

HIV-1 BASED VIRAL VECTOR DEVELOPMENT FOR GENE TRANSFER
TO THE CARDIOVASCULAR SYSTEM

By

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by

Matthew J. Huentelman

This work is dedicated to my family, my spouse, my fellow lab-mates, and my friends,
without whom none of my goals could be achieved.

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Abstract of Dissertation Presented to the Graduate School
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Cardiovascular disease (CVD) is the nation's number one killer. Several pharmacotherapies exist to combat CVD however its incidence and mortality rates continue to rise. Alternative treatment options must be explored in order to provide hope for the future treatment of this disease. Gene therapy has been suggested as one such alternative option.

Gene therapy involves the transfer of therapeutic nucleic acid into diseased cell types. The vector for such gene transfer is of major consideration when approaching any disorder. Viral vectors based on HIV-1 are an up and coming class of gene transfer vehicles first utilized in 1996 and already finding their way into human clinical trials. However the HIV-1 vectors, or lentiviral vectors, are poorly characterized in almost every major organ of the CV system. Lentiviral vectors possess many of the characteristics required for effective gene therapy for CVD including the ability to accommodate large payloads of nucleic acid, transduce non-dividing cells, direct long-

term transgene expression, and evoke a minuscule immune response. However, many challenges face lentiviral vectors including questions about their safety for use in humans and technical hurdles concerning their large-scale preparation. The primary goal of this study was to further develop the lentiviral vector system and characterize its efficacy in the CV system and its ability to prevent CVD.

Novel methods were developed for the production and concentration of the vector allowing for reproducible large-scale production. Upon delivery to neonatal rats, the lentiviral vector transduced every major target organ of the rat cardiovascular system including the heart, liver, brain, kidney, and adrenal gland. The optimal vector dose was determined, and additional studies using an angiotensin II type 2 receptor transgene illustrated the ability of the vector to prevent the development of hypertrophy in a spontaneous model of hypertension in the rat.

The methodology of lentiviral vector preparation was improved and its effectiveness in the CV system illustrated. Initial results using a therapeutic transgene show promise for the future study of cardiac hypertrophy. The findings here help to lay the foundation for future use of the lentiviral vector in the study and possible therapy of CVD.

CHAPTER 1

INTRODUCTION: BACKGROUND AND SIGNIFICANCE

Cardiovascular Disease and Hypertension

Importance and Impact

Cardiovascular disease (CVD) is the number one cause of mortality and morbidity in industrialized countries. An estimated 1 in 4 Americans suffer from some form of CVD and over 40% of all deaths in the United States are linked to CVD. Additionally, the economic impact on society is enormous. The National Center for Chronic Disease Prevention and Health Promotion reports that nearly \$300 billion is spent each year both managing this disease through health care costs and through lost workplace productivity. With our increasingly aging population, these statistics can only be predicted to worsen.

CVD in general encompasses many disorders including hypertension, heart disease, and atherosclerosis among others. In most cases, the treatment options for the CVD patient only serve to alleviate the symptoms not reverse or sometimes even halt the progress of the underlying disorder. As medicine advances into the next century there is great hope that new treatment options may emerge to remedy this.

The contributions of the renin-angiotensin system (RAS) in both the normal physiology of blood pressure (BP) regulation and the pathophysiology of hypertension are under intense study. Hypertension alone affects over 50 million Americans reaching epidemic proportions in the adult population where 1 in 5 have been diagnosed with the disorder. The primary symptom of this disease is chronic elevated blood pressure, but

patients often have an increased risk of stroke, heart disease, and renal damage. If left untreated, hypertension can adversely affect several major organs including the kidneys, heart, brain, vasculature, and eyes. Many antihypertensive pharmacotherapies exist; however, they primarily succeed in managing the symptoms of the disease.

The Renin-Angiotensin System

The Renin-Angiotensin System (RAS) is an endocrine system of major importance to the body with its key feature being the maintenance of body fluid homeostasis. The next section discusses the major players of the RAS individually. Figure 1-1 is a reference diagram of the RAS components discussed below.

Renin

The unusually specific aspartyl protease renin was first identified over 100 years ago by Tiegerstedt and Bergman in extracts from the kidneys of rabbits. In the circulation, renin is the rate limiting step of the RAS. The main source of the circulating enzyme is from the juxtaglomerular (JG) cells in the kidney (Gomez et al, 1990). The JG cells are modified smooth muscle cells containing the characteristic dense core secretory granules often found in other neuroendocrine cells (Hackenthal et al, 1990). However, in JG cells the “secretory granules” are atypical in appearance with a much greater similarity to the common lysosome. Therefore some researchers suggest that the JG cells do not contain the stereotypical granules found in many endocrine cells, but rather have adapted their lysosomes for the specialized role of processing and

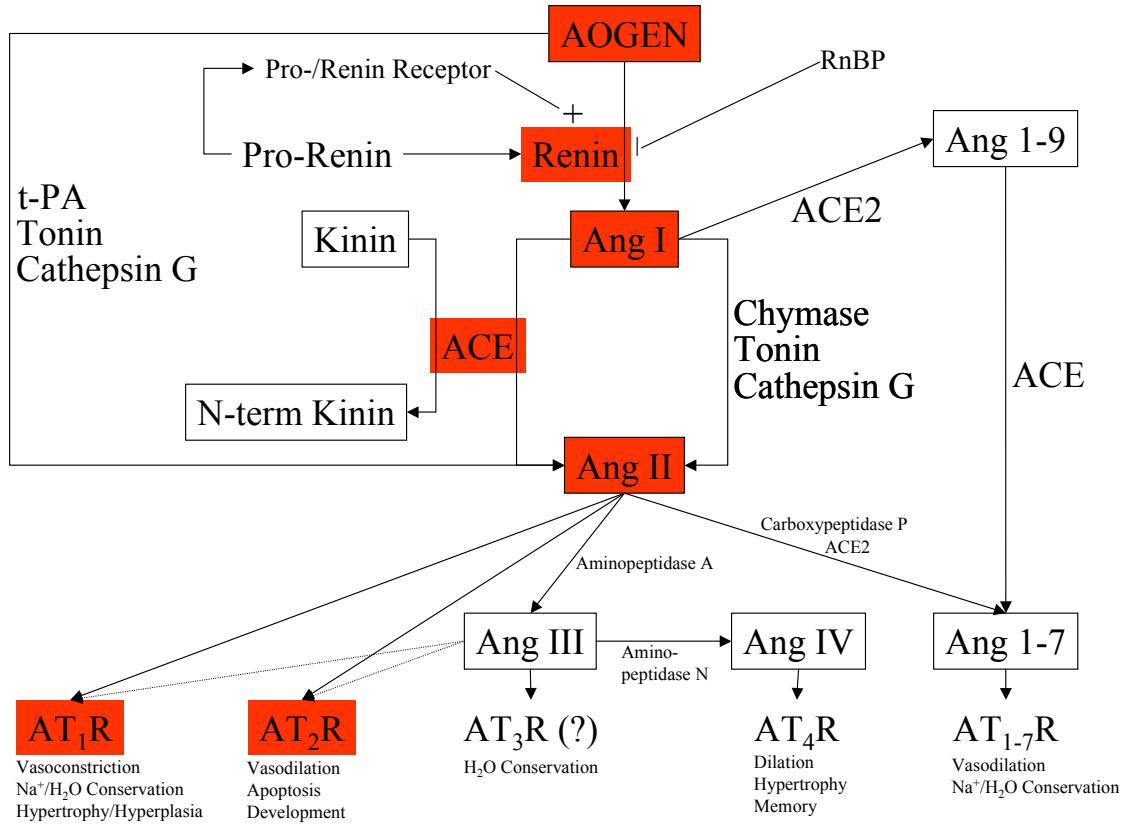


Figure 1-1. Major components of the Renin-Angiotensin System. Highlighted in red are the more thoroughly understood proteins and peptides of the system. All enzymes are written nearest an arrowed line connecting the substrate and products of the reaction they catalyze. Other components (proteins, peptides, and receptors) are listed at the tail or head of an arrow.

secuting renin (Bader and Ganten 2000). Renin is produced and secreted from these cells through the processing of its inactive precursor, prorenin, by the lysosomal protease cathepsin B. The JG cells are not the only source of renin in the organism. It has been shown that renin/prorenin is secreted from cells in the adrenal gland, heart, and brain and may therefore be assumed to participate in a local, tissue-based RAS (Dostal et al, 1992; Rong et al, 2001). Once in the circulation, renin may act on its circulating substrate, angiotensinogen.

Angiotensinogen

The 55-60 kDa globular protein angiotensinogen (AOGEN) is the single known precursor to angiotensin I synthesis. The bioavailability of AOGEN rate limits the activity of renin, and the primary source of circulating AOGEN is from the hepatocytes in the liver. In fact, within the AOGEN secreting hepatocytes a positive feedback loop has been established with the final bioactive product of the RAS, angiotensin II, acting at angiotensin II type 1 receptor sites on the cells to further upregulate AOGEN secretion (Brasier et al, 2000). Additionally, astrocytes, primarily located in the hypothalamus and brainstem nuclei, and adipocytes have been shown to secrete large amounts of AOGEN while AOGEN mRNA can be found in a wide variety of tissues and cell types in the body. This suggests that AOGEN synthesis can proceed in organ systems throughout the body as is the case with the renin enzyme and other components of the RAS.

There is no direct experimental evidence to show any intracellular processing of AOGEN, in fact the three major sources of AOGEN (hepatocytes, astrocytes, and adipocytes) lack basic cellular machinery to concentrate secretory proteins. Instead these cell types can only constitutively export the AOGEN to the extracellular fluid. The result is a circulating concentration of AOGEN of approximately $1\mu\text{M}$, essentially equal to the calculated K_m of the renin-AOGEN cleavage reaction.

The action of renin on the N-terminus of AOGEN liberates the invariant decapeptide, angiotensin I (Ang I), in addition to its globular chaperone, des(Ang I)-AOGEN. To date no direct physiological effects have been shown for AOGEN or des(Ang I)-AOGEN alone and the general consensus is that AOGEN functions purely as

an extracellular reservoir of Ang I. However, a recent publication suggests anti-angiogenic effects for both Aogen and des(Ang I)-Aogen (Corvol et al, 2003).

Angiotensin converting enzyme

The dipeptidyl carboxyl metallopeptidase, angiotensin converting enzyme (ACE), cleaves Ang I to form the vasoactive octapeptide angiotensin II (Ang II). ACE exists in both a soluble and endothelial cell bound form. The primary site of ACE action is found in the lungs, but ACE activity has been documented in several other organs including but not limited to the kidney, liver, heart, and blood vessels (Kohara et al, 1992; Balcells et al, 1997). Unlike renin, ACE is very non-selective in its mode of action. In fact, ACE has been documented to cleave substance P, luteinizing hormone releasing hormone, and the potent vasodilator bradykinin. It is also important to note that ACE action is not a rate controlling step in the RAS cascade.

The pharmacologic inhibition of ACE (ACEi) represents one of the early and most successful antihypertensive targets of the RAS. The first orally active ACE inhibitor, captopril, was reported in *Science* in 1977 (Ondetti et al, 1977). Since then, ACEi has been used to successfully treat hypertension, myocardial ischemia, and cardiac hypertrophy (Latini et al, 2000; Cuspidi et al, 2002). As touched on above, ACEi blocks both the formation of Ang II and the breakdown of bradykinin. In this manner ACEi serves to quiet the vasoconstrictive qualities of the RAS while increasing the circulating concentrations of the vasodilator bradykinin.

Along with touching off the pharmacologic crusade against hypertension, the study of ACE gene polymorphisms in the human population helped to initiate the modern day understandings of the genetic component of hypertension. The I/D ACE polymorphism was related to serum ACE concentrations. It was shown that those individuals

homozygous for the D allele presented with higher levels of serum ACE than heterozygotes or homozygous I/I patients (Rigat et al, 1990).

ACE action on Ang I drives the formation of the vasoactive octapeptide Ang II. The majority of Ang II's well studied cardiovascular (CV) actions are mediated by the type 1 receptor (AT₁R). The type 2 receptor (AT₂R) had classically been thought to only play a role during the development of the CV system due to its transient increased expression during embryogenesis, however many studies have suggested an AT₁R - antagonistic role for the AT₂R.

Angiotensin II type 1 receptor

The distinct AT₁R and AT₂R binding sites were first experimentally revealed in 1987. Through treatment with the thiol-reducing agent DTT two distinctly sensitive classes of receptors for Ang II were identified. The molecular cloning of the AT₁R in 1991 revealed it to be a part of the G-protein couple receptor (GPCR) super-family (Sasaki et al, 1991). Within two years the AT₁R was cloned from several animals including humans, rats, and mice. Also during this time a second subtype of the receptor, the AT_{1B}R, was isolated in rodents. The AT_{1B}R was found to predominate in the adrenal cortex and pituitary gland while AT_{1A}R was primarily expressed in vascular smooth muscle, liver, kidney, heart, and other organs (Kakar et al, 1992; Murphy et al, 1992). Both subtypes were found to bind Ang II with similar affinity and also couple to identical G-protein subtypes.

By GPCR family definition the AT₁R consists of seven transmembrane domains with its N-terminus located extracellularly and C-terminus intracellularly. Members of the GPCR family must also couple to cytosolic G-proteins, most commonly a complex of three G-proteins known as a heterotrimer. Three mechanisms exist for AT₁R -mediated

signal transduction: G-protein mediated, G-protein independent, and internalization (Figure 1-2).

AT₁R	AT₂R
G Protein	G Protein
Gα_{q11} or Gα_{12/13} : PLC activation, IP ₃ /DAG generation, Ca ²⁺ release, PKC activation, MAPK activation Gα_i : AC inhibition, decreased cAMP levels	Gα_i : Protein phosphatase activation (PP2A, MKP-1, SHP-1), MAPK inhibition, decreased cell growth, apoptosis, increased cGMP levels
Tyrosine Kinase	Tyrosine Kinase
JAK : STAT phosphorylation, transcriptional changes Src : PLC activation, IP ₃ /DAG generation, Ca ²⁺ release, PKC activation, MAPK activation FAK : Paxillin phosphorylation, cytoskeletal re-arrangement	No definitive evidence supporting any form of activation
Internalization	Internalization
PKC-dependent clathrin/caveolae process	No definitive evidence supporting any form of internalization

Figure 1-2. Signal transduction pathways of the AT₁R and AT₂R.

Ang II binding at the AT₁R produces IP₃ by activation of the Gα_{q11} or Gα₁₃β₁γ₃ pathways. Cytosolic loop number three has been shown to play the critical role in this action (Ohyama et al, 1992). Additionally the Gβγ subunits are known to activate phospholipase D. In fact, the AT₁R is known to mediate several varied effects depending on both cell type and distinct Gα subunit coupling. In general, the intracellular hallmarks of Ang II binding at the AT₁R and acting via G-protein mediated signal transduction are activation of phospholipase C (PLC), protein kinase C (PKC), mitogen activated protein kinase (MAPK), and a resultant release of intracellular calcium stores.

GPCRs also have the ability to transactivate growth factor receptors and the janus kinase (JAK) signal transduction activators of transcription (STAT) pathway (Wu and Cunnick 2002). This is termed the “tyrosine kinase” activity of the AT₁R. Through the activation of these membrane-bound and soluble tyrosine kinases, Ang II can exert effects on cellular proliferation and transcription (Marrero et al, 1997). The important site of action for these effects is a canonical “YIPP” motif located on the C-terminus of the molecule (Ali et al, 1997).

The C-terminus plays a critical role in rapid desensitization and internalization of the AT₁R (Hunyady et al, 2000). Upon ligand binding the receptor is phosphorylated by a PKC-dependent mechanism and internalized via clathrin and caveolae-mediated processes (Bkaily et al, 2003). It is possible that caveolae-internalized AT₁Rs can still transduce their intracellular signals. In fact, internalization of receptor into specific subcellular domains (nuclear and perinuclear) has been demonstrated in both neurons and smooth muscle cells (Yang et al, 1997; Adams et al, 1999).

Angiotensin II type 2 receptor

Type 2 receptors also belong to the GPCR superfamily and therefore exist in the membrane in a nearly identical form as the AT₁R. The AT₂R was cloned in 1993 and found to only share an approximate 30% sequence homology with the AT₁R (Kambayashi et al, 1993; Mukoyama et al, 1993). This is the lowest value known among all GPCR family subtypes. Current evidence suggests coupling to both pertussis toxin sensitive (G_{iα2} and G_{iα3}) and G_{αs} proteins. It was demonstrated that the AT₂R can couple and functionally signal after association with the G_{αs} alone (Feng et al, 2002). This suggests a novel mechanism for GPCR signaling because previously the paradigm of

GPCR members required heterotrimeric (α , β , and γ subunits) coupling prior to signal transduction.

The AT₂R is classically thought to play a role during development where it is expressed highly in the embryonic kidney and vasculature among other CV-relevant organs. In adults, the receptor is expressed in a limited number of tissues, but is up-regulated over 5-fold during times of tissue remodeling (Ohkubo et al, 1997). However, ongoing research from the past several years suggests an antagonistic role for the AT₂R on the classical AT₁R -mediated actions of Ang II. For example, the presence of AT₂R in Ang II pressor areas of the brain helps to decrease the resultant BP rise following an intracerebroventricular injection of Ang II (Li et al, 2003). Additionally, mice lacking the AT₂R have higher blood pressure compared to their wild-type littermates (Siragy et al, 1999).

Many of the intracellular signaling pathways of the AT₂R have yet to be fully characterized. However it has been shown that the AT₂R can signal through the activation of protein tyrosine (SHP-1) or protein serine/threonine (PP2A) phosphatases to inactivate the extracellular signal regulated kinases (ERKs) (Bedecs et al, 1997).

Recently it was shown that SHP-1 activation via the AT₂R occurs via a novel GPCR mechanism (Feng et al, 2002). Upon AT₂R stimulation, the G α_s protein alone activates the SHP-1 protein. In fact, the G $\beta\gamma$ subunits were shown to be inhibitory to this process presenting an alternate mechanism for GPCR signaling which challenges the obligatory heterotrimeric G-protein association.

Several studies suggesting a “constitutive” activity of the AT₂R have also been performed. One such study replaced the third cytoloop of the AT₂R with the same loop

from the AT₁R. Transfection of this chimera resulted in increased expression of the *c-fos* gene and calcium release, two hallmarks of AT₁R activation, in the absence of ligand (Wang et al, 1995). Secondly, modifications to any Ang II side chains was shown to have little effect on AT₂R function suggesting that the receptor already exists in the membrane in an active state (Noda et al, 1996).

The AT₂R has also been shown to induce apoptosis. Sadashiva Karnik's group from the Cleveland Clinic has shown that mere over-expression of the receptor, again in the absence of ligand, caused increased apoptosis in a vascular smooth muscle cell line (Miura and Karnik 2000). This process was shown to be mediated by increased caspase-3 and p38 MAPK activities. Additionally, the AT₂R doesn't seem to undergo internalization desensitization (Figure 1-2) (Ouali et al, 1997).

Pro-/renin receptor

A functional receptor for the renin enzyme was described in 2002 (Nguyen et al, 2002). The initial search for such a receptor was based on the evidence that pro-renin is taken up from the circulation and concentrated in certain tissues. The receptor was shown at high levels in the heart and brain while at lower levels in the liver and kidney. In the kidney it was shown to co-localize with renin primarily in smooth muscle cells. Renin bound to its receptor is fourfold more efficient at forming Ang I and it can also induce an intracellular signal via the MAPKs.

Angiotensin(1-7)

Angiotensin(1-7) is a biologically active peptide of the RAS that has been shown to counterbalance the hypertensive and hypertrophic actions of Ang II (Santos et al, 2000). Intracerebroventricular (i.c.v.) infusion of Ang(1-7) into the lateral ventricle has no effect on mean arterial pressure or heart rate, but it greatly facilitates the baroreflex control of

heart rate (Campagnole-Santos et al, 1992). This is in opposition to Ang II specific effects. In the kidney, Ang(1-7) can act at both proximal and distal sites in the nephron to cause natriuresis and diuresis (Benter et al, 1995). In the vasculature, Ang(1-7) is generally characterized as a vasodilator; however, some researchers have proposed vasoconstrictor and even dilator/constrictor properties to the peptide (Santos et al, 2000).

