eckhart AD, Yang N, Xin X, Faber JE. Characterization of the alpha1B-adrenergic receptor gene promoter region and hypoxia regulatory elements in vascular smooth muscle. Proc Natl Acad Sci USA 1997;94:9487–9492.
155. Webster KA, Gunning P, Hardeman E, Wallace DC, Kedes L. Coordinate reciprocal trends in glycolytic and mitochondrial transcript accumulations during the in vitro differentiation of human myoblasts. J Cell Physiol. 1990;142:566–573.
156. Semenza GL, Jiang BH, Leung SW, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 1996;271:32,529–32,537.
157. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604–4613.
158. Minchenko A, Salceda S, Bauer T, Caro J. Hypoxia regulatory elements of the human vascular endothelial growth factor gene. Cell Mol Biol Res 1994;40:35–39.
159. Yang BC, Phillips MI, Zhang YC, et al. Critical role of AT1 receptor expression after ischemia/reperfusion in isolated rat hearts: beneficial effect of antisense oligodeoxynucleotides directed at AT1 receptor mRNA. Circ Res 1998;83:552–559.
160. Chen H, Zhang YC, Li D, et al. Protection against myocardial dysfunction induced by global ischemia-reperfusion by antisense-oligodeoxynucleotides directed at beta(1)-adrenoceptor mRNA. J Pharmacol Exp Ther 2000;294:722–727.
161. Chen EP, Bittner HB, Akhter SA, Koch WJ, Davis RD. Myocardial recovery after ischemia and reperfusion injury is significantly impaired in hearts with transgenic overexpression of beta-adrenergic receptor kinase. Circulation 1998;98(19 Suppl):II249–II253.
162. Chen EP, Bittner HB, Davis RD, Folz RJ, Van Trigt P. Extracellular superoxide dismutase transgene overexpression preserves post ischemic myocardial function in isolated murine hearts. Cir 1996;94(9 Suppl):II412–II417.
163. Wang P, Chen H, Qin H, et al. Overexpression of human copper, zinc-superoxide dismutase (SOD1) prevents post ischemic injury. Proc Natl Acad Sci USA 1998;95:4556–4560.
164. Chen H, Mohuczy D, Li D, et al. Protection against ischemia/reperfusion injury and myocardial dysfunction by antisense-oligodeoxynucleotide directed at angiotensin-converting enzyme mRNA. Gene Ther 2001;8:804–810.
165. Divisova J, Vavrinkova H, Tutterova M, Kazdova L, Meschisvili E. Effect of ACE inhibitor captopril and L-arginine on the metabolism and on ischemia-reperfusion injury of the isolated rat heart. Physiol Res 2001;50:143–152.
166. Minchenko A, Salceda S, Bauer T, Caro J. Hypoxia regulatory elements of the human vascular endothelial growth factor gene. Cell Mol Biol Res 1994;40:35–39.
167. Vigna et al. (Vigina E, Cavalieri S, Ailles L, Geuna M, Loew R, Bujard H, Naldini L. Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. Mol Ther 2002;5:252–261.

168. Ryding ADS, Sharp MGF, Mullins JJ. Conditional transgenic technologies. J Endocrinol 2001;171:1–14.
169. Robertson A, Perea J, Tolmachova T, Thomas PK, Huxley C. Effects of mouse strain, position of integration and tetracycline analogue on the tetracycline conditional system in transgenic mice. Gene 2002;282:65–74.
170. Tang Y, Schmitt-Ott K, Qian K, Kagiyama S, Phillips MI. Vigilant vectors: adeno-associated virus with a biosensor to switch on amplified therapeutic genes in specific tissues in life-threatening diseases. Methods 2002;28:259–266.
171. Tang Y, Jackson M, Qian K, Phillips MI. Hypoxia inducible double plasmid system for myocardial ischemia gene therapy. Hypertension 2002;39(2 Pt 2):695–698.
