Cardiovascular Research

New Technologies, Methods, and Applications

Edited by

Gerard Pasterkamp

Dominique P.V. de Kleijn

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Springer

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Library of Congress Control Number: 2005933093

ISBN-10: 0-387-23328-8; e-ISBN: 0-387-23329-6 Printed on acid-free paper.
ISBN-13: 978-0387-23328-4

Printed in the United States of America. (TB/EB)

9 8 7 6 5 4 3 2 1

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Preface

In the last decade, technical improvements have changed the inventory of many research laboratories. New techniques and discoveries continuously give rise to observations that result in the definition of new research objectives. In the past, research departments were clearly demarcated. Nowadays, technology that is shared by all lines of research stimulates convergence of research interests. This also applies to cardiovascular research. Vascular occlusive disease is now core business for researchers employed by cardiology, vascular surgery, vascular medicine, radiology, cell biology, chemistry, physiology, and many other areas. Knowledge on actual research development is shared by researchers with different skills. It is sometimes difficult to acquire expertise when a researcher feels his experimental work could be improved by introducing a new research technique. In this book, the investigator will find an overview of recent developments that are relevant for research in general but cardiovascular research in particular. Genomics, proteomics, microarray, RNAi, stem cells, and progenitor cells are just some phrases that have become increasingly prevalent in literature in the last few years and that are recognized by many, but are fully understood by few. In this book, experts share the most appreciated new developments and techniques in cardiovascular research. We hope that this book will help the reader who is working in the field of cardiovascular research to understand and critically appreciate current research, and that it will help improve the quality of experimental work.

Dr G. Pasterkamp
Dr DPV de Kleijn

Utrecht, The Netherlands

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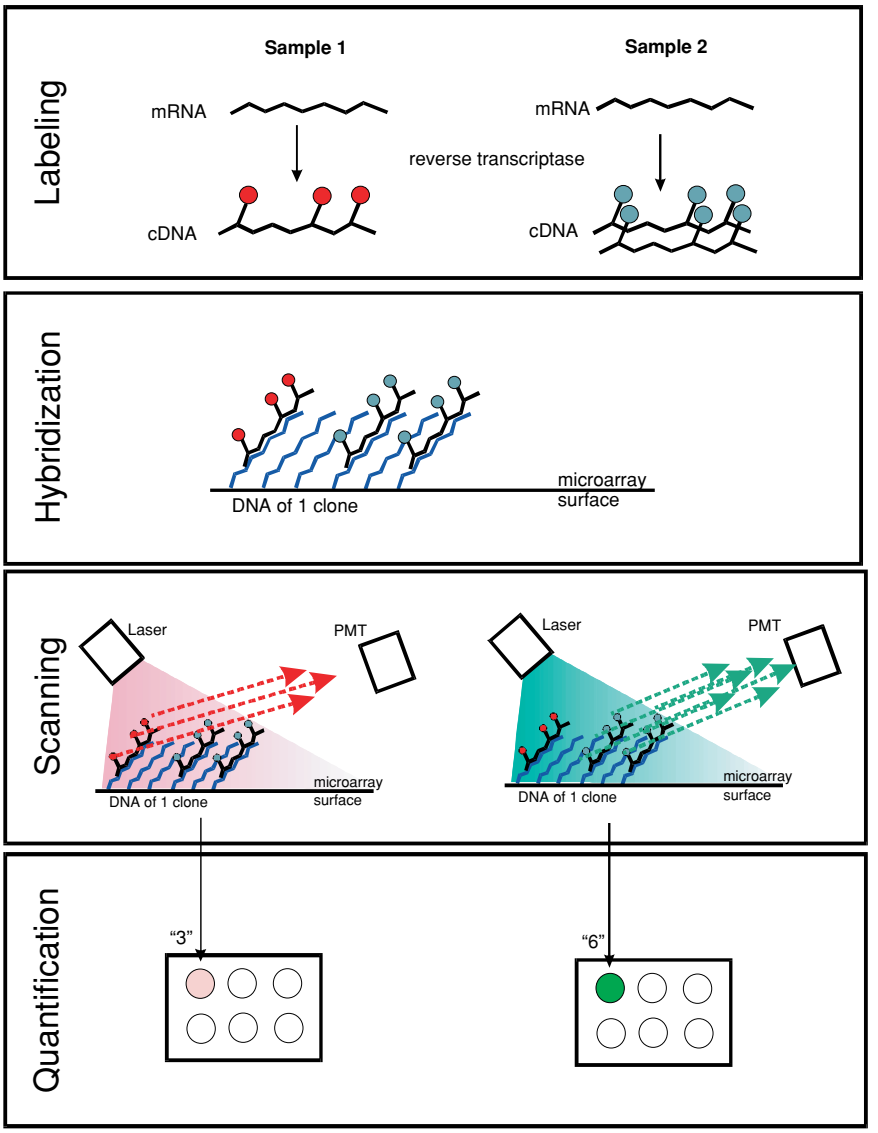

FIGURE 1 (page 6). Overview of the microarray procedure.

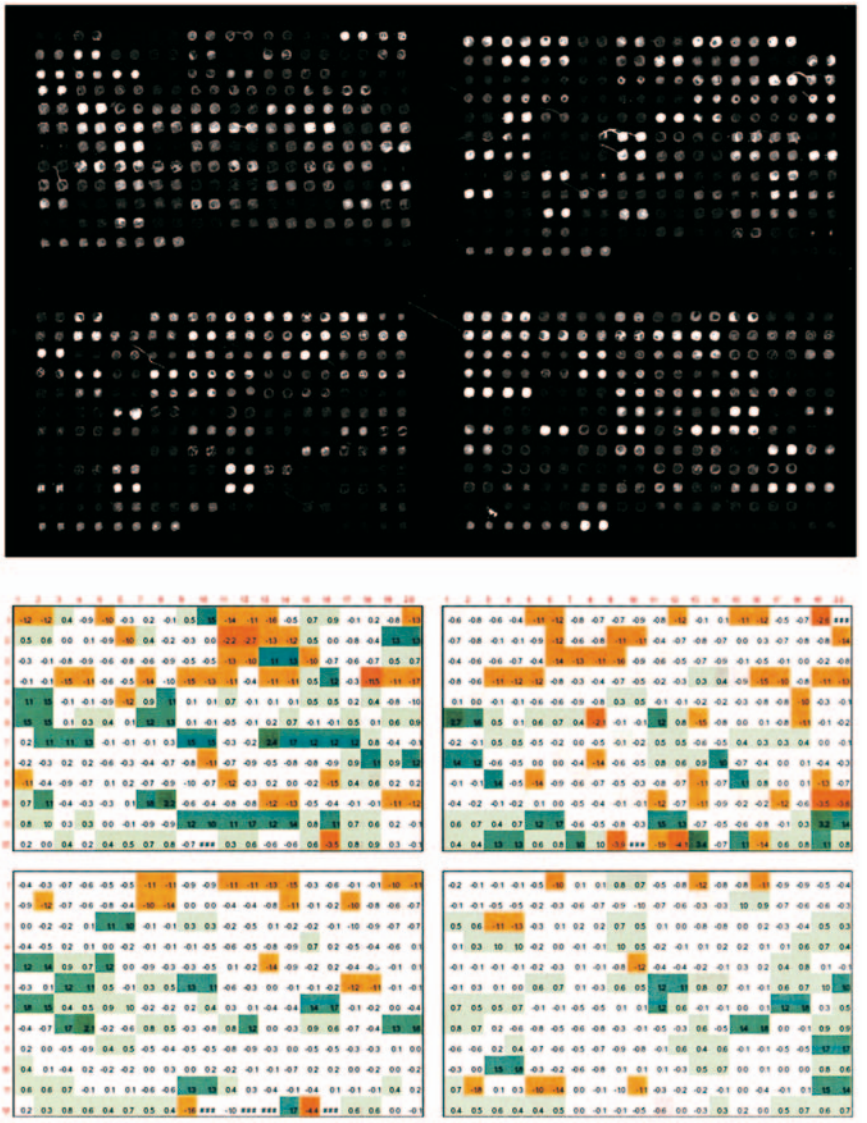

FIGURE 5 (page 21). Image of a microarray and the corresponding table with the $\log_2$ ratios. No location-related ratios can be detected.

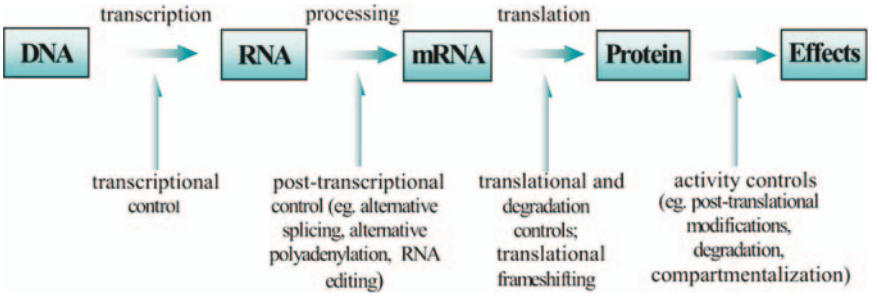

FIGURE 1 (page 46). The ways in which gene expression can be regulated or modified from transcription to posttranslation.

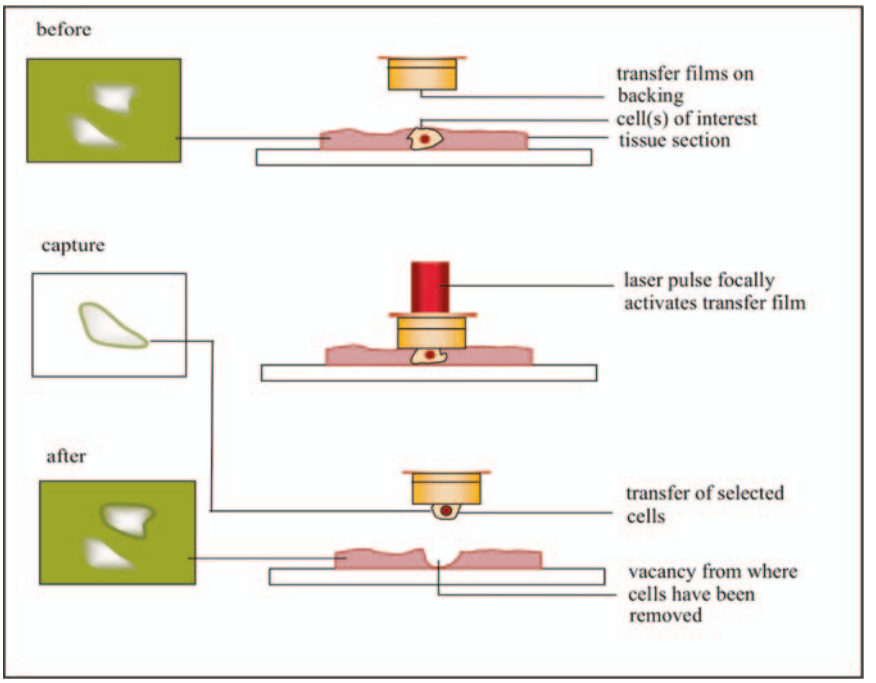

FIGURE 3 (page 48). Laser capture microdissection. A transparent polymer film is placed in direct contact with the surface of a heterogeneous tissue section. Laser energy is used to activate the polymer directly over the selected cells. The activated region captures the selected cells, which can be lifted away from unwanted tissue.

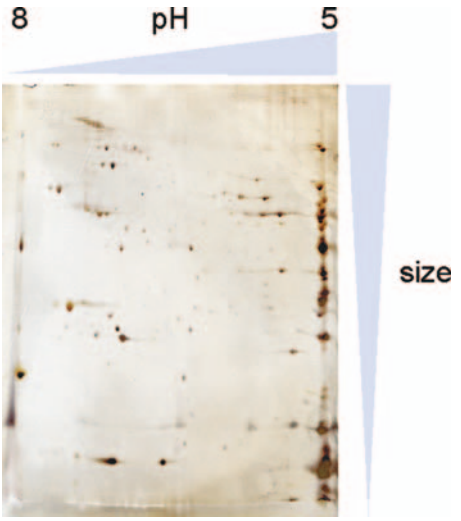

FIGURE 4 (page 50). Silver-stained 2D-PAGE gels of a mouse heart (left ventricle).

swiss 2D page : **P06576**

1 protein has been found in the clicked spot (2D-000TKM):

General information about the entry	
View entry in original SWISS-2DPAGE format	
Entry name	**ATPB_HUMAN**
Primary accession number	**P06576**
Entered in SWISS-2DPAGE in	Release 06, September 1997
Last modified in	Release 14, October 2001

NiceProt View of Swiss-Prot: P06576

[General] [Name and origin] [References] [Comments] [Cross-references] [Keywords] [Features] [Sequence] [Tools]

General information about the entry	
Entry name	**ATPB_HUMAN**
Primary accession number	**P06576**
Secondary accession number	Q14283
Entered in Swiss-Prot in	Release 06, January 1988
Sequence was last modified in	Release 14, April 1990
Annotations were last modified in	Release 41, February 2003

FIGURE 6 (page 56). Annotated protein in SWISS-2DPAGE and SWISS-PROTS databases.

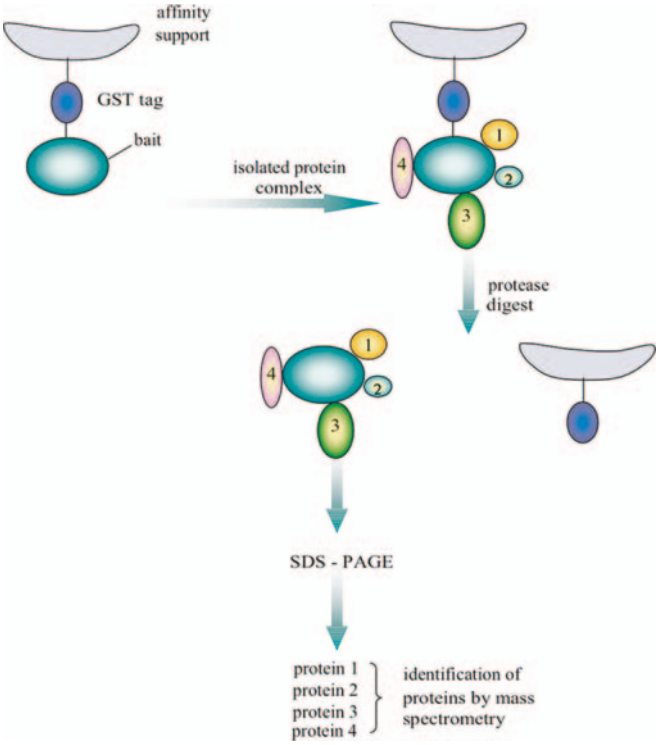

FIGURE 7 (page 58). Analysis of protein–protein interactions. The protein of interest is expressed as a fusion protein with a cleavable affinity tag to identify interacting proteins. It is immobilized onto agarose beads using a glutathione S-transferase tag. Nuclear cell extracts are incubated with the beads and the beads washed extensively. Thrombin is used to cleave between the glutathione S-transferase and the "bait" protein, which results in the elution of all proteins that are specifically bound to "bait" (Pandey and Mann, 2000). The eluted proteins are resolved by 2D-PAGE and analyzed by MS. The success of the above-mentioned strategies relies on sufficient affinity of the protein complex to the bait and on optimized conditions for purifications steps.

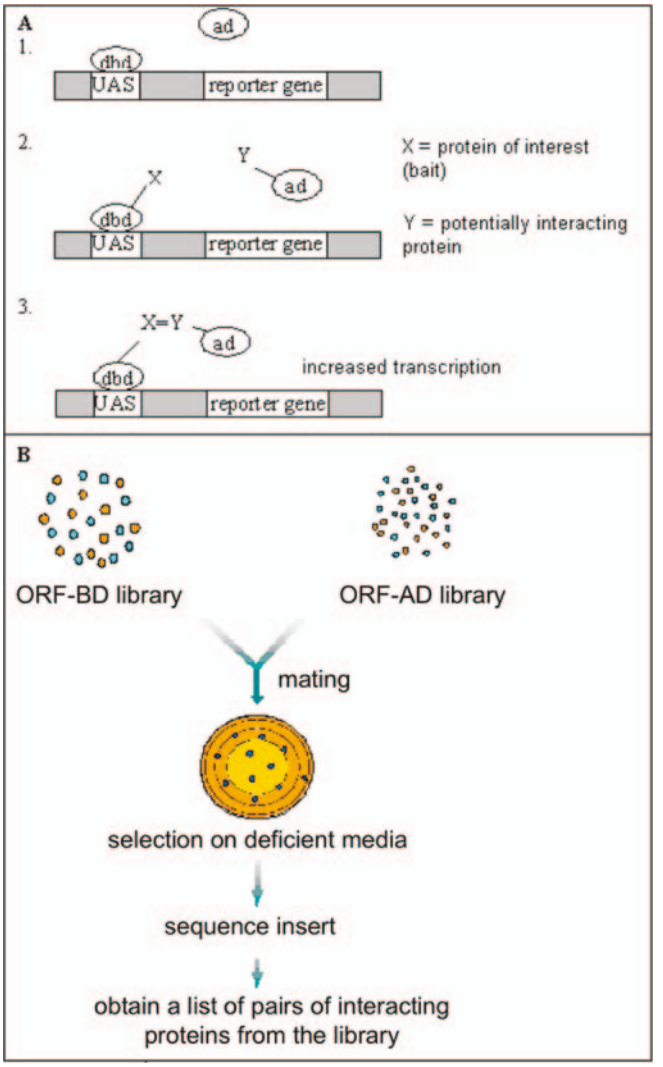

FIGURE 8 (page 59). The yeast two-hybrid system. (a) *Schematic representation of the yeast two-hybrid system*. (1) The two separated domains of a transcription factor are not functional and therefore do not induce transcription of the reporter gene. (2) The DBD and AD are fused to two proteins of interest and co-expressed in a yeast reporter strain. (3) If DBD-X and AD-Y interact, the fusion proteins are assembled at the binding site of the reporter gene, which leads to activation of transcription. (b) *Library-based yeast two-hybrid screening method*. In this strategy, two different yeast strains containing two different cDNA libraries are prepared. In one case, the open reading frames (ORFs) are expressed as GAL 4–BD fusions and in other case, they are expressed as GAL 4–AD fusions. The two yeast strains are then mated and diploids selected on deficient media. Thus, only the yeast cells expressing interacting proteins survive. The inserts from both the plasmids are then sequenced to obtain a pair of interacting genes. (Modified from Pandey and Mann, 2000).

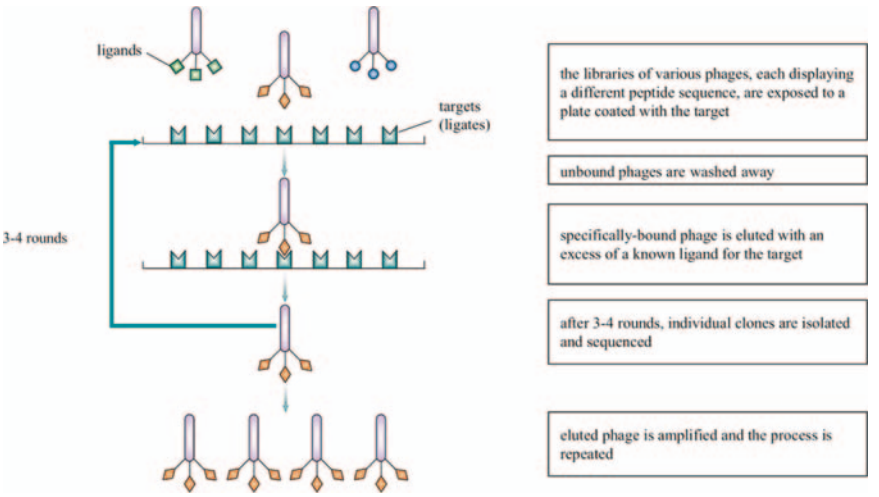

FIGURE 9 (page 60). Affinity-selection of ligands from phage display libraries. Different peptide sequences can be screened for binding to a specific target molecule. After several rounds only the clones with the peptides that bind to the target are isolated.

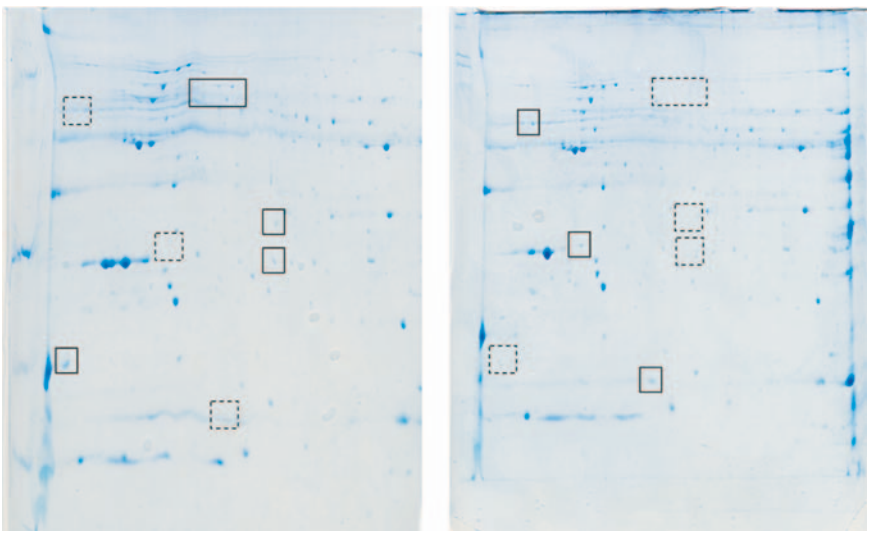

FIGURE 2 (page 72). Coomassie-stained 2D gels of proteins isolated from normal (left) and infarcted left ventricle (right). Spots displaying significant differences in staining intensity between normal and infarcted left ventricle are framed (interrupted frames indicate the positions of spots of reduced or even undetectable intensities).

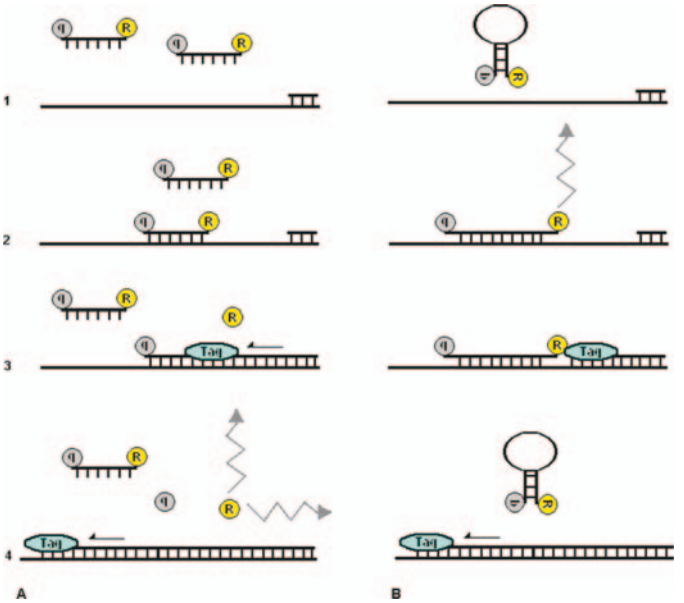

FIGURE 3 (page 79). Schematic picture of the TaqMan® assay and the molecular beacon approach. (A) In the TaqMan® assay, the probe is labeled at the 5′ end with a fluorescent reporter (R) and at the 3′ end with a quencher molecule (Q). The fluorescence of the reporter will be absorbed by the quencher when the probe is still intact. During the extension phase the Taq polymerase cleaves the hybridized probe and the reporter molecule is released from the quencher. The fluorescent signal can now be detected and will be increased during amplification when more and more probe will be bound and will be cleaved. (B) The molecular beacon is a hairpin-loop-shaped oligonucleotide with a fluorescent reporter (R) to one arm and a quencher molecule to the complementary other arm. When the probe binds to the template the reporter and quencher are separated and the fluorescent signal can be monitored. During amplification more and more probe will bind and an increased fluorescent signal is observed.

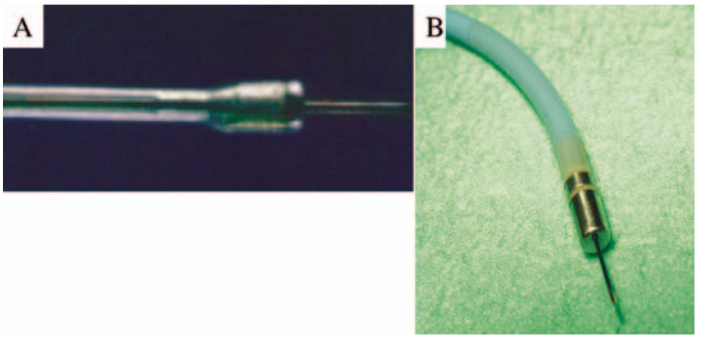

FIGURE 1 (page 91). Intramyocardial gene delivery catheters. (A) Stiletto® catheter for intramyocardial injection. A microscopic needle is on the tip of the catheter for the penetration of the endocardial border and for the infiltration of genes inside the myocardium. (B) NOGA® electromechanical mapping and injection catheter. The electrical measurements and the myocardial movements can be detected with the same catheter as the gene injection. A small needle is pushed out and pulled in during the gene injection.

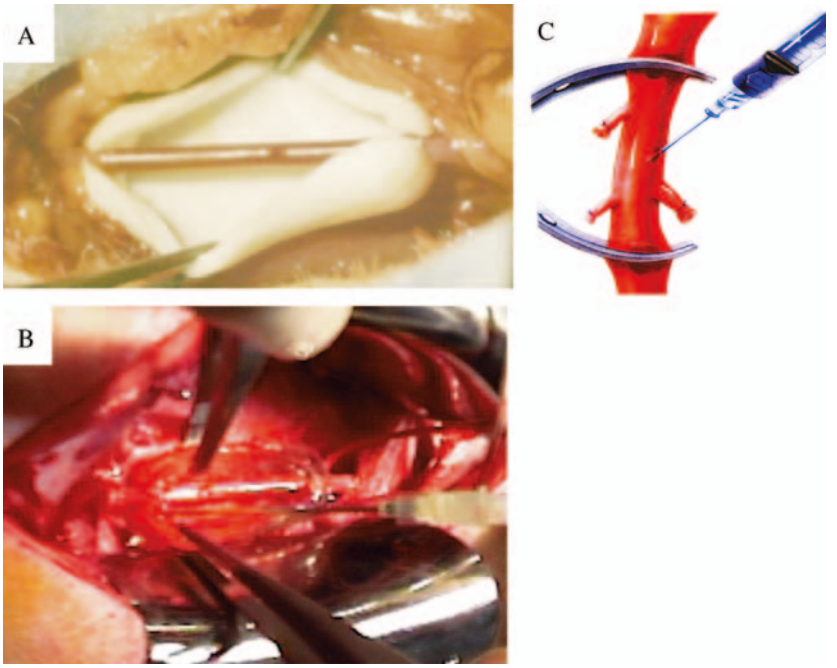

FIGURE 2 (page 92). Extravascular gene delivery methods. (A) A biologically inert collar installed around the rabbit carotid artery. Genes are injected into the collar so that they are in close contact with the vascular wall. (B) Biodegradable collar releasing therapeutic agents into the vessel wall. (C) Direct injection of genetic material into blood vessel.

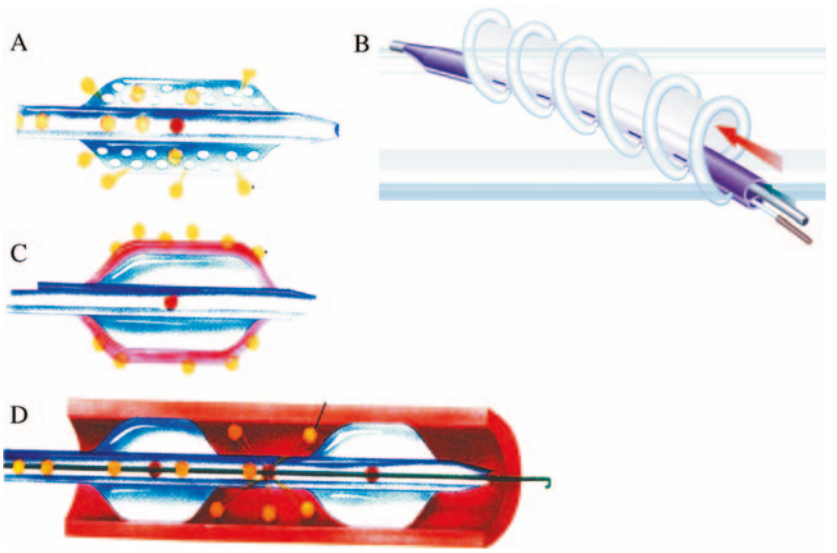

FIGURE 3 (page 93). Intravascular gene delivery catheters. (A) Channel balloon® with microscopic injection pores. Gene injection can be performed during angioplasty. (B) Dispatch® infusion–perfusion catheter. Continuous blood flow is allowed through the catheter during gene injection. (C) Hydrogel-coated balloon. The genes are applicated into the hydrogel material and released after balloon inflation. (D) Double balloon catheter. See details in the text.

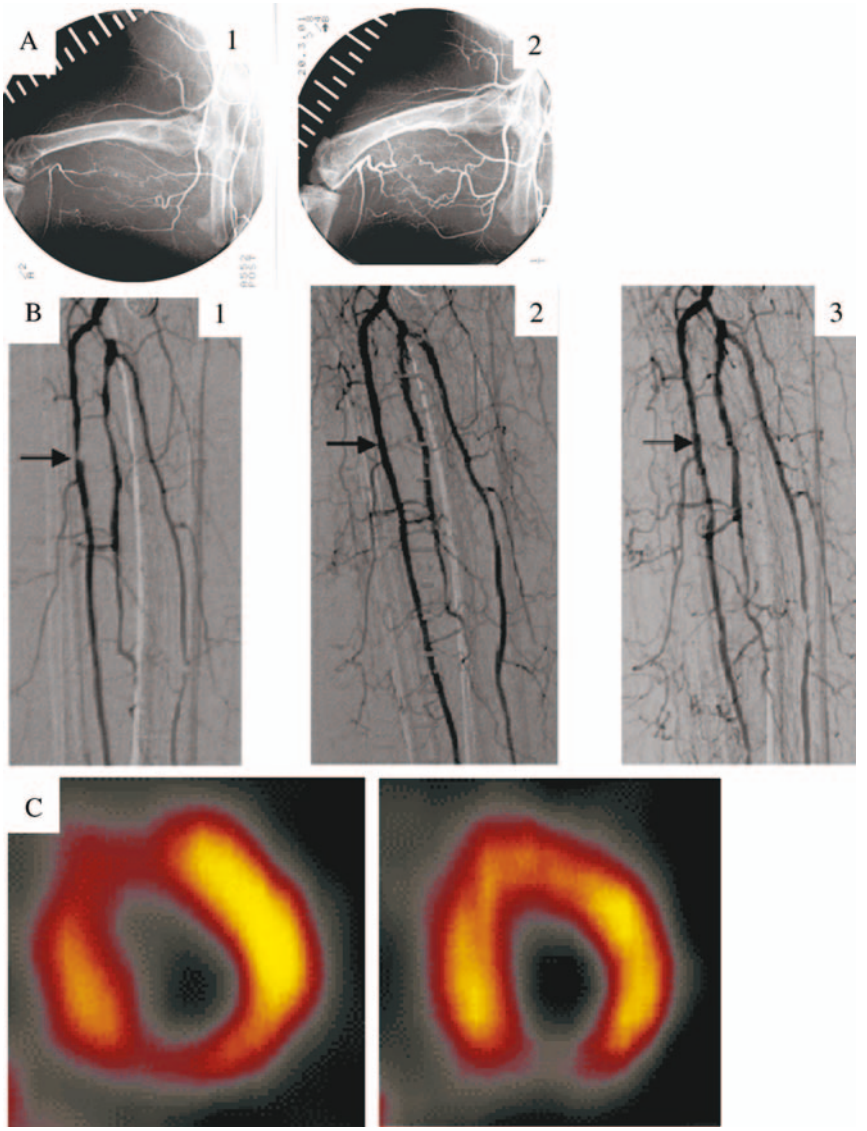

FIGURE 4 (page 96). *In vivo* examples of angiogenic gene therapy. (A) An animal model of a rabbit hind limb before (1) and 35 days after (2) intramuscular injections of VEGF gene in an adenovirus vector (Laitinen *et al.*, 1997). (B) Human truncus of leg arteries before (1), 3 months after (2), and 9 months after (3) angioplasty combined with local intra-arterial VEGF gene transfer (Laitinen *et al.*, 1998). (C) Myocardial scintigraphy of a human heart before and 6 months after local intracoronary adenoviral VEGF gene transfer performed during coronary angioplasty (Hedman *et al.*, 2003).

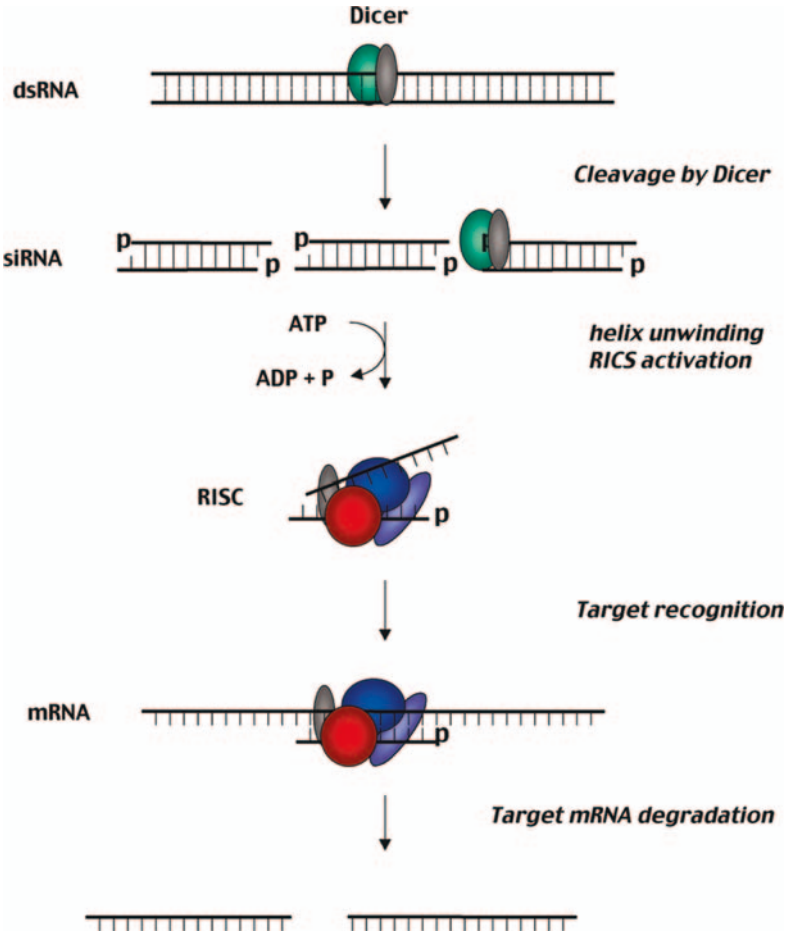

FIGURE 1 (page 123). Mechanism of RNAi. dsRNA is cleaved into 19–23 nucleotide fragments: siRNA. The RNA duplex is presented to the inactive RISC. RISC is activated by the reduction of ATP and causes unwinding of the RNA double helix. The antisense strand is incorporated in the RISC and is responsible for the recognition of sequence-specific mRNA. The sense strand is discarded. Upon recognition, the mRNA is cleaved and degraded.

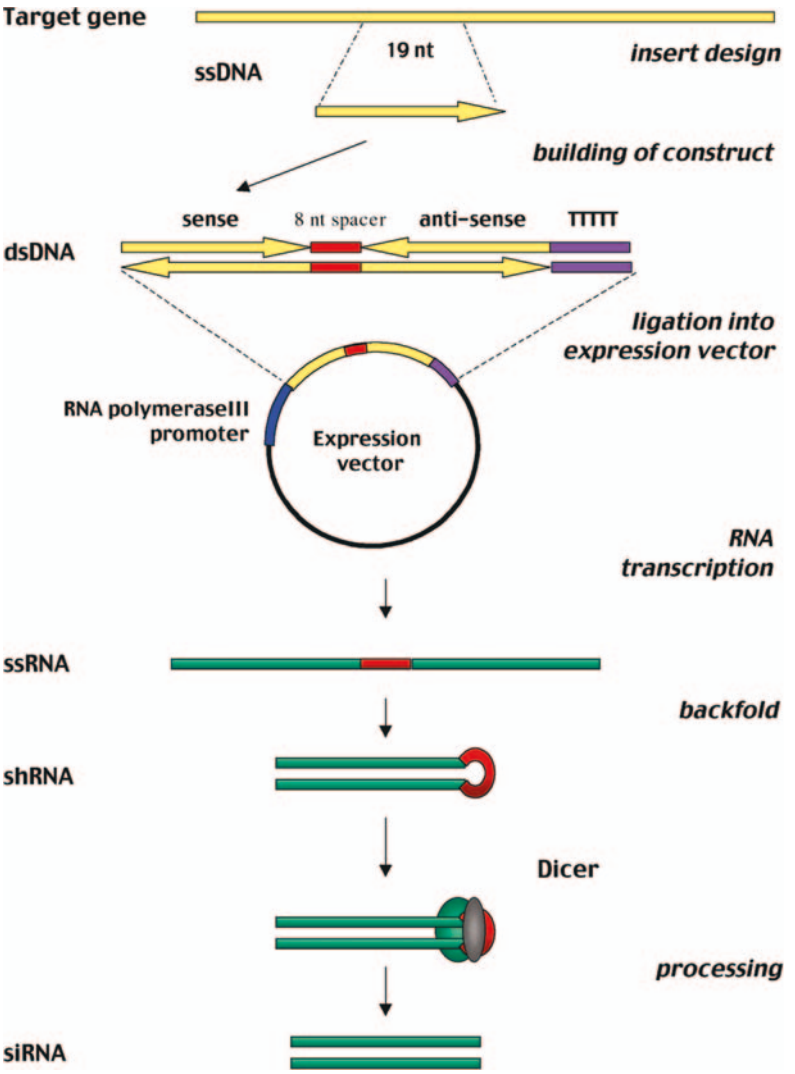

FIGURE 2 (page 125). Construction of siRNA by means of hairpin-expressing vectors. A 19 nucleotide DNA sequence is designed in homology to the gene of interest. A construct is created with this sequence followed by a short spacer and the same sequence in the antisense direction (inverted repeat) and a poly-T fragment. This construct is ligated into an expression vector containing a RNA polymerase III promoter. Transfection of the vector into eukaryotic cells initiates transcription of the construct into single-stranded RNA (ssRNA). The palindromic nature of the ssRNA leads to back folding of the ssRNA into shRNA. Dicer processes shRNA into functional siRNA.

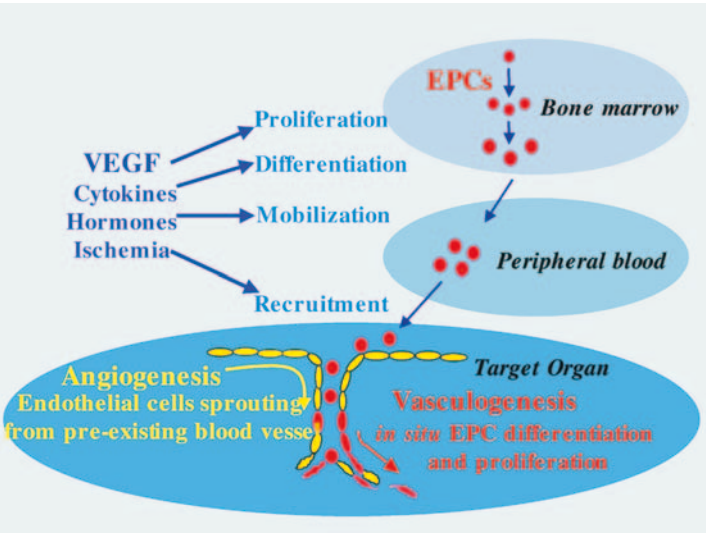

FIGURE 1 (page 176). Neovascularization encompasses both angiogenesis and vasculogenesis. Angiogenesis represents the classic paradigm for new vessel growth, as mature, differentiated endothelial cells (ECs) break free from their basement membrane and migrate as well as proliferate to form sprouts from parental vessels. Vasculogenesis involves participation of bone marrow-derived endothelial progenitor cells (BM-EPCs), which circulate to sites of neovascularization where they differentiate *in situ* into mature ECs. Growth factors, cytokines, or hormones released endogenously in response to tissue ischemia, or administered exogenously for therapeutic neovascularization, act to promote EPC proliferation, differentiation, and mobilization from BM (via the peripheral circulation) to finally home and incorporate into neovascular foci.

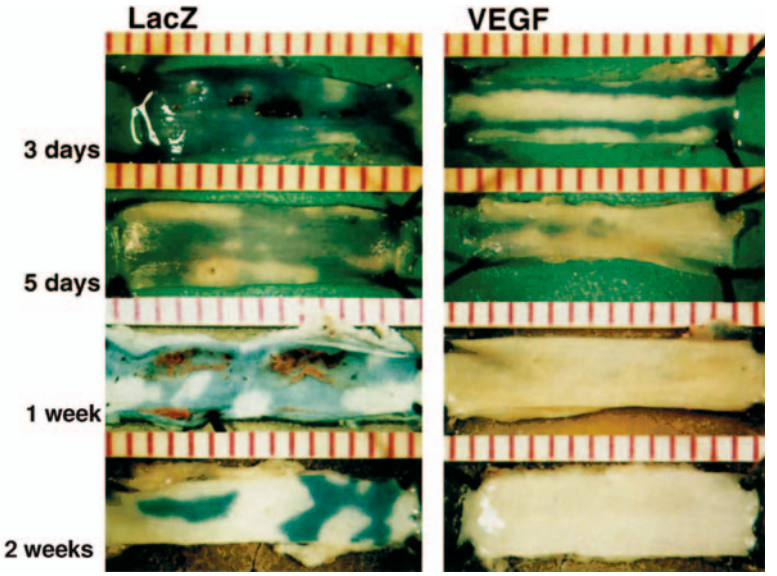

FIGURE 2 (page 178). Representative macroscopic photographs of balloon-injured rabbit iliac arteries transfected with plasmid DNA encoding for β-galactosidase (LacZ) or VEGF at 3 and 5 days and 1 and 2 weeks after transfection. The reendothelialized area does not stain with Evan's blue dye and appears white. $P < 0.01$.

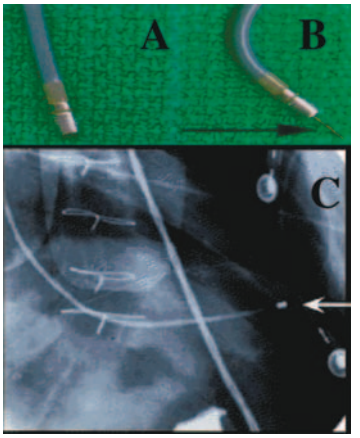

FIGURE 4 (page 189). Modified mapping-injecting catheter (Biosense™, Johnson & Johnson). (A) The electrode in the distal tip of the catheter allows annotation of electromechanical maps to document the sites of gene transfer by intramyocardial injection. (B) The 27-gauge needle has been advanced out of the distal tip of the catheter to simulate myocardial engagement in preparation for injection. (C) Posteroanterior view recorded during cine-fluoroscopy shows the distal tip of the catheter (arrow) against the endocardium of the left ventricular lateral wall in preparation for injection. The 27-gauge needle advanced into the myocardium is not visible.

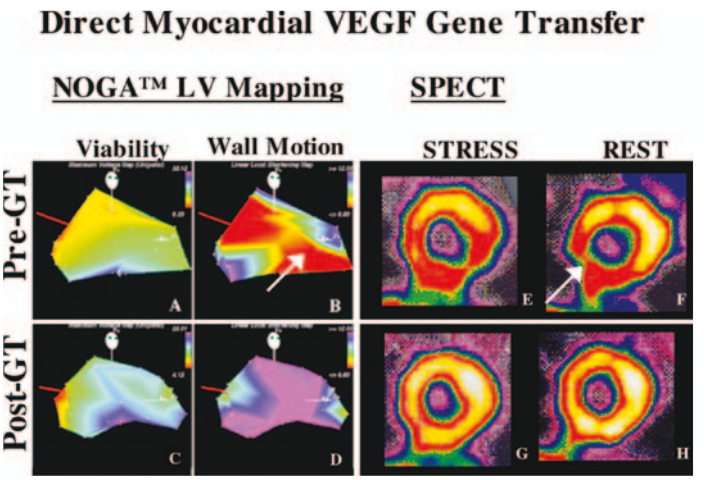

FIGURE 5 (page 190). NOGA™ left ventricular electromechanical mapping (LV EMM): Unipolar images (A) showing normal voltages suggestive of viable myocardium (purple/pink/blue/green) and linear local shortening (LLS) map (B) with large zone of abnormal wall motion (red, arrow) that represents electromechanical uncoupling suggestive of ischemic or hibernating myocardium involving the inferoseptal region, from a 48-year-old man before phVEGF$_{165}$ GTx. The red lines represent the long axis through apex. Vertical and horizontal axes (x, y, and z) are presented as white lines. Sixty days after GTx, unipolar (C) and LLS (D) images show complete resolution of ischemia (10.08 cm^2 before GTx vs 0.00 cm^2 after GTx) that corresponds to changes observed on SPECT scan. Persantine SPECT-sestamibi myocardial perfusion scanning images: White and yellow colors depict maximal uptake of radionuclide, and red depicts impaired uptake. Selected short-axis stress and resting images were taken before (E, F) and after (G, H) phVEGF165 GTx in the same patient. Pre-GTx scans (top) show reversible inferoseptal defect (arrows). Post-GTx scans (bottom) show complete normalization of resting perfusion and perfusion after pharmacological stress.

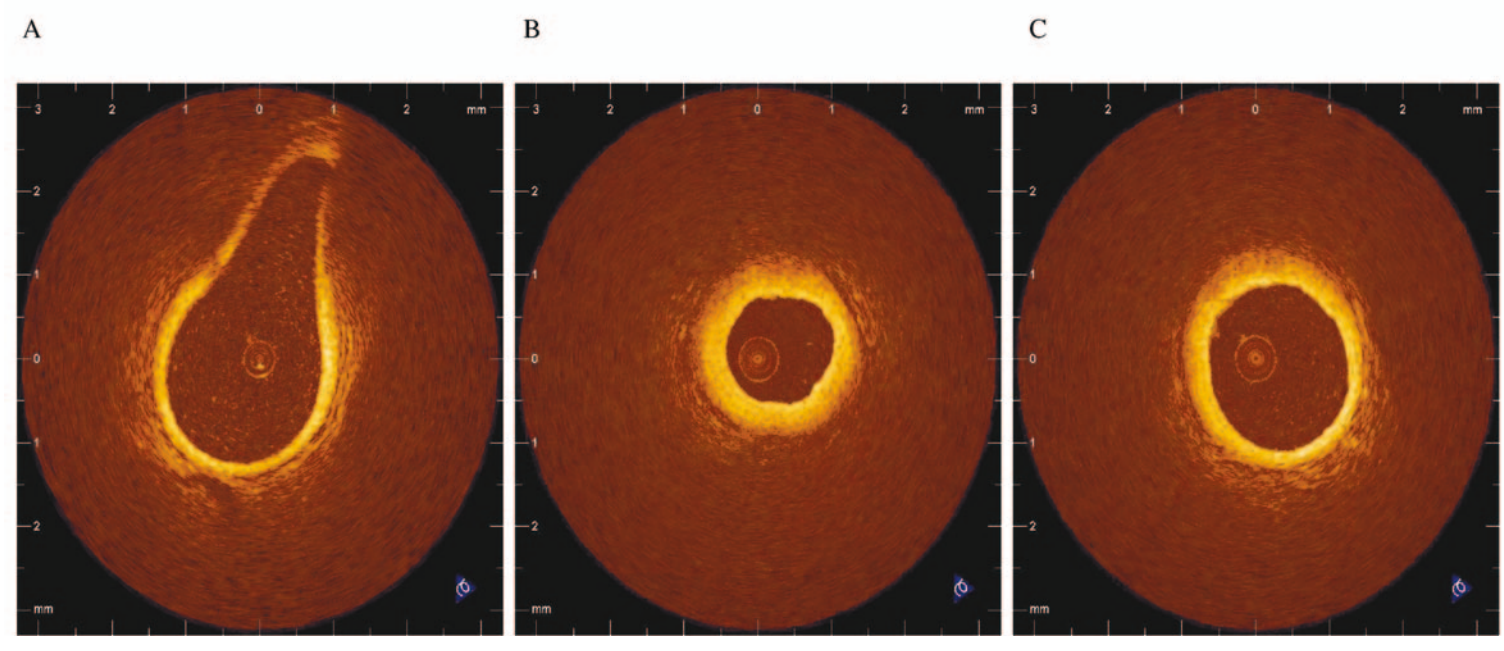

FIGURE 1 (page 225). OCT images obtained during a pullback of an atherosclerotic rabbit aorta. Bloodless conditions were created by balloon occlusion and flushing at a constant rate. Indicated are a normal segment (panel A), a plaque having features of fibrotic tissue (panel B), and a cross section of a plaque containing a lipid-rich plaque (panel C).

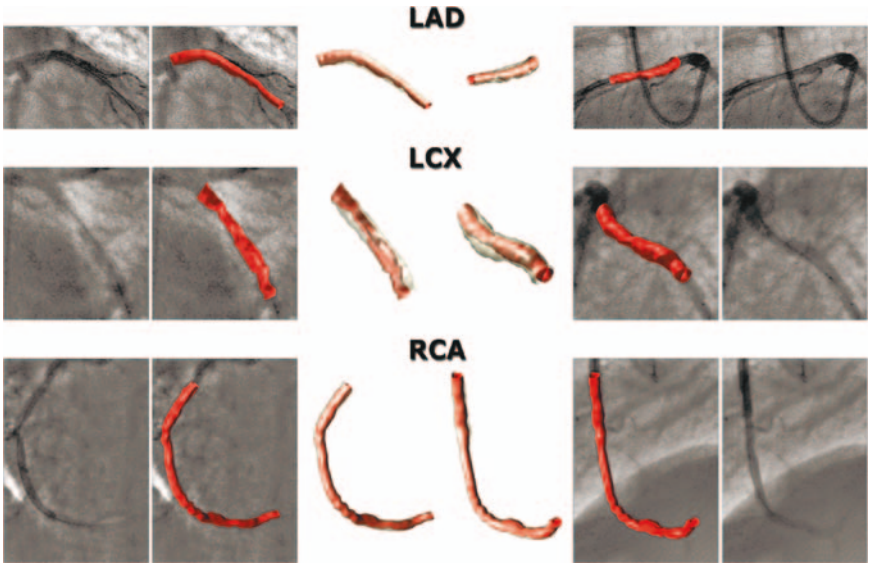

FIGURE 3 (page 229). Three-dimensional reconstruction of human coronary blood vessels based upon a combination of angiography and IVUS (ANGUS). Indicated are three vessels from three different patients.

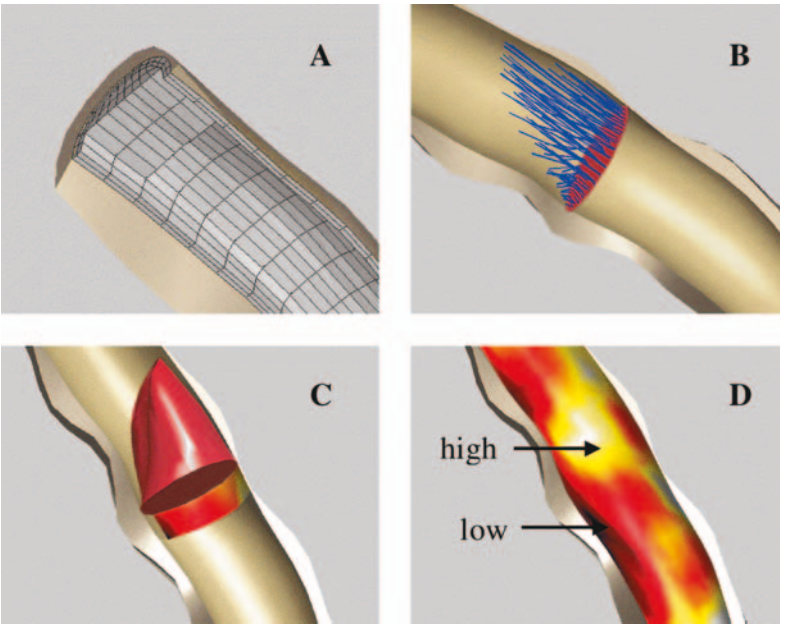

FIGURE 4 (page 230). Indication of the method to obtain a shear stress mapping. Panel A shows the filling of the lumen by finite elements, panel B the resulting velocity field for a cross section, panel C shows the envelope of the vectors, and panel D the resulting shear stress mapping on the endothelium of the 3D reconstructed blood vessel.

Part I

Methods

Chapter 1

Expression Profiling in Cardiovascular Disease Using Microarrays

Branko Braam and Hans Bluyssen

1. GENERAL INTRODUCTION

This chapter is focused on the application of genome-wide screening techniques, with the emphasis on microarrays to assess gene expression and the application in cardiovascular research. Why would one still want to look at gene expression when proteomics is also available? One point of view is that gene expression analysis deals with the perceptive site of the cell: how do extracellular signals affect the program of the cell. How does induction of second messengers lead to alterations in gene expression? Another viewpoint is that without the transcript there is no protein. There are as many examples of good correlations between transcription and protein expression as there are exceptions; however, the *no transcript, no protein* concept remains of important value. Good examples of close relations between gene and protein expression are formed by the components of the renin–angiotensin system: mice with one to four copies of the human renin gene have proportional protein expression (Smithies *et al*., 2000). A more defensive point of view is that proteomics is still associated with major problems, in particular with respect to the activation state and modifications of proteins. Also, there is only one genome in, from which multiple transcriptomes are derived, depending upon the state of the cell. The number of possible proteomes in a cell is, however, breathtaking.

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

The aim of the chapter is to briefly guide the reader through the technical backgrounds of genome-wide expression techniques, with a strong emphasis on the use of microarrays. Serial analysis of gene expression (SAGE) and differential display are also quite useful techniques; however, since microarrays are easier to apply, application of microarrays has become increasingly popular. We discuss the goals that one can set for gene expression studies and propose a work-up for the obtained data. At the end of the chapter we discuss several gene expression studies that have been directed to cardiovascular problems.

2. TECHNICALITIES IN A NUTSHELL

2.1. Tools for Differential Expression Analysis

From the start of the twentieth century, when William Bateson first introduced the term *gene*, the pace of progress in genetics has been astounding. That chromosomes contained the genetic material of the cell was discovered a decade later, and the structure of DNA was elucidated by Watson and Crick in 1953 (Watson and Crick, 1953). Less than 50 years later, the sequencing of the entire human and mouse genomes has become a fact (Lander *et al*., 2001; Venter *et al*., 2001) and announced with great hullabaloo. This marked the start of a new era in the field of genetics.

Current estimates suggest that there are about 30,000–35,000 genes in the human genome (Ewing and Green, 2000), twice the number in *Drosophila*. Given the relative modest increase in the number of genes expressed compared with increases in size and complexity from *Drosophila* to mouse to human being, the timing and levels of expression and alternative splicing of these genes likely underlie the complex function of the human body. Thus, the challenge of discovery has begun to shift from the identification of genes to the elucidation of their functions and their contribution to normal development, individual variability, and common diseases.

Based on the principle of hybridization of complementary nucleotides, application of microarrays for genome-wide expression analysis has first been described by the laboratory of Pat Brown in 1995 (Schena *et al*., 1995). The description of this technique included technical details about the development of spotting robots and hybridization procedures. At that time, two other techniques were operational, SAGE and differential display, which have been successfully applied, but are somewhat harder to perform (Lockhart *et al*., 1996; Schena *et al*., 1995). More recently, beads-based techniques have become available (Brenner *et al*., 2000). All of these techniques are targeting the same issue: when different states of cells or tissues are compared, how is this reflected in a change in gene expression?

2.1.1. Microarrays

DNA microarray is an array of DNA probes that are deposited on a carrier surface, usually a glass slide or a nylon membrane, in a high-density pattern.

Nucleotide arrays work by hybridization of labeled RNA or DNA in solution to the DNA molecules attached to the surface. The hybridization of a sample to an array is, in effect, a highly parallel search of each molecule for a matching partner on an "affinity matrix," with the eventual pairings of molecules on the surface governed by the rules of physicochemical interactions between molecules. In this way, different samples can be compared and the relative abundance of a gene or gene transcript in each sample can be quantitatively determined. Microarrays allow the monitoring of gene expression for thousands or tens of thousands of genes simultaneously in one hybridization experiment. Thus, DNA microarrays have revolutionized the collection and analysis of genetic information. The overall procedure is depicted in Figure 1.

2.1.2. Other Expression Profiling Strategies: SAGE and Differential Display

Another high-throughput technique for the measurement of gene expression, SAGE technology, was originally developed by Velculescu *et al*. (1995). Two basic principles underlie the SAGE methodology (Patino *et al*., 2002; Ye *et al*., 2002), which distinguish it from the microarray technology. First, it reduces cDNA molecules that have been reverse transcribed from polyadenylated mRNA by a series of enzymatic manipulations to tags of 10–13 bp. Each tag represents one mRNA. Second, tags are ligated to form concatemers that are cloned and sequenced. Comparing the sequence information from the tags to the GenBank database provides qualitative information about transcribed genes. The frequency of a specific tag within the SAGE tag population correlates with its relative abundance in the cell and gives quantitative information about expressed genes. The SAGE method therefore allows for cataloging and comparison of expressed genes under various physiological conditions.

Differential display, although a less high throughput technique, is also a powerful tool for the comparison between two or more mRNA populations (Liang and Pardee, 1992; Welsh *et al*., 1995) and based on three steps. First, RNAs extracted from the sources are reverse transcribed using an anchored primer to produce cDNA reflecting a subset of the mRNA component of the cell. Second, the cDNA subset is amplified in a PCR reaction using the anchored primer and a random primer to produce a set of cDNAs representing a smaller subset of RNA. The reaction is carried out in the presence of radiolabeled nucleotide that will incorporate in the accumulating PCR product. Third, the complex mixture of cDNAs is then resolved by electrophoresis through a denaturing polyacrylamide gel and visualized by autoradiography. Subsequently, differentially displayed bands are excised from the gels and reamplified by PCR in the absence of radionucleotide. The amplified products are then ligated into a plasmid vector, transformed into bacteria, and screened for the presence of insert DNA by PCR using flanking vector primer sequences. These cDNAs can be tested to confirm differential gene expression. Recombinant clones are selected and plasmid DNA extracted prior to sequencing and database searching. Recent developments have incorporated safer, nonradioactive methods,

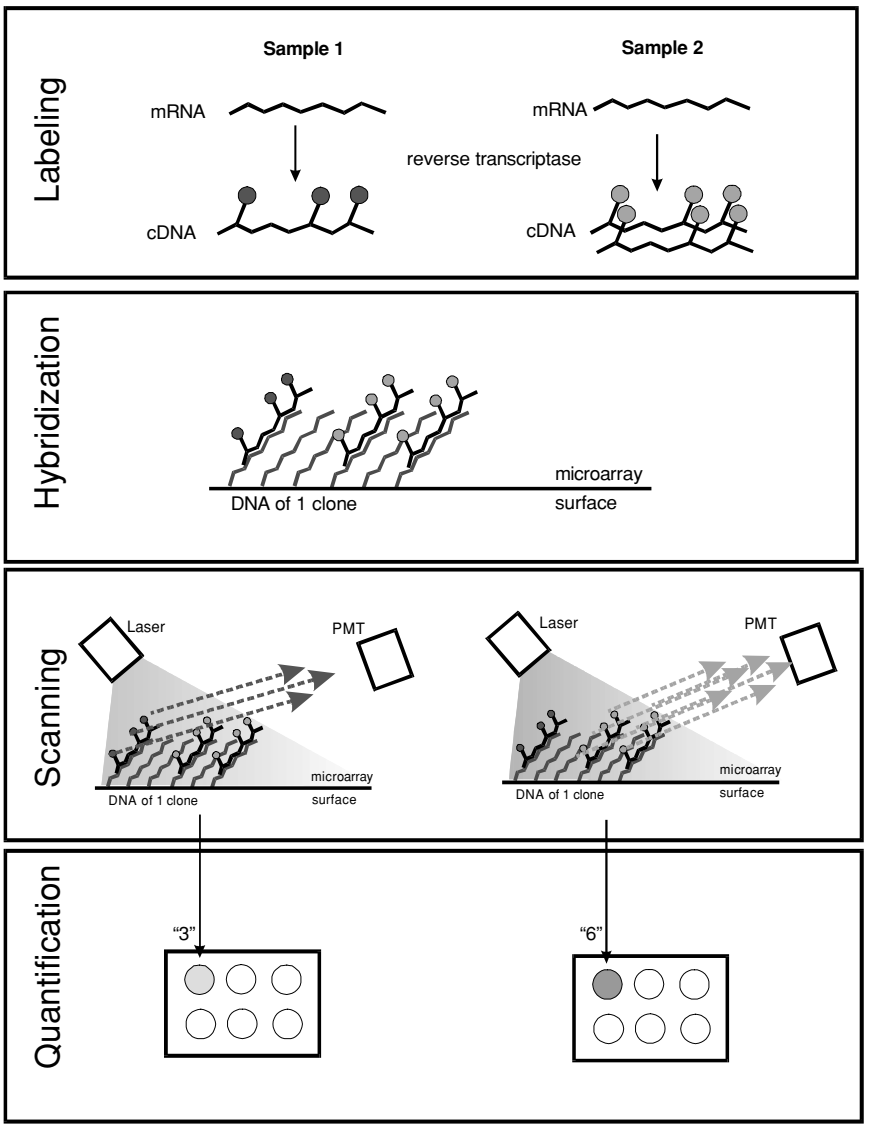

FIGURE 1. Overview of the microarray procedure. (*See Color Plate 1.*)

including silver staining for detection after gel electrophoresis, fluorescent-labeled oligonucleotides for PCR, and the use of biotinylated primers with streptavidin-coated beads for capture. Agarose gel electrophoresis in combination with ethidium bromide staining and UV detection of cDNAs is an alternative option.

One of the main differences between DNA microarray and SAGE is that microarray preparation requires prior knowledge of the sequence of the gene

Table 1
Classification of Microarrays

DNA on chip
Oligonucleotide arrays
cDNA arrays
Surface
Nylon
Glass
Labeling
Fluorescent dyes (glass slide arrays)
Radioactive dyes (filter arrays)
Purpose
Find new function for known genes
Find function for new genes
Dissect pathways of transcriptional regulation
Mutation detection

transcripts to be analyzed. This is a serious limitation, even for organisms with completely sequenced genomes such as humans, because genome annotation and gene prediction remain technical challenges. SAGE, however, can be used to analyze gene expression in organisms whose genomes are largely uncharacterized. Microarrays have advantages, because they are relatively easy to use and more suitable for high-throughput applications, and therefore, they are likely to be useful in clinical settings.

