ABSTRACT

Design, Optimization and Evaluation of Conditionally Active Gene Therapy Vectors

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It is the objective of this project to produce gene therapy vectors that are active and/or significantly up-regulated due to specific physiological conditions. The significance of such constructs is that it imparts a greater degree of control in the implementation of gene therapy. In general, it is desirable for a gene therapy vector to be active only when and where it is needed. The majority of gene therapy research to date has focused primarily on obtaining expression levels high enough to elicit a therapeutic response, as well as, distributing the vector to enough tissues to provide a corrective effect to the disorder being addressed. However, simply having a gene adequately delivered to enough cells to treat disease and having the gene product be produced in sufficient amounts to have a therapeutic effect cannot be the end of the story. Not unlike genes found naturally in the body, artificially delivered genes also need to be regulated. The construction of such vectors could prove useful for the treatment of disorders; such as Coronary Artery Disease (CAD) or even Cardiomyopathy, that occur in a specific tissue type or that are associated with an abnormal physiological state, such as hypoxia. Our vector constructs are a small step towards this ultimate goal. In this study, we

present data on DNA vectors that were designed, constructed and evaluated *in vitro* and *in vivo*; both qualitatively and quantitatively. We report success in the creation of vectors/plasmids that are primarily cardiac tissue specific (pMHCI, pMHCII), vectors that are regulated by cellular oxygen levels (pHAL, pHAM), and even some success in combining the two (pHMHC).

Design, Optimization and Evaluation of Conditionally Active Gene Therapy Vectors

by

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A Dissertation

Approved by the Institute of Biomedical Studies

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Robert R. Kane, Ph.D., Director

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1, 2-dipalmitoyl-sn-glycero-3-phosphatidylcholine	DPPC
1, 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine	DPPE
4',6-diamidino-2-phenylindole	DAPI
Activator Protein-1	AP1
Adeno-Associated Virus	AAV
American Type Culture Collection	ATCC
Angiopoietin-1	ANG-1
Angiopoietin-2	ANG-2
Angiotensin-Converting Enzyme	ACE
Aryl Hydrocarbon Receptor Nuclear Translocator Protein	ARNT
Atherosclerotic Heart Disease	AHD
basic Fibroblast Growth Factor	bFGF
Baylor University Medical Center	BUMC
Betacellulin	BTC
Bovine Serum Albumin	BSA
Calf-Intestinal Alkaline Phosphatase	CIAP
cAMP Response Element	CRE
cAMP Response Element Binding Proteins	CREB
Centers for Disease Control and Prevention	CDC
Congestive Heart Failure	CHF
Coronary Artery Bypass Grafting	CABG

Coronary Artery Disease	CAD
Coronary Heart Disease	CHD
CREB Binding Protein	CBP
Cytomegalovirus	CMV
Digoxigenin	DIG
Dimethyl sulfoxide	DMSO
Di-Nucleotide Tri-Phosphate	dNTP
Dual Luciferase Reporter	DLR
Dulbecco/Vogt Modified Eagle's Minimal Essential Medium	DMEM
Electrocardiogram	ECG
Erythropoietin	EPO
Ethanol	EtOH
Familial Hypertrophic Cardiomyopathy	FHC
Fetal Calf Serum	FCS
Fibroblast Growth Factor	FGF
Fluorescein Isothiocyanate	FITC
Glucose Transpoter-1	GSP-1
Hypoxia Inducible Factor-1α	HIF-1α
Hypoxia Inducible Factor-2α	HIF-2α
Hypoxia Inducible Factor-3α	HIF-3α
Hypoxia Inducible Factor-β	HIF-β
Hypoxia Responsive Element	HRE
Institutional Animal Care and Research Advisory Committee	IACUC

Intravenously	IV
Ischemic Heart Disease	IHD
Luciferase Assay Reagent II	LAR II
Luri Bertani	LB
Multiple Cloning Site	MCS
Myocardial Infarction	MI
Nitric Oxide Synthase	NOS
Pancreatic Duodenal Homeobox-1	PDX1
Peripheral Vascular Disease	PVD
Phosphate Buffered Saline	PBS
Platelet-Derived Growth Factor-ß	PDGF-ß
Polymerase Chain Reaction	PCR
Prolyl Hydroxylase 4	PH-4
Responsive Element	RE
Reverse Transcription Polymerase Chain Reaction	RT-PCR
Severe Combined Immune Deficiency	SCID
Simian Virus 40	SV40
Streptozotocin	STZ
Super Optimized Culture Medium	SOC
Transmyocardial Laser Revascularization	TMR
Tris/Acetate/EDTA	TAE
Tris/Borate/EDTA	TBE
Tris/EDTA	TE

U.S. Food and Drug Administration	FDA
Ultrasound Targeted Microbubble Destruction	UTMD
Vascular Endothelial Growth Factor	VEGF
von Hippel-Lindau	VHL
YinYang1	YY1
α-Myosin Heavy Chain	αMHC MYH6
β-Myosin Heavy Chain	βMHC MYH7

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DEDICATION

To my family

CHAPTER ONE

Introduction

Background and Introduction

Gene Therapy is an area of research that holds tremendous potential for the treatment of disease and restoration of health. Although still in its infancy, the realm of gene therapy research has already yielded an impressive wealth of data. Potential treatments for a wide variety of disorders have already been forthcoming in recent years. Gene therapy research has shown enticing evidence of what may be possible in future decades. The concept of gene therapy has advanced research into disorders as diverse as cancer, diabetes, heart disease and Alzheimer's; just to name a few. Indeed, there is now hope that a variety of genetic disorders, once thought incurable, may someday be treatable and even cured completely. In fact, such results are already beginning to become a reality.

Most notably, this is the case with clinical trials designed to treat Human X-linked Severe Combined Immune Deficiency (SCID) via *ex-vivo* gene therapy (Cavazzana-Calvo et al. 2000). Several patients suffering from X-linked SCID have already been cured using this gene therapy approach. Unfortunately, for the promise of gene therapy to be fully realized, many obstacles are still to be overcome. For example, in the X-linked SCID trials 3 out of 20 patients developed T-Cell Leukemia. This was possibly due to a lack of precision in inserting therapeutic DNA via a retroviral vector (Francis et al. 2001; Hammond et al. 2001).

Unintended blockage of tumor suppressor genes or activation of oncogenes may lead to induction of cancer in the patient. Additionally, otherwise normally functioning genes may be altered as well. Gene therapy first appeared to be a magic bullet in correcting X-linked SCID. However, when fired blindly into the dark of the genome, the unintended and perhaps inevitable consequences yielded devastating collateral damage. The primary hindrance to gene therapy being truly revolutionary is adequate control, delivery and sustainability of gene expression.

This dissertation is comprised of a series of studies, which addresses in part some of these major challenges to gene therapy. The introduction begins with a comprehensive overview of the current state of knowledge and introduction to the specific objectives of the studies presented in this dissertation. Next, we evaluated the effectiveness of our chosen site directed delivery technique, Ultrasound Targeted Microbubble Destruction (UTMD) (Chen et al. 2007), confirming the effectiveness of UTMD demonstrated in previous studies (Bekeredjian et al. 2004). Next, we designed and constructed cardiac tissue specific gene therapy vectors and demonstrated effective delivery and expression of these DNA plasmids via UTMD *in vivo*. The construction of such tissue specific vectors could prove useful for the treatment of tissue specific disorders such as Coronary Artery Disease (CAD), Congestive Heart Failure (CHF), Familial Hypertrophic Cardiomyopathy (FHC), and others. This might conceivably be accomplished by the administration of a variety of potential therapeutic genes delivered to the precise tissue of interest.

For example, CAD may be treated by correcting or compensating for the ischemic condition. This has been demonstrated by numerous groups (Korpanty, et al. 2005) *in*

vivo utilizing genes such as Vascular Endothelial Growth Factor (VEGF), Angiopoietin-1 (ANG-1), Angiopoietin-2 (ANG-2), Fibroblast Growth Factor (FGF) and others (Prentice, et al. 1997). Such intervention should subsequently act as a preventative measure against further degeneration of the myocardium, as well as, a possible Myocardial Infarction (MI). This may someday become an effective component of an overall treatment strategy. Thus, the first fundamental gene therapy control concept we considered vital was the following: Ideally a gene should only be expressed in the specific location it is needed and not elsewhere.

Additionally, we confirm that oxygen levels may be utilized to effectively control the expression of gene therapy vectors and expound upon the current state of knowledge (Binley et al. 1999). Regulation of gene expression via a hypoxia responsive mediator would be an ideal genetic switch for gene therapy vectors targeting hypoxic related diseases, such as Ischemic Heart Disease (IHD) and Peripheral Vascular Disease (PVD).

In recent years, Hypoxia Responsive Elements (HREs') have been reported in the promoter regions of genes such as: VEGF, Erythropoietin (EPO), Nitric Oxide Synthase (NOS), etc. (Kimura et al. 2000). All of these genes, along with other similar examples, are transcriptionally up-regulated in response to low cellular oxygen levels. We utilized carefully arrayed HREs' and other expression motifs (TATA, AP1/CRE, Kozak, etc.) to drive the Luciferase reporter gene *in vitro*. Previous attempts at creating hypoxia regulated vectors via HRE repeats have been tried by various groups (Boast et al. 1999; Tang et al. 2002; Su et al. 2004). However, results appear mixed and even contradictory. After numerous construct variants were designed, produced and tested; we were able to identify examples of vectors that were significantly active only under conditions of

hypoxia. These HRE vectors address yet another fundamental gene therapy control concept: A gene should ideally be expressed only when it is needed and not at any other time.

The ultimate goal, from our perspective, is to have total control over a gene therapy vector in both space and time. In other words, when and where the gene is expressed is of critical importance. In fact, it is arguably as important as the effectiveness of the therapeutic gene and its efficient delivery. Without proper control, gene therapy could potentially do more harm than good. In this dissertation, data is presented regarding enhancing the fundamental control and regulation of potential gene therapy vectors.

The Purpose Driven Vector

Tissue specificity in vector delivery and expression is a great challenge in the field of gene therapy. The majority of gene therapy research to date has focused primarily on obtaining expression levels high enough to elicit a therapeutic response, as well as, distributing the vector to enough tissues to provide a corrective effect to the disorder being addressed (Hawley-Nelson et al. 2008). However, simply having a gene adequately delivered to enough cells to treat disease and having the gene product be produced in sufficient amounts to have a therapeutic effect cannot be the end of the story. Not unlike genes found naturally in the body, artificially delivered genes also need to be regulated.

Uncontrolled, high yield expression may correct one problem, but left unchecked could create an even worse problem for an individual. For example, VEGF is a gene of great interest to researchers and justifiably so. VEGF is known to induce new blood

vessel growth through a process known as angiogenesis (Connolly et al. 1989). This is of great significance to researchers interested in treating ischemic diseases such as CHF or PVD. VEGF may one day be used as a standard therapeutic to treat disorders such as these. Unfortunately, VEGF and other angiogenics can be a double edged sword. Cancer researchers are quite interested in VEGF as well. The reason being, VEGF and other angiogenic factors are known to be secreted by many tumors, which allows vascularization and continued growth of the tumor (Folkman et al. 1985). Of course, the problem that arises for researchers using VEGF or other pro-angiogenic genes for the purpose of gene therapy quickly becomes apparent. Uncontrolled high-yield expression of VEGF or other pro-angiogenics could result in the unrestrained over-growth of blood vessels. This could lead to problems with the basic function of specific organs and could lead to systemic complications, even death. So, in effect, the potential cure may become worse than the disease that was to be treated in the first place. This is just one of many such problems with the use of unregulated gene therapy vectors.

In the long run, for many disorders, only vectors that have some means of being turned on and off are truly viable. For some disorders, the transient nature of current gene therapy methods may be effective enough in terms of regulating the cumulative expression level of the therapeutic gene. However, for any disorder that requires sustained treatment, this method of regulation simply will not be adequate. Our goal in this project was to design and evaluate gene therapy vectors, which could be controlled both spatially and temporally by the disease state itself.

Cardiovascular Disease

According to the Centers for Disease Control and Prevention (CDC) National Center for Health Statistics, as of their most recent 2004 study, cardiovascular disease was the leading cause of death of both men and women in the US. Cardiovascular diseases accounted for approximately 27.2% of all US deaths in 2004 (Miniño et al. 2007). This is despite numerous advances in the diagnosis and treatment of cardiovascular disease over the last few decades. Our understanding of cardiovascular disease has improved greatly and the development of new technologies and treatments has given us new weapons in the battle against heart disease. We are, without question, better able to deal with heart disease today than 40 years ago; however, there is still much to be learned. The number of deaths from heart disease has been on a steady increase since 1970 (Miniño et al. 2007). In part, the advancing age of "baby boomers" and the increasing life span of the general population are to blame for these numbers. However, also to blame is the steady rise of obesity in industrialized nations where heart disease remains the number one cause of fatalities. Causes of heart disease may be genetic, environmental or both. Certain genetic markers have been identified that are linked to various forms of heart disease. However, a variety of certain environmental factors such as diet, smoking, and stress levels play an important role in determining whether or not an individual will develop heart disease (Blenkinsop et al. 2003). CAD, also known as Atherosclerotic Heart Disease (AHD), IHD, or Coronary Heart Disease (CHD), is the most common form of heart disease and affects about 7 million Americans. Every year in the US 480,000 angioplasties and nearly 600,000 bypass surgeries are carried out in response to CAD. The end result of CAD is a MI, which leads to the death of about

500,000 Americans each year (Cooper et al. 2006). CAD occurs as a result of narrowing of the arteries and subsequent restriction of blood flow due to accumulation of fatty deposits (Figure 1-1). These fatty deposits or atheromatous plaques are comprised of cholesterol and various fats and build up over time along the inner wall of the coronary arteries. This resulting restriction of blood flow subsequently restricts oxygen and other vital nutrients along with it. This condition is known as ischemia and the resulting decrease in oxygen levels creates a state known as hypoxia. When an artery becomes completely blocked; the result is a MI, more commonly referred to as a heart attack. This final complete blockage may occur as a gradual build up, however, this is uncommon. The more frequent scenario involves the plaque rupturing leading to a sudden blockage within the blood vessel downstream.

Current Treatments for CAD. There are a number of treatments currently used to manage CAD. These may include such strategies as managing risk factors such as: high cholesterol, high blood pressure, obesity, smoking, sedentary lifestyle, and diabetes. Drug therapy is another common method used to treat CAD. Drug treatments may include: cholesterol lowering drugs to reduce plaque build-up; blood thinners, such as aspirin, to reduce the chance of thrombosis formation; drugs to regulate high blood pressure such as Angiotensin-Converting Enzyme (ACE) inhibitors and beta blockers; and others such as nitroglycerine and long acting nitrates that help increase blood flow to the heart. Conventional Coronary Artery Bypass Grafting (CABG) surgery may be used to re-route blood flow around the affected area of stenosis. This is achieved by taking a blood vessel from the leg or chest and grafting it to the cardiac blood vessel, thereby creating a course for arterial blood flow around the stenosis. Angioplasty is another

common treatment strategy. This involves threading a catheter from the groin to the heart, where a balloon then inflates at the tip of the catheter to compress the plaque and widen the artery. This is often accompanied by implantation of a mesh like cylinder, known as a stent, which holds the artery open and may be laced with therapeutics. This may be done in combination with 3D digital mapping systems such as NOGA® (Cordis). Brachytherapy is a more recent development in the treatment of CAD. This involves the application of radiation to a damaged blood vessel to help prevent re-narrowing of an artery. This is usually done after angioplasty and implantation of a stent. Another recent development is the use of Transmyocardial Laser Revascularization (TMR). TMR is implemented by treating the heart with a laser, which in turn stimulates angiogenesis. This re-vascularization may relieve the symptoms of CAD. This has primarily been used in patients who are not able to receive angioplasty or a CABG. In addition, there are many new developments on the horizon for the treatment of CAD. Perhaps the most promising is the long term potential of gene therapy (Cooper et al. 2006).

Promoters and Gene Regulation

Genes are regulated by stretches of DNA known as the gene "promoter." The promoter region is found upstream of the 5' end of the gene's coding region. The promoter region acts as a sort of molecular switch; turning transcription of the gene on or off (Lodish et al. 1999). In many instances, a dimmer switch might be a more precise analogy; in that transcriptional activity of a gene might be increased or decreased in a relative response to regulatory factors as opposed to an absolute one. Most genes include a core promoter region, which typically lies within 100 bases of the transcriptional start site. Frequently, these promoter regions include key common consensus sequences.

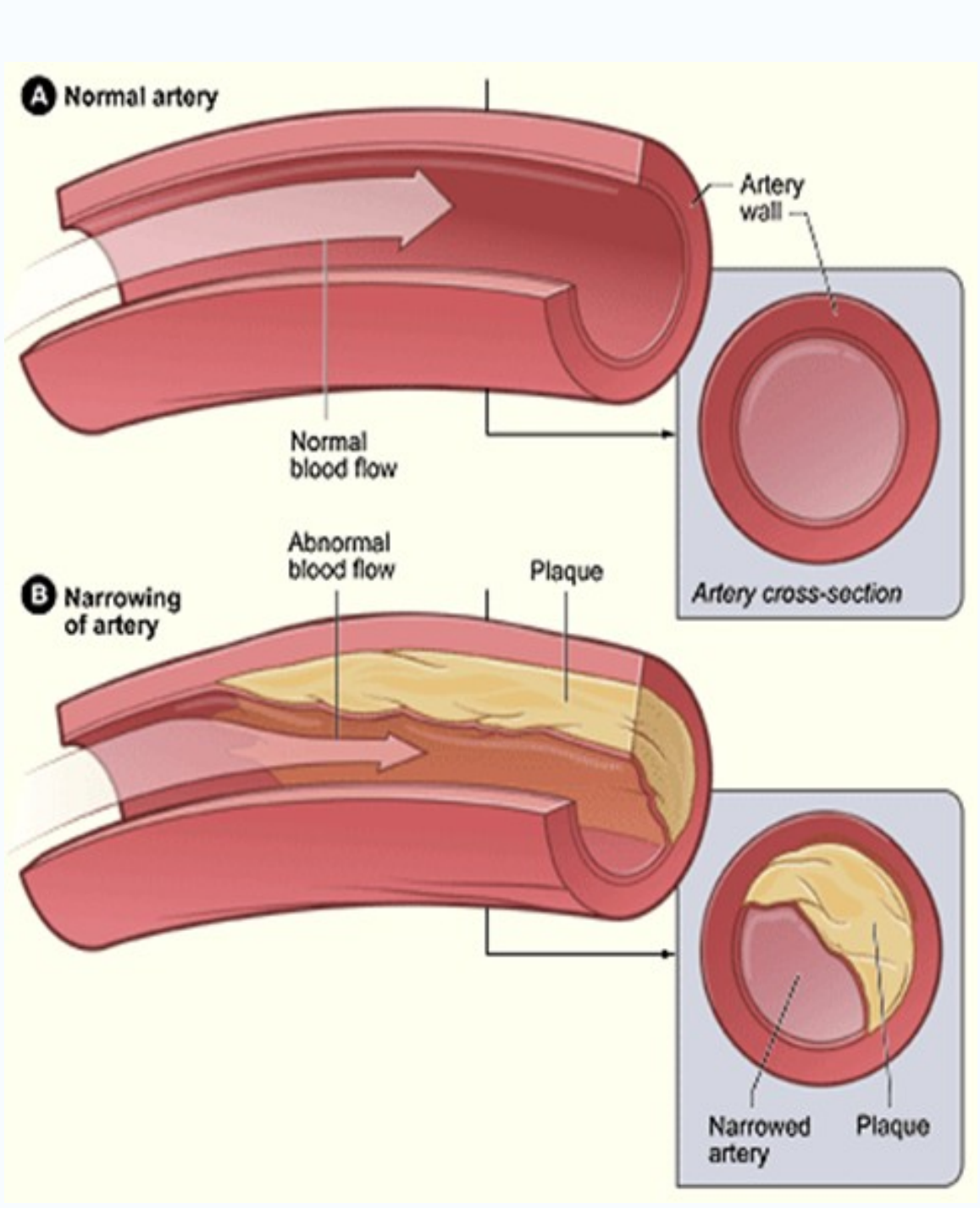

Figure 1-1 Coronary Artery Plaque Occlusion (Source: NHLBI)

Some notable examples of such sequences include: E-Box, TRE, TATA Box and the CAAT Box. Transcription factors then bind to these DNA sequences and form an initiation complex. It is the proper formation of this complex that will allow transcription to proceed. However, precise characterization of a gene's promoter region is difficult in eukaryotes (Cowell 1994). This is due to the fact that enhancers and repressors may be located thousands of base pairs away from the gene being regulated. To further convolute the situation, the regions that may be identified as the active promoter site may vary in different cell types. In other words, simply because a certain consensus sequence motif is identified as part of a promoter region, it does not mean that it will participate in regulation of that gene in all cell types or physiological conditions. It is for this reason that sophisticated computer programs designed to identify consensus sequences cannot pinpoint promoter boundaries. Rather, they reveal overlapping motifs and poorly defined promoter margins (Pedersen et al. 1999). This ambiguity exemplifies the complexity of Eukaryotic promoter regions and the difficulty in surmising their boundaries and points of origin. The consensus sequences that were of primary interest to us were those that imparted a high degree of specificity and control over the transcription of a gene.

Gene Therapy

Webster's Dictionary (2008) defines Gene Therapy as, "The insertion of, usually genetically altered, genes into cells, especially to replace defective genes in the treatment of genetic disorders or to provide a specialized disease-fighting function." Medline (2008) defines it this way: "Gene therapy is an experimental technique that uses genes to treat or prevent disease."

However, these definitions fail to encompass the full spectrum of what gene therapy might entail. Gene Therapy might be utilized for some purpose other than to correct a disease. For example, gene therapy might be used to enhance the growth of cattle by inducing a temporary or permanent up-regulation of growth hormone. Gene Therapy might be used to induce plants to store more of its sugars in the form of starch as opposed to cellulose.

To have a truly accurate definition of Gene Therapy we must even include the potential for misuse of the technology. For example, an athlete may wish to enhance their performance by increasing systemic levels of testosterone, HGF, EPO, etc. Gene Therapy holds the potential for such misuse in the future. The current state of technology may not allow for such scenarios to be a current day reality; nevertheless, it is no less practical or likely than the idea of correcting any number of medical disorders via Gene Therapy. If I may humbly make an assertion, a more precise definition might be the following: "Gene Therapy is the process of inserting foreign DNA into an organism for the purpose of introducing specific physiological modifications."

Gene Therapy may be applied through a number of approaches; these include: gene addition, gene deletion, gene replacement, gene enhancement, and gene repression. Additionally, there are numerous delivery approaches; these range from direct injection of DNA to complex viral vectors (Hammond et al. 2001). There is no single magic bullet; each strategy has its own pros and cons. It is unlikely that there will be a single grand unified approach for gene therapy in the foreseeable future. Most likely, there will be a constellation of methods utilized, often in combination, to fully realize the potential of gene therapy.

One such novel delivery method utilized by our group is UTMD (Korpanty et al. 2005). Gene therapy allows for the over-expression of a desired therapeutic gene. Ideally, this over-expression should occur exclusively in the desired spatial and temporal target. However, many gene therapy attempts thus far have been very poorly regulated (Lu et al. 2004). Although, there have been a number of successful studies that have regulated a genes expression to some degree. Some have tissue specific promoters to focus expression in the target tissue (Heine et al. 2005). Others focused on controlling the timing of gene expression by utilizing genetic "switches" activated by drugs such as tetracycline (Bertrand et al. 1984). Still others have used the presence of certain transcription factors to control the expression of the therapeutic gene (Rodriguez et al. 1990).

Researchers have attempted a wide variety of strategies in the endeavor to develop gene therapy treatments for cardiovascular disease (Cooper et al. 2006). There are essentially three major variables, which must be addressed in creating a gene therapy strategy. The first point which must be dealt with is the method of delivery. This may involve injection of naked DNA, viral encapsulation, liposomal delivery or others, such as UTMD. Next, a precise therapeutic target must be selected. (This must be done at a high enough efficiency and sustainability to effect treatment and specific enough so as to treat only the targeted tissues.) Finally, it must be established how the gene will be regulated. It must be determined what regulatory elements, if any, will be incorporated into the promoter.

Once the vector has been designed and constructed it must be evaluated. Key criteria may include the following: intensity of gene expression, duration of gene

expression, location of gene expression, control of gene expression, and ability to effectively treat a disorder. Generally, only a few of these criteria are focused on in any one particular study. However, all are ultimately important and will likely have to be seamlessly incorporated together to realize the full potential of any gene therapy strategy. It is likely that gene therapy will be most effective by using multiple therapeutic gene strategies, making use of numerous targets in a single treatment. Table 1-1 illustrates the wide variety of approaches and targets, which are being studied to treat a number of different cardiovascular diseases. However, each has its own positives and negatives; none are perfect.

Table 1-1 Gene Therapy Strategies for the Cardiovascular Diseases.

Disease	Therapeutic approach	Target
Atherosclerosis	Antiatherogenic	LCAT, apoAI, apoE mRNA
Vein graft failure, ischemia, thrombosis	Diffusible/secreted gene products	VEGF, FGF, eNOS, Antithrombotic agents, SOD, Heme Oxygenase
Vein graft failure	Inhibitors of smooth muscle cell migration/proliferation	p53, TIMPs, Rb, p21
Thrombosis	Prevention of thrombus formation	TFPI, tPA
Restenosis	Suicide genes	Tk
Hypertension	Antisense oligonucleotides	Angiotensinogen, AT_1 receptor, ACE
Vein graft failure	Decoys	Soluble VCAM, E2F
Hypertension	Reduction in blood pressure	Kallikrein, ANP, eNOS, endothelin

Gene Delivery Strategies in the Treatment of Cardiovascular Diseases

Gene therapy for the treatment of a variety of cardiovascular disorders has been an area of great interest and has shown much promise to researchers over the last several years. Numerous strategies have been attempted with mixed results (Lu et al. 2004). There are a number of gene therapy clinical trials currently underway including several for the treatment of cardiovascular disease (Clinicaltrials.gov 2008). Many of these show great potential, however, the U.S. Food and Drug Administration (FDA) has not yet approved any gene therapy product for standard clinical use in the United States. There are a number of gene therapy strategies currently being studied for the treatment of a variety of cardiac related disorders (Table 1-1). Additionally, there are a wide variety of gene delivery techniques (Table 1-2) that have been studied in myocardial tissue (Hammond et al. 2001). These include both viral and non-viral techniques and have had varying degrees of success. No delivery strategy has proven to be perfect: each has its own series of strengths and weaknesses. Direct injection of naked plasmid DNA is the earliest and most basic form of gene therapy. It is useful today in gauging the effectiveness of new vector designs and as a basis for comparison for new gene transfer techniques. Recombinant viral techniques have been the most popular viral gene transfer approach in the treatment of myocardial disorders (Binley et al. 1999); however, they have a number of limitations. These include slow onset of transgene expression, viral mutation, immune response and oncogenesis. Non-viral techniques have been limited as well. The primary limitations in non-viral techniques include short sustainability of therapeutic effect, cytotoxicity, and low transfer efficiency (Francis et al. 2001).

Table 1-2 Vectors Used for Cardiovascular Gene Transfer

Vector	Advantages	Disadvantages
Naked Plasmid DNA	Easy to produce safe	Very low transduction efficiency Transient expression
Adenovirus	High transduction efficiency Relatively high transgene capacity Easy to produce Transduces quiescent cells Tropism for multiple cells	Inflammation with high doses Transient expression
Adeno-associated virus	Long-term gene expression Moderate immune response Transduces quiescent cells Wild type does not cause disease in humans	Limited transgene capacity Difficult to produce in large quantities
Lentivirus	Long-term gene expression Transduces quiescent cells Relatively high transgene capacity Low immune response	Non-specific integration Low transduction efficiency Limited tropism Difficult to produce in large quantities
Retrovirus	Long-term gene expression Relatively easy to produce Low immune response	Non-specific integration Transduces only dividing cells Low transfection efficiency Limited tropism
Herpes simplex-virus	High transduction efficiency High Transgene Capacity Tropism for neuronal cells	Difficult to produce in large quantities Transduces only dividing cells Cytotoxicity
Epstein-Barr-virus	High transduction efficiency High transgene capacity Extra-chromosomal replication	Limited tropism Transduces only dividing cells Difficult to produce in large quantities
Baculovirus	High transgene capacity Easy to produce Rapid construction of recombinant baculoviruses Wild type does not cause disease in humans	Transient expression Inflammation with high doses Limited tropism
siRNA	More potent than antisense approach	High gene transfer efficiency required

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UTMD

UTMD as a gene delivery technique (Klibanov et al. 1998) is a new approach which involves using sonic waves to cause gas filled microbubbles to burst at a specific targeted location (Figure 1-2). This promising new technique was selected as our *in vivo* gene transfer strategy due its ability to transfect the myocardium, its minimally elicited immunological response, its availability, ease of use and the added control via site directed gene delivery (Chen et al. 2006). Also, UTMD has previously been evaluated by our lab in collaboration with Dr. Grayburn's lab and has been shown to non-invasively and selectively deliver VEGF165 (Figure 1-3) to the myocardium (Korpanty et al. 2005), as well as, functional genes to pancreas Figure 1-4) (Chen et al. 2007). It should be noted that the data cited from this particular study (Korpanty et al. 2005) were conducted on a normal animal model and VEGF165 was being expressed in a standard normoxic environment. It is our belief that this limited the expression levels and contributed to the transient nature of the VEGF165 gene expression. This is plausible because VEGF is known to be quickly degraded in the cell under conditions of normoxia causing the effects of VEGF become handicapped (Maynard et al. 2005).

In UTMD, cationic liposomes are loaded with the plasmid DNA under evaluation. This is then attached to the phospholipid shell of perfluoropropane gas filled microbubbles. These microbubble–liposome complexes are infused intravenously (IV) and destroyed within the microcirculation by low-frequency ultrasound (Figure 1-2). This allows for precise release of the plasmid within the body allowing for more localized expression of the therapeutic gene. It should also be noted that the exact molecular mechanism of how UTMD functions is not fully understood. (Korpanty et al. 2005).

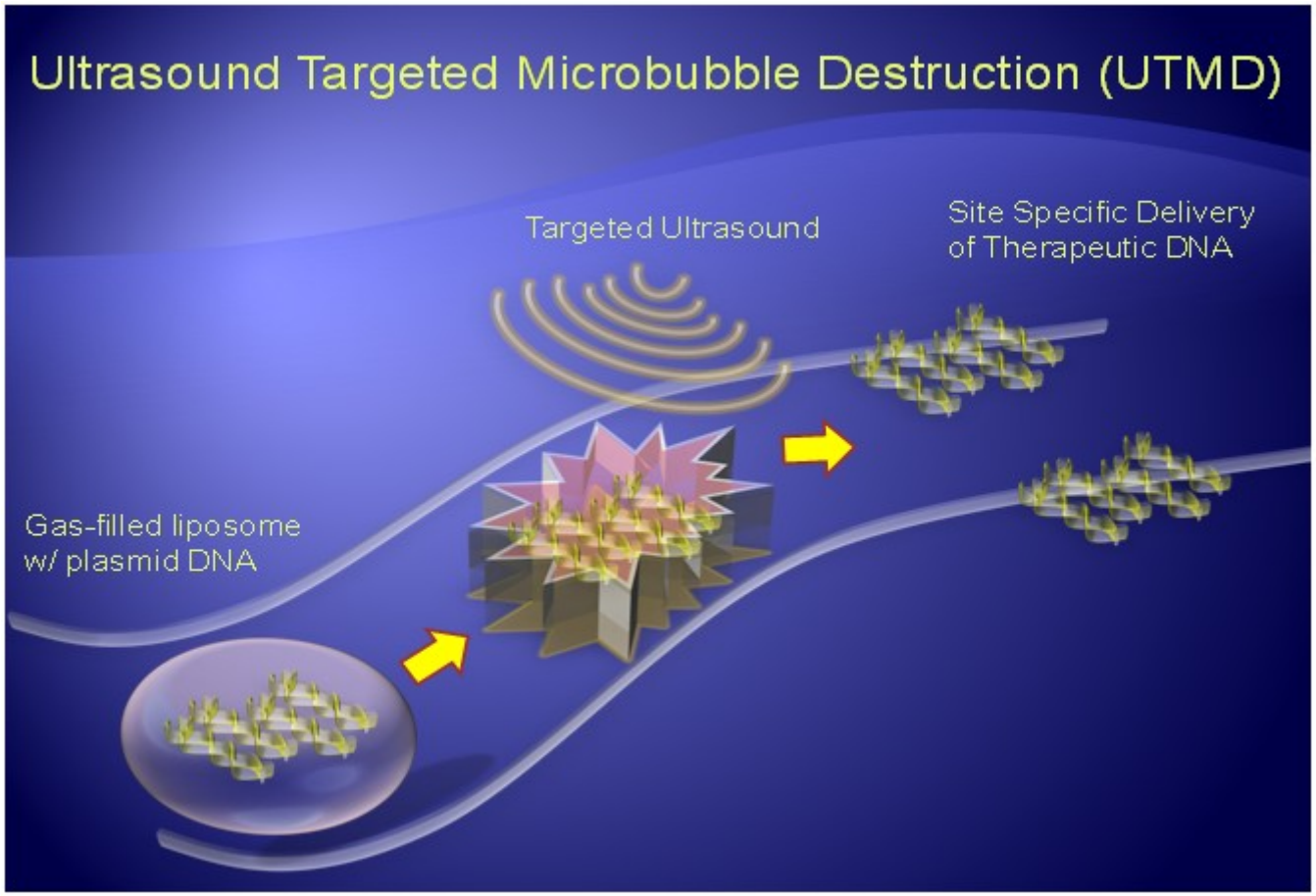

Figure 1-2 Delivery of plasmid DNA *in vivo* was accomplished via the use of a promising new method known as UTMD. Microbubbles composed of a high molecular weight perfluoropropane gas surrounded by a cationic liposomal shell are produced and integrated with DNA plasmid, which is believed to be bound to the liposomal shell. A microbubble/liposome complex is formed and infused intravenously (IV) and is destroyed by a precise low frequency ultrasonic signal, thus releasing the integrated DNA plasmid into the microvasculature of the surrounding tissue. UTMD allows for site specific delivery of DNA plasmids *in vivo* thus yielding more localized gene expression (Korpanty et al. 2005). This allows for precise release of the plasmid within the body and more localized expression of the therapeutic gene.

Plasmids were attached to microbubbles and efficient delivery to target organs was accomplished by destroying the microbubbles with a guided ultrasonic signal. There are numerous potential applications to this technology. A few of those currently being investigated by our lab include: VEGF delivery to ischemic tissue, insulin delivery to pancreatic islet cells, and gene expression modification upon endothelial and myocardial tissue.

Reversal of Streptozotocin-Induced Diabetes in Rats by Gene Therapy with Betacellulin and Pancreatic Duodenal Homeobox-1. We recently demonstrated that gene therapy could be utilized to target pancreatic islets in normal rats, using UTMD (Figure 1-4). The following is a brief synopsis of a study conducted by our group as evidence for the efficacy of UTMD as our selected means of *in vivo* transfection. UTMD was used to direct betacellulin (BTC) and pancreatic duodenal homeobox-1 (PDX1) to rat pancreas 48 h after islet destruction by Streptozotocin (STZ) (Chen et al. 2007). Sprague–Dawley rats were rendered diabetic by STZ injection. Controls included normal rats, STZ only without UTMD, and UTMD with DsRed reporter gene.

Blood glucose increased dramatically in all rats 48 h after STZ, and continued to rise after UTMD with BTC alone. Blood glucose declined from day 3 to day 10 after UTMD with PDX1, but remained elevated (26178 mg/dl). However, in rats treated with both BTC and PDX1, blood glucose remained below 200 mg/dl throughout day 10. This was accompanied by normalization of blood insulin and C-peptide. Histology demonstrated islet-like clusters of glucagon-staining cells in the rats treated with BTC and PDX1, but these clusters disappeared by 30 days after UTMD treatment.

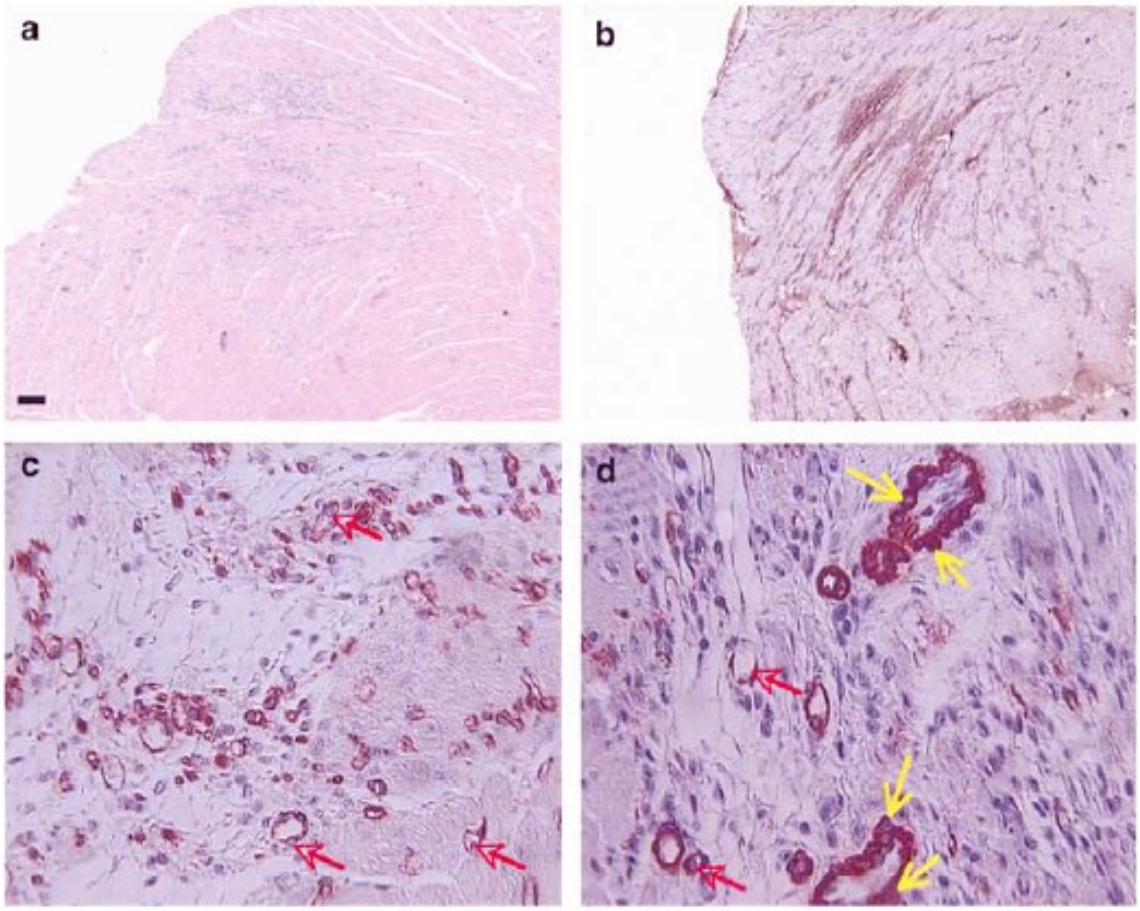

Figure 1-3 Histological sections of myocardium 10 days after UTMD treatment. (A) Low-power (100X) hematoxylin & eosin staining showing a hyper-cellular region of myocardium. (B) Low-power (100X) image of a hyper-cellular region stained with anti-VEGF antibody, confirming the presence of VEGF in the hyper-cellular region. (c) High-power image (400X) of hyper-cellular area stained with BS-lectin. Red arrows depict prominent nuclei in capillary endothelial cells, consistent with angiogenesis. There is also disorganized cellular architecture consistent with mild inflammation. (d) High-power (400X) image of hyper-cellular area stained with smooth muscle α-actin. Red arrows point to pericytes covering new blood vessels. Yellow arrows point to prominent nuclei on arteriolar smooth muscle cells. Bars indicate 100 mm (Korpanty, Ding et al. 2005) Reprinted by permission from Macmillan Publishers Ltd: [Gene Therapy] (Gene Ther. (17):1305-12.), copyright (2005)

.

Although regeneration of insulin-producing islets was not seen, diabetes was reversed for up to 15 days after a single UTMD treatment by ectopic insulin production by pancreatic acinar cells. These cells co-expressed amylase and insulin and demonstrated several β-cell markers by reverse transcription-PCR. Gene therapy by UTMD can reverse diabetes *in vivo* in adult rats by restoring pancreatic insulin production (Chen et al. 2007).

α-Myosin Heavy Chain Promoter

We wished to produce a vector that would be active only in the myocardium. To produce this vector we would need a promoter known to be active primarily within the myocardium. Numerous genes were evaluated; however, α-Myosin Heavy Chain (αMHC or MYH6) would prove to be the most appropriate. Both αMHC and β-Myosin Heavy Chain (βMHC) are expressed in significant quantities in cardiomyocytes. However, βMHC is also highly expressed in skeletal muscle, while αMHC expression is nominal. Other cardiac specific genes were evaluated; however, expression of these genes tended to be expressed less intensely or intermittently. We would need a promoter that would be active on a consistent basis. αMHC was selected based on its potential to provide strong, consistent, cardiac specific gene expression (Helmke et al. 2003). Additionally, αMHC had already been demonstrated to be a potentially effective gene therapy vector promoter (Franz et al. 1997).

