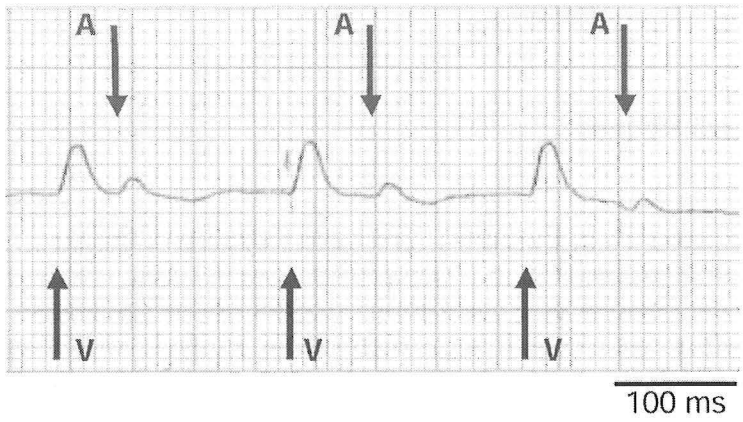
Cardiovascular Gene Therapy: Angiogenesis and Beyond



"Biological pacemaker created by gene transfer" Miake et al., *Nature* 419:132, 2002.

Internal Medicine Grand Rounds

June 5, 2003

Ralph V. Shohet, M.D.

Ralph Shohet, M.D. has no financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Shohet will not be discussing off-label uses in his presentation.

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Our lab investigates gene expression in the heart. We have developed tissue-specific microarrays to examine expression in a comprehensive fashion, and are applying these tools to both exogenous and transgenic models of cardiac stress. In addition to insights into pathophysiology, this transcriptional analysis identifies targets for genetic analysis, which we are evaluating in large numbers of patients with heart disease. We are also exploring a new way to modify gene expression, using ultrasound targeted microbubble destruction to place transgenes in the heart and other organs.

Recent advances in conventional therapy have produced gratifying improvements in the care of cardiovascular disease. Statins, beta-blockers and angiotensin converting enzyme inhibitors have clear and substantial benefits and the use of stents and internal mammary grafts have improved results with angioplasty and bypass grafting. However, many patients are not helped, even if they receive optimal care, and some are harmed. New strategies would be welcome. The rapid progess in our understanding of the mechanisms of cardiovascular pathophysiology, as well as dramatic advances in molecular biology and human genomic science, have produced many new targets, as well as improvements in the delivery mechanisms, for gene therapy. Here I will review the vectors and delivery methods that show the most promise for cardiovascular therapy. I will then examine the progress in this discipline for three of the main problems in cardiovascular medicine; angiogenesis, as an approach to atherosclerotic obstruction, specific therapies for heart failure, and potential applications for modulation of dysrhythmias.

Cardiovascular disease presents several tempting targets for gene therapy including refractory ischemia in the heart and limbs, restenosis, and advanced heart failure. There are a substantial number of patients with one of these syndromes who have no real options with current medical therapies other than narcotic analgesia. Gene therapy protocols are reasonable options for these patients and represent a moderate portion of the present trials, primarily for angiogenesis (Fig 1).

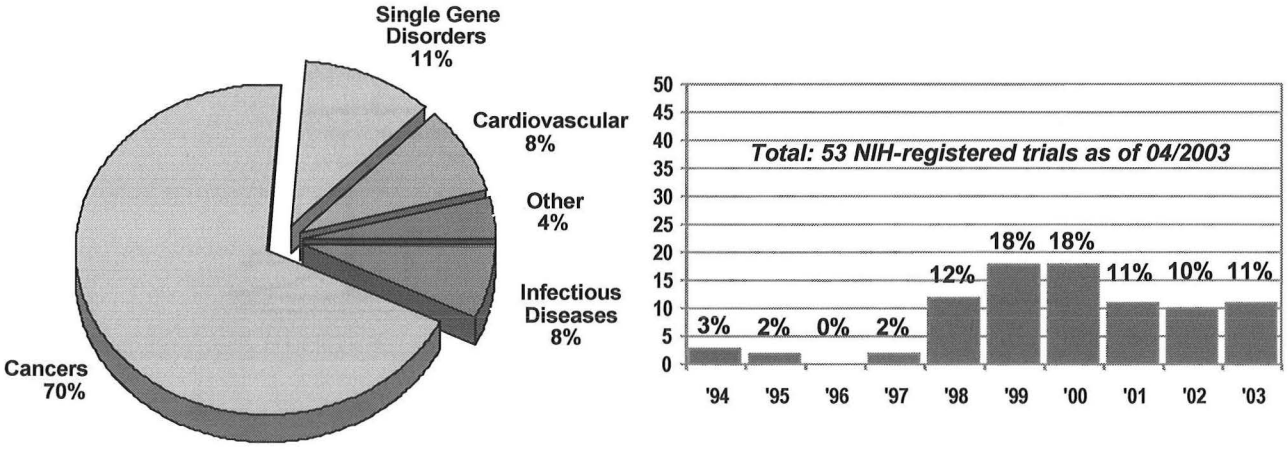


Fig. 1. The percentage of all gene therapy trials directed at specific disease processes is shown at left, the history of the percentage of the total made up of cardiovascular trials on the right. It may be that the growing enthusiasm for cardiovascular trials in 1999 and 2000 was blunted by the unimpressive results of large clinical studies of first generation angiogenic agents. Data is from the Office of Biotechnology Activites (OBA) of the NIH.

Gene therapy developed 15 years ago with great optimism as an approach for the correction of inherited genetic diseases such as adenosine deaminase deficiency, hemoglobinopathies, muscular dystrophy and cystic fibrosis. Gene therapy for these disorders is reasonably thought of as requiring lifelong replacement of a missing gene product, with high levels of expression, from an early age, to avoid early and progressive morbidity and mortality. The therapeutic successes in the field have been limited, and these studies have been recently further tarnished by the recognition of the unanticipated frequency of insertional mutagenesis by retroviral vectors[14].

The cardiovascular equivalent of this approach might be LDL receptor replacement for homozygous familial hypercholesterolemia[15,16]. By contrast, gene therapy for common cardiovascular disease appears to require relatively brief expression of transgenes to alter or augment the physiological response to a discreet insult. The molecules used for angiogenesis may be particularly felicitious agents for brief, low level expression; as signaling molecules their effects are amplified and the brevity of expression, apparently sufficient for induction of angiogenesis, may reduce the likelihood of untoward side effects.

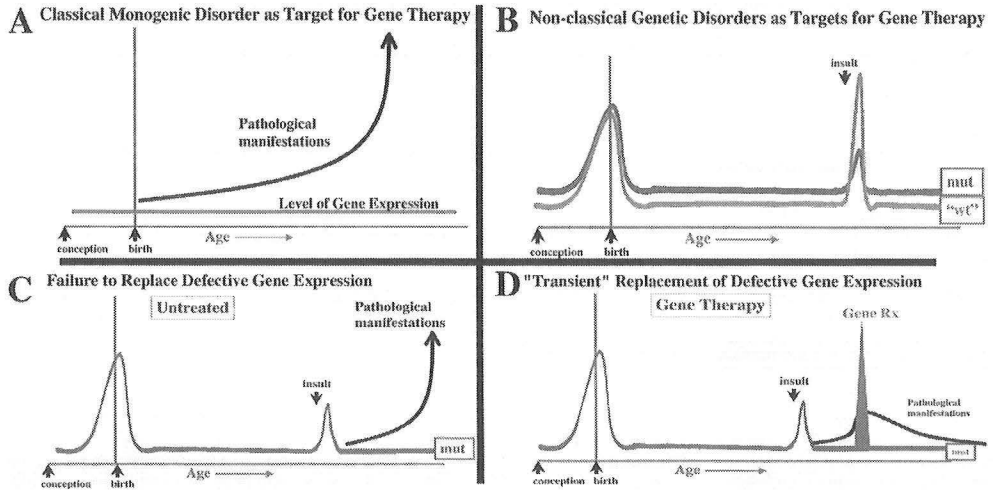


Figure 2. In the initial approach to gene therapy, lifelong, high level expression was required to avoid early, progressive morbidity from monogenic, inherited defects, as schematically presented in panel A. Panel B shows the theoretical situation in common cardiovascular disease, with insufficient expression of a crucial gene in response to a physiological insult in the mature patient. Panel C suggest how this insufficient response leads to onset and progression of pathology and panel D how the relatively brief expression of a transgene could ameliorate the course. Modified from Isner et al., Circ Research 89:389, 2001[7].

Gene therapy can include any placement of foreign genetic material to achieve a therapeutic result. However, this discussion will be limited to a rigorous and somewhat exclusive definition of gene therapy, namely placement of functional genes that directly influence some aspect of pathophysiology. This excludes many interesting approaches including genetic immunization[17-19] and modulation of transcription with antisense and RNAi strategies[20-22].

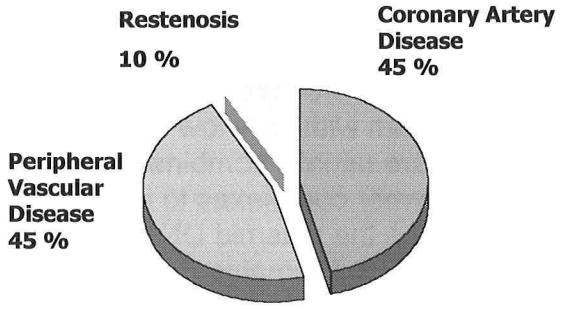


Figure 3. Target diseases of cardiovascular gene therapy trials to date. Data is from the OBA.

