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(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612–3550 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HAMMOND, H., Kirk [US/US]; 5770 Waverly Avenue, La Jolla, CA 92037 (US). KELLY, Tamsin, L. [US/US]; 5770 Waverly Avenue, La Jolla, CA 92037 (US).

(74) Agents: DYLAN, Tyler, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304–1018 (US).

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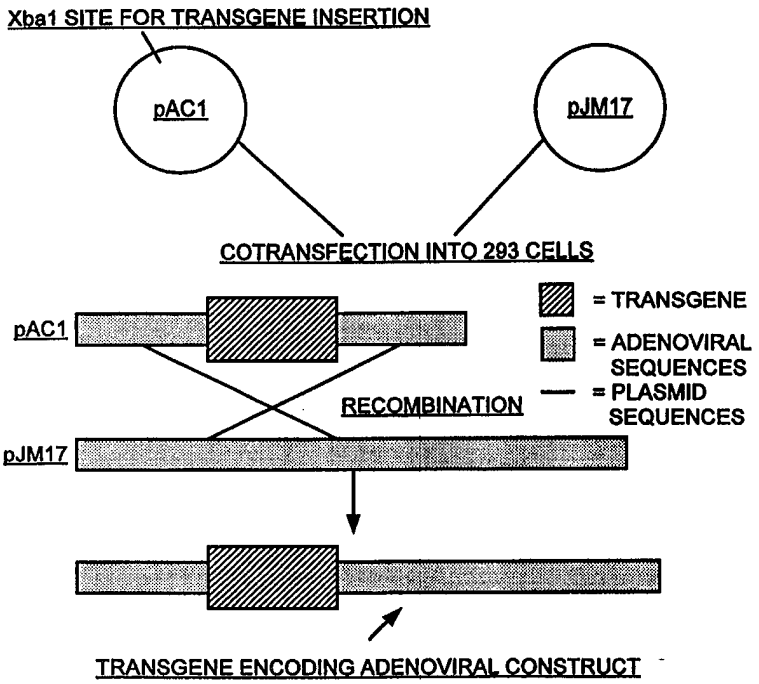
(57) Abstract

Methods are provided for treating patients with congestive heart failure (including dilated cardiomyopathy and congestive heart failure associated with severe coronary artery disease), and for preventing or alleviating deleterious ventricular remodeling after myocardial infarction. The preferred methods of the present invention involve *in vivo* delivery of genes encoding angiogenic proteins or peptides to the myocardium by direct injection of a vector containing the gene into a blood vessel supplying the heart.



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TECHNIQUES AND COMPOSITIONS FOR TREATING HEART FAILURE AND VENTRICULAR REMODELING BY *IN VIVO* DELIVERY OF ANGIOGENIC TRANSGENES

STATEMENT REGARDING GOVERNMENT-SPONSORED RESEARCH

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FIELD OF THE INVENTION

The present invention relates to methods and compositions for treating cardiovascular disease. More specifically, the present invention relates to techniques and polynucleotide constructs for treating heart failure and ventricular remodeling by *in vivo* delivery of angiogenic transgenes.

BACKGROUND OF THE INVENTION

It has been reported that 3-4 million adults in the United States have congestive heart failure (abbreviated "CHF" herein); and the incidence of CHF is increasing (see, e.g., Baughman, K., Cardiology Clinics 13: 27-34, 1995). Annually in US hospitals, CHF is the most frequent non-elective admission, and is the discharge diagnosis for 500,000 patients. Once symptoms of heart failure are moderately severe, the prognosis is worse than most cancers in that 50% of such patients are dead within 2 years (Braunwald, E. (ed), In: Heart Disease, W.B. Saunders, Philadelphia, page 471-485, 1988). Medical therapy can initially attenuate the symptoms of heart failure (e.g., edema, exercise intolerance and breathlessness), and in some cases prolong life. However, the prognosis for this disease, even with medical treatment, remains grim (see, e.g., Baughman, K., Cardiology Clinics 13: 27-34, 1995).

CHF is defined as abnormal heart function resulting in inadequate cardiac output to meet metabolic needs (Braunwald, E. (ed), In: Heart Disease, W.B. Saunders, Philadelphia, page 426, 1988). Symptoms include breathlessness, fatigue, weakness, leg swelling and exercise intolerance. On physical examination, patients with heart failure tend to have elevations in heart and respiratory rates, rales (an indication of fluid in the lungs), edema, jugular venous distension, and, in general, enlarged hearts. The most common cause of CHF is atherosclerosis which causes

blockages in the coronary arteries that supply blood to the heart muscle. Ultimately, such blockages can cause myocardial infarction (death of heart muscle) with subsequent decline in heart function and resultant heart failure. Other causes of CHF include valvular heart disease, hypertension, viral infections of the heart, alcohol and diabetes. Some cases of heart failure occur without clear etiology and are therefore called idiopathic.

Present treatments for CHF include pharmacological therapies, coronary revascularization procedures (e.g. coronary artery bypass surgery and angioplasty), and heart transplantation. Pharmacological therapies have been directed toward increasing the force of contraction of the heart (by using inotropic agents such as digitalis and β-adrenergic receptor agonists), reducing fluid accumulation in the lungs and elsewhere (by using diuretics), and reducing the work of the heart (by using agents that decrease systemic vascular resistance such as angiotensin converting enzyme inhibitors). β-adrenergic receptor antagonists have also been tested. While such pharmacological agents can improve symptoms, and potentially prolong life, the prognosis in most cases remains dismal.

Some patients with heart failure due to associated coronary artery disease can benefit, at least temporarily, by revascularization procedures such as coronary artery bypass surgery and angioplasty. Such procedures are of potential benefit when the heart muscle is not dead but may be dysfunctional because of inadequate blood flow. If normal coronary blood flow is restored, viable dysfunctional myocardium may contract more normally, and heart function may improve. However, because of an inadequate microvascular bed, revascularization rarely restores cardiac function to normal or near-normal levels in patients with CHF, even though mild improvements are sometimes noted.

Heart transplantation can be a suitable option for patients who have no other confounding diseases and are relatively young, but this is an option for only a small number of patients with heart failure, and only at great expense. In summary, CHF has a very poor prognosis and responds poorly to current therapies.

Further complicating the physiological conditions associated with CHF, are various natural adaptations that tend to occur in patients with dysfunctional hearts. Although these natural responses can initially improve heart function, they ultimately result in problems that can exacerbate CHF, confound treatment, and have adverse effects on survival. There are three such adaptive responses commonly observed in CHF: (i) volume retention induced by changes in sodium reabsorption, which expands plasma volume and initially improves cardiac output; (ii) cardiac enlargement (from dilation and hypertrophy) which can increase stroke volume while maintaining a relatively normal wall tension; and (iii) increased norepinephrine release from

adrenergic nerve terminals impinging on the heart which, by interacting with cardiac β-adrenergic receptors, tends to increase heart rate and force of contraction, thereby increasing cardiac output. However, each of these three natural adaptations tends ultimately to fail for various reasons. In particular, fluid retention tends to result in edema and retained fluid in the lungs that impairs breathing. Heart enlargement can lead to deleterious left ventricular remodeling with subsequent severe dilation and increased wall tension, thus exacerbating CHF. Finally, long-term exposure of the heart to norepinephrine tends to make the heart unresponsive to adrenergic stimulation and is linked with poor prognosis.

Angiogenesis refers generally to the development and differentiation of blood vessels. A number of proteins, typically referred to as "angiogenic proteins," are known to promote angiogenesis. Such angiogenic proteins include members of the fibroblast growth factor (FGF) family, the vascular endothelial growth factor (VEGF) family, the platelet-derived growth factor (PDGF) family, the insulin-like growth factor (IGF) family, and others (as described in more detail below and in the art). For example, the FGF and VEGF family members have been recognized as regulators of angiogenesis during growth and development. Their role in promoting angiogenesis in adult animals has recently been examined (as discussed below). The angiogenic activity of the FGF and VEGF families is reasonably well established in the setting of protein infusions. For example, it has been shown that acidic FGF ("aFGF") protein within a collagen-coated matrix, when placed in the peritoneal cavity of adult rats, resulted in a well vascularized and normally perfused structure (Thompson, *et al.*, *PNAS*, 86:7928-7932, 1989). Injection of basic FGF ("bFGF") protein into adult canine coronary arteries during coronary occlusion reportedly led to decreased myocardial dysfunction, smaller myocardial infarctions, and increased vascularity in the bed at risk (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403, 1992). Similar results have been reported in animal models of myocardial ischemia using bFGF protein (Harada, et al. *J. Clin. Invest.*, 94:623-630, 1994; Unger, et al., *Am. J. Physiol.*, 266:H1588-H-1595, 1994). An increase in collateral blood flow was shown in dogs treated with VEGF protein (Banai et al. Circulation 89:2183-2189, 1994). Difficulties associated with the potential use of such protein infusions to promote cardiac angiogenesis include: achieving proper localization, and ensuring that the protein is and remains in the proper form and concentration needed for uptake and the promotion of an angiogenic effect within cells of the myocardium. Another difficulty is the need for repeated infusion or injection of the protein.

Several groups have described *in vivo* gene transfer into the myocardium using plasmids, retrovirus, adenovirus and other vectors (see e.g., Barr et al., Supplement II, Circulation, 84(4):Abstract 1673, 1991; Barr et al., Gene Therapy, 1:51-58, 1994; French et al., Circulation,

90(5):2414-2424 (1994); French et al., Circulation 90:1517 Abstract No. 2785 (1994); Giordano et al., Clin. Res., 42:123A (1994); Giordano et al., J. Investigative Med., Supplement 2, 43:287A (1995); Guzman et al., Circulation Research, 73(6):1202-1207 (1993); Kass-Eisler et al., PNAS (USA), 90:11498-11502 (1993); Mühlhauser et al., Human Gene Therapy, 6:1457-1465 (1995); Mühlhauser et al. Circulation Research, 77(6):1077-1086 (1995); French et al., Circulation, 90(5):2402-2413 (1994); Rowland et al., Am. Thorac. Surg., 60(3):721-728 (1995). In general, these reported methods suffer from one or more of the following deficiencies: inadequate transduction efficiency and transgene expression; marked immune response to the vectors used, including inflammation and tissue necrosis; and importantly, a relative inability to target transduction and transgene expression to the heart (i.e., gene transfer resulted in the transgene also being delivered to non-cardiac sites such as liver, kidneys, lungs, brain and testes of the test animals). Inflammatory infiltrates were documented in the heart after direct intramyocardial injection through a needle inserted into the myocardial wall (French et al., Circulation, 90(5):2414-2424 (1994)).

A method for treating certain forms of congestive heart failure associated with β-adrenergic signaling has been demonstrated by Hammond et al. in commonly-owned U.S. Patent Application No. 08/708,661, filed September 5, 1996, incorporated herein in its entirety. That method involves the delivery of genes encoding elements of the β-adrenergic signaling pathway to the heart of a patient with heart disease associated with a reduction β-adrenergic signaling. A method for treating various forms of heart disease such as myocardial ischemia and peripheral vascular disease has been described by Hammond et al. in PCT publication WO96/26742, published 06 Sept. 1996.

One form of congestive heart failure is associated with coronary artery disease ("CAD") that is so severe in scope or abruptness that it results in the development of chronic or acute heart failure. In such patients, extensive and/or abrupt occlusion of one or more coronary arteries precludes adequate blood flow to the myocardium, resulting in severe ischemia and, in some cases, myocardial infarction or death of heart muscle. The consequent myocardial necrosis tends to be followed by progressive chronic heart failure or an acute low output state - both of which are associated with high mortality.

Some forms of congestive heart failure have been especially problematic to treat because the cause of the condition remains unknown or untreatable. One such example is "dilated cardiomyopathy" (or DCM as used herein). DCM is a condition of the heart typically diagnosed by the finding of a dilated, hypocontractile left and/or right ventricle in the absence of other characteristic forms of cardiac disease such as coronary occlusion or a history of myocardial

infarction. Patients with DCM can experience angina pectoris even though they do not have severe coronary artery disease. In some of these cases, the cause of DCM is known or suspected. Examples include familial cardiomyopathy (such as that associated with progressive muscular dystrophy, myotonic muscular dystrophy, Freidrich's ataxia, and hereditary dilated cardiomyopathy), infections resulting in myocardial inflammation (such as infections by various viruses, bacteria and other parasites), noninfectious inflammations (such as those due to autoimmune diseases, peripartum cardiomyopathy, hypersensitivity reactions or transplantation rejections), metabolic disturbances causing myocarditis (including nutritional, endocrinologic and electrolyte abnormalities) and exposure to toxic agents causing myocarditis (including alcohol, as well as certain chemotherapeutic drugs and catecholamines). In the majority of cases however, approximately 80%, the cause of DCM remains unknown and the condition is thus referred to as "idiopathic dilated cardiomyopathy." The occurrence of DCM poses several major therapeutic concerns, including progressive myocardial injury, hemodynamic inefficiencies associated with the dilated heart, the threat of systemic emboli, and the risk of ventricular arrhythmias. Traditional revascularization is not an option for treatment of dilated cardiomyopathy, because occlusive coronary disease is not the primary problem. Even for those patients for which the cause of DCM is known or suspected, the damage is typically not readily reversible. For example, in the case of adriamycin-induced cardiotoxicity, the cardiomyopathy is generally irreversible and results in death in over 60% of afflicted patients. For most patients with DCM, the cause itself is unknown. As a result, there are no generally applied treatments for DCM. Physicians have traditionally focused on alleviating the symptoms presented in a patient exhibiting DCM (e.g., by relieving fluid retention with diuretics, and/or reducing the demand of the heart muscle for oxygen and nutrients with angiotensin converting enzyme inhibitors). As a result, approximately 50% of the patients exhibiting DCM die within two years of diagnosis, often from sudden cardiac arrest associated with ventricular arrhythmias.

"Ventricular remodeling" often occurs after myocardial infarction, resulting in poor ventricular function. After a myocardial infarct heals, the heart tends to dilate and remodel. This dilating or remodeling often causes further impairment of ventricular function. Poor left ventricular function is the best single predictor of adverse outcome following myocardial infarction. Thus, preventing ventricular remodeling after myocardial infarction would be beneficial. One approach to try to prevent ventricular remodeling is to treat patients who have suffered a myocardial infarction with angiotensin converting enzyme ("ACE") inhibitors (see, e.g., McDonald, K.M., Trans. Assoc. Am. Physicians 103:229-235, 1990; Cohn, J. Clin. Cardiol. 18

(Suppl. IV) IV-4-IV-12, 1995). However, these agents are only somewhat effective at preventing deleterious ventricular remodeling and new therapies are needed.

SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for treating congestive heart failure (including dilated cardiomyopathy as well as congestive heart failure associated with severe coronary artery disease), and for preventing or alleviating deleterious ventricular remodeling, by introducing transgenes encoding angiogenic proteins into the myocardium of a patient. Various aspects of the present invention include the following:

A method for treating a patient suffering from congestive heart failure, comprising delivering a vector to the heart of said patient, the vector comprising a transgene encoding an angiogenic protein or peptide operably linked to a promoter for expression of the transgene. Among such patients suffering from congestive heart failure are those exhibiting dilated cardiomyopthy. Other patients suffering from congestive heart failure are those who have exhibited severe myocardial infarctions, typically associated with severe or occlusive coronary artery disease. Synthesis of the angiogenic protein in the myocardium promotes angiogenesis and increases blood flow. The vector is preferably introduced into a blood vessel supplying blood to the myocardium of the heart, so as to deliver the vector to cardiac myocytes. Preferably the vector is introduced into the lumen of a coronary artery, a saphenous vein graft, or an internal mammary artery graft; most preferably, the vector is introduced into the lumen of both the left and right coronary arteries. The intracoronary injection is preferably made relatively deeply within the artery, preferably at least about 1 cm into the lumens of the left and right coronary arteries. The intracoronary injection can also be made about 1 cm into the lumens of a saphenous vein graft and/or an internal mammary artery graft, which can be made in addition to intracoronary injection. The vector can be delivered in a single injection into each such vessel.

A method for preventing or alleviating deleterious ventricular remodeling in a patient who has suffered (or may suffer) a myocardial infarction, comprising delivering a vector to the heart of said patient, the vector comprising a gene encoding an angiogenic protein or peptide operably linked to a promoter for expression of the gene. Synthesis of the angiogenic protein in the myocardium induces angiogenesis, thus increasing blood flow and alleviating deleterious ventricular remodeling.

In preferred methods of promoting angiogenesis in the treatment of heart failure according to one of the preceding embodiments, the vector comprises at least one gene encoding an

angiogenic protein or peptide from the family of FGF (fibroblast growth factors), VEGF (vascular endothelial growth factors), PDGF (platelet-derived growth factors) or IGF (insulin-like growth factors). In certain preferred embodiments, the vector contains a gene encoding an FGF, including but not limited to FGF-4, FGF-5, FGF-6, aFGF (also known as FGF-1) and bFGF (also known as FGF-2). Preferred angiogenic proteins include those which are naturally secreted, or have been modified to enhance secretion. Presently preferred among such angiogenic proteins are forms of FGF-4, FGF-5, FGF-6, FGF-1 and FGF-2 that are naturally secreted or have been modified to enhance secretion. In other embodiments, the gene encodes a VEGF, including but not limited to members of the VEGF-A family (such as VEGF-121, VEGF-145, VEGF-165, VEGF-189 and VEGF-206), members of the VEGF-B family (such as VEGF-167 and VEGF-186), and members of the VEGF-C family; or a PDGF or an IGF.

The angiogenic protein-encoding gene is operably linked to a promoter that directs transcription and expression of the gene in a mammalian cell, preferably in a cardiac myocyte. One presently preferred promoter is a CMV promoter. In other preferred embodiments, the promoter is a tissue specific promoter, preferably a cardiac-specific promoter, more preferably a ventricular myocyte-specific promoter. Preferred examples of ventricular myocyte-specific promoters include a ventricular myosin light chain-2 promoter and a myosin heavy chain promoter. Preferably, the gene encoding the angiogenic factor is also operably linked to a polyadenylation signal.

In preferred methods of treating heart failure (including DCM) or alleviating deleterious ventricular remodeling (e.g. after myocardial infarction) according to the present invention, the vector is a viral vector or a lipid-based vector, preferably a viral vector. The vector can be a targeted vector, especially a vector that preferentially targets ventricular myocytes. Presently preferred viral vectors are derived from adenovirus (Ad) or adeno-associated virus (AAV). Both human and non-human viral vectors can be used but preferably the recombinant viral vector is replication-defective in humans. Where the vector is an adenovirus, it preferably comprises a polynucleotide having a promoter operably linked to a gene encoding and FGF (e.g. an FGF-1, FGF-2, FGF-4, FGF-5 or FGF-6), and is replication-defective in humans. Presently preferred replication-defective adenoviral vector have deletions that remove the E1A and E1B genes, or have deletions that remove the E1A, E1B and E4 genes. Preferably about 10^6 to 10^{14} adenovirus vector particles, more preferably about 10^8 to 10^{12} vector particles, are introduced into a blood vessel, preferably a blood vessel supplying the myocardium. Most preferably, an adenovirus vector comprising the angiogenic protein-encoding gene is introduced into the heart of the patient by intracoronary injection directly into the lumens of both coronary arteries.

For AAV vectors, the vector preferably comprises a polynucleotide having a promoter operably linked to a gene encoding an angiogenic protein flanked by AAV inverted terminal repeats (ITRs). Preferably, the AAV vector is replication-defective in humans. Presently preferred replication-defective AAV vectors have deletions affecting one or more AAV replication or encapsidation sequences. More preferably, the AAV *rep* and *cap* genes are both removed (and therefore must be supplied *in trans* to replicate and package AAV vectors, typically in a packaging cell line, as is known in the AAV art).

The vector can also be a lipid-based vector comprising a gene encoding an angiogenic protein. A variety of such lipid-based vectors, including liposomes, micelles and lipid-containing emulsions, have been described in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 graphically presents percent wall thickening during pacing in a porcine model of congestive heart failure. Percent wall thickening was assessed sequentially in the interventricular septum and lateral wall before pacing (day 0) and every 7 days as heart failure progressed (described in Example 1). Symbols represent mean values; error bars denote 1 SD. Two-way ANOVA (repeated measures) showed that percent wall thickening was affected by duration of pacing ($P< 0.001$) and by region ($P=.001$). Furthermore, the pattern of change in wall thickening was different between the two regions ($P<0.0001$). Mean values for percent wall thickening at each time point were tested for differences between the two regions post hoc by the Tukey method; *P* values for these analyses are shown beneath the error bars.

Figures 2A and 2B graphically present subendocardial blood flow during pacing in a porcine model of congestive heart failure, as described in Example 1.

For Figure 2A, subendocardial (endo) blood flow was assessed sequentially in the interventricular septum and lateral wall under the conditions listed along the x axis. Day refers to the day of sustained pacing that measurements were obtained (0, initiation of pacing; 14, 14 days; 21-28, 21 to 28 days). PACE refers to whether blood flow determinations were obtained with pacemaker activated (+) or inactivated (0). Pacemaker rate was 225 bpm. (See Table 3 herein for numerical values.) Symbols represent mean values; error bars denote 1 SD. Two-way ANOVA (repeated measures) showed that subendocardial blood flow was affected by duration of pacing ($P=.0001$) and by region ($P=.017$). Furthermore, the pattern of change in subendocardial blood flow was different between the two regions ($P<.006$). Mean values for subendocardial blood flow at each time point were tested for differences between the two regions post hoc by Tukey analyses; *P* values for these analyses are shown beneath the error bars.

For Figure 2B, subendocardial blood flow per beat was assessed sequentially in the interventricular septum and lateral wall under the conditions listed along the x axis. Symbols and conditions as in Figure 2A. (See Table 4 herein for numerical values.) Two-way ANOVA (repeated measures) showed that subendocardial blood flow per beat was affected by duration of pacing (P=.0001) and by region (P=.0198).

Figure 3A graphically presents meridional end-systolic wall stress as assessed sequentially in the interventricular septum and lateral wall before pacing (day 0) and every 7 days as heart failure progressed (described in Example 1). Two-way ANOVA (repeated measures) showed that systolic wall stress was affected by duration of pacing (P<.0001). However, the pattern of systolic wall stress was similar in both regions. Measurements were made with pacemakers inactivated.

Figure 3B graphically presents coronary vascular resistance during pacing in a porcine model of congestive heart failure, as described in Example 1. An index of coronary vascular resistance was assessed sequentially in the interventricular septum and lateral wall under the conditions listed along the x axis. Symbols and conditions are the same as in Fig 2. Two-way ANOVA (repeated measures) showed that the coronary vascular resistance index was affected by duration of pacing (P=.0001) and by regions (P=.013). Furthermore, the pattern of change in coronary vascular resistance was different between the two regions (P=.0012). Mean values for coronary vascular resistances at each time point were tested for differences between the two regions post hoc by Tukey analyses. This analysis showed that coronary vascular resistance was higher in the lateral wall than in the septum directly after the initiation of pacing (P value below error bar).

Figure 4 shows a schematic of the construction of an exemplary replication-defective recombinant adenovirus vector useful for gene transfer into cells and into the heart, as described in the Examples below.

Figure 5 is a schematic figure which shows rescue recombination construction of a transgene encoding adenovirus.

Figures 6A and 6B graphically presents the regional contractile function of the treated animals, as described in Example 5. Figure 6A shows results of animals examined 2 weeks post gene transfer and Figure 6B shows results 12 weeks post gene transfer.

Figures 7A, 7B and 7C show diagrams corresponding to myocardial contrast echocardiographs. White areas denote contrast enhancement (more blood flow) and dark areas denote decreased blood flow. Figure 7A illustrates acute LCx occlusion in a normal pig. Figure 7B illustrates the difference in contrast enhancement between IVS and LCx bed 14 days after gene transfer with lacZ, indicating different blood flows in two regions during atrial pacing (200 bpm).

In Figure 7C, contrast enhancement appears equivalent in IVS and LCx bed 14 days after gene transfer with FGF-5, indicating similar blood flows in the two regions during atrial pacing. These results are described in Example 5.

Figure 8 shows the peak contrast ratio (a correlate of blood flow) expressed as the ratio of the peak video intensity in the ischemic region (LCx bed) divided by the peak video intensity in the interventricular septum (IVS), measured from the video images using a computer-based video analysis program during atrial pacing (200 bpm) before and 14±1 days after gene transfer with lacZ (control gene) and with FGF-5, and in 5 animals, 12 weeks after FGF-5 gene transfer (described in Example 5). Blood flow to the ischemic bed remained 50% of normal after gene transfer with the control gene but increased 2-fold above normal after gene transfer with FGF-5 ($p=0.0018$), an effect that persisted for at least 12 weeks.

Figure 9 shows vessel number as quantitated by microscopic analysis in the ischemic and nonischemic regions after gene transfer with FGF-5 and with lacZ (described in Example 5). There was increased capillary number surrounding each fiber in the ischemic and nonischemic regions of animals that received FGF-5 gene transfer ($p<0.038$) compared to animals that received the lacZ gene.

Figures 10A, 10B and 10C are from gels documenting DNA, mRNA and protein expression after gene transfer of an angiogenic transgene to the myocardium according to the present invention (as described in Example 5). Figure 10D is from a gel following PCR amplification demonstrating the absence of any detectable gene transfer to the retina, liver or skeletal muscle of treated animals (as described in Example 5).

Figure 11 shows a comparison of wall thickening achieved with *in vivo* gene transfer using different angiogenic gene constructs, FGF-4, FGF-5 and FGF-2LI +/- sp (i.e. FGF-2LI plus or minus secretion signal peptide), as described in examples 6 and 7.