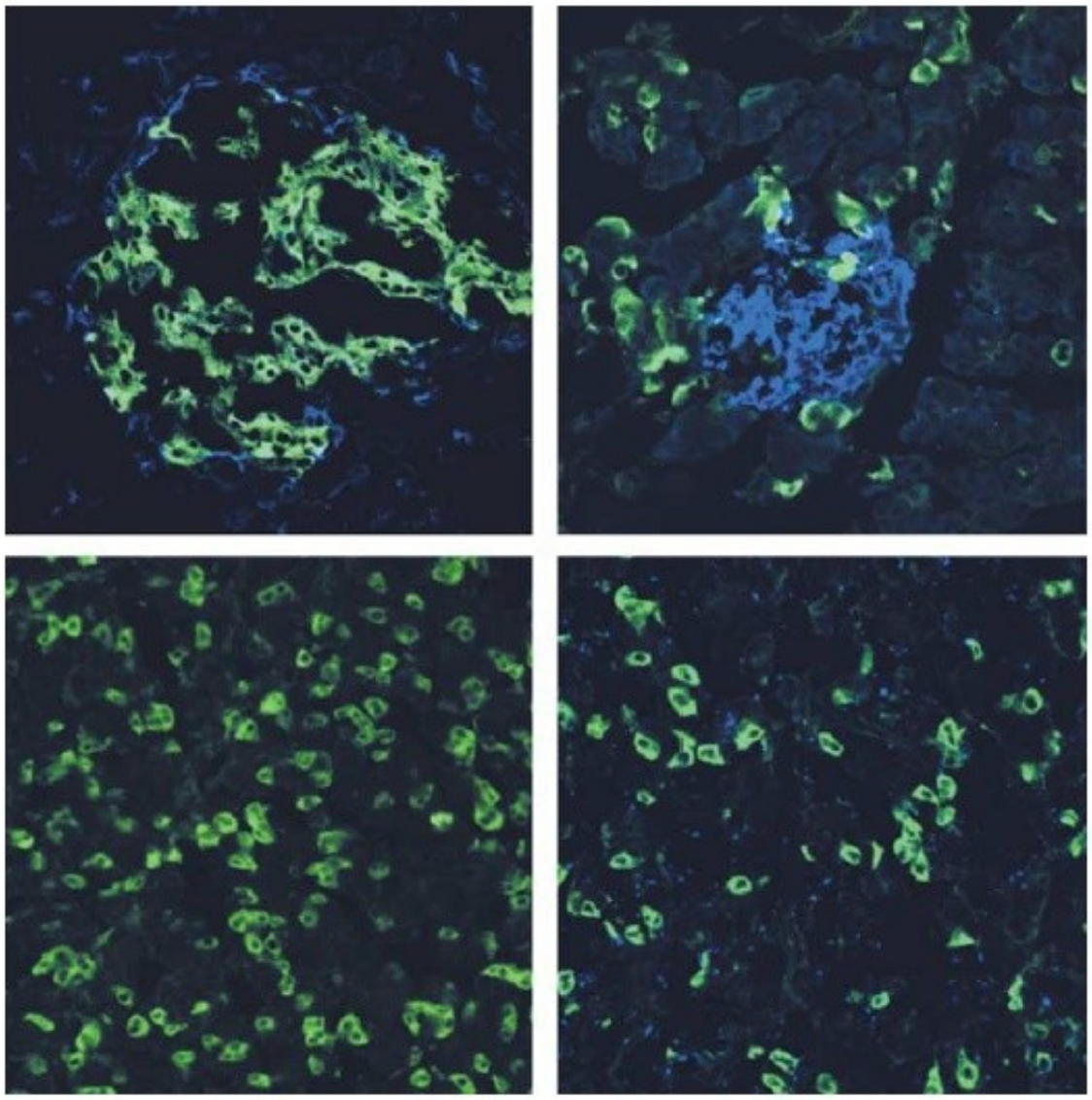

Figure 1-4 Representative histological sections of rat pancreas (day 10) stained with FITC-labeled anti-insulin (green) and CY5-labeled anti-glucagon (blue) antibodies. (Left/ upper panel) High power (X400) section from a normal control rat showing a typical islet with β cells in the center (green) and α-cells on the periphery (blue). There is some background autofluorescence in both colors. (Right/upper panel) High power section (X400) from a rat treated with PDX1 and betacellulin plasmids by UTMD. Atypical islet-like clusters of cells stain mostly with glucagon (blue). In addition, anti-insulin staining (green) appears to be present in the exocrine pancreas. (Left/lower panel) High power (X400) image from a rat treated with PDX1 and BTC plasmids showing prominent insulin staining in what appear to be acinar cells. (Right/lower panel) High power (X400) image from a rat treated with BTC and PDX1. FITC-labeled anti-insulin staining (green) shows insulin present in acinar cells. CY5-labeld anti-BTC staining (blue) shows BTC present in and around these cells within the exocrine. CY5-labeled anti-PDX1 had a nearly identical appearance (not shown). (Chen, Wood, Ding *et al.*)

Hypoxia

Hypoxia may best be defined as the sustained deprivation of oxygen levels necessary for aerobic respiration. Numerous genes and their associated pathways are regulated by cellular oxygen levels. These include such physiological processes as: basic metabolism, the ability to repair cellular/tissue damage, development from zygote to adulthood, adaptation to low oxygen environments such as high altitude, basic immunity, and angiogenesis (Ziello et al. 2007). Hypoxia is also known to affect the pathophysiology of diseases such as cancer, neurodegeneration, ischemic heart disease, stroke, PVD, and even diabetes. These are but a few of the processes that hypoxia is known to influence; many more are still likely to be elucidated in the years to come. VEGF, EPO and NOS are just a few examples of the many genes known to be regulated, at least in part, by cellular oxygen levels. The molecular basis for the effects of hypoxia is being demonstrated with ever increasing accuracy (Taylor et al. 2008). While many different molecular pathways are involved the pantheon of hypoxia related conditions, it has been demonstrated that there are key convergence points that these pathways tie into (Lee et al. 1994). One such key convergence point is the HIF-1α regulatory pathway nexus (Figure 1-5).

Hypoxia Responsive Element (HRE)

Once the HIFα/β complex is fully formed and stabilized, it is able to bind to a core HRE consensus sequence. This core HRE sequence is necessary, but not sufficient, for significant induction of hypoxia regulated gene expression (Wegner et al. 2005). HRE is a key element in hypoxia regulation; however, it does not operate in a vacuum. Rather, it is part of a complex array of promoter elements working in concert to

orchestrate tight control over gene expression. As noted during our own experimentation, while HIF will technically interact with a lone HRE site, it does so weakly. For proper complex formation, additional elements such as CREB and/or AP-1 must be present (Semenza et al. 2007). A caveat that should be mentioned is that hypoxia regulated expression has been reported by arranging several tandem HREs in expression vectors (Su et al. 2007). However, results reported using this technique varies greatly (Wegner et al. 2005). Our own experiments showed this technique to yield un-impressive results (Figures 3-1, 3-2, 3-3, and 3-4). Naturally occurring tandem HREs may be found in genes such as transferrin and glucose transpoter-1 (GSP-1); however, the promoters of these genes still include several other regulatory elements as well (Kimura et al. 2000). The core HRE sequence is 5' CGTG 3'. This sequence is present as the central region of all HREs. However, certain base pairs are more common than others on the flanking regions of HREs (Figure 1-6). Additionally, epigenetic modifications are known to regulate the basic functionality of HREs *in situ*. HRE expression has a direct correlation with the degree of CpG methylation. Consequently, most HIF dependent genes tend to be found within methylation-free CpG islands. (Wenger et al. 2005) It is suspected that an, as of yet, un-described secondary mechanism keeps these islands methyl free (Ziel et al. 2005). DNA footprinting has revealed that the HRE region is occupied even under conditions of normoxia, during which it is inactive and the HIF complex is not present. (Ziel et al. 2005)

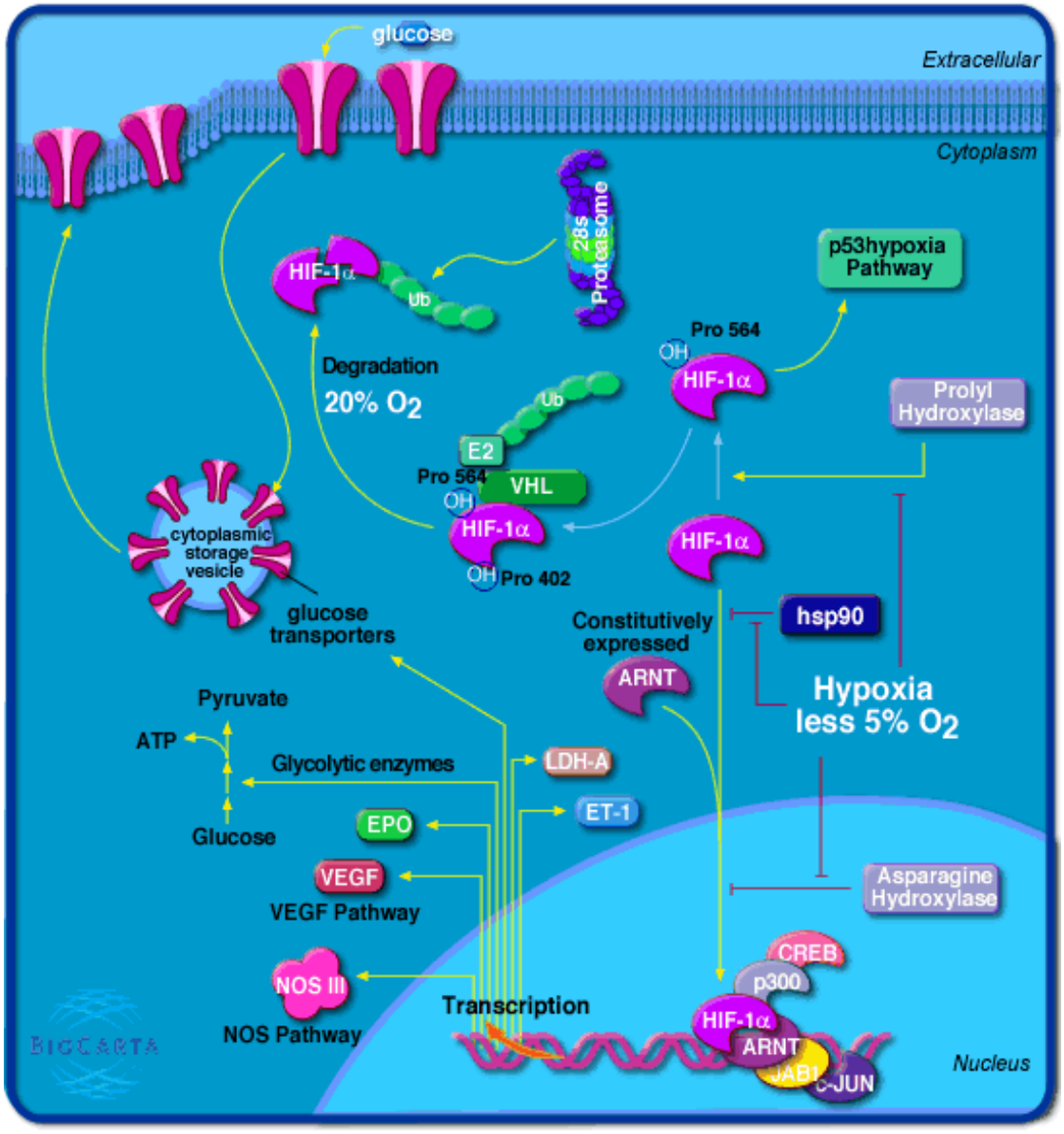

Figure 1-5 Hypoxia Signal Transduction: HIF-1 is stabilized prior to induction of VEGF expression during acute ischemia in the human heart. Second, pulmonary hypertension associated with chronic respiratory disorders results from persistent vasoconstriction and vascular remodeling. The primary molecular mechanism of gene activation during hypoxia is through HIF-1. Several genes involved in cellular differentiation are directly or indirectly regulated by hypoxia. These include EPO, LDH-A, ET-1, transferrin, transferrin receptor, VEGF, Flk-1, Flt-1, platelet-derived growth factor-ß (PDGF-ß), basic fibroblast growth factor (bFGF), genes affecting glycolysis, etc. (Reprinted with permission of Biocarta.com)

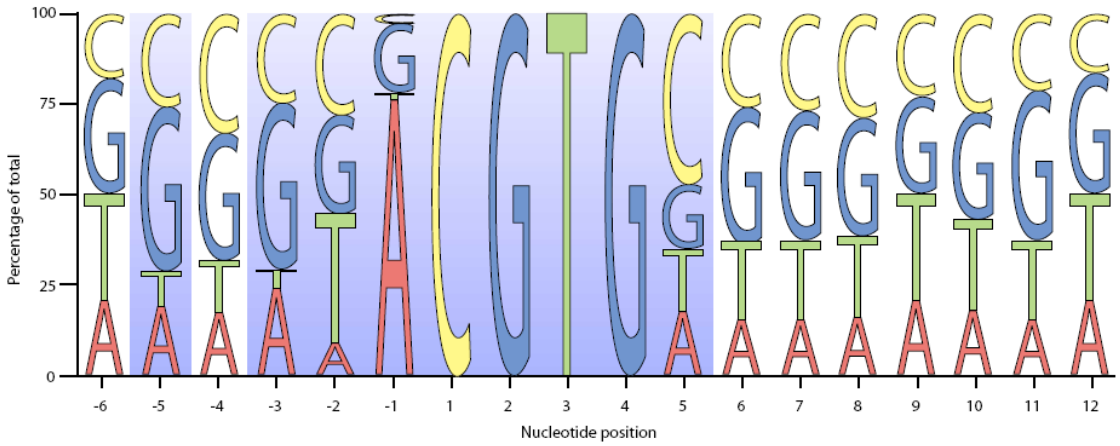

Figure 1-6 HRE Core Consensus Sequence and Relative Occurrence of Flanking Regions. (Wegner et al.) From Sci STKE. (306):re12. Review 2005. Reprinted with permission from AAAS.

Hypoxia Inducible Factor (HIF)

Hypoxia Inducible Factor-1 (HIF) is a transcription factor, which is known to be regulated by cellular oxygen concentrations. HIF was first discovered as a regulator of EPO in hypoxic tissues (Wang et al. 1995). Subsequently, HIF was determined to be a heterodimeric transcription factor consisting of a HIFβ subunit and one of three HIFα subunits: HIF-1α, HIF-2α, and HIF-3α (Semenza et al. 2001). HIF is now known to be involved in the regulation of over 100 genes; microarray studies indicate this number may be well over 200 (Greijer et al. 2005). These genes include within their promoters Hypoxia Responsive Elements. These HREs vary to some degree in their sequence, but many bind to HIF-1 to form a transcription initiation complex. HIF-1 is known to be involved in the regulation of processes as diverse as neurogenesis, angiogenesis, and circadian rhythms, in addition to, a wide array of metabolic functions (Wegner et al. 2005).

HIF-1β, also known as Aryl Hydrocarbon Receptor Nuclear Translocator Protein (ARNT), is constitutively expressed. Under typical conditions of normoxia HIF-1α is

ubiquinated and is rapidly hydroxylated by Prolyl Hydroxylase 4 (PH-4). Hydroxylation of HIF-1α allows it to interact with the von Hippel-Lindau (VHL) protein. This association of VHL with HIF-1α marks it for degradation by proteases and is an important regulatory feedback mechanism. Conversely, HIF-1α is stabilized by an array of various co-factors under hypoxic conditions (< 5.0% O_2). It is under these hypoxic conditions that HIF-1α is able to initiate the formation of the HIF transcription factor complex by binding to ARNT. This is the core of the complex that will bind to the HRE consensus sequences (Figure 1-7). The formation of this heterodimeric HIF complex is a regulatory mechanism, which is crucial for the direct control of oxygen regulated gene promoters (Ziello et al. 2007).

Additionally, there are a number of transcriptional co-activators, which may associate with this complex. Some of these co-activators include: AP-1 (C-Jun, C-Fos), CREB, CBP, p300, SRC-1, TIF-2, HDAC-7, Ref-1 (Ape1), HNF4, pRB, etc. There are a variety of combinations of these and other co-activators present between genes; however, the principle factors of the HIF complex remain constant between genes. Previous research has demonstrated that there is intercommunication in the form of binding AP-1/CREB proteins to AP-1/CRE binding sequences (Masquilier and Sassone-Corsi 1992; Hadman et al. 1993; Hai and Curran 1991; Pestell et al. 1994). Additionally, the ability of AP-1/CRE DNA binding proteins to form heterodimers has been demonstrated in multiple systems (Hai et al. 1989; Hai and Curran 1991; Chatton et al. 1994; Ryseck and Bravo 1991). AP1/CRE consensus sequences were found to be critical in our most successful vector designs.

Cardiac Specific Expression via a Modified α-Myosin Heavy Chain Promoters

Our objectives for this phase of the project were as follows: To design and construct tissue specific gene therapy vectors and to demonstrate effective delivery and expression of these vectors via UTMD *in vivo*. The construction of such tissue specific vectors could prove useful for the treatment of tissue specific disorders such as CAD, FHCM, etc. This may be accomplished by the administration of a variety of potential therapeutic genes delivered to the precise tissue of interest. For example, CAD may be treated by correcting or compensating for the ischemic condition. This has been demonstrated by numerous groups (Korpanty et al. 2005) *in vivo* utilizing genes such as VEGF, ANG-1, ANG-2 and others. Such intervention would subsequently act as a preventative measure against further degeneration of the myocardium, as well as, a possible MI.

Hypoxia Regulation via de Novo HRE and AP-1/CRE Based Promoters

The second objective of this project was to produce gene therapy vectors that would be significantly up-regulated only under specific physiological conditions. Specifically, we wished to produce vectors that would be regulated by cellular oxygen levels, be expressed only in certain tissues or both. The significance of such constructs is that it imparts a greater degree of control in the implementation of gene therapy. Such control is of vital importance for the future viability of actual clinical applications involving gene therapy strategies. In general, it is desirable for a gene therapy vector to be active only when and where it is needed. Our constructs are a step towards this ultimate goal.

Cardiac Specific and Hypoxia Regulated Luciferase Expression via a HRE/AP1 Modified α-Myosin Heavy Chain Promoter

The final phase of the project was intended to bring the previous two phases together in an attempt to create a single vector that would meet all of the above criteria for the treatment of cardiac related illness. In this dissertation, we report the design, construction and evaluation of just such vectors including: tissue specificity, hypoxia correlated gene expression levels, and both elements functioning within a single modified promoter.

CHAPTER TWO

Materials and Methods

Promoter/Plasmid Design and Constructions

There were two primary functions we wanted to include in the promoter designs; tissue specificity and oxygen regulation (Table 2-1). Independently and conjointly (if possible), vectors such as these could ultimately be a powerful weapon in the fight against heart disease. We began with both the pCI-neo (Figure 2-2) and pGL4.10 (Figure 2-3) vectors as the backbone of the constructs. Next, we designed a modified cardiac alpha myosin heavy chain (αMHC) promoter to drive reporter genes such as Luciferase and DsRed. These genes served as a demonstrative substitution for potential therapeutic genes such as VEGF165. The αMHC promoter was modified in a number of variations, such as by the removal of the YinYang1 (YY1) motif sequences and by the addition of Hypoxia Responsive Element (HRE) and Activator Protein-1 (AP1) consensus sequences. As our quantitative reporter gene we selected Luciferase to give a measurable value for the activity of various vector constructs. Expression levels of our final vector constructs were tested *in vitro*. The vectors' expression levels were examined under conditions of hypoxia and normoxia. The intent of our final design was to produce a cardiac specific vector that is regulated by local oxygen levels.

Several primary vector constructs were assembled: MYH6/DsRed, pCI/DsRed (Figure 2-2); HRE1/Luc, HRE2/Luc (Figure 2-5); HRE3/ Luc, HRE4/Luc, HRE5/Luc

Table 2-1 Gene Therapy Vector Summary List

Vector Name	Vector Backbone	Promoter	Gene Expressed	*In Vitro or In Vivo*	Vector Purpose
CMV	pCI-neo	CMV	Firefly Luciferase	Both	Positive Control
SV40	pGL3-control	SV40	Firefly Luciferase	*In Vitro*	Positive Control
pGL4.10	pGL4.10	None	Firefly Luciferase	*In Vitro*	Negative Control
pGL3-basic	pGL3-basic	None	Firefly Luciferase	*In Vivo*	Negative Control
pCI-DsRed	pCI-neo	CMV	DsRed	*In Vivo*	Positive Control
pMYH6-DsRed	pDsRed-Express-1	α-Myosin Heavy Chain	DsRed	*In Vivo*	Cardiac Exclusive Expression
HRE1	pCI-neo	8XHRE	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
HRE2	pCI-neo	16XHRE	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
HRE3	pCI-neo	8XHRE-CMV	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
HRE4	pCI-neo	CMV-8XHRE	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
HRE5	pCI-neo	8XHRE-CMV-8XHRE	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
HAL	pGL4.10	8XHRE/API	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
HAM	pGL4.23	8XHRE/API-TATA	Firefly Luciferase	*In Vitro*	Hypoxia Regulated Expression
MHCI	pGL4.10	α-Myosin Heavy Chain	Firefly Luciferase	*In Vitro*	Cardiac Exclusive Expression
MHCII	pCI-neo	α-Myosin Heavy Chain	Firefly Luciferase	*In Vitro*	Cardiac Exclusive Expression
HMHC	pGL4.10	8XHRE/API-αMHC	Firefly Luciferase	*In Vitro*	Cardiac Exclusive & Hypoxia Regulated Expression

(Figure 2-6); HAL/Luc, HAM/Luc (Figure 2-7); MHC2/Luc, HMHC/Luc (Figure 2-8); pCI/Luc, MHC1/Luc (Figure 2-10). More than 100 vectors were constructed over the course of my entire period of study. However, discussion in this dissertation will focus primarily on the vectors listed above, as they are the ones most pertinent to the topic at hand. These vectors were carefully designed, constructed and selected for testing based on their perceived potential for selective gene expression or as a control.

PCR

Polymerase Chain Reaction (PCR) was performed for all samples using a GeneAmp PCR System 9700 (PE ABI) in 50 µl volume containing Xµl DNA, 1.0µl of HotStarTaq Master Mix (Qiagen), and 25 pmol of each primer, Xµl of H_2O. After an initial hold at 95ºC (10 min), the PCR was carried out for 42+ cycles (94ºC for 30s, 57ºC for 30s (+1min / 1000 bp), and 72ºC for 1 min). At the end, a single incubation at 72ºC was added for 10 min. Amplification products were analyzed on 1-3% agarose gels or 12-20% acrylamide gels. PCR products were confirmed by sequencing.

Control Vectors

Control vectors were needed as a basis for comparison against experimental vectors. Positive control vectors included a strong modified Cytomegalovirus (CMV) promoter or a weak Simian Virus 40 (SV40) promoter and a reporter gene. These were designated pCI-Luciferase (Figure 2-10) & pCI DsRed, CMV (Figure 2-4) and pGL3 Control, SV40 (Figure 2-1). A promoterless negative control vector was also utilized, pGL3 basic (Figure 2-1). Control vectors were exposed to the same treatments and processes that experimental vectors underwent.

MYH6 Promoter Vectors

The -435/+215 bp fragment of the α- Cardiac Myosin Heavy Chain (αMHC) promoter region of a Sprague Dawley rat was PCR amplified and purified using a QIAquick Gel Extraction Kit (Qiagen). Primers were designed based on sequence data for *Rattus Norvegicus* MYH6 promoter region obtained from Genbank (NW_047454). The sequence below includes the αMHC core promoter sequence, as has been previously described (Molkentin et al. 1996). The likely promoter region for this gene was also predicted using the following programs: *Dragon Promoter finder v15*, *The Promoter 2.0 Prediction Server* at The Center for Biological Sequence Analysis, and *The WWW Promoter Scan* of the NIH Center for Information Technology. The sequence selected for use as the MYH6 promoter region was:

5'GAGGCTGGAATGGGAGCTTGTGTGTTGGAGACAGGGGACAAATATT
AGGCCCGTAAGAGAAGGTGACCCTTACCCAGTGTGTTCAACTCAGCCT
TCAGATTAAAAATAACTAAGGTAAGGGCCATGTGGGTAGGGGAGGTG
GTGTGAGACGGTCCTGTCTCTCCTCTATCTGCCCATCGGCCCTTTGGGG
AGGAGGAATGTGCCCAAGGACTAAAAAAAGGCCCTGGAGCCAGAGGG
GCGAGGGCAGCAGACCTTTCATGGGCAAACCTCAGGGCTGCTGTCCTC
CTGTCACCTCCAGAGCCAAGGGATCAAAGGAGGAGGAGCCAGGACAG
GAGGGATGGGAGGGAGGGTCCCAGCAGATGACTCCAAATTTAGGCAG
CAGGCACGTGGAATGAGCTATAAAGGGGCTGGAGCGCTGAGAGCTGT
CAGACCGAGATTTCTCCATCCCAGGTAAGAAGGAGTTTAGCGTGGGGG
CTCTCCAACCGCACCAGACCTGTCCCCACCTAGAGGGAA3'

The purified product was then cloned into restriction sites, as detailed later, of both the pCI-neo (Figure 2-2) expression vector (Promega), the pDsRed-Express-1 (Figure 2-2) expression vector (Clonetech) and the pGL41.0 expression vector (Promega). The final vectors produced by these measures include: pMHC1/Luc (Figure 2-10), this includes the αMHC promoter and Luciferase reporter gene tied into the pCI/Neo Vector Backbone; pMHC2/ Luc (Figure 2-8), this includes the αMHC promoter

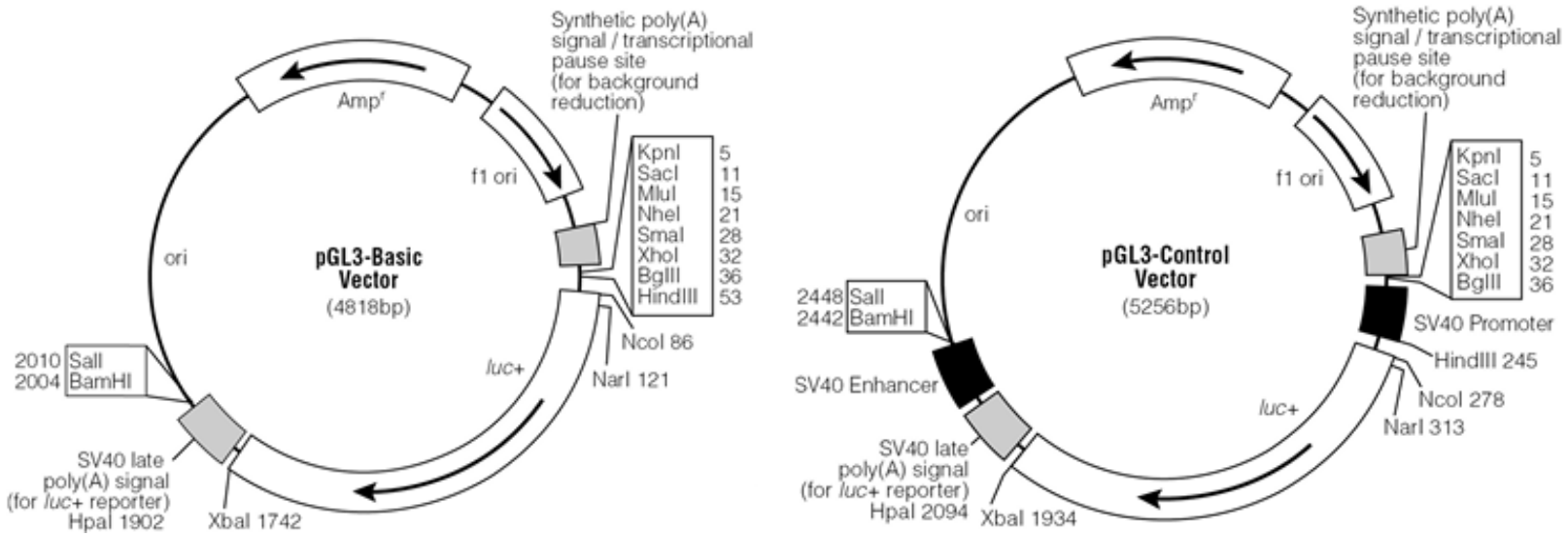

Figure 2-1 pGL3-Basic vector was used as a negative control in quantitative assays. pGL3-Control vector was used as an SV40 positive control vector during quantitative *in vitro* studies.(Images courtesy of Promega Corp.)

and Luciferase reporter gene tied into the pGL41.0 Vector Backbone; and pMYH6/DsRed (Figure 2-4), this consists of the αMHC gene inserted into the pDS-Express-1 vector (Figure 2-2). All vector constructs were sequenced using a 3100 Genetic Analyzer (Applied Biosystems) to confirm the identity and orientation of the insert and the accuracy of the sequence.

pCI-Neo + α-MHC Promoter

In order to prepare a cardiac specific vector utilizing the pCI-neo backbone, the CMV immediate-early enhancer/promoter was excised from the pCI-neo mammalian expression vector (Promega) and exchanged with the α-myosin heavy chain promoter, which had been amplified from rat genomic DNA. The CMV promoter was digested from the plasmid via two stage restriction enzyme digestion with *I-PpO* I and *Bgl II.*

Briefly, 10µl of pCI-neo vector was digested in a 20µl reaction containing 6.8µl H_2O, 2µl of 10X *I-Ppo I* Buffer, 1.0µl *I-PpO I*, and 0.2µl of BSA. The reaction was digested overnight at 37ºC. Following incubation, the reaction was heated at 65ºC for 15 minutes to deactivate the *I-Ppo I* enzyme. The reaction was then gel-purified on 1.5% agarose gel using a QiaQuick Gel Extraction Kit (QIAGEN). To complete the CMV promoter excision from the plasmid, 34.0µl of the gel-purified *I-PpO I* digestion was mixed with 4µl 10X React 3 Buffer, and 2µl of *Bgl II* and incubated overnight at 37ºC. Once again the reaction was terminated by heating the sample to 65ºC for 15 minutes.

The digested plasmid was then treated with Calf-Intestinal Alkaline Phosphatase (CIAP) to remove 5' phosphate groups, preventing self-ligation of digested ends. 40µl of the *Bgl II* digestion was mixed with 5µl of CIAP, 10X Buffer and 5µl of CIAP. The reaction was heated for 30 minutes at 37ºC. After the initial incubation, 5µl of fresh

CIAP was added and the incubation proceeded for 30 more minutes at 37ºC. The reaction was terminated by heating at 75ºC for 10 minutes in the presence of 5mM EDTA (2.3µl 125mM EDTA + 55µl CIAP Rxn). The reaction was then gel purified using QIAquick Gel Extraction kit (QIAGEN). Samples were eluted with 40µl EB Buffer and stored at 4ºC. The α-MHC promoter was amplified from rat genomic DNA using the following PCR primers:

Sense: 5'GGAGCTTGTGTGTTGGAGAC3'
Antisense: 5'GAGAAATCTCGGTCTGACAG3'

A sense primer was also designed that contained a built in *IPpO I* enzyme digestion site and an antisense primer containing a *Bgl II* digestion site. After amplification the PCR product was subcloned into the pCR 2.1-TOPO TA cloning vector (Invitrogen). The α-MHC promoter was excised using the restriction enzymes *I-PpO I* and *Bgl II*, corresponding to the same enzyme sites in the polylinker region of the pCI-neo vector (Figure 2-2). Briefly, a 60µl reaction comprised 50µl of pCR 2.1-TOPO TA + α-MHC promoter, 6.0µl 10X *I-Ppo I* buffer, 2.5µl of *I-PpO I*, 1.0µl H2O, and 0.5µl BSA. The reaction was incubated overnight at 37ºC. Afterwards, 60µl of H2O was added and a 150µl enzyme digest was set up for *Bgl II*. 15µl of React3 buffer, 7.5µl of *Bgl II*, and 7.5µl of H_2O were added to the previous reaction and 60µl of H_2O and Incubated for 8hrs at 37ºC. The digest was run on 1.5% agarose gel and a DNA band of ~570 bp was excised for gel purification.

The pCI-neo promoterless vector and α-MHC promoter fragments were ligated using the Fast-link ligation system (Epicentre). Reaction was incubated at 37ºC for 3 hours and 20 minutes. Following the incubation, the reaction was terminated at 70ºC for 20 minutes. 2µl of the ligation reaction was then used to transfect 1 vial of One-Shot

chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's protocol (Appendix B). From here the typical lab protocol for plasmid preparations were used (Appendix B). Plasmids were digested with *Bgl II* and *Xba I* to verify ligation of the α-MHC promoter ligation and plasmids containing the correct fragment length were purified for sequence verification. These vectors contain the pCI-neo vector backbone and an intact polylinker region with the cardiac specific rat α-MHC promoter.

pGL4.10 + αMHC Promoter (MHCI)

pGL4.10 vector digested with *Xho I* and *Hind III* 37ºC overnight. Vector was gel purified using the Qiagen gel purification kit (QIAGEN) according to the manufacturer's protocol. The purified vector was then treated with CIAP; 40µl plasmid + 5.0µl 10X Buffer + 5.0µl CIAP at 37ºC for 30 minutes followed by the addition of 5 more µl of CIAP and another 30 minute incubation at 37ºC. The reaction was terminated at 75ºC for 10 minutes in the presence of 5mM EDTA. Samples were again gel purified.

Next, α-MHC promoter was digested overnight from the TA vector containing the amplified αMHC promoter insert using *Xho I* and *Hind III*. A DNA band of approximately 650 bp was excised from gel and purified with Qiagen kit (QIAGEN).

The Phosphorylated-pGL4.10 vector and αMHC promoter fragments were ligated using the Fast-link ligation system (Epicentre). Reaction was incubated at 37ºC for 3 hours. Following the incubation, the reaction was terminated at 70ºC for 20 minutes. 2µl of the ligation reaction was then used to transfect 1 vial of One-Shot chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's protocol (Appendix B). Plasmid was then isolated according to standard protocol (Appendix B). One ml of overnight culture was saved for maxi-prep. Following typical plasmid purification and

sequencing, plasmids possessing the correct vector/insert sequence were selected for maxi-prep using the EndoFree Plasmid Maxi Kit (Qiagen) (Appendix B).

pCI-αMHC + Luciferase (MHCII)

This vector was designed to compare α-MHC promoter driven luciferase expression in the pCI-neo backbone to the pGL4.10-αMHC reporter. MHCII was constructed to evaluate variability in activity of the αMHC promoter due to inconsistency in the vector backbone. MHCII was an attempt to compare vector expression to the pCI-Luc control with as little variability as possible. In other words, pCI-Luc and MHCII were identical with the exception of the promoter region.

MHCII promoter vector was also digested with *Xho I* and *Xba I*. After digestion, the DNA fragment was gel purified with Qiagen kit (QIAGEN) and treated with CIAP (Invitrogen) as above. Luciferase gene was excised (~1700 bp) from the pGL3-basic vector using *Xho I* and *Xba I*. The DNA fragment was then gel purified with Qiagen kit (QIAGEN).

The de-phosphorylated pCI-αMHC vector and luciferase DNA fragments were ligated using the Fast-link ligation system (Epicentre). Reaction was incubated at 37ºC for 3 hours. Following the incubation, the reaction was terminated at 70ºC for 20 minutes. 2µl of the ligation reaction was then used to transfect 1 vial of One-Shot chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's protocol (Appendix B). Plasmid was then isolated according to standard protocol (Appendix B). One ml of overnight culture was saved for maxi-prep. Following typical plasmid purification and sequencing, plasmids possessing the correct vector/insert sequence were selected for max-prep using the EndoFree Plasmid Maxi Kit (QIAGEN).

Bacterial Transformation

Competent cells were put on ice for 5min to thaw. 5.0µl of plasmid DNA was added the cell suspension and mixed gently. Cells were kept on ice for 30min with occasional swirling. Cells were heat-shocked for 40 seconds at 42^{o}C. and then placed on ice for 1 min. 200µl of SOC was added and cells were incubated at 37^{o}C for 1 hr at 250rpm. Dilutions were plated at 1-200 µl/plate on (100 µl/ml) ampicillin plates and spread evenly. Cells were incubated overnight at 37^{o}C with dish upside down. Several colonies were picked for DNA analysis and glycerol stocks.

Modified Qiagen Miniprep Isolation of Plasmid DNA

A 7.0 ml culture is grown from a single colony of transformed *E. coli* (alternatively 50µl glycerol stock) by incubating at 37^{o}C and shaking at 250rpm overnight. in Luri Bertani (LB) media with antibiotic solution. 1.5ml of overnight culture was transferred into an Eppendorf tube and centrifuged at 10,000 rpm for 1min. Supernatant was discarded and pellet retained. 250µl of Buffer P1 solution containing RNase was re-suspended evenly by vortexing vigorously for 10 seconds. 250µl of Buffer P2 solution was added and mixed by inverting the tube 8 times and cells allowed to lyse at room temperature for 5 min. 350 µl of Buffer N3 was added and the tube inverted immediately 8 times to neutralize the lysis reaction. Solution was centrifuged at 13,000 rpm for 10 min. The supernatant was then added to the QIAprep spin column by decanting. Column was then centrifuged at 13,000 rpm for 60 seconds and the flow-through discarded. The column was rinsed by adding 600µl Buffer PE and centrifuged for 60 seconds at 13,000 rpm and the flow-through discarded. Wash was repeated with 600µl Buffer PE and centrifuged for 1 min at 13,000 rpm, the flow-through discarded and

put the column back in a different rotation angle so that there will be no solution retentate in the corner of the column. Solution was then centrifuged for an additional 1 min to remove residual wash buffer. Column was placed in a clean sterile Eppendorf tube. DNA was eluted by adding 50µl Buffer EB to the center of the column, letting it stand for 1min, and centrifuging for 1 min at 13,000 rpm. DNA solution was stored at 4^{o}C or -20 ^{o}C.

Modified Qiagen Maxiprep for Plasmid DNA

10 ml of culture (with appropriate selective antibiotic) from a single colony. were incubated for 8 hours at 37 ^{o}C. Two 500 ml media aliquots were inoculated 1ml of culture sample each along with appropriate antibiotic in 2L flasks and allowed to grow at 37^{o}C overnight with vigorous shaking at 300 rpm. Buffer P3 was placed on ice. Cells were harvested by centrifugation at 3000 rpm for 15 min at 4^{o}C in 1000ml centrifuge bottles. Supernatant was discarded and pellets re-suspended from 1000 ml culture in 30 ml buffer P1 with RNase A. Contents were transferred to a 250ml centrifuge bottle. 30 ml of buffer P2 was added while swirling the bottle gently to avoid forming clamps and incubated at ambient temperature for 5min. 30ml of chilled buffer P3 was added while swirling the bottle gently then incubated on ice for 30 min. Samples were then centrifuged at 25,000 X g for 30min at 4^{o}C. Supernatant was transferred into 2 X 50 ml centrifuge bottles and centrifuged at 28,000xg for 30min at 4ºC. Qiagen-tip was equilibrated with 10 ml of buffer QBT. Supernatant from step 7 was added to the QIAGEN-tip and allowed to enter the resin and go through by gravity flow. The tip was washed with 2X30ml of Buffer QC. DNA was eluted with 15 ml Buffer QF to a fresh 50ml centrifuge tube. DNA was precipitated by adding 11ml of ambient-temperature

isopropanol to the eluted DNA solution. Solution was mixed and centrifuged immediately at 25,000 X g for 30min at 4^{o}C and the supernatant discarded. DNA pellet was washed with 5ml of ambient-temperature 70% ethanol and centrifuged at 25,000 x g for 15min.and supernatant carefully discarded without disturbing the pellet. DNA pellet was vacuum dried for 30min. DNA was re-dissolved in 1.0ml of 10mM Tris, pH8 buffer. The Phenol/CIA-Ethanol precipitation protocol was then carried out to further purify the DNA sample before transfection (Appendix B).

Transfection of Mammalian Cells

Plasmids were prepared for transfection using Endofree Plasmid Kit (QIAGEN) to avoid toxicity problems. One day prior to transfection cells were subcultured and diluted to 0.5-2.0 x 10^7 cells per T-25 flask. On the day of transfection cells were harvested by trypsinization & centrifugation, medium was removed, and cells washed once with 1X PBS in a 15ml tube. Cells were seeded at 0.5-2.0 x 10^7 cells per 12-well plate in 12ml DMEM containing 10% Fetal Calf Serum (FCS) and antibiotics. 4µg of plasmid DNA was dissolved in TE buffer with the DNA-condensation buffer EC to a total volume of 600µl. 32µl of enhancer was added and vortexed for 1 second. Cells were then incubated for 5 minutes at ambient temperature and then centrifuged briefly to remove drops from top of tube. 120µl of Effectene transfection reagent (QIAGEN) was added and mixed by vortexing for 10 seconds. Incubation was carried out for 10 minutes at ambient temperature to allow transfection-complex formation. Transfection complexes were added to 15ml tubes containing 3.0ml DMEM and mixed by pipetting up and down twice then immediately adding the mixture drop-wise onto the cells in the 12-well plate. Cells were gently swirled in the wells to ensure uniform distribution of transfection

complexes. Cells were incubated with transfection complexes for 24hrs. under normal (20% O_2) or hypoxic (1% O_2) growth conditions. Cells were analyzed via Luciferase assay to confirm and quantify gene expression.

Sequence Reaction

Sequencing of target plasmids was carried out in 10µl reactions containing 100ng of plasmid template, 4.0µl of sequencing dye from the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and 1.3pmol of sequencing primer. The PCR program for sequencing reactions is: 25 cycles at 96ºC for 10sec, 50 ºC for 5sec, and 60ºC for 4min.

Big Dye Clean Up. Reaction products were purified using an EDTA/ethanol Big Dye clean up procedure as follows: Add 5µl of 125mM EDTA, 60µl of 100% ethanol to each sample, finger vortex, and incubate at ambient temperature for 15min. Spin samples at max speed for 20min at 4ºC. Aspirate of the supernatant. Add 60µl of 70% ethanol, spin sample at max speed for 20min at 4 ºC. Aspirate off supernatant and vacuum dry the samples for 30min.