Recently, research has emerged reporting the identification of a putative Ang(1-7) receptor molecule known as Mas (Santos et al. 2003). Previously an “orphan” GPCR, the authors illustrate that in the kidneys of Mas knockout mice there is a significant decrease in Ang(1-7) binding when compared to non-transgenic animals. Additionally, the Mas knockout mice showed a decrease in their ability to concentrate urine and vasodilate in response to Ang(1-7).

Angiotensin converting enzyme 2

The gene of a new angiotensin converting enzyme, ACE2, was identified in 2000 (Donoghue et al, 2000). ACE2 shares an approximate 40% identify with ACE, but it differs greatly in substrate specificity. Whereas ACE action promotes Ang II formation, ACE2 activity catalyzes the formation of the vasodilator molecule, Ang(1-7). Therefore ACE2 may act as a functional antagonist of Ang II action.

ACE2 is expressed in endothelial cells located in the heart, kidney, and testis (Tipnis et al, 2000). For this reason ACE2 has proposed roles in both cardiac and renal physiology.

ACE2 knockout mice (ACE2 -/-) have elevated plasma and tissue Ang II levels, normal basal blood pressure, decreased cardiac contractility, increased cardiac expression of hypoxia-inducible genes, normal kidney function, and unaltered female or male fertility (Crackower et al, 2002). This is in contrast to the ACE knockout mice which

exhibit low basal blood pressure, normal heart function, reduced male fertility, and the inability to produce concentrated urine (Bernstein 1998; Stec and Sigmund 2001).

However, the ACE/ACE2 double knockout mice have reduced basal blood pressure and completely normal heart, kidney, and reproductive function (Crackower et al, 2002).

From these findings it is possible to suggest that ACE2 expression is important to direct normal cardiac development. In the case of basal blood pressure regulation, it may be that in the absence of ACE2 another system compensates enough to keep blood pressure at a normal level.

Additionally it was shown that the ACE2 gene maps to reported quantitative trait loci identified in three diverse models of hypertension in the rat (Oudit et al, 2003). Located on the X chromosome, ACE2 was genetically linked with hypertension in Sabra salt-sensitive rats, stroke prone spontaneously hypertensive rats, and the spontaneously hypertensive rat (Hilbert et al, 1991; Kloting et al, 1998; Yagil et al, 1999).

The future study of the CV-relevance of ACE2 will be aided by the recent creation of novel peptide-based inhibitors (Huang et al, 2003).

Angiotensin III

The Angiotensin III (Ang III, 2-8 amino acid fragment of Ang II) heptapeptide is a documented physiological modulator of the RAS. To this date a specific Ang III receptor has yet to be identified; however, both the AT₁R and AT₂R can bind Ang III. There is some evidence that Ang III is the major effector peptide in the brain, not Ang II (Reaux et al, 2001).

Injection of Ang III directly into the supraoptic (SON) or paraventricular (PVN) nucleus of the brain induces the release of arginine vasopressin (AVP) into the blood. Co-injection of the AT1R and AT2R antagonists completely block this effect (Hogarty et

al, 1992). The conversion of Ang II to Ang III in the brain occurs quite rapidly and is mediated by aminopeptidase A (APA). If an APA inhibitor is co-injected with Ang II into the same brain nuclei the response to the Ang II is inhibited in a dose-dependent manner (Zini et al, 1998). This suggests that under these conditions Ang II conversion to Ang III is necessary to stimulate AVP release.

Ang III in the brain also exerts control over systemic blood pressure. Again there is evidence supporting the theory that Ang II must be converted to Ang III to elicit a rise in blood pressure via the CNS. The authors show that in spontaneously hypertensive rats (SHR) treated with an APA inhibitor the pressor response to i.c.v. delivered Ang II is blocked.

Angiotensin IV

Angiotensin IV (Ang IV, C-terminal 3-8 amino acid fragment of Ang II) is formed by the action of aminopeptidase B on Ang III. In 1992, Ang IV was found to bind to a specific site on adrenal membranes denoted as the AT₄R (Harding et al, 1992). In late 2001 the AT₄R was identified as the insulin-regulated aminopeptidase (IRAP) enzyme (Albiston et al, 2001). IRAP is a transmembrane, zinc-dependent peptidase found in the brain and periphery and Ang IV was shown to inhibit its activity. In the brain in particular IRAP localizes very closely with the glucose transporter 4 (GLUT4) molecule. The majority of physiological effects for Ang IV have focused on its ability to potentiate memory. It is proposed that Ang IV-mediated inhibition of IRAP in the CNS indirectly increases the half-life of neurotransmitters involved in memory and cognition, like substance P and somatostatin.

Gene Therapy and Cardiovascular Disease

Why Gene Therapy?

As discussed earlier, typical pharmacotherapy for hypertension treats only the symptoms of the disorder and usually does nothing to halt or reverse its progression. Other problems with current pharmacotherapies include the existence of side effects and the need for daily administration. These second two problems work together to cause patient drug regimen compliance to drop possibly as low as 60%. Clearly, more must be done to help alleviate these problems with antihypertensive therapy.

We chose to investigate gene therapy as an option to help us both understand hypertension in general and hopefully advance the treatment of this disease. It could be envisioned that a gene therapy approach to hypertension would result in more specific long-term treatment of the disorder. Additionally, in the ideal situation, therapeutic vector need only be delivered once thereby alleviating the problems associated with compliance.

The Perfect Vector

In order for a gene therapy vector to succeed it must have the following characteristics: (i) target both dividing and non-dividing cells; (ii) integrate into a known or “safe” location in the host genome; (iii) direct robust and stable gene expression free from positional silencing; (iv) invoke no long-term immune response in the recipient animal; (v) be produced reproducibly and at high concentrations; (vi) deliver a large payload of therapeutic nucleic acid that could theoretically contain cell-type specific promoter or regulatory elements; and (vii) achieve all of these goals without compromising the safety profile of the vector.

Gene Transfer Vectors

A major step in every gene therapy experiment comes at the very beginning where the investigator must choose the vehicle that will be used to transfer the therapeutic nucleic acid. Many times gene therapy experiments are hindered from the start simply by the improper choice of gene transfer vector.

Non-viral vectors

Non-viral vectors simply avoid using the virus life cycle to achieve gene transfer. In this case, the therapeutic nucleic acid may take many forms: circular plasmid DNA, linearized plasmid DNA, and short DNA or RNA oligonucleotides. The therapeutic nucleic acid may then be delivered “naked”, that is simply purified and injected, or it may be complexed with one of many available reagents.

Naked nucleic acid

Naked DNA injections for gene therapy primarily target either the skin, skeletal muscle, or liver. These organs are easily isolated and directly injected with DNA solution and they possess above average abilities to uptake DNA from the blood and interstitial fluid. Recently electroporation was employed to deliver plasmid DNA to skin and skeletal muscle with increased efficiency (Hartikka et al, 2001). Up to a 10-fold increase in expression was seen when employing such a technique. Efficient expression can be achieved in the liver by the simple delivery of DNA into the tail vein. On average, 10-15% of hepatocytes are transduced when delivering 10 μ g of plasmid DNA into the tail vein (Herweijer et al, 2001). However, the liver is not the only tissue that is transduced following such a procedure, low numbers of positive cells (100-fold less) can be found in the heart, spleen, and kidneys. Better targeting of the liver can be achieved by direct delivery into the portal circulation (Eastman et al, 2002). Cardiac muscle can

also be targeted by direct injection into the muscle wall or the coronary circulation. Expression from naked DNA injection is generally limited to 1-4 weeks in duration with the highest levels of expression lost within the first seven days.

Naked DNA for gene therapy is inexpensive to prepare, not constrained by gene size, and is very safe. However, its limited range of target tissues and very short term expression pattern generally preclude its usage from all but very specific gene therapy protocols.

Complexed nucleic acid

Nucleic acid is usually complexed with cationic lipids (lipoplexes) or cationic polymers (polyplexes) prior to delivery. The overall goal of the process of complexation is to spontaneously create small particles containing nucleic acid with an overall net positive charge. The positive charge helps to force an interaction with the surface of the cell where the small particles may then be endocytosed. Coating the therapeutic nucleic acid with cationic molecules helps to increase their stability *in vivo* and improves their tissue tropism and efficiency compared to naked DNA.

Complexed nucleic acid can effectively transduce every major organ in the body. Additionally, recent advances have shown the inclusion of other components into the cationic complexes could further increase their efficacy. Polyethylene glycol has been included to increase stability and allow for lyophilization and long-term room temperature storage of therapeutic complexes (Anchordoquy et al, 2001). Such a development is key for the future clinical use of these complexes. Additionally, the incorporation of basic peptides, called protein transduction domains, into the lipo- or polyplexes can markedly facilitate their efficacy (Nakanishi et al, 2003).

Complexed nucleic acid incorporates all the advantages of naked nucleic acid plus the added benefits of increased tropism and stability. However, this technique is still limited by relatively short transgene expression duration therefore it is an unlikely candidate for therapeutic use against a chronic disorder.

Viral vectors

Viral-based vectors exploit the natural process of viral infection to transfer therapeutic nucleic acid into the cell and therefore generally exhibit an increased efficiency over non-viral methods. There are a myriad of viral vector systems in existence, but this section will focus on the most popular vectors for gene transfer to non-dividing cells.

Adenovirus based

Adenovirus-based (Ad) vectors are the second most commonly utilized vector in human clinical trials. Ad vector usage in humans came under fire in 1999 when a patient died of vector-associated toxicity, but the vector was recently redeemed when its use in a phase II trial resulted in very promising results on lower limb angiogenesis (Makinen et al, 2002).

The Ad particle is non-enveloped and has a size of approximately 80nm. The early generation Ad vectors could only carry up to 8kb of DNA, but the newly developed helper-dependent Ad (HD-Ad) can carry 30kb. Ad particles bind a cell surface receptor molecule known as the coxsackievirus and Ad receptor (CAR) and are rapidly internalized. Once inside the cell the virus genome is transported into the nucleus where it exists in episomal form, un-integrated into the host genome. Ad vectors are generally easy to purify and perform efficiently *in vitro* and *in vivo* provided that the target cell expresses the CAR molecule.

Recently the area of the vector that confers the CAR binding specificity (the capsid) was modified to contain an RGD peptide motif instead thereby targeting the vector to integrin molecules (Okada et al, 2002). Other peptides have also been utilized to specifically target endothelial and smooth muscle cells (Nicklin et al, 2001). Alternatively, bi-specific antibodies, one end directed against the Ad vector and other to the target cell, were bound to the vector to achieve the same result (Levy et al, 2001).

The severe immunogenicity of early generation Ad vectors has also been addressed. Early generation Ad vectors elicit inflammation and immune response even in immune privileged organs (Mashhour et al, 1994). To address this, much of the Ad genome was deleted and vector production was achieved using helper virus. These HD-Ad vectors are the future of the Ad system since they can carry a greater payload (30kb versus 8kb for early generation Ad vectors), have a reduced immune response, and exhibit much longer transgene expression. Non HD-Ad vectors only manage expression for approximately thirty days while HD-Ad vectors have been shown to express for greater than 9 months (Reddy et al, 2002).

The current generation of HD-Ad vectors look very promising due to their large payload capacity, decreased immunogenicity, and long-term transgene expression abilities. However, target cells are limited to those expressing the CAR unless modifications are made to the Ad vector capsid protein. Also, the HD-Ad are more difficult to produce and every preparation of vector is contaminated at a low level with helper vector presenting a biosafety issue for the user.

Adeno-associated virus based

The adeno-associated virus (AAV) is a non-enveloped particle approximately 25nm in diameter with a single stranded genome (Hoggan 1970). The wild type virus integrates

in a specific location on human chromosome 19 and has yet been associated with any type of disease. The modified vector genome results in a loss in this specificity with the majority of vector existing in an episomal form. However, up to 90% of the population is seropositive for AAV thereby causing problems for the future use of this vector in the human clinic. Five major serotypes of AAV have been identified in humans therefore each patient must be screened for neutralizing antibodies and treated with a different serotype vector for the greatest efficacy.

The AAV genome is almost completely deleted, but the payload capacity of the virus remains quite low, between 4-5kb. Some investigators have worked their way around this potential problem by splitting a large expression cassette into two AAV vectors and then co-delivering them into target cells (Yan et al, 2002). This requires infection of a single target cell by each vector and the proper heterodimerization of the AAV genomes inside the cell.

The primary receptor for AAV is the widely expressed heparin sulfate proteoglycan molecule, and the vector has been used successfully in several species and cell types. The one major cell type that is refractory to AAV transduction is the hematopoietic stem cell. Targeting to specific cell types was achieved using the exact same techniques employed with the Ad virus: bi-specific antibody complexes and genetic manipulation of the capsid protein (Bartlett et al, 1999).

Although existing primarily as an episome the AAV directs long-term expression (over one year) of transgene, but, depending on the viral dose, transgene expression could take up to one month before reaching appreciable levels.

AAV is well suited to *in vivo* gene transfer due to its safety, broad tropism, and long-term gene expression. However, the vector is limited by its very small payload, the presence of neutralizing antibodies in the human population, and its slow start to transgene expression. Additionally, AAV is difficult to produce on a large scale and has somewhat limited effectiveness in rapidly dividing cells.

Human immunodeficiency virus based

The human immunodeficiency type 1 viral (HIV-1) vector is an enveloped virus approximately 120nm in diameter. HIV-1 consists of an RNA genome of approximately 9kb. However, two copies of the genome are packaged in each virion bringing the total space for therapeutic RNA to 18kb (Kumar et al, 2001).

While the wild type virus primarily transduces lymphocytes the HIV-1 vector has been adapted to accept a wide variety of envelope glycoproteins from different enveloped viruses. This process is called pseudotyping and is equivalent to the genetic modification of the Ad or AAV capsid proteins. For example, pseudotyping the vector with an Ebola virus envelope causes preferential transduction of airway epithelial cells. The most common pseudotype protein for the HIV-1 vector is the vesicular stomatitis virus glycoprotein (VSV-G). The VSV-G pseudotype helps to stabilize the vector particles and broadens the tropism of the vector since the receptor for VSV-G is a phospholipid.

As with AAV, the HIV-1 vector has been used successfully across many species and in almost every tissue in the body. The wild type HIV-1 genome consists of nine genes, but five of these are completely unnecessary for vector function. To increase safety all five of these genes are deleted from the vector and the other four genes are expressed in *trans* and then only during production of the vector. After entering the cell the vector genome is actively transported into the nucleus of both dividing and non-

dividing cells where it integrates permanently into the genome. The nuclear transport and integration process happens very quickly allowing transgene expression to proceed within 12-24 hours following infection. This integration is random with some preference to areas in the genome with relaxed chromatin structure. The vector's stable integration in the genome results in long-term (greater than one year) transgene expression.

The HIV-1 vector is very amenable to *in vivo* therapeutic gene transfer due to its large size, low immunogenicity, long-term expression, and ability to infect both dividing and non-dividing cell types. However, since this vector is based on a human pathogen there will always be concerns about its safety. Additionally, the vector is still difficult to produce in large quantities and because it is an integrating vector there exists the possibility for insertional mutagenesis.

Vector choice

Based on the above descriptions we made the decision to use the HIV-1 vector in our experiments. The reasons were three-fold. First, gene therapy for CVD will likely require long-term expression of transgene thereby eliminating naked and complexed DNA along with classical Ad vectors. The helper-dependent Ad vectors were not available at the start of these experiments, but they still would not have been chosen due to their tropism and contaminating helper virus issues. Additionally, we wanted the option of using large transgene cassettes with multiple regulatory elements and the possibility of using our therapeutic gene in situations where it must express immediately, like during restenosis or after myocardial infarction, was a possibility. Therefore, the only logical choice was the HIV-1 vector. Much of my work was spent improving the large-scale production methods.

State of the Field: Gene Therapy for Cardiovascular Disease

Gene therapy for cardiovascular disorders is of major clinical interest due to the impact of such diseases both economically and socially. Not surprisingly a myriad of vector systems and therapeutic genes have been utilized depending on the specific disorder being tackled. Three major areas for CVD gene therapy are covered below.

Hypertension

Antihypertensive gene therapies have focused on primarily either the introduction of a vasodilatory gene or the inhibition or knockdown of a vasoconstrictor molecule. Knockdown is classically achieved through the use of antisense technology, but recent advances in double stranded RNA inhibition appear to be promising alternatives to this approach (Brantl 2002). These techniques allow specific targeting of a constrictor gene's mRNA through the introduction of complementary nucleic acid. The resulting double stranded RNA is a target for degradation by endogenous cellular machinery. Viral and non-viral methods have made use of such an approach.

Antisense was used successfully against beta 1 adrenergic receptors, ACE, and the AT₁R. Ian Phillips' group delivered non-viral oligonucleotide antisense molecules to the beta 1 receptor into the systemic circulation of spontaneously hypertensive rats (SHR). A single dose of the antisense oligo was able to decrease systolic blood pressure by 35 mmHg for 30 days (Zhang et al, 2000). Cardiac contractility was significantly reduced during this period, and the authors suggest this as the major mechanism of action for the nucleic acid drug. However after 30 days the antisense effect began to reverse with the values for both parameters returning to baseline. While these findings are a significant step forward with regards to required daily administration of antihypertensive medication, there is still room for improvement.

To address this, Mohan Raizada's group at the University of Florida developed retroviral vectors encoding both ACE and AT₁R antisense. Systemic administration of the ACE antisense vector into the neonatal SHR resulted in an approximate 15 mmHg drop in blood pressure in the animals when measured in adulthood (92 days of age) (Wang et al, 1999). This finding illustrates the much longer term nature of viral-mediated antisense transduction versus oligonucleotide transfection. The AT₁R antisense vector showed more impressive results with a drop in blood pressure of >35 mmHg in adult animals for a period of at least 90 days (Iyer et al, 1996). Additional effects were also shown including decreased cardiac hypertrophy, improved endothelial cell function, and decreased perivascular and cardiac fibrosis (Martens et al, 1998).

The major "sense" approach to hypertension originates from the lab of Julie and Lee Chao at South Carolina. Using an adenoviral-based vector system expressing either human kallikrein or adrenomedullin, they were able to reduce blood pressure in a variety of models of hypertension. Kallikrein gene transfer into the quadriceps reduced blood pressure in the SHR for 5 weeks (Xiong et al, 1995). In an animal model of renal failure, kallikrein transduction again reduced blood pressure in Dahl salt-sensitive rats for four weeks while also significantly improving cardiac hypertrophy and fibrosis and renal function (Chao et al, 1998). Similar effects were also demonstrated in the Goldblatt model of hypertension (Yayama et al, 1998).

The impact of reactive oxygen on blood pressure and especially nitric oxide bioavailability has only recently been shown to play a significant role in pathophysiology. In fact, Donald Heistad's group at the University of Iowa was able to reduce mean arterial blood pressure and rescue impaired vasorelaxation in the SHR by

transferring the free radical scavenging enzyme, superoxide dismutase, via an adenoviral vector (Chu et al, 2003). The future of antioxidant gene therapy for hypertension looks very promising at this time.

Even the formidable disease of pulmonary hypertension has been attacked with gene therapy. No effective pharmacotherapy exists for this disorder, but gene transfer of the potassium channel Kv1.5 into the lung or prostacyclin synthase into the liver resulted in lowered pulmonary pressures (Suhara et al, 2002; Pozeg et al, 2003). These findings should stimulate more interest in addressing the problem of pulmonary hypertension via gene therapy.

