Toward Molecular Strategies for Heart Disease

— Past, Present, Future —

Kenneth R. Chien, MD, Ph D^{1,2}; Mitsuyuki Shimizu, MD, Ph D^{1*};
Masahiko Hoshijima, MD, Ph D¹; Susumu Minamisawa, MD, Ph D¹;
Andrew A. Grace, Ph D, MRCP^{1*}

The past two decades of cardiovascular biology and medicine have been based largely upon the consideration of the heart and vasculature as an integrated physiological system, a view that has resulted in major therapeutic advances. With the advent of developments in gene transfer, mouse and human genetics, genetic engineering of intact animals, and molecular and cellular technology, cardiovascular medicine is now on the threshold of a molecular therapeutic era! Major steps have been taken toward unraveling the molecular determinants of complex, integrative, and polygenic cardiovascular disease states, including atherogenesis, hypertension, cardiac hypertrophy and failure, congenital heart disease, and coronary restenosis following balloon angioplasty. Our improved understanding of the fundamental basis of these important cardiovascular disease processes has established a scientific foundation for diagnostic, prognostic, and therapeutic advances in the mainstream of cardiovascular medicine.

(*Jpn Circ J* 1997; **61**: 91–118)

Key Words: Genetics; Cardiology; Heart disease

This article, which is an abridged version of a chapter in the Fifth Edition of Braunwald's Textbook on Heart Disease, has been designed to provide a few selected examples that will highlight the breadth and growing impact of the molecular sciences on the practice of cardiovascular medicine. A set of principles of molecular and cellular cardiology will be discussed, including the practical development and application of new, recombinant proteins; approaches for rational drug design; isolation of human disease genes and modifiers by positional/candidate cloning; generation of disease models in genetically-engineered animals; and steps towards gene therapy. In addition, a synopsis of recent molecular advances in our understanding of the molecular biology of cardiac hypertrophy will be highlighted. A primer of basic molecular biology can also be found in a number of specialized texts and reviews devoted to molecular and cellular biology^{2–4}. Although the scope of this chapter does

not allow for a comprehensive analysis of this rapidly-expanding area an upcoming, companion text⁵ to Braunwald's Heart Disease will provide further background to the themes underlying the impact of molecular advances in cardiovascular medicine.

Recombinant Protein Therapy

The production of clinically valuable recombinant proteins and their subsequent manipulation using protein engineering is one of the major medical applications of recombinant DNA technology, and has given rise to the biotechnology industry, which has been based upon the need for large-scale protein isolation and purification⁶. In medical applications, the recombinant protein can be the therapeutic agent, designed for replacement therapy where disease is based upon an acquired or genetic deficiency of a specific protein, as in diabetes mellitus or hemophilia.

Department of Medicine¹ and Center for Molecular Genetics² University of California, San Diego, La Jolla, California 92093, USA

K.R.C. is supported by grants from the National Institutes of Health, National Heart, Lung, and Blood Institute

Address Correspondence to: Kenneth R. Chien, MD, Ph D, Professor of Medicine, University of California, San Diego, Department of Medicine, 0613-C, 9500 Gilman Drive, La Jolla, CA 92093-0613, USA

*Current Address: M. S.: The Jikei University School of Medicine, Department of Internal Medicine (III), 3-25-8, Nishishinbashi, Minato-ku, Tokyo 105, Japan; A.A.G.: University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge, CB21QW, UK

Alternatively, the therapeutic application may be based upon the promotion or inhibition of a specific biological pathway, eg, stimulating erythrocytosis in chronic renal failure.⁷ At present, a number of recombinant proteins have documented clinical utility, including insulin, factor VIII, erythropoietin, and hemopoietic growth factors.^{7,8} While tissue plasminogen activator was the first product of protein engineering designed for clinical use in the cardiovascular field,^{9,10} a number of other potentially valuable proteins will be tested in the next few years.^{11,12}

The techniques for large-scale production of proteins have necessitated industrial-scale approaches, but fundamentally rely on conventional molecular technology utilizing gene cloning, expression, and mutagenesis to manipulate and match protein structure to particular tasks. Producing a recombinant protein first requires the cloning of the gene encoding the protein of interest, construction of an expression vector, and transfer of the gene construct into a surrogate cell system that is programmed for large-scale synthesis.³

Further Applications of Recombinant Protein Engineering

The production of sufficient quantities of clinical-grade tPA for widespread use in thrombolysis represented a dramatic entrance for recombinant technology into the practice of cardiology. Second-generation plasminogen activators have followed, but truly rational protein design is dependent on 3-dimensional structural analysis,^{6,10} and it is not currently possible to precisely deduce tertiary protein structure from the primary amino acid sequences.⁶ However, there have been technical advances in both the practical methods of protein structure determination, using both X-ray crystallography and nuclear magnetic resonance spectroscopy (which has the advantage of allowing structure determination in solution), and parallel advances in computer modeling that now allow improved prediction of ternary structure that should enhance rational approaches to protein engineering, and which should prove particularly valuable in regard to multi-domain proteins such as tPA.⁶ Such approaches are likely to result in improvements in mutant design of existing molecules in addition to allowing the production of other recombinant molecules.

In this regard, several other recombinant proteins have been designed for cardiovascular use and are currently being tested in the appropriate clinical settings. Hirudin was initially isolated from the medicinal leech, *Hirudo Medicinalis*, but recognition of therapeutic potential coincided with the leech

becoming endangered!¹¹⁻¹³ Hirudin is the prototypic, direct thrombin inhibitor, binding both the active catalytic site and the substrate recognition site (anion exosite) of thrombin.¹²⁻¹⁴ It has several putative advantages over heparin, including an ability to inactivate clot-bound thrombin, and theoretical advantages of lack of inactivation by either heparinase or platelet factor 4, and no dependence on antithrombin-III.¹² The recombinant molecule has similar in vitro and in vivo anticoagulant, anti-platelet, and anti-thrombotic actions to those identified in the naturally-occurring molecule.¹¹

In addition to agents directed to the vessel wall, the development of recombinant peptide-derived growth factors that promote angiogenesis such as the fibroblast and vascular endothelial cell growth factors (FGF, VEGF)¹⁵; or stimulate specific hematopoietic lineages such as thrombopoietin⁸ have potential clinical utility, and will be examined in clinical studies in the next several years.

One other approach to rational antagonism uses genetically-engineered monoclonal antibodies produced from cell lines secreting single antibody species of desired specificity.^{16,17} This technology has been advanced by the development of mutant antibodies, antibodies of dual-specificity, and rodent antibodies humanized by linking rodent immunoglobulin variable regions to human constant regions (chimeric humanized antibodies), thereby serving to reduce immunogenicity.¹⁸ The 2 major categories of monoclonal antibodies applied in cardiovascular medicine, viz. anti-fibrin and anti-platelet antibodies ($\alpha_{IIb}\beta_3$ and thrombospondin), have, however, not yet found widespread application (see below).

Rational Design and Development of Pharmacological Antagonists

Pharmaceutical development has traditionally relied on large-scale screening of natural sources for the identification of candidate compounds¹⁹ but molecular technology now indicates a likely change of fundamental approach. Recombinant protein and therapeutic antibody technology has had a clear impact on the development of new synthetic agonists and antagonists aimed at specific molecular targets causally related to disease phenotypes.²⁰ The molecular cloning of target molecules has led to rapid throughput screening approaches to identify appropriate antagonists. Molecular modeling of the compound and protein targets can now be employed to maximize the efficiency of identifying structure-function relationships and designing families of related compounds.^{6,19}

$\alpha_{IIb}\beta_3$ Platelet-Specific Integrin

The $\alpha_{\text{IIb}}\beta_3$ complex, a member of the widely-distributed integrin supergene family of heterodimeric membrane proteins²⁹ is an early specific marker of the megakaryocyte lineage and the most abundant platelet integrin (density $\sim 50,000$ per platelet²¹). These integrins play key roles in cell adhesion events, and are important in the migration, proliferation, and differentiation of several cell types^{25,26}. Integrins are divided into 3 families, each possessing a common β sub-unit, but with different α sub-units; $\alpha_{\text{IIb}}\beta_3$ is a β_3 -cytoadherin family member along with the vitronectin receptor.²¹ The receptor complex of $\alpha_{\text{IIb}}\beta_3$ has 2 components: the α sub-unit (GPIIb) is a 136 kilodalton glycoprotein with light and heavy chains and a disulfide bridge (Fig 1). The light chain of 22 kilodaltons allows anchorage and the extracellular

Fig 1. Schematic of structure and function of platelet membrane integrin $\alpha_{IIb}\beta_3$. Notable features of β_3 are the extracellular $-\text{NH}_2$ terminus; disulphide loop which binds $-\text{NH}_2$ to the midregion; typical transmembrane domain; and short cytoplasmic tail. α_{IIb} consists of disulphide linked heavy and light chains. The heavy chain has 4 Ca^{2+} -binding repeats and the light chain has a transmembrane segment and a short cytoplasmic tail. Three fibrinogen binding sites are indicated: (1) β_3 109 to 130 has primary recognition for RGD sequences and secondary recognition for the fibrinogen γ -chain sequence; (2) α_{IIb} 294 to 314 has the primary recognition for the γ -chain sequence and secondary recognition for RGD; (3) β_3 211–222 recognition specificity has yet to be defined. The fibrinogen binding sites provide potential targets for planned pharmacological intervention.

In view of their role in cell interaction and in thrombus formation, molecular characterization of $\alpha_{\text{IIb}}\beta_3$, and the structural basis of the $\alpha_{\text{IIb}}\beta_3$ -ligand interaction, have become of great interest.²¹ It has been established that in unstimulated platelets, $\alpha_{\text{IIb}}\beta_3$ has a random distribution and recognizes only immobilized fibrinogen. The binding of adhesive proteins stimulates platelets, and activates $\alpha_{\text{IIb}}\beta_3$ with patches of $\alpha_{\text{IIb}}\beta_3$ becoming visible on the platelet surface, with a concomitant increase in platelet binding of fibrinogen, vWF, fibronectin, and thrombospondin. The dimeric structure of the molecule facilitates

binding to platelets leading to lattice formation and thrombus propagation²¹

There are 2 principal peptide recognition sequences on ligands that interact with the platelet $\alpha_{IIb}\beta_3$ receptor complex. The arg-gly-asp (RGD) sequence was originally defined as the fibronectin sequence responsible for cell adhesion interactions³⁰ and has been shown to interact with a sequence localized to the amino-terminal part of the β sub-unit at residues 109–171²⁸. Binding of RGD peptide recognition sequences in 2 molecules, vWF and fibrinogen, activates signals for the initiation of cell spreading, granular secretion, pro-coagulant activity, and conformational changes in the $\alpha_{IIb}\beta_3$ complex³¹. Conformational change results in exposure of the fibrinogen-binding site on the receptor complex. The second binding sequence is the lys-gln-ala-gly-asp-val sequence localised to the carboxy terminus of the γ chain of fibrinogen²⁸ and probably mediates the main binding mechanism for fibrinogen²¹ (Fig 1).

$\alpha_{IIb}\beta_3$ Inhibition

The approach to developing inhibitors of the $\alpha_{IIb}\beta_3$ system have employed a broad range of technologies, including the generation of monoclonal antibodies which can neutralize receptor function, identification of naturally-occurring antagonists³², and the development of synthetic antagonists based upon knowledge of the binding characteristics of the $\alpha_{IIb}\beta_3$ receptor RGD peptides which may also function as partial agonists to generate high affinity ligand-binding states.

Naturally-occurring peptide inhibitors (disintegrins: echistatin, trigramin, aploggin, kistrin, bitan, barbourin)^{32–34}; isolated from puff adder and viper venoms, contain proteins usually with the common RGD sequence that reversibly inhibits in vitro platelet aggregation. Binding is often rapid (kistrin) and with moderate affinity (K_d is $\sim 10^{-7}$ M). Barbourin displayed higher selectivity for $\alpha_{IIb}\beta_3$, which is conferred by a small change in the recognition sequence compared to other disintegrins³⁵. The switch of the Lys to the Arg (KGD sequence) is responsible for this high selectivity and the interaction of KGD with $\alpha_{IIb}\beta_3$ may be unique³⁵.

Recognition of natural inhibitors and delineation of their binding characteristics and, in some cases, structural features³⁶ led to the development of synthetic peptides or peptido-mimetics having higher affinity for the $\alpha_{IIb}\beta_3$ complex and enhanced inhibitory activity. The special properties of barbourin led to an investigation of KGD derivatives³⁵. The synthetic heptapeptide, integrelin, has a modified KGD sequence and a high affinity and specificity for

$\alpha_{IIb}\beta_3$ ³⁵. The drug is potent, with a rapid onset of action and short biologic half-life ($t_{1/2} \sim 10$ min), and both in vitro and in vivo, inhibits platelet aggregation. The clinical utility of this agent is being tested clinically^{21,37}. The possibility of the future development of peptide-specific inhibitors based on these approaches are likely to include synthetic peptidomimetic agents (eg Ro43-5054³⁸ which may be more potent as $\alpha_{IIb}\beta_3$ inhibitors than RGD-containing peptides). Two peptido-mimetics have been tested in clinical trials (MK-383; Ro4483), but appear to be less specific³⁸.

Vascular-targeted monoclonal antibodies to the $\alpha_{IIb}\beta_3$ receptor are more potent than aspirin both ex and in vivo²¹. The technology may however be limited by potential immunogenicity problems with repeated exposure, therefore confining use to single administrations²¹. However, clinical trials are assessing the value of a single shot monoclonal antibodies in the context of acute coronary angioplasty^{39,40}.

Localization of Cardiovascular Disease Genes

The ability to characterize the genetic modifiers which maintain complex cardiovascular function, as well as to identify specific cardiovascular disease genes in monogenic disorders, is a central goal in molecular cardiology. The accelerated application of molecular genetics to human cardiovascular disease is covered in chapter 49, Fifth Edition of Braunwald's Textbook on Heart Disease. Here, we review general principles, indicating how genetic information is extracted and then integrated with other data sources to allow mechanistic insights into cardiovascular physiology and pathology.