Electrophoresis and Data Collection. The dried pellet was re-suspended in 15µl formamide. The samples were then denatured at 95 ºC for 2min. and chilled on ice for at least 5min. Then samples were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

HRE Promoter Vectors

There are several HRE sequences, which have been reported in previous studies (Wegner et al. 2005). To select our particular HRE sequence we utilized the base pairs,

which are known to be most common in HRE sequences (Figure 1-6) including the crucial core HRE sequence; 5'CGTG3' (Wegner et al. 2005). The full sequence chosen as our representative HRE sequence is 5'GGGTACGTGCGT3'. Oligos were ordered containing 8 of these HRE repeats and a second with 8 HRE and AP1 repeats (Table 2-1) 5'GGGTACGTGCAGTCA3' in tandem (Integrated DNA Technologies). Primers were designed based on the oligo sequence data and used to PCR amplify the oligo:

Sense: 5'GTAGATCTGACGTCAGTAC3'
Antisense: 5'ACAGATCTCGCACGTACTG3'

Primer sequences included a restriction enzyme cut site to allow for insertion into the appropriate vector. The PCR amplified product was purified using a QIAquick Gel Extraction Kit (Qiagen). The 8X HRE sequence was used in the construction of vectors: HRE1 (Figure 2-5); HRE3, HRE4, and HRE5 (Figure 2-6). The 16X HRE sequence was used to construct HRE2 (Figure 2-5). The HRE/AP1 sequence was used to construct HAL, HAM (Figure 2-7) and HMHC (Figure 2-8). The purified product was then cloned into the *BGLII, XhoI*, or both sites of the following plasmids: pGL4.10, pGL4.23 (Figure 2-3); pGL3-basic, pGL3-control (Figure 2-1) vectors (Promega). The final vectors produced by this phase include: HAL (Figure 2-7), This includes the 8X HRE repeat consensus sequence and the Luciferase reporter gene tied into the pGL4.10 Vector Backbone; HAM (Figure 2-7), This includes the 8X HRE repeat consensus sequence and the Luciferase reporter gene tied into the pGL4.23 Vector Backbone; HRE1 (Figure 2-5).

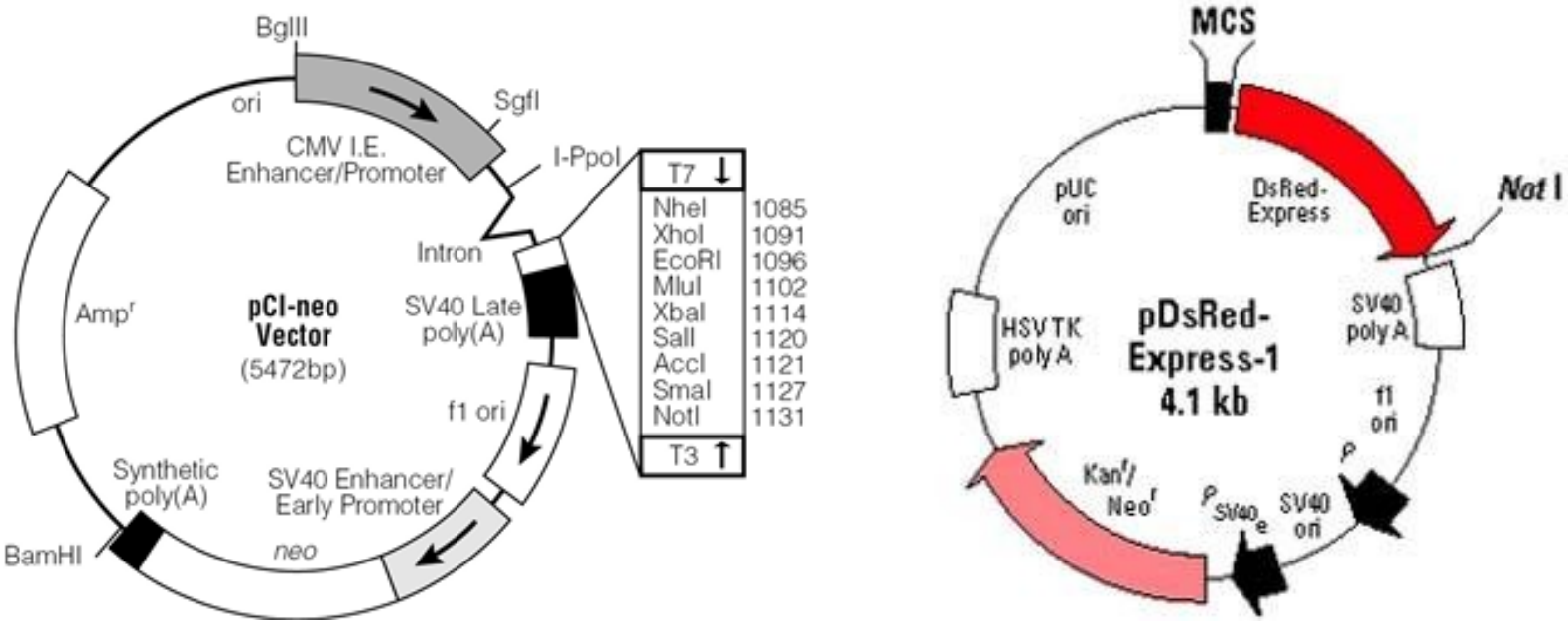

Figure 2-2 pCI-neo served as the plasmid backbone in multiple HRE and MHC vector constructs. pDsRed-Express-1 was utilized in the construction of vectors for the *in vivo* qualitative analysis. .(Images courtesy of Promega Corp.)

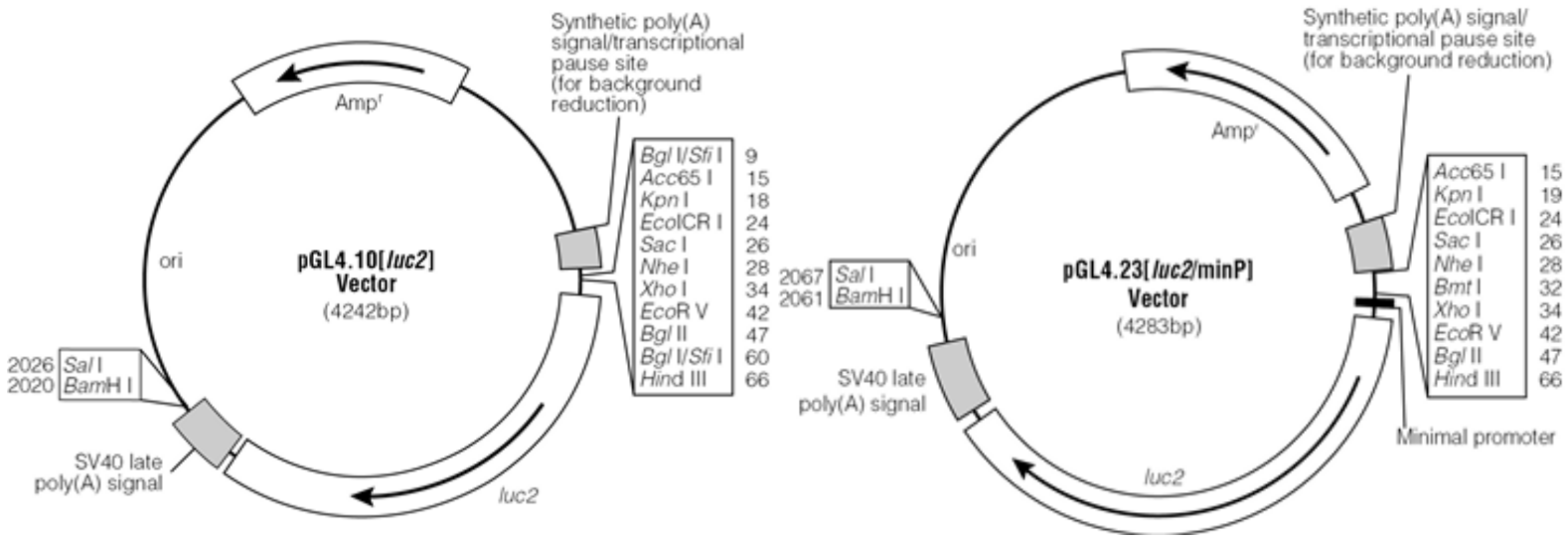

Figure 2-3 pGL4.10 and pGL4.23 were used in the construction of HAL, HAM and HMHC, which were part of the *in vitro* quantitative analysis. .(Images courtesy of Promega Corp.)

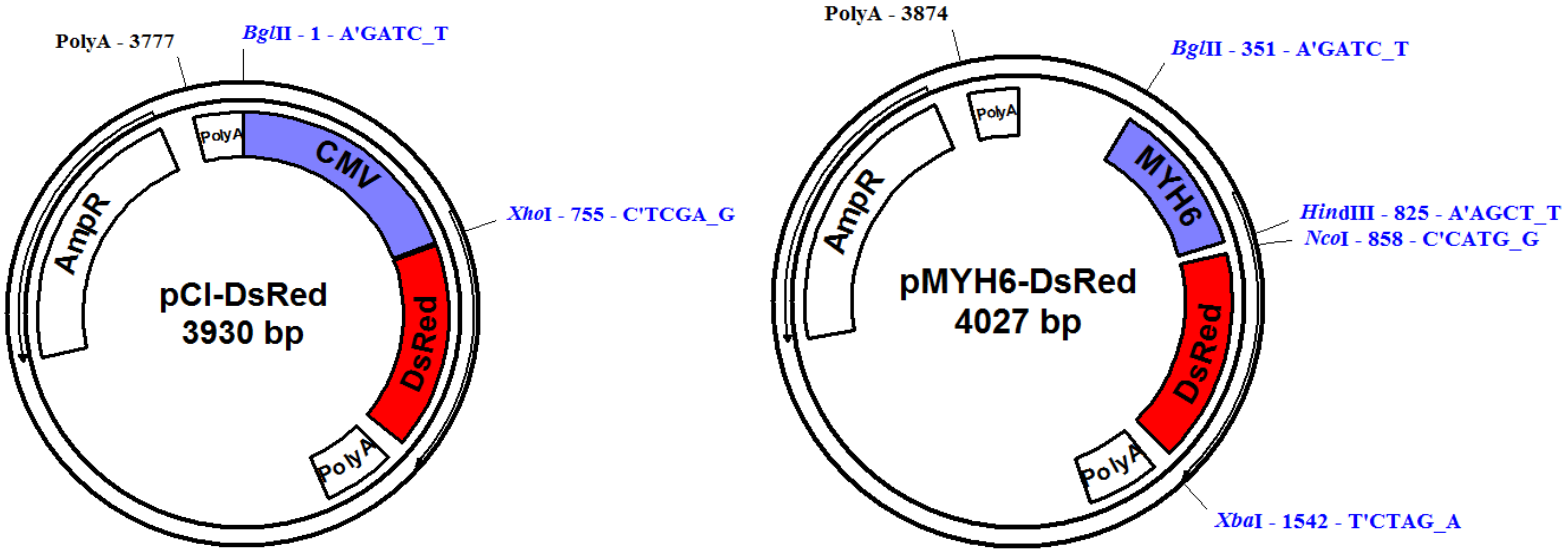

Figure 2-4 The pCI-DsRed and pMYH6-DsRed vectors allowed us to observe the tissue specificity of the MHC1 promoter as compared to the positive control pCI (CMV) and negative control (pGL3 basic) promoters. These vectors were delivered to the target tissue via UTMD. Tissue sections were produced then analyzed via confocal microscopy. This led to new insight with regard to the precise location of where the reporter gene was being expressed. Results indicated MHC1 was expressed exclusively in cardiac cells as expected; however, pCI was found primarily in endothelial cells of the myocardium. No pCI-DsRed expression was observed in cardiomyocytes as might have been expected.

Table 2-2 Primary HRE Oligos Used for DNA Vector Promoter Constructs

HRE Repeat Oligos
HRE Repeat Sequence (8X) 5'GGGTACGTGCGTGGGTACGTGCGT GGGTACGTGCGTGGGTACGTGCGT GGGTACGTGCGT GGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGT3'
HRE Repeat Sequence (16X) 5'GGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGT GGGTACGTGCGT GGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGT GGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGT GGGTACGTGCGT GGGTACGTGCGTGGGTACGTGCGTGGGTACGTGCGT 3'
HRE/AP1 Repeat Sequence (8X) 5'GGGTACGTGCAGTCAGGGTACGTGCAGTCAGGGTACGTGCAGTCAGGGTAC GTGCAGTCAGGGTACGTGCAGTCAGGGTACGTGCAGTCAGGGTACGTGCAGT CAGGGTACGTGCAGTCA 3'

This includes the 8X HRE repeat consensus sequence and the Luciferase reporter gene tied into the pGL3-basic Vector Backbone; HRE2 (Figure 2-5), which includes the 16X HRE repeat consensus sequence and the Luciferase reporter gene tied into the pGL3-basic Vector Backbone; HRE3 (Figure 2-6), which includes the 8X HRE repeat consensus sequence immediately upstream of the 5' end of the CMV promoter (*BGLII* site) and the Luciferase reporter gene tied into the pCI-Neo Vector Backbone; HRE4 (Figure 2-6), which includes the 8X HRE repeat consensus sequence immediately downstream of the 3' end of the CMV promoter (*XhoI* site) and the Luciferase reporter gene tied into the pCI-Neo Vector Backbone; HRE5 (Figure 2-6), This includes both the 8X HRE repeat consensus sequence immediately upstream of the 5' end of the CMV promoter (*BGLII* site) and an 8X HRE repeat consensus sequence immediately downstream of the 3' end of the CMV promoter (*XhoI* site), as well as, the Luciferase reporter gene tied into the pCI-Neo Vector Backbone. All vector constructs were

sequenced using a 3100 Genetic Analyzer (Applied Biosystems) to confirm the identity and orientation of the insert and the accuracy of the sequence.

In Vitro Quantitative Hypoxia Assay

To achieve a quantitative value *in vitro* for each of our final vectors, cells were grown under conditions of both normoxia (20% O_2) and hypoxia (1% O_2). Human Fibroblast, Rat2, L6, H9C2 and Ypen-1 (ATCC) cell types were tested under these conditions. Cells were thawed, cultured, frozen in N_2 to replenish stocks, and sub-cultured into 12 well plates all according to standard lab protocols (Appendix B). Each cell type was plated and grown in DMEM (Invitrogen) w, 10% FCS (Invitrogen), and antibiotic/antimycotic mix (Invitrogen). All cells were grown for 24 hrs at 37ºC and 5% CO_2 (Thermo/Forma Scientific Series I CO2 Water Jacketed Incubator Model 3110). Cell confluence of each cell type was assessed via hemacytometer (VWR). Once cells reached ~70% confluency they were considered fit for transfection. Media was removed and cells were rinsed 3X with 1X PBS (Invitrogen). A control group and an experimental group of cells were transfected simultaneously under the same conditions and with the same vectors and reagents. Effectene transfection reagent (Qiagen) was used according to the standard manufacturer protocol (Appendix B). Effectene was chosen after comparative trials between it and several other commercial products were carried out. After careful consideration, Effectene proved to be the best overall reagent for our particular cell and vector types *in vitro*. Control group cells were grown for 24 hrs at 37ºC, 5% CO_2 and 20% O_2. Experimental group cells were grown in a custom designed hypoxia chamber (Figure 2-9), which utilized an Oxygen regulator/meter (Biospherix ProOx). Experimental group cells were grown for 24 hrs at 37ºC, 5% CO_2 and 1-2% O_2.

Next, the Dual Luciferase Reporter (DLR) kit (Promega) was used according to the standard manufacturer protocol (Appendix C). The DLR kit was used in conjunction with a Luminometer (Turner Designs TD-20/20) to assess the degree of Luciferase expression for each sample well in Relative Light Units (RLU). A measurement for an internal control (*Renilla* Luciferase) was also measured. This allowed for normalization of all Luciferase data. Every measurement was repeated in triplicate. An R/C value (Firefly Luciferase, RLU divided by the internal control, *Renilla* Luciferase, RLU) was derived. The mean and standard deviation for each sample was then determined via standard calculation by Excel spreadsheet, (Microsoft). Data was then graphed showing both the raw RLU data and the normalized R/C data (Figures 3-1, 3-2, 3-9, 3-10, 3-11, and 3-12). Data was then analyzed via One-Way ANOVA (Appendix A).

In Vitro Dual Luciferase Assay

LAR II, Stop & Glo reagent and samples were warmed to ambient temperature prior to performing the Dual-Luciferase Assay. 100µl of LAR II was pre-dispensed into luminometer tubes to complete the desired number of DLR assays. Luminometer (Turner Designs) was set to perform a 2-second pre-measurement delay, followed by a 10-second measurement period for each reporter assay. 20µl of cell lysate was transferred into the luminometer tube containing LAR II, mixed by pipetting 2 or 3 times and placed in the luminometer. Luminometer was then initiated and Firefly Luciferase output recorded in an Excel (Microsoft) spreadsheet. 100µl of Stop & Glo Reagent was then added and mixed by pipetting up and down 5 times. The tube was then placed back in the luminometer, and the next reading initiated. The *Renilla* luciferase activity measurement was then recorded.

pMHCI-Luciferase and pCI-Luciferase Treatment of Rattus Norvegicus

Three experimental protocols were followed. In the first protocol, 18 rats were transfected with pCI-luciferase and pMHCI-luciferase plasmids with the use of UTMD. Ultrasound targeting to rat heart was conducted on two groups of 6 rats. Ultrasound targeting to right quadratus skeletal muscle was conducted on two groups of 6 rats. Ultrasound targeting to right kidney was conducted on two groups of 6 rats. Rats were sacrificed, and organs harvested 4 days after UTMD treatment.

For luciferase assays, the heart, left lung, liver (mid-portion), skeletal muscle (right and left M quadratus) and right and left kidney were obtained. The atria along with the anterior and posterior portions of ventricle were analyzed. 100-mg samples of the organs were disrupted in a (Polytron) homogenizer in 1 ml luciferase lysis buffer. The solution was cleared by centrifugation and 20 μL of supernatant was analyzed in a luminometer with the use of a commercial substrate DLR. Protein concentration was determined by the Lowry method with a commercial kit (Pierce Endogen) to allow calculation of relative luminescence units per mg protein per minute (RLU/mg per minute).

In the second protocol, 12 rats were divided into 4 groups: MHCI-DsRed plasmid group, pCI-DsRed plasmid group (Figure 2-4), microbubble without plasmids control group and normal rat control group. Hearts were harvested after 4 days (3 rats in each group) for DsRed signal; *in situ* PCR and immunohistology assay (Figure 3-9). Mean and standard deviation was calculated for all rats treated with luciferase plasmids. Differences in experimental and control luciferase activity between groups were compared by ANOVA for multiple group comparisons (Appendix A).

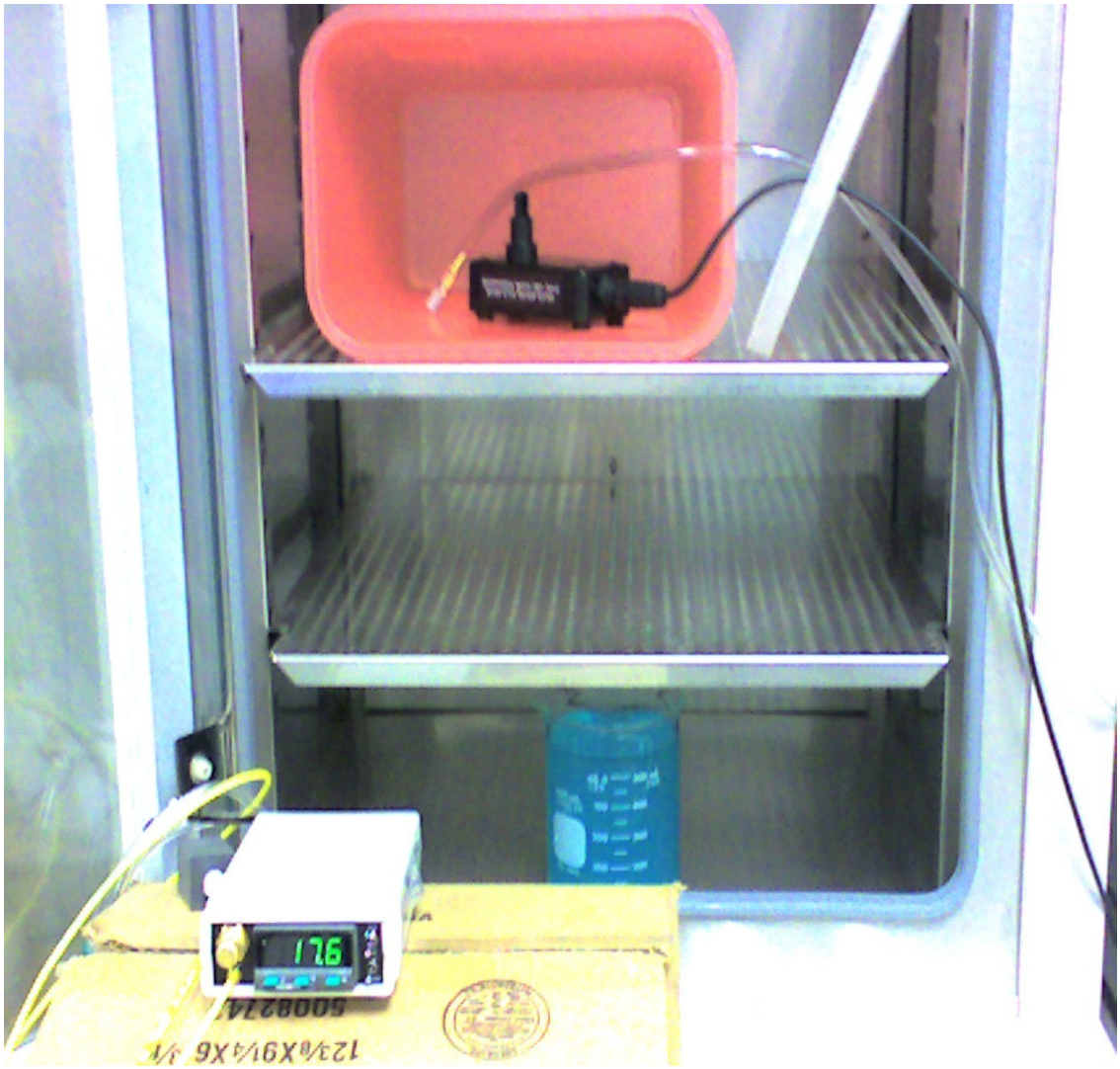

Figure 2-5 Hypoxia Chamber: Consists of Biospherix ProOx Oxygen regulator/monitor, Thermo/Forma Scientific Series I CO2 Water Jacketed Incubator Model 3110, Sharps Container, Tubing, Connectors, CO2 tank, N2 tank, 2 Gas Regulators.

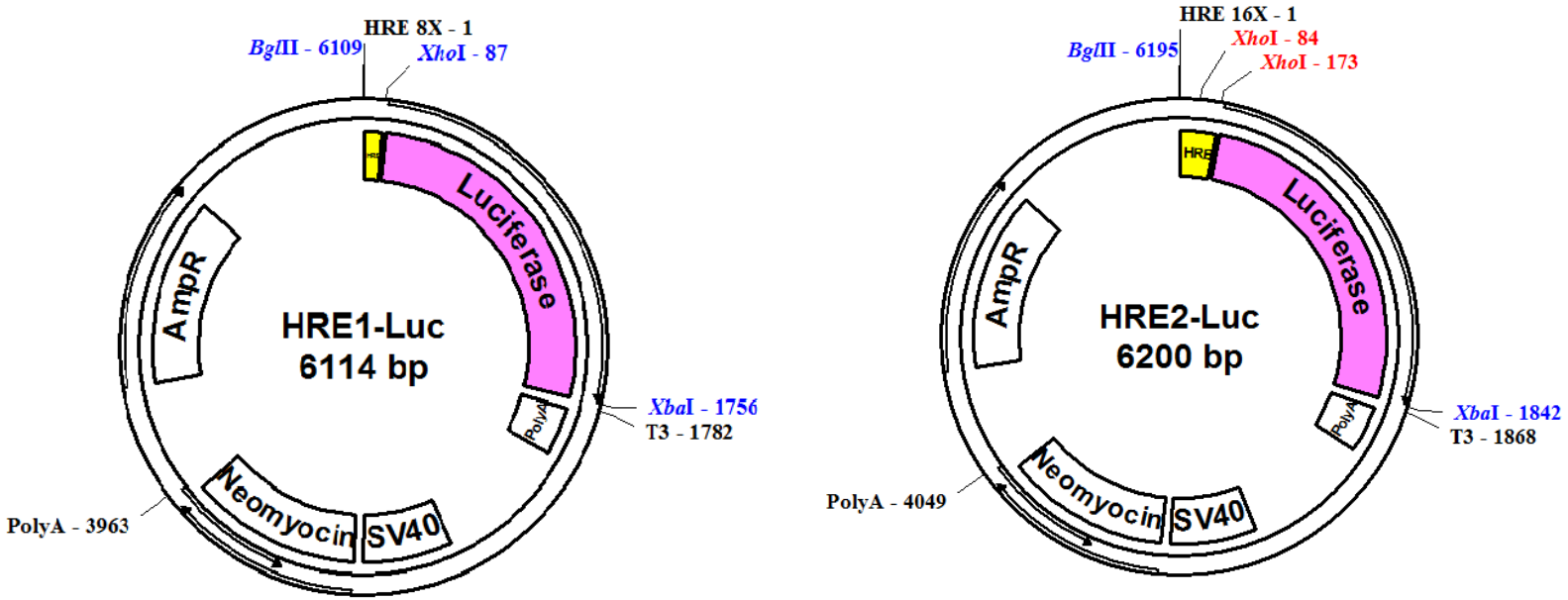

Figure 2-6 HRE1; Luciferase gene expression driven by an 8X HRE tandem repeat. HRE2 is identical to HRE1 except that there are 16X HRE tandem repeats. This was done in order to ascertain if there is an increase in gene expression that corresponds to an increase in the number of tandem repeats.

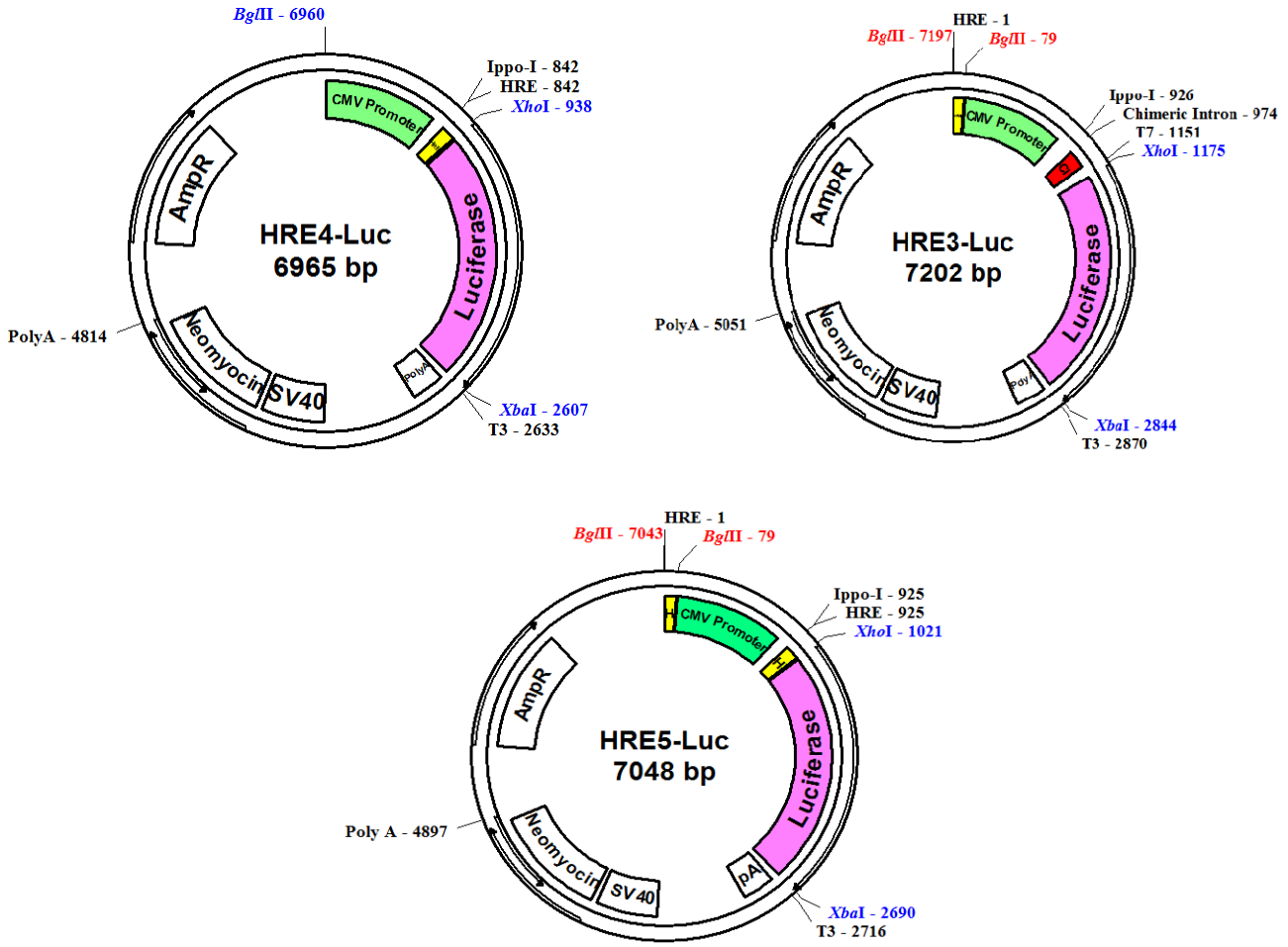

Figure 2-7 HRE3 includes an 8X HRE tandem repeat added to the 5' end of a CMV promoter. HRE4 to the 3' end. In the case of HRE5, this 8X HRE tandem repeat was added to both the 5' and 3' end of the CMV promoter.

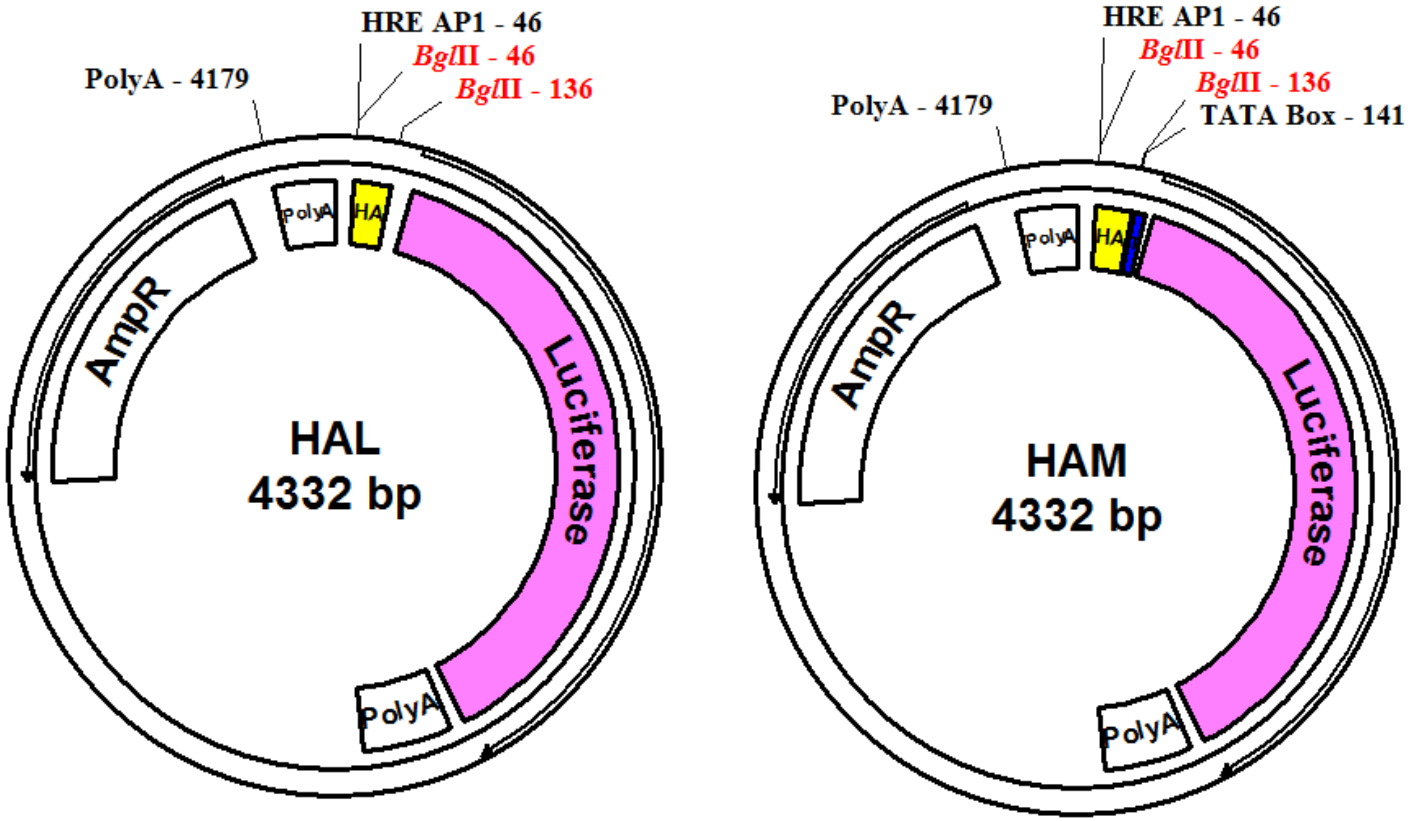

Figure 2-8 pHAL and pHAM both include an 8X HRE/AP1 tandem repeat in the promoter region. These promoter constructs were intended to better interact with the hypothesized HIF-1 complex. pHAM includes the addition of a TATA Box on the 3' end of the promoter region in an attempt to further facilitate transcription.

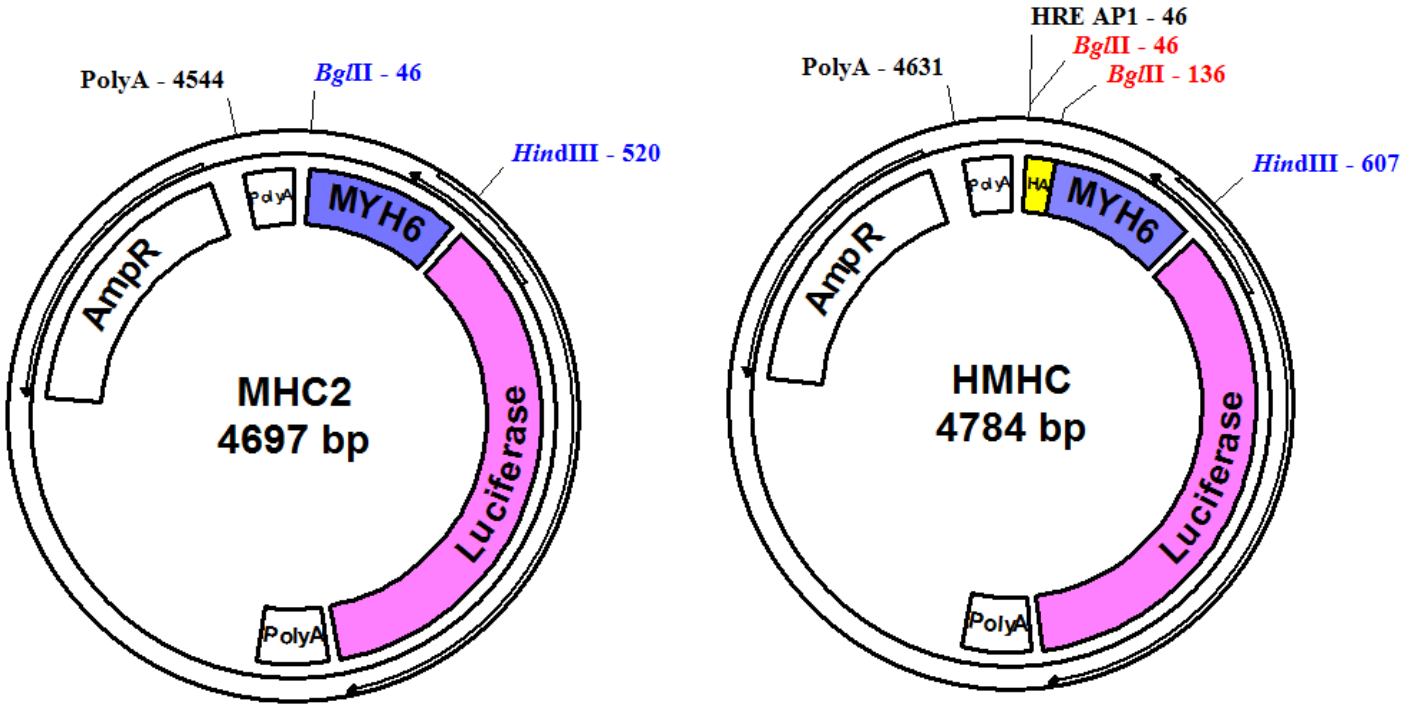

Figure 2-9 pMHC2 (Cardiac Specific Vector) and pHMHC (Cardiac Specific and Hypoxia Up-regulated)

In Situ PCR for Detection of DsRed DNA and mRNA on Rat Heart Slides

A single pair of DsRed primers, shown below, was directed against the DsRed fragment of the MYH6/pDsRed and pCI/pDsRed plasmid constructs (Figure 3-5).

DsRed 125^{+} (5'-GAGTTCATGCGCTTCAAGGTG-3')

DsRed 690^{-} (5'-TTGGAGTCCACGTAGTAGTAG-3').

Rats were anesthetized with Avertin. A polyethylene tube (PE50) was inserted into the right jugular artery by cutdown for intra-artery infusion of 200ml cool saline and washed out blood from jugular vein cutdown and perfusion fixed with 100 ml of 2% Para-formaldehyde (Sigma) and 0.4% Glutaric Dialdehyde (Sigma). Rat heart tissue was cut into 0.5 cm pieces and put into a 20% sucrose solution overnight at 4°C and then put into an OTC mold. Samples were then placed in –86°C and 5 μm cryostat sections were produced using a frozen microtome and mounted on silane coated slides (Sigma). 5 μm cryostat sections were fixed in 4% paraformaldehyde for 15 min at 4 °C, 10 mM glycine (Sigma) in PBS for 5 minutes and rinsed in 1X PBS for 5 min. Cells were then permeabilized 3 times with 0.5% Triton X-100 (Sigma) in 1X PBS for 10 min and rinsed with 1X PBS 10 min. A PCR DIG Probe Synthesis Kit (Roche) was utilized. Slides were covered with a 24×50 mm coverslip, which was anchored with a drop of nail polish at one side. The slide was then placed in an aluminum `boat' directly on the block of an *in situ* PCR thermocycler (Perkin-Elmer, GeneAmp system 1000). 50 μl of a cocktail solution (0.8 units of *Taq* DNA polymerase, 2μl of DsRed primers, 3 μl of DIG-dNTP, 5 μl of 10× Buffer and 40 μl of H_2O) was added to each slide.

In situ PCR was performed using an *in situ* PCR thermocycler as follows: after an initial hold at 94 °C (1 min) the PCR was carried out for 11 cycles (94°C for 1 min, 54 °C for 1 min, and 72 °C for 2 min). After amplification, the coverslip was lifted off and the slide was immersed in 2× SSC for 10 min and 0.5% paraformaldehyde for 5 min and twice in PBS for 5 min. The digoxigenin incorporated-DNA fragment was detected using a fluorescent antibody enhancer set for DIG detection, (Roche) followed by histochemical staining. First, the sections were incubated with 10% goat serum (ATCC) for 30 min. Then, the sections were incubated with 50 µl of anti-DIG solution (1:25) for 1 h at 37°C in a moisturized chamber. The slides were then washed with PBS three times with shaking, each for 5 min. Again, the slides were incubated with 50 µl of anti-mouse-lgG-digoxigenin antibody solution (1:25) for 1 hr at 37°C. The slides were washed with PBS three times with shaking, each for 5 min. The slides were incubated with 50 µl of anti-DIG-fluorescence solution (1:25) for 1 hr at 37°C. The slides were then washed with PBS three times with shaking, each for 5 min. Finally, the sections were dehydrated in 70% EtOH, 95% EtOH and 100% EtOH, each for 2 min, cleared in xylene (Sigma) and cover-slipped. The FITC signal is identified with a green filter at an excitable wavelength of 488 nm and an emission wavelength of 510-540 nm by confocal microscope (Leica SP1).