Figure 12 shows that improved function in the ischemic region after FGF-4 gene transfer (as indicated by wall thickening) was associated with improved regional perfusion.

Figure 13 shows a comparison of perfusion (blood flow) resulting from injection of FGF-4, FGF-5 or FGF-2LI +/- sp (= FGF-2LI plus or minus signal peptide), as described in Examples 6 and 7.

Figure 14 shows a comparison of wall thickening as a result of gene transfer with FGF-2 plus (FGF-2LI+sp) or minus secretion signal peptide (FGF-2LI-sp), as described in Example 7.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Definitions

"Heart failure" is clinically defined as a condition in which the heart does not provide adequate blood flow to meet metabolic demands. Symptoms include breathlessness, fatigue, weakness, leg swelling, and exercise intolerance. On physical examination, patients with heart failure tend to have elevations in heart and respiratory rates, rales (an indication of fluid in the lungs), edema, jugular venous distension, and, in general, enlarged hearts. Patients with heart failure suffer a high mortality; typically 50% of the patients die within two years of developing the condition. In some cases, heart failure is associated with severe coronary artery disease ("CAD"), typically resulting in myocardial infarction and either progressive chronic heart failure or an acute low output state, as described herein and in the art. In other cases, heart failure is associated with dilated cardiomyopathy (as described below) without associated severe coronary artery disease.

"Dilated cardiomyopathy" (or DCM as used herein) is a type of heart failure that is typically diagnosed by the finding of a dilated, hypocontractile left and/or right ventricle in the absence of other characteristic forms of cardiac disease such as coronary occlusion or a history of myocardial infarction. DCM is associated with poor ventricular function and symptoms of heart failure. In these patients, chamber dilation and wall thinning generally results in a high left ventricular wall tension. Although typical occlusive coronary artery disease is absent, these patients often complain of angina pectoris. Other symptoms include breathlessness, fatigue, weakness, leg swelling and exercise intolerance. Many such patients exhibit these symptoms even under mild exertion or at rest, and are thus characterized as exhibiting severe, i.e. "Type-III" or "Type-IV", heart failure, respectively. On physical examination, patients with DCM tend to have elevations in heart and respiratory rates, rales (an indication of fluid in the lungs), edema, jugular venous distension, and, in general, enlarged hearts. Echocardiography is typically employed in the diagnosis of DCM. Patients with DCM can experience angina pectoris, even though they do not have severe coronary artery disease. In some cases, the cause of DCM is known or suspected. Examples include familial cardiomyopathy (such as that associated with progressive muscular dystrophy, myotonic muscular dystrophy, Freidrich's ataxia, and hereditary dilated cardiomyopathy), infections resulting in myocardial inflammation, noninfectious inflammations (such as those due to autoimmune diseases, peripartum cardiomyopathy, hypersensitivity reactions or transplantation rejections), metabolic disturbances (including nutritional, endocrinologic and electrolyte abnormalities) and exposure to toxic agents (such alcohol, chemotherapeutic drugs and certain catecholamines). In the majority of cases (approximately 80%), the cause of DCM remains unknown and the condition is thus referred to as "idiopathic dilated cardiomyopathy."

As used herein, the terms "having therapeutic effect" and "successful treatment" carry essentially the same meaning. In particular, a patient suffering from heart failure (associated with severe CAD or DCM) is successfully "treated" for the condition if the patient shows observable and/or measurable reduction in or absence of one or more of the above-described symptoms after receiving an angiogenic factor transgene according to the methods of the present invention. Reduction of these signs or symptoms may also be felt by the patient. Thus, indicators of successful treatment of such heart failure conditions include the patient showing or feeling a reduction in any one of the symptoms of angina pectoris, fatigue, weakness, breathlessness, leg swelling, rales, heart or respiratory rates, edema or jugular venous distension. The patient may also show greater exercise tolerance, have a smaller heart with improved ventricular and cardiac function, and in general, require fewer hospital visits related to the heart condition. Preferably, the improvement in cardiac function is adequate to meet the metabolic needs of the patient and the patient does not exhibit symptoms under mild exertion or at rest. Many of these signs and symptoms are readily observable by eye and/or measurable by routine procedures familiar to a physician. One indicator of improved cardiac function increased blood flow in the heart especially in the previously ischemic region, is also an indicator of successful treatment. As described below, blood flow in a patient can be measured by thallium imaging (as described by Braunwald in Heart Disease, 4th ed., pp. 276-311 (Saunders, Philadelphia, 1992)) or by echocardiography (described in Examples 1 and 5 and in Sahn, DJ, et al., *Circulation.* 58:1072-1083, 1978). Blood flow before and after angiogenic gene transfer can be compared using these methods. Improved heart function is associated with decreased signs and symptoms, as noted above. In addition to echocardiography, one can measure ejection fraction (LV) by nuclear (non-invasive) techniques as is known in the art.

"Ventricular remodeling" often occurs after myocardial infarction and is typically characterized as follows. After a myocardial infarct heals, continued ischemia in the border region between the healed infarct and normal tissue and other factors leads to a dilation and/or remodeling of the remaining heart tissue, a condition formerly referred to as infarct expansion. Dilation of the whole heart occurs in about 50% of patients who have such infarcts. This dilating, or remodeling, causes further impairment of ventricular function. Remodeling usually develops within a few months after a myocardial infarction although it can occur as early as 1-2 weeks after the infarct.

"Deleterious left ventricular remodeling" (or "deleterious remodeling" for short), refers to chamber dilation after myocardial infarction, which can progress to severe heart failure. By employing the treatment methods described herein, it is expected that such deleterious remodeling can be prevented or at least substantially alleviated. Even if ventricular remodeling has already

initiated, it is still desirable to induce angiogenesis since the development of a microvascular bed and an increase in blood flow can still be effective to offset ventricular dysfunction. In a patient who has suffered a myocardial infarction, deleterious ventricular remodeling is "prevented" if the patient lacks chamber dilation and if symptoms of heart failure (described above under "heart failure") do not develop. Deleterious ventricular remodeling is alleviated if there is any observable or measurable reduction in an existing symptom of the heart failure. For example, the patient may show less breathlessness and improved exercise tolerance. Methods of assessing improvement in heart function and reduction of symptoms are essentially analogous to those described above for DCM. Prevention or alleviation of deleterious ventricular remodeling as a result of improved collateral blood flow and ventricular function is expected to be achieved within weeks after *in vivo* angiogenic gene transfer in the patient using methods as described herein.

A "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. This term refers to the primary structure of the molecule, and thus includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modified polynucleotides such as methylated and/or capped polynucleotides.

"Recombinant," as applied to a polynucleotide, means that the polynucleotide is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature.

A "gene" or "transgene" refers to a polynucleotide or portion of a polynucleotide comprising a sequence that encodes a protein. For most situations, it is desirable for the gene to also comprise a promoter operably linked to the coding sequence in order to effectively promote transcription. Enhancers, repressors and other regulatory sequences may also be included in order to modulate activity of the gene, as is well known in the art. (See, e.g., the references cited below).

The terms "polypeptide," "peptide," and "protein" are used interchangeably to refer to polymers of amino acids of any length. These terms also include proteins that are post-translationally modified through reactions that include glycosylation, acetylation and phosphorylation.

An "angiogenic peptide or protein" refers to any protein or peptide that promotes angiogenesis or angiogenic activity, i.e. blood vessel development. Blood vessels that develop in angiogenesis include capillaries which are the smallest caliber blood vessels having a diameter of about 8 microns, and larger caliber blood vessels that have a diameter of at least about 10 microns. Angiogenic activity can be determined by measuring blood flow, increase in myocardial function or the presence of blood vessels, using procedures known in the art or described herein. For

example, capillary number or density can be quantitated in an animal visually or by microscopic analysis of the tissue site (see Example 5). Myocardial blood flow can be determined by the radioactive microsphere technique as described in Roth, DM, et al., *Am. J. Physiol.* 253:H1279-H1288, 1987 or Roth, DM, et al., *Circulation* 82:1778-1789, 1990. Myocardial blood flow can also be quantitated, e.g., by thallium imaging, which involves perfusing the heart with the radionuclide thallium as described by Braunwald in Heart Disease, 4th ed., pp. 276-311 (Saunders, Philadelphia, 1992). The cells in the heart have an avidity for thallium. Uptake of thallium is positively correlated with blood flow. Thus, reduced uptake indicates reduced blood blow as occurs in ischemic conditions in which there is a perfusion deficit. In a conscious individual, angiogenic activity can be readily evaluated by contrast echocardiography such as described in Examples 1 and 5 and in Sahn, DJ, et al., *Circulation.* 58:1072-1083, 1978. Improved myocardial function can be determined by measuring wall thickening such as by transthoracic echocardiography. Angiogenesis is "promoted" if there is measurable increase in angiogenic activity in the heart of the patient after receiving the angiogenic transgene as compared to before treatment with the transgene. Angiogenic peptides include peptide precursors that are post-translationally processed into active peptides. Preferably, the angiogenic protein or peptide is a member of the fibroblast growth factor (FGF) family (including but not limited to aFGF (also known as FGF-1), bFGF (also known as FGF-2), FGF-4, FGF-5 and FGF-6), a member of the vascular endothelial growth factor (VEGF) family, including but not limited to members of the VEGF-A family (such as VEGF-121, VEGF-145, VEGF-165, VEGF-189 and VEGF-206), members of the VEGF-B family (such as VEGF-167 and VEGF-186), members of the VEGF-C family; a member of the platelet-derived growth factor (PDGF) family (e.g., PDGF A or PDGF B), or a member of the insulin-like growth factor (IGF) family. However, a gene encoding any protein or peptide that exhibits angiogenic activity measurable, e.g., by the above-described methods is likely to be useful in the methods of the present invention. Angiogenic peptides or proteins also include "derivatives" and "functional equivalents" of angiogenic proteins such as FGFs, VEGFs, PDGFs and IGFs. Derivatives of such an angiogenic protein or peptide are peptides having similar amino acid sequence and retaining, to some extent, one or more activities of the related angiogenic protein or peptide. Useful derivatives generally have substantial sequence similarity (at the amino acid level) in regions or domains of the protein associated with the angiogenic activity. "Sequence similarity" refers to similarity observed between amino acid sequences in two different proteins or peptides, irrespective of peptide origin. As is well known in the art, there are a number of amino acid replacements that are essentially "conservative" in nature in which the substituted amino acid is relatively similar (e.g. in size and charge) to the original amino acid. Substantial sequence

similarity typically involves at least 30% sequence identity (generally as measured over stretches of 10 or more amino acids), preferably 70%, more preferably 90%, and even more preferably 95% sequence similarity to the related angiogenic protein or peptide or subunit. As is also known in the art, conservative additions or deletions are generally those that are localized in regions or domains of the protein that do not appear to be involved in activity, in this case promoting angiogenesis. By "functional equivalent" is meant a protein or peptide that has an activity that can substitute for one or more activities of a particular angiogenic protein or peptide. Preferred functional equivalents retain all of the activities of a particular angiogenic protein or peptide; however, the functional equivalent may have an activity that, when measured quantitatively, is stronger or weaker than the wild-type peptide or protein as measured in angiogenesis assays. Preferred functional equivalents have activities that are within 1% to 10,000% of the activity of the related angiogenic protein or peptide or subunit, more preferably between 10% to 1000%, and more preferably within 50% to 200%. As is known in the art, a variety of techniques, including computer modeling, are available for assessing the likely impact of such variations; and prospective candidates can be readily screened for the retention of angiogenic potential using *in vitro* and/or *in vivo* assays as described herein.

A "heterologous" component refers to a component that is introduced into or produced within a different entity from that in which it is naturally located. For example, a polynucleotide derived from one organism and introduced by genetic engineering techniques into a different organism is a heterologous polynucleotide which, if expressed, can encode a heterologous polypeptide. Similarly, a promoter or enhancer that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous promoter or enhancer.

A "promoter," as used herein, refers to a polynucleotide sequence that controls transcription of a gene or coding sequence to which it is operably linked. A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources, are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources).

An "enhancer," as used herein, refers to a polynucleotide sequence that enhances transcription of a gene or coding sequence to which it is operably linked. A large number of enhancers, from a variety of different sources are well known in the art (and identified in databases such as GenBank) and available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of

polynucleotides comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences.

"Operably linked" refers to a juxtaposition of two or more components, wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter is operably linked to a gene or coding sequence if the promoter controls transcription of the gene or coding sequence. Although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within or downstream of coding sequences. A polyadenylation sequence is operably linked to a coding sequence if it is located at the downstream end of the coding sequence such that transcription proceeds through the coding sequence into the polyadenylation sequence.

A "replicon" refers to a polynucleotide comprising an origin of replication which allows for replication of the polynucleotide in an appropriate host cell. Examples include replicons of a target cell into which a heterologous nucleic acid might be integrated (e.g., nuclear and mitochondrial chromosomes), as well as extrachromosomal replicons (such as replicating plasmids and episomes).

"Gene delivery", "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stable or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

"In vivo" gene delivery, gene transfer, gene therapy and the like as used herein, are terms referring to the introduction of a vector comprising an exogenous polynucleotide directly into the body of an organism, such as a human or non-human mammal, whereby the exogenous polynucleotide is introduced into a cell of such organism *in vivo.*

A "vector" (sometimes referred to as a gene delivery or gene transfer "vehicle") refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either *in vitro* or *in vivo*. The polynucleotide to be delivered may comprise a coding sequence of interest in gene therapy. Vectors include, for example, viral vectors (such as adenoviruses (Ad), adeno-associated viruses (AAV), and retroviruses), liposomes and other lipid-containing complexes, and other macromolecular complexes capable of mediating delivery of a polynucleotide to a host cell. Vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. Such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that
influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such as agents mediating nuclear localization); and components that influence expression of the polynucleotide. Such components also include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities. A large variety of such vectors are known in the art and are generally available (see, e.g., the various references cited herein).

A "recombinant viral vector" refers to a viral vector comprising one or more heterologous genes or sequences. Since many viral vectors exhibit size-constraints associated with packaging, the heterologous genes or sequences are typically introduced by replacing one or more portions of the viral genome. Such viruses may become replication-defective, requiring the deleted function(s) to be provided *in trans* during viral replication and encapsulation (by using, e.g., a helper virus or a packaging cell line carrying genes necessary for replication and/or encapsulation) (see, e.g., the references and illustrations below). Modified viral vectors in which a polynucleotide to be delivered is carried on the outside of the viral particle have also been described (see, e.g., Curiel, DT, et al. PNAS 88: 8850-8854, 1991).

Viral "packaging" as used herein refers to a series of intracellular events that results in the synthesis and assembly of a viral vector. Packaging typically involves the replication of the "pro-viral genome", or a recombinant pro-vector typically referred to as a "vector plasmid" (which is a recombinant polynucleotide than can be packaged in an manner analogous to a viral genome, typically as a result of being flanked by appropriate viral "packaging sequences"), followed by

encapsulation or other coating of the nucleic acid. Thus, when a suitable vector plasmid is introduced into a packaging cell line under appropriate conditions, it can be replicated and assembled into a viral particle. Viral "rep" and "cap" genes, found in many viral genomes, are genes encoding replication and encapsulation proteins, respectively. A "replication-defective" or "replication-incompetent" viral vector refers to a viral vector in which one or more functions necessary for replication and/or packaging are missing or altered, rendering the viral vector incapable of initiating viral replication following uptake by a host cell. To produce stocks of such replication-defective viral vectors, the virus or pro-viral nucleic acid can be introduced into a "packaging cell line" that has been modified to contain genes encoding the missing functions which can be supplied *in trans*. For example, such packaging genes can be stably integrated into a replicon of the packaging cell line or they can be introduced by transfection with a 'packaging plasmid' or helper virus carrying genes encoding the missing functions.

A "detectable marker gene" is a gene that allows cells carrying the gene to be specifically detected (e.g., distinguished from cells which do not carry the marker gene). A large variety of such marker genes are known in the art. Preferred examples thereof include detectable marker genes which encode proteins appearing on cellular surfaces, thereby facilitating simplified and rapid detection and/or cellular sorting. By way of illustration, the lacZ gene encoding beta-galactosidase can be used as a detectable marker, allowing cells transduced with a vector carrying the lacZ gene to be detected by staining, as described below.

A "selectable marker gene" is a gene that allows cells carrying the gene to be specifically selected for or against, in the presence of a corresponding selective agent. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows a host cell to be positively selected for in the presence of the corresponding antibiotic. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow selection for cells carrying the marker, whereas negative selectable markers allow cells carrying the marker to be selectively eliminated. A variety of such marker genes have been described, including bifunctional (i.e. positive/negative) markers (see, e.g., WO 92/08796, published 29 May 1992, and WO 94/28143, published 8 December 1994), hereby incorporated by reference herein. Such marker genes can provide an added measure of control that can be advantageous in gene therapy contexts.

"Vasculature" or "vascular" are terms referring to the system of vessels carrying blood (as well as lymph fluids) throughout the mammalian body.

"Blood vessel" refers to any of the vessels of the mammalian vascular system, including arteries, arterioles, capillaries, venules, veins, sinuses, and vasa vasorum. In preferred aspects of the present invention, vectors comprising angiogenic transgenes are introduced directly into

vascular conduits supplying blood to the myocardium. Such vascular conduits include the coronary arteries as well as vessels such as saphenous veins or internal mammary artery grafts.

"Artery" refers to a blood vessel through which blood passes away from the heart. Coronary arteries supply the tissues of the heart itself, while other arteries supply the remaining organs of the body. The general structure of an artery consists of a lumen surrounded by a multi-layered arterial wall.

An "individual" or a "patient" refers to a mammal, preferably a large mammal, most preferably a human.

"Treatment" or "therapy" as used herein refers to administering, to an individual patient, agents that are capable of eliciting a prophylactic, curative or other beneficial effect on the individual.

"Gene therapy" as used herein refers to administering, to an individual patient, vectors comprising a therapeutic gene.

A "therapeutic polynucleotide" or "therapeutic gene" refers to a nucleotide sequence that is capable, when transferred to an individual, of eliciting a prophylactic, curative or other beneficial effect in the individual.

REFERENCES

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Molecular Cloning: A Laboratory Manual, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); Current Protocols in Molecular Biology (F. Ausubel et al. eds., 1987 and updated); Essential Molecular Biology (T. Brown ed., IRL Press 1991); Gene Expression Technology (Goeddel ed., Academic Press 1991); Methods for Cloning and Analysis of Eukaryotic Genes (A. Bothwell et al. eds., Bartlett Publ. 1990); Gene Transfer and Expression (M. Kriegler, Stockton Press 1990); Recombinant DNA Methodology (R. Wu et al. eds., Academic Press 1989); PCR: A Practical Approach (M. McPherson et al., IRL Press at Oxford University Press 1991); Cell Culture for Biochemists (R. Adams ed., Elsevier Science Publishers 1990); Gene Transfer Vectors for Mammalian Cells (J. Miller & M. Calos eds., 1987); Mammalian Cell Biotechnology (M. Butler ed., 1991); Animal Cell Culture (J. Pollard et al. eds., Humana Press 1990); Culture of Animal Cells, 2nd Ed. (R. Freshney et al. eds., Alan R. Liss 1987); Flow Cytometry and Sorting (M. Melamed et al. eds., Wiley-Liss 1990); the series Methods in Enzymology (Academic Press, Inc.); Techniques in Immunocytochemistry, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983,

1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); Cellular and Molecular Immunology (A. Abbas et al., W.B. Saunders Co. 1991, 1994); Current. Protocols in Immunology (J. Coligan et al. eds. 1991); the series Annual Review of Immunology; the series Advances in Immunology; Oligonucleotide Synthesis (M. Gait ed., 1984); and Animal Cell Culture (R. Freshney ed., IRL Press 1987).

Additional references describing delivery and logistics of surgery which may be used in the methods of the present invention include the following: Topol, EJ (ed.), The Textbook of Interventional Cardiology, 2nd Ed. (W.B. Saunders Co. 1994); Rutherford, RB, Vascular Surgery, 3rd Ed. (W.B. Saunders Co. 1989); The Cecil Textbook of Medicine, 19th Ed. (W.B. 1992); and Sabiston, D, The Textbook of Surgery, 14th Ed. (W.B. 1991). Additional references describing cell types found in the blood vessels, and those of the vasculature which may be useful in the methods of the present invention include the following: W. Bloom & D. Fawcett, A Textbook of Histology (V.B. Saunders Co. 1975).

Various publications have postulated on the uses of gene transfer for the prevention of disease, including heart disease. See, e.g., Methods in Virology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Weiss, Clifton, N.J., 1991; Mazur et al., Molecular and Cellular Biology, 21:104-111, 1994; French, Herz 18:222-229, 1993; Williams, Journal of Medical Sciences 306:129-136, 1993; and Schneider, Circulation 88:1937-1942, 1993.

The references cited in the above section are hereby incorporated by reference herein to the extent that these references teach techniques that are employed in the practice of the present invention.

INCORPORATION BY REFERENCE

References cited within this application, including patents, published applications and other publications, are hereby incorporated by reference.

DETAILED DESCRIPTION OF VARIOUS PREFERRED EMBODIMENTS

Various preferred aspects of the present invention are summarized below and further described and illustrated in the subsequent detailed descriptions and figures.

The present invention relates to methods and compositions for treating congestive heart failure, including dilated cardiomyopathy ("DCM") as well as severe heart failure associated with coronary artery disease ("CAD") and myocardial infarction, and for preventing or alleviating deleterious ventricular remodeling, by introducing transgenes encoding angiogenic proteins into the myocardium of a patient.

In the present method for treating congestive heart failure, a vector construct containing a gene encoding an angiogenic protein or peptide is targeted to the heart of a patient where synthesis of the exogenous angiogenic protein in the myocardium promotes angiogenesis, thus ameliorating cardiac dysfunction by improving blood flow through newly formed blood vessels. Improved heart function ultimately leads to the reduction or disappearance of symptoms of heart failure and prolonged life beyond the expected mortality within two years of developing severe CHF.

The preferred methods employ vector constructs and/or delivery methods that result in localized expression of an angiogenic protein that is relatively restricted to the myocardium of the patient. Examples of angiogenic proteins include members of the FGF family (including FGF-1, FGF-2, FGF-4, FGF-5 and FGF-6, more preferably FGF-2, FGF-4 and FGF-5); members of the vascular endothelial growth factor (VEGF) family, including but not limited to members of the VEGF-A family (such as VEGF-121, VEGF-145, VEGF-165, VEGF-189 and VEGF-206), members of the VEGF-B family (such as VEGF-167 and VEGF-186), and members of the VEGF-C family; platelet-derived growth factor (PDGF) family; and members of the insulin-like growth factor (IGF) family.

Preferred vectors for use in the present invention include viral vectors, lipid-based vectors and other vectors that are capable of delivering DNA to non-dividing cells *in vivo*. Presently preferred are viral vectors, particularly replication-defective viral vectors (including, for example replication-defective adenovirus (Ad) vectors and adeno-associated virus (AAV) vectors. For ease of production and use in the present invention, replication-defective adenovirus vectors are presently most preferred.

The presently preferred means of *in vivo* delivery (especially for vector constructs that are not otherwise targeted for delivery and/or expression that is restricted to the myocardium), is by injection of the vector into a blood vessel directly supplying the myocardium, preferably by injection into a coronary artery. Such injection is preferably achieved using a catheter introduced substantially deep (typically at least about 1 cm) within the lumen of one or both coronary arteries or one or more saphenous veins or internal mammary artery grafts or other conduits delivering blood to the myocardium. Injections that are not sufficiently deep in the coronary artery can result in systemic arterial distribution of the virus.

By injecting the viral vector stock, preferably containing no wild-type virus, deeply into the lumen of one or both coronary arteries (or grafts and other vascular conduits), preferably into both the right and left coronary arteries (or grafts and other vascular conduits), and preferably in an amount of 10^6-10^{14} viral particles as determined by optical densitometry (more preferably 10^8-10^{12} viral particles), it is possible to locally transfect a desired number of cells, especially cardiac

myocytes, with genes that encode proteins that promote angiogenesis in the affected myocardium, thereby maximizing therapeutic efficacy of gene transfer, and minimizing undesirable effects at extracardiac sites and the possibility of an inflammatory response to viral proteins. Vector constructs that are specifically targeted to the myocardium, such as vectors incorporating myocardial-specific binding or uptake components, and/or which incorporate angiogenic protein transgenes that are under the control of myocardial-specific transcriptional regulatory sequences (e.g., ventricular myocyte-specific promoters) can be used in place of or, preferably, in conjunction with such directed injection techniques as a means of further restricting expression to the myocardium, especially the ventricular myocytes. For vectors that can elicit an immune response, it is preferable to inject the vector directly into a blood vessel supplying the myocardium as described above, although the additional techniques for restricting the potential for extracardiac expression can also be employed.

The patient can be administered the angiogenic gene treatment at any time in the case of CHF but more preferably within about 1 to 4 weeks after the occurrence of myocardial infarction. The administration is preferably via a single (one time) intracoronary injection (e.g. into each coronary artery), although more than one injection may sometimes be necessary.

As described in detail below, we have shown that the use of such techniques with vectors carrying angiogenic protein transgenes can effectively promote coronary collateral vessel development and enhance function within the myocardium of a large mammal heart, without any observed effect on non-cardiac tissues and without generating an inflammatory reaction. No inflammatory cell infiltrates, cell necrosis or increased fibrosis were detected. Two weeks after angiogenic gene transfer, myocardial blood flow was improved and ischemia was alleviated, thus enhancing myocardial function. These beneficial effects persisted for at least 12 weeks. By way of illustration, we have used a single intracoronary injection (of about 10^{11} vector particles), which was observed to provide adequate expression of the angiogenic protein to produce a therapeutic benefit, without toxicity. In accordance with the enhancement of myocardial blood flow, our analyses revealed that high yield transduction of greater than 60% of the myocardial cells *in vivo* was achievable using this procedure.