2.2. Types of Arrays

Microarrays can be qualified on different bases: interrogating DNA material, used surface, construction, or purpose (Table 1). cDNA arrays have been at the basis of microarray development. Basically, 1000–2000 bp clones of the genes of interest are amplified using PCR. One issue of particular relevance is whether the clones indeed reflect the genes that are annotated. Unfortunately, skepticism has been shown to be in the right place (Knight, 2001). Affymetrix™ has set a standard for the use of smaller sequences; the technique encompasses several 25mer sequences to interrogate a specific gene, and the hybridization signals of the test sample are constituted to represent a quality score for the gene of interest. This method applies amplification of the RNA so that very small samples can be processed. Finally, 60–80mer oligonucleotide sequences are now frequently used as probes and have been shown to be very useful.

One main difference between used surfaces for microarrays is formed by nylon filters or glass. Filter arrays are exclusively used for radiolabeled samples, and glass arrays mainly for the use of fluorescent dyes. Glass surfaces allow a higher density of spots on the array, which is relevant for the amount of test sample needed to hybridize the array and the number of interrogated genes. There is a big difference between the numbers of genes that can be assessed by a microarray: the large

arrays are now capable of assessing 25,000 to 35,000 genes and expressed sequence tags (ESTs). There is no doubt that microarrays or similar techniques will be able to cover the whole genome including splice variants in the near future. The glass surfaces are modified by different processes to yield optimal hybridization; detailed information about this issue is beyond the scope of this text (Zammatteo *et al*., 2000).

Besides the surface, the method of nucleic acid deposition on the substrate can be different: robotically spotted (Schena *et al*., 1995) or *in situ* synthesized (Lipshutz *et al*., 1999). Historically, the robotically spotted microarrays were referred to as cDNA microarrays because the nucleic acids being spotted were PCR products amplified from cDNA libraries. However, more and more presynthesized long oligonucleotides (∼50–70mers) are used as an alternative in this format, to prepare spotted oligonucleotide microarrays (van de Peppel *et al*., 2003). A typical spotted array is printed onto a 60 mm by 20 mm glass microscope slide by a computer-controlled spotting robot; each spot is about 50–150 μm in diameter. Nucleotide arrays are usually spotted on glass slides but can also be produced on a nylon membrane with spots of slightly larger size in a lower density pattern. cDNA and long oligo clone sets of 15,000–20,000 mouse and 40,000 human genes are available for in-house arrays in addition to ready-made commercial chip sets. Although most commercial arrays contain 10,000–20,000 spots, arrays containing 80,000 spots can be created. Given the estimated ∼30,000–70,000 genes in the human genome (Lander *et al*., 2001), microarrays encoding all expressed genes of a particular organism will likely be available in the foreseeable future, thus enabling truly comprehensive analysis of transcription. Spotted arrays allow a certain degree of flexibility in the choice of arrayed elements, particularly for the preparation of smaller, customized arrays for specific investigations.

High-density-oligonucleotide microarrays can be produced *in situ*, either by photolithography onto silicon wafers (high-density-oligonucleotide arrays from Affymetrix, http://www.affimetrix.com) or by ink-jet technology (developed by Rosetta Inpharmatics, http://www.rii.com, and licensed by Agilent Technologies). With this type of array, shorter oligonucleotides (∼25mers) are placed on the arrays and each gene is represented by multiple oligos. Affimetrix has pioneered the use of the high-density-oligonucleotide array with the development of the Genechip. The Genechip series is designed with 11–20 different oligonucleotides to represent each gene on the array. Each oligonucleotide has a partner with a single base mismatch to control for nonspecific binding. Together, over 1,000,000 unique oligonucleotide features are present, covering 39,000 transcript variants, which in turn represent more than 33,000 of the best-characterized human genes. Methods based on synthetic oligonucleotides offer an advantage that production and handling of cDNAs is not required.

A relative newcomer to the array field, the self-assembled bead array, uses a combination of the attractive features of the above-mentioned global gene analysis techniques. Lynx Therapeutics has commercialized a highly advanced tool for comprehensive gene expression, called massively parallel signature sequencing (MPSS) (Brenner *et al*., 2000a, 2000b). MPSS, based on Megaclone technology, involves cloning each mRNA molecule onto the surface of a 5-μm bead. An

arbitrary but unique DNA combitag sequence is attached to a fragment of cDNA derived from each mRNA, and the tagged library is PCR amplified. The resulting cDNA library is hybridized to beads, each of which is decorated with 100,000 identical oligonucleotide strands complementary to one of the combitags. After hybridization, each of the beads displays amplified copies of one and only one starting mRNA molecule. The amplified cDNA copies on each bead originate from a single mRNA molecule. Thus, each bead is conceptually equivalent to a bacterial clone, with each clone (bead) harboring many copies of a single cDNA.

After hybridization, one million beads are immobilized in a flow cell for sequencing biochemistry and imaging, and the cDNA signature sequence on each bead is determined in parallel. The novel sequencing process involves repeatedly exposing four nucleotides by enzymatic digestion, ligating a family of encoded adapters, and decoding the sequence by sequential hybridization with fluorescent decoder probes. Comparison of the signature sequences with available databases reveals their genetic origin. The count of beads from each mRNA yields its frequency in the sample. The level of sensitivity provided by MPSS is critical for a variety of experiments because many important genes are expressed at low levels in the cell. MPSS has a routine sensitivity of a few molecules of mRNA per cell and the results are in a digital format that simplifies data management and analysis. MPSS results will be particularly useful for generating the type of complete data sets that will help to identify the functionally important genes in the sample of interest.

Microarrays can be used for different goals (see below). One of the most frequently used scientific goals is to investigate the relevance of genes for a specific physiological or pathophysiological state. One could call this curiosity-driven science. A second goal is to test the expression behavior of a specific set of genes, which is too large to test using techniques based on expression levels of individual genes.

2.3. RNA Isolation, Labeling, and Hybridization

The aim of a typical microarray experiment is to measure the amount of a large set of mRNA species (transcribed genes) in a tissue or cell type, for example from a control individual in comparison to a patient with cardiovascular disease (hypertension, atherosclerosis). To accomplish this, different steps in a microarray experiment are performed. In addition to microarray construction, these include (1) RNA extraction, (2) transformation of RNA into labeled cDNA, (3) array hybridization with labeled material, and (4) scanning and analysis of the hybridization pattern.

A microarray experiment starts with isolation of high-quality RNA. In this respect, total RNA or mRNA can be isolated. It is of note that a change in RNA isolation procedure may affect the microarray experiment. Next, the (m)RNA is transformed by reverse transcription into cDNA, which is more stable. mRNA or cDNA from a sample is applied to the array surface and allowed to hybridize. There is a fundamental difference in the experimental set-up between the robotically spotted arrays and *in situ* synthesized ones. In the robotically spotted array

experiments, the two samples under comparison are labeled (either directly or indirectly) with two different fluorescent dyes, for example Cy3 (green) and Cy5 (red), purified, pooled, and cohybridized to the same array (Fig. 1A). This is essentially a comparative hybridization experiment. Thus, by comparison of the hybridization of cDNA from a control individual with that from a patient with hypertension, labeled with Cy3 or Cy5 for detection, the relative amounts of all the genes present on the array and expressed in the two samples can be measured. In the photolithographically synthesized array experiments, the two samples under comparison are labeled with the same dye and individually hybridized to different arrays (Fig. 1B). Signal intensities of probe array element sets on different arrays are used to calculate relative abundance for the genes represented on the arrays. For a reliable microarray experiment, all steps in array construction, RNA extraction, labeling, and array hybridization have to be performed to the highest possible standards.

2.4. Scanning

In the following we will focus on dual-label hybridization techniques, in which Cy3 and Cy5 are the most frequently used dyes. To be able to extract the relative signal intensities and ratios of mRNA abundance for the genes represented on the spotted cDNA or long oligo array, hybridization is followed by high-resolution confocal fluorescence scanning of the array, with two different wavelengths corresponding to Cy3 and Cy5. Thus, two independent images are generated, one corresponding to the control and one to that of the test material. These images must then be analyzed to identify the arrayed spots and to measure the relative fluorescence intensities of each element. The choice of scanning parameters is of utmost importance. These include the laser, which illuminates and detects each pixel sequentially. The laser power can be varied to provide optimal excitation power. The photomultiplier tube (PMT) converts the signal into a digital image. In general, the PMT gain setting is fixed between 30 and 65%. The laser power (80–100%) can be adjusted during the scanning process to balance the overall intensities between the two channels (i.e. Cy3 and Cy5) as much as possible.

The choice of a suitable scanning resolution depends on the array specification. A rule of thumb is that (1) the resolution setting should be at least 10% of the spot diameter, (2) the number of spots with saturated pixels should be kept to a minimum to maximize the dynamic range usage of the scanner, and (3) excessive scanning of a slide should be avoided to prevent photobleaching. Therefore, it is recommended to scan directly after hybridization and washing and to keep the slides in the dark as much as possible. Images of high quality can be acquired routinely when all these factors are taken into consideration.

2.5. Image Analysis

The next step following image acquisition involves spot recognition (finding) or gridding. The goal of the spot finding operation is to locate the signal spots in the image and estimate the size of each spot. Microarray images consist of arrays

of spots arranged in grids. All the grids have the same number of rows and columns of spots. These subgrids are arranged with relatively equal spacing to each other, forming a complete array. Spot finding algorithms have been developed, which use a grid frame in combination with contemporary image analysis software to locate the signal spots in the image and estimate the size of each spot. In semiautomatic methods (developed by ImaGene, BioDiscovery) it is possible/necessary to adjust the grid for some spots manually afterwards. Completely automatic spot finding utilizes advanced computer vision algorithms to find the spots without the need for any human intervention (AutoGene, BioDiscovery). Many scientists, however, prefer to visually inspect the images for adjusting the grid and flagging low-quality spots instead of totally relying on software recognition.

After the spot location is determined in the image, a process called segmentation is used to differentiate the foreground pixels (i.e. the true signal) in a spot grid from the background pixels. There are several algorithms for segmentation, including fixed circle segmentation and adaptive circle segmentation, which assume that spots are circles and vary only in size. More sophisticated algorithms, like adaptive shape segmentation and histogram segmentation, can recognize spots that also vary in shape and morphology. There are also several algorithms for background estimation, for example constant background, local background, and morphological opening. For more detailed description the reader is referred to more specified reviews, including Yang and Buckley (2001) and references therein. The choice of appropriate algorithms obviously depends on the quality of the raw images. For example, the adaptive circle segmentation that estimates the diameter separately for each spot works best when all the spots are circular. However, variations in spot morphology in poor-quality images, high background, or other image imperfections require more sophisticated methods of segmentation (Yang and Buckley, 2001). Because it is much more robust for various algorithms to perform segmentation and background estimation processes on a high-quality image than on a low-quality one, it is crucial to produce a high-quality microarray and collect a high-quality image from it in the first place.

2.6. Bio-informatics

After the segmentation process, the pixel intensities within the foreground and background masks (i.e. the areas in the image defined as foreground and background by the software, respectively) are averaged separately to give the foreground and background intensities, respectively. Median or other intensity extraction methods can be used when there are extreme values in the spots that skew the distribution of pixel intensities. Subtracting the background intensity from the foreground intensity in each channel gives the spot intensity for calculating the expression ratio between the two channels.

The data extracted from the image analysis need to be preprocessed to exclude poor-quality spots. Any spot with intensity lower than the background plus two standard deviations should be excluded. The intensity ratios should also be

log-transformed so that upregulated and downregulated values are of the same scale and comparable (Smyth and Speed, 2003).

After image processing, it is necessary to normalize the relative fluorescence intensities in each of the two scanned channels. Normalization adjusts for differences in labeling and detection efficiencies for the fluorescent labels and for differences in the quantity of initial RNA from the two samples examined in the experiment. These problems can cause a shift in the average ratio of Cy5 to Cy3 and the intensities must be rescaled before an experiment can be properly analyzed.

There are different techniques that can be used to normalize gene expression data from a single microarray experiment: (1) total intensity normalization, which uses all genes on the array; (2) housekeeping genes normalization, which uses housekeeping genes and the assumption that they are constantly expressed; (3) external controls normalization, which uses known amounts of spiked-in external control RNAs added during cDNA preparation and labeling. Unfortunately, these normalization methods are inadequate because dye bias can depend on spot intensity, spatial location on the array, and even depend on the pin of the spotting robot. Housekeeping genes are not as constantly expressed as was previously assumed. As a result, using housekeeping genes normalization might introduce another potential source of error. Dye-swapping experiments are seen as a plausible solution to reduce the dye bias problem, but can be impractical because of the limited supply of certain precious samples.

Recently, several more sophisticated, nonlinear normalization methods have been developed on the basis of gene intensity and spatial information (Quackenbush, 2002; Yang and Speed, 2003), such as LOWESS (locally weighted scatterplot smoothing) or ratio statistics (Powell *et al*., 2002; Smyth and Speed, 2003). In this way (local) corrections can be made, for example, for variation between the different spotting pens and for nonlinear relationships between the dye intensities. The issue of selecting a postnormalization cutoff is discussed in Section 4.3.

2.7. Reporting Array Data MIAME

It is almost impossible to judge the validity of a result just by inspecting the expression changes or even the raw data, without a reliable standard (Perou, 2001). For example, the same increase or decrease in gene expression observed by two different laboratories might actually be different, especially when they are using different experimental protocols and data analysis methods. In this respect, the Microarray Gene Expression Data (MGED) Society (http://www.mged.org), an international initiative to develop standards for microarray data, has recently proposed a standard Minimum Information About a Microarray Experiment (MIAME) (http://www.mged.org/Workgroups/MIAME/miame.html) (Brazma *et al*., 2001). The research community has embraced it and many major journals now require compliance with MIAME for any new submission. It is therefore advisable to ensure that the experimental design, implementation, and data analysis comply with the MIAME standard.

MIAME represents the minimal information to be recorded that enables faithful experimental replication, the verification of the validity of the reported result, and the facilitation of the comparison among similar experiments. Besides, the information should be structured with controlled vocabularies and ontology to assist in developing database and automated data analysis. Currently, the minimal information includes six parts:

1. experimental design
2. array design
3. samples
4. hybridizations
5. measurements
6. normalization controls

A detailed description of each part and a convenient checklist are available on the MIAME Web site: http://www.mged.org/Workgroups/MIAME/miame_checklist.html.

3. DESIGN

Despite the fact that microarray experiments should be designed in a similar fashion as a regular experiment not evaluating mass-scale parameters, several considerations should be taken into account. Since resources are still expensive and since the experience is that once the microarray data are available researchers are overwhelmed by the potential ways to analyze the data, planning a design that fits the goals of a microarray experiment is of utmost importance (Fig. 2). Goals can be categorized into the search for major players or fishing for new genes (finding candidates), unraveling pathways (pathway analysis), and gene expression pattern analysis (clinical profile analysis). Designs can be transversal or longitudinal (time series) and may or may not include interventions. In addition, the choice of proper reference samples should be carefully evaluated. Unfortunately, the use of reference samples has not been addressed in many reports and the difficulty of a right choice for reference material (which involves careful planning)

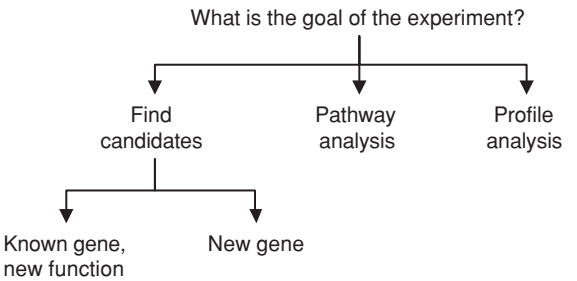

FIGURE 2. Goals for a microarray experiment.

is not sufficiently appreciated. In the following we will describe in more detail the different goals of a microarray experiment and elaborate on different designs that can meet these goals. Although the choice of cutoffs and normalization may seem trivial, we hope to evoke a critical view. In this chapter we do not include a detailed discussion about statistical analyses since excellent texts are now available (Draghici, 2003).

3.1. Setting Your Goals

3.1.1. Finding Candidates

The pleasure of the enlightenment of new functions for known genes or new genes can easily turn into the shadow side of differential profiling. Posing questions at the time of the design of the experiment may help toward interpretation of problems:

- Which candidates are already known markers for the experimental condition so that the experimental setup can be tested?
- Is the goal to find new genes or to find new functions for known genes, and is the applied microarray suitable for this purpose?
- In which biological or functional classes can differentially expressed genes be expected?
- How many differentially expressed genes can be expected?
- How is differential expression going to be confirmed and which criteria are going to be used to select which subset of differentially expressed genes is going to be confirmed?
- Will the generation of a list of candidates be sufficient to support a (patho)physiological role for these genes in the condition of interest, or need interventions to be planned?

In Figure 3 we present a proposal for a strategy to design an array experiment: define your goals, define validation targets, perform the microarray experiment, and first check your validation markers. In practice, the excitement of performing an array experiment has left many with a large number of expression ratios, which unfortunately did not confirm a previously known target, and as such, could not validate a previously known result. To illustrate such an experiment, we have compared amplified RNA obtained from human leukocytes exposed to IFNγ for 2 h and from untreated leukocytes. First, differential expression of IP10 and IRF1, which are known target genes of IFNγ, were confirmed using reverse-transcription PCR; this validated the setup. The RNA was labeled and subjected to 19K human 70mer microarrays, scanned, quantitated, and normalized. Analysis of the microarray data also confirmed the induction of IP10 and IRF1. After this confirmation, we continued with the further analysis of the microarray results.

The case in which one aims to find new genes versus the recognition of new function for known genes may demand different microarrays. As explained above, the probe set from which the microarray is printed may interrogate a large number

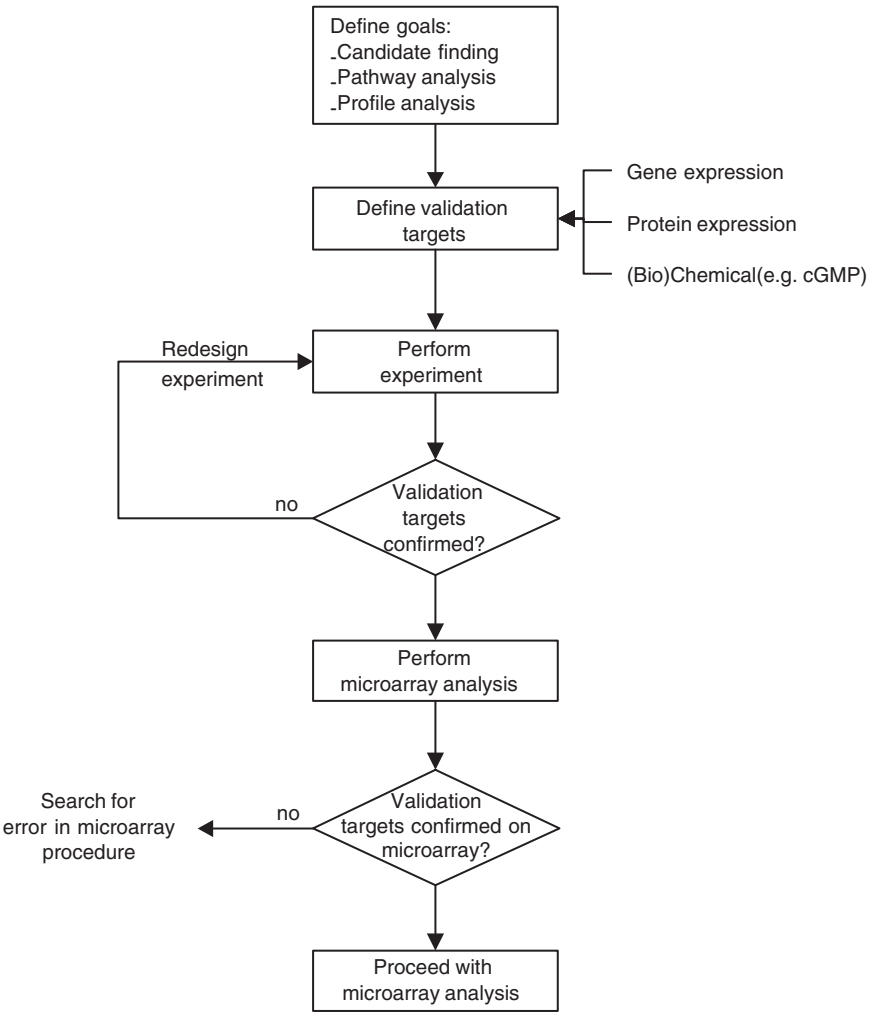

FIGURE 3. Diagram for validation approach.

of known genes and/or interrogate many ESTs. Let us start small: one wants to investigate the transcriptional behavior of a relatively small set of genes, e.g. cytokines, in a known pathway. For such purposes there are affordable commercial arrays available (and one could even imagine testing a set of genes with Northerns or real-time PCR). When the response to a stimulus is studied that is known to exert very broad expressional actions, but the aim is still to study the behavior of known genes, one can use a large microarray. If the specific aim is to identify new genes, the probe set should contain a large number of ESTs. One has to realize that pathophysiological states can evoke genes that are hardly ever expressed in a particular tissue. As such, it may be relevant to know from which source the ESTs originate: a set of brain ESTs may not be suitable to identify new genes of interest

in endothelial cells (ECs). Until microarrays (or other genome-wide techniques) are available that interrogate the full genome, such questions remain relevant.

3.1.2. Pathway Analysis

An approach gaining more and more interest is the investigation of signaling pathways in combination with expression profiles. A stimulus activates one or more signaling pathways. Within the cell, the transmission of the activation signal diverges through multiple pathways, which all arrive at transcription activators or repressors and ultimately modulate the transcriptome. Part of physiological genomics is now directed at deciphering this circuitry, that is to analyze which transcription factors are responsible for the transmission of information from second messengers to the genome. Such an approach starts with the research on specific regulatory networks that are involved in specific physiological responses of a cell and ends with the description of the cellular circuitry for very complex behavior of cells. For example, the EC is exposed to a variety of factors, among which shear stress is probably one of the most important. Shear induces NO release, and NO activates cyclic guanylyl cyclase (GC), which catalyzes the formation of 3′,5′-cyclic guanosine monophosphate (cGMP) from GTP. cGMP initiates a large number of reactions by induction of cGMP-dependent kinases (Bogdan, 2001). NO can also directly activate or repress the activity of transcription factors, such as NF-κB (Bogdan, 2001), most likely by nitrosylation. Finally, NO can interfere with the action of other signaling cascades (Schindler and Bogdan, 2001). Briefly, the net response of this activation is anti-inflammation, antiadhesion, and antiproliferation. Sequential intervention in the signaling cascade can be performed to decipher which part of signaling cascade is responsible for which set of gene activation, and regulatory motifs can then be searched for in the promoter areas of the genes of interest. At the end of the spectrum is automated discovery analysis combined with decision-making strategies to describe large network of regulatory genes. How this pathway analysis is related to system's biology is discussed in Section 5.6.

3.1.3. Profile Analysis and Disease Classification

It has now been clearly established that neoplasms can leave signatures in the transcriptome that can be used for diagnosis and classification. Several studies have now been published that could identify a group of genes that were differentially expressed in cancer patients and were successfully used to predict outcome (Alizadeh *et al*., 2000; van 't Veer *et al*., 2002). Alizadeh *et al*. (2000) investigated the transcription profiles of non-Hodgkin lymphoma, and was able to classify four subgroups of profiles using hierarchical clustering corresponding to different subgroups according to pathological classifications. The different clusters were predictive for the prognosis of the patients, which has not been achieved using conventional pathological diagnosis (Alizadeh *et al*., 2000). van 't Veer reported that by the use of a supervised learning technique to classify breast cancer, using a subset of 70 genes, they were able to predict the outcome (metastatic disease versus disease free) in 85% of the cases, an accuracy that cannot be met by conventional

classification methods for breast cancer (van 't Veer *et al*., 2002). Remarkably, there have been extremely few attempts to classify cardiovascular disease from gene expression profiles.

3.2. Define the Experiment

3.2.1. Cross-sectional Experiments versus Time Course Experiments

Cross-sectional experiments are relatively simple and extremely suitable for comparisons where one (or more) factor(s) is unambiguously present or absent. Such comparisons have been applied in oncology to test cancerous versus healthy tissue or to compare tissues from genetically different animals. In cardiovascular research this setup has been used to compare, for example, left ventricles from humans (Yang *et al*., 2000) and animals (Ueno *et al*., 2003) with and without heart failure and from animals with and without myocardial infarction (Stanton *et al*., 2000). When more than two conditions are compared, interpretation problems can arise. Imagine the complexity of a study where samples from left ventricles of animals with and without heart failure and in the presence and absence of angiotensin-converting enzyme would be compared.

Experimental designs that evaluate gene expression at a number of time points obviously enable the analysis of regulatory networks. One of the first reported time course microarray experiments investigated the response of fibroblasts to serum after a period of starvation and was able to discern regulatory programs sequentially being activated (Iyer *et al*., 1999). In the design of a time course experiment, the following parameters should be included:

- the number of meaningful time points: t
- the number of replicates for the experimental procedure: R_1
- the number of replicates for the microarray procedure: R_2
- the choice of reference time points

It may be helpful to realize that the number of arrays for such an experiment can be high: $t \times R_1 \times R_2 \times 2$. If one applies Affymetrix chips, reference samples have to be processed separately, whereas dual-label microarrays will demand a dye-swap approach, explaining the multiplication by 2. An ideal design is shown in Figure 4A; in this design each time point has its separate reference. This allows for the detection of changes in treatment that are independent of changes in the reference. In case there is clear indication that the reference does not change during the time course of interest, one reference may be sufficient (Fig. 4B). Obviously, this limits the amount of starting material, limits the number of arrays, and reduces the complexity of (time-consuming) data analysis.

3.2.2. One versus Multiple Comparisons and Reference Samples

The addition of extra comparisons in an experimental setup will increase the complexity of the attained data matrix. While two samples do not leave many options, adding a third sample increases possibilities for comparisons. One can

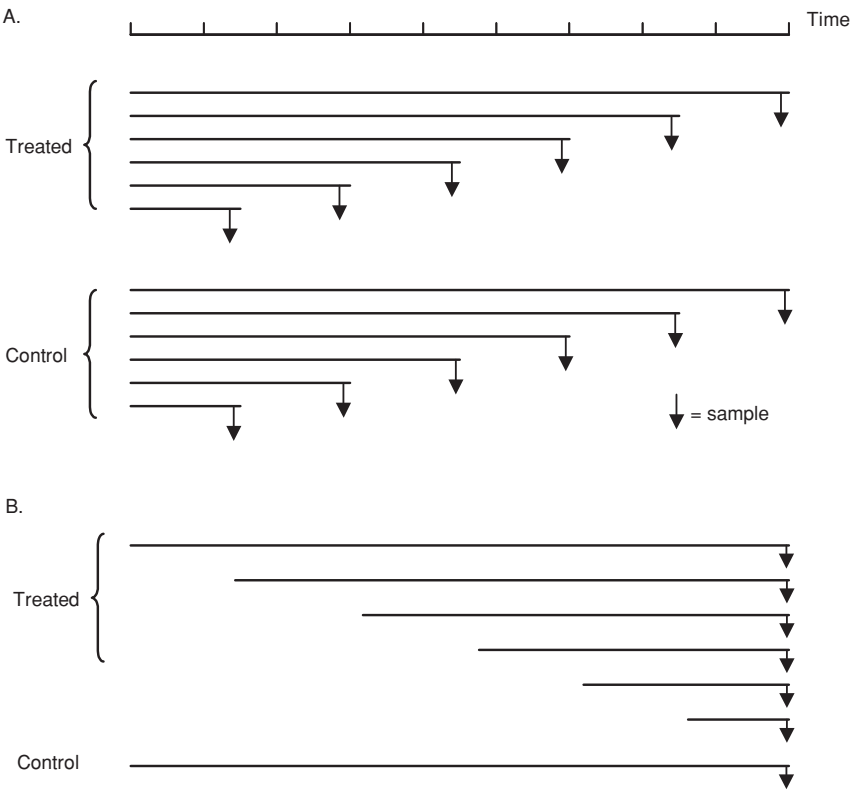

FIGURE 4. Designs of time course experiments.

compare two samples to a third sample (the so-called reference design) or compare each sample to both the other samples (loop design) (Chu *et al*., 1998). Inevitably, a dye-swap approach will further increase the number of arrays. When using a loop design, one should realize that *each comparison has its own summed error*. Such considerations require that you

- plan the comparisons,
- acquire plenty of high-quality reference material, and
- decide on the applied technique to compare ratios.

When using a reference design, the following designs can be considered:

$$\text{Test-to-matched reference:} \quad \frac{T_1}{R_1} \cdots \frac{T_n}{R_n}$$

$$\text{Test-to-pooled reference:} \quad \frac{T_1 \cdots T_n}{R_{\text{pool}}}$$

Here, T denotes a test sample and R a reference sample. The test-to-matched reference is particularly useful in patient studies. Test-to-pooled reference is an

implementation of *blocking* (Draghici, 2003), that is, creating a subset of experimental conditions which is more homogeneous than the individual samples.

3.2.3. How Many Replications of Experiments and Arrays Are Useful and Necessary?

For some time, the cost of microarrays has placed a burden on replication. Now performing microarrays is becoming affordable, and the issue of *replication* has explicit consequences. Replications can involve replication of spots on the microarray interrogating the same gene, replication of the microarray procedure *per se* (including a dye swap), or replication of the experiment itself. Several reports are now available that have addressed the usefulness of replication at these different levels. Lee *et al*. (2000) analyzed the multiplications of spots interrogating a gene and developed an error model using a relatively small cDNA array with a certain number of known positive targets. Single spots resulted in a relatively large number of false-positive gene calls in particular, and replication of the spots strongly reduced the false positives and negatives. In a joint report from two laboratories, nonbiological sources of error, intralaboratory variation and interlaboratory variation, were investigated using Affymetrix microarrays and different culture conditions for *Saccharomyces cerevisiae*. This analysis indicated low variability in case the experiment was performed in triplicate and a cutoff for the expression ratios of three times up or down was employed (Piper *et al*., 2002). A similar conclusion has been reached for cDNA arrays, be it that a dye-swap approach yielded more reproducibility than just a replicate of the same Cy3/Cy5 configuration (Liang *et al*., 2003). This study employed a walk-through method to determine cutoff levels, rather than a fixed cutoff. Pavlidis *et al*. (2003) analyzed microarray data of a large number of studies with respect to power (selected genes), recovery (percentage of genes that were significant in a subset, also found in the complete set), and order (order of the selected genes maintained in a subset). Most of the studies involved comparisons between neoplastic and healthy tissue samples. They concluded that large variation existed between the mentioned quality parameters. Since the analyses included studies performed with highly heterogeneous tissue samples, their advice to perform 8–15 replicates is not surprising. We would prefer to state that in such a clinical setup, with so many variables, number of *replicates* should be denoted as number of *subjects*.

4. FROM RAW DATA TO CLEAN DATA

4.1. Sources of Error

The potential sources of error in a microarray experiment are numerous. We have chosen not to describe all these errors in detail. Table 2 lists potential errors varying from biological errors in the experiment to technical issues concerning scanning and bio-informatics. Although biological variations and errors

Table 2
Sources of Error in Microarray Experiments

Step in Procedure	Resulting Variation
RNA extraction and storage	Tissue collection Degradation and impurity of RNA
Reverse transcription	Low cDNA yield
Labeling	Degradation of dyes Low labeling efficacy Free dyes in sample after cleanup
Microarrays	Missing or malformed spots Low amounts of deposited probe Irregularity of glass slides Other batch-to-batch differences in arrays
Hybridization	Physical or chemical damage to microarray Nonspecific hybridization Fading (bleaching) of dye Suboptimal hybridization conditions
Scanning	Nonlinearity of photomultiplier or laser Fading (bleaching) of dye Imperfect laser focus
Image analysis	Imperfect alignment of grid Imperfect spot finding
Normalization	Degradation of external controls Calculation errors

introduced during RNA isolation are not specific to the microarray experiment, the consequences may dramatically affect the outcome of transcription analysis. Moreover, such enumeration can be helpful in troubleshooting.

4.2. Inspect Every Array and Image

An important and not so frequently mentioned source of error is physicochemical damage of the microarray surface. Scratches on microarrays can occur during production, and dust and dirt may be introduced at many steps in the microarray procedure. Unfortunately, cleanup procedures of microarrays can leave salts on the surface that are sometimes hard to remove. A neat list of expression ratios in a spreadsheet may mislead a researcher and introduce an error that can be prevented by visually inspecting the array surface at all possible moments: after prehybridization, after hybridization, and before scanning. Similarly, grid-by-grid inspection of the scanned image will help to identify spotting problems and physicochemical damage. Finally, visualization of expression ratios in the format of the microarray grids may reveal areas with high number of significant values, pointing to a potential problem during the hybridization. To illustrate this point, we have written a small macro that places the expression ratios of a microarray in the format of the array and applies a color scheme to the background related to the ratio (Fig. 5).

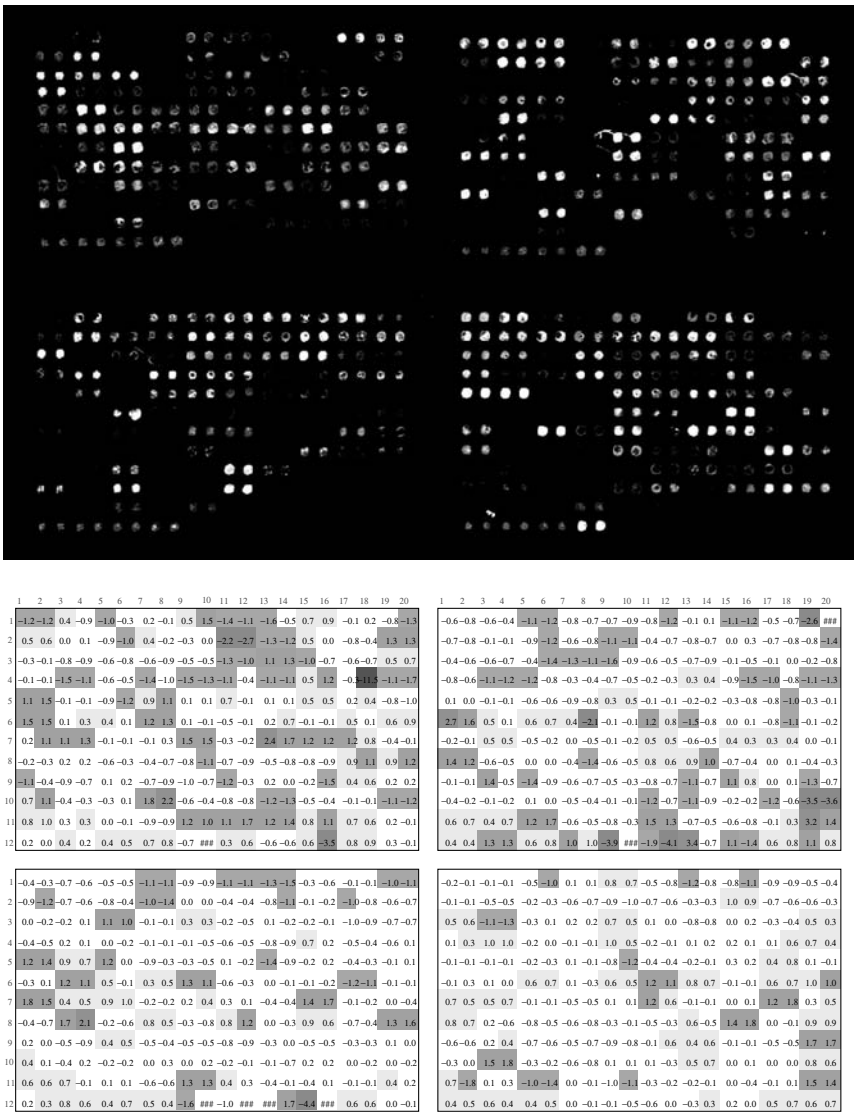

FIGURE 5. Image of a microarray and the corresponding table with the $\log_2$ ratios. No location-related ratios can be detected. (*See Color Plate 2.*)

4.3. Selecting Genes That Are Differentially Expressed

One of the most frequently used ways of selecting genes that are differentially expressed is by use of a threshold. Absolute values for thresholds of $\log_2$ transformed expression ratios in the literature vary from 0.6 to 1.5. Such an approach is very simple however, but has two major drawbacks: one is that the cutoff is arbitrary, and a second is that scatter in the data increases with decreasing signal intensities, so that a fixed cutoff is not logical. Several other strategies are currently

available. A simple method is to calculate the standard deviation; 95% of the data will fall between the mean $\pm$ 2 $\times$ standard deviation. This so-called "unusual ratio" (Draghici, 2003) method is also not taking into respect the higher variation at lower signal intensities, and, more seriously, will report 5% of the genes to be regulated even if there are many more regulated genes or none at all. An adaptation of the "unusual ratio" method is to divide the data into an arbitrary number of bins, and calculate the standard deviation for each bin or fit a function to the point in each bin. Both procedures can then be applied to calculate a signal-dependent 95% confidence limit (Draghici, 2003).

Procedures that are suitable only for larger data sets are based on the variation of a ratio for a specific gene in repetitive experiments in different experimental groups, such as *t* tests. Since multiple comparisons are involved, and spots on an array are dependent, one has to protect for false positives. Bonferroni protection is too conservative since it was not designed for tens of thousands of comparisons. There are several publications dealing with the quality and details of the various techniques, which include the false-discovery rate (Reiner *et al*., 2003) and significance analysis of microarrays (Tusher *et al*., 2001). More detailed information and discussion about this subject can be found in Draghici (2003).

4.4. What Is Normal (Variation)?

In a setting where many different conditions have to be evaluated, the effort to build up a data set that defines the variation of your whole procedure for every spot on the array should be considered. Such a data set can then serve as a reference and for each gene represented on the chip a variance can be obtained. Obviously, considerations about multiple comparisons still apply. Interestingly, in this respect is a paper that describes variation of gene expression using cDNA microarrays in mice: in particular, components of the immune response and genes that have been shown to be regulated by cytokine signaling show a higher variance than normal (Pritchard *et al*., 2001). The authors warned that interpretation of differential expression is hampered when the normal variation of expression is unknown, and that this can introduce the suggestion that genes are linked to a specific disease or condition, while in fact they are just highly variable. A similar observation of high differences in gene expression has been reported for human tissues using Affymetrix arrays (Hsiao *et al*., 2001). Larger sample sizes and insight into the total of biological variation and technical error by using a control of control pool of course protect this phenomenon.

5. FROM DATA TO INTERPRETATION

5.1. Getting an Overview

Once you have obtained a normalized data set of expression ratios, the work to find an interpretation starts. We propose a schematic approach of data interpretation

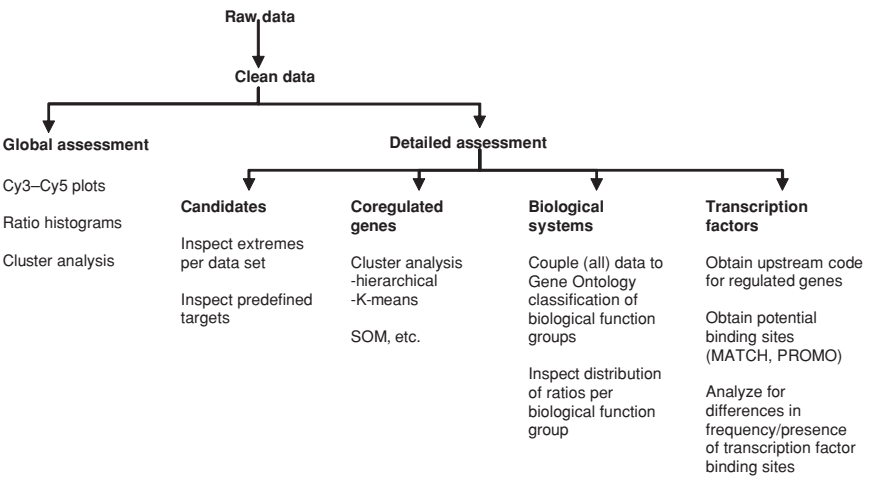

FIGURE 6. A strategy for microarray data analysis.

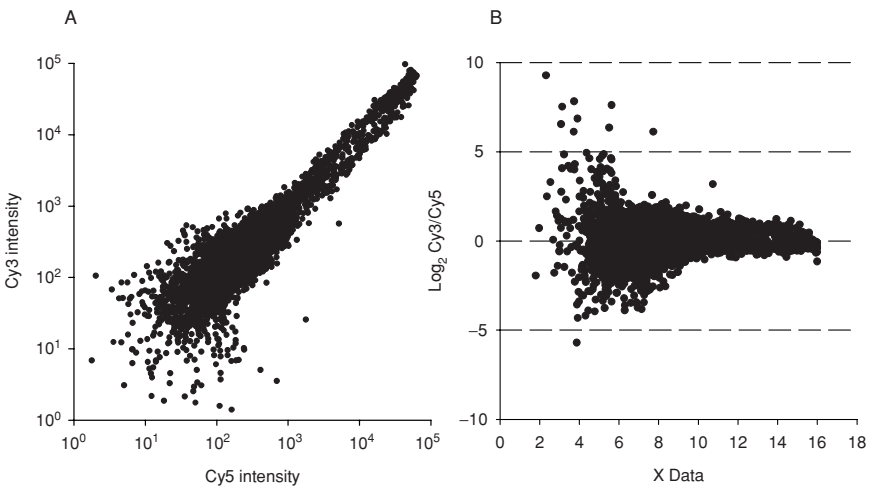

FIGURE 7. Scatter plots of microarray data. A: Scatter plot of the intensity of the Cy5 channel versus the Cy3 channel; B: Ratio versus intensity plot.

in Figure 6. In each step, the data are considered in more detail. The global assessment of the data sets will give indications about the quality of the data. Scatter plots with the Log_2 ratios have frequently been presented with the signal intensity of the one dye on the x-axis and the other on the y-axis (example in Fig. 7A). The other widely used format is to plot the log ratio versus the log average intensity (example in Fig. 7B). Since

$$\frac{\log_2 \mathrm{Cy3} + \log_2 \mathrm{Cy5}}{2} = \log\sqrt{\mathrm{Cy3}\,\mathrm{Cy5}}$$

where Cy3 and Cy5 are the intensities of the Cy channels, respectively, one can view these plots as log ratio versus log product plots. Similarly, ratio histograms give quick indications as to how conditions affect the variance of the $\log_2$ ratio around zero. Besides these visualization tools, several useful parameters can easily be calculated:

- Numbers of differentially expressed genes (i.e. with $\log_2$ ratios >1 and < -1 or identified by a more sophisticated method)
- Variance of $\log_2$ ratio
- Maximum and minimum $\log_2$ ratios
- Number of genes below background threshold
- Number of genes exceeding high cutoff ("maxed-out")

Since it is sometimes hard to judge whether the array hybridization was of good quality, such parameters can be used as quality control and can be used to qualify arrays as outliers when sample size is large enough. Finally, cluster analysis can be used as a quick way to evaluate variation between experiments, without the specific goals that are stated below.

5.2. Sorting Is Powerful

A quick way that does not require any bio-informatics skills to gain insight into up- and downregulated genes and coregulation between different data sets is accomplished by column-by-column sorting of the ratios for each of the experimental conditions (or even for each array). In fact, such a manual analysis gives immediate insight into consistency and does not involve sophisticated software. To underscore and illustrate this point, Table 3 gives a short list of regulated genes in control samples and samples from a nitro-L-arginine treatment without (L-NNA) and with (L-NNA+VitE) addition of vitamin E. The complete set of 7500 genes was sorted on ratio for the L-NNA group. Without any further elaborate methods, it already becomes clear that a subset of genes is not regulated anymore during vitamin E treatment and that 4 day treatment with L-NNA does not cause the same change in gene expression as 21 days of treatment.

5.3. Cluster Analysis

Cluster analysis, which has first been developed in the beginning of the twentieth century, stems from the more general area of multivariate analysis. Methods in this area aim to group variables that are highly correlated and similar and are applied to problems where taxonomy is searched for. Hierarchical cluster analysis groups variables by their *distance* using a *linkage rule*. To illustrate this, imagine that gene expression is measured in two different conditions (or time moments), so that for each gene two ratios are present; one could consider these ratios as coordinates of a point, plot these points in a plane, and calculate the distance between the points. These distances can be measured in a classical Euclidean way,

Table 3
Sorting as a Powerful Tool to Evaluate Microarray Data[a]

GenBank	Description	Control vs Control	L-NNA 21d vs Control	L-NNA 21d + VitE vs Control
AF035963	Rattus norvegicus kidney injury molecule-1 (KIM-1)	0.08	5.14	3.97
NM_012881	Secreted phosphoprotein 1	0.07	3.64	3.12
AF184983	Glycoprotein (transmembrane) nmb	0.05	3.03	1.30
NM_024400	A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS-1)	0.01	2.75	1.30
NM_012679	Clusterin	0.06	2.64	1.32
NM_022279	Meiotic recombination 11 homologue A (*S. cerevisiae*)	0.10	2.59	1.21
AF157026	Solute carrier family 34 (sodium phosphate), member 2	0.12	2.40	0.80
NM_031832	Lectin, galactose binding, soluble 3	−0.31	2.22	1.00
U42719	Complement component 4	0.06	2.16	1.00
NM_031114	S-100 related protein, clone 42C	−0.06	2.03	1.10
NM_031642	Core promoter element binding protein	0.14	2.00	0.76
NM_012603	V-myc avian myelocytomatosis viral oncogene homologue	−0.12	1.99	1.00
NM_017055	Transferrin	−0.05	1.97	−0.04
NM_021261	Thymosin, beta 10	−0.11	1.95	1.08
NM_019905	Calpactin I heavy chain	0.07	1.95	1.06
NM_031010	Arachidonate 12-lipoxygenase	−0.09	1.89	0.27
NM_022542	*RhoB* gene	0.16	1.88	1.12
U96138	Cell surface glycoprotein CD44 (hyaluronate binding protein)	−0.08	1.81	0.37
NM_013043	Transforming growth factor beta stimulated clone 22	−0.04	1.71	0.89
NM_012696	T-kininogen	0.08	1.70	1.27
X13295	Lipocalin 2	0.14	1.70	1.61
NM_017286	Cytochrome P450, subfamily 11A	0.00	1.61	0.93
NM_012580	Heme oxygenase	−0.02	1.60	2.64
NM_012912	Activating transcription factor 3	0.08	1.53	0.69

[a] Rats treated with the NO synthase inhibitor L-NNA for 21 days (21d), in the absence and presence of cotreatment with vitamin E, were compared to control animals. From the $\log_2$ ratios it is obvious that vitamin E does attenuate some of the changes of 21 days L-NNA treatment.

or in a number of other ways, one of them also illustrated in Figure 8, the so-called city-block distance. A linkage rule is then used to decide on how genes will be clustered. For example, one can first select the genes that are closest, bundle them, then look at the next pair that is closest, and finally sort the clusters. Different distance measures and linkage rule can lead to different dendrograms; the issue as to which distance measure and which linkage rule can be best applied to your design is still under dispute. Some guidelines can be found in Draghici (2003). Application of cluster analysis for microarray data has first been described by Eisen *et al.* (1998). Clustering has since been applied successfully in several experiments, and

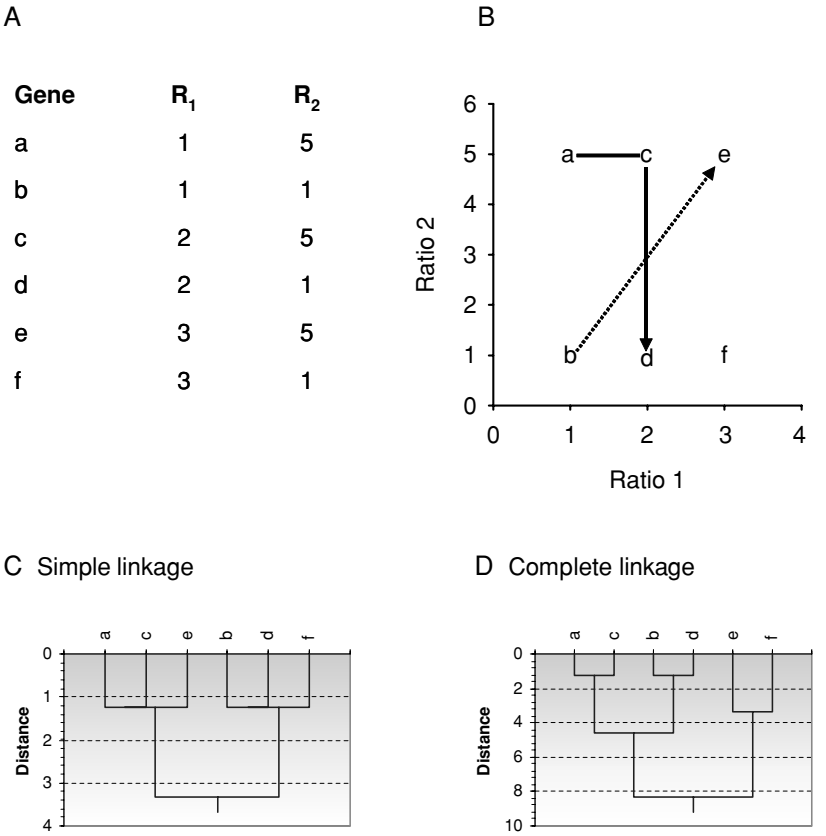

FIGURE 8. Illustration of the basics of cluster analysis.

has pointed to interesting groups of coregulated genes. Nevertheless, we feel that the application of cluster analysis is overemphasized. k-means clustering is highly related to the clustering mentioned above, be it that it is not completely unsupervised. If one can predict the number of clusters for a particular data set, k-means clustering will group the data to match that number of profiles. Such an approach can be particularly useful in pathway analysis (see below). Alternative methods for automated clustering are self-organized maps and principle component analysis. Details of these methods are beyond the scope of this chapter but can be found in Baldi and Hatfield (2002) and Draghici (2003).

5.4. Link Your Data to Gene Ontology

The meaning of microarray data sets can be greatly enhanced by linking the gene expression ratios to biological function groups. The Gene Ontology (GO) Consortium has eased such a link, by classifying a large number of genes into

biological processes, molecular function, and cellular localization (Consortium, 2001). Briefly, the goal of the GO classification is (1) to create a structured vocabulary of terms in molecular biology, (2) to describe biological objects using these terms, (3) to provide tools to query and manipulate these vocabularies, and (4) to link assigned GO terms to biological objects (such as microarrays). An interesting aspect of the GO classification is its structure of so-called directed acyclic graphs. In this structure a child object is bound to a parent object either by a *is-a* relationship or by a *is-part-of* relationship (Consortium, 2001). A child has one or more parents so that, for example, a protein can be defined as being relevant for more than one process.

Although there are a number of commercial tools available to link your data to this classification, data can also be linked using publicly available sources. One option is to use MAPPFinder software (Doniger *et al*., 2003). You can link your microarray definition to the program, which then displays in which categories of genes the changes are most pronounced. A similar approach is followed by the GOMiner program, which is platform independent and has a very potent linking to public databases (Zeeberg *et al*., 2003), FatiGO (Herrero *et al*., 2003), and Onto-Express (Khatri *et al*., 2002). It may be useful to realize that there is also the option to couple data to GO classes yourself by uploading GenBank IDs to the SOURCE Web site, which has the option to return GO classes (Diehn *et al*., 2003). This option does not fully use the capabilities of GO classes, but can push the interpretation of data into a certain biological direction.

5.5. Pathway Analysis and Interrogating Promoter Areas

A first step in deciphering regulatory networks by use of gene expression analysis is to combine cluster analysis and biological annotation of the data with analysis of the upstream regions of the coregulated genes for common binding sites of transcription factors. Such analyses may seem farfetched; however, public tools are available for (limited) promoter-site analyses. In yeast, a first approach to identify global regulatory networks by analyzing regulatory motifs from large-scale gene expression data was presented by Pilpel *et al.* (2001). Such an approach may be more complicated in human studies since the promoter areas are so much more complex and interactions between transcription factors are only partially understood.

Two approaches can be followed after promoter areas of differentially expressed and coregulated genes, or genes that are biologically related, have been obtained (Qiu, 2003). The first is to align the promoter areas and search for overrepresented common motifs, irrespective as to whether or not these motifs have been identified as transcription factor binding sites (TFBSs). There are tools available to assist in this task such as AlignACE and Consensus (Qiu, 2003). The second approach is to search for known TFBSs in the upstream regions of the coregulated genes. The TRANSFAC database is an example of a collection of a large number of transcription factors and their binding sites (Wingendet *et al*., 2000). Using the MATCH tool that is linked to the TRANSFAC database, TFBS can

be identified and the stringency of the selection can be adjusted. A commercial version of MATCH (Biobase Inc.) has additional options to display regulatory networks associated with transcription factors in the database. PROMO (Messeguer *et al*., 2002) is a Web-based application with similar capabilities. It should be emphasized that identification of a high frequency of occurrence of a TFBS in a particular set of coregulated genes using such analyses does not necessarily imply a biological role for the associated transcription factor. Nevertheless, it can be foreseen that application of promoter analyses will be extremely important in understanding regulatory networks.

5.6. Systems Biology and Genomics

Now that large-scale techniques have entered biological research, the need to understand the interactions between factors is increasing. A framework is formed by "Systems Biology," the focus of which is on understanding the structure and dynamics of the interactions between genes and proteins (Kitano, 2002). A basic strategy is supplied by the group of Hood (Ideker *et al*., 2001):

1. Define all the structural components (genes, proteins, compartments)
2. Perturb each component through a series of genetic or environmental manipulations and record the global response
3. Build a global model, generate a testable hypothesis, and reiterate 1 and 2

This strategy has been tested in *S. cerevisiae* for galactose metabolism, of which a large part of the components and interactions is known (step A), using the presence and absence of galactose as perturbation and microarrays and mass spectroscopy for global RNA and protein determination (step B). Using this approach, coregulation of several genes in the network was detected that suggested an interaction at the protein level and had not been recognized before. Analysis of upstream regions of the coregulated genes indicated a higher presence of the Gal4P-binding sites. The experiments identified a strong inhibitory effect of two genes (*gal7* and *gal10*) on the expression levels of other GAL genes that had not previously been recognized. These observations are expanding the known network and lead to new hypotheses (step C). To go further into regulatory networks in *S. cerevisiae* is beyond the scope of this chapter; however, a beautiful example of a similar perturbation analysis and coupling to functional regulation is the study of Hughes *et al*. (2000). Attempts to apply such an approach to subjects with cardiovascular disease are under way. Stoll *et al*. (2001) investigated a large number of physiological responses and morphological variations in rats to the genetic background (Dahl Salt Sensitive and Brown Norway). By correlating blood pressure increase to i.v. administration of NE and of Ang II in both strains, they could identify a region on chromosome II harboring inducible NO synthase variants that predicted the response. Further, characterization of blood pressure regulating systems is ongoing.

To conclude this paragraph, the reader should not be discouraged by these high-tech approaches of Systems Biology. A start is made to such an approach by formulating metabolic, protein, or regulatory networks from present knowledge

Table 4
Software Packages to Support Microarray Analysis Freely Available on the Internet

	Platform	Web Address
Database software		
TIGR MADAM	Java	http://www.tigr.org/software/tm4/index.html
AMAD	Perl	http://www.microarrays.org/software.html
SMD	Perl/Oracle	
Image analysis		
Scanalyze	Windows	http://rana.lbl.gov/EisenSoftware.htm
TIGR Spotfinder	Windows	http://www.tigr.org/software/tm4/index.html
Dapple	Unix	http://www.cs.wustl.edu/%7Ejbuhler//research/dapple/
Data analysis		
TIGR MIDAS	Java	http://www.tigr.org/software/tm4/index.html
TIGR MEV	Java	http://www.tigr.org/software/tm4/index.html
GEPAS Tools	Web-based	http://gepas.bioinfo.cnio.es/index.html
Expression Profiler		http://ep.ebi.ac.uk/EP/
Biological annotation		
GOMiner	Java	http://discover.nci.nih.gov/gominer/index.jsp
Onto-Express	Web	http://vortex.cs.wayne.edu/Projects.html
MAPPFinder	Windows	http://www.genmapp.org/MAPPFinder.html
FatiGO	Web-based	http://gepas.bioinfo.cnio.es/index.html
Motif finder		
AlignACE	Web-based	http://atlas.med.harvard.edu/
TFBS analysis		
PROMO	Web-based	http://www.lsi.upc.es/∼alggen/
MATCH	Web-based	http://www.gene-regulation.com

[a] Rats treated with the NO synthase inhibitor L-NNA for 21 days (21d), in the absence and presence of cotreatment with vitamin E, were compared to control animals. From the $\log_2$ ratios it is obvious that vitamin E does attenuate some of the changes of 21 days L-NNA treatment.

and fitting obtained microarray data to these models. Despite the limitations of graphical representations of networks (e.g. the inability to display time-dependent events), they may help to generate new hypotheses. For a further discussion of the representation of networks, the reader is referred to Baldi and Hatfield (2002).

5.7. Software to Support Data Analysis

The number of software packages is increasing at a very high pace. We have attempted to produce a small list with software packages or Web applications that are freely available on the Internet (Table 4). This list contains software for data storage, for image and data analysis, and for biological annotation. There are many commercial packages currently available. Of these, we mention the Genespring package that covers most of the process of initial data analysis to biological interpretation (Conway, 2003) and is relatively easy to use.

6. APPLICATIONS IN CARDIOVASCULAR RESEARCH

Recently, there has been a literal explosion of gene expression studies in cardiovascular biology, using the microarray technology (Barrans *et al*., 2001, 2002; Friddle *et al*., 2000; Geraci *et al*., 2001, 2002; Haase *et al*., 2002; Hwang *et al*., 2000, 2002; Lowes *et al*., 2002; Paoni and Lowe, 2001; Stanton *et al*., 2000; Yang *et al*., 2000). "Cardiovascular genomics" covers a wide range of subjects, including EC (dys)function, the atherosclerotic process, myocardial infarction, and heart failure and arrhythmia. There are a number of large cardiovascular genomics initiatives using microarray technology in progress across the United States, such as CardioGenomics (www.cardiogenomics.org) and PhysGen (www.brc.mcw.edu) projects. In the discussion that follows, we do not intend to summarize every paper that used microarrays to study cardiovascular disease. We focus on a few topics that exemplify progress in understanding the basic mechanism of EC (dys)function and the pathophysiology of atherosclerosis and heart failure and identification of biomarkers for diagnostic and treatment purposes.

6.1. Understanding the Transcriptional Programs of ECs

The endothelium is the single-cell layer that covers the surface of blood vessels. The strategic location that it occupies allows it to act both as a sensor and as a modulator within the vessel. The endothelium plays a key role in vascular homeostasis through the release of a variety of autocrine and paracrine substances, the best characterized being NO. ECs are normally exposed to continuous flow, inducing shear stress. Adaptation of these cells to continuous flow during their entire life span is crucial for correct functioning in their physiological context. Endothelial injury, resulting from chemical, mechanical, and/or biological insults, is believed to be an important mechanism initiating the pathogenesis of atherosclerosis. Disruption of the delicate balance of the NO system, especially by increased vascular production of reactive oxygen species, promotes the development of EC dysfunction. Although the risk factors for atherosclerosis are of a systemic nature, the localization of lesions is confined to specific and reproducible positions in the arterial tree in areas of turbulent flow and low fluid shear stress. Besides the role of the EC in the initiation and progression of atherosclerosis, the EC layer has a pivotal role in remodeling of resistance vessels, a phenomenon that contributes to the pathogenesis and maintenance of hypertension.

6.1.1. Gene Expression Responses of the EC to Exogenous NO

Substantial evidence is now available on the actions of NO in the vasculature (Behrendt and Ganz, 2002). The by now already classical paradigm is that NO, released from ECs, acts on the vascular smooth muscle cell (VSMC) by activating soluble GC, resulting in cGMP formation and vasodilation (Behrendt and Ganz, 2002). In addition, NO affects multiple other processes in the VSMC, i.e. inhibits

proliferation (Yu *et al*., 1997) and modulates expression of genes with vasoactive function (Ichiki *et al*., 1998). NO also affects EC function in an autocrine fashion and thereby inhibits adhesion, inflammation, and the generation of proliferative stimuli. Gene expression responses of VSMCs to NO have been extensively investigated; the actions of NO on EC gene expression, however, are less well defined. Recently, in our laboratory a study was designed to test the hypothesis that NO causes widespread changes in gene expression in ECs.

In this study, human umbilical veins endothelial cells (HUVECs) were exposed to 250 μM of the NO donor DETA-NONOate for 2, 4, 8, and 24 h. Also, cells were subjected to 4 h of DETA-NONOate application in the presence and absence of the GC inhibitor 10 μM ODQ. RNA was isolated, reverse transcribed, Cy3- and Cy5-labeled, and analyzed using cDNA microarrays. DETA-NONOate caused a wave of expression changes that were observed with a maximum at 4 h. Remarkable was the downregulation of a number of transcription factors by DETA-NONOate, in particular of MSX1, RELB, and Egr-1. In DETA-NONOate administered cells, gene expression responses had faded after 24 h; however, cGMP in the medium of the treated cells was still increased after 24 h of SNP administration. Coadministration of ODQ decreased many, but not all, of the expression changes in response to DETA-NONOate. Thus, NO exerts substantial influence of EC gene expression, in particular on transcription factors. These effects of NO are not sustained despite continued cGMP in the medium. The observation that many, but not all, transcriptional changes induced by NO could be inhibited by inhibition of GC indicates the presence of GC-independent NO actions on gene expression. This study demonstrates that EC gene expression is responsive to changes in NO, but, that during a sustained elevation, the transcriptional response fades (Braam *et al*., 1998).

6.1.2. Risk Factors of EC Dysfunction

Several studies have been aimed at identifying endothelial genes that are regulated by risk factors for EC dysfunction and atherosclerotic disease. Hyperhomocysteinemia (HHCy) is an independent and graded risk factor of cardiovascular disease such as stroke, myocardial infarction, peripheral vascular disease, and atherosclerosis. In search for the molecular link of HHCy to the atherosclerotic process, HUVECs were screened for the gene expression modified by HCy (Li *et al*., 2002). Using a cDNA microarray consisting of 600 cardiovascular-relevant genes, the data disclosed an unexpected link between HCy and cholesterol dysregulation based on the finding of increased expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol biosynthesis. The increase in free cholesterol in EC by HCy was associated with increased caveolin-1 and a decreased NO release, which could be prevented by cotreatment with simvastatin. Because of this remarkable finding, this study could also provide experimental evidence demonstrating that statins, which can decrease cardiovascular morbidity and mortality by lowering serum lipid levels and restoring

endothelial function, possess an additional effect. This includes amelioration of EC dysfunction induced by elevated levels of HCy, and presents the rationale for the use of statins in HHCy.

Cigarette smoking is a major risk factor for cardiovascular diseases and causes EC dysfunction. Nicotine, a major constituent of cigarette smoke, has shown to alter gene expression in ECs. In search for the regulatory pathways involved, a cDNA microarray approach ascertained the expression of over 4000 genes in primary Human Coronary Artery Endothelial Cells (HCAEC) stimulated with nicotine (10 μM) in comparison to untreated cells (Zhang *et al*., 2001). Among the nicotine-modulated genes were phosphatidylinositol phosphate kinase and diacylglycerol kinase. In addition, changes were detected for the transcription factors cAMP response element binding protein and nuclear factor κB, of which the activities have previously been shown to be affected in nicotine-stimulated cells. Together, the data suggested that the inositol phospholipid pathway is likely to be involved in mediating the actions of nicotine on vascular endothelium. Interestingly, this pathway has previously been implicated in the pathogenesis of atherosclerosis, particularly in response of ECs and VSMCs to endothelin-1, angiotensin II, HCy, fluid shear stress, and various cytokines. Thus, by using microarray analysis, it appeared that nicotine shares a common signaling mechanism with other atherogenic factors.