In Situ RT-PCR for Detection of DsRed mRNA

DNase treatment. 50 µl of cocktail solution (Invitrogen) (5.0µl of DNaseI, 5.0µl of 10X DNase buffer, and 40µl of H_2O) was placed on each slide, covered with a

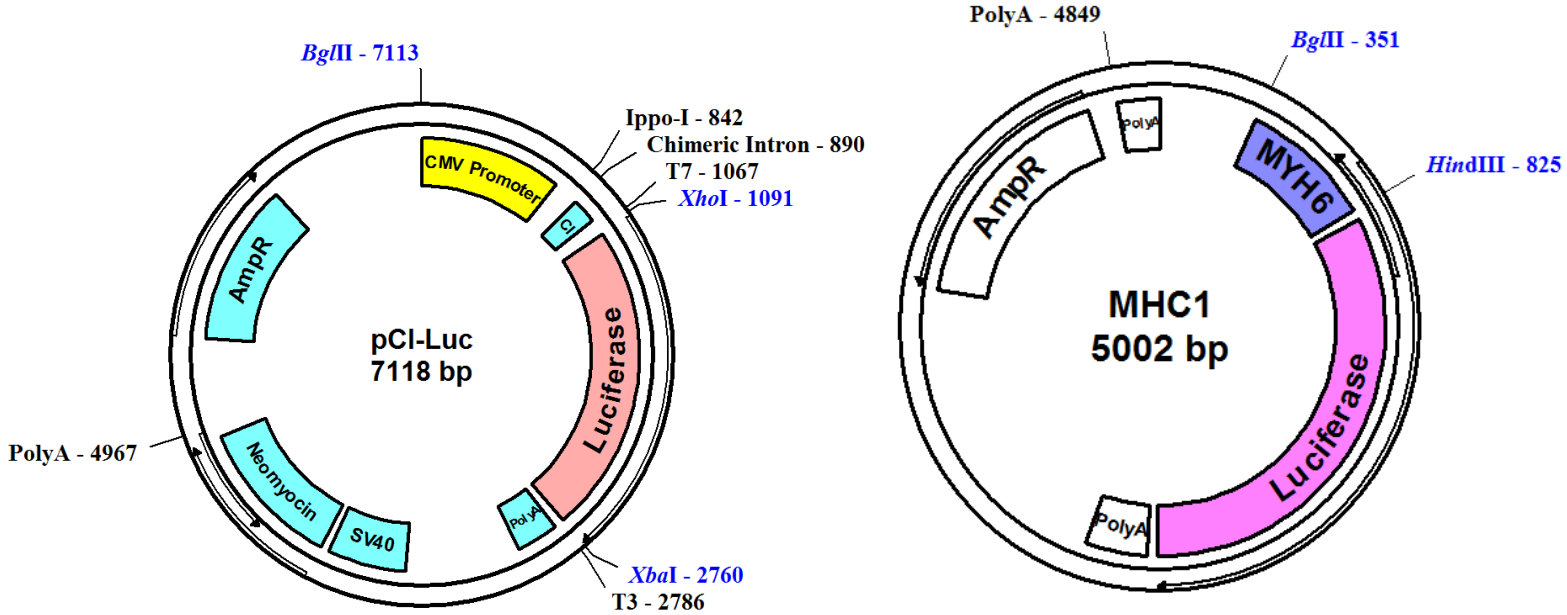

Figure 2-10 The pCI-Luc and pMHC1 vectors were constructed to test the effectiveness of UTMD as our mode of site directed delivery of the vector *in vivo*. The Luciferase reporter gene produced a quantitative value to measure. These two vectors also allowed us to observe the tissue specificity of the MHC1 promoter as compared to the positive control pCI (CMV) promoter.

coverslip, incubated at 25°C overnight, and then washed twice with 1X PBS 5 min. Reverse transcription: First-strand cDNA synthesis was performed on the slide with a 50µl total volume along with 50µl of cocktail solution (Invitrogen, Superscript II First-Strand Synthesis System).

RT-PCR. 1.0µl of DsRed primers, 5.0µl of DTT solution, 2.5µl of dNTP's, 5.0µl of 10X buffer, 5.0µl of 25 mM MgCl, 29µl of H_2O and 2.5µl of SuperScript II RT (Invitrogen) on each slide. The slide was then covered with a coverslip, incubated at 42 °C for 2 hrs, washed with 1X PBS 5 min 2 times, treated with 100% ETOH for 1 min and allowed to dry. *In situ* PCR procedure was then carried out (Figure 3-6).

In Vivo Luciferase Assay

To detect expression of the transgene, the atria, anterior wall and posterior wall of ventricle of the heart and skeletal muscle were each pulverized in a Polytron and incubated with luciferase lysis buffer (Promega), 0.1% NP-40, and 0.5% deoxycholate and proteinase inhibitors (Sigma). The resulting homogenate was centrifuged at 10,000 g for 10 minutes and 100µl of luciferase reaction buffer (Promega) was added to 20µl of the clear supernatant. Light emission was measured by a luminometer (TD-20/20, Turner Designs) in RLU. Total protein content was determined by the Lowry method (BCA protein assay reagent, Pierce) from an aliquot of each sample. Luciferase activity was expressed as RLU/mg protein/min (Table 3-1, 3-2; Figure 3-1, 3-2).

Detection of DsRed protein signal and Immunohistology Study

5 µm cryostat sections were fixed in 4% paraformaldehyde for 15 min. at 4°C, treated with 10 mM glycine in 1X PBS for 5 min., and rinsed in 1X PBS for 5 min then

mounted using mounting medium. The DsRed protein is identified with a red filter at an excitable wavelength of 568nm and an emission wavelength of 590-610nm by confocal microscope (Leica SP-1). Sections were then permeabilized 3X in 0.5% Triton X-100 in 1X PBS for 10 min. and rinsed with 1X PBS for 10 min. Sections were then blocked with 10% goat serum at 37°C for 1hr and washed 3X with 1X PBS for 5 min. Primary antibody (Sigma) (1:50 dilution in block solution) was then placed on slides and incubated at 4°C overnight. Slides were then washed with 1X PBS three times for 5 min. Next, secondary antibody (Sigma, anti-mouse lgG conjugated with FITC) (1:50 dilution in block solution), was placed on the slides and incubated for 1 hr at 37°C. Slides were then washed with 1X PBS for 10 min, 5 times, and then mounted using mounting medium. The FITC signal is identified with a green filter at an excitable wavelength of 488 nm and an emission wavelength of 510-540 nm by confocal microscope (Figure 3-6).

UTMD

Preparation of Liposome Microbubbles Containing Plasmid DNA

Liposome microbubbles were prepared using a modification of the method described by (Korpanty et al. 2005) A solution of DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, Sigma) 2.5 mg/ml, DPPE (1, 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine, Sigma) 0.5 mg/ml, and 10% glycerol was mixed with 2 mg of MYH6-pDsRed in a 2:1 ratio. Aliquots of 0.5 ml of this liposome-plasmid solution were placed in 1.5 ml clear vials; the remaining headspace was filled with the perfluoropropane gas (Air Products). Each vial was incubated at 40°C for 30 min. and then shaken for 20 seconds by a dental amalgamator (Dupont). The mean diameter and

concentration of the microbubbles in the upper layer were measured by Coulter counter and were 1.9 ± 0.2 μm and 5.2 ± 0.3 × 10^9 bubbles/ml, respectively. The dosage of plasmid attached to the liposome microbubbles was 250 ± 10 μg/ml.

Animal Preparation and Ultrasound Microbubble Destruction

Sprague-Dawley rats (200 to 250g; Charles River) were anesthetized with 100 mg/kg ketamine (IP) and 5 mg/kg xylazine (IP). A polyethylene tube (PE 50, Becton Dickinson) was inserted into the right internal jugular vein by cutdown. One milliliter of the microbubble solution was infused at a constant rate of 3 ml/h for 20 minutes. Microbubble destruction was obtained by using a Sonos 5500 machine (Agilent Technologies) with an S3 transducer operating in ultraharmonic mode (transmit, 1.3 MHz/receive, 3.6 MHz) with a mechanical index of 1.2-1.4 and a depth of 4 cm. The transducer was placed on the thorax in a mid short-axis view. After an adequate window was obtained, the transducer was held in place with a clamp during the infusion of microbubbles. Sonography was ECG-triggered (at 80 ms after the peak of the R wave) to deliver a burst of 6 frames of ultrasound every 4 cardiac cycles. At the end of the experiment, the jugular vein was tied off and the skin closed. The research protocol conformed to institutional guidelines for animal research and was approved by the Institutional Animal Care and Research Advisory Committee (IACUC) at the Baylor University Medical Center (BUMC) at Dallas.

Data Analysis

Data was analyzed with Excel 2007 and Statview software (SAS). The results are expressed as mean ± one standard deviation. Differences were analyzed by One-Way ANOVA; $\alpha = 0.1$.

CHAPTER THREE

Results and Discussion

Overview

The objective of this project was to produce gene therapy vectors that would be significantly up-regulated only under certain physiological conditions. Specifically, we wished to produce vectors that would be regulated by cellular oxygen levels, be expressed exclusively in cardiac tissues or both. The significance of such constructs is that it imparts a greater degree of control in the implementation of gene therapy. In general, it is desirable for a gene therapy vector to be active only when and where it is needed. Our constructs are a small step towards this ultimate goal.

Gene therapy vectors, which incorporated variants of the α-Myosin Heavy Chain promoter region, were utilized to produce cardiac tissue specific gene expression. Additionally, we constructed gene therapy vectors, which included HREs' and various other transcriptional elements arranged in an array of combinations. These unique arrangements of consensus sequences were designed *de novo* with the intended purpose of increased expression levels under the specific physiological conditions of hypoxia. One of our primary goals was to construct vectors that would be directly regulated by cellular oxygen levels. Such vectors would have long term implications in the eventual treatment of ischemic diseases such as CAD. Several variations of these vectors were constructed and analyzed. Constituents of our final and most effective designs included HRE and AP1 repeat consensus sequences (HAL, HAM, and HMHC). In some cell

lines, the addition of a TATA box downstream of all other consensus sequence elements enhanced gene expression.

During the *in vivo* study, we verified that delivery of vectors exclusively to cardiac tissue could be carried out via UTMD. We then demonstrated that cardiac tissue specific expression by a modified α-MHC promoter delivered via UTMD was possible. Furthermore, we found expression by a pCI (modified CMV) promoter to be limited to endothelial cells of the myocardium when delivered via UTMD *in vivo*.

Our *in vitro* study results indicate that significant gene expression is possible via a UTMD delivered hypoxia reactive promoter. These results indicate that a combination of HRE and AP-1 repeats in a common promoter significantly up-regulate gene expression. Additionally, *in vitro* analysis indicates that it may be possible to achieve both tissue specificity and hypoxia reactivity from a single promoter construct. This data indicates that a further degree of control over *in vivo* vector gene expression may be possible. Such an improvement in control over gene expression is a potentially invaluable tool for future gene therapy efforts.

Results

HRE Promoter Vectors

We constructed gene therapy vectors with a variety of transcriptional elements arranged in an array of combinations. These unique arrangements of consensus sequences were designed *de novo* with the intended purpose of increased expression levels under specific physiological conditions. One of our primary goals was to construct vectors that would be directly regulated by cellular oxygen levels. (Such vectors would have long term implications in the eventual treatment of ischemic diseases such as CAD.)

Several variations of these vectors were constructed and analyzed (HRE 1-5; HAL, HAM, HMHC). Several of our vectors contain a chain of HRE consensus sequence repeats (Figures 2-5, 2-6, 2-7, and 2-8). The sequence used was carefully chosen. There are a variety of known HRE sequences (Wegner et al. 2005). The sequence chosen encompassed the base pairs most commonly found in such HRE regions (Figure 1-6).

HRE/AP-1 Expression Vectors

Constituents of our final and most effective designs included Hypoxia Responsive Element (HRE) and Activator Protein-1(AP1) repeat consensus sequences. In some cell lines, the addition of a TATA box downstream of all other consensus sequence elements significantly enhanced gene expression.

Previous attempts at creating vector promoters regulated by hypoxia generally consisted of simply stringing together 2 or more HRE sequences in tandem. This was used in place of the promoter entirely or placed immediately upstream of a core SV40 promoter. These attempts demonstrated mixed results (Binley et al. 1999). This is not surprising considering the lack of consistency between the designs of various groups (Su et al. 2002). Some of the variables that are likely to have affected the expression levels of these vectors include:

The specific type of HRE chosen and what sequence is even considered to be an HRE, HRE number, distance between repeats, distance from the transcriptional start site, secondary and tertiary DNA conformation, vector backbone, expression system, cell type, transfection method, cell confluence at transfection, oxygen level at various phases and cell growth conditions; just to name a few. One consistent omission in all previous studies reviewed is the fact that no other HIF1 binding consensus sequence elements

were included with the HRE vector promoter constructs. This appears to be a glaring omission based on the fact that the HIF complex should not be able to fully form with the presence of the HRE element alone. Co-factors of the HIF complex, such as AP-1 and CREB, must also bind the DNA for the complex to be fully active. HRE alone is necessary, but not sufficient to produce a significant hypoxia based up-regulation in gene expression (Figure 1-7). Nevertheless, our data indicates a possible marginal increase in expression under hypoxic conditions when the HRE sequence is used exclusively as an expression vector promoter, as compared to a control SV40 promoter. However, when we constructed a promoter sequence that alternated a HRE sequence with an AP-1 sequence, expression under hypoxic conditions improved dramatically (Figures 3-9, 3-10, 3-11, and 3-12).

Two particularly noteworthy vectors include our (pHAL) and (pHAM) constructs (Figure 2-7). The two vectors are identical except that the HAM vector includes a TATA box and the HAL vector does not. Both vectors are built on the pGL4 system backbone, which includes Luciferase as its reporter gene. Both constructs include alternating HRE and AP-1 sequences in tandem upstream of the function gene. These two elements were selected because they are known to interact with Hypoxia Inducible Factor-1α (Hif-1α) to form an initiator complex. As mentioned previously, past attempts at HRE based expression vectors relied on tandem repeats of HRE elements alone (Su et al. 2002), which when replicated by our lab (HRE 1-5) produced marginal results. Our pHAL and pHAM vectors remedy the issue of an incomplete HIF docking point and have produced significantly increased expression levels under hypoxia (Figures 3-9, 3-10, 3-11, and 3-12). This type of construct is a promising first step towards a gene therapy vector suited

to treat ischemic diseases, being highly active only under hypoxic conditions when its therapeutic gene would need to be expressed and inactive under normoxia when it may do more harm than good.

Quantitative In Vitro Assessment of HRE / AP-1 Modified αMHC Based Vector Designs

The αMHC promoter was incorporated into both a pCI and a pGL4 backbone in an attempt to further enhance expression levels. Expression levels were compared via Luciferase analysis; however, differences in expression were not significant. (Appendix B) Furthermore, several attempts at producing a vector that would be both cardiac tissue specific and hypoxia regulated were made. The most successful of these variants was our (pHMHC) vector (Figure 2-8). This and other designs attempted to add HRE and AP1 components to the αMHC core promoter. Expression was significant under hypoxia in a rat fetal cardiomyocyte cell line, although not to levels exceeding that of our CMV positive control promoter (Figures 3-9, 3-10, 3-11, 3-12). However, expression under normoxia was significantly lowered and expression under hypoxia remained comparable to vector constructs that included the αMHC core promoter region only (MHC1 & MHC2).

Luciferase assays were carried out and the data was recorded. All assays were carried out in triplicate under conditions of both normoxia (20% O_2) and hypoxia (1% O_2). The entire experiment was then replicated an additional two times for a total of three trials consisting of 3 separate samples. Each sample was read 3 times and the data averaged to obtain the particular data point. So, for each vector used on each cell type a total of (3x3x3x2 = 54) separate measurements were made. The samples within each

major trial were averaged together and compared via one-way ANOVA (Appendix A). The results of which are summarized in (Table 4-1).

The data for cells kept in a hypoxic state were compared to those grown under standard normoxic conditions. The Null hypothesis (HO) states, "There will be no difference in luciferase expression levels between hypoxic and normoxic cells." Conversely, the alternative hypothesis (HA) states, "There will be a difference in luciferase expression levels between hypoxic and normoxic cells." This same experiment was carried out on the following cell lines: Human fibroblast cell line, rat fibroblast cell line (Rat2), rat skeletal muscle cell line (L6), rat fetal cardiomyocyte cell line (H9C2), and rat endothelial cell line (YPEN-1).

Discussion

The human and rat fibroblast cell lines were used as controls to compare the other cell lines to. H9C2 is of particular interest because it is a cardiac cell line. However, it is not expected to be directly analogous to an *in vivo* test conducted on adult cardiomyocytes. This is due to two primary reasons. First, different regions of the heart express very different ratios of αMHC and βMHC. This cannot easily be duplicated *in vitro*. Second, expression ratios of αMHC and βMHC change during maturation from the fetal to the adult stage (Hixon et al. 1989). Unfortunately, adult cardiomyocytes do not divide and cannot be grown in culture. It is possible to isolate a few adult cells and keep them alive for a few days in culture without dividing. However, this method is very cumbersome, yields a very small amount of cells, and is a mixture of various cardiac regions. Therefore, it did not seem practical or purposeful to attempt this method.

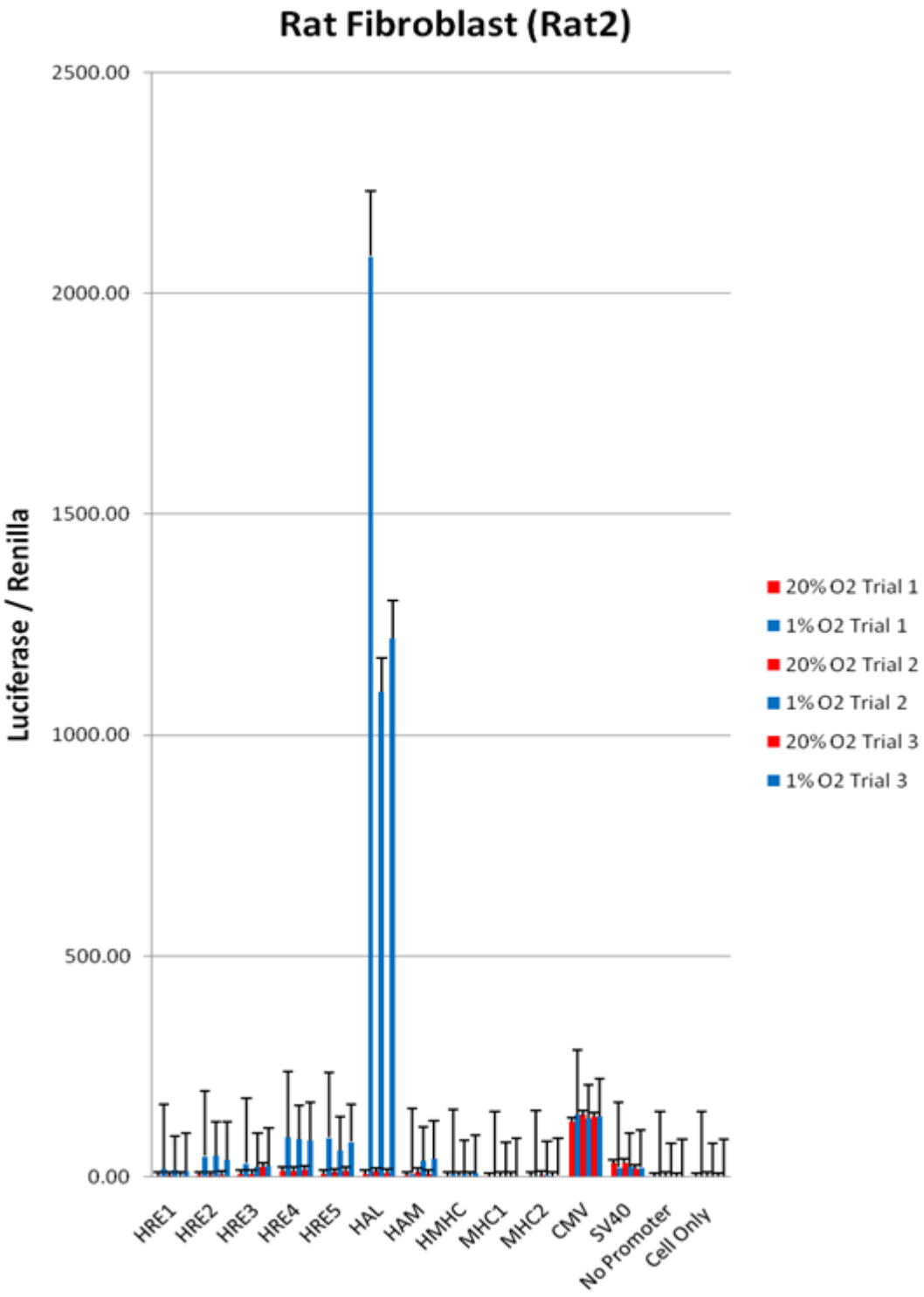

Figure 3-1 Normalized Luciferase expression in rat fibroblasts under normoxic and hypoxic conditions. HAL was observed to be greatly up-regulated in this cell line under hypoxic conditions.

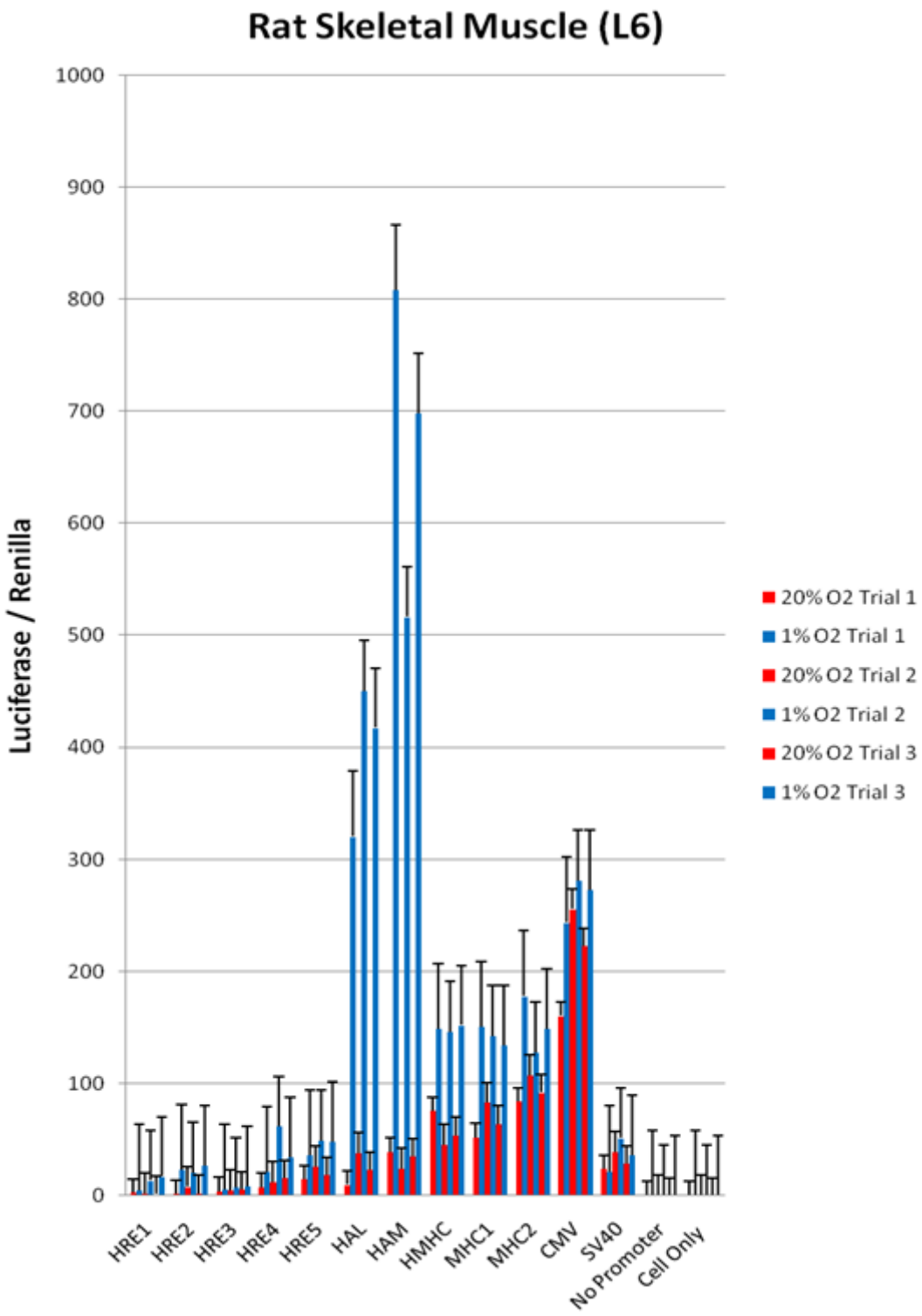

Figure 3-2 Normalized Luciferase expression in rat L6 skeletal muscle cells under normoxic and hypoxic conditions. HAL and HAM were both significantly up-regulated in this cell line under hypoxic conditions.

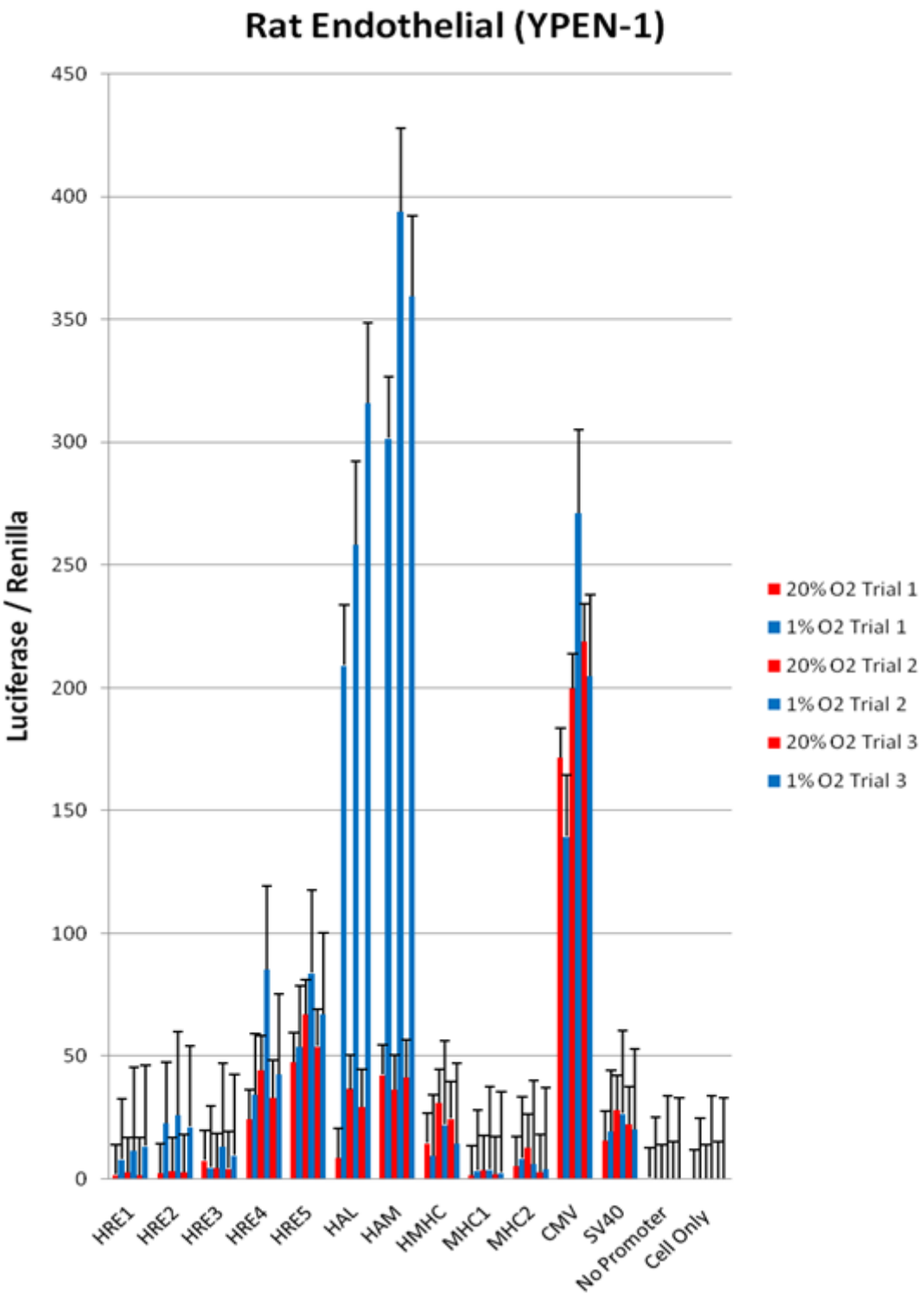

Figure 3-3 Normalized Luciferase expression in rat YPEN-1 endothelial cells under normoxic and hypoxic conditions. HAL and HAM were both significantly up-regulated in this cell line under hypoxic conditions.

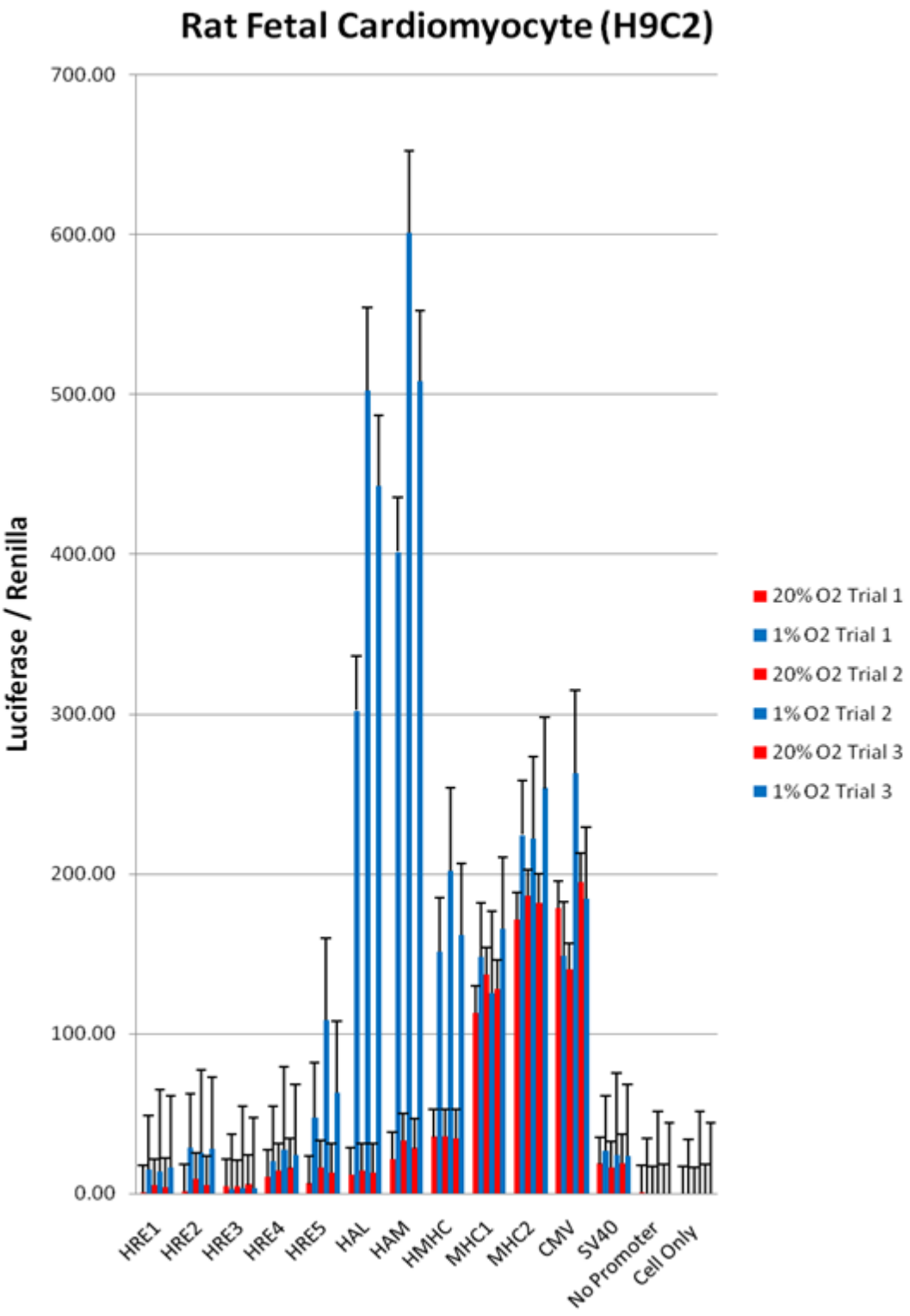

Figure 3-4 Normalized Luciferase expression in rat H9C2 fetal cardiomyocyte cells under normoxic and hypoxic conditions. HAL and HAM were both significantly up-regulated in this cell line under hypoxic conditions.

Indeed, it was more sensible and practical to move directly to the *in vivo* study. (For all *in vivo* procedures, vector constructions were prepared by David R. Wood of Dr. Ding's Lab and the UTMD animal study was conducted by Dr. Grayburn's lab as part of a collaborative effort. Confocal images were prepared and taken by members of both labs and with the assistance of BRI confocal core staff.)

L6 was an important cell line because it is skeletal muscle and shares many commonalities with cardiac tissue. There are high levels of βMHC and very small amounts of αMHC. This is important to allow an assessment of tissue specificity (Diederich et al. 1989). We would expect skeletal muscle αMHC promoter activity to be second only to cardiac tissue; however, expression should be quite low compared to that of cardiac tissue. Expression in all other tissue types should be negligible. In fact, this is exactly what our results indicated in both the *in vitro* and *in vivo* analysis (Table 3-1, 3-2; Figures 3-9, 3-10, 3-11, 3-12). Ypen-1 was used in the *in vitro* assay because endothelial cells are a primary component of the vasculature, including that of the heart. It would be useful to know how this cell type responds to each vector.

Ypen-1 could be a very appealing secondary tissue target in the highly vascularized heart. Additionally, we wanted to compare the *in vitro* assay results to the earlier *in vivo* study. Figure 3-11 reveals a data trend in the YPEN-1 cells very much in agreement with the *in vivo* study. MHC1 and MHC2 showed negligible expression as expected. Expression of the pCI-Luc (CMV) vector was also quite high as expected, ≈13,000 RLU (Appendix A). However, the H9C2 assays did not reveal such a clear cut picture. Expression for MHC1, MHC2, and CMV were all comparable (Figure 3-12). This is completely in line with early expectations (before the *in vivo* study); however, the

CMV expression levels in H9C2 are possibly a contradiction to the qualitative *in vivo* study (Figures 3-1, 3-2, 3-12).

The quantitative and qualitative results are in agreement; however, the quantitative *in vivo* assay is a mixture of cardiomyocytes and the endothelial cells of the vasculature (Table 3-1). So, a direct comparison cannot be made. There are a number of possible explanations for this result. First, fetal and adult cardiomyocytes do not have identical gene expression profiles (Hixon et al. 1989). Additionally, preliminary results of *in vivo* analysis of Luciferase in the myocardium were relatively low and perhaps even to low for confidence in the accuracy of the readings. I strongly suspect that difference in α/β expression profiles is involved in this discrepancy. Although, exactly how is currently unknown. Additionally, UTMD is a new technique and transfecting the tough tissue of the myocardium still needs to be perfected. This may be done by altering liposomal structures and other microbubble components in the future, as well as, modification of the procedure itself such as perfecting the sonic frequency precision and accuracy. Perhaps, the wispy immature fetal cardiomyocytes are simply more easily transfected than the dense fibrous tissue of the adult myocardium. It may be that the fact that the fetal cardiomyocytes are actively dividing and the adult cardiomyocytes are not plays some role. It is also conceivable that the mature myocardium (being in need of a high degree of protection since it cannot regenerate) recognizes the viral heritage of the modified CMV promoter and has mounted a defense against the CMV DNA in vivo, whereas the αMHC DNA may be seen as generally indigenous. Of course there is always the minute possibility of human error as well. These are but a few proposed explanations for this discrepancy.

CREB & AP-1

It is known that transcription factors such as AP-1/CREB, CBP and p300 are involved in gene regulation perpetuated by conditions of hypoxia (Ziello et al. 2007). cAMP Response Element Binding Protein (CREB) binds the cAMP Response Element (CRE) consensus sequence 5'TGACGTCA3' (Carlezon et al. 2005). This is considered by some to be a specific variant of AP-1 and its slightly more general consensus sequence 5'TGA [G/C] TCA3' (Shih et al. 2001). CRE/AP-1 sites are known oxygen based modulators of gene expression. CRE/AP-1 sequences are commonly found within the promoter of oxygen regulated genes such as VEGF and EPO. For example, four potential AP-1 binding sites are known to occur within the VEGF promoter (Komatsu et al. 2004). Co-factors such as these appear to be a vital component in the assembly of a complete and fully functioning HIF complex; although, the complex does not always occur in the exact same configuration from one gene to the next (Ziello et al. 2007; Komatsu et al. 2004; Shih et al. 2001; Tuomisto et al. 2004; Menendez et al. 2005).

Ap-1 is known to interact with many Responsive Elements (REs') within genomic regulatory regions (Rabbi et al. 1997; Bianchi et al. 2002). These include consensus sequence binding sites such as: TRE, CRE, MRE, and ARE. TRE and CRE are perhaps the most frequent targets of AP-1 (Wegner et al. 2005). Specifically, AP-1 has been shown to be a necessary component of the hypoxia induced activation of VEGF and NDRG-1/Cap43 genes (Salinkow et al. 2002). This seems logical given that AP-1 is sensationally sensitive to cellular oxygen concentrations and is observed in numerous regulatory regions of genes influenced by hypoxia (Michiels et al. 2001).

CREB in its dimerized form is an AP1 and has also shown to be a vital component to the activation of certain hypoxia regulated genes. For example, the hypoxic induction of VEGF gene expression has been shown to stop when CREB is blocked; indicating a critical role for CREB (Wu et al. 2007). Taken together, these results along with those of our own lab (presented later in this dissertation) begin to paint a picture of the full scope of HIF regulation. Therefore, I have proposed a hypothetical modified HIF activation complex model for use in the rational design of a gene therapy promoter (Figure 1-7). The crux of this model is as follows: Hif-1α and ARNT complexing with one or more HREs is necessary, but not sufficient to induce proper gene expression in VEGF and other genes regulated by hypoxia (Semenza et al. 2007). An additional consensus sequence such as CRE/AP-1 binding sequence appears to be needed for robust transcription to occur. Additional study is needed to verify the extent to which this model is accurate. Such a model is already indicated, at least in part, within the peer review literature (Gray et al. 2005). Specifically, AP-1 has been shown to interact with HIF-1 during the hypoxic expression of VEGF (Michiels et al. 2001). So, this model may not seem to be any great leap in cognition; however, all known previous attempts to produce hypoxia driven vectors appear to have been designed with the notion that HIF and HRE interact fairly independently in order to initiate transcription: current research indicates otherwise (Phillips et al. 2002; Su et al. 2007; Tang et al. 2002).

Quantitative In Vivo Assessment of α-Myosin Heavy Chain Promoter Vector Designs

As described in the previous section, an α-Myosin Heavy Chain Promoter was selected and used to produce the α-MHCI-Luc (MHC1) vector (Figure 2-10).

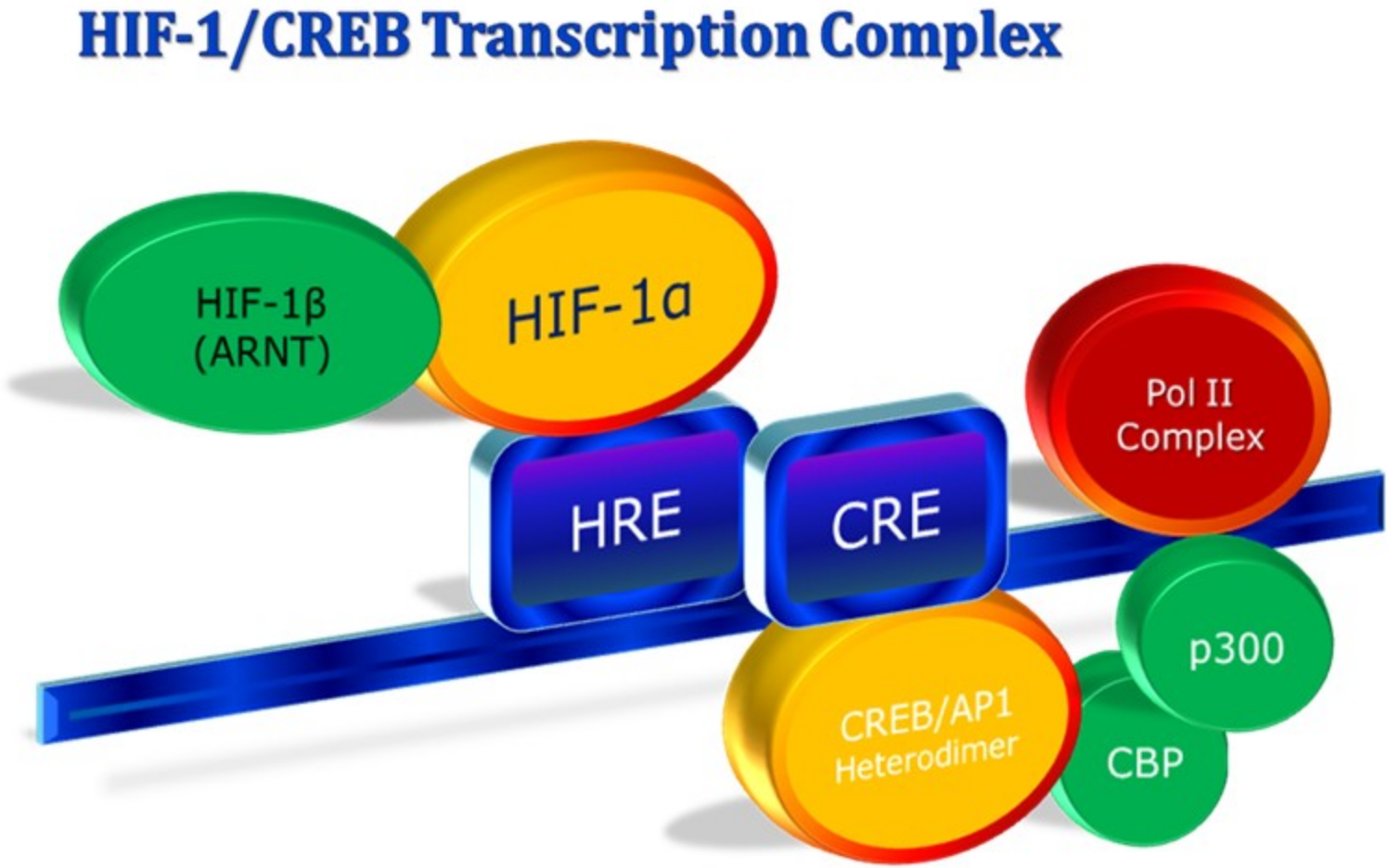

Figure 3-5 Proposed Hif-1/CREB Transcription Complex for HRE/AP1 Vectors.