Adenovirus-mediated gene transfer generally does not result in stable incorporation of the transgene into the host genome and therefore transgene expression is relatively transient (although vectors have been shown to be present for up to 5 months). However, in the present treatment methods for the aforementioned heart conditions, transient expression of the angiogenic transgene is generally adequate for achieving the therapeutic goal. In fact, for the present purposes, transient expression is generally desirable over stable integration from the perspective of safety since

disruption of the host genome can be avoided. As the experimental examples below demonstrated, transgene expression was maintained sufficiently long to allow collateral vessel development and concomitant restoration of normal heart function. Thus, the angiogenic factor gene does not have to be present in the transfected cell for more than a few weeks to produce a therapeutic effect. Once the new blood vessels have formed, continued expression of the exogenous angiogenic protein may not required to maintain the new vascular structure and increased blood flow.

The techniques of *in vivo* angiogenic transgene delivery provided by the present invention thus effectively circumvent the need for multiple injections or infusions of angiogenic proteins. The localized transduction and transgene expression achieved with these present treatment methods also avoid the potentially harmful effects of angiogenesis in non-cardiac tissues such as the retina.

Another aspect of the present invention is a method for preventing or alleviating ventricular remodeling in post-myocardial infarction patients, the method involving targeting *in vivo* delivery of a vector construct containing a gene encoding an angiogenic protein or peptide to the patient's heart to promote angiogenesis. The increased vascularity and blood flow achieved using the methods of the present invention will tend to reduce ischemia and therefore alleviate progression to deleterious ventricular remodeling. To rectify these disease conditions, patients are preferably treated soon after an infarct has occurred, preferably within one month post-infarct and even more preferably, within one to three weeks of the infarct. The present invention can also be employed, in a preventive manner, in patients likely to exhibit such infarcts. Unless otherwise indicated, the techniques and vector compositions for affecting targeted gene transfer in the heart of a patient to prevent or alleviate ventricular remodeling are essentially the same as for the treatment of DCM.

Animal Models

Important prerequisites for successful studies on gene therapy are (a) constitution of an animal model that is applicable to clinical congestive heart failure that can provide useful data regarding mechanisms for angiogenesis in the setting of CHF, and (b) accurate evaluation of the effects of gene transfer. In the present invention, the pig models fulfill these prerequisites. The pig is a particularly suitable model for studying heart diseases of humans because of its relevance to human physiology. The pig heart closely resembles the human heart in the following ways. The pig has a native coronary circulation very similar to that of humans, including the absence of native coronary collateral vessels. Secondly, the size of the pig heart, as a percentage of total body weight, is similar to that of the human heart. Additionally, the pig is a large animal model,

therefore allowing more accurate extrapolation on various parameters such as effective vector dosages, toxicity, etc. In contrast, the hearts of animals such as dogs and members of the murine family have a lot of endogenous collateral vessels. Relative to total body weight, the size of the dog heart is twice that of the human heart.

Two porcine models were used in the present invention. The first model, described herein in Example 1, mimics clinical congestive heart failure. In this model, continuous rapid ventricular pacing over a period of 3 to 4 weeks induces heart failure which shows similarities with many features of heart failure (including DCM as well as heart failure associated with severe coronary artery disease). Such pacing results in left ventricular dilation with impaired systolic function analogous to regional functional abnormalities seen in heart failure (including those associated with severe coronary artery disease (CAD), or DCM). Thus, this model can be used to determine whether delivery of a vector construct coding for an angiogenic peptide or protein is effective to alleviate the cardiac dysfunctions associated with these conditions. This model is particularly useful in providing some of the parameters by which to assess the effectiveness of *in vivo* gene therapy for the treatment of congestive heart failure and ventricular remodeling.

The second model, a model of myocardial ischemia, is described herein in Example 5. Using this second model, it was demonstrated that adenovirus-mediated delivery of a gene encoding an angiogenic protein alleviated myocardial ischemia by stimulating collateral vessel development and enhancing blood flow in the ischemic region. By way of illustration, we have successfully demonstrated these gene transfer techniques with three different angiogenic proteins, including both native forms and muteins (as described in detail in the Examples below).

In the second model, placement of an ameroid constrictor around the left circumflex (LCx) coronary artery results in gradual complete closure (within 7 days of placement) with minimal infarction (1% of the left ventricle, 4 ± 1% of the LCx bed) (Roth, et al., *Circulation,* 82:1778, 1990; Roth, et al., *Am. J. Physiol.,* 235:1-11279, 1987; White, et al., *Circ. Res.,* 71:1490, 1992; Hammond, et al., *Cardiol.,* 23:475, 1994; and Hammond, et al., *J. Clin. Invest.,* 92:2644, 1993). Myocardial function and blood flow are normal at rest in the region previously perfused by the occluded artery (referred to as the ischemic region), due to limited endogenous collateral vessel development, but blood flow reserve is insufficient to prevent ischemia when myocardial oxygen demands increase. Thus, the LCx bed is subject to episodic ischemia, analogous to clinical *angina pectoris.* Collateral vessel development and flow-function relationships are stable within 21 days of ameroid placement, and remain unchanged for four months (Roth, et al., *Circulation,* 82:1778, 1990; Roth, et al., *Am. J. Physiol.,* 235:H1279, 1987; White, et al., *Circ. Res.,* 71:1490, 1992). It has been documented by telemetry that animals have period ischemic dysfunction in the bed at

risk, throughout the day, related to abrupt increases in heart rate during feeding, interruptions by personnel, etc. Thus, the model has a bed with stable but inadequate collateral vessels, and is subject to periodic ischemia. Another distinct advantage of the model is that there is a normally perfused and functioning region (the LAD bed) adjacent to an abnormally perfused and functioning region (the LCx bed), thereby offering a control bed within each animal.

Myocardial contrast echocardiography was used to estimate regional myocardial perfusion. The contrast material is composed of microaggregates of galactose and increases the echogenicity (whiteness) of the image. The microaggregates distribute into the coronary arteries and myocardial walls in a manner that is proportional to blood flow (Skyba, et al., *Circulation,* 90:1513-1521, 1994). It has been shown that peak intensity of contrast is closely correlated with myocardial blood flow as measured by microspheres (Skyba, et al., *Circulation,* 90:1513-1521, 1994). To document that the echocardiographic images employed in the present invention were accurately identifying the LCx bed, and that myocardial contrast echocardiography could be used to evaluate myocardial blood flow, a hydraulic cuff occluder was placed around the proximal LCx adjacent to the ameroid.

In the present study, when animals were sacrificed, the hearts were perfusion-fixed (glutaraldehyde, physiological pressures, *in situ)* in order to quantitate capillary growth by microscopy. PCR was used to detect angiogenic protein DNA and mRNA in myocardium from animals that had received gene transfer. As described below, two weeks after gene transfer, myocardial samples from all five lacZ-transduced animals showed substantial β-galactosidase activity on histological inspection. Finally, angiogenic protein expression in cells and myocardium from animals that had received gene transfer was demonstrated using a polyclonal antibody to the angiogenic protein.

The strategy for therapeutic studies included the timing of transgene delivery, the route of administration of the transgene, and choice of the angiogenic gene. In the ameroid model of myocardial ischemia, gene transfer was performed after stable but insufficient collateral vessels had developed. Previous studies using the ameroid model involved delivery of angiogenic peptides during the closure of the ameroid, prior to the development of ischemia and collateral vessels. However, this strategy was not employed for several reasons. First, previous studies are not suitable for closely duplicating the conditions that would be present in the treatment of clinical myocardial ischemia in which gene transfer would be given in the setting of ongoing myocardial ischemia; previous studies are analogous to providing the peptide in anticipation of ischemia, and are therefore less relevant. Second, it was presumed, based upon previous studies in cell culture, that an ischemic stimulus in conjunction with the angiogenic peptide would be the optimal milieu

for the stimulation of angiogenesis. Angiogenesis could optimally be achieved by delivery of the transgene at a time when the onset of heart failure (associated with DCM or severe coronary artery disease) or ventricular remodeling was already present. Linked to these decisions was the selection of the method to achieve transgene delivery.

The pig model provided an excellent means to follow regional blood flow and function before and after gene delivery. The use of control animals that received the same recombinant adenovirus construct but with a reporter gene provided a control for these studies. Based on the foregoing, and previous published studies, those skilled in the art will appreciate that the results described below in pigs are expected to be predictive of results in humans.

Transgenes Encoding Angiogenic Proteins and Peptides

In the present invention, one or more transgenes encoding an angiogenic protein or peptide factor that can promote angiogenic activity in the heart can be used. Any protein or peptide that exhibits "angiogenic activity" (defined above) measurable by the methods described herein and in the art can be potentially employed in connection with the present invention. Suitable angiogenic proteins or peptides are exemplified by members of the family of fibroblast growth factors (FGF), vascular endothelial growth factors (VEGF), platelet-derived growth factors (PDGF), insulin-like growth factors (IGF), and others. Members of the FGF family include, but are not limited to, aFGF (FGF-1), bFGF (FGF-2), FGF-4 (also known as "hst/KS3"), FGF-5, FGF-6. VEGF has been shown to be expressed by cardiac myocytes in response to ischemia *in vitro* and *in vivo*; it is a regulator of angiogenesis under physiological conditions as well as during the adaptive response to pathological states ((Banai et al. Circulation 89:2183-2189, 1994). The VEGF family, includes, but is not limited to, members of the VEGF-A sub-family (e.g. VEGF-121, VEGF-145, VEGF-165, VEGF-189 and VEGF-206), as well as members of the VEGF-B sub-family (e.g. VEGF-167 and VEGF-186) and the VEGF-C sub-family. PDGF includes PDGF A and PDGF B. The nucleotide sequences of the genes encoding these proteins, and the corresponding amino acid sequences are known in the art (see, e.g., the GENBANK sequence database for these and other sequences). For the FGF family, see, e.g., Burgess, Ann. N.Y. Acad. Sci. 638: 89-97, 1991; Burgess et al. Annu. Rev. Biochem 58:575-606, 1989; Muhlhauser et al. Hum. Gene Therapy 6:1457-1465, 1995; Zhan et al., Mol. Cell. Biol., 8:3487, 1988; Seddon et al. Ann. N.Y. Acad. Sci. 638:98-108, 1991. For human hst/KS3 (i.e. FGF-4), see Taira et al. Proc. Natl. Acad. Sci. USA 84:2980-2984, 1987. For human VEGF, see e.g., Tischer et al. J. Biol. Chem. 206:11947-11954, 1991, and references therein; Muhlhauser et al., Cir. Res. 77:1077-1086, 1995). Sequence information for such genes and encoded polypeptides is readily obtainable from sequence

databases such as GenBank or EMBL. Polynucleotides encoding these proteins can also be obtained from gene libraries, e.g., by using PCR or hybridization techniques routine in the art.

Success of the gene transfer approach requires both synthesis of the gene product and secretion from the transfected cell. From this point of view, a gene encoding FGF-4, FGF-5, or FGF-6 is preferred since these proteins contain functional secretory signal sequences and are readily secreted from cells. Many if not most human VEGF proteins (including but not limited to VEGF-121 and VEGF-165) also are readily secreted and diffusable after secretion. Thus, when expressed, these angiogenic proteins can readily access the cardiac interstitium and induce angiogenesis.

With other angiogenic proteins such as aFGF (FGF-1) and bFGF (FGF-2) that lack a native secretory signal sequence, fusion proteins having secretory signal sequences can be recombinantly produced using standard recombinant DNA methodology familiar to one of skill in the art. It is believed that both aFGF and bFGF are naturally secreted to some degree; however, inclusion of an additional secretion signal sequence can be used to enhance secretion of the protein. The secretory signal sequence would typically be positioned at the N-terminus of the desired protein but can be placed at any position suitable to allow secretion of the angiogenic factor. For example, a polynucleotide containing a suitable signal sequence can be fused 5' to the first codon of the selected angiogenic protein gene. Suitable secretory signal sequences include signal sequences of the FGF-4, FGF-5, FGF-6 genes or a signal sequence of a different secreted protein such as IL-1β. Example 7 below exemplifies one type of modification of an angiogenic protein to contain a signal sequence from another protein, the modification achieved by replacement of residues in the angiogenic protein with residues that direct secretion of the secreted second protein. A signal sequence derived from a protein that is normally secreted from cardiac myocytes can be used.

For treating humans, genes encoding angiogenic proteins of human origin are preferred although angiogenic proteins of other mammalian origin, that exhibit cross-species activity i.e. having angiogenic activity in humans, can also be used.

Vectors for Gene Delivery In Vivo

In general, the gene of interest is transferred to the heart, including cardiac myocytes, *in vivo,* and directs production of the encoded protein. Preferably such production is constitutive (although inducible expression systems can also be employed).

Vectors useful in the present invention include viral vectors, lipid-based vectors (e.g., liposomes) and other vectors that are capable of delivering DNA to non-dividing cells *in vivo.*

Presently preferred are viral vectors, particularly replication-defective viral vectors including, for example, replication-defective adenovirus vectors and adeno-associated virus (AAV) vectors. For ease of production and use in the present invention, replication-defective adenovirus vectors are presently most preferred. In contrast to retrovirus, adenovirus generally does not require host cell replication for gene expression because integration is not a component of the adenoviral life cycle. Adenovirus efficiently infects nondividing cells and is therefor useful for expressing recombinant genes in the myocardium because of the nonreplicative nature of cardiac myoctes.

A variety of other vectors suitable for *in vivo* gene therapy can readily be employed to deliver angiogenic protein transgenes for use in the present invention. Such other vectors include other viral vectors (such as AAV), non-viral protein-based delivery platforms, as well as lipid-based vectors. References describing a these and other gene delivery vectors are known in the art, some of which are cited herein.

As described above and in the cited references, vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. Such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such as agents mediating nuclear localization); and components that influence expression of the polynucleotide. Such components also might include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow selection for cells carrying the marker, whereas negative selectable markers allow cells carrying the marker to be selectively eliminated. A variety of such marker genes have been described, including bifunctional (i.e. positive/negative) markers (see, e.g., Lupton, S., WO 92/08796, published 29 May 1992; and Lupton, S., WO 94/28143, published 8 December 1994). Such marker genes can provide an added measure of control that can be advantageous in gene therapy contexts. A large variety of such vectors are known in the art and are generally available (see, e.g., the various references cited above).

References describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., Adenoviridae and Their

Replication, in Fields, B., et al.. (eds.) Virology, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F., et al., pp. 109128 in Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al., FASEB Journal 9: 190-199, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68: 145-159, 1994; Schneider and French, Circulation 88:1937-1942, 1993; Curiel D.T., et al., Human Gene Therapy 3: 147-154, 1992; Graham, F.L., et al., WO 95/00655 (5 January 1995); Falck-Pedersen, E.S., WO 95/16772 (22 June 1995); Denefle, P. et al., WO 95/23867 (8 September 1995); Haddada, H. et al., WO 94/26914 (24 November 1994); Perricaudet, M. et al., WO 95/02697 (26 January 1995); Zhang, W., et al., WO 95/25071 (12 October 1995). A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996).

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Carter, B., Handbook of Parvoviruses, vol. 1, pp. 169-228, 1990; Berns, Virology, pp. 1743-1764 (Raven Press 1990); Carter, B., Curr. Opin. Biotechnol., 3: 533-539, 1992; Muzyczka, N., Current Topics in Microbiology and Immunology, 158: 92-129, 1992; Flotte, T.R., et al., Am. J. Respir. Cell Mol. Biol. 7:349-356, 1992; Chatterjee et al., Ann. NY Acad. Sci., 770: 79-90, 1995; Flotte, T.R., et al., WO 95/13365 (18 May 1995); Trempe, J.P., et al., WO 95/13392 (18 May 1995); Kotin, R., Human Gene Therapy, 5: 793-801, 1994; Flotte, T.R., et al., Gene Therapy 2:357-362, 1995; Allen, J.M., WO 96/17947 (13 June 1996); and Du et al., Gene Therapy 3: 254261, 1996.

References describing non-viral vectors which could be used in the methods of the present invention include the following: Ledley, FD, Human Gene Therapy 6: 11 29-1144, 1995; Miller, N., et al., FASEB Journal 9: 190-199, 1995; Chonn, A., et al., Curr. Opin. in Biotech. 6: 698-708, 1995; Schofield, JP, et al., British Med. Bull. 51: 56-71, -1995; Brigham, K. L., et al., J. Liposome Res. 3: 31 49, 1993; Brigham, K.L., WO 91/06309 (16 May 1991); Felgner, P.L., et al., WO 91/17424 (14 November 1991); Solodin et al., Biochemistry 34: 13537-13544, 1995; WO 93/19768 (14 October 1993); Debs et al., WO 93125673; Felgner, P.L., et al., U.S. Patent 5,264,618 (November 23, 1993); Epand, R.M., et al., U.S. Patent 5,283,185 (February 1, 1994); Gebeyehu et al., U.S. Patent 5,334,761 (August 2, 1994); Felgner, P.L., et al., U.S. Patent 5,459,127 (October 17, 1995); Overell, R.W., et al., WO 95/28494 (26 October 1995); Jessee, WO 95/02698 (26 January 1995); Haces and Ciccarone, WO 95/17373 (29 June 1995); Lin et al., WO 96/01840 (25 January 1996).

An Exemplary Adenoviral Vector that is Helper-Independent and Replication-Deficient in Humans

In general, the gene of interest is transferred to the heart including cardiac myocytes, *in vivo* and directs production of the encoded protein. Several different gene transfer approaches are feasible. Presently preferred is a helper-independent replication-deficient system based on human adenovirus 5. Using this system, we have demonstrated transfection greater than 60% of myocardial cells *in vivo* by a single intracoronary injection (Giordano and Hammond, *Clin. Res.*, 42:123A, 1994). Non-replicative recombinant adenoviral vectors are particularly useful in transfecting coronary endothelium and cardiac myocytes resulting in highly efficient transfection after intracoronary injection.

As demonstrated herein, the helper-independent replication-defective human adenovirus 5 system can be used effectively transfect a large percentage of myocardial cells *in vivo* by a single intracoronary injection. We have also shown that such a delivery technique can be used to effectively target vectors to the myocardium of a large mammal heart. Additional means of targeting vectors to particular cells or tissue types are described below and in the art.

In various illustrations described below, the recombinant adenovirus vectors used are based on the human adenovirus 5 (as described by McGrory WJ et al., Virology 163:614-617, 1988) which are missing essential early genes from the adenovirus genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products *in trans*. In place of the missing adenovirus genomic sequences, a transgene of interest can be cloned and expressed in tissue/cells infected with the replication-defective adenovirus. Although adenovirus-based gene transfer does not generally result in stable integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), adenovirus vectors can be propagated in high titer and transfect non-replicating cells; and, although the transgene is not passed to daughter cells, this is suitable for gene transfer to adult cardiac myocytes, which do not actively divide. Retrovirus vectors provide stable gene transfer, and high titers are now obtainable via retrovirus pseudotyping (Burns, et al., Proc Natl. Acad. Sci. (USA) 90: 8033-8037, 1993), but current retrovirus vectors are generally unable to efficiently transduce nonreplicating cells (e.g., cardiac myocytes) efficiently. In addition, the potential hazards of transgene incorporation into host DNA are not warranted if short-term gene transfer is sufficient.

Indeed, we have demonstrated that a limited duration in the expression of an angiogenic protein is sufficient for substantial angiogenesis (see Example 5). Thus, transient gene transfer is therapeutically adequate for treating such cardiovascular conditions. Within 14 days after gene

transfer of FGF-5 into the myocardium, blood flow to the ischemic bed had increased two-fold and the effect persisted for at least 12 weeks (Example 5 and Figure 8). The increased blood flow correlated with an increase in the number of capillaries in the heart (see Example 5). Wall thickening also increased within two weeks after gene transfer and persisted for at least 12 weeks. Thus, the angiogenic factor gene does not have to be present in the infected cell for more than a few weeks to produce a therapeutic effect. Once the blood vessels have developed, continued expression of the exogenous angiogenic protein may not be required to maintain the new vascular structure and increased blood flow.

An advantage associated with nondividing cells such as myocytes is that the viral vector is not readily "diluted out" by host cell division. However, if it is necessary or desirable to further enhance duration of transgene expression in the heart, it is also possible to employ various second generation adenovirus vectors that have both E1 and E4 deletions, which can be used in conjunction with cyclophosphamide administration (See, e.g., Dai et al., Proc. Nat'l Acad. Sci. (USA) 92: 1401-1405, 1995). To further increase the extent of initial gene transfer, additional infusions, or infusion in an isolated coronary circuit can also be employed.

Human 293 cells (Accession No. ATCC CRL1573; Rockville, MD), which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines for the production of such replication-defective vectors. However, other cell lines which allow replication-defective adenovirus vectors to propagate therein can also be used, such as HeLa cells.

Construction of Recombinant Adenovirus Vectors

Adenoviral vectors used in the present invention can be constructed by the rescue recombination technique described in McGrory W.J. et al., *Virology*, 163:614-617, 1988. Briefly, the transgene of interest is cloned into a shuttle vector that contains a promoter, polylinker and an adenoviral sequence containing a deletion in the E1A/E1B genes that are essential for viral replication. The transgene can be cloned into a convenient restriction site within the polylinker. Suitable shuttle vectors include plasmid pAC1 (McGrory et al., *Virology*, 163:614-617, 1988) (or an analog) which encodes portions of the left end of the human adenovirus 5 genome *(Virology*, 163:614-617, 1988) minus the early protein-encoding EIA and EIB sequences, and plasmid ACCMVpLpA (Gomez-Foix et al. *J. Biol. Chem.*, 267:25129-25134, 1992). pACCMVpLpA contains the 5' end of the adenovirus serotype 5 genome (map units 0 to 17) where the E1 region has been substituted with the human cytomegalovirus enhancer-promoter (CMV promoter) followed by the multiple cloning site (polylinker) from pUC 19 (plasmid well known in the art),

followed by the SV40 polyadenylation signal. The use of plasmid pAC1 or ACCMVPLA facilitates the cloning process.

The shuttle vector can then be co-transfected with a plasmid which contains the entire human adenoviral 5 genome with a length too large to be encapsidated, into 293 cells. Co-transfection can be conducted by any means commonly used in the art, such as by calcium phosphate precipitation or lipofection (see e.g., Biotechniques, 15:868-872, 1993).

As an illustrative plasmid for co-transfection, plasmid "JM17"encodes the entire human adenovirus 5 genome plus portions of the vector pBR322 including the gene for ampicillin resistance (4.3 kb). Although JM17 encodes all of the adenoviral proteins necessary to make mature viral particles, it is too large to be encapsidated into mature virions (40 kb versus 36 kb for wild type).

In a small subset of co-transfected cells, "rescue recombination" occurs between the transgene-containing shuttle vector (such as plasmid pAC1) and the plasmid having the entire adenoviral 5 genome (such as plasmid pJM17) which generates a recombinant genome that contains the transgene of interest in place of the deleted E1A/E1B sequences, and that secondarily loses the additional sequence (such as the pBR322 sequences) during recombination, thereby being small enough to be encapsidated (see Figures 4 and 5; Giordano et al., *Nature Medicine* 2(5):534–538, 1996; Giordano et al., *Circulation,* 88:I-139, 1993; and Giordano and Hammond, *Clin. Res.,* 42:123A, 1994). The CMV promoter driven β-galactosidase gene in adenovirus HCMVSP11acZ *(Clin. Res.,* 42:123A, 1994), with the use of X-gal treatment can be used to evaluate efficiency of gene transfer.

Illustrative examples demonstrating the preparation and use of such vectors are provided below. The advantages of these vectors include the ability to effect high efficiency gene transfer (more than 60% of target organ cells transfected *in vivo),* the ease of obtaining high titer viral stocks and the ability of these vectors to effect gene transfer into cells such as cardiac myocytes which do not divide.

Targeted Angiogenic Protein Vector Constructs

Limiting expression of the angiogenic transgene to the heart, or to particular cell types within the heart (e.g. cardiac myocytes) can provide certain advantages, as discussed below.

The present invention contemplates the use of cell targeting not only by delivery of the transgene into the coronary artery, for example, but also by use of targeted vector constructs having features that tend to target gene delivery and/or gene expression to particular host cells or host cell types (e.g. cardiac myocytes). Such targeted vector constructs would thus include

targeted delivery vectors and/or targeted vectors, as described in more detail below and in the published art. Restricting delivery and/or expression can be beneficial as a means of further focusing the potential effects of gene therapy. The potential usefulness of further restricting delivery/expression depends in large part on the type of vector being used and the method and place of introduction of such vector. As described herein, delivery of viral vectors via intracoronary injection to the myocardium has been observed to provide, in itself, highly targeted gene delivery (see the Examples below). In addition, using vectors that do not result in transgene integration into a replicon of the host cell (such as adenovirus and numerous other vectors), cardiac myocytes are expected to exhibit relatively long transgene expression since the cells do not generally replicate. In contrast, expression in rapidly dividing cells such as endothelial cells would tend to be decreased by cell division and turnover. However, other means of limiting delivery and/or expression can also be employed, in addition to or in place of the illustrated delivery method, as described herein.