Principles of Gene Localization and Gene Product Analysis

The starting point for the identification of the gene responsible for a particular phenotype is the precise definition of that phenotype. The genetic study of human disease poses several problems, but has the advantage of large numbers of well-characterized wild-type and mutant phenotypes^{41,42}. The identification of human disease genes can follow one of 2 basic strategies (Fig 2). Functional cloning allows the identification of the disease gene on the basis of functional knowledge, and the isolation of the gene is based upon the definition of a precise, usually biochemical, defect⁴¹. This method is not applicable to most mutations of the cardiovascular system, which have no biochemical or cytogenetic correlate that can be readily assayed from a clinical phenotype⁴³. The major

Fig 2. Paradigms for functional and positional cloning. Comparison of the functional and positional cloning of disease genes. In functional cloning, the study of the gene function precedes gene identification. In positional cloning, gene mapping precedes gene identification. The last step in each case (gene mapping and defining gene function, respectively) are not critical for the isolation of the disease gene itself. (Modified and used with permission from Collins, FS. Positional cloning; lets not call it reverse anymore. *Nature Genet.* 1992, 1:3; reprinted with permission.)

cardiovascular disease phenotypes each represent a complex interaction between genetic and environmental influences⁴¹

Positional cloning assumes no functional information and is instead dependent on defining the position of the gene on a map of the entire human genome, the identification of the primary structure of the gene, and a determination of the structure, tissue-distribution, and expression of the gene product^{41,43}. This approach had recently become dominant, but as information derived from the human genome project becomes available, the identification of candidate genes within a given region should greatly facilitate disease gene isolation (positional candidate approach)^{41,43}. It has been predicted that this modified approach, combining elements of both functional and positional cloning, will become the principal mode of disease gene identification⁴³. This, in conjunction with appropriate biological assays for candidate gene expression, should allow further rapid advances in identifying single gene diseases that affect the cardiovascular system (Table 1).

The human genome. The first step in the characterisation of a human gene usually involves the identification of the position of that gene in the genome, the overall structure of which is best described by a map⁴¹. The premise upon which positional cloning is based suggests that the gene is located at a specific location on one of the chromosomes⁴². The requirement for the map is imposed by the size of the human genome⁴⁴. The map may be physical or genetic, and is constructed by observing the inheritance of markers that can be used as points of reference in offspring after well-characterized matings⁴¹. The quality and density

of markers is the major determinant of its potential utility but until recently markers were not ideal and the map of the human genome was characterized as being like a desert with only the occasional polymorphic oasis⁴⁵. The first routinely used markers identified points of cleavage by restriction enzymes, known to vary within the population, and termed restriction fragment length polymorphisms, with the acronym RFLP^{41,46,47}. These are resolved following the separation of endonuclease generated fragments by gel electrophoresis and hybridization with appropriate probes using Southern blotting⁴¹.

The situation has been dramatically changed by the recognition that clusters of repeated sequences are present throughout the genome, allowing the construction of higher resolution micro-satellite based maps⁴⁵. These new maps, constructed around such short repeats, most notably containing cytosine and adenine (CA repeats) that occur approximately every 25–100 kb along the genome, are highly polymorphic⁴⁵. In addition, they are reproducible, easily scored, and readily analysed using amplification with the polymerase chain reaction and simple gel electrophoresis^{41,45}.

Positional assignment. Mapping the position of a gene depends on defining the inheritance of genetic markers, along with the genes underlying the phenotype of interest. These will show co-inheritance (genetic linkage) with markers in the immediate vicinity^{41,42}. Conversely, genes that are far apart will segregate independently with an approximately linear relationship between the distance between genes and the probability of recombination⁴¹. In an individual family, the inheritance of a marker repeatedly with the disease phenotype implies a high probability that

Table 1 Genetic Basis of Some Cardiovascular Disease Phenotypes

Disease	Cardiac phenotype	Designation	Gene			Probable mechanism
			Location	Mutations	Product	
Myocardial disease						
Familial hypertrophic cardiomyopathy	Cardiac hypertrophy, diastolic heart failure, arrhythmias, sudden cardiac death	CHM1	14q12	>25 missense	Cardiac β -MHC	Mutations all dominant-negative; mechanisms for hypertrophy, disarray, and fibrosis unknown
		CHM2	1q3	>6 missense	Cardiac TnT	
		CHM3	15q2	>2 missense	α -tropomyosin	
		CHM4	11	—	—	
Duchenne/Becker muscular dystrophy	Dilated cardiomyopathy, arrhythmias	DMD	Xp21	Deletion promoter	Dystrophin	Modified ion transport
Myotonic dystrophy	<10% clinical heart failure	MD	19	CTG repeat	Myotinin	Unknown
Long Q-T syndromes	Abnormal ECG, syncope, sudden death	LQ-T1	11p15.5	—	—	—
		LQ-T2	7q35–36	Missense/ID	HERG	↓ Repolarizing current
		LQ-T3	3p21–24	In frame ID	SCN5A	↑ Duration depolarizing current
Arrhythmogenic RV dysplasia	Monomorphic VT, Sudden cardiac death	ARVD	14q23–q24	—	—	Unknown
Vascular disease						
Marfan syndrome	Aortic root disease, valvar regurgitation	FBN1	15q21	>30 mutations	Fibrillin	↓ Fibrillin content
Supravalvular aortic stenosis (SVAS)	SVAS, peripheral pulmonary artery stenosis	ELN	7q11.23	ID/translocation	Elastin	↓ Elastin content with development
William's syndrome	SVAS, hypertension, systemic features	—	7q11.23	De novo deletion	Elastin	Contiguous gene disorder
Ehlers-Danlos IV	Aortic dilatation and aneurysms	COL3A1	2q24.3–q31	—	Type III collagen	Defects of type III collagen
Rendu-Weber-Osler syndrome	Telangiectasia, arteriovenous fistulae	—	9q33.34	—	—	Unknown

Data from Schwartz, K et al, Molecular basis of familial cardiomyopathies. *Circ* 1995; 91: 532–540; Towbin, J et al, X-linked dilated cardiomyopathy: molecular genetic evidence of linkage to the Duchenne muscular dystrophy (Dystrophin) gene at the Xp21 locus. *Circulation* 1993; 87: 1854–1865; Keating, MT. Genetic approaches to cardiovascular disease. *Circ* 1995; 92: 142–147; Rampazzo, A et al, The gene for arrhythmogenic right ventricular cardiomyopathy maps to chromosome 14q23–q24. *Hum Mol Gen* 1994; 3: 959–962; Shovlin, CL et al, A gene for hereditary haemorrhagic telangiectasia maps to chromosome 9q3. *Nature Genet* 1994; 6: 205–209; Dietz, HC and Pyeritz, RE. Molecular genetic approaches to the study of human cardiovascular disease. *Annu Rev Physiol* 1994; 56: 763–796. ID=intragenic deletion; MHC=myosin heavy chain; TnT=tropomyosin T.

the marker is in the vicinity of the culprit gene. The concepts underlying localisation are therefore simple, although in humans achieving linkage depends on pro-saic considerations, particularly the size of a family pedigree, and the quality of the available map.^{41,42} The ideal is to develop a high resolution map that covers the entire human genome, a goal of the Human Genome Project.⁴⁸ The delineation of such a map will allow the definition of the position of any gene and will thereby facilitate the analysis of the expression of that gene, and, subsequently, of the gene product.^{43,48} Present strategies require tracking a gene down to a chromosomal location of <1–3 million base pairs of DNA, containing only a few genes likely to be expressed in the relevant tissues. Once linkage is established, and now based on some knowledge of the disease it is usually possible to directly search for the disease gene of interest with some likelihood of success.^{41,42}

Gene characterization. The identification of a specific gene location allows the nature of the genetic defect to be determined.⁴¹ This is achieved either by identification of appropriate candidates, if known genes exist in that location, or by deriving the sequence of the gene in individuals having the disease phenotype and comparing these genotypes with normals. Sequencing can be manual, but more recently has been automated with fluorescent markers and laser detection.⁴⁹ The mutation in the gene can cause the loss or change of function of a given gene via several mechanisms (deletions and duplications, base substitutions, expanding tri-nucleotide repeats) and these mutations provide important information, not only for the aberrant gene, but also regarding normal function and regulation.⁴¹ Insights are then obtained into structure-function relationships of gene products and provide means to detect and analyse gene distribution in given families or in the general population.

This section provides 2 examples of the identification of important human cardiovascular disease genes, and discusses recent insights gained into the relationship between the structure of the mutant protein and the acquisition of the disease phenotype.

Molecular Genetics of Long Q-T Syndromes

The clinical presentation of long Q-T syndromes is often in the young with syncope, sudden cardiac death, abnormalities of ventricular repolarisation, and Torsade de pointes.^{50,51} The condition is familial with an autosomal dominant pattern of inheritance and high penetrance.⁵² However, the incidence remains unknown, as minimal symptoms may go unrecognized, particularly in conjunction with a normal Q-T interval.⁵³ The gene frequency may therefore be much

higher than suspected. Until recently, the electrophysiological mechanism responsible for this syndrome was unclear, and as a result, management was empirical rather than rational.⁵¹ This lack of a rational approach has nevertheless resulted in clinically-effective approaches that have served to reduce the risks of the disease in those individuals that have been identified as being at risk.⁵¹

Within a single family, considerable heterogeneity in clinical presentation and electrocardiographic manifestations can be evident, thereby producing a problem in identifying affected individuals; the prelude to genetic dissection.^{51,53} The application of a particularly stringent set of clinical criteria is usually required before successful linkage studies can be undertaken.⁴¹ Published studies have used conservative approaches to phenotypic assignment, characterizing patients in affected families as normal (if Q-T_c interval <410 msec); abnormal (if Q-T_c interval >450 msec and symptoms; or asymptomatic and Q-T_c interval >470 msec); all other patients were classified as being of uncertain phenotype.⁵⁴

Positional assignment of the first gene locus for the long Q-T syndrome was facilitated by the availability of a large, well-characterized family with the condition.^{54,55} The family had been based in a small town in Utah, since the arrival of 2 brothers from the Netherlands in the mid-nineteenth century.⁵⁴ The lineage tree was constructed and DNA from the relatives examined using restriction digestion to establish RFLP linkage. Linkage of the long Q-T locus 1 to the H-ras marker on the short arm of the chromosome 11 (11p15.5) with a Lod score of +16.43 was determined (the logarithm of the odds ratio for linkage (Lod) score is a logarithmic index of the likelihood that a disease gene is not linked to a particular genetic marker; here 1/10^{16.43}). This finding pointed strongly to the H-ras gene as a potential candidate and also provided a putative mechanistic explanation in that both the p21 ras 1 protein and GAP (GTPase-activating protein) regulate cardiac muscarinic potassium channels.⁵⁴ However, H-ras was subsequently excluded for LQ-T₁, both by exclusion of this candidate gene from the region containing the disease gene⁵⁶ and with the sequencing of the entire segment containing H-ras showing no mutations. Moreover, this 1 million base region contains channel (KCNA4 and KCNC1) and dopamine receptor (DRD4) genes that have also been excluded. Further analysis of the telomeric location of this region of chromosome 11 should reveal the defective gene.

Early reports showed linkage to 11p15.5 in all families investigated.⁵⁷ Further reports however identified other multi-generational families with autosomal

dominant long Q-T syndrome that were not linked to this locus⁵⁸⁻⁶⁰. To date, 2 additional loci have been reported with LQ-T₂ on chromosome 7q35-36, and LQ-T₃ on 3p21-24.⁶¹ In addition, 3 further families not linked to any of these loci indicated the presence of additional loci, which, although initially unexpected serve to reflect the complexity of the process of ventricular repolarization and also imply different, albeit related, mechanisms leading to a similar phenotype.⁶² The availability of the map locations opened the possibility of the identification of the disease genes, using in this case, the candidate gene approach as the phenotype of abnormal repolarisation pointed to genes encoding ion channels or modulators as likely and reasonable candidates.^{61,62}

The candidates for the second locus (LQ-T₂) at chromosome localisation 7q35-36, included a skeletal muscle chloride channel and the cardiac muscarinic ACh receptor, which were both excluded by linkage studies.⁶² In *Drosophila melanogaster*, the ether-a-go-go (*eag*) gene encodes a Ca²⁺-modulated K⁺ channel and following screening of a human hippocampal cDNA library with a mouse homologue to *eag* resulted in localisation of the human equivalent gene to the short arm of chromosome 7.⁶³ The Human Ether-a-go-go-Related Gene (*HERG*) therefore became a candidate for LQ-T₂. *HERG* mutations were identified as a cause of LQ-T₂, as there was linkage of 7q35-36, and *HERG* within affected families; *HERG* was strongly expressed in heart and *HERG* mutations associated with LQ-T₂ arose de novo.⁶² Three classes of mutations were identified including intragenic, point, and de novo. The *HERG* protein was later expressed in *Xenopus* oocytes and investigated by patch-clamp analysis.⁶⁴ The biophysical properties were those of the cardiac rapidly-activating delayed rectifier (*I_{Kr}*; K⁺ selective; activated with K⁺_o; blocked by lanthanum)^{64,65}. Further evidence of mechanism were obtained as the channel was not blocked by the methanesulfonamides (E-4031; MK-499) suggesting a separate, pharmacologically-sensitive modulating element.⁶⁴ Pharmacological sensitivity thereby resembled that of the recently cloned cardiac K_{ATP} channel where an additional sulphonylurea-responsive sub-unit is required to confer pharmacological sensitivity.⁶⁶

The third locus at 3p21-24 (Long Q-T locus 3; LQ-T₃) initially suggested 2 candidates, the Ca²⁺ channel gene (*CACNL1A2*) and the K⁺ channel modulating GTP-binding protein gene (*GNA12*), which were both subsequently excluded.⁶⁷ The mutation was however localized to another gene in this region with an intragenic deletion of the Na⁺ channel gene, *SCN5A*, which was tightly linked to LQ-T₃ in 3 unrelated

Fig 3. Mutation in the chromosome 3-linked long Q-T syndrome (LQ-T). Schematic representation of predicted topology of the protein encoded by *SCN5A* and the location of the LQ-T deletion. Genetic linkage was established between LQ-T₃ and polymorphisms within *SCN5A*, the cardiac sodium channel. Single strand conformation polymorphism and DNA sequence analyses revealed identical intragenic deletions in *SCN5A* in affected members of 2 unrelated LQ-T families. The 9 base pair deletion disrupts the coding sequence with a corresponding deletion of 3 conserved amino acids (Lys 1505-Pro 1506-Gln 1507; KPO). The deleted sequences reside in the region important for channel inactivation, with the predicted phenotype being prolonged depolarizing currents accounting for the electrocardiographic phenotype of Q-T prolongation. (From Wang et al, *SCN5A* mutations associated with an inherited cardiac arrhythmia, long QT syndrome. *Cell* 1995, 80: 805; reprinted with permission.)

families.⁶⁷ This finding again provided a mechanistic basis, since the deletions of the disrupted sequences, between D3 and D4, were known to be important in channel inactivation, delayed Na⁺ channel inactivation, and altered voltage dependence of channel inactivation (Fig 3). Thus, the deletion suggested that the LQ-T₃ phenotype may be based upon a delay in Na⁺ channel inactivation. The deletion therefore is not the result of a dominant-negative electrophysiological effect and loss of function, but is essentially an enhancement of normal function.^{67,68}

The long Q-T syndromes are therefore apparently due to genetically heterogeneous anomalies of ion channels.^{62,67,68} Phenotypic expression with delayed repolarisation and predisposition to polymorphic ventricular tachycardia are, however, generally similar.⁵¹