The pCI-Luc (CMV) vector (Figure 2-10) was also utilized as a positive control. UTMD was used to deliver the vectors to the myocardium as well as, control tissues. A second trial was conducted; this time UTMD was used to specifically target skeletal muscle. This was carried out because, while α-Myosin Heavy Chain is a cardiac specific gene, it is still known to be present in very small amounts in skeletal muscle. (Heine et al. 2005)

As seen in Figure 3-1 and Table 3-1, the positive control vector (pCI-Luc) showed high level Luciferase expression in all areas of the target region including: the atria, anterior ventricle and posterior ventricle. Areas not targeted by UTMD (skeletal muscle, lung, and liver) showed low, base line luciferase expression levels. This is precisely what was expected of the pCI positive control vector. pMHC1-Luc experimental vector was administered in the same fashion targeting the myocardium via UTMD (Table 3-1; Figure 3-1). In a fashion similar to the results of pCI-Luc vector the pMYHC1-Luc vector showed low, base line luciferase expression levels in the regions not targeted by UTMD. Expression levels in the myocardium, while not matching those of pCI-Luc, were significantly higher than base line Luciferase levels. However, unlike pCI-Luc, expression levels were not consistently high throughout the three regions of the heart evaluated (Table 3-1; Figure 3-1). This is not unexpected. The distribution of α-Myosin Heavy Chain is known to be present in different concentrations in various regions of the heart. α-Myosin Heavy Chain is present in all regions of the myocardium in a balanced ratio with β-Myosin Heavy Chain. In other words, as the concentration of one increases the other decreases and vice versa (Molkentin et al. 1996). In fact, by the end of fetal development β-Myosin Heavy Chain dominates the landscape of the myocardium.

However, there is a dramatic shift after birth from a β-Myosin Heavy Chain dominant cardiac ratio to an α-Myosin Heavy Chain dominant ratio, at least in the majority of the myocardium (Hixon et al. 1989).

Next, the entire process was repeated; this time specifically targeting only the skeletal muscle (Right *Quadratus Femoris*) by UTMD. As seen before, the negative control tissues (lung, liver and left *Quadratus Femoris*) all displayed low, baseline Luciferase expression for both pCI-Luc and pMHC1-Luc as expected (Figure 3-2). However, Luciferase expression levels produced by pCI-Luc found in the target tissue (Right *Quadratus Femoris*) were significantly higher. Expression levels yielded by pMHC1-Luc, while higher than the negative control tissues, was still nominal (Table 3-2; Figure 3-2).

These results indicate the following: UTMD is able to effectively target, deliver and transfect tissues with DNA plasmid to specific regions of the body. MHC1 is active in the myocardium, expressed in nominal amounts in skeletal muscle, and is undetectable in other tissues observed.

Quantitative In Vivo Analysis of the pCI (CMV) and MYH6 (MHC) Promoter

In order to establish a more intricate understanding of the expression of pMHCI within the myocardium *in vivo* analysis was conducted. Positive and Negative control vectors, as well as, pMHC1 retained the original promoter and vector backbone used in the quantitative *in vivo* study. Luciferase was replaced by the DsRed reporter gene to allow for a visual interpretation of our data. DsRed was specifically chosen over other fluorescent proteins because of certain properties that make its use preferable. Cardiac tissue is notoriously difficult to analyze via immunohistology due to high background

levels, particularly in the yellow/green spectrum. For this reason, the classic choice of Fluorescein Isothiocyanate (FITC) was abandoned in favor of DsRed in the hopes that imaging would be clearer. DsRed (Molecular Probes) is brighter and longer lasting than other products available within the same approximate spectrum such as Rhodamine or Texas Red. Results were favorable; we found that with the confocal imaging system background could be greatly negated and clearly distinguished from the actual DsRed protein. In fact, we would later test and use FITC with little difficulty and produce some of our most impressive and enlightening images to date. Representative photos have been selected to illustrate the progress made at each stage of the *in vivo* qualitative analysis (Figures 3-3, 3-4, 3-5, 3-6, 3-7, and 3-8).

One of our earlier attempts is exemplified by Figure 3-3, which shows a transverse section of myocardium. The fluorescent signature from the presence of pMHCI-DsRed is clearly visible in the top two images. The bottom two images are negative controls: bottom right (No vector/No transfection), bottom left (pGL3 basic/Promoterless). As you can see the background luminescence is clearly identified when compared to the negative control slides.

Once it was confirmed that indeed our plasmid had been effectively delivered to the myocardium and the MHCI promoter was actively driving the DsRed reporter gene; we wanted to determine exactly where this expression was occurring. Of course, the myocardium is made up of many cells types in addition to cardiomyocytes. These include: endothelial cells, fibroblasts, adipocytes, smooth muscle, immune cells, stem cells etc. We wanted to visually confirm that our plasmid was active in cardiomyocytes and not a secondary cell type. We suspected the major secondary cell type would be the

endothelial cells of the myocardial vasculature. During this round of testing we would use pCI-DsRed as a positive control vector in addition to the pGL3 basic negative control vector. Immunohistology was used to tag CD31 (a marker for endothelial cells) with FITC. This allowed us to more clearly identify the vasculature of the myocardium slide preparations (Figure 3-4).

Our expectation was that pCI-DsRed would be expressed in all cell types, being that the promoter is CMV derived. However, expression appeared to be limited to endothelial cells having little to no expression in cardiomyocytes. Therefore, we wished to establish if pCI-DsRed was present, but inactive in cardiomyocytes or if it was simply not present at all. *In situ* PCR of the DNA plasmid was conducted to locate the precise location of both pCI-DsRed and MHCI-DsRed (Figure 3-5). Once slides were analyzed via confocal microscopy, there was a starkly apparent difference in the location of the pMHCI-DsRed. Currently, we have little evidence to support any explanation for this phenomenon, although it has been confirmed in subsequent trials. There is little information regarding this matter in the peer reviewed literature. This is most likely due to the notoriously difficult nature of transfecting cardiomyocytes *in vivo*. Most successes have been via complex Adeno-Associated Viral (AAV) constructs. Thus far, only a scant few mentioning's of observed CMV promoter masking in cardiomyocytes can be found in the peer-reviewed literature (Bauer et al. 2005). Next, we wanted to examine the actual expressed mRNA of the plasmid. To achieve this, we performed *in situ* RT-PCR (Figure 3-6). The results appeared to be very consistent to that of the plasmid DNA based *in situ* PCR. Subsequent trials revealed the same results. Confocal immunohistology continued in an attempt to verify findings and to produce as clear and

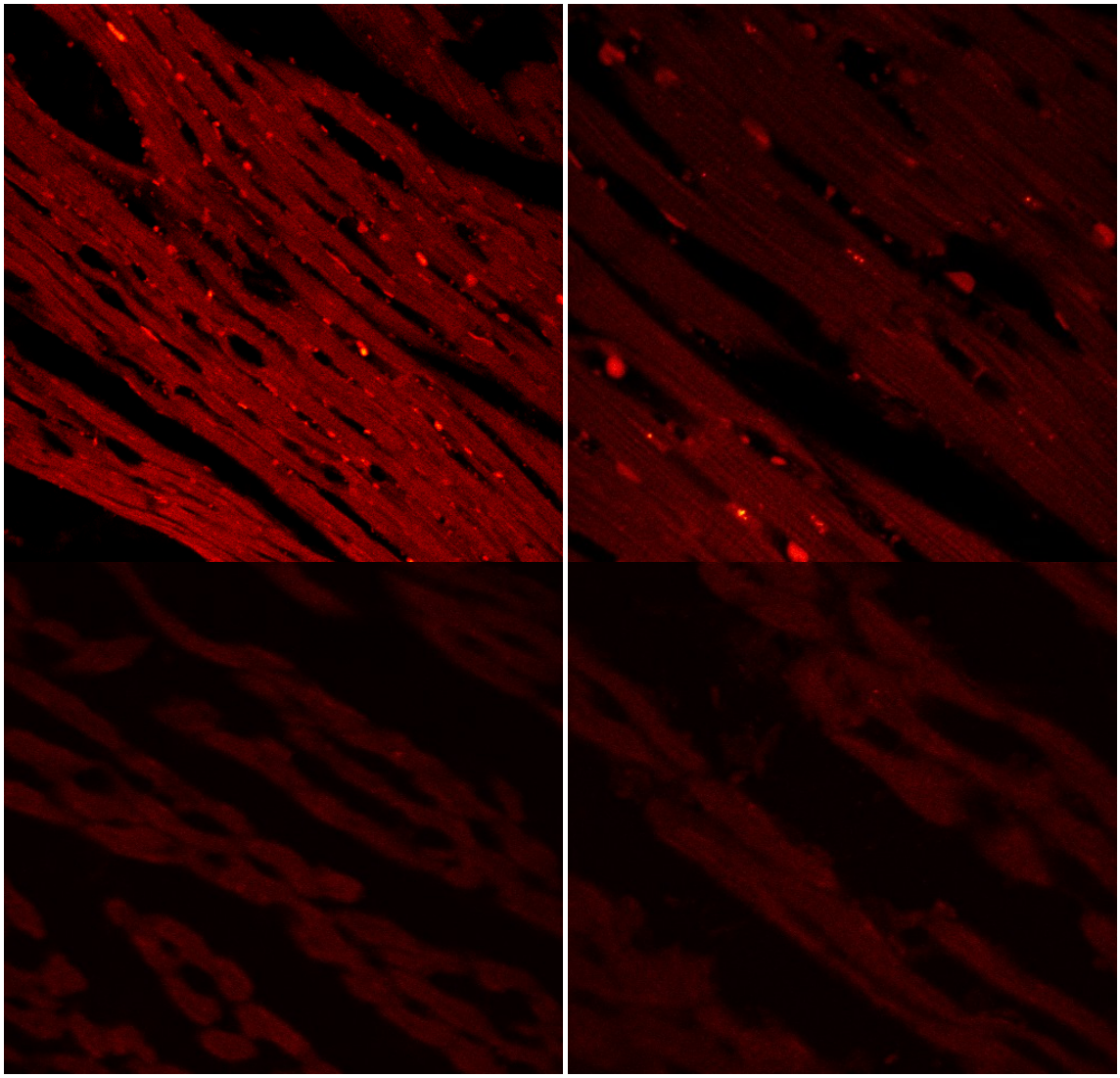

Figure 3-6 pMHCI-DsRed fluorescent protein expressed in rat myocardium via UTMD. Confocal DsRed Signal: 560nm/590nm. (Top/Left) 400X. (Top/Right) 1000X; (Lower/Left) pGL3 basic, no promoter. (Negative Control) No Expression; (Lower/Right) (Normal Control, No Transfection) No Expression.

reliable an image as possible (Figure 3-7, 3-8). Final images show the location of the pCI or MHCI DsRed vector as well as markers for endothelial cells (CD31) and cardiomyocytes (MF20 or Cardiac Actin). Our qualitative findings, specifically regarding pMHCI-DsRed, are probably best summarized in (Figure 3-8). This overlay gives a clear picture of the location and activity of pMHCI-DsRed.

CHAPTER FOUR

Conclusions

Our objectives for this project were as follows: To design and construct tissue specific gene therapy vectors and to demonstrate effective delivery and expression of these vectors. The second objective of this project was to produce gene therapy vectors that would be significantly up-regulated only under unique physiological conditions. Specifically, we wished to produce vectors that would be regulated by cellular oxygen levels. As noted earlier, the significance of such constructs is that it imparts a greater degree of control in the implementation of gene therapy. The final phase of the project was intended to bring the previous two phases together in an attempt to create a single vector that would meet all of the above criteria. To meet our design goals, the gene therapy vector should be both cardiac tissue specific and regulated by hypoxia.

All project goals were achieved to varying degrees. Specifically, MHC1, MHC2, and HMHC vectors all demonstrated preferential expression in the H9C2 cell line. Luciferase expression levels were nominal in RAT2 and YPEN-1 cell lines. Expression in L6 cells was present, but greatly reduced as compared with H9C2. (Figure 3-9, 3-10, 3-11, and 3-12) Additionally, MHC1 was found to be active in significant amounts only in cardiomyocytes during the preliminary collaborative *in vivo* study. The hypoxia study revealed significantly up-regulated Luciferase expression by HAL, HAM, and HMHC in low oxygen conditions of certain cell types (Figures 3-9, 3-10, 3-11, 3-12). HAL and HAM were not designed with tissue specificity in mind, but rather the intent of their

Table 4-1 Final Results of Hypothesis Tests Based on One Way ANOVA

Vector	Null and Alternative Hypothesis	Human Fibroblast	Rat2	L6	H9C2	YPEN-1
HRE1	H0: $\mu1 = \mu2 = \mu3$	Reject	Reject	Reject	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Accept	Accept	Accept	Reject
HRE2	H0: $\mu1 = \mu2 = \mu3$	Reject	Reject	Reject	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Accept	Accept	Accept	Reject
HRE3	H0: $\mu1 = \mu2 = \mu3$	Accept	Accept	Accept	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject	Reject	Reject	Accept	Reject
HRE4	H0: $\mu1 = \mu2 = \mu3$	Accept	Reject	Reject	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject	Accept	Accept	Accept	Reject
HRE5	H0: $\mu1 = \mu2 = \mu3$	Reject	Reject	Reject	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Accept	Accept	Accept	Reject
HAL	H0: $\mu1 = \mu2 = \mu3$	Reject	Reject	Reject	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Accept	Accept	Accept	Reject
HAM	H0: $\mu1 = \mu2 = \mu3$	Accept	Accept	Accept	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject	Reject	Reject	Accept	Reject
HMHC	H0: $\mu1 = \mu2 = \mu3$	Reject	Reject	Reject	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Accept	Accept	Accept	Reject
MHC1	H0: $\mu1 = \mu2 = \mu3$	Reject	Reject	Reject	Accept	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Accept	Accept	Reject	Reject
MHC2	H0: $\mu1 = \mu2 = \mu3$	Reject	Accept	Accept	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Reject	Reject	Accept	Reject
CMV	H0: $\mu1 = \mu2 = \mu3$	Accept	Accept	Accept	Accept	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject	Reject	Reject	Reject	Reject
SV40	H0: $\mu1 = \mu2 = \mu3$	Reject	Accept	Accept	Reject	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept	Reject	Reject	Accept	Reject
No Promoter	H0: $\mu1 = \mu2 = \mu3$	Accept	Accept	Accept	Accept	Reject
	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject	Reject	Reject	Reject	Accept
Cell Only	H0: $\mu1 = \mu2 = \mu3$	Accept	Accept	Accept	Accept	Accept
	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject	Reject	Reject	Reject	Reject

design was to focus on up- regulation of gene expression under hypoxia. HMHC was an attempt to produce a vector that would be both cardiac tissue specific and up-regulated under hypoxic conditions. HAL was found to be significantly up-regulated under hypoxic conditions in Rat2, L6, YPEN-1, H9C2 and human fibroblast cell lines. HAM was found to be significantly up-regulated under hypoxic conditions in L6, YPEN-1and H9C2 cell lines. HMHC was found to be significantly up-regulated under hypoxic conditions in L6 and H9C2 cell lines (Figures 3-9, 3-10, 3-11, 3-12). Statistical significance results are listed in Appendix A and results are summarized in table 4-1

APPENDICES

APPENDIX A1

Human Fibroblast Statistical Data

Human Fibroblast	R/C	A	B	C		
HRE1	20% O2	1.48	1.60	1.63	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	14.43	11.62	16.59	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE2	20% O2	1.89	1.92	1.92	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	25.37	42.20	21.94	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE3	20% O2	3.01	10.70	5.06	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	5.97	10.90	16.13	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE4	20% O2	9.16	23.00	15.79	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	12.17	27.30	18.72	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE5	20% O2	2.86	9.56	13.62	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	17.4	39.90	21.82	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAL	20% O2	6.92	11.90	19.77	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	843.75	750.60	619.48	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAM	20% O2	2.8	7.70	14.36	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	3.23	33.47	43.47	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HMHC	20% O2	1.45	2.80	4.81	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	7.04	8.79	9.65	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC1	20% O2	0.44	1.50	1.55	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	2.88	4.60	2.38	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC2	20% O2	0.87	1.70	1.84	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	3.04	5.40	3.13	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
CMV	20% O2	222.16	182.20	199.02	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	188.8	298.20	279.93	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
SV40	20% O2	42.3	29.90	27.12	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	21.7	23.72	19.51	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
	20% O2	0	0.00	0	H0: $\mu1 = \mu2 = \mu3$	Accept
No Promoter	1% O2	0.17	0.09	0	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject

Anova: Single Factor — HRE1 — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	4.71	1.57	0.0063
1% O2	3	42.64	14.213333	6.2104333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	239.78081	1	239.78081	77.140454	0.00093	4.544770
Within Groups	12.433466	4	3.1083666			
Total	252.21428	5				

Anova: Single Factor — HRE2 — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.73	1.91	0.0003
1% O2	3	89.51	29.836666	117.58023

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1169.8480	1	1169.8480	19.898669	0.01115	4.544770
Within Groups	235.16106	4	58.790266			
Total	1405.0091	5				

Anova: Single Factor — HRE3 — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	18.77	6.2566666	15.858033
1% O2	3	33	11	25.8139

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	33.748816	1	33.748816	1.6197384	0.27207	4.544770
Within Groups	83.343866	4	20.835966			
Total	117.09268	5				

Anova: Single Factor — HRE4 — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	47.95	15.983333	47.914433
1% O2	3	58.19	19.396666	57.572633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.476266	1	17.476266	0.3313442	0.5957	4.544770
Within Groups	210.97413	4	52.743533			
Total	228.4504	5				

Anova: Single Factor — HRE5 — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	26.04	8.68	29.5252
1% O2	3	79.12	26.373333	142.11213

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	469.58106	1	469.58106	5.4717823	0.07945	4.544770
Within Groups	343.27466	4	85.818666			
Total	812.85573	5				

Anova: Single Factor — HAL — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	38.59	12.863333	41.976633
1% O2	3	2213.83	737.94333	12694.401

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	788611.50	1	788611.50	123.83606	0.00037	4.544770
Within Groups	25472.756	4	6368.1891			
Total	814084.26	5				

Anova: Single Factor — HAM — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	24.86	8.2866666	33.666533
1% O2	3	80.17	26.723333	438.95253

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	509.86601	1	509.86601	2.1576193	0.21579	4.544770
Within Groups	945.23813	4	236.30953			
Total	1455.1041	5				

Anova: Single Factor — HMHC — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	9.06	3.02	2.8587
1% O2	3	25.48	8.4933333	1.7690333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	44.936066	1	44.936066	19.420335	0.01163	4.544770
Within Groups	9.2554666	4	2.3138666			
Total	54.191533	5				

Anova: Single Factor — MHC1 — 20% O2 Vs. 1% O2 — α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	3.49	1.1633333	0.3930333
1% O2	3	9.86	3.2866666	1.3561333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.7628166	1	6.7628166	7.7326155	0.04978	4.544770
Within Groups	3.4983333	4	0.8745833			
Total	10.26115	5				

Anova: Single Factor | MHC2 | 20% O2 Vs. 1% O2 | α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	4.41	1.47	0.2749
1% O2	3	11.57	3.8566666	1.7884333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.5442666	1	8.5442666	8.2820032	0.04511	4.544770
Within Groups	4.1266666	4	1.0316666			
Total	12.670933	5				

Anova: Single Factor | CMV | 20% O2 Vs. 1% O2 | α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	603.38	201.12666	402.52893
1% O2	3	766.93	255.64333	3434.4716

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4458.1004	1	4458.1004	2.3237423	0.20209	4.544770
Within Groups	7674.0011	4	1918.5002			
Total	12132.101	5				

Anova: Single Factor | SV40 | 20% O2 Vs. 1% O2 | α = 0.1

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	99.32	33.106666	65.320133
1% O2	3	64.93	21.643333	4.4334333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	197.11201	1	197.11201	5.6516684	0.07621	4.544770
Within Groups	139.50713	4	34.876783			
Total	336.61915	5				

Anova: Single Factor	No Promoter	20% O2 Vs. 1% O2	α = 0.1	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	0	0	0
1% O2	3	0.26	0.0866666	0.0072333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0112666	1	0.0112666	3.1152073	0.15233	4.544770
Within Groups	0.0144666	4	0.0036166			
Total	0.0257333	5				

APPENDIX A2

Rat Fibroblast Statistical Data

Rat Fibroblast	R/C	A	B	C		
HRE1	20% O2	1.76	2.20	1.61	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	13.52	17.72	15.64	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE2	20% O2	2.96	2.23	3.68	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	38.95	47.54	49.10	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE3	20% O2	6.04	5.94	21.87	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	25.73	31.30	22.90	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE4	20% O2	12.74	12.97	14.88	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	83.04	90.66	84.80	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE5	20% O2	6.55	9.01	12.79	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	79.8	88.72	60.20	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAL	20% O2	5.60	11.28	8.67	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	1219.15	2084.76	1097.70	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAM	20% O2	2.97	9.24	4.98	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	40.97	8.02	37.00	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HMHC	20% O2	1.91	1.04	2.1	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	8.54	6.27	5.53	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC1	20% O2	0.77	0.42	1.01	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	2.06	1.86	1.60	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC2	20% O2	1.71	2.36	1.47	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	2.77	2.27	3.60	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
CMV	20% O2	124.95	139.70	136.63	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	136.1	140.68	131.50	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
SV40	20% O2	29.90	31.70	17.67	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	21.51	22.04	22.10	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
	20% O2	0.00	0.00	0	H0: $\mu1 = \mu2 = \mu3$	Accept
No Promoter	1% O2	0.02	0.07	0.00	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject

Anova: Single Factor | HRE1 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.57	1.8566666	0.0940333
1% O2	3	46.88	15.626666	4.4101333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	284.41935	1	284.41935	126.29166	0.00036	7.708647
Within Groups	9.0083333	4	2.2520833			
Total	293.42768	5				

Anova: Single Factor | HRE2 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	8.87	2.9566666	0.5256333
1% O2	3	135.59	45.196666	29.874033

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2676.3264	1	2676.3264	176.07603	0.00019	7.708647
Within Groups	60.799333	4	15.199833			
Total	2737.1257	5				

Anova: Single Factor | HRE3 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	33.85	11.283333	84.060633
1% O2	3	79.93	26.643333	18.265633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	353.8944	1	353.8944	6.9169805	0.05819	7.708647
Within Groups	204.65253	4	51.163133			
Total	558.54693	5				

Anova: Single Factor | HRE4 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	40.59	13.53	1.3801
1% O2	3	258.5	86.166666	15.916933

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7914.1280	1	7914.1280	915.08501	7.1E-06	7.708647
Within Groups	34.594066	4	8.6485166			
Total	7948.7220	5				

Anova: Single Factor | HRE5 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	28.35	9.45	9.8796
1% O2	3	228.72	76.24	212.8528

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6691.3561	1	6691.3561	60.084263	0.00149	7.708647
Within Groups	445.4648	4	111.3662			
Total	7136.8209	5				

Anova: Single | HAL | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	25.55	8.5166666	8.0832333
1% O2	3	4401.61	1467.2033	289719.70

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3191650.1	1	3191650.1	22.032061	0.00935	7.708647
Within Groups	579455.57	4	144863.89			
Total	3771105.7	5				

Anova: Single Factor | HAM | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	17.19	5.73	10.2501
1% O2	3	85.99	28.663333	323.55063

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	788.90666	1	788.90666	4.7268120	0.09537	7.708647
Within Groups	667.60146	4	166.90036			
Total	1456.5081	5				

Anova: Single Factor | HMHC | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.05	1.6833333	0.3194333
1% O2	3	20.34	6.78	2.4601

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	38.964016	1	38.964016	28.036373	0.00611	7.708647
Within Groups	5.5590666	4	1.3897666			
Total	44.523083	5				

Anova: Single Factor | MHC1 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	2.2	0.7333333	0.0880333
1% O2	3	5.52	1.84	0.0532

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.8370666	1	1.8370666	26.014633	0.00698	7.708647
Within Groups	0.2824666	4	0.0706166			
Total	2.1195333	5				

Anova: Single Factor | MHC2 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.54	1.8466666	0.2120333
1% O2	3	8.64	2.88	0.4513

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.6016666	1	1.6016666	4.8291457	0.09291	7.708647
Within Groups	1.3266666	4	0.3316666			
Total	2.9283333	5				

Anova: Single Factor | CMV | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	401.28	133.76	60.5683
1% O2	3	408.28	136.09333	21.068133

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.1666666	1	8.1666666	0.2000740	0.67781	7.708647
Within Groups	163.27286	4	40.818216			
Total	171.43953	5				

Anova: Single Factor | SV40 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	79.27	26.423333	58.275633
1% O2	3	65.65	21.883333	0.1054333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30.9174	1	30.9174	1.0591584	0.36157	7.708647
Within Groups	116.76213	4	29.190533			
Total	147.67953	5				

Anova: Single Factor	No Promoter	20% O2 Vs. 1% O2	α = 0.05	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	0	0	0
1% O2	3	0.09	0.03	0.0013

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00135	1	0.00135	2.0769230	0.22298	7.708647
Within Groups	0.0026	4	0.00065			
Total	0.00395	5				

APPENDIX A3

Rat Skeletal Statistical Data

Rat Skeletal	R/C	A	B	C		
HRE1	20% O2	1.76	2.20	1.61	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	13.52	17.72	15.64	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE2	20% O2	2.96	2.23	3.68	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	38.95	47.54	49.10	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE3	20% O2	6.04	5.94	21.87	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	25.73	31.30	22.90	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE4	20% O2	12.74	12.97	14.88	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	83.04	90.66	84.80	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE5	20% O2	6.55	9.01	12.79	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	79.8	88.72	60.20	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAL	20% O2	5.60	11.28	8.67	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	1219.15	2084.76	1097.70	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAM	20% O2	2.97	9.24	4.98	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	40.97	8.02	37.00	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HMHC	20% O2	1.91	1.04	2.1	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	8.54	6.27	5.53	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC1	20% O2	0.77	0.42	1.01	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	2.06	1.86	1.60	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC2	20% O2	1.71	2.36	1.47	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	2.77	2.27	3.60	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
CMV	20% O2	124.95	139.70	136.63	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	136.1	140.68	131.50	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
SV40	20% O2	29.90	31.70	17.67	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	21.51	22.04	22.10	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
	20% O2	0.00	0.00	0	H0: $\mu1 = \mu2 = \mu3$	Accept
No Promoter	1% O2	0.02	0.07	0.00	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject

Anova: Single Factor | HRE1 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.57	1.8566666	0.0940333
1% O2	3	46.88	15.626666	4.4101333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	284.41935	1	284.41935	126.29166	0.00036	7.708647
Within Groups	9.0083333	4	2.2520833			
Total	293.42768	5				

Anova: Single Factor | HRE2 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	8.87	2.9566666	0.5256333
1% O2	3	135.59	45.196666	29.874033

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2676.3264	1	2676.3264	176.07603	0.00019	7.708647
Within Groups	60.799333	4	15.199833			
Total	2737.1257	5				

Anova: Single Factor | HRE3 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	33.85	11.283333	84.060633
1% O2	3	79.93	26.643333	18.265633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	353.8944	1	353.8944	6.9169805	0.05819	7.708647
Within Groups	204.65253	4	51.163133			
Total	558.54693	5				

Anova: Single Factor | HRE4 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	40.59	13.53	1.3801
1% O2	3	258.5	86.166666	15.916933

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7914.1280	1	7914.1280	915.08501	7.1E-06	7.708647
Within Groups	34.594066	4	8.6485166			
Total	7948.7220	5				

Anova: Single Factor | HRE5 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	28.35	9.45	9.8796
1% O2	3	228.72	76.24	212.8528

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6691.3561	1	6691.3561	60.084263	0.00149	7.708647
Within Groups	445.4648	4	111.3662			
Total	7136.8209	5				

Anova: Single Factor | HAL | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	25.55	8.5166666	8.0832333
1% O2	3	4401.61	1467.2033	289719.70

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3191650.1	1	3191650.1	22.032061	0.00935	7.708647
Within Groups	579455.57	4	144863.89			
Total	3771105.7	5				

Anova: Single Factor — HAM — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	17.19	5.73	10.2501
1% O2	3	85.99	28.663333	323.55063

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	788.90666	1	788.90666	4.7268120	0.09537	7.708647
Within Groups	667.60146	4	166.90036			
Total	1456.5081	5				

Anova: Single Factor — HMHC — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.05	1.6833333	0.3194333
1% O2	3	20.34	6.78	2.4601

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	38.964016	1	38.964016	28.036373	0.00611	7.708647
Within Groups	5.5590666	4	1.3897666			
Total	44.523083	5				

Anova: Single — MHC1 — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	2.2	0.7333333	0.0880333
1% O2	3	5.52	1.84	0.0532

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.8370666	1	1.8370666	26.014633	0.00698	7.708647
Within Groups	0.2824666	4	0.0706166			
Total	2.1195333	5				

Anova: Single Factor — MHC2 — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	5.54	1.8466666	0.2120333
1% O2	3	8.64	2.88	0.4513

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.6016666	1	1.6016666	4.8291457	0.09291	7.708647
Within Groups	1.3266666	4	0.3316666			
Total	2.9283333	5				

Anova: Single Factor — CMV — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	401.28	133.76	60.5683
1% O2	3	408.28	136.09333	21.068133

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.166666	1	8.1666666	0.2000740	0.67781	7.708647
Within Groups	163.27286	4	40.818216			
Total	171.43953	5				

Anova: Single Factor — SV40 — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	79.27	26.423333	58.275633
1% O2	3	65.65	21.883333	0.1054333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30.9174	1	30.9174	1.0591584	0.36157	7.708647
Within Groups	116.76213	4	29.190533			
Total	147.67953	5				

Anova: Single Factor	No Promoter	20% O2 Vs. 1% O2	α = 0.05	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	0	0	0
1% O2	3	0.09	0.03	0.0013

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00135	1	0.00135	2.0769230	0.22298	7.708647
Within Groups	0.0026	4	0.00065			
Total	0.00395	5				

APPENDIX A4

Rat Fetal Cardiomyocyte Statistical Data

Rat Fetal H9C2	R/C	A	B	C		
HRE1	20% O2	0.56	3.97	5.30	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	13.76	16.36	15.12	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE2	20% O2	1.47	5.11	9.20	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	25.70	28.12	28.70	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE3	20% O2	4.70	5.62	4.50	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	3.36	3.27	2.97	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE4	20% O2	10.40	16.05	14.50	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	27.70	23.97	20.50	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HRE5	20% O2	6.20	13.04	16.50	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	108.30	63.17	47.70	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAL	20% O2	11.60	13.13	14.70	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	502.74	442.63	302.30	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HAM	20% O2	21.50	28.39	33.40	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	601.23	508.13	401.70	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
HMHC	20% O2	35.50	34.61	35.90	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	202.30	161.84	151.40	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
MHC1	20% O2	112.90	127.93	137.30	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	125.20	165.75	148.10	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
MHC2	20% O2	171.50	181.94	186.30	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	222.20	253.6	224.50	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
CMV	20% O2	178.60	194.78	140.20	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	263.30	184.54	148.90	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
SV40	20% O2	18.50	19.14	16.34	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	24.30	23.59	27.00	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept
No Promoter	20% O2	0.78	0	0.22	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	0.12	0	0.31	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject

Anova: Single Factor HRE1 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	9.83	3.276666667	5.97743333
1% O2	3	45.24	15.08	1.6912

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	208.9780167	1	208.9780167	54.5020234	0.00179	7.7086474
Within Groups	15.33726667	4	3.834316667			
Total	224.3152833	5				

Anova: Single Factor HRE2 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	15.78	5.26	14.9551
1% O2	3	82.52	27.50666667	2.53213333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	742.37126	1	742.371266	84.9043702	0.00077	7.7086474
Within Groups	34.974466	4	8.743616667			
Total	777.34573	5				

Anova: Single Factor HRE3 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	14.82	4.94	0.3568
1% O2	3	9.6	3.2	0.0417

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.5414	1	4.5414	22.7924718	0.00881	7.70864
Within Groups	0.797	4	0.19925			
Total	5.3384	5				

Anova: Single Factor HRE4 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	40.95	13.65	8.5225
1% O2	3	72.17	24.05666667	12.9656333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	162.4480667	1	162.4480667	15.1197933	0.01771	7.7086
Within Groups	42.97626667	4	10.74406667			
Total	205.4243333	5				

Anova: Single Factor HRE5 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	35.74	11.91333333	27.4745333
1% O2	3	219.17	73.0566666	991.399633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5607.760817	1	5607.760817	11.0077593	0.02944	7.70864
Within Groups	2037.748333	4	509.4370833			
Total	7645.5091	5				

Anova: Single Factor HAL 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	39.43	13.1433333	2.40263333
1% O2	3	1247.67	415.89	10580.3191

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	243307.3163	1	243307.3163	45.9819926	0.00247	7.70864
Within Groups	21165.44347	4	5291.360867			
Total	264472.7597	5				

Anova: Single Factor HAM 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	83.29	27.76333333	35.6970333
1% O2	3	1511.06	503.6866667	9967.86263

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	339754.5288	1	339754.5288	67.9267261	0.00118	7.70864
Within Groups	20007.11933	4	5001.779833			
Total	359761.6482	5				

Anova: Single Factor HMHC 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	106.01	35.33666667	0.43603333
1% O2	3	515.54	171.8466667	722.802533

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27952.47015	1	27952.47015	77.298063	0.00092	7.70864
Within Groups	1446.477133	4	361.6192833			
Total	29398.94728	5				

Anova: Single Factor MHC1 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	378.13	126.0433333	151.509633
1% O2	3	439.05	146.35	413.3725

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	618.5410667	1	618.5410667	2.18998276	0.21301	7.70864
Within Groups	1129.764267	4	282.4410667			
Total	1748.305333	5				

Anova: Single Factor MHC2 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	539.74	179.9133333	57.8405333
1% O2	3	700.3	233.4333333	306.343333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4296.5856	1	4296.5856	23.5956943	0.00829	7.70864
Within Groups	728.3677333	4	182.0919333			
Total	5024.953333	5				

Anova: Single Factor CMV 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	513.58	171.1933333	785.888133
1% O2	3	596.74	198.9133333	3426.78453

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1152.5976	1	1152.5976	0.54720492	0.50052	7.70864
Within Groups	8425.345333	4	2106.336333			
Total	9577.942933	5				

Anova: Single Factor SV40 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	53.98	17.99333333	2.15253333
1% O2	3	74.89	24.96333333	3.23703333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72.87135	1	72.87135	27.041636	0.00652	7.70864
Within Groups	10.77913333	4	2.694783333			
Total	83.65048333	5				

Anova: Single Factor — No Promoter — 20% O2 Vs. 1% O2 — α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	1	0.333333333	0.16173333
1% O2	3	0.43	0.143333333	0.02443333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.05415	1	0.05415	0.58173679	0.48813	7.70864 7421
Within Groups	0.372333333	4	0.093083333			
Total	0.426483333	5				

APPENDIX A5

Endothelial Cell Statistical Data

Rat Endothelial	R/C	A	B	C		
HRE1	20% O2	1.76	8	2.85	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	11.55	1.68	13.37	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE2	20% O2	2.35	22.6	2.98	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	26.16	2.91	21.33	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE3	20% O2	7.64	4.7	4.41	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	13.20	4.31	9.61	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE4	20% O2	24.3	34.2	44.24	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	85.31	33.15	42.67	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HRE5	20% O2	47.5	53.7	67.12	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	83.93	53.99	67.23	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HAL	20% O2	8.59	209.1	36.52	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	258.39	29.36	315.96	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HAM	20% O2	42.5	301.7	36.40	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	393.98	41.33	359.49	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
HMHC	20% O2	14.6	9.3	30.90	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	22.18	24.66	14.43	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
MHC1	20% O2	1.53	3.4	3.61	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	3.89	2.09	2.58	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
MHC2	20% O2	5.34	8.44	12.58	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	6.14	2.71	4.17	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
CMV	20% O2	171.6	139.5	199.83	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	271.27	218.99	204.92	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
SV40	20% O2	15.75	19.4	28.12	H0: $\mu1 = \mu2 = \mu3$	Accept
	1% O2	26.68	22.19	20.28	HA: $\mu1 \neq \mu2 \neq \mu3$	Reject
No Promoter	20% O2	0.59	0.38	0.21	H0: $\mu1 = \mu2 = \mu3$	Reject
	1% O2	0.03	0	0	HA: $\mu1 \neq \mu2 \neq \mu3$	Accept

Anova: Single Factor HRE1 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	12.61	4.203333333	11.108033
1% O2	3	26.6	8.866666667	39.564233

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	32.620016	1	32.62001667	1.2874899	0.3199	7.7086
Within Groups	101.34453	4	25.33613333			
Total	133.96455	5				

Anova: Single Factor HRE2 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	27.93	9.31	132.5673
1% O2	3	50.4	16.8	150.5313

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	84.15015	1	84.15015	0.5944935	0.4837	7.7086
Within Groups	566.1972	4	141.5493			
Total	650.34735	5				

Anova: Single Factor HRE3 20% O2 Vs. 1% O2 α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	16.75	5.583333333	3.19343333
1% O2	3	27.12	9.04	20.0017

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.922816	1	17.92281667	1.5453945	0.2817	7.7086
Within Groups	46.390266	4	11.59756667			
Total	64.313083	5				

Anova: Single Factor	HRE4	20% O2 Vs. 1% O2	α = 0.05	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	102.74	34.24666667	99.402533 3
1% O2	3	161.13	53.71	771.5776

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	568.23201	1	568.2320167	1.3048105	0.31707	7.7086
Within Groups	1741.9602	4	435.4900667			
Total	2310.1922	5				

HRE5		20% O2 Vs. 1% O2	α = 0.05	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	168.32	56.10666667	100.58013 3
1% O2	3	205.15	68.38333333	225.09853 3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	226.07481	1	226.0748167	1.3883305	0.304	7.7086
Within Groups	651.35733	4	162.8393333			
Total	877.43215	5				

Anova: Single	HAL	20% O2 Vs. 1% O2	α = 0.05	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	254.21	84.73666667	11794.700
1% O2	3	603.71	201.2366667	22984.767

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20358.375	1	20358.375	1.1707122	0.34012	7.7086
Within Groups	69558.935	4	17389.73393			
Total	89917.310	5				

Anova: Single Factor | HAM | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	380.6	126.8666667	22934.323
1% O2	3	794.8	264.9333333	37796.228

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	28593.606	1	28593.60667	0.9416547	0.3868	7.7086
Within Groups	121461.10	4	30365.27568			
Total	150054.70	5				

Anova: Single Factor | HMHC | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	54.8	18.26666667	126.72333
1% O2	3	61.27	20.42333333	28.477633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.9768166	1	6.976816667	0.0899068	0.77923	7.7086
Within Groups	310.40193	4	77.60048333			
Total	317.37875	5				

Anova: Single Factor | MHC1 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	8.54	2.846666667	1.3112333
1% O2	3	8.56	2.853333333	0.8660333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.667E-05	1	6.66667E-05	6.123E-05	0.99413	7.7086
Within Groups	4.3545333	4	1.088633333			
Total	4.3546	5				

Anova: Single Factor | MHC2 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	26.36	8.786666667	13.194533
1% O2	3	13.02	4.34	2.9629

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29.659266	1	29.65926667	3.6712844	0.12785	7.7086
Within Groups	32.314866	4	8.078716667			
Total	61.974133	5				

Anova: Single Factor | CMV | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	510.93	170.31	911.1753
1% O2	3	695.18	231.7266667	1222.2476

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5658.0104	1	5658.010417	5.3041619	0.08266	7.7086
Within Groups	4266.8458	4	1066.711467			
Total	9924.8562	5				

Anova: Single Factor | SV40 | 20% O2 Vs. 1% O2 | α = 0.05

Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	63.27	21.09	40.3963
1% O2	3	69.15	23.05	10.7947

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.7624	1	5.7624	0.2251333	0.6599	7.7086
Within Groups	102.382	4	25.5955			
Total	108.1444	5				

Anova: Single Factor	No Promoter	20% O2 Vs. 1% O2	α = 0.05	
Groups	*Count*	*Sum*	*Average*	*Variance*
20% O2	3	1.18	0.393333333	0.0362333 3
1% O2	3	0.03	0.01	0.0003

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.2204166	1	0.220416667	12.066605	0.0255	7.7086
Within Groups	0.0730666	4	0.018266667			
Total	0.2934833	5				

APPENDIX B

Protocols

Modified Qiagen PCR Amplification w/ HotStarTaq DNA Polymerase

Thaw 10x PCR Buffer, dNTP mix, primer solutions, and 25 mM MgCl2. It is important to mix the solutions completely before use to avoid localized concentrations of salts. Prepare a reaction mix. It is not necessary to keep reaction vessels on ice since HotStarTaq DNA Polymerase is inactive at room temperature. The reaction mix typically contains all the components needed for PCR except the template DNA. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. A negative control (without template DNA) should always be included.

Note: The Mg2+ concentration provided by the supplied PCR Buffer will produce satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg2+ concentration.

Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes. Mix gently (e.g., by pipetting the reaction mix up and down a few times). It is not necessary to keep PCR tubes on ice as nonspecific DNA synthesis cannot occur at room temperature due to the inactive state of HotStarTaq DNA Polymerase. Add template DNA (<1 µg/100 µl reaction) to the individual tubes containing the reaction mix. For RT-PCR, add an aliquot from the reverse transcriptase reaction. This should not exceed 10% of the final PCR volume.

