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Epigenetic and gene therapy in cardiovascular diseases: an appraisal

Review Article

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Abbreviations: adeno-associated virus, (AAV); atrial natriuretic peptide, (ANP); antisense angiotensin type I receptor, (asAT1R); atrioventricular, (AV); α2-adrenergic receptor, (α2-AR); β-adrenergic receptor kinase, (β-ARK); coronary artery disease, (CAD); cardiovascular disease, (CVD); double-stranded RNA, (dsRNA); cardiac troponin T, (cTNT); experimental autoimmune myocarditis, (EAM); endothelial nitric oxide synthase, (eNOS); electron transport chain, (ETC); fibroblast growth factor, (FGF); histone acetyltransferase, (HAT); histone deacetylase, (HDAC); hypoxia inducible factor 1α, (HIF-1α); heme oxygenase 1, (H0-1); homeodomain-only protein, (HOP); hypoxia response element, (HRE); heat shock protein 70, (HSP 70); inducible nitric oxide synthase, (iNOS); potassium voltage-gated long QT syndrome 1 channel, (KVLQT1); myocyte enhancing factor 2, (MEF2); ventricle-specific myosin light chain-2, (mlc-2v); mitochondrial DNA, (mtDNA); mitochondrial transcription factor A, (mtTFA); nuclear factor κ B, (NFκB); nuclear respiratory factor 1, (NRF1); prostaglandin I synthase, (PGIS); Project in Ex-Vivo graft Engineering via Transfection, (PREVENT); peripheral vascular disease, (PVD); renin-angiotensin system, (RAS); Restenosis Gene Therapy trial, (REGENT); reactive oxygen species, (ROS); RNA inteference, (RNAi); s-adenosylhomocysteine, (SAH); sarcoplasmic reticulum Ca^{++} ATPase, (SERCA); spontaneously hypertensive rats, (SHR); superoxide dismutase, (SOD); silent information regulator 2, (SIR2); serum response factor, (SRF); sarcoplasmic reticulum, (SR); tumor necrosis factor α, (TNF α); vascular endothelial growth factor, (VEGF);

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Summary

Modifications of DNA and its nuclear environment (i.e. chromatin) may underlie cardiovascular diseases (CVD) and aging. Epigenetic alterations in chromatin (e.g. histone acetylation) have been directly implicated in the modulation of myocardial gene expression in progressive CVD, including cardiac hypertrophy. Cytosine methylation which can lead to gene inactivation has also been linked to increased levels of homocysteine, an important CVD disease risk factor. Advances in the identification of genes affected in CVD has lead to improved therapies either by the use of gene replacement and/or gene suppression (silencing) methodologies. Preclinical studies have shown that therapeutic gene transfer can provide beneficial results in treating heart failure, myocardial protection, hypertension, hypertrophy, cardiac arrhythmias and myocarditis as well as in disorders of the vascular wall where drug therapy has often proved to be of limited value. While early phases of clinical gene therapy trials for CVD have shown promising results in particular with therapeutic angiogenesis and restenosis treatment, the development of improved vectors, methods of delivery, and the acquisition of safety and toxicity data are critically needed before the use of these therapies can be indicated in a clinical setting. In this review, we will survey the current information available on the role of epigenetic modifications in CVD, and examine the present status and prospects for clinical use of epigenetic and gene therapy.

I. Introduction

Modifications of DNA and its nuclear environment (i.e. chromatin) may underlie CVD and aging. Epigenetic alterations in the chromatin structure (e.g. histone acetylation) have been shown to pivotally regulate myocardial gene expression and may be implicated in the progression of CVD as well as playing a contributory role in modulating the heart's responses to physiological insult. Changes in DNA (e.g. cytosine methylation) which can lead to gene inactivation or silencing have also been recently linked to increased levels of homocysteine, an important risk factor associated with CVD.

Moreover, advances in the identification and mapping of genes in the human genome that are affected in CVD may lead to improved therapies either by the use of gene replacement and/or gene suppression (silencing). Preclinical studies have demonstrated that therapeutic gene transfer can have beneficial results in the treatment of heart failure, myocardial protection, hypertension, hypertrophy, cardiac arrhythmias and myocarditis as well

as in disorders of the vascular wall where the success of drug therapy has often proved to be limited. Early phases of clinical gene therapy trials for CVD have shown promising results in particular with therapeutic angiogenesis and the treatment of restenosis. However, the development of improved vectors, methods of delivery, and the acquisition of safety and toxicity data are critically needed before the use of these therapies can be indicated in a clinical setting.

In this review, we will critically examine the information available on the role of epigenetic modifications, as well as survey the current status and prospects for epigenetic and gene therapy in humans with CVD.

II. Epigenetics and heart disease

A. Histone/chromatin modifications; epigenetic control of gene expression

The amino termini of histones contain a diversity of posttranslational modifications. The most prominent are acetylation and methylation of lysine residues in the highly conserved N termini of histones H3 and H4. Increased acetylation invariably correlates with up-regulation of transcriptional activity whereas decreased acetylation correlates with transcriptional repressed states (the heterochromatic state is associated with hypoacetylation of histones). Interestingly, the inactivation of genes in heterochromatin contrasts with euchromatin characterized by genes with robust gene expression and activation as shown in **Figure 1A**.

The regulation of heterochromatin assembly is also mediated by modifying enzymes that act directly on histones or by factors that bind them (Grewal and Moazed 2003) as depicted in **Figure 1B**. The status of histone acetylation, at a given promoter, is determined by the balanced action of histone acetyltransferases (HATs) and histone deacetylases (HDACs). These include the NAD-dependent HDAC (SIR2), a key mediator of heterochromatin assembly and gene silencing. Also, there is ample evidence for the role of repetitive DNA elements and noncoding RNAs in the regional targeting (and propagation) of heterochromatin complexes. For instance, RNAi is involved in post-transcriptional gene silencing and in initiating heterochromatin complexes at repetitive DNA. Moreover, centromeric repeat sequences produce small double-stranded RNAs (dsRNA) that are sufficient to recruit heterochromatin (Reinhardt and Bartel 2002). Small RNAs are involved in dosage compensation and genomic imprinting (Volpe et al, 2003) and dsRNAs complementary to promoter regions can cause gene silencing mediated via DNA methylation (Mette et al, 2000).