This molecular genetic approach therefore provides 3 openings in this condition: a basis for family screening; an advanced understanding of the mechanistic basis; and a point from which to devise improved management strategies. The syndrome also has become a paradigm for the molecular understanding for the genesis of arrhythmias due to ion channel dysfunction which might be present, in partial form, in some acquired arrhythmias.^{69,70}

Molecular Genetics of Factor V Mutation

The Factor V mutation as seen in patients with familial thrombosis provides a contemporary example of the functional candidate approach in the identification of a gene which confers susceptibility to a venous thrombosis and pulmonary thromboembolism⁷¹⁻⁷³ The molecular basis of familial thrombosis was, until recently, unclear in over 90% of affected kindreds, with several potential hypotheses, including resistance to activated protein C (APC), being advanced as potential thrombogenic mechanisms. Following the development of a clinically applicable assay for APC, inherited resistance to APC was identified as a significant cause of thrombophilia.^{71,74,75} In the assay, the normal response to the addition of exogenous APC is increased clotting time, with APC resistance being associated with no change, following APC addition.^{74,76}

The mechanism for APC resistance in affected families was initially unclear.⁷⁶ APC activity is due to specific cleavage and inactivation of the phospholipid binding factors, Va and VIIIa, resulting in the inhibition of coagulation^{76,77} (Fig 4). Several possible mechanisms were excluded, including mutated protease inhibitors functioning as APC inhibitors, functional protein S deficiency, mutation of the Factor VIII gene, antibodies to protein C, and anti-phospholipid antibodies.⁷⁶ The exclusion of these candidate molecules led to 2 outstanding possibilities: deficient function of an unknown APC co-factor, or a Factor V mutation. The correction of the defect following the addition of wild-type Factor V and the finding that Factor V from APC-resistant plasma transmitted the defect in vitro implicated Factor V as the molecular determinants of APC resistance.⁷⁶ This notion was subsequently supported by linkage studies in 2 large families that demonstrated microsatellite markers near the known location of the Factor V gene.^{72,76,78,79} Sequencing of the Factor V gene then demonstrated the mutation and the replacement of Arg 506 with a glutamine residue. The site of the Factor V mutation corresponded to one of the known APC cleavage sites in Factor Va; APC cleavage of Arg 506 correlates with the loss of Factor Va activity and inhibition of

Fig 4. Activation and inactivation of factor V and role of mutation factor V. Factor V (top) is activated by excision of the B domain by thrombin leading to the 2-chain polypeptide, Factor Va (middle). On the platelet surface, Factor Va markedly enhances the activation of prothrombin (factor II) by Factor Xa. When thrombin binds to its receptor, thrombomodulin (TM), on the surface of endothelial cells, it activates protein C (PC). Factor Va augments this action. Activated protein C (APC) inactivates Factor Va, which becomes factor Vi (bottom) in the presence of protein S by first cleaving the single peptide bond, Arg⁵⁰⁶-Gly⁵⁰⁷ (dotted line) in Factor Va, leading on to the cleavage at Arg³⁰⁶ responsible for inactivation (not shown). The mutation Arg⁵⁰⁶ to Gln reduces the sensitivity to APC, thereby perpetuating the procoagulant effects of Factor Va and predisposing to thrombosis (APC resistance). (From Hajjar, KA. Factor V Leiden - an unselfish gene? *N Engl J Med* 1994, 331: 1585; reprinted with permission of author and the New England Journal of Medicine, Massachusetts Medical Society.)

coagulation.^{72,76,78}

These findings have had a major clinical impact. Most cases of APC resistance are caused by this single-point mutation of the Factor V gene being found in >50% of all patients with inherited thrombophilia.⁷⁶ The thrombotic tendency is however variable, but estimates suggest heterozygosity in 3-7% population and homozygosity in 0.02-0.12%.^{71,80,81} The mutation therefore represents the most prevalent, well-defined

genetic defect associated with human disease⁷⁶ The finding suggests that the identification of at risk groups are now possible prospectively, prior to high-risk situations, pointing to the need for possible directed therapy.^{76,77} Despite the defect being responsible for venous thrombosis, it does not appear to confer an increased risk for arterial thrombosis.⁸²

Future Impact of Molecular Genetics in Cardiovascular Disease

The long Q-T syndromes and Factor V mutation both represent single gene defects and provide useful illustrations of the dissection of monogenic disorders. However, in general, cardiovascular disease phenotypes are complex, integrative, and polygenic^{41,83} Several genes are operative, each with a small individual contribution, and many times no single gene exerts a dominant influence. In addition, gene-environment interactions can complicate the appearance of a given disease phenotype, either positively or negatively.⁴² Thus, there is no simple correspondence between genotype and phenotype, making positional cloning difficult. In addition, there are other problems with variable penetrance and phenocopy, genetic heterogeneity, polygenic inheritance, a high frequency of disease-causing alleles, and variable transmission^{41,83} However, such complex traits in humans and model organisms are now targets for genetic analysis.⁸³ The problem is being approached with the development of new techniques (genomic mismatch scanning, representational difference analysis) allowing rapid genome analysis, computer analysis; and the use of more sophisticated polymorphic markers.⁴⁹

One other way forward in the analysis of complex traits is by the use of parallel studies in model organisms in whom planned breeding of naturally-occurring wild-type animal traits can produce models of human disease phenotypes. This approach to polygenic analysis has proven useful in hypertension with the spontaneously hypertensive rat (SHR), the stroke-prone SHR (SHRSP), and the Dahl salt-sensitive rat being representative examples.⁸⁴ In mice, strains with phenocopies of atherosclerosis^{85,86} and obesity⁸⁷ have also been generated. The major effort to generate inbred strains of mice with obesity has culminated in the isolation of a gene with a human homologue that may influence body fat stores.⁸⁸

Obesity-diabetes syndrome. Using similar methods, at least 6 locations on 5 chromosomes producing obesity-diabetes syndromes with both recessive (diabetes (db), tubby (tub), fat (fat)), and dominant (agouti (AyAvy) adult obesity /diabetes (db)) mutations have been identified. The ob gene is the best

characterized and has been cloned and sequenced.⁸⁷ Ob homozygous mice have morbid obesity with a 3-fold increase in body weight vs controls. Positional assignment of the ob gene initially was to a 650 kilobase region on chromosome 6, containing 6 potential candidate genes, one of which was found to be expressed in adipose tissue of normal mice but not in the obese mice.⁸⁸ The gene ob encodes a 4.5 kilobase mRNA with a highly conserved 167 amino acid open-reading frame.

Obese mice have a premature stop codon, resulting in the truncation of the translated protein. The predicted gene product has not been identified, but sequence data predict a secreted protein.⁸⁸ In addition, there is an 84% identity with the human homologous sequence.⁸⁸ The data fit well with the well described physiological models and based on available information it is therefore suggested there is a signaling pathway from adipose tissue with hypothalamic action with long-term inhibition of the desire to eat regulating size of the body fat depot.⁸⁸ Ob mice have deficient function of the secreted protein. The function would appear to be highly conserved and naturally leads on to the analysis of the function of the ob gene in humans.^{87,89,90}

Information obtained using animal models will be interpreted in conjunction with existing information on physiology and further studies of the human genotype.⁸³ Ultimately, the application of information accruing from the availability of the human genetic map will make a significant difference in the isolation of disease genes. The generation of a catalog of genes will transform positional cloning to a systematic analysis of candidate genes.^{41,43} Rigorous analysis and association studies could then be used to correlate functionally relevant allelic variations with disease risk. The initial intention is to obtain a high-resolution map of the human genome by the end of the decade, but the definition of the sequence map will remain some way off with <0.1% determined to date.^{43,48}

Genetic Models of Human Cardiovascular Disease

The study of the mechanisms of human disease and the design of new treatments depends, to a significant extent, on the application of animal research. Spontaneously-occurring animal models of human disease^{91,92} may be difficult to obtain in the numbers needed for controlled experiments, have complex phenotypes based on the actions of multiple genes, and are often expensive to raise to the point where the phenotype is tractable. Each of these characteristics

Table 2 Some Murine Transgenic Models of Atherosclerosis*

<i>Intervention</i>	<i>Phenotypic features</i>
<i>Atherosclerosis-prone</i>	
<i>Human apoAII overexpression</i>	<i>ApoAII 20% HDL protein content. Phenotype has no elevation in HDL-C. Despite normal HDL-C risk of atherosclerosis increases with fatty streak development even on low-fat diet. Indicates qualitative features of HDL-C are also important.</i>
<i>Human CIII overexpression</i>	<i>First animal model of primary hypertriglyceridemia. Triglyceride level proportional to CIII expression. Primary abnormality decreased VLDL fractional catabolic rate.</i>
<i>Human AI, CIII, CETP overexpression</i>	<i>High triglyceride, low HDL-C phenotype. Comparable to most common lipoprotein disorder conferring susceptibility to CAD in humans.</i>
<i>Human apo (a) overexpression</i>	<i>Apo(a) mice developed 20 times greater area lipid-rich lesions than control mice (outbred genetic background 3 months on atherogenic diet). <5% apo(a) associated with lipoprotein; suggested apo (a) produces pathology independently of LDL.</i>
<i>Apo E deficient</i>	<i>See text</i>
<i>LDL-receptor knockout</i>	<i>Homozygous mice have T_{chol} > twice normal litter mates with 7–9 times increase in LDL and IDL. Normal triglycerides. Correction with adenovirus-mediated gene transfer LDL-R protein. Increased atherosclerosis.</i>
<i>Atherosclerosis resistant</i>	
<i>ApoE overexpression</i>	<i>See text</i>
<i>Human LPL overexpression</i>	<i>LPL directed by the chick β-actin promoter produced accelerated VLDL clearance and resistance to diet-induced hypercholesterolemia</i>
<i>Human LDL receptor overexpression</i>	<i>Radiolabelled LDL clearance increased 8–10 times compared to control mice. Resistant to high-fat high-cholesterol diet.</i>
<i>Human ApoAI overexpression</i>	<i>Major (>70%) HDL protein. Selective doubling HDL-C. Useful model for examining effects of diet and drugs on HDL-C/apoA-L. Resistant to fatty streak development induced by atherogenic diet.</i>

*Approximately 20 genes involved in human lipid transport have been overexpressed or knocked out in transgenic mice. Data from Breslow, JL. Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc Natl Acad Sci* 1993; 90: 8314; Ishibashi, S et al, hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 1993; 92: 883–893; Lawn, RM et al, Atherogenesis in transgenic mice expressing human apolipoprotein (a). *Nature* 1992; 360: 670–672

make them non-ideal as experimental systems.

The development of genetic models of disease therefore represents one of the most dramatic examples of the power of molecular approaches to increase understanding of cardiovascular disease mechanisms.^{93–97} Planned alterations of the murine genome allow the introduction of exogenous human disease genes, or their equivalents, or the deletion of endogenous genes.^{98,99} The resulting models of deranged normal physiology, or of disease, allow not only the investiga-

tion of underlying mechanisms, but also the effects of specific interventions on disease processes to be defined!¹⁰⁰ Significant advances in coupling these techniques to the important problems of cardiovascular biology and medicine using genetically-based models of developmental defects,¹⁰¹ atherosclerosis,^{86,102} hypertension,¹⁰³ hypertrophy and cardiac failure¹⁰⁴ in conjunction with traditional analytical techniques, can provide the details of integrated physiological mechanisms.^{97,104–108}

Transgenic Phenotypes

Several cardiovascular diseases have been modeled using transgenic and gene-targeting technology.⁹⁵ Although other species are amenable to transgenic techniques, such as swine,¹⁰⁹ rabbit¹¹⁰ and rat,¹⁰³ most have been created in mice due to several intrinsic advantages including the availability of ES cell technology, inbred strains, and the wealth of information on the mouse genome.¹¹¹ The development of apoE-deficient mice provides an example of the potential value of utilising transgenic technology to engineer animal models of known cardiovascular disease.

Mouse Models of Atherosclerosis

The wild-type mouse, with some exceptions (eg strain C57BL/6), is generally resistant to atherosclerosis.^{86,102,112} Nevertheless, due to the creation of models with powerful analogies to human atherogenesis (Table 2), the mouse has become an important system for increasing our understanding of the human disease.^{86,102} The generation of mouse models has been facilitated by the fact that human genes involved in lipoprotein metabolism are usually single-copy and have often been sequenced and mapped.^{113,114} In addition to increasing understanding of the underlying processes⁸⁶ these mice can also be used in the development of drugs directed against the atherosclerotic process that can be used to predict efficacy in clinical trials¹⁰⁰; and also for testing the applicability of other interventions directed at these processes.

The most atherogenic mouse strain is the apolipoprotein E (apoE)-deficient mouse, where the apoE gene has been disrupted by homologous recombination.^{115,116} apoE is the ligand that is responsible for LDL and chylomicron remnant uptake, playing an important role in the clearance of lipoprotein particles from the circulation.⁸⁶ The knockout of the gene for apoE results in a phenotype that is a true null mutation, with no expression of apoE but has the considerable advantage that the homozygotes are both viable and fertile.^{115,116} The pattern of disease is diet-responsive and the homozygotes fed a standard laboratory chow diet (0.01% cholesterol, 4.5% fat) have a serum cholesterol concentration of 400–500 mg per deciliter with the development of foam cells in the aortic sinus at 10 weeks, whereas on a diet similar to that consumed in the United States (0.15% cholesterol 0.15% and fat 20%) serum cholesterol rises to ~ 1800 mg per deciliter, this being mostly in the VLDL and IDL fractions with triglycerides being minimally raised.^{115,116}

Animals consuming the western diet develop advanced lesions of atherosclerosis with their distribution

and histology being almost indistinguishable from human disease.^{117,118} These mice also provide a model for the study of lipoprotein oxidation with lesion oxidation-specific epitopes and antibodies to malondialdehyde-lysine in serum.^{119,120} Heterozygotes have diminished apoE with normal fasting lipids and slightly delayed post-prandial lipid clearance consistent with half normal levels of E2 being sufficient to maintain normal serum lipids.¹¹⁵ Interestingly, crossing these animals with mice which display overexpression of human transgene AI, leads to an elevation of apoAI, and HDL, and apparently alleviates atherogenicity, as it does also with apo(a) over-expressing mice.¹²¹

The physiological role of apoE is exemplified in 2 other models. The human apoE3 Leiden variant (tandem duplication amino acids 120–126) leads to type III hyperlipoproteinemia^{122,123}; overexpression of this gene in so-called E3 Leiden mice leads to a phenotype of the human disease.¹²² The apoE lipoprotein has also been over-expressed in transgenic mice leading to 4-fold increase in apoE levels. These animals have a several-fold increase in radio-labeled VLDL/LDL clearance and are resistant to diet-induced hypercholesterolemia.¹²⁴

Gene Therapy

The concept of gene therapy encompasses a broad range of diverse technologies having as a common aim the transfer and expression of specific genes to alleviate the fundamental consequences of a disease.^{125,126} These aims are inextricably linked to the development of other molecular technologies which are required for the efficient, localized and long-term expression of the transferred gene. In addition, molecular approaches are often required to identify candidate genes that have potential therapeutic value in experimental model systems.¹²⁵ While the potential impact of gene therapy for cardiovascular medicine is clear, to date, there has been no direct demonstration of the practical use for disease targets, cardiovascular or otherwise, within the clinical context.^{127,128}

Indeed significant challenges remain if gene therapy is to become a standard form of treatment for a subset of cardiovascular diseases.^{127,128} The major contemporary problem is devising technically feasible methods for gene delivery, a task which will require the co-ordinate development of several individual technologies with proven value in appropriate animal models that have fidelity to human disease.^{127,128} Once established, the specific questions that balance efficacy and safety, in comparison to existing treatments, will also need to be addressed. The denominators of the risk-

benefit equation are clearly going to be different in non-terminal diseases with high morbidity, such as cardiovascular disease vs terminal diseases, such as the acquired immunodeficiency syndrome and cancer.^{127,128} In addition, the minimal requirement for gene therapy must bear comparison to those of existing treatments having appropriate risk-benefit profiles.^{125,129} Herein, we consider the technical issues involved in the application of cardiovascular gene therapy and early steps towards its implementation.