172. Gibson, SA, Shillitoe EJ. Ribozymes. Their functions and strategies for their use. Mol Biotechnol 1997;7:125–137.
173. Shuey DJ, McCallus DE, Giordano T. RNAi: gene-silencing in therapeutic intervention. Drug Discov Today 2002;7:1040–1046.
174. Guru T. A silence that speaks volumes. Nature 2000;404:804–808.
175. Ingelbrecht I, Van Houdt H, English J, Que Q, Napoli CA. Chalcone synthase co suppression phenotypes in petunia flowers: comparison of sense and antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol Biol 1994;31:957–973.
176. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806–811.
177. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494–498.
178. Walters DK, Jelinek DF. The effectiveness of double stranded short inhibitory RNAs (siRNAs) may depend on the method of transfection. Antisense Nucleic Acid Drug Dev 2002;12:411–418.
179. Tuschl T, Borkhardt A. Small Interfering RNAs: a revolutionary tool for the analysis of gene function and gene therapy. Mol Intervent 2002;2:158–167.
180. Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenes by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci USA 2002;99:2140–2145.
181. Shen C, Buck AK, Liu X, Winkler M, Reske SN. Gene silencing by adenovirus-delivered siRNA. FEBS Lett 2003;539:111–114.
182. Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet 2002;32:107–108.
183. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue specific expression of transgenes derived from lentiviral vectors. Science 2002;295:868–872.
184. Cheng JC, Moore TB, Sakamoto KM. RNA interference and human disease. Mol Genet Metabol 2003;80:121–128.
185. Boguski MS, McIntosh MW. Biomedical informatics for proteomics. Nature 2003;422:233–237.
186. Templin MF, Stoll D, Schrenk M, Traub PC, Vohringer CF, Joos TO. Protein microarray technology. Drug Discov Today 2002;7:815–822.
187. Ward R. Familial aggregation and genetic epidemiology of blood pressure. In: Hypertension: pathophysiology, diagnosis and management. (Laragh JH, Brenner BM, eds). Raven, New York: 1990;81–100.
188. Luft FC. Hypertension as a complex genetic trait. Semin Nephrol 2002;22:115–126.
189. Zhu X, Cooper RS. Linkage disequilibrium analysis of the renin-angiotensin system genes. Curr Hypertens Rep 2003;5:40–46. .
190. Hamet P, Pausova Z, Adarichev V, Adaricheva K, Tremblay J. Hypertension: genes and environment. J Hypertens 1998;16:397–418.
191. Pravenec, M., Krenova D, Kren V, et al. Congenic strains for genetic analysis of hypertension and dyslipidemia in the spontaneously hypertensive rat. Transplant Proc 1999;31:1555–1556.
192. Rapp JP. Genetic analysis of inherited hypertension in the rat. Physiol Rev 2000;80:135–172.
193. Stoll M, Kwitek-Black AE, Cowley AW Jr, et al. New target regions for human hypertension via comparative genomics. Genome Res 2000;10:473–482.
194. Cicila GT, Garrett MR, Lee SJ, Liu J, Dene H, Rapp, JP. High-resolution mapping of the blood pressure QTL on chromosome 7 using Dahl rat congenic strains. Genomics 2001;72:51–60.
195. Cook SA, Rosenzweig A. DNA microarrays: implications for cardiovascular medicine. Circ Res 2002;91:559–564.
196. Patino WD, Mian OY, Hwang P M. Serial analysis of gene expression: technical considerations and applications to cardiovascular biology. Circ Res 2002;91:565–569.

197. Nadon R, Shoemaker J. Statistical issues with microarrays: processing and analysis. Trends Genet 2002;18:265–271.
198. Quackenbush J. Computational analysis of microarray data. Nat Rev Genet 2001;2:418–427.
199. Pravenec M, Wallace C, Aitman, TJ, Kurtz, TW. Gene Expression profiling in hypertensive research. A critical perspective. Hypertension 2003;41:3–8.