6.1.3. Shear Stress and EC Function

Evidence from both animal models of atherosclerosis and human subjects demonstrates that thickening of the intima and plaque localization occur predominantly in regions of the vasculature characterized by low-wall shear stress and complex flow patterns that fluctuate in direction. In atherosclerosis, plaque lesions are generally found at sites of vessel bifurcations and curvatures, leading to the opinion that low and/or turbulent shear stress are conducive to atherosclerosis. Thus, one may envision both lesion-prone and lesion-protected areas within the vascular tree. The notion that complex transcriptional events occur in response to increases in shear stress preceded the days of expression analysis using microarrays (Braddock *et al*., 1998). Nevertheless, the knowledge of EC responses to shear has been recently expanded by *in vitro* microarray studies identifying endothelial genes that are regulated by arterial levels of shear stress (Brooks *et al*., 2002; Chen *et al*., 2001, 2003; Dekker *et al*., 2002; Garcia-Cardena *et al*., 2001; McCormick *et al*., 2001).

A number of microarray experiments have been performed to assess gene expression responses to shear stress. Chen *et al*. (2001) investigated gene expression responses of cultured human aortic endothelial cells to 24 h of laminar shear stress at 12 dyn/cm^2, using an Atlas 1.2 Array microarray. No normalization between arrays was applied. McCormick *et al*. (2001) subjected HUVEC to high shear stress (25 dyn/cm^2) for 6 and 24 h using a parallel plate chamber and analyzed gene expression using the Human Named Genes Genefilters microarray (Research Genetics). Garcia-Gardena *et al*. (2001) reported transcriptional responses of HUVEC

to 24 h laminar and turbulent shear stress of 10 dyn/cm^2 using Research Genetics GeneFilter microarrays. In a very elegant study, Dekker *et al*. (2002) subjected HUVEC in a parallel plate system to steady 25 dyn/cm^2 or pulsatile 12 ± 7 dyn/cm^2 shear stress and analyzed expressional responses with a Gene Discovery Array (Insight). After this preanalysis, they have focused further on a subset of genes, in particular the transcription factor Krüppel-like factor-2 (KLF2) using a homemade array, and compared gene expression of cells exposed to shear stress with that of cells exposed to TGFβ, thrombin, VEGF, and IL1β. Using this approach, they could identify KLF2 as a gene not regulated via one of those pathways (Dekker *et al*., 2002).

Shear stress modulates gene expression in EC partly through NO, acting via enhanced cGMP formation by GC. Recently, NO has underlined protein nitrosylation as an optional, potentially important mechanism of transcription modulation (Stamler *et al*., 2001). We addressed whether significant non-cGMP-mediated transcriptional responses to shear stress could be identified by subjecting HUVEC to shear stress in the absence and presence of the GC inhibitor ODQ. Gene expression was analyzed using a 19K 70mer oligonucleotide array (van de Peppel *et al*., 2003). A general dampening effect of NO on EC function could be demonstrated. Indeed, there was significant non-GC-mediated transcriptional regulation in response to shear. Interestingly, heme oxygenase 1 was very consistently induced by flow, and not dependent upon NOS or GC. *In silico* promoter analysis of the shear- sensitive and NO-dependent and NO-independent genes revealed prominent presence of SOX5 and SOX9 binding sites in NO-sensitive genes (Braam *et al.*, submitted).

We have performed a concise comparison of the responses to shear stress so as to identify patterns of gene regulation that may have escaped attention from the individual analyses (Fig. 9). This analysis shows gene responses that are expected, such as upregulation of eNOS and downregulation of caveolin-1. Interestingly, there is prominent regulation of redox state-related genes such as heme oxygenase-1, thioredoxin reductase, and gluthathion synthase. Such "meta-analysis" may overcome the bias introduced by experimental variation and by selective attention for specific groups of genes of the researchers.

6.1.4. Egr-1 Regulated Gene Expression in HUVEC

Recent findings suggest that early growth response factor-1 (Egr-1) is a key transcriptional regulator in orchestrating the functional characteristics of the vessel wall after injury. Egr-1 was originally identified as an immediate-early gene that is rapidly induced in response to a variety of stimuli, including growth factors, cytokines, hypoxia, physical forces, and injury, all of which are implicated in the progression of vascular diseases. As a transcription factor, Egr-1 can induce expression of a set of vasculature genes, such as PDGF A and B chain, bFGF, TGFβ, TNFα, and intracellular adhesion molecule-1 (Khachigian and Collins, 1997).

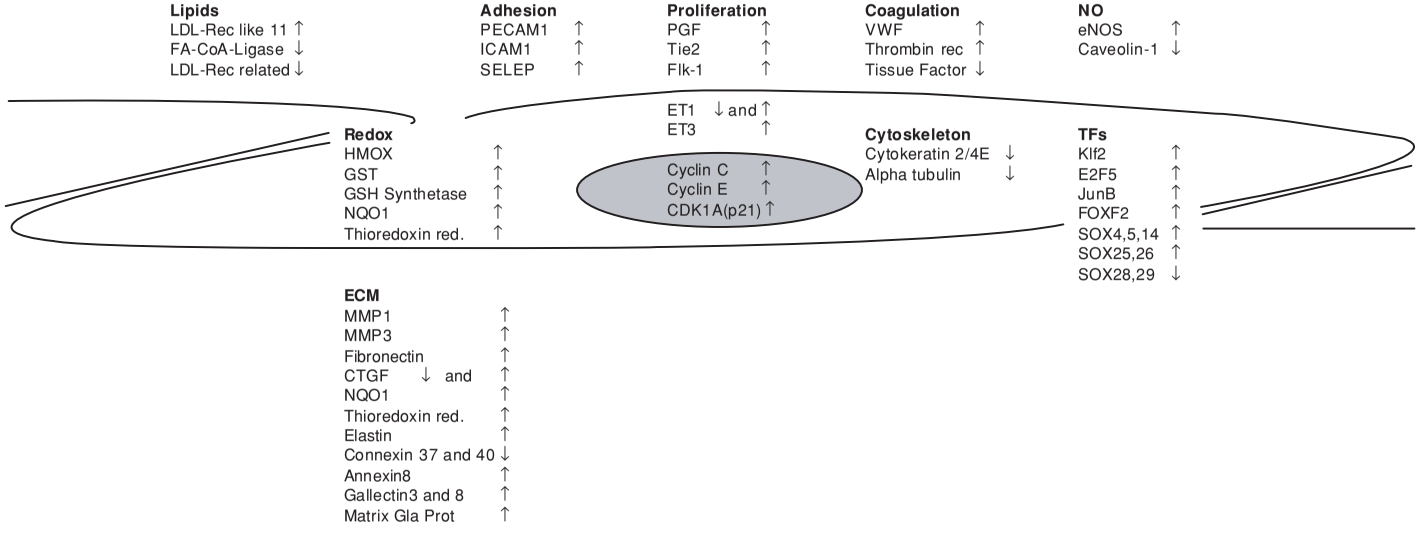

FIGURE 9. Summary of the observations from microarray studies assessing the influence of shear stress on gene expression.

To better understand Egr-1 in the vasculature, Fu *et al*. (2003) profiled Egr-1 target genes in HUVEC using adenoviral gene transfer and human Affymetrix oligonucleotide-based microarray U95Av2 GeneChip. More than 300 genes were regulated greater than threefold with Egr-1 overexpression and, partially, confirmed by Northern and Western blotting, including genes for transcriptional regulators, signaling proteins, cell cycle regulatory proteins, growth factors, and cytokines. Among them, several genes had been previously identified as Egr-1 target genes, such as IGF-2, TGFβ, VEGF, $p57^{kip2}$, fibronectin, and PPARγ. However, most of them were novel Egr-1 target genes. These included Rad, which encodes a Ras homologue that was originally identified as overexpressed in the muscle of patients with type II diabetes. Furthermore, thymus-expressed chemokine and IP-30 were dramatically induced by Egr-1, whereas TNFα-related apoptosis inducing ligand was significantly repressed by Egr-1. The identification of these novel Egr-1-induced genes in vasculature will provide novel targets for studying inflammation related to atherogenesis.

6.2. What Happens in Atherosclerotic Lesions?

The first studies using global expression analysis in atherosclerotic arteries and in neo intima formation in stents in arteries have now been published. Durier *et al*. (2003) analyzed ascending aorta biopsies obtained during coronary bypass surgery using Affymetrix microarrays. In nine samples, they found 330 genes that were significantly expressed in three or more samples. Interestingly, a relatively large percentage of these genes were related to signaling, communication, and gene expression. Unfortunately, the distribution of this classification for other tissues is not supplied. In the same group of patients, pulse wave velocity (PWV), a known measure of aortic stiffness, was assessed. The PWV values were then correlated with the gene expression data: remarkably, of those genes that were related to PWV, many were related to signaling and communication (see for further details, Durier *et al*., 2003). A limitation of the study that was recognized by the authors is the heterogeneity of the sampling site and the absence of a confirmation method. Nevertheless, this study is the first to couple directly gene expression data to a functional measure.

Martinet *et al*. first investigated human endarterectomy specimens using gene expression analysis (Martinet *et al*., 2002, 2003), which was later followed by a protein expression study (Martinet *et al*., 2003). They identified death-associated protein kinase (DAPK) in the first study to be differentially expressed as compared to nonatherosclerotic mammary arteries. Stimulation of aortic vascular smooth with C_6-ceramide increased DAPK expression at RNA and protein level. Using the protein array, another proapoptotic protein was identified to be downregulated, the antiapoptotic gene *apoptosis-linked gene-2* (*ALG2*). These two studies indicate induction of apoptosis in atherosclerotic plaques.

Both examples indicate that gene (and protein) expression profiling may help to identify new players in atherosclerotic disease. Unfortunately, there is currently no high-throughput system to test the physiological relevance of candidates in such studies.

6.3. From Emerging Left Ventricular Hypertrophy to Heart Failure

Using human tissue and animal disease models, initial cardiovascular microarray studies identified transcriptional changes associated with cardiac hypertrophy (Friddle *et al*., 2000; Weinberg *et al*., 2003), myocardial infarction (Sehl *et al*., 2003; Stanton *et al*., 2000), and heart failure (Tan *et al*., 2002). In the following sections, we summarize these first characterizations of these processes using microarrays.

6.3.1. Cardiac Hypertrophy

Using the pharmacologic inducers Ang II and isoproterenol to study the molecular mechanisms involved in induction and regression of cardiac hypertrophy in mice (Friddle *et al*., 2000), in combination with a small microarray (∼4000 genes), 32 genes showed changes in expression only during the induction phase. These included genes encoding secretion proteins (ANP, BNP, and PAI-2), receptors, intracellular signaling molecules (ECE-2, COMT, and PKC-bp), structural proteins (α-actin), and those participating in energy metabolism (ATP synthase A chain, ATP synthase D chain, carbonyl reductase, and fructose biphosphate aldolase B). These changes correlate with early functional changes, known to take place in the early stages of cardiac hypertrophy. An important finding of this study was the identification of a distinct set of genes specifically altered during the regression of hypertrophy. This supports the concept that regression involves a separate transcriptional program.

A study by Weinberg *et al*. (2003), in which male and female mice underwent transverse aortic constriction to characterize responses of the left ventricle to pressure overload, focused more on the overlap between acute and chronic phase of cardiac hypertrophy. At 1 day and 30 weeks, the left ventricle free wall was harvested and RNA isolated from 27 individual ventricles was analyzed on Mu74Av2 GeneChips (Affymetrix). The number of genes changing in response to acute pressure overload (269 males, 110 females) was greater than the number of genes changing in response to chronic pressure overload (53 males, 56 females). Low overlap also suggested that acute and chronic genomic responses to overload are distinct. Sex modified these responses. Hierarchical clustering analysis revealed the presence of different expression profiles. Of these clusters, the majority contained genes that were regulated in response to either acute overload or both acute and chronic overload. In their analysis they focused more on the latter group. The known hypertrophic marker genes, such as BNP and skeletal actin, were upregulated in both males and females. According to function, immunity, inflammation, and stress response, extracellular matrix and cytoskeletal genes are well represented during the acute response and even further regulated in response to chronic overload. These included transcripts recently reported to be increased following myocardial infarction (Stanton *et al*., 2000). Chronic response genes also showed overlap with those affected in human heart failure (Tan *et al*., 2002).

6.3.2. Myocardial Infarction

The changes that take place in the ventricle after myocardial infarction comprise scar formation, myocyte hypertrophy, dilation, and extracellular matrix deposition, with an overall effect of worsening of the contractile function. In an elegant study by Stanton *et al*. (2000), approximately 7000 cDNAs collected from rat heart cDNA libraries were printed onto microarrays and profiled for expression in the left ventricle free wall and interventricular septum at 2, 4, 8, 12, and 16 weeks after surgically induced myocardial infarction in the rat.

Thus, the upregulation of extracellular matrix genes (several types of collagens, fibronectin, laminin, fibrillin, fibulin, thrombospondin-4, osteopontin, SPARC, SCI/ECM2, decorin, TIMP-3) was observed. The same was true for several cytoskeleton assembly genes (thymosin, moesin, transgelin, the Arp 2/3 actin assembly complex). In addition, certain genes that encode proteins related to the muscle contractile apparatus were either elevated in expression (α/cardiac myosin heavy chain, atrial myosin light chain) or repressed (titin, tropomyosin 4, troponin 1, telethonin). Together, this implicates that these genes exemplify a possible functional role in the alteration of contractility, which correlates with the disease process. Among the upregulated genes were also several transcription factors, including Egr-1, TSC-22, and cardiac ankyrin repeat protein, the latter implicated as a regulator of cardiac gene expression. It is tempting to speculate in this respect that coordinate changes in gene expression of many genes may be orchestrated by a few transcription factors. Very obvious was also the upregulation of several ribosomal proteins, involved in protein expression.

Many of the differentially regulated genes encoded enzymes of energy metabolism. Interestingly, those involved in catabolism of fatty acids were mostly repressed genes, including enoyl-coenzyme A (CoA) isomerase, dienoyl CoA reductase, hydroxyacyl-CoA dehydrogenase, long-chain acyl-CoA synthase, and ketoacyl-CoA thiolase. Their coordinate repression indicates a shift of the primary energy source from fatty acids to glucose. It also implicates that there is a long-term adaptation in response to myocardial infarction in expression of fatty acid metabolism genes as a means to accomplish this metabolic reprogramming. Also downregulated are genes encoding stress-response proteins, such as glutathione S-transferase and thioredoxin peroxidase, implicating an impaired ability of the infarcted myocardium to protect against oxidative stress. Genes that are known to be involved in reduction of the load on the failing heart were also found to be elevated (ANP, BNP, ANF), a sign of a more restricted response to failure *per se*.

6.3.3. Heart Failure

In a study using RNA from left ventricular wall, obtained from explanted failing (F) hearts of 15 transplant recipients or unmatched organ donors (NF) (Tan *et al*., 2002), in combination with the Affymetrix HuFI 6800 GeneChip, 103 genes were found to be differentially expressed in F and NF hearts. All of these genes were divided into 10 functional groups. Biomarkers of heart failure included

ANF, ANF precursor, and BNP. Cardiac α-actin was found to be downregulated and myosin heavy chain-β upregulated in the F hearts, which is consistent with the fact that during development of heart failure, myocytes revert to a pattern of fetal gene expression, e.g., increased expression of myosin heavy chain-β and replacement of cardiac α-actin by skeletal α-actin. Genes encoding extracellular matrix/cytoskeletal proteins, including increased expression of collagen types I and III, fibromodulin, t-plastin, fibronectin, and desmosome-associated protein, represented another group with a significant increase in expression in F hearts. Considering the maladaptive remodeling process that occurs in F hearts, this was not unexpected.

Consistent with the fact that proteolysis and activation of a stress response occurs in F hearts in response to neurohormonal and mechanical injury, the majority of genes encoding proteolysis/stress-related proteins showed increased expression. In this respect, it was interesting to note the upregulation of thrombospondin-4 in F hearts, since association of thrombospondin-4 with cardiac disease was highlighted by a recent report indicating that a missense variant (A387P) is associated with premature myocardial infarction (Topol *et al*., 2001). Strikingly, many genes encoding proteins involved in metabolism were differentially expressed. Genes encoding proteins involved in fatty acid metabolism, e.g., apoliprotein D, fatty acid synthase, phospholipid transfer protein, were exclusively downregulated. However, genes related to glucose metabolism, such as fructose-1, 6-biphosphatase and mitochondrial NADP(+)-dependent malic enzyme, were upregulated. These observations may indicate the fact that the primary myocardial energy source switches from fatty acid to glucose in heart failure (Sack and Kelly, 1998). Consistent with observations of increased apoptosis in F human hearts, decreased expression of α1-antichymotrypsin, metallothionein, and metallothionein 1L may indicate that the antiapoptosis pathway is inhibited in F hearts. Finally, unsupervised clustering segregated 2 of the 15 failing hearts with different diagnoses, thus offering the exciting possibility of identifying dilated cardiomyopathy hearts of distinct etiologies.

6.4. Systemic Changes in Essential Hypertension

We assessed whether large-scale expression profiling of leukocytes of patients with essential hypertension reflects characteristics of systemic disease and whether such changes are responsive to antihypertensive therapy. Total RNAs from leukocytes were obtained from untreated ($n = 6$) and treated ($n = 6$) hypertensive patients without apparent end-organ damage and from normotensive controls ($n = 9$). RNA was reverse transcribed and labeled and gene expression analyzed using a 19K oligonucleotide microarray using dye swaps. Samples of untreated and treated patients were pooled for each sex and compared to age- and sex-matched controls. In untreated patients, 680 genes were differentially regulated (314 up and 366 down). In the treated patients, these changes were virtually absent (4 genes up, 3 genes down). A myriad of changes was observed in pathways involved in inflammation. Inflammation-dampening interleukin receptors were decreased in expression. Intriguingly, inhibitors of cytokine signaling

(the PIASses) were differentially expressed. The expression of several genes that are involved in regulation of blood pressure were also differentially expressed: the angiotensin AT1 receptor, the ANP-A receptor, endothelin-2, and three of the serotonin receptors were increased, while endothelin-converting enzyme-1 was decreased. Strikingly, virtually no changes in gene expression could be detected in hypertensive patients that had become normotensive with treatment. This observation substantiates the longstanding idea that hypertension is associated with a complex systemic response involving inflammation-related genes. Furthermore, leukocytes display differential gene expression that is of importance in blood pressure control. Importantly, treatment of blood pressure to normal values can virtually correct such disturbances (Chon *et al.*, 2004).

7. CONCLUSION

We have attempted to guide the reader into the exciting new world of global expression analysis, with the focus on microarrays. It is clear that this technique is a first firm attempt to access transcriptional programs and applications in cardiovascular research and medicine are appearing. A lot of open space is still to be explored. The technique still has technical problems and limitations and there is no standard frame for data analysis. The use of gene expression profiling in cardiovascular research has already revealed a myriad of new function for known genes and has shifted paradigms in several areas. This is just a start.

ACKNOWLEDGMENT. B. Braam is supported by a Fellowship from the Royal Dutch Academy of Arts and Sciences. Support for the research of Drs Braam and Bluyssen is obtained from the Dutch Kidney Foundation (NS6013, C03.2062), from the Department of Economic Affairs (Project "Nutrition and Vascular Aging"), and from the University of Utrecht (Project "Healthy Aging").

REFERENCES

Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X., Powell, J. I., Yang, L., Marti, G. E., Moore, T., Hudson, Jr., J., Lu, L., Lewis, D. B., Tibshirani, R., Sherlock, G., Chan, W. C., Greiner, T. C., Weisenburger, D. D., Armitage, J. O., Warnke, R., Levy, R., Wilson, W., Grever, M. R., Byrd, J. C., Botstein, D., Brown, P. O., and Staudt, L. M., 2000, Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* **403**:503–511.

Baldi, P., and Hatfield, G. W., 2002, DNA microarrays and gene expression: from experiments to data analysis and modelling. (Cambridge University Press, Cambridge).

Barrans, J. D., Allen, P. D., Stamatiou, D., Dzau, V. J., and Liew, C. C., 2002, Global gene expression profiling of end-stage dilated cardiomyopathy using a human cardiovascular-based cDNA microarray. *Am. J. Pathol.* **160**:2035–2043.

Barrans, J. D., Stamatiou, D., and Liew, C., 2001, Construction of a human cardiovascular cDNA microarray: portrait of the failing heart. *Biochem. Biophys. Res. Commun.* **280**:964–969.

Behrendt, D., and Ganz, P., 2002, Endothelial function. From vascular biology to clinical applications. *Am. J. Cardiol.* **90**:40L–48L.

Bogdan, C., 2001, Nitric oxide and the regulation of gene expression. *Trends. Cell. Biol.* **11**:66–75.

Braam, B., de Roos, R., Bluyssen, H., Kemmeren, P., Holstege, F., Joles, J. A., and Koomans, H., 2005, Nitric Oxide-Dependent and Nitric Oxide-Independent Transcriptional Responses to High Shear Stress in Endothelial Cells. *Hypertension*.

Braam, B., de Roos, R., Dijk, A., Boer, P., Post, J. A., Kemmeren, P. P., Holstege, F. C., Bluysen, H. A., and Koomans, H. A., 2004, Nitric oxide donor induces temporal and dose-dependent reduction of gene expression in human endothelial cells. *Am. J. Physiol. Heart. Circ. Physiol.* **287**:H1977–1986.

Braddock, M., Schwachtgen, J. L., Houston, P., Dickson, M. C., Lee, M. J., and Campbell, C. J., 1998, Fluid Shear Stress Modulation of Gene Expression in Endothelial Cells. *News Physiol. Sci.* **13**:241–246.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C., Kim, I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M., 2001, Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat. Genet.* **29**:365–371.

Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D. H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M., Roth, R., George, D., Eletr, S., Albrecht, G., Vermaas, E., Williams, S. R., Moon, K., Burcham, T., Pallas, M., DuBridge, R. B., Kirchner, J., Fearon, K., Mao, J., and Corcoran, K., 2000a, Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat. Biotechnol.* **18**:630–634.

Brenner, S., Williams, S. R., Vermaas, E. H., Storck, T., Moon, K., McCollum, C., Mao, J. I., Luo, S., Kirchner, J. J., Eletr, S., DuBridge, R. B., Burcham, T., and Albrecht, G., 2000b, In vitro cloning of complex mixtures of DNA on microbeads: physical separation of differentially expressed cDNAs. *Proc. Natl. Acad. Sci. U.S.A.* **97**:1665–1670.

Brooks, A. R., Lelkes, P. I., and Rubanyi, G. M., 2002, Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. *Physiol. Genomics.* **9**:27–41.

Chen, B. P., Li, Y. S., Zhao, Y., Chen, K. D., Li, S., Lao, J., Yuan, S., Shyy, J. Y., and Chien, S., 2001, DNA microarray analysis of gene expression in endothelial cells in response to 24-h shear stress. *Physiol. Genomics.* **7**:55–63.

Chen, X.-L., Varner, S. E., Rao, A. S., Grey, J. Y., Thomas, S., Cook, C. K., Wasserman, M. A., Medford, R. M., Jaiswal, A. K., and Kunsch, C., 2003, Laminar flow induction of antioxidant response element-mediated genes in endothelial cells: a novel anti-inflammatory mechanism. *J. Biol. Chem.* **278**:703–711.

Chon, H., Gaillard, C. A., van der Meijden, B. B., Dijstelbloem, H. M., Kraaijenhagen, R. J., van Leenen, D., Holstege, F. C., Joles, J. A., Bluyssen, H. A., Koomans, H. A., and Braam, B., 2004 (May), Broadly altered gene expression in blood leukocytes in essential hypertension is absent during treatment. *Hypertension* **43**:947–951.

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O., and Herskowitz, I., 1998, The transcriptional program of sporulation in budding yeast. *Science* **282**:699–705.

Consortium, T. G. O., 2001, Creating the gene ontology resource: design and implementation. *Genome. Res.* **11**:1425–1433.

Conway, A. R., 2003, Genespring (Silicon Genetics, Redwood City).

Dekker, R. J., van Soest, S., Fontijn, R. D., Salamanca, S., de Groot, P. G., VanBavel, E., Pannekoek, H., and Horrevoets, A. J., 2002, Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2). *Blood* **100**:1689–1698.

Diehn, M., Sherlock, G., Binkley, G., Jin, H., Matese, J. C., Hernandez-Boussard, T., Rees, C. A., Cherry, J. M., Botstein, D., Brown, P. O., and Alizadeh, A. A., 2003, SOURCE: a unified genomic resource of functional annotations, ontologies, and gene expression data. *Nucleic. Acids. Res.* **31**:219–223.

Doniger, S. W., Salomonis, N., Dahlquist, K. D., Vranizan, K., Lawlor, S. C., and Conklin, B. R., 2003, MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome. Biol.* **4**:R7.

Draghici, S., 2003, Data analysis tools for DNA microarrays (Chapmann and Hall/CRC Press, Boca Raton).

Durier, S., Fassot, C., Laurent, S., Boutouyrie, P., Couetil, J. P., Fine, E., Lacolley, P., Dzau, V. J., and Pratt, R. E., 2003, Physiological genomics of human arteries: quantitative relationship between gene expression and arterial stiffness. *Circulation* **108**:1845–1851.

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D., 1998, Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U.S.A.* **95**:14863–14868.

Ewing, B., and Green, P., 2000, Analysis of expressed sequence tags indicates 35,000 human genes. *Nat. Genet.* **25**:232–234.

Friddle, C. J., Koga, T., Rubin, E. M., and Bristow, J., 2000, Expression profiling reveals distinct sets of genes altered during induction and regression of cardiac hypertrophy. *Proc. Natl. Acad. Sci. U.S.A.* **97**:6745–6750.

Fu, M., Zhu, X., Zhang, J., Liang, J., Lin, Y., Zhao, L., Ehrengruber, M. U., and Chen, Y. E., 2003, Egr-1 target genes in human endothelial cells identified by microarray analysis. *Gene* **315**:33–41.

Garcia-Cardena, G., Comander, J., Anderson, K. R., Blackman, B. R., and Gimbrone, Jr., M. A., 2001, Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. *Proc. Natl. Acad. Sci. U.S.A.* **98**:4478–4485.

Geraci, M. W., Gao, B., Hoshikawa, Y., Yeager, M. E., Tuder, R. M., and Voelkel, N. F., 2001, Genomic approaches to research in pulmonary hypertension. *Respir. Res.* **2**:210–215.

Geraci, M. W., Hoshikawa, Y., Yeager, M., Golpon, H., Gesell, T., Tuder, R. M., and Voelkel, N. F., 2002, Gene expression profiles in pulmonary hypertension. *Chest* **121**:104S–105S.

Haase, D., Lehmann, M. H., Korner, M. M., Korfer, R., Sigusch, H. H., and Figulla, H. R., 2002, Identification and validation of selective upregulation of ventricular myosin light chain type 2 mRNA in idiopathic dilated cardiomyopathy. *Eur. J. Heart. Fail.* **4**:23–31.

Herrero, J., Al-Shahrour, F., Diaz-Uriarte, R., Mateos, A., Vaquerizas, J. M., Santoyo, J., and Dopazo, J., 2003, GEPAS: A web-based resource for microarray gene expression data analysis. *Nucleic. Acids. Res.* **31**:3461–3467.

Hsiao, L. L., Dangond, F., Yoshida, T., Hong, R., Jensen, R. V., Misra, J., Dillon, W., Lee, K. F., Clark, K. E., Haverty, P., Weng, Z., Mutter, G. L., Frosch, M. P., Macdonald, M. E., Milford, E. L., Crum, C. P., Bueno, R., Pratt, R. E., Mahadevappa, M., Warrington, J. A., Stephanopoulos, G., and Gullans, S. R., 2001, A compendium of gene expression in normal human tissues. *Physiol. Genomics.* **7**:97–104.

Hughes, T. R., Marton, M. J., Jones, A. R., Roberts, C. J., Stoughton, R., Armour, C. D., Bennett, H. A., Coffey, E., Dai, H., He, Y. D., Kidd, M. J., King, A. M., Meyer, M. R., Slade, D., Lum, P. Y., Stepaniants, S. B., Shoemaker, D. D., Gachotte, D., Chakraburtty, K., Simon, J., Bard, M., and Friend, S. H., 2000, Functional discovery via a compendium of expression profiles. *Cell* **102**:109–126.

Hwang, D. M., Dempsey, A. A., Lee, C. Y., and Liew, C. C., 2000, Identification of differentially expressed genes in cardiac hypertrophy by analysis of expressed sequence tags. *Genomics* **66**:1–14.

Hwang, J. J., Allen, P. D., Tseng, G. C., Lam, C. W., Fananapazir, L., Dzau, V. J., and Liew, C. C., 2002, Microarray gene expression profiles in dilated and hypertrophic cardiomyopathic end-stage heart failure. *Physiol. Genomics.* **10**:31–44.

Ichiki, T., Usui, M., Kato, M., Funakoshi, Y., Ito, K., Egashira, K., and Takeshita, A., 1998, Down-regulation of angiotensin II type 1 receptor gene transcription by nitric oxide. *Hypertension* **31**:342–348.

Ideker, T., Thorsson, V., Ranish, J. A., Christmas, R., Buhler, J., Eng, J. K., Bumgarner, R., Goodlett, D. R., Aebersold, R., and Hood, L., 2001, Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* **292**:929–934.

Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C., Trent, J. M., Staudt, L. M., Hudson, Jr., J., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O., 1999, The transcriptional program in the response of human fibroblasts to serum. *Science* **283**:83–87.

Khachigian, L. M., and Collins, T. 1997, Inducible expression of Egr-1-dependent genes. A paradigm of transcriptional activation in vascular endothelium. *Circ. Res.* **81**:457–461.

Khatri, P., Draghici, S., Ostermeier, G. C., and Krawetz, S. A., 2002, Profiling gene expression using onto-express. *Genomics* **79**:266–270.

Kitano, H., 2002, Systems biology: a brief overview. *Science* **295**:1662–1664.

Knight, J., 2001, When the chips are down. *Nature* **410**:860–861.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., and Frazier, M., 2001, Initial sequencing and analysis of the human genome. *Nature* **409**:860–921.

Lee, M. L., Kuo, F. C., Whitmore, G. A., and Sklar, J. 2000, Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc. Natl. Acad. Sci. U.S.A.* **97**:9834–9839.

Li, H., Lewis, A., Brodsky, S., Rieger, R., Iden, C., and Goligorsky, M. S., 2002, Homocysteine induces 3-hydroxy-3-methylglutaryl coenzyme a reductase in vascular endothelial cells: a mechanism for development of atherosclerosis? *Circulation* **105**:1037–1043.

Liang, M., Briggs, A. G., Rute, E., Greene, A. S., and Cowley, Jr., A. W., 2003, Quantitative assessment of the importance of dye switching and biological replication in cDNA microarray studies. *Physiol. Genomics.* **14**:199–207.

Liang, P., and Pardee, A. B., 1992, Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**:967–971.

Lipshutz, R. J., Fodor, S. P., Gingeras, T. R., and Lockhart, D. J., 1999, High density synthetic oligonucleotide arrays. *Nat. Genet.* **21**:20–24.

Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E. L., 1996, Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* **14**:1675–1680.

Lowes, B. D., Gilbert, E. M., Abraham, W. T., Minobe, W. A., Larrabee, P., Ferguson, D., Wolfel, E. E., Lindenfeld, J., Tsvetkova, T., Robertson, A. D., Quaife, R. A., and Bristow, M. R., 2002, Myocardial gene expression in dilated cardiomyopathy treated with beta-blocking agents. *N. Engl. J. Med.* **346**:1357–1365.

Martinet, W., Schrijvers, D. M., De Meyer, G. R., Herman, A. G., and Kockx, M. M., 2003, Western array analysis of human atherosclerotic plaques: downregulation of apoptosis-linked gene 2. *Cardiovasc. Res.* **60**:259–267.

Martinet, W., Schrijvers, D. M., De Meyer, G. R., Thielemans, J., Knaapen, M. W., Herman, A. G. and Kockx, M. M., 2002, Gene expression profiling of apoptosis-related genes in human atherosclerosis: upregulation of death-associated protein kinase. *Arterioscler. Thromb. Vasc. Biol.* **22**:2023–2029.

McCormick, S. M., Eskin, S. G., McIntire, L. V., Teng, C. L., Lu, C. M., Russell, C. G., and Chittur, K. K., 2001, DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **98**:8955–8960.

Messeguer, X., Escudero, R., Farre, D., Nunez, O., Martinez, J., and Alba, M. M., 2002, PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* **18**:333–334.

Paoni, N. F., and Lowe, D. G., 2001, Expression profiling techniques for cardiac molecular phenotyping. *Trends. Cardiovasc. Med.* 11:218–221.

Patino, W. D., Mian, O. Y., and Hwang, P. M., 2002, Serial analysis of gene expression: technical considerations and applications to cardiovascular biology. *Circ. Res.* **91**:565–569.

Pavlidis, P., Li, Q., and Noble, W. S., 2003, The effect of replication on gene expression microarray experiments. *Bioinformatics* **19**:1620–1627.

Perou, C. M., 2001, Show me the data! *Nat. Genet.* **29**:373.

Pilpel, Y., Sudarsanam, P., and Church, G. M., 2001, Identifying regulatory networks by combinatorial analysis of promoter elements. *Nat. Genet.* **29**:153–159.

Piper, M. D., Daran-Lapujade, P., Bro, C., Regenberg, B., Knudsen, S., Nielsen, J., and Pronk, J. T., 2002, Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of Saccharomyces cerevisiae. *J. Biol. Chem.* **277**:37001–37008.

Powell, D. A., Anderson, L. M., Cheng, R. Y., and Alvord, W. G., 2002, Robustness of the Chen-Dougherty-Bittner procedure against non-normality and heterogeneity in the coefficient of variation. *J. Biomed. Opt.* **7**:650–660.

Pritchard, C. C., Hsu, L., Delrow, J., and Nelson, P. S., 2001, Project normal: defining normal variance in mouse gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **98**:13266–13271.

Qiu, P., 2003, Recent advances in computational promoter analysis in understanding the transcriptional regulatory network. *Biochem. Biophys. Res. Commun.* **309**:495–501.

Quackenbush, J., 2002, Microarray data normalization and transformation. *Nat. Genet.* 32 Suppl, 496–501.

Reiner, A., Yekutieli, D., and Benjamini, Y., 2003, Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* **19**:368–375.

Sack, M. N., and Kelly, D. P., 1998, The energy substrate switch during development of heart failure: gene regulatory mechanisms (Review). *Int. J. Mol. Med.* **1**:17–24.

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O., 1995, Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**:467–470.

Schindler, H., and Bogdan, C., 2001, NO as a signaling molecule: effects on kinases. *Int. Immunopharmacol.* **1**:1443–1455.

Sehl, P. D., Tai, J. T., Hillan, K. J., Brown, L. A., Goddard, A., Yang, R., Jin, H., and Lowe, D. G., 2000, Application of cDNA microarrays in determining molecular phenotype in cardiac growth, development, and response to injury. *Circulation* **101**:1990–1999.

Smithies, O., Kim, H. S., Takahashi, N., and Edgell, M. H., 2000, Importance of quantitative genetic variations in the etiology of hypertension. *Kidney. Int.* **58**:2265–2280.

Smyth, G. K., and Speed, T., 2003, Normalization of cDNA microarray data. *Methods* **31**:265–273.

Stamler, J. S., Lamas, S., and Fang, F. C., 2001, Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* **106**:675–683.

Stanton, L. W., Garrard, L. J., Damm, D., Garrick, B. L., Lam, A., Kapoun, A. M., Zheng, Q., Protter, A. A., Schreiner, G. F., and White, R. T., 2000, Altered patterns of gene expression in response to myocardial infarction. *Circ. Res.* **86**:939–945.

Stoll, M., Cowley, Jr., A. W., Tonellato, P. J., Greene, A. S., Kaldunski, M. L., Roman, R. J., Dumas, P., Schork, N. J., Wang, Z., and Jacob, H. J., 2001, A genomic-systems biology map for cardiovascular function. *Science* **294**:1723–1726.

Tan, F. L., Moravec, C. S., Li, J., Apperson-Hansen, C., McCarthy, P. M., Young, J. B., and Bond, M., 2002, The gene expression fingerprint of human heart failure. *Proc. Natl. Acad. Sci. U.S.A.* **99**:11387–11392.

Topol, E. J., McCarthy, J., Gabriel, S., Moliterno, D. J., Rogers, W. J., Newby, L. K., Freedman, M., Metivier, J., Cannata, R., O'Donnell, C. J., Kottke-Marchant, K., Murugesan, G., Plow, E. F., Stenina, O., and Daley, G. Q., 2001, Single nucleotide polymorphisms in multiple novel thrombospondin genes may be associated with familial premature myocardial infarction. *Circulation* **104**:2641–2644.

Tusher, V. G., Tibshirani, R., and Chu, G., 2001, Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U.S.A.* **98**:5116–5121.

Ueno, S., Ohki, R., Hashimoto, T., Takizawa, T., Takeuchi, K., Yamashita, Y., Ota, J., Choi, Y.L., Wada, T., Koinuma, K., Yamamoto, K., Ikeda, U., Shimada, K., and Mano, H., 2003, DNA microarray analysis of in vivo progression mechanism of heart failure. *Biochem. Biophys. Res. Commun.* **307**:771–777.

van de Peppel, J., Kemmeren, P., van Bakel, H., Radonjic, M., van Leenen, D., and Holstege, F. C., 2003, Monitoring global messenger RNA changes in externally controlled microarray experiments. *EMBO Rep.* **4**:387–393.

van't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H., 2002, Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**:530–536.

Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W., 1995, Serial analysis of gene expression. *Science* **270**:484–487.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V., Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., and Yan, C., 2001, The sequence of the human genome. *Science* **291**:1304–1351.

Watson, J. D., and Crick, F. H., 1953, Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**:737–738.

Weinberg, E. O., Mirotsou, M., Gannon, J., Dzau, V. J., Lee, R. T., and Pratt, R. E., 2003, Sex dependence and temporal dependence of the left ventricular genomic response to pressure overload. *Physiol. Genomics.* **12**:113–127.

Welsh, J., Rampino, N., McClelland, M., and Perucho, M., 1995, Nucleic acid fingerprinting by PCR-based methods: applications to problems in aging and mutagenesis. *Mutat. Res.* **338**:215–229.

Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I., and Schacherer, F., 2000, TRANSFAC: an integrated system for gene expression regulation. *Nucleic. Acids. Res.* **28**:316–319.

Yang, J., Moravec, C. S., Sussman, M. A., DiPaola, N. R., Fu, D., Hawthorn, L., Mitchell, C. A., Young, J. B., Francis, G. S., McCarthy, P. M., and Bond, M., 2000, Decreased SLIM1 expression and increased gelsolin expression in failing human hearts measured by high-density oligonucleotide arrays. *Circulation* **102**:3046–3052.

Yang, Y. H., Buckley, M. J., and Speed, T. P., 2001, Analysis of cDNA microarray images. *Brief. Bioinform.* **2**:341–349.

Yang, Y. H., and Speed, T., 2002, Design issues for cDNA microarray experiments. *Nat. Rev. Genet.* **3**:579–588.

Ye, S. Q., Lavoie, T., Usher, D. C., and Zhang, L. Q., 2002, Microarray, SAGE and their applications to cardiovascular diseases. *Cell. Res.* **12**:105–115.

Yu, S. M., Hung, L. M., and Lin, C. C., 1997, cGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signaling pathway. *Circulation* **95**:1269–1277.

Zammatteo, N., Jeanmart, L., Hamels, S., Courtois, S., Louette, P., Hevesi, L., and Remacle, J., 2000, Comparison between different strategies of covalent attachment of DNA to glass surfaces to build DNA microarrays. *Anal. Biochem.* **280**:143–150.

Zeeberg, B. R., Feng, W., Wang, G., Wang, M. D., Fojo, A. T., Sunshine, M., Narasimhan, S., Kane, D. W., Reinhold, W. C., Lababidi, S., Bussey, K. J., Riss, J., Barrett, J. C., and Weinstein, J. N., 2003, GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome. Biol.* **4**:R28.

Zhang, S., Day, I. N., and Ye, S., 2001, Microarray analysis of nicotine-induced changes in gene expression in endothelial cells. *Physiol. Genomics.* **5**:187–192.

Chapter 2

Proteomics: State of the Art and Its Relevance for Gene Therapy

Sergiu Scobioala, Rainer Klocke, Günter Michel, and Sigrid Nikol

The molecular mechanisms underlying most diseases, including those of the cardiovascular system, are widely unknown. Basically, pathological changes in the organism arise from protein alterations. Proteomics comprises a set of tools allowing the identification of protein alterations, i.e. changes of protein abundance and posttranslational modifications, associated with diseases. The linkage of information about such protein changes with functional alterations as revealed by physiological studies constitutes functional proteomics that enables the disclosure of disease mechanisms.

Disease-linked protein alterations include those of suitable candidates for drug targets and disease biomarkers as well as therapeutic proteins/peptides. Since gene therapy depends on the function of a therapeutic protein encoded by a "therapeutic" gene, proteomic analyses provide the basis for the design and application of gene therapies.

The storage and administration of experimental data obtained by the application of proteomic analyses is supported by species- and tissue-specific protein databases and specific software. Publications in this field are reviewed in this chapter.

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

1. INTRODUCTION

1.1. Definitions

The term *proteome* was created by Wasinger *et al.* (1995) and Wilkins *et al.* (1996a) and defined as the cell- and tissue-specific patterns of proteins expressed by the genome of an organism. Proteomics provides the technologies to investigate the proteome. Traditionally, proteomic analyses or "proteomics" means the separation of a large number of proteins from a cell line or organism at a given time point in 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the cataloging of the spots to create databases. Today, proteomics also comprises the identification of proteins and their posttranslational modifications (PTMs) by mass spectrometry (MS), their functional analysis, e.g., by interaction studies using techniques such as the yeast two-hybrid system or the phage display method, and their subcellular localization.

1.2. Importance of Proteomics

Because of different mechanisms, e.g. differential splicing and alternative codon usage, one gene can encode various proteins. Preliminary studies suggest an average number of protein variants per gene of three to more than six in human beings (Wilkins *et al.*, 1996b). Thus, the human organism may contain more than half a million proteins, which is not evident from the genome sequence. This complexity is further enhanced by different types of modifications contributing to the regulation of protein activity at various levels of protein expression (Fig. 1). Examples are PTMs, e.g. phosphorylation, glycosylation, peptide cleavage, and complex formation of the proteins. Consequently, the functional complexity of an organism significantly exceeds that indicated by its genome sequence.

Since proteins are the main functional entities of the cell they should be expected to provide the most relevant information regarding cellular functions. Together with an often poor correlation between mRNA abundance and the quantity of the corresponding protein present within a cell, a recent study analyzing human liver samples determined the correlation coefficient between the amount of mRNA

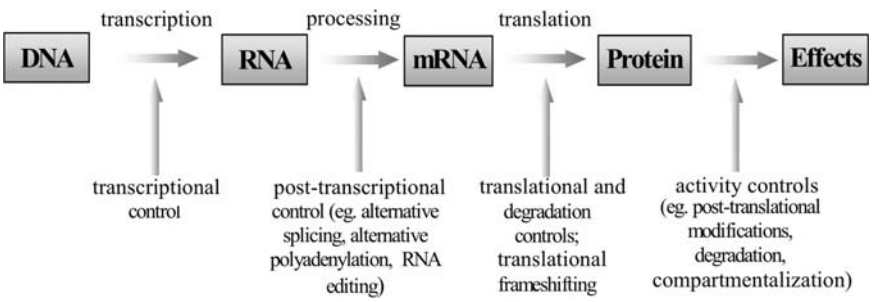

FIGURE 1. The ways in which gene expression can be regulated or modified from transcription to posttranslation. (*See Color Plate 3.*)

present and the corresponding protein abundance to be 0.48 only (Seilhamer and Anderson, 1999); this indicates the need for large-scale proteomics studies that complement the widely performed transcriptome analyses by DNA microarray hybridization technologies.

Because of the central importance of proteins for the functions of cells, tissues, organs, and whole organisms, disease processes are predominantly caused by changes in protein expression, subcellular localization, and PTM. Accordingly, the identification of such changes by proteomics is essential to define candidates for drug targets, therapeutic proteins, and disease biomarkers. Since gene therapy, although primarily based on the delivery of a "therapeutic" gene to the diseased organ, is an approach whose effect is finally due to the function of the therapeutic protein encoded by the "therapeutic" gene, proteomics provides the basis required for the design and application of gene therapies. In addition, as it is the case for conventional types of therapies too, proteomics enables the molecular monitoring of therapeutic effects and undesirable side effects by providing and exploiting disease biomarkers.

2. TECHNOLOGIES

Proteomics comprises a vast number of techniques, which basically enables sample preparation, separation, imaging, and identification of proteins, as well as the determination of the nature and position of protein modifications (Fig. 2)

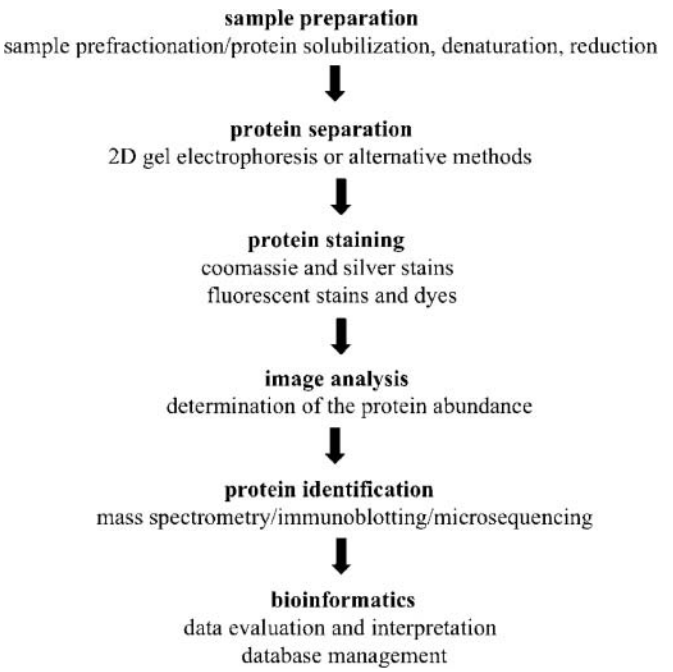

FIGURE 2. Elements of proteomics-based studies.

(Westbrook *et al.*, 2001) and the identification of protein–protein interactions. 2D-PAGE and MS are currently the central proteome analysis techniques.

2.1. Protein Separation by 2D-PAGE

2.1.1. Sample Preparation

Sample preparation involves the isolation and homogenization of cells or tissues, protein dissociation into its polypeptide subunits, solubilization, denaturation (unfolding of secondary and tertiary structures), and reduction of internal disulfide bonds to break secondary structures.

Tissue samples are usually isolated by microsurgery. However, since tissue samples often contain many different contaminating cell types, e.g. cells of connective tissue (fibroblasts), only a subset of the cells present will actually contribute proteins that are relevant for the study. To circumvent this problem laser capture microdissection was developed (Fig. 3) (Simone *et al.*, 2000a). Specific cell subpopulations isolated by laser capture microdissection from frozen or fixed tissue sections under direct microscopic visualization have been used for highly

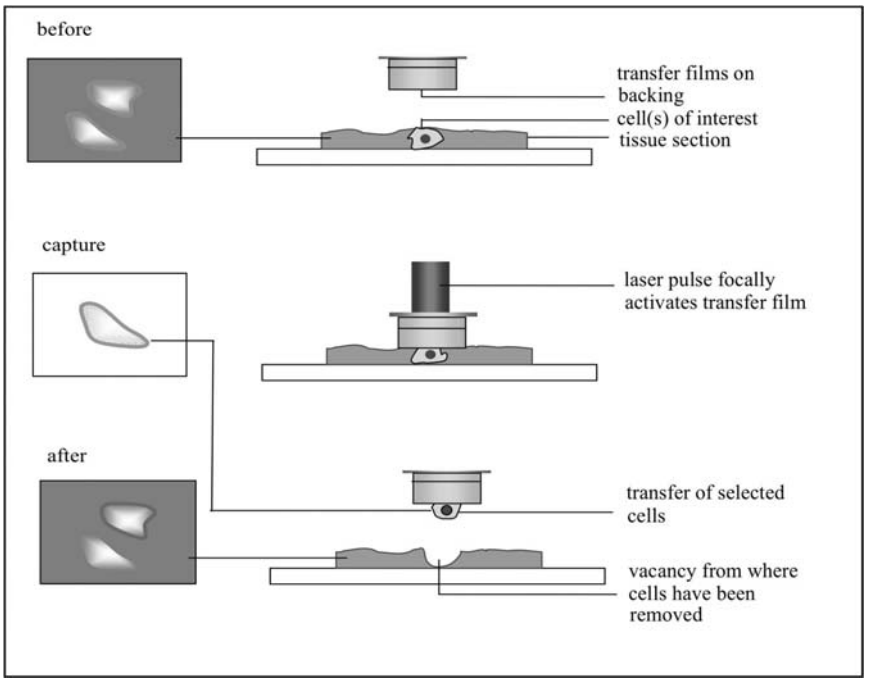

FIGURE 3. Laser capture microdissection. A transparent polymer film is placed in direct contact with the surface of a heterogeneous tissue section. Laser energy is used to activate the polymer directly over the selected cells. The activated region captures the selected cells, which can be lifted away from unwanted tissue. (*See Color Plate 3.*)

Table 1
Methods for Protein Prefractionation

Prefractionation Method	Principle	Reference
High-speed centrifugation	Differential sedimentation under centrifugal force	Celis, 1998
Affinity-based purification	Sterical interaction of proteins with immobilized receptors on specific surface	Ianello and Jeffrey, 1990
Liquid-phase separation	Separation of proteins in the liquid phase according to their physical characteristics—charge, size, etc.	Bier, 1998
Precipitation	Differential solubility of proteins	Görg *et al.*, 1997
Selective "clearing"	Selective filtration of proteins of interest	Walsh *et al.*, 1984
Sequential extraction	Differential solubility of proteins	Weiss *et al.*, 1992

sensitive and reproducible proteomic analysis using 2D-PAGE and other analytical methods (Simone *et al.*, 2000a, 2000b).

Deep-frozen cells or tissues are homogenized by different techniques such as grinding in a liquid nitrogen-cooled mortar, use of microdismembrators, sonication, or shearing-based methods. Because of the high diversity of proteins in eukaryotic tissues, it is sometimes advisable to carry out a prefractionation step to enrich the desired proteins (e.g. low abundant proteins or alkaline proteins) on the basis of their physical characteristics—e.g. isoelectric point (pI), molecular weight, hydrophobicity. Prefractionation of proteins can be achieved by various methods (Table 1).

The most frequently used buffer for sample solubilization is based on O'Farrel's lysis buffer and contains the chaotropic agent urea, the nonionic detergent NP-40, and the reducing agent dithiothreitol (O'Farrel, 1975). For the solubilization of certain classes of proteins, in particular membrane, membrane-associated, or other hydrophobic proteins, modified variants of O'Farrell's lysis buffer containing various combinations of reducing agents, chaotropic agents, and detergents (Herbert, 1999; Herbert and Molloy, 1998; Herbert and Sanchez, 1997) are available. Although the presence of the relatively high concentrations of chaotropic agents and detergents in the lysis buffer usually minimizes protease activity for the duration of the sample-processing period (Rabilloud, 1996), it is sometimes advisable to include protease inhibitors to protect sensitive proteins from proteolytic degradation.

2.1.2. 2D-PAGE

The first step of proteome analysis consists in the separation of complex mixtures of proteins obtained from cells, tissues, and organs.

Currently, the best method for separating complex protein mixtures is 2D-PAGE. One can routinely resolve 2000–3000 proteins from a single sample of a protein extract. Using large-scale gels (Jungblut *et al.*, 1994) even up to 10,000 proteins can be resolved. 2D-PAGE involves the separation of proteins on the basis of their isoelectric point by isoelectric focusing (IEF) in the first dimension and based on molecular mass in the second dimension. The result is a gel

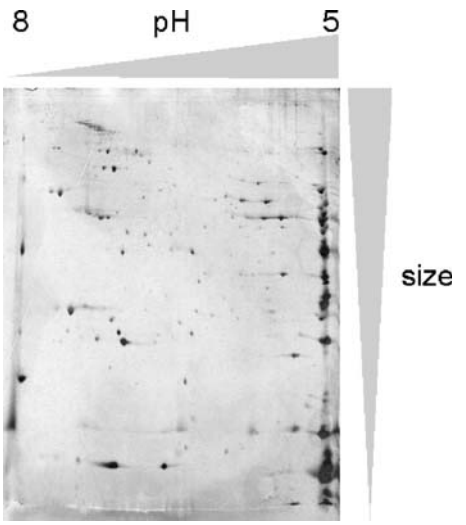

FIGURE 4. Silver-stained 2D-PAGE gels of a mouse heart (left ventricle). (*See Color Plate 4.*)

which, when stained, reveals a large number of spots (Fig. 4). Any given spot may contain more than one protein and any given protein may be contained in multiple spots depending on, for example, its posttranslational modification states.

2.1.2a. Separation in the First Dimension Proteins are amphoteric molecules and hence will migrate in the presence of an electric field based on the total sum of the charges present on the molecule at a given pH. Following application of current, the charged polypeptides will migrate in a polyacrylamide gel strip or a tube gel that contains a pH gradient until they reach the pH at which their overall charge is neutral (pI), hence producing a gel strip containing discrete protein bands along its length.

The currently preferred approach for protein separation in the first dimension consists in the use of Immobiline reagent-based (Amersham Pharmacia Biotech) immobilized pH gradients (IPGs). IPG strips are composed of acidic and basic buffering groups covalently linked to the polyacrylamide matrix. They are available in various lengths and pH ranges. Wide pH (3–10) gradients can be used to provide an overview of the protein diversity in a sample. Narrow-range IPG strips covering a pH range of three pH steps are used to achieve an optimum resolution in order to avoid the presence of multiple proteins in a single spot for unambiguous protein identification (Dunn, 2000).

Before separation in the second dimension, iodoacetamide is added for *S*-carboxymethylation in order to prevent S–S bridges from re-forming after they have been reduced using DDT or mercaptoethanol.

2.1.2b. Separation in the Second Dimension Since the IPG strips containing the separated, carboxymethylated proteins are placed on an SDS polyacrylamide gel, the proteins will migrate in a direction rectangular to their

Table 2
Comparison of Staining Techniques

Parameter	Silver Stain	Coomassie Blue Dye	Fluorescent Dye
Sensitivity	+++[a]	++[b]	+++[a]
Reproducibility	+++[a]	++	+++[a]
Compatibility with mass spectrometry	++[b]	+++[d]	+++[a]
Linearity of staining intensity	++[c]	+++	+++[a]
Simplicity and rapidity of staining procedure	+	++	++[a]

[a] Görg *et al.*, 2000.
[b] Hames, 1990.
[c] Giometti *et al.*, 1991.
[d] Macri *et al.*, 2000.

former movement when current is applied. Specific gel characteristics such as size, thickness, and percentage of acrylamide, the inclusion of a stacking gel as well as the buffering conditions may be varied according to the specific requirements (Macri and Rapundalo, 2001). Second dimension 2D-PAGE systems are available in both horizontal and vertical configurations. (Görg *et al.*, 1995).

2.1.3. Protein Staining and Labeling

Protein spots in 2D gels can be detected by Coomassie staining, zinc or silver staining, ^{32}P or ^{35}S radiolabeling, fluorescent staining, and/or immunodetection.

Although radiolabeling is the most sensitive detection method, silver chemistry and Coomassie staining are the most commonly applied (Table 2). Introduction of new fluorescent dyes with a variety of detection wavelengths and sensitivities has great application potential.

2.1.3a. Coomassie Stains Stains with the Coomassie blue dyes G-250 or R-250 cannot be used to quantify proteins and the sensitivity is clearly below silver and fluorescent stains (Hames, 1990). However, in contrast to most silver stain methods, Coomassie blue staining does not greatly interfere with subsequent protein identification by mass spectrometry (Macri and Rapundalo, 2001).

2.1.3b. Silver Stain With a sensitivity 10–50 times that of Coomassie blue, silver stain allows the detection of as little as 0.1 ng of protein per spot. Either silver diammine (alkaline method) or silver nitrate (acidic method) is mainly used as the silvering agent (Rabilloud, 1992).

2.1.3c. Fluorescent Stains and Dyes For fluorescent staining three approaches are possible (Görg *et al.*, 2000): (1) pre-electrophoretic derivatization of proteins, where proteins are coupled with a fluorescent dye, e.g. monobromobimane and 2-methoxy-2, 4-diphenyl-3(2*H*)-furanone, prior to the IEF step of 2D-PAGE, (2) labeling of proteins after the first dimension, and (3) postelectrophoretic labeling after 2D-PAGE with dyes like SYPRO Orange, SYPRO Red, or

SYPRO Ruby. The fluorescently labeled proteins can be imaged with a UV transilluminator, a blue-light transilluminator, or laser-scanning instruments (Macri and Rapundalo, 2001). The best results were obtained with SYPRO Ruby staining. The patterns obtained with silver staining and SYPRO Ruby staining are similar, but not identical (Görg *et al.*, 2000; Görg and Weiss, 2000).

2.1.4. Technical Problems and Limitations of Protein Separation by 2D-PAGE and Their Detection

Streaks are one of the most common technical problems associated with 2D-PAGE (Görg and Weiss, 2000). Horizontal streaks are localized in different areas of the 2D gels and are caused by (1) reduced solubility of certain proteins during IEF due to protein overload or protein–contaminant interaction, (2) considerable over focusing times, or (3) depletion of DDT during IEF (Görg *et al.*, 1995).

Vertical "empty" lines can be caused by urea crystals formed during IEF on the surface of the IPG strip (cf. Görg *et al.*, 2000).

Background smear can be due to poor quality of chemicals used for sample preparation or electrophoresis (cf. Görg *et al.*, 2000).

There are still technical problems to resolve all the proteins within a proteome by 2D-PAGE. Two circumstances hampering total proteome resolution by 2D-PAGE are insufficient sensitivity to detect low-abundance proteins and the underrepresentation of certain classes of proteins, e.g. basic and membrane proteins. To overcome these problems sample prefractionation methods (see above) can be applied to achieve subproteomic enrichment (Table 1).

2.1.5. Image Analysis

Image analysis follows 2D-PAGE and protein staining. Image acquisition is accomplished with an imaging densitometer, document scanner, charged-coupled device camera, or storage phosphoimager (Macri and Rapundalo, 2001). The resulting image either is acquired digitally or has to be converted to a digital format. Several computer software packages are available for the analysis of 2D-PAGE gel images (Table 3). These software packages accomplish the detection and quantification of spots and they enable both multiple image alignments and image comparisons. The data obtained by quantitative analysis of matched protein spots are generally exported to a spreadsheet program for statistical analysis. Such export files usually contain quantitative measurements as well as protein spot characteristics such as x/y coordinates, area, and volume of specific protein spots (Macri and Rapundalo, 2001).

2.2. Alternative Analytical Protein Separation Technologies

2D-PAGE is the most popular analytical protein separation technique for proteome analysis. However, other separation methods have been developed and may be used for proteome analysis, provided that they achieve a sufficient degree of

Table 3
Software for the Analysis of 2D-PAGE

Software	Company	Web site
Proteomweaver	Definiens	www.definiens.com
Delta 2D	Decodon	www.decodon.de
Gellab	Scanalytics	www.scanalytics.com
ImageMaster 2-D Elite	Amersham Biosciences	www.amershambiosciences.com
Imagep1Q	Proteome Systems	www.proteomesystems.com
Investigator 2-D	BST Scientific	www.bst-asia.com
Kepler	Large Scale Biology	www.lsbc.com
Melanie	Geneva Bioinformatics	www.genebio.com
PDQuest	Bio-Rad	www.discover.bio-rad.com
Phoretix 2D/Progenesis	Nonlinear Dynamics	www.nonlinear.com
ProteinMime	Scimagix	www.scimagix.com
Z3	Compugen	www.2dgels.com

resolution or may be suitable to isolate and display a subproteome not amenable to separation by 2D-PAGE. Examples for such technologies are capillary zone electrophoresis (Jensen *et al.*, 2000; Shen *et al.*, 2000), affinity chromatography (Damer *et al.*, 1998; Ping *et al.*, 2001), or the recently developed protein chip method (Fung *et al.*, 2000, 2001; Nelson *et al.*, 2000; von Eggeling *et al.*, 2000; Weinberger *et al.*, 2001; Williams and Addona, 2000).

2.3. Protein Identification and Characterization by MS

2D-PAGE does not provide direct clues to the identities of the separated proteins. The current method of choice for the identification of proteins is MS since it is faster, cheaper, and more accurate than earlier techniques like microsequencing by automated Edman degradation. In addition, MS is much more sensitive, can deal with protein mixtures, offers much higher throughput, and is available to automation. MS-based mass determination is based on the conversion of proteins or peptides into gas-phase ions with an ionization source. Mass spectrometers are classified according to the ionization source and mass analyzer employed. Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization are the most widely used ionization techniques. Basically, ionized peptides are separated according to their mass-to-charge ratio and detected by mass analyzers. MALDI-derived peptides are usually analyzed in so-called time-of-flight analyzers.

Protein identification by MS is usually accomplished by two main approaches. The first technique is called mass fingerprinting and was initially suggested by Henzel *et al.* (1993). It is generally employed to identify proteins purified by 2D-PAGE and relies on in-gel digestion of gel-separated proteins into peptides by sequence-specific proteases (i.e. trypsin) or chemical reagents (i.e. CNBr). These sequence-specific protein digests provide characteristic mass profiles or "fingerprints" since most amino acid residues have a unique mass (Fig. 5).

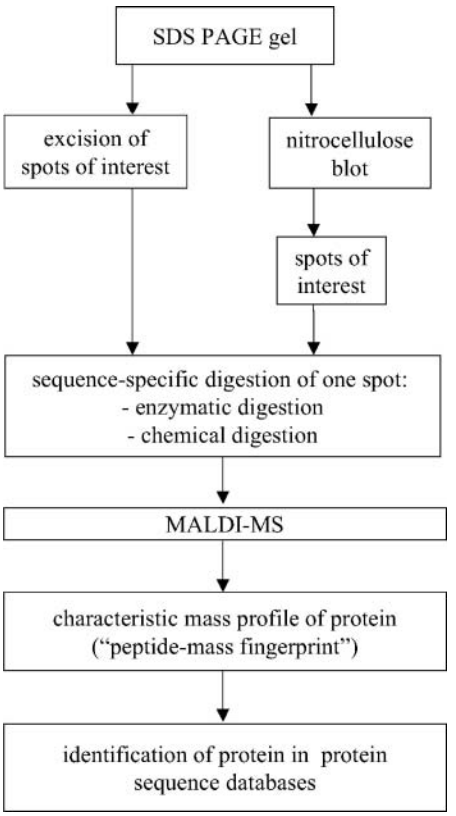

FIGURE 5. Mass spectrometric protein identification separated by 2D-PAGE.

The success of peptide mass fingerprinting (PMF) also depends on the existence of comprehensive, searchable databases for the species under investigation. Since the presence of full length cDNA sequences in the database is at least instrumental, PMF works effectively for species whose genomes are completely sequenced.

The main reason for analyzing peptides rather than proteins is that the molecular weight of proteins is usually not sufficient for database identification. However, even PMF databases are often insufficient for unambiguous protein identification. Therefore, PMF frequently has to be combined with additional MS techniques to obtain further information. Tandem mass spectrometry (MS/MS) can be instrumental to achieve unambiguous protein identification. In MS/MS, an ionized peptide is selected by the primary MS and fragmented by collision with inert gas. The resulting fragments are then analyzed by the supplementary MS to identify and locate the amino acids in the peptide. The identity and location within the peptide of only two amino acids can be sufficient to identify the peptide by comparison with the databases.