Technical Aspects

Before gene therapy will be achieved in the clinical setting, it is necessary to document the feasibility of the controlled, sustained expression at the appropriate level of therapeutic gene/product for the required duration in the chosen location.^{125,129} Two general approaches to somatic gene therapy have been defined. First, gene transfer *ex vivo* with removal of cells, followed by gene transduction and the *in vivo* re-implantation of the modified cells. Second, *in vivo* gene transfer employing a variety of gene delivery approaches and vectors that have been applied in animal models.^{125,128,129} In the context of the cardiovascular system, such an approach implies transvascular delivery in which regard, specialized catheters have been designed.¹³⁰ This could be applied for a local effect with delivery to the vessel wall, into myocardial vasculature to facilitate improved coronary flow, or might provide a source for the release of cell-derived products for systemic gene replacement therapy *eg*, Factor VIII delivery.¹²⁸

Vector Development

The vector is the agent carrying the gene to the desired site of action, and vector choice and optimization are therefore fundamental to achieve effective gene transfer.^{125,129} The ideal vector would be relatively safe, highly efficient, display tissue- or cell-type tropism, and would afford high but controllable levels of expression for a long duration. Vectors fall into 2 general categories, *viz.* viral and non-viral, with other approaches using a combination of these delivery modes.

The main viruses considered are retroviruses and adenoviruses, with most others having confounding problems with limited host range, antigenicity, pathogenicity, problems with integration and size constraints.^{125,128,129} Replication-defective retroviruses have the advantages of a high (~100%) transduction rate of transported DNA into the host genome. However, they are relatively labile and inactivated *in vivo* in humans by complement activation. In addition,

the host cell range is limited by the requirement for cell division to allow viral integration, and not all mammalian cell types are permissive for retroviral infection.^{125,128,129} There is also an additional concern about long-term expression with the risk of oncogene activation and neoplasia.^{125,128} To date, the single most important advance in improving retroviral vectors to date has been the development of packaging cell lines able to produce high viral titers.

Adenoviruses have become important in view of the possibility of their use *in vivo*.^{125,128,131} The 2 serotypes most commonly used (Ad2, Ad5) are minimally pathogenic and have been made replication-defective by the deletion of E1A/E1B gene components.^{125,128} The viruses attach to the adenoviral glycoprotein receptor and undergo receptor-mediated endocytosis, escaping lysosomal degradation, to reach the nucleus where the delivered DNA persists un-integrated.^{125,128} The adenoviral vectors display several advantages, including an ability to infect non-dividing cells, and the capability to transfer genes *in situ*. In addition, recombinant adenoviruses can accommodate large amounts of DNA (36 kilobase-pair genome), can be produced in high titers (10^{11} ml⁻¹) and do not integrate into the genome, thereby reducing the risk of malignant transformation. The main disadvantages are of transient expression due to the humoral response following *in vivo* use facilitated by the presence of neutralizing antibodies in >20% normal individuals. Humoral immunity reduces efficacy and increases pathogenicity with tissue inflammation being a significant problem.^{125,128}

Two other viruses have been considered in cardiovascular applications. Adeno-associated viruses (AAV) are relatively small, stable, non-pathogenic and defective viruses that cannot go into the lytic stage, and have broad host-range with site-specific integration (chromosome 19) *cf.* retrovirus.¹³² It is proposed they have applications for both *in vitro* packaging and for *ex vivo* use, although producing sufficiently high titres remains a problem.¹³² Haemagglutinin virus of Japan (HJV) is an inactivated paramyxovirus which has been used with a liposome-complex (viral conjugate vector) entrapping DNA and has been suggested as an efficient way of introducing DNA into vascular tissues.¹³³

Physical methods exploit natural endocytosis mechanisms to deliver the DNA-ligand complexes targeted to cell-type specific receptors.^{125,134} Cationic liposomes are able to form large complexes with DNA, have low toxicity and are non-immunogenic allowing the possibility of repeated high-level dosing, but have problems with low efficacy and transient expression.¹³⁴

In an effort to circumvent some of these, conjugates have employed the whole virus or fusogenic viral peptides to disrupt endosomes, or modifications of liposomal composition.¹³⁴ Direct injection of DNA has also been shown to be capable of allowing recombinant gene expression, although this has been in <1% of cells around myocardial injection sites.^{128,135} The advantages suggested are that this is not *ex vivo*; there are no infectious vectors; plasmid DNA does not integrate, and there is, in general, muscle-specific gene expression.¹³⁵ Polymer gels applied to the adventitial aspect of blood vessels have been used for the application of antisense oligonucleotides.^{136,137}

Approaches to Specific Problems

Several potential clinical targets have been identified for cardiovascular gene therapy. However, the only clinical studies with direct cardiovascular relevance have been those related to the *ex vivo* therapy of monogenic familial hypercholesterolemia.^{138,139} Below, 2 additional targets are considered, on which some experimental data are available:

Angiogenesis: Angiogenesis, with new vessel formation and restoration of physiological blood flow, has been identified as a potential therapeutic option both in chronic coronary artery¹⁴⁰ and peripheral vascular disease.^{141,142} The agenda here is clearly different than in neoplastic disease and metastasis, where the aim is inhibition of angiogenesis.¹⁴³ Two approaches to achieving new vessel growth have been identified. First, the administration of recombinant angiogenic growth factor formulations directly to the vessels related to the underperfused region, to expedite or augment collateral development.^{140,144} The second approach is based upon gene transfer^{141,142} which has the theoretical advantage that the secreted gene product could have significant biological effects, with only relatively few cells transfected, following site-specific arterial gene transfer.¹²⁸ The genes encoding angiogenic factors, such as FGF, VEGF, have been cloned and mechanisms of action have been established *in vitro*.¹¹⁰ However, *in vivo* efficacy has yet to be definitively established. The expression of PDGF-B, FGF-1 and TGF β_1 genes following direct transfer into porcine arteries has been shown to induce intimal hyperplasia and neo-intimal angiogenesis.¹⁴¹ Exploring the *in vivo* feasibility and efficacy of this angiogenic approach will undoubtedly be a major area of cardiovascular research in coming years.

Inhibition of vascular smooth muscle cell proliferation: Vascular smooth muscle cell proliferation following PTCA is generally perceived as being a target for gene delivery, an agenda that found particular urgency

in the significant absence of proven pharmacological therapy.^{21,127,128,145} Inhibition of vascular smooth muscle cell proliferation has been the principal aim with other studies being designed to define the signaling pathways which mediate *in vivo* proliferation of vascular smooth muscle cells.^{128,146}

The feasibility of gene transfection *in vivo* has been suggested following studies with retroviruses¹⁴⁷ and liposomes, although both methods have a low (<1%) efficiency of transfer.¹²⁸ Adenoviruses have been reported to display an increased efficiency of reporter-gene expression (10–100 fold), but transient (7–14 days) expression is likely to limit utility.^{128,148} One of the major determinants of gene transfer with both vector systems is the state of the vessel wall, with transfection in normal vessels being restricted to vascular endothelial cells,¹⁴⁸ while in balloon-injured vessels expression in medial cells is also seen. The 2 approaches currently in use include direct gene transfer with balloon delivery *in vivo* or the implantation of grafts post *ex vivo* gene transfer.¹²⁸

Two novel methods for the selective inhibition of proliferation of actively dividing cells have been reported.^{149,150} The first approach employed a herpes virus-associated thymidine kinase suicide gene in an adenoviral vector which has been delivered into the injured pig femoral artery. The expression of thymidine kinase gene selectively in dividing cells converts gancyclovir to an active toxic form resulting in cell death.¹⁵⁰ The second approach targeted another component of the nuclear cell cycle regulatory pathway.¹⁴⁹ The expression of the non-phosphorylatable, constitutively-active form of the retinoblastoma (Rb) gene product limited vascular smooth muscle cell proliferation in both rat carotid and pig femoral arteries at >3 weeks following vascular injury.¹⁴⁹

Antisense oligonucleotides: One further potential approach to vascular proliferation is the use of antisense oligonucleotides.^{151,152} These are short chain nucleic acids (10–39 residues) which bind to a targeted complementary region of mRNA (sense strand) and prevent translation, thereby blocking the expression of a specific gene.^{151,153} This approach has the theoretical advantage of the inhibition of abnormal genes, while leaving normal genes intact, but their use has been associated with several technical difficulties.¹⁵³ The oligonucleotides cross the cell membrane with low efficiency, they are degraded rapidly in the circulation, and no efficient method of drug delivery has been devised.¹⁵¹ In addition, the antisense oligonucleotides often display non-specific activity at high concentrations, which complicates interpretation of experimental results.¹⁵³

Fig 5. Signaling mechanisms of cardiac hypertrophy as identified in in vitro model. Three principal putative mechanisms (GP130; Ras; Gq) leading to the hypertrophic phenotype are shown along with the retinoid suppressor pathway defined in vitro. Details are indicated in text. GP130, glycoprotein cytokine signaling molecule; JAK, janus kinase; IGF₁R, insulin like growth factor-1 receptor; TK, tyrosine kinase; α_{1a} -R, alpha-1 adrenoceptor; G_q, G protein (G_s) subunit; PKC, protein kinase C; Ras, signaling protein responsible for translocation Raf to cell membrane; Raf, principal effector of the ras pathway; ERK, mitogen activated protein kinase; RXR, retinoid X receptor; RAR, retinoid A receptor; RA, retinoids: all trans- and 9-cis retinoic acid.

The published studies have thus far reported biological effects, but no direct relevant effects on the production of either the protein or the mRNA.¹⁵¹ The use of antisense oligonucleotides to the proto-oncogenes c-myc,¹³⁶ c-myc,¹³⁷ cdc2 kinase, and proliferating cell nuclear antigen¹³³ have been reported. The improvement of stability facilitating administration (chemical modification, liposomal conjugates etc.), increases in in vivo biological half-life, and improved specificity will all be required before more widespread application.¹⁵¹

Prospects for Gene Therapy

Although currently immense difficulties need to be addressed to allow routine use of in vivo gene therapy in the cardiovascular system, this approach nevertheless represents one of the areas in which the molecular sciences could impact cardiology in the next millennium. Most likely, the first application of gene therapy will be in gene replacement therapy for patients harboring specific genetic deficiencies of circulating proteins, such as Factor VIII deficiency (hemophilia). Vascular targets may continue to be attractive but

will require critical comparison to other therapeutic approaches with regard to the inhibition of restenosis.¹²⁸ Cardiac muscle diseases may be more difficult to approach, given the requirement for the transfer and long-term expression of a given gene in sufficient numbers of muscle cells to effect a global change in chamber function, and the potentially arrhythmogenic effects of heterogeneous expression.¹³⁵

Molecular Advances in Cardiac Hypertrophy

The past decade has seen the accumulation of a large body of evidence from both basic and clinical research that is beginning to reshape our view of the failing heart.¹⁵⁴ The result is that the heart failure syndrome can no longer be viewed, strictly, as a problem of altered peripheral hemodynamics and depressed cardiac contractility.¹⁵⁴ There are 2 basic elements underlying this change in perspective. The first is the realization that heart failure is heterogeneous, at both the molecular and cellular level, and this heterogeneity has significant physiological and clinical correlations. The second is that the course of both experimental

Table 3 In Vitro Stimuli, Signaling Molecules, and Gene Expression That Characterize the Hypertrophic Response

<i>Agonists</i>	<i>Transduction pathways</i>	<i>Kinases</i>	<i>Nuclear factors</i>	<i>Induction of immediate early genes</i>	<i>Induction of embryonic genes</i>	<i>Induction of constitutive contractile protein genes</i>
<i>α-adrenergic</i>	<i>ras</i>	<i>Protein kinase C</i>	<i>HF-1a/HF-1b</i>	<i>c-fos</i>	<i>ANF</i>	<i>MLC-2v</i>
<i>Angiotensin II</i>	<i>Gq</i>	<i>Raf-1 kinase</i>	<i>TEF-1</i>	<i>c-jun</i>	<i>Skeletal α-actin</i>	<i>Cardiac α-actin</i>
<i>Endothelin-I</i>	<i>gp130</i>	<i>S6 kinase</i>	<i>SRF</i>	<i>Egr-1</i>	<i>β-MHC</i>	
<i>FGF</i>		<i>MAP kinases</i>		<i>Jun-B</i>		
<i>TGF-β</i>		<i>JAK kinases</i>		<i>Nur-77</i>		
<i>Cardiotrophin-1</i>						
<i>LIF</i>						
<i>Stretch</i>						

and clinical heart failure is characterised by a series of physiological transitions.¹⁵⁴

The activation of the cardiac hypertrophic response is characterized, and essentially defined, by increases in the size and contractile protein content of individual cardiac muscle cells.¹⁵⁴ The development of hypertrophy allows the heart to maintain cardiac work and follows as a response to several mechanical or hormonal cues.¹⁵⁶ It is an early milestone during the clinical course of heart failure and a significant risk factor for subsequent morbidity and mortality.¹⁵⁷ Agents that blunt the structural and clinical manifestations of hypertrophy may have beneficial effects on these outcomes.¹⁵⁷ The pattern of hypertrophy is dependent on the context within which it develops. In pressure-overload hypertrophy (eg hypertensive heart disease, aortic stenosis), for example, additional sarcomeric proteins are assembled in parallel and the heart acquires a concentric pattern of hypertrophy with increased wall thickness, relative preservation of chamber volume, and maintained systolic function.¹⁵⁸ Conversely, in hypertrophy developing after chronic volume-overload (eg aortic or mitral insufficiency), sarcomeric units become assembled in series, with increased individual myocyte length, global ventricular dilatation, and early-onset systolic dysfunction.¹⁵⁸ Given such distinct morphological and physiological features, it is entirely possible that divergent signaling pathways mediate the development of these individual phenotypes^{154,155} (Fig 5).