Targeted delivery vectors include, for example, vectors (such as viruses, non-viral protein-based vectors and lipid-based vectors) having surface components (such as a member of a ligand-receptor pair, the other half of which is found on a host cell to be targeted) or other features that mediate preferential binding and/or gene delivery to particular host cells or host cell types. As is known in the art, a number of vectors of both viral and non-viral origin have inherent properties facilitating such preferential binding and/or have been modified to effect preferential targeting (see, e.g., Douglas et al., Nature Biotechnology 14:1574-1578, 1996; Kasahara, N. et al. Science 266:1373-1376, 1994; Miller, N., et al., FASEB Journal 9: 190-199, 1995; Chonn, A., et al., Curr. Opin. in Biotech. 6: 698-708, 1995; Schofield, JP, et al., British Med. Bull. 51: 56-71, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68: 145-159, 1994; Ledley, F.D., Human Gene Therapy 6: 1129-1144, 1995; Conary, J.T., et al., WO 95/34647 (21 December 1995); Overell, R.W., et al., WO 95/28494 (26 October 1995); and Truong, V.L. et al., WO 96/00295 (4 January 1996)).

Targeted vectors include vectors (such as viruses, non-viral protein-based vectors and lipid-based vectors) in which delivery results in transgene expression that is relatively limited to particular host cells or host cell types. By way of illustration, angiogenic transgenes to be delivered according to the present invention can be operably linked to heterologous tissue-specific promoters thereby restricting expression to cells in that particular tissue.

For example, tissue-specific transcriptional control sequences derived from a gene encoding left ventricular myosin light chain-2 (MLC2V) or myosin heavy chain (MHC) can be fused to a transgene such as an FGF gene within a vector such as the adenovirus constructs described above. Expression of the transgene can therefore be relatively restricted to ventricular

cardiac myocytes. The efficacy of gene expression and degree of specificity provided by MLC_{2V} and MHC promoters with lacZ have been determined (using a recombinant adenovirus system such as that exemplified herein); and cardiac-specific expression has been reported (see, e.g., Lee et al., J. Biol Chem 267:15875-15885,1992).

Since the MLC2V promoter comprises only about 250 bp, it will fit easily within even size-restricted delivery vectors such as the adenovirus-5 packaging system exemplified herein. The myosin heavy chain promoter, known to be a vigorous promoter of transcription, provides another alternative cardiac-specific promoter and comprises less than 300 bp. Other promoters, such as the troponin-C promoter, while highly efficacious and sufficiently small, do not provide such tissue specificity.

In the present methods for the treatment of heart disease, targeting gene transfer to the heart by intracoronary injection with a high titer of the vector is presently preferred.

Propagation and Purification of Adenovirus Vectors

Recombinant viral vector, such as adenoviral vectors, can be plaque purified according to standard methods. By way of illustration, the resulting recombinant adenoviral viral vectors can be propagated in human 293 cells (which provide E1A and E1B functions in trans) to titers in the preferred range of about 10^{10}-10^{12} viral particles/ml. Propagation and purification techniques have been described for a variety of viral vectors that can be used in conjunction with the present invention. Adenoviral vectors are exemplified herein but other viral vectors such as AAV can also be employed. For adenovirus, cells can be infected at 80% confluence and harvested 48 hours later. After 3 freeze-thaw cycles of the infected cells, the cellular debris is pelleted by centrifugation and the virus purified by CsCl gradient ultracentrifugation (double CsCl gradient ultracentrifugation is preferred). Prior to *in vivo* injection, the viral stocks can be desalted (e.g., by gel filtration through Sepharose columns such as Sephadex G25). The desalted viral stock can also be filtered through a 0.3 micron filter if desired. We typically concentrate and purify the viral stock by double CsCl ultracentrifugation, followed by chromatography on Sephadex G25 equilabrated with phosphate buffered saline (PBS). The resulting viral stock typically has a final viral titer that is at least about 10^{10}-10^{12} viral particles/ml.

Preferably, the recombinant adenovirus is highly purified and is substantially free of wild-type (potentially replicative) virus. For these reasons, propagation and purification can be conducted to exclude contaminants and wild-type virus by, for example, identifying successful recombinant virus with PCR using appropriate primers, conducting two rounds of plaque purification, and double CsCl gradient ultracentrifugation.

Delivery of Vectors Carrying an Angiogenic Transgene

The means and compositions which are used to deliver the vectors carrying angiogenic protein transgenes depend on the particular vector employed as is well known in the art. Typically, however, a vector can be in the form of an injectable preparation containing a pharmaceutically acceptable carrier/diluent such as phosphate buffered saline, for example. Other pharmaceutical carriers, formulations and dosages are described below.

The presently preferred means of *in vivo* delivery (especially for vector constructs that are not otherwise targeted for delivery and/or expression that is restricted to the myocardium), is by injection of the vector into a blood vessel directly supplying the myocardium, preferably by injection into one or both coronary arteries. Such injection is preferably achieved by catheter introduced substantially (typically at least about 1 cm) within the lumen of one or both coronary arteries or one or more saphenous veins or internal mammary artery grafts or other conduits delivering blood to the myocardium. Preferably the injection is made in both coronary arteries to provide general distribution to all areas of the heart.

The vectors are delivered in an amount sufficient for the transgene to be expressed and to provide a therapeutic benefit. For viral vectors (such as adenovirus), the final titer of the virus in the injectable preparation is preferably in the range of about 10^6-10^{14} viral particles which allows for effective gene transfer. An adenovirus vector stock preferably free of wild-type virus can be injected deeply into the lumen of one or both coronary arteries (or grafts), preferably into both the right and left coronary arteries (or grafts), and preferably in an amount of 10^8 - 10^{12} viral particles as determined by optical densitometry. Preferably the vector is delivered in a single injection into each conduit (e.g. into each coronary artery).

By injecting the vector composition directly into the lumen of the coronary artery by coronary catheters, it is possible to target the gene rather effectively, and to minimize loss of the recombinant vectors to the proximal aorta during injection. This type of injection enables local transfection of a desired number of cells, especially cardiac myocytes, in the affected myocardium with angiogenic protein- or peptide-encoding genes, thereby maximizing therapeutic efficacy of gene transfer, and minimizing undesirable angiogenesis at extracardiac sites.

Vector constructs that are specifically targeted to the myocardium, such as vectors incorporating myocardial-specific binding or uptake components, and/or which incorporate angiogenic protein transgenes that are under the control of myocardial-specific transcriptional regulatory sequences (e.g., ventricular myocyte-specific promoters) can be used in place of or, preferably, in conjunction with such directed injection techniques as a means of further restricting

expression to the myocardium, especially the ventricular myocytes. For vectors that can elicit an immune response, it is preferable to inject the vector directly into a blood vessel supplying the myocardium as described above, although the additional techniques for restricting the potential for extracardiac expression can also be employed.

As described in detail below, it was demonstrated that using such techniques for *in vivo* delivery of a viral vector containing the angiogenic transgene, transgene expression did not occur in hepatocytes and viral RNA could not be found in the urine at any time after intracoronary injection. In addition, no evidence of extracardiac gene expression in the eye, liver, or skeletal muscle could be detected by PCR two weeks after intracoronary delivery of transgenes in this manner.

A variety of catheters and delivery routes can be used to achieve intracoronary delivery, as is known in the art. Direct intracoronary (or graft vessel) injection can be performed using standard percutaneous catheter based methods under fluoroscopic guidance. Any variety of coronary catheter, or a Stack perfusion catheter, for example, can be used in the present invention. For example, a variety of general purpose catheters, as well as modified catheters, suitable for use in the present invention are available from commercial suppliers such as Advanced Cardiovascular Systems (ACS), Target Therapeutics, Boston Scientific and Cordis. Also, where delivery to the myocardium is achieved by injection directly into a coronary artery (which is presently most preferred), a number of approaches can be used to introduce a catheter into the coronary artery, as is known in the art. By way of illustration, a catheter can be conveniently introduced into a femoral artery and threaded retrograde through the iliac artery and abdominal aorta and into a coronary artery. Alternatively, a catheter can be first introduced into a brachial or carotid artery and threaded retrograde to a coronary artery. Detailed descriptions of these and other techniques can be found in the art (see, e.g., the references cited above, including: Topol, EJ (ed.), The Textbook of Interventional Cardiology, 2nd Ed. (W.B. Saunders Co. 1994); Rutherford, RB, Vascular Surgery, 3rd Ed. (W.B. Saunders Co. 1989); Wyngaarden JB et al. (eds.), The Cecil Textbook of Medicine, 19th Ed. (W.B. Saunders, 1992); and Sabiston, D, The Textbook of Surgery, 14th Ed. (W.B. Saunders Co. 1991)).

Targeted Gene Expression

An unexpected finding of the present invention is that the recombinant adenovirus is taken up very efficiently in the first vascular bed that it encounters. Indeed, in the animal model of Example 4, the efficiency of the uptake of the virus in the heart after intracoronary injection, was 98%, i.e., 98% of the virus was removed in the first pass of the virus through the myocardial

vascular bed. Furthermore, serum taken from the animals during the injection was incapable of growing viral plaques (Graham, *Virology,* 163:614-617, 1988) until diluted 200-fold, suggesting the presence of a serum factor (or binding protein) that inhibits viral propagation. These two factors (efficient first pass attachment of virus and the possibility of a serum binding protein) may act together to limit gene expression to the first vascular bed encountered by the virus.

To further evaluate the extent to which gene transfer was limited to the heart following intracoronary gene transfer, polymerase chain reaction (PCR) was used to see whether there was evidence for extracardiac presence of viral DNA two weeks after gene transfer in two treated animals (Example 4 below). Animals showed the presence of viral DNA in their hearts but not in their retinas, skeletal muscles, or livers. The sensitivity of the PCR is such that a single DNA sequence per 5,000,000 cells would be detectable. Therefore these data demonstrated that no viral DNA was present in extracardiac tissues two weeks after gene delivery. These results were further confirmed using other angiogenic proteins and derivatives as described below. These findings are extremely important because they confirm the concept of cardiac transgene targeting (i.e. providing expression of the transgene in the heart, but not elsewhere). The localized transgene delivery and expression provide the advantage of safety, further enhancing the use of the present methods in the treatment of patients.

Therapeutic Applications

The present method of *in vivo* transfer of a transgene encoding an angiogenic protein can be applied to the treatment of congestive heart failure (including that associated with dilated cardiomyopathy (DCM) as well as that associated with severe coronary artery disease (CAD)), and to prevent or alleviate ventricular remodeling. As the data below shows, expression of an exogenously-provided angiogenic transgene in the myocardium promotes angiogenesis resulting in increased blood flow and function in the ischemic and non-ischemic regions of the heart. This increased vascularity will alleviate congestive heart failure and will offset the ventricular dysfunction associated with ventricular remodeling.

As described herein, a number of different vectors can be employed to deliver the angiogenic protein transgenes *in vivo* according to the methods of the present invention. By way of illustration, the replication-deficient recombinant adenovirus vectors exemplified herein achieved highly efficient gene transfer *in vivo* without cytopathic effect or inflammation in the areas of gene expression.

In treating congestive heart failure, gene transfer of an angiogenic protein encoding transgene can be conducted, for example, after diagnosis of heart failure or the likely development

of heart failure. For treating ventricular remodeling, gene transfer can be performed any time after the patient has suffered an infarct, preferably within 30 days and even more preferably within 7-20 days after an infarct.

Compositions or products of the invention may conveniently be provided in the form of formulations suitable for administration into the blood stream (e.g. by intracoronary injection). A suitable administration format may best be determined by a medical practitioner for each patient individually. Suitable pharmaceutically acceptable carriers and their formulation are described in standard formulations treatises, *e.g., Remington's Pharmaceuticals Sciences* by E.W. Martin. *See also* Wang, Y.J. and Hanson, M.A., "Parental Formulations of Proteins and Peptides: Stability and Stabilizers", *Journals of Parental Sciences and Technology,* Technical Report No. 10, Supp. 42:2S (1988). Vectors of the present invention should preferably be formulated in solution at neutral pH, for example, about pH 6.5 to about pH 8.5, more preferably from about pH 7 to 8, with an excipient to bring the solution to about isotonicity, for example, 4.5% mannitol or 0.9% sodium chloride, pH buffered with art-known buffer solutions, such as sodium phosphate, that are generally regarded as safe, together with an accepted preservative such as metacresol 0.1% to 0.75%, more preferably from 0.15% to 0.4% metacresol. The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions. If desired, solutions of the above compositions also can be prepared to enhance shelf life and stability. The therapeutically useful compositions of the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be mixed to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water and/or a buffer to control pH or an additional solute to control tonicity.

For use by the physician, the compositions will be provided in dosage form containing an amount of a vector of the invention which will be effective in one or multiple doses to induce angiogenesis sufficient to provide a therapeutic effect. As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age and weight of the patient, the patient's physical condition, and the level of angiogenesis to be obtained, and other factors.

The effective dose of the compounds of this invention will typically be in the range of about 10^6 - 10^{14} viral particles, preferably about 10^8 - 10^{12} viral particles. The number of viral particles may, but preferably does not exceed 10^{14}. As noted, the exact dose to be administered is

determined by the attending clinician, but is preferably in 5 ml or less of physiologically buffered solution (such as phosphate buffered saline), more preferably in 1-3 ml.

The presently preferred mode of administration in the case of heart disease is by intracoronary injection to one or both coronary arteries (or to one or more saphenous vein or internal mammary artery grafts) using an appropriate coronary catheter.

The following Examples are provided to further assist those of ordinary skill in the art. Such examples are intended to be illustrative and therefore should not be regarded as limiting the invention. A number of exemplary modifications and variations are described in this application and others will become apparent to those of skill in this art. Such variations are considered to fall within the scope of the invention as described and claimed herein.

EXAMPLES

The preferred methods of the present invention involve the treatment of patients with heart failure and/or ventricular remodeling.

Example 1 describes a model of heart failure induced by prolonged (over a 3- to 4- week period) rapid ventricular pacing. The Example demonstrates that in an animal model of heart failure, left ventricular myocardial blood flow is abnormally low when the left ventricle has dilated and signs of heart failure are present. In this model, myocardial demand often supersedes myocardial blood flow (oxygen supply), and leads to myocardial ischemia.

Example 2 demonstrates the construction of the adenovirus vectors used in the present invention.

Example 3 describes preliminary *in vitro* experiments to establish the efficacy of an adenovirus vector of the present invention. A control (β-galactosidase-encoding) adenoviral vector (AdlacZ) was also tested. The results indicated that the present recombinant adenovirus vectors can indeed infect adult cardiomyocytes and provide expression of the transgene with high efficiency. *In vivo* studies then followed.

Example 4 demonstrates *in vivo* infection of porcine myocardium with the adenoviral vector encoding β-galactosidase (AdLacZ) and expression of β-gal in the myocardium.

Example 5 demonstrates that virus-mediated delivery of an FGF-5 angiogenic transgene to the myocardium *in vivo* can improve regional blood flow and ameliorate regional myocardial contractile dysfunction.

Example 6 demonstrates that virus-mediated delivery of an FGF-4 angiogenic transgene to the myocardium *in vivo* can improve regional blood flow and ameliorate regional myocardial contractile dysfunction.

Example 7 demonstrates that virus-mediated delivery of an FGF-2 angiogenic transgene to the myocardium *in vivo* can improve regional blood flow and ameliorate regional myocardial contractile dysfunction.

EXAMPLE 1: PORCINE MODEL OF CONGESTIVE HEART FAILURE AND ASSOCIATED MYOCARDIAL ISCHEMIA

1-A. Animals and Surgical Procedure

Nine Yorkshire pigs (*Sus scrofa*) weighing 40±6 kg were anesthetized with ketamine (50 mg/kg IM) and atropine sulfate (0.1 mg/kg IM) followed by sodium amytal (100 mg/kg IV). After endotracheal intubation, halothane (0.5% to 1.5%) was delivered by a pressure-cycled ventilator throughout the procedure. At left thoracotomy, catheters were placed in the aorta, pulmonary artery, and left atrium. A Konigsberg micromanometer was placed into the left ventricular apex, and an epicardial unipolar lead was placed 1.0 cm below the atrioventricular groove in the lateral wall of the left ventricle. The power generator (Spectrax 5985; Medtronic, Inc.) was inserted into a subcutaneous pocket in the abdomen. Four animals were instrumented with a flow probe (Transonic, Inc.) around the main pulmonary artery. The pericardium was loosely approximated and the chest closed. Seven to 10 days after thoracotomy, baseline measures of hemodynamics, left ventricular function, and myocardial blood flow were made. Ventricular pacing then was initiated (220±9 bpm (beats per minute) for 26±4 days). The stimulus amplitude was 2.5 V, the pulse duration 0.5 ms. Nine additional pigs (40±7 kg) were used as controls; five underwent thoracotomy and instrumentation without pacing and were killed 30±7 days after initial thoracotomy. Data regarding right and left ventricular mass were similar in the control animals whether they had undergone thoracotomy or not, so their data were pooled into a single control group.

1-B. Hemodynamic Studies

Hemodynamic data were obtained from conscious, unsedated animals after the pacemaker had been inactivated for at least 1 hour and animals were in a basal state. All data were obtained in each animal at 7-day intervals. Pressures were obtained from the left atrium, pulmonary artery, and aorta. Left ventricular dP/dt was obtained from the high-fidelity left ventricular pressure.

Pulmonary artery flow was recorded. Aortic and pulmonary blood samples were obtained for calculation of arteriovenous oxygen content difference.

1-C. Echocardiographic Studies

Echocardiography is a method of measuring regional myocardial blood flow which involves injection of a contrast material into the individual or animal. Contrast material (microaggregates of galactose) increase the echogenicity ("whiteness") of the image after left atrial injection. The microaggregates distribute into the coronary arteries and myocardial walls in a manner that is proportional to blood flow (Skyba, et al., *Circulation,* 90:1513-1521, 1994). The peak intensity of contrast enhancement is correlated with myocardial blood flow as measured by microspheres (Skyba, et al., *Circulation,* 90:1513-1521, 1994).

Two-dimensional and M-mode images were obtained with a Hewlett Packard Sonos 1500 imaging system. Images were obtained from a right parasternal approach at the mid-papillary muscle level and recorded on VHS tape. Measurements were made according to criteria of the American Society of Echocardiography (Sahn, DJ, et al., *Circulation.* 58:1072-1083 (1978)). Because of the midline orientation of the porcine interventricular septum (IVS) and use of the right parasternal view, short-axis M-mode measures were made through the IVS and the anatomic lateral wall. All parameters, including end-diastolic dimension (EDD), end-systolic dimension (ESD), and wall thickness, were measured on at least five random end-expiratory beats and averaged. End-diastolic dimension was obtained at the onset of the QRS complex. End-systolic dimension was taken at the instant of maximum lateral position of the IVS or at the end of the T wave. Left ventricular systolic function was assessed by use of fractional shortening, FS=[(EDD-ESD)/EDD]x100. Percent wall thickening (%WTh) was calculated as %WTh=[(ESWTh-EDWTh)/EDWTh]x100. To demonstrate reproducibility of echocardiographic measurements, animals were imaged on 2 consecutive days before the pacing protocol was initiated. The data from the separate determinations were highly reproducible (fractional shortening, R^2=.94, P=.006; lateral wall thickening, R^2=.90, P=.005). All of these measurements were obtained with pacemakers inactivated.

1-D. Myocardial Blood Flow

Myocardial blood flow was determined by the radioactive microsphere technique as described in detail in previously (Roth, DM, et al., *Am. J. Physiol.* 253:H1279-H1288, 1987; Roth, DM, et al., *Circulation* 82:1778-1789, 1990). Transmural samples from the left ventricular lateral wall and IVS were divided into endocardial, midwall, and epicardial thirds, and blood flow to each

third and transmural flow were determined. Transmural sections were taken from regions in which echocardiographic measures had been made so that blood flow and functional measurements corresponded within each bed. Microspheres were injected in the control state (unpaced), at the initiation of ventricular pacing (225 bpm), and then at 7-day intervals during ventricular pacing at 225 bpm; microspheres were also injected with the pacemakers inactivated at 14 days (n=4) and 21 to 28 days (n=3). Myocardial blood flow per beat was calculated by dividing myocardial blood flow by the heart rate (recorded during microsphere injection) (Indolfi, C., et al., *Circulation* 80:933-993 (1989)). Mean left atrial and mean arterial pressures were recorded during microsphere injection so that an estimate of coronary vascular resistance could be calculated; coronary vascular resistance index equals mean arterial pressure minus mean left atrial pressure divided by transmural coronary blood flow.

1-E. Systolic Wall Stress

Circumferential systolic wall stress could not be determined because we could not obtain a suitable view to estimate the long axis of the left ventricle. Therefore, we calculated meridional end-systolic wall stress (Riechek, N., et al., *Circulation* 65:99-108 (1982)) using the equation meridional end-systolic wall stress (dynes) = (0.334xPxD)÷[h(I-h/D)], where P is left ventricular end-systolic pressure in dynes, D is left ventricular end-systolic diameter in cm, and h is end-systolic wall thickness. Meridional end-systolic wall stress was calculated for both lateral wall and IVS before the initiation of pacing and subsequently at weekly intervals (pacemaker off).

1-F. Terminal Surgery

After 26±2 days of continuous pacing, animals were anesthetized and intubated, and midline sternotomies were made. The still-beating hearts were submerged in saline (4°C), the coronary arteries were rapidly perfused with saline (4°C), the right ventricle and left ventricle (including IVS) were weighed, and transmural samples from each region were rapidly frozen in liquid nitrogen and stored at a temperature of -70°C.

1-G. Adenine Nucleotides

ATP and ADP were measured in transmural samples of the IVS and lateral wall in four animals with heart failure (paced 28 days) and four control animals. The samples from the animals with heart failure were obtained with the pacemakers off (60 minutes) on the day the animals were

killed. Samples were obtained identically in all animals. ATP and ADP were measured in a Waters high-performance liquid chromatograph as previously described (Pilz, R.B., et al., *J. Biol. Chem.* 259:2927-2935 (1984)).

1-H. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Specific measurements obtained in the control (prepaced) state and at 1-week intervals during pacing were compared by repeated measures ANOVA (Crunch4, Crunch Software Corp.). In some comparisons (lateral wall versus IVS, for example), two-way ANOVA was used. Post hoc comparisons were performed with the "Tukey method" as described in the art. Nine animals survived 21 days of pacing; six of these survived 28 days of pacing. Data from animals surviving 28 days were statistically indistinguishable from those who survived only 21 days. ANOVA was conducted, therefore, on nine animals at four time points: control (prepacing), 7 days, 14 days, and 21 to 28 days. The null hypothesis was rejected when $P<.05$ (two-tailed).

Results

1-I. Hemodynamic Studies

Rapid ventricular pacing resulted in changes in hemodynamics that were significant after 7 to 14 days of pacing. At 7 days, animals had increased mean left atrial and pulmonary arterial pressures. These pressures became increasingly abnormal with additional weeks of pacing (Table 1). Signs of circulatory congestion (tachypnea, ascites, and tachycardia) were evident by 14 to 21 days. Pulmonary arterial flow (cardiac output) had decreased by 21 days of pacing (control, 3.3±0.1 L/min; 21 days, 1.9±0.4 L/min; $P<.05$).

Table 1. HEMODYNAMICS AND LEFT VENTRICULAR FUNCTION

	n	Control	7d	14d	21-28d	p
HR (bpm)	9	122±16	136±15	149±13[b]	157±15[c,g]	.0004
MAP (mm Hg)	9	103±8	99±6	98±7	102±14	.52
PA (mm Hg)	7	24±7	37±4[b]	42±9[c]	48±8[c,g,i]	.0001
LA (mm Hg)	8	13±3	25±5[c]	30±7[c]	36±6[c,e]	.0001
AVO_2D(ml/dl)	7	3.6±1.1	3.5±0.9	5.2±1.5	6.2±1.5[b,h]	.0005
EDD (cm)	9	3.9±0.4	4.4±0.5	4.9±0.6[c]	5.8±0.6[c,f,i]	.0001
FS (%)	9	39±3	26±5[c]	18±6[c,e]	13±4[c,i]	.0001
LV dP/dt (mm Hg/s)	4	2849±278	2408±460	1847±381[c,d]	1072±123[c,e,i]	.0001

Analysis of variance (repeated measures) was used to determine whether duration of pacing affected a specific variable; p-values from ANOVA are listed in the rightward column. *Post hoc* testing was performed by the Tukey method: [a]p<0.05; [b]p<0.01; [c]p<0.001 (versus control value for the same variable); [d]p<0.05; [e]p<0.01; [f]p,0.001 (vs. previous week); [g]p<0.05; [h]p<0.01; [i]p<0.001 (vs. 7d value); *post-hoc* testing by Tukey method. Measurements were made with pacemakers inactivated and represent mean ±SD. 7d: 7 days of pacing; 14d: 14 days of pacing; 21-28d: 21-28 days of pacing.

1-J. Global Left Ventricular Function

Left ventricular function was assessed by echocardiography and hemodynamic variables after pacemakers had been inactivated. Fractional shortening was progressively reduced with duration of pacing (*P*=.0001; Table 1), reaching its lowest value at 21 to 28 days (control, 39±3%; 21 to 28 days 13±4%; *P*<.0002). Left ventricular end-diastolic dimension progressively increased during pacing (*P*<.0001; Table 1), reaching its maximal value at 21 to 28 days (control, 3.9±0.4 cm; 21 to 28 days, 5.8±0.6 cm; *P*=.0002). Left ventricular peak positive dP/dt also decreased throughout the study (*P*=.0001; Table 1). The progressive fall in peak dP/dt was accompanied by increasing left ventricular end-diastolic pressure, documenting decreased left ventricular

contractility, since increased preload normally augments left ventricular peak dP/dt. (Mahler, F., et al., *Am. J. Cardiol.* 35:626-634 (1975))

1-K. Left Ventricular Regional Function

With the pacemaker inactivated, regional left ventricular function was assessed by measurement of percent wall thickening of the left ventricular lateral wall and IVS. Ventricular pacing from the lateral wall caused significant deterioration in function of the lateral wall compared with the IVS (P=.001; Fig 1 and Table 2). This difference was significant at 7 days and increased further at 21 to 28 days as lateral wall function deteriorated. The IVS showed an insignificant decrease in wall thickening over the course of the study. End-diastolic wall thickness showed progressive thinning during the study that was more severe in the lateral wall (Table 2).