Access to the cardiovascular system can be as simple as venapuncture or as complex as left ventricular catheter injections guided by electromechanical mapping[11]. Transgenes can be simply released into the blood stream. This is not a reasonable strategy for naked DNA because of abundant DNAses that rapidly digest unprotected DNA. However, when coated with lipid, or incorporated into a virus, transgenes can survive the rigors of the circulation. Unfortunately, some anatomical localization, often to a very specific region, is usually required and is difficult following systemic release. Engineering of viral vectors for trophism to an organ of interest[23], or tissue specific promoters that will only permit transcription in a limited setting[24] are two possible solutions for this difficulty but neither has yet shown practical application in humans. Since the death of Jesse Gelsinger[25], in a gene therapy trial involving systemic adenovirus, only targeted approaches have received further attention in clinical studies.

Vectors

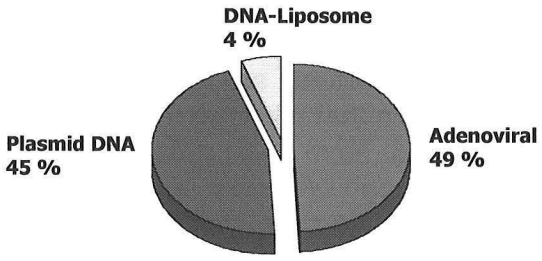


Figure 4. Distribution of vectors in cardiovascular gene therapy trials. Data is from the Office of Biotechnology Activites (OBA) of the NIH.

The choice of gene therapy vector depends primarily on the level and duration of transgene expression required and the role of the immune response. Other

considerations include the tissue to be targeted. Viral vectors are chosen for long-term expression and retroviruses were most highly represented in the early efforts at treatment of monogenic disorders.
For the brief, lower levels of expression required for angiogenesis, both nonviral vectors and adenovirus seem appropriate.

Plasmid

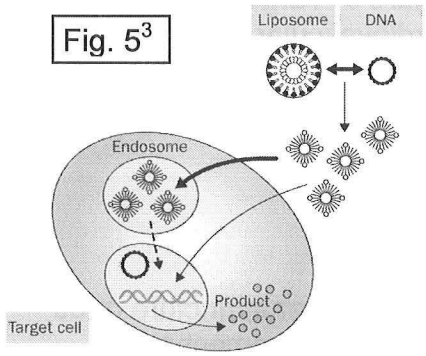


Fig. 5[3]

Naked plasmid DNA crosses into cells by unclear mechanism with very low efficiency. Thus plasmids are usually combined with liposome or polymer complexes to improve transfection. Once the plasmid DNA finds its way into the nucleus it remains extrachromosomal and directs transcription. The advantages of plasmid DNA include: no limitation on the size of transgenes, the absence of potentially immunogenic viral proteins, and ease of preparation, new constructs can be created quickly and easily, facilitating experimental evaluations. The main limitation of this approach is low efficiency of transfections and rapid clearance of the transgene. Also the plasmid vector has to be delivered to the site of action protected from DNAses abundantly present in plasma.

Viral – Adenovirus

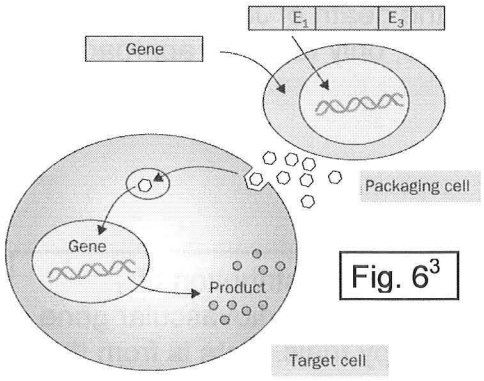


Fig. 6[3]

Adenovirus enters the cell via specific receptor-mediated endocytosis. They can then disrupt the enveloping lysosome to which they are targeted obtaining release into the cytoplasm with subsequent transport to the nucleus. There they remain extrachromosomal and direct transient expression of their transgene. Replication – deficient adenoviruses are made in packaging cells that produce the viral gene products which have been deleted to make room for the transgene. Advantages include: virus can be easily created and prepared at high titer, it will readily infect nonreplicating cells, and it produces high efficiency transduction and high level expression of transgenes. Disadvantages are transient expression, in large part due to a robust immune response to viral proteins. Other problems include hepatic trophism with resulting hepatitis.

Targeting adenovirus - Two modifications of adenovirus are required for targeting to a specific anatomic or pathologic site. Interaction with native receptors must be removed and new tissue-specific ligands added. This is being done in two ways: 1. a single molecule, such as a bispecific antibody, that blocks receptor interaction and confers a new affinity, 2. genetic engineering of the virus to remove native receptor interactions and to introduce a new ligand affinity[23,26,27]. Ultimately this approach could be combined with new maximally deleted adenoviral backbones[28] and tissue-specific or pathology-specific promoters to generate an optimal gene therapy vector that delivers its transgene with high specificity, minimal immunogenicity and expresses it precisely where and when it is required.

Viral – Adeno Associated Virus

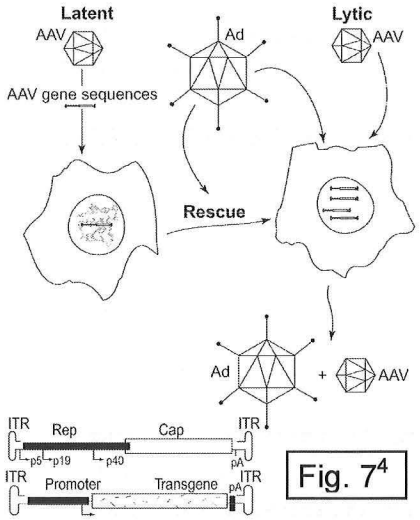


Fig. 7[4]

Adeno-associated virus was first identified as a contaminant in stocks of adenovirus. It is a non-pathogenic Parvovirus that requires other helper viruses for replication. It has a single stranded DNA genome that can integrate into host chromosomal DNA (when helper virus is not available to create the setting for lytic infection). Remarkably, the wild-type virus has a preferred site of integration, on chromosome 19. Unfortunately, initial excitement about the potential use of this site-specificity for non-toxic long-term integration was dispelled by the finding that site-specific integration required the Rep protein of the virus, which had to be removed to create room for a transgene. In fact, the entire genome of the native virus can be removed in creating a gene therapy vector, except for the inverted terminal repeat at the ends of the genome. These are the only sequence elements required for packaging and integration, and also act as origins of replication. Advantages of AAV for gene therapy are: 1. the potential for long lasting gene expression from integrated transgenes, even in nonreplicating cells, 2. the virus efficiently transduces a broad range of cells – one receptor is the relatively widely expressed heparan sulfate, and 3. the paucity of required viral genes reduces the response of the host immune response, which contributes to the increased duration of expression. Disadvantages include: 1. very small packaging capacity (<4.3kb), 2. risk of insertional mutagenesis, and 3. it is logistically difficult to produce large amounts of virus.

Viral - Retrovirus

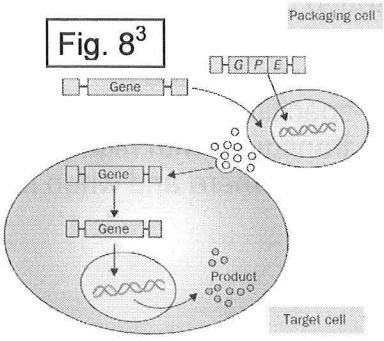


Fig. 8[3]

Retroviruses enter cells via specific receptors whereupon their genomic RNA is reverse transcribed into DNA that is stably integrated into the host genome. This permits long-lasting expression of transgenes. However, transcription of the retroviral genome requires cell division and this vector is only appropriate for proliferative tissues, substantially reducing its utility for most cardiovascular indications, and limiting its clinical use to treatment of restenosis where proliferation of smooth muscle cells is sufficient to sustain integration. Replication defective retroviruses are made in cell culture by packaging cells that synthesize the coat proteins that have been deleted from the genome of the therapeutic retrovirus to make room for the transgene. This results in two other limitations of retroviruses – a limited size for the inserted transgene and difficulty in producing high titers of therapeutic retroviruses for treatment.

Delivery methods

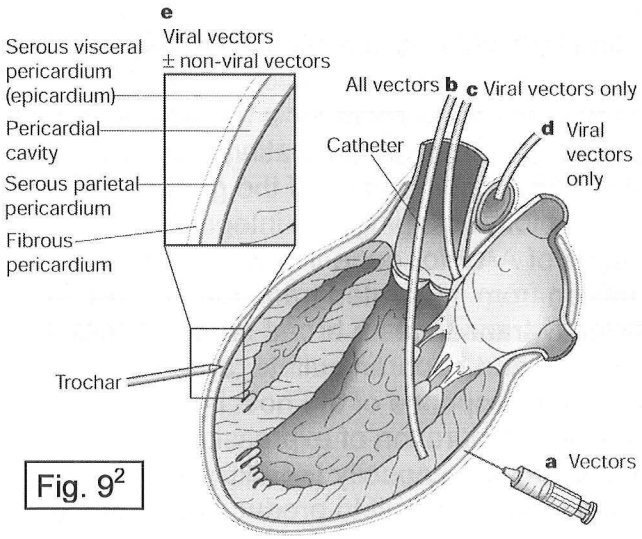


Fig. 9[2]

Both direct injection of vectors into the heart (at the time of thoracic surgery)[29] and into skeletal muscle (distal to atherosclerotic obstruction) have been used for human therapy. Transfection tends to be inefficient and transient and expression is limited to the region very close to the track of the injecting needle. This may be sufficient for a secreted protein that acts locally. There is a special situation, during coronary artery bypass surgery, when the saphenous vein is available for genetic modification outside of the body. At that time one can potentially improve the subsequent performance of the vein-graft by engineering of the endothelium [30-33]. A special anatomic characteristic of the heart is the pericardial sack, which would seem to be a particularly appropriate way to confine a gene therapy to the epicardial surface (a potentially desirable location for arteriogenesis). Attempts at

gene therapy by this route have shown good and specific epicardial transfection, although not the hoped for angiogenesis[34].