In addition to protein identification MS can be used to identify PTMs, e.g. phosphorylation, glycosylation, and sulfation. Since PTMs can determine activity, stability, localization, and turnover of proteins, their identification is very important for the determination of protein functions. In comparison with standard biochemical methods (see below), the use of MS for PTM identification is more powerful since it provides information about the type of a modification and its localization within the protein.

Efforts have been made to miniaturize and automate protein preparation using microfabricated "chips." Such surface-enhanced laser desorption/ionization chips combine protein chip technology with MS (Fung *et al.*, 2000). Samples are first prepared and fractionated on chips with varying chromatographic properties, e.g. anion exchange, cation exchange, metal affinity, or reverse phase. After the chips are washed to remove unbound proteins, the bound proteins are identified in a time-of-flight mass spectrometer.

2.4. Technologies to Analyze PTMs

Besides MS (see above) further technologies including a number of classic biochemical techniques may be used to investigate PTMs. The use of biochemical methods as lectin binding, acid/Schiff staining, or enzymatic deglycosylation allows the identification of glycosylated proteins (Baker *et al.*, 1992; Ianello and Jeffrey, 1990). Phosphorylated proteins have been identified by ^{32}P radiolabeling in living cells (Hansen and Moller, 1993; Mason *et al.*, 1998), enzymatic dephosphorylation (Arrell *et al.*, 2000), or the use of antiphosphospecific antibodies (Soskic *et al.*, 1999).

Proteolysis, another type of PTM, may play a role in the etiology or pathology of cardiovascular diseases. For example, using direct Edman sequencing, ischemia/reperfusion-associated myosin light chain 1 proteolysis at amino acid residue 19 has been detected (Arrell *et al.*, 2001a).

2.5. Protein Bioinformatics

Bioinformatics is of central importance for proteomics. Special software packages enable the quantitative analysis and database collection of the results of 2D-PAGE proteome analysis (Dunn, 2000). In addition, special bioinformatics tools have been developed for the detailed characterization of proteins, e.g. for the calculation of their physicochemical properties (e.g. pI, M_r) and the prediction of their potential posttranslational modifications (i.e. MOD, FIND, BOLD, NetPhas) (Banks *et al.*, 2000). Most of these tools are available via proteomics servers like NIH (http://www.ncbi.nlm.nih.gov), EBI (http://www.ebi.ac.uk), and ExPASY (http://www.expasy.ch/tools/).

SWISS-PROT and SWISS-2DPAGE are typical examples of annotated protein and two-dimensional electrophoresis databases (Fig. 6). Such databases ideally should provide a visual image of a 2D-PAGE gel from which a protein spot of interest

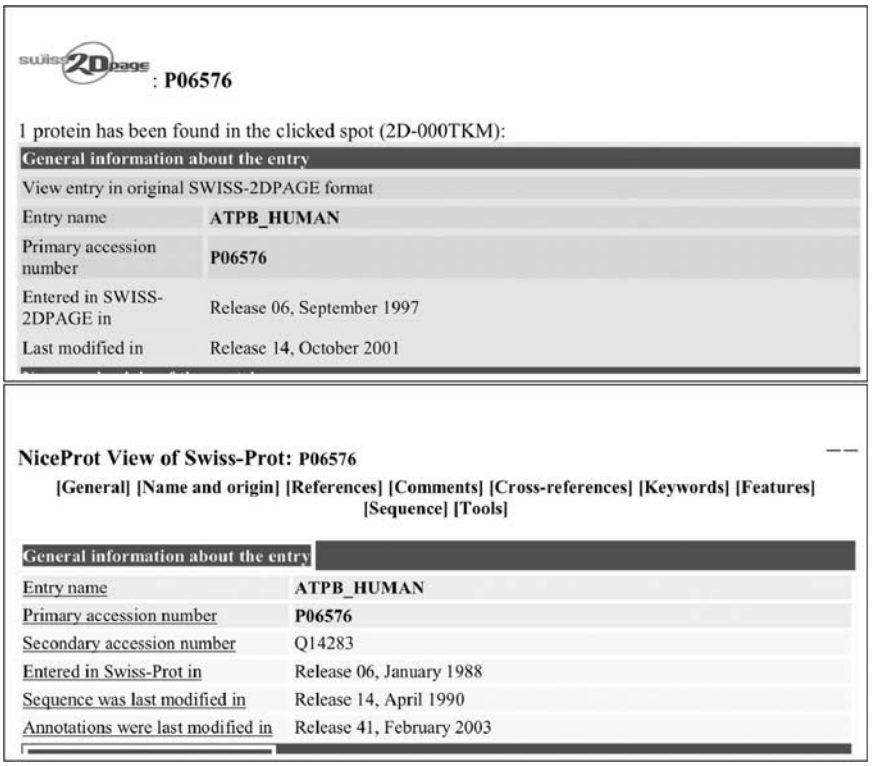

FIGURE 6. Annotated protein in SWISS-2DPAGE and SWISS-PROTS databases. (*See Color Plate 4.*)

can be selected to obtain specific information about that protein (e.g. M_r, pI, amino acid sequence if available, function, any PTMs) (Arrell *et al.*, 2001b). In addition, complex experimental details, relevant biochemical pathways, and disease implications should be highlighted (Banks *et al.*, 2000).

Special databases of human cardiac proteins are HSC-2DPAGE (Evans *et al.*, 1997), HEART-2DPAGE, HP-2DPAGE, and RAT HEART-2DPAGE. These databases are accessible through www.expasy.ch/ch2d/2d-index.html (Table 4). They contain information on several hundreds of cardiac proteins.

2.6. Technologies to Analyze Protein–Protein Interactions

Protein–protein interactions have a high impact on the determination of biological functions and their knowledge can potentially be exploited for therapeutic purposes. Currently, the most important approaches to study protein–protein interactions are the purification of protein complexes, the yeast two-hybrid system, and the phage display method.

Table 4
2D-PAGE Protein Databases for the Heart Accessible via the World Wide Web

Database	Web Address	Source	Protein Spots Separated	Protein Species Separated	Organization
HSC-2DPAGE	http://www.harefield.nthames.nhs.uk/nhli/protein	Human heart (LV)	∼1500	∼153	Heart Science Centre, Harefield Hospital
		Dog heart (LV)	1212	80	
		Rat heart (LV)	1188	30	
HEART-2DPAGE	http://www.chemie.fu-berlin.de/user/pleiss/dhzb.html	Human heart (RA, RV)	3300	150	German Heart Institute, Berlin
HP-2DPAGE	http://www.mdc-berlin.de/∼emu/heart/	Human heart (RV)	∼ 3300	70	MDC, Berlin
RAT HEART-2D-PAGE	http://gelmatching.inf.fu-berlin.de/∼pleiss/2d/	Rat heart	>3000	64	German Heart Institute, Berlin

2.6.1. Purification of Protein Complexes

Protein–protein interactions can be studied by the purification of entire multiprotein complexes by affinity-based methods and their subsequent analysis by 2D-PAGE in combination with MS (Pandey and Mann, 2000). One way to identify the interaction partners of a protein consists in the coimmunoprecipitation of the protein and its interaction partners with an antibody. Usually, the protein is genetically tagged with an epitope for which an antibody is commercially available. The recombinant full length cDNA encoding the tagged protein is then transfected into a suitable cell. Subsequently, the overexpressed protein is immunoprecipitated from the cell extract together with its interaction partners.

Alternatively, a multiprotein complex can be purified based on its interaction with a "bait" fusion protein which is immobilized on a solid support. After washing off the proteins that interact nonspecifically, the protein complex is eluted and analyzed by 2D-PAGE in combination with subsequent MS for protein identification (Fig. 7). Such studies provide insight into mechanisms. Since no assumptions are made about the complex, unsuspected connections between cellular processes may be recognized.

2.6.2. Yeast Two-Hybrid System

The yeast two-hybrid system is a powerful tool to study protein–protein interactions (Fields and Song, 1989). It is based on the modular structure of the yeast transcription factors that contain a DNA-binding domain (DBD) and an activation domain (AD) (Auerbach *et al.*, 2001). Using DNA cloning techniques, a protein

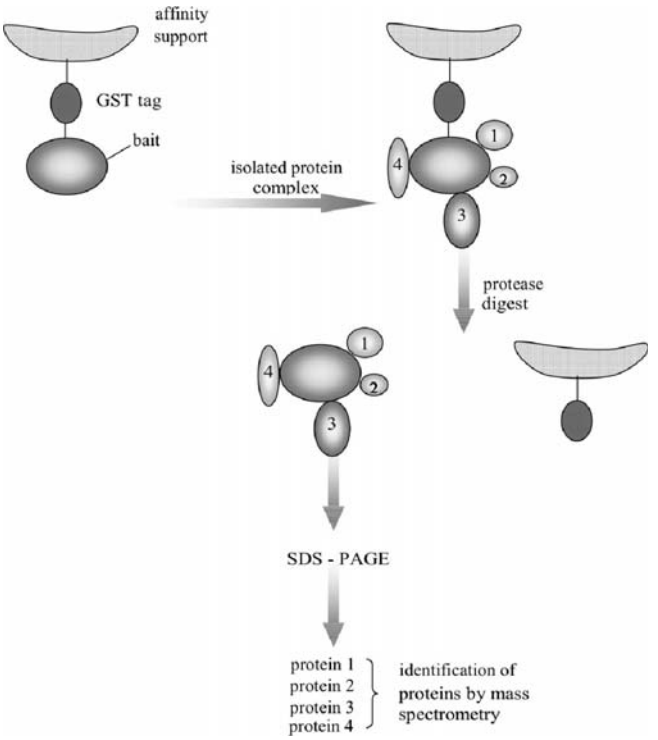

FIGURE 7. Analysis of protein–protein interactions. The protein of interest is expressed as a fusion protein with a cleavable affinity tag to identify interacting proteins. It is immobilized onto agarose beads using a glutathione S-transferase tag. Nuclear cell extracts are incubated with the beads and the beads washed extensively. Thrombin is used to cleave between the glutathione S-transferase and the "bait" protein, which results in the elution of all proteins that are specifically bound to "bait" (Pandey and Mann, 2000). The eluted proteins are resolved by 2D-PAGE and analyzed by MS. The success of the above-mentioned strategies relies on sufficient affinity of the protein complex to the bait and on optimized conditions for purifications steps. (*See Color Plate 5.*)

of interest X ("bait") is fused to the DBD of a transcription factor whereas a potentially interacting protein Y ("prey") is fused to the AD. The DBD-X and AD-Y fusion proteins are then expressed in a yeast strain that contains a plasmid with the binding site for the DBD upstream of a reporter gene. In the case of physical interaction of DBD-X and AD-Y, the DNA-binding and activation domains will come into close proximity, resulting in the reconstitution of a functional transcription factor that will activate transcription of the reporter gene (Fig. 8a).

In principle, no previous knowledge about the interacting proteins is necessary for a screen to be performed. Thus, the yeast two-hybrid system is suitable to identify protein–protein interactions in a genome-wide scale. Basically, two libraries of cDNAs comprising open reading frames (ORFs) fused to the DBD or AD of a transcription factor are used. Two different amino acid-deficient yeast strains containing the different libraries are generated. After mating the haploid strains

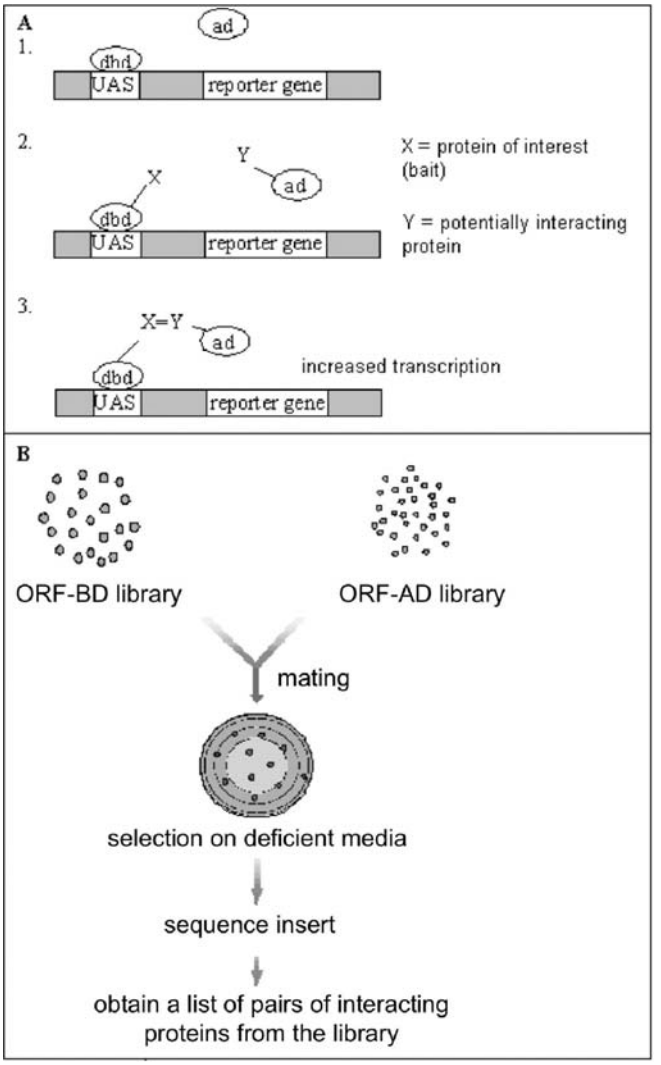

FIGURE 8. The yeast two-hybrid system. (a) *Schematic representation of the yeast two-hybrid system.* (1) The two separated domains of a transcription factor are not functional and therefore do not induce transcription of the reporter gene. (2) The DBD and AD are fused to two proteins of interest and co-expressed in a yeast reporter strain. (3) If DBD-X and AD-Y interact, the fusion proteins are assembled at the binding site of the reporter gene, which leads to activation of transcription. (b) *Library-based yeast two-hybrid screening method.* In this strategy, two different yeast strains containing two different cDNA libraries are prepared. In one case, the open reading frames (ORFs) are expressed as GAL 4–BD fusions and in other case, they are expressed as GAL 4–AD fusions. The two yeast strains are then mated and diploids selected on deficient media. Thus, only the yeast cells expressing interacting proteins survive. The inserts from both the plasmids are then sequenced to obtain a pair of interacting genes. (Modified from Pandey and Mann, 2000). (*See Color Plate 6.*)

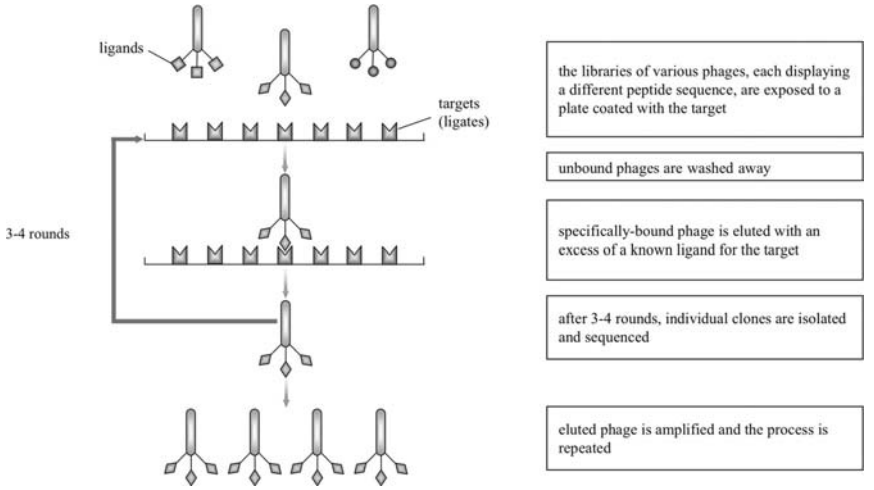

FIGURE 9. Affinity-selection of ligands from phage display libraries. Different peptide sequences can be screened for binding to a specific target molecule. After several rounds only the clones with the peptides that bind to the target are isolated. (*See Color Plate 7.*)

diploids are selected on deficient media. Thus, only the yeast cells expressing interacting proteins survive (Fig. 8b). The ORF cDNAs encoding the interacting proteins are identified by sequencing the relevant clones (Pandey and Mann, 2000).

The range of the conventional yeast two-hybrid system is limited by the fact that hydrophobic transmembrane proteins and membrane-associated proteins may not enter into the nucleus. Therefore, alternative membrane-associated two-hybrid systems have been developed (reviewed in Auerbach *et al.*, 2002).

2.6.3. Phage Display

The phage display method is based on bacteriophage particles expressing a peptide or protein of interest fused to a capsid or coat protein. The DNA encoding the virus proteins and also the fusion protein is localized within the protein coat of the virus particle. Consequently, the virus provides a physical link between the protein/peptide and the genetic information that encodes it. Libraries of peptides can be screened for binding to a variety of target molecules (antibodies, enzymes, cell-surface receptors) by incubating the phage library with the target molecules. Whereas the nonbinding phage is washed away, the remaining phages are amplified. After several rounds of binding under increasing selection pressure, individual clones are characterized by DNA sequencing (Fig. 9).

Phage display can be used to screen for peptide epitopes, peptide ligands, enzyme substrates, or single-chain antibody fragments in solution. Like the two-hybrid system it is simple and can be performed in an automated high-throughput setting (Pandey and Mann, 2000). Whether the former or the latter is preferable

depends on the particular class of proteins being studied, since the interactions take place in solution as opposed to the nucleus of the yeast cell. In addition, phage display is applicable to transcription factors, in contrast to the yeast two-hybrid system.

3. CONCLUSIONS AND PERSPECTIVES

Pathological changes within an organism arise from protein alterations. Proteomics provides a powerful set of tools for the large-scale study of protein expression and PTM. Accordingly, the application of proteomics is the strategy of choice to elucidate the mechanisms of diseases and to identify protein-based drug targets, disease biomarkers and therapeutic peptides and proteins, thus providing the basis of pharmacological and gene-based therapies.

2D-PAGE and MS are still the basic methods of protein identification, enabling the establishment of protein databases. Many complementary technologies have been developed recently or are currently being developed. These include protein arrays, the yeast-two hybrid system, phage-display libraries, surface-enhanced laser desorption/ionization (Auerbach *et al.*, 2001; Pandey and Mann, 2000). Either alone or in combination they will gain important roles in proteomics. The further development of high-quality bioinformatic tools and disease- and species-specific databases will be necessary to complement proteomics technologies (Jager *et al.*, 2002).

ACKNOWLEDGMENT. We thank the Interdisciplinary Center for Clinical Research (IZKF, project B15) for supporting Dr Sergiu Scobioala.

REFERENCES

Arrell, D. K., Au, B. P. Y., Fraser, H., Sasaki, N., and Van Eyk, J. E., 2000, Pharmacological preconditioning mediates altered phosphorylation of the "unphosphorylatable" myosin light chain 1, *Circulation* **102**(Suppl. II):II-202.

Arrell, D. K., Neverova, I., Fraser, H., Marban, E., and Van Eyk, J. E., 2001a, Proteomic analysis of pharmacologically preconditioned cardiomyocytes reveals novel phosphorylation of myosin light chain 1, *Circulation Res.* **14**:480–487.

Arrell, D. K., Neverova, I., and Van Eyk, J. E., 2001b, Cardiovascular proteomics. Evolution and potential, *Circulation Res.* **88**:763–773.

Auerbach, D., Galeuchet-Schenk, B., Hottiger, M. O., and Stagljar, I., 2002, Genetic approaches to the identification of interactions between membrane proteins in yeast,
J. Recept. Signal Transduct. Res. **22**(1–4):471–481.

Auerbach, D., Schenk, B., Hübner, B., Meier, M., Hottiger, M. O., Hübscher, U., and Stagljar, I., 2001, Proteomics in vivo: using yeast as a genetic tool to study protein–protein interactions, *BIOforum Int.* **4**:171–173.

Baker, C. S., Corbett, J. M., May, A. J., Yacoub, M. H., and Dunn, M. J., 1992, A human myocardial two-dimensional electrophoresis database: protein characterisation by microsequencing and immunoblotting, *Electrophoresis* **13**(9–10):723–726.

Banks, R. E., Dunn, M. J., Hochstrasser, D. E., Sanchez, J. C., Blackstock, W., Pappin, D. J., and Selby, P. J., 2000, Proteomics: new perspectives, new biomedical opportunities, *Lancet* **356**(9243):1749–1756.

Bier, M., 1998, Recycling isoelectric focusing and isotachophoresis, *Electrophoresis* **19**(7):1057–1063.

Celis, J., 1998, *Cell Biology: A Laboratory Handbook*, 2nd edn. San Diego: Academic Press.

Damer, C. K., Partridge, J., Pearson, W. R., and Haystead, T. A., 1998, Rapid identification of protein phosphatase 1-binding proteins by mixed peptide sequencing and data base searching. Characterization of a novel holoenzymic form of protein phosphatase 1, *J. Biol. Chem.* **273**(38): 24396–24405.

Dunn, M. J., 2000, Studying heart disease using the proteomic research, *Drug Dis. Today* **2**:76–84.

Evans, G., Wheeler, C. H., Corbett, J. M., and Dunn, M. J., 1997, Construction of HSC-2DPAGE: a two-dimensional gel electrophoresis database of heart proteins, *Electrophoresis* **18**(3–4):471–479.

Fields, S., and Song, O. K., 1989, A novel genetic system to detect protein–protein interactions, *Nature* **340**(6230):245–246.

Fung, E. T., Thulasiraman, V., Weinberger, S. R., and Dalmasso, E. A., 2001, Protein biochips for differential profiling, *Curr. Opin. Biotechnol.* **12**(1):65–69.

Fung, E. T., Wright, G. L., and Dalmasso, E. A., 2000, Proteomic strategies for biomarker identification: progress and challenges, *Curr. Opin. Mol. Therap.* **2**, 643–649.

Giometti, C. S., Gemmell, M. A., Tollaksen, S. L., and Taylor, J., 1991, Quantitation of human leukocyte proteins after silver staining: a study with two-dimensional electrophoresis, *Electrophoresis* **12**:536–543.

Görg, A., Obermaier, C., Boguth, G., Csordas, A., Diaz, J. J., and Madjar, J. J., 1995, Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins, *Electrophoresis* **18**(3–4):328–337.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., and Weiss, W., 2000, The current state of two-dimensional electrophoresis with immobilized pH gradients, *Electrophoresis* **21**(6):1037–1053.

Görg, A., Obermaier, C., Boguth, G., and Weiss W., 1999, Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures, *Electrophoresis* **20**(4–5):712–717.

Görg, A., and Weiss, W., 2000, *Proteome Research: Two-Dimensional Electrophoresis and Identification Methods*, pp. 57–106. Berlin: Springer- Verlag.

Hames, B. D., 1990, *A Practical Approach*, pp. 51–85. New York: Oxford University Press.

Hansen, K., and Moller, J. V., 1993, Application of two-dimensional gel analysis to identification and characterization of tyrosine phosphorylated substrates for growth factor receptors, *Electrophoresis* **14**:112–126.

Henzel, W. J., Billeci, T. M., Stults, J. T., Wong, S. C., Grimley, C., and Watanabe, C., 1993, Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases,*Proc. Natl. Acad. Sci. U.S.A.* **90**(11):5011–5015.

Herbert, B. R., 1999, Advances in protein solubilisation for two-dimensional electrophoresis, *Electrophoresis* **20**:660–663.

Herbert, B. R., Molloy, M. P., Gooley, A. A., Walsh, B. J., Bryson, W. G., and Williams, K. L., 1998, Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent, *Electrophoresis* **19**(5):845–851.

Herbert, B. R., and Sanchez, J. C., 1997, *Proteome Research: New Frontiers in Functional Genomics*. Heidelberg: Springer-Verlag.

Ianello, R. C., and Jeffrey, P. L., 1990, Glycoproteins of rat skeletal muscle sarcolemma: characterization by two-dimensional gel electrophoresis and effect of denervation, *Exp. Neurol.* **108**:156–161.

Jager, D., Jungblut, P. R., and Muller-Werdan, U., 2002, Separation and identification of human heart proteins, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **771**:131–153.

Jensen, P. K., Pasa-Tolic, L., Peden, K. K., Martinovic, S., Lipton, M. S., Anderson, G. A., Tolic, N., Wong, K. K., and Smith, R. D., 2000, Mass spectrometric detection for capillary isoelectric focusing separations of complex protein mixtures, *Electrophoresis* **21**(7):1372–1380.

Jungblut, P., Otto, A., Zeindl-Eberhart, E., Plessner, K. P., Knecht, M., Regitz-Zagrosek, V., Fleck, E., and Wittmann-Liebold, B., 1994, Protein composition of the human heart: the construction of a myocardial two-dimensional electrophoresis database, *Electrophoresis* **15**(5):685–707.

Macri, J., McGee, B., Thomas, J. N., Du, P., Stevenson, T. I., Kilby, G. W., and Rapundalo, S. T., 2000, Cardiac sarcoplasmic reticulum and sarcolemmal proteins separated by two-dimensional electrophoresis: surfactant effects on membrane solubilization, *Electrophoresis* **21**(9):1685–1693.

Macri, J., and Rapundalo, S. T., 2001, Application of proteomics to the study of cardiovascular research, *Trends Cardiovasc. Med.* **2**:66–75.

Mason, G. G., Murray, R. Z., Pappin, D., and Rivett, A. J., 1998, Phosphorylation of ATPase subunits of the 26S proteasome, *FEBS Lett.* **430**(3):269–274.

Nelson, R. W., Nedelkov, D., and Tubbs, K. A., 2000, Biosensor chip mass spectrometry: a chip-based proteomics approach, *Electrophoresis* **21**(6):1155–1163.

O'Farrel, P. H., 1975, High resolution two-dimensional electrophoresis of protein, *J. Biol. Chem.* **250**:4007–4021.

Pandey, A., and Mann, M., 2000, Proteomics to study genes and genomes, *Nature* **405**:321–329.

Ping, P., Zhang, J., Pierce, W. M., Jr., and Bolli, R., 2001, Functional proteomic analysis of protein kinase C epsilon signaling complexes in the normal heart and during cardioprotection, *Circulation Res.* **88**(1):59–62.

Rabilloud, T., 1992, A comparison between low background silver diammine and silver nitrate protein stains, *Electrophoresis* **13**:429–439.

Rabilloud, T., 1996, Solubilization of proteins for electrophoretic analyses. *Electrophoresis* **17**:813–29.

Seilhamer, J., and Anderson, L., 1999, A comparison of selected m-RNA and protein abundances in human liver, *Electrophoresis* **18**:533–537.

Shen, Y., Berger, S. J., Anderson, G. A., and Smith, R. D., 2000, High-efficiency capillary isoelectric focusing of peptides, *Anal. Chem.* **72**(9):2154–2159.

Simone, N. L., Paweletz, C. P., Charboneau, L., Petricoin, E. F., III, and Liotta, L. A., 2000a, Laser capture microdissection: beyond functional genomics to proteomics, *Mol. Diagn.* **5**(4): 301–307.

Simone, N. L., Remaley, A. T., Charboneau, L., Petricoin, E. F., III, Glickman, J. W., Emmert-Buck, M. R., Fleisher, T. A., and Liotta, L. A., 2000b, Sensitive immunoassay of tissue cell proteins procured by laser capture microdissection, *Am. J. Pathol.* **156**(2):445–452.

Soskic, V., Gorlach, M., Poznanovic, S., Boehmer, F.-D., and Godovac-Zimmermann, J., 1999, Functional proteomics analysis of signal transduction pathways of the PDGF beta receptor, *Biochemistry* **38**:1757–1764.

von Eggeling, F., Davies, H., Lomas, L., Fiedler, W., Junker, K., Claussen, U., and Ernst, G., 2000, Tissue-specific microdissection coupled with ProteinChip array technologies: applications in cancer research, *Biotechnologies* **29**:1066–1070.

Walsh, M. J., Limos, L., and Tourtellotte, W. W., 1984, Two-dimensional electrophoresis of cerebrospinal fluid and ventricular fluid proteins, identification of enriched and unique proteins, and comparison with serum, *J. Neurochem.* **43** 1277–1285.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L., and Humphery-Smith, I., 1995, Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*, *Electrophoresis* **16**(7):1090–1094.

Weinberger, S. R., Dalmasso, E. A., and Fung, E. T., 2001, Current achievements using protein chip array technology, *Curr. Opin. Chem. Biol.* **6**:86–91.

Weiss, W., Postel, W., and Görg, A., 1992, Application of sequential extraction procedures and glycoprotein blotting for the characterization of the 2-D polypeptide patterns of barley seed proteins, *Electrophoresis* **13**:770–773.

Westbrook, J. A., Jun, X. Y., Wait, R., and Dunn, M. J., 2001, A combined radiolabelling and silver staining technique for improved visualisation, localization and identification of proteins separated by two-dimensional gel electrophoresis, *Proteomics* **1**:370–376.

Wilkins, M. R., Sanchez, J. C., Gooley, A. A., Appel, R. D., Humphrey-Smith, I., Hochstrasser, D. F., and Williams, K. L., 1996a, Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it, *Biotechnol. Genet. Eng. Rev.* **13**:19–50.

Wilkins, M. R., Sanchez, J. C., Williams, K. L., and Hochstrasser, D. F., 1996b, Current challenges and future applications for protein maps and posttranslational vector maps in proteome projects, *Electrophoresis* **17**:830–838.

Williams, C., and Addona, T. A., 2000, The integration of SPR biosensors with mass spectrometry: possible application for proteome analysis, *Trends Biotechnol.* **18**:45–48.

Chapter 3

Cardiovascular Proteomics

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Traditional cardiovascular proteomics includes the comparative large-scale determination of protein profiles and the identification of individual proteins of myocardial tissue from different species including humans. The goal of these studies consisted, and still consists, in the establishment of comprehensive proteome databases. By comparison with protein profiles from diseased tissues such databases will enable to an increasing degree the fast identification of protein alterations accompanying or underlying cardiovascular diseases. Examples demonstrating the feasibility of this approach are already available. In combination with pathophysiological data the knowledge about protein alterations in diseased tissues will allow the identification of disease mechanisms and of candidates for therapeutic proteins, drug targets, and disease biomarkers. Thereby it provides the basis for therapies, including gene therapies, of cardiovascular diseases. These efforts will be supported by the use of animal models displaying significant degrees of similarity to human cardiovascular diseases.

1. INTRODUCTION

Traditional proteomic technologies have been used for several years to identify and characterize individual proteins mediating the normal functions of the cardiovascular system. They are now being increasingly used on a large scale to

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

reveal protein alterations accompanying or potentially underlying the development of cardiovascular disease processes. Together with functional analyses, proteomics will lead to a deepened understanding of the mechanisms of cardiovascular diseases. The knowledge of the molecular disease mechanisms constitutes the basis for the development of gene therapies since it allows the definition of candidates for therapeutic proteins, i.e. the proteins encoded by the "therapeutic" transgenes applied in gene therapy. In addition, suitable candidates for disease biomarkers, enabling the monitoring of clinical trials and later perhaps even routine therapies, may be uncovered by the application of proteomics.

2. PROTEOMICS IN BASIC CARDIOVASCULAR RESEARCH

Traditional proteome analysis provides a protein inventory for a cell or tissue during a particular condition or at a given moment. Many of the early studies that were focused on 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation of cardiac proteins (Baker *et al.*, 1992; Jungblut *et al.*, 1994) demonstrated the feasibility of simultaneously separating and detecting hundreds of proteins. The goal of these early studies was to create databases containing the molecular weight, pI, and, in some instances, functional data of the identified proteins. Today, several databases of human cardiac proteins are available: HSC-2DPAGE (Evans *et al.*, 1997), HEART-2DPAGE, HP-2DPAGE, and RAT HEART-2DPAGE. These databases are accessible through www.expasy.ch/ch2d/2d-index.html and conform to the rules for federated two-dimensional gel electrophoresis (2-DE). These databases contain information on several hundreds of cardiac proteins. Some of these databases provide a visual image of a 2-DE gel, from which one may select a protein spot of interest and obtain information about that protein [i.e. M_r, pI, amino acid sequence if available, function, posttranslational modifications (PTMs)] (Arrell *et al.*, 2001).

The inclusion of further species in the large-scale identification of cardiac proteins is of high importance for the progress of basic cardiovascular research and the elucidation of the molecular mechanisms underlying cardiovascular diseases. Heart protein databases for several animals are currently being constructed. For example, a recent report described the identification of 64 proteins from more than 3300 proteins resolved by 2D-PAGE of rat myocardial tissue (Li *et al.*, 1999). To create a database of rat myocardial proteins the authors used the Make2ddb software package, which can be downloaded from the ExPASy server. In the future, the existing databases will not only be expanded by the inclusion of more and more proteins but will probably be subdivided to represent chamber- or organelle-specific proteomes and will be complemented by protein profiles from hearts or *in vitro* cultivated cardiomyocytes representing different stages of development or differentiation, respectively (cf. Macri and Rapundalo, 2001).

To demonstrate the value of the cross-species comparative approach, myocardial protein profiles from dog, rat, and mouse were established by 2D-PAGE and compared with the protein profile of human myocardium. Many potentially

homologous proteins between these species were tentatively identified on the basis of the similarity of their migration properties in 2D-PAGE with known human proteins (Corbett *et al.*, 1995).

Recently, the analysis of the myocardial proteome has profited from several technical innovations. The most crucial improvement was the introduction of immobilized pH gradients (IPG) in isoelectric focusing (see Chapter 2), since IPG gels provide reliable pH gradients. They are available in a wide variety of gradients, from pH 3 to 12, thus enabling increased protein resolution and detection of both low-abundance proteins and protein modifications that might otherwise remain undetected (Görg *et al.*, 1999).

New methods have been developed to enrich certain classes of proteins to reduce the complexity of the proteome to be analyzed (subproteomic approach), allowing for the detection of both lower abundance proteins and subtle alterations in PTMs. For example, a selective extraction method, called "IN sequence," recently led to the detection and quantification of a very subtle change in phosphorylation of the myosin light chain 1 (MLC 1) protein, which was undetectable in whole-cell homogenates (Arrell *et al.*, 2000, 2001).

By altering the protein solubilization conditions, Macri *et al.*, (2000) improved the resolution and detection of membrane proteins from cardiac sarcoplasmic reticulum and sarcolemmal fractions. Nevertheless, not all membrane proteins could be detected by 2-DE, e.g. sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, indicating that further improvement of such techniques is required.

3. MODIFICATIONS OF CARDIOVASCULAR PROTEOMES UNDER CHRONIC EXPERIMENTAL OR PATHOLOGICAL CONDITIONS

Pathological changes of the cardiovascular system arise from protein alterations and have the potential to alter proteomes of tissues within the whole organism. Such changes may be elucidated through an integrated series of proteome analysis technologies. Global changes in protein expression levels of the myocardium in response to various conditions such as ischemia, hypertension, hypertrophy, heart failure, and infarction can be identified by protein profiling.

Modification of the proteome under chronic experimental or pathological conditions may be caused by up- or downregulation of specific genes, isoform switching, *de novo* protein synthesis, and aberrant PTMs. For example, an increased abundance of heat shock proteins (HSPs) and mitochondrial proteins was found in a number of heart failure models (Heinke *et al.*, 1998, 1999; Pleissner and Regitz-Zagrosek, 1995; Portig *et al.*, 1996; Scheler *et al.*, 1997; Weekes *et al.*, 1999). Isoform switching has been identified in the case of expression of MLC 1 and MLC 2 in neonatal rat myocytes from a phenylephrine-stimulated hypertrophy model (Table 1) (Arnott *et al.*, 1998). *De novo* synthesis of HSP 72 was shown to occur in heat-stressed endothelial cells (Portig et al., 1996) and the significance of PTMs for proteome modification has been exemplified by analyses of HSP 27 and troponin I PTMs (see below).

Table 1
Proteins Displaying Similarly Altered Expression in Human and Animal DCM or Hypertrophy

Protein	Species	Alteration in Expression	Reference
Cytoskeletal and myofibrillar proteins			
Desmin	Dog	Decrease	Heinke *et al.*, 1998
	Human	Decrease	Corbett *et al.*, 1998
MLC 2 (ventricular isoform)	Human	Variable, possible PTM	Thiede *et al.*, 1996
	Rat[a]	Increase, possible PTM	Arnott *et al.*, 1998
MLC 1 (atrial isoform)	Human	Increase, atypical expression in LV	Pleissner *et al.*, 1995
	Rat[a]	Increase, atypical expression in LV	Arnott *et al.*, 1998
Proteins associated with mitochondria and energy production			
ATPase δ chain, ATP synthase	Dog	Decrease	Heinke *et al.*, 1998
	Human	Variable	Corbett *et al.* 1998; Thiede *et al.*, 1996
Dihydrolipoamide dehydrogenase	Dog	Decrease	Heinke *et al.*, 1998
	Human	Decrease	Corbett *et al.*, 1998
Creatine kinase M-chain	Dog	Minor decrease	Heinke *et al.*, 1998, 1999
	Human	Decrease	Corbett *et al.*, 1998
Proteins associated with stress responses			
Heat shock protein 70 (inducible)	Dog	Decrease	Heinke *et al.*, 1998
	Human	Decrease	Corbett *et al.*, 1998
Heat shock protein 60	Dog	Decrease	Heinke *et al.*, 1998
	Human	Decrease	Corbett *et al.*, 1998
Heat shock protein 27	Rat[a]	Increase	Arnott *et al.*, 1998
	Human	Variable, possible PTM	Scheler *et al.*, 1999
αB-Crystallin	Dog	Variable, possible PTM	Heinke *et al.*, 1998
	Human	Variable, possible PTM	Corbett *et al.*, 1998; Thiede *et al.*, 1996

Note: PTM, posttranslational modification; DCM, dilated cardiomyopathy; LV, left ventricle.
[a]Phenylephrine-induced hypertrophy; in all other cases DCM.

Up to now proteomics of human heart diseases have concentrated on the analysis of dilated cardiomyopathy (DCM). Approximately 100 proteins displaying altered expression in this disease have been observed. The majority of these proteins display less abundance in the diseased tissue (Corbett *et al.*, 1995; Li *et al.*, 1999; Pleissner *et al.*, 1995). Many of these proteins have been identified (e.g. Pleissner *et al.*, 1995; Thiede *et al.*, 1996) and they can be assigned to three functional classes, i.e. cytoskeletal and myofibrillar proteins, proteins associated with mitochondria and energy production, and proteins associated with stress responses. Similar changes were found in animal models of DCM and heart failure (see below and Table 1). These changes are in agreement with the pathophysiology of the disease that includes contractile dysfunction and an impairment of myocardial energetics. Examples of proteins exhibiting decreased expression in

human DCM are desmin, ATP synthase, creatine kinase, HSP 60, and HSP 70 (Table 1).

PTMs probably constitute the primary mechanism of fast protein alterations under conditions of acute stress. To date, there are only few cases of PTMs identified by cardiovascular proteomics. For example, Van Eyk and Murphy (2001) published the proteolysis of the myofilament protein troponin I during myocardial stunning. A detailed analysis of the expression of HSP 27 in human DCM has been performed by Scheler *et al.* (1997, 1999). Of nearly 60 HSP 27 protein spots found in 2D-PAGE gels by immunostaining, 9 were finally confirmed to belong to HSP 27. Although none of these were analyzed for the presence of PTMs in detail, this demonstrates the potentially large number of PTMs possible for a single protein (Scheler *et al.*, 1997).

In several studies only a subproteome has been analyzed. For example, new downstream targets of protein kinase C signaling during myocardial preconditioning have been identified using monoclonal antibodies (Ping *et al.*, 2001). A similar approach was used to identify cardiac-specific antigens that elicit antibody responses in heart diseases and following cardiac transplantation. In this approach, serum samples from patients were used to probe Western blots of 2D-PAGE separations of myocardial proteins. Several cardiac antigens have been identified that are associated with antibody responses following cardiac transplantation and might be involved in acute (Latif *et al.*, 1995) and chronic (Wheeler *et al.*, 1995) rejection. In addition, cardiac antigens have been identified that are reactive with autoantibodies in DCM (Latif *et al.*, 1993; Pohlner *et al.*, 1997) and myocarditis (Pankuweit *et al.*, 1997).

4. PROTEOMICS OF ANIMAL MODELS OF HEART DISEASES

Investigations of human diseased tissues by proteomic technologies are complicated by factors such as tissue heterogeneity, genetic variability, the disease stage, and the patient's medical therapy. In principle, these problems can be circumvented by the investigation of large numbers of human samples. However, this is expensive and requires large (international) cooperations. An alternative approach consists in the use of appropriate animal models of human diseases.

The results of two recently published studies of heart failure in large animals revealed similarities with those of a proteome analysis of human DCM (see above; Heinke *et al.*, 1998, 1999). Significant alterations in the abundance of several proteins associated with the cytoskeleton and myofibrills, with the mitochondria and energy production, and with the stress responses were found to accompany pacing-induced heart failure in canines (Table 1) (Heinke *et al.*, 1998, 1999) and human DCM (Corbett *et al.*, 1998; Thiede *et al.*, 1996) as well. Similar changes were also identified in bovine hereditary DCM (Weekes *et al.*, 1999) that is considered to be a valuable model of the human disease (Weil *et al.*, 1997). A sevenfold increase in ubiquitin carboxyl-terminal hydrolase (UCH) was interpreted as the most important change in the bovine model. Since UCH is responsible for the maintenance of the cytoplasmic pool of free ubiquitin, it was hypothesized that

its increase could lead to an increased degree of protein ubiquination in the diseased state, causing enhanced proteolysis via the 26S proteasome pathway. This is in agreement with the former assumption that inappropriate ubiquitination and subsequent degradation of proteins by the proteasome pathway may contribute to the development of heart failure (Field and Clark, 1997) and it was recently supported by the findings that UCH is also elevated in human DCM hearts (Weekes *et al.*, 2003) and that such proteins that were previously found to be reduced in abundance in DCM display an enhanced degree of ubiquitination (cf. McGregor and Dunn, 2003).

There are also several models of myocardial infarction, cardiac hypertrophy, and heart failure in small animals, particularly the rabbit, rat, and mouse. Proteome analyses of cardiac tissue from these animals were performed in the past to investigate protein profile changes in response to alcohol consumption (Patel *et al.*, 2000; Preedy *et al.*, 1997), lead application (Toraason *et al.*, 1997), and retrovirus infection (Weekes *et al.*, 2003).

Recently, the alterations of protein expression following myocardial ischemia and reperfusion (MI/R) in rabbits were analyzed by Schwertz *et al.* (2002). In addition to sham-operated animals and animals that were subjected to MI/R, they investigated MI/R-treated animals that were additionally treated with the synthetic serine protease inhibitor Futhan/nafamstat mesilate (FUT-175). FUT-175 is known to inhibit both the classical and alternative complement pathways, thereby significantly reducing myocardial necrosis following MI/R. On average 509 protein spots were found in 2D gels of myocardial proteins of the three groups. Two of 10 proteins displaying different levels of expression in sham- and MI/R-treated animals were identified as superoxide dismutase or αB-crystallin, respectively. In the hearts of individuals subjected to MI/R and additionally treated with FUT-175, the expression levels of both proteins were significantly preserved in comparison with sham-operated individuals. These results impressively indicate the potential of proteomics to identify new disease biomarkers suitable to monitor therapeutic effects on the molecular level (Fig. 1).

In a rat model of ischemic heart disease induced by coronary artery ligation the alterations of protein expression were analyzed by 2D-PAGE (cf. McGregor and Dunn, 2003). Most of the identified 22 differentially expressed proteins were myofibrillar or energy metabolism-related proteins.

It was stated that a potential problem in these small animal models is that their cardiac physiology and their normal pattern of gene expression (i.e. isoforms of the major cardiac contractile proteins) is different from that in larger mammals such as humans. However, in a recently performed proteome analysis of a coronary artery ligation-induced mouse model of myocardial infarction, we identified differentially expressed proteins (Fig. 2), indicating the presence of stem cell-based regeneration, compensatory mechanisms of blood pressure regulation, and extracellular matrix remodeling. Since these processes are known to take place in human myocardial infarction as well and probably will significantly contribute to its outcome, it is reasonable to assume that the mouse model of myocardial infarction will be instrumental to elucidate the molecular mechanisms of these processes in humans.

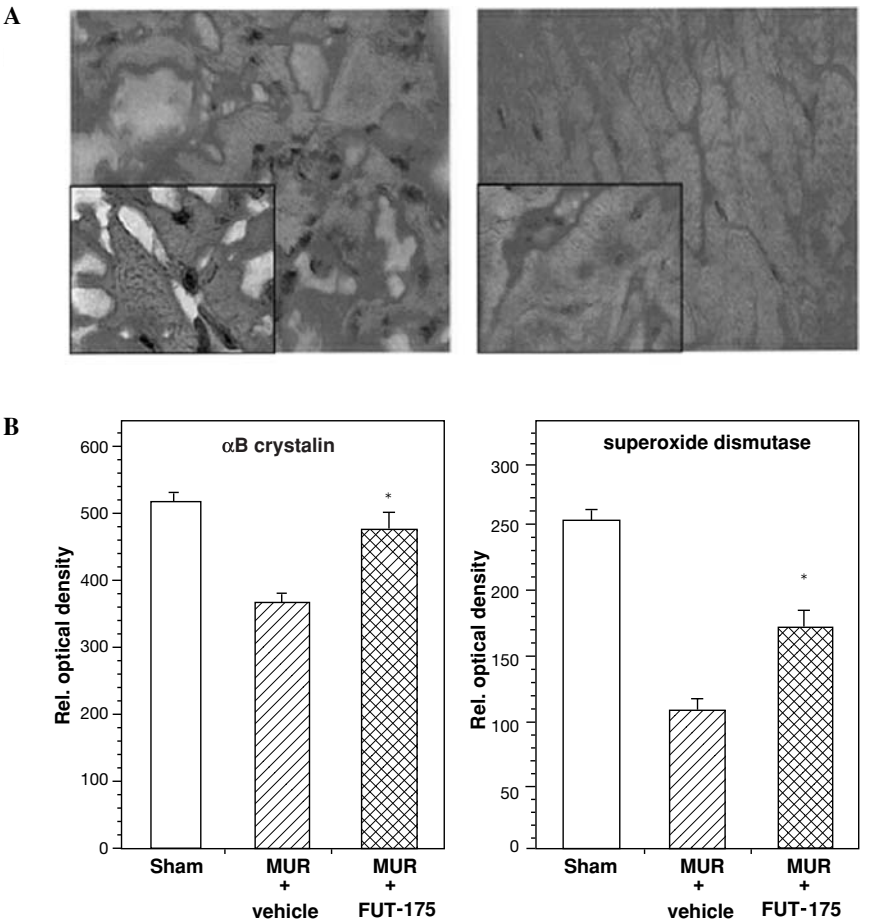

FIGURE 1. FUT-175 treatment protects the myocardium from MI/R-induced necrosis and significantly preserves the expression of proteins. (Modified according to Schwertz *et al.*, 2002.) (A) Photomicrographs of MI/R-treated hearts. Left: vehicle-treated MI/R heart; infiltrating neutrophils and myocardial cell damage is clearly visible. Right: FUT-175-treated MI/R heart; decreased tissue injury indicated by the preserved cross-striation of the cardiac myocytes and lower neutrophil accumulation are obvious. (B) Expression levels of αB-crystallin (left) and superoxide dismutase (right) in control hearts (sham-operated individuals), hearts of MI/R plus vehicle-treated individuals and hearts of MI/R plus FUT-175-treated individuals.

As already mentioned above, Arnott *et al.* (1998) detected aberrant expression of MLC 1 and MLC 2 in phenylephrine-stimulated hypertrophic neonatal rat cardiomyocytes. They also found increased levels of HSP 27 in the hypertrophic cells. The fact that similar changes were found in human DCM (Table 1) suggests that not only small animals but even *in vitro* cultivated cardiomyocytes of small animals may serve as suitable model systems for the identification of certain molecular changes associated with human heart diseases.

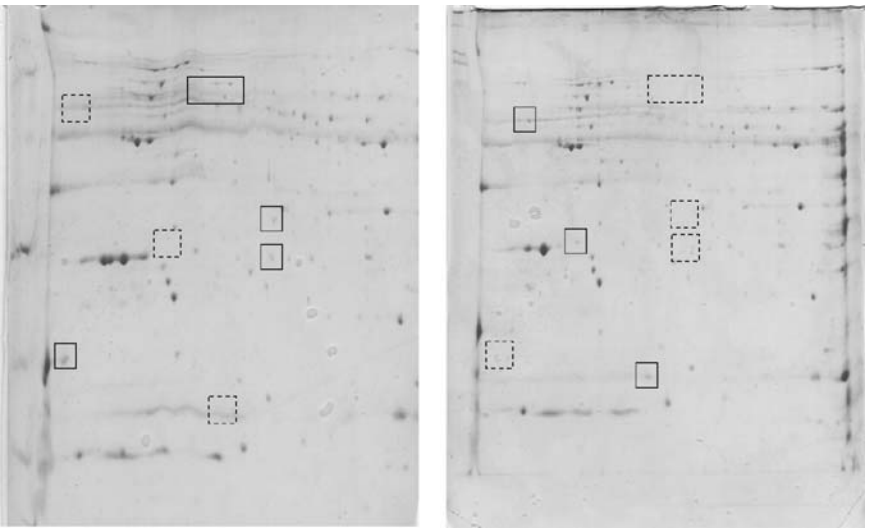

FIGURE 2. Coomassie-stained 2D gels of proteins isolated from normal (left) and infarcted left ventricle (right). Spots displaying significant differences in staining intensity between normal and infarcted left ventricle are framed (interrupted frames indicate the positions of spots of reduced or even undetectable intensities). (*See Color Plate 7.*)

5. CONCLUSIONS AND PERSPECTIVES

In combination with physiological analyses the application of proteomics is the strategy of choice to elucidate the mechanisms of cardiovascular diseases and to identify protein-based drug targets, disease biomarkers, and therapeutic peptides and proteins, thus providing the basis for pharmacological and gene therapies.

Many protein alterations have been already identified in human diseased myocardium. Further experiments have to reveal whether some of them may concern suitable drug targets, therapeutic proteins/peptides, or disease biomarkers. However, the investigation of human diseases is complicated by tissue heterogeneity, disease complexity and stage, multimorbidity and age of patients, as well as drug interferences. Accordingly, animal models of human cardiovascular diseases will become increasingly important to understand these disorders. In addition, *in vitro* model systems are in use and will be more and more used to study certain aspects of cardiovascular diseases by the application of proteomic technologies.

ACKNOWLEDGMENT. We thank Dr Michael Kuhlmann for help with the figures and the Interdisciplinary Center for Clinical Research (IZKF; Project B15) for supporting Dr Sergiu Scobioala.

REFERENCES

Arnott, D., O'Connell, K. L., King, K. L., and Stults, J. T., 1998, An integrated approach to proteome analysis: identification of proteins associated with cardiac hypertrophy, *Anal. Biochem.* **258**(1): 1–18.

Arrell, D. K., Au, B. P. Y., Fraser, H., Sasaki, N., and Van Eyk, J. E., 2000, Pharmacological preconditioning mediates altered phosphorylation of the "unphosphorylatable" myosin light chain 1, *Circulation* **102**(Suppl. II):II-202.

Arrell, D. K., Neverova, I., Fraser, H., Marban, E., and Van Eyk, J. E., 2001, Proteomic analysis of pharmacologically preconditioned cardiomyocytes reveals novel phosphorylation of myosin light chain 1, *Circ. Res.* **14**:480–487.

Arrell, D. K., Neverova, I., and Van Eyk, J. E., 2001, Cardiovascular proteomics. Evolution and potential, *Circ. Res.* **88**:763–773.

Baker, C. S., Corbett, J. M., May, A. J., Yacoub, M. H., and Dunn, M. J., 1992, A human myocardial two-dimensional electrophoresis database: protein characterisation by microsequencing and immunoblotting, *Electrophoresis* **13**(9–10):723–726.

Corbett, J. M., Wheeler, C. H., and Dunn, M. J., 1995, Coelectrophoresis of cardiac tissue from human, dog, rat and mouse: towards the establishment of an integrated two-dimensional protein database, *Electrophoresis* **16**(8):1524–1529.

Corbett, J. M., Why, H. J., Wheeler, C. H., Richardson, P. J., Archard, L. C., Yacoub, M. H., and Dunn, M. J., 1998, Cardiac protein abnormalities in dilated cardiomyopathy detected by two-dimensional polyacrylamide gel electrophoresis, *Electrophoresis* **19**(11):2031–2042.

Evans, G., Wheeler, C. H., Corbett, J. M., and Dunn, M. J., 1997, Construction of HSC-2DPAGE: a two-dimensional gel electrophoresis database of heart proteins, *Electrophoresis* **18**(3–4):471–479.

Field, M., and Clark, J., 1997, Inappropriate ubiquitin conjugation: a proposed mechanism contributing to heart failure, *Cardiovasc. Res.* **33**:8–12.

Görg, A., Obermaier, C., Boguth, G., and Weiss, W., 1999, Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures, *Electrophoresis* **20**(4–5):712–717.

Heinke, M. Y., Wheeler, C. H., Chang, D., Einstein, R., Drake-Holland, A., Dunn, M. J., and dos Remedios, C. G., 1998, Protein changes observed in pacing-induced heart failure using two-dimensional electrophoresis, *Electrophoresis* **19**(11):2021–2030.

Heinke, M. Y., Wheeler, C. H., Yan, J. X., Amin, V., Chang, D., Einstein, R., Dunn, M. J., and dos Remedios, C. G., 1999, Changes in myocardial protein expression in pacing-induced heart failure, *Electrophoresis* **20**:2086–2093.

Jungblut, P., Otto, A., Zeindl-Eberhart, E., Plessner, K. P., Knecht, M., Regitz-Zagrosek, V., Fleck, E., and Wittmann-Liebold, B., 1994, Protein composition of the human heart: the construction of a myocardial two-dimensional electrophoresis database, *Electrophoresis* **15**(5):685–707.

Latif, N., Baker, C. S., Dunn, M. J., Rose, M. L., Brady, P., and Yacoub, M. H., 1993, Frequency and specificity of antiheart antibodies in patients with dilated cardiomyopathy detected using SDS-PAGE and Western blotting, *J. Am. Coll. Cardiol.* **22**(5):1378–1384.

Latif, N., Rose, M. L., Yacoub, M. H., and Dunn, M. J., 1995, Association of pretransplantation antiheart antibodies with clinical course after heart transplantation, *J. Heart Lung Transplant.* **14**:119–126.

Li, X. P., Pleissner, K. P., Scheler, C., Regitz-Zagrosek, V., Salnikow, J., and Jungblut, P. R., 1999, A two-dimensional electrophoresis database of rat heart proteins, *Electrophoresis* **20**(4–5): 891–897.

Macri, J., McGee, B., Thomas, J. N., Du, P., Stevenson, T. I., Kilby, G. W., and Rapundalo, S. T., 2000, Cardiac sarcoplasmic reticulum and sarcolemmal proteins separated by two-dimensional electrophoresis: surfactant effects on membrane solubilization, *Electrophoresis* **21**(9):1685–1693.

Macri, J., and Rapundalo, S. T., 2001, Application of proteomics to the study of cardiovascular biology, *Trends Cardiovasc. Med.* **11**(2):66–75.

McGregor, E., and Dunn, M. J., 2003, Proteomics of heart disease, *Hum. Mol. Genet.* **12**(2):135–144.

Pankuweit, S., Portig, I., Lottspeich, F., and Maisch, B., 1997, Autoantibodies in sera of patients with myocarditis: characterization of the corresponding proteins by isoelectric focusing and N-terminal sequence analysis, *J. Mol. Cell. Cardiol.* **29**(1):77–84.

Patel, V. B., Sandhu, G., Corbett, J. M., Dunn, M. J., Rodrigues, L. M., Griffiths, J. R., Wassif, W., Sherwood, R. A., Richardson, P. J., and Preedy, V. R., 2000, A comparative investigation into the effect of chronic alcohol feeding on the myocardium of normotensive and hypertensive rats: an electrophoretic and biochemical study, *Electrophoresis* **21**(12):2454– 2462.

Ping, P., Zhang, J., Pierce, W. M., Jr., and Bolli, R., 2001, Functional proteomic analysis of protein kinase C epsilon signaling complexes in the normal heart and during cardioprotection, *Circ. Res.* **88**(1):59–62.

Pleissner, K. P., Regitz-Zagrosek, V., Weise, C., Neuss, M., Krudewagen, B., Soding, P., Buchner, K., Hucho, F., Hildebrandt, A., and Fleck, E., 1995, Chamber-specific expression of human myocardial proteins detected by two-dimensional gel electrophoresis, *Electrophoresis* **16**:841–850.

Pohlner, K., Portig, I., Pankuweit, S., Lottspeich, F., and Maisch, B., 1997, Identification of mitochondrial antigens recognized by antibodies in sera of patients with idiopathic dilated cardiomyopathy by two-dimensional gel electrophoresis and protein sequencing, *Am. J. Cardiol.* **80**(8):1040–1045.

Portig, I., Pankuweit, S., Lottspeich, F., and Maisch, B., 1996, Identification of stress proteins in endothelial cells, *Electrophoresis* **17**(4):803–808.

Preedy, V. R., Macallan, D. C., Griffin, G. E., Cook, E. B., Palmer, T. N., and Peters, T. J., 1997, Total contractile protein contents and gene expression in skeletal muscle in response to chronic ethanol consumption in the rat, *Alcohol* **14**(6):545–549.

Scheler, C., Li, X. P., Salnikow, J., Dunn, M. J., and Jungblut, P. R., 1999, Comparison of two-dimensional electrophoresis patterns of heat shock protein Hsp27 species in normal and cardiomyopathic hearts, *Electrophoresis* **20**(18):3623–3628.

Scheler, C., Muller, E. C., Stahl, J., Muller-Werdan, U., Salnikow, J., and Jungblut, P., 1997, Identification and characterization of heat shock protein 27 protein species in human myocardial two-dimensional electrophoresis patterns, *Electrophoresis* **18**(15):2823–2831.

Schwertz, H., Langin, T., Platsch, H., Richert, J., Bomm, S., Schmidt, M., Hillen, H., Blaschke, G., Meyer, J., Darius, H., and Buerke, M., 2002, Two-dimensional analysis of myocardial protein expression following myocardial ischemia and reperfusion in rabbits, *Proteomics* **2**(8):988–995.

Thiede, B., Otto, A., Zimny-Arndt, U., Muller, E. C., and Jungblut, P., 1996, Identification of human myocardial proteins separated by two-dimensional electrophoresis with matrix-assisted laser desorption/ionization mass spectrometry, *Electrophoresis* **17**(3):588– 599.

Toraason, M., Moorman, W., Mathias, P. I., Fultz, C., and Witzmann, F., 1997, Two-dimensional electrophoretic analysis of myocardial proteins from lead-exposed rabbits, *Electrophoresis* **18**(15):2978–2982.

Van Eyk, J. E., and Murphy, A. M., 2001, The role of troponin abnormalities as a cause for stunned myocardium,*Coron. Artery Dis.* **12**:343–347.

Weekes, J., Morrison, K., Mullen, A., Wait, R., Barton, P., and Dunn, M. J., 2003, Hyperubiquitination of proteins in dilated cardiomyopathy, *Proteomics* **3**(2):208–216.

Weekes, J., Wheeler, C. H., Yan, X. J., Eschenhagen, T., Scholtysik, G., and Dunn, M. J., 1999, Bovine dilated cardiomyopathy: proteomic analysis of an animal model of human dilated cardiomyopathy, *Electrophoresis* **20**:898–906.

Weil, J., Eschenhagen, T., Magnussen, O., Mittmann, C., Orthey, E., Scholz, H., Schafer, H., and Scholtysik, G., 1997, Reduction of myocardial myoglobin in bovine dilated cardiomyopathy, *J. Mol. Cell. Cardiol.* **29**:743– 751.

Wheeler, C. H., Collins, A., Dunn, M. J., Crisp, S. J., Yacoub, M. H., and Rose, M. L., 1995, Characterization of endothelial antigens associated with transplant-associated coronary artery disease, *J. Heart Lung Transplant.* **14**:188–197.

Chapter 4

Quantitative Real-Time PCR

J. P. G. Sluijter, G. Pasterkamp, and D. P. V. de Kleijn

Changes in mRNA expression levels occur during physiological and pathological processes in the cardiovascular system. An increase in DNA transcription results in increased mRNA levels and will subsequently result in increased protein levels that regulate processes inside and outside the cell. To determine alterations in mRNA levels, traditional methods such as Northern blot and ribonuclease protection assay can be used; however, large amounts of RNA are necessary and the methods are very labor intensive. In this chapter, we focus on the newest advancements in reverse transcription polymerase chain reaction (RT-PCR) technology, the real-time PCR or quantitative PCR, using small amounts of RNA to determine expression levels. We discuss the technique in general and describe two different approaches.

1. INTRODUCTION

During physiological and pathological processes expression levels of mRNA and proteins are changed to achieve biological effects. For this, studying mRNA levels will help us to understand the underlying processes, since alteration of mRNA levels often precedes a change in protein levels, which is necessary to obtain the desired effect. Traditional methods to quantify mRNA expression levels are Northern blotting, *in situ* hybridization, ribonuclease protection, cDNA arrays, and reverse transcription polymerase chain reaction (RT-PCR). Northern analysis

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

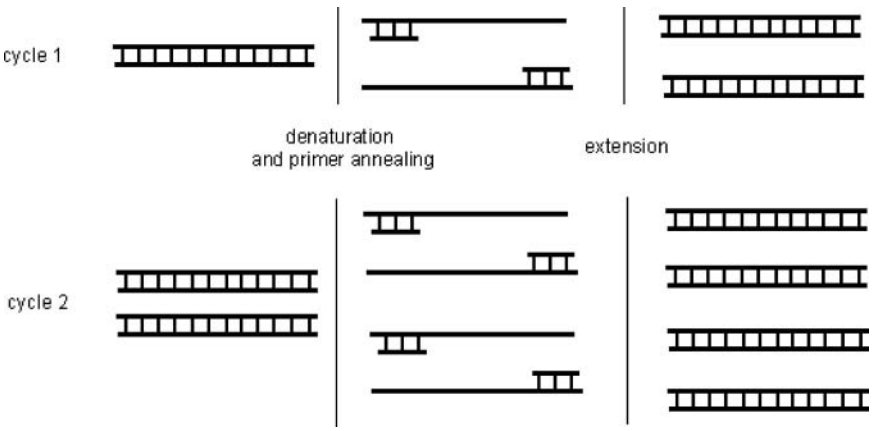

FIGURE 1. Schematic picture of DNA amplification during the PCR. Each cycle starts with a double-stranded template and denaturation is needed to create two single-strand molecules to which the primer can anneal. This serves as the starting point of the DNA polymerase and extension can occur when the right conditions are present, resulting in a doubling of the DNA template. The cycle of denaturation–annealing–extension is repeated and amplification of the template will occur.

provides information about mRNA size, alternative splicing, and the integrity of the RNA samples. The *in situ* hybridization is the only one that allows localization of the produced mRNAs (Parker and Barnes, 1999). Ribonuclease protection (RNAse protection assay) is most useful for mapping transcript initiation and termination sites (Hod, 1992). The cDNA arrays are still limited in its use by cost considerations (Bucher, 1999). The main limitation of these methods is the low sensitivity. RT-PCR is the most sensitive and accurate of the quantification methods, but needs intensive and laborious post-PCR manipulations, makes use of hazardous chemicals, and carries a potential risk for laboratory contamination.