Table 2. SEQUENTIAL LEFT VENTRICULAR WALL THICKENING

	CONTROL	7d	14d	21-28d	p(ANOVA)
IVS EDTh (cm)	.8±.1	.7±.1	.7±.1	.6±.1	time: .0001
LAT EDTh (cm)	.8±.1	.7±.1	.6±.1	.5±.1	region: .039
p (IVS vs. LAT)	ns	ns	ns	ns	inter: .027
IVS WTh (%)	33±4	33±5	28±3	28±6	time: .0001
LAT WTh (%)	35±5	25±4	19±8	14±6	region: .001
p (IVS vs. LAT)	ns	.02	.007	.0001	inter: .0001

Two-way analysis of variance (repeated measures) was used to determine whether end-diastolic wall thickness (EDTh) or % wall thickening (WTh) was affected by duration of pacing (time), or region (lateral wall, LAT; or interventricular septum, IVS), or whether the change in EDTh or WTh% was different between the two regions (inter). Mean values for EDTh and WTh% at each time point were tested for differences between the two regions *post-hoc* by Tukey analyses. Values represent mean ±SD. 7d: Seven days of pacing; 14d: 14 days of pacing; 21-28d: 21-28 days of pacing. n=9.

1-L. Left Ventricular Regional Blood Flow

Subendocardial blood flow per minute increased more in the IVS than in the lateral wall when pacing was initiated (Fig 2 and Table 3). This difference in regional blood flow during pacing persisted for the duration of the study, and the pattern of change in blood flow was different between the two regions (P=.006). The pattern of change in blood flow per minute between the two regions during pacing was consistent whether measured in endocardial (P=.006), midwall (P=.002), epicardial (P=.016), or transmural (P=.003) sections (Table 3). In contrast, when the pacemaker was inactivated, subendocardial blood flow showed no regional differences whether measured in the control state, at 14 days, or at 21 to 28 days (Fig 2 and Table 3).

Table 3. SEQUENTIAL MYOCARDIAL BLOOD FLOW

	DAY 0		DAY 14		DAY 21-28		
	OFF	ON	OFF	ON	OFF	ON	p(ANOVA)
IVS ENDO (ml/min/g)	1.41±.26	1.96±.38	1.68±.22	2.35±.46	1.88±.18	2.67±.39	time: .0001
LAT ENDO (ml/min/g)	1.40±.33	1.11±.14	1.50±.35	1.65±.25	1.73±.05	2.05±.16	region:.017
p (IVS vs. LAT)	ns	.001	ns	.002	ns	.006	inter: .006
IVS MID (ml/min/g)	1.56±.20	2.11±.33	1.84±.29	2.48±.31	2.04±.09	2.98±.46	time: .0001
LAT MID (ml/min/g)	1.66±.28	1.53±.17	1.50±.43	1.77±.29	1.76±.39	2.12±.06	region:.019
p (IVS vs. LAT)	ns	.01	ns	ns	ns	.001	inter: .002
IVS EPI (ml/min/g)	1.13±.27	1.50±.24	1.54±.38	1.91±.48	1.79±.14	2.53±.38	time: .0001
LAT EPI (ml/min/g)	1.37±.22	1.48±.31	1.24±.24	1.55±.25	1.50±.04	1.92±.08	region:.17
p (IVS vs. LAT)	ns	ns	ns	ns	.049	ns	inter: .0016
IVS TRANS (ml/min/g)	1.36±.21	1.85±.27	1.69±.30	2.24±.46	1.90±.09	2.73±.40	time: .0001
LAT TRANS (ml/min/g)	1.47±.27	1.38±.22	1.41±.33	1.65±.25	1.66±.02	2.03±.07	region:.019
p (IVS vs. LAT)	ns	.001	ns	ns	ns	.001	inter: .003
IVS ENDO/ EPI	1.30±.3	1.32±.23	1.13±.20	1.27±.26	1.05±.20	1.06±.10	time: .058
LAT ENDO/ EPI	1.01±.07	0.77±.10	1.21±.06	1.07±.11	1.15±.02	1.07±.10	region:.054
p (IVS vs. LAT)	ns	.0002	ns	ns	ns	ns	inter: .0008

Two-way analysis of variance (repeated measures) was used to determine whether subendocardial (ENDO) or transmural (TRANS) blood flow was affected by duration of pacing (time), or region (lateral wall, LAT; or interventricular septum, IVS), or whether the pattern of change in blood flow was different between the two regions (inter). Mean values for blood flows at each time point were tested for differences between the two regions *post-hoc* by Tukey analyses. Values represent mean ±SD from 5 animals. ON: microspheres injected during ventricular pacing (225 bpm). OFF: Pacemaker inactivated. Day O=Control; Day 14: 14 days of pacing; Day 21-28: 21-28 days of pacing.

Endocardial-to-epicardial blood flow ratios did not change significantly as heart failure progressed (P=.058). However, with the initiation of pacing, the endocardial-to-epicardial ratio was substantially lower in the lateral wall than in the IVS (IVS, 1.32±0.23; lateral wall, 0.77±0.10; P=.0002; Table 3). Ratios in both regions were >1.0 throughout the rest of the study. Endocardial blood flow per beat (Fig 2 and Table 4) was similar in both regions before the initiation of pacing (IVS, 0.013±0.003 mL · min^{-1} · g^{-1} · $beat^{-1}$; lateral wall, 0.012±0.004mL · min^{-1} · g^{-1} · $beat^{-1}$; P=NS). On initiation of ventricular pacing (225 bpm), there was a regional deficit in endocardial blood flow per beat in the lateral wall but not in the IVS (IVS, 0.009±0.002 mL · min^{-1} · g^{-1} · $beat^{-1}$; lateral wall, 0.005±0.001 mL · min^{-1} · g^{-1} · $beat^{-1}$; P=.001). At 14 days and 21 to 28 days, endocardial flow per beat was less in the lateral wall than in the IVS during pacing (Fig 2 and Table 4). These data indicate that myocardial hypoperfusion in the lateral wall began with the onset of pacing, and this relative ischemia persisted. However, endocardial blood flows per beat remained normal in both regions with the pacemaker off (Fig. 2 and Table 4).

Blood flow in both regions tended to increase during the final week of pacing (Fig 2 and Table 3). This pattern was associated with a progressive fall in the coronary vascular resistance index (Fig 3), suggesting that alterations in coronary vascular structure and function may accompany left ventricular remodeling as heart failure progresses. The coronary vascular resistance index was significantly greater in the lateral wall than in the IVS at the initiation of pacing, and the pattern of change in coronary vascular resistance was different between the two regions (P=.0012) (Fig 3). These findings may indicate an effect of altered electrical activation on myocardial perfusion.

Table 4. ENDOCARDIAL BLOOD FLOW PER BEAT

MODEL		ENDOCARDIAL FLOW PER BEAT (ml/min/gram/beat)	
PORCINE AMAROID ISCHEMIA (HR 220 bpm; n=6)			
NONISCHEMIC BED		0.012±0.004	
ISCHEMIC BED		0.006±0.002	
		$p < 0.001$*	
PORCINE LV PACING-INDUCED CHF (HR 225 bpm; n=5)			
		PACER ON	**PACER OFF**
DAY 0	IVS	0.009±0.002	0.013±0.003 (HR 122 bpm)
	LATERAL WALL	0.005±0.001	0.012±0.004
		$p = 0.001$	ns
DAY 14	IVS	0.010±0.003	0.011±0.002 (HR 149 bpm)
	LATERAL WALL	0.007±0.001	0.010±0.003
		$p = 0.008$	ns
DAY 21-28	IVS	0.012±0.002	0.013±0.003 (HR 157 bpm)
	LATERAL WALL	0.009±0.001	0.012±0.002
		$p < 0.024$	ns

Data from ameroid ischemia model have been previously published from our laboratory (Hammond, H.K. and McKiman, M.D., J. Am. Coll. Cardiol., 23:475-82 (1994)). Values represent mean ± 1 SD. These data show that the collateral-dependent (ischemic region) of the ameroid model and the lateral wall of the left ventricular pacing-induced heart failure model have similar deficits in endocardial blood flow per beat compared with myocardial regions that are normally perfused.

1-M. Left Ventricular End-Systolic Wall Stress

There was a significant increase in estimated meridional end-systolic wall stress with respect to duration of pacing ($P<.0001$), but the pattern of change in wall stress was similar for the lateral wall and IVS ($P=33$), and post hoc testing failed to show any regional differences in systolic wall stress at any specific time point (Fig 3). The increase in end-systolic wall stress was roughly threefold in the lateral wall (control, $168\pm40x10^3$ dynes; 28 days, $412\pm143x10^3$ dynes; $P=.0001$) and in the IVS (control, $159\pm35xi0^3$ dynes; 28 days, $480\pm225x10^3$ dynes; $P=.0001$).

1-N. Necropsy

At necropsy, animals with heart failure had ascites (mean amount, 1809 mL; range, 300 to 3500 mL) and dilated, thin-walled hearts, with all four chambers appearing grossly enlarged. Ratios of ventricular weight to body weight suggested hypertrophy of the right ventricle only, confirming data from a previous study using this model. (Roth, D.A., et al., *J. Clin. Invest.* 91:939-949 (1993)) Compared with weight-matched control animals, there was no change in left ventricular mass associated with heart failure (control, 112±10 g; heart failure, 114±17 g); ratios of left ventricular weight to body weight were also similar in both groups (control, 2.8±0.3 g/kg; heart failure, 2.9±0.3 g/kg). In contrast, heart failure was associated with increased right ventricular weight (control, 38±3 g; heart failure, 52±11 g; $P=.003$) and ratios of right ventricular weight to body weight (control, 0.09±0.1 g/kg; heart failure, 1.3±0.3 g/kg; $P<.003$). Paced animals gained 4 kg during the course of the study, an amount accounted for in part by ascites accumulation. If the initial body weight is used to calculate the ratio of left ventricular weight to body weight, the ratio still is not significantly higher than that from weight-matched control animals. These data confirm that there was no substantive increase in left ventricular mass during the course of the study.

1-O. Adenine Nucleotides

Control animals showed normal ATP/ADP ratios, similar to those reported in pig heart collected by drill biopsies followed by immediate submersion in liquid nitrogen, (White, F.C., and Boss, G., *J. Cardiovasc. Pathol.* 3:225-236 (1990)) documenting that the sampling techniques used were suitable. Animals with heart failure showed a marked reduction in ATP/ADP ratio in samples taken from the IVS (control, 14.8±1.1; heart failure, 2.4±0.3; $P<.0001$, n=4 both groups) and from the lateral wall (control, 14.3±4.0; heart failure, 2.4±0.9; $P=.0012$, n=4 both groups). This confirms an imbalance between myocardial oxygen supply and demand.

1-P. Myocardial Blood Flow

Regional variations in myocardial blood flow, an immediate consequence of rapid ventricular pacing, may play a role in the pathogenesis of regional and global dysfunction in pacing-induced heart failure. During pacing, a difference was found in myocardial blood flow per minute between the left ventricular lateral wall (adjacent to the site of stimulation) and the IVS. Reduced blood flow was present in the lateral wall immediately on the initiation of pacing and remained for 21 to 28 days. The left ventricular lateral wall, receiving less blood flow than the IVS during pacing, showed progressive reduction in wall thickening (pacer off) during 21 to 28 days of pacing. In contrast, the IVS, receiving greater blood flow during pacing, maintained relatively normal wall thickening through 21 to 28 days of pacing.

Since myocardial blood flow per minute does not readily permit assessment of relative myocardial ischemia, we also expressed coronary flow as endocardial blood flow per beat. The physiological basis for such an analysis lies in previous experiments showing that regional subendocardial blood flow per minute (rather than outer wall of transmural flow) is the primary determinant of regional myocardial contraction under conditions of progressive coronary artery stenosis (Gallagher, K.P., et al., *Am. J. Physiol.* 16:H727-H738 (1984)) and that increases in heart rate shift this flow-function relation downward, with lower regional function at any level of subendocardial blood flow. (Delbaas, T., et al., *J. Physiol* 477:481-496 (1990)) However, if the flow-function relation is plotted as regional function versus endocardial blood flow per beat, to correct for heart rate effects, there is a single relation at different heart rates, indicating that endocardial blood flow per beat primarily determines the level of wall function when coronary blood flow is reduced. (Indolfi, C., et al., *Circulation* 80:933-993 (1989); Ross, J., *Circulation* 83:1076-1083 (1991)) With the initiation of pacing, there was a >50% reduction in endocardial blood flow per beat in the lateral wall compared with the IVS ($P<.001$; Table 4)

In prior studies in the conscious pig, we have documented that a 50% reduction in endocardial blood flow caused a 50% reduction of regional function and was associated with a subendocardial flow per beat similar to that observed in the lateral wall in the present studies (Table 4). The reduction in blood flow in the lateral wall during pacing persisted throughout the study. These data provide evidence for myocardial ischemia in the lateral wall on initiation of ventricular pacing. In contrast, IVS function and endocardial flow per beat remained relatively normal. With the pacemaker off, subendocardial blood flow per beat remained normal in both regions throughout the study, while regional dysfunction persisted in the lateral wall, consistent

with the occurrence of myocardial stunning in that region. Thus, we postulate that sustained ischemia of the lateral wall has a significant effect on global function during and after pacing.

EXAMPLE 2: PREPARATION OF ADENOVIRAL CONSTRUCTS

A helper independent replication deficient human adenovirus-5 system was used. As an initial illustration of vector constructs, we used the genes encoding β-galactosidase and FGF-5. Recombinant adenoviruses encoding β-galactosidase or FGF-5 were constructed using full length cDNAs. The system used to generate recombinant adenoviruses imposed a packing limit of about 5kb for transgene inserts. Each of the β-gal and FGF-5 genes operably linked to the CMV promoter and with the SV40 polyadenylation sequences were less than 4 kb, well within the packaging constraints.

The full length cDNA for human FGF-5 was released from plasmid pLTR122E (Zhan et al., Mol. Cell. Biol., 8:3487, 1988) as a 1.1 kb ECOR1 fragment which includes 981 bp of the open reading frame of the gene and cloned into the polylinker of shuttle vector plasmid ACCMVpLpA. The nucleotide and amino acid sequence of FGF-5 is disclosed in Figure 1 of Zhan et al., Mol. Cell. Biol., 8:3487, 1988. pACCMVpLpA is described in Gomez-Foix et al. J. Biol. Chem., 267:25129-25134, 1992. pACCMVpLpA contains the 5' end of the adenovirus serotype 5 genome (map units 0 to 17) where the E1 region has been substituted with the human cytomegalovirus enhancer-promoter (CMV promoter) followed by the multiple cloning site (polylinker) from pUC 19 (plasmid well known in the art), followed by the SV40 polyadenylation signal. The lacZ-encoding control adenovirus is based on a EIA /EIB deletion from map unit 1 to 9.8. The FGF-5-encoding adenovirus (Ad.FGF-5) is based on a EIA /EIB deletion from map unit 1.3 to 9.3. Both of these vectors eliminate the entire E1A coding sequences and most of the E1B coding sequences. Both of the vectors have the transgene inserts cloned in an anti-sense orientation relative to the adenovirus sequences. Therefore, in the unlikely event of read-through transcription, the adenovirus transcript would be antisense and would not express viral proteins.

The FGF-5 gene-containing plasmid was co-transfected (using calcium phosphate precipitation) into 293 cells with plasmid JM17 (pJM17) which contains the entire human adenovirus 5 genome with an additional 4.3 kb insert, making pJM17 too large to be encapsidated into mature adenovirus virions. The cells were then overlaid with nutrient agarose. Infectious viral particles containing the angiogenic gene were generated by homologous rescue recombination in the 293 cells and were isolated as single plaques 10-12 days later. (Identification of successful recombinant virus also can be done by co-transfection by lipofection and directly looking for cytopathic effect microscopically as described in Zhang et al. Biotechniques 15(5):868-

872, 1993). The resultant adenoviral vectors contain the transgene but are devoid of E1A/E1B sequences and are therefore replication-deficient. Adenovirus vector carrying the FGF-5 gene is also referred to herein as Ad.FGF-5.

Although these recombinant adenovirus were nonreplicative in mammalian cells, they could propagate in 293 cells which had been transformed with E1A/E1B and provided these essential gene products *in trans*. Recombinant virus from individual plaques was propagated in 293 cells and viral DNA was characterized by restriction analysis.

Successful recombinant virus then underwent two rounds of plaque purification using standard procedures. Viral stocks were propagated in 293 cells to titers in the range of 10^{10} and 10^{12} viral particles per milliliter (ml) as determined by optical densitometry. Human 293 cells were infected at 80% confluence and culture supernatant was harvested at 36-48 hours. After subjecting the virus-containing supernatant to freeze-thaw cycles, the cellular debris was pelleted by standard centrifugation and the virus further purified by two cesium chloride (CsCl) gradient ultracentrifugations (discontinuous 1.33/1.45 CsCl gradient; CsCl prepared in 5 mM Tris, 1 mM EDTA (pH 7.8); 90,000 x g (2 hr), 105,000 x g (18 hr)). Prior to *in vivo* injection, the viral stocks were desalted by gel filtration through Sepharose columns (e.g. G25 Sephadex equilibrated with PBS). Final viral concentrations were about 10^{11} viral particles per milliliter (ml), as determined by optical densitometry. Viral stocks can be conveniently stored in cells in media at minus 70 degrees C. For injections, purified virus is preferably resuspended in saline. The adenoviral vector preparation was highly purified, with no wild-type (potentially replicative) virus. Thus adenoviral infection and inflammatory infiltration in the heart were minimized.

EXAMPLE 3: GENE TRANSFER IN ADULT RAT CARDIOMYOCYTES

Adult rat cardiomyocytes were prepared by Langendorf perfusion with a collagenase containing perfusate according to standard methods. Rod shaped cells were cultured on laminin coated plates and at 24 hours, were infected with the β-galactosidase-encoding adenovirus obtained in the above Example 2, at a multiplicity of infection of 1:1. After a further 36 hour period, the cells were fixed with glutaraldehyde and incubated with X-gal. Consistently 70-90% of adult myocytes expressed the β-galactosidase transgene after infection with the recombinant adenovirus. At a multiplicity of infection of 1-2:1 there was no cytotoxicity observed.

EXAMPLE 4: *IN VIVO* GENE TRANSFER INTO PORCINE MYOCARDIUM

The β-galactosidase-encoding adenoviral vector obtained in Example 2 was propagated in permissive 293 cells and purified by CsCl gradient ultracentrifugation with a final viral titer of 1.5

x 10^{10} viral particles, based on the procedures of Example 2. An anesthetized, ventilated 40 kg pig underwent thoracotomy. A 26 gauge butterfly needle was inserted into the mid left anterior descending (LAD) coronary artery and the vector (1.5 x 10^{10} viral particles) was injected in a 2 ml volume in phosphate buffered saline. The chest was closed and the animal allowed to recover. On the fourth post-injection day the animal was killed. The heart was fixed with glutaraldehyde, sectioned and incubated with X-gal for 16.5 hours. After imbedding and sectioning, the tissue was counterstained with eosin.

Microscopic analysis of tissue sections (transmural sections of LAD bed 96 hours after intracoronary injection of adenovirus containing lacZ) revealed a significant magnitude of gene transfer observed in the LAD coronary bed with many tissue sections demonstrating a substantial proportion of the cells staining positively for β-galactosidase. Areas of the myocardium remote from the LAD circulatory bed did not demonstrate X-gal staining and served as a negative control, while diffuse expression of a gene was observed in myocytes and in endothelial cells. A substantial proportion of myocytes showed β-galactosidase activity (blue stain), and, in subsequent studies using closed chest intracoronary injection, similar activity was present 14 days after gene transfer (n=8). There was no evidence of inflammation or necrosis in areas of gene expression.

EXAMPLE 5: PORCINE MODEL OF ANGIOGENESIS-MEDIATED GENE THERAPY (USING AN FGF-5 TRANSGENE)

In this pig model for myocardial ischemia and heart failure, animals were subjected to stress by atrial electrical stimulation (pacing). The degree of stress-induced myocardial dysfunction and inadequate regional blood flow was quantified and then gene transfer was performed by intracoronary injection of an illustrative recombinant adenovirus expressing FGF-5. Gene transfer was performed after stable but limited endogenous angiogenesis had developed, and inducible ischemia, analogous to angina pectoris in patients, was present. The animals had no ischemia at rest but developed ischemia during activity or atrial pacing. Control animals received a recombinant adenovirus expressing lacZ (β-gal) to exclude the possibility that the adenovirus itself, independent of FGF-5, was stimulating new blood vessel formation. This also controlled for possible continued collateral vessel development unrelated to gene transfer. Two weeks after gene transfer, stress-induced cardiac dysfunction and regional blood flow were again measured.

Pigs receiving lacZ showed a similar degree of pacing-induced dysfunction in the ischemic region before and two weeks after gene transfer. In contrast, two weeks after receiving the FGF-5 gene, the animals showed increase in wall thickening and improved blood flow in the ischemic region during pacing. The results demonstrated that gene transfer of an angiogenic transgene

(FGF-5) was effective to ameliorate regional myocardial contractile dysfunction by improving regional blood flow through newly-formed blood vessels.

5

Methods

Animals and model.

Yorkshire domestic pigs *(Sus scrofa, n = 27)* weight 47 ± 9 kg were used. Two animals underwent intracoronary injection of a recombinant adenovirus expressing *lacZ* (10^{11} viral
10 particles in 2.0 ml saline) and were killed 3 or 5 days after injection. The remaining 25 animals had catheters placed in the left atrium, pulmonary artery and aorta, providing a means to measure regional blood flow, and to monitor pressures. Wires were sutured on the left atrium to permit ECG recording and atrial pacing. An ameroid constrictor placed around the proximal left circumflex coronary artery. The ameroid material is hygroscopic and slowly swells, leading
15 gradually to complete closure of the artery 10 days after placement, with minimal infarction (<1% of the left ventricle) because of the development of collateral blood vessels. Myocardial function and blood flow are normal at rest in the region previously perfused by the occluded artery (the ischemic region), but blood flow is insufficient to prevent ischemia when myocardial oxygen demands increase. Collateral vessel development is complete within 21 days of ameroid
20 placement and remains unchanged for at least 4 months (Roth et al., Am. J. Physiol. 253: H1279–H1288, 1987). A hydraulic cuff was also placed around the artery, adjacent but distal to the ameroid. These procedures have been described in detail elsewhere (Hammond et al., J. Am. Coll. Cardiol. 23: 475–482, 1994 and Hammond et al., J. Clin. Invest. 92: 2644–2652, 1993). Two animals died 5 and 7 days after ameroid placement. Thirty-eight (± 2) days after ameroid
25 placement, when limited collateral circulation had developed and stabilized, animals underwent studies to define pacing-induced regional function and blood flow and then received recombinant adenovirus expressing either *lacZ* (n = 7, control animals) or FGF-5 (n = 16, treatment group) delivered by intracoronary injection. Then, 14 ± 1 days later, studies to define pacing-induced regional function and blood flow were repeated. The following day, AdlacZ (n = 7) and AdFGF-5
30 (n = 11) animals were killed and tissues collected. Five AdFGF-5 animals were studied 12 weeks after gene transfer and then killed.

Recombinant adenovirus and transgene delivery.

A helper-independent replication-deficient human adenovirus-5 system was prepared as described in Example 2 above.

For intracoronary delivery of the transgene, animals were anesthetized, and a 5F arterial sheath placed into the carotid artery. A 5F multipurpose (A2) coronary catheter was inserted through the sheath and into the coronary arteries. Closure of the ameroid was confirmed in all animals by contrast injection into the left main coronary artery. The catheter tip was then placed deeply within the arterial lumen so that minimal material would be lost to the proximal aorta during injection. Four milliliters containing 2×10^{11} viral particles of recombinant adenovirus was delivered by slowly injecting 2.0 ml into both the left and right coronary arteries.

Assays:

(i) **Regional contractile function and perfusion.** Two-dimensional and M-mode images were obtained from a right parasternal approach at the papillary muscle level using a Hewlett Packard ultrasound imaging system (Hewlett-Packard Sonos 1000). Conscious animals were studied suspended in a comfortable sling to minimize body movement. Images were recorded on VHS tape with animals in a basal state and again during left atrial pacing (heart rate = 200 beats per min). These studies were performed 1 day before gene transfer and repeated 14 ± 1 days later. Five animals were examined again 12 weeks after gene transfer with FGF-5 to determine whether the effect on improved function was persistent. Rate-pressure products and left atrial pressures were similar in both groups before and after gene transfer, indicating similar myocardial oxygen demands and loading conditions. Echocardiographic measurements were made using standardized criteria (Sahn et al., Circulation 58: 1072–1083, 1978). To demonstrate reproducibility of echocardiographic measurements, animals *(n = 5)* were imaged on two consecutive days. The data from the separate determinations were highly reproducible (lateral wall thickening: $r^2 = 0.90$; $P = 0.005$). The percent decrease in function measured by transthoracic echocardiography and sonomicrometry in this model are similar (Hammond et al., J., Am. Coll. Cardiol. 23: 475–482, 1994 and Hammond et al. J. Clin. Invest. 92: 2644–2652, 1993), documenting the accuracy of echocardiography for evaluation of ischemic dysfunction. Analysis was performed without knowledge of treatment group.