The central importance of hypertrophy to the clinical phenotype of chronic heart failure indicates that unraveling the molecular signals that first trigger and then maintain the hypertrophic response may eventually hold the key to understanding the pathogenesis and mechanisms of progression of heart failure. Such understanding could lead to the identification of new therapeutic targets, the interruption of which may halt

clinical deterioration.

Activation of Cardiac Muscle Genes During Hypertrophy

The activation of specific subsets of cardiac muscle genes is one of the first detectable phenotypic changes in the development of the hypertrophic response (Table 3) with the pattern of gene activation being related to the hypertrophic stimulus (pressure-overload, volume-overload, exercise-induced hypertrophy, hypertrophic cardiomyopathy).¹⁵⁴ These findings are consistent with the existence of distinct molecular phenotypes underlying clinical expressions of hypertrophy.¹⁵⁴ Under certain conditions, down-regulation of a subset of cardiac muscle genes has also been observed, including the gene encoding the sarcoplasmic reticulum Ca^{2+} -ATPase which plays a central role in diastolic relaxation.¹⁵⁹

Cardiac hypertrophy results in the induction of constitutively-expressed sarcomeric protein genes accounting for the increase in contractile protein content that is a necessary component of the phenotype.^{154,156,158} In addition, the hypertrophic response is accompanied by ventricular re-expression of genes, which are ordinarily expressed in this location only in the fetus. In the embryonic heart, for example, ANF gene expression is observed in the atrium and ventricle.¹⁶⁰ Following birth there is selective down-regulation of the ventricular gene leading to restricted atrial expression.¹⁶⁰ However hypertrophy invariably results in a rapid re-induction of ventricular ANF gene expression and natriuretic peptide expression is increased in both hypertrophic¹⁶¹ and dilated cardiomyopathy.¹⁶² The constitutive activation of ANF in the hypertrophied heart is also thought to reflect the switching-on of other embryonic genes.^{163–165} In view of these findings, the ANF gene has become established as a well-characterized marker of the

Table 4 Experimental Strategy to Map Signaling Pathways

1. Identify candidate signaling molecules and pathways in *in vitro* cultured myocardial cell model systems
2. Develop and characterize a mouse based model of hypertrophy
3. Utilize miniaturized technology to monitor *in vitro* cardiac physiology in the living mouse
4. Develop strategies for ventricular chamber specific expression of transgenes
5. Utilize combination of transgenic and gene-targeting approaches to assess role of individual candidate signaling molecules in *in vivo* hypertrophy in the mouse

Adapted from K. R. Chien: Cannon Award Lecture. Cardiac muscle diseases in genetically engineered mice: the evolution of molecular physiology. Am J Physiol 1995; 269: H753–H766

hypertrophic response in both *in vitro* and *in vivo* model systems and has been extensively used to map signaling pathways responsible for this adaptive physiological response.^{105,160,166–169} These systems have also been used to identify the role of new and known growth factors in the activation of the hypertrophic response.^{160,168,169}

In vitro Assay System for Myocardial Cell Hypertrophy

Several laboratories have employed *in vitro* myocardial cell assays to determine which of the many phenotypic features of hypertrophy are under the control of particular signaling pathways (Table 4). The availability of a well-characterized cultured myocardial cell systems that displays morphological and genetic features of the *in vivo* hypertrophic response^{163,164,167,170–175} has led to the identification of a number of defined hormonal stimuli which can activate the hypertrophic phenotype *in vitro*, including α -adrenergic agonists,^{171,172} angiotensin II,^{173,176} endothelin-1¹⁶⁷ and cardiotrophin-1.^{174,175} These systems have also been adapted to characterize specific elements of the hypertrophic response. For example, the development of reproducible methods to, both actively and passively, stretch such cells has been successfully used to characterize signaling pathways activated following mechanical stimuli.^{177,178} In addition, the *in vitro* myocardial cell assay system has been used to identify new paracrine sources of hypertrophic factors derived from cardiac fibroblasts¹⁷⁹; and following transfer of the assay system to a rapid throughput 96-well format has also allowed the isolation and cloning of novel cytokines.^{180,181}

Combinations of co-transfection and micro-injection techniques have been used to induce or block specific component molecules and led to a skeleton plan of the downstream signaling pathways capable of activating phenotypic features of hypertrophy¹⁸² (Fig 5). However, while clearly valuable in identifying, known and novel, candidate genes for the hypertrophic process, such *in vitro* studies do not address the ques-

tion as to whether activation of any of these signaling pathways is sufficient to promote a hypertrophic response *in vivo*. For example, whether the complex *in vivo* physiological stimulus of pressure-overload can be adequately modeled by simple mechanical stretching of myocytes *in vitro* must remain an open question until correlative *in vivo* data are available.¹⁷⁸ Ultimately, the detailed modeling and evaluation of the role of specific signaling molecules in clinical hypertrophy and the cardiomyopathies, and in the transition from hypertrophy to dilatation and systolic failure require study *in vivo*.⁹⁴

In vivo Cardiac Hypertrophy Assay System in Genetically-Manipulated Mice

One difficulty faced in defining signaling pathways in *in vivo* models of hypertrophy has been the limited ability to manipulate or control individual elements of the *in vivo* physiology of myocardial hypertrophy.¹⁶⁶ Inhibitors of various surface receptors and enzyme systems have been used to study the development of hypertrophy, but in many cases use of the inhibitors, being non-specific, have led to secondary physiological effects that confound clear interpretation of results.¹⁵⁴ Thus, the ability to genetically manipulate an *in vivo* animal hypertrophy model represents a significant advantage.¹⁰⁴ The final aim has been to document how mechanical triggers orchestrate a specific set of genetic events in the adult cardiac muscle cell within a multicellular context.¹⁶⁶ This endpoint could be achieved by the expression of a dominantly-acting gene product, or the neutralization of the activity of a specific signaling molecule without, ideally, interference with other components of a highly integrated network.⁹⁴ Subsequently, assessment can be made of the effects of such genetic alteration on the acquisition of specific molecular and cellular features of hypertrophy (increase in contractile protein content, activation of embryonic genes, increase in myocyte size etc) in addition to other hallmarks of the integrated physiological phenotype.^{105,166,183}

Advances in mouse genetics and transgenic/gene-

targeting technology has resulted in the mouse finding an important role as a model system to study cardiac hypertrophy and failure.^{105,166} The use of micro-surgical approaches have led to a reproducible model of

Fig 6. Microsurgical induction of transverse aortic constriction and associated cardiac hypertrophy in the mouse. Method for the production of stable transverse aortic constriction in mouse (see text for details). (From: Rockman, H. A., Ross, R. S., Harris, A. et al: Segregation of atrial specific and inducible expression of an ANF transgene in an in vitro murine model of cardiac hypertrophy. *Proc Natl Acad Sci* 1991, 88: 8277; reprinted with permission.)

pressure-overload hypertrophy despite the diminutive size of the murine heart and great vessels (Fig 6).^{105,166} Creating a constriction in the ascending thoracic aorta produces a stable 35 mmHg gradient and a 35% increase in heart weight with >20-fold increase in steady-state myocardial ANF mRNA expression.¹⁰⁵ The pattern of immediate early gene expression during pressure-overload hypertrophy in murine myocardium is identical with that elicited in other in vivo and in vitro model systems of hypertrophy.¹⁰⁵ In addition, the mouse hypertrophy model recapitulates other characteristic features of ventricular hypertrophy seen both in vitro systems and clinically.^{105,166,184} The onset of hypertrophy in the human setting, for example, is accompanied by a well-defined set of in vivo physiological phenotypes. In the initial stages of human hypertension increased ventricular wall thickness is associated with decreased diastolic compliance and relative preservation of systolic function.¹⁸⁵ Subsequently left ventricular ejection fraction decreases with ventricular dilatation and symptomatic heart failure associated with blunted β -adrenergic responsiveness as assessed by agonist-mediated increases in $LVdp/dt$.^{186,187} The ultimate utility of the mouse as a model system to study hypertrophic heart disease and heart failure rests upon documenting that these physiological phenotypes of impaired diastolic compliance, decreased systolic function and basal and

Fig 7. Digitized ventricular angiography for the in vivo assessment of hemodynamic function in the mouse. Cardiac catheter inserted from right jugular vein imaged in left anterior oblique; in addition to intracardiac opacification renal shadow and bladder are visualized by contrast; tracheal cannula in situ. (From: Chien, KR: Molecular advances in cardiovascular biology. *Science* 1993, 260: 916; reprinted with permission, American Association for the Advancement of Science.)

Table 5 Growth Factors Implicated in Activating a Hypertrophic Response

Growth factor	Activity
Angiotensin II	<p>↑ release in <i>in vitro</i> stretch-induced hypertrophy</p> <p>↑ ANF, immediate early genes and skeletal α-actin expression in <i>in vitro</i> model</p> <p>ACE inhibition or AT₁ receptor antagonists block hypertrophy</p>
Endothelin	<p>Presence of binding sites on cardiac myocytes</p> <p>↑ mRNA pre-pro-endothelin-1 during cardiac hypertrophy</p> <p>↑ immediate early genes, ANF, and MLC_{2v} in <i>in vitro</i> model</p> <p>ET_A receptor antagonists block hypertrophy</p>
TGF- β	<p>↑ mRNA during cardiac hypertrophy</p> <p>↑ β-MHC, α-skeletal actin, α-smooth muscle actin, ANF</p> <p>↓ α-MHC</p>
IGF-I	<p>↑ IGF-I mRNA and protein during hypertrophy</p> <p>↑ β-MHC, MLC_{2v}, troponin I</p> <p>↑ number of nascent myofibrils</p> <p>IGF-I knockout mice show phenotype with impaired cardiac and skeletal muscle growth</p> <p>↑ Cardiac mass following <i>in vivo</i> administration</p>

ANF=atrial natriuretic factor; AT=angiotensin; MLC=myosin light chain; MHC=myosin heavy chain. Adapted from G. Lembo, J.J. Hunter, and K. R. Chien: Signaling pathways for cardiac growth and hypertrophy in Recent advances and prospects for growth factor therapy. *Ann NY Acad Sci* 1995; 752: 115–127

agonist-mediated increases in cardiac contractility and relaxation can be quantitatively assayed in the *in vivo* mouse context.^{108,154} Miniaturized catheterization¹⁸³ with microangiography technology¹⁰⁶ to provide such quantitative assays is now available and has been routinely applied to characterize such physiological phenotypes in both transgenic and gene-targeted mice (Fig 7).^{104,188}

Using observations from the *in vitro* model systems of hypertrophy, experimental strategies have been devised, that should ultimately allow a definitive assessment of the roles of particular signaling molecules in the pathophysiology of cardiac hypertrophy and failure (Table 3). Candidate genes are identified in the *in vitro* model system, and their subsequent role in the *in vivo* context is evaluated in genetically-manipulated mice produced with cardiac-specific promoters.^{95,189} With the advent of tissue-specific knockout, conditional transgene expression,^{190,191} and a variety of rescue strategies, future refinements in mouse genetic technology should eventually allow discrimination between primary and secondary events during the course of cardiac growth and development.¹⁵⁴

Identification of Cardiac Growth Factors and Cytokines

The precise means by which hemodynamic stress is sensed by cardiac myocytes, and the mechanisms of the subsequent activation of those growth-related signaling pathways that regulate the cardiac muscle gene program during myocardial hypertrophy are unknown.¹⁵⁴ The relative absence of hypertrophy in

the right heart following aortic constriction¹⁰⁵ and the selective growth of the right ventricle after pulmonary artery banding¹⁰⁶ count against systemic circulating factors as principal mediators. The current general hypothesis is that growth factors are produced by cardiac non-muscle cells, or by myocytes themselves, in response to hemodynamic stress and, through specific signaling cascades, selectively regulate the transcription of genes leading to cardiomyocyte growth.^{154,192}

The recent isolation and molecular cloning of numerous growth factors, the description of their expression in the heart, and the demonstration that cardiac myocytes are targets for peptide-derived growth factors have all supported a role for such locally-produced diffusible factors initially in cardiac growth and later in myocardial failure^{172,192,193} (Table 5). According to this perspective, the transition between compensated hypertrophy and overt cardiac dysfunction may reflect the action of different subsets of growth factors and/or cytokines. At any one time, the net structure and function of the heart might be the result of the integration of diverse paracrine stimuli that mediate distinct physiological phenotypes via control of various subsets of the cardiac gene program.¹⁵⁴ Under such circumstances, the pathogenesis of cardiac muscle failure could be viewed as a classical problem in growth and development, analogous to the process of cardiogenesis where specific molecular cues guide chamber morphogenesis with tight spatial and temporal regulation.^{194,195} If such is the case, then new therapeutic approaches to heart failure could rest on

Fig 8. Location of cardiac MHC mutations in familial hypertrophic cardiomyopathy. The myosin heavy-chain dimer and the corresponding primary amino acid structure are shown in a linear format (amino acids 1 through 2002). 9 of the amino acid substitutions known to cause familial hypertrophic cardiomyopathy are shown (>29 β -MHC mutations have been described). (Adapted with permission from: Kelly, DP and Strauss, AW; Inherited cardiomyopathies. *N Engl J Med* 1993, 330: 913; reprinted with permission of author and the New England Journal of Medicine, Massachusetts Medical Society.)

the identification and manipulation of factors which promote physiological hypertrophy and inhibition of stimuli that activate phenotypic features of the failing heart!⁵⁴

The absolute number of distinct growth factors expressed in the myocardium is not yet known, and therefore the identification of cardiac growth factors and their receptors remains an expanding area in cardiovascular research. Among those that have been identified in the heart include transforming growth factor-beta (TGF- β), insulin-like growth factor-1 (IGF-1), endothelin I, angiotensin II, and a burgeoning number of new growth factors and cytokines!^{172,180,192,193} Some of the candidate growth factors which have been implicated in activating a hypertrophic response in various cardiac model systems are discussed below (Table 5).