200. Cook SA, Rosenzweig A. DNA microarrays: implications for cardiovascular medicine. Circ Res 2002;91:559–564.
201. Aitman TJ, Glazier AM, Wallace CA, et al. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. Nat Genet 1999;21:76–83.
202. McBride MW, Carr FJ, Graham D, et al. Microarray analysis of rat chromosome 2 congenic strains. Hypertension 2003;41:847–853.
203. Okuda T, Sumiya T, Iwai N, Miyata T. Difference of gene expression profiles in spontaneous hypertensive rats and Wistar-Kyoto rats from two sources. Biochem Biophys Res Commun 2002;296:537–543.
204. Okuda T, Sumiya T, Mizutani K, et al. Analyses of differential gene expression in genetic hypertensive rats by microarray. Hypertens Res 2002;25:249–255.
205. Veerasingham SJ, Sellers KW, Raizada MK. Functional genomics as an emerging strategy for the investigation of central mechanisms in experimental hypertension. Prog Biophys Mol Biol 2004;84:107–123.
206. Yang H, Francis SC, Sellers K, et al. Hypertension-linked decrease in the expression of brain gamma-adducin. Circ Res 2002;91:633–639.
207. Raizada MK, Lu D, Tang W, Kurian P, Sumners C. Increased angiotensin II type-1 receptor gene expression in neuronal cultures from spontaneously hypertensive rats. Endocrinology 1993;132:1715–1722.
208. Matsuoka Y, Li X, Bennett V. Adducin: structure, function and regulation. Cell Mol Life Sci 2000;57:884–895.
209. Bianchi G, Tripodi G, Casari G, et al. Two point mutations within the adducin genes are involved in blood pressure variation. Proc Natl Acad Sci USA 1994;91;3999–4003.
210. Wang J G, Staessen JA, Barlassina C, et al. Association between hypertension and variation in the alpha- and beta- adducin genes in a white population. Kidney Int 2002;62:2152–2159.
211. Zagato L, Modica R, Florio M, et al Genetic mapping of blood pressure quantitative trait loci in Milan hypertensive rats. Hypertension 2000;36:734–739.
212. Marro ML, Scremin OU, Jordan MC, et al. Hypertension in beta-adducin-deficient mice. Hypertension 2000;36,449–453.
213. Yang H, Reaves PY, Katovich MJ, Raizada MK. Decreases in hypothalamic gamma adducin in rat models of hypertension. Hypertension 2004;43:324–328.
214. Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, Gonzalez FJ. Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. J Biol Chem. 2000;275:40,504–40,510.
215. Baudin B, Berard M, Carrier JL, Legrand Y, Drouet L. Vascular origin determines angiotensin I-converting enzyme expression in endothelial cells. Endothelium 1997;5:73–84.
216. Schulz WW, Hagler HK, Buja LM, Erdos EG. Ultrastructural localization of angiotensin I-converting enzyme (EC 3.4.15.1) and neutral metalloendopeptidase (EC 3.4.24.11) in the proximal tubule of the human kidney. Lab Invest 1988;59:789–797.
217. McKinley MJ, Albiston AL, Allen AM, et al. The brain renin-angiotensin system: location and physiological roles. Int J Biochem Cell Biol 2003;35:901–918.
218. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie, G. A human homolog of angiotensin-converting enzyme. J Biol Chem 2000;275;33,238–33,243.
219. Donoghue M, Hsieh F, Baronas E, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angtiotensin 1–9. Circ Res 2000;87:1–9.
220. Harmer D, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE2, a novel homologue of Angiotensin converting enzyme. FEBS Lett 2002;532:107–110.
221. Stegbauer J, Vonend O, Oberhauser V, Rump, LC. Effects of Angiotensin-(1–7) and other bioactive components of the renin-angiotensin system on vascular resistance and noradrenaline release in rat kidney. J Hypertens 2003;21:1391–1399.
222. Zhu Z, Zhong J, Zhu S, Liu D, van der Giet M, Tepel M. Angiotensin 1-7 inhibits angiotensin II inducted signal transduction. J Cardiovasc Pharmacol 2002;40:693–700.