1. Histone acetylation/ chromatin remodeling plays a critical role in the triggering/ progression of cardiac hypertrophy

Among the best-characterized control points for gene regulation in hypertrophic myocardium is histone acetylation (Metzger 2002). Together with other histone modifications, the change in chromatin structure and remodeling is a prerequisite for access of transcription factors to their target DNA. The essential role of a HAT protein in cardiac muscle was first proven by deletion of the coactivator p300, which perturbed heart development and cell proliferation (Gusterson et al, 2003). Class II HDACs can act as signal-responsive repressors of cardiac hypertrophy, inhibiting gene expression that is dependent on myocyte enhancer factor-2 (Zhang et al, 2002). In addition, overexpression of the transcriptional corepressor homeodomain-only protein (Hop) causes cardiac hypertrophy by the recruitment of a class I HDAC. In addition, the activity of different HDACs can act, in some contexts, as repressors of cardiac hypertrophy by inhibiting the gene expression of pro-hypertrophic genes (**Figure 1C**) and in other contexts (e.g. recruitment by HOP) by inhibiting the expression of a novel growth-suppressing anti-hypertrophic transcriptional pathway (**Figure 1D**), HDAC activity effectively contributes to cardiac hypertrophy (Hamamori and Schneider 2003; Kook et al, 2003).

2. Other regulators of chromatin remodeling have been recently identified which may be operative in the heart

In a genome-wide search for cardiogenic genes, the *simjang* gene which encodes a protein component of the chromatin remodeling complex recruited by methyl-CpG-DNA binding proteins was found, suggesting hat epigenetic information may be crucially involved in early cardiac development (Kim et al, 2004).

Can these data be used for therapeutic purposes?. At first glance, the large scale and global nature of the transcriptional suppression engendered by this type of chromatin modification/remodeling might be difficult to apply to modulating specific pathways without compromising the gene expression, that is essential for cardiac function; however, the possibility that this modification could be finely targeted by the appropriate administration of specific small RNAs and/or repeated elements, opens the door for the directed use of global transcriptional inactivation reagents in the treatment of CVD.

B. DNA modification-methylation

DNA methylation is a key epigenetic mechanism implicated in genomic imprinting, gene regulation, chromatin structure, genome stability and disease, and is now the focus of a human epigenome project (Novik et al, 2002).

It is well established that a major mechanism for down-regulation of gene expression involves the methylation of a cytosine and guanosine rich area in the promoter region of genes termed the CpG island (see **Figure 2**). This promoter associated CpG methylation has been associated with the permanent inactivation of gene transcription (Bird 1986). This process has also been shown to be involved in the inactivation of the X

chromosome in which promoter methylation is critical in maintaining the silenced state, and where demethylation results in renewed gene expression. (Beggs and Migeon 1989). Promoter methylation is also involved in genomic imprinting in which the silenced state of the affected allele is determined by methylation of the promoter regions for numerous imprinted genes, and demethylation results in bi-allelic gene expression (Ferguson-Smith et al, 1993).

Thus far, there has been limited systematic study addressing the relationship of DNA methylation to the expression of cardiac genes either during myocardial development, normal physiological transition or during cardiac disease. Studies have demonstrated that the stability and expression of the cardiac troponin gene associated with cardiac contractility function (and disease) is affected by cytosine methylation (D'Cruz et al, 2000). Recently, it has also been demonstrated that the expression of genes known to be essential in maintaining homeostatic cardiac physiology can be modulated by targeted DNA methylation, e.g. the KVLQT1 gene involved in cardiac membrane transport is subject to regulation by DNA methylation which alters its expression. (Smilinich et al, 1999; Cerrato et al, 2002). Interestingly, defects in this gene are associated with long QT syndrome, cardiac arrhythmias and sudden cardiac death. Moreover, the mtTFA gene associated with mitochondrial biogenesis, is also regulated by DNA methylation (Choi et al, 2001).