Vascular disease

Blood vessels, and the myriad of cell types they are comprised of, represent major targets for cardiovascular disease gene therapy. Anna Dominiczak's group at the University of Glasgow in the United Kingdom used an adenovirus encoding superoxide dismutase to improve endothelial dysfunction in the stroke prone SHR (Fennell et al, 2002). Other groups have explored ways to improve new blood vessel growth, or angiogenesis, in response to ischemia. Human tissue kallikrein gene delivery was shown to augment ischemia-induced angiogenesis in the SHR (Emanueli et al, 2001). Perhaps the most well known and by far the most clinically explored gene therapy for angiogenesis originates from the late Jeffery Isner's group at Tufts University. Their work has focused on plasmid-based delivery of vascular endothelial growth factor (VEGF) to aid in collateral angiogenesis in peripheral arterial disease (PAD) and myocardial ischemia (MI). In fact this plasmid-based approach was used in the clinic beginning in 1994. The trial for PAD exceeded all expectations following the demonstration of the growth of countless numbers of new collateral blood vessels in the

limbs of patients suffering from PAD (Baumgartner et al, 1998). The trial using direct myocardial muscle delivery of the VEGF encoding DNA produced impressive results also. In over 30 patients, who previously failed all conventional therapy, the VEGF encoding plasmid successfully decreased the number of angina attacks and nitroglycerin tablet consumption by over 15-fold (Losordo et al, 1998). The success of VEGF therapy resulted in further successful angiogenic experiments with fibroblast and hepatocyte growth factors in animal models of disease.

Cardiac disease

Cardiomyocytes themselves are another important target for anti-CVD gene therapy. Antioxidant therapy with an adenovirus encoding superoxide dismutase was shown to protect rabbit myocytes *in vivo* against MI, reducing the infarct size by 50% when given prior to the MI (Li et al, 2001). Victor Dzau's group from the Harvard Medical School pre-delivered another antioxidant gene, heme-oxygenase 1 (HO-1), to adult rats in an adeno-associated vector and prevented over 75% of the infarct zone following MI (Melo et al, 2002). Eduardo Marban's group at Johns Hopkins focuses on heart failure and arrhythmia. Recently they showed the ability to create pacemaker activity in non-nodal cardiomyocytes through the use of an adenoviral vector encoding a dominant negative inward rectifier potassium channel (Kir2.1) (Miake et al, 2002). In the future it may be possible to use such manipulation in place of mechanical pacemaker implantation.

Lentiviral Vectors

Development and Discovery

The first non-replicating lentiviral vector was reported in 1990 and was designed to aid investigations into wild-type HIV-1 biology (Sakai et al, 1990). The first

“pseudotyped” (viruses enveloped with a non-native protein) particles were reported later that year after infectious virus was successfully produced using the murine leukemia virus 4070A amphotropic envelope glycoprotein (Trono and Baltimore 1990). The quantitative titers of these initial vectors were approximately 10^5 infectious units per milliliter (IU/mL).

However, the gene transfer potential of these vectors went unnoticed until Buchshacker and Panganiban hypothesized in 1992 that such defective lentivectors may be useful therapeutics for the treatment of AIDS (Buchsacher and Panganiban 1992). The modern age of lentivector use and development really began in 1996 after Naldini *et al* published their seminal paper in *Science* showing successful transduction of brain tissue *in vivo* (1996) . Their manuscript was key for several reasons: (i) the first deletions were made in the virus to improve safety without sacrificing efficacy; (ii) the particles were pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) resulting in titers of 5×10^5 before and 5×10^8 IU/mL after concentration; and (iii) most importantly, they were the first group to use the lentivector system in a non-dividing cell type (neurons). This set the stage for the future of the lentivector. The theme with each new “generation” of vector is ever increasing safety through further deletions, versatility through altered envelope pseudotyping, and efficacy through re-engineered production/concentration protocols.

The lentivector is attractive to many investigators due to its inherent abilities to transduce both dividing and non-dividing cells, direct long term expression, and produce little to no immune response (Thomas et al, 2003). These traits coupled to the ability to

produce the vector in large quantities and at high concentrations results in a vector system that is almost ideally suited for therapeutic gene transfer.

Lentiviral Vector Design and Molecular Biology

The HIV-1 virus is a so-called “complex” retrovirus belonging to the *Lentivirinae* family. This means it shares conserved structural and enzymatic genes encoding the Gag, Pol, and Env proteins with other retroviruses while at the same time the virus encodes for an additional two regulatory genes (tat and rev) and four accessory genes (vif, vpu, vpr, and nef). It is these additional genes which set apart the lentiviruses from the oncoviruses. The major genes of HIV-1 biology and their importance to lentiviral vector development are detailed below (Figure 1-3).

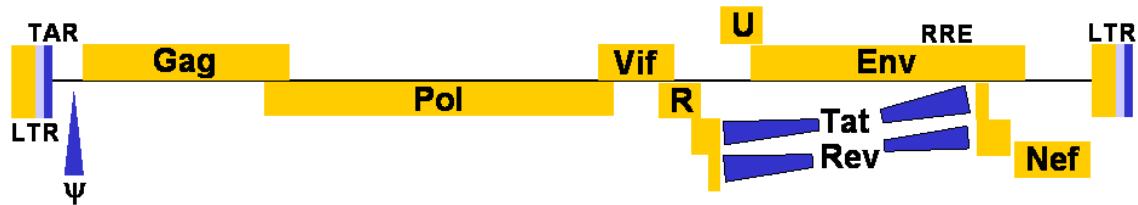


Figure 1-3. Wild-type HIV-1 genome organization.

Gag-Pol

The Gag-Pol fusion protein (p160) is created by a ribosomal frameshift during normal Gag translation. During virus maturation a virally encoded protease cleaves the Pol segment away from Gag and further digests Pol into 4 additional proteins: Protease (Pro), Reverse-Transcriptase (RT), RNase H, and Integrase (IN).

Pro is an aspartyl protease dimer whose activity is required for the cleavage of the Gag and Gag-Pol polyprotein. The RT protein has both an RNA and DNA-dependent polymerase activity. Functional RT can be found in the capsid of budded virions and because of this viral DNA can be completely synthesized in 6 hours following

transduction. The RNase H protein is necessary during the action of RT. RNase H digests the original RNA template from the newly created first strand of DNA allowing synthesis to begin on the complementary strand. Integrase mediates the insertion of the viral genome into the transduced cell. Gag-Pol expression is required during viral vector production and packaging.

Env

This 160 kDa glycosylated protein (gp160) is further processed by the host cell into 120 (gp120) and 41 kDa (gp41) proteins. The gp120 protein translocates to the cell surface and interacts with gp41 to aid in its incorporation into budding virions. The gp120 dictates the tropism of the virus toward T-cells and primary macrophages by its ability to bind the CD4 molecule on these cell's surfaces. The gp41 protein has been shown to aid in viral fusion with the cell membrane (Huang et al, 2003).

Env expression is completely dispensable with regards to vector production. In fact, one of the major benefits of the lentiviral vector is the fact that it is “pseudotypeable”. This means Env proteins from different viruses can efficiently be substituted for the native HIV-1 Env. The most commonly used pseudotype envelope is based on the vesicular stomatitis virus glycoprotein (VSV-G) (Yee et al, 1994). The incorporation of the VSV-G into the coat of the vector has been shown to greatly improve the stability of the vector, and because the purported receptor molecule for VSV-G is a phospholipid, it broadens the tropism of the vector to include every cell type in the body (Guibinga et al, 2002).

In fact these pseudotype Env proteins can do more than just alter stability of the particle; they can be used to specifically direct the vector to cell types normally transduced by the envelope protein's parent virus. For example, lentivector particles

pseudotyped with the rabies glycoprotein have been shown to undergo neuronal retrograde transport in the same manner as the native rabies virus (Mazarakis et al, 2001). This feat is achieved by simply exchanging the outer envelope protein. Additionally, pseudotyping with the Zaire Ebola virus glycoprotein preferentially transduces airway epithelial cells versus VSV-G pseudotyped vector, a potential advantage for the treatment of cystic fibrosis and other epithelial disorders. Lentiviral vectors have been successfully pseudotyped with several envelope glycoproteins including those mentioned above along with the murine leukemia virus (MuLV), lymphocytic choriomenigitus virus (LCMV), and Mokola virus envelope glycoproteins (Watson et al, 2002; Duisit et al, 2002).

Rev

Rev protein is expressed to circumvent the normal process of intronic splicing and is therefore crucial for virus production. Rev is known to bind a multi-stem loop structure on the viral RNA known as the Rev-response element (RRE) in a multimeric fashion (Lesnik et al, 2002). This binding in turn exposes a region in Rev known as the nuclear export signal (NES). The NES is then free to interact with nucleoporins, nuclear export receptors, and other components of the infected cells native export machinery to facilitate export of partially or un-spliced viral transcripts. Rev function is required for the cytoplasmic translation of the splice site containing Gag-Pol mRNA.

For these reasons the Rev protein, or some functional replacement of it, must be expressed during lentiviral vector production. Additionally, the RRE should be present on any transcript destined to be packaged into therapeutic virus. To further improve biosafety, some groups have exchanged the HIV-1 based Rev/RRE system for other similar spliceosome-evasive strategies of other viruses. The use of such systems has not

become widespread due to the approximately 10-fold lower vector titers obtained from these systems (Wagner et al, 2000).

Tat

The Tat protein is a transcriptional transactivator that acts at the 5' long terminal repeat (LTR) region. Tat forms a complex with other host proteins at a nascent viral RNA hairpin known as the trans-activating response element (TAR). Once bound, the Tat complexes direct hyper-phosphorylation of RNA Polymerase II and enhance transcriptional activity by 100 to 500-fold (Roebuck and Saifuddin 1999).

In lentiviral vector design the Tat protein is dispensable. Chimeric 5' LTR regions able to direct robust tat-independent transcription have replaced the native 5' LTR promoter sequence (Mitta et al, 2002). However, the TAR motif is essential for initiation of reverse transcription in the transduced cell and therefore should be present on the therapeutic construct.

Vpr

The Vpr protein is carried into infected cells inside the virus and plays a role in the nuclear import of the viral genome (Krichevsky et al, 2003). Additionally, Vpr has been shown to cause G2 phase cell cycle arrest. It is unnecessary in the lentiviral vector.

Vpu

This 16 kDa polypeptide has been shown to both downregulate the CD4 molecule (the HIV-1 receptor) and to enhance the release of infectious virions (Piguet et al, 1999). In the absence of Vpu the majority of newly formed virions merely accumulate at the cell surface and never successfully bud. It has been shown to be dispensable with regards to the lentiviral vector.

Vif

Vif is a 23 kDa polypeptide essential for replication of HIV-1 in certain cell types. In most cells some unidentified endogenous protein can complement Vif function (Lake et al, 2003). Vif is incorporated into virions. This peptide is also unnecessary for proper lentivector function.

Nef

Negative factor (Nef) is a 27 kDa myristolated protein whose primary action is to post-translationally decrease the expression of CD4 in infected cells (Fackler and Baur 2002). This action has been shown to actually increase virus production. Nef is packaged into virions and is the first viral protein to accumulate following HIV infection. Nef is not needed for proper lentivector performance.

State of the Field: Lentiviral-Based Vectors

Since their first *in vivo* usage in 1996 the lentiviral vector development and utility has been improving and expanding by leaps and bounds. This next section details the current state of the art.

Alternative lentiviruses

The efficacy of lentivirus-based vectors is of no question, however, from their inception much emphasis was placed on the overall safety profile of the vector. To this end many molecular “safety valves” were ingressed into the vector system, but to some investigators these improvements simply did not change the fact that the entire system was based on a human pathogen.

To these groups the solution to this problem was to make use of other *Lentivirinae* family members not shown to cause human disease. Good transfer efficacy was achieved using equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV)

derived vectors (Olsen 2001; Curran and Nolan 2002). Both vector types performed similarly to HIV-1 based lentiviral vectors and were used to transduce equivalent target cells. Even the less virulent HIV-2 strain was developed as a lentiviral gene transfer vector (Gilbert and Wong-Staal 2001).

However, the highest titers of these vector systems remain up to 100-fold lower than the HIV-1 lentivectors. Additionally, for the very reason that HIV-1 is a human pathogen the scientific and medical community have much more knowledge about its biology. There are simple and fast diagnostic tests for HIV infection and the clinic is arrayed with multiple antiretroviral pharmacotherapies specifically targeted at HIV. For these reasons the HIV-1 based lentivectors lead the field and will most probably be the first type of lentiviral vectors used in the human clinic. However, the further development of the “alternative” lentivirus systems should continue. As we learn more about these other lentiviruses they may soon approach the overall performance of the HIV-1 based systems.

***In vivo* usage**

Since its first *in vivo* usage in 1996 the lentiviral vector has been utilized by numerous investigators to transduce a myriad of target cells. Organs successfully transduced with a targeted injection of lentiviral vector include the heart, brain, liver, eye, blood, pancreas, spleen, kidney, skeletal muscle, and skin among others. In the work detailed here I will illustrate that non-targeted delivery of lentivector into the systemic circulation is additionally capable of transducing lung, adrenal gland, and even the testes (Coleman et al, 2003). These results indicate the potential for this vector system in a wide range of organ systems. Additionally, the lentivector was used to correct a number of defects in animal models of human genetic disorders including cancer, cystic fibrosis,

parkinson's disease, sickle cell, huntington's disease, thalassemia, retinitis pigmentosa, diabetes, porphyria, ischemic myocardium, and hemophelia. Lentiviral vectors have also been used to combat HIV infection and even to help slow the progression of aging. Recently, lentiviral vectors were used to efficiently create transgenic rats, a species traditionally refractory to the often used microinjection technique used to make transgenic mice (Hamra et al, 2002; Lois et al, 2002).

Surprisingly, very few examples of cardiovascular disease models have been targeted with lentivirus-derived vectors. In fact, most of the cardiovascular system work with the lentivector was performed in the last two years. In 2002, it was reported that lentivirus expressing a fragment of matrix metalloproteinase 2 was capable of inhibiting angiogenesis while earlier that year a group from New York illustrated that lentivector infused into the ureter efficiently transduced cells in the kidney, a traditionally difficult target to gene transfer (Gusella et al, 2002). In 2003 we published our findings on effective transfer into several organs of the cardiovascular system of neonatal animals while two other groups demonstrated tremendous (80-90%) *in vivo* transduction of cardiomyocytes in adult mice (Bonci et al, 2003; Fleury et al, 2003). While all of these findings contribute evidence that targeting of the CV system is possible with the lentivector no group has yet prevented or reversed any form of CVD with the lentivector.

The first proposal using an HIV-1 vector in a clinical trial was submitted for approval in 2001 (MacGregor 2001). The goal of this study was to use antisense technology to suppress the expression of the HIV env gene in T cells of seropositive patients currently failing antiretroviral therapy. If approved, this trial would begin less than ten years from the date the first generation of lentiviral vectors were used *in vivo*.

Such a short time frame from discovery to clinic is a testament to the utility of the lentiviral vector system.

Aims and Rationale

As detailed in this first chapter, CVD is the number one killer of Americans and management of the disease has enormous socioeconomic impacts on this country. Hypertension is the most common CVD with over 50 million Americans currently diagnosed. Modern antihypertensive pharmacotherapy is lacking in two main aspects: (i) It only treats the symptoms of the disease while not efficiently reversing the physiological damage already done, and (ii) The requirement for a daily drug regimen and drug related side effects results in low patient compliance leading to a general mismanagement of the disease. For these reasons, radical new methods for the management and possible cure of hypertension must be elucidated.

Successful gene therapy for the prevention and reversal of hypertension has already been demonstrated. However, in each case a certain aspect was lacking. The ultimate goal of antihypertensive therapy should be to reverse the disease once it is established. For this to be achieved the ideal gene transfer vector must be utilized. The ideal vector would efficiently target quiescent cells since this disease is generally found in the adult population. Additionally the vector must be easily produced and be as safe as possible for use in humans. Site specific genomic integration and a large nucleic acid payload capacity are also important. The lentivirus-based vector system satisfies the majority of these requirements. The overall goal of the work described here was to develop a user-friendly lentivector system for systemic gene transfer to the cardiovascular system. This would set the foundation for future work using the vector system in the general study and possibly treatment of hypertension. The specific aims for this work are detailed below.

Aim 1: Create a more user friendly lentiviral vector system.

- (a) Modify the core cloning constructs to include multiple unique site and various bicistronic marker gene cassettes.
- (b) Develop a reproducible transfection protocol using decreased amounts of DNA without compromising vector titer.
- (c) Engineer simple concentration methods permitting the large scale production of high titer vector stocks.

Aim 2: Characterize the efficacy of the lentiviral vector system in non-dividing cells *in vitro*.

- (a) Transduce growth-arrested and primary cells relevant to the cardiovascular system.
- (b) Assess transgene expression and determine transduction efficiency.

Aim 3: Deliver the lentiviral vector systemically into the cardiovascular system and characterize its efficacy.

- (a) Determine transduction efficiency.
- (b) Assay duration of transgene expression.

Aim 4: Prevent the development of cardiovascular disease in the SHR using systemic delivery of the lentiviral vector.

Monitor blood pressure along with other hypertensive pathophysiology following the systemic administration of the vector.

CHAPTER 2

IMPROVING LENTIVIRAL VECTOR PRODUCTION RESULTS IN THE ABILITY TO CONSISTENTLY PRODUCE VECTOR ON A LARGE SCALE.

Introduction

As discussed previously, it is quite clear that lentiviral vectors are fast becoming the vector of choice for long-term gene transfer into dividing and non-dividing cells. This fact is due to the many benefits associated with the lentiviral vector systems including large payload capacity, low immunogenicity, and wide tropism. This increased interest has given rise to a need for efficient and reproducible methods to produce large quantities of vector. As more investigative genes are cloned into the lentivirus, there is an additional need for utilitarian cloning constructs thereby allowing ease in cloning and also the possibility for the bicistronic expression of marker genes for the identification of transduced cells.

The traditional production of lentiviral vector involves the co-transfection of human cell lines with plasmid DNA encoding the viral components required for packaging. The transient transfection of these cell lines is usually achieved using the conventional calcium phosphate co-precipitation methodology. Disadvantages of this method include: (1) the large amount of plasmid DNA that is required for transfection; (2) the difficulties associated with scaling up the precipitation reaction; and (3) the high degree of variability observed in transfection efficiency and viral production. Recently, several groups have developed packaging cell lines that facilitate the production of lentiviral vectors by reducing the need for multi-plasmid transfections. Although the use

of packaging cell lines has streamlined the packaging procedure, the resulting viral titers have not been significantly higher than those obtained using transient co-transfection methods. In addition, the advantages of these new cell lines are often offset by the need to develop new lines for each generation of improved lentiviral vector. To achieve large-scale production of high-titer lentiviral vector it is critical that transfection of the virus-producing cell cultures be both efficient and reproducible; however, little effort has been put forth to optimize this step in vector production.

To target a disease like hypertension with gene therapy it may be critical to target many different cells or cell types distributed throughout the body. To achieve this using a single dose of vector would require its systemic administration into the circulation. Reaching this goal with any gene therapy vector would require methods to easily produce it on a large scale. Therefore to reach such a level with the lentivirus we developed methods utilizing a dendrimer-based transfection reagent, SuperFect (Qiagen), coupled with a novel concentration protocol. The result was the reproducible production of higher titer virus using one-third less the amount of plasmid DNA traditionally required when using calcium phosphate co-precipitation. Additionally, we designed a wide array of cloning constructs used to produce the lentivector. These were designed in such a way to simplify the cloning of new genes of interest into the vector system and also enable the bicistronic expression of a marker gene to simultaneously identify transduced cells. Finally, vectors produced using these new methods were assayed for *in vivo* efficacy in adult rat brain.