Contrast material (Levovist; microaggregates of galactose) increases the echogenicity (whiteness) of the image after left atrial injection. The microaggregates distribute into the coronary arteries and myocardial walls in a manner that is proportional to blood flow. The peak intensity of contrast enhancement is correlated with myocardial blood flow as measured by microspheres (Skyba et al., Circulation 58: 1072–1083, 1978). Thirty-eight (± 2) days after

ameroid placement, well after ameroid closure, but before gene transfer, contrast echocardiographic studies were performed during atrial pacing (200 bpm). Studies were repeated 14 ± 1 days after gene transfer, and, in five animals, 12 weeks after gene transfer with FGF-5. Peak contrast intensity was measured from the video images with a computer-based video analysis program (Color Vue II, Nova Microsonics, Indianapolis, Indiana), that provided an objective measure of video intensity. Data were expressed as the ratio of the peak video intensity in the ischemic region (LCx bed) divided by the peak video intensity in the interventricular septum (IVS, a region receiving normal blood flow through the unoccluded left anterior descending coronary artery). The differences in regional blood flow during atrial pacing measured by contrast echocardiography were similar to the differences measured by microspheres in this same model in our laboratory, documenting the accuracy of echocardiography for the evaluation of regional myocardial blood flow. The contrast studies were analyzed without knowledge of which gene the animals had received.

(ii) Assessment of angiogenesis.

The brachiocephalic artery was cannulated and other great vessels ligated. After intravenous injection of heparin (10,000 IU), papaverine (60 mg), and then potassium chloride (to induce diastolic cardiac arrest), the aorta was cross-clamped and the coronary vasculature perfused. Glutaraldehyde solution (6.25%, 0.1 M cacodylate buffer) was perfused at 120 mm Hg pressure; the heart was removed; the beds were identified using color-coded dyes injected anterograde through the left anterior descending, left circumflex and right coronary arteries; and the ameroid was examined to confirm closure. Samples taken from the normally perfused and ischemic regions (endocardial and epicardial thirds) were plastic-embedded and prepared for microscopic analysis of capillary number. Four 1-μm-thick transverse sections were taken from each subsample (endocardium and epicardium of each region) as previously described (Mathieu-Costello, Microvasc. Res. 33: 98–117, 1987 and Poole & Mathieu-Costello, Am. J. Physiol. 259: H204–H210, 1990). The number of capillaries around each fiber and fiber cross-sectional area in each of eight fields in each subsample (randomly selected by systematic sampling) were measured with an image analyzer (Videometric 150, American Innovision) at X1400. The number of capillaries around a total of 325 ± 18 fibers was measured. Capillary density (number per fiber cross-sectional area) was estimated by point counting 15 ± 1 fields per subsample. The relative standard errors of capillary number around a fiber, fiber cross-sectional area and capillary density were 1.4, 4.1 and 4.2% respectively. Capillary-to-fiber ratio was calculated as the product of capillary density and fiber cross-sectional area. There was no significant difference in fiber cross-

sectional area in myocardial samples from either group. Bromodeoxyuridine (50 mg/kg) was injected into the peritoneal space of five animals: a control animal (no ameroid); two animals with ameroid occluders that received *lacZ* gene transfer 2 weeks before; and two animals with ameroid occluders that received FGF-5 gene transfer 2 weeks before. Thirty-six hours after BRDU injection the animals were killed, and the tissue was prepared for analysis using methods previously described (Kajstura et al., Circ. Res. 74: 383–400, 1994). Sections of duodenum were used as positive controls.

(iii) DNA, mRNA and protein expression.

Following gene transfer, left ventricular homogenates underwent studies to document transgene presence and expression. The polymerase chain reaction (PCR), using a sense primer to the CMV promoter (GCAGAGCTCGTTTAGTGAAC; SEQ ID. NO. 1) and an antisense to the internal FGF-5 sequence (GAAAATGGGTAGAGATATGCT; SEQ ID NO. 2) amplified the expected 500-bp fragment. Using a sense primer to the beginning of the FGF-5 sequence (ATGAGCTTGTCCTTCCTCCTC; SEQ ID NO. 3) and an antisense primer to the internal FGF-5 sequence (i.e., SEQ ID NO. 2), RT-PCR amplified the expected 400-bp fragment. Primers directed against the adenovirus DNA E2 region were used to detect wild-type or recombinant viral DNA in tissues (TCGTTTCTCAGCAGCTGTTG; SEQ ID NO. 4) and (CATCTGAACTCAAAGCGTGG; SEQ ID NO. 5). The expected 900-bp fragment was amplified from the recombinant virus. These studies were conducted on 200-mg tissue samples from myocardium and other tissues. PCR detection sensitivity was 1 viral sequence per 5 million cells. A polyclonal antibody directed against FGF-5 (Kitaoka et al., Invest. Ophthalmol. Vis. Sci. 35:3189-3198, 1994) was used in immunoblots of protein from the medium of cultured rat cardiac fibroblasts 48 h after the gene transfer of FGF-5 or *lacZ*. FGF-5 protein was found in conditioned media after gene transfer of FGF-5, but not after gene transfer of *lacZ*. Methods for PCR and western blotting have been described in detail elsewhere (Hammond et al., J. Clin. Invest. 92: 2644–2652, 1993, Roth et al. J. Clin. Invest. 91: 939–949, 1993, and Tsai et al. Am. J. Physiol. 267:H2079–H2085, 1994). To examine the transgene for mitogenic activity *in vitro*, adult rat cardiac fibroblasts were infected with adenovirus-encoding FGF-5 or with adenovirus-encoding lacZ, or were not infected. Media from these cell cultures were incubated with NIH 3T3 mouse fibroblasts, and tritiated thymidine incorporation was measured (Tsai et al., Endocrinology 136: 3831–3838, 1995).

(iv) Adenovirus release during intracoronary delivery.

Pulmonary arterial blood was withdrawn continuously during intracoronary injection of recombinant adenovirus in three animals. Serum from each sample was used in a standard plaque assay. Undiluted serum (0.5 ml) was added to subconfluent H293 cells; 10 days later, no plaques had formed. However, when 0.5 ml serum was diluted 200- to 8000-fold with DMEM (2% FBS), viral plaques formed by day 9. A single vascular bed (myocardial) separates the coronary and pulmonary arteries. If no virus attaches in this bed after injection into the coronary artery, then the pulmonary artery concentration of virus should reflect the dilution of coronary sinus blood by systemic venous blood over the time of the injection. Measurements from our laboratory indicate that coronary flow represents 5% of pulmonary artery flow. Using this dilution factor (20-fold), the duration of coronary injection, and the amount of adenovirus injected, we calculated the amount of adenovirus delivered to the pulmonary artery, assuming no adenovirus escape or attachment. This estimate was compared to the measured amount and the difference used as an estimate of the amount of virus cleared by the myocardial vascular bed.

(v) Assessment of inflammation.

Hematoxylin/eosin and Masson's trichrome stains were used to detect inflammatory cell infiltrates, cell necrosis and fibrosis. Mouse ascites, porcine anti-CD4 and anti-CD8 monoclonal antibodies (1.0 mg/ml; VMRD, Inc., Pullman, Washington) were used to detect CD4 and CD8 markers on T lymphocytes in frozen sections (6 μm) of spleen (positive control) and heart. These studies were performed on transmural samples of hearts of six animals that had received ameroid occluders 50 days before being killed: two animals received no gene transfer, two received FGF-5 gene transfer 2 weeks before, and two received *lacZ* gene transfer 2 weeks before. Analysis was conducted without knowledge of treatment group.

(vi) Statistical analysis.

Data are expressed as means ± 1 s.e.m. Measurements made before and after gene transfer with FGF-5 and *lacZ* were compared using two-way analysis of variance (Crunch4, Crunch Software Corporation, Oakland, California). Data from angiogenesis studies also underwent two-way analysis of variance. The null hypothesis was rejected when $P < 0.05$.

RESULTS USING AN FGF-5 TRANSGENE

Three measurements were used to assess whether gene transfer of FGF-5 was effective in treating myocardial ischemia: regional contractile function and perfusion (assessed before and

after gene transfer) and capillary number. All measurements were conducted without knowledge of which gene the animals had received (FGF-5 versus *lacZ*).

Regional contractile function and blood flow. Thirty-eight days after ameroid placement, animals showed impaired wall thickening during atrial electrical stimulation (pacing). Pigs receiving *lacZ* showed a similar degree of pacing-induced dysfunction in the ischemic region before and two weeks after gene transfer. In contrast, two weeks after FGF-5 gene transfer there was a 2.7-fold increase in wall thickening in the ischemic region during pacing ($P < 0.0001$; Fig. 6). Wall thickening in the normally perfused region (the interventricular septum) was normal during pacing and unaffected by gene transfer (% wall thickening: *lacZ*: pre gene transfer 53 ± 8%, post gene transfer 51 ± 6%; FGF-5: pre gene transfer 59 ± 4%, post gene transfer 59 ± 6%).

Associated with improved function in the ischemic region was improved regional blood flow. Two weeks after *lacZ* gene transfer there was a persistent flow deficit in the ischemic region during pacing (Fig. 8). Animals receiving FGF-5 gene transfer, however, showed homogeneous contrast enhancement in the two regions two weeks later, indicating improved blood flow in the ischemic region ($P = 0.0001$). To determine whether improved function and perfusion in the ischemic bed were long lasting, five animals were examined again 12 weeks after FGF-5 gene transfer. Each animal showed persistent improvements in function ($P = 0.005$; Fig. 6) and perfusion ($P = 0.001$; Fig. 8).

Angiogenesis. Uninfected ameroid-constricted animals (no gene transfer performed) had identical physiological responses to animals receiving lacZ-encoding adenovirus, indicating that the lacZ vector did not adversely affect native angiogenesis. To assess angiogenesis, myocardial capillary number was quantified using microscopic analysis of perfusion-fixed hearts (Fig. 9). The number of capillaries surrounding each myocardial fiber was greater in the endocardium of the ischemic and nonischemic regions in animals that received gene transfer with FGF-5 when compared with the same regions of the hearts of animals that had received gene transfer with *lacZ* ($P = 0.038$). Thus, improved regional function and perfusion were associated with capillary angiogenesis two weeks after FGF-5 gene transfer. Increased capillary number around each fiber tended to increase in the epicardial portion of the wall after FGF-5 gene transfer ($P = 0.13$). Other measures of capillarity such as capillary number per fiber cross-sectional area and capillary number per fiber number were not changed in endocardium or epicardium.

DNA, mRNA and protein expression. Having established favorable effects of FGF-5 gene transfer on function, prefusion and capillary number around each fiber, it was imperative to demonstrate presence and expression of the transgene in the heart. Polymerase chain reaction (PCR) and reverse transcriptase coupled with PCR (RT-PCR) were used to detect transgenic FGF-5 DNA and mRNA in myocardium from animals that had received FGF-5 gene transfer.

Following gene transfer, left ventricular samples were examined to document transgene incorporation and expression. Briefly, 3 days after intracoronary gene transfer of *lacZ*, myocardium was treated with X-gal, and then counterstained with Eosin X120. Examination using standard histological techniques revealed that the majority of myocytes showed β-galactosidase activity (blue stain). Activity was also seen 14 ± 1 days after gene transfer in all animals that had received *lacZ* gene transfer. Higher magnification demonstrated cross striations in cells containing β-galactosidase activity, confirming gene expression in myocytes. PCR analysis using a sense primer directed against the CMV promoter and an antisense primer directed against an internal FGF-*5* sequence, was performed to confirm the presence of recombinant adenovirus DNA encoding FGF-*5* in the ischemic (LCx) and nonischemic (LAD) regions of three animals that received FGF-*5* gene transfer. The results, shown in Figure 10A confirmed the presence of the expected 500-bp fragments. FGF-*5* mRNA expression was then examined 14 days after gene transfer. As shown in Figure 10B, the RT-PCR-amplified 400-bp fragment was present in both regions from two animals, whereas control animals showed no signal. A polyclonal antibody directed against FGF-5 was used in immunmoblots of protein from the medium of cultured rat cardiac fibroblasts 48 hours after gene transfer of FGF-*5* or lacZ. As shown in Figure 10C, FGF-5 protein was found after gene transfer of FGF-5 (F), but not after gene transfer of *lacZ* (β), demonstrating protein expression and extracellular secretion after FGF-5 gene transfer. Finally, PCR, using a set of primers directed against adenovirus DNA (E2 region), was performed to determine whether adenovirus DNA was present in retina, liver, or skeletal muscle of two animals that received intracoronary injection of adenovirus 14 days before. As shown in Figure 10D, the expected 900-bp amplified fragment was only found in a control lane (+) containing recombinant adenovirus (as a positive control), and not in the lanes derived from the retina (r), liver (l), or skeletal muscle (m) of the treated animals.

Successful gene transfer was documented in both the ischemic and nonischemic regions. Immunoblotting showed FGF-5 protein in myocardium from animals that received FGF-5 gene transfer. In additional experiments using cultured fibroblasts, we documented that gene transfer of FGF-5 conferred the ability of these cells to synthesize and secrete FGF-5 extracellularly. Media from cultured cells infected with recombinant adenovirus expressing FGF-5 showed a mitogenic

response (14-fold increase versus control; $P = 0.005$). Finally, two weeks after gene transfer, myocardial samples (but not liver samples) from *lacZ*-infected animals showed β-galactosidase activity on histological inspection. These studies confirm successful *in vivo* gene transfer and expression, and demonstrate the biological activity of the transgene product.

Two weeks after intracoronary injection of recombinant adenovirus, we were unable to detect viral DNA in liver, retina or skeletal muscle using PCR despite the presence of viral DNA in myocardium. Furthermore, viral DNA was undetectable in urine 2–24 hours after intracoronary injection. These experiments indicated that intracoronary delivery of the adenoviral vector minimized systemic arterial distribution of the virus to a level below the detection limits of the PCR methods. This technique might be difficult to achieve in animals with smaller coronary artery size such as rabbits.

To assess the efficiency of myocardial uptake of adenovirus, we measured the amount of adenovirus released from the heart by sampling pulmonary arterial blood during intracoronary injection. A surprising 98.7% of the virus was cleared by the heart on the first pass. Undiluted serum obtained from the pulmonary artery during intracoronary delivery of virus was incapable of forming viral plaques in appropriate conditions. Thus the present invention effectively provides a cardiac-specific gene delivery system.

Assessment of inflammation. Microscopic inspection of transmural sections of hearts of animals that had received recombinant adenovirus did not show inflammatory cell infiltrates, cell necrosis or increased fibrosis. As an additional evaluation for an adenovirus-induced cytopathic effect, we conducted immuno-histological studies to detect CD4 and CD8 antigens that would indicate the presence of cytotoxic T cells. These studies showed rare positive cells on transmural sections of heart from uninfected animals ($n = 2$) or animals that had received recombinant adenovirus ($n = 4$). The liver also was free from inflammation.

EXAMPLE 6: GENE-MEDIATED ANGIOGENESIS USING AN FGF-4 TRANSGENE

This experimental example demonstrated successful gene therapy using a different angiogenic protein-encoding gene, FGF-4. The protocol for FGF-4 gene therapy was essentially as described in Example 5 above for FGF-5.

The human FGF-4 gene was isolated from a cDNA library which was constructed from mRNA of Kaposi's Sarcoma DNA transformed-NIH3T3 cells. The FGF-4 cDNA is about 1.2 kb in length and encodes a polypeptide of 206 amino acids including a 30 amino acid signal peptide at the N-terminal (Dell Bovi et al. *Cell* 50:729-737, 1987; Bellosta et al. *J. Cell Biol.* 121:705-713,

1993). We subcloned the FGF-4 cDNA as an essentially full-length 1.2 kb EcoR1 fragment, into the EcoR1 site in adenovirus vector pACCMVpLpASR (pACSR for simplicity). The 5' start site was at 243 basepairs and the 3' end at 863 basepairs. Recombinant adenovirus encoding FGF-4 (also referred to herein as Ad.FGF-4) was made as described in Example 2 for making the FGF-5 adenovirus.

Expression of FGF-4 in cardiac tissue (and a lack of expression in other tissues including the liver, skeletal muscle and eye) was confirmed by Western-blot analysis using anti-FGF-4 antibody for detection. The mitogenic effect of FGF-4 on proliferation of endothelial cells *in vitro* was also tested.

Forty-five days after ameroid placement, animals underwent studies to define stress-induced regional function and blood flow and then received recombinant adenovirus expressing FGF-4 (n=6 animals) delivered by intracoronary injection. Thirteen days later, studies to define stress-induced regional function and blood flow were repeated. The following day, animals were killed and tissues collected.

Transgene Delivery

As with FGF-5, gene transfer was performed after endogenous angiogenesis was quiescent and inducible myocardial ischemia, analogous to *angina pectoris* in patients, was present. For intracoronary delivery of the transgene, animals were anesthetized, and a 5F arterial sheath placed into the carotid artery. A 5F multipurpose coronary catheter was inserted through the sheath and into the coronary arteries. Closure of the ameroid was confirmed in all animals by contrast injection into the left main coronary artery. The catheter tip was then placed 1 cm within the arterial lumen so that minimal material would be lost to the proximal aorta during injection. Five ml containing $1.5x10^{12}$ viral particles of recombinant adenovirus expressing FGF-4 were delivered by slowly injecting 3.0 ml into the left and 2.0 ml into the right coronary arteries.

RESULTS USING AN FGF-4 TRANSGENE

Regional Function and Perfusion

Forty-five days after ameroid placement, animals showed impaired wall thickening during atrial pacing. In contrast, two weeks after FGF-4 gene transfer there was a 2.7 fold increase in wall thickening in the ischemic region during pacing ($p<0.0001$; Figures 11 and 12). Wall thickening in the normally perfused region (the interventricular septum) was normal during pacing and unaffected by gene transfer. The improvement in function after FGF-4 gene transfer was statistically indistinguishable from the improvement obtained following gene transfer with FGF-5

or FGF-2LI + signal peptide ("sp") (Figure 11). Improved function in the ischemic region was associated with improved regional perfusion (Figure 12). As shown in Figure 12, prior to FGF-4 gene transfer there was a flow deficit in the ischemic region during pacing. Two weeks after gene transfer with FGF-4, homogeneous contrast enhancement was seen in the two regions, indicating improved flow in the ischemic region (p=0.0001). Results with FGF-4 were statistically indistinguishable from results obtained with FGF-5 and FGF-2LI + sp (Figure 13). FGF-2LI+sp is described in Example 7 and refers to FGF-2 containing a signal sequence.

Transgene Expression

Exclusive expression of FGF-4 in the heart was confirmed by performing PCR and RT-PCR using primers specific for sequences encoding the transgene. FGF-4 DNA and mRNA were found in the heart, but absent in the eye, liver, and skeletal muscle. These data confirm data derived from the use of Ad.FGF-5 (n=2) and Ad.FGF-2LI + sp (n=1). Thus exclusive expression of the transgene in the heart was confirmed in all four animals which had received adenoviral vectors containing different angiogenic protein-encoding genes.

Absence of Myocardial Inflammation

Transmural myocardial biopsies from three consecutive animals that received Ad.FGF-4 have been examined. The animals were killed 2 weeks after gene transfer. There was no evidence of inflammatory cell infiltrates, necrosis, or increased fibrosis in these sections compared to control ameroid animals that received no adenovirus. This was true in both the LAD and LCx beds. These slides were reviewed by a pathologist who made a blind-sample assessment and commented that there was no evidence for myocarditis in any section.

EXAMPLE 7: GENE-MEDIATED ANGIOGENESIS USING AN FGF-2 MUTEIN

This experimental example demonstrated successful gene therapy using a third angiogenic protein-encoding gene, FGF-2. This experiment also demonstrates how an angiogenic protein can be modified to increase secretion and potentially improve efficacy of angiogenic gene therapy in enhancing blood flow and cardiac function within the heart. The protocol used for human FGF-2 gene therapy was virtually identical to that employed for FGF-5 and FGF-4 above.

Acidic FGF (aFGF, FGF-1) and basic FGF (FGF-2) lack a native secretory signal sequence; although some protein secretion may occur. An alternate secretary pathway, not involving the Golgi apparatus, has been described for acidic FGF. Two FGF-2 constructs (FGF-2LI +sp and FGF-2LI

-sp) were made, one with a sequence encoding a signal peptide (FGF-2LI +sp) for the classic protein secretary pathway and one without the signal peptide encoding sequence (FGF-2LI -sp) to test for improved efficacy of FGF-2 having an added signal peptide over the same protein without the added signal peptide.

As shown below, FGF-2 has a five-residue loop structure which extends from amino acid residue 118 to residue 122. This loop structure was replaced by cassette directed mutagenesis, with the corresponding five-residue loop from interleukin-1β to produce FGF-2LI loop replacement mutants. Briefly, the gene encoding human $Glu^{3,5}$FGF-2 (Seddon et al. *Ann. N.Y. Acad. Sci.* 638:98-108, 1991) was cloned into T7 expression vector pET-3a (M13), a derivative of pET-3a (Rosenberg et al. *Gene* 56:125-135, 1987), between restriction sites *Ndel* and *Bam*H1. The unique restriction endonuclease sites, *Bst*Bl and *Spl*1, were introduced into the gene in such a way as to produce no change in the encoded amino acids (i.e. silent mutations) at positions that flank the codons encoding the segment Ser117-Trp123 of FGF-2.

Structured alignment[1] of the β9-β10 loops in FGF-1, FGF-2, and IL-1β.

```
          110   115  120 125 130
FGF-1 ENHYNTYISKKHAEKHWFVGLKKNG          (SEQ ID NO. 6)

      110  115  120    125  130
FGF-2 SNNYNTYRSRKY..TSWYVALKRTG          (SEQ ID NO. 7)

        110    115   120  125
IL-1β  NNKLEFESAQF..PNWYISTSQAE          (SEQ ID NO. 8)
```

[1] Numbering for FGF-1 and FGF-2 is from amino acid residue 1 deduced from the cDNA sequence encoding the 155-residue form (as described in Seddon et al. *Ann. N.Y. Acad. Sci.* 638:98-108, 1991), and that for IL-1β is from residue 1 of the mature 153-residue polypeptide (id.).

Replacement of residues Arg118-Lys119-Tyr120-Thr121-Ser122 of FGF-2 with the human sequence Ala-Gln-Phe-Pro-Asn from the corresponding loop of the structural analogue IL-1β (115-119) was essentially performed as follows:

The plasmid DNA, pET-3a (M13), was subjected to *Bst*B1 and *Spl*1 digestion, and the resulting larger DNA fragment was isolated using agarose gel electrophoresis. The DNA fragment

was ligated, using T_4 DNA ligase, to a double-stranded DNA obtained by annealing two synthetic oligonucleotides: 5"-CGAACGATTG GAATCTAATA ACTACAATAC GTACCGGTCT GCGCAGTTTC CTAACTGGTA TGTGGCACTT AAGC-3' (SEQ ID NO. 9) and 5' GTACGCTTAA GTGCCACATA CCAGTTAGGA AACTGCGCAG ACCGGTACGT ATTGTAGTTA TTAGATTCCA ATCGTT-3' (SEQ ID NO. 10), that contain termini compatible with those generated by *Bst*B1 and *Spl*1 digestion. The ligation product was used to transfer *Escherichia coli* (strain DH5α) cells. The desired mutant plasmid (FGF-2LI) was selected for on the basis of susceptibility to cleavage at the newly introduced Afl2 restriction site (underlined above).

FGF-2LI with and without signal peptide were constructed by using a polymerase chain reaction (PCR)-based method. In order to add the FGF-4 signal peptide sequences to the 5' of FGF-2LI and to ensure that the signal peptide will be cleaved from FGF-2LI protein, the gene cassette used by Forough R. et al for getting the secreted FGF-1 was employed. Using a primer (pF1B: 5'- CGGGATCCGC CCATGGCGGG GCCCGGGACG GC-3' (SEQ ID NO. 11) matching the 5' portion of the FGF-4 signal peptide and a second primer (pF2R: 5'-CGGAATTCTG TGAAGGTGGT GATTTCCC-3') (SEQ ID NO. 12) to the 5' portion of FGF-1, we synthesized, by PCR, a DNA fragment containing a Bam HI site at the 5' end of the FGF-4 signal peptide sequences followed by the first ten amino acids of FGF-1 and an EcoRI site at the 3' end. Using another pair of primers (pF3R: 5'-CGGAATTCAT GGCTGAAGGG GAAATCACC-3' (SEQ ID NO. 13) and pF4HA: 5'-GCTCTAGATT AGGCGTAGTC TGGGACGTCG TATGGGTAGC TCTTAGCAGA CATTGGAAGA AAAAG-3' (SEQ ID NO. 14)) matching the sequences of 5'- and 3'-of FGF-2LI, respectively, we obtained a second DNA fragment which has an EcoRI site at the 5' end and an influenzae hemoagglutinin (HA) tag plus an XbaI site at the 3' end of the FGF-2LI. These two fragments were then subcloned into pcDNA3 vector at a BamHI and XbaI site by three molecule ligation. The plasmid pFGF-2LI/cDNA3 which was similar to pSPFGF-2LI/cDNA3 except that it has no signal peptide was subcloned in a similar manner. Both plasmids were then sequenced to confirm the correction of the inserts. Both FGF-2LI fragments were then released from pcDNA3 by digestion with BamHI and XbaI and subcloned into pACCMVpLpASR(+) (pACSR for simplicity) which is a shuttle vector for making recombinant virus. Recombinant virus and injectable vector were prepared essentially as described in Example 2. Gene transfer was performed as described in Example 5 (using 8 animals for FGF-2LI sp+ and 6 animals for FGF-2LI sp-, with the lacZ vector serving as a control, all with 10^{11} to 10^{12} viral particles).