Here we focus on one of the newest advancements in PCR technology: the real-time PCR or quantitative PCR. The introduction of this new procedure is based on fluorescence-kinetic RT-PCR and allows quantification of the PCR product in "real time." It measures PCR product accumulation during the exponential phase of the reaction. The technique is much faster than the previous endpoint RT-PCR as it is designed to provide information as rapidly as the amplification process itself, thus requiring no post-PCR manipulations.

The basic principle of the real-time PCR is the classical PCR. In short, the PCR, developed by Dr Mullis (Mullis *et al.*, 1986; Mullis and Faloona, 1987), is a rapid *in vitro* enzymatic amplification of a specific DNA region (see Fig. 1). The formation of complementary strands is the basic principle of PCR. First, the double-stranded template is denaturated to form single-stranded molecules (denaturation). Next, synthetic oligonucleotide primers, which flank the target DNA sequence that has to be amplified, hybridize to the single-stranded template and serve as the starting point of the DNA polymerase (annealing). Finally, DNA polymerase synthesizes a complementary single-stranded DNA matching the template in a process called extension.

Repeated denaturation–annealing–extension cycles will accumulate the target sequence exponentially. The reaction will continue as long as there is an excess of primers, oligonucleotides, and an active polymerase and will occur only in the 3′ direction. During the PCR, DNA amplification is exponential, eventually reaching a plateau. At any point during the exponential amplification phase for a reaction the amount of DNA can be calculated. The key is to perform this calculation before the reaction leaves the exponential phase. Modifications of the classical PCR are needed to use this technique for quantifying mRNA levels. RNA cannot serve as a template for PCR, and therefore, an essential step in determining mRNA levels is the conversion of RNA to a complementary DNA template (cDNA) by the enzyme reverse transcriptase. This cDNA is now used as the template in the PCR. The classical PCR needs analysis afterwards, which is time-consuming and not quantitative, and contaminations can occur.

Real-time PCR makes it possible to quantify the amount of target mRNA at the beginning of the PCR. This technique is very accurate, reproducible, and sensitive, with a high throughput and in which contaminations are reduced to a minimum. A well-designed real-time PCR assay can allow for the quantification of initial DNA concentration over a dynamic range of six or more orders. In real-time PCRs, fluorescent molecules are used to monitor the reaction while amplification is taking place. It is characterized by the point during amplification when the accumulation of PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles.

Real-time PCR is a very powerful technique and therefore a wide expanding technology with a range of possibilities in instrumentation and chemistries. Different competing PCR systems are developed with their own characteristics and sensitivities, e.g. the ABI Prism 7700 (Perkin-Elmer-Applied Biosytems), the Lightcycler (Roche Molecular Biochemicals), the i-Cycler iQ (Bio-Rad Laboratories) (Bustin, 2000; Giulietti *et al.*, 2001).

For detection of the amplified product, nonspecific reporter molecules (e.g. SYBR® green, ethidium bromide) as well as specific probes (e.g. molecular beacons, TaqMan™, dual-oligo FRET pairs, Scorpions™/Amplifluor™) can be used:

1. The use of nonspecific molecules will be fast and cheap and can be used for every primer set, but controls are needed to demonstrate specificity of the monitored fluorescent signal.
2. A more sensitive and specific way to monitor the amplificated signal is the use of specific hybridizing probes; however, to set up this assay is more time-consuming and more expensive.

2. NONSPECIFIC FLUORESCENT QUANTIFICATION

The use of nonspecific fluorescent molecules is a relatively simple technique and can be used for any PCR. It is based on the binding of intercalating dyes,

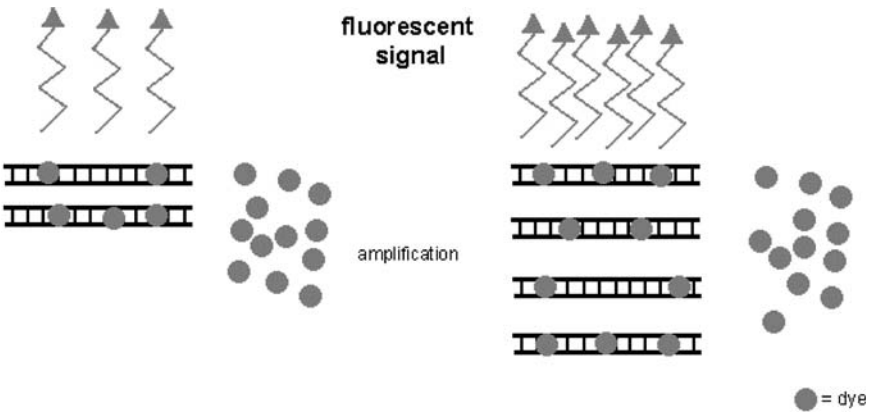

FIGURE 2. Nonspecific intercalating dye binding during amplification. Dyes bind to double-stranded DNA and when bound they will give a fluorescent emission signal. The free dye will give no signal when excited. During amplification more and more double-stranded product is formed and increasing amount of dye can bind, increasing the observed fluorescent signal.

e.g. SYBR® green and ethidium bromide, that can insert between two adjacent base pairs in a molecule of double-stranded DNA, distorting the architecture of the double helix (Morrison *et al.*, 1998). Only dye bound to DNA will give a fluorescent emission signal, and not the free dyes that are in the mixture (see Fig. 2). SYBR® green fluoresces 50 times brighter than ethidium bromide when bound to double-stranded DNA. During the PCR cycles more and more double-stranded DNA is formed; thus the amount of the dye that can bind will increase, resulting in an increased fluorescent signal. The increase in fluorescent signal will be caused by all amplified DNA products formed in your reactions, which are not necessarily specific for the desired product. When using this approach, primers must be selected carefully and PCRs must be optimized to avoid formation of nondesired products. Post-PCR correction is sometimes necessary to obtain accurate quantitative information. With this kind of DNA amplification you can measure relatively quickly and easily the expression of several genes, without the use of specific probes.

Smeets *et al.* (2003) used the nonspecific molecule SYBR® green in real-time PCR to quantify haptoglobin mRNA expression levels after balloon dilation in rabbits. Haptoglobin is an acute phase glycoprotein, mainly produced in the liver and secreted in the serum. But increasing evidence shows that haptoglobin is produced in extrahepatic tissues, e.g. the arterial wall. Haptoglobin is thought to play a role in cell migration and arterial restructuring. In this study they found an early increase in haptoglobin mRNA levels, which could be measured in the picogram range. Furthermore, they found that arterial haptoglobin consists of a unique set of glycoforms, with other oligosaccharides attached, compared to haptoglobin produced in the liver. Alterations in glycosylation are known to modify, e.g., function; thus, it is conceivable that arterial haptoglobin has a different function compared to liver-produced haptoglobin.

3. SPECIFIC FLUORESCENT QUANTIFICATION

To ensure that the increase in fluorescent signal is more sensitive and specific, you can use fluorescent oligonucleotide hybridization probes. Setting up this kind of analysis will cost some more time, but for very large studies it will be worth the investment.

One strategy often used is the TaqMan® assay (see Fig. 3A) and needs the annealing of three oligonucleotides to the DNA: two template specific primers, and a third probe that hybridizes to the target sequence (Gut *et al.*, 1999). In this

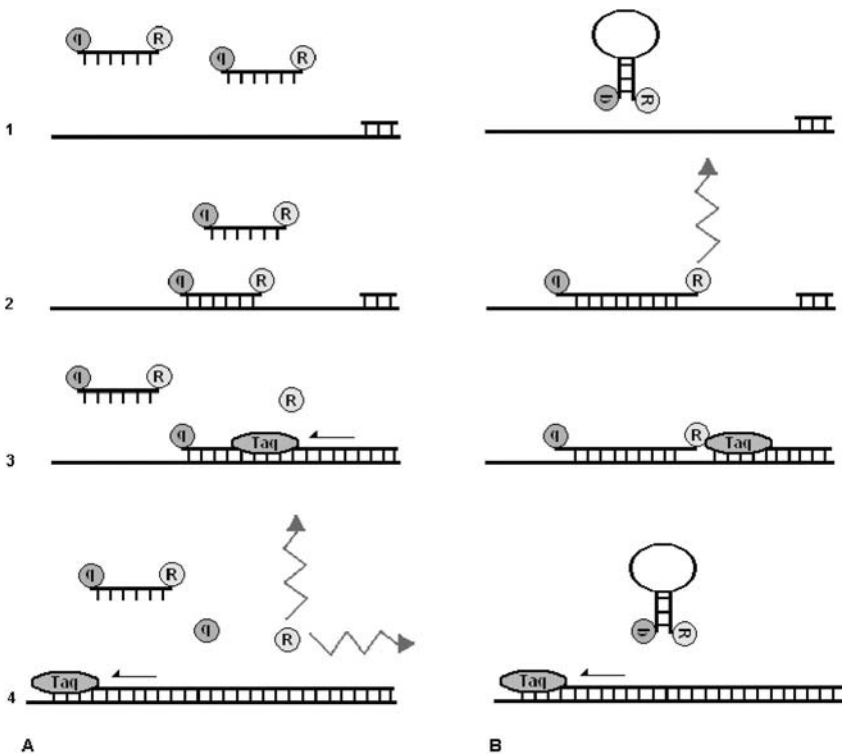

FIGURE 3. Schematic picture of the TaqMan® assay and the molecular beacon approach. (A) In the TaqMan® assay, the probe is labeled at the 5′ end with a fluorescent reporter (R) and at the 3′ end with a quencher molecule (Q). The fluorescence of the reporter will be absorbed by the quencher when the probe is still intact. During the extension phase the Taq polymerase cleaves the hybridized probe and the reporter molecule is released from the quencher. The fluorescent signal can now be detected and will be increased during amplification when more and more probe will be bound and will be cleaved. (B) The molecular beacon is a hairpin-loop-shaped oligonucleotide with a fluorescent reporter (R) to one arm and a quencher molecule to the complementary other arm. When the probe binds to the template the reporter and quencher are separated and the fluorescent signal can be monitored. During amplification more and more probe will bind and an increased fluorescent signal is observed. (*See Color Plate 8.*)

assay, which uses the 5′-nuclease activity of the DNA polymerase, the probe is labeled at the 5′ end with a fluorescent reporter and at the 3′ end with a quencher molecule and is complementary to the target DNA. The quencher molecule absorbs the energy emitted by a fluorophore and emits the energy at a different wavelength, reducing the fluorescence of the fluorophore. When the probe is still intact, the reporter and quencher are near each other and the fluorescence of the 5′-reporter is absorbed by the 3′-quencher and no signal is detected. PCR amplification of your target DNA will increase the number of probes that hybridize with the complementary template. During the extension phase of a reaction, the Taq polymerase cleaves the labeled probe which is hybridized. The reporter molecule is then released from the quencher and the fluorescent signal can be detected. Accumulation of the released reporter molecules during the amplification cycles results in an increasing fluorescent signal and is correlated to the amount of the target DNA present.

Another hybridization probe is the molecular beacon (Bonnet *et al.*, 1999; Tyagi and Kramer, 1996), which is a hairpin-loop-shaped single-stranded oligonucleotide (see Fig. 3B). It consists of a probe sequence, homologous to the target sequence, but is flanked by complimentary arm sequences, which are homologous to one another but not to the target sequence. One arm contains a fluorescent reporter and the other a nonfluorescent quencher. At room temperature, the molecular beacon assumes the hairpin formation, bringing the reporter and quencher into intimate contact; the fluorescent signal will be captured by the quencher; and no fluorescence is detected from the reporter. During the annealing step of the amplification cycle, thermodynamics favors the binding of the molecular beacon to its target rather than the formation of the hairpin structure. When the probe binds to the target sequence this will result in a separation of the reporter and quencher molecule and the fluorescent signal can be monitored. Because more and more probes will bind to the DNA, the fluorescent signal will increase during amplification. The main drawback with molecular beacons is associated with the design and costs of the hybridization probe. Large background signals are produced when the beacon folds in alternate conformations that do not place the fluorophore near the quencher or when the stem is too strong that the annealing to the target sequence will be disturbed.

Ortiz-Pallardo *et al.* (2000) used real-time PCR to identify the PiZ (proteinase inhibitor) mutations and to determine hetero- and homozygeous carrier status in whole blood and from paraffin-embedded specimens. A mutation in the α_1-antitrypsin (AAT), with the structural gene locus encoding AAT called Pi, resulting in AAT deficiency is a common inherited cause of emphysema and cirrhotic liver disease. Current diagnostic techniques are time-consuming and have a limited accuracy. They compared the results of the RT-PCR with single-strand conformational polymorphism and direct DNA sequencing, which are used nowadays. They found that the results of the RT-PCR were confirmed by the other two techniques in all cases, but allowed a higher throughput level.

4. SETTING UP A QUANTITATIVE PCR

Amplification in a real-time PCR will increase the target DNA and therefore also the observed fluorescent signal. In the early cycles of amplification, the change in fluorescence of the reporter is usually undetectable, but at some point during amplification, the accumulation of product results in a measurable change in the fluorescence of the reaction mixture. The higher the starting amount of the target DNA in your sample, the sooner a significant increase in the fluorescent signal will be registered. The cycle number observed when the fluorescent signal rises above background levels will be used to quantify the amount of DNA, and is called the threshold cycle (Gibson *et al.*, 1996). This point will always occur during the exponential phase of amplification, and therefore, quantification is not affected by any reaction components becoming limited in the plateau phase. There is a linear relationship between the threshold cycle during real-time PCR and the log of the starting amount of a template. Quantification of your target will be performed by comparison with a standard curve of your target reference standard and can be either relative or absolute. Relative quantification determines the change compared to the calibrator (standard used in dilution series) as containing either more or less mRNA. The absolute quantification allows the precise determination of copy numbers per, e.g., cell or unit mass of tissue. In most cases, the lower detection limit is 30–500 copies of target mRNA.

Computerized analysis of the data is needed to quantify the starting amounts in your samples. A grid of the reaction plate is installed to let the computer know which part of the signal represents the well that is recorded by the CCD camera. The amplification plot (see Fig. 4) displays the relative fluorescence for each well at every cycle. To determine threshold cycles and the standard curve, a PCR baseline subtraction is needed. This will correct for the fluorescent background level, the noise in your samples, and is automatically calculated from the optimal baseline cycles for each well individually or can be applied manually. After this, the standard curve can be generated and the amount of DNA in the unknown samples can be calculated. From the created standard curve, a correlation coefficient and the efficiency can be calculated. The correlation coefficient tells you something about the accuracy of the experimental data compared to the expected values, and thus how well a sample fits to the created standard curve. The efficiency of the reactions is directly related to the slope of the standard curve and gives you information about the PCR itself. An efficiency of 100% represents a doubling of your template after each cycle and corresponds with the theoretical perfect PCR. You will need equal efficiencies in the different PCRs to be able to compare different PCR runs and also normalization of your samples is needed for a relevant housekeeping gene.

5. CONCLUDING REMARKS

Using real-time PCR, one is able to monitor different expression levels of different mRNAs in one reaction (real-time multiplex gene expression) when using

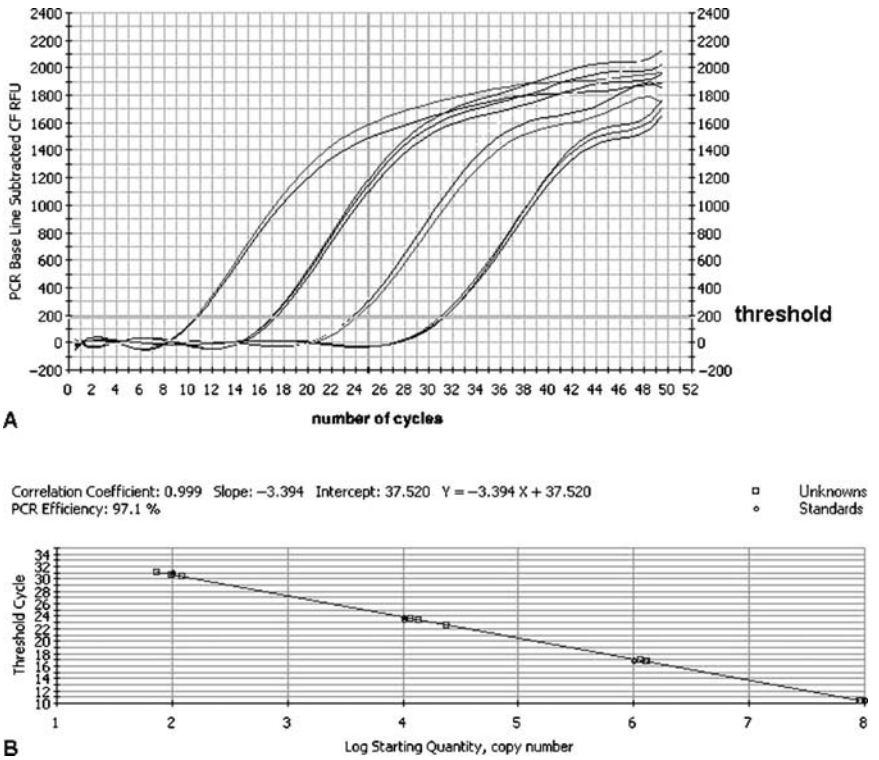

FIGURE 4. Amplification plot and standard curve generated with the iCycler iQ^{TM} software (Bio-Rad Laboratories). (A) The amplification plot displays the observed increased fluorescent signal for each well (one line represents one well). The threshold is determined by the background signal found in the first cycles and the cycle, where the observed fluorescence rises above this threshold, is called the threshold cycle and will be used in the analysis. (B) The standard curve is generated from the target reference samples and the DNA concentrations of the unknowns are calculated. The correlation coefficient is a measure of the accuracy of the data compared to the generated curve. The slope of the curve represents the PCR efficiency.

different probes with different fluorescent signals. Hybridization probes that do not cleave can identify mutations, even a single base mutation, within the PCR product. Hybridization probes can also determine the distribution of genotypes within a population compared to known genotypes and are able to detect mRNA splice variants. The real-time PCR can also be used to verify quickly the data obtained by microarray.

Quantitative, or real-time, PCR is an accurate, sensitive, and high-throughput technique that can be used for several purposes. The goal of the study determines which kind of tools is used to obtain the fluorescent data. The analysis of these data and interpretation of the results need some experience, but will be worth the investment.

REFERENCES

Bonnet, G., Tyagi, S., Libchaber, A., and Kramer, F. R., 1999, Thermodynamic basis of the enhanced specificity of structured DNA probes, *Proc. Natl. Acad. Sci. U.S.A.* **96**:6171–6176.

Bucher, P., 1999, Regulatory elements and expression profiles, *Curr. Opin. Struct. Biol.* **9**:400–407.

Bustin, S. A., 2000, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, *J. Mol. Endocrinol.* **25**:169–193.

Gibson, U. E., Heid, C. A., and Williams, P. M., 1996, A novel method for real time quantitative RT-PCR, *Genome Res.* **6**:995–1001.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C., 2001, An overview of real-time quantitative PCR: applications to quantify cytokine gene expression, *Methods* **25**:386–401.

Gut, M., Leutenegger, C. M., Huder, J. B., Pedersen, N. C., and Lutz, H., 1999, One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses, *J. Virol. Methods* **77**:37–46.

Hod, Y., 1992, A simplified ribonuclease protection assay, *Biotechniques* **13**:852–854.

Morrison, T. B., Weis, J. J., and Wittwer, C. T., 1998, Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification, *Biotechniques* **24**:954-958, 960, 962.

Mullis, K. B., and Faloona, F., 1987, A specific synthesis of DNA in vitro via a polymerase catalysed chain reaction, *Methods Enzymol.* **155**:335–350.

Mullis, K. B., Faloona, F. A., Scharf, S. J., Saiki, R. K., Horn, G. T., and Erlich, H. A., 1986, Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction, *Cold Spring Harbor Symp. Quant. Biol.* **51**:263–273.

Ortiz-Pallardo, M. E., Zhou, H., Fischer, H. P., Neuhaus, T., Sachinidis, A., Vetter, H., Bruning, T., and Ko, Y., 2000, Rapid analysis of alpha1-antitrypsin PiZ genotype by a real-time PCR approach, *J. Mol. Med.* **78**:212–216.

Parker, R. M., and Barnes, N. M., 1999, mRNA: detection by in situ and northern hybridization, *Methods Mol. Biol.* **106**:247–283.

Smeets, M. B., Sluijter, J. P. G., Donners, M. P. C., Velema, E., Heeneman, S., Pasterkamp, G., and de Kleijn, D. P., 2003, Increased arterial expression of a glycosylated haptoglobin isoform after balloon dilation, *Cardiovasc. Res.* **58**:689–695.

Tyagi, S., and Kramer, F. R., 1996, Molecular beacons: probes that fluoresce upon hybridization, *Nat. Biotechnol.* **14**:303–308.

Chapter 5

New Technologies in Cardiovascular Research
Gene Therapy

Marja Hedman, Mikko P. Turunen,
and Seppo Ylä-Herttuala

1. INTRODUCTION

Gene therapy has become an alternative possibility for the treatment of cardiovascular diseases. The studies in cardiovascular gene therapy are mainly focused on inducing therapeutic angiogenesis and cytoprotection and on the inhibition of vascular graft restenosis. Also, disorders of lipid metabolism and the whole process of atherosclerosis are under intensive exploration. On the other hand, as the pathogenesis of different multifactorial vascular diseases has been explored, new targets have emerged for cardiovascular gene therapy. Currently, the main obstacles of gene therapy are the low efficacy of different gene delivery vectors and the difficulty of targeting genes into specific cells. Studies focused on developing not only the appropriate and optimal gene delivery techniques but also suitable models for human diseases are essential for the further development of cardiovascular gene therapy.

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

1.1. Principles of Gene Transfer

Therapeutic gene transfer can be defined as the transfer of nucleic acids to specific somatic cells in order to obtain a therapeutic effect (Ylä-Herttuala and Alitalo, 2003). Blood vessels are among the easiest targets for gene therapy. The target cells in arteries and other organs are usually easily reached using intra- or perivascular approach or a direct injection into tissues (Hedman *et al.*, 2003; Tripathy *et al.*, 1996; Turunen *et al.*, 1999; Ylä-Herttuala and Alitalo, 2003). In addition to direct injection methods sufficient gene expression level can also be reached by using delivery of transduced cells (*ex vivo* gene transfer) (Hiltunen *et al.*, 2000b). The effectiveness of gene transfer is determined by the efficacy of gene delivery to the target tissue, the entry of genetic material into cells, and the expression level of the transduced gene in the cells (Ylä-Herttuala and Alitalo, 2003). The first human gene transfer was performed in lymphocytes using retroviruses in 1989 (Rosenberg *et al.*, 1990), and a very first therapeutic gene transfer study was done in patients suffering from adenosinedeaminase deficiency in 1990 (Blaese *et al.*, 1995). Since then over 600 clinical gene therapy trials have been performed for different mono- and multifactorial diseases (Ylä-Herttuala and Alitalo, 2003).

1.2. Vectors for Gene Therapy

Different gene transfer vectors have been used to transfer therapeutic genes into the target cells. Target cells in the vascular system consist of endothelial cells, smooth muscle cells, and fibroblasts as well as monocytes and macrophages (Ylä-Herttuala and Alitalo, 2003). Other target cells when designing gene therapy for cardiovascular diseases would be myocardial and peripheral muscle cells and liver cells (Baumgartner *et al.*, 1998; Pakkanen and Ylä-Herttuala, 2000; Vajanto *et al.*, 2002; Ylä-Herttuala and Alitalo, 2003). In vascular gene therapy, the most widely used vectors have been plasmid DNA and adenoviruses. Naked plasmid DNA suffers from very low gene transfer efficacy due to the poor uptake of plasmid DNA into cells and breakdown of plasmids in lysosomes. In the cells, plasmids remain extrachromosomal and lead to a transient gene expression lasting 1–2 weeks in most tissues (Tripathy *et al.*, 1996). Carrier molecules such as liposome complexes, polyethyleneimines, and polyamidoamine dendrimers can be used to increase the transfection efficiency of plasmid DNA (Laitinen *et al.*, 1997a; Turunen *et al.*, 1999). Liposomes are self-assembled colloidal particles with a covering lipid mono-, bi-, or polylayer. The lipids either fuse with plasma membrane or enter via endocytosis, thereby facilitating the nucleic acid delivery into cells (Jacobsen, 1997). Liposomes are easily synthetically prepared and are safe, but they lead only to a transient short-time gene expression lasting from a few days to two weeks, and they may cause some toxic effects *in vivo* (Hedman *et al.*, 2003; Ylä-Herttuala and Alitalo, 2003). Polyethyleneimine is an organic polymer with a potential for high cationic charge yielding high transfection efficiency (Turunen *et al.*, 1999). Dendrimers are a new class of synthetic macromolecules used for DNA delivery. Although dendrimers bind DNA and deliver genes to cultured cells and animal tissues, their use for human gene therapy has not yet been demonstrated

(Service, 1995). Antisense and decoy oligonucleotides as well as inhibitory and small interfering RNAs can also be used for gene transfer (Dykxhoorn *et al.*, 2003; Ylä-Herttuala and Alitalo, 2003). An advantage of plasmids and carrier molecules is that they are easy to manufacture in large quantities. The use of plasmid vectors for clinical trials has been approved in several countries and several clinical trials with naked DNA and plasmid–liposome complexes have already been performed with varying results (Ylä-Herttuala and Alitalo, 2003).

Other means to improve the efficacy of plasmid-mediated gene therapy have also been introduced. Tissue damage and regeneration caused by mechanical trauma, toxic agents, or ischemia enhances transfection, presumably via permeabilization of cell and nuclear membranes (Pakkanen *et al.*, 1999; Takeshita *et al.*, 1996; Vitadello *et al.*, 1994). Ultrasound and electric currents have also been used to aid the entry of plasmids into target cells (Yamashita *et al.*, 2002).

Because gene transfer efficiency with plasmid-based systems is relatively low, viral vectors have been used to increase the efficiency of gene transfer. For the cardiovascular gene therapy, the most commonly used viral vector is adenovirus, which can transduce both dividing and nondividing cells (Kootstra and Verma, 2002; Laitinen *et al.*, 1998). Gene transfer efficiencies of 10–30% have been achieved with adenoviral vectors even in human tissues (Laitinen *et al.*, 1997a, 1998; Ylä-Herttuala and Alitalo, 2003). Adenoviruses are composed of a linear double-stranded DNA of approximately 36 kbp surrounded by a capsid (Kovesdi *et al.*, 1997). Wild- type human adenoviruses exist in at least 47 different serotypes divided in six subgroups and are a general cause of respiratory, gastrointestinal, and other infections. They can be produced in high titers and they enter cells through a specific receptor (Coxsackie-adenovirus receptor) (Bergelson, 1999). In human trials, adenoviral vectors have caused inflammatory reactions, formation of antiadenovirus antibodies, transient fever and increases in liver transaminases (Hedman *et al.*, 2003; Mäkinen *et al.*, 2002; Ylä-Herttuala and Alitalo, 2003). Although the only death directly associated in gene therapy was caused by a very high dose of adenovirus in an immunocompromised patient (Lehrman, 1999), clinical phase I/II trials suggest that adenoviruses are safe and well tolerated and they have been approved as a gene therapy vector for several clinical trials by US Food and Drug Administration and other regulatory authorities (Hedman *et al.*, 2003; Mäkinen *et al.*, 2002; Rajagopalan *et al.*, 2003; Ylä- Herttuala and Alitalo, 2003).

The efficiency of retroviruses has been very low in cardiovascular gene therapy studies. They have been used in both preclinical and clinical cardiovascular studies (Grossman *et al.*, 1995; Laitinen *et al.*, 1997a; Pakkanen *et al.*, 1999). The gene transfer efficiency in the vascular wall has been shown to be less than 0.1% (Laitinen *et al.*, 1997a). Still, some properties of retroviral vectors would be of great benefit when treating inherited cardiovascular diseases, such as familial hypercholesterolemia, or when long-term gene therapy effect would be needed (Grossman *et al.*, 1995; Pakkanen *et al.*, 1999; Pakkanen and Ylä-Herttuala, 2000; Ylä-Herttuala and Alitalo, 2003). Retroviruses, lentiviruses, and adeno-associated viruses (AAVs) integrate into the host genome and provide long-lasting transgene expression (Grossman *et al.*, 1995; Kessler *et al.*, 1996; Kootstra and Verma, 2002; Naldini *et al.*, 1996; Pajusola *et al.*, 2002; Pakkanen *et al.*, 1999; Pakkanen and

Ylä-Herttuala, 2000; Ylä-Herttuala and Alitalo, 2003). AAV is a small single-stranded DNA parvovirus that exists at least in seven different serotypes and is not known to cause diseases in humans (Kessler *et al.*, 1996; Pajusola *et al.*, 2002). Unlike retroviruses, AAVs can also transduce nondividing cells. Lentiviruses (human, simian, equine, and feline immunodeficiency viruses) have many appealing properties for therapeutic purposes. They seem to be safer than retroviruses and they can also transduce nondividing cells. Even though the transduction efficiency of skeletal muscle and myocardium with lentiviruses seems to be low, they have shown relatively high transduction efficiencies in the central nervous system and liver (Kang *et al.*, 2002; Naldini *et al.*, 1996). Other vectors used for gene transfer include herpes simplex virus (Mesri and Federoff, 1995), Sendai virus (Shiotani *et al.*, 2001), and baculovirus (Airenne *et al.*, 2000). More details about the gene delivery vectors are given in Table 1.

Table 1
Gene Transfer Vehicles

	Description	Advantages	Disadvantages
Viral vectors			
Adenovirus	Nonenveloped double-stranded DNA virus, serotypes 2 and 5 most widely used	High efficacy, high titers (10^{11} to 10^{12} pfu), tropism for multiple cell types, trans duction of nondividing cells, relatively safe, large cloning capacity	Episomal, transient expression (<2 weeks) immune and inflammatory reactions, safety concerns
Retrovirus	Enveloped positive strand RNA virus	Stable gene expression integrates into the host genome	Low titers (10^5 to 10^6 cfu), labile virus particles if not pseudotyped
Lentivirus	Enveloped RNA virus usually pseudotyped with VSV-G	High titers ($>10^{10}$ TU/ml), long expression, integrates into the host genome, transduction of nondividing cells	Low efficacy in some cell types
Adeno-associated virus	Small parvovirus	High titers (10^{10} to 10^{12} pfu), long expression, integrates into the host genome, transduction of nondividing cells	Immunogenicity, limited capacity, difficult production
Baculovirus	Insect DNA virus	High capacity, easy to produce in high titers not shown to cause disease in mammals	Transient expression, limited tropism

(*cont.*)

Table 1
(*cont.*)

	Description	Advantages	Disadvantages
Herpes simplex virus	DNA virus	High efficiency in some cells, high capacity, easy to produce	Cytotoxicity, unable to transduce non dividing cells
Sendai virus	Negative strand RNA virus	High efficiency. Not pathogenic in humans. Used also with liposomes	imited target tropism Nonintegrative
Non-viral methods			
Naked plasmid DNA	Circular plasmid	Safe, easy to prepare	Very low gene transfer efficiency, transient gene expression
Plasmid–liposome complex	Synthetic lipids, interaction with plasmid based on molecular charge	Safe, easy to prepare	Low gene transfer efficiency, toxicity
Plasmid–polymer complex	Synthetic polymers, interaction with plasmid based on molecular charge	Easy to prepare	Low gene transfer efficiency, toxicity
Antisense oligo nucleotides	Synthetic oligonucleotides	Safe, easy to prepare	Interferon response, Low specific efficiency
siRNA	Double-stranded RNA	Targeted gene shutdown	Difficult to prepare (but commercially available)

1.3. siRNA as a Tool for Gene Therapy

In the past few years, it has been discovered that small double-stranded RNAs are key regulators of gene expression in plants, animals, and most fungi (Shi, 2003). The most well-known RNA silencing process is RNA interference (RNAi), which leads to sequence-specific degradation of mRNAs that are identical in sequence to the initiator dsRNA (Elbashir *et al.*, 2001). The guiding RNAs in RNAi are called small interfering RNAs (siRNAs), which form ribonucleoprotein complexes that mediate target mRNA cleavage. The use of long dsRNAs has been limited primarily because the introduction of dsRNA longer than 30 nt induces a nonspecific interferon response. However, it has now been shown that siRNAs can be introduced into mammalian cells, which leads to sequence-specific degradation of mRNA without inducing an interferon response (Dykxhoorn and Novina, 2003). For gene therapy applications, this new method has a huge potential, as expression of specific genes can be efficiently turned off in most cell lines and *in vivo* in animals. Also, transgenic animals expressing siRNAs have been developed (Tiscornia *et al.*, 2003). Most widely applied method is to use synthetic siRNAs

for gene silencing, which leads to a transient response. Plasmid-based expression systems as well as viral vectors have also been used for delivery of siRNAs both *in vitro* and *in vivo*. Lentiviral vectors show great promise for siRNA delivery, as the transgene is integrated into target cell genome and therefore expression of siRNAs is stable (Tiscornia *et al.*, 2003). Regulatable systems can be designed as well as tissue-specific lentiviral vectors (Kafri *et al.*, 2000).

2. GENE TRANSFER ROUTES

Various potential routes for administration of nucleic acids have been used in experimental animals as well as in clinical studies. Intravascular gene transfer is easily performed during angioplasty, stenting, and other intravascular manipulations (Ylä-Herttuala and Alitalo, 2003). Limitations for intravascular gene transfer are the presence of anatomical barriers, such as the internal elastic lamina and calcified atherosclerotic lesions (Laitinen *et al.*, 1998; Rome *et al.*, 1994), and the presence in plasma of virus-inactivating complement system (Plank *et al.*, 1996). The most commonly used route for angiogenic gene therapy is direct intramuscular injection (Baumgartner *et al.*, 1998; Vajanto *et al.*, 2002). Intramyocardial injections are more difficult to perform than injections into skeletal muscle, because thoracotomy is usually required, but may be practical during bypass surgery. However, the development of modern endocardial injection catheters have made percutaneous intramyocardial gene transfer possible (Kornowski *et al.*, 2000; Marshall *et al.*, 2000). Perivascular gene transfer can be used for the delivery of therapeutic genes into the arterial wall during bypass operations, prosthesis and anastomosis surgery, and endarterectomies (Ylä-Herttuala and Alitalo, 2003).

2.1. Intramyocardial Gene Injection

Intramyocardial gene injections have traditionally been performed by using an open thoracotomy (Fortuin *et al.*, 2003; Mann and Whittemore, 1999). Feasibility of the intramyocardial gene injections has been substantially improved by the introduction of percutaneous endomyocardial catheter-mediated injection systems such as NOGA® (Johnson & Johnson, Cordis, USA) (Kornowski *et al.*, 2000) and Stiletto® (Boston Scientific, Boston, USA) (Marshall *et al.*, 2000). Myocardial gene transfer guided by the NOGA system has been reported to be as efficient as direct injections at thoracotomy (Kornowski *et al.*, 2000). In addition to an electromechanical sensor apparatus there is a 28-gauge needle in the end of the NOGA catheter. At the target site of the myocardium the needle can be pushed out from the catheter for a gene injection (Kornowski *et al.*, 2000) and thereafter the needle is pulled in again. An 8 F NOGA injection catheter is usually needed for the studies in domestic pigs (Rutanen *et al.*, in press). The depth of the intramyocardial injections is 5–6 mm. The Stiletto catheter contains an infusion lumen attached to a retractable stainless steel needle that can be used to inject vector

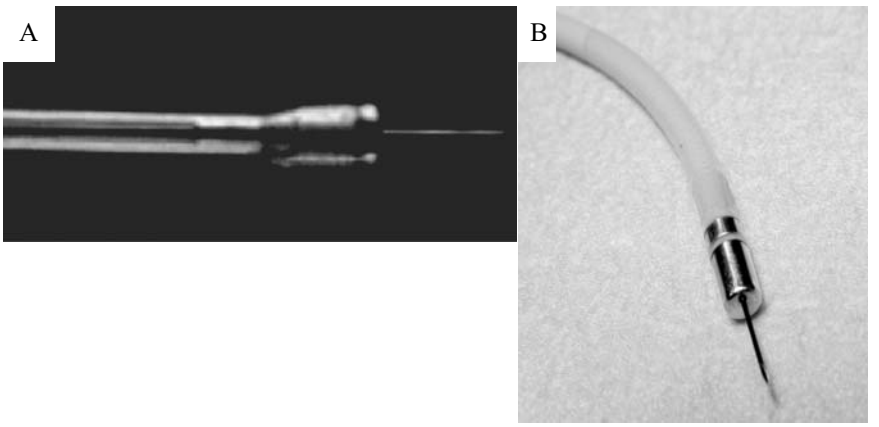

FIGURE 1. Intramyocardial gene delivery catheters. (A) Stiletto® catheter for intramyocardial injection. A microscopic needle is on the tip of the catheter for the penetration of the endocardial border and for the infiltration of genes inside the myocardium. (B) NOGA® electromechanical mapping and injection catheter. The electrical measurements and the myocardial movements can be detected with the same catheter as the gene injection. A small needle is pushed out and pulled in during the gene injection. (*See Color Plate 8.*)

solutions into the myocardium (Marshall *et al.*, 2000). Several injections can be made to cover the target area in the myocardium. Manipulation of the myocardium may cause ventricular arrhythmias and for the prevention of arrhythmias several pre- and perioperative medications have been used in animal studies (fentanyl 10 μg/kg/h, metoprolol 0.15–0.2 mg/kg, $MgSO_4$ 20 mg/kg, lidocain 5 mg/kg, and amiodarone 10 mg/kg) (Rutanen *et al.*, in press). To ensure the safety and the best possible efficiency of the gene transfer, the virus preparations and plasmids need to be carefully tested and analyzed for the absence of helper viruses, bacteriological contaminants, and lipopolysaccharides (Hedman *et al.*, 2003; Ylä- Herttuala and Alitalo, 2003). Catheters for intramyocardial gene injections are presented in Figure 1. Pericardial gene delivery is also a possibility, but it does not result in effective transduction of the myocardium without a concomitant treatment with proteases (Fromes *et al.*, 1999). In addition, gene delivery via coronary veins and coronary sinus can be used for intramyocardial gene delivery (Ylä-Herttuala and Alitalo, 2003).

2.2. Perivascular Gene Transfer

For studying periadventitial gene transfer a local inert perivascular collar or biodegradable gene releasing material can be used (Airenne *et al.*, 2000; Laitinen *et al.*, 1997a, 1997b; Pakkanen *et al.*, 2000; Turunen *et al.*, 1999). A direct injection of genes into the vascular wall is also possible. When a gene transfer vector is administered on the adventitial surface of the vessels, it can stay in close contact with the target cells for a long time (Laitinen *et al.*, 1997a). The limitation of this

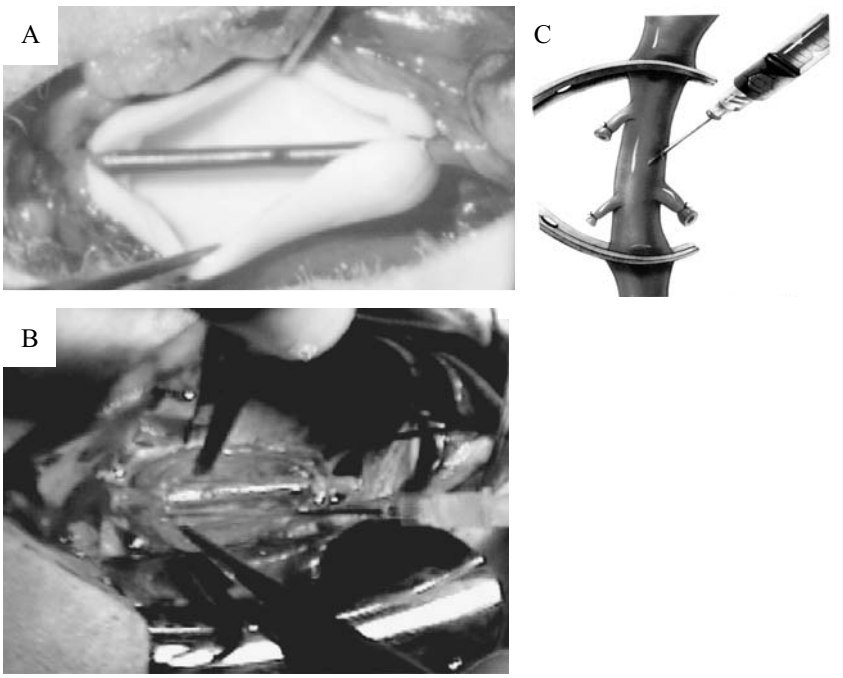

FIGURE 2. Extravascular gene delivery methods. (A) A biologically inert collar installed around the rabbit carotid artery. Genes are injected into the collar so that they are in close contact with the vascular wall. (B) Biodegradable collar releasing therapeutic agents into the vessel wall. (C) Direct injection of genetic material into blood vessel. (*See Color Plate 9.*)

method is the difficulty of the gene transfer vector or the secreted/diffusible gene product to reach the inner layers of the vascular wall.

For the preclinical collar studies, models with rabbit carotid or femoral arteries or carotid arteries of domestic pigs or mice have been used (Airenne *et al.*, 2000; Laitinen *et al.*, 1997a, 1997b; Pakkanen *et al.*, 2000; Turunen *et al.*, 1999). Collar installation into the carotid artery is usually performed through a midline neck incision exposing the carotid arteries between the sternohyoid and sternocleidomastoid muscles. The gene transfer is performed either by injecting the gene solution inside the collar, where it can stay in contact with the vascular wall, or by having the genes applied into a biodegradable material from which a long-term gene–cell interaction can be achieved. Examples of a collar and a biodegradable gel material are presented in Figure 2.

2.3. Intravascular Gene Delivery

Intra-arterial gene delivery route has been used in both preclinical and clinical gene therapy studies (Hedman *et al.*, 2003; Hiltunen *et al.*, 2000a, 2000c;

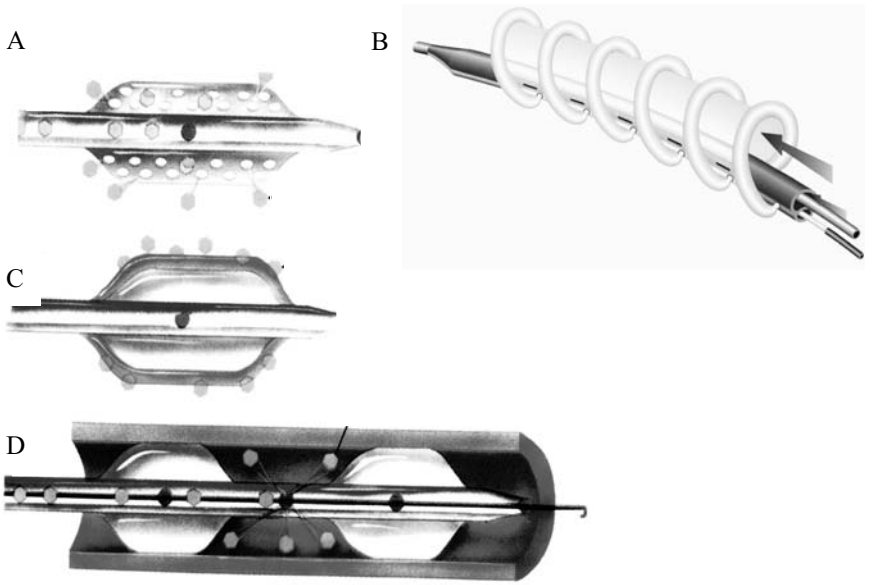

FIGURE 3. Intravascular gene delivery catheters. (A) Channel balloon® with microscopic injection pores. Gene injection can be performed during angioplasty. (B) Dispatch® infusion–perfusion catheter. Continuous blood flow is allowed through the catheter during gene injection. (C) Hydrogel-coated balloon. The genes are applicated into the hydrogel material and released after balloon inflation. (D) Double balloon catheter. See details in the text. (*See Color Plate 9.*)

Laitinen et al., 1997a; Mäkinen *et al.*, 2002; Mesri *et al.*, 1995; Rome *et al.*, 1994; Ylä-Herttuala and Alitalo, 2003). Even though there are several factors impairing the efficacy of intravascular gene transfer (see above), detectable gene expression and signs of biological effects both in animals and in humans have been achieved. Wolinsky *et al.* published the first demonstration showing that administration of drugs delivered locally within the vascular lumen could diffuse into the arterial wall and exhibit a biological effect (Wolinsky and Thung, 1990). On the other hand, intra-arterially administered vectors lead to a more extensive biodistribution than direct injections into tissues (Hiltunen *et al.*, 2000c). However, it may also provide a more widespread transduction of the target tissue than local intramuscular injections. The systemic release of intra-arterially injected gene material can be limited by using local injection catheters (Figure 3) instead of systemic injections. Several types of catheters have been reported to permit a relatively efficient gene transfer into vascular endothelium and cells in tunica media (Willard *et al.*, 1994). The double balloon catheter is made of two latex balloons, which, when inflated inside the target artery, delineates a transfection chamber into which the gene solution can be infused. It allows a close contact for the genes and the vessel wall but because of blockage of the blood flow during the gene transfer, its use is limited to small peripheral arteries and animal experiments (Willard *et al.*, 1994). When continuous tissue perfusion is needed, like in coronary

arteries, infusion–perfusion catheters are more suitable for the gene delivery. They allow a continuous blood flow through an inner channel during the balloon inflation and gene injection. The Dispatch® catheter consists of a spiral inflatable balloon forming separate compartments adjacent to the vessel wall into which the genes are injected. Prolonged gene infusion can be performed since blood flow occurs through the central channel of the catheter. This system has been successfully used to achieve substantial gene delivery into the endothelium and medial layers even in atherosclerotic human arteries (Laitinen *et al.*, 1998). Several types of catheters resembling conventional angioplasty balloon catheters have also been developed for intravascular injections. Hydrogel-coated balloon catheter has an inflatable angioplasty balloon covered with a gene-releasing hydrogel coat. Balloon catheters containing microscopical pores have also been used for intra-arterial gene delivery. The channeled balloon catheter has 24 longitudinal channels, each containing 100-μm pores, and also allows a continuous blood flow into peripheral tissues. Iontophoretic catheters are based on electroporation technique joined with a balloon system. Other catheters for intravascular gene transfer include transport catheter, stented porous balloon catheter, and nipple infusion catheter. Examples of gene delivery catheters used for intravascular gene transfer are presented in Figure 3.

3. ANIMAL MODELS FOR STUDYING CARDIOVASCULAR DISEASES

Genetically modified animal models can be used to study pathogenesis of different cardiovascular disorders and possible ways to interfere the disease process by means of gene therapy. Gene knockout technologies have provided researchers a powerful tool for studying the effects of single genes in some cardiovascular diseases. For example, apolipoprotein functions have been studied using knockout mice, and also several knockout models for different vascular growth factors have been created (Ylä-Herttuala and Alitalo, 2003). Also, animals with naturally occurring gene defects, such as Watanabe heritable hyperlipidemic (WHHL) rabbits with LDL-receptor mutation, have been successfully used for studies of atherosclerotic diseases (Pakkanen *et al.*, 1999). Possibility of creating knockout models using lentivirus vector-mediated delivery of siRNA nucleotides (see above) is a new technique that will allow quick and convenient method for creating novel transgenic animals.

Many restenosis studies have been carried out in rat, rabbit, canine, and pig carotid or femoral artery models where the target arteries are subjected to balloon injury, leading to pathologic neointimal cell proliferation. A cholesterol diet can be used to enhance the process of cell proliferation and accumulation of lipids and macrophages inside the vessel wall (Feldman *et al.*, 1996; Hiltunen *et al.*, 2000a). Ischemia can be induced in peripheral tissues by ligating major arteries (Vajanto *et al.*, 2002). Animal models for refractory angina pectoris or end-stage coronary artery disease have also been developed. They are usually based on peri- (ameroid constrictor) or intracoronary (stents, coils) interventions in pig coronary arteries

(Hughes *et al.*, 2003). Ischemia models are used to explore cellular mechanisms of the ischemic tissue injury and to perform studies with therapeutic approaches, such as with vascular growth factors (Vajanto *et al.*, 2002; Takeshita *et al.*, 1996).

3.1. *In Vivo* Examples of Cardiovascular Gene Therapy

3.1.1. Therapeutic Angiogenesis

Since ischemia is due to lack of oxygen, all the effort put on increasing the number of blood vessels within the ischemic tissue is logical. Currently, several vascular growth factors are known to induce angio- and arteriogenesis (Ylä-Herttuala and Alitalo, 2003). The family of vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) are the mostly widely explored angiogenic growth factors. VEGFs are angiogenic factors that have significant roles in the development of vascular structures, in vasculogenesis, and in arteriogenesis and angiogenesis (Ferrara *et al.*, 2003). Actions of VEGFs include vasodilatation, induction of arterial sprouting, and vascular permeability. In addition to direct angiogenic effects, some VEGFs induce other vasoactive agents, such as nitric oxide and prostacyclin (Laitinen *et al.*, 1997b; Murohara *et al.*, 1998). They can also inhibit apoptosis in endothelial cells by inducing expression of antiapoptotic genes (Gerber *et al.*, 1998). Furthermore, some VEGFs are also responsible for lymphangiogenesis (Karkkainen *et al.*, 2001). FGFs are multifunctional proteins stimulating the proliferation of a variety of cell types, including endothelial cells, smooth muscle cells, and myofibroblasts (Galzie *et al.*, 1997). They are involved in embryonic development, tissue regeneration, cell transformation, tumor growth, and angiogenesis. Other factors involved in the process of angiogenesis and potentially applicable for therapeutic angiogenesis include angiopoietins, insulin-like growth factors, hepatocyte growth factor, platelet-derived growth factors, and some transcription factors (HIF-1α, EGR-1, etc.)

Numerous preclinical angiogenesis studies with or without induction of tissue ischemia have shown a clearly detectable increase in the number of functional blood vessels both in peripheral muscles and in myocardium (Rutanen *et al.*, in press; Vajanto *et al.*, 2002). In addition to these preclinical findings, several human trials of therapeutic angiogenesis have shown positive results (Baumgartner *et al.*, 1998; Hedman *et al.*, 2003; Mäkinen *et al.*, 2002; Rajagopalan *et al.*, 2003; Ylä-Herttuala and Alitalo, 2003). Examples of angiogenic gene therapy are illustrated in Figure 4.

3.1.2. Arterial Cytoprotection and Prevention of Graft Restenosis

Cardiovascular gene therapy strategies are mostly directed toward the inhibition of smooth muscle cell migration and proliferation, formation of connective tissue, and undesirable growth factor effects (Ylä-Herttuala and Alitalo, 2003). In the wall of arteries, VEGF, nitric oxide, and prostaglandin I_2 are cytoprotective

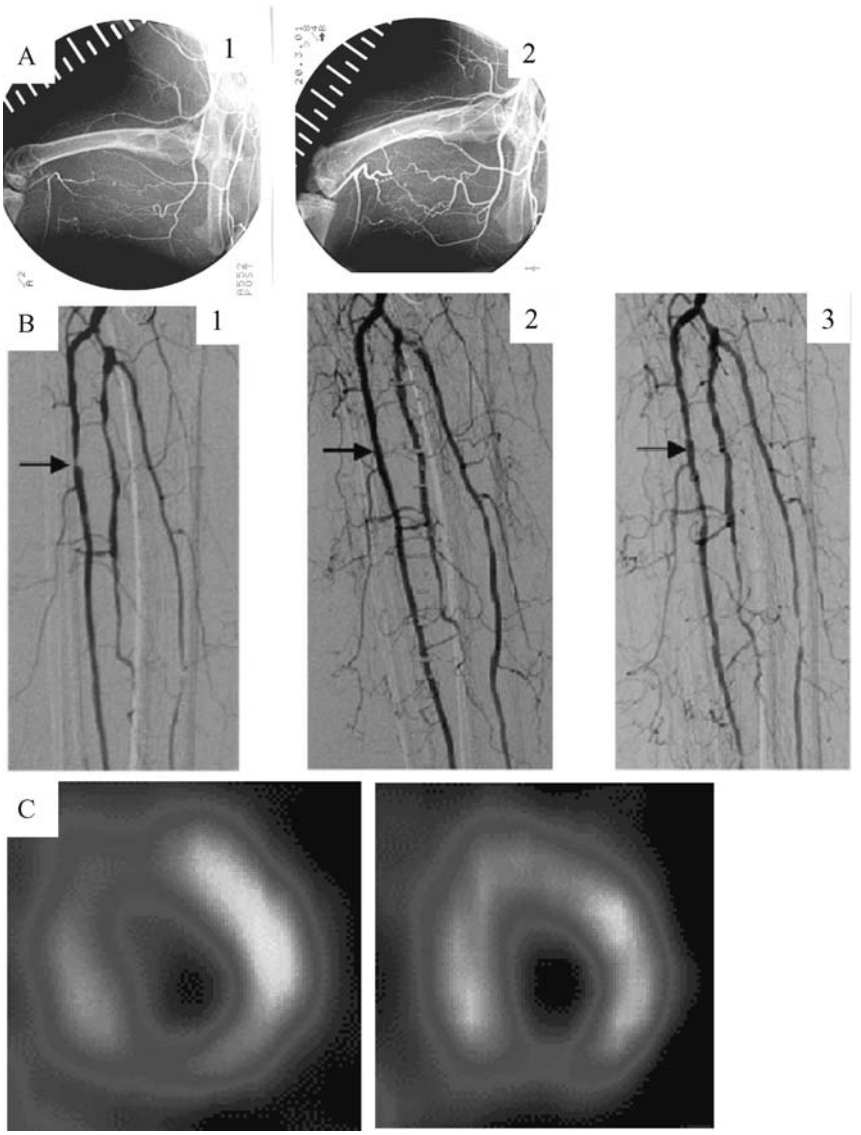

FIGURE 4. *In vivo* examples of angiogenic gene therapy. (A) An animal model of a rabbit hind limb before (1) and 35 days after (2) intramuscular injections of VEGF gene in an adenovirus vector (Laitinen *et al.*, 1997). (B) Human truncus of leg arteries before (1), 3 months after (2), and 9 months after (3) angioplasty combined with local intra-arterial VEGF gene transfer (Laitinen *et al.*, 1998). (C) Myocardial scintigraphy of a human heart before and 6 months after local intracoronary adenoviral VEGF gene transfer performed during coronary angioplasty (Hedman *et al.*, 2003). (*See Color Plate 10.*)

compounds, since they stimulate endothelial repair and survival, prevent platelet aggregation, improve endothelial survival, and control smooth muscle cell proliferation (Ylä-Herttuala and Alitalo, 2003). In rabbit carotid arteries transfected with $VEGF_{165}$gene by using the collar model, inhibition of smooth muscle cell proliferation and neointima formation was detected (Laitinen *et al.*, 1997b). This biological effect was at least partially explained by the finding that VEGF dramatically stimulated nitric oxide production in endothelial cells. In clinical gene therapy trials targeted to prevent restenosis both positive and negative results have been reported. When a local intracoronary VEGF gene transfer was performed during angioplasty and stenting, no differences in clinical restenosis rate or in angiographical minimal lumen diameter were detected (Hedman *et al.*, 2003). On the other hand, when cell growth inhibition was induced by applying E2F oligonucleotide decoy into bypass grafts, a significant improvement was detected regarding the graft failures and occlusions after 6 months (Mann *et al.*, 1999).

3.1.3. Prevention of Atherogenesis

Hypercholesterolemia is a well-known risk factor for cardiovascular diseases and therefore lipoprotein metabolism plays a critical role in the pathogenesis of atherosclerosis (Ylä-Herttuala and Alitalo, 2003). In preclinical studies transfer of LDL-receptor gene, VLDL-receptor gene, soluble scavenger receptor gene, as well as genes regulating the production of lipoprotein lipase, hepatic lipase, and several apolipoproteins have been used (Pakkanen *et al.*, 1999; Ylä-Herttuala and Alitalo, 2003). Also, clinical trials for the treatment of familial hypercholesterolemia have been performed (Grossman *et al.*, 1995; Ylä-Herttuala and Alitalo, 2003). LDL-oxidation and accumulation of cholesterol in macrophages and vascular wall are also processes that could be interfered by means of gene therapy.

4. SUMMARY

Vascular gene therapy is a new area in which only a few preliminary results from human trials are available (Ylä-Herttuala and Alitalo, 2003). Several sophisticated animal models are currently in use for the preclinical gene therapy studies. One of the most important difficulties in gene therapy is the low transfection efficiency in target tissues (Laitinen *et al.*, 1998). This efficiency needs to be improved with the development of better expression vectors and gene delivery methods. Improvement of gene delivery catheters and optimization of other technical aspects regarding gene injections are under investigation. In the future, imaging of the myocardium or vascular lesions might be possible with magnetic resonance imaging or transcutaneous or intravascular ultrasonography, which will help to elucidate the nature of the underlying pathology and vascular lesions (Ylä-Herttuala and Alitalo, 2003). Once the pathophysiology of vascular diseases is better understood, new therapeutic targets and genes may become available. Also, combinations of

differently acting genes would be possible. DNA array and other screening methods for the underlying genetic disorders will also help to define better targets for gene therapy. The development of gene therapy in the cardiovascular system is proceeding rapidly, because of the great importance of the heart and blood vessels for human health.

REFERENCES

Airenne, K. J., Hiltunen, M. O., Turunen, M. P., Turunen, A. M., Laitinen, O. H., Kulomaa, M. S., and Ylä-Herttuala, S., 2000, Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery, *Gene Ther.* **7**:1499–1504.

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

Bergelson, J. M., 1999, Receptors mediating adenovirus attachment and internationalization. *Biochem. Pharmacol*. **57**:975–979.

Blaese, R. M., Culver, K.W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., and Tolstoshev, P. T., 1995, lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* **270**:475–480.

Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A., 2003, Killing the messenger: short RNAs that silence gene expression. *Nat. Rev. Mol. Cell Biol.* **4**:457–467.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T., 2001, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 24*, **411**:494–498.

Feldman, L. J., Tahlil, O., and Steg, G., 1996, Perspectives of arterial gene therapy for the prevention of restenosis. *Cardiovasc. Res.* **32**:194–207.

Ferrara, N, Gerber, H. P., and LeCouter, J., 2003, The biology of VEGF and its receptors. *Nat. Med.* **9**:669–676.

Fortuin, F. D., Vale, P., Losordo, D. W., Symes, J., DeLaria, G. A., Tyner, J. J., Schaer, G. L., March, R., Snell, R. J., Henry, T. D., Van Camp, J., Lopez, J. J., Richenbacher, W., Isner, J. M., and Schatz, R. A., 2003, One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. *Am. J. Cardiol.* **92**:436–439.

Fromes, Y, Salmon, A., Wang, X., Collin, H., Rouche, A., Hagege, A., Schwartz, K., and Fiszman, M. Y., 1999, Gene delivery to the myocardium by intrapericardial injection. *Gene Ther.* **6**:683–688.

Galzie, Z., Kinsella, A. R., and Smith, J.A., 1997, Fibroblast growth factors and their receptors. *Biochem. Cell Biol*. **75**:669–685.

Gerber, H. P., Dixit, V., and Ferrara, N., 1998. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bc1-2 and A1 in vascular endothelial cells. *J. Biol. Chem*. **273**:13313–13316.

Grossman, M., Rader, D. J., Muller, D. M. W., Kolansky, D. M., Kozarsky, K., Clark, B. J. 3rd, Stein, E. A., Lupien, P.J., Brewer, H. B. Jr, Raper, S. E., and Wilson, J. M., 1995, A pilot study of *ex vivo* gene therapy for homozygous familial hypercholesterolemia. *Nat. Med*. **1**:1148–1154.

Hedman, M., Hartikainen, J., Syvänne, M., Stjernvall, J., Hedman, A., Kivelä, A., Vanninen, E., Mussalo, H., Kauppila, E., Simula, S., Närvänen, O., Rantala, A., Peuhkurinen, K., Nieminen, M. S., Laakso, M., and Ylä-Herttuala, S., 2003, Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT), *Circulation*. **107**:2677–2683.

Hiltunen, M. O, Laitinen, M., Turunen, M., Hartikainen, J., Häkkinen, T., Kivelä, A., Rissanen, T., Mansukoski, H., Turunen, A., Alitalo, K., and Ylä-Herttuala, S., 2000a, Intravascular

adenovirus-mediated VEGF-C gene transfer inhibits neointima formation in balloon-denuded rabbit aorta. *Circulation*. **102**:2262–2268.

Hiltunen, M. O, Turunen, M. P, Laitinen, M., and Ylä-Herttuala, S., 2000b, Insights into the molecular pathogenesis of atherosclerosis and therapeutic strategies using gene transfer. *Vasc. Med*. 5:41–48.

Hiltunen, M. O, Turunen, M. P, Turunen, A. M, Rissanen, T. T, Laitinen, M., Kosma, V. M., and Ylä-Herttuala, S., 2000c, Biodistribution of adenoviral vector to nontarget tissues after local *in vivo* gene transfer to arterial wall using intravascular and periadventitial gene delivery methods. *FASEB J*. **14**:2230–2236.

Hughes, G. C, Post, M. J, Simons, M., and Annex, B. H., 2003, Translational physiology. Porcine models of human coronary artery disease: implications for preclinical trials of therapeutic angiogenesis. *J. Appl. Physiol*. **94**:1689–1701.

Jacobsen, L. B., 1997, Plasmid and other non-viral vectors. Gene transfer in the cardiovascular system. In *Experimental Approaches and Therapeutic Implications*, ed. K. L. March, pp. 85–110. Dodrecht: Kluwer Academic Publishers.

Kafri, T., van Praag, H., Gage, F. H., and Verma, I.M., 2000, Lentiviral vectors: regulated gene expression. *Mol. Ther*. 1:516–521.

Kang, Y., Stein, C. S., Heth, J. A., Sinn, P. L., Penisten, A. K., Staber, P. D., Ratliff, K. L., Shen, H., Barker, C. K., Martins, I., Sharkey, C. M., Sanders, D. A., McCray, P. B. Jr., and Davidson, B. L., 2002, *In vivo* gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. *J. Virol*. **76**:9378–9388.

Karkkainen, M. J, Saaristo, A., Jussila, L., Karila, K. A., Lawrence, E. C., Pajusola, K., Bueler, H., Eichmann, A., Kauppinen, R., Kettunen, M. I., Ylä-Herttuala, S., Finegold, D.N., Ferrell, R.E., and Alitalo, K., 2001. A model for gene therapy of human hereditary lymphedema. *Proc. Natl. Acad. Sci. U.S.A*. **98**:12677–12682.

Kessler, P. D., Podsakoff, G. M., Chen, X., McQuiston, S. A., Colosi, P. C., Matelis, L. A., Kutzman, G. J., and Byrne, B. J., 1996, Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. U.S.A*. **93**:14082–14087.

Kootstra, N. A., and Verma, I. M., 2002, Gene therapy with viral vectors. *Annu. Rev. Pharmacol. Toxicol*. **43**:413–439.

Kornowski, R., Leon, M. B., Fuchs, S., Vodovotz, Y., Flynn, M. A., Gordon, D. A., Pierre, A., Kovesdi, I., Keiser, J. A., and Epstein, S. E., 2000, Electromagnetic guidance for catheter-based transendocardial injection: a platform for intramyocardial angiogenesis therapy. Results in normal and ischemic porcine models. *J. Am. Coll. Cardiol*. **35**:1031–1039.

Kovesdi, I., Brough, D. E., Bruder, J. T., and Wickham, T. J., 1997, Adenoviral vectors for gene transfer. *Curr. Opin. Biotechnol*. **8**:583–589.

Laitinen, M., Mäkinen, K., Manninen, H., Matsi, P., Kossila, M., Agrawal, R., Pakkanen, T., Luoma, J. S., Viita, H., Hartikainen, J., Alhava, E., Laakso, and M., Ylä-Herttuala, S., 1998, Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. *Hum. Gene Ther*. **9**:1481–1486.