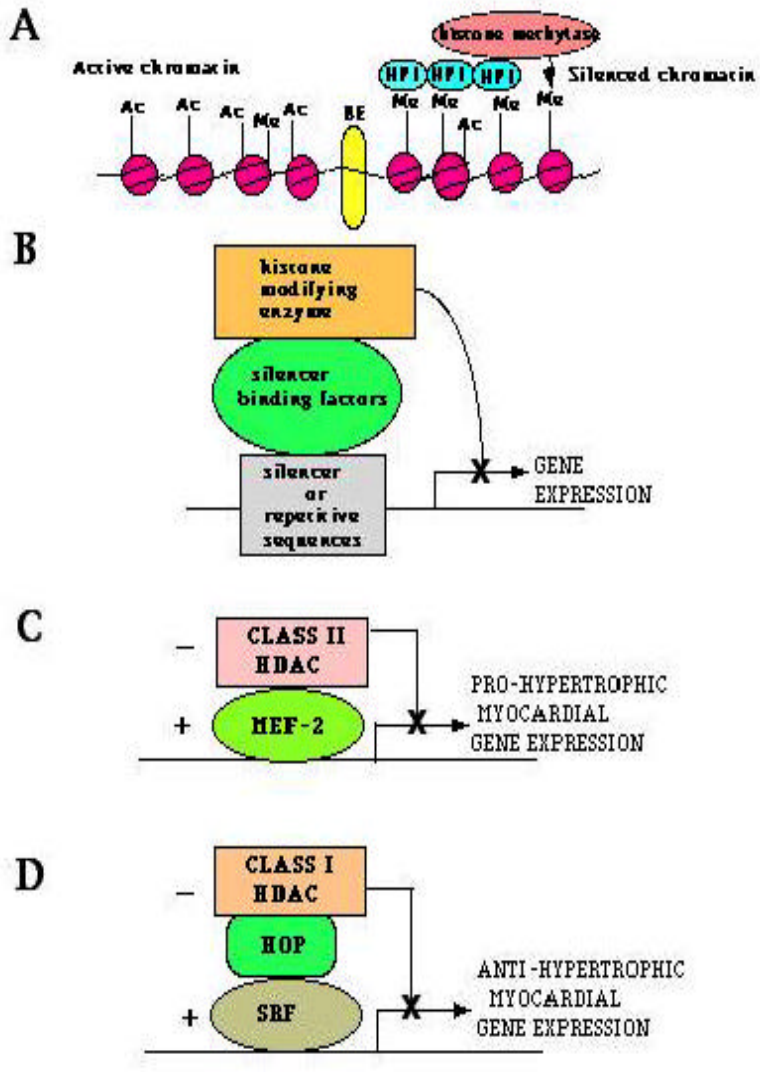

Figure 1. Epigenetic chromatin histone modifications and gene expression.
A. Representation of transcriptionally active (euchromatin) and inactive chromatin (heterochromatin) with acetylated (Ac) and methylated (ME) histone N-termini. HP1 is a transcription-inhibiting protein recruited by methylated histone residues. BE is a boundary element which separates areas of active and inactive chromatin.
B. Model of the formation of heterochromatic gene silencing.
C. Chromatin modification can block cardiac hypertrophy. A pro-hypertrophic transcriptional program mediated by the binding of myocyte enhancing factor–2 (MEF-2) is repressed with the recruitment and functional modification of chromatin by specific histone deacetylases (class II HDAC).
D. Other settings of chromatin can promote cardiac hypertrophy.
An anti-hypertrophic transcriptional program mediated by the binding of SRF (serum response factor) is repressed by the binding of the HOP protein and the recruitment of class I HDAC, promoting hypertrophic growth response.

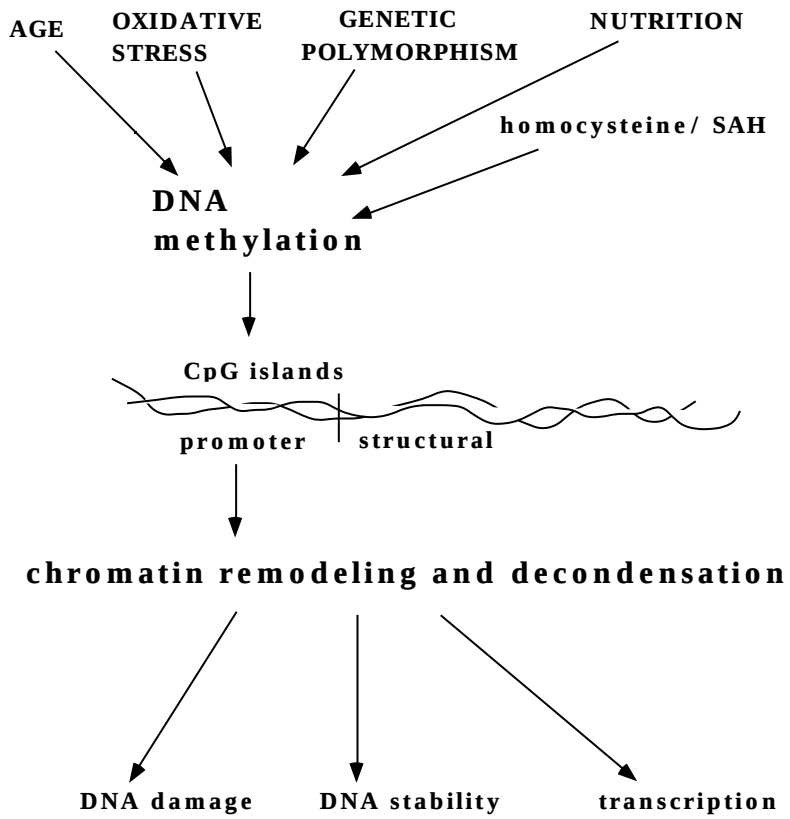

Figure 2. Model of the epigenetic role of DNA methylation in mediating gene expression, DNA damage and stability.

The promoter of human mtTFA contains 67 CpG dinucleotides particularly evident at its NRF1 binding site. *In vitro* methylation of NRF-1 site by HhaI methylase abolished the mtTFA promoter activity up to 90%, implying that the CpG methylation of NRF-1 site inactivate mtTFA promoter-driven transcriptional activity. The significance of a normal functioning mtTFA has been recently demonstrated in a transgenic mice who developed dilated cardiomyopathy by harboring a deleted mtTFA allele.

Recent studies have also reported that methylation of the estrogen receptor gene was prevalent with a non-uniform distribution in human cardiovascular tissues including right atria, saphenous veins and the proximal aorta (Post et al, 1999). In addition, elevated levels of DNA methylation were found to be age and not gender-dependent, and were more prevalent in coronary atherosclerotic plaques as compared to normal tissues.