RESULTS USING FGF-2 MUTEINS

Two weeks after gene transfer with FGF-2LI +sp, the peak contrast ratio (LCx/LV) during pacing stress at 200 bpm was significantly improved compared to pre-gene transfer. Figure 13 shows results using intracoronary gene transfer of recombinant adenovirus expressing lacZ, FGF-5, FGF-2LI +sp, FGF-2LI -sp, and FGF-4 for comparison. The black bar on the right side in Figure 13 shows the normal flow ratio using this method. FGF-2LI +sp normalized peak contrast flow ratio in these animals.

Percent wall thickening was also improved two weeks after intracoronary delivery of a recombinant adenovirus expressing FGF-2LI +sp. Figure 11 shows results using intracoronary gene transfer of recombinant adenovirus expressing lacZ, FGF-5, FGF-2LI +sp, FGF-2LI -sp, and FGF-4 for comparison. The black bar on the right side in Figure 11 shows the normal percent wall thickening before pacing-induced stress. FGF-2LI +sp improved regional function to a degree that was statistically indistinguishable from FGF-5. Although there was some improvement noted after gene transfer with FGF-2LI -sp, the improvement with the signal peptide containing transgene was superior (Figure 13).

CLAIMS

We claim:

1. A method for treating a patient suffering from congestive heart failure, comprising delivering a vector to the heart of said patient, said vector comprising a gene encoding an angiogenic protein or peptide operably linked to a promoter for expression of the gene.

2. The method of claim 1, wherein the congestive heart failure is associated with severe coronary artery disease and myocardial ischemia.

3. The method of claim 1, wherein the congestive heart failure is associated with a dilated cardiomyopathy.

4. The method of claim 1, wherein the vector is delivered to a blood vessel supplying blood to the myocardium of the heart.

5. The method of claim 4, wherein said blood vessel supplying blood to the myocardium is a coronary artery, a saphenous vein graft, or an internal mammary artery graft.

6. The method of claim 4, wherein the vector is delivered by intracoronary injection directly into the left and/or right coronary arteries.

7. The method of claim 6, wherein said intracoronary injection is conducted about 1 cm into the lumens of the left and/or right coronary arteries.

8. The method of claim 1, wherein the angiogenic protein or peptide is a member of the fibroblast growth factor (FGF) family.

9 . The method of claim 8, wherein the angiogenic protein or peptide is an FGF-4, FGF-5, FGF-6, FGF-1 or FGF-2.

10. The method of claim 9, wherein the angiogenic protein is an FGF-4.

11. The method of claim 9, wherein the angiogenic protein is an FGF-5.

12. The method of claim 10, wherein the angiogenic protein is an FGF-1.

13. The method of claim 10, wherein the angiogenic protein is an FGF-2.

14. The method of claim 1, wherein the angiogenic protein comprises a secretion signal sequence.

15. The method of claim 14, wherein the angiogenic protein is an FGF-2 modified by inclusion of a heterologous secretion signal sequence.

16. The method of claim 1, wherein the angiogenic protein or peptide is a member of the vascular endothelial growth factor (VEGF) family.

17. The method of claim 16, wherein the angiogenic protein or peptide is a VEGF-121, VEGF-145, VEGF-165, VEGF-189, VEGF-206, VEGF-167, VEGF-186 or a VEGF-C.

18. The method of claim 1, wherein the angiogenic protein or peptide is a member of the platelet-derived growth factor (PDGF) family.

19. The method of claim 18, wherein the angiogenic protein or peptide is a PDGF-A or a PDGF-B.

20. The method of claim 1, wherein the angiogenic protein or peptide is a member of the insulin-like growth factor (IGF) family.

21. The method of claim 1, wherein said promoter is a CMV promoter.

22. The method of claim 1, wherein said promoter is tissue-specific for cardiac myocytes.

23. The method of claim 22, wherein said tissue-specific promoter is a ventricular myocyte-specific promoter.

24. The method of claim 23, wherein said ventricular myocyte-specific promoter is a ventricular myosin light chain-2 promoter or a ventricular myosin heavy chain promoter.

25. The method of claim 1, wherein the vector is a viral vector or a lipid-based vector.

26. The method of claim 25, wherein the vector is a viral particle.

27. The method of claim 26, wherein the viral particle is a replication-defective adenovirus (Ad).

28. The method of claim 26, wherein the viral particle is a replication-defective adeno-associated virus (AAV).

29. The method of claim 26, wherein about 10^6-10^{14} virus vector particles are delivered in the injection.

30. The method of claim 27, wherein about 10^8-10^{12} adenovirus vector particles are delivered in the injection.

31. A method for preventing or alleviating ventricular remodeling in a patient who has suffered a myocardial infarction, comprising delivering a vector to the heart of said patient, said vector comprising a gene encoding an angiogenic protein or peptide operably linked to a promoter for expression of the gene, thereby increasing myocardial blood flow and alleviating deleterious ventricular remodeling.

32. The method of claim 31, wherein the vector is delivered to a blood vessel supplying blood to the myocardium of the heart.

33. The method of claim 32, wherein said blood vessel supplying blood to the myocardium is a coronary artery, a saphenous vein graft, or an internal mammary artery graft.

34. The method of claim 32, wherein the vector is delivered by intracoronary injection directly into the left and/or right coronary arteries.

35. The method of claim 34, wherein said intracoronary injection is conducted about 1 cm into the lumens of the left and/or right coronary arteries.

36. The method of claim 31, wherein the angiogenic protein or peptide is a member of the fibroblast growth factor (FGF) family.

37. The method of claim 36, wherein the angiogenic protein or peptide is an FGF-4, FGF-5, FGF-6, FGF-1 or FGF-2.

38. The method of claim 37, wherein the angiogenic protein is an FGF-4.

39. The method of claim 37, wherein the angiogenic protein is an FGF-5.

40. The method of claim 39, wherein the angiogenic protein is an FGF-1.

41. The method of claim 39, wherein the angiogenic protein is an FGF-2.

42. The method of claim 31, wherein the angiogenic protein comprises a secretion signal sequence.

43. The method of claim 42, wherein the angiogenic protein is an FGF-2 modified by inclusion of a heterologous secretion signal sequence.

44. The method of claim 31, wherein the angiogenic protein or peptide is a member of the vascular endothelial growth factor (VEGF) family.

45. The method of claim 44, wherein the angiogenic protein or peptide is a VEGF-121, VEGF-145, VEGF-165, VEGF-189, VEGF-206, VEGF-167, VEGF-186 or a VEGF-C.

46. The method of claim 31, wherein the angiogenic protein or peptide is a member of the platelet-derived growth factor (PDGF) family.

47. The method of claim 46, wherein the angiogenic protein or peptide is a PDGF-A or a PDGF-B.

48. The method of claim 31, wherein the angiogenic protein or peptide is a member of the insulin-like growth factor (IGF) family.

49. The method of claim 31, wherein said promoter is a CMV promoter.

50. The method of claim 31, wherein said promoter is tissue-specific for cardiac myocytes.

51. The method of claim 50, wherein said tissue-specific promoter is a ventricular myocyte-specific promoter.

52. The method of claim 51, wherein said ventricular myocyte-specific promoter is a ventricular myosin light chain-2 promoter or a ventricular myosin heavy chain promoter.

53. The method of claim 31, wherein the vector is a viral vector or a lipid-based vector.

54. The method of claim 53, wherein the vector is a viral particle.

55. The method of claim 54, wherein the viral particle is a replication-defective adenovirus (Ad).

56. The method of claim 54, wherein the viral particle is a replication-defective adeno-associated virus (AAV).

57. The method of claim 54, wherein about 10^6-10^{14} virus vector particles are delivered in the injection.

58. The method of claim 55, wherein about 10^8-10^{12} adenovirus vector particles are delivered in the injection.

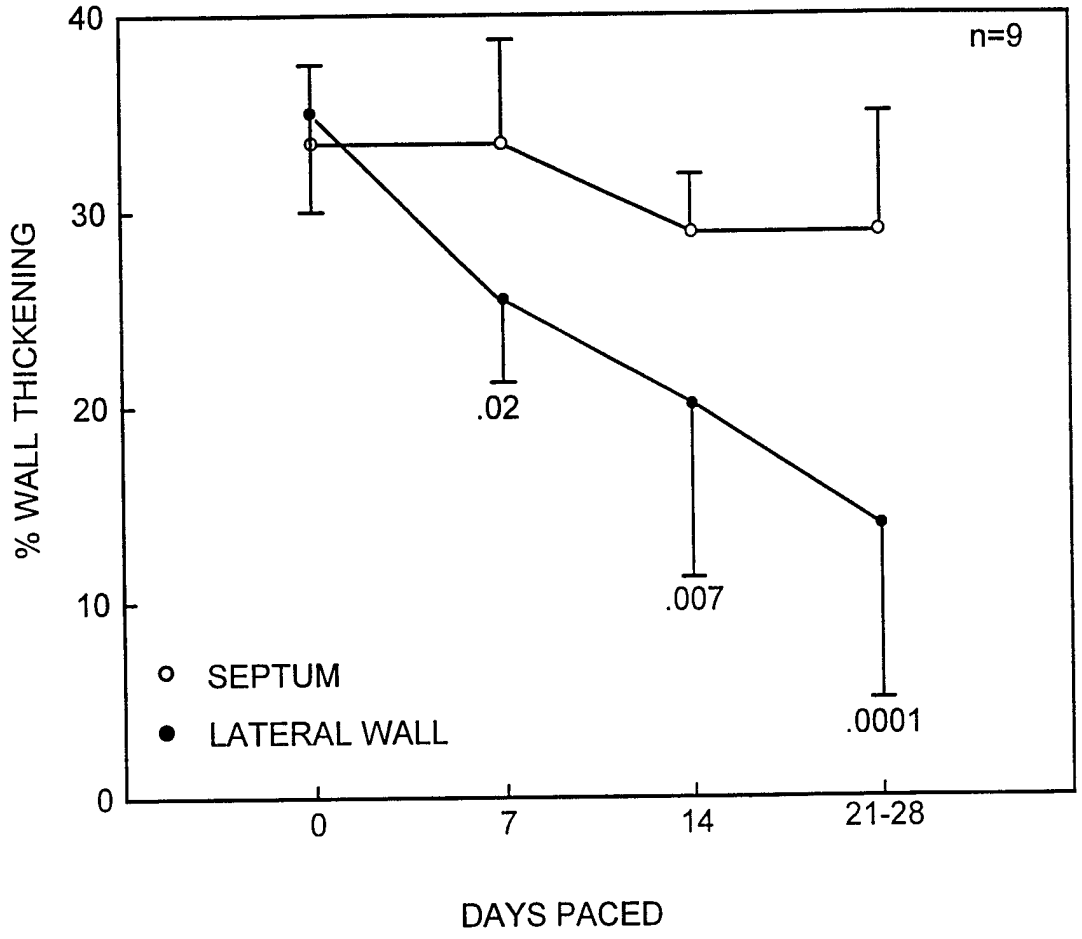

40
30
20
10
0
% WALL THICKENING
n=9
.02
.007
.0001
SEPTUM
LATERAL WALL
0
7
14
21-28
DAYS PACED

FIG. 1

FIG. 2A

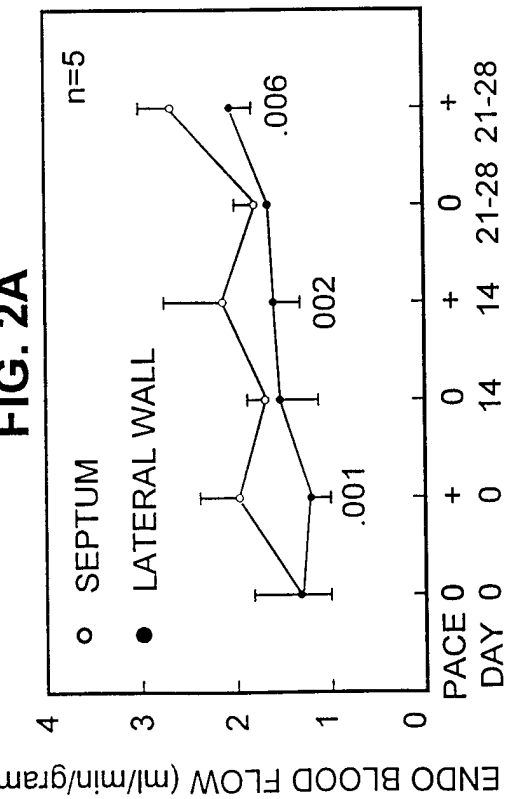


FIG. 2B

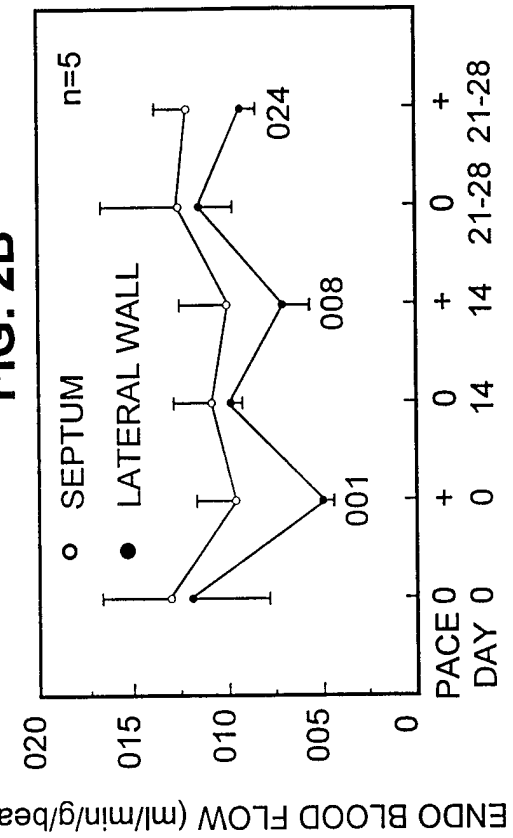


FIG. 3A

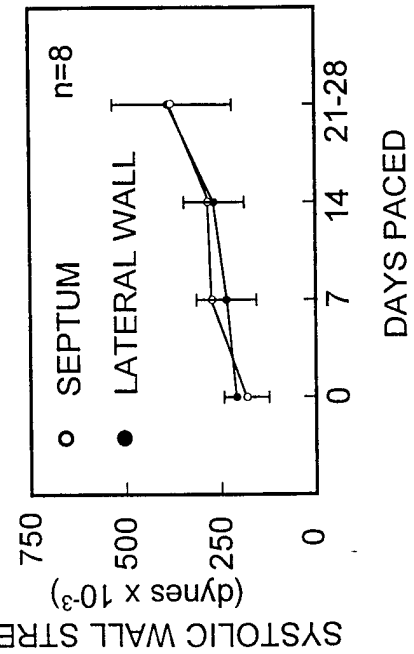
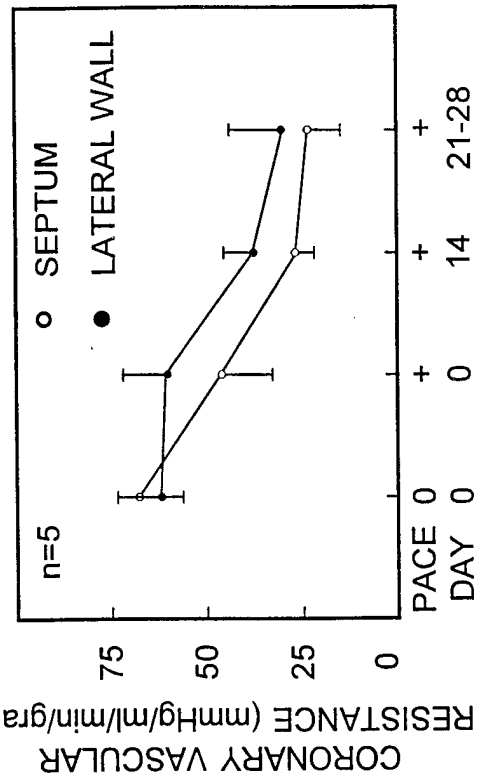