Component Volume/reaction Final Concentration Reaction mix

10x PCR Buffer 10 µl
1x 25 mM MgCl2
dNTP mix (10 mM of each) 2 µl 200 µM of each dNTP
Primer A Variable 0.1–0.75 µM
Primer B Variable 0.1–0.75 µM
HotStarTaq DNA 0.5 µl 3.5 units/reaction
Distilled water Variable
Template DNA, Variable ≤1 µg/100 ml reaction added at step 4
Total volume 100 µl

Place the PCR tubes in the thermal cycler and start the cycling program.

Initial activation step: 15 min 95°C HotStarTaq DNA Polymerase is activated by this heating step.

Denaturation: 0.5–1 min 94°C
Annealing: 0.5–1 min 50–68°C (Approximately 5°C below Tm of primers)
Extension: 1 min 72°C (For PCR products longer than 1 kb, use an extension time of approximately 1 min per kb DNA.)
Number of cycles: 30–45
Final extension: 10 min 72°C

Agarose Gel Electrophoresis

Place gel in electrophoresis box making certain that wells are closest to negative electrodes (black). Fill the box with 1X TAE buffer, covering gel completely.

1. Add 11 µl of 10X loading buffer to each 100 µl sample.
2. Carefully load all 111 µl into the large well.
3. Skipping the smaller wells, fill the gel with the samples.
4. Load 3 µl of low molecular weight marker into final small well.
5. Run gel between 80 and 90 volts for 45 minutes.

DNA Extraction from Agarose Gel (Qiagen QiaQuick Gel Extract Kit)

This protocol is designed to extract and purify DNA of 70 bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions

using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the new MinElute Reaction Cleanup Kit.

1. Excise the DNA band from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing excess agarose. Use a 1.5-ml microcentrifuge tube for processing up to 250 mg agarose.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel. For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.
3. Incubate at 50°C for 10 minutes (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing every 2-3 minutes during the incubation. Important: Dissolve agarose completely. For >2% gels, increase incubation time.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The absorption of DNA to the QIAquick membrane is efficient only at pH ≤ 7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
5. Add 1 gel volume of isopropanol to the sample and mix. For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. Place a QIAquick spin column in a provided 3 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube. Collection tubes are re-used to reduce plastic waste.
9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 minute. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 minute. Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2-5 minutes after addition of Buffer PE, before centrifuging.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at 13,000 rpm. Important: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or HO to the center of the QIAquick membrane and centrifuge the column for 1 minute. Alternatively, for increased DNA concentration, add 30 µl of elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute, and then centrifuge for 1

minute. Important: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluted volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within buffering agent. The purified DNA can also be eluted in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Modified Epicentre Ligation of DNA Molecules with Cohesive or Blunt Ends

This protocol is suitable for the ligation of insert DNAs with cohesive or blunt ends into plasmid vectors with like ends.

1. Assemble the reaction in a micro-centrifuge tube at room temperature as outlined; adding the ligase last (Numbers in parentheses indicate molar ratios.):

Ligations of Insert DNA with Cohesive Ends

1.5 µl 10X Fast-Link Ligation Buffer
1.5 µl 10 mM ATP
X µl vector DNA (1)
X µl insert DNA (3)
X µl sterile water to a volume of 14 µl
1.0 µl Fast-Link DNA Ligase
15 µl Total reaction volume

Ligations of Insert DNA with Blunt Ends

1.5 µl 10X Fast-Link Ligation Buffer
0.75 µl 10 mM ATP
X µl vector DNA (1)
X µl insert DNA (10)
X µl sterile water to a volume of 14 µl
3 µl Fast-Link DNA Ligase
15 µl Total reaction volume

2. Incubate the reaction 1 hour at room temperature for cohesive ends and overnight for blunt ends.

3. Transfer the reaction to 70ºC for 15 minutes to inactivate the Fast-Link DNA ligase; failure to inactivate the ligase may decrease transformation efficiencies.

4. Spin briefly in a micro-centrifuge.

5. Transform competent *E. coli* cells with 1/10 of the ligation reaction, keeping the volume of the ligation to no more than 5% of the volume of competent cells, or follow the manufacturer's recommendations. If electroporating the ligated molecules, use no more than 2 µl of the ligation reaction with 50 µl of electro-competent cells.

6. To determine the extent of ligation, inactivate the ligase and run 5 µl of the ligation reaction on an agarose gel and visualize.

Bacterial Transformation

1. Put competent cells on ice for about 5min to thaw.
2. Add Plasmid DNA or ligation products, no more than 15µl into the cell suspension and mix gently.
3. Keep on ice for 30min with occasional swirling.
4. Heat 40 seconds at 42^{o}C.
5. Put on ice for 1 min.
6. Add 200 µl SOC Incubate at 37^{o}C for 1 hr at 250rpm.
7. Plate 1-200 µl on (100 µl/ml) ampicillin plates and spread evenly.
8. Incubate overnight at 37^{o}C with dish upside down.
9. Pick up several colonies for DNA analysis and glycerol stocks.

Modified Qiagen Miniprep Isolation of Plasmid DNA

1. Grow 7 ml culture from a single colony of transformed E. coli (alternatively 50µl glycerol stock but not recommended) by incubating at 37^{o}C and shaking at 250rpm overnight.
2. Transfer 1.5 ml overnight culture into an Eppendorf tube. Spin at 10,000rpm for 1min. Discard the supernatant. If the pellet is too small, add another 1.5 ml culture and spin again until there is a large enough pellet.
3. Add 250 µl Buffer P1 solution (in 4^{o}C refrigerate) containing RNase A re-suspend evenly by pipetting or vortexing vigorously.
4. Add 250 µl Buffer P2 solution (room temperature) and mix by inverting the tube 4-8 times. Lyse cells at room temperature for 5 min.
5. Add 350 µl Buffer N3 and invert the tube immediately 4-8 times to neutralize the lysis reaction.
6. Spin at 13,000 rpm for 10 min.
7. Apply the supernatant to the QIAprep spin column by decanting.
8. Spin for 30–60 s. Discard the flow-through.
9. Wash the column by adding 600 µl Buffer PE and spin for 30 second at 13,000 rpm. Discard the flow-through.
10. Wash again with 200 µl Buffer PE and spin for 1 min at 13,000 rpm. Discard the flow-through and put the column back in a different rotation angle so that there will be no solution re-tentate in the corner of the column. Spin for an additional 1 min to remove residual wash buffer.
11. Place the column in a clean sterile Eppendorf tube.

12. To elute DNA, add 50 µl Buffer EB to the center of the column, let stand for 1-5 min, and centrifuge for 1 min at 13,000 rpm.
13. Store DNA solution at 4°C or 20 °C.

Modified Qiagen Maxiprep for Low Copy Number Plasmid DNA

1. Start 5-10 ml culture (with appropriate selective antibiotic) from a single colony.
2. Incubate for 8 hours at 37 °C.
3. Inoculate 0.5 ml each in two 500 ml media with appropriate antibiotic in 2L flasks and grow the culture at 37°C overnight with vigorous shaking at 300 rpm.
4. In the next morning, put buffer P3 on ice. Harvest cells by centrifugation at 3000 rpm for 15 min at 4°C in 1000ml centrifuge bottles.
5. Decant supernatant and re-suspend pellets from 1000 ml culture in 30 ml buffer P1 with RNase A. Transfer to 250ml centrifuge bottle.
6. Add 30 ml buffer P2 while swirling the bottle gently to avoid forming clamps and incubate at room temperature for 5 min.
7. Add 30 ml chilled buffer P3 while swirling the bottle gently then incubate on ice for 20-30 min.
8. Centrifuge at 25,000 X g for 30min at 4°C.
9. Transfer the supernatant into 2 X 50 ml centrifuge bottles, centrifuge at 28,000xg for 30min at 4ºC.
10. Equilibrate the Qiagen-tip with 10 ml buffer QBT.
11. Apply the supernatant from step 7 to the QIAGEN-tip and allow it to enter the resin and go through by gravity flow.
12. Wash the tip with 2X30ml Buffer QC.

13. Elute DNA with 15 ml Buffer QF to a fresh 50ml centrifuge tube.
14. Precipitate DNA by adding 10.5ml (~11ml) room-temperature isopropanol to the eluted DNA solution. Mix and centrifuge immediately at 25,000 X g for 30min at 4^{o}C. Carefully decant the supernatant.
15. Wash DNA pellet with 5ml of room-temperature 70% ethanol and centrifuge at 25,000 x g for 15min. Carefully decant the supernatant without disturbing the pellet.
16. Air-dry the pellet for 30min. Re-dissolve the DNA in a suitable volume (0.5-1.0ml) of 10mM Tris, pH8 buffer.
17. Carry out the Phenol/CIA-Ethanol precipitation protocol.

Phenol- CIA / Ethanol Purification of DNA

1. Dissolve dry pellet in 5ml 10mM Tris, pH8 buffer.
2. Extract with 5ml 1:1 phenol/CIA.
3. Separate phases at 3000 rpm for 10 min at RT (in 15 ml centrifuge tube)
4. Remove aqueous phase to fresh 50 ml centrifuge tube
5. Add 250µl 10M ammonium acetate to make 0.5M ammonium acetate final concentration, mix
6. Add 2 volumes of 200 proof ethanol.
7. Vortex. Put in -80^{o}C freezer for >=2 hr w/ 1M ammonium acetate.
8. Spin 15 min at RT at >=15,000 X g.
9. Decant ethanol
10. Add 15ml 80% ethanol, vortex
11. Re-pellet by centrifugation.

12. Remove ethanol completely
13. Lyophilize in SpeedVac.
14. Re-dissolve pellet in 0.5 ml 10mM Tris, pH8 buffer.
15. Store at 4^{o}C

Sequencing

Turn on computer by pushing the on/off button located on the tower. Once the log on box appears, press ctrl+ alt+ del at the same time. When the log on appears, click okay. No password is required. Once the computer has booted up, turn on the 3100 Genetic Analyzer. Let the machine completely turn on before you launch any software. Open the 3100 Data Collection software by double clicking on the icon on the desktop.

Setting Up the 3100 Genetic Analyzer for Sequencing

If polymer has been on machine longer than one week, refer to Instrument Maintenance in the Preparation for Analytical procedure. Push the tray button on the front of the 3100 Genetic Analyzer. Once the auto-sampler has completely stopped moving, remove the two water and one buffer reservoirs. Remove the anode buffer reservoir (shot glass). Empty each reservoir. Using distilled water, rinse out each reservoir. Completely dry reservoir using Kimwipes, lint free paper. Rinse each septum with distilled water and dry by gently tapping on a paper towel. Refill the water reservoirs with distilled water at room temperature. (Note: DO NOT use autoclaved water!) Refill the buffer reservoir and the anode buffer with room temperature 1X EDTA buffer. (Refer to reagents list for recipe for 1X EDTA buffer) Replace septa to each reservoir and replace reservoirs to their appropriate positions. (Buffer, position 1; waters, positions 2 and 4) Close instrument doors.

Preparation of Sample for Sequencing

Once samples have been dried, add 20 µl of HiDi Formamide to the dried sample in a 0.2 ml PCR tube. Denature the sample by heat for 5 minutes at 95°C. Immediately put on ice for at least 2 minutes. Obtain a 96-well optical plate. Pipette 20 µl from the 0.2ml PCR tube into the desired well on the 96-well plate. Continue to do this for all of the samples. For any empty wells within a run, add 20 µl of HiDi formamide. A run includes 16 wells, example, well A1-H2 is one run. Place a 96-well gray plate septa on the 96-well plate. Place the white 96-well plate retainer on top of the plate with the septa. Be sure that the plate retainer is on straight and locks into position. Turn on the computer and the instrument. Double click on the 3100 Data Collection Software icon on the desktop. Push the tray button on the instrument. When the auto-sampler comes to a complete stop, place the plate assembly on the machine. Be sure the plate sits flush with the reservoirs. Check the software to ensure plate position has been identified by software. Plate area on the Plate View page of the software will turn yellow.

Creating a Plate Record

Click the Plate View tab on the 3100 Data Collection Software window to go to the Plate View page. On the Plate View page, click New. Or, double-click the Plate Editor button on the toolbar. The Plate Editor dialog box opens. Name your plate and ensure that sequencing has been highlighted. Click Finish. The Plate Editor spreadsheet opens. Type the names of all the samples in the Sample Name column as they correspond to the well position you put your samples. Select the appropriate Dye Set from the drop-down list. Use Dye Set E for BigDye Terminator version 1.0 and Dye Set Z for BigDye Terminator version 3.0. Select the appropriate Mobility File from the drop

down list. Use DT3100POP6 {BDv3} v1.mob for BigDye version 3.0 and use DT3100POP6 {BD} v2.mob for BigDye version 1.0. Select 3100_Project1 under the BioLiMS Project. For each sample, select the appropriate Run Module from the drop-down list. Use StdSeq50_POP6DefaultModule for standard DNA sequencing. For each sample, select the appropriate Analysis Module from the drop-down list. Use BC-3100SR_SeqOffFtOff.saz for standard sequencing. Check to make sure the plate record is correct and click "OK." This will save the information a put the plate record under the pending plate records. Click the on the Pending Plate Records to highlight your plate. Click on the plate (yellow) you want to link that plate record to. This links the plate to the plate record. Plate will turn green when plate record has been linked. The Run Instrument arrow will become green. Click on the green arrow to begin sequencing run. Click the Run View tab to view the run schedule. To monitor run, click the Status View tab to monitor the status of the instrument during the run.

Transforming One-shot Competent Cells

1. Deactivate the ligation reaction at 70°C for15min in PCR machine.
2. Equilibrate a water bath to 42°C and warm the vial of S.O.C. medium from Box to room temperature.
3. Thaw on ice 1 vial of One Shot Cells for each transformation.
4. Add 4µl of the TOPO TA Cloning reaction result in step 2 into a vial of One Shot Chemically Competent *E.coil* and mix gently.
5. Incubate on ice for 5 to 30mins, usually 30min.
6. Heat-shock the cells for 40sec in a 42°C water bath, without shaking.
7. Immediately transfer the tubes to ice.

8. Add 250µl of room temperature S.O.C. Medium, no need to mix.
9. Shake the tube horizontally (200rpm) at 37°C for 1hour.
10. During the 1hour shaking time, warm selective LB plates at 37°C for 30 min, 1 transformation use 3 plates; also put the X-gal in DMF on ice at the same time.
11. Spread 40µl of 40µg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
12. Spread 10-50µl from each transformation on a pre-warmed selective plate and incubate at 37°C overnight without shaking.

    NOTE:

    1. This time we use LB plates containing 75µg/ml amp, so we use more than 50 µl transformation, we use three plates for each transformation, use 50µl, 75µl, 100µl transformation for three plates.
    2. To ensure even spreading of small volumes, add 20µl of S.O.C. medium. We recommend use different volumes on each plate to ensure that at least one plate will have well-spaced colonies.
    3. The plates should be put up-side down in the incubator
13. An efficient TOPO TA Cloning reaction should produce several hundred colonies. Pick about 10 for analysis.

Luciferase Assay (In Vitro)

Passive Lysis of Cells Cultured in Multiwell Plates

1. Determine transfection parameters (i.e., plated cell density and subsequent incubation time) such that cells are no more than 95% confluent at the desired time of lysate preparation. Remove the growth medium from the cultured cells, and gently apply a sufficient volume of phosphate buffered saline (PBS) to wash the surface of the culture vessel. Swirl the vessel briefly to remove detached cells and residual growth medium. Completely remove the rinse solution before applying PLB reagent.

2. Dispense into each culture well the minimum volume of 1X PLB required to completely cover the cell monolayer. The recommended volumes of PLB to add per well are as follows:

Multiwell Plate	1X PLB
6-well culture plate	500µl
12-well culture plate	250µl
24-well culture plate	100µl
48-well culture plate	65µl
96-well culture plate	20µl

3. Place the culture plates on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of the cell monolayer with 1X PLB. Rock the culture plates at room temperature for 15 minutes.

4. Transfer the lysate to a tube or vial for further handling and storage.

Alternatively, reporter assays may be performed directly in the wells of the culture plate. In general, it is unnecessary to clear lysates of residual cell debris prior to performing the DLR assay. However, if subsequent protein determinations are to be made, we recommend clearing the lysate samples for 30 seconds by centrifugation at top speed in a

refrigerated microcentrifuge. Transfer cleared lysates to a new tube prior to reporter enzyme analyses.

Notes:

1. Cultures that are overgrown are often more resistant to complete lysis and typically require an increased volume of PLB and/or an extended treatment period to ensure complete passive lysis. Firefly and *Renilla* Luciferases are stable in cell lysates prepared with PLB; therefore, extending the period of passive lysis treatment will not compromise reporter activities.

2. Microscopic inspection of different cell types treated for passive lysis may reveal seemingly different lysis results. Treatment of many types of cultured cells with PLB produces complete dissolution of their structure within a 15-minute period. However, PLB treatment of some cell types may result in discernible cell silhouettes on the surface of the culture well or large accumulations of floating debris. Despite the appearance of such cell remnants, we typically find complete dissolution of both Luciferase reporter enzymes within a 15-minute treatment period. However, some types of cultured cells may exhibit greater inherent resistance to lysis, and optimizing the treatment conditions may be required.

Preparation of Luciferase Assay Reagent II

Prepare Luciferase Assay Reagent II (LAR II) by re-suspending the provided lyophilized Luciferase Assay Substrate in 10ml of the supplied Luciferase Assay Buffer II. Once the substrates and buffer have been mixed, write LAR II on the existing vial label for easy identification. LAR II is stable for one month at -20°C or for one year when stored at -70°C. Do not substitute Luciferase Assay Reagent (Cat. # E1483) included in the

Luciferase Assay Systems (Cat. # E1500, E1501, E4030, E4530, E4550), for LAR II. The Luciferase Assay Reagent supplied with these systems is not designed for use with the DLR assay System.

Notes:

1. Repeated freeze-thawing of this reagent may decrease assay performance. We recommend that LAR II be dispensed into aliquots for each experimental use (e.g., a 1ml aliquot will provide 10 assays).

2. The components of LAR II are heat-labile. Frozen aliquots of this reagent should be thawed in a water bath at room temperature.

3. The process of thawing generates both density and composition gradients within LAR II. Mix the thawed reagent prior to use by inverting the vial several times or by gentle vortexing.

Preparation of Stop & Glo® Reagent

Prepare an adequate volume to perform the desired number of DLR. Assays (100µl reagent per assay). Stop & Glo® Substrate is supplied at a 50X concentration. Add 1 volume of 50X Stop & Glo® Substrate to 50 volumes of Stop & Glo® Buffer in a glass or siliconized polypropylene tube. Stop & Glo® Reagent (Substrate + Buffer) is best when prepared just before use. If stored at 22°C for 48 hours, the reagents activity decreases by 8%. If necessary, Stop & Glo® Reagent may be stored at -20°C for 15 days with no decrease in activity. It may be thawed at room temperature up to 6 times with ≤15% decrease in activity.

Note: Reagents that have been prepared and stored frozen should be thawed in a room temperature water bath. Always mix the reagent prior to use because thawing generates density and composition gradients.

Standard Protocol

Note: The LAR II, Stop & Glo® Reagent and samples should be at ambient temperature prior to performing the Dual-Luciferase® Assay. Prior to beginning this protocol, verify that the LAR II and the Stop & Glo® Reagent have been warmed to room temperature. The assays for firefly luciferase activity and *Renilla* luciferase activity are performed sequentially using one reaction tube. The following protocol is designed for use with a manual luminometer or a luminometer fitted with one reagent injector.

Note: In some instances, it may be desirable to measure only *Renilla* luciferase reporter activity in the lysates of pGL4 Vector-transfected cells. For this application, we recommend the Renilla Luciferase Assay System (Cat. # E2810, E2820). If the DLR assay System is used to measure only Renilla luciferase activity, it is still necessary to combine 100µl of both LAR II and Stop & Glo® Reagent with 20µl cell lysate to achieve optimal Renilla luciferase assay conditions.

1. Pre-dispense 100µl of LAR II into the appropriate number of luminometer tubes to complete the desired number of DLR assays.

2. Program the luminometer to perform a 2-second pre-measurement delay, followed by a 10-second measurement period for each reporter assay.

3. Carefully transfer up to 20µl of cell lysate into the luminometer tube containing LAR II; mix by pipetting 2 or 3 times. Do not vortex. Place the tube in the luminometer and initiate reading.

Note: We do not recommend vortexing the solution at Step 3. Vortexing may coat the sides of the tube with a microfilm of luminescent solution, which can escape mixing with the subsequently added volume of Stop & Glo® Reagent. This is of particular concern if Stop & Glo® Reagent is delivered into the tube by automatic injection.

4. If the luminometer is not connected to a printer or computer, record the firefly luciferase activity measurement.

5. If available, use a reagent injector to dispense 100µl of Stop & Glo® Reagent. If using a manual luminometer, remove the sample tube from the luminometer; add 100µl of Stop & Glo® Reagent and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.

Note: It is possible to prime auto-injector systems with little or no loss of DLR assay reagents. Before priming injectors with LAR II or Stop & Glo® assay reagents, we recommend first purging all storage liquid (i.e., deionized water or ethanol wash solution) from the injector system. Priming assay reagent through an empty injector system prevents dilution and contamination of the primed reagent. Thus, the volume of primed reagent may be recovered and returned to the reservoir of bulk reagent.

6. If the luminometer is not connected to a printer or computer, record the *Renilla* luciferase activity measurement

7. Discard the reaction tube, and proceed to the next DLR assay.

Mammalian Cell Culture

Cell reconstitution

1. Pre-warm medium to room temperature
2. Take a vial of cells from liquid nitrogen storage tank, and quickly thaw the vial on a 37^{o}C water bath.
3. Wipe the vial with 70% ethanol and let dry in hood.
4. Uncap the vial; transfer the cell suspension into a T25 flask. Discard the empty vial in a biohazard container.
5. Using a new pipette, add 5ml culture medium to the flask and pipette up and down several times to break cell clumps and mix the suspension.
6. Put the flask in incubator and replace fresh medium the next day.
7. Change medium twice a week until the cells are sub-confluent.
8. When sub-confluent, the cells can be used for experiment, subcultured or frozen for future usage.

Cell propagation

1. Aspirate the medium in the flask with Pasteur pipette.
2. Wash cells 1-2 times by adding 5 ml sterile PBS and aspirating off.
3. Add 3.5ml trypsin-EDTA; shake to cover the whole cell surface. Incubate at 37ºC for 3-5min. depending on cell lines.
4. Once the cells detach from the flask, neutralize the trypsin by rinsing cells with 5ml culture medium and pipette up and down to break cell clumps.
5. Dilute cells with culture medium at 1:3-20 depend on cell line and purpose.
6. Plate 7ml each in new T25 flasks and incubate at 37ºC with 5% CO2.

7. Harvest cells and freeze for long-term storage.

Freezing cells

1. Trypsinize cells as above and transfer cell suspension into 50 ml sterile falcon tubes.
2. Centrifuge at 200 x g for 5 min to pellet cells.
3. Mark cryogenic vials indicating: Cell name, passage, date.
4. Aspirate media.
5. For each flask of cells, Re-suspend cells in 1 ml 7.5% DMSO freezing media for each flask of cells and transfer into 2 ml cryogenic vials at 0.5ml/tube.
6. Place vials in Nalgene Cryo 1^{o}C Freezing Container and put in -80^{o}C freezer.
7. The next day, transfer vials to liquid nitrogen tank and record the cells information and locations in log book.

Hemacytometer Cell Counting

1. Trypsinize the cells and break the clumps to single cells.
2. Withdraw a sample of the cell suspension and prepare a series of dilutions, as required, in PBS. The optimal concentration of cells for counting is 5-10x10 5 cells/ml (50-100 cells per large square) after dilution in the Trypan Blue solution.
3. Add Trypan Blue stock so that the final concentration is 0.1%. Or dilute the Trypan Blue stock to 0.2% solution and add to cell suspension at 1:1.
4. After being stained with Trypan Blue, the cells should be counted within 3 minutes: after that time the cells will begin to take up the dye.
5. Using a Pasteur pipette, withdraw a small amount of the stained cell suspension and place the tip of the pipette onto the slot of a clean hemacytometer with

coverslip. The cell suspension will pass under the coverslip by capillary action. Fill the opposite chamber with the second diluted sample. Do not overfill. Do not lift or move the coverslip.

6. Place the hemacytometer on the stage of an inverted microscope. Adjust focus and power until a single counting square fills the field.
7. Count under the microscope two 1x1x0.1 mm areas (delimited by a double line; nine small squares), from two chambers. Ideally, count more than 200 cells and less than 500 per chamber.
8. The number of cells per 1x1x0.1 mm areas = cells x $1*10^{-4}$ cm^3 ($1*10^{-4}$ ml). With a 1:20 dilution the easy count is: number of cells in two 1mm squares divided by 10 = cells x 10^6 / ml.

   (2 squares / 2 x 20 x10,000 / 1,000 = 10^6 cells / ml)
   (2 squares x 10 x 10,000 / 1,000 = 10^6 cells / ml)
   (2 squares x 100 = 10^6 cells / ml)
   (2 squares / 10 = millions / ml)
9. If cell suspension is way above 500 / square, count as many smallest squares you can, calculate the mean: this number multiplied by 0.9 will approximate the number of millions / ml.

Immunofluorescent Labeling of Monolayer Cells

1. Trypsinize cells to get cell suspension in culture medium. Determine cell density.
2. Sterilize cover slips. Put in 6-well plates.
3. Add cell suspension in the well. Incubate at 37^{o}C with 5% CO_2 until the cells reach to proper density.

4. Cool cells on ice. Pipette off culture medium and wash in 4°C PBS. Pipette off PBS.
5. If cell surface antigens are being studied, fix 30 min in 10% TCA fixative at 4°C. If cytoplasmic antigens are being studied, fix 30 min in 10% TCA containing 0.1 % Triton X-100 at 4°C.
6. Pipette off fixative and wash 3 times with PBS.
7. Incubate with blocking solution for 30~60 min.
8. Microcentrifuge diluted primary antibody 2 min at 13,500 x *g*.
9. Layer primary antibody onto cover slips such that cells are just covered and incubate 1 hr. at appropriate temp. Wash five times with PBS.
10. Microcentrifuge diluted secondary antibody 2 min at 13,500 x *g*.
11. Layer secondary antibody onto cover slips and incubate 1 hr. at appropriate temp. Wash five times with PBS. 4',6-diamidino-2-phenylindole DAPI solution can be used at the second wash if counter stain is needed.
12. Rinse briefly with H2O and let dry. (It's better to remove the cover slips when they are in H2O. If aspirate first, it would be difficult to move out).
13. Write down the sample information on the white part of the slides. Mount the cover slips onto slides with the cells inside with antifade mounting medium. Seal with nail polish.
14. Check under fluorescent microscope with appropriate emission / excitation filter.

Immunostaining of Tissue Sections

1. Cool the paraffin blocks on ice for 10-15min.

2. Cut paraffin blocks at the desired thickness (5-8um) on the microtome (cut off the outer part until you see the flat face of the tissue. Cut a few section at different depth).
3. Float the sections on water bath for a few minutes to help remove wrinkles.
4. Pick up the section onto a glass microscopic slide.
5. Place the glass slides in a warm oven for about 15 minutes to help the section adhere to the slide.
6. Arrange the slides in a slide rack and put in a staining dish heat in oven at 60^{o}C for one hour to melt the paraffin.
7. De-paraffinize slides in 2 changes of xylene for 5 min each.
8. Transfer slides to absolute ethanol, 2 changes for 3min each.
9. Transfer slides to 95% ethanol for 3 min.
10. Rinse in PBS, 2 changes for 5min each.
11. Put slides in TE buffer in a glass container covered with lid loosely, microwave 3 cycles of 5 min each to retrieve antigens.
12. Rinse in PBS, 2 changes for 5min each.
13. Block slides in 6% Bovine Serum Albumin (BSA) for 1 hour.
14. Incubate with primary antibody solution at room temperature for 1 hr.
15. Wash slides with PBS three times for 10 min each.
16. Incubate slides with secondary antibody for 1 hr.
17. Wash slides with PBS three times for 10 min each.
18. Rinse briefly with MilliQ H_2O and let dry.

19. Put a drop of mounting solution on the stained area. Gently put a coverslip on it. Seal with nail polish.
20. Check under fluorescent microscope with appropriate emission/excitation filter.

Transfection of Mammalian Cells with Qiagen Effectene

1. Prepare plasmids for transfection using Endofree Plasmid Kit to avoid toxicity problems.
2. Day before transfection: split cells, need 0.5-2.0 x 10^7 cells per 100 mm dish.
3. Day of transfection: harvest cells by trypsinization & centrifugation, remove the medium, and wash cells once with PBS in a 15 ml tube.
4. Seed 0.5-2.0 x 10^7 cells per 100 mm dish in 7 ml growth medium containing serum and antibiotics.
5. Dilute 4 µg DNA (dissolved in TE buffer) with the DNA-condensation buffer EC to a total volume of 600 µl.
6. Add 32 µl Enhancer and vortex for 1 second.
7. Incubate 2-5 minutes at RT, centrifuge briefly to remove drops from top of tube.
8. Add 120 µl Effectene Transfection Reagent. Mix by pipetting up and down 5 times or vortexing for 10 seconds.
9. Incubate 5-10 minutes at RT to allow transfection-complex formation.
10. Add transfection complexes to tube containing 3 ml growth medium. Mix by pipetting up and down twice then immediately add mixture drop-wise onto the cells in the 100 mm dish.
11. Gently swirl the dish to ensure uniform distribution of transfection complexes.

12. Incubate cells with transfection complexes under normal growth conditions for an appropriate time for expression of the transfected gene.
13. Assay cells to confirm gene expression.

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BIBLIOGRAPHY

Adelman, D. M., E. Maltepe, and M. C. Simon. 1999. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes Dev. 13:2478-2483.

Aikawa, R, Huggins, GS and Snyder, RO 2002. Cardiomyocyte-specific gene expression following recombinant adeno-associated viral vector transduction. J Biol Chem 277: 18979–18985.

Aoki, M, Morishita, R, Taniyama, Y, Kida, I, Moriguchi, A, Matsumoto, K et al. (2000). Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: up-regulation of essential transcription factor for angiogenesis, ets. Gene Ther 7: 417–427.

Arany, Z., L. E. Huang, R. Eckner, S. Bhattacharya, C. Jiang, M. A. Goldberg, H. F. Bunn, and D. M. Livingston. 1996. An essential role for p300/CBP in the cellular response to hypoxia. Proc. Natl. Acad. Sci. USA 93:12969-12973.

Arsic, N, Zentilin, L, Zacchigna, S, Santoro, D, Stanta, G, Salvi, A et al (2003). Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. Mol Ther 7: 450–459.

Ausoni, S., M. Campione, A. Picard, P. Moretti, M. Vitadello, C. D. Nardi, and S. Schiaffino. 1994. Structure and regulation of the mouse cardiac troponin I gene. J. Biol. Chem. 269:33 346

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology, vol. 1. Green Publishing Associates, Inc., and J. Wiley & Sons, Inc., New York, N.Y.

Ay T, Havaux X, Van Camp G, et al. Destruction of contrast microbubbles by ultrasound: effects on myocardial function, coronary perfusion pressure, and microvascular integrity Circulation 2001;104:461-466.

Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L. In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 2005; 48: 49–57.

Bao S, Thrall BD, Miller DL. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro Ultrasound Med Biol 1997;23:953-957.

Barnett NB, Ter Haar GR, Ziskin MC, Nyborg WL, Maeda K, Bang J. Current status of research on biophysical effects of ultrasound Ultrasound Med Biol 1994;20:205-218.

Bauer S, Maier SK, Neyses L, Maass AH. Optimization of gene transfer into neonatal rat cardiomyocytes and unmasking of cytomegalovirus promoter silencing. DNA Cell Biol. 2005 Jun;24(6):381-7.

Baumgartner, I, Pieczek, A, Manor, O, Blair, R, Kearney, M, Walsh, K et al. (1998). Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 97: 1114–1123.

Beck I, Weinmann R, Caro J. Characterization of hypoxia-responsive enhancer in the human erythropoietin gene shows presence of hypoxia-inducible 120-Kd nuclear DNA-binding protein in erythropoietin-producing and nonproducing cells. *Blood* 1993; 82: 704–711.

Bekeredjian R, Chen S, Frenkel PA, Grayburn PA, Shohet RV. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart Circulation 2003;108:1022-1026.

Bekeredjian R, Chen S, Grayburn PA, Shohet RV. Augmentation of cardiac protein delivery using ultrasound targeted microbubble destruction. Ultrasound Med Biol 2005. May;31(5):687-91.

Bekeredjian R, Chen S, Pan W, Grayburn PA, Shohet RV. Effects of ultrasound targeted microbubble destruction on cardiac gene expression Ultrasound Med Biol 2004;30:539-543.

Bekeredjian R, Shohet RV. Cardiovascular gene therapy: angiogenesis and beyond Am J Med Sci 2004;327:139-148.

Bertrand KP, Postle K, Wray LV Jr, Reznikoff WS. Construction of a single-copy promoter vector and its use in analysis of regulation of the transposon Tn10 tetracycline resistance determinant. J Bacteriol. 1984 Jun;158(3):910-9.

Bianchi L, Tacchini L, Matteucci E, Desiderio MA. A cluster region of AP-1 responsive elements is required for transcriptional activity of mouse ODC gene by hepatocyte growth factor. Arch Biochem Biophys. 2002 May 1;401(1):115-23.

Binley K *et al*. An adenoviral vector regulated by hypoxia for the treatment of ischaemic disease and cancer. *Gene Therapy* 1999; 6: 1721-1727.

Binley K, Iqball S, Kingsman A, Kingsman S, Naylor S. An adenoviral vector regulated by hypoxia for the treatment of ischaemic disease and cancer. Gene Ther. 1999 Oct;6(10):1721-7.

Bishop, DL, and Milton RL. The effects of denervation location on fiber type mix in self-reinnervated mouse soleus muscles. *Exp Neurol* 147: 151-158, 1997.

Bishopric, N. E., and L. Kedes. 1991. Adrenergic regulation of the skeletal α-actin gene promoter during myocardial cell hypertrophy. Proc. Natl. Acad. Sci. USA 88:2132-2136

Blenkinsopp A, Anderson C, Armstrong M. Systematic review of the effectiveness of community pharmacy-based interventions to reduce risk behaviours and risk factors for coronary heart disease. J Public Health Med. 2003 Jun;25(2):144-53. Review.

Bleske BE, Shea MJ. Current concepts of silent myocardial ischmiea. *Clin Pharm.* 1990; 9: 339–357.

Boast K *et al*. Characterization of physiologically regulated vectors for the treatment of ischaemic disease. *Hum Gene Ther* 1999; 10: 2197-2208.

Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE. A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 1993; 42: 1715–1720.

Bonner-Weir S, Wier GC. New sources of pancreatic b-cells. Nat Biotechnol 2005; 23: 857–861.

Borges AC, Walde T, Reibis RK, et al. Does contrast echocardiography with Optison induce myocardial necrosis in humans? J Am Soc Echocardiogr 2002;15:1080-1086.

Brown JM. Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Mol Med Today* 2000; 6: 157-162.

Bruick, R. K. 2003. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes Dev. 17:2614-2623.

Caiozzo, VJ, Baker MJ, McCue SA, and Baldwin KM. Single-fiber and whole muscle analyses of MHC isoform plasticity: interaction between T_3 and unloading. *Am J Physiol Cell Physiol* 273: C944-C952, 1997.

Cao, R, Brakenhielm, E, Pawliuk, R, Wariaro, D, Post, MJ, Wahlberg, E et al. (2003). Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med 9: 604–613

Carlezon WA Jr, Duman RS, Nestler EJ. The many faces of CREB. Trends Neurosci. 2005 Aug;28(8):436-45. Review.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000 Apr 28;288(5466):669-72.

Chatterjee, S, Stewart, AS, Bish, LT, Jayasankar, V, Kim, EM, Pirolli, T et al. (2002). Viral gene transfer of the antiapoptotic factor Bcl-2 protects against chronic postischemic heart failure. Circulation 106 (Suppl 1): I212–I217.

Chen S, Ding J, Yu C, Yang B, Wood DR, Grayburn PA. Reversal of streptozotocin-induced diabetes in rats by gene therapy with betacellulin and pancreatic duodenal homeobox-1. Gene Ther. 2007 Jul;14(14):1102-10. Epub 2007 Apr 26.

Chen S, Ding JH, Bekeredjian R, Yang BZ, Shohet RV, Johnston SA et al. Efficient gene delivery to pancreatic islets with ultrasonic microbubble destruction technology. Proc Natl Acad Sci USA 2006; 103: 8469–8474.

Chen S, Kroll MH, Shohet RV, Frenkel P, Mayer SA, Grayburn PA. Bioeffects of myocardial contrast microbubble destruction by echocardiography Echocardiography 2002;19:495-500.

Chen S, Shohet RV, Bekeredjian R, Frenkel P, Grayburn PA. Optimization of ultrasound parameters for cardiac gene delivery of adenoviral or plasmid deoxyribonucleic acid by ultrasound-targeted microbubble destruction J Am Coll Cardiol 2003;42:301-308.

Chin, ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Weiguang Z, Bassel-Duby R, and Williams RS. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* 12: 2499-2509, 1998.

Chiu-Pinheiro, CK, O'Brien, T, Katusic, ZS, Bonilla, LF, Hamner, CE and Schaff, HV (2002). Gene transfer to coronary artery bypass conduits. Ann Thorac Surg 74: 1161–1166.

Cho, CH, Kim, KE, Byun, J, Jang, HS, Kim, DK, Baluk, P et al. (2005). Long-term and sustained COMP-Ang1 induces long-lasting vascular enlargement and enhanced blood flow. Circ Res 97: 86–94.

Chomas JE, Dayton P, Allen J, Morgan K, Ferrara KW. Mechanisms of contrast agent destruction IEEE Trans Ultrason Ferroelectr Control 2001;48:232-248.

Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles Ultrasound Med Biol 2003;29:1759-1767.

Ciccarone V, Hawley-Nelson P, Jessee J. Cationic liposome-mediated transfection: effect of serum on expression and efficiency. *Focus* 1993; 15: 80-83.

Cichon, G, Willnow, T, Herwig, S, Uckert, W, Loser, P, Schmidt, HH et al. (2004). Non-physiological overexpression of the low density lipoprotein receptor (LDLr) gene in the liver induces pathological intracellular lipid and cholesterol storage. J Gene Med 6: 166–175.

ClinicalTrials.gov (2006). NOGA angiogenesis revascularization therapy: evaluation by radionuclide imaging—the northern trial. ClinicalTrials.gov. <http://www.clinicaltrials.gov>.

ClinicalTrials.gov (2006). Safety and efficacy study of Ad2/hypoxia inducible factor (HIF)-1a/VP16 gene transfer in patients with intermittent claudication. ClinicalTrials.gov. <http://www.clinicaltrials.gov>.

ClinicalTrials.gov (2007). Angiogenesis in women with angina pectoris who are not candidates for revascularization (AWARE). ClinicalTrials.gov. <http://www.clinicaltrials.gov>.

Coffin, RS, Howard, MK, Cumming, DV, Dollery, CM, McEwan, J, Yellon, DM et al (1996). Gene delivery to the heart in vivo and to cardiac myocytes and vascular smooth muscle cells in vitro using herpes virus vectors. Gene Ther 3: 560–566.

Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest. 1989 Nov;84(5):1470-8.

Cooper K, Brailsford SC, Davies R, Raftery J. A review of health care models for coronary heart disease interventions. Health Care Manag Sci. 2006 Nov;9(4):311-24. Review.

Cowell, I. G. 1994. Repression versus activation in the control of gene transcription. Trends Biochem. Sci. 19:38-92.

Culp WC, Porter TR, McCowan TC, et al. Microbubble-augmented ultrasound declotting of thrombosed arteriovenous dialysis grafts in dogs J Vasc Interv Radiol 2003;14:343-347.

Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ *et al.* Targeting gene expression to hypoxic tumor cells. *Nat Med* 1997; 3: 515–520.

Dai Y, Xu M, Wang Y, Pasha Z, Li T, Ashraf M. HIF-1alpha induced-VEGF overexpression in bone marrow stem cells protects cardiomyocytes against ischemia. J Mol Cell Cardiol. 2007 Jun;42(6):1036-44. Epub 2007 Apr 6.

Dai, Q, Huang, J, Klitzman, B, Dong, C, Goldschmidt-Clermont, PJ, March, KL et al. (2004). Engineered zinc finger-activating vascular endothelial growth factor transcription factor plasmd DiNA induces therapeutic angiogenesis in rabbits with hindlimb ischemia. Circulation 110: 2467–2475.

Dames, S. A., M. Martinez-Yamout, R. N. De Guzman, H. J. Dyson, and P. E. Wright. 2002. Structural basis for Hif-1 alpha /CBP recognition in the cellular hypoxic response. Proc. Natl. Acad. Sci. USA 99:5271-5276.

D'Angelo G, Ladoux A, Frelin C. Hypoxia-induced transcriptional activation of vascular endothelial growth factor is inhibited by serum. Biochem Biophys Res Commun. 2000 Jan 7;267(1):334-8.

Dayton PA, Morgan KE, Klibanov AL, Brandenburger GH, Ferrara KW. Optical and acoustical observations on the effects of ultrasound on contrast agents IEEE Trans Ultrason Ferroelectr Control 1999;46;:220-232.

Dhar, M., E. M. Mascareno, and M. A. Q. Siddiqui. 1997. Two distinct factor-binding DNA elements in cardiac myosin light chain-2 gene are essential for repression of its expression in skeletal muscle. J. Biol. Chem. 272:18490-18497.

Dhond MR, Nguyen TT, Dolan C, Pulido G, Bommer WJ. Ultrasound-enhanced thrombolysis at 20 kHz with air-filled and perfluorocarbon-filled contrast biospheres J Am Soc Echocardiogr 2000;13:1025-1029.