Also, recently it has been reported that high levels of homocysteine correlated with decreased levels of DNA methylation (or increased hypomethylation). Hyperhomocysteinemia has been implicated in several CVD including its participation in the pathogenesis of occlusive CVD (Chen et al, 1999). The mechanism of the relationship between DNA methylation and homocysteine levels has been recently clarified (James et al, 2002). Chronic elevation in homocysteine levels results in parallel increases in intracellular s-adenosylhomocysteine (SAH) with the consequent inhibition of DNA methyl-transferases (Cox et al, 1977). Hence, elevated levels of DNA hypomethylation is associated with increased levels of homocysteine. Increased SAH-mediated DNA hypomethylation and associated alterations in gene expression and chromatin structure may prove informative in understanding the pathogenesis of diseases related to homocysteinemia including CVD.

A linkage of SAH-mediated DNA hypomethylation with increased oxidative damage to DNA has also been proposed (James et al, 2002). This hypothesis suggests that DNA hypomethylation increases the DNA's vulnerability and sensitivity to free radical attack. It is well established that DNA hypomethylation is associated with elevated levels of hyperacetylated and decondensed chromatin, due to decreased binding of methyl-sensitive proteins such as methyl CpG binding protein and histone deacetylase. This is supported by the promotion of chromatin decondensation by hypomethylating agents such as SAH and 5- azacytidine. A more open DNA conformation associated with hypomethylated chromatin would constitute an easier target for endonuclease digestion and increased DNA strand breaks.

The potential therapeutic application of these findings is unclear at this time. While DNA methylation-mediated regulation is generally “broad-brush” or global (similar to that described above with altered chromatin in relation to heterochromatic gene expression), site-specificity might be directed by the introduction of CpG islands into non-coding regions of introduced genetic constructs in the cardiovascular system**.**

III. Gene therapy and CVD

A. Why gene therapy for CVD?

Gene therapy enables therapeutic concentrations of a gene product to be accumulated and maintained at

optimally high levels at a localized target site of action. It also offers the possibility of minimizing systemic side effects by avoiding high plasma levels of the gene product. (Dzau, 2003).

B. Vectors and targets

Thus far, both vascular and cardiac tissues have served as targets for gene therapy (Nabel et al, 1989). For myocardial gene therapy**,** the methods of gene delivery includes (a) direct injection into the epicardium, (b) catheter-mediated injection into the endocardium or (c) into coronary arteries, (d) retroperfusion of coronary veins, and (e) intrapericardial delivery via catheter (Isner 2002; Hajjar et al, 2000). The mechanics of delivery are continuously being optimized, and are dependent on the vector used; for instance, viral rather than non-viral vectors can be effectively delivered via coronary circulation approaches ((c) and (d) above). In addition, ultrasound exposure has been shown to greatly enhance the uptake and expression of plasmid DNA in both arteries and myocardium (Lawrie et al, 2000; Chen et al, 2003).

Both viral vectors and naked plasmid DNAs have been employed in preclinical and clinical cardiovascular gene transfer studies. Naked plasmid DNA has been shown to have good entry and expression in normal and ischemic muscle (Baumgartner and Isner 2001). However, the use of plasmid vectors pose limitations with lower efficiency of transfection. Features of the viral vector predetermine both the range of host cells that can be transduced as well as the efficiency, level and duration of transgene expression. Adenoviral vectors can transduce both dividing and non-dividing cells and are particularly efficient in transfecting postmitotic cells including cardiomyocytes and to a lesser extent vascular cells, and have been the viral vector of choice primarily used thus far. A limitation of the adenoviral vectors is their provision of transient rather than prolonged transgene expression. Moreover, adenoviral vectors pose additional safety concerns; these vectors produce increased inflammation, and long term cell- and antibody-mediated immune responses have been reported in several studies (Isner et al, 2001; Pislau et al, 2002). Nevertheless, to date no evidence of serious adverse effects have been reported in clinical trials of cardiovascular gene therapy using adenoviral vector-mediated involving over 150 subjects (Isner et al, 2001). Other viral vectors are being considered for future use in cardiovascular therapies included (lentivirus) and recombinant adeno-associated virus (AAV). AAV is taken up more slowly into myocardial cells and transgene expression levels are lower compared to adenovirus, but transgene expression can be longer term, being sustained in rodent myocardium for 9 to 12 months and AAV vectors have a lower potential to induce unwanted immunocytotoxicity or inflammation (Dzau, 2003). In addition, liposomal carriers are also used, albeit less frequently.

Another alternative gene transfer approach uses oligonucleotides (e.g. antisense oligonucleotides) that regulate transcription of targeted endogenous genes and inhibit their expression. In addition, double stranded oligonucleotides homologous to the *cis* regulatory sequences of the promoter of the gene of interest can be similarly employed. These function as decoys to bind transcription factors, and therefore block expression of genes requiring those transcription factors (Morishita et al, 1998). Transcription factors are attractive targets for gene therapy since they mediate the expression of a large number of genes involved in a coordinated cellular program. Competively inhibiting the binding and activity of critical transcription factors such as NFKB and E2f involved in cell-cycle progression, cellular proliferation and inflammatory responses using the decoy approach has proved highly effective in treating myocarditis and intimal hyperplasia in preclinical studies as discussed in later sections**.**