FIG. 3B



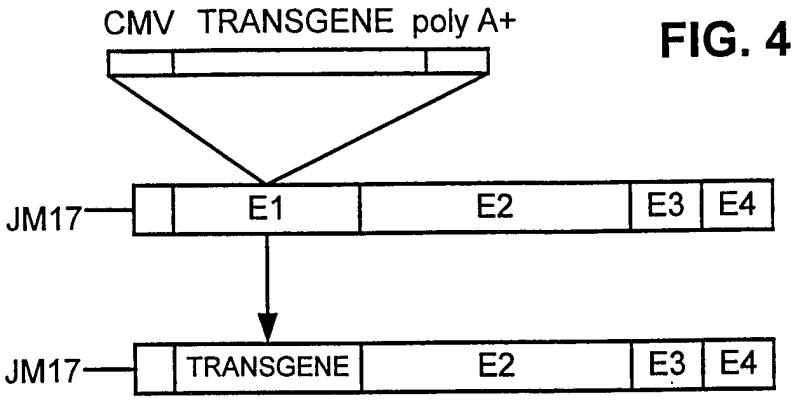


FIG. 4

E1 - DELETED RECOMBINANT ADENOVIRUS

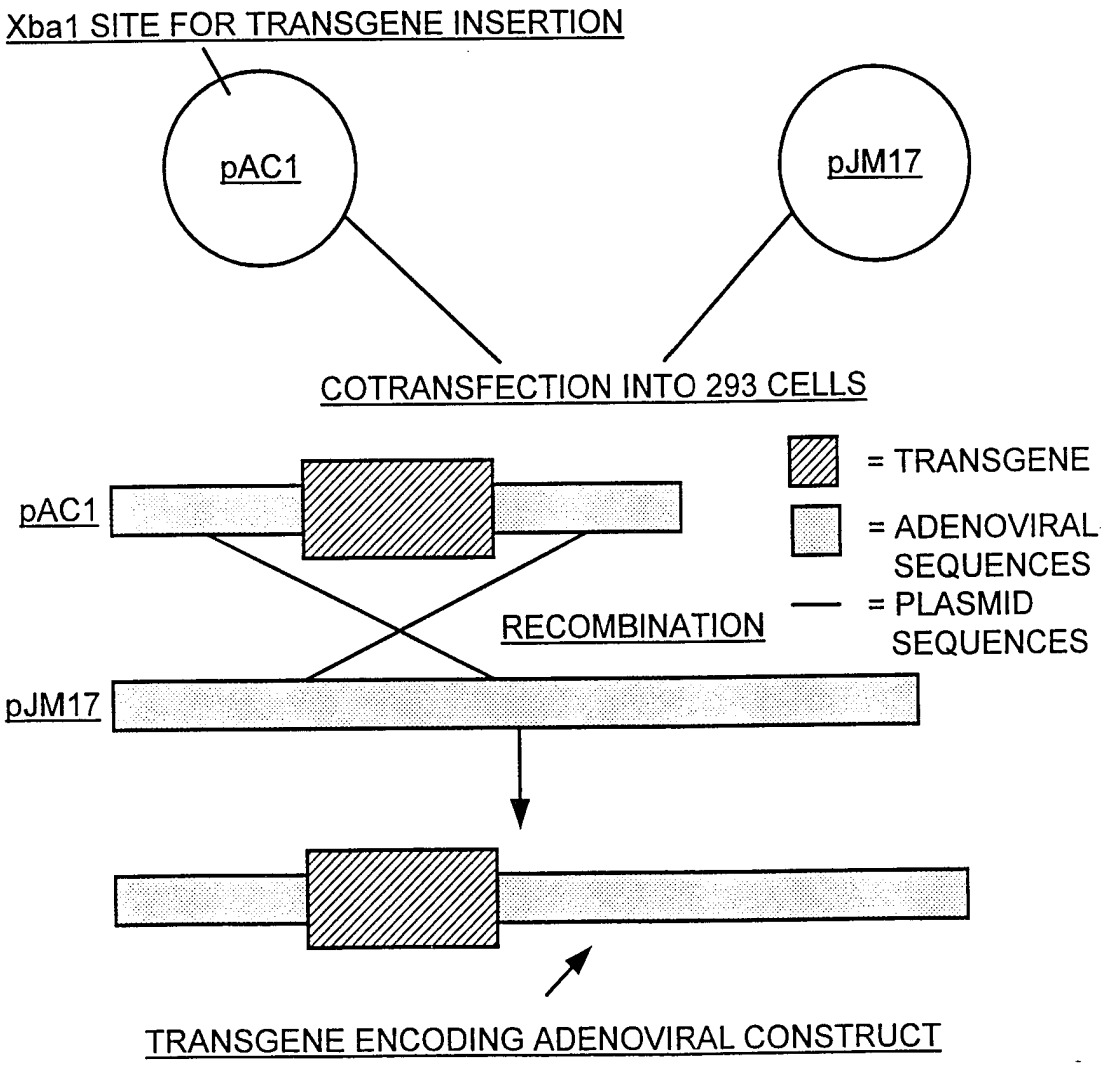


FIG. 5

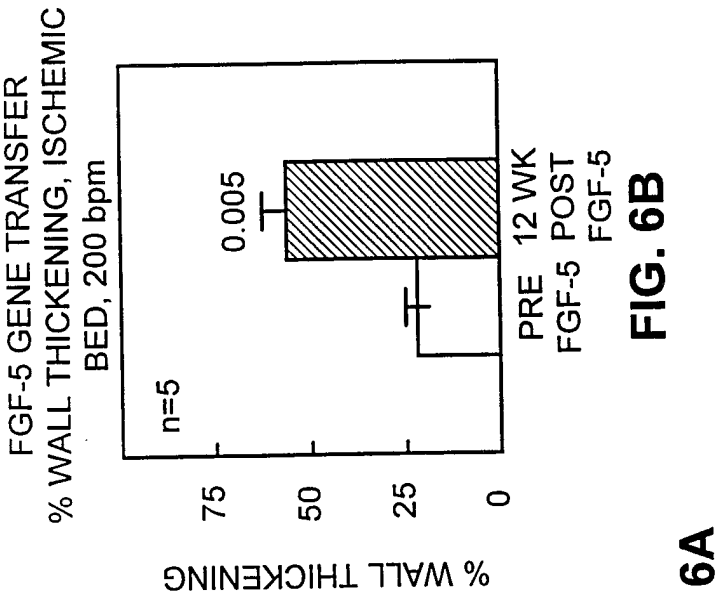


FIG. 6B

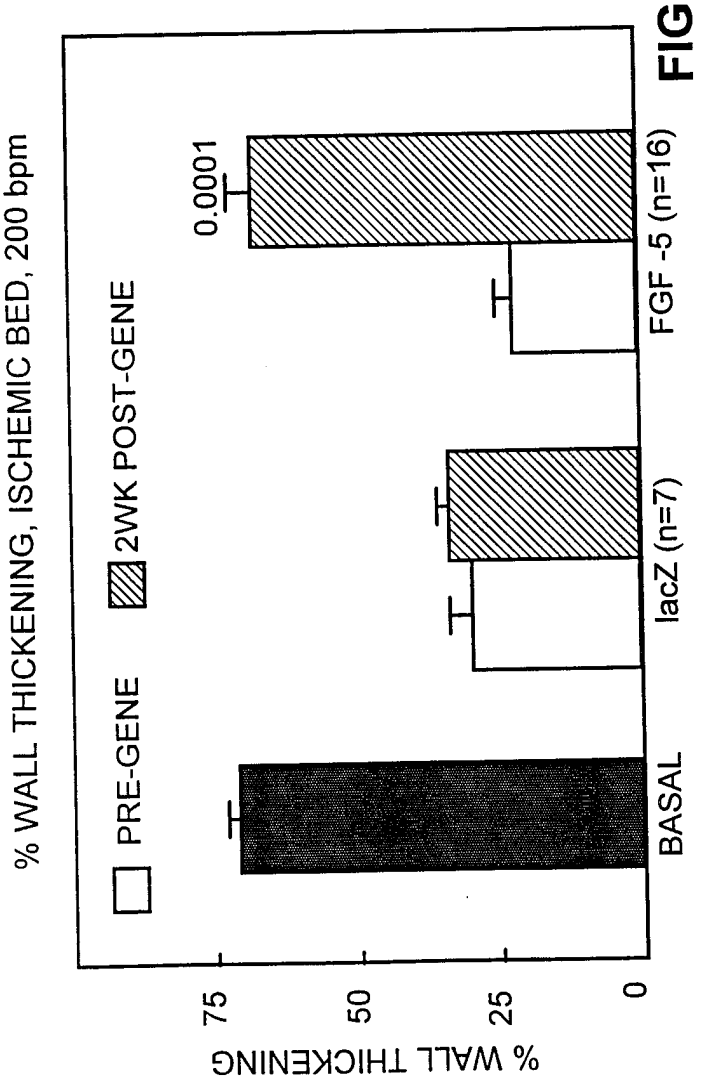


FIG. 6A

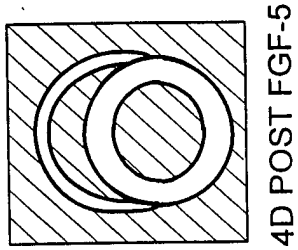


FIG. 7C

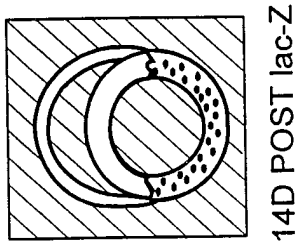


FIG. 7B

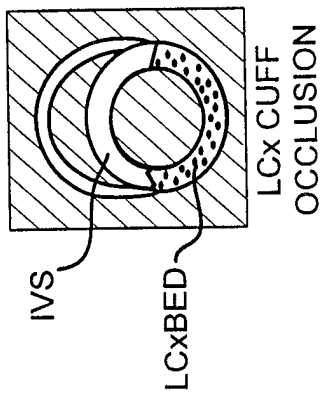


FIG. 7A

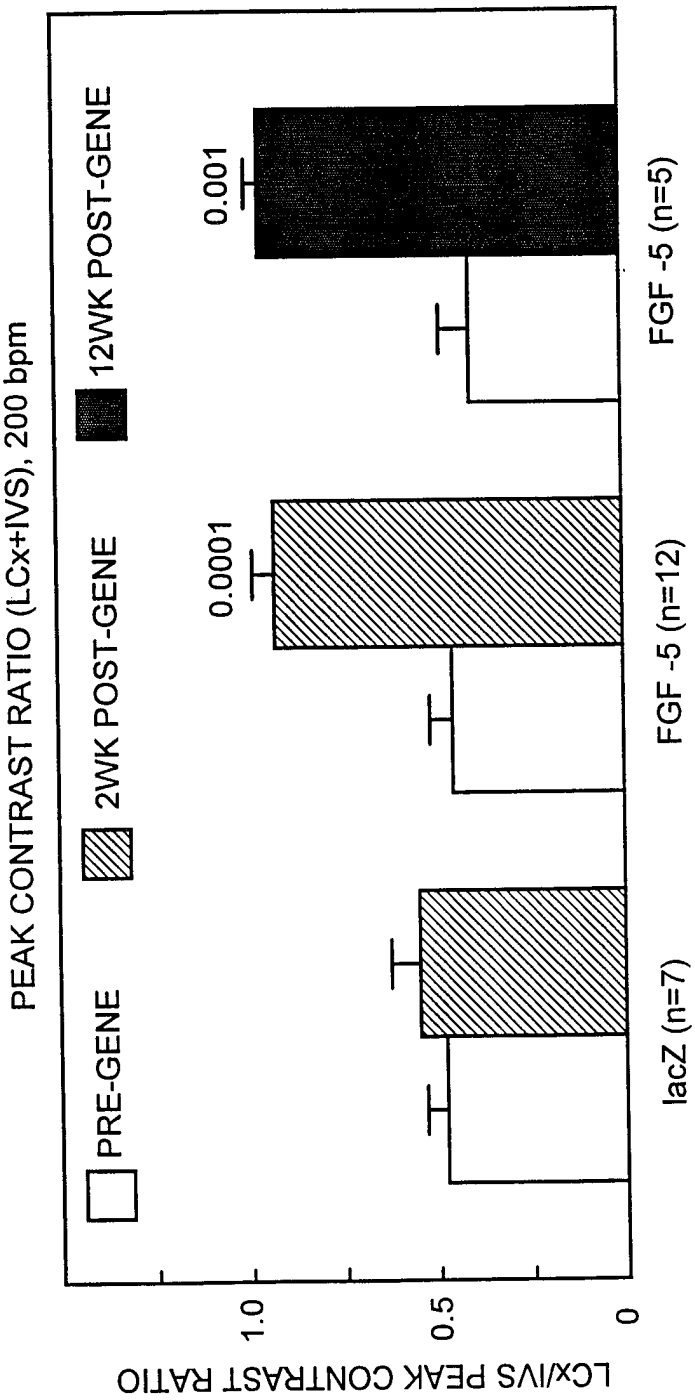

PEAK CONTRAST RATIO (LCx+IVS), 200 bpm
PRE-GENE
2WK POST-GENE
12WK POST-GENE
0.001
0.0001
FGF -5 (n=5)
FGF -5 (n=12)
lacZ (n=7)
LCx/IVS PEAK CONTRAST RATIO
1.0
0.5
0

FIG. 8

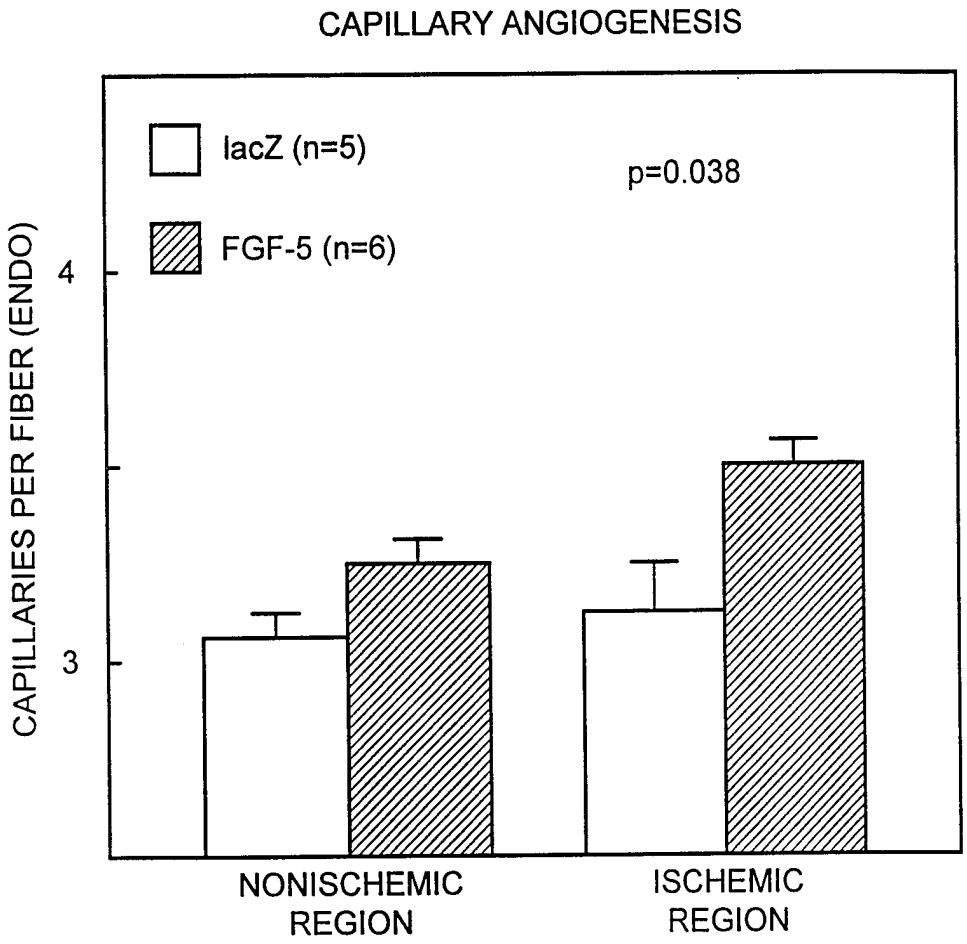

CAPILLARY ANGIOGENESIS
lacZ (n=5)
FGF-5 (n=6)
p=0.038
CAPILLARIES PER FIBER (ENDO)
4
3
NONISCHEMIC REGION
ISCHEMIC REGION

FIG. 9

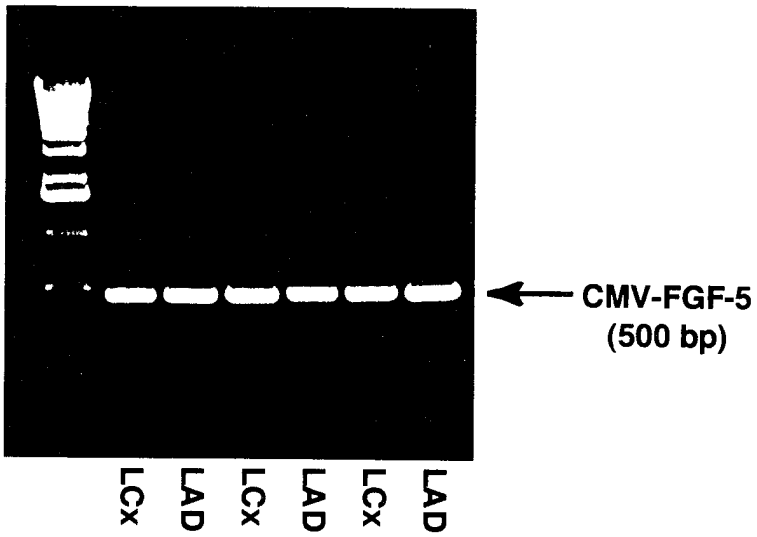


Figure 10A

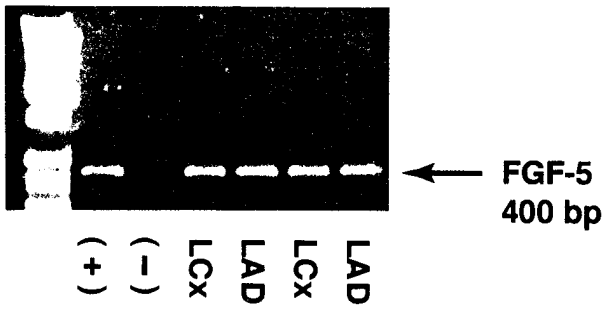


Figure 10B

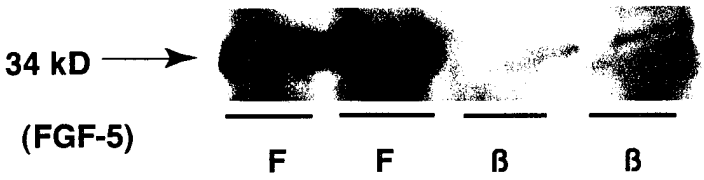


Figure 10C

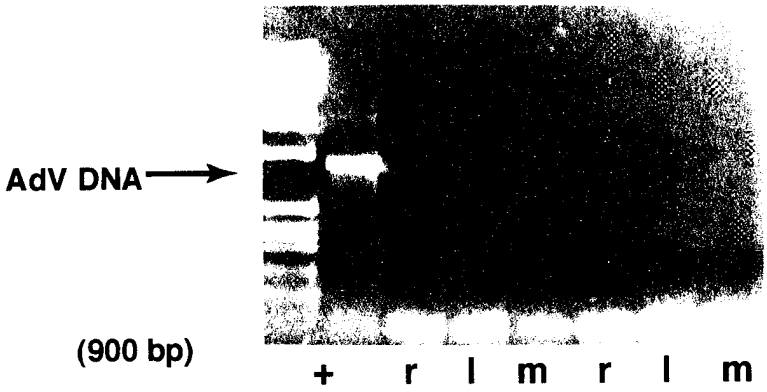


Figure 10D

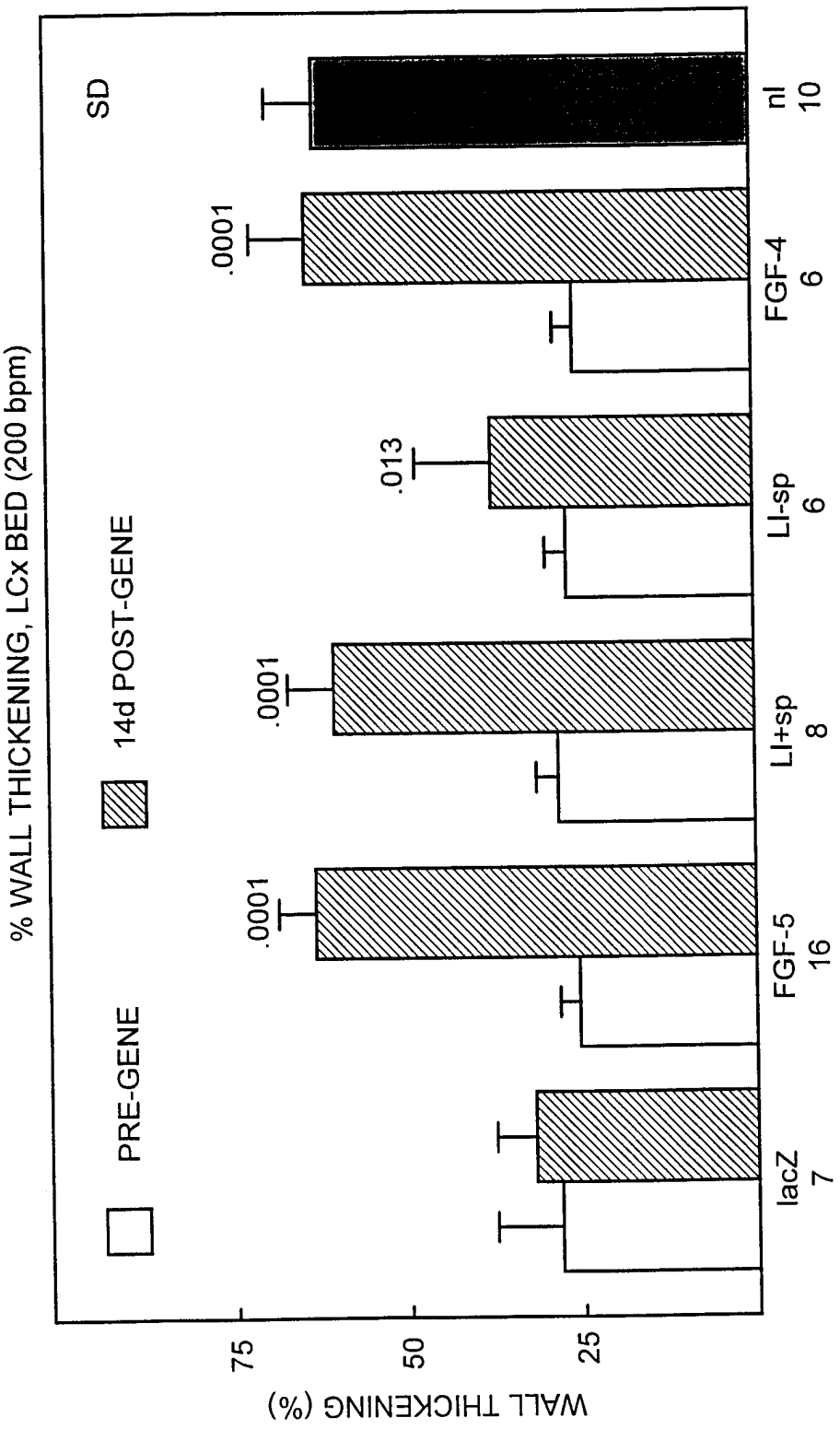

% WALL THICKENING, LCx BED (200 bpm)
SD
PRE-GENE
14d POST-GENE
WALL THICKENING (%)
75
50
25
.0001
.0001
.013
.0001
lacZ 7
FGF-5 16
LI+sp 8
LI-sp 6
FGF-4 6
nl 10

FIG. 11

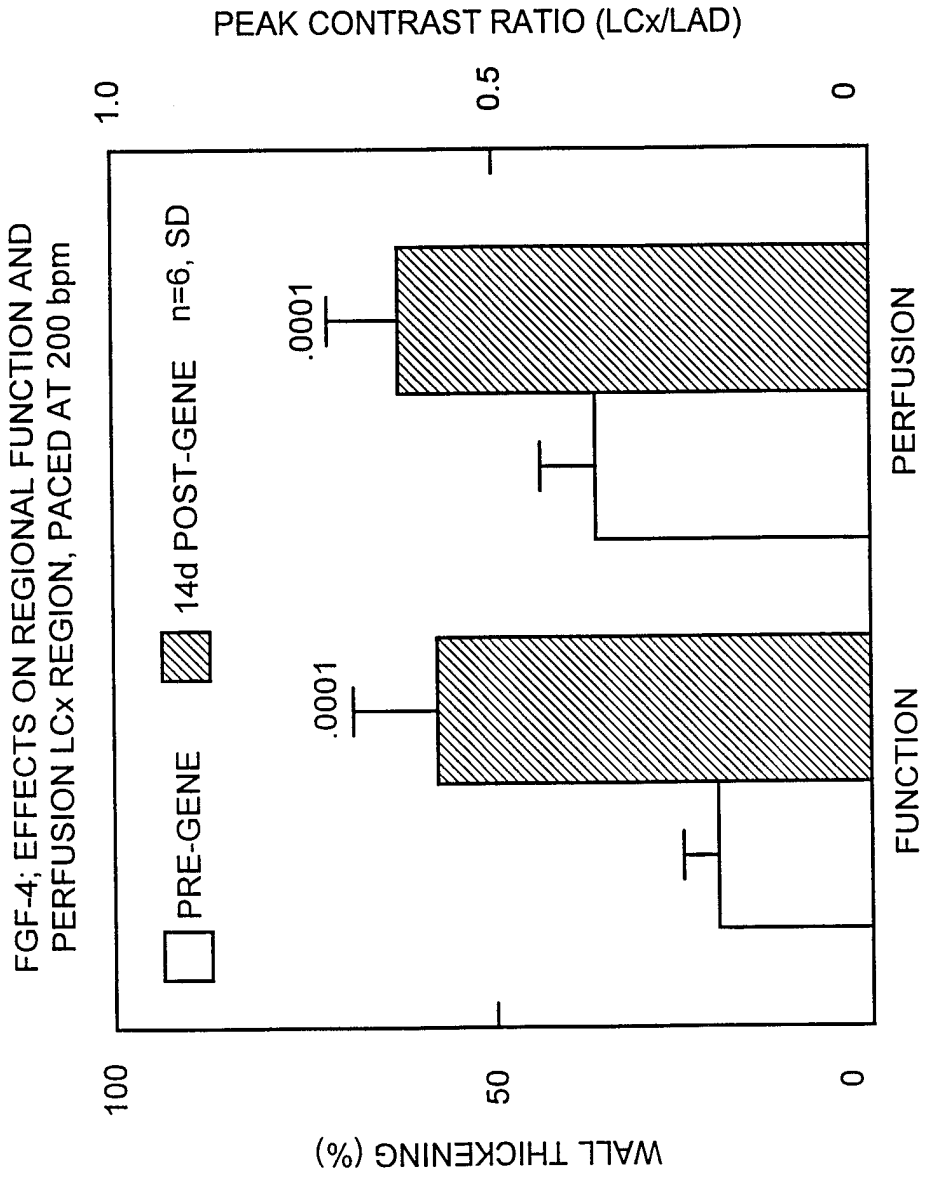

FGF-4; EFFECTS ON REGIONAL FUNCTION AND PERFUSION LCx REGION, PACED AT 200 bpm
PEAK CONTRAST RATIO (LCx/LAD)
1.0
0.5
0
PRE-GENE
14d POST-GENE n=6, SD
.0001
.0001
PERFUSION
FUNCTION
100
50
0
WALL THICKENING (%)

FIG. 12

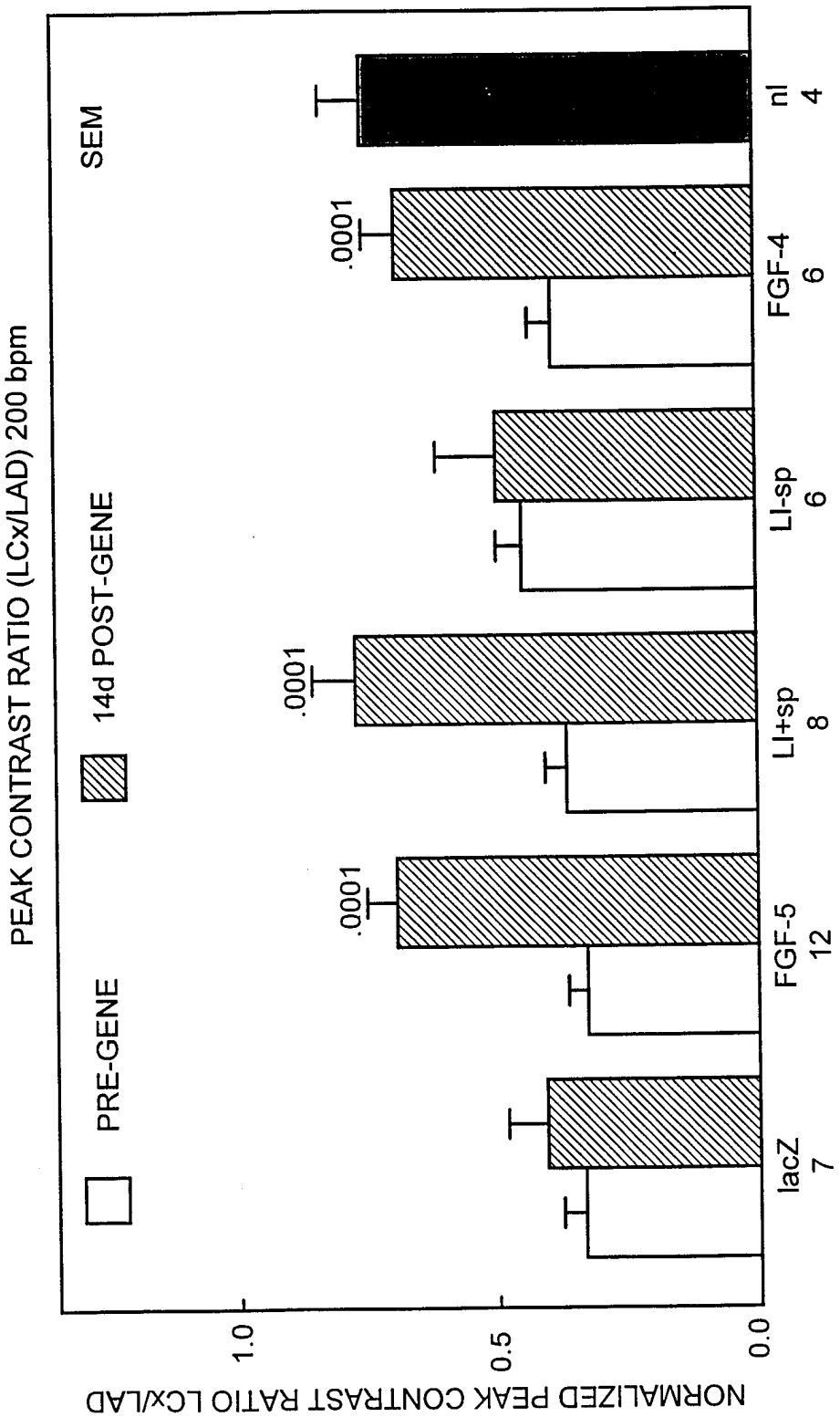

PEAK CONTRAST RATIO (LCx/LAD) 200 bpm
PRE-GENE
14d POST-GENE
SEM
NORMALIZED PEAK CONTRAST RATIO LCx/LAD
1.0
0.5
0.0
.0001
.0001
.0001
.0001
lacZ
7
FGF-5
12
LI+sp
8
LI-sp
6
FGF-4
6
nl
4

FIG. 13

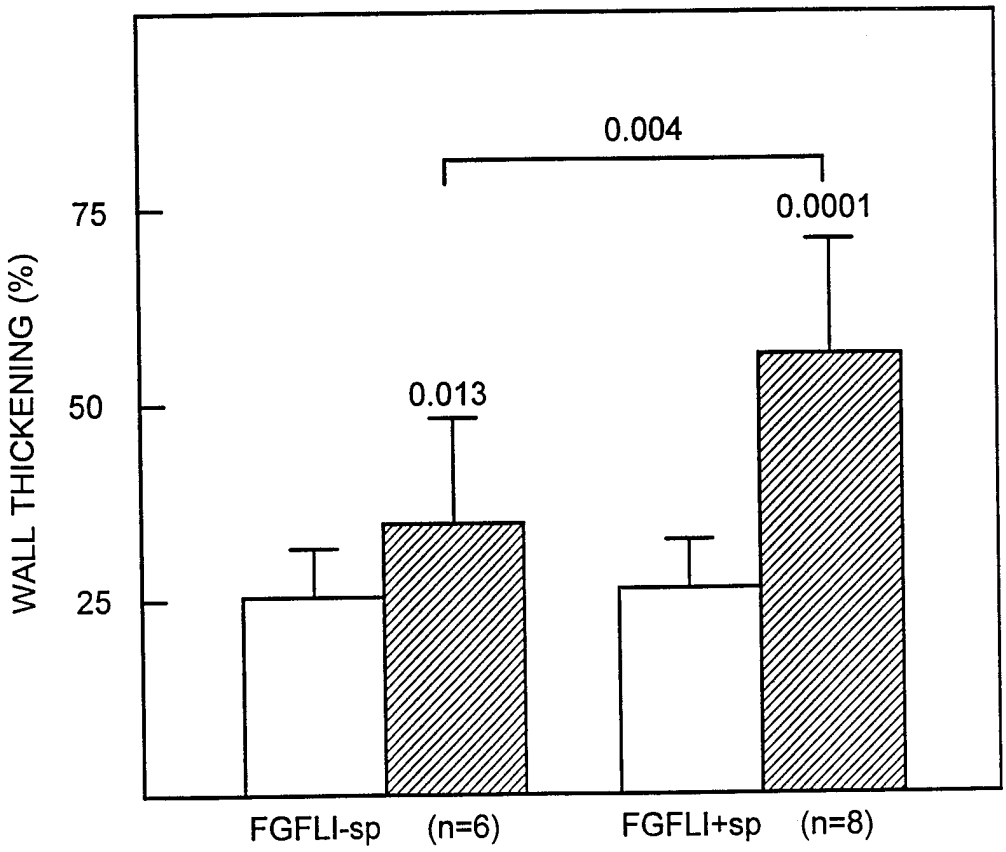

0.004
0.0001
0.013
75
50
25
WALL THICKENING (%)
FGFLI-sp (n=6)
FGFLI+sp (n=8)

FIG. 14