Much effort and interest has revolved around catheter-based tools for placement of gene-therapies at the site of atherosclerotic plaque, down a coronary, or into the myocardium by injection. The main lesson of such catheter studies has been that a healthy endothelium presents a substantial barrier to transfection, and increases in transgene delivery tend to be directly related to the degree to which the endothelium is disrupted or impaired[35].

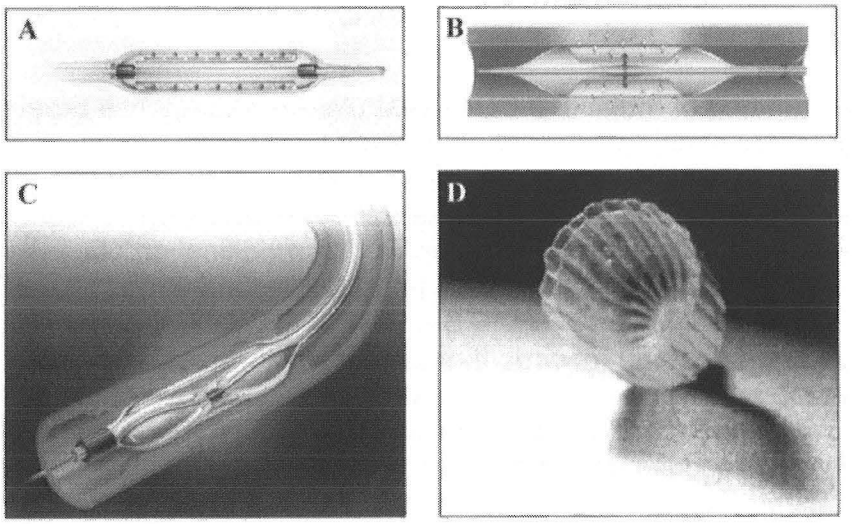


Fig. 10. Catheters for gene delivery. Panel A: the "Infiltrator" has a PTCA balloon surrounded by 21 microinjector ports. Panel B: the "Crescendo" balloon has an inner balloon covered by a micropoous membrane. Panel C: the "InfusaSleeve" can be positioned over standard PTCA balloons, with subsequent infusion through the sleeve. Panel D: the "Remedy" balloon has both a high pressure PTCA balloon and drug infusion capacity in the surrounding channels. Modified from Varenne et al., Human Gene Ther. 10,1105, 1999[3].

Agents for Angiogenesis

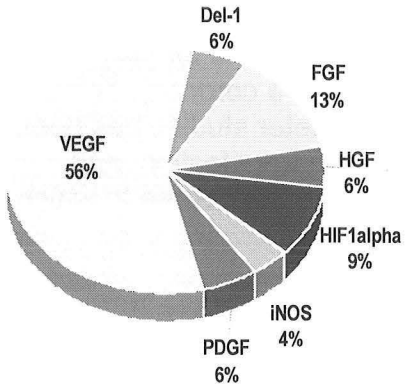


Fig. 11. The specific genes used in all cardiovascular gene therapy trials. Data is from the Office of Biotechnology Activites (OBA) of the NIH.

As our understanding of molecular cardiovascular physiology has progressed the number of potential candidates for gene therapy has rapidly grown. In laboratory animals these include strategies to modify lipid profiles[36,37], systemic hypertension[38], primary pulmonary hypertension[39], modulation of vascular reactivity in stroke[40], efforts to limit restenosis[41-43], modification of cardiac contractility and apoptosis to limit or treat heart failure and even initial efforts to modify the cardiac conduction system. But 90% of the gene therapy protocols in humans have been directed towards therapeutic angiogenesis for atherosclerotic vascular disease and we will focus on those studies. Only two of the potential therapeutic genes have reached substantial clinical tests, Fibroblast Growth Factor (FGF) and Vascular endothelial growth factor (VEGF). These have been studied both as proteins and as viral and plasmid gene therapy vectors.

Fibroblast Growth Factor

The first trial of gene therapy directed toward a cardiovascular disease began in 1994 with an effort at angiogenesis for peripheral arterial obstruction using intramuscular injection of plasmids encoding Fibroblast Growth Factor (FGF)[44]. These signaling molecules have also been used in the largest clinical studies[1]. They are a family of, presently, 22 related proteins that share structural homology and bind to FGF receptors[45]. The 4 receptors have extracellular immunoglobulin-like domains and cytoplasmic tyrosine kinase activity[46]. Alternative splicing confers further variation. FGF1 and FGF2 lack signal peptides that direct the other FGFs to the extracellular matrix. They have been most studied for their angiogenic potential, although FGF3,4 and 5 also promote angiogenesis in animal models. The apparent redundancy of FGFs and their multiple receptors produce a potential for complementation that has interfered with the analysis of specific differences in their biological activity. This may be why transgenic knock-out of the genes for specific FGFs leads to only mild phenotypes[47].

The precise mechanisms of angiogenesis induced by FGFs are incompletely understood. The best studied, FGF2 ("basic FGF") stimulates proliferation and migration of endothelial cells and is also mitogenic for smooth muscle and

macrophages. This may result in formation of more robust vessels than agents that only stimulate endothelium[48]. Encouraging results with FGF 1,2,4 and 5 in dogs and pigs[49] led to phase one studies in patients with myocardial or peripheral arterial ischemic syndromes. These initial studies were designed to assess safety and dose with various delivery strategies and focused on use of the peptide rather than gene. Intramyocardial injection of peptide at the distal enastomosis during CABG suggested increased collateralization[50] and epicardial placement of slowly released peptide appeared to reduce symptoms and the severity of ischemia, with benefits lasting at least 3 years[51]. Even simple intracoronary infusion of FGF2 appeared to improve exercise capacity and improved wall motion[52]. However, when these claims were tested in a large double-blind phase II trial[53], the FIRST trial, (FGF Initiating RevaScularization Trial), FGF2 treatment was no better than placebo. Both groups showed substantial improvement in subjective angina and exercise tolerance. This substantial placebo effect is an important feature of angiogenesis trials (and will be discussed below).

FGF	Formulation	Dose (μg/kg)	Delivery	Design	Patients (n)	Follow-up	Primary endpoint	Reference
FGF1	Peptide	10	im	[I] open-label	20	12 weeks	DS angiography	Schumacher et al. 1998
FGF2	Peptide	0–100	ic, single	[I] open-label	25	29 d	ETT, angiography	Unger et al. 2000
FGF2	Peptide	0.3 → 48	ic, single	[I] open-label	52	29 d, 57 d, 180 d	SAQ, ETT, MRI	Laham et al. 2000
FGF2	Peptide	0, 10, 100	Hep-alg	[II] DBR	24	90 d, 33 mo	CCS, SPECT	Laham et al. 1999c Ruel et al. 2002
FGF2	Peptide	0.3 → 48	ic, iv	[I] open-label	59	29 d, 57 d, 180 d	ETT	Udelson et al. 2000
FGF2	Peptide	0, 10, 30	ia single, double	[I] DBR	19	4 weeks, 24 weeks	Plethysmography	Lazarous et al. 2000
FGF1	plasmid DNA	500–16,000 μg	im	[I] open-label	51	12 weeks	Calf arteriography	Comerota et al. 2002
FGF2	Peptide	0, 0.3, 3, 30	ic, single	[II] DBR	337	90 d, 180 d	SAQ, ETT, SPECT	Simons et al. 2002
FGF4	Adenoviral	3.3×10^8–10^9	ic	[I/II] DBR	79	30 d, 90 d	ETT	Grines et al. 2002
FGF2	Peptide	0, 0.3, 3, 30	ia, single, double	[II] DBR	190	90 d	ABI	Lederman et al. 2002

ABI, ankle-brachial index; CCS, Canadian Cardiovascular Society; DBR, double-blind, randomized; DS, digital subtraction; ETT, exercise tolerance test; FGF, fibroblast growth factor; FGFR, FGF receptor; ia, intra-arterial; ic, intracoronary; im, intramyocardial (muscular); SAQ, Seattle Angina Questionnaire; SPECT, single photon emission CT.

Table 1. Summary of human trials of FGF. The early emphasis on peptide is evident. (Khurana & Simon, TCM, 13:116, 2003[1].)

Concerns that the brief half-life of angiogenic proteins in the circulation produced the disappointments of placebo controlled studies contributed to enthusiasm for viral delivery of gene therapy. Previous studies had suggested relatively long lasting expression and evidence of angiogenesis after adenoviral delivery of FGF5 via intracoronary infusion[54]. (Although more recent data revealed the very low efficiency of this approach[55].)

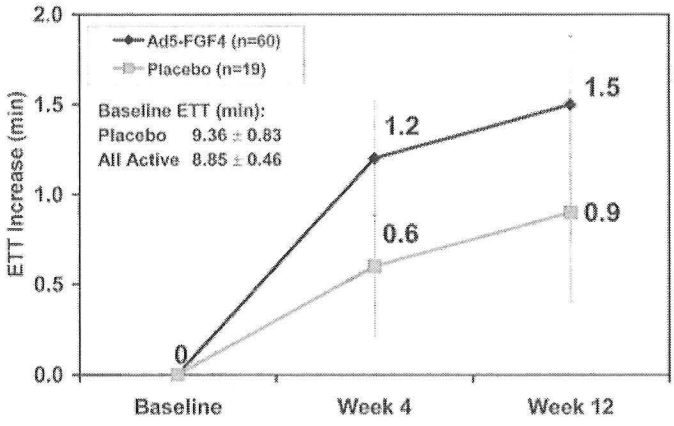


Fig. 12. Improvement in exercise tolerance in both treatment and placebo arms of the AGENT trial. Grines et al., Circ, 105:1291, 2002[8].