Which genes to use in gene therapy is markedly dependent on the specific disorder to be treated, as well as which cell-type is being targeted. In the next section, we review a large assortment of genes thus far used. These range from SERCA, plasma membrane channel proteins, cytokines, transcription factors, and signaling pathway components as depicted in **Figure 3**. Early experiments found that the transfer of genes encoding extracellular (secreted proteins) rather than intracellular proteins (active secretion can either be mediated by a native or ligated signal sequence) was advantageous since a limited number of injections/ transfections was needed to produce a large enough quantity of protein; this was particularly evident with the vascular-treatments involving therapeutic angiogenesis (e.g. VEGF, FGF). The duration and level of transgene expression is also an important variable to be considered. An advantage in the treatment of some CVD is that many genes require shorter-term expression (transgene expression is required only during a period of defined risk such as remodeling after myocardial infarct, or in preventing myocardial ischemia); short-term expression (e.g. 2-3 weeks) appears to be sufficient for promoting neovascularization or inhibiting restenosis (Isner et al, 2001; Barbato et al, 2003). The regulation of transgene expression can be modulated by the addition of the appropriate regulatory sequences within the genetic construct to be introduced. These generally use constitutive promoters and enhancers. Promoter elements that are inducible in response to a variety of endogenous or exogenous molecular signals (e.g. steroid hormones, cytokines, growth factors) are also available. Moreover, by the addition of specific peptide presequences to the transgene, targeting of the gene product to the appropriate cellular compartment can also be directed. Also, prevention of unwanted transgene expression in non-target cells can be achieved by the incorporation of tissue-specific regulatory elements such as myocyte-specific promoter sequences of ventricle-specific myosin light chain-2 (MLC-2v) and cardiac troponin T (cTNT). Aiming gene therapies directly at the myocardium and at vascular tissues, transfection of cells with specific transgenes *ex vivo* followed by *in vivo* delivery of the transfected cells has broadened the possibilities associated

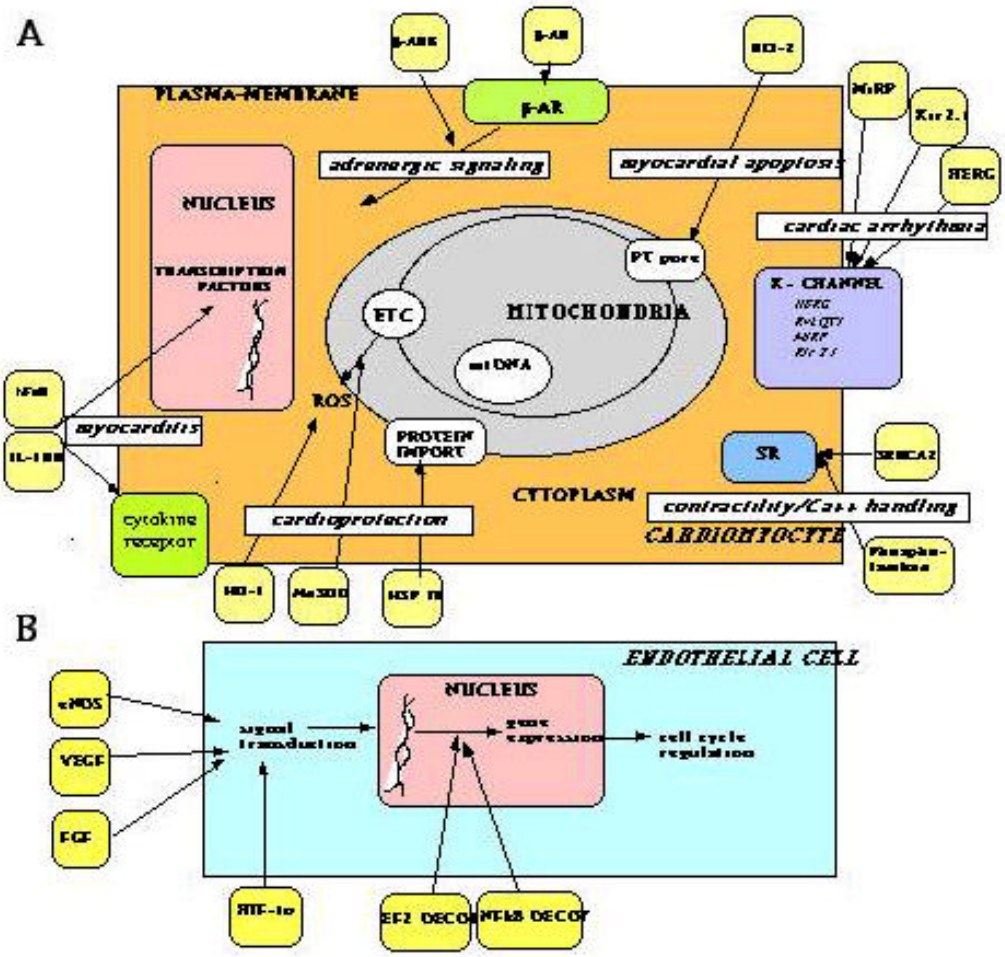

Figure 3. **Specific cellular targets of gene therapy in the cardiomyocyte and endothelial cell.** A broad array of sub-cellular loci can be effective targets of cardiovascular gene therapy including (in cardiomyocyte as depicted in Panel A): transcription factors of the nucleus, plasma membrane associated signaling pathways of cytokine, α-adrenergic signaling pathway and K+ channel, mitochondria,the primary site of energy and ROS generation by the electron transport chain (ETC), and the sarcoplasmic reticulum (SR) involved in Ca++ cycling. Targets shown in the endothelial cell (Panel B) include the nucleus and signal transduction pathways which regulate cell cycle progression and endothelial cell proliferation. For each cell type, specific genes (yellow boxes) that have been effectively used in cardiovascular therapies are shown and are more fully described in the text and in **Table I**.