Results

“TYF” Cloning Vector Improvements

The plasmid DNA constructs used to produce the lentiviral vector are denoted as pNHP, phEF1.VSV.G, and pTYF (Figure 2-1). The pNHP plasmid encodes all the viral

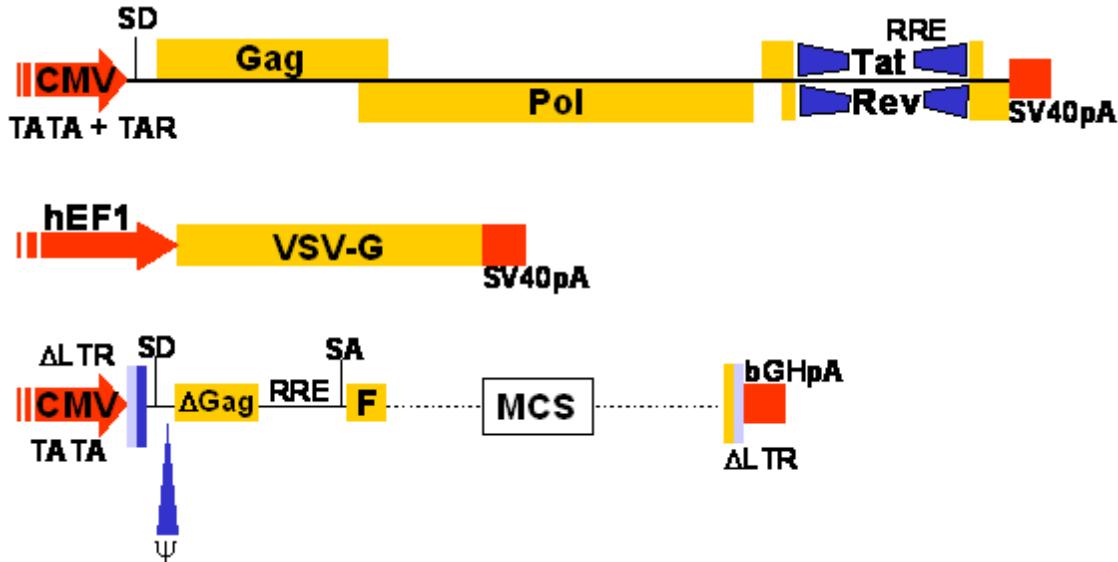


Figure 2-1. Three plasmid system used to produce recombinant lentiviral vector. From top to bottom, pNHP, phEF1.VSV.G, and pTYF backbone.

proteins necessary for the production of infectious virus in trans. It is important to note the open reading frame deletions of all the unnecessary accessory genes (vif, vpr, vpu, and nef) along with the native env gene. Expression of the pseudotype env gene is achieved by the co-transfection of the phEF1.VSV.G construct. This plasmid expresses the vesicular stomatitis virus glycoprotein (VSV-G) under the control of the constitutive human elongation factor 1 alpha promoter (hEF1). The TYF family of vectors are the only component of the system containing an intact psi (Ψ) packaging signal and fully functional long terminal repeats (LTR). The therapeutic gene is cloned into this construct, therefore, modifications were made to this plasmid in order to facilitate cloning and improve the overall utility of the vector system. The newly created family of

constructs are illustrated in Figure 2-2. It is important to note the large number of unique cloning sites and the further inclusion of an internal ribosome entry site (IRES) based bicistronic cassette. The IRES-containing vectors have been successfully used to create stable cell lines *in vitro* (IRES.NEO), assay live cell transduction *in vitro* (IRES.EGFP), and to localize transduction *in vivo* (IRES.PLAP).

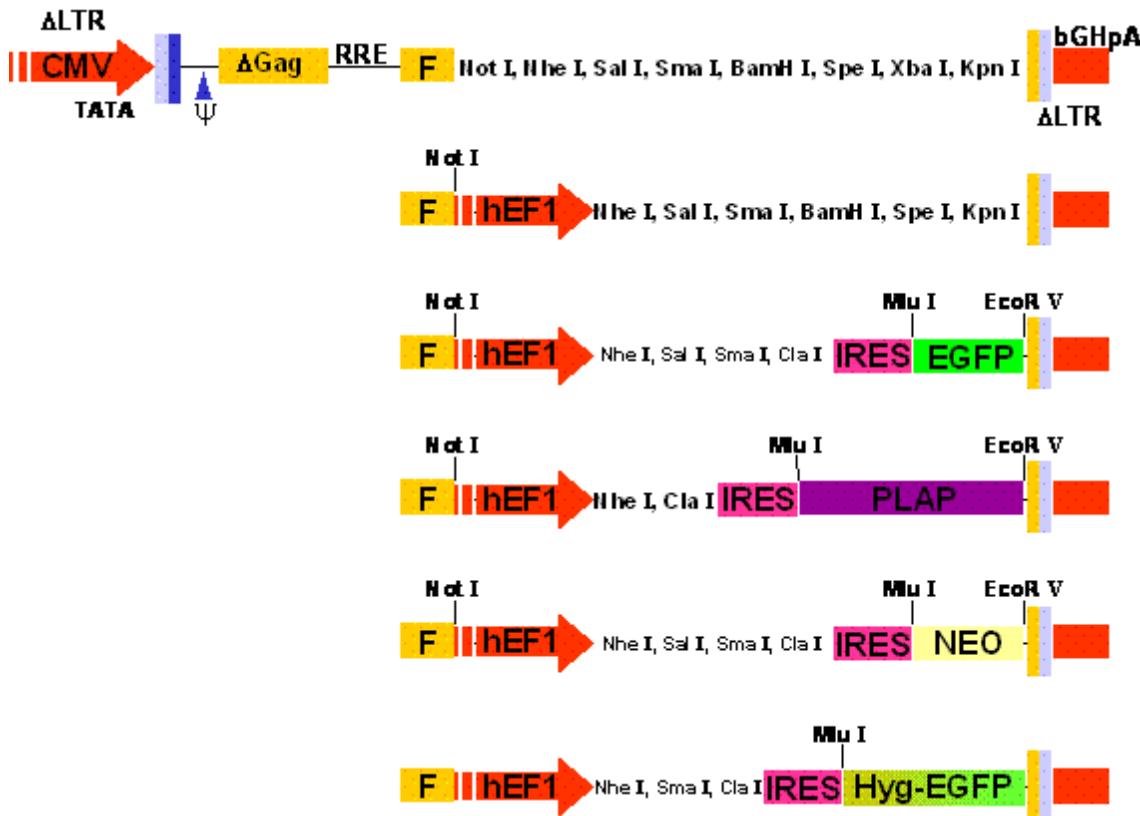


Figure 2-2. The pTYF family of modified cloning vectors. Only the unique cloning sites are indicated for each construct. From top to bottom, pTYF.Linker, pTYF.hEF1.Linker, pTYF.hEF1.IRES.EGFP, pTYF.hEF1.IRES.PLAP, pTYF.hEF1.IRES.NEO, and pTYF.hEF1.IRES.Hyg-EGFP

Vector Production Modifications

The goals of our first series of experiments were to determine the optimum ratio of total plasmid DNA to Superfect reagent that produced the highest titer virus and the optimum time for viral harvest. This ratio was determined to be 1:2 (ratios of 1:1, 1:1.5,

1:2, 1:5, and 1:10 were tested; data not shown). The titers of virus-containing media harvested directly from transfected 293T cultures were determined 30, 45, 60, and 70 hours post-transfection to identify the timeframe during which virus production by these cultures is at maximum levels (Figure 2-3). The average titer values were 8.0×10^6 , 6.8×10^6 , 2.6×10^6 and 0.8×10^6 transducing units (TU) per ml at 30, 45, 60 and 70 h post-transfection, respectively. Therefore, we collected culture media 30 and 45 hours post-transfection for subsequent experiments. It should also be noted that 293T cells passaged between 2 and 60 times were used for transfections and that passage number did not significantly affect transfection efficiency or final vector titers.

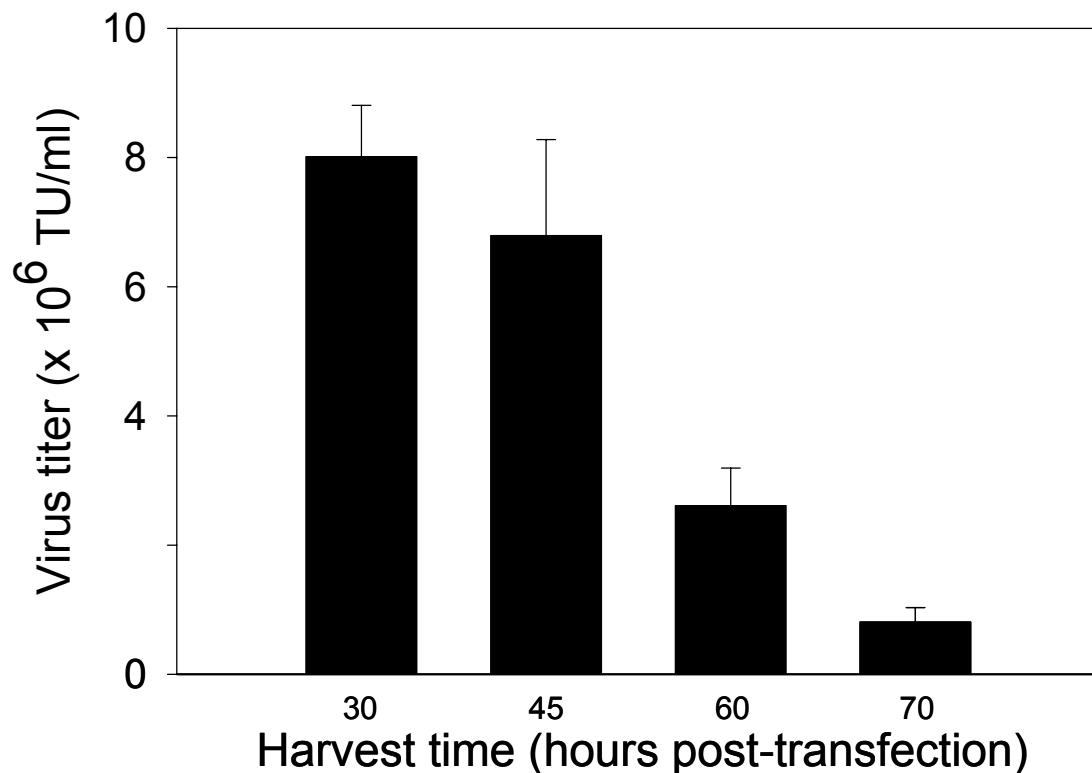


Figure 2-3. Lentivector production is greatest within the first 48 hours following transfection. Packaging cells were transfected with the appropriate plasmids to produce TYF.hEF1.PLAP vector. Un-concentrated supernatants were collected at the indicated times and titered on TE671 cells (n=3).

Concentration Protocol Modifications

The goal of our second series of experiments was to develop a concentration protocol that would minimize virus loss and yield the highest titer virus in the smallest possible volume. The concentration procedure and results are summarized in Figure 2-4. The average starting titer of the virus-containing media (Figure 2-4, Steps 1-3) was $1.40 \pm 0.35 \times 10^7$ TU/ml. The next step in the concentration procedure (Figure 2-4, Step 4) yielded an average titer of $3.59 \pm 0.70 \times 10^8$ TU/ml in a volume of ~3.0 ml, resulting in a 33-fold increase in titer and an average recovery of 84%. Further concentration of the virus stock by low-speed centrifugation (Figure 2-4, Steps 5c and 6) yielded $1.40 \pm 0.44 \times 10^{10}$ TU/ml, a 958-fold increase over the average starting titer. The average overall percent recovery of the virus was 40%.

Lentivector Performance in the Brain *In Vivo*

Lentiviral vector carrying an hEF1-EGFP transgene was delivered into the paraventricular (PVN) and the caudal nucleus of the solitary tract (NTS) of the adult rat brain. Examination of transverse sections cut from the brains of animals either 7 days (PVN) or 30 days (NTS) after injection revealed that the vector transduced a high proportion of cells in both nuclei as evidenced by the presence GFP-positive cells (Figure 2-5). Many GFP-positive cells exhibited a neuronal phenotype (Figure 2-5, C) and in many cases fluorescent axons could be seen hundreds of microns away from the area of concentration of fluorescent cell bodies. Although not shown, robust expression was shown for up to 90 days, the duration of the experiment.

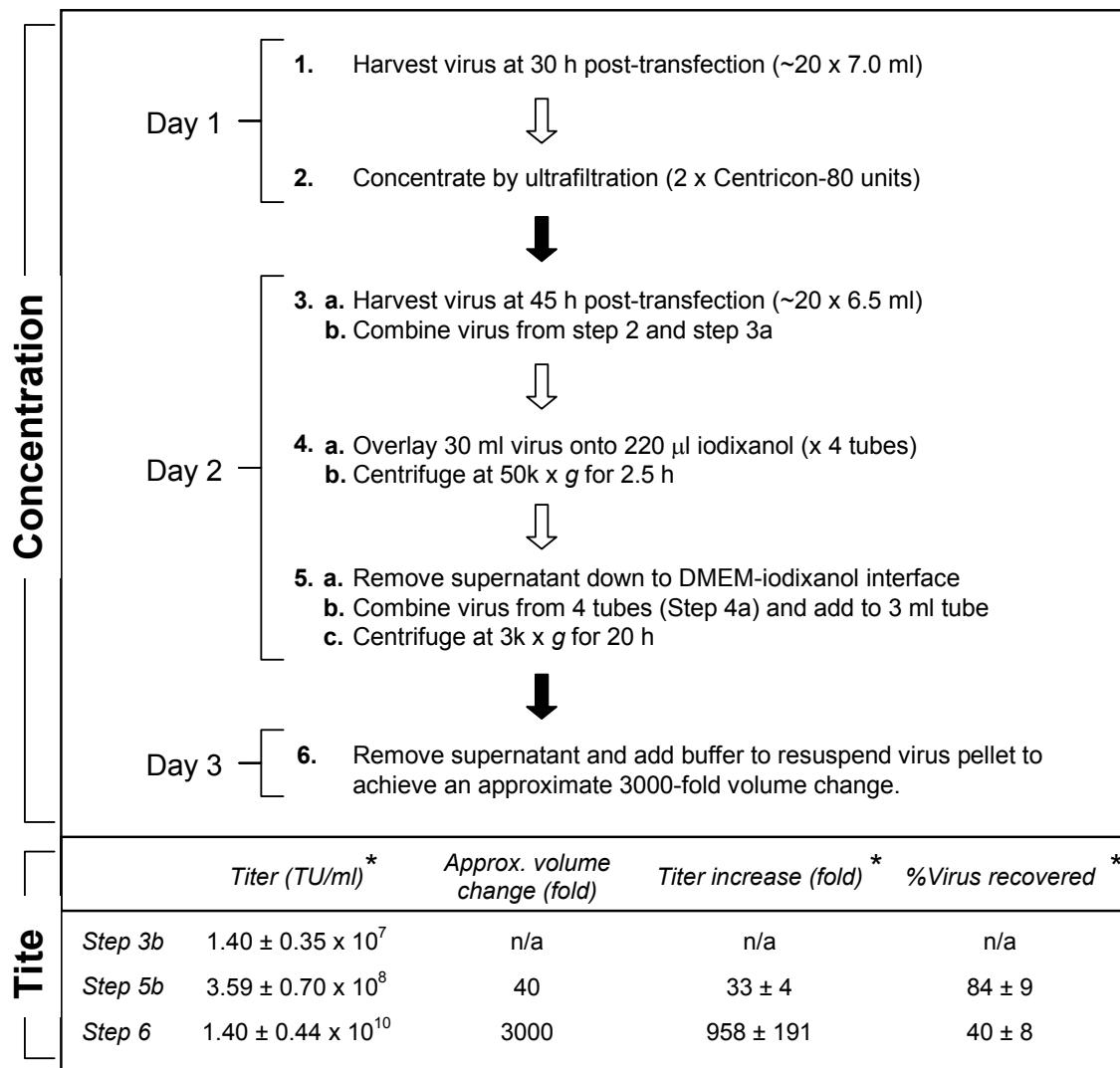


Figure 2-4. A modified lentivector concentration protocol results in higher final titers. Multiple preparations of TYF.hEF1.PLAP lentiviral vector were concentrated as indicated and titered on TE671 cells.

Discussion

By optimizing both the DNA transfection and viral concentration steps for production of lentiviral vector, we have overcome many of the problems that we had previously encountered in our efforts to produce large volumes of high-titer lentiviral vector in a consistent manner. We found that SuperFect-mediated transfection of viral packaging cells consistently yielded large-scale vector stocks (~ 120 ml) with starting

titors averaging $>1.0 \times 10^7$ TU/ml, titers that were comparable to vector stocks prepared using other transfection reagents (Curran et al, 2002; Fleury et al, 2003; Gusella et al, 2002). Use of SuperFect greatly simplified the transfection protocol and significantly reduced the amount of plasmid DNA required for the procedure. The viral concentration protocol that we developed consistently increased the titers of the viruses by approximately 1000-fold ($\sim 1 \times 10^{10}$ TU/ml). Lentiviral vectors produced using these novel methods were shown to be able to efficiently transduce non-dividing cells (neurons) *in vivo*.

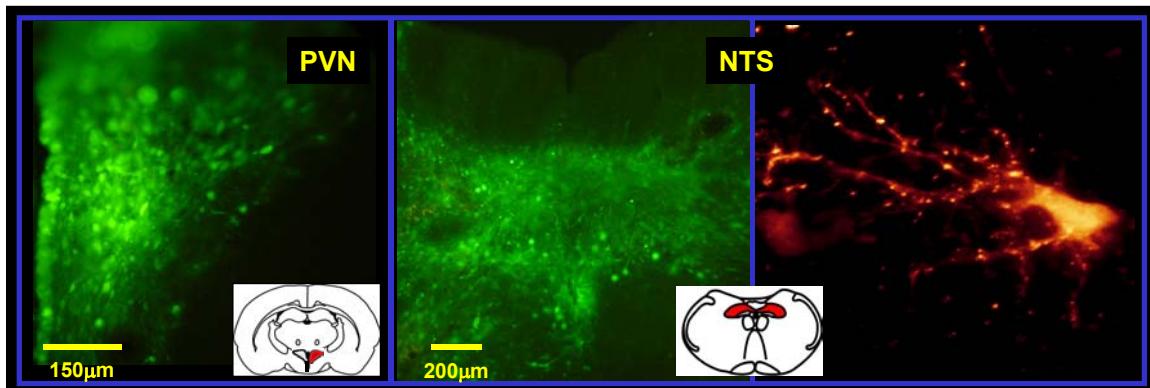


Figure 2-5. Lentiviral vector efficiently transduces neurons in the adult rat brain *in vivo*. Vector encoding EGFP (TYF.hEF1.EGFP, see Chapter 3) was prepared using the transfection and concentration schemes outlined above. The left-most panel illustrates green fluorescence in the paraventricular nucleus (PVN) 7 days after the delivery of 5×10^5 transducing units of vector (n=2). The PVN is highlighted in red in the inset drawing. The center panel illustrates similar results achieved in the nucleus of the solitary tract (NTS) 30 days after the bilateral delivery of 3×10^5 transducing units (n=2). Inset illustrates in red the location of the NTS. The right-most panel is a magnified, pseudocolored image of an EGFP-positive neuron from the NTS.

Additionally, we illustrated some modifications introduced into the TYF vectors allowing an increased ease in cloning new genes of interest into the lentivector system. Bicistronic expression cassettes were also created to allow the simultaneous expression of marker protein or second therapeutic gene.

In summary, the transfection and concentration protocols outlined here allow efficient, reproducible production of high-titer lentiviral vectors that exhibit robust transduction properties *in vivo*. The transfection protocol itself is simple and can be easily implemented by investigators interested in producing lentiviral vector in their laboratories. Furthermore, the methods can be easily adapted to large-scale lentiviral production protocols that are currently being developed for use in large animal studies or for possible use in clinical studies.

CHAPTER 3
SYSTEMICALLY ADMINISTERED LENTIVIRAL VECTOR TRANSDUCES
SEVERAL TISSUES IN THE RAT RELEVANT TO THE CARDIOVASCULAR
SYSTEM.

Introduction

Having shown that it is possible to produce lentivector reproducibly and in large quantities it is necessary to characterize the vector system in cardiovascular (CV) target cells *in vitro* and *in vivo*.

Lentiviral vectors have documented success in very few CV relevant cell types *in vitro*. Successful transfer of marker genes was demonstrated in primary cultured neurons and cardiomyocytes. To investigate or treat a systemic disorder like hypertension it may be necessary to transduce these cell types in addition to endothelial, smooth muscle, liver, and kidney cells. Although vector pseudotyped with the VSV-G envelope would be predicted to efficiently transduce these types of cells it has not been demonstrated empirically.

Through the simple transduction of such cell types *in vitro* it cannot be inferred to translate directly to the *in vivo* performance of the vector. Such *in vivo* investigations into the tropism of the vector are necessary to perform. Additionally, when moving to the whole animal it is important to determine what vector dose is necessary to transduce a majority of target tissues.