Laitinen, M., Pakkanen, T., Donetti, E., Baetta, R., Luoma, J., Lehtolainen, P., Viita, H., Agrawal, R., Miyanohara, A., Friedmann, T., Risau, W., Martin, J. F., Soma, M., and Ylä-Herttuala, S., 1997a, Gene transfer into the carotid artery using an adventitial collar: comparison of the effectiveness of the plasmid–liposome complexes, retroviruses, pseudotyped retroviruses, and adenoviruses. *Hum. Gene Ther*. **8**:1645–1650.

Laitinen, M., Zachary, I., Breier, G., Pakkanen, T., Häkkinen, T., Luoma, J., Abedi, H., Risau, W., Soma, M., Laakso, M., Martin, J. F., and Ylä-Herttuala, S., 1997b, VEGF gene transfer reduces intimal thickening via increased production of nitric oxide in carotid arteries. *Hum. Gene Ther*. **8**:1737–1744.

Lehrman, S., 1999, Virus treatment questioned after gene therapy death. *Nature*. **401**:517–518.

Mäkinen, K., Manninen, H., Hedman, M., Matsi, P., Mussalo, H., Alhava, E., and Ylä-Herttuala, S., 2002, Increased vascularity detected after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. *Mol. Ther*. **6**:127–133.

Mann, M. J., Whittemore, A. D., Donaldson, M. C., Belkin, M., Conte, M. S., Polak, J. F., Orav, E. J., Ehsan, A., Dell'Acqua, G., and Dzau, V. J., 1999, *Ex-vivo* gene therapy of human vascular by-pass grafts with E2F decoy: the PREVENT single-centre, randomized, controlled trial. *Lancet* **354**:1493–1498.

Marshall, D. J, Palasis, M., Lepore, J. J., and Leiden, J. M., 2000, Biocompatibility of cardiovascular gene delivery catheters with adenovirus vectors: an important determinant of the efficiency of cardiovascular gene transfer. *Mol. Ther*. **5**:423–429.

Mesri, E. A, Federoff, H. J., and Brownlee, M., 1995, Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice. *Circ. Res*. **76**:161–167.

Murohara, T., Horowitz, J. R., Silver, M., Tsurumi, Y., Chen, D., Sullivan, A., and Isner, J. M., 1998, Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation* **97**:99–107.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., and Trono, D., 1996, *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**:263–267.

Pajusola, K., Gruchala, M., Joch, H., Luscher, T. F., Ylä-Herttuala, S., and Bueler, H., 2002, Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. *J. Virol*. **76**:11530–11540.

Pakkanen, T. M., Laitinen, M., Hippeläinen, M., Hiltunen, M. O., Alhava, E., and Ylä-Herttuala, S., 2000, Periadventitial lacZ gene transfer to pig carotid arteries using a biodegradable collar or a wrap of collagen sheet with adenoviruses and plasmid–liposome complexes. *J. Gene Med*. **2**:52–60.

Pakkanen, T. M., Laitinen, M., Hippeläinen, M., Kallionpää, H., Lehtolainen, P., Leppänen, P., Luoma, J. S., Tarvainen, R., Alhava, E., and Ylä-Herttuala, S., 1999, Enhanced plasma cholesterol lowering effect of retrovirus-mediated LDL receptor gene transfer to WHHL rabbit liver after improved surgical technique and stimulation of hepatocyte proliferation by combined partial liver resection and thymidine kinase ganciclovir treatment. *Gene Ther*. **6**:34–41.

Pakkanen, T., and Ylä-Herttuala, S., 2000, Gene therapy for dyslipidaemias. *Eur. Heart J. Suppl*. **2**:D62–D64.

Plank, C., Mechtler, K., Szoka, F. C. Jr., and Wagner, E., 1996, Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther*. **7**:1437–1446.

Rajagopalan, S., Mohler, E. R. III, Lederman, R. J., Mendelsohn, F. O., Saucedo, J. F., Goldman, C. K., Blabea, J., Macko, J., Kessler, P. D., Rasmussen, H. S., and Annex, B. H., 2003, Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* **108**:1933–1938.

Rome, J. J., Shayani, V., Flugelman, M. Y., Newman, K. D., Farb, A., Virmani, R., and Dichek, D. A., 1994, Anatomic barriers influence the distribution of *in vivo* gene transfer into the arterial wall. Arterioscler. *Thromb*. **14**:148–161.

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M. T., Yang, J. C., and Topalian, S. L., 1990, Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med*. **323**:570–578.

Rutanen, J., Rissanen, T. T., Markkanen, J. E., Gruchala, M., Silvennoinen, P., Kivelä, A., Hedman, A., Hedman, M., Heikura, T., Orden, M.-R., Stacker, S. A., Achen, M. G., Hartikainen, J., and Ylä-Herttuala, S., 2004, Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of VEGF-D induces transmural angiogenesis in porcine heart. *Circulation.* **109**: 1029–35.

Service, R. F., 1955, Dendrimer: dream molecules approach real applications. *Science* **267**:458–459.

Shi, Y., 2003, Mammalian RNAi for the masses. *Trends Genet*. **19**:9–12.

Shiotani, A., Fukumura, M., Maeda, M., Hou, X., Inoue, M., Kanamori, T., Komaba, S., Washizawa, K., Fujikawa, S., Yamamoto, T., Kadono, C., Watabe, K., Fukuda, H., Saito, K., Sakai, Y., Nagai,

Y., Kanzaki, J., and Hasegawa, M., 2001, Skeletal muscle regeneration after insulin-like growth factor I gene transfer by recombinant Sendai virus vector. *Gene Ther*. **8**:1043–1050.

Takeshita, S., Isshiki, T., and Sato, T., 1996, Increased expression of direct gene transfer into skeletal muscles observed after acute ischemic injury in*rats. Lab. Invest*. **74**:1061–1065.

Tiscornia, G., Singer, O., Ikawa, M., and Verma, I. M., 2003. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. U.S.A. 18* **100**:1844–1848.

Tripathy, S. K., Svensson, E. C., Black, H. B., Goldwasser, E., Margalith, M., Hobart, P. M., and Leiden, J. M., 1996, Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. *Proc. Natl. Acad. Sci. U.S.A*. **93**:10876–10880.

Turunen, M. P., Hiltunen, M. O., Ruponen, M., Virkamäki, L., Szoka, F. C. J., Urtti, A., and Ylä-Herttuala, S., 1999, Efficient adventitial gene delivery to rabbit carotid artery with cationic polymer–plasmid complexes. *Gene Ther*. **6**:6–11.

Vajanto, I., Rissanen, T. T., Rutanen, J., Hiltunen, M. O., Tuomisto, T. T., Arve, K., Närvänen, O., Manninen, H., Räsänen, H., Hippeläinen, M., Alhava, E., and Ylä-Herttuala, S., 2002, Evaluation of angiogenesis and side effects in ischemic rabbit hind limbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. *J. Gene Med*. **4**:371–380.

Vitadello, M., Schiaffino, M. V., Picard, A., Scarpa, M., and Schiffiano, S., 1994, Gene transfer in regenerating muscle. *Hum. Gene Ther*. **5**:11–18.

Willard, J. E., Landau, C., Glamann, D. B., Burns, D., Jessen, M. E., Pirwitz, M. J., Gerard, R. D., and Meidell, R. S., 1994, Genetic modification of the vessel wall. Comparison of surgical and catheter-based techniques for delivery of recombinant adenovirus. *Circulation* **89**:2190–2197.

Wolinsky, H. and Thung, S. N., 1990, Use of perforated balloon catheter to deliver concentrated heparin into the wall of the normal canine artery. *J. Am. Coll. Cardiol*. **15**:475–481.

Yamashita, Y., Shimada, M., Tachibana, K., Harimoto, N., Tsujita, E., Shirabe, K., Miyazaki, J., and Sugimachi, K. 2002, *In vivo* gene transfer into muscle via electro-sonoporation. *Hum. Gene Ther*. **13**:2079–2084.

Ylä-Herttuala, S. and Alitalo, K., 2003, Gene transfer as a tool to induce therapeutic vascular growth, *Nat. Med.*, **9**:694–701.

Chapter 6

The Role of Bioinformatics in Genomic Medicine

A. H. C. van Kampen and A. J. G. Horrevoets

1. INTRODUCTION

For more than a century vast progress has been made in genetics and molecular biology. During the last decade new high-throughput experimental techniques have rapidly emerged. The automation of DNA sequencing has set the stage for the Human Genome Project in 1990 (Collins *et al.*, 1998), which has led to genomics (the branch of genetics that studies organisms in terms of their full DNA sequences) and a range of related disciplines such as transcriptomics (the study of the complete gene expression state), proteomics (the study of the full set of proteins encoded by a genome), and metabolomics (the study of comprehensive metabolite profiles). In the remainder of this chapter these domains will all be referred to as genomics. Genomics has greatly accelerated fundamental research in molecular biology as it enables the measurement of molecular processes globally and from different points of view. This led to a range of applications in the biomedical sector and increasingly affects patient care (Collins *et al.*, 2003; Subramanian *et al.*, 2001; van Ommen, 2002).

One of the main bottlenecks that prevents (large-scale) implementation of genomics in health care is the problems related to the management, analysis, and interpretation of large amounts of heterogeneous data that are measured for

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

(patient) samples. The necessity to deal with these problems initially triggered the emergence of bioinformatics, which is the discipline that focuses on the conversion of experimental data into biomedical knowledge and hypotheses (Bayat, 2002; Luscombe *et al.*, 2001). Bioinformatics develops and applies mathematical and informatics tools to analyze and interpret genomics data at the level of interactions between molecules, pathways, and cells. Moreover, applications of genomics in clinical medicine (i.e. genomic medicine) will force bioinformatics researchers to consider medically related issues such as the analysis and integration of clinical data. Consequently, it is important to realize that for successful application of genomics in health care one also needs support of medical informatics, which complements bioinformatics in many ways. Unmistakably, bioinformatics and medical informatics are rapidly converging (Maojo and Kulikowski, 2003). In addition, the fields of informatics, (bio)statistics, and epidemiology will provide further expertise to advance genomic medicine.

Current bioinformatics covers a wide range of scientific research that reflects the breadth of biological research. Active areas of bioinformatics research include sequence analysis (including, e.g., sequence alignment, gene finding, phylogenetics), three-dimensional protein structure determination, visualization, pathway analysis and reconstruction, modeling and simulation of molecular processes, construction of genome maps, statistical analysis of experimental data, development of ontologies, and development of databases. This work, which is conducted by both bioinformatics and other research groups, is crucial for successful application of genomics in genomic medicine. Many bioinformatics resources that result from this research become freely available via the Internet (see Table 1; this table lists the Web sites of resources mentioned throughout this chapter). In the bioinformatics community it is almost common practice that research groups and institutes make their databases and software freely accessible, which is probably the main reason that bioinformatics was able to evolve quickly into an established scientific discipline. The National Center of Biotechnology Information (NCBI) and the European Bioinformatics Institute (EBI) are two major providers of bioinformatics tools and biological databases, and play an important role in the further development of bioinformatics. Many other institutes complement these two major bioinformatics centers, and the "Pedro's Biomolecular Research Tools" list provides many pointers to available tools and databases.

In this chapter, we will discuss a selection of bioinformatics aspects that directly or indirectly play a role in genomic medicine. We will provide examples to illustrate several of these issues. In addition, we discuss the current role of bioinformatics in cardiovascular research.

2. PUBLIC BIOLOGICAL DATABASES

Much of the data that are produced by the genomics community become freely available and this resulted in the development of many biological databases

Table 1
Bioinformatics and Medical Informatics Resources Referred to in the Text

Acronym	Name	Web Address
Ascenta	Gene expression database	www.genelogic.com
Bodymap	Data bank of expression information	bodymap.ims.u-tokyo.ac.jp
CaGE	Cardiac Gene Expression knowledge base	www.cage.wbmei.jhu.edu
EBI (SRS, MIAME, MAGE)	European Bioinformatics Institute	www.ebi.ac.uk
Genew	Human Gene Nomenclature database	www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl
GenomeVista	Comparative genome analysis	pipeline.lbl.gov/cgi-bin/GenomeVista
GO	Gene Ontology	www.geneontology.org
HTM	Human Transcriptome Map	Bioinfo.amc.uva.nl
NCBI (Entrez, Genbank, UniGene, PubMed, BLAST, dbSNP, OMIM)	National Center for Biotechnology Information	www.ncbi.nlm.nih.gov
OBO	Open Biological Ontologies	obo.sourceforge.net
Pedro's Biomolecular Research Tools	Pointers to tools and databases	www.biophys.uni-duesseldorf.de/BioNet/Pedro/research_tools.html
SwissProt	Protein database	http://www.expasy.org/

(Baxevanis, 2003) that can be accessed through the Internet with key word-based search systems like SRS (Zdobnov *et al.*, 2002) or Entrez (Wheeler *et al.*, 2003). These databases contain a wealth of information about, e.g., nucleotide sequences, protein sequences, metabolic pathways, single-nucleotide polymorphisms (SNPs), mutations, physical and genetic maps, protein structure, transcription factors, and ontologies. Several of these databases have direct clinical relevance such as OMIM (Online Mendelian Inheritance in Man) (Hamosh *et al.*, 2002), which is a catalog of human genes and genetic disorders. The dbSNP database (Thorisson and Stein, 2003) is an important resource for pharmacogenomics (Altman and Klein, 2002) and cardiovascular research, as will be discussed below. Many of these databases are linked to literature databases (Pubmed) and are complementary to literature. Moreover, the biological databases are generally cross-linked, which makes it possible to gather information about the gene, protein, or pathway of interest relatively easily. One of the main uses of the nucleotide and sequence databases

is sequence-similarity searches in which BLAST is used to identify sequences that are homologous to the input sequence (Altschul *et al.*, 1990, 1997).

In addition to the key word-based and sequence-similarity searches, the biological databases are frequently used for *in silico* experiments preliminary to wet-lab experiments to generate or validate new hypotheses. For example, the Human Transcriptome Map (Caron *et al.*, 2001; Versteeg *et al.*, 2003) integrates the human genomic DNA sequence with gene expression profiles obtained with Serial Analysis of Gene Expression (SAGE) (Velculescu *et al.*, 1995). This provides novel and fundamental insight into the higher order structure of the human genome. The integrated databases allowed constructing chromosome profiles for gene expression levels, gene density, intron length, GC content, and LINE/SINE repeats. These maps clearly revealed correlated domains on the genome, which resulted in new hypotheses with respect to gene expression regulation. Such information can only be obtained if public databases are integrated locally, if errors and inconsistencies are removed (see below), and if these databases are complemented with appropriate (statistical) methods.

Biological databases also play an important role in the analysis and interpretation of experimental data. It is, for example, routine to integrate experimental data obtained with DNA microarrays with data from the Gene Ontology database (Herrero *et al.*, 2003; Zhong *et al.*, 2003) or pathway databases (Dahlquist *et al.*, 2002; Doniger *et al.*, 2003; Nakao *et al.*, 1999) to reveal the function of the genes in the system under study. Also, the Human Transcriptome Map integrates experimental data with biological databases, which enabled the identification of genes involved in neuroblastoma (Spieker *et al.*, 2001).

2.1. Potential Pitfalls with Applications of Public Biological Databases

Although biological databases have proven important for many applications in genomics, one has to be cautious when using these data. Some of the databases like GenBank (Wheeler *et al.*, 2003) are open to submission by essentially everyone while other databases like, e.g., SwissProt (Gasteiger *et al.*, 2003) are curated by experts, and consequently, information in these repositories is generally considered to be more reliable. However, also the quality of SwissProt has been the subject of discussion (Apweiler *et al.*, 2001; Karp *et al.*, 2001). It is important to realize that most biological databases contain erroneous and inconsistent data (Karp, 1998; Stein, 2003). The first type of error is spelling mistakes in, e.g., gene names, which may cause problems in, e.g., database searches. Similar problems may be caused by differences in US vs UK spellings or problems of representation of international characters (Bork and Bairoch, 1996). Another obvious type of error is the sequencing errors in nucleotide sequences, which complicate their use in specific applications (Caron *et al.*, 2001; Clark and Whittam, 1992). Sequence databases also include contaminating sequences, which are pieces of foreign sequence that intentionally or accidentally were introduced at various steps of the cloning procedure or by recombination events in yeast or bacteria (Anderson, 1993; Binns, 1993; Dean and Allikmets, 1995; Gonzalez and Sylvester, 1997;

Wenger and Gassmann, 1995; Yoshikawa *et al.*, 1997). These contaminations may cause problems for, e.g., sequence analysis and database searching (Miller *et al.*, 1999; Seluja *et al.*, 1999; Versteeg *et al.*, 2003). Another problem is nucleotide and protein sequence annotation. Many sequences are annotated automatically by computer programs and this process is likely to introduce errors in the databases (Karp, 1998). In addition, it is occasionally difficult to track how annotation was derived and whether or not it was experimentally verified. Although the scope of this problem is unknown, it again complicates the use of these databases. Furthermore, if one realizes that novel sequences are frequently annotated on the basis of annotation currently present in the sequence databases, this may cause an explosion of incorrectly annotated sequences. Naming conventions for genes and proteins cause additional problems. A single gene may have more than one name, or alternatively, two genes may share the same name (Stein, 2003). The Genew database (Wain *et al.*, 2002) attempts to standardize naming conventions for genes.

Several other types of problems exist, most of which are database specific. In general, these problems are difficult to identify and solve. However, these problems will require the attention from database developers, curators, and users.

2.2. Database Standardization and Ontologies

To ensure optimal benefit from biological databases, it is crucial to define exactly what information should be stored so that future use of these data is secured. This issue is far from trivial, which was recently clearly illustrated by the efforts to define MIAME (Minimal Information About a Microarray Experiment) (Brazma *et al.*, 2001), which specifies the minimal set of information that should accompany microarray data and includes experimental conditions, quality control parameters, a list of genes on the array, and a description of array layout. This also enabled the development of related standards such as the data-exchange standard MAGE-ML (Microarray and Gene Expression Markup Language) (Spellman *et al.*, 2002), which should ensure seamless data exchange between databases and software applications.

Equally important is the development and use of biomedical ontologies in databases. An ontology is a means of capturing knowledge about a domain such that it can be used by both humans and computers. There are many ways of representing ontologies, including word lists (e.g. the key words used in SwissProt and the Human Gene Nomenclature database Genew), taxonomies (e.g. the organism taxonomy of the NCBI), database schemes (MAGE-OM), and acyclic graphs (e.g. the gene ontology). The "Open Biological Ontologies" provides an entry point to biological ontologies. An important effort comprises the development of the Unified Medical Language System (UMLS) (Lindberg, 1990; Lindberg *et al.*, 1993; Sarkar *et al.*, 2003; Yu *et al.*, 1999), the purpose of which is to enable the development of systems that help health professionals and researchers retrieve and integrate electronic biomedical information. The UMLS connects over

60 vocabularies (e.g. SNOMED), thesauri (e.g. MeSH), classifications (e.g. ICD-10), drug sources, alternative medicine, gene ontology, etc.

Specifications like MIAME and biomedical ontologies will further standardize the way in which data are stored in databases. This will help to remove inconsistencies between databases, allow more powerful queries, and will facilitate data integration (Stein, 2003).

3. DEVELOPMENT OF (STATISTICAL) ALGORITHMS

Another important area of bioinformatics research involves the development and application of (statistical) algorithms for the analysis and interpretation of data. These algorithms cover a broad range of domains such as sequence analysis, visualization (pathway maps, genomic maps, protein structure), statistical analysis of experimental data, and modeling and simulation of cellular processes. Many of these algorithms are developed to deal with new types of questions and problems arising from the production of genome-wide data sets.

One particular challenge is the development of algorithms that can handle large amounts of (heterogeneous) data. Traditional algorithms may face difficulties when applied to large genomic data sets. For example, cluster algorithms that are used for the identification of related groups of genes in DNA microarray experiments cannot be applied to full-sized data sets that contain, say, over 15,000 genes. To overcome this problem new algorithms are developed. For example, Herwig and coworkers developed a sequential k-means algorithm to cluster data from cDNA fingerprinting, which is a method for simultaneous determination of expression levels for every active gene of a specific tissue (Herwig *et al.*, 1999).

Another active field of research is the classification of tumors (or disease) for diagnosis on the basis of gene or protein profiles ('t Veer *et al.*, 2002). Here, statistical methods are challenged by the fact that in genomics experiments the number of measurements (e.g. gene expression levels) greatly exceeds the number of samples. Consequently, application of current classification algorithms may result in poorly behaving statistical models (Harrell *et al.*, 1984). This again prompts for the development of new statistical approaches that simultaneously should address related problems such as the estimation of the number of samples to be analyzed and the maximum number of genes to be included in the classification model for the given number of samples.

4. EXPERIMENTAL DESIGN FOR GENOMICS EXPERIMENTS

In analogy with other areas of scientific research, it is good practice to think carefully about experimental design for genomics experiment before starting an experiment. This will not only make clear whether the expected results are realistic, but it will also result in a maximized amount of information for the conducted experiment. Inappropriate experimental design may lead to disappointments afterwards,

as it may turn out that the research question cannot be answered. For genomics experiments, however, the selection of a particular experimental design and the choice for the subsequent data analysis approach can be complicated for several reasons. First, genomics experiments are complex and not always fully understood in all their experimental details. At many stages of the experiment, systematic bias can be introduced in the measurements and not all of these bias sources can always be (easy) controlled. Second, most genomics experiments are expensive and time-consuming. This often prevents a researcher from doing the necessary replicates. On the other hand, this would be the main argument to think carefully about experimental design. SAGE provides a clear example. In most cases only a single gene expression profile for each sample is constructed. Consequently, no information is available on the experimental variability for the gene expression measurements. Fortunately, for SAGE data one can safely make assumptions about the distribution of the data to obtain information about the variability of the data (Kal *et al.*, 2002). This opens the door to experimental design considerations and statistical data analysis. Unfortunately, this approach is not feasible for other types of genomics experiments where information about data variability can only be obtained through replication.

Example: Experimental Design and Statistics for DNA Microarray Experiments

The field of gene expression profiling with DNA microarrays provides the clearest examples of how experimental design can be applied to set up experiments (Kerr and Churchill, 2001; Kerr *et al.*, 2000; Yang and Speed, 2002). Nevertheless, this area is still subject of lively research and debate and new insights and approaches toward experimental design for microarrays are likely to appear.

Experiments conducted with spotted DNA microarrays are subjected to several experimental factors that may cause systematic bias and noise in the gene expression measurements. Sources of these unwanted experimental effects are, e.g., positional biases caused by print tips, nonuniform hybridization, existence of a gene specific bias for the incorporation of one dye over the second dye, spot size and shape variances, scanner nonlinearities, and variances in background due to dye sticking on the arrays or contaminations. Accounting for these systematic effects during data analysis requires careful consideration of the experimental design prior to the actual experiment. However, because of the complexity of the experiments and experimental constraints this is not easy and several designs have been proposed (Yang *et al.*, 2002). Each design has its advantages and disadvantages and some designs are dedicated to specific types of experiments such as time series experiments.

Once the data are obtained the data analysis poses a next problem. The analysis of microarray data is also an unsettled area of research and, consequently, many methods are developed and proposed for data processing and statistical analysis (Dudoit *et al.*, 2002; Rajagopalan, 2003; Smyth *et al.*, 2003). Analysis of variance (ANOVA), which has a very tight link to experimental design, seems to be very

powerful for microarray data analysis (Kerr *et al.*, 2000). ANOVA decomposes the gene expression measurements into different components, which include the true (but unknown) biological effect and one or more components for systematic bias. Effectively, after decomposition of the signals one obtains measures for the biological effects. Subsequently, bootstrapping can be used to establish the statistical significance of these effects.

In addition to the choice of the experimental design and data analysis method, the choice for a specific gene expression profiling platform may (largely) affect the outcome of the study. Several profiling platforms are available and include SAGE (Velculescu *et al.*, 1995), radioactively labeled arrays (Donovan and Becker, 2002), cDNA microarrays (Schena *et al.*, 1995), oligonucleotide microarrays (Lockhart *et al.*, 1996), and Affymetrix GeneChips (Yap, 2002). SAGE, radioactively labeled arrays, and Affymetrix are believed to produce quantitative data while the other types of arrays produce relative expression levels. There is lively debate about whether or not (and how) these platforms can be compared. Bioinformatics plays an important role in the required analysis (Huminiecki *et al.*, 2003; Ishii *et al.*, 2000; Kuo *et al.*, 2002; Staal *et al.*, 2003).

5. GENOMIC MEDICINE

It is a common belief that genomics accelerates the development of new tools for application in health care (Collins *et al.*, 2003; Subramanian *et al.*, 2001; van Ommen, 2002). Bioinformatics and medical informatics largely contribute to the development of these tools but it is imperative that physicians, basic scientists, bioinformatics investigators, and medical investigators should collaborate more closely. The two most important applications of genomic medicine are as follows:

- The identification of genes and pathways and the elucidation of their role in health and disease. This will enhance our basic understanding of the molecular processes underlying disease and identify new targets for drug development.
- Development of genomics-based diagnostic methods for the (presymptomatic) prediction of illness and the prediction of drug response. The development of diagnostic methods will force us to think about accurate molecular classifications of disease.

Genomics is already a basic tool to identify and study genes and pathways and their role in health and disease. For example, the transcriptional changes in brain after ischemia in rats were studied with oligonucleotide arrays and several pathways involved in this pathological condition were identified (Lu *et al.*, 2003). In the field of genomics-based diagnostics, several examples demonstrated the possibilities. For example, DNA microarrays have been applied to the classification of breast tumors resulting in an association of different gene expression profiles of breast tumors with their clinical prognosis ('t Veer *et al.*, 2002). Another example is the use of SELDI mass spectroscopy (Hutchens and Yip, 1993) to distinguish

neoplastic from nonneoplastic disease within the ovary (Petricoin *et al.*, 2002). Pharmacogenomics plays an important role in the development of new treatments and drugs and cardiovascular research is one of the target domains (Anderson *et al.*, 2003; Mooser *et al.*, 2003; Siest *et al.*, 2003).

Despite these first successes much work remains to be done. To systematically implement and enable genomic medicine in a hospital environment, the acquisition, storage, and integration of genomics and clinical data are crucial. However, the requirements are still beyond the scope of today's information systems. In addition, it will not always be easy to decide which molecular and clinical data need to be integrated to answer specific research questions. In addition, we need to consider issues such as the privacy of data and the acquisition and storage of patient samples.

If we are serious to further establish the field of genomic medicine then access to detailed and standardized clinical data becomes increasingly important. Unfortunately, in contrast to the many public biological databases, clinical data are not readily available. One of the exceptions is the Shared Tumor Expression Profiler (Becich, 2000), which combines pathology data, clinical information, and microarray data. The proprietary Ascenta database is an example in which clinical data are combined with Affymetrix gene expression profiles. However, such databases are not readily available to the academic community because of the high costs involved.

Finally, as we have already mentioned above, the (statistical) analysis of genomics data sets is complex and results largely depend on the quality of the data. However, the production of genomics data can in general be well controlled and will therefore be of relatively good quality. Consequently, the analysis of genomics data may be easier than the analysis of clinical data. Clinical data, other than for controlled clinical trials, are often incomplete, noisy, and difficult to reproduce because of individual variability and the subjective nature of many clinical observations. This makes the analysis of retrospective patient data difficult and results not easy to replicate or to apply in daily health care. The assembly and analysis of data sets in which molecular and clinical data are combined will be the main challenge for the near future, and their progress will dictate the pace of implementation of genomic medicine.

6. GENOMICS AND BIOINFORMATICS IN CARDIOVASCULAR RESEARCH

Compared to cancer research, only limited integrated databases have been established in the cardiovascular field, possibly by the higher complexity of the latter. Integrated research is hampered by the relatively poor availability of well-characterized and annotated patient tissue biopsies and the complexity of the diseases as clinical symptoms often develop over several decades (Lusis, 2000), hampering the performance of prospective studies. Furthermore, each of the cardiovascular subfields, like atherothrombotic disease, congenital or failing heart disease, stroke, hypertension, and interrelated fields like diabetes and metabolic

syndromes has a different focus and deals with multiple tissues and organs. Therefore, individual researchers tend to combine the different tools and databases that become available through the more general biological approaches for their personal use. A publicly available example is the Cardiac Gene Expression knowledge base (CaGE) (Bober *et al.*, 2002), which uses information in the Toronto Cardiac Gene Unit library (www.tcgu.med.utoronto.ca) and the general BodyMap database that describes tissue-specific gene expression in human and mouse, to define gene expression in left ventricle, right atria, and embryonic mouse heart. A recent review paper from Winslow and Boguski (2003) gives a good overview of current genomics and bioinformatics applications in cardiovascular medicine.

At present, cardiovascular genomics and bioinformatics research is focused on establishing gene-expression profiles for a multitude of *ex vivo* and animal model systems and limited collections of human material, to identify potential molecular diagnostics markers and new targets for drug development (Cook and Rosenzweig, 2002; Dekker *et al.*, 2002; Horrevoets *et al.*, 1999). This gene expression profiling is a formidable task and will be of limited direct importance to clinical practice in the immediate future. Two basic problems are the progressive changes in multiple gene expression profiles over the course of disease development (Doevendans *et al.*, 2001) and the limited direct relationship of genotype to phenotype (Herrmann and Paul, 2002).

6.1. Gene–Environment Interactions and Cardiovascular Disease

Cardiovascular disease has a clear-cut genetic component, with family history being a strong predictor for future outcome. Even for cardiovascular monogenetic disorders, it is well established that carriers of the same disease allele within a single family can experience quite disparate disease progression and outcome. This has been attributed to so-called modifier genes and to alleles, which in turn may modulate the disease phenotype in various ways (Corvol *et al.*, 1999; Herrmann *et al.*, 2002). Therefore, most successful classical genetic approaches have been limited to cases where disease phenotype can be accurately established and quantified, like in the case of arrythmia's (Tan *et al.*, 2001, 2003) or plasma lipid profiles (Brooks-Wilson *et al.*, 1999). In the latter case, the causative defect for Tangiers disease proved to be dysfunctional ABCA1, but genomic analysis of the transcriptome of ABCA–/– cells showed that in response to the primary defect several hundred genes show altered levels of expression, each of which might be considered potential modifier genes (Lawn *et al.*, 1999). As many complex phenotypes have been unsuccessfully challenged by classical genetics, focus for common diseases has shifted to genomics. In particular, single nucleotide polymorphisms that predispose to, rather than directly cause, a certain complex phenotype like hypertension are studied (Halushka *et al.*, 1999). A comparison of any pair of human chromosomes will reveal sites of genetic variation approximately once every 1250 bases, some of which are responsible for inherited differences in susceptibility to disease and in quantitative traits, the best-known example being Factor V Leiden, which leads to an increased risk of deep venous thrombosis (Bertina *et al.*,

1994). Many researchers use the public catalogs of SNPs (e.g. dbSNP) in combination with large-scale resequencing of candidate genes, e.g., at the cardiogenomics laboratories of Harvard (cardiogenomics.med.harvard.edu) or Inserm's Genopole (www.pasteur-lille.fr) (Blankenberg *et al.*, 2003; Cambien *et al.*, 1999). Others rely on the private SNP Consortium, which is dedicated to cataloging 300,000 SNPs within the next few years (Thorisson *et al.*, 2003) that can be accessed through the SNP Consortium Web site (snp.cshl.org). In addition to the enormous numbers of SNPs, an intrinsic complexity is caused by the fact that actual penetrance of each SNP is related to lifestyle, also known as gene–environment interactions. Therefore, genetic predisposition is usually manifested only in combination with environmental lifestyle-associated risk factors, which enormously complicates statistical analyses. For example, only common variants of the lipoprotein lipase gene in combination with unhealthy diets lead to disease (Humphries *et al.*, 1998).

6.2. Impact of Genomic Variation on Cardiovascular Medicine

As a result of the complexity of the impact of genetic variation on outcome of cardiovascular disease, many researchers have turned to inbred mouse models of vascular disease. Indeed, different strains of mice show quite different susceptibility to cardiovascular disease, even when single alterations are made to individual genes by transgenesis or knockout technology (Weiler *et al.*, 2001). This approach enables a thorough analysis of whole genome impact on common diseases like atherosclerosis through various genetic approaches combined with high-throughput sequencing of candidate regions (Allayee *et al.*, 2003; Bodnar *et al.*, 2002). A repository of all the different cardiovascular research mouse models is contained in the JAX Mice database of the Jackson Laboratories (jaxmice.jax.org). As the sequences of both human and mouse genomes have been completed, finding predisposing genes in mice can be directly transferred to human studies (Pennacchio and Rubin, 2003; Rubin and Tall, 2000). Recently, using such comparative genome sequence alignment with GenomeVista, a novel human gene was discovered, apolipoprotein AV, that has a strong inverse correlation with plasma triglyceride levels in mice. Next, genetic polymorphisms in the *APO-AV* locus in humans turned out to be significantly associated with plasma triglyceride levels in humans. Despite the power of such cross-species genomic approaches, one has to remain careful, as rodent genotype–phenotype relations not always correspond well to human ones (Corvol *et al.*, 1999). Furthermore, several differences do exist between mouse and human genomes, especially with respect to lipid homeostasis, evidenced by the lack of mouse orthologs for human proteins like apolipoprotein L (Monajemi *et al.*, 2002) and cholesteryl ester transfer protein (CETP). Variations within CETP turned out to be a strong predictor of future cardiovascular complications in humans (Blankenberg *et al.*, 2003; Klerkx *et al.*, 2003) and, moreover, show a strict correlation with patient's response to statin therapy (Kuivenhoven *et al.*, 1998). Again, like with lipoprotein L, disease outcome relies on combination of SNP with lifestyle and diet (Singaraja *et al.*, 2003). These latter studies and many other association studies between genomic

variation, SNPs, and cardiovascular disease progression or patients response to therapy emphasize that for common diseases with a clear genetic component, the application of genomics to cardiovascular research holds great promise for fast implication into clinical practice (Doevendans *et al.*, 2001; Rubin *et al.*, 2000). At present, the hunt for candidate genes involved in cardiovascular disease, using techniques like SAGE, differential display, and microarray analysis, is proceeding at a high pace (Cook *et al.*, 2002; Dekker *et al.*, 2002; Horrevoets *et al.*, 1999). These candidate genes can be used as possible targets for genomic SNP linkage analysis (Cambien *et al.*, 1999; Halushka *et al.*, 1999). The development of bioinformatics infrastructure and statistical analyses needed to link hundreds of thousands of SNPs or common variants to clear-cut and well-defined clinical phenotypes at the full genome level is still in its infancy (Kruglyak, 1999; Ohashi and Tokunaga, 2001; Tiret *et al.*, 2002). The effects of lifestyle and diet on the actual outcome of the genetic predisposition also have to be taken into account (Fisher *et al.*, 1997; Humphries *et al.*, 1998).

6.3. Future of Genomic Cardiovascular Medicine

It shall be clear that genomics offers great new possibilities for fundamental cardiovascular research and holds great promise for improved health care. Many research tools and unambiguous quantitative clinical phenotyping need to be developed before genome-wide SNP association studies can be routinely performed on sufficiently large human sample collections. In this respect, it will be crucial to make use of possibilities offered by supporting disciplines such as bioinformatics and medical informatics, not only in analyzing results but also in the design of future studies. Large integrated databases in which molecular and clinical data are combined are needed to enable an integration of the information and biomedical technologies into genomic medicine. Thus, the synergy between informatics and biomedical research will fundamentally change the current "general risk factor"-centered evidence-based medicine into tailor-made individual genomic medicine.

ACKNOWLEDGMENT. A. J. G. Horrevoets is an established investigator of the Netherlands Heart Foundation, supported through the Molecular Cardiology Program (NHS Grant 1993M007). We thank L. A. Gilhuijs-Pederson, B. D. C. van Schaik, and P. D. Moerland for providing many useful comments.

REFERENCES

Allayee, H., Ghazalpour, A., and Lusis, A. J., 2003, Using mice to dissect genetic factors in atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.* **23**:1501–1509.

Altman, R. B. and Klein, T. E., 2002, Challenges for biomedical informatics and pharmacogenomics, *Annu. Rev. Pharmacol. Toxicol.* **42**:113–133.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J., 1990, Basic local alignment search tool, *J. Mol. Biol.* **215**:403–410.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J., 1997, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* **25**:3389–3402.

Anderson, C., 1993, Genome shortcut leads to problems, *Science* **259**:1684–1687.

Anderson, J. L., Carlquist, J. F., Horne, B. D., and Muhlestein, J. B., 2003, Cardiovascular pharmacogenomics: current status, future prospects, *J. Cardiovasc. Pharmacol. Ther.* **8**:71–83.

Apweiler, R., Kersey, P., Junker, V., and Bairoch, A., 2001, Technical comment to "Database verification studies of Swiss-Prot and GenBank" BY Karp et al., *Bioinformatics* **17**:533–534.

Baxevanis, A. D., 2003, The molecular biology database collection: 2003 update, *Nucleic Acids Res.* **31**:1–12.

Bayat, A., 2002, Science, medicine, and the future: bioinformatics, *BMJ* **324**:1018–1022.

Becich, M. J., 2000, The role of the pathologist as tissue refiner and data miner: the impact of functional genomics on the modern pathology laboratory and the critical roles of pathology informatics and bioinformatics, *Mol. Diagn.* **5**:287–299.

Bertina, R. M., Koeleman, B. P., Koster, T., Rosendaal, F. R., Dirven, R. J., De Ronde, H., Van Der Velden, P. A., and Reitsma, P. H., 1994, Mutation in blood coagulation factor V associated with resistance to activated protein C, *Nature* **369**:64–67.

Binns, M., 1993, Contamination of DNA database sequence entries with *Escherichia coli* insertion sequences, *Nucleic Acids Res.* **21**:779.

Blankenberg, S., Rupprecht, H. J., Bickel, C., Jiang, X. C., Poirier, O., Lackner, K. J., Meyer, J., Cambien, F., and Tiret, L., 2003, Common genetic variation of the cholesteryl ester transfer protein gene strongly predicts future cardiovascular death in patients with coronary artery disease, *J. Am. Coll. Cardiol.* **41**:1983–1989.

Bober, M., Wiehe, K., Yung, C., Onal, S. T., Lin, M., Baumgartner, W., Jr., and Winslow, R., 2002, CaGE: cardiac gene expression knowledgebase, *Bioinformatics* **18**:1013–1014.

Bodnar, J. S., Chatterjee, A., Castellani, L. W., Ross, D. A., Ohmen, J., Cavalcoli, J., Wu, C., Dains, K. M., Catanese, J., Chu, M., Sheth, S. S., Charugundla, K., Demant, P., West, D. B., De Jong, P., and Lusis, A. J., 2002, Positional cloning of the combined hyperlipidemia gene *Hyplip1*, *Nat. Genet.* **30**:110–116.

Bork, P., and Bairoch, A., 1996, go hunting in sequence databases but watch out for the traps, *Trends Genet.* **12**:425–427.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C. A., Causton, H. C., Gaasterland, T., Glenisson, P., Holstege, F. C., Kim, I. F., Markowitz, V., Matese, J. C., Parkinson, H., Robinson, A., Sarkans, U., Schulze–Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M., 2001, Minimum information about a microarray experiment (MIAME)—toward standards for microarray data, *Nat. Genet.* **29**:365–371.

Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L. H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O., Loubser, O., Ouelette, B. F., Fichter, K., Ashbourne-Excoffon, K. J., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J., and Hayden, M. R., 1999 (Aug), Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency, *Nat. Genet.* **22**(4):336–45.

Cambien, F., Poirier, O., Nicaud, V., Herrmann, S. M., Mallet, C., Ricard, S., Behague, I., Hallet, V., Blanc, H., Loukaci, V., Thillet, J., Evans, A., Ruidavets, J. B., Arveiler, D., Luc, G., and Tiret, L., 1999, Sequence diversity in 36 candidate genes for cardiovascular disorders, *Am. J. Hum. Genet.* **65**:183–191.

Caron, H., Van Schaik, B., Van Der, M. M., Baas, F., Riggins, G., Van Sluis, P., Hermus, M. C., Van Asperen, R., Boon, K., Voute, P. A., Heisterkamp, S., Van Kampen, A., and Versteeg, R., 2001, The human transcriptome map: clustering of highly expressed genes in chromosomal domains, *Science* **291**:1289–1292.

Clark, A. G., and Whittam, T. S., 1992, Sequencing errors and molecular evolutionary analysis, *Mol. Biol. Evol.* **9**:744–752.

Collins, F. S., Green, E. D., Guttmacher, A. E., and Guyer, M. S., 2003, A vision for the future of genomics research, *Nature* **422**:835–847.

Collins, F. S., Patrinos, A., Jordan, E., Chakravarti, A., Gesteland, R., and Walters, L., 1998, New goals for the U.S. Human Genome Project: 1998–2003, *Science* **282**:682–689.

Cook, S. A., and Rosenzweig, A., 2002, DNA microarrays: implications for cardiovascular medicine, *Circ. Res.* **91**:559–564.

Corvol, P., Persu, A., Gimenez-Roqueplo, A. P., and Jeunemaitre, X., 1999, Seven lessons from two candidate genes in human essential hypertension: angiotensinogen and epithelial sodium channel, *Hypertension* **33**:1324–1331.

Dahlquist, K. D., Salomonis, N., Vranizan, K., Lawlor, S. C., and Conklin, B. R., 2002, Genmapp, a new tool for viewing and analyzing microarray data on biological pathways, *Nat. Genet.* **31**:19–20.

Dean, M., and Allikmets, R., 1995, Contamination of cDNA—libraries and expressed-sequence-tags databases, *Am. J. Hum. Genet.* **57**:1254–1255.

Dekker, R. J., Van Soest, S., Fontijn, R. D., Salamanca, S., De Groot, P. G., Vanbavel, E., Pannekoek, H., and Horrevoets, A. J., 2002, Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (Klf2), *Blood* **100**:1689–1698.

Doevendans, P. A., Jukema, W., Spiering, W., Defesche, J. C., and Kastelein, J. J., 2001, Molecular genetics and gene expression in atherosclerosis, *Int. J. Cardiol.* **80**:161–172.

Doniger, S. W., Salomonis, N., Dahlquist, K. D., Vranizan, K., Lawlor, S. C., and Conklin, B. R., 2003, MAPPFinder: using gene ontology and GenMAPP to create a global gene-expression profile from microarray data, *Genome Biol.* **4**:R7.

Donovan, D. M., and Becker, K. G., 2002, Double round hybridization of membrane based cDNA arrays: improved background reduction and data replication, *J. Neurosci. Methods* **118**:59–62.

Dudoit, S., Yang, Y. H., Cakkiw, M. J., and Speed, T. P., 2002, Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments, *Stat. Sin.* **12**:111–139.

Fisher, R. M., Humphries, S. E., and Talmud, P. J., 1997, Common variation in the lipoprotein lipase gene: effects on plasma lipids and risk of atherosclerosis, *Atherosclerosis* **135**:145–159.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A., 2003, Expasy: the proteomics server for in-depth protein knowledge and analysis, *Nucleic Acids Res.* **31**: 3784–3788.

Gonzalez, I. L., and Sylvester, J. E., 1997, Incognito Rrna and Rdna in databases and libraries, *Genome Res.* **7**:65–70.

Halushka, M. K., Fan, J. B., Bentley, K., Hsie, L., Shen, N., Weder, A., Cooper, R., Lipshutz, R., and Chakravarti, A., 1999, Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis, *Nat. Genet.* **22**:239–247.

Hamosh, A., Scott, A. F., Amberger, J., Bocchini, C., Valle, D., and Mckusick, V. A., 2002, Online Mendelian inheritance in man (OMIM), a knowledgebase of human genes and genetic disorders, *Nucleic Acids Res.* **30**:52–55.

Harrell, F. E., Jr., Lee, K. L., Califf, R. M., Pryor, D. B., and Rosati, R. A., 1984, Regression modelling strategies for improved prognostic prediction, *Stat. Med.* **3**:143–152.

Herrero, J., Al Shahrour, F., Diaz-Uriarte, R., Mateos, A., Vaquerizas, J. M., Santoyo, J., and Dopazo, J., 2003, GEPAS: a web-based resource for microarray gene expression data analysis, *Nucleic Acids Res.* **31**:3461–3467.

Herrmann, S. M., and Paul, M., 2002, Studying genotype–phenotype relationships: cardiovascular disease as an example, *J. Mol. Med.* **80**:282–289.

Herwig, R., Poustka, A. J., Muller, C., Bull, C., Lehrach, H., and O'brien, J., 1999, Large-scale clustering of cDNA—fingerprinting data, *Genome Res.* **9**:1093–1105.

Horrevoets, A. J., Fontijn, R. D., Van Zonneveld, A. J., De Vries, C. J., Ten Cate, J. W., and Pannekoek, H., 1999, Vascular endothelial genes that are responsive to tumor necrosis factor-alpha in vitro are expressed in atherosclerotic lesions, including inhibitor of apoptosis protein-1, stannin, and two novel genes, *Blood* **93**:3418–3431.

Huminiecki, L., Lloyd, A. T., and Wolfe, K. H., 2003, Congruence of tissue expression profiles from gene expression Atlas, SAGEmap and TissueInfo databases, *BMC Genomics* **4**:31.

Humphries, S. E., Nicaud, V., Margalef, J., Tiret, L., and Talmud, P. J., 1998, Lipoprotein lipase gene variation is associated with a paternal history of premature coronary artery disease and fasting and postprandial plasma triglycerides: the European Atherosclerosis Research Study (EARS), *Arterioscler. Thromb. Vasc. Biol.* **18**:526–534.

Hutchens, T. W., and Yip, T.-T., 1993, New desorption strategies for the mass spectrometric analysis of macromolecules, *Rapid Commun. Mass Spectrom.* **7:**576–580.

Ishii, M., Hashimoto, S., Tsutsumi, S., Wada, Y., Matsushima, K., Kodama, T., and Aburatani, H., 2000, Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis, *Genomics* **68**:136–143.

Kal, A. J., Van Zonneveld, A. J., Benes, V., Van Den, B. M., Koerkamp, M. G., Albermann, K., Strack, N., Ruijter, J. M., Richter, A., Dujon, B., Ansorge, W., and Tabak, H. F., 1999, Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources, *Mol. Biol. Cell* **10**:1859–1872.

Karp, P. D., 1998, What we do not know about sequence analysis and sequence databases, *Bioinformatics* **14**:753–754.

Karp, P. D., Paley, S., and Zhu, J., 2001, Database verification studies of Swiss-Prot and Genbank, *Bioinformatics* **17**:526–532.

Kerr, M. K., and Churchill, G. A., 2001, Experimental design for gene expression microarrays, *Biostatistics* **2**:183–201.

Kerr, M. K., Martin, M., and Churchill, G. A., 2000, Analysis of variance for gene expression microarray data, *J. Comput. Biol.* **7**:819–837.

Klerkx, A. H., Tanck, M. W., Kastelein, J. J., Molhuizen, H. O., Jukema, J. W., Zwinderman, A. H., and Kuivenhoven, J. A., 2003, Haplotype analysis of the CETP gene: not TaqIB, but the closely linked -629c–>a polymorphism and a novel promoter variant are independently associated with CETP concentration, *Hum. Mol. Genet.* **12**:111–123.

Kruglyak, L., 1999, Prospects for whole-genome linkage disequilibrium mapping of common disease genes, *Nat. Genet.* **22**:139–144.

Kuivenhoven, J. A., Jukema, J. W., Zwinderman, A. H., De Knijff, P., Mcpherson, R., Bruschke, A. V., Lie, K. I., and Kastelein, J. J., 1998, The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. *N. Engl. J. Med.* **338**:86–93.

Kuo, W. P., Jenssen, T. K., Butte, A. J., Ohno-Machado, L., and Kohane, I. S., 2002, Analysis of matched mrna measurements from two different microarray technologies, *Bioinformatics* **18**:405–412.

Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., Seilhamer, J. J., Vaughan, A. M., and Oram, J. F., 1999, The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway, *J. Clin. Invest.* **104**:R25–31.

Lindberg, C., 1990, The Unified Medical Language System (UMLS) of the National Library of Medicine, *J. Am. Med. Rec. Assoc.* **61:**40–42.

Lindberg, D. A., Humphreys, B. L., and Mccray, A. T., 1993, The Unified Medical Language System, *Methods Inf. Med.* **32**:281–291.

Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E. L., 1996, Expression monitoring by hybridization to high-density oligonucleotide arrays, *Nat. Biotechnol.* **14**:1675–1680.

Lu, A., Tang, Y., Ran, R., Clark, J. F., Aronow, B. J., and Sharp, F. R., 2003, Genomics of the periinfarction cortex after focal cerebral ischemia, *J. Cereb. Blood Flow Metab.* **23**:786–810.

Luscombe, N. M., Greenbaum, D., and Gerstein, M., 2001, What is bioinformatics? A proposed definition and overview of the field, *Methods Inf. Med.* **40**:346–358.

Lusis, A. J., 2000, Atherosclerosis, *Nature* **407**:233–241.

Maojo, V., and Kulikowski, C. A., 2003, Bioinformatics and medical informatics: collaboration on the road to genomic medicine? *J. Am. Med. Inform. Assoc*. **10**:515–22.

Miller, C., Gurd, J., and Brass, A., 1999, A rapid algorithm for sequence database comparisons: application to the identification of vector contamination in the EMBL databases, *Bioinformatics* **15**:111–121.

Monajemi, H., Fontijn, R. D., Pannekoek, H., and Horrevoets, A. J., 2002, The apolipoprotein L gene cluster has emerged recently in evolution and is expressed in human vascular tissue, *Genomics* **79**:539–546.

Mooser, V., Waterworth, D. M., Isenhour, T., and Middleton, L., 2003, Cardiovascular pharmacogenetics in the SNP era, *J. Thromb. Haemost.* **1**:1398–1402.

Nakao, M., Bono, H., Kawashima, S., Kamiya, T., Sato, K., Goto, S., and Kanehisa, M., 1999, Genome-scale gene expression analysis and pathway reconstruction in KEGG, *Genome Inform. Ser. Workshop Genome Inform.* **10**:94–103.

Ohashi, J., and Tokunaga, K., 2001, The power of genome-wide association studies of complex disease genes: statistical limitations of indirect approaches using SNP markers, *J. Hum. Genet.* **46**:478–482.

Pennacchio, L. A., and Rubin, E. M., 2003, Comparative genomic tools and databases: providing insights into the human genome, *J. Clin. Invest.* **111**:1099–1106.

Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C., and Liotta, L. A., 2002, Use of proteomic patterns in serum to identify ovarian cancer, *Lancet* **359**:572–577.

Rajagopalan, D., 2003, A comparison of statistical methods for analysis of high density oligonucleotide array data, *Bioinformatics* **19**:1469–1476.

Rubin, E. M., and Tall, A., 2000, Perspectives for vascular genomics, *Nature* **407**:265–269.

Ruijter, J. M., Van Kampen, A. H., and Baas, F., 2002, Statistical evaluation of sage libraries: consequences for experimental design, *Physiol. Genomics* **11**:37–44.

Sarkar, I. N., Cantor, M. N., Gelman, R., Hartel, F., and Lussier, Y. A., 2003, Linking biomedical language information and knowledge resources: GO and UMLS, *Pac. Symp. Biocomput*. 439–450.

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O., 1995, Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science* **270**:467–470.

Seluja, G. A., Farmer, A., Mcleod, M., Harger, C., and Schad, P. A., 1999, Establishing a method of vector contamination identification in database sequences, *Bioinformatics* **15**:106–110.

Siest, G., Ferrari, L., Accaoui, M. J., Batt, A. M., and Visvikis, S., 2003, Pharmacogenomics of drugs affecting the cardiovascular system, *Clin. Chem. Lab. Med.* **41**:590–599.

Singaraja, R. R., Brunham, L. R., Visscher, H., Kastelein, J. J., and Hayden, M. R., 2003, Efflux and atherosclerosis: the clinical and biochemical impact of variations in the *Abca1* gene, *Arterioscler. Thromb. Vasc. Biol.* **23**:1322–1332.

Smyth, G. K., Yang, Y. H., and Speed, T., 2003, Statistical issues in cDNA microarray data analysis, *Methods Mol. Biol.* **224**:111–136.

Spellman, P. T., Miller, M., Stewart, J., Troup, C., Sarkans, U., Chervitz, S., Bernhart, D., Sherlock, G., Ball, C., Lepage, M., Swiatek, M., Marks, W. L., Goncalves, J., Markel, S., Iordan, D., Shojatalab, M., Pizarro, A., White, J., Hubley, R., Deutsch, E., Senger, M., Aronow, B. J., Robinson, A., Bassett, D., Stoeckert, C. J., Jr., and Brazma, A., 2002, Design and implementation of microarray gene expression markup language (MAGE-ML), *Genome Biology* **3**:research0046.1–0046.9.

Spieker, N., Van Sluis, P., Beitsma, M., Boon, K., Van Schaik, B. D., Van Kampen, A. H., Caron, H., and Versteeg, R., 2001, The *Meis1* oncogene is highly expressed in neuroblastoma and amplified in cell line Imr32, *Genomics* **71**:214–221.

Staal, F. J., Van Der, B. M., Wessels, L. F., Barendregt, B. H., Baert, M. R., Van Den Burg, C. M., Van Huffel, C., Langerak, A. W., Van, D. V., V, Reinders, M. J., and Van Dongen, J. J., 2003, Dna microarrays for comparison of gene expression profiles between diagnosis and relapse in precursor-B acute lymphoblastic leukemia: choice of technique and purification influence the identification of potential diagnostic markers, *Leukemia* **17**:1324–1332.

Stein, L. D., 2003, Integrating biological databases, *Nat. Rev. Genet.* **4**:337–345.

Subramanian, G., Adams, M. D., Venter, J. C., and Broder, S., 2001, Implications of the human genome for understanding human biology and medicine, *JAMA* **286**:2296–2307.

Tan, H. L., Bezzina, C. R., Smits, J. P., Verkerk, A. O., and Wilde, A. A., 2003, Genetic control of sodium channel function, *Cardiovasc. Res.* **57**:961–973.

Tan, H. L., Bink-Boelkens, M. T., Bezzina, C. R., Viswanathan, P. C., Beaufort-Krol, G. C., Van Tintelen, P. J., Van Den Berg, M. P., Wilde, A. A., and Balser, J. R., 2001, A sodium-channel mutation causes isolated cardiac conduction disease, *Nature* **409**:1043–1047.

Thorisson, G. A., and Stein, L. D., 2003, The SNP Consortium Website: past, present and future, *Nucleic Acids Res.* **31**:124–127.

Tiret, L., Poirier, O., Nicaud, V., Barbaux, S., Herrmann, S. M., Perret, C., Raoux, S., Francomme, C., Lebard, G., Tregouet, D., and Cambien, F., 2002, Heterogeneity of linkage disequilibrium in human genes has implications for association studies of common diseases, *Hum. Mol. Genet.* **11**:419–429.

't Veer, L. J., Dai, H., Van De Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., Van Der, K. K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H., 2002, Gene expression profiling predicts clinical outcome of breast cancer, *Nature* **415:**530–536.

Van Ommen, G. J., 2002, The human genome project and the future of diagnostics, treatment and prevention, *J. Inherit. Metab. Dis.* **25**:183–188.

Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995). Serial Analysis Of Gene Expression. *Science, 270,* 484–487.

Versteeg, R., Van Schaik, B. D., Van Batenburg, M. F., Roos, M., Monajemi, R., Caron, H., Bussemaker, H. J., and Van Kampen, A. H., 2003, The human transcriptome map reveals extremes in gene density, intron length, Gc content, and repeat pattern for domains of highly and weakly expressed genes, *Genome Res.* **13**:1998–2004.

Wain, H. M., Lush, M., Ducluzeau, F., and Povey, S., 2002, Genew: the human gene nomenclature database, *Nucleic Acids Res.* **30**:169–171.

Weiler, H., Lindner, V., Kerlin, B., Isermann, B. H., Hendrickson, S. B., Cooley, B. C., Meh, D. A., Mosesson, M. W., Shworak, N. W., Post, M. J., Conway, E. M., Ulfman, L. H., Von Andrian, U. H., and Weitz, J. I., 2001, Characterization of a mouse model for thrombomodulin deficiency, *Arterioscler. Thromb. Vasc. Biol.* **21**:1531–1537.

Wenger, R. H., and Gassmann, M., 1995, Mitochondria contaminate databases, *Trends Genet.* **11**:167–168.

Wheeler, D. L., Church, D. M., Federhen, S., Lash, A. E., Madden, T. L., Pontius, J. U., Schuler, G. D., Schriml, L. M., Sequeira, E., Tatusova, T. A., and Wagner, L., 2003, Database resources of the national center for biotechnology, *Nucleic Acids Res.* **31**:28–33.

Winslow, R. L., and Boguski, M. S., 2003, Genome informatics: current status and future prospects, *Circ. Res.* **92**:953–961.

Yang, Y. H., and Speed, T., 2002, Design issues for cDNA microarray experiments, *Nat. Rev. Genet.* **3**:579–588.

Yap, G., 2002, Affymetrix, Inc., *Pharmacogenomics* **3**:709–711.

Yoshikawa, T., Sanders, A. R., and Detera-Wadleigh, S. D., 1997, Contamination of sequence databases with adaptor sequences, *Am. J. Hum. Genet.* **60**:463–466.

Yu, H., Friedman, C., Rhzetsky, A., and Kra, P., 1999, Representing genomic knowledge in the Umls semantic network, *Proc. AMIA Symp.* 181–185.

Zdobnov, E. M., Lopez, R., Apweiler, R., and Etzold, T., 2002, The Ebi Srs server—new features, *Bioinformatics* **18**:1149–1150.

Zhong, S., Li, C., and Wong, W. H., 2003, Chipinfo: software for extracting gene annotation and gene ontology information for microarray analysis, *Nucleic Acids Res.* **31**:3483–3486.

Chapter 7

RNA Interference
Mechanisms and Therapeutic Applications

Anne E. Lamers and Dominique P. V. de Kleijn

RNA interference (RNAi) is a technology developed after the recent discovery of well-conserved cellular processes that induce posttranscriptional gene silencing triggered by small fragments of double-stranded RNA. This technique is rapidly developing into a promising tool used for functional genetics and therapeutic applications. We focus here on the aspects concerning RNAi mechanism, applications in mammals, and construct design and delivery. We summarize some therapeutic applications in general and speculate on the relevance in cardiovascular medicine.

1. INTRODUCTION

An ancient process for defense against viral infections and transposons, and in higher developed organisms an endogenous process that regulates gene expression, triggered by double-stranded RNA (dsRNA) was recently revealed (for reviews see Carrington and Ambros, 2003; Hammond *et al*., 2001; Sharp, 2001). This discovery presented the scientific community with a powerful tool for sequence-specific silencing of gene expression: RNA interference (RNAi). RNAi is a technique in which any gene of which the sequence is known can be silenced in a

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

sequence-specific way by the introduction of complementary dsRNA. This newly developed technique is now widely used to conduct functional genetic studies in a fast, easy, and inexpensive way. Using the right means of transfection, the constructs can be built into the genome. However, as an advantage over genetically engineered knockout organisms, where genes are systemically and permanently knocked out, RNAi permits the spatial and temporal silencing of genes in specific tissues or organs. It also allows specific knock-down genes in human cells and in animals larger and less easily bred than mice.

Compared to the related gene-specific inhibition using "antisense" technique, ribozymes and DNAzymes, RNAi proved to be far more efficient. It can therefore be used at much lower concentrations with fewer side effects (Grunweller *et al*., 2003; Scherer and Rossi, 2003).

The final goal to develop this technique into therapeutic applications has advanced quickly to the point where the production of intruding viruses is successfully inhibited (Joost Haasnoot *et al.*, 2003), specific genes are shut down in cultured cells, and genes are knocked down *in vivo* in mice and rats (Backman *et al*., 2003; Hommel *et al*., 2003).

2. RNAi: GENERAL MECHANISMS

The main characteristic of RNAi is the sequence-specific silencing of genes by dsRNA (elaborately reviewed by Agrawal *et al*., 2003; Tijsterman *et al*., 2002). The silencing process of RNAi is initiated by the introduction of dsRNA into the cell (Fig. 1). This dsRNA is either produced within the cell or artificially transfected into the cell. An endogenous enzyme called Dicer processes the RNA hybrid into 21–23 base pairs fragments. Dicer is an RNase III-like enzyme that completes the fragments with a 2-nucleotide overhang at the 3′ end and a phosphate at the 5′ end (Bernstein *et al*., 2001). These modifications seem to be crucial for successful RNAi (Nykanen *et al.*, 2001). The obtained small dsRNA fragments are called small interfering RNAs (siRNAs) and are the pivotal elements in the interfering process.

Carrier units of Dicer present the siRNAs to another endogenous enzyme complex called RNA-induced silencing complex (RISC). In the presence of ATP, RISC incorporates the siRNA, unwinding the hybrid in the process (Nykanen *et al.*, 2001). The antisense strand, that is complementary to mRNA present in the cell, is bound to the enzyme complex; the sense strand is degraded. The RISC has become active and with the antisense RNA fragment as a probe, it recognizes the homologous sequences in circulating mRNA in the cell. The thus captured mRNA is then endonucleolytically degraded by the digesting units of RISC (Nykanen *et al.*, 2001), leading to downregulation of protein translation and consequently of the expression of the homologous gene. After cleavage of the target mRNA, the RISC complex may be recycled for another round of RNAi.

The silencing of the gene is sequence-specific as the sequence of the siRNA must be the exact complement of the target mRNA. A single mismatch appears to dramatically impede the efficiency of the silencing (Elbashir *et al*., 2001).

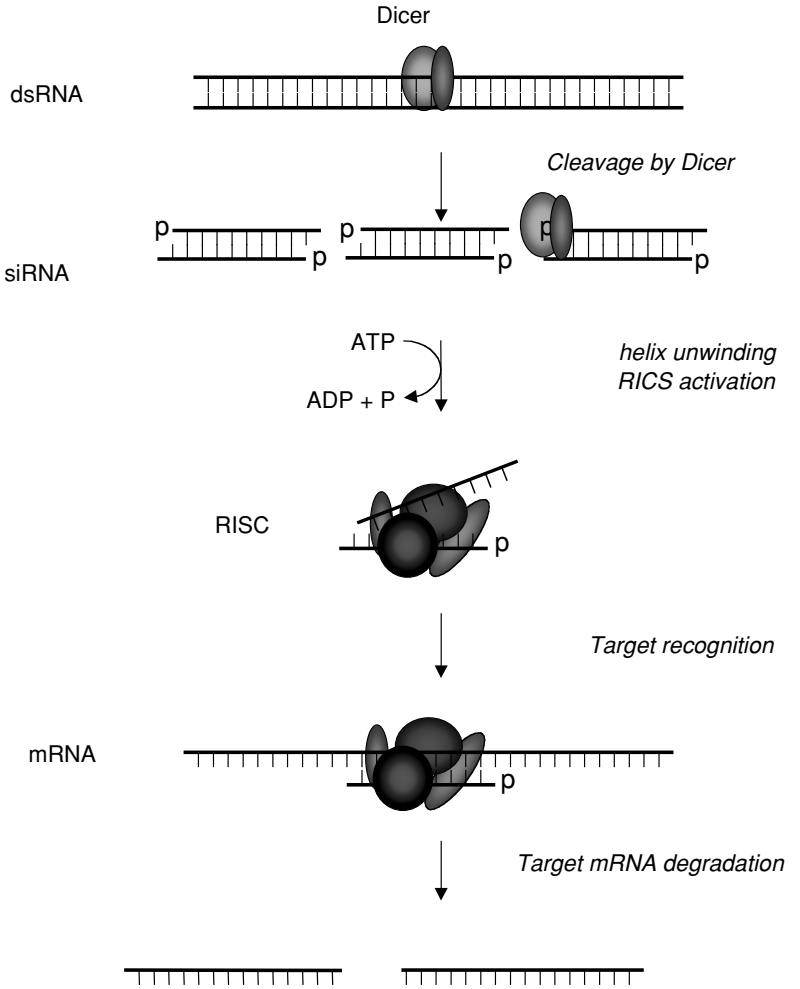

FIGURE 1. Mechanism of RNAi. dsRNA is cleaved into 19–23 nucleotide fragments: siRNA. The RNA duplex is presented to the inactive RISC. RISC is activated by the reduction of ATP and causes unwinding of the RNA double helix. The antisense strand is incorporated in the RISC and is responsible for the recognition of sequence-specific mRNA. The sense strand is discarded. Upon recognition, the mRNA is cleaved and degraded. (*See Color Plate 11.*)

Next to silencing at this posttranscriptional level, RNAi is thought to act on transcriptional level as it can hamper the actual translation of the mRNA molecule at the ribosome (Wightman *et al.*, 1993). On the level of transcription, it is reported that the siRNA when complexed with RITS (RNA-induced initiation of transcriptional gene silencing) complex can alter conformation of the genomic DNA by methylation (Wassenegger *et al.*, 1994) or condensation of chromatin and consequential silencing of specific chromosomal loci (Pal-Bhadra *et al.*, 2002; Verdel *et al.*, 2004).