Table I: Preclinical studies of gene therapy

Cardiovascular target	Gene used	Vector	Animal model	References
Restenosis	NOS	adenovirus	pig	Kibbe, 2001
	as-E2F decoy	oligos	rabbit	Mann, 1999
Therapeutic angiogenesis/ischemia	VEGF	plasmid	rabbit	Isner, 1996
	FGF	plasmid	rabbit	Tabata, 1997
	HIF-1α	plasmid	rabbit	Vincent, 2000
Hypertension	As-α-AR	liposome	SHR rat	Zhang, 2000
	as-AT1R	AAV	SHR rat	Kimura, 2001
	kallikrein	adenovirus	rat	Agata, 2002
	ANP	adenovirus	Dahl rat	Chao, 1998
	adrenomedullin	adenovirus	SHR rat	Chao, 2001
	eNOS	plasmid	SHR rat	Lin, 1997
Cardiac arrhythmia	ShK	adenovirus	Dog myocyte	Nuss, 1996
	KIR2.5/SERCA	adenovirus	guinea pig	Ennis, 2001
	MiRP	plasmid	pig	Burton, 2003
	Gαi2	adenovirus	pig	Donahue, 2000
Myocarditis	IL-10	plasmid	Rat (EAM)	Watanabe, 2001
	NFκB/cis decoy	oligos	Rat (EAM)	Yokoseki, 2001
Heart failure	α-AR K inhibitor	adenovirus	rabbit	Shah, 2001
	SERCA2	adenovirus	rat	Miyamoto, 2000

Myocardial protection	HO-1	AAV	rat	Melo, 2002
	SOD	adenovirus	rat	Abunasra, 2001
	HSP 70	Liposome	rat	Jayakumar, 2001
	BCl-2	adenovirus	rabbit	Chatterjee, 2002
Pulmonary hypertension	eNOS	adenovirus	mouse	Champion, 1999
	Kvi.5	adenovirus	rat	Pozeg, 2003
	PGIS	adenovirus	rat	Suhara, 2002

Table II: Clinical studies of gene therapy

Cardiovascular target	Gene used	Vector	Mode of delivery	References
Restenosis	VEGF	Plasmid DNA	Balloon catheter	Vale, 1998
	E2F decoy	Oligonucleotide	pressure-mediated transfection of vein grafts	Mann ,1999
CAD	VEGF-A	Naked DNA	Intramyocardial injection	Lasordo, 1998
	VEGF	Liposome/adenovirus	Infusion/perfusion	Laitinen 2000
	VEGF	Adenovirus	Intramyocardial injection	Rosengart, 1999
	FGF-4	Adenovirus	Intracoronary injection	Hammond, 2001
	VEGF-C	Naked DNA	Intramyocardial injection	Yla-Herttula, 2000
	VEGF	Plasmid DNA	Intramyocardial injection	Vale, 2000

with gene therapy and potentially sidestepped a number of the safety concerns. Gene transfer of VEGF has been shown to augment the proliferative activity of bone marrow-derived endothelial progenitor cells which increase neovascularization when administered to animals with limb or myocardial ischemia (Kalka et al, 2000; Kawamoto et al, 2001). Future approaches might use gene therapy to enhance the homing and engrafting of bone marrow derived progenitor cells to the heart into areas damaged by ischemia promoting neovascularization for tissue repair.

C. Specific categorized examples of cardiovascular gene therapy-Preclinical studies

1. Restenosis

One of the initial CVD targeted for gene therapy was restenosis following coronary stenting and vein graft occlusion (Isner et al, 1996; Kibbe et al, 1999; Kullo et al, 1999). Vasculoproliferation, characteristic of these disorders, has been effectively inhibited in a variety of preclinical studies by the transfer and overexpression of genes, including isoforms of nitric oxide synthase (Kibbe et al, 2001) as well as by targeting gene expression of cell cycle progression, including the use of decoy and antisense genetic constructs to knock-out vasculoproliferative gene expression in vascular smooth muscle (Gascon-Irun et al, 2003). While advances in non-genetic treatments of these disorders, and concerns with their efficacy and safety have been raised, clinical trials for the use of gene therapy in restenosis are currently in place and are described in a later section (Dzau, 2003).

2. Therapeutic angiogenesis (increasing proliferation of blood vessels) to limit ischemia

The pioneering studies of the Isner laboratory (Isner et al, 1996, 1996a; Baumgartner et al, 1998; Losordo et al, 1998; Vale et al, 2000) demonstrated that the delivery of angiogenic peptides by gene transfer could enhance blood flow in ischemic tissue. In a number of animal models, vascular endothelial growth factor (VEGF), a secreted endothelial mitogen, markedly induced angiogenesis. Interestingly, gene transfer of HIF-1α, a transcription factor known to regulate the transcription of hypoxia-inducible genes also induces angiogenesis at ischemic sites (Vincent et al, 2000). Clinical trials using plasmid delivery of VEGF into ischemic limbs (Fortuin et al, 2003; Grines et al, 2003), have initially proved promising and have been extended to include other angiogenic transgenes including other VEGF isoforms and fibroblast growth factor (FGF) as described in a later section.

3. Hypertension

Gene therapy has been effectively applied in preclinical studies with both systemic and pulmonary hypertension. Two major approaches to gene therapy for treatment of systemic hypertension include the overexpression of vasodilator genes and reduction of vasoconstriction gene expression (usually accomplished by antisense inhibition). Preclinical studies in rat have demonstrated that the transfer and expression of genes, including an O2-sensitive voltage-gated potassium channel (Kv1.5), endothelial nitric oxide synthase (eNOS) and prostaglandin I synthase, (PGIS) markedly improved pulmonary hypertension (Suhara et al, 2002; Pozeg et al, 2003).

Because antihypertensive treatments often are not aimed at a specific identifiable causes, traditional pharmacological therapy has focused on elements such as the renin-angiotensin system (RAS), that are known to be directly involved in the control of blood pressure (Gelband et al, 2000). The development of gene therapy directed at RAS represents a significant advance toward managing high blood pressure and reversing its associated pathophysiology. Delivery of antisense constructs to the angiotensin II type 1 receptor (AT1R) successfully prevented blood pressure elevation, alterations in calcium

homeostasis, ion channel activity and cardiovascular ultrastructure changes (for up to 18 months) in spontaneously hypertensive rats (SHRs) when compared to control rats. These results, in animal models, demonstrate that antisense gene delivery may be useful in the long-term treatment of hypertension. In addition to the angiotensin receptor, another genetic component of RAS targeted in the therapeutic treatment of hypertension by deploying antisense oligonucleotide therapies is angiotensinogen (Tomita et al, 1995, Phillips, 1997, Kimura et al, 2001). Antisense oligonucleotides used in combination with an adeno-associated virus delivery system resulted in a significantly prolonged reduction of hypertension in adult rats and transgenic mice, with a single dose administration of AAV-antisense constructs (Phillips, 2002). Moreover, it has been shown that concomitant with the reduction of hypertension, the angiotensinogen and angiotensin-receptor antisense therapies resulted in significant attenuation of cardiac hypertrophy [Kimura et al, 2001, Reaves et al, 2003].