Diederich KW, Eisele I, Ried T, Jaenicke T, Lichter P, Vosberg HP. Isolation and characterization of the complete human beta-myosin heavy chain gene. Hum Genet. 1989 Feb;81(3):214-20.

Diffee, GM, McCue SA, LaRosa A, Herrick RE, and Baldwin KM. Interaction of various mechanical activity models in regulation of myosin heavy chain isoform expression. *J Appl Physiol* 74: 2517-2522, 1993.

Dishart, KL, Denby, L, George, SJ, Nicklin, SA, Yendluri, S, Tuerk, MJ et al. (2003). Third-generation lentivirus vectors efficiently transduce and phenotypically modify vascular cells: implications for gene therapy. J Mol Cell Cardiol 35: 739–748.

Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta cells are formed by self-duplication rather than stem cell differentiation. Nature 2004; 429: 41–46.

Dor, Y, Djonov, V, Abramovitch, R, Itin, A, Fishman, GI, Carmeliet, P et al. (2002). Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. EMBO J 21: 1939–1947.

Duan D, Yue Y, Yan Z, Engelhardt JF. A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat Med.* 2000; 6: 595–598.

Durand RE, Raleigh JA. Identification of nonproliferating but viable hypoxic tumor cells *in vivo*. *Cancer Res* 1998; 58: 3547–3550.

E. Teiger, I. Deprez, V. Fataccioli, S. Champagne, J.L. Dubois-Randé, M. Eloit, S. Adnot. Gene therapy in heart disease. *Biomed Pharmacother* 2001 ; 55 : 148-54

Edwards, JG, Bahl JJ, Flink IL, Milavetz J, Goldman S, and Morkin E. A repressor region in the human β-myosin heavy chain gene that has a partial position dependency. *Biochem Biophys Res Commun* 189: 504-510, 1992.

Eitenmuller, I, Volger, O, Kluge, A, Troidl, K, Barancik, M, Cai, WJ et al. (2006). The range of adaptation by collateral vessels after femoral artery occlusion. Circ Res 99: 656–662.

Endoh M, Koibuchi N, Sato M, Morishita R, Kanzaki T, Murata Y, Kaneda Y. Fetal gene transfer by intrauterine injection with microbubble-enhanced ultrasound Mol Ther 2002;5:501-508.

Erikson JM, Freeman GL, Chandrasekar B. Ultrasound-targeted antisense oligonucleotide attenuates ischemia/reperfusion-induced myocardial tumor necrosis factor-alpha J Mol Cell Cardiol 2003;35:119-130.

Eslami, MH, Gangadharan, SP, Sui, X, Rhynhart, KK, Snyder, RO and Conte, MS (2000). Gene delivery to in situ veins: differential effects of adenovirus and adeno-associated viral vectors. J Vasc Surg 31: 1149–1159.

Farrance, IKG, Mar JH, and Ordahl CP. M-CAT binding factor is related to the SV40 enhancer binding factor, TEF-1. *J Biol Chem* 267: 17234-17240, 1992.

Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I et al. Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 2000; 6: 568–572

Fichou, Y and Ferec, C (2006). The potential of oligonucleotides for therapeutic applications. Trends Biotechnol 24: 563–570.

Firschke C, Lindner JR, Wei K, Goodman NC, Skyba DM, Kaul S. Myocardial perfusion imaging in the setting of coronary artery stenosis and acute myocardial infarction using venous injection of a second-generation echocardiographic contrast agent Circulation 1997;96:959-967.

Firth, J. D., B. L. Ebert, and P. J. Ratcliffe. 1995. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J. Biol. Chem. 270:21021-21027.

Firth, S. M., and R. C. Baxter. 2002. Cellular actions of the insulin-like growth factor binding proteins. Endocr. Rev. 23:824-854.

Flink, IL, and Morkin E. Alternatively processed isoforms of cellular nucleic acid-binding protein interact with a suppressor region of the human β-myosin heavy chain gene. *J Biol Chem* 270: 6959-6965, 1995.

Flink, IL, Edwards JG, Bahl JJ, Liew C-C, Sole M, and Morkin E. Characterization of a strong positive *cis*-acting element of the human β-myosin heavy chain gene in fetal rat heart cells. *J Biol Chem* 267: 9917-9924, 1992.

Folkman J.Tumor angiogenesis. Adv Cancer Res. 1985;43:175-203. Review.

Fortuin, FD, Vale, P, Losordo, DW, Symes, J, DeLaria, GA, Tyner, JJ et al. (2003). One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. Am J Cardiol 92: 436–439.

Francis SC, Katovich MJ, Gelband CH, Raizada MK. Gene therapy in cardiovascular disease. Current status. Am J Pharmacogenomics. 2001;1(1):55-66. Review.

Franz WM, Rothmann T, Frey N, Katus HA. Analysis of tissue-specific gene delivery by recombinant adenoviruses containing cardiac-specific promoters. *Cardiovasc Res.* 1997; 35: 560–566.

Frenkel PA, Chen S, Thai T, Shohet RV, Grayburn PA. DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection in vitro Ultrasound Med Biol 2002;28:817-822.

Furukoji, E, Matsumoto, M, Yamashita, A, Yagi, H, Sakurai, Y, Marutsuka, K et al. (2005). Adenovirus-mediated transfer of human placental ectonucleoside triphosphate diphosphohydrolase to vascular smooth muscle cells suppresses platelet aggregation in vitro and arterial thrombus formation in vivo. Circulation 111: 808–815.

Gagandeep S, Brew R, Green B, Christmas SE, Klatzmann D, Poston GJ *et al.* Prodrug-activated gene therapy: involvement of an immunological component in the 'bystander effect'. *Cancer Gene Ther* 1996; 3: 83–88.

Galson, D. L., T. Tsuchiya, D. S. Tendler, L. E. Huang, Y. Ren, T. Ogura, and H. F. Bunn. 1995. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol. Cell. Biol. 15:2135-2144.

Geisterfer-Lawiance AAT, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, Seidman JG. Molecular basis for familial hypertrophic cardiomyopathy: a cardiac myosin heavy chain gene missense mutation. *Cell.* 1990; 12: 999–1006.

Gelling RW, Du XQ, Dichmann DS, Romer J, Huang H, Cui L et al. Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagons receptor knockout mice. Proc Natl Acad Sci USA 2003; 100: 1438–1443.

Giordano, FJ, Ping, P, McKirnan, MD, Nozaki, S, DeMaria, AN, Dillmann, WH et al. (1996). Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nat Med 2: 534–539.

Gordon, T, and Pattullo MC. Plasticity of muscle fibers and moter unit types. *Exerc Sport Sci Rev* 21: 331-362, 1993.

Gowdak, LH, Poliakova, L, Wang, X, Kovesdi, I, Fishbein, KW, Zacheo, A et al. (2000). Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. Circulation 102: 565–571.

Gracey, A. Y., J. V. Troll, and G. N. Somero. 2001. Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. Proc. Natl. Acad. Sci. USA 98:1993-1998.

Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, Gallick GE. HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene. 2005 Apr 28;24(19):3110-20.

Greco O, Marples B, Dachs GU, Williams KJ, Patterson AV, Scott SD. Novel chimeric gene promoters responsive to hypoxia and ionizing radiation. *Gene Therapy* 2002; 9: 1403–1411.

Greco O, Marples B, Joiner MC, Scott SD. How to overcome (and exploit) tumor hypoxia for targeted gene therapy. *J Cell Physiol* 2003; 197: 312–325.

Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, van de Wiel MA, Belien JA, van Diest PJ, van der Wall E. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol. 2005 Jul;206(3):291-304.

Greve, JM, Chico, TJ, Goldman, H, Bunting, S, Peale, FV Jr., Daugherty, A et al. (2006). Magnetic resonance angiography reveals therapeutic enlargement of collateral vessels induced by VEGF in a murine model of peripheral arterial disease. J Magn Reson Imaging 24: 1124–1132.

Grines, CL, Watkins, MW, Helmer, G, Penny, W, Brinker, J, Marmur, JD et al. (2002). Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. Circulation 105: 1291–1297.

Grines, CL, Watkins, MW, Mahmarian, JJ, Iskandrian, AE, Rade, JJ, Marrott, P et al. (2003). A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina. J Am Coll Cardiol 42: 1339–1347.

Griscelli F, Gilardi-Hebenstreit P, Hanania N, Franz WM, Opolon P, Perricaudet M, Ragot T. Heart-specific targeting of beta-galactosidase by the ventricle-specific cardiac myosin light chain 2 promoter using adenovirus vectors. *Hum Gene Ther.* 1998; 9: 1919–1928.

Grossman, M, Rader, DJ, Muller, DW, Kolansky, DM, Kozarsky, K, Clark, BJ 3rd et al. (1995). A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. Nat Med 1: 1148–1154.

Gruchala, M, Bhardwaj, S, Pajusola, K, Roy, H, Rissanen, TT, Kokina, I et al. (2004). Gene transfer into rabbit arteries with adeno-associated virus and adenovirus vectors. J Gene Med 6: 545–554.

Guillemin, K., and M. A. Krasnow. 1997. The hypoxic response: huffing and HIFing. Cell 89:9-12.

Gulick J., Subramaniam A. Neumann J, Robbins J. *et al*. Isolation and Characterization of the Mouse Cardiac Myosin Heavy Chain Genes. *The Journal of Biological Chemistry*. 1991; 266: 9180-9185

Gupta, M. P., C. S. Amin, M. Gupta, N. Hay, and R. Zak. 1997. Transcription enhancer factor-1 interacts with a basic helix-loop-helix protein, Max, for positive regulation of cardiac α-myosin heavy chain gene expression. Mol. Cell. Biol. 17:3924-3936.

Gupta, M. P., M. Gupta, and R. Zak. 1994. An E-box/M-CAT hybrid motif and cognate binding protein regulate the basal muscle-specific and cAMP-inducible expression of the rat cardiac α-myosin heavy chain gene. J. Biol. Chem. 269:29677-29687.

Gupta, M. P., M. Gupta, R. Zak, and V. P. Sukhatme. 1991. Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac α-myosin heavy chain gene. J. Biol. Chem. 266:12813-12816.

Guz Y, Nasir I, Teitelman G. Regeneration of pancreatic beta cells from intra-islet precursor cells in an experimental model of diabetes. Endocrinology 2001; 142: 4956–4968.

Haddad, F, Arnold C, Zeng M, and Baldwin KM. Interaction of thyroid state and denervation on skeletal myosin heavy chain expression. *Muscle Nerve* 20: 1487-1496, 1997.

Haddad, F, Herrick RE, Adams GR, and Baldwin KM. Myosin heavy chain expression in rodent skeletal muscle: effects of exposure to zero gravity. *J Appl Physiol* 75: 2471-2477, 1997.

Haddad, F, Qin A, McCue SA, and Baldwin KM. Thyroid receptor plasticity in striated muscle-types: effects of altered thyroid state. *Am J Physiol Endocrinol Metab* 274: E1018-E1026, 1998.

Haddad, F, Qin A, Zeng M, McCue SA, and Baldwin KM. Interaction of hyperthyroidism and hindlimb suspension on skeletal myosin heavy chain expression. *J Appl Physiol* 85: 2227-2266, 1998.

Hagemeier, C., A. J. Bannister, A. Cook, and T. Kouzarides. 1993. The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID *in vitro*: RB shows sequence similarity to TFIID and TFIIB. Proc. Natl. Acad. Sci. USA 90:1580-1584.

Hambleton, M, Hahn, H, Pleger, ST, Kuhn, MC, Klevitsky, R, Carr, AN et al. (2006). Pharmacological- and gene therapy-based inhibition of protein kinase Calpha/beta enhances cardiac contractility and attenuates heart failure. Circulation 114: 574–582.

Hammond HK, McKirnan MD. Angiogenic gene therapy for heart disease: a review of animal studies and clinical trials. Cardiovasc Res. 2001 Feb 16;49(3):561-7.

Hao E, Tyrberg B, Itkin-Ansari P, Lakey J, Geron I, Monosov EZ et al. Beta-cell differentiation from non-endocrine epithelial cells of the adult human pancreas. Nat Med 2006; 12: 310–316.

Hao, X, Mansson-Broberg, A, Grinnemo, KH, Siddiqui, AJ, Dellgren, G, Brodin, LA et al. (2007). Myocardial angiogenesis after plasmid or adenoviral VEGF-A(165) gene transfer in rat myocardial infarction model. Cardiovasc Res 73: 481–487.

Hashiya N, Aoki M, Tachibana K, et al. Local delivery of E2F decoy oligodeoxynucleotides using ultrasound with microbubble agent (Optison) inhibits intimal hyperplasia after balloon injury in rat carotid artery model Biochem Biophys Res Commun 2004;317:508-514.

Hawley-Nelson P, Ciccarone V, Moore ML. Transfection of cultured eukaryotic cells using cationic lipid reagents. Curr Protoc Mol Biol. 2008 Jan;Chapter 9:Unit 9.4.

Hedman, M, Hartikainen, J, Syvanne, M, Stjernvall, J, Hedman, A, Kivela, A et al. (2003). Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). Circulation 107: 2677–2683.

Heine HL, Leong HS, Rossi FM, McManus BM, Podor TJ. Strategies of conditional gene expression in myocardium: an overview. Methods Mol Med. 2005;112:109-54. Review.

Helmke SM, Robbins J, et al. Analysis of Myosin Heavy Chain Functionality in the Heart. *The Journal of Biological Chemistry*. 2003; 278: 17466-17474

Henderson SA, Spencer M, Sen A, Kumar C, Siddiqui MAQ, Chien KR. Structure, organization, and expression of the rat cardiac myosin light chain-2 gene. Identification of a 250-based pair fragment which confers cardiac-specific expression. *J Biol Chem.* 1989; 264: 18142–18148.

Henry, TD, Annex, BH, McKendall, GR, Azrin, MA, Lopez, JJ, Giordano, FJ et al. (2003). The VIVA trial: vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation 107: 1359–1365.

Henry, TD, Mendelsohn, F, Comerota, A, Pham, E, Grek, V, Coleman, M et al. (2006). Dose and regimen effects of intramuscular NV1FGF in patients with critical limb ischemia: a randomized, double-blind, placebo controlled study. Eur Heart J 27 (Suppl 1): 235.

Hiasa, K, Ishibashi, M, Ohtani, K, Inoue, S, Zhao, Q, Kitamoto, S et al. (2004). Gene transfer of stromal cell-derived factor-1α enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization. Circulation 109: 2454–2461.

Hiltunen, MO, Laitinen, M, Turunen, MP, Jeltsch, M, Hartikainen, J, Rissanen, TT et al (2000). Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta. Circulation 102: 2262–2268.

Hiltunen, MO, Ruuskanen, M, Huuskonen, J, Mahonen, AJ, Ahonen, M, Rutanen, J et al. (2003). Adenovirus-mediated VEGF-A gene transfer induces bone formation in vivo. FASEB J 17: 1147–1149.

Hixson JE, Henkel RD, Britten ML, Vernier DT, deLemos RA, VandeBerg JL, Walsh RA. Alpha-myosin heavy chain cDNA structure and gene expression in adult, fetal, and premature baboon myocardium. J Mol Cell Cardiol. 1989 Oct;21(10):1073-86.

Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996; 56: 4509–4515.

Hockel M, Schlenger K, Hockel S, Vaupel P. Hypoxic cervical cancers with low apoptotic index are highly aggressive. *Cancer Res* 1999; 59: 4525–4528.

Hockel M, Vaupel P. Biological consequences of tumor hypoxia. *Semin Oncol* 2001; 28: 36–41.

Holt RG, Roy RA. Measurements of bubble-enhanced heating from focused, MHz-frequency ultrasound in a tissue-mimicking material Ultrasound Med Biol 2001;27:1399-1412.

Hoshijima, M, Ikeda, Y, Iwanaga, Y, Minamisawa, S, Date, MO, Gu, Y et al. (2002). Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. Nat Med 8: 864–871.

Hu, M. C., S. B. Sharp, and N. Davidson. 1986. The complete sequence of the mouse skeletal α-actin gene reveals several conserved and inverted repeat sequence outside of the protein-coding region. Mol. Cell. Biol. 6:15-25.

Huang LE, Gu J, Schau M, Bunn HR. Regulation of hypoxia-inducbile factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A.* 1998; 95: 7987–7992.

Huang, S. T., K. C. Vo, D. J. Lyell, G. H. Faessen, S. Tulac, R. Tibshirani, A. J. Giaccia, and L. C. Giudice. 2004. Developmental response to hypoxia. FASEB J. 18:1348-1365.

Huber PE, Pfisterer P. In vitro and in vivo transfection of plasmid DNA in the Dunning prostate tumor R3327-AT1 is enhanced by focused ultrasound Gene Ther 2000;7:1516-1525.

Iannello, R. C., J. H. Mar, and C. P. Ordahl. 1991. Characterization of a promoter element required for transcription in myocardial cells. J. Biol. Chem. 266:3309-3316.

Immonen, A, Vapalahti, M, Tyynela, K, Hurskainen, H, Sandmair, A, Vanninen, R et al. (2004). AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. Mol Ther10: 967–972.

Isner, JM, Baumgartner, I, Rauh, G, Schainfeld, R, Blair, R, Manor, O et al. (1998). Treatment of thromboangitis obliterans (Buerger's disease) by intramuscular gene transfer of vascular endothelial growth factor: preliminary clinical results. J Vasc Surg 28: 964–973.

Ito, WD, Arras, M, Winkler, B, Scholz, D, Schaper, J and Schaper, W (1997). Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. Circ Res 80: 829–837.

Ivan, M., K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane, and W. G. Kaelin, Jr. 2001. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 292:464-468.

Iyer, N. V., L. E. Kotch, F. Agani, S. W. Leung, E. Laughner, R. H. Wenger, M. Gassmann, J. D. Gearhart, A. M. Lawler, A. Y. Yu, and G. L. Semenza. 1998. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 12:149-162.

Izumo, S., B. Nadal-Ginard, and V. Mahdavi. 1986. All members of the myosin heavy chain multigene family respond to thyroid hormone in a highly tissue-specific manner. Science 231:597-600.

Jaakkola, P., D. R. Mole, Y. M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. Kriegsheim, H. F. Hebestreit, M. Mukherji, C. J. Schofield, P. H. Maxwell, C. W. Pugh, and P. J. Ratcliffe. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292:468-472.

Jalkanen, J, Leppanen, P, Pajusola, K, Narvanen, O, Mahonen, A, Vahakangas, E et al. (2003). Adeno-associated virus-mediated gene transfer of a secreted decoy human macrophage scavenger receptor reduces atherosclerotic lesion formation in LDL receptor knockout mice. Mol Ther 8: 903–910.

Janssens, S, Flaherty, D, Nong, Z, Varenne, O, Van Pelt, N, Haustermans, C et al. (1998). Human endothelial nitric oxide synthase gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation after balloon injury in rats. Circulation 97: 1274–1281.

Jiang B, Dong H, Zhang Z, Wang W, Zhang Y, Xu X. Hypoxic response elements control expression of human vascular endothelial growth factor(165) genes transferred to ischemia myocardium in vivo and in vitro. J Gene Med. 2007 Sep;9(9):788-96.

Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am J Physiol.* 1996; 271: C2272–C1180.

Jiang, H, Pierce, GF, Ozelo, MC, de Paula, EV, Vargas, JA, Smith, P et al. (2006). Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. Mol Ther 14: 452–455.

Joukov, V, Pajusola, K, Kaipainen, A, Chilov, D, Lahtinen, I, Kukk, E et al. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15: 290–298.

June Yin, Michael G. Kaplitt, Ann D. Kwong and Donald W. Pfaff . In situ PCR for in vivo detection of foreign genes transferred into rat brain. Brain research 1998, 783(2):347-54.

Kaczmarczyk SJ, Green JE. A single vector containing modified cre recombinase and LOX recombination sequences for inducible tissue-specific amplification of gene expression. *Nucleic Acids Res* 2001; 29 (12, e56): 1–13.

Kajimura, S., K. Aida, and C. Duan. 2005. Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. Proc. Natl. Acad. Sci. USA 102:1240-1245.

Kallio, P. J., K. Okamoto, S. O'Brien, P. Carrero, Y. Makino, H. Tanaka, and L. Poellinger. 1998. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1α. EMBO J. 17:6573-6586.

Kankkonen, HM, Turunen, MP, Hiltunen, MO, Lehtolainen, P, Koponen, J, Leppanen, P et al. (2004). Feline immunodeficiency virus and retrovirus-mediated adventitial ex vivo gene transfer to rabbit carotid artery using autologous vascular smooth muscle cells. J Mol Cell Cardiol 36: 333–341.

Kankkonen, HM, Vahakangas, E, Marr, RA, Pakkanen, T, Laurema, A, Leppanen, P et al. (2004). Long-term lowering of plasma cholesterol levels in LDL-receptor-deficient WHHL rabbits by gene therapy. Mol Ther 9: 548–556.

Kariya, K, Farrance IKG, and Simpson PC. Transcriptional enhancer factor-1 in cardiac myocytes interacts with an α_1-adrenergic- and β-protein kinase C-inducible element in the rat β-myosin heavy chain promoter. *J Biol Chem* 268: 26658-26662, 1993.

Kariya, K, Karns LR, and Simpson PC. An enhancer core element mediates stimulation of the rat β-myosin heavy chain promoter by an α_1-adrenergic agonist and activated β-protein kinase C in hypertrophy of cardiac myocytes. *J Biol Chem* 269: 3775-3782, 1994.

Kashiwakura, Y, Cho, HC, Barth, AS, Azene, E and Marban, E (2006). Gene transfer of a synthetic pacemaker channel into the heart: a novel strategy for biological pacing. Circulation 114: 1682–1686.

Kastelein, JJ, Wedel, MK, Baker, BF, Su, J, Bradley, JD, Yu, RZ et al. (2006). Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. Circulation 114: 1729–1735.

Kastrup, J, Jorgensen, E, Ruck, A, Tagil, K, Glogar, D, Ruzyllo, W et al. (2005). Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. J Am Coll Cardiol 45: 982–988.

Kate L. Dishart, Lorraine M. Work, Laura Denby, and Andrew H. Baker. Gene Therapy for Cardiovascular Disease. *Jounal of Biomed Biotechnol*. 2003; 2003(2): 138–148.

Kawamoto, S, Shi, Q, Nitta, Y, Miyazaki, J and Allen, MD (2005). Widespread and early myocardial gene expression by adeno-associated virus vector type 6 with a beta-actin hybrid promoter. Mol Ther 11: 980–985.

Kibbe, MR, Tzeng, E, Gleixner, SL, Watkins, SC, Kovesdi, I, Lizonova, A et al. (2001). Adenovirus-mediated gene transfer of human inducible nitric oxide synthase in porcine vein grafts inhibits intimal hyperplasia. J Vasc Surg 34: 156–165.

Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. *Exp Cell Res.* 1976; 98: 367–381.

Kimura H, Weisz A, Kurashima Y, Hashimoto K, Ogura T, D'Acquisto F, Addeo R, Makuuchi M, Esumi H. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood. 2000 Jan 1;95(1):189-97.

Kimura, H., A. Weisz, T. Ogura, Y. Hitomi, Y. Kurashima, K. Hashimoto, F. D'Acquisto, M. Makuuchi, and H. Esumi. 2001. Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. J. Biol. Chem. 276:2292-2298.

Kipshidze NN, Porter TR, Dangas G, et al. Systemic targeted delivery of antisense with perflourobutane gas microbubble carrier reduced neointimal formation in the porcine coronary restenosis model Cardiovasc Radiat Med 2003;4:152-159.

Kitajima, K, Marchadier, DH, Miller, GC, Gao, GP, Wilson, JM and Rader, DJ (2006). Complete prevention of atherosclerosis in apoE-deficient mice by hepatic human apoE gene transfer with adeno-associated virus serotypes 7 and 8. Arterioscler Thromb Vasc Biol 26: 1852–1857.

Klibanov AL, Ferrara KW, Hughes MS, Wible JH Jr, Wojdyla JK, Dayton PA, Morgan KE, Brandenburger GH. Direct video-microscopic observation of the dynamic effects of medical ultrasound on ultrasound contrast microspheres. Invest Radiol. 1998 Dec;33(12):863-70.

Knisely JP, Rockwell S. Importance of hypoxia in the biology and treatment of brain tumors. *Neuroimaging Clin N Am* 2002; 12: 525–536.

Knotts, S, Rindt H, and Robbins J. Position independent expression and developmental regulation is directed by the β myosin heavy chain gene's 5' upstream region in transgenic mice. *Nucleic Acids Res* 23: 3301-3309, 1995.

Knotts, S, Rindt H, Neumann J, and Robbins J. In vivo regulation of the mouse β myosin heavy chain gene. *J Biol Chem* 269: 31275-31282, 1994.

Kojima H, Fujimaya M, Matsumura K, Younan P, Imaeda H, Maeda M et al. NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 2003; 9: 596–603.

Kojima H, Nakamura T, Fujita Y, Kishi A, Fujimiya M, Yamada S et al. Combined expression of pancreatic duodenal homeobox 1 and islet factor 1 induces immature enterocytes to produce insulin. Diabetes 2002; 51: 1398–1408.

Komatsu DE, Hadjiargyrou M.Activation of the transcription factor HIF-1 and its target genes, VEGF, HO-1, iNOS, during fracture repair. Bone. 2004 Apr;34(4):680-8.

Kondo I, Ohmori K, Oshita A, et al. Treatment of acute myocardial infarction by hepatocyte growth factor gene transfer J Am Coll Cardiol 2004;44:644-653.

Korpanty G, Chen S, Shohet RV, Ding J, Yang B, Frenkel PA, Grayburn PA. Targeting of VEGF-mediated angiogenesis to rat myocardium using ultrasonic destruction of microbubbles. *Gene Ther.* 2005 Sep;12(17):1305-12.

Koshikawa N, Takenaga K, Tagawa M, Sakiyama S. Therapeutic efficacy of the suicide gene driven by the promoter of vascular endothelial growth factor gene against hypoxic tumor cell. *Cancer Res* 2000; 60: 2936-2941.

Kowabata K, Takakura Y, Hashida M. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake Pharm Res 1995;12:825-830.

Kusano, KF, Pola, R, Murayama, T, Curry, C, Kawamoto, A, Iwakura, A et al. (2005). Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. Nat Med 11: 1197–1204.

Kusumanto, YH, Weel, VV, Mulder, NH, Smit, AJ, Dungen, JJ, Hooymans, JM et al. (2006). Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. Hum Gene Ther 17: 683–691.

Kutryk, MJ, Foley, DP, van den Brand, M, Hamburger, JN, van der Giessen, WJ, deFeyter, PJ et al. (2002). Local intracoronary administration of antisense oligonucleotide against c-myc for the prevention of in-stent restenosis: results of the randomized investigation by the thoraxcenter of antisense DNA using local delivery and IVUS after coronary stenting (ITALICS) trial. J Am Coll Cardiol 39: 281–287.

Kvietikova, I., R. H. Wenger, H. H. Marti, and M. Gassmann. 1995. The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. Nucleic Acids Res. 23:4542-4550.

Labarca, C, and Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 102: 344-352, 1980.

Lai, NC, Roth, DM, Gao, MH, Tang, T, Dalton, N, Lai, YY et al. (2004). Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure. Circulation 110: 330–336.

Laitinen, M, Hartikainen, J, Hiltunen, MO, Eränen, J, Kiviniemi, M, Närvänen, O et al. (2000). Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. Hum Gene Ther 11: 263–270.

Laitinen, M, Mäkinen, K, Manninen, H, Matsi, P, Kossila, M, Agrawal, RS et al. (1998). Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. Hum Gene Ther 9: 1481–1486.

Laitinen, M, Pakkanen, T, Donetti, E, Baetta, R, Luoma, J, Lehtolainen, P et al. (1997). Gene transfer into the carotid artery using an adventitial collar: comparison of the effectiveness of the plasmid- liposome complexes, retroviruses, pseudotyped retroviruses, and adenoviruses. Hum Gene Ther 8: 1645–1650.

Laugwitz, KL, Moretti, A, Weig, HJ, Gillitzer, A, Pinkernell, K, Ott, T et al. (2001). Blocking caspase-activated apoptosis improves contractility in failing myocardium. Hum Gene Ther 12: 2051–2063.

Laukkanen, MO, Kivela, A, Rissanen, T, Rutanen, J, Karkkainen, MK, Leppanen, O et al. (2002). Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta. Circulation 106: 1999–2003.

Lawrence MS *et al*. Herpes simplex viral vectors expressing Bcl-2 are neuroprotective when delivered after a stroke. *J Cereb Blood Flow Metab* 1997; 17: 740-744.

Lawrie A, Brisken AF, Francis SE, Cumberland DC, Crossman DC, Newman CM. Microbubble-enhanced ultrasound for vascular gene delivery Gene Ther 2000;7:2023-2027.

Lazarous, DF, Shou, M, Stiber, JA, Hodge, E, Thirumurti, V, Goncalves, L et al. (1999). Adenoviral-mediated gene transfer induces sustained pericardial VEGF expression in dogs: effect on myocardial angiogenesis. Cardiovasc Res 44: 294–302.

Lebherz, C, Gao, G, Louboutin, JP, Millar, J, Rader, D and Wilson, JM (2004). Gene therapy with novel adeno-associated virus vectors substantially diminishes atherosclerosis in a murine model of familial hypercholesterolemia. J Gene Med 6: 663–672.

Lederman, RJ, Mendelsohn, FO, Anderson, RD, Saucedo, JF, Tenaglia, AN, Hermiller, JB et al. (2002). Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. Lancet 359: 2053–2058.

Lee KJ, Hickey R, Zhu H, Chien KR. Positive regulatory elements (HF-1a) and HF-1b) and a novel negative regulatory element (HF-3) mediate ventricular muscle-specific expression of myosin light chain 2-luciferase fusion genes in transgene mice. *Mol Cell Biol.* 1994; 14: 1220–1229.

Lee KJ, Ross RS, Rockman HA, Harris AN, O'Brien TX, Van Bilsen M, Shubeita HE, Kandolf R, Brem G, Price J, Evans SM, Zhu H, Franz WM, Chien KR. Myosin light chain-2 luciferase transgenic mice reveal distinct regulatory program for cardiac and skeletal muscle-specific expression of a single contractile protein gene. *J Biol Chem.* 1992; 267: 15875–15885.

Lee, LY, Patel, SR, Hackett, NR, Mack, CA, Polce, DR, El-Sawy, T et al. (2000). Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endothelial growth factor 121. Ann Thorac Surg 69: 14–23.

Lee, RJ, Springer, ML, Blanco-Bose, WE, Shaw, R, Ursell, PC and Blau, HM (2000). VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation 102: 898–901.

Lee, Y., B. Nadal-Ginard, V. Mahdavi, and S. Izumo. 1997. Myocyte-specific enhancer factor-2 and thyroid hormone receptor associate and synergistically activate the α -myosin heavy chain gene. Mol. Cell. Biol. 17:2745-2755.

Lemstrom, KB, Krebs, R, Nykanen, AI, Tikkanen, JM, Sihvola, RK, Aaltola, EM et al. (2002). Vascular endothelial growth factor enhances cardiac allograft arteriosclerosis. Circulation 105: 2524–2530.

Leong-Poi H, Le E, Rim SJ, Sakuma T, Kaul S, Wei K. Quantification of myocardial perfusion and determination of coronary stenosis severity during hyperemia using real-time myocardial contrast echocardiography J Am Soc Echocardiogr 2001;14:1173-1182.

Leotta, E, Patejunas, G, Murphy, G, Szokol, J, McGregor, L, Carbray, J et al. (2002). Gene therapy with adenovirus-mediated myocardial transfer of vascular endothelial growth factor 121 improves cardiac performance in a pacing model of congestive heart failure. J Thorac Cardiovasc Surg 123: 1101–1113.

Li P, Cao LQ, Dou CY, Armstrong WF, Miller D. Impact of myocardial contrast echocardiography on vascular permeability: an in vivo dose response study of delivery mode, pressure amplitude and contrast dose Ultrasound Med Biol 2003;29:1341-1349.

Li Q, Bolli R, Qui Y, Tang XL, Murphree SS, French BA. Gene therapy with extracellular superoxide dismutase attenuates myocardial stunning in conscious rabbits. *Circulation.* 1998; 98: 1438–1448.

Li, X, Tjwa, M, Moons, L, Fons, P, Noel, A, Ny, A et al. (2005). Revascularization of ischemic tissues by PDGF-CC via effects on endothelial cells and their progenitors. J Clin Invest 115: 118–127.

Liew, C. C., M. J. Sole, K. Y. Takihara, B. Kellan, D. H. Anderson, L. Lin, and J. C. Liew. 1990. Complete sequence and organization of the human cardiac β-myosin heavy chain gene. Nucleic Acids Res. 18:3647-3651.

Lipsett M, Aikin R, Castellarin M, Hanley S, Jamal A, Laganiere S et al. Islet neogenesis: a potential therapeutic tool in type 1 diabetes. Int J Biochem Cell Biol 2006; 38: 498–503.

Liu, Y., S. R. Cox, T. Morita, and S. Kourembanas. 1995. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ. Res. 77:638-643.

Lobov, IB, Brooks, PC and Lang, RA (2002). Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc Natl Acad Sci USA 99: 11205–11210.

Lodish, Harvey; Berk, Arnold; Zipursky, S. Lawrence; Matsudaira, Paul; Baltimore, David; Darnell, James E. Molecular Cell Biology: New York: W. H. Freeman & Co. ; c1999

Losordo, DW, Vale, PR, Hendel, RC, Milliken, CE, Fortuin, FD, Cummings, N et al. (2002). Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. Circulation 105: 2012–2018.

Losordo, DW, Vale, PR, Symes, JF, Dunnington, CH, Esakof, DD, Maysky, M et al. (1998). Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF(165) as sole therapy for myocardial ischemia. Circulation 98: 2800–2804.

Lu QL, Liang HD, Partridge T, Blomley MJ. Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage Gene Ther 2003;10:396-405.

Lu Y. Recombinant adeno-associated virus as delivery vector for gene therapy--a review. Stem Cells Dev. 2004 Feb;13(1):133-45. Review.

Lyons, G. E., S. Schiaffino, D. Sassoon, P. Barton, and M. Buckingham. 1990. Developmental regulation of myosin heavy chain gene expression in mouse cardiac muscle. J. Cell Biol. 111:2427-2436.

Mack, CA, Patel, SR, Schwarz, EA, Zanzonico, P, Hahn, RT, Ilercil, A et al. (1998). Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. J Thorac Cardiovasc Surg 115: 168–176.

Mahdavi, V., A. P. Chambers, and B. Nadal-Ginard. 1984. Cardiac α- and β-myosin heavy chain genes are organized in tandem. Proc. Natl. Acad. Sci. USA 81:2628-2630.

Makinen, K, Manninen, H, Hedman, M, Matsi, P, Mussalo, H, Alhava, E et al. (2002). Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. Mol Ther 6: 127–133.

Maltepe, E., J. V. Schmidt, D. Baunoch, C. A. Bradfield, and M. C. Simon. 1997. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature 386:403-407.

Marmottant P, Hilgenfeldt S. Controlled vesicle deformation and lysis by single oscillating bubbles Nature 2003;423:153-156.

Masaki, I, Yonemitsu, Y, Komori, K, Ueno, H, Nakashima, Y, Nakagawa, K et al. (2001). Recombinant Sendai virus-mediated gene transfer to vasculature: a new class of efficient gene transfer vector to the vascular system. FASEB J 15: 1294–1296.

Masson, N., C. Willam, P. H. Maxwell, C. W. Pugh, and P. J. Ratcliffe. 2001. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J. 20:5197-5206.

Mathiesen, I (1999). Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther 6: 508–514.

Maurice, JP, Hata, JA, Shah, AS, White, DC, McDonald, PH, Dolber, PC et al. (1999). Enhancement of cardiac function after adenoviral-mediated in vivo intracoronary beta2-adrenergic receptor gene delivery. J Clin Invest 104: 21–29.

Maxwell, P. H., M. S. Wiesener, G. W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher, and P. J. Ratcliffe. 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271-275.

Maynard MA, Ohh M. Molecular targets from VHL studies into the oxygen-sensing pathway. Curr Cancer Drug Targets. 2005 Aug;5(5):345-56. Review.

McCarthy, JJ, Fox M, Tsika GL, Gao L, and Tsika RW. β-MHC transgene expression in suspended and mechanically overloaded/suspended soleus muscle of transgenic mice. *Am J Physiol Regulatory Integrative Comp Physiol* 272: R1552-R1561, 1997.

McCarthy, JJ, Vyas D, Tsika GL, and Tsika RW. Segregated regulatory elements direct β-myosin heavy chain expression in response to altered muscle activity. *J Biol Chem* 274: 14270-14279, 1999.

McCully, J. D., R. X. Wang, B. Kellam, M. J. Sole, and C. C. Liew. 1991. Isolation and characterization of a previously unrecognized myosin heavy chain gene present in the Syrian hamster. J. Mol. Biol. 218:657-665.

McLellan, K. C., S. B. Hooper, A. D. Bocking, P. J. Delhanty, I. D. Phillips, D. J. Hill, and V. K. Han. 1992. Prolonged hypoxia induced by the reduction of maternal uterine blood flow alters insulin-like growth factor-binding protein-1 (IGFBP-1) and IGFBP-2 gene expression in the ovine fetus. Endocrinology 131:1619-1628.

McMahon, JM, Wells, KE, Bamfo, JE, Cartwright, MA and Wells, DJ (1998). Inflammatory responses following direct injection of plasmid DNA into skeletal muscle. Gene Ther 5: 1283–1290.

McMurtry, MS, Archer, SL, Altieri, DC, Bonnet, S, Haromy, A, Harry, G et al. (2005). Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension. J Clin Invest 115: 1479–1491.

Medline Plus Online. "Gene Therapy." 2008 http://www.nlm.nih.gov/medlineplus (2 Jan. 2008)

Melo, LG, Agrawal, R, Zhang, L, Rezvani, M, Mangi, AA, Ehsan, A et al. (2002). Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene. Circulation 105: 602–607.

Menendez JA, Vellon L, Oza BP, Lupu R. Does endogenous fatty acid metabolism allow cancer cells to sense hypoxia and mediate hypoxic vasodilatation? Characterization of a novel molecular connection between fatty acid synthase (FAS) and hypoxia-inducible factor-1alpha (HIF-1alpha)-related expression of vascular endothelial growth factor (VEGF) in cancer cells overexpressing her 2/neu oncogene. J Cell Biochem. 2005 Apr 1;94(5):857-63.

Merriam-Webster Online Dictionary. "Gene Therapy." 2008. http://www.merriamwebster.com (2 Jan. 2008).

Michiels C, Minet E, Michel G, Mottet D, Piret JP, Raes M. HIF-1 and AP-1 cooperate to increase gene expression in hypoxia: role of MAP kinases. IUBMB Life. 2001 Jul;52(1-2):49-53. Review.

Miller DL, Bao S, Gres RA, Thrall BD. Ultrasonic enhancement of gene transfection in murine melanoma tumors Ultrasound Med Biol 1999;25:1425-1430.

Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation Somat Cell Mol Genet 2002;27:115-134.

Miller DL, Quddus J. Diagnostic ultrasound activation of contrast agent gas bodies induces capillary rupture in mice Proc Natl Acad Sci USA 2000;97:10179-10184.

Miller DL, Quddus J. Sonoporation of monolayer cells by diagnostic ultrasound activation of contrast agent gas bodies Ultrasound Med Biol 2000;26:661-667.

Miller, DL, Ortega, S, Bashayan, O, Basch, R and Basilico, C (2000). Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. Mol Cell Biol 20: 2260–2268.

Miller, WH, Brosnan, MJ, Graham, D, Nicol, CG, Morecroft, I, Channon, KM et al. (2005). Targeting endothelial cells with adenovirus expressing nitric oxide synthase prevents elevation of blood pressure in stroke-prone spontaneously hypertensive rats. Mol Ther 12: 321–327.

Miniño AM, Heron MP, Murphy SL, Kochankek, KD. Deaths: Final Data for 2004. National vital statistics reports; vol 55 no 19. Hyattsville, MD: National Center for Health Statistics. 2007.

Minty, A., and L. Kedes. 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. 6:2125-2136.

Miyamoto, MI, del Monte, F, Schmidt, U, DiSalvo, TS, Kang, ZB, Matsui, T et al. (2000). Adenoviral gene transfer of SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. Proc Natl Acad Sci USA 97: 793–798.

Mizushige K, Kondo I, Ohmori K, Hirao K, Matsuo H. Enhancement of ultrasound-accelerated thrombolysis by echo contrast agents: dependence on microbubble structure Ultrasound Med Biol 1999;25:1431-1437.

Molkentin JD, Jobe SM, Markham BE. Alpha-myosin heavy chain gene regulation: delineation and characterization of the cardiac muscle-specific enhancer and muscle-specific promoter. J Mol Cell Cardiol. 1996 Jun;28(6):1211-25.

Molkentin, J. D., and B. E. Markham. 1994. An M-CAT binding factor and an RSRF-related A-rich binding factor positively regulate expression of the α-cardiac myosin heavy chain gene in vivo. Mol. Cell. Biol. 14:5056-5065.

Molkentin, J. D., D. V. Klavakolann, and B. E. Markham. 1994. Transcription factor GATA-4 regulates cardiac muscle specific expression of the α-myosin heavy chain gene. Mol. Cell. Biol. 14:4947-4957.

Molkentin, J. D., J. Lu, C. Antos, B. E. Markham, J. Richardson, J. Robbins, S. Grant, and E. Olson. 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93:215-228.

Mortimer I *et al*. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Therapy* 1999; 6: 403-411.

Most, P, Pleger, ST, Volkers, M, Heidt, B, Boerries, M, Weichenhan, D et al. (2004). Cardiac adenoviral S100A1 gene delivery rescues failing myocardium. J Clin Invest 114: 1550–1563.