The finding of this principle was soon translated into techniques to create loss-of-function phenotypes for functional genetic studies and for the development of new therapeutic techniques.

3. RNAi IN MAMMALIAN CELLS

Although the silencing by dsRNA seems to be a general phenomenon in all living cells, from plants to mammals, there are certain restrictions to consider when working with mammalian cells: In nematode worms and plants, the RNAi is distributed all through the organism and even to the next generation, when applied locally, administered in the food, or injected (Palauqui *et al.*, 1997; Vionnet and Baulcombe, 1997). In mammals it seems that RNAi is not spread systemically, but is only expressed transiently in the cells to which the dsRNA was applied.

The introduction of long molecules of dsRNA in mammalian cells initiates an interferon response, inducing nonspecific inhibition of protein synthesis (Grant *et al*., 1995; Jacobs and Langland, 1996), and cytotoxic reactions leading to cell death (Der *et al*., 1997). dsRNA fragments of 19–23 base pairs seem to be able to induce RNAi without provoking programmed death of the host cells. However, short dsRNAs are short-lived and have relatively low transfection efficiency. Furthermore, longer fragments seem to be more effective than short RNA particles, because they are more efficiently processed into more different siRNAs.

The convenient method of introducing small dsRNA fragments into the cell by hairpin-expressing plasmids (Fig. 2) can overcome these disadvantages (Kawasaki and Taira, 2003; Yu *et al*., 2002). The plasmid vector contains an expression cassette with an RNA polymerase promoter. This promoter initiates the transcription of a 19–23 nucleotide DNA sequence coding for a specific gene. This sequence is followed by a short (7–9 nucleotides) spacer and the same sequence in the antisense direction (inverted repeat). When transcribed into a single-stranded palindromic RNA, it will fold back into a hairpin structure, thus forming a short hairpin RNA (shRNA): a 19–23 base pair dsRNA with a loop at its end. This shRNA will then be a substrate for Dicer, and acquire the 3′ two-nucleotide overhang and a phosphate group at the 5′ end. Hairpin-expressing plasmids appear to produce a longer lasting RNAi effect but it is still time-limited. Additionally, the transfection rate of plasmid vectors is about 80% at the most, therefore not giving complete silencing of the gene of interest.

In our laboratory we are now cloning hairpin-expressing constructs into viral vectors. Retroviruses, adenoviruses, and lentiviruses penetrate into a wide range of target cells at a 100% efficiency rate. In the case of retroviruses (Liu *et al*., 2004), adeno-associated viruses (Hommel *et al*., 2003), and lentiviruses (Scherr *et al*., 2003; Van Den Haute *et al*., 2003), the expression cassette can be stably incorporated into the genome. This may expand the possibilities of RNAi in producing stable knock-down mammals. However, before applying these virus constructs to living mammals, probable immunological obstacles related to viral infections and,

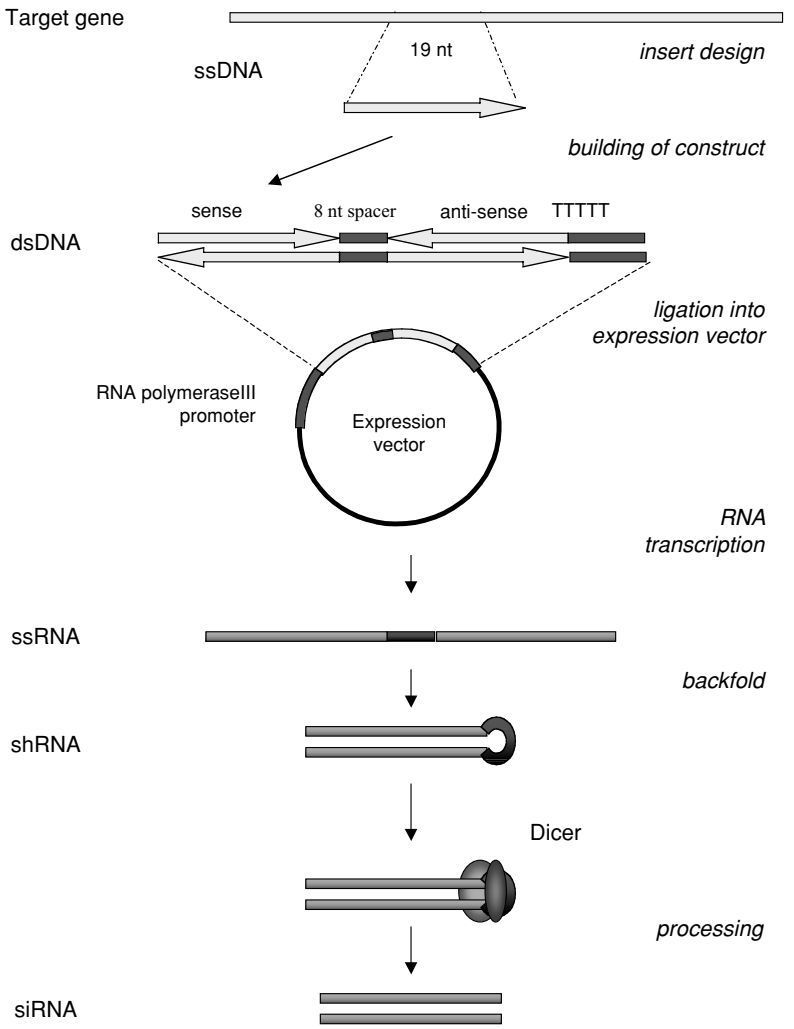

FIGURE 2. Construction of siRNA by means of hairpin-expressing vectors. A 19 nucleotide DNA sequence is designed in homology to the gene of interest. A construct is created with this sequence followed by a short spacer and the same sequence in the antisense direction (inverted repeat) and a poly-T fragment. This construct is ligated into an expression vector containing a RNA polymerase III promoter. Transfection of the vector into eukaryotic cells initiates transcription of the construct into single-stranded RNA (ssRNA). The palindromic nature of the ssRNA leads to back folding of the ssRNA into shRNA. Dicer processes shRNA into functional siRNA. (*See Color Plate 12.*)

in the case of retro- and lentiviruses, the risk of insertional mutagenesis (Trono, 2003) may have to be overcome first.

The primary effects of RNAi should be monitored at the level of mRNA. Real-time PCR, which measures amounts of mRNA of a certain gene in tissue samples, is an excellent way of determining the effect of RNAi (Sluijter *et al.*,

2003). However, the ultimate goal of RNAi is the silencing of the gene at the protein level. A limiting factor might be the turnover rate of the protein. In case a protein has an *in vivo* turnover rate of several weeks, little or no silencing might be observed at the protein level.

4. CONSIDERATIONS FOR THE DESIGN OF RNAi CONSTRUCTS

To design a successful hairpin-forming construct, the sequence of the 19-nucleotide fragment should follow a few important rules:

A 19-nucleotide sequence is preferably picked from the coding region of the gene. Sequences with relatively low cytosine/guanine percentage appear to produce most successful constructs. The fragment should have a CG content of 35–55%. Poly-T stretches in the 19-nucleotide sequence have to be avoided, for these will trigger premature termination of transcription that is initiated by RNA polymerase III promoters like U6, H1, or the modified tRNA polymerase promoter MTP.

The sequence should be 100% complementary to the target mRNA, since a single mismatch will considerably decrease the effectiveness of the construct (Elbashir *et al*., 2001). On the other hand, the sequence should have as little as possible homology to other genes to limit off-target reactions, i.e. unwanted silencing of genes that are not targeted (Jackson *et al*., 2003). For the same reason, sequences from highly conserved domains in multigene families should be avoided. It is advised to run a BLAST search (see http://www.ncbi.nlm.nih.gov/BLAST) to screen for homology with other genes. If there is a substantial homology with related or unrelated genes, the sequence should be excluded.

RISC assembles favorably the siRNA strand whose 5′ end has the greater tendency to fray (Hohjoh, 2004; Schwarz *et al.*, 2003). This translates into a design in which the 5′ end of the antisense has a base pair that is not as tightly joined as the 3′ end. A C–G bond is thermodynamically stronger than an A–U bond. The 19-nucleotide sequence, then, should preferably begin with C or G and end with A or T or even with a mismatch (A–C, A–G, T–C, T–G).

The choice of RNA polymerase promoters appears to be critical for the effective production of shRNA. RNA polymerase III promoters like U6 and H1 appear to be strong promoters for the stable expression of shRNAs. A comparative study of RNA polymerase III promoters by Boden *et al*. (2003) showed that the modified $tRNA^{met}$ derived (MTD) promoter was more effective in producing shRNA than did other polymerase III promoters such as H1 and U6, although the efficiency of the U6 promoter could be increased when extended by 27 nucleotides. Boden *et al.* (2003) also showed that a successful production of shRNA does not necessarily mean an equally successful destruction of complementary mRNA. What should be taken into account is that the U6 promoter is active in the nucleus whereas Dicer is a cytoplasm enzyme. MTD, on the other hand, is activated in the cytoplasm (Kawasaki and Taira, 2003) and therefore delivers the shRNA into the direct vicinity of Dicer, making it a more available target for processing into siRNA.

Even if these general rules are followed it seems that some designs are more efficient than others. It is therefore advised to choose several different sites (two to four) along the target mRNA sequence.

5. RNAi AS A THERAPEUTIC TOOL

The first reports have appeared of successful RNAi in mammals using various means of delivery into the cell. Several studies have used RNAi to inhibit replication of viruses such as HIV (Novina *et al.*, 2002; Park *et al*., 2003), hepatitis B and C, polio, dengue, influenza A (reviewed by Joost Haasnoot *et al*., 2003), and the SARS associated corona virus (He *et al*., 2003) in cultured cells. Also, oncology has also rapidly embraced RNAi technology. Duursma and Agami (2003) showed the stable suppression of oncogenic K-Ras in tumor cells by RNAi.

Next to this, RNAi appeared to be successful in repressing viruses in live mammals: McCaffrey *et al.* (2002) showed an *in vivo* inhibition of reproduction of hepatitis C virus proteins in adult mice by intravenously injecting plasmids promoting siRNA production. Zhang *et al.* (2003) showed *in vivo* knock-down of gene expression in rat brain tumor. Tumorigenecity was stably suppressed in mice by Brummelkamp *et al.* (2003) and tyroxine hydroxilase gene was locally knocked down in the midbrain of adult mice (Backman *et al*., 2003; Hommel *et al*., 2003). At the same time, the first transgenic siRNA expressing knock-down mice and rats have been reported (Hasuwa *et al*., 2002; Hommel *et al.*, 2003).

However, with the efficiency of inhibition of gene expression still not 100%, and since all target cells should be transfected to obtain maximal inhibition, the way to clinical applications is still a long way ahead. Therefore, it seems that the delivery of RNAi inducing vectors is the time-limiting step toward the use of RNAi in gene therapy in humans in the near future.

The use of tissue-specific promoters may allow a more localized application to tissue-specific disorders. For instance, the Tie-2 promoter is specifically activated in endothelial tissue (Teng *et al*., 2002). Using this Tie-2 promoter in an expression cassette could initiate the transcription of an RNAi construct exclusively in endothelial tissue. However, one has to take into account that the RNA polymerase II-type Tie-2 promoter is less efficient in transcribing small stem-loop transcripts like shRNAs than RNA polymerase III promoters. Since arteries and the heart can be reached by catheters, it might be possible to treat vascular diseases like stenosis and atherosclerosis with RNAi inducing vectors locally at the intimal side. Additionally, RNAi vectors can be applied locally at the adventitional side of the artery in a cuff placed around the vessel during surgical intervention. In this way RNAi constructs can specifically interfere with the development of pathological processes like neo intima formation and arterial shrinkage after bypass surgery or arterio-venous shunting for dialysis patients.

6. CONCLUSION

Although the development of RNAi in therapeutic field appears to be in the fast lane, there are still several problems to overcome before the technique can be applied to human patients. The RNAi technology, however, is so promising that it challenges scientists to work on an efficient and safe system to deliver siRNA into mammalian cells and live organisms to make the evaluation possible in clinical trials. Overall, it seems that RNAi is rapidly developing into a technology that can help us better understand and eventually treat cardiovascular diseases.

REFERENCES

Agrawal, N., Dasaradhi, P. V., Mohmmed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K., 2003, RNA interference: biology, mechanism, and applications, *Microbiol. Mol. Biol. Rev.* **67**:657–685.

Backman, C., Zhang, Y., Hoffer, B. J., and Tomac, A. C., 2003, Short interfering RNAs (siRNAs) for reducing dopaminergic phenotypic markers, *J. Neurosci. Methods* **131**:51–56.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J., 2001, Role for a bidentate ribonuclease in the initiation step of RNA interference, *Nature* **409**:363–366.

Boden, D., Pusch, O., Lee, F., Tucker, L., Shank, P. R., and Ramratnam, B., 2003, Promoter choice affects the potency of HIV-1 specific RNA interference, *Nucleic Acids Res.* **31**:5033–5038.

Brummelkamp, T. R., Bernards , R., and Agami, R., 2002, Stable suppression of tumorigenicity by virus-mediated RNA interference, *Cancer Cell* **2**:243–247.

Carrington, J. C., and Ambros, V., 2003, Role of micro RNAs in plant and animal development, *Science* **301**:336–338.

Der, S. D., Yang, Y. L., Weissmann, C., and Williams, B. R. G., 1997, A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis, *Proc. Natl. Acad. Sci. U.S.A.* **94**:279–3283.

Duursma, A. M., and Agami, R., 2003, Ras interference as cancer therapy, *Sem. Cancer Biol.* **13**:267–273.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T., 2001 (May 24), Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* **411**(6836):494–8.

Grant, C. E., Vasa, M. Z., and Deeley, R. G., 1995, cIRF-3, a new member of the interferon regulatory factor (IRF) family that is rapidly and transiently induced by dsRNA, *Nucleic Acids Res.* **23**:2137–2146.

Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A., and Kurreck, J., 2003, Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-*O*-methyl RNA, phosphorothioates and small interfering RNA, *Nucleic Acids Res.* **31**:3185–3193.

Hammond, S. M., Caudy, A. A., and Hannon, G. J., 2001, Post-transcriptional gene silencing by double-stranded RNA, *Nat. Rev. Genet.* **2**:110–119.

Hasuwa, H., Kaseda, K., Einarsdottir, T., and Okabe, M., 2002, Small interfering RNA and gene silencing in transgenic mice and rats, *Fed. Eur. Biochem. Soc. Lett.* **532**:227–230.

He, M. L., Zheng, B., Peng, Y., Peiris, J. S., Poon, L. L., Yuen, K. Y., Lin, M. C., Kung, H. F., and Guan, Y., 2003, Inhibition of SARS-associated coronavirus infection and replication by RNA interference, *J. Am. Med. Assoc.* **290**:2665–2666.

Hohjoh, H., 2004, Enhancement of RNAi activity by improved siRNA duplexes, *Fed. Eur. Biochem. Soc. Lett.* **557**:193–198.

Hommel, J. D., Sears, R. M., Georgescu, D., Simmons, D. L., and DiLeone, R. J., 2003, Local gene knockdown in the brain using viral-mediated RNA interference, *Nat. Med.* **9**:1539–1544.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S., 2003, Expression profiling reveals off-target gene regulation by RNAi, *Nat. Biotechnol.* **21**:635–637.

Jacobs, B. L., and Langland, J. O., 1996, When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA, *Virology* **219**:339–349.

Joost Haasnoot, P. C., Cupac, D., and Berkhout, B., 2003, Inhibition of virus replication by RNA interference, *J. Biomed. Sci.* **10**:607–616.

Kawasaki, H., and Taira, K., 2003, Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells, *Nucleic Acids Res.* **31**:700–707.

Liu, C. M., Liu, D. P., Dong, W. J., and Liang, C. C., 2004, Retrovirus vector-mediated stable gene silencing in human cell, *Biochem. Biophys. Res. Commun.* **313**:716–720.

McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A., 2002, RNA interference in adult mice, *Nature* **418**:38–39.

Novina, C. D., Murray, M. F., Dykxhoorn, D. M., Beresford, P. J., Riess, J., Lee, S. K., Collman, R. G., Lieberman, J., Shankar, P., and Sharp, P. A., 2002, siRNA-directed inhibition of HIV-1 infection, *Nat. Med.* **8**:681–686.

Nykanen, A., Haley, B., and Zamore, P. D., 2001, ATP requirements and small interfering RNA structure in the RNA interference pathway, *Cell* **107**:309–321.

Palauqui, J. C., Elmayan, T., Pollien, J. M., and Vaucheret, H., 1997, Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions, *Eur. Mol. Biol. Organ. J.* **16**:4738–4745.

Pal-Bhadra, M., Bhadra, U., and Birchler, J. A., 2002, RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*, *Mol. Cell* **9**:315–327.

Park, W. S., Hayafune, M., Miyano-Kurosaki, N., and Takaku, H., 2003, Specific HIV-1 env gene silencing by small interfering RNAs in human peripheral blood mononuclear cells, *Gene Ther.* **10**:2046–2050.

Scherer, L. J., and Rossi, J. J., 2003, Approaches for the sequence-specific knockdown of mRNA, *Nat. Biotechnol.* **21**:1457–1465.

Scherr, M., Battmer, K., Ganser, A., and Eder, M., 2003, Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA, *Cell Cycle* **2**:251–257.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D., 2003, Asymmetry in the assembly of the RNAi enzyme complex, *Cell* **115**:199–208.

Sharp, P. A., 2001, RNA interference—2001, *Genes Dev.* **15**:485–490.

Sluijter, J. P. G., Smeets, M. B., Pasterkamp, G., and de Kleijn, D. P. V., 2003, Methods in molecular cardiology: quantitative real-time polymerase chain reaction, *Neth. Heart J.* **11**:401–404.

Teng, P. I., Dichiara, M. R., Komuves, L. G., Abe, K., Quertermous, T., and Topper, J. N., 2002, Inducible and selective transgene expression in murine vascular endothelium, *Physiol. Genomics* **11**:99–107.

Tijsterman, M., Ketting, R. F., and Plasterk, R. H., 2002, The genetics of RNA silencing, *Annu. Rev. Genet.* **36**:489–519.

Trono, D., 2003, Virology. Picking the right spot, *Science* **300**:1670–1671.

Van Den Haute, C., Eggermont, K., Nuttin, B., Debyser, Z., and Baekelandt, V., 2003, Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain, *Hum. Gene Ther.* **14**:1799–1807.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I., and Moazed, D., 2004, RNAi-mediated targeting of heterochromatin by the RITS complex, *Science* **303**(5658):672–6.

Vionnet, O., and Baulcombe, D. C., 1997, Systemic signalling in gene silencing, *Nature* **389**(6651):553.

Wassenegger, M., Heimes, S., Riedel, L., and Sanger, H. L., 1994, RNA-directed de novo methylation of genomic sequences in plants, *Cell* **76**:567–576.

Wightman, B., Ha, I., and Ruvkun, G., 1993, Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*, *Cell* **75**:855–862.

Yu, J. Y., DeRuiter, S. L., and Turner, D. L., 2002, RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells, *Proc. Natl. Acad. Sci. U.S.A.* **99**:6047–6052.

Zhang, Y., Boado, R. J., and Pardridge, W. M., 2003, In vivo knockdown of gene expression in brain cancer with intravenous RNAi in adult rats, *J. Gene Med.* **5**:1039–1045.

Part II

Applications

Chapter 8

Stem Cells and Cardiomyocytes

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1. INTRODUCTION

Chronic heart diseases such as ischemia and hypertensive heart pathologies are characterized by irreversible loss of cardiomyocytes. Although terminally differentiated cardiomyocytes have been described as showing some evidence of mitotic division in the heart (Soonpaa and Field, 1994), the generally accepted paradigm in contemporary cardiology is that adult cardiomyocytes lack the ability to regenerate the myocardium because they proliferate only up to the time of birth. The concept has been questioned recently by two reports (Beltrami *et al.*, 2003; Urbanek *et al.*, 2003) describing a resident stem cell population in human and rodent hearts that proliferate and, at least in the case of the rat, are multipotent and able to give rise to cardiomyocytes, vascular smooth muscle cells, and endothelial cells. While the robustness of these findings still requires independent verification, cell transplantation for the treatment of cardiac disease remains an attractive concept and finding the most suitable source of cells for this purpose is one of the most exciting challenges of experimental cardiology at the present time. Stem cells are a much-discussed source of cells for transplantation, derived either from adult human tissues ("adult stem cells") or from human embryos ("embryonic stem cells").

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

Both are considered as options deserving further research (reviewed in Passier and Mummery, 2003). However, in the light of ethical reservations about the use of human embryonic stem (ES) cells in many countries, it has been difficult in the ensuing debate to obtain a balanced view of scientific developments. Here, we provide a brief introduction to adult and ES cells, describing the advantages and disadvantages of each with respect to transplantation, and provide the scientific background for understanding the current strategies being used to produce and identify the most suitable cells for restoring cardiac function. Understanding how cardiac cell fate is specified by extracellular cues and how the electrical phenotype of stem cell-derived cardiomyocytes compares with that of the normal counterparts is essential if cell transplantation therapy is to become useful in restoring cardiac function.

1.1. Embryonic and Adult Stem Cells

Stem cells are primitive cells present in all organisms that can divide and give rise to more stem cells or become more specialized cells, such as those of heart, brain, muscle, and kidney. Stem cells in early embryos give rise to cells of all tissues of the adult body and are therefore termed *pluripotent*. Stem cells of adult tissues, involved in tissue replacement and repair, usually give rise only to cell types already present in the surrounding tissues from which they are derived. Stem cells of the bone marrow, for example, give rise to hematopoietic cells. These adult stem cells are generally regarded as *multipotent*, although research driven by the attractive possibility that autologous transplantation could result from the use of adult stem cells has suggested that adult stem cells may be more "plastic" than originally thought and able to undergo translineage differentiation, at least *in vivo*. However these findings are still controversial. Other (adult) tissues that contain a resident stem cell population include the intestine, adipose tissue, hair follicles of skin, parts of the brain, and peripheral blood, which includes umbilical cord blood.

The first ES cells were isolated more than 20 years ago from mouse preimplantation embryos 3.5 days after fertilization. The background to the discovery lay in the study of teratocarcinomas, spontaneous tumors of the testis in mice and humans, consisting of tissues as diverse as hair, muscle, bone, and even complete teeth and resembling a disorganized fetus. In the mid-1970s, developmental biologists discovered that teratocarcinomas could be induced in mice by transferring embryos to extrauterine sites and that they contained undifferentiated stem cells. These embryonal carcinoma (EC) stem cells could be isolated and grown in culture without losing the capacity to differentiate. This was most strikingly demonstrated by introducing them into embryos; if derived from a brown mouse and placed in a blastocyst from an albino, the pups delivered by the foster mother were brown and white. The stem cells had formed "chimeras" and had contributed to all somatic tissues, most obviously in the skin. After differentiation, EC cells are no longer malignant; they therefore became useful not only for studying development, but also for studying the loss of tumorigenic potential. Among the mouse EC cell lines derived at this time, P19 EC cells have proven a particularly useful model system for studying differentiation of stem cells to cardiomyocytes [induced by aggregation of the cells in the presence of dimethylsulfoxide (DMSO)] and neural

cells (induced by aggregation in the presence of retinoic acid) (McBurney *et al.*, 1982; for review of P19 EC cells, see van der Heyden and Defize, 2003). Developmental biologists then tried to isolate stem cells directly from mouse embryos, without an intermediate teratocarcinoma stage. In 1981, two groups succeeded in establishing mouse ES cell lines (Evans and Kaufman, 1981; Martin, 1981).

In view of the similarities between mice and human teratocarcinomas, it was predicted that ES cells could be isolated from human embryos. The motivation initially was for studying early human development but later, the perspectives for cell transplantation therapies became evident. First attempts were made in the mid-1980s, when embryos could not be cryopreserved and excess were discarded after *in vitro* fertilization. The attempts were unsuccessful and were mostly discontinued when freezing of embryos became common practice. Exceptionally, Thomson in the United States continued, first in primates and then in humans; in 1998 he succeeded. The markers developed by pathologists to diagnose EC stem cells in human tumors provided the first evidence of their undifferentiated phenotype; their capacity to form teratocarcinomas containing many tissue types in immunodeficient mice confirmed their pluripotency (Thomson *et al.*, 1998).

A second group (Reubinoff *et al.*, 2000) later described the isolation of two human ES cell lines independently. Since then, a handful of publications have described their differentiation to various cell types in culture in response to cytokines, hormones, and growth conditions. These include neural cells (neurons, glia, and oligodendrocytes) and, most recently, insulin-producing pancreas cells, cartilage and bone, cardiomyocytes, hematopoietic cells, and hepatocytes.

1.2. Stem Cells and Tissue Repair

The ability of human ES cells to differentiate to cells from all three germ layers (ectoderm, endoderm, and mesoderm) in culture is still a long way from contributing to regenerative medicine. Speculation here is being driven primarily by experiments in rodents with a variety of experimentally induced or genetic lesions. Mouse ES-derived cardiomyocytes have been transplanted to the heart and survived (Klug *et al*., 1996), rats with spinal cord lesions receiving mouse ES cells were again able to bear their own weight (McDonald *et al*., 1999), transplantation of ES-derived neural cells resulted in functional improvement in mice with Parkinson-like lesions (Kim *et al.*, 2002), and myelin became detectable around axons in mutant rats lacking myelin after transfer of mouse ES-derived glial cells (Brustle *et al.*, 1999), suggesting a potential treatment for multiple sclerosis (reviewed in Passier and Mummery, 2003).

Success stories have recently been extended to adult stem cells, particularly from bone marrow. Simple injection of selected bone marrow cells to rodents with myocardial infarction spectacularly restored short-term function and survival (Orlic *et al.*, 2001). Bone marrow cells have also been shown to become neuron-like, and most recently, a single bone marrow cell has been shown to multiply and contribute to lung tissue, liver, intestine, and skin, albeit in very low numbers (Krause *et al.*, 2001). Fetal, neonatal, mouse ES cell-derived, and skeletal myoblasts have also been shown to engraft in the myocardium (reviewed Dowell

et al., 2003) although in the case of mES-derived cardiomyocytes, survival after transplantation to an infarcted mouse heart has been problematic (Johkura *et al.*, 2003; Klug *et al.*, 1996). In the studies of skeletal myoblasts transplanted to rat infarcted myocardium, the cells were functionally isolated from their host heart tissue (Leobon *et al.*, 2003); this may explain in part the episodes of ventricular tachycardia recently observed in myoblast transplantation in humans (Menasche *et al.*, 2003) and is perhaps a warning against clinical trials prior to completion of extensive animal experimentation.

Most recent evidence suggests that direct injection of undifferentiated bone marrow cells into animal leads to *apparent* transdifferentiation but in fact all differentiated cells are the result of cell fusion only, at least in liver, brain, and heart (Alvarez-Dolado *et al.*, 2003). This would not then appear to be a useful strategy. The question thus arises whether adult stem cells from any source can be adequately expanded and induced to differentiate in culture to produce sufficient cell numbers for cell therapy on patients. Expansion of adult stem cells in culture is reasonably successful for rodents, but much less so for humans. Nevertheless, this is where research on human embryonic and adult stem cells will need to go hand-in-hand: in both cases, expansion of the cells in culture ("upscale") and efficient induction of differentiation to the required cell type (ventricular cardiomyocytes) is a prerequisite for any cell-based cardiac therapy. As many as 10^9 cardiomyocytes would be required to replace those lost after myocardial infarction. In the following section we will review our understanding of the molecular mechanisms controlling heart cell development in the embryo in order to develop strategies for inducing cardiomyocyte differentiation of stem cells.

2. SIGNALING PATHWAYS AND SPECIFICATION OF CARDIAC CELL FATE BY EXTRACELLULAR CUES

The process of forming a heart in a developing embryo requires many signals, some of which originate outside the precardiac mesoderm and some of which originate in the developing heart. The fruit fly *Drosophila melangster* has been a particularly useful source of insights into the molecular control of heart development in mammals. In the following sections we will focus on the three main signal transduction pathways that emerged from these studies as being essential for heart development in *Drosophila* and proved to have a similar importance in heart development in mammals. These signaling pathways are those activated by the bone morphogenetic protein (BMP), Wnt, and fibroblast growth factor (FGF) families of ligands.

2.1. BMPs

BMPs are members of the transforming growth factor β superfamily with multiple functions in the control of growth and differentiation in development (reviewed in Hogan, 1996). More than 30 different ligands of this family are now known. In combination with leukemia inhibitory factor, BMP4 has recently been

shown to be sufficient to keep mES cells pluripotent and undifferentiated in culture, provided the medium is serum-free (Chambers *et al.*, 2003). *Drosophila* have only one BMP gene, *decaptaplegic* or *dpp*, which is expressed in the developing dorsal mesoderm and in cells that are known to induce heart development (Frasch, 1995). Ectopic expression of *dpp* in the noncardiac mesoderm results in ectopic differentiation of dorsal vessel cells (*Drosophila* have an open circulatory system and thus do not have a true heart but instead have a dorsal vessel responsible for pumping hemolyph throughout the body), illustrating its cardiogenic activity (Yin and Frasch, 1998). *Drosophila* lacking *dpp* develop neither dorsal vessel cells nor heart precursors (Frasch, 1995). *dpp* acts through SMAD-binding sites to activate *tinman*, a master transcription factor in heart development which when mutated results in flies lacking hearts (Frasch, 1995; Xu *et al.*, 1998). Although *dpp* is required for cardiomyogenesis, it is however not sufficient for heart formation in *Drosophila* (Yin and Frasch, 1998).

In mammalian cells, BMPs control several major cardiac-specific transcription factors, including Nkx 2-5, the mammalian homologue of *tinman*, through SMAD-binding sites (Brand, 2003; Liberatore *et al.*, 2002; Lien *et al.*, 2002). BMP2, 4, and 7 are co-expressed spatially and temporally with GATA-4 and Nkx 2-5 in the precardiac mesoderm, again suggesting they may control the expression of cardiac transcription factors (Andree *et al.*, 1998; Schultheiss *et al.*, 1997;). As in *Drosophila*, treatment of mammalian embryos with BMP results in the ectopic expression of Nkx 2-5 and GATA-4 (Schultheiss *et al.*, 1997). The addition of noggin, a BMP antagonist, to developing embryos inhibits differentiation of cardiac cells (Schlange *et al.*, 2000; Schultheiss *et al.*, 1997). Mouse *BMP2*$^{-/-}$ mutants have abnormal heart development, but heart precursors develop normally suggesting that BMP2 is not required for the induction of cardiomyocytes, but for their subsequent organization (Zhang and Bradley, 1996). BMP2 is expressed in endoderm, a tissue adjacent to the heart-forming region and containing the most cardiac inductive activity (Schultheiss *et al.*, 1997). BMP5 and 7 double knockout mice have severely delayed cardiac morphogenesis (Solloway and Robertson, 1999). DMSO-induced cardiac differentiation of P19 EC cells appears to be mediated by BMP2 and 4 since stable expression of noggin blocks the formation of beating muscle (Monzen *et al.*, 1999, 2001). In P19Cl6 EC cells, a clonal line derived from P19 EC cells with an enhanced ability to form cardiomyocytes (Habara-Ohkubo, 1996), BMP2 and 4 are already expressed in the undifferentiated cells and their expression remains detectable throughout DMSO-induced differentiation, perhaps contributing to their enhanced cardiogenic potential (Monzen *et al.*, 1999). As seen *in vivo* in developing mouse embryos and in P19 EC cells, overexpression of noggin in P19Cl6 inhibits the DMSO-induced differentiation into cardiomyocytes (Monzen *et al.*, 1999).

2.2. Wnts

Drosophila has only one Wnt gene, *wingless* (*wg*), which is required for all aspects of cardiomyogenesis; in mutants lacking wingless, no heart precursors form (Wu *et al.*, 1995). *wg* and *dpp* appear to cooperate in heart development, since

cardiomyocyte precursors form only where *dpp* and *wg* are co-expressed (Wu *et al.*, 1995) and this in turn coincides with upregulation of *tinman* (Wu *et al.*, 1995). *wg* has two known targets in *Drosophila*: *sloppy paired* (*slp*) and *even-skipped* (Halfon *et al.*, 2000; Knirr and Frasch, 2001; Park *et al.*, 1996; Lee and Frasch, 2000). Although it has been shown that loss of *slp* mimics the complete lack of cardiac precursors in *wg* mutants, the exact mechanism through which this occurs is still unknown (Lee and Frasch, 2000). *Even-skipped* contains SMAD-, *tinman*-, and *wg*-binding sites and is a homeobox gene involved in cardiomyogenesis, although its role is poorly understood (Halfon *et al.*, 2000; Knirr and Frasch, 2001).

Wnt signaling in vertebrate cardiomyogenesis is more complex. The "canonical" Wnts 1, 3A, and 8, acting by inhibition of GSK3 allowing nuclear localization of β-catenin, appear to inhibit cardiac differentiation, whereas the noncanonical Wnt 11, involving protein kinase C, seems to enhance cardiac differentiation (reviewed by Olson and Schneider, 2003). Supporting this, inhibition of the canonical Wnt signaling increases cardiac differentiation (Schneider and Mercola, 2001) and that conditional deletion of β-catenin from the definitive endoderm of the mouse results in the formation of multiple ectopic hearts (Lickert *et al.*, 2002). The presence of crescent, dkk-1, or other Wnt antagonists induce cardiomyocyte differentiation in nonheart producing mesoderm (Marvin *et al.*, 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). Wnts 3A and 8 block Nkx 2-5 and Tbx expression and therefore cardiomyocyte differentiation (Marvin *et al.*, 2001; Schneider and Mercola, 2001). Recent findings showing that β-catenin signaling must be blocked for heart valve formation in zebrafish also demonstrate a negative role for Wnts in the development of the heart (Hurlstone *et al.*, 2003). However, the role of the canonical Wnts as a cardiac inhibitor has been questioned recently by studies showing that Wnts 3A and 8, in addition to β-catenin, are required for cardiomyocyte differentiation of P19Cl6 cells (Nakamura *et al.*, 2003). Wnt 11, a noncanonical Wnt, can induce cardiomyogenesis in the non-precardiac mesoderm of chick embryos (Eisenberg and Eisenberg, 1999). This may be a result of its ability to block canonical Wnt signaling by sequestering cytoplasmic β-catenin and inducing cardiomyogenesis in P19Cl6 (Pandur *et al.*, 2002). In the light of recent data showing that on the one hand Wnts can act as growth factors for stem cells (Reya *et al.*, 2003; Willert *et al.*, 2003) yet on the other can control differentiation in the positive or negative sense at later stages of development, it may be assumed that the response of an individual cell depends on the combination of cofactors (inhibitors and activators) also expressed at the time at which the signal is received.

2.3. FGFs

Drosophila have only two FGF receptors and only one of them, *heartless* (*htl*), is expressed in the mesoderm in early gastrulation and during differentiation; it is required for normal cardiac development (Gisselbrecht *et al.*, 1996; Michelson *et al.*, 1998; Narasimha and Leptin, 2000). *htl* mutants have no dorsal vessel (Narasimha and Leptin, 2000). This is probably due in part to a role in directing

the migration of cardiac precursors in the mesoderm (reviewed in Narasimha and Leptin, 2000). However, in addition to the ability of *htl* to direct migration, it also plays a direct role in cardiomyogenic specification; blocking *htl* function after migration has occurred also blocks cardiac precursor specification (Michelson *et al.*, 1998).

Although there are nine FGF family members in vertebrates, not all have been implicated in heart development (Slavin, 1995). FGFs 1, 2, and 4 have been identified as being secreted by the portion of the developing embryo that is necessary for cardiac development (Parlow *et al.*, 1991; Zhu *et al.*, 1996), although their exact role is unclear. What is clear is that they cooperate with BMPs to induce cardiomyogenesis (Barron *et al.*, 2000). In chick embryos, FGF2 and 4 induce cardiomyogenesis in non-precardiac mesoderm, but the differentiation is much more efficient if BMP2 or 4 is also present (Barron *et al.*, 2000; Ladd *et al.*, 1998). Another family member, FGF8, is expressed in the cardiac mesoderm of mice, chicks, and zebrafish (Alsan and Schultheiss, 2002; Crossley and Martin, 1995; Reifers *et al.*, 2000). Again, BMPs seem to cooperate with FGF8 in heart induction and morphogenesis; for example, In chick embryos BMP2 concentration regulates FGF8 expression (Alsan and Schultheiss, 2002). Zebrafish with FGF8 mutations do not express Nkx 2-5 or GATA-4 in the precardiac mesoderm and have severely deformed ventricles (Reifers *et al.*, 2000). This may be directly or indirectly due to the fact that these mutant zebrafish have less Nkx 2-5 and GATA-4 expression (Reifers *et al.*, 2000).

3. TRANSCRIPTION FACTORS

The transcription factors utilized to form the heart of a developing embryo are tied together in a complex signaling pathway (Fig. 1). Without correctly timed expression of several families of transcription factors, the developing embryo would quickly die (reviewed in Bruneau, 2002; Cripps and Olson, 2002; Doevendans and van Bilsen, 1996). In brief, GATA4, Nkx 2-5, and MEF2 are thought to regulate each other and in turn regulate the more specific muscle transcription factors and proteins necessary for muscle contraction (Black and Olson, 1998). Evidence of the ability of GATA-4, Nkx 2-5, and MEF2 to regulate each other is seen in P19 EC cells where the overexpression of Nkx 2-5 leads to an upregulation of GATA-4 and MEF2C, and likewise the overexpression of MEF2c leads to an upregulation of Nkx 2-5 and GATA-4 (Skerjanc *et al.*, 1998).

3.1. GATA Family

The GATA family of transcription factors is one of the first to be expressed in cells that can be identified as precardiac (Grèpin *et al.*, 1997; Heikinheimo *et al.*, 1994). *Drosophila* have only one GATA gene, *pannier*, which is required for cardiomyogenesis, and embryos lacking *pannier* do not form any heart structures (Gajewski *et al.*, 1999). Conversely, the overexpression of *pannier* leads to an

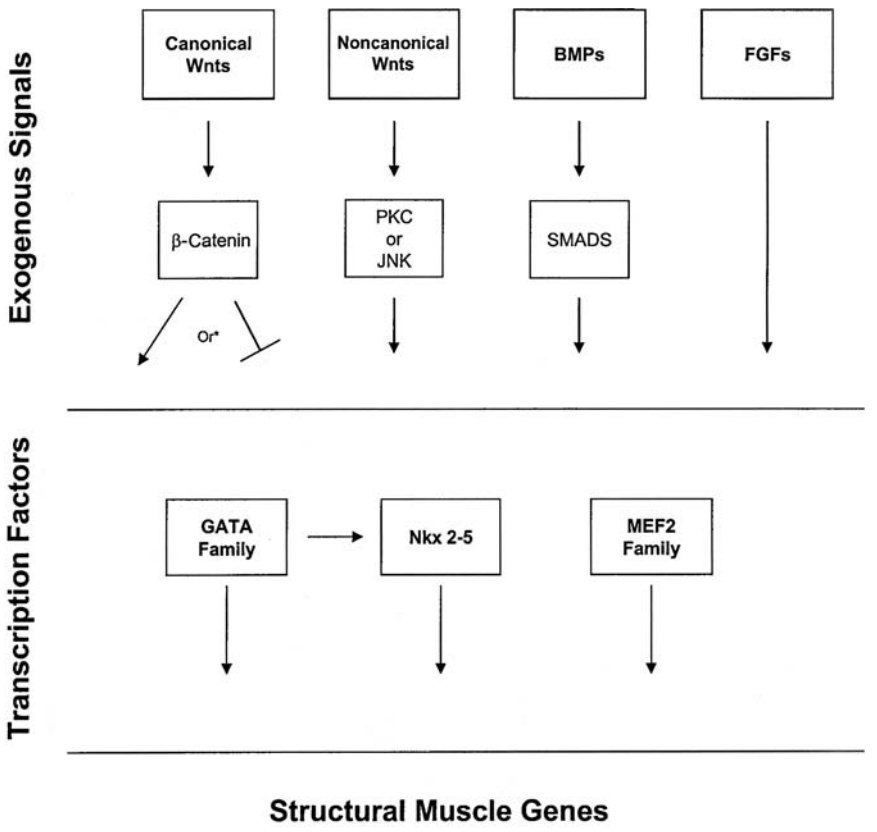

FIGURE 1. Schematic diagram representing some of the major exogenous signaling factors and important transcription factors in cardiac development.

embryo that contains excess cardiomyocytes and lacks other cell types derived from the dorsal mesoderm (Gajewski *et al.*, 1999).

Although there are six GATA family members in mammals, only GATA-4 to -6 are expressed in the heart (Molkentin, 2000). During mouse embryogenesis, GATA-4 is first expressed in the early precardiac mesoderm and is then expressed in the heart throughout development (Heikinheimo *et al.*, 1994; Jiang and Evans, 1996; Laverriere *et al.*, 1994). Mice embryos lacking GATA-4 have reduced numbers of cardiomyocytes and bilateral heart tubes (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). Analysis of mouse ES and EC cells lacking GATA-4 has shown that endoderm and early mesoderm markers are unchanged, but that areas of beating muscle are never formed (Grèpin *et al.*, 1995, 1997; Soudais *et al.*, 1995). As seen in *Drosophila*, overexpression of GATA-4 increases the efficiency of beating

muscle formation in P19 EC cells and mouse ES cells, but only when the cells are aggregated (Arceci *et al.*, 1993; Grèpin *et al.*, 1997).

Like GATA-4 null mice, mutant GATA-5 zebrafish have bilateral hearts (Reiter *et al.*, 1999). Although GATA-5 null mice have been generated, their hearts were normal perhaps due to compensation by GATA-4 or -6 (Molkentin *et al.*, 2000). GATA-6 knockout mice are embryonic lethal, dying soon after implantation (Morrisey *et al.*, 1998). To understand the phenotype of GATA-6 null mice better, GATA-$6^{-/-}$ ES cells were generated; when aggregated to form embryoid bodies they lacked the usual outer layer of visceral endoderm and never expressed the typical early and late endoderm markers (Morrisey *et al.*, 1998).

3.2. Nkx 2-5

The transcription factor Nkx 2-5 is the earliest heart-specific transcription factor (Lints *et al.*, 1993; Schultheiss *et al.*, 1995) and is the mammalian homologue of *tinman* in *Drosophila*. It is essential for heart formation (Bodmer, 1993); for example, flies without *tinman* lack the dorsal vessel and visceral endoderm. It is first expressed in the presumptive mesoderm just before gastrulation (Bodmer *et al.*, 1990) and expression is maintained throughout gastrulation and organization of the mesoderm into a bilayer (Harvey, 1996). *tinman* is then inactivated except in cells destined to become cardiac muscle (Bodmer *et al.*, 1990).

In the mouse, Nkx 2-5 is first expressed at day 7.5 pc in cardiac progenitors, which is several hours ahead of other myogenic markers, and remains present in the heart throughout embryogenesis (Lints *et al.*, 1993). Unlike *Drosophila*, vertebrate Nkx 2-5 is not expressed in nascent mesoderm (Harvey, 1996). Mice lacking Nkx 2-5 form a heart tube but cardiac looping never occurs (Lyons *et al.*, 1995). Although Nkx 2-5 does not thus appear to be necessary for cardiac recruitment and heart tube formation, it is compensated by Nkx 2-6, a highly similar gene expressed at the same time (Lyons *et al.*, 1995; Tanaka *et al.*, 2000). Recent work has shown that Nkx 2-5 may be required for the formation of the left ventricle chamber (Yamagishi *et al.*, 2001). Nkx 2-5 mutants have normal MLC-2a expression but lack MLC-2v expression (Lyons *et al.*, 1995).

In mouse EC cells, Nkx 2-5 is required for the formation of areas of beating muscle (Jamali *et al.*, 2001) and its overexpression results in beating muscle in aggregated P19 EC cells without the usual requirement for DMSO (Skerjanc *et al.*, 1998). Experiments with a dominant-negative Nkx 2-5 in P19 EC cells have shown that mesoderm induction is not affected by lack of functional Nkx 2-5 because Brachyury T, Wnt 5b, and Wnt 3a are unchanged (Jamali *et al.*, 2001). In these experiments the dominant-negative-Nkx 2-5-containing cells do not up regulate cardiac markers such as GATA-4 and MEF2C (Jamali *et al.*, 2001).

3.3. MEF-2 Family

Unlike mammals that have four MEF2 genes that can be differentially spliced into several MEF2 products (Yu *et al.*, 1992), *Drosophila* have only one MEF2

gene product (Lilly *et al.*, 1995), which is first expressed in the early mesoderm (Taylor *et al.*, 1995), followed later in development by increased expression in the dorsal mesoderm (Nguyen and Xu, 1998). Loss of function of MEF2 in *Drosophila* is embryonic lethal; the embryos lack cells of all myogenic lineages (i.e. cardiac, somatic, and visceral muscle cells), although muscle precursors form normally (Bour *et al.*, 1995). The *Drosophila* MEF2 mutants also lack structural genes such as myosin, which may suggest that MEF2 is involved indifferentiating the mature muscle cells from the myoblasts (Bour *et al.*, 1995; Cripps *et al.*, 1998; Lilly *et al.*, 1995).

Although mammalian MEF2 transcripts are ubiquitously expressed (Doevendans and van Bilsen, 1996), MEF2 protein seems to be restricted to the brain and muscle (Gossett *et al.*, 1989; Yu *et al.*, 1992). MEF2c is the first of the MEF2 family members expressed and is initially seen in the early mesoderm (Edmondson *et al.*, 1994). MEF2c expression is then followed by the other MEF2s (Black and Olson, 1998). MEF2c null mice were normal until embryonic day 9.5, when they began to show indications of cardiac insufficiency (Lin *et al.*, 1997). Additionally, the heart tube showed no signs of looping and the future right ventricle region does not form (Lin *et al.*, 1997). Interestingly, the MEF2b null mice have no cardiac phenotype, but this may be due to other MEF2s compensating (Lin *et al.*, 1997). As for GATA-4 and Nkx 2-5, overexpression of MEF2c in mouse EC cells leads to the formation of beating muscle without the need for prior aggregation (Skerjanc *et al.*, 1998). The overexpression of MEF2c in fibroblasts causes their conversion into skeletal muscle (Kaushal *et al.*, 1994).

3.4. Other Transcription Factors

Once the cells destined to become the heart are fated, they must still be organized in the correct manner and the transcription factors required for the contracting of the heart must be activated. This is done by several transcription factors including eHAND, dHAND, Tbx5, and the myosin light and heavy chain proteins. dHAND and eHAND are first expressed throughout the entire linear heart tube, but become restricted to the right and left ventricles (Srivastava *et al.*, 1995). Mutational analysis of these genes has shown that they are responsible for the patterning of the left and right ventricles in mammalian embryos (Srivastava *et al.*, 1997). Likewise in zebrafish, where there is only a single ventricle, dHAND (the only HAND gene in zebrafish), it is required for ventricle formation (Yelon *et al.*, 2000). The T-box transcription factor Tbx5 is expressed in the developing heart, and loss of function mutations in mice have atrial and ventricular septal defects (Bruneau *et al.*, 2001). Another T-box gene that may be involved in chamber specification is Tbx2. Recent data have shown that the cooperation of Tbx2 and Nkx 2-5 in the atrioventricular canal is a mechanism that inhibits gene activity in this region (Habets *et al.*, 2002). This suggests that the mechanism of Tbx2 might be to prevent the positive chamber forming effects of Tbx5 in the atrioventricular canal (Habets *et al.*, 2002). Transcription of the major proteins responsible for the contracting apparatus of the heart, the myosin heavy and light chains, are

first expressed on embryonic days 7.5–8 (Lyons *et al.*, 1990). Soon after this the ventricle and atrial forms of each are restricted to their respective future chambers in mice, even though chamber formation has not yet begun (Kubalak *et al.*, 1994; Morkin, 2000; O'Brien *et al.*, 1993). This may, however, not be the case in humans.

In summary, in order for a stem cell to become a cardiomyocyte, it is likely that it will be necessary to express the major cardiac transcription factors, perhaps in the order that this takes place in normal development. This could be achieved by controlled ectopic expression of the genes, expression of the most upstream gene leading to upregulation of its downstream targets, or by the addition of exogenous factors (such as BMPs, Wnts, and FGFs) in an appropriate sequence and combination with the major cardiac transcription factors that are activated by physiological stimuli in the correct order and magnitude. This strategy may be appropriate for adult and ES cells and is likely to be conserved cross species. We now need to address what types of cardiomyocytes we can expect to obtain in culture following these strategies and which properties would be most appropriate for successful transplantation.

4. ELECTROPHYSIOLOGY OF PRIMARY AND ES-DERIVED CARDIOMYOCYTES

Contractions of the heart are initiated in the sinus node, from which the excitation spreads through the heart. Pump function is directly related to the quality of impulse formation, conduction, and the presence or absence of factors that interfere with the signal. It is important that cell transplantation therapies for the heart do not cause arrhythmias (Menasche *et al.*, 2003; Zhang *et al.*, 2002). Matching of action potential format and gap-junctional conduction of stem cell-derived cardiomyocytes with the host environment is likely to decrease the risk of introducing an arrhythmic substrate. It is therefore important to characterize the electrophysiology of stem cell cardiomyocytes before transplantation and compare this with their normal counterparts. While this information is readily available for rodent cardiomyocytes (Maltsev *et al.*, 1993, 1994; Wobus *et al.*, 1995), there are relatively few descriptions of the electrical properties of human cardiomyocytes of adult, fetal, or stem cell origin. Here, we will review the literature in rodents in terms of ionic currents, action potentials and gap junctions, as well as their underlying expression of protein and mRNA, as a basis for understanding and comparing their human counterparts.

4.1. Fetal and ES Cell-Derived Mouse Cardiomyocytes

Maltsev and coworkers (Maltsev *et al.*, 1993) were among the first to demonstrate that spontaneous action potentials of mES cell-derived cardiomyocytes early during their differentiation program that were uniform, with an upstroke velocity between 1 and 10 V/s and an APD_{90}(action potential duration) between 70 and

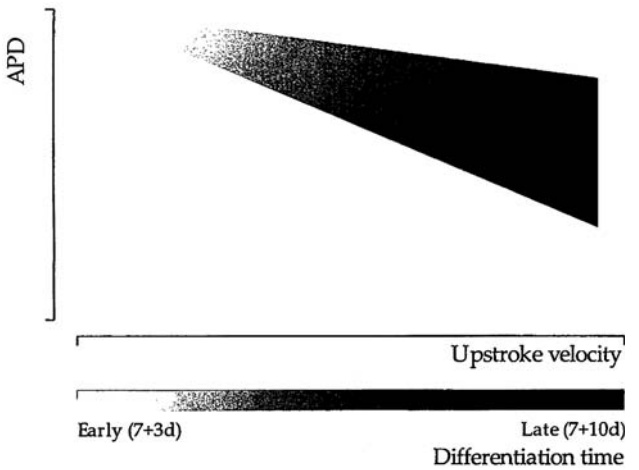

FIGURE 2. Schematic diagram showing the relationship between action potential duration (APD) and upstroke velocity in differentiating cardiomyocytes of murine origin (mES, P19, and embryonic heart). Differentiation of mES and P19 EC cells involves aggregation in suspension followed by replating (Davies *et al.*, 1996; Fijnvandraat *et al.*, 2003; van der Heyden *et al.*, 2003).

100 ms. In later differentiation, two types of spontaneous action potentials were found, with an upstroke velocity of 10–50 V/s with an APD_{90} of 100–200 ms, or 2–10 V/s with an APD_{90} of 70 ms, respectively. These "late" cardiomyocytes had a stable resting membrane potential of −74.5 mV, more negative than "early" cardiomyocytes (about −50 mV). Electrical triggering of these cells revealed upstroke velocities of 227 and 235 V/s for different action potential formats respectively (Maltsev *et al.*, 1993) and APD_{90}s of 124 and 148 ms. In P19 EC cell-derived cardiomyocytes, a decrease in APD was also found during differentiation (van der Heyden *et al.*, 2003), although more pronounced than in mES cells (Fig. 2). This mimics observations in primary mouse embryonic ventricular myocytes, where a shortening of APD and an increase in upstroke velocity are also observed during development (Wang *et al.*, 1996).

Analysis of ion currents in cardiomyocytes isolated from mouse embryos at different developmental stages demonstrated that L-type calcium currents exert dominant control over early cardiomyocyte electrophysiology while inward sodium currents increase dramatically just before birth and dominate thereafter (Davies *et al.*, 1996). In the same study, inward rectifier currents were only seen in late stages of murine development. These *in vivo* data resemble observations in differentiating P19 and mES cells, where L-type calcium currents are present in virtually all cardiomyocytes, while fast inward channels (such as sodium channels) were less frequently expressed (van der Heyden *et al.*, 2003). Moreover, the inward rectifier, responsible for the ability of sodium and/or T-type calcium channel activation and the cause of a more negative resting membrane potential, were observed only in some late stage cardiomyocytes.

4.2. Electrophysiology of the Developing Human Heart

More relevant to potential cardiomyocyte transplantation therapies in human of course are the electrical properties of the human embryonic, fetal, and adult heart. Of the few studies to date, most have focused on fetal cardiomyocytes, collected between 7 and 22 weeks of gestation, the period in which abortion is legal in many countries. This period is characterized by maturation of the four-chamber morphology of the heart. Here we will review the data available to date on action potential shape, response to various pharmacological compounds, and calcium currents.

4.3. Action Potentials in Human Fetal Ventricle and Atrium

The beating rate of excised intact 7- to 12-week human embryonic hearts is 50–132 (mean 91) beats/min and is determined by the sinus node (Tuganowski and Cekanski, 1971). No correlation was found between heart rate and development between 7 and 12 weeks, although it is known that *in vivo* the heart rate in the fetus peaks at ~180 beats/min at 9 weeks of gestation, increasing from ~140 beats/min at 6 weeks (Doubilet *et al.*, 2000; Ursem *et al.*, 1998; Van Lith *et al.*, 1992;). In this period, action potential amplitude and duration were also not correlated with maturation in noninnervated hearts (Fig. 3).

A feature of adult cardiomyocytes is that APD is frequency sensitive; i.e., with increasing heart rate the action potentials shorten. Interestingly, in 8- to 12-week ventricles, it was shown that APD shortening occurs when stimulation frequency is increased, or when an extrasystolic stimulus is given (Jezek *et al.*, 1982). The highest frequency sensitivity was found during the plateau phase. Shortening of APD was also observed after introducing a pause in the pacing of 9- to 11-week ventricles (Jezek *et al.*, 1985). The effect is proportional to the duration of the pause and involves calcium currents, since lowering of the extracellular calcium concentration increases the shortening effect. Therefore, the intrinsic action potential properties are already developed during an early embryonic phase.

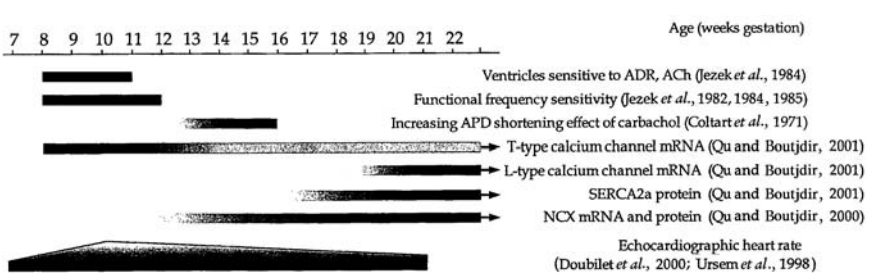

FIGURE 3. Overview of electrophysiological parameters during early human heart development. Bars indicate the periods investigated. For further explanation, see text. ADR: adrenaline, ACh: acetylcholine, APD: action potential duration, SERCA2a: sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), and NCX: Na^{+}/Ca^{2+} exchanger.

Adrenaline released from sympathetic nerves increases the beating rate of adult hearts, but also affects embryonic hearts. In 8- to 11-week ventricles, adrenaline increases action potential amplitude while decreasing the APD. Acetylcholine increases APD, but does not affect the amplitude of the action potential (Jezek *et al.*, 1984). The adrenaline response can only be partly blocked by β-blocker propanolol, indicating alternative pathways for the adrenaline effect. Atropine can completely block the acetylcholine effect (Jezek *et al.*, 1984).

Carbachol, a muscarinic receptor agonist, shortens the APD. Carbachol-induced shortening of APD, however, becomes more efficient with maturation of the heart. In atrial preparations from 12- to 13-week hearts, no shortening was observed, while 14- and 16-week hearts respond increasingly (21 and 50% decrease of 90% repolarization time, respectively) (Coltart *et al.*, 1971). Simultaneous measurement of contractile force and action potentials showed that contractile force responds to carbachol earlier in development than the APD shortening. Additionally, the attenuating effect of acetylcholine on APD shortening in human fetal hearts is smaller than in the human adult heart, where acetylcholine can cause arrest of spontaneous activity at lower doses (Trautwein *et al.*, 1962). These are all parameters that may be usefully applicable to determining the differentiation and maturation state of stem cell-derived cardiomyocytes.

4.4. Currents Underlying Action Potential Generation

Action potentials from embryonic atrial appendages of 7- to 9-week human hearts show a separation of the initial fast depolarization and subsequent repolarization phases with decreasing temperature (37–20° C). Temperatures below 20° C result in a stronger separation and arrest of spontaneous activity, which is completely reversible (Tuganowski and Tendera, 1973). Pacemaker frequency of spontaneously beating isolated fetal hearts was found to decrease by approximately 50% when lowering temperature from 37 to 27° C (Gennser and Nilsson, 1970).

The currents involved in the generation of atrial action potentials are mediated by Na^+, K^+, and Ca^{2+} ions. The initial fast depolarization involves both Na^+ and Ca^{2+} currents, as both decreased Na^+ concentration and addition of Mn^{2+}, a Ca^{2+} current blocker, resulted in a decreased depolarization. Auricular resting membrane potential can be increased by increasing $[K^+]$ (Tuganowski and Tendera, 1973), as observed in the ventricle by Gennser and Nilsson (1970), which indicates a role for potassium membrane conductance in the maintenance of the resting membrane potential.

The L-type calcium current plays an important role in cardiomyocyte excitation–contraction coupling by triggering ryanodine receptor gated calcium release from the sarcolemma, which facilitates contraction. Expression of L-type, T-type, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), and Na^+/Ca^{2+} exchanger (NCX) during human embryonic development have been determined (Qu *et al.*, 2000; Qu and Boutjdir, 2001). It was found that in the period between 8 weeks of gestation and adulthood, L-type channel mRNA expression increased, T-type channel mRNA expression decreased, and that SERCA protein, but not mRNA

expression, increased. NCX protein and mRNA expression decreased in the period between 9 weeks of gestation and adulthood.

Ventricular myocytes from 17- to 20-week-old hearts express both L- and T-type or solely L-type calcium currents (Bkaily *et al.*, 1992). L-type but not T-type currents could be dose-dependently and reversibly blocked with apamin. Furthermore, L-type current was decreased by Mn^{2+} and L-type channel blockers (nifedipine and PN200-110), while T-type current was only affected by Mn^{2+}. Single channel analysis of 13- to 22-week ventricular cardiomyocytes showed that the L-type current can be blocked by several L-type specific blockers (Bay K8644, nisoldipine) (Chen *et al.*, 1999).

Ryanodine receptor gated release of calcium from intracellular stores occurs in human auricle cardiomyocytes from 3-day-old to 4-year-old patients suffering from congenital heart defects (Hatem *et al.*, 1995). It was shown that membrane depolarization, ryanodine, or caffeine application can elicit myoplasmic Ca^{2+} transients in fura-2 loaded myocytes. fura-2 monitors intracellular Ca^{2+} concentrations. An age dependence in release rate was found, with faster release in older individuals. Additionally, isoprenaline increased both calcium current and release.

4.5. hES and Human Fetal Cardiomyocytes Compared

Cardiomyocytes from dissociated beating aggregates of the hES2 cell line (Reubinoff *et al.*, 2000) show predominantly ventricular-like action potentials, but some atrial-like, pacemaker-like, and vascular smooth muscle-like action potentials have also been observed (Mummery *et al.*, 2003). This particular cell line requires coculture with endoderm-like cells to form cardiomyocytes although other hES cell lines will form cardiomyocytes spontaneously in aggregates, just as mES and P19 EC cells (Kehat *et al.*, 2001; Gepstein, 2002; Nir *et al.*, 2003), and there appears to be no bias toward ventricular differentiation. It is not clear at present whether this is an intrinsic difference between the cell lines or the result of the method of inducing differentiation. The resting membrane potentials found for the hES2-derived atrial-like cells and human fetal atrial cardiomyocytes were comparable (-38.7 ± 0.6 versus -34.9 ± 1.6 mV). hES cell-derived ventricular cardiomyocytes show a slightly more negative membrane potential than human fetal ventricular cardiomyocytes (-48.0 ± 1.7 versus -38.5 ± 1.6 mV), but have a comparable upstroke velocity (7.0 ± 0.8 versus 8.9 ± 4.3 V/s). Although others report resting membrane potentials in human fetal cardiomyocytes ranging between -70 and -85 mV (Gennser and Nilsson, 1970; Kolossov *et al.*, 1998; Jezek *et al.*, 1982), these authors used intact or dissected myocardium, whereas Mummery *et al.* (2003) characterized cardiomyocytes dissociated from fetal myocardium and maintained under culture conditions similar to the hES cell-derived cardiomyocytes.

Remarkably, only plateau-type action potentials were observed in hES-derived atrial-like cells, whereas in 7- to 16-week fetal atrium non-plateau-type action potentials are also found (Janse *et al.*, 1976; Tertoolen, Hassink, Brutel de la Riviere, 2004; Tuganowski and Cekanski, 1971). Whether only "plateau-type"

atrial-like cardiomyocytes are present in hES cell-derived beating aggregates and whether the cells display APD shortening remains to be elucidated. Overall, hES cell-derived cardiomyocytes appear to be comparable with their human fetal counterparts and distinct from human adult cardiomyocytes largely in terms of the magnitude of action potential and resting membrane potentials. It is of note that the action potentials of both hES cell-derived and human fetal cardiomyocytes are inhibited by Verapamil, blocks L-type Ca^{2+}, as in adult cells (Mummery *et al.*, 2003). This contrasts with mouse fetal myocytes and mouse ES cell-derived cardiomyocytes, in which early cells are nonresponsive despite the presence of L-type Ca^{2+} channels. Here the lack of cAMP-dependent kinase appears to be a limiting factor (An *et al.*, 1996; Doevendans *et al.*, 2000). Thus, although hES-derived cardiomyocytes and early human fetal cardiomyocytes show some features in common with early mouse cardiomyocytes, their calcium channel modulation resembles that in the *adult* mouse and human. HES cells may thus represent an excellent system for studying changes in calcium channel function during early human development, which appears to differ from that in mice.