To answer the questions regarding the *in vitro* and *in vivo* efficacy of the vector we utilized a construct expressing marker genes (PLAP, EGFP, nlacZ, or DsRED). The goal of these experiments was to determine if and under what conditions the lentivector could

successfully transduce cell types and tissues of the CV system. We were able to show that the lentivector very efficiently transduces cells *in vitro* relevant to the CV system including endothelial, neuronal, vascular smooth muscle, and liver cells. *In vivo* it was shown that the highest dose of vector examined, 2.5×10^8 total particles, was able to transduce a myriad of tissues including heart, liver, kidney, lung, adrenal glands, and testes when administered into the systemic circulation of a 5-day-old rat.

Results

In Vitro Efficacy

All vectors were produced using the transfection and concentration protocols detailed in the previous chapter. The PLAP and EGFP TYF constructs used in these studies are illustrated in Figure 3-1. Two additional TYF constructs were also utilized, one expressing a nuclear localized form of bacterial beta-galactosidase (*nlacZ*) and a second one encoding a red-shifted commercially available fluorescent protein (DsRED, Clontech).

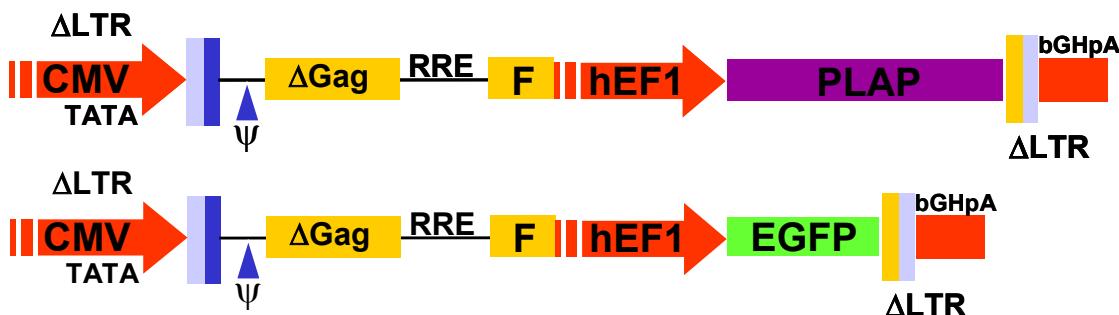


Figure 3-1. The pTYF lentiviral vector reporter gene constructs. The top vector encodes the PLAP gene and is denoted as pTYF.hEF1.PLAP. The vector illustrated on the bottom expresses the EGFP gene and is denoted as pTYF.hEF1.EGFP.

The goal of the *in vitro* experiments was to simply assay the efficacy of the lentivector in quiescent, CV-relevant cell types. The first cell type tested was a cell line

of rat aortic endothelial cells. The cells were growth-arrested at confluence and transduced with a TYF.hEF1.nlacZ vector. Staining at 48 hours post-transduction revealed that at 1 multiplicity of infection (MOI) approximately 50% of the cells were transduced. Two cell lines of vascular smooth muscle (A.10 and A7r5) were treated in the same manner and 20% of the cells were able to be transduced. A hepatocyte cell line (HepG2) was also growth-arrested and instead incubated with a vector expressing enhanced GFP (TYF.hEF1.EGFP). When infected at 5 MOI 80% of the cells were positive for EGFP expression 48 hours following viral incubation.

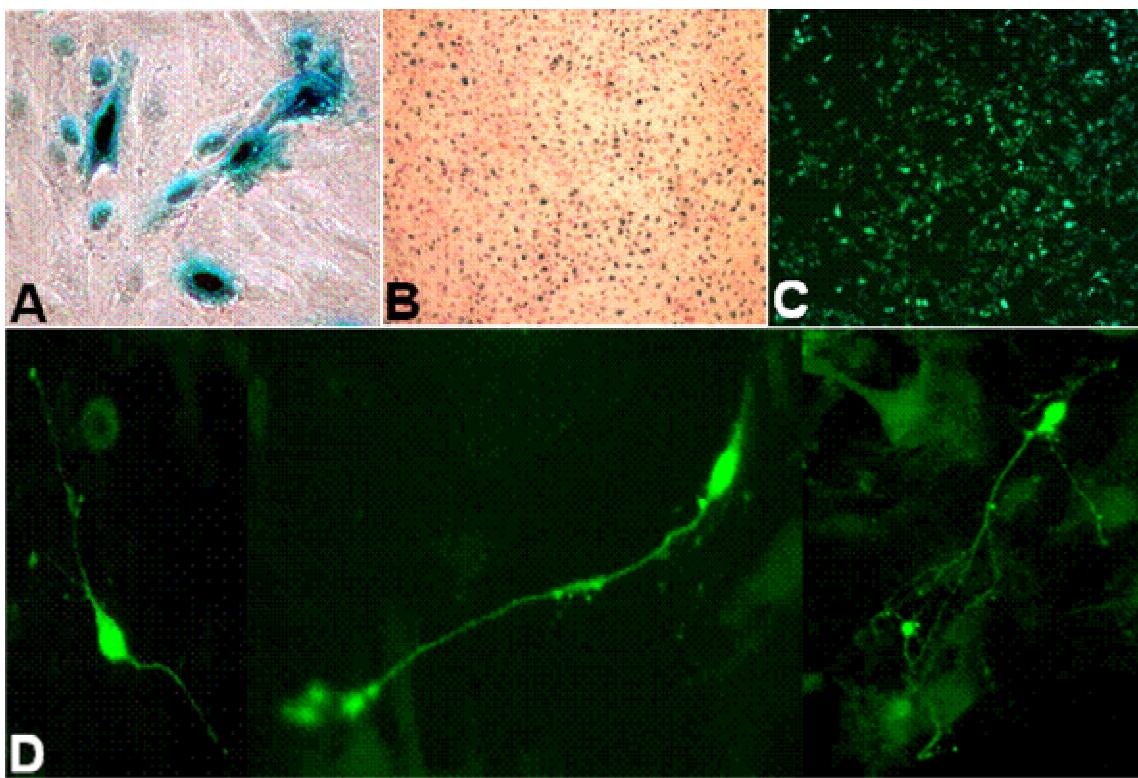


Figure 3-2. Lentiviral vector efficiently transduces quiescent cells relevant to the cardiovascular system *in vitro*. A) Growth-arrested A-10 vascular smooth muscle cells transduced with lacZ encoding vector. B) Growth-arrested pulmonary artery endothelial cells transduced with lacZ encoding vector and counterstained with nuclear fast red. C) Growth-arrested HepG2 cells transduced with EGFP vector. D) Primary cultures of hypothalamus / brainstem neurons transduced with EGFP vector.

Primary cultures of neurons from the hypothalamus and brainstem regions were also transduced with TYF.hEF1.EGFP viral vector. At 10 days post-infection ~20% of the neurons were positive for EGFP expression when transduced at 10 MOI. A second DsRED expressing vector was used in the neurons to illustrate the ability of the lentivirus to infect the same cell multiple times thus transferring genes encoding for EGFP and DsRED (Figure 3-3). In all instances control treated cells were incubated with an equivalent amount of viral re-suspension buffer and examined for background activity.

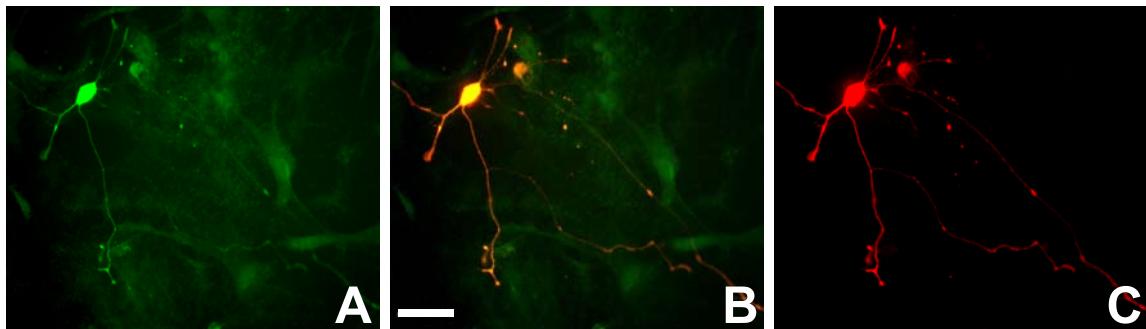


Figure 3-3. Separate lentiviral vectors are able to transduce the same cell in succession. Primary neuron cultures were transduced at 10 MOI with EGFP expressing vector and 5 MOI with DsRED vector. Ten days following vector exposure the cells were assayed by direct fluorescence. A) Neuron viewed using a filter specific for EGFP, B) A composite picture of the same neuron merging the green and red fluorescent images, and C) Neuron viewed using a filter for the DsRED fluorescence. Bar = 50 μ m

In Vivo Efficacy

For the *in vivo* studies all vectors were prepared using the methods detailed in Chapter 2 and only the PLAP expressing construct (TYF.hEF1.PLAP) was utilized. The objective of this second set of experiments was to determine three key points regarding the *in vivo* performance of the vector: (i) The dose of vector needed to transduce a wide variety of CV tissues; (ii) The duration of transgene expression; and (iii) Elucidate what relevant tissues are targets of systemically delivered vector. In all cases the vector was

delivered into the systemic circulation of neonatal rats through direct injection into the left ventricular space.

Dose-response

Equal volumes of three doses (2.5×10^8 , 5×10^7 , and 2.5×10^7 total infectious particles) were injected into 5-day-old rat pups in replicates of four along with virus re-suspension buffer (a-CSF) injected controls. Animal were sacrificed and assayed for PLAP expression in the heart, liver, and lung at 3 (n=1 per group), 30 (n=2), and 60 (n=1) days post-delivery. At all time points and in every tissue the highest dose of lentivector resulted in the greatest degree of PLAP histochemical staining (Figure 3-4). No obvious adverse affects on the general health of the animals was noted. Based on these findings all *in vivo* work from here onward was conducted using the maximum dose of 2.5×10^8 particles.

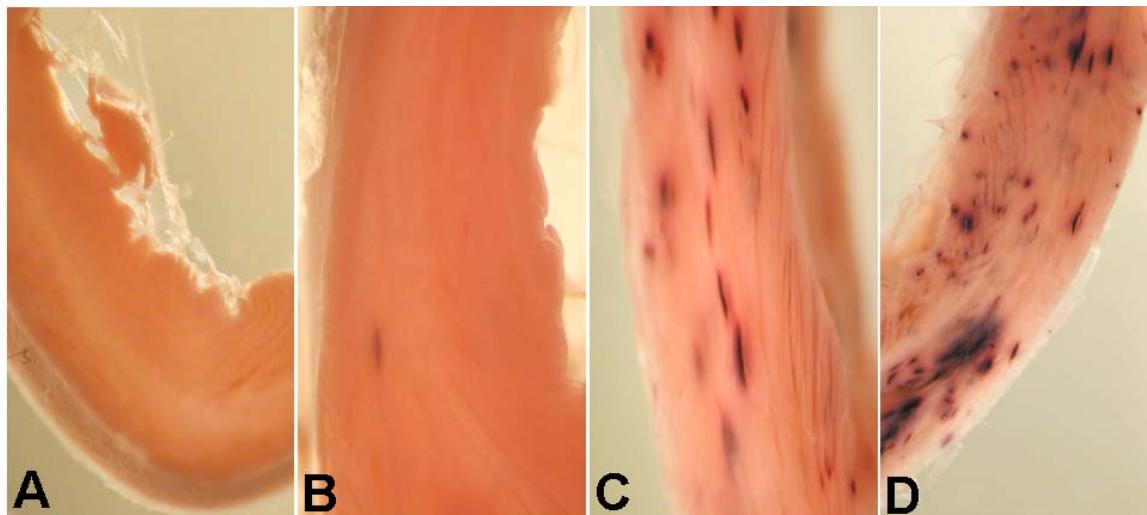


Figure 3-4. Increasing doses of lentiviral vector results in higher transduction efficiency *in vivo*. Neonatal Sprague-Dawley rats were injected with control (A) saline, 2.5×10^7 (B), 5×10^7 (C), or 2.5×10^8 (D) total infectious particles of PLAP encoding lentiviral vector. The ventricles of the heart were grossly dissected 30 days post-delivery of vector and stained for PLAP activity (n=2 per group for time point shown). Images were acquired at low magnification through a dissecting microscope.

Transgene expression duration

Six neonatal rats were transduced with 2.5×10^8 particles of PLAP expressing lentivector along with an equal number of control injected (a-CSF) animals. Animals were sacrificed at 3 (n=1 per group), 30 (n=3), and 120 (n=2) days following the delivery of the vector. Heart, lung, liver, and kidney samples were collected and assayed for PLAP expression using histochemical staining. At all time points and in all three tissues PLAP expression was detected in the vector injected animals and could not be identified in their respective controls. The results for the kidney are illustrated (Figure 3-5). Expression levels at three days post-delivery were lower than all other time points. Among the other time points no significant difference in expression was noted.

Biodistribution of vector

The final set of experiments utilized a PLAP encoding vector. A total of 2.5×10^8 infectious particles were administered into the circulation of 5-day-old rat pups. A total of eight animals were injected, 4 experimental and 4 receiving a control injection (a-CSF). At 30 and 120 days post-delivery, 2 animals from each group were sacrificed and several organs were removed to undergo PLAP staining. At both time points in the virus injected animals only PLAP staining was seen in every tissue collected including heart, liver, lung, brain, aorta, kidney, adrenal gland, and spleen (Figure 3-6). Liver was the highest transduced tissue (~60%) followed by the heart (~30%). Within the heart 90-95% of the positive cells were identified morphologically as cardiomyocytes (Figure 3-7). In the 30 day old animal occasional single positive cells were found in the testes, however, in the 120 day old animal it was evident that some of the transduced cells in the tubule of the testes were indeed germ cells (Figure 3-8). No background staining was seen in any tissue of the control treated rats.

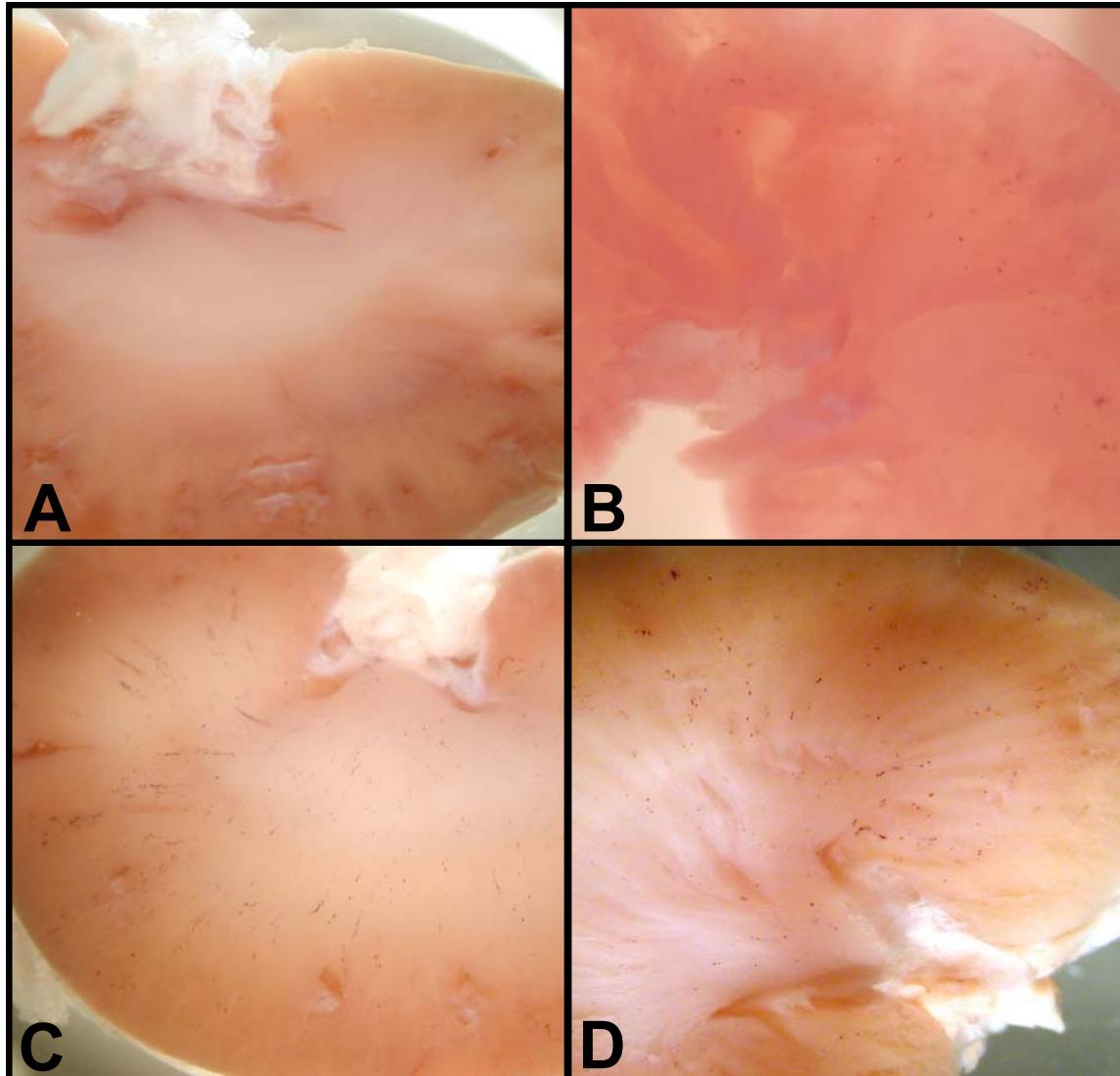


Figure 3-5. Lentiviral vector expresses transgene for at least 120 days *in vivo*. Neonatal rats were injected with 2.5×10^8 total infectious particles of PLAP encoding lentiviral vector. Kidneys were removed and stained for PLAP activity at either 3, 30, or 120 days following viral delivery. Panel A illustrates a control animal of the same age. Images were acquired at low magnification through a dissection microscope.

Discussion

Knowledge regarding the use of lentivirus-derived vectors in the cardiovascular system is largely lacking. We report here several studies highlighting the use of lentivector for gene transfer into cardiovascularly relevant cells *in vitro* and *in vivo*.

Lentivector was able to efficiently (from 1-10 MOI) transduce non-dividing endothelial, vascular smooth muscle, neuronal, and liver-derived cells *in vitro* (Figure 3-2). Gene expression occurred rapidly (within 48 hours) and lasted for at least 10 days in the case of the primary neuron cultures. These *in vitro* studies re-iterated the utility of the lentivector system, but in some novel cell types not previously transduced with lentivector.