Movsas B, Chapman JD, Hanlon AL, Horwitz EM, Greenberg RE, Stobbe C *et al.* Hypoxic prostate/muscle pO_2 ratio predicts for biochemical failure in patients with prostate cancer: preliminary findings. *Urology* 2002; 60: 634–639.

Muhlhauser, J, Pili, R, Merrill, MJ, Maeda, H, Passaniti, A, Crystal, RG et al. (1995). In vivo angiogenesis induced by recombinant adenovirus vectors coding either for secreted or nonsecreted forms of acidic fibroblast growth factor. Hum Gene Ther 6: 1457–1465.

Mukherjee D, Wong J, Griffin B, et al. Ten-fold augmentation of endothelial uptake of vascular endothelial growth factor with ultrasound after systemic administration J Am Coll Cardiol 2000;35:1678-1686.

Nagaya, N, Yokoyama, C, Kyotani, S, Shimonishi, M, Morishita, R, Uematsu, M et al. (2000). Gene transfer of human prostacyclin synthase ameliorates monocrotaline-induced pulmonary hypertension in rats. Circulation 102: 2005–2010.

Nakao, K., W. Minobe, R. Roden, R. M. Bristow, and L. A. Leinwand. 1997. Myosin heavy chain gene expression in the human heart failure. J. Clin. Investig. 100:2362-2370.

Nalbantoglu, J, Pari, G, Karpati, G and Holland, PC (1999). Expression of the primary coxsackie and adenovirus receptor is downregulated during skeletal muscle maturation and limits the efficacy of adenovirus-mediated gene delivery to muscle cells. Hum Gene Ther 10: 1009–1019.

Nicklin, SA, White, SJ, Nicol, CG, Von Seggern, DJ and Baker, AH (2004). In vitro and in vivo characterisation of endothelial cell selective adenoviral vectors. J Gene Med 6: 300–308.

Nikinmaa, M., and B. B. Rees. 2005. Oxygen-dependent gene expression in fishes. Am. J. Physiol. Regul Integr Comp. Physiol. 288:R1079-R1090.

Nikol, S, Baumgartner, I, Van Belle, E, Diehm, C, Visona, A, Capogrossi, MC et al. (2006). Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. Late breaking clinical trial. American College of Cardiology 55th Annual Scientific Session.

Nishiyama, K, Takaji, K, Kataoka, K, Kurihara, Y, Yoshimura, M, Kato, A et al. (2005). Id1 gene transfer confers angiogenic property on fully differentiated endothelial cells and contributes to therapeutic angiogenesis. Circulation 112: 2840–2850.

Nor JE *et al*. Up-regulation of Bcl-2 in microvascular endothelial cells enhances intratumoral angiogenesis and accelerates tumor growth. *Cancer Res* 2001; 61: 2183-2188.

Norris, M. L., and D. E. Millhorn. 1995. Hypoxia-induced protein binding to O_2-responsive sequences on the tyrosine hydroxylase gene. J. Biol. Chem. 270:23774-23779.

Numaguchi, Y, Naruse, K, Harada, M, Osanai, H, Mokuno, S, Murase, K et al. (1999). Prostacyclin synthase gene transfer accelerates reendothelialization and inhibits neointimal formation in rat carotid arteries after balloon injury. Arterioscler Thromb Vasc Biol 19: 727–733.

Nykanen, AI, Krebs, R, Saaristo, A, Turunen, P, Alitalo, K, Yla-Herttuala, S et al. (2003). Angiopoietin-1 protects against the development of cardiac allograft arteriosclerosis. Circulation 107: 1308–1314.

Nykanen, AI, Pajusola, K, Krebs, R, Keranen, MA, Raisky, O, Koskinen, PK et al. (2006). Common protective and diverse smooth muscle cell effects of AAV-mediated angiopoietin-1 and -2 expression in rat cardiac allograft vasculopathy. Circ Res 98: 1373–1380.

Odorisio, T, Schietroma, C, Zaccaria, ML, Cianfarani, F, Tiveron, C, Tatangelo, L et al. (2002). Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. J Cell Sci 115: 2559–2567.

Ogawa, S, Oku, A, Sawano, A, Yamaguchi, S, Yazaki, Y and Shibuya, M (1998). A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J Biol Chem 273: 31273–31282.

Ohh, M., C. W. Park, M. Ivan, M. A. Hoffman, T. Y. Kim, L. E. Huang, N. Pavletich, V. Chau, and W. G. Kaelin. 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat. Cell Biol. 2:423-427.

Ohno, N, Itoh, H, Ikeda, T, Ueyama, K, Yamahara, K, Doi, K et al. (2002). Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. Circulation 105: 1623–1626.

Ohta S, Suzuki K, Tachibana K, Yamada G. Microbubble-enhanced sonoporation: efficient gene transduction technique for chick embryos. Genesis 2003;37:91-101.

Ojamaa, K., A. M. Samarel, and I. Klein. 1996. Identification of a contractile response element in the cardiac α-myosin heavy chain gene. J. Biol. Chem. 270:31276-31281.

Pacak, CA, Mah, CS, Thattaliyath, BD, Conlon, TJ, Lewis, MA, Cloutier, DE et al. (2006). Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction in vivo. Circ Res 99: e3–e9.

Pakkanen, TM, Laitinen, M, Hippelainen, M, Kallionpaa, H, Lehtolainen, P, Leppanen, P et al. (1999). Enhanced plasma cholesterol lowering effect of retrovirus-mediated LDL receptor gene transfer to WHHL rabbit liver after improved surgical technique and stimulation of hepatocyte proliferation by combined partial liver resection and thymidine kinase ganciclovir treatment. Gene Ther 6: 34–41.

Parmacek, M. S., and J. M. Leiden. 1989. Structure and expression of the murine slow/cardiac troponin C gene. J. Biol. Chem. 264:13217-13225.

Parsons-Wingerter, P, Chandrasekharan, UM, McKay, TL, Radhakrishnan, K, DiCorleto, PE, Albarran, B et al. (2006). A VEGF165-induced phenotypic switch from increased vessel density to increased vessel diameter and increased endothelial NOS activity. Microvasc Res 72: 91–100.

Patel, TH, Kimura, H, Weiss, CR, Semenza, GL and Hofmann, LV (2005). Constitutively active HIF-1alpha improves perfusion and arterial remodeling in an endovascular model of limb ischemia. Cardiovasc Res 68: 144–154.

Pedersen AG, Baldi P, Chauvin Y, Brunak S. The biology of eukaryotic promoter prediction--a review. Comput Chem. 1999 Jun 15;23(3-4):191-207. Review.

Pettersson, A, Nagy, JA, Brown, LF, Sundberg, C, Morgan, E, Jungles, S et al. (2000). Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. Lab Invest 80: 99–115.

Phillips MI, Tang Y, Schmidt-Ott K, Qian K, Kagiyama S.Vigilant vector: heart-specific promoter in an adeno-associated virus vector for cardioprotection. Hypertension. 2002 Feb;39(2 Pt 2):651-5.

Pires P *et al*. Interaction of cationic liposomes and their DNA complexes with monocytic leukemia cells. *Biochim Biophys Acta* 1999; 1418: 71-84.

Pislaru SV, Pislaru C, Kinnick RR, et al. Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities Eur Heart J 2003;24:1690-1698.

Poliakova, L, Kovesdi, I, Wang, X, Capogrossi, MC and Talan, M (1999). Vascular permeability effect of adenovirus-mediated vascular endothelial growth factor gene transfer to the rabbit and rat skeletal muscle. J Thorac Cardiovasc Surg 118: 339–347.

Ponten, A, Li, X, Thoren, P, Aase, K, Sjoblom, T, Ostman, A et al. (2003). Transgenic overexpression of platelet-derived growth factor-C in the mouse heart induces cardiac fibrosis, hypertrophy, and dilated cardiomyopathy. Am J Pathol 163: 673–682.

Post DE, Van Meir EG.Generation of bidirectional hypoxia/HIF-responsive expression vectors to target gene expression to hypoxic cells. Gene Ther. 2001 Dec;8(23):1801-7

Powell, RJ, Dormandy, J, Simons, M, Morishita, R and Annex, BH (2004). Therapeutic angiogenesis for critical limb ischemia: design of the hepatocyte growth factor therapeutic angiogenesis clinical trial. Vasc Med 9: 193–198.

Prentice H *et al*. Regulated expression of a foreign gene targeted to the ischaemic myocardium. *Cardiovasc Res* 1997; 35: 567-574.

Price RJ, Skyba DM, Kaul S, Skalak TC. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound Circulation 1998;98:1264-1267.

Proc Natl Acad Sci U S A. 2002 Jul 9;99(14):9480-5. Epub 2002 Jun 25.

Puhakka, HL, Turunen, P, Rutanen, J, Hiltunen, MO, Turunen, MP and Yla-Herttuala, S (2005). Tissue inhibitor of metalloproteinase 1 adenoviral gene therapy alone is equally effective in reducing restenosis as combination gene therapy in a rabbit restenosis model. J Vasc Res 42: 361–367.

Rabbi MF, Saifuddin M, Gu DS, Kagnoff MF, Roebuck KA. U5 region of the human immunodeficiency virus type 1 long terminal repeat contains TRE-like cAMP responsive elements that bind both AP-1 and CREB/ATF proteins. Virology. 1997 Jun 23;233(1):235-45.

Raizada, MK and Der Sarkissian, S (2006). Potential of gene therapy strategy for the treatment of hypertension. Hypertension 47: 6–9.

Rajagopalan, S, Mohler, ER 3rd, Lederman, RJ, Mendelsohn, FO, Saucedo, JF, Goldman, CK et al. (2003). Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. Circulation 108: 1933–1938.

Rajagopalan, S, Olin, J, Deitcher, S, Pieczek, A, Laird, J, Grossman, PM et al. (2007). Use of a constitutively active hypoxia-inducible factor-1{∝} transgene as a therapeutic strategy in no-option critical limb ischemia patients. Phase I dose-escalation experience. Circulation 115: 1234–1243.

Rajagopalan, S, Shah, M, Luciano, A, Crystal, R and Nabel, EG (2001). Adenovirus-mediated gene transfer of VEGF(121) improves lower-extremity endothelial function and flow reserve. Circulation 104: 753–755.

Rajkumar, K., D. Barron, M. S. Lewitt, and L. J. Murphy. 1995. Growth retardation and hyperglycemia in insulin-like growth factor binding protein-1 transgenic mice. Endocrinology 136:4029-4034.

Raleigh JA, Calkins-Adams DP, Rinker LH, Ballenger CA, Weissler MC, Fowler Jr WC *et al.* Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. *Cancer Res* 1998; 58: 3765–3768.

Reilly, JP, Grise, MA, Fortuin, FD, Vale, PR, Schaer, GL, Lopez, J et al. (2005). Long-term (2-year) clinical events following transthoracic intramyocardial gene transfer of VEGF-2 in no-option patients. J Interv Cardiol 18: 27–31.

Rindt, H., J. Gulick, S. Knott, J. Neumann, and J. Robbins. 1993. *In vivo* analysis of the murine β-myosin heavy chain gene promoter. J. Biol. Chem. 268:5332-5338.

Ripa, RS, Wang, Y, Jorgensen, E, Johnsen, HE, Hesse, B and Kastrup, J (2006). Intramyocardial injection of vascular endothelial growth factor-A165 plasmid followed by granulocyte-colony stimulating factor to induce angiogenesis in patients with severe chronic ischaemic heart disease. Eur Heart J 27: 1785–1792.

Rissanen, T. Herttuala, S. Current Status of Cardiovascular Gene Therapy. Molecular Therapy (2007) 15 7, 1233–1247.

Rissanen, TT, Korpisalo, P, Markkanen, JE, Liimatainen, T, Orden, MR, Kholova, I et al. (2005). Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterialization and sprouting angiogenesis. Circulation 112: 3937–3946.

Rissanen, TT, Markkanen, JE, Arve, K, Rutanen, J, Kettunen, MI, Vajanto, I et al. (2003). Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. FASEB J 17: 100–102.

Rissanen, TT, Markkanen, JE, Gruchala, M, Heikura, T, Puranen, A, Kettunen, MI et al. (2003). VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res 92: 1098–1106.

Robbins, J. 1996. Regulation of cardiac gene expression during development. Cardiovasc. Res. 31:E2-E16.

Rodriguez JF, Smith GL. Inducible gene expression from vaccinia virus vectors.

Rooman I, Bouwens L. Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycemia in C57BL/6J mice treated with alloxan. Diabetologia 2004; 47: 259–265.

Rooman I, Heremans Y, Heimberg H, Bouwens L. Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. Diabetologia 2000; 43: 907–914

Rosen, MR, Brink, PR, Cohen, IS and Robinson, RB (2004). Genes, stem cells and biological pacemakers. Cardiovasc Res 64: 12–23.

Roy, H, Bhardwaj, S, Babu, M, Jauhiainen, S, Herzig, KH, Bellu, AR et al. (2005). Adenovirus-mediated gene transfer of placental growth factor to perivascular tissue induces angiogenesis via upregulation of the expression of endogenous vascular endothelial growth factor-A. Hum Gene Ther 16: 1422–1428.

Ruan H *et al*. Hypoxia-inducible expression of BAX: application in tumor-targeted gene therapy. *Neurosurg Focus* 2000; 8: 1-7.

Rutanen, J, Rissanen, TT, Markkanen, JE, Gruchala, M, Silvennoinen, P, Kivela, A et al. (2004). Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of vascular endothelial growth factor-D induces transmural angiogenesis in porcine heart. Circulation 109: 1029–1035.

Rutanen, J, Turunen, AM, Teittinen, M, Rissanen, TT, Heikura, T, Koponen, JK et al. (2005). Gene transfer using the mature form of VEGF-D reduces neointimal thickening through nitric oxide-dependent mechanism. Gene Ther 12: 980–987.

Ryu, JK, Cho, CH, Shin, HY, Song, SU, Oh, SM, Lee, M et al. (2006). Combined angiopoietin-1 and vascular endothelial growth factor gene transfer restores cavernous angiogenesis and erectile function in a rat model of hypercholesterolemia. Mol Ther 13: 705–715.

Saaristo, A, Tammela, T, Timonen, J, Yla-Herttuala, S, Tukiainen, E, Asko-Seljavaara, S et al. (2004). Vascular endothelial growth factor-C gene therapy restores lymphatic flow across incision wounds. FASEB J 18: 1707–1709.

Sadoshima, J., and S. Izumo. 1997. The cellular and molecular response of cardiac myocytes to mechanical stress. Annu. Rev. Physiol. 59:551-571.

Salceda S, Caro J. Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem.* 1997; 272: 22642–22647.

Salloum RM, Mauceri HJ, Hanna NN, Gorski DH, Posner MC, Weichselbaum RR. Dual induction of the Epo-Egr-TNF-alpha- plasmid in hypoxic human colon adenocarcinoma produces tumor growth delay. *Am Surg* 2003; 69: 24–27.

Samson S, Chan L. Gene therapy for diabetes: reinventing the islet. Trends Endocrinol Metab 2005; 17: 92–100.

Schepers, A, Eefting, D, Bonta, PI, Grimbergen, JM, de Vries, MR, van Weel, V et al. (2006). Anti-MCP-1 gene therapy inhibits vascular smooth muscle cells proliferation and attenuates vein graft thickening both in vitro and in vivo. Arterioscler Thromb Vasc Biol 26: 2063–2069.

Schiaffino, S, and Reggiani C. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 76: 371-423, 1996.

Schiaffino, S, and Reggiani C. Myosin isoforms in mammalian skeletal muscle. *J Appl Physiol* 77: 493-501, 1994.

Schillinger, KJ, Tsai, SY, Taffet, GE, Reddy, AK, Marian, AJ, Entman, ML et al. (2005). Regulatable atrial natriuretic peptide gene therapy for hypertension. Proc Natl Acad Sci USA 102: 13789–13794.

Schnepp, BC, Clark, KR, Klemanski, DL, Pacak, CA and Johnson, PR (2003). Genetic fate of recombinant adeno-associated virus vector genomes in muscle. J Virol 77: 3495–3504.

Schug, J., and G. C. Overton. 1997. TESS: transcription element search software on the WWW. Technical report CBIL-TR-1997-1001-v0.0. Computational Biology and Informatics Laboratory, University of Pennsylvania, Philadelphia.

Scott SD, Marples B, Hendry JH, Lashford LS, Embleton MJ, Hunter RD *et al.* A radiation-controlled molecular switch for use in gene therapy of cancer. *Gene Therapy* 2000; 7: 1121–1125.

Seemann S, Hauff P, Schultze-Mosgau M, Lehmann C, Reszka R. Pharmaceutical evaluation of gas-filled microparticles as gene delivery system Pharm Res 2002;19:250-257.

Semenza G. Perspectives on oxygen sensing. *Cell* 1999; 98: 281-284

Semenza GL. HIF-1 and mechanisms of hypoxia sensing. Curr Opin Cell Biol. 2001 Apr;13(2):167-71. Review.

Semenza GL. Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE. 2007 Oct 9;2007(407):cm8. Review.

Semenza, G. L., and G. L. Wang. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. 12:5447-5454.

Semenza, G. L., B. H. Jiang, S. W. Leung, R. Passantino, J. P. Concordet, P. Maire, and A. Giallongo. 1996. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J. Biol. Chem. 271:32529-32537.

Seta, K. A., and D. E. Millhorn. 2004. Functional genomics approach to hypoxia signaling. J. Appl. Physiol. 96:765-773.

Sharif, F, Hynes, SO, McMahon, J, Cooney, R, Conroy, S, Dockery, P et al. (2006). Gene-eluting stents: comparison of adenoviral and adeno- associated viral gene delivery to the blood vessel wall in vivo. Hum Gene Ther 17: 741–750.

Shen, F, Su, H, Fan, Y, Chen, Y, Zhu, Y, Liu, W et al. (2006). Adeno-associated viral-vector-mediated hypoxia-inducible vascular endothelial growth factor gene expression attenuates ischemic brain injury after focal cerebral ischemia in mice. Stroke 37: 2601–2606.

Shen, R., S. K. Goswami, E. Mascareno, A. Kumar, and M. A. Q. Siddiqui. 1991. Tissue-specific transcription of the cardiac myosin light chain-2 gene is regulated by an upstream repressor element. Mol. Cell. Biol. 11:1676-1683.

Shibata T *et al*. Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. *Int J Rad Oncol Biol Phys* 1998; 42: 913-916.

Shibata T, Giaccia AJ, Brown JM. Development of a hypoxia-responsive vector for tumor specific gene therapy. *Gene Therapy* 2000; 7: 493-498.

Shih SC, Claffey KP .Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression. Growth Factors. 2001;19(1):19-34.

Shimazaki K *et al*. Adeno-associated virus vector-mediated bcl-2 gene transfer into post-ischemic gerbil brain *in vivo*: prospects for gene therapy of ischemia-induced neuronal death. *Gene Therapy* 2000; 7: 1244-1249.

Shimizu, N., G. Prior, P. K. Umeda, and R. Zak. 1992. Cis-acting elements responsible for muscle-specific expression of the myosin heavy chain β gene. Nucleic Acids Res. 20:1793-1799.

Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K et al. Betacellulin: a mitogen from pancreatic beta cell tumors. Science 1993; 259: 2681–2689.

Shohet RV, Chen S, Zhou YT, Wang Z, Meidell RS, Unger RH, Grayburn PA. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium Circulation 2000;101:2554-2556.

Shohet RV, Garcia JA. Keeping the engine primed: HIF factors as key regulators of cardiac metabolism and angiogenesis during ischemia. J Mol Med. 2007 Dec;85(12):1309-15. Epub 2007 Nov 20.

Shyu, KG, Chang, H, Wang, BW and Kuan, P (2003). Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. Am J Med 114: 85–92.

Silvestre, JS, Tamarat, R, Ebrahimian, TG, Le-Roux, A, Clergue, M, Emmanuel, F et al. (2003). Vascular endothelial growth factor-B promotes in vivo angiogenesis. Circ Res 93: 114–123.

Simoes S *et al*. Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum. *Biochim Biophys Acta* 2000; 1463: 459-469.

Skyba DM, Price RJ, Linka AZ, Skalak TC, Kaul S. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue Circulation 1998;98:290-293.

Smith, RSJr.,Lin, KF, Agata, J, Chao, L and Chao, J (2002). Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. Arterioscler Thromb Vasc Biol 22: 1279–1285.

Song J, Chappell JC, Qi M, VanGieson EJ, Kaul S, Price RJ. Influence of injection site, microvascular pressure and ultrasound variables on microbubble-mediated delivery of microspheres to muscle J Am Coll Cardiol 2002;39:726-731.

Song J, Qi M, Kaul S, Price RJ. Stimulation of arteriogenesis in skeletal muscle by microbubble destruction with ultrasound Circulation 2002;106:1550-1555.

Song S, Embury J, Laipis PJ, Berns KI, Crawford JM, Flotte TR. Stable therapeutic serum levels of human alpha-1 antitrypsin (hAAT) after protein vein injection of recombinant adeno-associated viral (rAAV) vectors. *Gene Therapy.* 2001; 8: 1299–1306.

Springer, ML, Chen, AS, Kraft, PE, Bednarski, M and Blau, HM (1998). VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Mol Cell 2: 549–558.

Springer, ML, Ozawa, CR, Banfi, A, Kraft, PE, Ip, TK, Brazelton, TR et al. (2003). Localized arteriole formation directly adjacent to the site of VEGF-induced angiogenesis in muscle. Mol Ther 7: 441–449.

Starritt HC, Duck FA, Humphrey VF. An experimental investigation of streaming in pulsed diagnostic ultrasound beams Ultrasound Med Biol 1989;15:363-373.

Stewart, DJ, Hilton, JD, Arnold, JM, Gregoire, J, Rivard, A, Archer, SL et al. (2006). Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. Gene Ther 13: 1503–1511.

Su H, Kan YW.Adeno-associated viral vector-delivered hypoxia-inducible gene expression in ischemic hearts. Methods Mol Biol. 2007;366:331-42.

Su, H, Huang, Y, Takagawa, J, Barcena, A, Arakawa-Hoyt, J, Ye, J et al. (2006). AAV serotype-1 mediates early onset of gene expression in mouse hearts and results in better therapeutic effect. Gene Ther 13: 1495–1502.

Su, H, Joho, S, Huang, Y, Barcena, A, Arakawa-Hoyt, J, Grossman, W et al. (2004). Adeno-associated viral vector delivers cardiac-specific and hypoxia-inducible VEGF expression in ischemic mouse hearts. Proc Natl Acad Sci USA 101: 16280–16285.

Subramaniam, A., W. K. Jones, J. Gulick, S. Wert, J. Neumann, and J. Robbins. 1991. Tissue-specific regulation of the α-myosin heavy chain gene promoter in transgenic mice. J. Biol. Chem. 266:24613-24620.

Sun L, Li J, Xiao X. Overcoming adeno-associated virus vector size limitation through viral DNA heterodimerization. *Nat Med.* 2000; 6: 599–602.

Svet-Moldavsky, GJ and Chimishkyan, KL (1977). Tumour angiogenesis factor for revascularisation in ischaemia and myocardial infarction. Lancet 1: 913.

Swoap, SJ, Haddad F, Bodell P, and Baldwin KM. Control of β-myosin heavy chain expression in systemic hypertension and caloric restriction in the rat heart. *Am J Physiol Cell Physiol* 269: C1025-C1033, 1995.

Swoap, SJ, Haddad F, Bodell P, and Baldwin KM. Effect of chronic energy deprivation on cardiac thyroid hormone receptor and myosin isoform expression. *Am J Physiol Endocrinol Metab* 266: E254-E260, 1994.

Swoap, SJ, Haddad F, Caiozzo VJ, Herrick RE, McCue SA, and Baldwin KM. Interaction of thyroid hormone and functional overload on skeletal muscle isomyosin expression. *J Appl Physiol* 77: 621-629, 1994.

Swoap, SJ. In vivo analysis of the myosin heavy chain IIB promoter region. *Am J Physiol Cell Physiol* 274: C681-C687, 1998.

Sylven, C, Sarkar, N, Ruck, A, Drvota, V, Hassan, SY, Lind, B et al. (2001). Myocardial Doppler tissue velocity improves following myocardial gene therapy with VEGF-A165 plasmid in patients with inoperable angina pectoris. Coron Artery Dis 12: 239–243.

Tachibana K, Tachibana S. Albumin microbubble echo-contrast material as an enhancer for ultrasound accelerated thrombolysis Circulation 1995;92:1148-1150.

Tada H, Sasada R, Kawaguchi Y, Kojima I, Gullick WJ, Salomon DS et al. Processing and juxtacrine activity of membrane-anchored betacellulin. J Cell Biochem 1999; 72: 423–434.

Takihara, K. Y., M. J. Sole, J. Liew, D. Ing, and C. C. Liew. 1989. Characterization of human cardiac myosin heavy chain genes. Proc. Natl. Acad. Sci. USA 86:3416-3417.

Tang Y, Jackson M, Qian K, Phillips MI. Hypoxia inducible double plasmid system for myocardial ischemia gene therapy. Hypertension. 2002 Feb;39(2 Pt 2):695-8.

Taniyama Y, Tachibana K, Hiraoka K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle Gene Ther 2002;9:372-380.

Taniyama Y, Tachibana K, Hiraoka K, et al. Local delivery of plasmid DNA into rat carotid artery using ultrasound Circulation 2002;105:1233-1239.

Taylor CT. Mitochondria and cellular oxygen sensing in the HIF pathway. Biochem J. 2008 Jan 1;409(1):19-26. Review.

Tazuke, S. I., N. M. Mazure, J. Sugawara, G. Carland, G. H. Faessen, L. F. Suen, J. C. Irwin, D. R. Powell, A. J. Giaccia, and L. C. Giudice. 1998. Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia. Proc. Natl. Acad. Sci. USA 95:10188-10193.

Tenhunen, O, Soini, Y, Ilves, M, Rysa, J, Tuukkanen, J, Serpi, R et al. (2006). p38 Kinase rescues failing myocardium after myocardial infarction: evidence for angiogenic and anti-apoptotic mechanisms. FASEB J 20: 1907–1909.

Teupe C, Richter S, Fisslthaler B, et al. Vascular gene transfer of phosphomimetic endothelial nitric oxide synthase (S1177D) using ultrasound-enhanced destruction of plasmid-loaded microbubbles improves vasoreactivity Circulation 2002;105:1104-1109.

Thirion, C, Lochmuller, H, Ruzsics, Z, Boelhauve, M, Konig, C, Thedieck, C et al. (2006). Adenovirus vectors based on human adenovirus type 19a have high potential for human muscle-directed gene therapy. Hum Gene Ther 17: 193–205.

Thompson, W. R., B. Nadal-Ginard, and V. Mahdavi. 1991. A MyoD-1-independent muscle-specific enhancer controls the expression of the β-myosin heavy chain gene in skeletal and cardiac muscle cells. J. Biol. Chem. 266:22678-22688.

Thorsell A, Fox E, Heilig M. Lipid mediated gene delivery in the adult rat brain: quantitative analysis of expression. *Neurochem Int* 1999; 35: 65-71.

Tokunaga, N, Nagaya, N, Shirai, M, Tanaka, E, Ishibashi-Ueda, H, Harada-Shiba, M et al. (2004). Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector, gelatin. Circulation 109: 526–531.

Trivedi RA, Dickson G. Liposome-mediated gene transfer into normal and dystrophin-deficient mouse myoblasts. *J Neurochem* 1995; 64: 2230-2238.

Tsika, GL, Weidenman JL, Gao L, McCarthy JJ, Sheriff-Carter K, Rivera-Rivera ID, and Tsika RW. Induction of β-MHC transgene in overloaded skeletal muscle is not eliminated by mutation of conserved elements. *Am J Physiol Cell Physiol* 271: C690-C699, 1996.

Tsika, R. W., J. J. Gahl, L. A. Leinwand, and E. Morkin. 1990. Thyroid hormone regulates expression of a transfected human α-myosin heavy chain fusion gene in fetal rat heart cells. Proc. Natl. Acad. Sci. USA 87:379-383.

Tsurumi, Y, Takeshita, S, Chen, D, Kearney, M, Rossow, ST, Passeri, J et al. (1996). Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation 94: 3281–3290.

Tuomisto TT, Rissanen TT, Vajanto I, Korkeela A, Rutanen J, Ylä-Herttuala S. HIF-VEGF-VEGFR-2, TNF-alpha and IGF pathways are upregulated in critical human skeletal muscle ischemia as studied with DNA array. Atherosclerosis. 2004 May;174(1):111-20.

Turunen, P, Puhakka, HL, Heikura, T, Romppanen, E, Inkala, M, Leppanen, O et al. (2006). Extracellular superoxide dismutase with vaccinia virus anti-inflammatory protein 35K or tissue inhibitor of metalloproteinase-1: combination gene therapy in the treatment of vein graft stenosis in rabbits. Hum Gene Ther 17: 405–414.

Ueno, H, Li, JJ, Masuda, S, Qi, Z, Yamamoto, H and Takeshita, A (1997). Adenovirus-mediated expression of the secreted form of basic fibroblast growth factor (FGF-2) induces cellular proliferation and angiogenesis in vivo. Arterioscler Thromb Vasc Biol 17: 2453–2460.

Unger EC, Matsunaga TO, McCreery T, Schumann P, Sweitzer R, Quigley R. Therapeutic applications of microbubbles Eur J Radiol 2002;42:160-168.

Vajanto, I, Rissanen, TT, Rutanen, J, Hiltunen, MO, Tuomisto, TT, Arve, K et al. (2002). Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. J Gene Med 4: 371–380.

Vale, PR, Losordo, DW, Milliken, CE, Maysky, M, Esakof, DD, Symes, JF et al. (2000). Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. Circulation 102: 965–974.

Vale, PR, Losordo, DW, Milliken, CE, McDonald, MC, Gravelin, LM, Curry, CM et al. (2001).Randomized, single-blind, placebo-controlled pilot study of catheter-based myocardial gene transfer for therapeutic angiogenesis using left ventricular electromechanical mapping in patients with chronic myocardial ischemia. Circulation 103: 2138–2143.

Van Wamel A, Bouakaz A, Bernard B, Ten Cate F, De Jong N. Radionuclide tumour therapy with ultrasound contrast microbubbles Ultrasonics 2004;42:903-906.

Vannan M, McCreery T, Li P, et al. Ultrasound-mediated transfection of canine myocardium by intravenous administration of cationic microbubble-linked plasmid DNA J Am Soc Echocardiogr 2002;15:214-218.

Varenne, O, Pislaru, S, Gillijns, H, Van Pelt, N, Gerard, RD, Zoldhelyi, P et al. (1998). Local adenovirus-mediated transfer of human endothelial nitric oxide synthase reduces luminal narrowing after coronary angioplasty in pigs. Circulation 98: 919–926.

Vincent, KA, Shyu, KG, Luo, Y, Magner, M, Tio, RA, Jiang, C et al. (2000). Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1α/VP16 hybrid transcription factor. Circulation 102: 2255–2261. Virology. 1990 Jul;177(1):239-50.

Vitiello L *et al*. Condensation of plasmid DNA with polylysine improves liposome-mediated gene transfer into established and primary muscle cells. *Gene Therapy* 1996; 3: 396-404.

Von Bibra H, Voigt JU, Froman M, Bone D, Wranne B, Juhlin-Dannfeldt A. Interaction of microbubbles with ultrasound Echocardiography 1999;16:733-741.

Walter, DH, Cejna, M, Diaz-Sandoval, L, Willis, S, Kirkwood, L, Stratford, PW et al. (2004). Local gene transfer of phVEGF-2 plasmid by gene-eluting stents: an alternative strategy for inhibition of restenosis. Circulation 110: 36–45.

Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A*. 1993; 90: 4304–4308.

Wang Y, O'Malley BW, Jr, Tsai SY, O'Malley BW. A regulatory system for use in gene transfer. *Proc Natl Acad Sci U S A*. 1994; 91: 8180–8184.

Wang, G. L., and G. L. Semenza. 1995. Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem. 270:1230-1237.

Wang, G. L., B. H. Jiang, E. A. Rue, and G. L. Semenza. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. USA 92:5510-5514.

Wang, R., M. J. Sole, E. Cukerman, and C. C. Liew. 1994. Characterization and nucleotide sequence of the cardiac myosin heavy chain gene from Syrian hamster. J. Mol. Cell. Cardiol. 26:1155-1165.

Wang, T, Li, H, Zhao, C, Chen, C, Li, J, Chao, J et al. (2004). Recombinant adeno-associated virus-mediated kallikrein gene therapy reduces hypertension and attenuates its cardiovascular injuries. Gene Ther 11: 1342–1350.

Wang, Z, Zhu, T, Qiao, C, Zhou, L, Wang, B, Zhang, J et al. (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat Biotechnol 23: 321–328.

Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin. 1990. The c-Ets proto-oncogene encodes transcription factors that co-operate with c-fos and c-Jun for transcriptional activation. Nature 346:191-193.

Wasylyk, B., S. L. Hahn, and A. Giovane. 1993. The Ets family of transcription factors. Eur. J. Biochem. 211:7-18.

Waugh, JM, Kattash, M, Li, J, Yuksel, E, Kuo, MD, Lussier, M et al. (1999). Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. Proc Natl Acad Sci USA 96: 1065–1070.

Waugh, JM, Yuksel, E, Li, J, Kuo, MD, Kattash, M, Saxena, R et al. (1999). Local overexpression of thrombomodulin for in vivo prevention of arterial thrombosis in a rabbit model. Circ Res 84: 84–92.

Wei K, Jayaweera AR, Firoozan S, Linka A, Skyba DM, Kaul S. Basis for detection of stenosis using venous administration of microbubbles during myocardial contrast echocardiography: bolus or continuous infusion? J Am Coll Cardiol 1998;32:252-260

Weidenman, JL, Tsika GL, Gao L, McCarthy JJ, Rivera-Rivera ID, Vyas D, Sheriff-Carter K, and Tsika RW. Muscle-specific and inducible expression of 293-base pair β-myosin heavy chain promoter in transgenic mice. *Am J Physiol Regulatory Integrative Comp Physiol* 271: R688-R695, 1996.

Wen, S, Graf, S, Massey, PG and Dichek, DA (2004). Improved vascular gene transfer with a helper-dependent adenoviral vector. Circulation 110: 1484–1491.

Whitlock, PR, Hackett, NR, Leopold, PL, Rosengart, TK and Crystal, RG (2004). Adenovirus-mediated transfer of a minigene expressing multiple isoforms of VEGF is more effective at inducing angiogenesis than comparable vectors expressing individual VEGF cDNAs. Mol Ther 9: 67–75.

Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis. 1995. Integration of MAP-kinase signal transduction pathways at the serum response element. Science 269:403-407.

Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27: 1047–1053.

Wilson, BD, Ii, M, Park, KW, Suli, A, Sorensen, LK, Larrieu-Lahargue, F et al. (2006). Netrins promote developmental and therapeutic angiogenesis. Science 313: 640–644.

Winoto, A., and D. Baltimore. 1989. α,β lineage-specific expression of the α T cell receptor gene by nearby silencers. Cell 59:649-655.

Wolff, JA, Williams P, Acsadi G, Jiao S, Jani A, and Chong W. Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* 11: 474-485, 1991.

Woo, YJ, Panlilio, CM, Cheng, RK, Liao, GP, Atluri, P, Hsu, VM et al. (2006). Therapeutic delivery of cyclin A2 induces myocardial regeneration and enhances cardiac function in ischemic heart failure. Circulation 114 (Suppl 1): I206–I213.

Work, LM, Buning, H, Hunt, E, Nicklin, SA, Denby, L, Britton, N et al. (2006). Vascular bed-targeted in vivo gene delivery using tropism-modified adeno-associated viruses. Mol Ther 13: 683–693.

Wright, CE, Haddad F, Bodell PW, and Baldwin KM. In vivo regulation of the β myosin heavy chain gene in rodent heart: role of thyroid hormone and evidence for an upstream enhancer. *Am J Physiol Cell Physiol* 276: C883-C891, 1999.

Wright, MJ, Rosenthal, E, Stewart, L, Wightman, LM, Miller, AD, Latchman, DS et al. (1998). β-Galactosidase staining following intracoronary infusion of cationic liposomes in the in vivo rabbit heart is produced by microinfarction rather than effective gene transfer: a cautionary tale. Gene Ther 5: 301–308.

Wright, MJ, Wightman, LM, Latchman, DS and Marber, MS (2001). In vivo myocardial gene transfer: optimization and evaluation of intracoronary gene delivery in vivo. Gene Ther 8: 1833–1839.

Wright, MJ, Wightman, LM, Lilley, C, de Alwis, M, Hart, SL, Miller, A et al. (2001). In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors. Basic Res Cardiol 96: 227–236.

Wu J. Theoretical study on shear stress generated by microstreaming surrounding contrast agents attached to living cells Ultrasound Med Biol 2002;28:125-129.

Yamashita, Y, Shimada, M, Tachibana, K, Harimoto, N, Tsujita, E, Shirabe, K et al. (2002). In vivo gene transfer into muscle via electro-sonoporation. Hum Gene Ther 13: 2079–2084.

Yanagisawa-Miwa, A, Uchida, Y, Nakamura, F, Tomaru, T, Kido, H, Kamijo, T et al. (1992). Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. Science 257: 1401–1403.

Yang BC, Phillips MI, Ambuehl PEJ, Shen LP, Mehta P, Mehta JL. Increase in angiotensin II type 1 receptor expression immediately after ischemia-reperfusion in isolated rat hearts. *Circulation.* 1997; 96: 922–926.

Yet SF, Tian R, Layne MD, Wang ZY, Maemura K, Solovyeva M, Ith B, Melo LG, Zhang L, Ingwall JS, Dzau VJ, Lee ME, Perella MA. Cardiac-specific expression of heme oxygenase-2 protects against ischemia and reperfusion injury in transgenic mice. *Circ Res.* 2001; 89: 205–107.

Yla-Herttuala, S and Alitalo, K (2003). Gene transfer as a tool to induce therapeutic vascular growth. Nat Med 9: 694–701.

Yla-Herttuala, S and Martin, JF (2000). Cardiovascular gene therapy. Lancet 355: 213–222.

Yla-Herttuala, S, Markkanen, JE and Rissanen, TT (2004). Gene therapy for ischemic cardiovascular diseases: some lessons learned from the first clinical trials. Trends Cardiovasc Med 14: 295–300.

Yla-Herttuala, S, Rissanen, TT, Vajanto, I and Hartikainen, J (2006). Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 49: 1015–1026.

Zacchigna, S, Papa, G, Antonini, A, Novati, F, Moimas, S, Carrer, A et al. (2005). Improved survival of ischemic cutaneous and musculocutaneous flaps after vascular endothelial growth factor gene transfer using adeno-associated virus vectors. Am J Pathol 167: 981–991.

Zelphati O, Uyechi LS, Barron LG, Szoka Jr FC. Effect of serum components on the physico-chemical properties of cationic lipid-oligonucleotide complexes and on their interactions with cells. *Biochem Biophys Acta* 1998; 1390: 119-133.

Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF 1alpha/ARNT. EMBO J. 1998 Sep 1;17(17):5085-94.

Zhan Y, Brady JL, Johnston AM, Lew AM. Predominate transgene expression in exocrine pancreas directed by the CMV promoter. DNA Cell Biol 2000; 19: 639 645.

Zhang W, Couldwell WT, Simard MF, Song H, Lin JH, Nedergaard M. Direct gap junction communication between malignant glioma cells and astrocytes. *Cancer Res* 1999; 59: 1994–2003.

Zheng, Y, Murakami, M, Takahashi, H, Yamauchi, M, Kiba, A, Yamaguchi, S et al. (2006). Chimeric VEGF-E(NZ7)/PlGF promotes angiogenesis via VEGFR-2 without significant enhancement of vascular permeability and inflammation. Arterioscler Thromb Vasc Biol 26: 2019–2026.

Zhong P, Lin H, Xi X, Zhu S, Bhoghte ES. Shock wave-inertial microbubble interaction: methodology, physical characterization, and bioeffect study J Acoust Soc Am 1999;105:1997-2009.

Zhong, J, Eliceiri, B, Stupack, D, Penta, K, Sakamoto, G, Quertermous, T et al. (2003). Neovascularization of ischemic tissues by gene delivery of the extracellular matrix protein Del-1. J Clin Invest 112: 30–41.

Zhu H, Bunn H. Oxygen sensing and signaling: impact on the regulation of physiologically important genes. *Resp Physiol* 1999; 115: 239-247

Zhu H, Garcia AV, Ross RS, Evans SM, Chien KR. A conversed 28-base-pair element (HF-1) in the rat cardiac myosin light-chain-2 gene confers cardiac-specific and alpha-adrenergic-inducible expression in cultured neonatal rat myocardial cells. *Mol Cell Biol.* 1991; 11: 2273–2281.

Ziel KA, Campbell CC, Wilson GL, Gillespie MN. Ref-1/Ape is critical for formation of the hypoxia-inducible transcriptional complex on the hypoxic response element of the rat pulmonary artery endothelial cell VEGF gene. FASEB J. 2004 Jun;18(9):986-8. Epub 2004 Apr 14.

Ziel KA, Grishko V, Campbell CC, Breit JF, Wilson GL, Gillespie MN. Oxidants in signal transduction: impact on DNA integrity and gene expression. FASEB J. 2005 Mar;19(3):387-94.

Ziello JE, Jovin IS, Huang Y. Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. Yale J Biol Med. 2007 Jun;80(2):51-60. Review.

Zoldhelyi, P, McNatt, J, Shelat, HS, Yamamoto, Y, Chen, ZQ and Willerson, JT (2000). Thromboresistance of balloon-injured porcine carotid arteries after local gene transfer of human tissue factor pathway inhibitor. Circulation 101: 289–295.

Zoldhelyi, P, McNatt, J, Xu, XM, Loose-Mitchell, D, Meidell, RS, Clubb, FJ Jr. 1996. Prevention of arterial thrombosis by adenovirus-mediated transfer of cyclooxygenase gene. *Circulation* 93: 10–17.