Hypertrophy Suppressor Pathways

Recent studies have uncovered the presence of hypertrophy suppressor pathways that can act to suppress the onset of features of hypertrophy in the in vitro model system!¹⁹⁶ The first example of the existence of hypertrophy suppressor pathways has been provided by documenting that retinoids can suppress either α -adrenergic- or endothelin receptor-mediated hypertrophy in cultured cardiac muscle cells in vitro!¹⁹⁶ The suppressor pathway appears to utilize an RXR/RAR heterodimer pathway and requires the transcriptional induction of downstream cardiac muscle

genes, since co-transfection of the dominant-negative receptor effectively alleviates suppressor activity!¹⁹⁶ These studies provide evidence for cross-talk between the retinoid signaling pathways!¹⁹⁷ and G protein-coupled receptor pathways!¹⁹⁸ in cardiac muscle cells, and could lead to the identification of a panel of retinoid-inducible hypertrophy suppressor genes. Whether these in vitro observations have any relevance to the in vivo context remains unproven, but is clearly a source for future work, given the documented importance of retinoid pathways for in vivo embryonic cardiac muscle growth!^{101,107} It will also become of interest to determine whether any other defined agonist or ligands can be purified on the basis of their suppressor activity in the same model system.

Familial Hypertrophic Cardiomyopathy

One of the major advances in the entire field of molecular cardiology has been the identification of the genetic defects responsible for familial hypertrophic cardiomyopathy (FHC)!^{199–203} It is now clear that FHC is a genetically heterogeneous disease, and that although several loci remain to be identified many of the disease loci fall into the class of sarcomeric proteins!^{200,201} To date, most of the work has focused on the β -myosin heavy chain mutations, most of which appear to be missense mutations localized in the head and head-rod junction!^{202,203} (Fig 8). Since this mutation results in the expression of a myosin heavy chain with impaired function!²⁰⁴ a scenario for how

Table 6 Altered Expression of Cardiac Genes in the Failing Human Heart

<i>Gene product</i>	<i>Expression</i>	<i>Characteristics</i>
<i>Contractile proteins</i>		
<i>Myosin heavy chain</i>	↔	No ventricular isoform shift Shift α MHC to β MHC in atrium
<i>Myosin light chain</i>	↑↓	↑ Ventricular MLC _{2a} in DCM ↓ MLC _{2v} in DCM
<i>Actin</i>	↔	Ratio α -skeletal to α -cardiac actin mRNA unchanged in DCM
<i>Troponin T</i>	↑↓	↑ TnT ₂ expression
<i>Sarcolemmal proteins</i>		
β -adrenoceptor	↓	↓ β_1 radioligand binding ↓ mRNA in DCM
<i>G_{1a} protein</i>	↑	↑ mRNA in DCM ↑ protein expression
<i>G_{sa} protein</i>	↔	Unchanged
<i>L-type Ca²⁺ channel</i>	↑↓	Controversial
<i>Na⁺, K⁺-ATPase</i>	↔	Unchanged
<i>Na⁺/Ca²⁺ exchanger</i>	↑	↑ mRNA in DCM ↑ protein expression
<i>SR-associated proteins</i>		
<i>Ryanodine receptor</i>	↑↓	Controversial
<i>Ca²⁺-ATPase</i>	↓	↓ mRNA in DCM ↓ protein expression
<i>Phospholamban</i>	↓	↓ mRNA in DCM
<i>Calsequestrin</i>	↔	↔ mRNA in DCM

DCM=Dilated cardiomyopathy; MLC2a=atrial myosin light chain; MLC2v=ventricular myosin light chain; MHC=myosin heavy chains. Adapted from G. Hasenfuss and H. Just: Myocardial phenotype changes in heart failure: cellular and subcellular adaptations and their functional significance. *Br Heart J* 1994; 72: S10–17

hypertrophy develops in this entity can now be constructed. In this manner, the expression of these mutant β -myosin heavy chains has been proposed to serve as dominant negatives, which would act to impair cardiac function in a subtle manner by their assembly with normal sarcomeric units.²⁰⁴ A secondary massive hypertrophic response in the myocardium, and markedly increased chamber pressures, would eventually lead to myocyte dropout and accompanying fibrosis.

This working model would account for much of our current clinical knowledge of this disorder, including the sporadic nature of hypertrophy, the non-uniform correlation between hypertrophy and the disease genotype, the evidence of cardiac fibrosis, and recent

studies demonstrating that a phenocopy of hypertrophic cardiomyopathy can be developed by expressing a dominantly-acting signaling molecule for hypertrophy in the ventricular chamber in the in vivo context in transgenic mice.¹⁰⁴ While far from being proven, the possibility exists that hypertrophy in the setting of FHC may be a secondary, rather than a primary phenomenon, and that hypertrophy in the setting of FHC may utilize a subset of the pathways that are responsible for activating hypertrophy in other forms of the acquired disease, such as those activated during the course of chronic hypertension.¹⁵⁴

Towards New Therapeutic Strategies for Heart Failure

As noted earlier, a large body of evidence suggests that cardiac muscle cells, in response to distinct hormonal, mechanical, and physiological stimuli can activate defined subsets of the cardiac muscle gene program. In many cases, such patterns of activation may be closely linked to the distinct physiological phenotypes which are found in hypertrophied heart.¹⁵⁴ Initially, these changes may be adaptive, but subsequently may become maladaptive. The cardiac muscle cell is endowed with a series of complex signaling cascades that orchestrate these various elements into defined responses, and may also harbor pathways to suppress individual signaling pathways that could lead to the appearance of distinct phenotypes. One important challenge will be defining which particular subsets of factors and pathways mediate distinct phenotypes during the variable course between initiation of cardiac hypertrophy and the appearance of overt cardiac muscle failure (Table 6).

It is becoming increasingly apparent that the course of cardiac muscle failure with different manifestations seen may largely represent a problem in cardiac growth and morphogenesis, with the morphogenic form taken being a predictor of the clinical outcome for a particular patient. Viewing this complex cardiovascular problem in this manner sets up analogies with the process of cardiogenesis itself, where a particular signaling pathway may very well have different phenotypic outcomes depending upon the time and the place of its activation during the course of the developmental process.^{194,195} In this manner, it may be appropriate to consider the problem of the heart failure syndrome after it has been broken down into stages. Individual components would then be viewed as 4 stages respectively: initiation, maturation, maintenance and finally decompensation of the hypertrophic response. The design of new molecular therapeutic approaches will be dependent upon elucidating the factors and the downstream signaling pathways which orchestrate transitions between stages. Given the current armamentarium of molecular, cellular, and genetic technology, there is little doubt that these definition of the characteristics of these switches will lead to molecular advances in the treatment of heart failure, that should be fully apparent within the next decade.

Future Perspectives

'The major scientific advances in medicine in the early twentieth century originated in scientific discoveries not aimed at the management of human diseases Present day cardiology has been similarly underpinned by curiosity-driven science, the fruits of

which have been taken up by physiologists and cardiologists and applied directly to clinical practice.'

David Weatherall²⁰⁵

The major clinical problems of interest to the cardiologist have remained largely unchanged over the course of 3 decades. Clearly, there has never been a greater opportunity for the generation of new, biologically-targeted therapeutic strategies to attack fundamental problems in atherogenesis, hypertension, vascular disease, congenital heart disease, and cardiac hypertrophy and failure. The prospects for modern biology to impact mainstream interests of clinical cardiologists is evidenced by the discovery of the disease genes for several monogenic human cardiovascular disorders; the identification of genes which confer risk for cardiovascular disease (eg, obesity and diabetes; the potential utility of structural biology for rational drug design; the large-scale production of low abundance recombinant proteins for gene replacement disorders (hemophilia), selected acquired diseases (erythropoietin, GM-CSF) and the promise of others (thrombopoietin), the engineering of second generation recombinant proteins that display improved specificity, efficacy, or a lower incidence of side effects; and the generation of new, genetically-manipulated animal models that offer the opportunity to intersect genetics with complex, integrative, and polygenic disorders which account for the large majority of human cardiovascular disease.^{93,206}

The impending elucidation of a complete set of human cDNAs offers the prospect of mining the human genome to identify genes which could be causative in specific cardiovascular diseases in surrogate model systems. As so aptly noted by David Weatherall,²⁰⁵ these considerable advances would not be possible without the tools that have been generated through several decades of fundamental science²⁰⁶ that initially was not designed for the study of human cardiovascular disease, but has nevertheless laid a substantial scientific foundation for molecular cardiology.

Acknowledgments

This article has been adapted, in part (with permission), from the chapter, Principles of Cardiovascular Molecular and Cellular Biology, previously printed in Braunwald E, editor. Heart Disease: A Textbook of Cardiovascular Medicine, 5th Edition, W. B. Saunders Company, Philadelphia, PA, chapter 48, pp 1626–1649, 1996.

References

1. Chien KR: Molecular advances in cardiovascular Biology. *Science* 1993; **260**: 916–917

2. Lewin B: Genes V. (5th edition) Oxford: Oxford University Press, 1994
3. Watson JD, Gilman M, Witkowski J, Zoller M: Recombinant DNA. (2nd edition) New York: Scientific American Books, 1992
4. Rosenthal N: General principles of molecular biology. In: Chien KR, editor. Molecular basis of heart disease. Philadelphia: W. B. Saunders Company, 1997
5. Chien KR, editor. Molecular basis of heart disease (in preparation). (1st edition) Philadelphia: W. B. Saunders Company, 1998

Recombinant Protein Therapy

6. Ward WHJ, Timms D, Fersht AR: Protein engineering and the study of structure-function relationships in receptors. *Trends Pharmacol Sci* 1990; **11**: 280–284
7. Erslev A: Erythropoietin. *N Engl J Med* 1991; **324**: 1339–1344
8. Schick BP: Hope for treatment of thrombocytopenia. *N Engl J Med* 1994; **331**: 875–876
9. Gerard RD., Chien KR, Meidell RS: Molecular biology of tissue plasminogen activator and endogenous inhibitors. *Mol Biol Med* 1986; **3**: 449–457
10. Collen D, Lijnen HR: Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* 1991; **78**: 3114–3124
11. Deutsch E, Rao AK, Colman RW: Selective thrombin inhibitors: the next generation of anticoagulants. *J Am Coll Cardiol* 1993; **22**: 1089–1092
12. Lefkowitz J, Topol EJ: Direct thrombin inhibitors in cardiovascular medicine. *Circulation* 1994; **90**: 1522–1536
13. Coughlin SR: Thrombin receptor function and cardiovascular disease. *Trends Cardiovasc Med* 1994; **4**: 77–83
14. Badimon L, Merino A, Badimon J, Chesebro JH, Fuster V: Hirudin and other thrombin inhibitors. Experimental results and potential clinical applications. *Trends Cardiovasc Med* 1991; **1**: 261–267
15. Ferrara N: Vascular endothelial growth factor. *Trends Cardiovasc Med* 1993; **3**: 244–250
16. Winter G, Milstein C: Man-made antibodies. *Nature* 1991; **349**: 293–299
17. Ritter MA, and Ladyman HM, editors. Monoclonal antibodies. Production, Engineering and Clinical application. Cambridge: Cambridge University Press, 1995
18. Riechmann L, Clark M, Waldmann H, Winter G: Reshaping human antibodies for therapy. *Nature* 1988; **332**: 323–327
19. Brugge JS: New intracellular targets for therapeutic drug design. *Science* 1993; **260**: 918–919
20. Goody RS: Rational drug design and HIV: hopes and limitations. *Nature Med* 1995; **1**: 519–520
21. Lefkowitz J, Plow EF, Topol EJ: Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med* 1995; **332**: 1553–1559
22. Shattil S, Ruggieri Z: Platelets. In: Chien KR, editor. Molecular basis of heart disease. Philadelphia: WB Saunders Co, 1997
23. Jang Y, Lincoff AM, Plow EF, Topol EJ: Cell adhesion molecules in coronary artery disease. *J Am Coll Cardiol* 1994; **24**: 1591–1601
24. Pytela R, Pierschbacher MD, Ginsberg MH, Plow EF, Ruoslahti E: Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science* 1986; **231**: 1559–1562
25. Hynes RO: Integrins: A family of cell surface receptors. *Cell* 1987; **48**: 549–554
26. Hynes RO: Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 1992; **69**: 11–25
27. Phillips DR, Charo IF, Scarborough RM: GPIIb-IIIa: the responsive integrin. *Cell* 1991; **65**: 359–362
28. Calvete JJ: Clues for understanding the structure and function of a prototypic human integrin: The platelet glycoprotein IIb/IIIa complex. *Thromb Hemostas* 1994; **72**: 1–15
29. Baldwin HS, Buck CA: Integrins and other cell adhesion molecules in cardiac development. *Trends Cardiovasc Med* 1994; **4**: 178–187
30. Pierschbacher MD, Ruoslahti E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 1984; **309**: 30–33
31. Du X, Plow EF, Frelinger 3rd AL, O'Toole TE, Loftus JC, Ginsberg MH: Ligands "activate" integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). *Cell* 1991; **65**: 409–416
32. Dennis MS, Henzel WJ, Pitti RM, Lipari MT, Napier MA, Deisher TA, et al: Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: evidence for a family of platelet aggregation inhibitors. *Proc Natl Acad Sci* 1990; **87**: 2471–2475
33. Shebuski RJ, Ramjit DR., Bencen GH, Polokoff MA: Characterisation and platelet inhibitory activity of bitistatin, a potent arginine-glycine-aspartic acid-containing peptide from the venom of the viper Bitis arietans. *J Biol Chem* 1989; **264**: 21550–21556
34. Bangs NU, Clayman MD: Antithrombotic agents from salivary glands of hematophagous animals. *Trends Cardiovasc Med* 1992; **2**: 183–188
35. Scarborough RM, Naughton MA, Teng W, Rose JW, Phillips DR, Nannizzi L, et al: Design of potent and specific integrin antagonists. *J Biol Chem* 1993; **268**: 1066–1073
36. Adler M, Lazarus RA, Dennis MS, Wagner G: Solution structure of kistrin, a potent platelet aggregation inhibitor and GPIIb-IIIa antagonist. *Science* 1991; **253**: 445–448
37. Tchong JE, Harrington RA, Kottke-Marchant K, Kleiman NS, Ellis SG, Kereiakes DJ, Mick MJ, Navetta FI, Smith JE, Worley SJ, Miller JA, Joseph DM, Sigmon KN, Kitt MM, du Mee CP, Califf RM, Topol EJ: Multicenter, randomized, double-blind, placebo-controlled trial of platelet integrin glycoprotein IIb/IIIa blocker intergrilin in elective coronary intervention. *Circulation* 1995; **91**: 2151–2157
38. Kouns WC, Kirchhofer D, Hadvary P, Edenhofer A, Weller T, Pfenninger G, Baumgartner HR, Jennings LK, Steiner B: Reversible conformational changes induced in glycoprotein IIb-IIIa by a potent and selective peptidomimetic inhibitor. *Blood* 1992; **80**: 2539–2547
39. The EPIC investigators: Use of monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. *N Engl J Med* 1994; **330**: 956–961
40. Harker LA: Platelets and vascular thrombosis. *N Engl J*