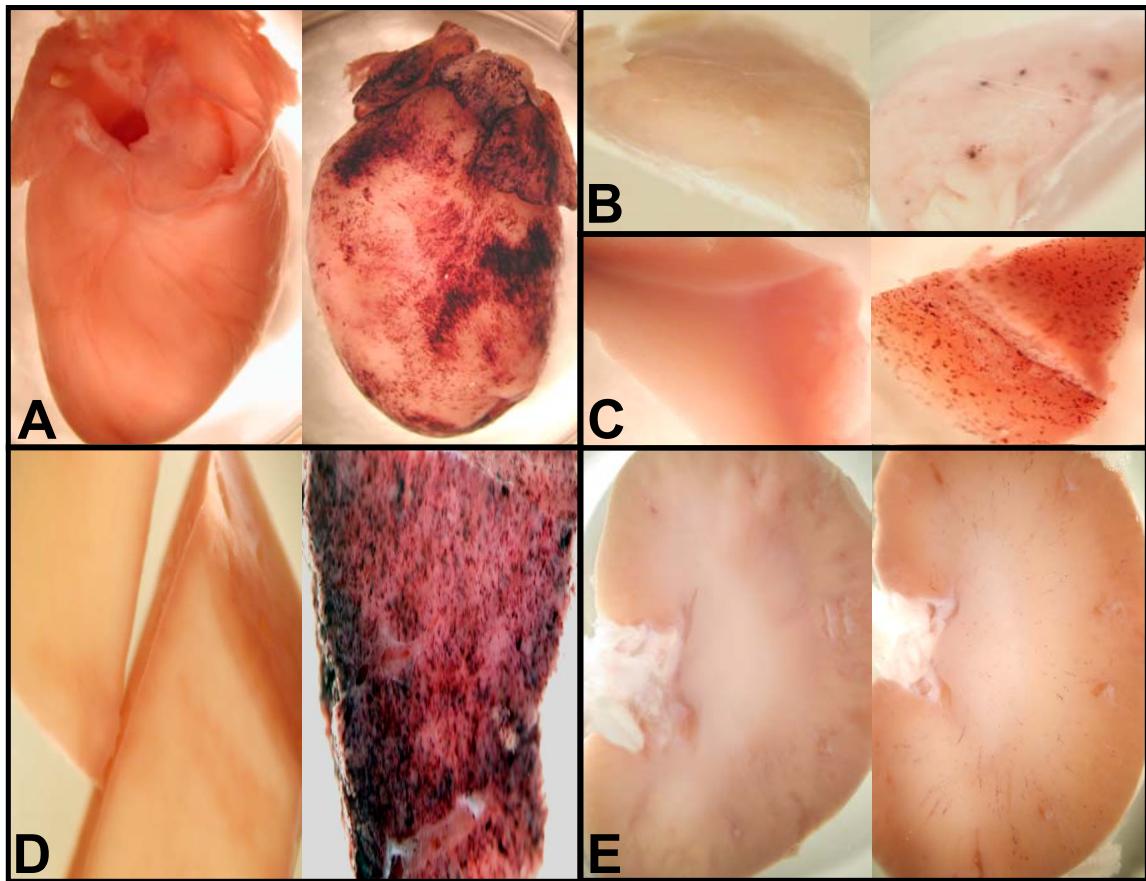


Figure 3-6. Systemically delivered lentiviral vector transduces multiple tissues relevant to the cardiovascular system. A total of 2.5×10^8 infectious particles were delivered to neonatal rats and tissues were collected and stained for PLAP activity ($n=4$ for each treatment group). In each lettered panel tissue from control animals is shown on the left: A) Heart, B) Adrenal Gland, C) Lung, D) Liver, and E) Kidney. Pictures were acquired at low magnification through a dissection microscope.

The second set of experiments focused on the *in vivo* efficacy of the vector system. When delivered into the circulation of 5-day-old rat pups it was shown that the higher the

dose of virus given the greater the amount of transduction (Figure 3-4). This finding seems intuitive, but many times gene transfer efficiency is shown to reach a plateau at a point where additional vector doesn't necessarily result in increased transduction. I think in our case this plateau is much higher than under "normal" circumstances due to the fact that our vector is injected and then disseminated throughout the entire blood stream of the animal. We are delivering the maximum dose currently possible. In the future, if higher titers can be obtained, I believe that systemically administered virus will transduce at an even higher level.

We also examined the length of transgene expression following the similar delivery of the highest dose of vector. In this study gene expression was found up to 120 days, the duration of the examination period (Figure 3-5). In another study not detailed here expression was documented at 200 days post-delivery. Such results are not surprising due to the well documented long-term nature of lentivirus-mediated transgene expression (Barker and Planelles 2003). It could be expected that virus administered to a five-day-old animal will continue to robustly express transgene for the life of that animal.

Lastly, and most importantly, we examined the biodistribution of systemically administered lentivector. To our surprise, lentivector-mediated transgene expression was found in varying extents in every tissue examined (Figure 3-6). The heart and liver were particularly well transduced at approximately 60 and 30% respectively. Additionally, in the heart upwards of 95% of the positive cells expressed cardiomyocyte morphology (Figure 3-7). These results illustrate the overwhelming potential of the lentivector to transduce a wide array of tissue and cell types *in vivo*. It also illustrates a potential problem. When vector is administered this way it may be important to limit transgene

expression through the use of cell specific promoter elements. In this way it becomes non-consequential if a cardiomyocyte promoter is used in the vector, but its major target happens to be hepatocytes.

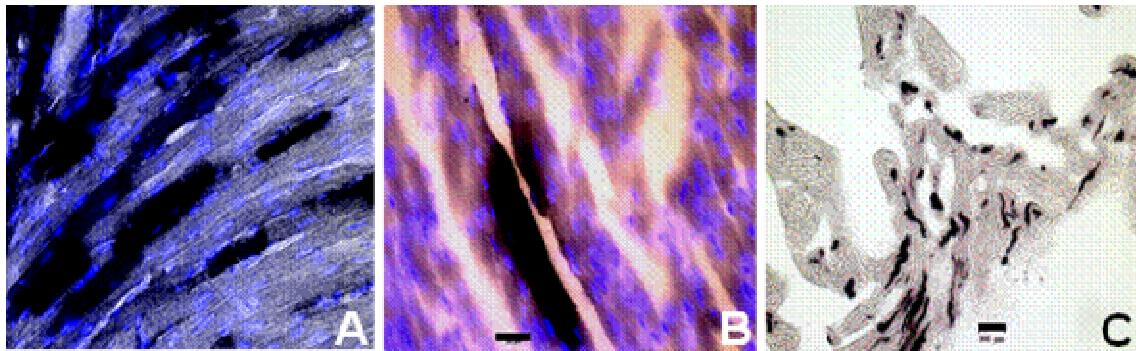


Figure 3-7. Lentivector effectively transduces cardiomyocytes *in vivo*. PLAP encoding vector was delivered into neonatal rats and ten micrometer thin sections were stained for PLAP activity. A) and B) Left ventricle sections with DAPI co-staining as a nuclear marker (Bar = 25 μ m). C) Left atria section (Bar = 100 μ m).

Surprisingly, our studies also showed that the systemically delivered lentiviral vector transduced germ cells in the male rat (Figure 3-8). To our knowledge, this is the first example of viral vector-mediated germ cell transduction in which transgene expression was detected using histochemical methods rather than PCR-based detection methods. Expression of PLAP was seen within the testicular tubule spermatogonia and included PLAP-positive spermatocyte, spermatid, and mature spermatozoa.

Our observation of transduced germ cells in male rats, while intriguing, must be interpreted with caution with regard to its potential impact on the use of lentiviral vectors for gene therapy. We believe that the transduction we observed could be attributed to the poorly developed blood-testicular barrier that is present in 5-day-old rats. It is our hypothesis that injections of lentivirus after this barrier has matured will not result in transduction of germ cells. It would be interesting to determine if our hypothesis is correct by conducting these experiments in adult animals.

The results detailed here shed some light onto the use of the lentivector in the cardiovascular system. Vector dose along with duration of expression and biodistribution were examined. These parameters set the stage for the future use of a cardiovascularly relevant therapeutic gene in the lentivector.

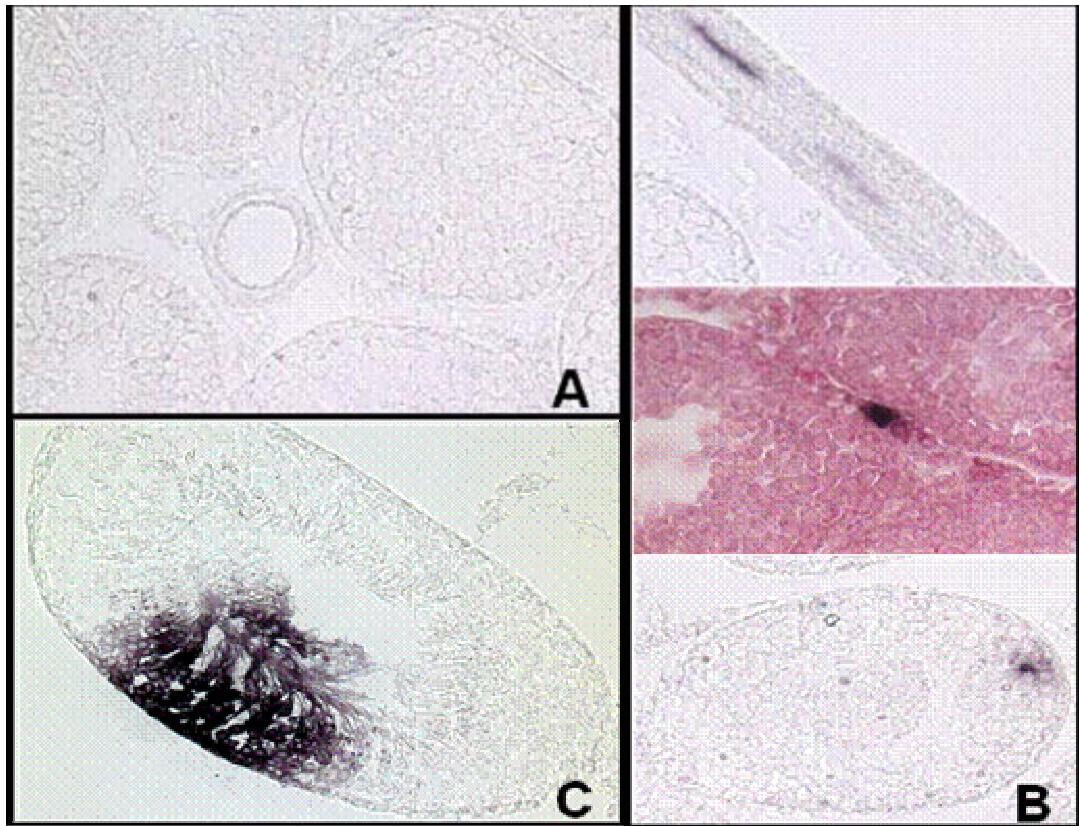


Figure 3-8. Systemically administered lentiviral vector is capable of transducing germ cells. Lentivector encoding PLAP was delivered into the circulation of neonatal rats. The testes were removed at various times and stained *in toto* for PLAP expression. Frozen thin sections were then prepared and analyzed. Samples are as follows: A) Control injected animal at 30 days post-injection, B) Three examples of different cell types within the testes in a vector injected animal at 30 days post-injection, C) Germ cell transduction in a 120 day old animal. Note the purple staining mature sperm cells in the lumen of the tubule.

CHAPTER 4
**ANGIOTENSIN II TYPE 2 RECEPTOR GENE TRANSFER ATTENUATES THE
DEVELOPMENT OF CARDIAC HYPERTROPHY IN THE SPONTANEOUSLY
HYPERTENSIVE RAT.**

Introduction

The role of Ang II actions at cardiac AT₁R s is well characterized during both physiological and pathophysiological conditions. Under pathological concentrations of Ang II the cardiac AT₁R s increase left ventricular hypertrophy, interstitial fibrosis, collagen deposition, and up-regulate extracellular matrix gene expression (Wagenaar et al, 2002).

If AT₁R sites are pharmacologically blocked during pathophysiological exposure of the heart to Ang II these effects of the AT₁R are avoided. Interestingly if AT₂R sites are blocked the effects via the AT₁R are enhanced. For this reason, among others, many investigators propose that during Ang II mediated cardiac disease the AT₂R s are up-regulated in an attempt to suppress the actions of the over-stimulated AT₁R s.

However, the role of the AT₂R in the heart is still poorly misunderstood if not controversial. As an example, one can simply investigate the currently available transgenic mouse models.

One mouse line was engineered to be completely devoid of AT₂R expression in every tissue of the body (AT₂R -/-). The AT₂R -/- mice were shown to have increased sensitivity to Ang II infusion exhibiting higher blood pressures (BP) and lower sodium secretion rates compared to wild-type littermates (Siragy et al, 1999). Lower levels of bradykinin and cGMP were also indicated. Abdominal aortic banding resulted in similar

increases in BP between strains, but the AT₂R -/- animals exhibited increased coronary artery wall thickening and increased perivascular fibrosis (Akishita et al, 2000).

However, in this study no effect on cardiac hypertrophy was noted between the two strains.

Using the same strain, another group showed that chronic Ang II infusion was unable to induce cardiac hypertrophy only in the AT₂R -/- animals while normal littermates did indeed develop hypertrophy (Ichihara et al, 2001). Together, these studies suggest two very differing roles for the AT₂R with regards to cardiac hypertrophy. On one side of the coin, the AT₂R appears to be essential for induction of hypertrophy while the other side suggests no role at all for the AT₂R in hypertrophy.

A second transgenic mouse exists over-expressing the AT₂R only in cardiomyocytes (Masaki et al, 1998). Upon chronic Ang II infusion in these mice a number of effects were noted both systemically and on the heart including increased BP, increased bradykinin and nitric oxide levels, decreased MAPK activity, and no effect on apoptosis in relation to control animals. Additionally, Ang II caused increased cardiac hypertrophy and atrial natriuretic peptide expression, a well accepted marker gene for hypertrophy. In contrast to the findings in the AT₂R -/- mice, a decreased perivascular fibrosis was noted in the cardiomyocyte-specific AT₂R over-expressing mice (Kurisu et al, 2003). The findings with these mice support the hypothesis that the AT₂R is involved in stimulating hypertrophy.

Transgenic mouse models are extremely useful, but they suffer from the major complication of possible developmental effects due to the deletion or over-expression of the gene of interest. In the case of the AT₂R, it has already been implicated in the

development of the CV system due to its high levels of expression during fetal life and concomitant decrease following parturition. It is possible that alterations in AT₂R expression levels or patterns through transgenesis could result in the improper development of the CV system. This fact did not escape the authors of the transgenic studies because they reported no observed super-structural CV abnormalities between transgenic and control mice. However, it cannot be ruled out that certain unforeseen molecular problems exist in the transgenic animals. These molecular abnormalities could manifest themselves as the changes reported in the studies highlighted above. To avoid developmental affects associated with AT₂R gene perturbation it is necessary to allow cardiovascular development to proceed as normal in the presence of unaltered levels of AT₂R expression. To achieve this a conditional transgenic animal must be created that will delete or over-express the AT₂R on demand and after fetal development, or the AT₂R gene could be regulated up or down through the use of gene transfer in neonatal or adult animals.

The goal of this study was to elucidate the role of the AT₂R on cardiac hypertrophy in the SHR following normal fetal development of the CV system. Such an approach will help determine the true role of the AT₂R in hypertrophy.

Lentiviral vector expressing the AT₂R gene was created and characterized in vitro. Delivery of this vector into the systemic circulation of 5-day-old SHR prevented the development of cardiac hypertrophy versus control injected animals. Blood pressures of treated and control animals remained elevated and equivalent. These findings support a role for the AT₂R in the attenuation of cardiac hypertrophy irrespective of blood pressure changes.

Results

Lentiviral Vector Design and *In Vitro* Assay

Bicistronic lentivector TYF constructs were created expressing both the rat AT₂R and either PLAP, Neo, or EGFP (Figure 4-1) from the hEF1 promoter. All constructs were shown to express both the individual genes *in vitro* (data not shown). AT₂R - specific binding was assayed in stably transduced Chinese hamster ovary (CHO) cells. Using the TYF.hEF1.AT₂R.IRES.Neo virus, CHO cells were infected at 1MOI and selected with G418 (1,000ug/mL) for 14 days. After selection, binding was conducted using radiolabelled Ang II (Sar-Ile-¹²⁵I-Ang II) and selective AT₁R (1μM Losartan) and AT₂R (1μM PD123,319) antagonists (Figure 4-2). Scatchard analysis revealed a Kd of 0.82 nM (Figure 4-2). Specific activity equal to 4.85 pmol/mg of protein indicated robust expression of the AT₂R transgene (Figure 4-2). Although not shown here, microarray experiments conducted by Beverly Metcalfe in Mohan Raizada's laboratory indicate that over-expression of the AT₂R using the identical lentivector was able to induce elevated expression of both caspase-3 and PP2A without ligand. These two proteins are known downstream targets of the AT₂R signal transduction cascade. Together these experiments illustrate that the TYF-hEF1.AT₂R.IRES.Neo lentivector can successfully transfer both the AT₂R and Neo genes *in vitro* and the AT₂R protein produced in transduced cells exhibits specific cell surface binding and appears to couple to its respective intracellular signaling cascade.

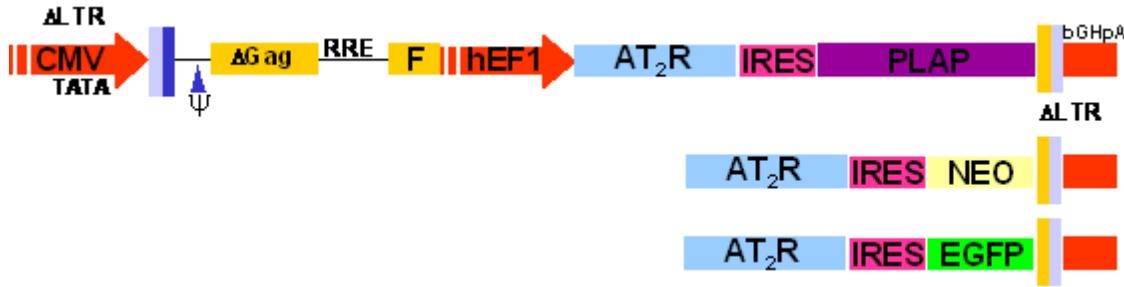


Figure 4-1. Lentiviral vector constructs encoding the AT₂R.

In Vivo Delivery of TYF.hEF1.AT₂R.IRES.Neo

Large-scale preparations of TYF.hEF1.AT₂R.IRES.Neo lentivector were prepared and 1.5 X 10⁸ total particles were delivered into the left ventricular space of 5-day-old male SHR (n=5). Control animals received an injection of virus resuspension buffer (a-CSF, n=6). At 11 weeks of age control echocardiography was performed on each animal. The initial measurement of left ventricular (LV) wall thickness indicated neither group of animals were hypertrophic (p=0.57, Figure 4-3). Additional echocardiograms were measured 5 weeks later (at 16 weeks of age) and revealed a LV wall thickness of 0.155cm +/- 0.003cm for treated and 0.178mm +/- 0.006cm for control animals (p<0.01, Figure 4-3). A third set of echos were measured at 20 weeks of age and indicated a LV wall thickness of 0.158cm +/- 0.003cm in the treated animals and 0.186mm +/- 0.019cm in control animals (p=0.24, Figure 4-3). When the findings are compared using repeated measures the p value is just short of significance (p=0.06, Figure 4-3). The individual, and overall, change in wall thickness was also compared. From the echos measure at 11 weeks to those measured at 16 weeks of age the treated animal's wall thickness increased by 0.15cm +/- 0.003cm and the control animal's increased by over double that number at 0.38cm +/- 0.06cm (p<0.05, Figure 4-4). From 16 to 20 weeks the treated animals showed a 0.05cm +/- 0.05cm increase while the control animals increased by 0.43cm +/-

0.17cm ($p=0.05$, Figure 4-4). When compared using repeated measures the p value obtained significance ($p<0.01$, Figure 4-4). After the echocardiograms were performed at 16 and 20 weeks the animals were allowed to recover and indirect systolic blood pressures were measured. At 16 weeks the virus treated animals had a blood pressure of $186 +/- 7$ mmHg while the control animals had a pressure of $184 +/- 5$ mmHg ($p=0.84$, Figure 4-5). At 20 weeks the treated animals had a pressure of $180 +/- 5$ mmHg while the control animals had pressures of $188 +/- 7$ mmHg ($p=0.51$, Figure 4-5).