The AGENT trial (Angiogenic GENe Therapy) was a placebo controlled, double-blind dose-ranging trial of adenoviral FGF-4 delivered by a single intracoronary infusion to 79 patients with Canadian class 2-3 angina[8]. This was the first multicenter, controlled trial of angiogenic gene therapy. At the behest of the FDA, only the highest dose tested was in the range that had shown physiological effect in animal studies. One patient in the treatment group had a substantial increase in SGPT that returned to normal after 4 weeks. Two subjects in the treatment group had fatal cancers diagnosed 69 and 267 days after treatment. Clinical evaluation showed no significant overall differences between the treated and placebo subjects in ETT time or stress-induced wall motion abnormalities. There was a small difference in ETT time when those with initial times >10 min were excluded from the analysis.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) was identified by Ferrara in 1989[56]. Unlike FGF and PDGF, this 45kD protein is mitogenic only for endothelial cells. Concurrently, Connolly identified the angiogenic characteristics of tumor vascular permeability factor (VPF)[57] and, in simultaneously published papers[58,59], the two proteins were noted to have the same sequence. Multiple isoforms derived from alternative splicing generate VEGF of 121, 165, 189 and 206 amino acids. Only $VEGF_{121}$ and $VEGF_{165}$ are diffusible and thus became the focus of angiogenic therapy. Two VEGF receptors have been identified, Flt-1 (VEGFR-1) and FLK-1KDR (VEGFR-2). They are found only on endothelial cells and macrophages and account for the specificity of VEGF action. VEGF, as a secreted, diffusible protein, can exert a relatively large paracrine effect on endothelial proliferation, and was therefore considered an excellent candidate for gene therapy trials, given the transient and low level expression obtained with the early generation of vectors.

Early animal studies showed dose-dependent increased collateralization in $VEGF_{165}$ treated ischemic limbs, delivered as protein, or plasmid[60]. Studies in the hearts of dogs and pigs were encouraging but encountered a worrisome acute

hypotension when large doses of VEGF protein were injected into coronaries. This effect, which appears to be mediated by nitric oxide, and can be ameliorated by NO synthase inhibitors without effect on angiogenesis[61-63], has not been a problem with the much lower and more gradually supplied doses provided by gene therapy.

Heartened by the animal studies, initial Phase 1 human trials began, in patients with peripheral vascular disease and ischemic heart disease, using a broad range of delivery methods and vectors[64,65]. As in the phase 1 FGF trials, clinical responses in uncontrolled studies were encouraging. Three placebo controlled studies have subsequently been completed and, again as in the FGF experience, although safety appeared to be good, the clinical responses were unimpressive.

In the VIVA trial[66] (Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis) 178 patients with stable exertional angina and coronary anatomy unsuitable for revascularization were randomized to placebo or a low or high dose of recombinant $VEGF_{165}$ infused at the time of catheterization followed by intravenous infusion after 3, 6 and 9 days. There was modest improvement in all groups in ETT duration and angina after 60 days. Only the high dose treatment group maintained improvement at 120 days. Three patients, all in the placebo group, were diagnosed with cancer during the 120 days of follow-up. One patient in the high dose group did have an episode of severe hypotension, responsive to fluids, after the infusion. A pertinent conclusion of the authors was that the results suggested the potential benefit of gene transfer technologies to obtain longer lasting expression of the angiogenic stimulus.

There have been several small trials of direct injection of transgene into ischemic myocardium at the time of thoracotomy. Although these demonstrated feasibility, and the usual underpowered suggestion of clinical benefit, it was impossible to obtain placebo controls under these circumstances. In an interesting effort to design a study that did allow such crucial controls, plasmid encoding VEGF2, (a related protein with similar biological activity) under the regulation of the CMV promoter was injected directly into the myocardium through a steerable catheter equipped with an injection apparatus[11]. Subjects had class 3 or 4 refractory angina, multivessel disease not amenable to revascularization and reversible ischemia on stress nuclear imaging. Ischemic area were identified by electromechanical mapping and 6 sites per ventricle were injected with 1 ml. of fluid containing 200, 800 or, in a single patient, 2000 μg of VEGF2 plasmid. There were no major complications of the procedure. Interestingly, a statistically significant improvement in anginal class was found with the treated patients enjoying an average decrease of 1.3 versus 0.1 in the placebo group ($p=.04$). Once again, there was improvement in reported anginal episodes in both the placebo and treatment group although only in the treatment group did it achieve statistical significance. Other clinical outcomes included trends favoring the treatment group but the number of nitroglycerin used per week was actually lower in the placebo group.

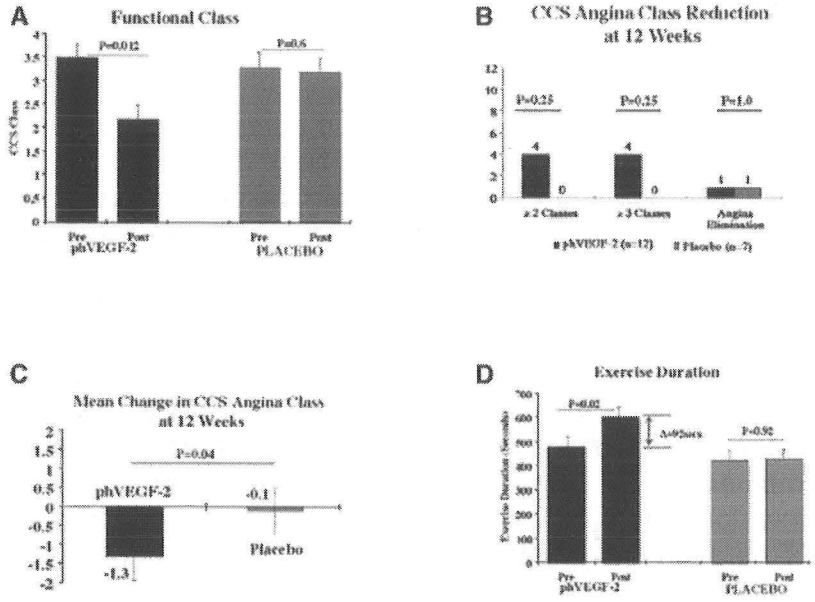


Fig. 13. Clinical endpoints after direct myocardial injections of VEGF2. Functional class (panel A), patients showing substantial improvement (panel B), change in anginal class (panel C) and exercise duration (panel D) all showed trends toward greater improvement in the treated patients. Note that not all enrolled patients are included in the analysis. Losordo et al., Circ, 105:2012, 2002[11].

In the recently published Kuopio Angiogenesis Trial (KAT), 103 patients with Canadian class 2-3 angina were randomized to treatment with PTCA and coronary delivery of VEGF-adenovirus, VEGF-liposomes, or saline. After 6 months myocardial perfusion was assessed by radionucleotide scans and exercise capacity by ETT. As seen in previous studies, all groups, including the saline treated patients, showed improvement in ventricular perfusion and exercise time, but only in the VEGF-adenovirus group was this difference statistically significant.

Study	Number of patients	Delivery method	Factor	Dose	Primary outcomes
Losordo et al. (1998)	5	Direct myocardial injection	$phVEGF_{165}$	125 μg	Angina frequency Dobutamine SPECT Coronary angiography
Symes et al. (1999)	20	Direct myocardial injection	$phVEGF_{165}$	125 μg 250 μg	Angina frequency Dobutamine SPECT Coronary angiography
Vale et al. (1999)	13	Direct myocardial injection	$phVEGF_{165}$	250 μg 500 μg	Angina frequency Dobutamine SPECT LVEMM (NOGA)
Rosengart et al. (1999)	21	Direct myocardial injection	$AD_{GV}VEGF121.10$	4×10^8 to 4×10^{10} pu	Angina frequency Dobutamine SPECT Coronary angiography Exercise treadmill Serial 2D Echo
Hendel et al. (2000)	14	Selective coronary injection	$rhVEGF_{165}$	0.005 μg/kg 0.017 μg/kg 0.05 μg/kg 0.167 μg/kg	Dobutamine, exercise, or dipyridamole stress SPECT
VIVA Trial (Henry et al. 1999 and 2001)	178	Selective coronary injection, intravenous infusion	$rhVEGF_{165}$	17 ng/kg/min 50 ng/kg/min Placebo	Angina frequency Quality of life Exercise treadmill Ejection fraction SPECT

Table 2. Summary of human trials of VEGF. Koransky et al., TCM 12:108, 2002[5].

There are two important advances that have come out of the initial handful of placebo-controlled studies of angiogenesis. Safety has been demonstrated in almost all studies, as were the importance of placebo controls, which may have special pertinence in these studies. The principal safety considerations of angiogenic therapy, and the ways in which clinical studies address these concerns, are reviewed here.

Mortality

The patient population in most early studies has been very sick with either inoperable coronary disease or limb threatening peripheral vascular disease. There has been no increased mortality in these studies compared to historical controls, nor has there been any periprocedural death. For example, among the first 100 subjects treated with VEGF for PVD at St. Elizabeth's Hospital, there were 9 deaths in the first 7 years, a 9% mortality that compares favorably with one historical estimate of a 32% 2-year mortality in patients with critical limb ischemia[67].

Morbidity

Vascular malformations - Hemangiomas have been produced by expression of VEGF in transplanted myoblasts[68] and heart and a large dose of VEGF plasmid produced similar results in heart[69]. However, at the lower doses obtained with typical clinical studies there has been only a single report of transient telangectasia[44] .

Neoplasms – Angiogenesis was first recognized as an attribute of tumors that appeared to be necessary for their growth[70]. This has led to the concern that angiogenic therapy might somehow either enable or stimulate tumor growth or metastasis. However, in the relatively brief clinical trials performed so far there is little evidence for such an effect. In the predominately elderly patients who have received gene therapy for cardiovascular indications there have been only a handful of tumors diagnosed. These include 3 of 88 patients treated with VEGF gene therapy for limb ischemia and 2 of 85 treated for cardiac ischemia[7]. Two subjects undergoing Adenoviral-FGF4 transfer to coronaries developed tumors months after therapy. In the VIVA (VEGF) and TRAFFIC (FGF-2) trials using recombinant factors the new cancers detected were limited to the placebo arms of the trials. With the present approaches to cardiovascular gene therapy the circulating levels of the transgene products are very low and the duration of detectable expression is less than a month. It remains a possibility that more effective or durable transgene expression could create a permissive environment for tumor growth.