Med 1994; **330**: 1006–1007

Localization of Cardiovascular Disease Genes

41. Beaudet AL, Scriver CR, Sly WS, Valle D: Genetics, biochemistry, and molecular basis of variant human phenotypes. In: Scriver C, Beaudet A, Sly W, Vale D, editors. The metabolic and molecular bases of inherited disease. (7th edition) New York: McGraw-Hill, 1995; 53. vol 3
42. White R, Caskey CT: The human as an experimental system in molecular genetics. *Science* 1988; **240**: 1483–1488
43. Collins FS: Positional cloning moves from perditional to traditional. *Nature Genetics* 1995; **9**: 347–350
44. Fields C, Adams MD, White O, Venter JC: How many genes in the human genome? *Nature Genet* 1994; **7**: 345–346
45. Goodfellow PN: Microsatellites and the new genetic maps. *Curr Biol* 1993; **3**: 149–151
46. Botstein D, White RL, Skolnick M, Davis RW: Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Gen* 1980; **32**: 314–331
47. Housman D: Human DNA polymorphism. *N Engl J Med* 1995; **332**: 318–320
48. Green ED, Cox DR, Myers RM: The human genome project and its impact on the study of human disease. In: Scriver CR, Beaudet AL, Sly WS, Vale D, editors. The metabolic and molecular bases of inherited disease. (7th edition) New York: McGraw-Hill, 1995: 401. vol 3.
49. Aldhous P: Fast track to disease genes. *Science* 1994; **265**: 2008–2010
50. Schwartz PJ, Moss AJ, Vincent GM, Crampton RS: Diagnostic criteria for the long QT syndrome. An update. *Circulation* 1993; **88**: 782–784
51. Schwartz PJ, Locati EH, Napolitano C, Priori SG: The long QT syndrome. In: Zipes DP, Jalife J, editors. Cardiac Electrophysiology: from cell to bedside. Philadelphia: WB Saunders Co, 1995: 788
52. Moss AJ, Robinson JL: The long-QT syndrome. Genetic considerations. *Trends Cardiovasc Med* 1992; **2**: 81–83
53. Vincent GM, Timothy KW, Leppert M, Keating MT: The spectrum of symptoms and QT intervals in carriers of the gene for the long-QT syndrome. *N Engl J Med* 1992; **327**: 846–852
54. Keating MT: Linkage analysis and long QT syndrome. Using genetics to study cardiovascular disease. *Circulation* 1992; **85**: 1973–1986
55. Keating MT, Atkinson D, Dunn C, Timothy K, Vincent GM, Leppert M: Linkage of a cardiac arrhythmia, the long QT syndrome, and the Harvey ras-1 gene. *Science* 1991; **252**: 704–706
56. Roy N, Kahlem P, Dausse E, Bennaceur M, Faure S, Weissenbach J, Komadja M, Denjoy I, Coumel P, Schwartz K, Guicheney P: Exclusion of Hras from long QT locus. *Nature Genet* 1994; **8**: 113–114
57. Keating MT, Dunn C, Atkinson D, Timothy K, Vincent GM, Leppert M: Consistent linkage of the long-QT syndrome to the Harvey ras-1 locus on chromosome 11. *Am J Hum Genet* 1991; **49**: 1335–1339
58. Benhorin J, Kalman YM, Medina A, Towbin JY, Rave-Harel N, Dye TD, et al: Evidence of genetic heterogeneity in the long QT syndrome. *Science* 1993; **260**: 1960–1962
59. Curran M, Atkinson D, Timothy K, Vincent GM, Moss AJ, Leppert M, et al: Locus heterogeneity of autosomal dominant long QT syndrome. *J Clin Invest* 1993; **92**: 799–803
60. Towbin JA, Li H, Taggart RT, Lehmann MH, Schwartz PJ, Satler CA, et al: Evidence of genetic heterogeneity in Romano-Ward long QT syndrome. Analysis of 23 families. *Circulation* 1994; **90**: 2635–2644
61. Jiang C, Atkinson D, Towbin JA, Splawski I, Lehmann MH, Li H, et al: Two long QT syndrome loci map to chromosomes 3 and 7 with evidence for further heterogeneity. *Nature Genet* 1994; **8**: 141–147
62. Curran ME, Splawski I, Timothy KW, Vincent GM, Green ED, Keating MT: A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell* 1995; **80**: 795–803
63. Warmke JW, Ganetzky B: A family of potassium channels related to eag in Drosophila and mammals. *Proc Natl Acad Sci USA* 1994; **91**: 3438–3442
64. Sanguinetti MC, Jiang C, Curran ME, Keating MT: A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{Kr} potassium channel. *Cell* 1995; **81**: 299–307
65. Kass RS, Freeman LC: Potassium channels in the heart. Cellular, molecular and clinical implications. *Trends Cardiovasc Med* 1993; **3**: 149–159
66. Ashford ML, Bond CT, Blair TA, Adelman JP: Cloning and functional expression of a rat heart K_{ATP} channel. *Nature* 1994; **370**: 456–469
67. Wang Q, Shen J, Splawski I, Atkinson D, Li Z, Robinson JL, et al: SCN5A mutations associated with an inherited cardiac arrhythmia, long QT syndrome. *Cell* 1995; **80**: 805–811
68. Bennett PB, Yazawa K, Makita N, George AL: Molecular mechanism for an inherited cardiac arrhythmia. *Nature* 1995; **376**: 683–685
69. Grace AA, Chien KR: Congenital long QT syndromes. Toward molecular dissection of arrhythmia substrates. *Circulation* 1995; **92**: 2786–2789
70. Marban E: Molecular approaches to arrhythmogenesis. In: Chien, KR, editor. Molecular Basis of Heart Disease. Philadelphia, W.B. Saunders Company, 1998
71. Bauer KA: Hypercoagulability-a new cofactor in the protein C anticoagulant pathway. *N Engl J Med* 1994; **330**: 566–567
72. Majerus PW: Human genetics: bad blood by mutation. *Nature* 1994; **369**: 14–15
73. Mann K, Rosenberg RD: Thrombosis. In: Chien KR, editor. Molecular basis of heart disease. Philadelphia: WB Saunders Co, 1998
74. Dahlback B, Carlsson M, Svensson PJ: Familial thrombophilia due to a previously unrecognized mechanism characterised by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004–1008
75. Castellino FJ: Human protein C and activated protein C. Components of the human anticoagulation system. *Trends Cardiovasc Med* 1995; **5**: 55–62
76. Dahlback B: Inherited thrombophilia: resistance to activated protein C as a pathogenic factor of venous thrombo-

- embolism. *Blood* 1995; **85**: 607–614
77. Hajjar K: Factor V Leiden — an unselfish gene? *N Engl J Med* 1994; **331**: 1585–1587
 78. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al: Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64–67
 79. Zoller B, Dahlback B: Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. *Lancet* 1994; **343**: 1536–1538
 80. Greengard JS, Eichinger S, Griffin JH, Bauer KA: Variability of thrombosis among homozygous siblings with resistance to activated protein C due to an Arg $\Rightarrow$ Gln mutation in the gene for factor V. *N Engl J Med* 1994; **331**: 1559–1562
 81. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM: Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden thrombophilia study. *Lancet* 1993; **342**: 1503–1506
 82. Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Eisenberg PR, Miletich JP: Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke and venous thrombosis in apparently healthy men. *N Engl J Med* 1995; **332**: 912–917
 83. Lander ES, Schork NJ: Genetic dissection of complex traits. *Science* 1994; **265**: 2037–2048
 84. Pattengale PK, Steart TA, Leder A, Sinn E, Muller W, Tepler I, et al: Animal models of human disease. Pathology and molecular biology of spontaneous neoplasms occurring in transgenic mice carrying and expressing activated cellular oncogenes. *Am J Pathol* 1989; **135**: 39–61
 85. Paigen B, Morrow A, Brandon C, Mitchell D, Holmes P: Variation in the susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis* 1985; **57**: 65–73
 86. Breslow JL: Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc Natl Acad Sci USA* 1993; **90**: 8314–8318
 87. Keightley PD: Chewing the fat. *Nature Genet* 1995; **10**: 125–126
 88. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425–432
 89. Rink TJ: Genetics. In search of a satiety factor. *Nature* 1994; **372**: 406–407
 90. Bennett WI: Beyond overeating. *N Engl J Med* 1995; **332**: 673–674
- Genetic Models of Human Cardiovascular Disease*
91. Yamamoto T, Bishop RW, Brown MS, Goldstein JL, Russell DW: Deletion in cysteine-rich region of LDL receptor impedes transport to cell surface in WHHL rabbits. *Science* 1986; **232**: 1230–1237
 92. Rapacz J, Hasler-Rapacz J, Taylor KM, Checovich WJ, Attie AD: Lipoprotein mutations in pigs are associated with elevated plasma cholesterol and atherosclerosis. *Science* 1986; **234**: 1573–1577
 93. Chien KR: Genes and physiology: Molecular physiology in genetically engineered animals. *J Clin Invest* 1996; **97**: 901–909
 94. Chien KR: Cannon Award Lecture: Cardiac muscle diseases in genetically engineered mice: the evolution of molecular physiology. *Am J Physiol* 1995; **269**: H753–H766
 95. Doevedans PA, Hunter JJ, Lembo G, Wollert KC, Chien KR: Strategies for studying cardiovascular diseases in transgenic and gene-targeted mice. In: Monastersky GM, Robl JM, editors. *Strategies in transgenic animal science*. Washington DC: American Society of Microbiology, 1995: 107–144
 96. Field LJ: Transgenic mice in cardiovascular research. *Annu Rev Physiol* 1993; **55**: 97–114
 97. Lin MC, Rockman HA, Chien KR: Heart and lung disease in genetically engineered mice: the evolution of molecular physiology. *Nature Med* 1995; **1**: 749–751
 98. Jaenisch R: Transgenic animals. *Science* 1988; **240**: 1468–1474
 99. Bronson SK, Smithies O: Altering mice by homologous recombination using embryonic stem cells. *J Biol Chem* 1994; **269**: 27155–27158
 100. Tangirala RK, Casanada F, Miller E, Witzum JL, Steinberg D, Palinski W: Effect of the antioxidant N, N'-diphenyl 1,4-phenylenediamine (DPPD) on atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 1995; **15**: 1625–1630
 101. Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR, Evans RM: RXR α mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev* 1994; **8**: 1007–1018
 102. Stoltzfus L, Rubin EM: Atherogenesis: insights from the study of transgenic and gene targeted mice. *Trends Cardiovasc Med* 1993; **3**: 130–134
 103. Paul M, Wagner J, Hoffmann S, Urata H, Ganten D: Transgenic rats: new experimental models for the study of candidate genes in hypertension research. *Annu Rev Physiol* 1994; **56**: 811–829
 104. Hunter JJ, Tanaka N, Rockman HA, Ross J Jr, Chien KR: Ventricular expression of a MLC-2v-Ras fusion gene induces cardiac hypertrophy and selective diastolic dysfunction in transgenic mice. *J Biol Chem* 1995; **270**: 23173–23178
 105. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhilber ME, Field LJ, et al: Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. *Proc Natl Acad Sci USA* 1991; **88**: 8277–8281
 106. Rockman HA, Ono S, Ross RS, Jones LR, Karimi M, Bhargava V, et al: Molecular and physiological alterations in murine ventricular dysfunction. *Proc Natl Acad Sci USA* 1994; **91**: 2694–2698
 107. Dyson E, Sucov HM, Kubalak SW, Schmid-Schönbein G, Delano F, Evans RM, et al: Atrial-like phenotype is associated with embryonic ventricular failure in RXR α -/- mice. *Proc Natl Acad Sci USA* 1995; **92**: 7386–7390
 108. Kubalak SW, Doevedans PA, Rockman HA, Hunter JJ, Tanaka N, Ross J Jr, et al: Molecular analysis of cardiac muscle diseases via mouse genetics. In: Adolph KW, editor. *Methods in Molecular Genetics*. Orlando: Academic Press, 1996; 470–487
 109. White D, Wallwork J: Xenografting: probability, possibility, or pipe dream? *Lancet* 1993; **342**: 879–880
 110. Fan J, Wang J, Bensadoun A, Lauer SJ, Dang Q, Mahley RW, Taylor JM: Overexpression of hepatic lipase in trans-

- genic rabbits leads to a marked reduction of plasma high density lipoproteins and intermediate density lipoproteins. *Proc Natl Acad Sci USA* 1994; **91**: 8724–8728
111. Paigen K: A miracle enough: the power of mice. *Nature Medicine* 1995; **1**: 215–220
 112. Lusis AJ: The mouse model for atherosclerosis. *Trends Cardiovasc Med* 1993; **3**: 135–143
 113. Tall A, Breslow J: Disorders of lipoprotein metabolism. In: Chien KR, editor. *Molecular basis of heart disease*. Philadelphia: WB Saunders Co, 1998
 114. Tabas I, Krieger M: Disorders of cellular cholesterol metabolism. In: Chien KR, editor. *Molecular basis of heart disease*. Philadelphia: WB Saunders Co, 1998
 115. Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstuyft JG, et al: Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992; **71**: 343–353
 116. Zhang SH, Reddick RL, Piedrahita JA, Maeda N: Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992; **258**: 468–471
 117. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R: ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb* 1994; **14**: 133–140
 118. Reddick RL, Zhang SH, Maeda N: Atherosclerosis in mice lacking apo E: evaluation of lesion development and progression. *Arterioscler Thromb* 1994; **14**: 141–147
 119. Palinski W, Ord VA, Plump AS, Breslow JL, Steinberg D, Witztum JL: ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of auto antibodies to malondialdehyde-lysine in serum. *Arterioscler Thromb* 1994; **14**: 605–616
 120. Steinberg D, Witztum J: The role of oxidized lipoproteins in atherogenesis. In: Chien KR, editor. *Molecular basis of heart disease*. Philadelphia: WB Saunders Co, 1998
 121. Paszty C, Maeda N, Verstuyft J, Rubin EM: Apolipoprotein AI transgene corrects apolipoprotein E deficiency-induced atherosclerosis in mice. *J Clin Invest* 1994; **94**: 899–903
 122. Van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, et al: Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol Chem* 1993; **268**: 10540–10545
 123. Fazio S: Recent insights into the pathogenesis of type III hyperlipoproteinemia. *Trends Cardiovasc Med* 1993; **3**: 191–196
 124. Shimano H, Yamada N, Katsuki M, Shimada M, Gotoda T, Harada K, et al: Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia. *Proc Natl Acad Sci USA* 1992; **89**: 1750–1754
 - 1995; **51**: 1
 127. Barr E, Leiden JM: Somatic gene therapy for cardiovascular disease. Recent advances. *Trends Cardiovasc Med* 1994; **4**: 57–63
 128. Nabel E: Gene therapy for cardiovascular disease. *Circulation* 1995; **91**: 541–548
 129. Miller AD: Human gene therapy comes of age. *Nature* 1992; **357**: 455–460
 130. Riessen R, Isner JM: Prospects for site-specific delivery of pharmacologic and molecular therapies. *J Am Coll Cardiol* 1994; **23**: 1234–1244
 131. Gerard RD, Meidell RS: Adenovirus-mediated gene transfer. *Trends Cardiovasc Med* 1993; **3**: 171–177
 132. Muzyczka N: Adeno-associated virus (AAV) vectors: will they work? *J Clin Invest* 1994; **94**: 1351
 133. Morishita R, Gibbons GH, Ellison KE, Nakajima M, Zhang L, Kaneda Y, et al: Single intraluminal delivery of antisense cdc2 kinase and proliferating cell-nuclear antigen oligonucleotides result in chronic inhibition of neointimal hyperplasia. *Proc Natl Acad Sci USA* 1993; **90**: 8474–8478
 134. Lasic D, Papahadjopoulos D: Liposomes revisited. *Science* 1995; **267**: 1275–1276
 135. Leiden JM, Barr E: In Vivo gene transfer into the heart. In: Wolff JA, editor. *Gene therapeutics: methods and applications of direct gene transfer*. Boston: Birkhauser, 1994; 363
 136. Simons M, Edelman ER, Dekeyser JL, Langer R, Rosenberg RD: Antisense c-myc oligonucleotides inhibit intimal smooth muscle cell accumulation in vivo. *Nature* 1992; **359**: 67–70
 137. Bennett MR, Anglin S, McEwan JR, Jagoe R, Newby AC, Evan GI: Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by C-myc antisense oligodeoxynucleotides. *J Clin Invest* 1994; **93**: 820–828
 138. Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, et al: Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolemia. *Nature Genet* 1994; **6**: 335–341
 139. Brown MS, Goldstein JL, Havel RJ, Steinberg D: Gene therapy for cholesterol. *Nature Genet* 1994; **7**: 349–350
 140. Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, et al: Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science* 1992; **257**: 1401–1403
 141. Nabel EG, Yang Z-Y, Plautz G, Forough R, Zhan X, Haudenschild CC, et al: Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis in arteries in vivo. *Nature* 1993; **362**: 844–846
 142. Isner JM, Walsh K, Symes J, Pieczek A, Takeshita S, Lowry J et al: Arterial gene therapy for therapeutic angiogenesis in patients with peripheral arterial disease. *Circulation* 1995; **91**: 2687–2692
 143. Folkman J: Clinical applications of research on angiogenesis. *N Engl J Med* 1995; **333**: 1757–1763
 144. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu L-Q, Bunting S, et al: Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994; **93**: 662–670
 145. Cantley L, Deuel T, Rosenberg RD: The molecular biology