The α-adrenergic signaling system is another viable target for gene therapy, designed to stem hypertension by utilizing antisense technology. Transfer of antisense oligonucleotides against rat (β1)-adrenergic receptor (β(1)-AR) mRNA provided a significant and prolonged reduction in blood pressure in the SHR model (Zhang et al, 2000).

Genes involved in vasodilation regulation have also proved to be excellent targets for gene therapy of systemic hypertension. Several studies in adult rats demonstrated that systemic hypertension can be significantly reversed by transfer and overexpression of genes encoding atrial natriuretic peptide, kallikrein, adrenomedullin, and endothelial nitric oxide synthase (eNOS) over a range of a 6 to 12 week period (Lin et al, 1997; Chao et al, 1998; Chao et al, 2001; Agata et al, 2002). Delivery of these genes used either non-viral (naked DNA) or adenoviral vectors with assorted rat models of hypertension (including SHR and Dahl salt-sensitive rats). Interestingly, the decrease in blood pressure mediated by gene transfer and overexpression of kallikrein and adrenomedullin was also accompanied by an attenuation of both cardiac hypertrophy and myocardial apoptosis.

4. Cardiac arrhythmias

The treatment of cardiac arrhythmias using gene therapy may provide a significant advance since current treatment options are limited. Several well defined genetic loci have been characterized, which can lead to ventricular arrhythmias, including gene defects in the membrane transporters associated with potassium channels.

Adenoviral-mediated transfection of a potassium channel gene has been performed with cardiomyocytes derived from failing dog hearts (Nuss et al, 1996). Interestingly, a moderate level of transgene expression increased the K channel current reversing the K channel deficiency and mimicking the non-disease phenotype. However, a robust level of transgene expression adversely impacted cardiomyocyte excitation-contraction coupling.

A dual gene therapy strategy was implemented to reverse K channel deficiency or down-regulation without depressing contractility (Ennis et al, 2002). An adenoviral vector was constructed which enabled the co-expression of two genes driven by a single promoter. The genes encoding the potassium channel (Kir2.1) and SERCA1, to boost contractility, were directly injected into the myocardium. Both genes were amply expressed and myocytes from transfected hearts exhibited significantly shorter action potentials and larger calcium transients, as well as unimpaired contractile function.

Recently, plasmid-mediated gene transfer of specific mutant alleles of potassium channel genes has been used (Burton et al, 2003). A channel gene abnormality associated with the long QT syndrome (Q9E-hMiRP1) was introduced into human cell lines and implanted directly into the pig atria. Results of these *in vitro* and *in vivo* studies have demonstrated significant levels of transgene expression and altered myocyte electrophysiological phenotype supporting the feasibility of site-specific gene transfer in the treatment of atrial arrhythmias.

Successful intracoronary adenoviral-mediated gene transfer of an inhibitory component (Gαi2) of the α-adrenergic pathway, directed to the atrioventricular (AV) node to suppress AV node conduction, has also been reported (Donahue et al, 2000).

5. Myocarditis

It is well established that the inflammatory cytokines play a critical role in the pathogenesis of viral myocarditis. In the rat model of experimental autoimmune myocarditis (EAM), introduction of plasmid DNA containing the gene encoded murine interleukin IL-10 into striated muscle (tibialis anterior) by electroporation, significantly affected survival rates, attenuated myocardial damage and improved hemodynamic parameters (Watanabe et al, 2001).

The transcription factor NFkB, by modulating the expression of TNF-α, inducible nitric oxide synthase (iNOS) as well as adhesion molecule (iCAM) genes, represents a potential target to reduce myocarditis using the same rat model of EAM. Reducing the expression of NFkB, by introducing into the rat coronary artery a decoy sequence directed to the cis-regulatory sequence within the NFkB promoter, reduced the areas of myocarditis as well as myocardial gene expression of iNOS, iCAM and TNF-α (Yokoseki et al, 2001).

6. Heart failure

In both human and experimental models of heart failure, sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) activity is decreased, resulting in abnormal calcium handling thought to contribute to severe reduction in cardiac contractility. Myocardial SERCA2 Ca^{2+} pumping activity is inhibited by phospholamban. Adenovirus-mediated overexpression of SERCA2 by *in vivo* gene transfer in a pressure-overload rat model of heart failure restored cardiac function including improved Ca^{2+} cycling and contractility (Miyamoto et al, 2000). Moreover, adenoviral-mediated transfer and overexpression of an antisense phospholamban construct or a dominant-negative mutant allele of phospholamban enhanced both

SERCA2 activity and contractility in myocytes derived from failing rat and human hearts (He et al, 1999, Del Monte et al, 2002). Caution must be used in applying these findings to human clinical trials since the models of heart failure are different (Dorn and Molkentin, 2004). Ablation of the phospholamban allele in some mouse models of heart failure reversed cardiac dysfunction whereas in other models no beneficial effect was found (Minamisawa et al, 1999; Dorn and Molkentin 2004). Moreover, polymorphic null-alleles of phospholamban have been recently identified in individuals with lethal dilated cardiomyopathy (Haghighi et al, 2003).