Retinopathy – VEGF and FGF-2 are found in increased concentration in the vitreous fluid and retinas of patients with proliferative retinopathies[71-73] and transgenic mice that overexpress VEGF in photoreceptors develop neovascularization[74]. There has, however, been no evidence in the clinical trials of exacerbation of retinal pathology. Diabetes is a common co-morbidity in the patients enrolled in angiogenic gene therapy trials but even in this high-risk group, and even among patients with pre-existing retinopathy, there has been no evidence of progression with careful follow-up[7].

Edema – The initial name for VEGF was vascular permeability factor[75] and it potently enhances vascular leakiness, and thereby contributes to the development of ascites in malignancies. In recent transgenic experiments, overexpression of VEGF in the skin produced leaky vessels (ameliorated by concommittent expression of Angiopoeitin-1[76,77]). Lower extremity edema has been recognized in clinical trials of VEGF gene therapy for PVD and appears to be potentiated by tissue ischemia, as it was more common and profound in subjects with more severe disease[78]. Edema responded well to diuresis and resolved after a few weeks, coincident with the fall in VEGF gene expression. At present, enhanced vascular permeability remains a potential limitation of VEGF gene therapy, as well as therapies, such as HIF1α, that lead to VEGF expression.

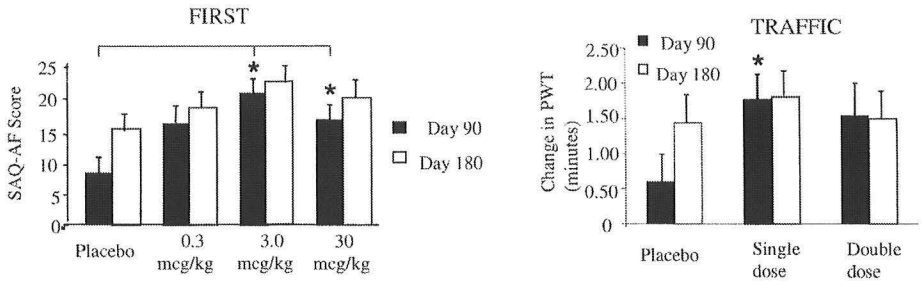


Fig.14. Improvements in the placebo group in the FGF initiating RevaScularization Trial (FIRST) and the Therapeutic Angiogenesis with Recombinannt Fibroblast Growth Factor-2 for Intermittent Claudication (TRAFFIC) trials. Khurana et al., Trends in Cardiovasc. Med. 13:116, 2003[1].

Placebo Effect(s)

A remarkable finding in many of the double-blind studies of angiogenesis is a substantial placebo effect. This has also been seen in trial of laser myocardial revascularization[79]. It is perhaps not surprising that relatively subjective endpoints, such as frequency of angina or claudication, would manifest a placebo effect given the inevitable hype and optimism that surrounds such high technology studies in patients lacking other therapeutic options. But a substantial placebo effect is also seen in objective measurements of ischemia such as positron emission tomography and single photon emission CT determinations of perfusion, suggesting a biological basis for improvement in the untreated patients. This could include improved compliance with other medicines in the study subjects or increased physical activity due to study-related optimism. Both of these could promote improved perfusion or angiogenesis. This augmented placebo effect must be considered in the design and interpretation of angiogenesis trials.

In summary, a fair reading of five recent placebo controlled clinical trial of angiogenic therapy - intracoronary FGF-2, intracoronary FGF-4 gene therapy, intramyocardial VEGF-2 gene therapy, combined intracoronary and systemic VEGF, and intracoronary infusion of adenoviral-VEGF or liposome-plasmid VEGF[80] - suggests that at the doses used these therapies are safe and provide modest, if any, clinical benefits. Moreover, it appears that either direct infusion of proteins or delivery of transgene, by viral vectors or plasmids, is similarly safe and ineffective. The basic understanding of angiogenesis has quickly advanced, even as these clinical studies have been underway. It should be possible to apply this new biological understanding with greater confidence and efficiency given what has been learned about safety and study design in these initial trials.

Second generation angiogenic agents

There is a firm consensus that angiogenesis, or for cardiovascular applications, the even more elusive arteriogenesis, will require not a single angiogenic factor but rather the coordinated expression of multiple factors that each contribute crucial elements to a new vessel. These will likely include VEGF - perhaps with appropriate splice variant isoforms expressed at the right times and places, angiopoeitins 1 and 2 - which may have crucial roles in degrading existing vessels to allow budding and enastomosis as well as recruitment of endothelial cells, pericytes - which have incompletely understood effects on smooth muscle cells, and modulators of the perivascular adventitia - for optimal strength of the new vessel. Our knowledge of all of these processes is incomplete, and a fair criticism of the clinical exploration of angiogenesis is that it has proceeded without adequate understanding of the underlying biology. For example, no one would today choose an FGF as the principal candidate for a gene therapy trial. But there does appear to be a short cut to a first level of understanding of new vessel formation. The regulatory factors that coordinate regulation of downstream angiogenic factors are now better understood[81,82]. We may be able to take advantage of their master regulatory role without understanding all of the nuances of the direct effector molecules.

One potential regulatory gene is Hypoxia Inducible Factor-1 alpha (HIF1α). This rapidly degraded transcription factor is stabilized by hypoxia with resulting expression of many genes important in angiogenesis as well as glycolytic metabolism.

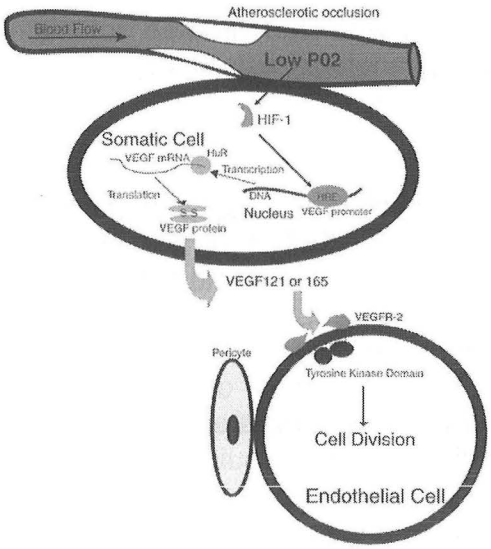


Fig. 15. Hypoxia induced angiogenesis. Hypoxia stabilizes the normally trasient HIF1a mRNA. Its expression regulates a host of hypoxia responsive genes including many angiogenic factors. VEGF is processed and secreted where it binds to its receptor on endothelial cells inducing a cascade of signals leading to cell division, increased migration and new vessel formation. Modified from Koransky et al. TCM 12:108, 2002[5].

A deeper understanding of HIF1α is now leading to design of mutated molecules that are resistant to degradation in normoxic conditions yet retain their ability to

activate angiogenic genes in a coordinated fashion. This pathway has been most compellingly investigated in transgenic mice where these mutant molecules orchestrate angiogenic responses in much the way we might wish for our patients. Eventually, these advances in molecular physiology will guide us to more intelligent design of gene therapy. We will also likely be able to take advantage of tricks used to regulate gene expression to improve the targeting, duration and extent of our stimulus. For example, when high level expression of VEGF is obtained, by engineering it into transplanted cells, the result is fatal angioma formation. An already available solution for this kind of problem, (not presently an issue with inefficient direct gene therapy), is to add an exogenously regulatable promoter to the transgene.

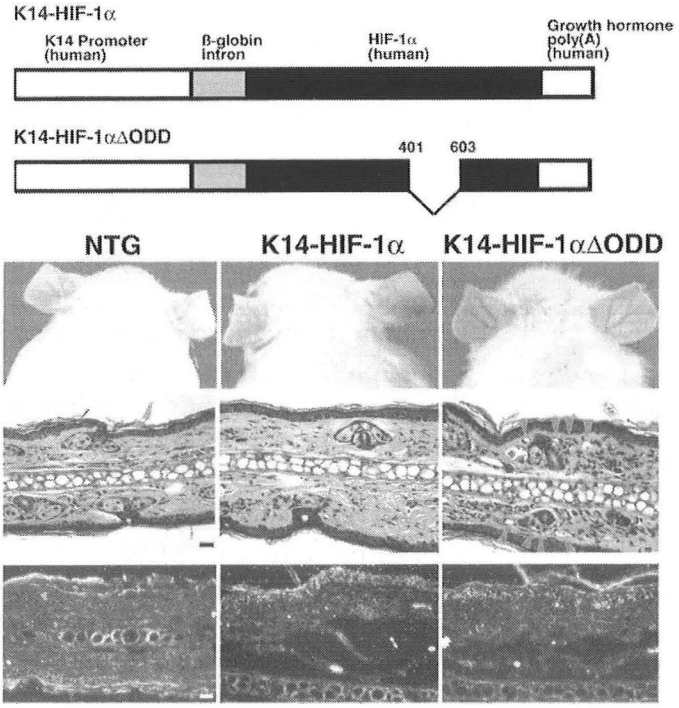


Fig. 16. Evaluation of a deletion mutation of HIF1α that removes the oxygen degradation domain (ΔODD). The wild-type (left panels) and normal HIF1 α transgenic (middle panels) have normal vascularization of their ears, (despite expression of the HIF1α message in the skin, under the regulation of the keratin promoter K14, as shown in the middle in situ darkfield image). The transgenic animal with the Hif1α ΔODD transgene expresses message and stable protein, thus obtaining inappropriately abundant, and mature, vessels in the skin (obvious in the ears and identified by green arrows). Elson et al., Genes & Devel. 15:2520, 2001[12].