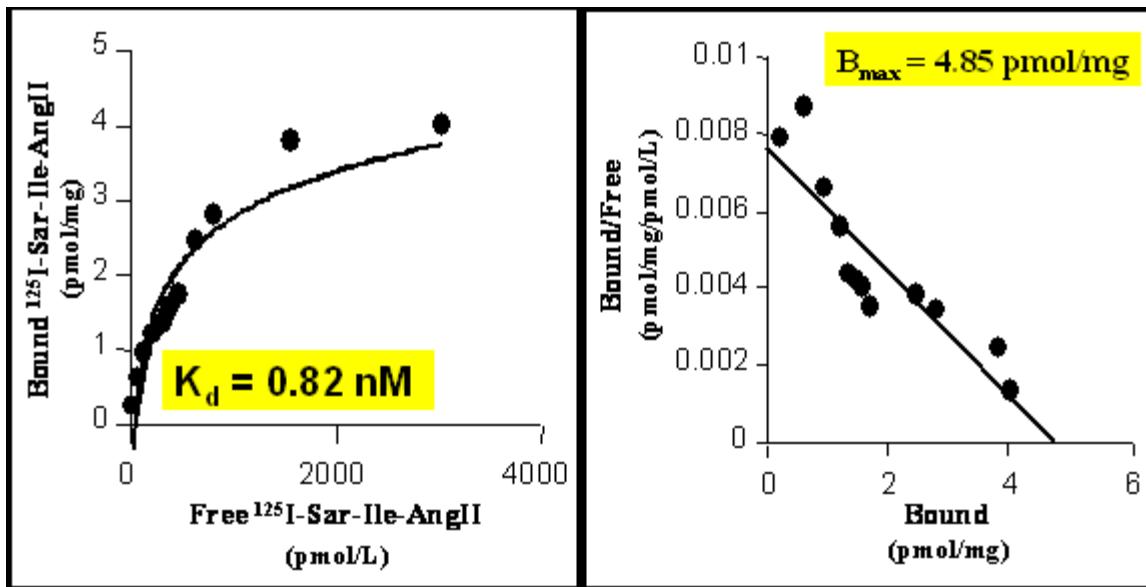


Figure 4-2. Binding characteristics of the AT_2R expressed from the lentiviral vector in CHO cells. CHO cells were transduced at 1MOI with a lentiviral vector expressing the AT_2R and NEO genes. After drug selection, whole cell binding was performed with ^{125}I -Sar-Ile-AngII.

Discussion

The role of the cardiac AT_2R under pathologic loads of Ang II is poorly understood. For example, through the use of transgenic animals researchers have reported conflicting results with regards to the importance of the AT_2R in the development of cardiac hypertrophy and perivascular fibrosis. The AT_2R plays a

demonstrated role in development therefore such transgenic models may be inherently flawed through the simple up or down regulation of this receptor during the developmental period.

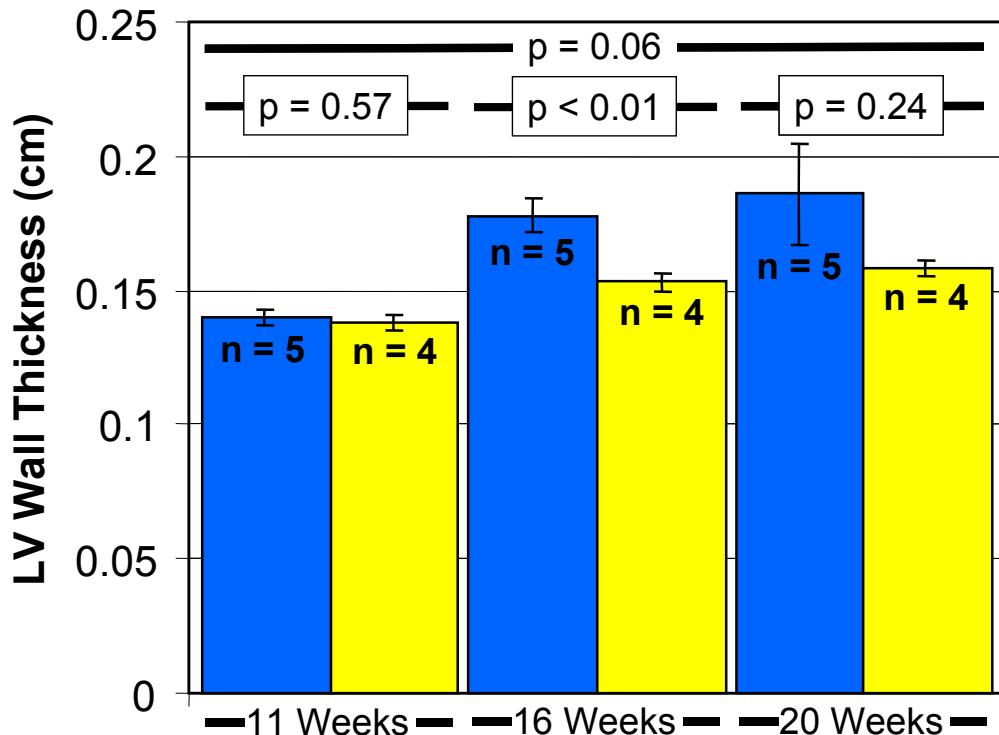


Figure 4-3. AT₂R gene transfer results in decreased cardiac hypertrophy in the SHR. Lentivector encoding AT₂R was transferred into neonatal SHR. At 11, 16, and 20 weeks of age echocardiography was performed. Illustrated is the left ventricular free wall thickness for control (blue) and vector treated (yellow) animals. P values are indicated above each respective set of bars. The repeated measures p value is indicated on top of the figure.

To avoid any unnecessary alterations in development we created a lentiviral vector expressing the AT₂R and delivered it into the circulation of neonatal SH rats. Through the use of such an approach we were able to demonstrate a clear inhibitory effect of AT₂R over-expression on cardiac hypertrophy. These results were demonstrated in the SHR, a hypertensive model known to express an overly active RAS, but were seen in the

absence of any alteration in blood pressure. Therefore, the AT₂R gene appears to be altering only the cardiac tissue-based RAS. This is not surprising based on the rather impressive cardiac transduction observed when delivering the lentivector as we did in this study into the left ventricular cavity (see Chapter 3).

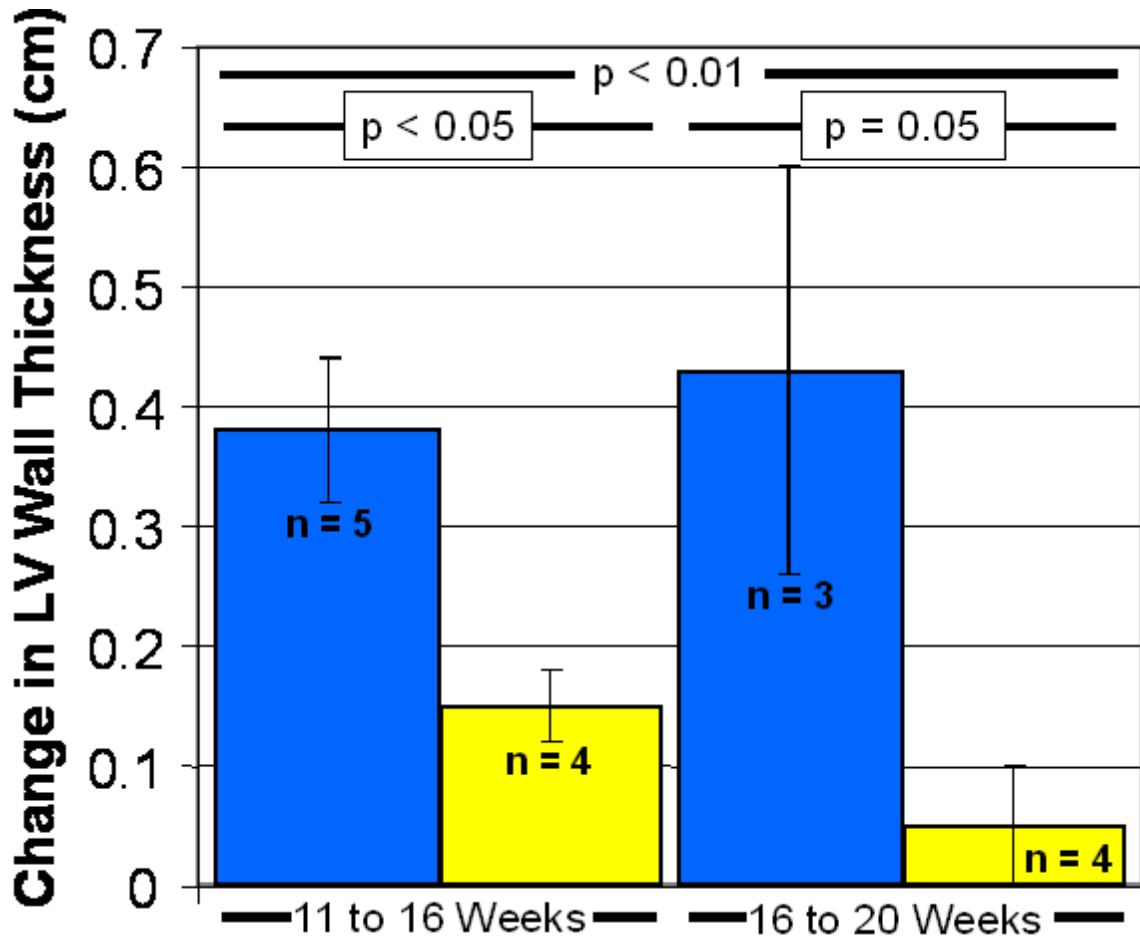


Figure 4-4. AT₂R gene transfer significantly decreases the increase in left ventricular wall thickness for up to 9 weeks. Lentivector encoding AT₂R was delivered into neonatal SHR. At 11, 16, and 20 weeks of age echocardiography was performed on the animals. The changes reflected here relate to the measured increase in left ventricular wall thickness when compared to the previous echocardiogram. The results from the control animals are in blue while the vector treated animals are in yellow. P values are indicated for each set of experiments and a repeated measures p value is shown at the top of the figure.

The basic finding revealed in this study, the AT₂R is a negative regulator of cardiac hypertrophy in the SHR, is the first genetically-based demonstration of this in an animal with “normal” CV development. The term “normal” CV development is used because it is not entirely clear that the SHR model itself undergoes what could be considered normal fetal development. We know that the SHR is a polygenic model of hypertension therefore it is plausible that it may express certain genes that could make it more amenable to cardiac directed therapy with the AT₂R gene. To investigate this, future experiments should be conducted using a myriad of high Ang II models of hypertension (i.e. chronic Ang II infusion, Ren2 transgenic rat, etc...)

The true cardiac specific effects of these findings could also be called into question. As shown in Chapter 3, lentivector encoding a hEF1.PLAP transgene delivered in exactly the same manner as in this experiment transduces many more tissues other than the heart. The similar blood pressures that were observed between treated and control animals suggests a truly cardiac specific effect for the AT₂R transgene, but it does not rule out peripheral effects from any of the other transduced tissues and cell types. To alleviate this problem, future experiments should use a lentivector encoding cardiomyocyte-specific promoter elements (i.e. alpha myosin heavy chain) driving expression of the AT₂R. In this manner, although other cell types will be transduced they will not express transgene and the direct effect of the AT₂R over-expression on the myocyte can be investigated.

Another area that could be improved is the age of the animal at the time of lentivector delivery. Although the cardiomyocyte is characteristically a quiescent cell type at all ages this vector system was really developed to be used in the adult animal.

Recently two independent research groups delivered lentivector into adult mice hearts *in vivo* and demonstrated efficient gene transfer using approximately the same number of infectious particles as our experiments in neonatal rats (Fleury et al, 2003). I feel it would be of great interest to deliver AT₂R expressing vector to adult rats just before or during the development of hypertrophy. This could more closely mimic the clinical situation where hypertensive patients already expressing LV hypertrophy may benefit from enhanced AT₂R expression or stimulation. More knowledge may also be gained by delivering vector after hypertrophy, but before complete failure of the heart. It is an important step to successfully block the development of hypertrophy, but actual reversal of the disease process should remain the ultimate goal.

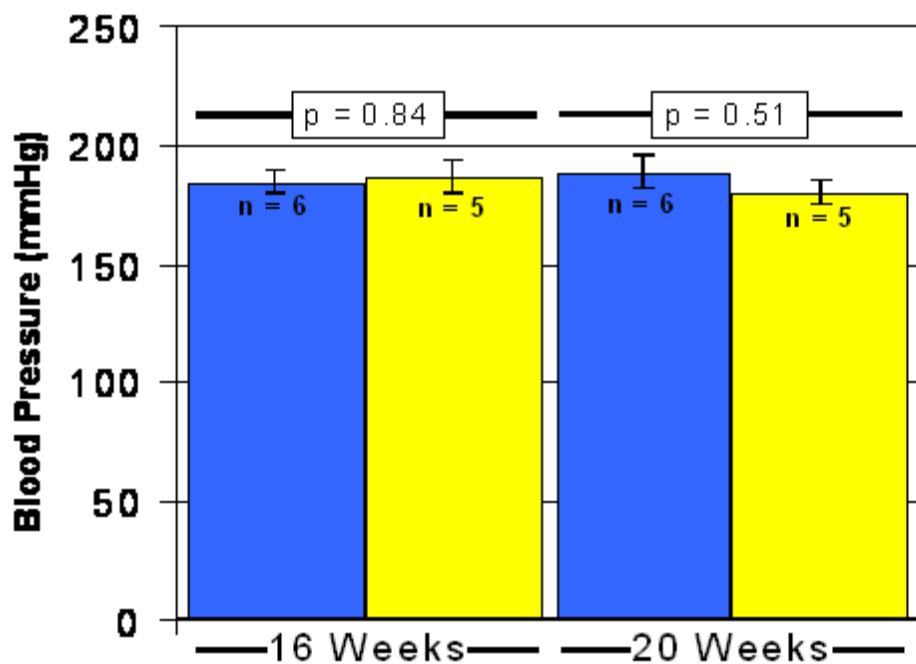


Figure 4-5. AT₂R gene transfer has no effect on systolic blood pressure in the SHR. Lentivector encoding the AT₂R was delivered into the systemic circulation of neonatal SHR. Indirect blood pressures were taken at the indicated times. Blue bars represent control treated animals while the yellow bars reflect the vector injected rats. P values are indicated above each set.

Our results illustrate another potential benefit of AT₂R stimulation in patients taking AT₁R blockers (ARBs). Patients on ARBs are known to have elevated circulating levels of Ang II . The endogenous AT₂Rs on cardiomyocytes, while extremely low in expression level, are nonetheless viable targets for this Ang II. Certainly ARBs slow the progression of cardiac hypertrophy, but much of this effect is presumably through their potent BP lowering abilities. The findings in this study occur separately from any changes in blood pressure. It may be interesting to inhibit or partially inhibit the AT₁Rs and see if the anti-hypertrophic effects of the AT₂R gene transfer are augmented.

In summary, our findings suggest a role for the AT₂R in the prevention of cardiac hypertrophy in the SHR. These findings are novel in that they were investigated in the rat after normal CV development. Although a relatively small and preliminary study the findings set the stage for future use of both the AT₂R gene and the lentivector in general in the heart.

CHAPTER 5 CONCLUSIONS AND DIRECTIONS

Chapter 2 and 3 illustrate the improvements made in the lentivector system and the resultant efficiency in CV-relevant tissues and cell types. When these studies were began very little was known about the *in vivo* performance of this vector system in tissues other than the brain. Even today, only two studies have been published detailing *in vitro* performance of the vector in cardiomyocytes and only three studies of its *in vivo* performance in the heart. Therefore, there is still much more to be learned about lentivector performance in the cardiovascular system. Our novel production and concentration schema represent a major advancement in the field. Our final titers are anywhere from 10-100 times greater than those currently reported in the literature using standard protocols. While a definite improvement, there is still more work to be done regarding the production and purity of the lentivector. Finally, Chapter 4 presents a preliminary study into the consequences of AT₂R over-expression in the heart of SH rats. As discussed, there is a lot of debate regarding the consequences of AT₂R gene activation in the cardiomyocyte. The work presented here seems to suggest that in the SHR the AT₂R may play a role in dampening the hypertrophic response of the cardiac muscle. Future evaluation of these findings in the SHR and other models of hypertension are necessary.

Lentivector Production and Performance

Further improvements in the technology of lentivector production can help keep this virus at the forefront of gene therapy vectors. Our final titers of 1×10^{10} TU/ml are high, but every small improvement in the production of this vector is a big help.

One avenue that should still be investigated is the creation of inducible packaging cell lines. Some systems have been created, however, they yield lower titers and require the laborious creation of new cell lines with every improvement in vector design (like the discovery of the cPPT/CTS DNA flap benefits). It is quite clear that the creation of a robust packaging cell line would greatly increase the “user friendliness” of the vector and therefore increase its usage. The use of alternative envelope proteins instead of the VSV-G will aid in the packaging cell line establishment. One group recently used the baculovirus GP64 protein in a packaging cell line with promising results (Kumar et al, 2003).

Cell free lentiviral vector production may one day become a reality. Recently a Canadian group was able to produce infectious encephalomyocarditis virus using cellular extract in a test tube (Sirven et al, 2000). This would certainly be the safest way to engineer lentivector and should be quite easily scaled up. All necessary proteins could be produced and then mixed with human cell membranes and vector genome. Lentivector could then be directly purified thereby significantly decreasing production time. Such protocols are possibly many years away, but I think it represents a major direction where viral vector production should be heading.

The overall purity of the lentiviral vector preparation should also be considered. With “cleaner” vector preps it may be possible that the overall infectivity of the vector could increase. This was demonstrated recently by a group who used anion exchange

HPLC to purify lentivector and observed a 2-fold increase in transduction (Yamada et al, 2003). Column purification of the lentivector should be explored further. Simple size exclusion or ligand columns may be alternative methods.

Magnetic purification of the vector should also be explored. In 2001 a group from the United Kingdom prepared classical retroviral vector labeled with biotin and later purified this vector using streptavidin coupled magnetic beads (Hughes et al, 2001). The overall purity of the vector preparation wasn't directly assayed, but the procedure improved their titers over 10-fold. Such a technique could easily be employed to lentivector production and the benefits may be increased infectivity and purity.

Lastly, work in the field of directed integration has advanced greatly recently. One group used a bacteriophage derived integrase to insert plasmid DNA into specific sites in the mouse genome (Olivares et al, 2001). In the human there are limited number of integration sites for this phage integrase. If incorporated into the lentivector system it may be possible to actually direct transgene integration into innocuous sites in the human genome and therefore relieve the fears of insertional mutagenesis. Such an advancement would represent the single most major accomplishment in the retroviral field in the past decade.

The AT₂R and Other Targets of the RAS

The AT₂R results, while interesting, are still preliminary due to the relatively small sample size. I think the next thing to be done with the AT₂R expressing vector is to repeat the experiment in SH rats to increase sample size and also to use Sprague-Dawley (SD) rats in a separate experiment. The SD rats have the added advantage of being much “cleaner” with regards to their genetics. Hypertrophy would have to be induced in the SD animals with chronic Ang II infusion.

Next, I would develop surgical methods to deliver the lentivector into the hearts of adult rats. As discussed earlier, one group has already achieved great success using the lentivector in the hearts of adult mice with transduction efficiencies approaching 80% (Bonci et al, 2003). Such methods will allow us to use the vector in adult animals as was intended at the inception of our experiments. This situation also more closely mimics what would take place in human therapy using the AT₂R. Additionally, we can begin to more fully dissect the AT₂R's role in hypertrophy by delivering the vector directly into the heart of animals at different stages of hypertrophic growth. Can the AT₂R reverse hypertrophy or can it only play a therapeutic role if present before the process begins?

After these initial experiments, I think future experiments should utilize a cardiomyocyte specific promoter, like alpha myosin heavy chain, to direct expression of the AT₂R. In this manner it will become much clearer if the therapeutic effects are due to the genetic manipulation of the myocytes, as we propose, or through some other mechanism.

Additionally, it would be advantageous to use an inducible promoter system, like the commonly used tetracycline-based system, to turn on or off the AT₂R gene. If this were achieved we could then transduce the animals at a young age and then induce expression of the AT₂R at different stages of hypertrophy or heart failure as they aged. The ultimate inducible promoter would respond to the actual disease state we are trying to prevent. Work is ongoing in our laboratory to synthesize an Ang II inducible promoter so that when the myocytes are exposed to high levels of Ang II, as is the case during hypertrophy, they will automatically turn on the therapeutic transgene.

Finally, the discovery of new components in the RAS is very exciting. The Mas receptor, ACE2 enzyme, and renin receptor should be inserted into the lentivector as quickly as possible so that we may use this unique technology to help elucidate the role of these newly discovered proteins.

APPENDIX METHODS

Chapter 2

Lentiviral Vector Constructs: pNHP, phEF1.VSV.G, and the pTYF “family”

The pNHP construct used to produce all vectors utilized in the studies described here is a modified version of the pHp plasmid (Chang, Urlacher et al. 1999). The pNHP has additional deletions of all remaining accessory proteins in addition to the deletions already highlighted in the original pHp plasmid. The pNHP plasmid was a kind gift of Lung-Ji Chang, University of Florida. The phEF1.VSV.G construct was fully detailed elsewhere (Iwakuma, Cui et al. 1999). This plasmid was another gift of Lung-Ji Chang.