It is well established that both experimental models and human heart failure exhibit marked abnormalities in β-adrenergic signaling including the downregulation of β-adrenergic receptors (β-AR), their uncoupling with second messenger pathways and modulation by upregulated β-AR kinase (β-ARK). Intracoronary adenoviral transfer of an adenovirus encoding a peptide inhibitor of β-ARK, reversed cardiac dysfunction in both rabbit and mouse models of heart failure (Rockman et al, 1998; Shah et al, 2001). However, side-effects with this approach have been reported that may result in sustained adrenergic stimulation, which can be both cardiotoxic and arrhythmogenic (Hajjar et al, 2000).

7. Myocardial protection

Short-term protection of the heart from ischemia can be provided by gene transfer and overexpression of cardioprotective genes such as superoxide dismutase (SOD) or heme oxygenase (HO-1). Administration of a myocardial protective gene such as HO-1, employing a recombinant AAV vector, significantly reduced infarct size in a rat model of ischemia and reperfusion when introduced into myocardium prior to ligation (Melo et al, 2002). The resulting cardioprotective effect was maintained over 5 days, as gauged by echocardiography. In addition, gene-mediated cardioprotection against myocardial ischemia has been achieved by introducing and overexpressing genes for anti-oxidant scavenger SOD (Abunasra et al, 2001), the heat shock protein HSP70 (Jayakumar et al, 2001) and the anti-apoptotic protein BCl-2 (Chatterjee et al, 2002). It remains to be seen whether these vectors and genes can provide longer term cardioprotection against repeated, chronic forms of ischemic insult.

As a methodology for acute intervention in cardiac insults (e.g. myocardial ischemia), myocardial gene therapy may have limited efficacy. A period of time is clearly needed for transgene introduction, transcription, translation and processing of the transgene product. Gene therapy in advance of specific insults, which prepares the cardiomyocyte to mount a protective response to an adverse event, may be more rewarding. Recently, a prototype cardioprotective vector has been developed called the “vigilant vector", designed to be expressed specifically in the heart and switch on therapeutic transgenes only during hypoxia (Phillips et al, 2002). It utilizes several elements including a cardiac-specific promoter (MLC-2v), a hypoxia response element (HRE), a therapeutic transgene and a reporter gene (green fluorescence protein) incorporated into an AAV vector. High levels of cardiac-specific transgene expression (AT1R) have been achieved with this vector with transfected cardiomyocytes (H9C2 cells), and *in vivo* with mice challenged with hypoxia.

D. Clinical studies of cardiovascular gene therapy thus far

Several small phase 1 and 2 studies have been conducted with adenovirus- and plasmid-based VEGF and FGF gene constructs to provide therapeutic angiogenesis in coronary artery disease (CAD) and peripheral vascular disease (PVD). While recent studies have reported that new vessels are formed and functional, the potential efficacy have not yet been ascertained. Similarly, data are not yet available from large-scale randomized trials of VEGF treatment in patients with CAD and PVD; parenthetically, symptomatic relief in some patients with severe CAD has been reported. Obviously, prior to routine clinical use additional data to develop the optimal dosage and delivery protocol are necessary. Moreover, concerns about the potential for retinal complications and tumor growth enhancement, due to increased vascularization will have to be further addressed reinforcing the need for critical subject selection and caution in VEGF use (Ratko 2003).

The Restenosis Gene Therapy (REGENT) trial, a phase 1 study, is in progress and should provide information about dosage and safety.

Inhibition of vasculoproliferation by targeting cell-cycle activation is also the rationale in clinical trials of antiproliferative gene therapy to prevent bypass vein graft failure after coronary artery bypass surgery (Mann et al, 1999). Data from the Project in Ex-Vivo Vein Graft Engineering via Transfection (PREVENT I and II), randomized, placebo-controlled studies have shown the safety and feasibility of using a synthetic DNA decoy to sequester the E2F family of transcription factors and arrest cells at the gap period (G1) checkpoint in the cell cycle. This mechanism can prevent intimal hyperplasia, associated with atherosclerosis and coronary graft failure, and phase III trials are presently in progress (Dzau 2003).

E. Future directions

Clearly, while the promise of effective gene therapy for a variety of cardiovascular disorders appears closer to being realized, with over 40 clinical trials currently in progress (Barbato et al, 2003), more data concerning the safety, efficacy of delivery and long range consequences of cardiovascular gene therapy is critically needed. The utilization of epigenetic mechanisms of gene regulation to reversibly modulate the expression of cardiovascular transgenes in distinct fashion from endogenous gene expression, offers a potential added value for future therapeutic approaches.

A number of cardiovascular disorders have not yet been examined with regards to either preclinical or clinical studies of gene therapy. Increasingly, cardiomyopathies with a pronounced mitochondrial-based cytopathy and bioenergetic dysfunction have been reported, a subset of which are due to defined mtDNA or nuclear DNA

mutations (Marín-García et al, 2000,). Potential gene therapy has been proposed for these mitochondrial cardiomyopathies utilizing *ex vivo* transfection of stem cells with their subsequent introduction to the diseased heart (Marín-García and Goldenthal 2002). This approach would employ the introduction of mtDNA-repaired stem cells into a patient harboring a mtDNA mutation, thereby potentially transforming a bioenergetically dysfunctional heart into a healthy one. The mtDNA-repaired cell can be derived from the patient's own cells or from embryonic stem cells grown *in vitro*, whose endogenous defective mtDNA genome has been entirely eliminated by treatment with ethidium bromide, and replaced by entirely wild-type mtDNA genes. Similarly, a patient's nuclear DNA mutations might be replaced in *ex vivo* grown stem cells with wild-type alleles using site-specific homologous recombination or containing transplanted nuclei. While these scenarios have not yet been tested, they have become more feasible with the recent identification of cardiac-specific stem cells, which can be grown *in vitro*, and successfully transplanted into the heart. (Beltrami et al, 2003).

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