223. Cesari M, Rossi GP, Pessina AC. Biological properties of the Angiotensin peptides other than Angiotensin II: implications for hypertension and other cardiovascular diseases. J Hypertens 2002;20:793–799.
224. Reaux A, Fournie-Zaluski MC, Lloren-Cortes C. Angiotensin III: a central regulator of vasopressin release and blood pressure. Trends Endocrinol Metab 2001;12:157–162.

225. Roks AJM, Henning RH. Angiotensin peptides: ready to re(de)fine the Angiotensin system? J Hypertens 2003;21:1269–1271.
226. Tom B, Dendorfer A, Danser AHJ. Bradykinin, angiotensin 1-7 and ACE inhibitors: how do they interact. Int. J Biochem Cell Biol 2003;35:792–801.
227. Allred AJ, Donoghue M, Acton S, Coffman TM. Regulation of blood pressure by the Angiotensin converting enzyme homologue ACE2. 35th annual meeting of the American Soc Nephrol Nov 1–4, 2002.
228. Donoghue M, Wakimoto H, Maguire CT, et al. Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins. J Mol Cell Cardiol 2003;35:1043–1053.
229. Chung O, Stoll M, Unger T. Physiological and pharmacological implications of AT1 versus AT2 receptors. Blood Press Suppl 1996;2:47–52.
230. Matsukawa T, Ichikawa I. Biological function of angiotensin and its receptors. Ann Rev Physiol 1997;59:395–412.
231. Gallinat S, Busche S, Raizada MK, Sumners C. The angiotensin II type 2 receptor: an enigma with multiple variations. Am J Physiol Endocrinol Metab 2000;278:E357–E374.
232. DeGasparo M, Catt K, Inagami J, Wright JW, Unger T. International Union of Pharmacology. XVIII. The Angiotensin II receptors. Pharmacol Rev 2000;52:415–472.
233. Unger T, The angiotensin type 2 receptor. Variations on an enigmatic theme. J Hypertens 1999;17:1775–1786.
234. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioral and cardiovascular effects of disrupting the angiotensin II type 2 receptor gene in mice. Nature 1995;377:744–747.
235. Ichiki T, Labosky PA, Shiota C, et al. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. Nature 1995;377:748–750.
236. Miura S, Karnik SS. Ligand-independent signals from angiotensin II type 2 receptor induce apoptosis. Embro J 2000;19:4026–4035.
237. Dzau, V.J. Tissue renin-angiotensin system in myocardial hypertrophy and failure. Arch Intern Med 1993;153:937–942.
238. Dzau, V.J. Circulating versus local renin-angiotensin system in cardiovascular homeostasis. Circulation 1988;77:(6 Pt 2):I4–I13.
239. Crockcroft JR, O'Kane KPJ, Webb DJ. Tissue angiotensin generation and regulation of vascular tone. Pharmacol Ther 1995;65:193–213.
240. Bader M, Peters J, Baltatu O, Muller DN, Luft FC, Ganten D. Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research. J Mol Med 2001;79:76–102.
241. Crockcroft JR, O'Kane KPJ, Webb DJ. Tissue angiotensin generation and regulation of vascular tone. Pharmacol Ther 1995;65:193–213.
242. Bader M, Peters J, Baltatu O, Muller DN, Luft FC, Ganten D. Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research. J Mol Med 2001;79:76–102.
243. Blaufarb IS, Sonnenblick EH. The renin angiotensin system in left ventricular remodeling. Am J Cardiol 1996;77:8C–16C.
244. Yu Z, Xu F, Huse LM, et al. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. Circ Res. 2000;87:992–998.
245. Fornage M, Hinojos CA, Nurowska BW, et al. Polymorphism in soluble epoxide hydrolase and blood pressure in spontaneously hypertensive rats. Hypertension 2000;40:485–490.
246. Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol 2002;20:1006–1010.