Gene Therapy

125. Mulligan R: The basic science of gene therapy. *Science* 1993; **260**: 926–932
126. Lever AML, Goodfellow PN. Gene therapy: *Br Med Bull*

- of restenosis. In: Chien KR, editor. Molecular basis of heart disease. Philadelphia: WB Saunders Co, 1998
146. Casscells W, Lappi DA, Baird A: Molecular atherectomy for restenosis. *Trends Cardiovasc Med* 1993; **3**: 235–243
 147. Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ: Recombinant gene expression in vivo within endothelial cells of the arterial wall. *Science* 1989; **244**: 1342–1344
 148. Lemarchand P, Jones M, Yamada I, Crystal RG: In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. *Circ Res* 1993; **72**: 1132–1138
 149. Chang MW, Barr E, Seltzer J, Jiang Y-Q, Nabel GJ, Nabel EG, et al: Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* 1995; **267**: 518–522
 150. Ohno T, Gordon D, San H, Pompili VJ, Imperiale MJ, Nabel GJ, Nabel EG: Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science* 1994; **265**: 781–784
 151. Wagner R: Gene inhibition using antisense oligodeoxynucleotides. *Nature* 1994; **372**: 333–335
 152. Davis AR: Current potential of antisense oligonucleotides as therapeutic drugs. *Trends Cardiovasc Med* 1994; **4**: 51–55
 153. Stein CA, Cheng Y-C: Antisense oligonucleotides as therapeutic agents - is the bullet really magical? *Science* 1993; **261**: 1004–1012
- Molecular Advances in Cardiac Hypertrophy*
154. Chien KR, Grace AA, Hunter JJ: Molecular basis of cardiac hypertrophy and heart failure. In: Chien KR, editor. Molecular basis of heart disease. Philadelphia: WB Saunders Co, 1998
 155. Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JN, Chien KR: Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. *J Biol Chem*, in press, 1997
 156. Morgan HE, Baker KM: Cardiac hypertrophy: mechanical, neural, and endocrine dependence. *Circulation* 1991; **83**: 13–25
 157. Katz AM: Scientific insights from clinical studies of converting-enzyme inhibitors in the failing heart. *Trends Cardiovasc Med* 1995; **5**: 37–44
 158. Spudich J, Leinwand L: Contractile structure and function. In: Chien KR, editor. Molecular basis of heart disease. Philadelphia: WB Saunders Co, 1998
 159. Arai M, Alpert NR, MacLennan DH, Barton P, Periasamy M: Alterations in sarcoplasmic reticulum gene expression in human heart failure. A possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. *Circ Res* 1993; **72**: 463–469
 160. Rosenzweig A, Seidman CE: Atrial natriuretic factor and related peptide hormones. *Annu Rev Biochem* 1991; **60**: 229–255
 161. Hasegawa K, Fujiwara H, Doyama K, Miyama M, Fujiwara T, Suga S, et al: Ventricular expression of brain natriuretic peptide in hypertrophic cardiomyopathy. *Circulation* 1993; **88**: 372–380
 162. Hasegawa K, Fujiwara H, Doyama K, Mukoyama M, Nakao K, Fujiwara T, et al: Ventricular expression of atrial and brain natriuretic peptides in dilated cardiomyopathy. An immunohistochemical study of the endomyocardial biopsy specimens using specific monoclonal antibodies. *Am J Pathol* 1993; **142**: 107–116
 163. Iwaki K, Sukhatme VP, Shubeita HE, Chien KR: α and β adrenergic stimulation induce distinct patterns of immediate early gene expression in neonatal rat myocardial cells: fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an α_1 mediated response. *J Biol Chem* 1990; **265**: 13809–13817
 164. Knowlton KU, Baracchini E, Ross RS, Harris AN, Henderson SA, Evans SM, et al: Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 gene during α -adrenergic stimulation of neonatal rat ventricular cells. *J Biol Chem* 1991; **266**: 7759–7768
 165. Takahashi T, Allen PD, Izumo S: Expression of A-, B-, and C-type natriuretic peptide genes in failing and developing human ventricles. Correlation with expression of the Ca^{2+} -ATPase gene. *Circ Res* 1992; **71**: 9–17
 166. Rockman HA, Knowlton KU, Ross J, Chien KR: In vivo murine cardiac hypertrophy. A novel model to identify genetic signaling mechanisms that activate an adaptive physiological response. *Circulation* 1993; **87**: VII14–VII21
 167. Shubeita HE, McDonough PM, Harris AN, Knowlton KU, Glembotski CC, Brown JH, et al: Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes: A paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem* 1990; **265**: 20555–20562
 168. Knowlton KU, Rockman HA, Itani M, Vovan A, Seidman CE, Chien KR: Divergent pathways mediate the induction of ANF transgenes in neonatal and hypertrophic ventricular myocardium. *J Clin Invest* 1995; **96**: 1311–1318
 169. Wollert KC, Taga T, Saito M, Narazaki M, Kishimoto T, Glembotski CC, et al: Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy: Assembly of sarcomeric units in series via gp130/leukemia inhibitory factor receptor dependent pathways. *J Biol Chem* 1996; **271**: 9535–9545
 170. Lee HR, Henderson SA, Reynolds R, Dunnmon P, Yuan D, Chien KR: Alpha-1 α_1 adrenergic stimulation of cardiac gene transcription in neonatal rat myocardial cells: Effects on myosin light chain-2 gene expression. *J Biol Chem* 1988; **263**: 7352–7358
 171. Meidell RS, Sen A, Henderson SA, Slahetka MF, Chien KR: Coordinate increases in myofibrillar protein synthesis during α_1 adrenergic stimulation of cultured rat myocardial cells. *Am J Physiol* 1986; **251**: H1076–H1084
 172. Parker TG, Schneider MD: Growth factors, proto-oncogenes, and plasticity of the cardiac phenotype. *Annu Rev Physiol* 1991; **53**: 179–200
 173. Sadoshima J, Izumo S: Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. *Circ Res* 1993; **73**: 413–423
 174. Bogoyevitch MA, Glennon PE, Sugden PH: Endothelin-1, phorbol esters and phenylephrine stimulate MAP kinase activities in ventricular cardiomyocytes. *FEBS Lett* 1993; **317**: 271–275
 175. Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A,

- Lazou A, Marshall CJ, et al: Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes: the potential role of the cascade in the integration of 2 signaling pathways leading to myocyte hypertrophy. *J Biol Chem* 1994; **269**: 1110–1119
176. Sadoshima J, Izumo S: Signal transduction pathways of angiotensin II-induced c-fos gene expression in cardiac myocytes in vitro. Roles of phospholipid-derived second messengers. *Circ Res* 1993; **73**: 424–438
 177. Sadoshima J, Takahashi T, Jahn L, Izumo S: Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. *Proc Natl Acad Sci USA* 1992; **89**: 9905–9909
 178. Komuro I, Yazaki Y: Intracellular signaling pathways in cardiac myocytes induced by mechanical stress. *Trends Cardiovasc Med* 1994; **4**: 117–121
 179. Long CS, Henrich CJ, Simpson PC: A growth factor for cardiac myocytes is produced by cardiac nonmyocytes. *Cell Regul* 1991; **2**: 1081–1095
 180. Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh S-M, et al: Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci USA* 1995; **92**: 1142–1146
 181. Sheng Z, Pennica D, Wood WI, Chien KR: Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. *Development* 1996; **122**: 419–428
 182. Thorburn A, Thorburn J, Chen S-Y, Powers S, Shubeita HE, Feramisco JR, et al: HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. *J Biol Chem* 1993; **268**: 2244–2249
 183. Milano CA, Allen LF, Rockman HA, Dolber PC, McMinn TR, Chien KR, et al: Enhanced myocardial function in transgenic mice overexpressing the β_2 -adrenergic receptor. *Science* 1994; **264**: 582–586
 184. Chien KR, Zhu H, Knowlton KU, Miller-Hance W, van Bilsen M, O'Brien TX, et al: Transcriptional regulation during cardiac growth and development. *Annu Rev Physiol* 1993; **55**: 77–95
 185. Little WC, Downes TR: Clinical evaluation of left ventricular diastolic performance. *Prog Cardiovasc Dis* 1990; **32**: 273–290
 186. Kiuchi K, Shannon RP, Komamura K, Cohen DJ, Bianchi C, Homcy CJ, et al: Myocardial beta-adrenergic receptor function during the development of pacing-induced heart failure. *J Clin Invest* 1993; **91**: 907–914
 187. Muntz KH, Zhao M, Miller JC: Downregulation of myocardial β -adrenergic receptors. Receptor subtypes selectivity. *Circ Res* 1994; **74**: 369–375
 188. Lembo G, Rockman HA, Hunter JJ, Warburton C, Koch WJ, Ma L, et al: Elevated blood pressure and enhanced myocardial contractility in mice with severe IGF-1 deficiency. *J Clin Invest* 1996; **98**: 2648–2655
 189. Hunter JJ, Zhu H, Lee KJ, Kubalak SW, Chien KR: Targeting gene expression to specific cardiovascular cell types in transgenic mice. *Hypertension* 1993; **22**: 608–617
 190. Sauer B, Henderson N: Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* 1988; **85**: 5166–5170
 191. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K: Deletion of DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* 1994; **265**: 103–106
 192. Lembo G, Hunter JJ, Chien KR: Signaling pathways for cardiac growth and hypertrophy. Recent advances and prospects for growth factor therapy. In: Claycomb WC, Di Nardo P, editors. *Annals NY Academy Science: Cardiac growth and regeneration*. New York: New Academy of Sciences, 1995; 752: 115–127
 193. Schneider MD, Parker TG: Cardiac growth factors. *Prog Growth Factor Res* 1991; **3**: 1–26
 194. Fishman MC, Stainier DYR: Cardiovascular development. *Prospects for a genetic approach*. *Circ Res* 1994; **74**: 757–763
 195. Chien KR: Molecular analysis of cardiac developmental phenotypes: Problems, progress and prospects. In: Clark EB, Markwald RR, Takao A, editors. *Developmental mechanisms of heart disease*. New York: Futura Press, 1995; 3–27
 196. Zhou MD, Sucov HM, Evans RM, Chien KR: Retinoid dependent pathways suppress myocardial cell hypertrophy. *Proc Natl Acad Sci USA* 1995; **92**: 7391–7395
 197. Chambon P: The retinoid signaling pathway: molecular and genetic analyses. *Sem Cell Biol* 1994; **5**: 115–125
 198. Neer EJ, Clapham DE: Signal transduction through G proteins in the cardiac myocyte. *Trends Cardiovasc Med* 1992; **2**: 6–11
 199. Geisterfer-Lowrance AA, Kass S, Tanigawa G, Vosberg H-P, McKenna WJ, Seidman CE, et al: A molecular basis for familial hypertrophic cardiomyopathy: A beta cardiac myosin heavy chain gene missense mutation. *Cell* 1990; **62**: 999–1006
 200. Watkins H, McKenna WJ, Thierfelder L, Suk HJ, Anan R, O'Donoghue A, et al: Mutations in the genes for cardiac troponin T and α -tropomyosin in hypertrophic cardiomyopathy. *N Engl J Med* 1995; **332**: 1058–1064
 201. Thierfelder L, Watkins H, MacRae CA, Lamas R, McKenna WJ, Vosberg H-P, et al: α -tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell* 1994; **77**: 701–712
 202. McKenna WJ, Watkins H: Hypertrophic cardiomyopathy. In: Scriver C, Beaudet A, Sly W, Valle D, editors. *The metabolic and molecular basis of inherited disease*. New York: McGraw Hill, 1995; 4253–4272
 203. Schwartz K, Carrier L, Guicheney P, Komajda M: Molecular basis of familial cardiomyopathies. *Circulation* 1995; **91**: 532–540
 204. Sweeney HL, Straceski AJ, Leinwand LA, Tikunov BA, Faust L: Heterologous expression of a cardiomyopathic myosin that is defective in its actin interaction. *J Biol Chem* 1994; **269**: 1603–1605
 205. Weatherall D: *Science and the quiet art: The role of medical research in health care*. New York: WW Norton, 1995
 206. Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, Perriard J-C, Chen KR, Caroni P: MLP-deficient mice exhibit a disruption of cardiac myofibrillar organization, dilated cardiomyopathy, and heart failure. *Cell* 1997, in press