Heart Failure

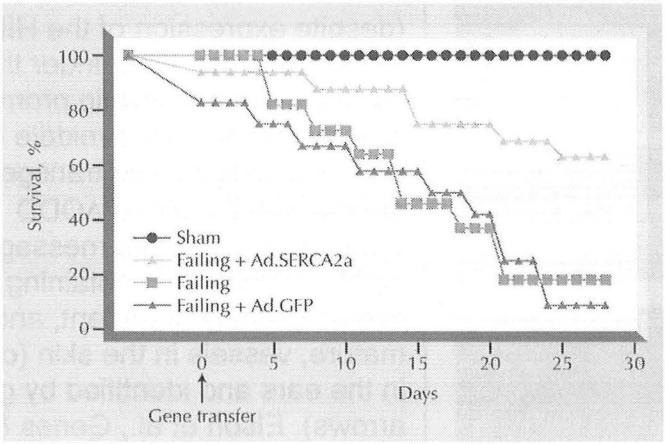
Angiogenesis is the most clinically mature domain of cardiovascular gene therapy, and the only area where clinical trials have progressed to large, placebo controlled efforts. The applications of gene therapy to heart failure are still primarily at the stage of animal studies. Perhaps this will allow these applications to benefit from the rapid advance of basic knowledge and avoid some of the pitfalls that have been encountered in the precipitate approach to angiogenesis. There is at least as great a need for such therapies. While angiogenesis is presently primarily viewed as applicable to patients with nonrevascularizable atherosclerotic disease, the number of such patients is tiny compared to the frequency of heart failure. There are about 300,000 deaths from heart failure each year in the U.S.. This is the largest number of critically ill people with a

single relatively homogeneous diagnosis in the developed world, and presents an obvious target for novel therapy.

Heart failure is a multifactorial disease but there is substantial overlap of key molecular changes between heart failure of different etiologies. These underlying similarities are being revealed with the advent of comprehensive transcriptional and proteomic methods. Three of the common findings are changes in: 1. energetics – with reversion to fetal, glycolytic metabolism, 2. ion channels – with disordered calcium homeostasis due to dysregulation of SERCA and ryanodine receptors, and 3. neurohormonal status – with elevated catecholamines and cytokines and resulting down-regulation of beta adrenergic receptor function. These and other observations lead to gene therapy approaches to heart failure that include: calcium handling, the ß-adrenergic receptor, and apoptosis.

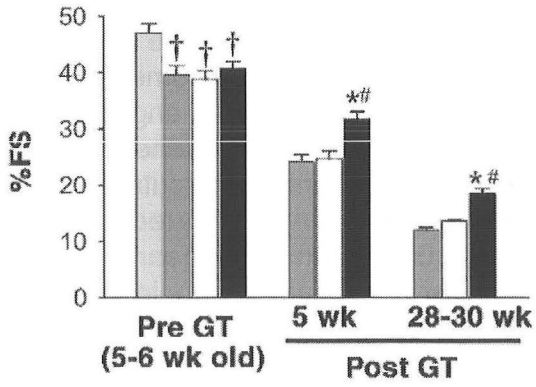
Gene targets in heart failure

Calcium handling



The sarcoplasmic endoplasmic reticulum calcium ATPase plays a crucial role in calcium reuptake with each cardiac contraction. Overexpression of SERCA by an adenoviral transgene restores function and prolongs life in pressure-overload failure in rats. Similarly, inhibition of phospholamban, an important inhibitor of SERCA, by a dominant negative strategy preserves function in cardiomyopathic hamsters.

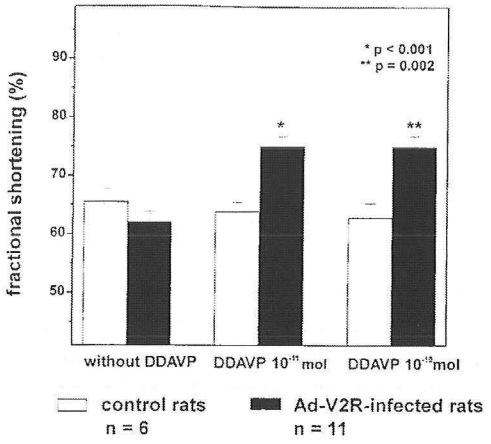
Fig. 17. Survival in aortic banded rats following gene therapy with SERCA directed to the myocardium. From del Monte et al, Circ 104:1424, 2001[10].



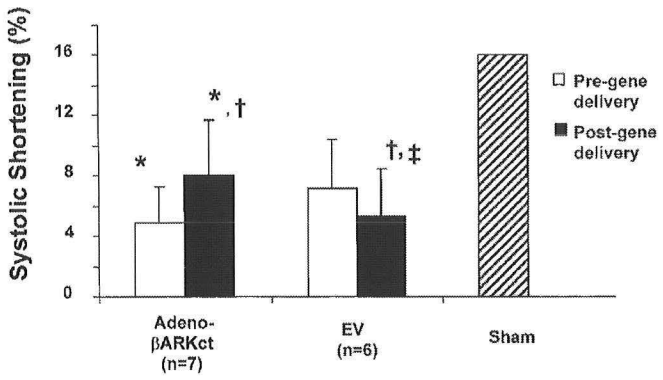
Maintenance of ventricular function in cardiomyopathic (CM) hamsters after myocardial transfection mediated by AAV encoding a dominant negative version of phospholamban. (in black) vs nl (light gray), untreated CM (dark gray) and LacZ treated CM hamsters (white). Hoshijima et al., Nat Med 8:864, 2002[13].

ß-adrenergic receptor

Observed abnormalities of beta-adrenergic receptor (ßAR) signaling in heart failure include elevated plasma norepinephrine, down-regulation of $ß_1$AR, uncoupling of $ß_2$AR, and upregulation of the ßAR kinase. When taken in addition to the well demonstrated clinical benefits of beta-blockade, these findings suggest the ßAR is a good target for gene therapy efforts. These have included adenoviral transfection of peptide inhibitor of ßARK which produces higher ßAR stimulated adenylate cyclase activity, adenoviral transfection of vasopressin receptors generating higher V_2R agonist- stimulated adenylate cyclase activity, and over-expression of adenylate cyclase.



Adenoviral transfection of vasopressin 2 receptors. Effect of bolus injection of vasopression on rats that had received cardiac injection of V_2R or ß-Gal adenoviri. Weig et al., Circ 101:1578, 2000[9].

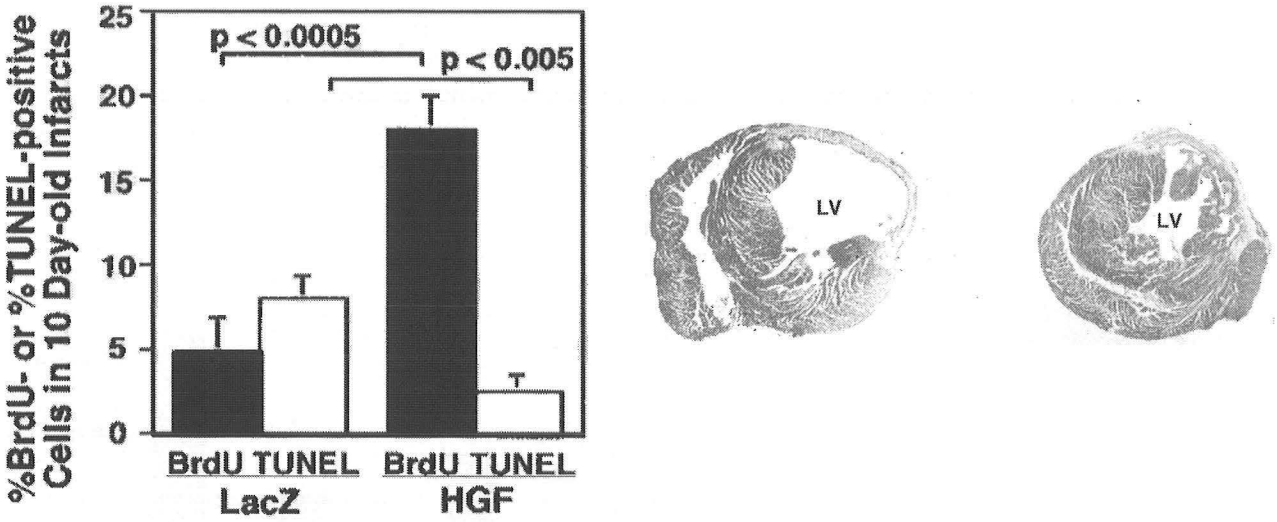


Adenoviral transfection of ßARK inhibitor. Effect of intracoronary adenoviral-mediated transfection of β-ARK inhibitor on systolic shortening (determined with sonomicrometry) 3 weeks after MI in rabbits. Shah et al., Circ 103:1311, 2001[6].

Apoptosis

A potentially unifying pathway in many etiologies of heart failure is programmed cell death. Transgenic expression of proapoptotic molecules commonly produces a dilated cardiomyopathy and evidence of apoptosis is easily found in many different animal models of cardiomyopathy, as well as patients. Trangenic mice that overexpress the anti-apoptotic molecule BCL-2 in the heart maintain ventricular function better than wild-type mice after infarction[83]. Adenoviral mediated overexpression of the antiapoptotic kinase Akt protects the heart against ischemia reperfusion injury[84], and elevated kinin levels obtained with kallikrein gene delivery reduce apoptosis and ventricular dilation after ischemia or infarction[85]. Hepatocyte growth factor has been implicated in tissue regeneration,

angiogenesis and reduction of apoptosis. In an interesting approach to gene therapy of the heart, adenoviral HGF was injected into skeletal muscle, producing sufficient peptide to reduce apoptosis and improve remodeling after a subsequently induced infarction[86].