The transducing vector used in our experiments was derived from a previously described self-inactivating vector (Iwakuma, Cui et al. 1999). The pTY vector was modified by inserting a cPPT-DNA FLAP element upstream of the multiple cloning site, an element that has been shown to significantly improve the transduction efficiency of recombinant lentiviral vectors *in vitro* and *in vivo* (Zennou, Petit et al. 2000). The original pTY vector was detailed elsewhere and was a generous gift of Lung-Ji Chang (Iwakuma, Cui et al. 1999). A 186-bp fragment containing the cPPT-DNA FLAP sequence was amplified from the pNHP vector using the polymerase chain reaction and the same core primers that have been previously described (Sirven, Pflumio et al. 2000). *Eag1* and *Not1* restriction sites were added to the sense and antisense primers, respectively. The resulting fragment was cloned into the *Not1* site of the pTY vector in the sense orientation creating the pTYF vector. The integrity of this modification was verified by DNA sequencing.

The TYF “family” of vectors were produced on the backbone of this original pTYF construct (known as pTYF.Linker).

The hEF1 alpha promoter was amplified from phEF1.VSV.G (Upper primer: 5' GCGCGGCCGCTTGGAGCTAACGCCAGCAAT, Lower primer: 5' GCGCTAGCA
TCGATTCACGACACCTGAAATGG) and cloned into the 5' *Not I* and 3' *Nhe I* sites in the pTYF.Linker. This new construct was denoted as pTYF.hEF1.Linker. The pTYF.hEF1.PLAP construct was made through the removal of the PLAP gene from the pRISAP construct (gift from Susan Semple-Rowland, UF) using 5' *Pme I* and 3' *Kpn I* digestions. This gene was directionally cloned into pTYF.hEF1.Linker at the 5' *Sma I* and 3' *Kpn I* sites. The pTYF.hEF1.EGFP vector was made by using PCR directed against the EGFP gene (Upper: 5' GCGTTAACGATCCACCGGTCGCCACCAT, Lower: 5'GCGGTACCCGCTTACTTGTACAGCTCGTC) in the pEGFP-N1 plasmid (Clontech). The amplified gene was cloned directionally into pTYF-hEF1.Linker at 5' *Sma I* and 3' *Kpn I*.

The bicistronic IRES constructs were created using a three fragment ligation approach. pTYF.hEF1.Linker was opened with 5' *Nhe I* and 3' *Kpn I*. The IRES, PLAP, Neo, and EGFP genes were amplified using the primers below:

IRES: Upper 5' GCGTCGACGTTAACATCGGAGCTAAAAC, Lower 5'GCACG CGTCCGCAATCCAA

PLAP: Upper 5' GCACCGTGCGTCGACAATA, Lower 5' GCGGTACCGATA TCTGGCCGTCTCCAG

Neo: Upper 5' GCACCGTGCCACCATGATTGAACAAGA, Lower 5' GCGGTA CCGATATCCGCTCAGAAGAACTCGTCAA

EGFP: Upper 5' GCACGCGTGATCCACCGGTGCCACCAT, Lower 5' GCGG
TACCGATATCCGCTTACTTGTACAGCTCGTC

All the fragments were individually purified and ligated at 1:1:1 molar ratios to create pTYF.hEF1.IRES.PLAP, pTYF.hEF1.IRES.Neo, and pTYF.hEF1.IRES.EGFP.

pTYF.hEF1.IRES.HygEGFP was created by moving the HygEGFP gene from pHygEGFP (Clontech) with 5' *Mlu I* and 3' *Sma I*. This fragment was ligated directionally into pTYF.hEF1.IRES.Neo cut open with 5' *Mlu I* and 3' *EcoRV*.

DNA Preparation

Plasmid DNA was prepared in either one of two methods. One method involved the use of Mega Plasmid Prep kit from Qiagen while the second method involved a modified alkaline lysis protocol followed by CsCl-EtBr gradient banding of the DNA (from Maniatis et. al).

Production of Lentivector: Transfection

293FT cells (Invitrogen Corporation, #R70007) were seeded in 75 cm² (T-75) culture flasks at a density of 1 x 10⁷ cells per flask and grown in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum and antibiotics (130 U/ml penicillin and 130 µg/ml streptomycin; growth medium). Prior to cell seeding the culture flasks were coated with poly-D-lysine in PBS for 1-2 hours at 37°C. Culture flasks were then rinsed 1X with PBS and stored dry overnight at room temperature. The cultures were maintained at 37°C in 5% CO₂ throughout the virus production period.

On the following day, or when the cultures reached 90-95% confluency, the transfection was performed. For one large-scale preparation of virus, 27 T-75 flasks of 293FT cells were transfected as follows: Transfection mixture for all 27 flasks was prepared by gently mixing 192 µg pNHP, 95 µg pTYF and 76 µg pHEF.VSVG plasmid

DNA and 8.0 ml DMEM in one 50 ml polystyrene tube. After mixing, 756 µl of Superfect was added to the DNA solution. The contents of the tube were gently mixed and incubated at room temperature for 10 min.

Next, these transfection complexes were diluted into 140 ml of pre-warmed growth medium. The cultures that were seeded ~24 hours ago were then removed from the incubator, their medium was aspirated, and then replaced with 5 mls of growth medium containing the transfection complexes. The flasks were then incubated for 4-8 hours in a 37°C/5% CO₂ incubator.

Following the incubation period, the medium containing the transfection mixture was replaced with 6.0 ml of fresh growth medium. The next day, the media containing the first batch of virus was harvested from each flask and 5.0 ml of fresh growth medium was added to the cells. This should result in two collections of vector, one at ~30 hours post-transfection and a second at ~45 hours.

To prepare transfection mixture sufficient for one T-75 flask, the amounts of DNA, DMEM and Superfect were each divided by 27 to scale the reaction down. We have also found that viral vector can be produced in larger or smaller cell culture flasks or plates by simply scaling cell numbers and the amount of DNA, DMEM and Superfect linearly with respect to the cell growth area.

Production of Lentivector: Concentration

The two collections of vector are handled independently. Each collection of vector is performed in 50 ml polystyrene tubes on ice. Each tube is then centrifuged at 2000 x g for 10 minutes then filtered through a 0.45 micron low protein binding membrane (Nalgene, PES). Concentration steps are outlined in Figure 2-4.

For ultrafiltration, the virus stock collected from 27 T-75 flasks at 30 h post-transfection (~120 ml) was divided into two 60 ml aliquots and centrifuged through Centricon-80 ultrafiltration columns (Millipore) for 1 h in 4°C at 2500 x g. The retentate was retrieved by centrifuging the inverted column for 1 min in 4°C at 990 x g and was stored at 4°C until further processing. On the following day, the virus-containing retentate was added to the ~120 ml of virus-containing media collected at 45 h post-transfection. Four 30 ml conical-bottom tubes (polyallomer Konical tubes; Beckman), each containing a 220 µl cushion of 60% iodixanol solution (used directly from the Optiprep stock solution obtained from Axis-Shield) were prepared. Iodixanol was used because of its demonstrated safety in human clinical trials.

Media containing virus (30 ml) was gently pipetted into each tube, taking care not to disturb the iodixanol, and the samples were centrifuged at 50,000 x g for 2.5 h at 4°C using a Beckman SW-28 swinging bucket rotor. The media just above the media/iodixanol interface was carefully removed from each tube and discarded, leaving ~750 µl of the solution in each tube (220 µl of iodixanol plus ~500 µl of media). The residual media containing virus and the iodixanol were mixed gently. The resulting mixtures were pooled into one 3 ml conical-bottom tube (polyallomer Konical Tubes; Beckman) and centrifuged at 6100 x g for 22-24 h at 4°C using a Beckman SW-50.1 swinging bucket rotor.

The resulting supernatant was removed and discarded and the remaining pellet was resuspended in 30-50 µl of artificial cerebrospinal fluid by incubating the virus at 4°C for 10-14 h. The final viral vector was gently mixed by pipetting, aliquoted and stored at -80°C until use.

Lentivector Titration: PLAP

Infectious titers of the TYF.hEF1.PLAP virus were determined by incubating 1.75×10^5 TE671 cells seeded in 12-well plates with limiting dilutions of the viral stock (1/10, 1/100 and 1/1000) in the presence of 8 µg/ml polybrene. After an overnight incubation period, the vector containing medium was removed and fresh medium was added directly to the cells. After 48 hours, cultures were rinsed 2X with PBS, fixed in 4% paraformaldehyde for 15 minutes at room temperature, rinsed 3X with PBS, and heated in PBS at 72°C for 60 minutes. The plate was then allowed to cool to room temperature with the lid removed. BCIP pre-inhibition buffer is then added to the cells and incubated for 30 minutes at room temperature. The pre-inhibition buffer is removed and replaced with BCIP reaction buffer. The cells are then incubated in the dark at room temperature for 1 hour and then moved to 4°C overnight. The following morning, the cells are rinsed with PBS containing 50mM EDTA and finally overlayed with 1ml of PBS + 50mM EDTA. Ten random areas from each well are scored for the total number of PLAP positive (purple) cells when viewed using the 10X objective. Final titer is determined using the following equation:

$$\frac{(\text{Number of PLAP positive cells} / \text{number of areas examined}) * 152}{\text{mL of virus added to the well}}$$

The number of transducing units (TU; defined as an infectious particle) was expressed as TU/ml which is equivalent to the number of PLAP positive cells per ml of virus.

Delivery of TYF.hEF1.EGFP Vector to Brain Nuclei

Male Wistar rats were anesthetized with a mixture of ketamine (60 mg/kg) and medetomidine (250 µg/kg) and placed in a stereotaxic frame. Vector delivered to the

brain was suspended in artificial cerebrospinal fluid. For the paraventricular nucleus (PVN) injections 275 g rats were used and the head of the animal was flexed 5 mm below the interaural line. The microinjection pipette was angled ten degrees relative to the midline to avoid the mid-sagittal sinus. A slow injection of 500 nl (5×10^5 TU) of virus was performed at the following coordinates: 1.8 mm lateral, 1.8 mm caudal to the bregma and 7.5 mm below the surface. The caudal nucleus of the solitary tract (NTS) was also injected bilaterally with 3 injections per side for a total of 300 nl (3×10^5 TU). The site of injection was within 0 to -500 μm relative to *calamus scriptorius*, 350-700 μm from midline and 500-600 μm below the dorsal surface of the medulla. The head of the animal was flexed 10 mm below the interaural line. The animals were sacrificed either 7 days (PVN, n = 2) or 30 days (NTS, n = 2) following the injections and fixed by intracardial perfusion with 4% paraformaldehyde in PBS. Brains were removed, cryoprotected in 30% sucrose, 60 μm thick brain sections were cut on a cryostat and confocal microscopy (Leica SP) was used to visualize GFP fluorescence.

Cell Culture

Human embryonic kidney cells (293FT) were obtained from Invitrogen Corporation (#R70007). Human medulloblastoma cells (TE671) were obtained from the European Collection of Cell Cultures (#89071904).

Solutions

Artificial cerebrospinal fluid (virus resuspension buffer) was made as described on <http://www.alzet.com> with the addition of 0.1% heat-inactivated (56°C , 40 minutes) fetal bovine serum + 1X fungizone and 1X penicillin/streptomycin. D-MEM (high glucose) was purchased from Invitrogen. Iodixanol was used as provided from Axis-

Shield (Oslo, Norway). Polybrene (Hexadimethrin bromide) was prepared as a 100X solution (800 μ g/ml) in Hank's balanced salt solution. BCIP pre-inhibititon buffer: 100mM Tris Base, 100mM sodium chloride, 50mM magnesium chloride, 0.5mM Levamisole, pH 9.5. BCIP reaction buffer: 100mM Tris Base, 100mM sodium chloride, 50mM magnesium chloride, 0.5mM Levamisole, 1mg/ml nitro blue tetrazolium, 0.1mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (also known as X-Phos or BCIP), pH 9.5.

Chapter 3

TYF Constructs

Additional TYF constructs used included pTYF.hEF1.DsRED and pTYF.hEF1.nlacZ. The DsRED construct was made by directly inserting the DsRED gene (Clontech) into pTYF.hEF1.Linker. The nlacZ construct was made by inserting the bacterial beta-galactosidase gene into pTYF.hEF1.Linker. Both constructs were generous gifts from Susan Semple-Rowland's laboratory, UF.

Growth Arrest and Transduction

All cell types (A.10, A7r5, HepG2, and RPAEC) were grown to confluency and then growth-arrested with aphidicolin at a concentration of 15 μ g/ml. At the same time as growth-arrest, the cells were overlaid with virus and incubated overnight. The following morning the vector containing media was removed and replaced with growth medium containing aphidicolin. The cells were either imaged or stained the next day.

Systemic Delivery of Lentivector to Neonatal Rat

Five-day-old animals were removed from their mothers and lightly anesthetized with methoxyfluorane. The animals are then placed on a heated surface and the heart visualized through the translucent chest wall. A standard insulin syringe needle was

inserted into the apex of the heart and angled into the left ventricular cavity. A small volume of blood was withdrawn into the syringe barrel to indicate the correct positioning of the needle and the 20-35 μ l volume of vector or control was delivered in a slow bolus. The needle was quickly removed and the animal allowed to recover in a heated location. Animals were then lightly coated with peanut oil and returned to their respective mothers. In all cases the investigator delivering the vector or control solution was blinded with respect to the composition of the sample being injected.

Cell Staining for lacZ

To assay for lacZ expression cells were first rinsed 2X with PBS then fixed in 4% paraformaldehyde for 10 minutes at room temperature. The fixed cells were then rinsed 3X with PBS and overlaid with lacZ staining buffer: 35mM potassium ferrocyanide, 35mM potassium ferricyanide, 2mM MgCl₂, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 1mg/ml X-gal, pH 8.0. Cells were then incubated at 37C until color developed.

Tissue Histochemistry for PLAP

The entire animal was perfused intracardially with 60mls of ice-cold PBS followed by 60mls of ice-cold 4% paraformaldehyde in PBS. Organs were then harvested into 4% paraformaldehyde and post-fixed for two hours on ice. Whole organs were then rinsed at room temperature in PBS 4 times for 15 minutes each rinse. The tissue was then heated in PBS at 72°C for 2.5 hours. After cooling to room temperature, the tissues were equilibrated in BCIP pre-inhibition buffer for one hour. The pre-inhibition buffer was then replaced with BCIP reaction buffer and the tissues were stained for one hour at room temperature. After this time they were moved to 4°C to develop a deep purple color overnight. In the morning the organs were rinsed 2X with

PBS + 50mM EDTA and stored in the same solution prior to cryoprotection and sectioning or direct microscopic analysis.

Cell Culture

All cell lines were acquired from American Tissue Culture Collection (ATCC) as follows: A-10 (CRL-1476), A7r5 (CRL-1444), HepG2 (HB-8065). Cells were cultured according to instructions provided by the supplier.

Chapter 4

AT₂R Lentivector Construction

AT2R coding region was generously provided by Jeffrey Harrison, UF. To create all three pTYF.hEF1.AT₂R.IRES vectors the AT₂R gene was excised from pLNSV-AT₂R with 5' *Avr II* and 3' *Cla I* and ligated into the pTYF.hEF1.IRES constructs cut open with 5' *Nhe I* and 3' *Cla I*.

Lentivector Titration: G418 Resistance

TE671 cells were split into 12-well plates at a density of 175,000 cells per well. After 24 hours they were transduced with 0.0001μl of vector in the presence of 8μg/ml polybrene in a total volume of 500μl in duplicate. Cells were then returned to the incubator for 24 hours after which time each well was rinsed with 1ml of trypsin solution and then overlaid with 500μl of trypsin solution. After a 5 minute incubation at 37°C to ensure complete dissociation of the cells from the bottom of each well, 500μl of growth medium (D-MEM + 10% FBS) was added to each well. The contents of each well were pipetted up and down to assure total de-aggregation of the trypsinized cells. Twenty microliters (a 1:50 dilution) of each cell suspension was then transferred to a well in a 6-well plate containing 2mls of growth medium in duplicate. This results in 4 total wells

used per 6-well plate: 2 wells containing duplicate dilutions of each originally transduced well of the 12-well plate. Control (non-transduced) cells were handled in the same manner. After another 24 hour period (now 48 hours from exposure to virus) the cells were incubated in growth medium containing 400 μ g/ml G418. Every 24 hour period the G418 concentration was increased by 400 μ g/ml until a final concentration of 1,200 μ g/ml was achieved. The cells were then incubated in the presence of 1,200 μ g/ml of G418 for a total of 10 more days. Fresh G418 media was added every other day to the cultures. At the end of this period the cells were rinsed 2X with room temperature PBS and then incubated in 0.1% crystal violet in 10% ethanol in water for 20 minutes. After this incubation, each well was rinsed 2X with 10% ethanol in water to remove an extra staining solution. The resulting purple staining, G418 resistant colonies were then counted and the titer of the virus calculated as infectious units (colony forming units) per ml of viral vector.

¹²⁵I-Angiotensin II Binding

Cells were grown to ~80% confluence in 12-well plates taking at least three days to reach this level following exposure to trypsin. Cells were removed from the incubator and rinsed 2X with room temperature PBS. Binding reaction mixtures consisting of 1% BSA, varied amounts of ¹²⁵I-Sar-Ile-AngII, and the presence or absence of specific inhibitors (losartan (1 μ M), PD123,319 (1 μ M), or cold AngII (100nM)) were then overlaid onto the cells. Binding was allowed to proceed at 37C for 30 minutes. After this time the cells were washed quickly 4X with ice-cold PBS to remove any unbound ligand. Cells were then lysed in 0.1N sodium hydroxide by incubating at room

temperature for 1 hour. This lysate was collected, each well was washed with distilled water, and then counted for total specific ^{125}I decay. Each sample was read in triplicate.

Cardiac Echocardiography

After mild sedation, echo readings were taken using an S12 probe and a clinical ultrasound machine at a depth of 2cm. The cardiac papillary muscles were used as a landmark for echocardiogram recording. Wall thickness was measured using an on-screen electronic micrometer. The investigator performing the echos was Dr. Leonard Parilak with assistance from David Taylor. In all cases, both investigator and assistant were blinded as to the animal's identification and treatment group.

Cell Culture

Chinese hamster ovary (CHO) cells were a generous gift from Peter Sayeski, UF.

Solutions

Losartan, PD123,319, and Angiotensin II were made up in sterile PBS at the indicated concentrations. G418 (Invitrogen) was made up in D-MEM without fetal bovine serum and sterile filtered before use.

Statistics

Repeated measures ANOVA and ANOVA were used. Outliers were determined using Grubb's Outlier test at a confidence level of $p<0.05$. Statistical analyses were performed using StatView (Version 5.0, SAS Institute Inc.).

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BIOGRAPHICAL SKETCH

Matthew John Huentelman was born on November 4th, 1975, in Hamilton, Ohio. Matthew's interest in science began in an advanced chemistry class at Hamilton High School taught by Susan King. Motivated to pursue a degree in chemistry, Matthew enrolled at Ohio University in Athens, Ohio. In this beautiful southeastern Ohio town he began to question his future as a chemist and as a result approached a faculty member in the Department of Chemistry, Dr. Peter Johnson, for some direction. Discovering his true interest lay in the biological applications of chemistry, his education path at Ohio University was altered. After four years, including two spent working in the lab with Dr. Johnson, Matthew graduated with a BS degree in biochemistry. Additionally, during his senior year a loving relationship was forged with a fellow Chemistry Department student, Heather Myers, who would later become his wife in 2002. Following undergraduate graduation Matthew spent three months working in the lab of Dr. Alexander Boldyrev, a close colleague of Dr. Johnson, in Moscow, Russia. Matthew's graduate thesis work began in 1998 following his enrollment in the University of Florida's interdisciplinary program in the biomedical sciences. Following the initial year of course work, he was excited to enter the laboratory of Dr. Mohan Raizada to pursue his Ph.D. degree in a stimulating and progressive thinking lab environment.