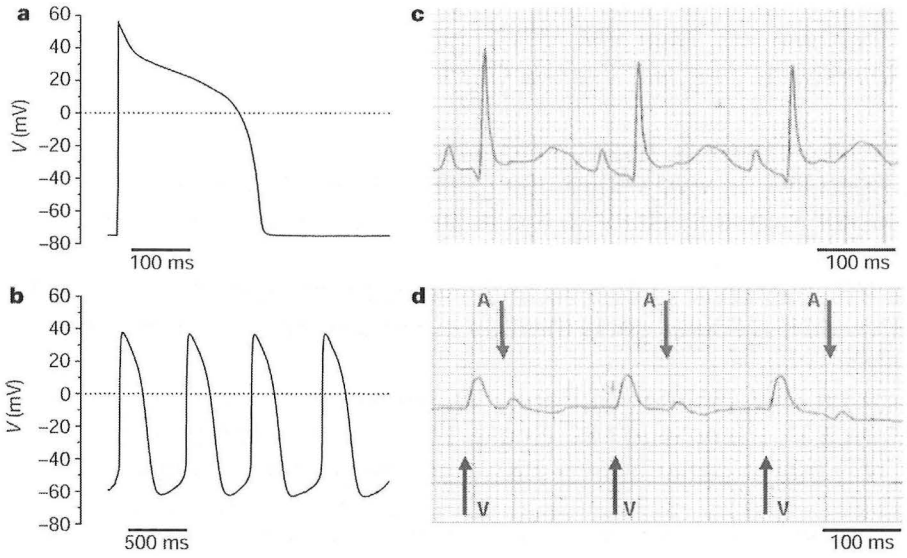
Mice treated with intramuscular Ad-HGF 3 days after MI and evaluated 4 weeks later show less apoptosis and more proliferation in the infarct area. These mice also have thicker walls in the infarcted region (heart on the right). Li et al., Circ, 107:2499, 2003.

Cardiac Dysrhythmias

There has been little work so far on gene therapy approaches to primary cardiac rhythm disturbances. However, potential benefits are compelling. The recent MADIT II data suggest the widespread utility of implantable defibrillators, to an extent that would strain available resources. In cell culture experiments, adenoviral transduction of the delayed rectifier potassium channel HERG to cardiac myocytes supresses afterdepolarizations that are thought to be a principal contributor to the development of ventricular fibrillation[87].

Other modifications of cardiac channels have an even more surprising result. In the early developing heart cardiomyocytes have intrinsic pacemaker activity. It is only with subsequent differentiation that such cells become quiescent and allow nodal pacemaker cells to determine the rate of depolarization. This stabilization is obtained by the inward-rectifier potassium current that is present in differentiated atrial and ventricular myocytes but not the nodal cells. One of the genes that encodes a channel responsible for this current is Kir2.1. An adenoviral vector was used to transfect a dominant negative form of Kir2.1 into the

ventricles of mice and latent pacemaker activity was unmasked[88]. Although the pacemaker activity was scattered throughout the ventricle a dominant ventricular rhythm was obtained in many animals. It would be relatively easy to direct delivery of this gene therapy to a specific location in the heart in a way that would recapitulate the function of a pacemaker. There would be several theoretical advantages to such a biological pacemaker: 1. it could respond to endogenous signaling providing authentic biological heart rate response, 2. it would avoid the use of hardware with attendant risk of infection, and 3. it could be easily removed, or modified, by catheter ablation.



Suppression of Kir2.1 with a dominant negative construct delivered by LV chamber injection of adenoviral vector. Control action potential and electrocardiogram are shown in panels A and C. Reduction of inward-rectifier potassium current (I_{K1}) produces spontaneous action potentials and a ventricularly paced rhythm shown in panels B and D.

Conclusion

Present therapies have dramatically improved the care of some patients with cardiovascular disease. Unfortunately, many patients are not helped by these approaches and new options would be welcome. Gene therapy offers a new world of treatments only now glimpsed. Despite the disappointments of initial clinical trials, the rapid progress in molecular understanding of physiology and identification of gene targets, as well as improvements in vectors, tissue targeting, and regulation of gene expression, will deliver a potent new set of therapies over the next 25 years.

References

1. Khurana R, Simons M. Insights from angiogenesis trials using fibroblast growth factor for advanced arteriosclerotic disease. *Trends Cardiovasc Med.* 2003;13:116-22.
2. Isner JM. Myocardial gene therapy. *Nature.* 2002;415:234-9.
3. Yla-Herttuala S, Martin JF. Cardiovascular gene therapy. *Lancet.* 2000;355:213-22.
4. Monahan PE, Samulski RJ. Adeno-associated virus vectors for gene therapy: more pros than cons? *Mol Med Today.* 2000;6:433-40.
5. Koransky ML, Robbins RC, Blau HM. VEGF gene delivery for treatment of ischemic cardiovascular disease. *Trends Cardiovasc Med.* 2002;12:108-14.
6. Shah AS, White DC, Emani S, Kypson AP, Lilly RE, Wilson K, Glower DD, Lefkowitz RJ, Koch WJ. In vivo ventricular gene delivery of a beta-adrenergic receptor kinase inhibitor to the failing heart reverses cardiac dysfunction. *Circulation.* 2001;103:1311-6.
7. Isner JM, Vale PR, Symes JF, Losordo DW. Assessment of risks associated with cardiovascular gene therapy in human subjects. *Circ Res.* 2001;89:389-400.
8. Grines CL, Watkins MW, Helmer G, Penny W, Brinker J, Marmur JD, West A, Rade JJ, Marrott P, Hammond HK, Engler RL. Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. *Circulation.* 2002;105:1291-7.
9. Weig HJ, Laugwitz KL, Moretti A, Kronsbein K, Stadele C, Bruning S, Seyfarth M, Brill T, Schomig A, Ungerer M. Enhanced cardiac contractility after gene transfer of V2 vasopressin receptors In vivo by ultrasound-guided injection or transcoronary delivery. *Circulation.* 2000;101:1578-85.
10. del Monte F, Williams E, Lebeche D, Schmidt U, Rosenzweig A, Gwathmey JK, Lewandowski ED, Hajjar RJ. Improvement in survival and cardiac metabolism after gene transfer of sarcoplasmic reticulum Ca(2+)-ATPase in a rat model of heart failure. *Circulation.* 2001;104:1424-9.
11. Losordo DW, Vale PR, Hendel RC, Milliken CE, Fortuin FD, Cummings N, Schatz RA, Asahara T, Isner JM, Kuntz RE. 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.* 2002;105:2012-8.
12. Elson DA, Thurston G, Huang LE, Ginzinger DG, McDonald DM, Johnson RS, Arbeit JM. Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha. *Genes Dev.* 2001;15:2520-32.
13. Hoshijima M, Ikeda Y, Iwanaga Y, Minamisawa S, Date MO, Gu Y, Iwatate M, Li M, Wang L, Wilson JM, Wang Y, Ross J, Jr., Chien KR. Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat Med.* 2002;8:864-71.

14. Kaiser J. Gene therapy. Seeking the cause of induced leukemias in X-SCID trial. *Science*. 2003;299:495.
15. Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, Lupien PJ, Wilson JM. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nat Genet*. 1994;6:335-41.
16. Grossman M, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ, 3rd, Stein EA, Lupien PJ, Brewer HB, Jr., Raper SE, et al. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. *Nat Med*. 1995;1:1148-54.
17. Thalhamer J, Leitner W, Hammerl P, Brtko J. Designing immune responses with genetic immunization and immunostimulatory DNA sequences. *Endocr Regul*. 2001;35:143-66.
18. Fomsgaard A. [Genetic immunization--"the biological equivalent of cold fusion"?]. *Ugeskr Laeger*. 1995;157:4932-6.
19. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature*. 1992;356:152-4.
20. Quarck R, Holvoet P. Restenosis and gene therapy. *Expert Opin Biol Ther*. 2001;1:79-91.
21. Santiago FS, Khachigian LM. Nucleic acid based strategies as potential therapeutic tools: mechanistic considerations and implications to restenosis. *J Mol Med*. 2001;79:695-706.
22. Shuey DJ, McCallus DE, Giordano T. RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today*. 2002;7:1040-6.
23. Havenga MJ, Lemckert AA, Grimbergen JM, Vogels R, Huisman LG, Valerio D, Bout A, Quax PH. Improved adenovirus vectors for infection of cardiovascular tissues. *J Virol*. 2001;75:3335-42.
24. Rossi FM, Kringstein AM, Spicher A, Guicherit OM, Blau HM. Transcriptional control: rheostat converted to on/off switch. *Mol Cell*. 2000;6:723-8.
25. Somia N, Verma IM. Gene therapy: trials and tribulations. *Nat Rev Genet*. 2000;1:91-9.
26. Wickham TJ. Targeting adenovirus. *Gene Ther*. 2000;7:110-4.
27. Young LS, Mautner V. The promise and potential hazards of adenovirus gene therapy. *Gut*. 2001;48:733-6.
28. Qian HS, Channon K, Nepliouéva V, Wang Q, Finer M, Tsui L, George SE, McArthur J. Improved adenoviral vector for vascular gene therapy : beneficial effects on vascular function and inflammation. *Circulation Research*. 2001;88:911-7.
29. Symes JF, Losordo DW, Vale PR, Lathi KG, Esakof DD, Mayskiy M, Isner JM. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann Thorac Surg*. 1999;68:830-6; discussion 836-7.
30. Cable DG, Caccitolo JA, Caplice N, O'Brien T, Simari RD, Daly RC, Dearani JA, Mullany CJ, Orszulak TA, Schaff HV. The role of gene therapy for intimal hyperplasia of bypass grafts. *Circulation*. 1999;100:II392-6.

31. Mangi AA, Dzau VJ. Gene therapy for human bypass grafts. *Ann Med.* 2001;33:153-5.
32. Chiu-Pinheiro CK, O'Brien T, Katusic ZS, Bonilla LF, Hamner CE, Schaff HV. Gene transfer to coronary artery bypass conduits. *Ann Thorac Surg.* 2002;74:1161-6; discussion 1166.
33. White SJ, Newby AC. Gene therapy for all aspects of vein-graft disease. *J Card Surg.* 2002;17:549-55.
34. Lazarous DF, Shou M, Stiber JA, Hodge E, Thirumurti V, Goncalves L, Unger EF. Adenoviral-mediated gene transfer induces sustained pericardial VEGF expression in dogs: effect on myocardial angiogenesis. *Cardiovasc Res.* 1999;44:294-302.
35. Varenne O, Gerard RD, Sinnaeve P, Gillijns H, Collen D, Janssens S. Percutaneous adenoviral gene transfer into porcine coronary arteries: is catheter-based gene delivery adapted to coronary circulation? *Hum Gene Ther.* 1999;10:1105-15.
36. Zsigmond E, Kobayashi K, Tzung KW, Li L, Fuke Y, Chan L. Adenovirus-mediated gene transfer of human lipoprotein lipase ameliorates the hyperlipidemias associated with apolipoprotein E and LDL receptor deficiencies in mice. *Hum Gene Ther.* 1997;8:1921-33.
37. Applebaum-Bowden D, Kobayashi J, Kashyap VS, Brown DR, Berard A, Meyn S, Parrott C, Maeda N, Shamburek R, Brewer HB, Jr., Santamarina-Fojo S. Hepatic lipase gene therapy in hepatic lipase-deficient mice. Adenovirus-mediated replacement of a lipolytic enzyme to the vascular endothelium. *J Clin Invest.* 1996;97:799-805.
38. Khurana R, Martin JF, Zachary I. Gene therapy for cardiovascular disease: a case for cautious optimism. *Hypertension.* 2001;38:1210-6.
39. Budts W, Pokreisz P, Nong Z, Van Pelt N, Gillijns H, Gerard R, Lyons R, Collen D, Bloch KD, Janssens S. Aerosol gene transfer with inducible nitric oxide synthase reduces hypoxic pulmonary hypertension and pulmonary vascular remodeling in rats. *Circulation.* 2000;102:2880-5.
40. Gunnett CA, Heistad DD. The future of gene therapy for stroke. *Curr Hypertens Rep.* 2001;3:36-40.
41. Mann MJ, Whittemore AD, Donaldson MC, Belkin M, Conte MS, Polak JF, Orav EJ, Ehsan A, Dell'Acqua G, Dzau VJ. Ex-vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. *Lancet.* 1999;354:1493-8.
42. Simons M, Edelman ER, DeKeyser JL, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature.* 1992;359:67-70.
43. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, Parmacek MS, Leiden JM. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science.* 1995;267:518-22.
44. Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes JF. Clinical evidence of

angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet*. 1996;348:370-4.
45. Ornitz DM, Itoh N. Fibroblast growth factors. *Genome Biol*. 2001;2:REVIEWS3005.
46. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer*. 2000;7:165-97.
47. Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haudenschild CC, Yin M, Coffin JD, Kong L, Kranias EG, Luo W, Boivin GP, Duffy JJ, Pawlowski SA, Doetschman T. Fibroblast growth factor 2 control of vascular tone. *Nat Med*. 1998;4:201-7.
48. Scholz D, Cai WJ, Schaper W. Arteriogenesis, a new concept of vascular adaptation in occlusive disease. *Angiogenesis*. 2001;4:247-57.
49. Post MJ, Laham R, Sellke FW, Simons M. Therapeutic angiogenesis in cardiology using protein formulations. *Cardiovasc Res*. 2001;49:522-31.
50. Schumacher B, Pecher P, von Specht BU, Stegmann T. Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation*. 1998;97:645-50.
51. Ruel M, Laham RJ, Parker JA, Post MJ, Ware JA, Simons M, Sellke FW. Long-term effects of surgical angiogenic therapy with fibroblast growth factor 2 protein. *J Thorac Cardiovasc Surg*. 2002;124:28-34.
52. Laham RJ, Chronos NA, Pike M, Leimbach ME, Udelson JE, Pearlman JD, Pettigrew RI, Whitehouse MJ, Yoshizawa C, Simons M. Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study. *J Am Coll Cardiol*. 2000;36:2132-9.
53. Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, Udelson JE, Gervino EV, Pike M, Whitehouse MJ, Moon T, Chronos NA. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. *Circulation*. 2002;105:788-93.
54. Giordano FJ, Ping P, McKirnan MD, Nozaki S, DeMaria AN, Dillmann WH, Mathieu-Costello O, Hammond HK. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. *Nat Med*. 1996;2:534-9.
55. Wright MJ, Wightman LM, Lilley C, de Alwis M, Hart SL, Miller A, Coffin RS, Thrasher A, Latchman DS, Marber MS. In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors. *Basic Res Cardiol*. 2001;96:227-36.
56. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun*. 1989;161:851-8.
57. 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;84:1470-8.

58. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science*. 1989;246:1309-12.
59. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*. 1989;246:1306-9.
60. Takeshita S, Pu LQ, Stein LA, Sniderman AD, Bunting S, Ferrara N, Isner JM, Symes JF. Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. *Circulation*. 1994;90:II228-34.
61. Hariawala MD, Horowitz JR, Esakof D, Sheriff DD, Walter DH, Keyt B, Isner JM, Symes JF. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J Surg Res*. 1996;63:77-82.
62. Lopez JJ, Laham RJ, Carrozza JP, Tofukuji M, Sellke FW, Bunting S, Simons M. Hemodynamic effects of intracoronary VEGF delivery: evidence of tachyphylaxis and NO dependence of response. *Am J Physiol*. 1997;273:H1317-23.
63. Sato K, Wu T, Laham RJ, Johnson RB, Douglas P, Li J, Sellke FW, Bunting S, Simons M, Post MJ. Efficacy of intracoronary or intravenous VEGF165 in a pig model of chronic myocardial ischemia. *J Am Coll Cardiol*. 2001;37:616-23.
64. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation*. 1998;97:1114-23.
65. Rosengart TK, Lee LY, Patel SR, Sanborn TA, Parikh M, Bergman GW, Hachamovitch R, Szulc M, Kligfield PD, Okin PM, Hahn RT, Devereux RB, Post MR, Hackett NR, Foster T, Grasso TM, Lesser ML, Isom OW, Crystal RG. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation*. 1999;100:468-74.
66. Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. *Circulation*. 2003;107:1359-65.
67. (TASC) TI-SC. Management of peripheral arterial disease. *J Vasc Surg*. 2000;31:S1-S296.
68. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol Cell*. 1998;2:549-58.
69. Kloner RA, Dow J, Chung G, Kedes LH. Intramyocardial injection of DNA encoding vascular endothelial growth factor in a myocardial infarction model. *J Thromb Thrombolysis*. 2000;10:285-9.

70. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med.* 1971;285:1182-6.
71. Frank RN, Amin RH, Eliott D, Puklin JE, Abrams GW. Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. *Am J Ophthalmol.* 1996;122:393-403.
72. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med.* 1994;331:1480-7.
73. Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo TK, Yeo KT. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol.* 1994;118:445-50.
74. Okamoto N, Tobe T, Hackett SF, Ozaki H, Vinores MA, LaRochelle W, Zack DJ, Campochiaro PA. Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. *Am J Pathol.* 1997;151:281-91.
75. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science.* 1983;219:983-5.
76. Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science.* 1999;286:2511-4.
77. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med.* 2000;6:460-3.
78. Baumgartner I, Rauh G, Pieczek A, Wuensch D, Magner M, Kearney M, Schainfeld R, Isner JM. Lower-extremity edema associated with gene transfer of naked DNA encoding vascular endothelial growth factor. *Ann Intern Med.* 2000;132:880-4.
79. Fuchs S, Baffour R, Vodovotz Y, Shou M, Stabile E, Tio FO, Leon MB, Kornowski R. Laser myocardial revascularization modulates expression of angiogenic, neuronal, and inflammatory cytokines in a porcine model of chronic myocardial ischemia. *J Card Surg.* 2002;17:413-24.
80. Hedman M, Hartikainen J, Syvanne M, Stjernvall J, Hedman A, Kivela A, Vanninen E, Mussalo H, Kauppila E, Simula S, Narvanen O, Rantala A, Peuhkurinen K, Nieminen MS, Laakso M, Yla-Herttuala S. 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.* 2003;107:2677-2683.
81. Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. The morphogen

Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med.* 2001;7:706-11.

82. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A.* 1998;95:7987-92.
83. Brocheriou V, Hagege AA, Oubenaissa A, Lambert M, Mallet VO, Duriez M, Wassef M, Kahn A, Menasche P, Gilgenkrantz H. Cardiac functional improvement by a human Bcl-2 transgene in a mouse model of ischemia/reperfusion injury. *J Gene Med.* 2000;2:326-33.
84. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation.* 2000;101:660-7.
85. Agata J, Chao L, Chao J. Kallikrein gene delivery improves cardiac reserve and attenuates remodeling after myocardial infarction. *Hypertension.* 2002;40:653-9.
86. Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M, Goto K, Takahashi T, Hayakawa K, Koda M, Kawase Y, Maruyama R, Okada H, Minatoguchi S, Mizuguchi H, Fujiwara T, Fujiwara H. Postinfarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. *Circulation.* 2003;107:2499-506.
87. Nuss HB, Marban E, Johns DC. Overexpression of a human potassium channel suppresses cardiac hyperexcitability in rabbit ventricular myocytes. *J Clin Invest.* 1999;103:889-96.
88. Miake J, Marban E, Nuss HB. Biological pacemaker created by gene transfer. *Nature.* 2002;419:132-3.