5. PERSPECTIVES FOR TRANSPLANTATION

Taken together, the data show that hES cells differentiate in culture to cell with a fetal phenotype, just like their murine counterparts. Direct injection of adult (bone marrow-derived) stem cells into the heart of circulation seems to result in incorporation of cells into the heart as cardiomyocytes but as the result of fusion; this is a rare event and could not be expected to result in sufficient cells adopting a cardiomyocyte phenotype to restore cardiac function. However, bone marrow does contain a population of endothelial precursor cells, which in animal models contribute significantly to neovascularization. This may explain to some extent patient benefit reported following injection of bone marrow cells immediately postinfarct: rapid neovascularization may limit ischemic damage (Aicher *et al.*, 2003; Urbich *et al.*, 2003). The ultimate therapy for myocardial infarct patients may then involve injection of bone marrow immediately postinfarct followed later, and depending on the degree of damage, by cardiomyocyte replacement. Continued research will determine whether adult or ES cells are most suitable for this purpose on the basis of their ability to communicate electrically with the host heart cells and mature *in situ.*

REFERENCES

Aicher, A., Brenner, W., Zuhayra, M., Badorff, C., Massoudi, S., Assmus, B., Eckey, T., Henze, E., Zeiher, A. M., and Dimmeler, S., 2003, Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling, *Circulation* **107**:2134–2139.

Alsan, B. H., and Schultheiss, T. M., 2002, Regulation of avian cardiogenesis by Fgf8 signaling, *Development* **129**:1935–1943.

Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J. M., Fike, J. R., Lee, H. O., Pfeffer, K., Lois, C., Morrison, S. J., and Alvarez-Buylla, A., 2003, Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes, *Nature* **425**:968–973.

An, R. H., Davies, M. P., Doevendans, P. A., Kubalak, S. W., Bangalore, R., Chien, K. R., and Kass, R. S., 1996, Developmental changes in beta-adrenergic modulation of L-type Ca^{2+} channels in embryonic mouse heart, *Circ. Res.* **78**:371–378.

Andree, B., Duprez, D., Vorbusch, B., Arnold, H. H., and Brand, T., 1998, BMP-2 induces ectopic expression of cardiac lineage markers and interferes with somite formation in chicken embryos, *Mech. Dev.* **70**:119–131.

Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H., and Wilson, D. B., 1993, Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart, *Mol. Cell Biol.* **13**:2235–2246.

Barron, M., Gao, M., and Lough, J., 2000, Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative, *Dev. Dyn.* **218**:383–393.

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., Leri, A., Kajstura, J., Nadal-Ginard, B., and Anversa, P., 2003, Adult cardiac stem cells are multipotent and support myocardial regeneration, *Cell* **114**:763–776.

Bkaily, G., Sculptoreanu, A., Jacques, D., Economos, D., and Menard, D., 1992, Apamin, a highly potent fetal L-type Ca^{2+} current blocker in single heart cells, *Am. J. Physiol.* **262**:H463–H471.

Black, B. L., and Olson, E. N., 1998, Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins, *Annu. Rev. Cell Dev. Biol.* **14**:167–196.

Bodmer, R., 1993, The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*, *Development* **118**:719–729.

Bodmer, R., Jan, L. Y., and Jan, Y. N., 1990, A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation of *Drosophila, Development* **110**:661–669.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M., and Nguyen, H. T., 1995, *Drosophila* MEF2, a transcription factor that is essential for myogenesis, *Genes Dev.* **9**:730–741.

Brand, T., 2003, Heart development: molecular insights into cardiac specification and early morphogenesis, *Dev. Biol.* **258**:1–19.

Bruneau, B. G., 2002, Transcriptional regulation of vertebrate cardiac morphogenesis, *Circ. Res.* **90**:509–519.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E., and Seidman, J. G., 2001, A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease, *Cell* **106**:709–721.

Brustle, O., Jones, K. N., Learish, R. D., Karram, K., Choudhary, K., Wiestler, O. D., Duncan, I. D., and McKay, R. D., 1999, Embryonic stem cell-derived glial precursors: a source of myelinating transplants, *Science* **285**:754–756.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A., 2003, Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells, *Cell* **113**:643–655.

Chen, L., El Sherif, N., and Boutjdir, M., 1999, Unitary current analysis of L-type Ca^{2+} channels in human fetal ventricular myocytes, *J. Cardiovasc. Electrophysiol.* **10**:692–700.

Coltart, D. J., Meldrum, S. J., Royds, R. B., and Spilker, B. A., 1971, Study of the contractile and electrophysiological maturation responses of the human foetal myocardium, *Br. J. Pharmacol.* **42**:653P–654P.

Cripps, R. M., Black, B. L., Zhao, B., Lien, C. L., Schulz, R. A., and Olson, E. N., 1998, The myogenic regulatory gene *Mef2* is a direct target for transcriptional activation by Twist during *Drosophila* myogenesis, *Genes Dev.* **12**:422–434.

Cripps, R. M., and Olson, E. N., 2002, Control of cardiac development by an evolutionarily conserved transcriptional network, *Dev. Biol.* **246**:14–28.

Crossley, P. H., and Martin, G. R., 1995, The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo, *Development* **121**:439–451.

Davies, M. P., An, R. H., Doevendans, P., Kubalak, S., Chien, K. R., and Kass, R. S., 1996, Developmental changes in ionic channel activity in the embryonic murine heart, *Circ. Res.* **78**:15–25.

Doevendans, P. A., Kubalak, S. W., An, R. H., Becker, D. K., Chien, K. R., and Kass, R. S., 2000, Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes, *J. Mol. Cell Cardiol.* **32**:839–851.

Doevendans, P. A., and van Bilsen, M., 1996, Transcription factors and the cardiac gene programme, *Int. J. Biochem. Cell Biol.* **28**:387–403.

Doubilet, P. M., Benson, C. B., and Chow, J. S., 2000, Outcome of pregnancies with rapid embryonic heart rates in the early first trimester, *AJR Am. J. Roentgenol.* **175**:67–69.

Dowell, J. D., Rubart, M., Pasumarthi, K. B., Soonpaa, M. H., and Field, L. J., 2003, Myocyte and myogenic stem cell transplantation in the heart, *Cardiovasc. Res.* **58**:336–350.

Edmondson, D. G., Lyons, G. E., Martin, J. F., and Olson, E. N., 1994, *Mef 2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis, *Development* **120**:1251–1263.

Eisenberg, C. A., and Eisenberg, L. M., 1999, WNT11 promotes cardiac tissue formation of early mesoderm, *Dev. Dyn.* **216**:45–58.

Evans, M. J., and Kaufman, M. H., 1981, Establishment in culture of pluripotential cells from mouse embryos, *Nature* **292**:154–156.

Fijnvandraat, A. C., van Ginneken, A. C., de Boer, P. A., Ruijter, J. M., Christoffels, V. M., Moorman, A. F., and Lekanne Deprez, R. H., 2003, Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube, *Cardiovasc. Res.* **58**:399–409.

Frasch, M., 1995, Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo, *Nature* **374**:464–467.

Gajewski, K., Fossett, N., Molkentin, J. D., and Schulz, R. A., 1999, The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*, *Development* **126**:5679–5688.

Gennser, G., and Nilsson, E., 1970, Excitation and impulse conduction in the human fetal heart, *Acta Physiol. Scand.* **79**:305–320.

Gepstein, L., 2002, Derivation and potential applications of human embryonic stem cells, *Circ. Res.* **91**:866–876.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q., and Michelson, A. M., 1996, Heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo, *Genes Dev.* **10**:3003–3017.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N., 1989, A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes, *Mol. Cell Biol.* **9**:5022–5033.

Grèpin, C., Robitaille, L., Antakly, T., and Nemer, M., 1995, Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation, *Mol. Cell Biol.* **15**:4095–4102.

Grèpin, C., Nemer, G., and Nemer, M., 1997, Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor, *Development* **124**:2387–2395.

Habara-Ohkubo, A., 1996, Differentiation of beating cardiac muscle cells from a derivative of P19 embryonal carcinoma cells, *Cell Struct. Funct.* **21**:101–110.

Habets, P. E., Moorman, A. F., Clout, D. E., van Roon, M. A., Lingbeek, M., van Lohuizen, M., Campione, M., and Christoffels, V. M., 2002, Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation, *Genes Dev.* **16**:1234–1246.

Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jimenez, F., Baylies, M. K., and Michelson, A. M., 2000, Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors, *Cell* **103**:63–74.

Harvey, R. P., 1996, NK-2 homeobox genes and heart development, *Dev. Biol.* **178**:203–216.

Hatem, S. N., Sweeten, T., Vetter, V., and Morad, M., 1995, Evidence for presence of Ca^{2+} channel-gated Ca^{2+} stores in neonatal human atrial myocytes, *Am. J. Physiol.* **268**:H1195–H1201.

Heikinheimo, M., Scandrett, J. M., and Wilson, D. B., 1994, Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development, *Dev. Biol.* **164**:361–373.

Hogan, B. L., 1996, Bone morphogenetic proteins in development, *Curr. Opin. Genet. Dev.* **6**:432–438.

Hurlstone, A. F., Haramis, A. P., Wienholds, E., Begthel, H., Korving, J., Van Eeden, F., Cuppen, E., Zivkovic, D., Plasterk, R. H., and Clevers, H., 2003, The Wnt/beta-catenin pathway regulates cardiac valve formation, *Nature* **425**:633–637.

Jamali, M., Rogerson, P. J., Wilton, S., and Skerjanc, I. S., 2001, Nkx2−5 activity is essential for cardiomyogenesis, *J. Biol. Chem.* **276**:42252–42258.

Janse, M. K., Anderson, R. H., van Capelle, F. J., and Durrer, D., 1976, A combined electrophysiological and anatomical study of the human fetal heart, *Am. Heart J.* **91**:556–562.

Jezek, K., Knaizl, Z., Kralicek, P., Pucelik, P., and Sauer, J., 1985, Effect of a pause on action potentials of the working ventricular myocardium of human embryos, *Physiol. Bohemoslov.* **34**:69–76.

Jezek, K., Pucelik, P., Sauer, J., and Bartak, F., 1982, Basic electrophysiological parameters and frequency sensitivity of the ventricular myocardium of human embryos, *Physiol. Bohemoslov.* **31**:11–19.

Jezek, K., Pucelik, P., Sauer, J., and Bartak, F., 1984, Effect of acetylcholine and adrenaline on ventricular action potentials of the human embryonic myocardium, *Physiol. Bohemoslov.* **33**:463–469.

Jiang, Y., and Evans, T., 1996, The *Xenopus* GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis, *Dev. Biol.* **174**:258–270.

Johkura, K., Cui, L., Suzuki, A., Teng, R., Kamiyoshi, A., Okamura, S., Kubota, S., Zhao, X., Asanuma, K., Okouchi, Y., Ogiwara, N., Tagawa, Y., and Sasaki, K., 2003, Survival and function of mouse embryonic stem cell-derived cardiomyocytes in ectopic transplants, *Cardiovasc. Res.* **58**:435–443.

Kaushal, S., Schneider, J. W., Nadal-Ginard, B., and Mahdavi, V., 1994, Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD, *Science* **266**:1236–1240.

Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and Gepstein, L., 2001, Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes, *J. Clin. Invest.* **108**:407–414.

Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., and McKay, R., 2002, Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease, *Nature* **418**:50–56.

Klug, M. G., Soonpaa, M. H., Koh, G. Y., and Field, L. J., 1996, Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts, *J. Clin. Invest.* **98**:216–224.

Knirr, S., and Frasch, M., 2001, Molecular integration of inductive and mesoderm-intrinsic inputs governs even-skipped enhancer activity in a subset of pericardial and dorsal muscle progenitors, *Dev. Biol.* **238**:13–26.

Kolossov, E., Fleischmann, B. K., Liu, Q., Bloch, W., Viatchenko-Karpinski, S., Manzke, O., Ji, G. I., Bohlen, H., Addicks, K., and Hescheler, J., 1998, Functional characteritics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein, *J. Cell Biol.* **143**:2045–2056.

Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S. J., 2001, Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, *Cell* **105**:369–377.

Kubalak, S. W., Miller-Hance, W. C., O'Brien, T. X., Dyson, E., and Chien, K. R., 1994, Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis, *J. Biol. Chem.* **269**:16961–16970.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C., and Leiden, J. M., 1997, GATA4 transcription factor is required for ventral morphogenesis and heart tube formation, *Genes Dev.* **11**:1048–1060.

Ladd, A. N., Yatskievych, T. A., and Antin, P. B., 1998, Regulation of avian cardiac myogenesis by activin/TGFbeta and bone morphogenetic proteins, *Dev. Biol.* **204**:407–419.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B., and Evans, T., 1994, GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut, *J. Biol. Chem.* **269**:23177–23184.

Lee, H. H., and Frasch, M., 2000, Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target sloppy paired, *Development* **127**:5497–5508.

Leobon, B., Garcin, I., Menasche, P., Vilquin, J. T., Audinat, E., and Charpak, S., 2003, Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host, *Proc. Natl. Acad. Sci. U.S.A.* **100**:7808–7811.

Liberatore, C. M., Searcy-Schrick, R. D., Vincent, E. B., and Yutzey, K. E., 2002, Nkx-2.5 gene induction in mice is mediated by a Smad consensus regulatory region, *Dev. Biol.* **244**:243–256.

Lickert, H., Kutsch, S., Kanzler, B., Tamai, Y., Taketo, M. M., and Kemler, R., 2002, Formation of multiple hearts in mice following deletion of beta-catenin in the embryonic endoderm, *Dev. Cell* **3**:171–181.

Lien, C. L., McAnally, J., Richardson, J. A., and Olson, E. N., 2002, Cardiac-specific activity of an Nkx2−5 enhancer requires an evolutionarily conserved Smad binding site, *Dev. Biol.* **244**:257–266.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A., and Olson, E. N., 1995, Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*, *Science* **267**:688–693.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E. N., 1997, Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C, *Science* **276**:1404–1407.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P., 1993, Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants, *Development* **119**:419–431.

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

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P., 1995, Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene Nkx2−5, *Genes Dev.* **9**:1654–1666.

Maltsev, V. A., Rohwedel, J., Hescheler, J., and Wobus, A. M., 1993, Embryonic stem cells differentiate *in vitro* into cardiomyocytes representing sinusnodal, atrial and ventricular cell types, *Mech. Dev.* **44**:41–50.

Maltsev, V. A., Wobus, A. M., Rohwedel, J., Bader, M., and Hescheler, J., 1994, Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents, *Circ. Res.* **75**:233–244.

Martin, G. R., 1981, Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells, *Proc. Natl. Acad. Sci. U.S.A.* **78**:7634–7638.

Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M., and Lassar, A. B., 2001, Inhibition of Wnt activity induces heart formation from posterior mesoderm, *Genes Dev.* **15**:316–327.

McBurney, M. W., Jones-Villeneuve, E. M., Edwards, M. K., and Anderson, P. J., 1982, Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line, *Nature* **299**:165–167.

McDonald, J. W., Liu, X. Z., Qu, Y., Liu, S., Mickey, S. K., Turetsky, D., Gottlieb, D. I., and Choi, D. W., 1999, Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord, *Nat. Med.* **5**:1410–1412.

Menasche, P., Hagege, A. A., Vilquin, J. T., Desnos, M., Abergel, E., Pouzet, B., Bel, A., Sarateanu, S., Scorsin, M., Schwartz, K., Bruneval, P., Benbunan, M., Marolleau, J. P., and Duboc, D., 2003,

Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction, *J. Am. Coll. Cardiol.* **41**:1078–1083.

Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K. H., and Buff, E. M., 1998, Dual functions of the heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm, *Dev. Genet.* **22**:212–229.

Molkentin, J. D., 2000, The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression, *J. Biol. Chem.* **275**:38949–38952.

Molkentin, J. D., Lin, Q., Duncan, S. A., and Olson, E. N., 1997, Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis, *Genes Dev.* **11**:1061–1072.

Molkentin, J. D., Tymitz, K. M., Richardson, J. A., and Olson, E. N., 2000, Abnormalities of the genitourinary tract in female mice lacking GATA5, *Mol. Cell Biol.* **20**:5256–5260.

Monzen, K., Hiroi, Y., Kudoh, S., Akazawa, H., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Kawabata, M., Miyazono, K., Ishii, S., Yazaki, Y., Nagai, R., and Komuro, I., 2001, Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation, *J. Cell Biol.* **153**:687–698.

Monzen, K., Shiojima, I., Hiroi, Y., Kudoh, S., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Habara-Ohkubo, A., Nakaoka, T., Fujita, T., Yazaki, Y., and Komuro, I., 1999, Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4, *Mol. Cell Biol.* **19**:7096–7105.

Morkin, E., 2000, Control of cardiac myosin heavy chain gene expression, *Microsc. Res. Tech.* **50**:522–531.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S., and Parmacek, M. S., 1998, GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo, *Genes Dev.* **12**:3579–3590.

Mummery, C., Ward-Van Oostwaard, D., Doevendans, P., Spijker, R., Van Den, Brink S., Hassink, R., Van Der, Heyden M., Opthof, T., Pera, M., De La Riviere, A. B., Passier, R., and Tertoolen, L., 2003, Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells, *Circulation* **107**:2733–2740.

Nakamura, T., Sano, M., Songyang, Z., and Schneider, M. D., 2003, A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis, *Proc. Natl. Acad. Sci. U.S.A.* **100**(10):5834–9

Narasimha, M., and Leptin, M., 2000, Cell movements during gastrulation: come in and be induced, *Trends Cell Biol.* **10**:169–172.

Nguyen, H. T., and Xu, X., 1998, *Drosophila* mef2 expression during mesoderm development is controlled by a complex array of cis-acting regulatory modules, *Dev. Biol.* **204**:550–566.

Nir, S. G., David, R., Zaruba, M., Franz, W. M., and Itskovitz-Eldor, J., 2003, Human embryonic stem cells for cardiovascular repair, *Cardiovasc. Res.* **58**:313–323.

O'Brien, T. X., Lee, K. J., and Chien, K. R., 1993, Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube, *Proc. Natl. Acad. Sci. U.S.A.* **90**:5157–5161.

Olson, E. N., and Schneider, M. D., 2003, Sizing up the heart: development redux in disease, *Genes Dev.* **17**:1937–1956.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M., Leri, A., and Anversa, P., 2001, Bone marrow cells regenerate infarcted myocardium, *Nature* **410**:701–705.

Pandur, P., Läsche, M., Eisenberg, L. M., and Kühl, M., 2002, Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis, *Nature* **418**:636–641.

Park, M., Wu, X., Golden, K., Axelrod, J. D., and Bodmer, R., 1996, The wingless signaling pathway is directly involved in *Drosophila* heart development, *Dev. Biol.* **177**:104–116.

Parlow, M. H., Bolender, D. L., Kokan-Moore, N. P., and Lough, J., 1991, Localization of bFGF-like proteins as punctate inclusions in the preseptation myocardium of the chicken embryo, *Dev. Biol.* **146**:139–147.

Passier, R., and Mummery, C., 2003, Origin and use of embryonic and adult stem cells in differentiation and tissue repair, *Cardiovasc. Res.* **58**:324–335.

Qu, Y., and Boutjdir, M., 2001, Gene expression of SERCA2a and L- and T-type Ca channels during human heart development, *Pediatr. Res.* **50**:569–574.

Qu, Y., Ghatpande, A., El Sherif, N., and Boutjdir, M., 2000, Gene expression of Na^+/Ca^{2+} exchanger during development in human heart, *Cardiovasc. Res.* **45**:866–873.

Reifers, F., Walsh, E. C., Leger, S., Stainier, D. Y., and Brand, M., 2000, Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar), *Development* **127**:225–235.

Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N., and Stainier, D. Y., 1999, Gata5 is required for the development of the heart and endoderm in zebrafish, *Genes Dev.* **13**:2983–2995.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A., 2000, Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro, *Nat. Biotechnol.* **18**:399–404.

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L., 2003, A role for Wnt signalling in self-renewal of haematopoietic stem cells, *Nature* **423**:409–414.

Schlange, T., Andree, B., Arnold, H. H., and Brand, T., 2000, BMP2 is required for early heart development during a distinct time period, *Mech. Dev.* **91**:259–270.

Schneider, V. A., and Mercola, M., 2001, Wnt antagonism initiates cardiogenesis in *Xenopus laevis*, *Genes Dev.* **15**:304–315.

Schultheiss, T. M., Burch, J. B., and Lassar, A. B., 1997, A role for bone morphogenetic proteins in the induction of cardiac myogenesis, *Genes Dev.* **11**:451–462.

Schultheiss, T. M., Xydas, S., and Lassar, A. B., 1995, Induction of avian cardiac myogenesis by anterior endoderm, *Development* **121**:4203–4214.

Skerjanc, I. S., Petropoulos, H., Ridgeway, A. G., and Wilton, S., 1998, Myocyte enhancer factor 2C and Nkx2−5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells, *J. Biol. Chem.* **273**:34904–34910.

Slavin, J., 1995, Fibroblast growth factors: at the heart of angiogenesis, *Cell Biol. Int.* **19**:431–444.

Solloway, M. J., and Robertson, E. J., 1999, Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup, *Development* **126**:1753–1768.

Soonpaa, M. H., and Field, L. J., 1994, Assessment of cardiomyocyte DNA synthesis during hypertrophy in adult mice, *Am. J. Physiol.* **266**:H1439–H1445.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M., and Wilson, D. B., 1995, Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro, *Development* **121**:3877–3888.

Srivastava, D., Cserjesi, P., and Olson, E. N., 1995, A subclass of bHLH proteins required for cardiac morphogenesis, *Science* **270**:1995–1999.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D., and Olson, E. N., 1997, Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND, *Nat. Genet.* **16**:154–160.

Tanaka, M., Yamasaki, N., and Izumo, S., 2000, Phenotypic characterization of the murine Nkx2.6 homeobox gene by gene targeting, *Mol. Cell Biol.* **20**:2874–2879.

Taylor, M. V., Beatty, K. E., Hunter, H. K., and Baylies, M. K., 1995, *Drosophila* MEF2 is regulated by twist and is expressed in both the primordia and differentiated cells of the embryonic somatic, visceral and heart musculature, *Mech. Dev.* **50**:29–41.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M., 1998, Embryonic stem cell lines derived from human blastocysts, *Science* **282**:1145–1147.

Trautwein, W., Kassebaum, D. G., Nelson, R. M., and Hecht, H. H., 1962, Electrophysiological study of human heart muscle, *Circ. Res.* **10**:306–312.

Tuganowski, W., and Cekanski, A., 1971, Electrical activity of a single fibre of the human embryonic heart, *Pflugers Arch.* **323**:21–26.

Tuganowski, W., and Tendera, M., 1973, Components of the action potential of human embryonic auricle, *Am. J. Physiol.* **224**:803–808.

Tzahor, E., and Lassar, A. B., 2001, Wnt signals from the neural tube block ectopic cardiogenesis, *Genes Dev.* **15**:255–260.

Urbanek, K., Quaini, F., Tasca, G., Torella, D., Castaldo, C., Nadal-Ginard, B., Leri, A., Kajstura, J., Quaini, E., and Anversa, P., 2003, Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy, *Proc. Natl. Acad. Sci. U.S.A.* **100**:10440–10445.

Urbich, C., Heeschen, C., Aicher, A., Dernbach, E., Zeiher, A. M., and Dimmeler, S., 2003, Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells, *Circulation.*[Q8]

Ursem, N. T., Struijk, P. C., Hop, W. C., Clark, E. B., Keller, B. B., and Wladimiroff, J. W., 1998, Heart rate and flow velocity variability as determined from umbilical Doppler velocimetry at 10–20 weeks of gestation, *Clin. Sci. (Lond.)* **95**:539–545.

van der Heyden, M. A., and Defize, L. H., 2003, Twenty one years of P19 cells: what an embryonal carcinoma cell line taught us about cardiomyocyte differentiation, *Cardiovasc. Res.* **58**:292–302.

van der Heyden, M. A., van Kempen, M. J., Tsuji, Y., Rook, M. B., Jongsma, H. J., and Opthof, T., 2003, P19 embryonal carcinoma cells: a suitable model system for cardiac electrophysiological differentiation at the molecular and functional level, *Cardiovasc. Res.* **58**:410–422.

Van Lith, J. M., Visser, G. H., Mantingh, A., and Beekhuis, J. R., 1992, Fetal heart rate in early pregnancy and chromosomal disorders, *Br. J. Obstet. Gynaecol.* **99**:741–744.

Wang, L., Feng, Z. P., Kondo, C. S., Sheldon, R. S., and Duff, H. J., 1996, Developmental changes in the delayed rectifier K^+ channels in mouse heart, *Circ. Res.* **79**:79–85.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., III, and Nusse, R., 2003, Wnt proteins are lipid-modified and can act as stem cell growth factors, *Nature* **423**:448–452.

Wobus, A. M., Rohwedel, J., Maltsev, V., and Hescheler, J., 1995, Development of cardiomyocytes expressing cardiac-specific genes, action potentials, and ionic channels during embryonic stem cell-derived cardiogenesis, *Ann. N. Y. Acad. Sci.* **752**:460–469.

Wu, X., Golden, K., and Bodmer, R., 1995, Heart development in *Drosophila* requires the segment polarity gene *wingless*, *Dev. Biol.* **169**:619–628.

Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L., and Frasch, M., 1998, Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm, *Genes Dev.* **12**:2354–2370.

Yamagishi, H., Yamagishi, C., Nakagawa, O., Harvey, R. P., Olson, E. N., and Srivastava, D., 2001, The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation, *Dev. Biol.* **239**:190–203.

Yelon, D., Ticho, B., Halpern, M. E., Ruvinsky, I., Ho, R. K., Silver, L. M., and Stainier, D. Y., 2000, The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development, *Development* **127**:2573–2582.

Yin, Z., and Frasch, M., 1998, Regulation and function of *tinman* during dorsal mesoderm induction and heart specification in *Drosophila*, *Dev. Genet.* **22**:187–200.

Yu, Y. T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B., 1992, Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors, *Genes Dev.* **6**:1783–1798.

Zhang, H., and Bradley, A., 1996, Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development, *Development* **122**:2977–2986.

Zhang, Y. M., Hartzell, C., Narlow, M., and Dudley, S. C., Jr., 2002, Stem cell-derived cardiomyocytes demonstrate arrhythmic potential, *Circulation* **106**:1294–1299.

Zhu, X., Sasse, J., McAllister, D., and Lough, J., 1996, Evidence that fibroblast growth factors 1 and 4 participate in regulation of cardiogenesis, *Dev. Dyn.* **207**:429–438.

Chapter 9

Can Somatic Stem Cells Regenerate Myocardial Tissue?

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Somatic stem cells can be obtained from various sources and their differentiation potential is not restricted only to the differentiated cell phenotypes of the source tissue. Therefore, somatic stem cells offer a great promise for cell replacement therapy in diseased hearts. Yet, much confusion has been created concerning the use of somatic stem cells. Different methods have been used to isolate and recognize stem cells, and the nomenclature is not standardized and often based on the method of cell isolation, making it difficult to compare the different studies. In humans the situation is worse as the scientific approach to the stem cell issue is overtaken by clinical applications of somatic stem cells or approaches to mobilize endogenous stem cells. Here we discuss the current knowledge on somatic stem cells in animal models and humans. In addition, the solid proof for beneficial effects of stem cell mobilization is critically reviewed and finally the question whether cardiomyoblasts exist is addressed. This chapter is written to provide clarity on different classifications of somatic stem cells and to raise hypothesis for future research.

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

1. INTRODUCTION

Stem cell research is a fascinating field as the potential applications are almost unlimited (Hassink *et al.*, 2003a, 2003b; Zwaginga and Doevendans, 2003). This statement is probably even more valuable for the scientific questions that can be answered through the use of stem cells and the analysis of their *in vitro* differentiation. With the introduction of the term somatic stem cells, all multipotent cells are indicated that can be derived from a living organism, excluding the pluripotent embryonic stem cells and primordial germ cells (see Chapter 8). The distinction is based on the wider differentiation potential of embryonic stem cells and primordial germ cells, including the formation of gametes (Gezondheidsraad, unpublished work, 2002). Although somatic stem cells appear to be more restricted in their differentiation potential and primarily involved in self-renewal of that specific tissue, the plasticity of these cells has been shown to be surprisingly great. Bone marrow, as well as more specifically defined populations of stem cells, has the potential to differentiate into vascular cells and possibly into cardiomyocytes *in vitro* and *in vivo*. The reason why somatic stem cells are not pluripotent has not yet been resolved, but there are also baseline differences in the differentiation prevalence in embryonic stem cells. For instance the D3 embryonic stem cell line produced by Doetschman has a high tendency to form cardiac muscle during embryoid body formation (Doetschman *et al.*, 1985) and also the human embryonic stem cell lines show differences in differentiation potential. It is likely that this preferential differentiation tendency is even more outspoken in somatic stem cells.

The potential advantages of somatic stem cells over embryonic stem cells are numerous, including immunocompatibility. The limited differentiation window of somatic stem cells is only one of the features that make these cells interesting, since we will eliminate the risk of tumorgenicity. The question remains which somatic stem cell(s) we need to isolate to be able to restore the blood supply and replace the damaged myocardium, since the bone marrow contains cells of multiple lineage and phenotype.

2. SOMATIC STEM CELLS

The innovative concept and potential application of stem cells comes from the hematological field where bone marrow and hematopoietic stem cells (HSCs) have been used successfully for more than 30 years to treat diseases like leukemia (McCulloch *et al.*, 1965). First chemotherapy will destroy the cancerous or ill bone marrow, after which intravenous injection of prepared stem cells from a healthy donor will repopulate the bone marrow and save critically ill patients suffering from a hematological malignancy. Despite the extensive knowledge on bone marrow-derived (hematopoietic) stem cells and the regenerative potential of circulating stem cells, here lies also an important source for confusion. Potentially, from every organ, cells with stem cell characteristics can be isolated. This led to the speculation that every organ harbors its own specific stem cells. Many somatic

stem cells, however, are not organ specific but merely caught by coincidence from the circulation and currently the proof for organ-specific pluripotent stem cells is lacking.

3. MOBILIZATION OF ENDOGENOUS STEM CELLS

One option to deliver stem cells to the heart might be via stimulation and mobilization of endogenous stem cells, assuming they will migrate and home to the injured tissue. The numbers of stem cells in peripheral blood can be increased by cytokine stimulation of the animal or patient. In fact the response to cytokine treatment in potential bone marrow human donors can be such that sufficient cells become available from peripheral blood to make transplantation feasible. This procedure has been shown to be effective and safe in humans (Cavallaro *et al.*, 2000). The presence and importance of endogenous stem cells originated from the bone marrow under pathophysiological conditions *in vivo* was demonstrated by analyzing posttransplant organs. Recent studies in human transplanted hearts revealed the presence of male recipient-derived cardiomyocytes and vascular cells in both the atrium and the ventricle of the female donor. According to one study, up to 17% of the cells in the donor heart were from the recipient. These primitive cells expressed c-Kit, mdr-1, and Sca-1, and a subpopulation was also positive for cardiomyocyte proteins (Anversa and Nadal-Ginard, 2002). The cells are presumably derived from the bone marrow/peripheral blood of the recipient of the heart (Quaini *et al.*, 2002). However, later studies failed to confirm these numbers and indicated a limited contribution of recipient stem cells to the vasculature, but hardly any cardiomyocytes (0.016%) (Laflamme *et al.*, 2002). These studies did however raise the interest for studying endogenous mobilization of stem cells after myocardial infarction.

Myocardial infarction is accompanied with an inflammatory reaction that induces cardiac dysfunction and remodeling including hypertrophy. The mechanisms by which the cytokine cascade is activated in the infarcted myocardium include various hematopoietic growth factors including interleukin-3 (IL-3), IL-6, granulocyte-macrophage colony-stimulating factors (GM-CSF), granulocyte-colony stimulating factor (G-CSF), and stem cell factor (SCF). G-CSF plays a critical role in regulation of proliferation, differentiation, and survival of myeloid progenitor cells. G-CSF also causes a marked increase in the release of HSCs into the peripheral blood circulation, a process termed mobilization. Therefore, if HCSs could transdifferentiate into cardiomyocytes, G-CSF could contribute to myocardial regeneration. This hypothesis was studied in mice, treating them post-myocardial infarction with SCF and G-CSF (Orlic *et al.*, 2001c). In this study a marked improvement both histologically and functionally was documented, which was combined with enhanced survival of the mice. The results in this mouse myocardial infarction model boosted research in primates. Baboons were either pretreated with SCF and G-CSF or treated post-myocardial damage. The control group was not treated with cytokines. The treatment did indeed increase the number of

circulating stem cells and was shown to enhance perfusion of the infarcted area. However the infarct size was unchanged, showing a contribution of circulating stem cells only to vessel formation, but not to myocardial repair (Norol *et al.*, 2003).

Before the rationale of cytokine treatment after myocardial infarction is considered in clinical trials, it is important to investigate what the natural course of cytokine release is (Takano *et al.*, 2003). Knowledge of the natural behavior would guide a rational and temporal supplementation of crucial factors. During the meeting of the European Heart Society 2003, the group of Massa *et al*. (2005) reported on the mobilization of CD34 cells after myocardial infarction in humans. In addition, increased levels of VEGF and SCF were reported shortly after the ischemic event with a gradual decrease of the levels back to normal in 8 weeks. Furthermore, we will have to uncover which (if any) of the bone marrow stem cells should be mobilized and what the optimal window for treatment will be. Also side effects should be analyzed. In a recent study, Kang *et al.* (2004) showed in patients post-myocardial infarction that the incidence of in-stent restenosis increased in the group treated with G-CSF.

4. INJECTION OF STEM CELLS INTO THE INJURED HEART

Another option to deliver cells to the damaged tissue is to inject cells directly into the heart. This method is feasible only if somatic stem cells can be isolated and safely reintroduced.

4.1. Isolation of Somatic Stem Cells

Isolation of somatic stem cells often starts with the purification of the mononuclear cell (MNC) fraction using a Ficoll gradient. This method isolates cells on the basis of their density. The mononuclear cell fraction contains cells of multiple lineages and phenotypes, e.g. the mesenchymal stem cell (MSC), the HSC, and the endothelial precursor cell (EPC). If this method is used with a bone marrow sample, a relatively large number of stem cells is isolated but if the method is applied to peripheral blood samples the number of stem cells is much lower (0.1% of the MNCs) (Cottler-Fox *et al.*, 1992; Oertel *et al.*, 1998).

For the isolation of more specific somatic stem cells, three methods are being applied frequently. The first method was described by Goodell and coworkers. This method relies on the differential ability of stem cells to efflux the Hoechst dye. Bone marrow cells were stained with the fluorescent DNA-binding dye Hoechst 33342 and analyzed using dual-wavelength flow cytometry. This selection defines an extremely small and rather homogeneous population of cells (termed side population [SP] cells), based on their position in the spectrum (Fig. 1). The SP fraction of bone marrow stem cells is about 0.1% of total MNCs. This method has been used to isolate transplantable SP cells in mice, apes, and humans (Bellantuono, 2004; Goodell, 1999; Goodell *et al.*, 1997, 2001). Besides from the bone marrow, SP

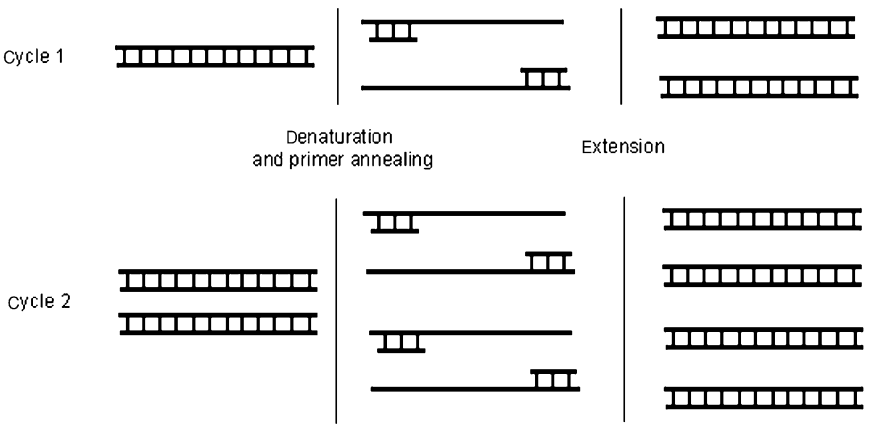

FIGURE 1. Example of detection of SP cells using their capacity to efflux Hoechst. The SP cells are located within the box.

cells have been isolated form various other sources including lung, aortic wall, and heart. The characteristics of SP cells, based on membrane protein expression, are variable, but in general SP cells are considered to be Lin−, CD34−, Sca-1+, and CD45+ (Table 1). Interestingly, SP cells have been shown to contribute to regeneration of ischemic myocardial tissue in mice by differentiating into endothelial cells and possibly cardiomyocytes (Goodell *et al.*, 2001).

A second approach is the isolation of MSCs from bone marrow. MSCs are isolated by seeding the MNC fraction on plastic and culture them. This plastic-adherent population of the marrow stromal cells is CD34− and CD45−/low, differentiating them from the HSC lineage. MSCs are able to differentiate into the adipocytic, chondrocytic, or osteocytic lineages and possibly into cardiomyocytes. MSCs are also present in the liver, brain, and even in lipoaspirates from human fat (Zuk *et al.*, 2002). As their counterparts in bone marrow, these lipoaspirate-derived cells have been shown to be multipotential and to differentiate into a skeletal muscle lineage (MyoD and myosin heavy chain expression) within 6 weeks (Mizuno *et al.*, 2002) and sporadically into cardiomyocytes (Planat-Benard *et al.*, 2004).

Table 1
Somatic Stem Cells and Their Expression

Cell Type	Gene Expression	Species	Reference
SP	Lin−, CD34−, Sca−1+, CD45+	M,h	Goodell *et al.*, 1997
EPC	CD34+, VEGFR−2+, CD133+	M,h	Rafii and Lyden, 2003
MSC	CD34−, CD14−, CD45−	M,H	Toma *et al.*, 2002
CPC	c−kit+, MDR1+, Sca−1+, c−Kit	M,H	Beltrami *et al.*, 2003; Oh *et al.*, 2003
MAPC	CD34−, CD45−, GpA−	M,H	Reyes and Verfaillie, 2001
HSC	CD34+, Sca−1, c−Kit	M,h	Bellantuono, 2004

Interestingly, Verfaillie and coworkers identified a specific cell within the bone marrow stromal cells of mice and humans, with the apparent ability to give rise to a wide range of cell types. These cells have been termed multipotent adult precursor cells (MAPCs) (Reyes *et al.*, 2001; Reyes and Verfaillie, 2001). The MAPCs are CD45− and glycophorin A−, and the frequency of MAPC has been determined to be 10^{-7} of the MNC fraction of the bone marrow. Very few of these cells can be retrieved from peripheral blood, under physiologic conditions. MAPCs are purified by prolonged cell culture (±3 months) after which a renewal growth speed is observed, indicating a transition in these cells to the stem cell phenotype. MAPCs have been kept in culture for many passages, without changes in karyotype or loss of their high telomerase activity. Most important, despite tedious culture demands, once established they will differentiate to somatic cells of most lineages in culture including cardiomyocytes (Reyes *et al.*, 2001). Although there are some differences in the behavior of MAPCs derived from the marrow versus peripheral blood, both appear to differentiate extensively (Verfaillie *et al.*, 2000). The major limitation of this stem cell population is the prolonged culture protocol required.

The third approach is based on the application of membrane proteins indicative of the stem cell phenotype. Cells can be sorted on the basis of antibody–epitope interaction. Markers used to isolate stem cells include CD34, AC133, Sca-1, c-Kit, Lin, SSEA-3, SSEA-4, TRA1-60, and TRA1-81 (http://stemcells.nih.gov/stemcell/stemCellRegistry.asp).

5. SOMATIC STEM CELLS AND THEIR DIFFERENTIATION CAPACITY

5.1. Bone Marrow

Among all presently known somatic stem or progenitor cells, bone marrow cells have shown the highest potential with respect to multilineage differentiation and functional engraftment into host animals. Bone marrow-derived stem cells are a potential source of cells for myocardial repair (Jackson *et al.*, 2001; Orlic *et al.*, 2001a, 2001b). Their differentiation into cardiomyocytes was observed for the first time in a sex-mismatched bone marrow transplantation study using dystrophic mdx mice, which suffer from cardiac muscle degeneration. Bittner *et al.* demonstrated the presence of single cardiomyocytes containing bone marrow-derived Y chromosomes incorporated into the myocardium (Bittner *et al.*, 1999), indicating that cardiac muscle is capable of undergoing regeneration by recruiting circulating bone marrow-derived progenitor cells.

5.2. Mesenchymal Stem Cells

Others used a more defined stem cell population of bone marrow cells, the MSC. MSCs were shown to be able to differentiate into cardiomyocytes *in vitro*.

Interestingly, by treatment of MSCs with the cytosine analog 5-azacytidine, acetylation of DNA and subsequently gene expression is altered such that Makino *et al.* were able to establish cell lines with a high rate of cardiomyocyte formation (Makino *et al.*, 1999). This group suggested that these cells are cardiomyocyte precursor cells or possibly cardiomyoblasts and have designated them cardiomyogenic (CMG) cells (Fukuda, 2001; Makino *et al.*, 1999). CMG cells start expressing β-adrenergic and muscarinic receptors upon differentiation (Hakuno *et al.*, 2002). In addition, these cells can be stably transfected and have been used in transplantation experiments. CMG cells expressing green fluorescent protein (GFP) driven by a CMV promoter were shown to survive, couple with neighboring cells, and contract for more than 3 weeks postinjection (Doevendans *et al.*, 1996; Fukuda, 2001). Injection of fetal and neonatal cardiomyocytes in cryoinjured murine hearts showed similar results, including the expression of cadherin and connexin-43. Despite these promising results, cell growth was uncontrollable and also long-term cell survival *in vivo* has not been achieved (Reinecke *et al.*, 1999). Currently, no human CMG-like cells have been identified.

The cardiogenic potential of MSCs was further demonstrated *in vivo* by injecting early-passage human MSCs, expressing β-galactosidase, into the left ventricle of SCID mice. Although only a small percentage of donor cells could be detected in the heart (0.44%), the engrafted cells did express several cardiac markers, such as cardiac troponin-T and α-actinin (Toma *et al.*, 2002). The problem with this study is that the definition of MSC is unclear. The cells were isolated by density gradient, without additional characterization, leaving all MNCs, not only the MSC. Pluripotent cells derived from rat liver were also shown to differentiate into cardiomyocytes following injection into healthy tissue. The genetically marked transplanted cells were detected by X-gal staining and their origin confirmed by Y chromosome detection in female hearts. Furthermore, the cardiomyocyte phenotype was confirmed by immunohistochemistry and ultrastructural analysis (Malouf *et al.*, 2001). Mangi *et al.* (2003) isolated cells using differential plating and selection of CD34− cells and labeled this fraction as the MSC. They transfected these cells with a survival gene (*Akt*), injected the cells after experimental-induced myocardial infarction, and showed myocardial regeneration as well as improved left ventricular function in mice. They observed the incorporation of LacZ+ cells in the heart, which costained with specific cardiac markers. All these studies indicate a potential of MSCs to transdifferentiate into cardiac muscle. However, the reported frequencies of the differentiation events are limited, suggesting a possible role for cell fusion.

5.3. Hematopoietic Stem Cells

Not only MSCs, but also a population of HSCs were able to differentiate into cardiomyocytes. Using a model of mouse myocardial infarction, Lin−, c-Kit+ hematopoietic stem cells were injected into the border zone of acute infarcts. Significant repair of the damaged myocardium was observed in about 45% of the mice and the injected cells were shown to contribute to the regeneration of

myocardial tissue and were able to differentiate into cardiomyocytes, fibroblasts, and endothelial cells. The histological evidence of myocardial regeneration was further supported by increased survival of the mice and recovery of cardiac function (Orlic *et al.*, 2001b). In contrast, several independent groups showed that these HSCs do not contribute to myocardial repair. They either transplanted GFP+ HSCs into lethally irradiated nontransgenic recipients (Wagers *et al.*, 2002), transfused the GFP-tagged HSCs into the circulation, or injected LacZ-tagged HSC directly into the damaged heart following ischemic myocardial injury (Balsam *et al.*, 2004; Murry *et al.*, 2004). Therefore it is still unclear what the physiologic contribution of injected HSCs to the repair of injured myocardium really is.

5.4. Cell Fusion

It has been questioned whether the expression of tissue-specific markers by a transplanted stem cell actually represents trans/dedifferentiation or is based on a rare fusion event between the stem cells and the adjacent somatic cells. Fusion results in tetraploid hybrid cells with the hybrid cells expressing somatic cell markers. The connection between cell fusion and stem cell plasticity was first proposed in reports of hybrid cell formation from cocultures of tissue-derived stem cells and embryonic stem cells (Terada *et al.*, 2002; Ying *et al.*, 2002). More recently, it was shown that cell fusion may account for the contribution of circulating cells to the repair of damaged liver tissue (Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003). The small numbers of differentiated cardiomyocytes identified *in vivo* might be explained by this phenomenon. Although there are multiple reports demonstrating the capacity of HSCs to directly generate cardiac myocytes *in vitro*, this does not exclude fusion of donor cells *in vivo*. Using a simple method based on Cre/lox recombination to detect cell fusion events, Alvarez-Dolado and coworkers demonstrate that bone-marrow-derived cells fuse spontaneously with cardiac muscle in the heart, resulting in the formation of multinucleated cells (Alvarez-Dolado *et al.*, 2003). No evidence of transdifferentiation without fusion was observed. These observations raise the possibility that cell fusion may contribute to the development or maintenance of cardiomyocytes. It is still possible that donor-derived cells underwent cell fusion after undergoing transdifferentiation into a cardiomyocyte or a cardiomyoblast.

5.5. Clinical Trials

Several studies have been reported on the intracoronary infusions of stem cells post-myocardial infarction. The initial studies were designed as safety trials and thus far no major side effects were reported after stem cell administration. No new infarcts or arrhythmias have been reported. Although the initial studies are small and not well controlled, they all report beneficial effects on myocardial perfusion and left ventricular function. The TOP-Care AMI study randomized patients to either infusion of EPCs or crude bone marrow-derived MNCs (containing stem cells). A marked increase in coronary flow reserve was observed in both treatment

arms compared to a historical control group. Also a modest improvement in left ventricular function was reported (Assmus *et al.*, 2002). A randomized trial was also reported by Wollert *et al.* (2004) during the 2003 American Heart Association meeting. In this study 60 patients were either treated with MNCs or not treated. Also here improvement of left ventricular function was observed in the cell infusion group. Myocardial infarct size and left ventricular function were imaged by state-of-the-art MRI technology, but no reduction of infarct size was observed. This is in contrast to a study by Strauer *et al.* (2002) suggesting also a reduction in infarct size. However, the imaging technique applied here was not optimal. Another recent study randomized patients to cell infusion, cytokine treatment, or no treatment and again the treated patients showed improved perfusion and preservation of left ventricular function (Kang *et al.*, 2004), but there was no evidence for reduction of infarct size or increase in myocardial mass.

6. CARDIOMYOBLASTS: DO THEY EXIST?

The question remains if there is a "cardiomyoblast" capable of differentiating into cardiomyocytes and replacing lost heart muscle. Throughout the years, there were several reports suggesting that cells within the myocardium undergo cell division. The documentation of cycling myocytes undergoing mitosis and cytokinesis suggests that new myocardial cells may arise in the adult from stem cells resident in the heart or homed to the injured myocardium from the circulation. Recent studies have identified cardiac stem cell clusters in the adult heart of both rodents and humans. These undifferentiated and early committed cells express stem cell markers like Sca-1, c-Kit, and mdr-1 and harbor telomerase activity. Telomerase is a reverse transcriptase that during the cell cycle synthesizes telomeric repeats at the end of chromosomes, preventing loss of DNA. Telomerase is present in dividing cells and is absent from resting and terminally differentiated cells (Greider, 1998). Besides being expressed in primitive cells, telomerase was also detected in differentiating myocytes, expressing cardiac markers like GATA-4, MEF2, and myosin. The presence of stem cell antigens and cardiac differentiation factors suggests the existence of a sequential lineage commitment of primitive cells into cardiac progenitor cells (CPCs). These studies indicate that the adult heart is a self-renewing organ that possesses an intrinsic growth reserve capable of responding, at least in part, to the physiological and pathological demands of the myocardium.

The potential of the identified CPCs has been studied in more detail in mouse (Oh *et al.*, 2003) and rat (Beltrami *et al.*, 2003). Oh and coworkers have isolated cardiac Sca-1+ cells from mouse heart. The Sca-1+ cells do not express the HSC marker CD45 and the EPC marker CD34 or c-Kit, but do express telomerase. Furthermore, they do not express myocyte, endothelial cell, or smooth muscle cell genes, nor the stem cell marker Oct-4, but do express cardiogenic transcription factors, cell cycle mediators, and transcriptional repressors. Beltrami and coworkers isolated a Lin-c-Kit+ cell population from rat hearts. Freshly isolated c-kit+ cells have an expression profile comparable with the Sca-1+ cell population, but

unfortunately, Sca-1 was not analyzed. The c-Kit+ cells are CD45− and CD34−, but do express Nkx2.5, GATA-4, and MEF2 in about 10% of the cells, with occasionally a cell expressing sarcomeric proteins (<0.5%), suggesting that some of them are already committed to the cardiac myogenic lineage. The lack of CD45 in both Sca-1+ and c-Kit+ CPCs suggested that the isolated cells are of bone marrow origin and have lost the hematopoietic lineage markers residing in the myocardium, or represent cells present in the myocardium since fetal life.

Upon 5-aza stimulation *in vitro*, the Sca-1+ cells differentiate into cardiomyocytes expressing sarcomeric α-actin and cardiac troponin-I in a BMP-dependent manner (Oh *et al.*, 2003). Using a delicate Cre/lox approach, they demonstrated that the i.v. injected Sca-1+ cells are able to home to the infarcted border zone after ischemia/reperfusion injury in mice and differentiate into an αMHC, cardiac troponin-I, and connexin-43 expressing cell. The engraftment of these cells in the left ventricle was approximately 3%, 150-fold greater than reported for marrow-derived SP cells (Jackson *et al.*, 2001). However, in roughly half of the myocytes fusion had taken place. More important, half of the cells differentiated autonomous of fusion. The differentiation capacity of the rat c-Kit+ cardiac cells *in vitro* was studied after growing the cells in medium containing 10^{-8}M dexamethasone (Beltrami *et al.*, 2003). The c-Kit+ cells differentiated into primitive myocytes, smooth muscle cells, and endothelial cells. Injection of these primitive cell types into the border zone of a 5-h-old infarcted rat heart showed that the c-Kit+ cells *in vivo* differentiate into all three cell types, suggesting that the c-Kit+ population is a CPC. Whether fusion takes place has not been as definitely addressed as for the Sca-1+ cells. Fusion alone cannot explain the number of new cells formed in the myocardial infarct model, but cannot be excluded. The different *in vivo* models used, ischemia/reperfusion injury versus myocardial infarction, might give different answers to whether or not fusion takes place and the capacity of the cardiac stem cells to differentiate into only cardiomyocytes or also smooth muscle cells and endothelial cells.

Whether the two CPC populations have the same origin is not clear. The identification of c-Kit+ and Sca-1+ cells in human sections might suggest a common origin. Does this define the cardiomyoblast? One thing that is clear from these studies is that, at least for rodent cells, both cell populations are able to differentiate into cardiomyocytes *in vitro* as well as *in vivo*.

7. CONCLUSION

Repair of the human heart will require restoration of an adequate blood supply to areas of ischemia and replacement of lost or poorly functioning cardiomyocytes. With respect to cell types already used in clinical studies, the predominant *in vivo* effect of bone marrow stem cells or EPCs may be neoangiogenesis or arteriogenesis and not cardiomyocyte differentiation (Assmus *et al.*, 2002). It is extremely difficult to assess the specific effects of myocyte versus nonmyocyte (e.g. endothelial cell)

differentiation on the functional improvement in myocardial performance after injury. Therefore, we have to be careful in attributing benefit to a specific cell while the bone marrow contains cells of multiple lineage and phenotype, not to mention the host of potential regulatory factors released by these cells. The good news from the finalized clinical studies is that the strategies to administer stem cells appear to be safe and all these small studies hint at improved left ventricular function. The bad news is that the clinical studies reported thus far lack the appropriate control groups and there is not a single shred of evidence that stem cell administration to the damaged heart contributes to myocardial regeneration. The quest to identify the CPC capable of cardiomyocyte differentiation is a global research goal. Although various authors claim the quest is over and that we have CPCs, we like to consider this conclusion premature (Badorff *et al.*, 2003; Condorelli *et al.*, 2001). First we will have to fully characterize the progenitor cells and before clinical applications should be considered these cells should proof their value in long-term functional studies in large animals (Chien, 2004).

REFERENCES

Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J. M., Fike, J. R., Lee, H. O., Pfeffer, K., Lois, C., Morrison, S. J., and Alvarez-Buylla, A., 2003, Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes, *Nature* **425**:968–973.

Anversa, P., and Nadal-Ginard, B., 2002, Myocyte renewal and ventricular remodelling, *Nature* **415**:240–243.

Assmus, B., Schachinger, V., Teupe, C., Britten, M., Lehmann, R., Dobert, N., Grunwald, F., Aicher, A., Urbich, C., Martin, H., Hoelzer, D., Dimmeler, S., and Zeiher, A. M., 2002, Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOP-Care AMI). *Circulation* **106**:3009–3017.

Badorff, C., Brandes, R. P., Popp, R., Rupp, S., Urbich, C., Aicher, A., Fleming, I., Busse, R., Zeiher, A. M., and Dimmeler, S., 2003, Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation* **107**:1024–1032.

Balsam, L. B., Wagers, A. J., Christensen, J. L., Kofidis, T., Weissman, I. L., and Robbins, R. C., 2004, Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* **428**:668–673.

Bellantuono, I., 2004, Haemopoietic stem cells. *Int. J. Biochem. Cell Biol.* **36**:607–620.

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., Leri, A., Kajstura, J., Nadal-Ginard, B., and Anversa, P., 2003, Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**:763–776.

Bittner, R. E., Schofer, C., Weipoltshammer, K., Ivanova, S., Streubel, B., Hauser, E., Freilinger, M., Hoger, H., Elbe-Burger, A., and Wachtler, F., 1999, Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat. Embryol. (Berlin)* **199**:391–396.

Cavallaro, A. M., Lilleby, K., Majolino, I., Storb, R., Appelbaum, F. R., Rowley, S. D., and Bensinger, W. I., 2000, Three to six year follow-up of normal donors who received recombinant human granulocyte colony-stimulating factor. *Bone Marrow Transplant.* **25**:85–89.

Chien, K. R. 2004, Stem cells: lost in translation. *Nature* **428**:607–608.

Condorelli, G., Borello, U., De Angelis, L., Latronico, M., Sirabella, D., Coletta, M., Galli, R., Balconi, G., Follenzi, A., Frati, G., Cusella De Angelis, M. G., Gioglio, L., Amuchastegui, S., Adorini, L., Naldini, L., Vescovi, A., Dejana, E., and Cossu, G., 2001, Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. *Proc. Natl. Acad. Sci. U.S.A.* **98**:10733–10738.

Cottler-Fox, M., Read, E. J., Yu, M., Carter, C., and Klein, H. G. 1992, Mononuclear and Cd34+ stem cell recovery after automated Ficoll processing of marrow or peripheral blood stem cells for transplantation. *Prog. Clin. Biol. Res.* **377**:569–573.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. 1985, The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**:27–45.

Doevendans, P. A., Becker, D. A., An, R., and Kass, R. S. 1996, The utility of fluorescent *in vivo* reporter genes in molecular cardiology. *Biochem. Biophys. Res. Commun.* **222**:352–358.

Fukuda, K. 2001, Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. *Artif. Organs* **25**:187–193.

Goodell, M. A. 1999, Introduction: focus on hematology. Cd34(+) or Cd34(−): does it really matter? *Blood* **94**:2545–2547.

Goodell, M. A., Jackson, K. A., Majka, S. M., Mi, T., Wang, H., Pocius, J., Hartley, C. J., Majesky, M. W., Entman, M. L., Michael, L. H., and Hirschi, K. K., 2001, Stem cell plasticity in muscle and bone marrow. *Ann. N. Y. Acad. Sci.* **938**:208–218.

Goodell, M. A., Rosenzweig, M., Kim, H., Marks, D. F., Demaria, M., Paradis, G., Grupp, S. A., Sieff, C. A., Mulligan, R. C., and Johnson, R. P., 1997, Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of Cd34 antigen exist in multiple species. *Nat. Med.* **3**:1337–1345.

Greider, C. W. 1998, Telomerase activity, cell proliferation, and cancer. *Proc. Natl. Acad. Sci. U.S.A.* **95**:90–92.

Hakuno, D., Fukuda, K., Makino, S., Konishi, F., Tomita, Y., Manabe, T., Suzuki, Y., Umezawa, A., and Ogawa, S., 2002, Bone marrow-derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and muscarinic receptors. *Circulation* **105**:380–386.

Hassink, R. J., Brutel, D. L. R., Mummery, C. L., and Doevendans, P. A. 2003a, Transplantation of cells for cardiac repair. *J. Am. Coll. Cardiol.* **41**:711–717.

Hassink, R. J., Dowell, J. D., Brutel, D. L. R., Doevendans, P. A., and Field, L. J. 2003b, Stem cell therapy for ischemic heart disease. *Trends Mol. Med.* **9**:436–441.

Jackson, K. A., Majka, S. M., Wang, H., Pocius, J., Hartley, C. J., Majesky, M. W., Entman, M. L., Michael, L. H., Hirschi, K. K., and Goodell, M. A., 2001, Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* **107**:1395–1402.

Kang, H. J., Kim, H. S., Zhang, S. Y., Park, K. W., Cho, H. J., Koo, B. K., Kim, Y. J., Soo Lee, D., Sohn, D. W., Han, K. S., Oh, B. H., Lee, M. M., and Park, Y. B., 2004, Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the Magic Cell Randomised Clinical Trial. *Lancet* **363**:751–756.

Laflamme, M. A., Myerson, D., Saffitz, J. E., and Murry, C. E. 2002, Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ. Res.* **90**:634–640.

Makino, S., Fukuda, K., Miyoshi, S., Konishi, F., Kodama, H., Pan, J., Sano, M., Takahashi, T., Hori, S., Abe, H., Hata, J., Umezawa, A., and Ogawa, S., 1999, Cardiomyocytes can be generated from marrow stromal cells in vitro. *J. Clin. Invest.* **103**:697–705.

Malouf, N. N., Coleman, W. B., Grisham, J. W., Lininger, R. A., Madden, V. J., Sproul, M., and Anderson, P. A., 2001, Adult-derived stem cells from the liver become myocytes in the heart in vivo. *Am. J. Pathol.* **158**:1929–1935.

Mangi, A. A., Noiseux, N., Kong, D., He, H., Rezvani, M., Ingwall, J. S., and Dzau, V. J., 2003, Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat. Med.* **9**:1195–1201.

Massa, M., Rosti, V., Ferrario, M., Campanelli, R., Ramajoli, I., Rosso, R., De Ferrari, G. M., Ferlini, M., Goffredo, L., Bertoletti, A., Klersy, C., Pecci, A., Moratti, R., and Tavazzi, L., 2005, Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. *Blood* **105**(1):199–206.

Mcculloch, E. A., Till, J. E., and Siminovitch, L. 1965, The role of independent and dependent stem cells in the control of hemopoietic and immunologic responses. *Wistar Inst. Symp. Monogr.* **4**:61–68.

Mizuno, H., Zuk, P. A., Zhu, M., Lorenz, H. P., Benhaim, P., and Hedrick, A. M. 2002, Myogenic differentiation by human processed lipoaspirate cells. *Plast. Reconstr. Surg.* **109**:199–209.

Murry, C. E., Soonpaa, M. H., Reinecke, H., Nakajima, H., Nakajima, H. O., Rubart, M., Pasumarthi, K. B., Virag, J. I., Bartelmez, S. H., Poppa, V., Bradford, G., Dowell, J. D., Williams, D. A., and Field, L. J., 2004, Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* **428**:664–668.

Norol, F., Merlet, P., Isnard, R., Sebillon, P., Bonnet, N., Cailliot, C., Carrion, C., Ribeiro, M., Charlotte, F., Pradeau, P., Mayol, J. F., Peinnequin, A., Drouet, M., Safsafi, K., Vernant, J. P., and Herodin, F., 2003, Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model. *Blood* **102**:4361–4368.

Oertel, J., Oertel, B., Schleicher, J., and Huhn, D. 1998, Detection of small numbers of immature cells in the blood of healthy subjects. *J. Clin. Pathol.* **51**:886–890.

Oh, H., Bradfute, S. B., Gallardo, T. D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L. H., Behringer, R. R., Garry, D. J., Entman, M. L., and Schneider, M. D., 2003, Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc. Natl. Acad. Sci. U.S.A.* **100**:12313–12318.

Orlic, D., Kajstura, J., Chimenti, S., Bodine, D. M., Leri, A., and Anversa, P. 2001a, Transplanted adult bone marrow cells repair myocardial infarcts in mice. *Ann. N. Y. Acad. Sci.* **938**:221–229.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M., Leri, A., and Anversa, P., 2001b, Bone marrow cells regenerate infarcted myocardium. *Nature* **410**:701–705.

Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D. M., Leri, A., and Anversa, P., 2001c, Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc. Natl. Acad. Sci. U.S.A.* **98**:10344–10349.

Planat-Benard, V., Menard, C., Andre, M., Puceat, M., Perez, A., Garcia-Verdugo, J. M., Penicaud, L., and Casteilla, L., 2004, Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. *Circ. Res.* **94**:223–229.

Quaini, F., Urbanek, K., Beltrami, A. P., Finato, N., Beltrami, C. A., Nadal-Ginard, B., Kajstura, J., Leri, A., and Anversa, P., 2002, Chimerism of the transplanted heart. *N. Engl. J. Med.* **346**:5–15.

Rafii, S., and Lyden, D. 2003, Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat. Med.* **9**:702–712.

Reinecke, H., Zhang, M., Bartosek, T., and Murry, C. E. 1999, Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation* **100**:193–202.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L., and Verfaillie, C. M. 2001, Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**:2615–2625.

Reyes, M., and Verfaillie, C. M. 2001, Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. *Ann. N. Y. Acad. Sci.* **938**:231–233.

Strauer, B. E., Brehm, M., Zeus, T., Kostering, M., Hernandez, A., Sorg, R. V., Kogler, G., and Wernet, P., 2002 (Oct 8), Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* **106**(15):1913–8.

Takano, H., Ohtsuka, M., Akazawa, H., Toko, H., Harada, M., Hasegawa, H., Nagai, T., and Komuro, I., 2003, Pleiotropic effects of cytokines on acute myocardial infarction: G-Csf as a novel therapy for acute myocardial infarction. *Curr. Pharm. Des.* **9**:1121–1127.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W., 2002 (Apr 4), Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416**(6880):542–5.

Toma, C., Pittenger, M. F., Cahill, K. S., Byrne, B. J., and Kessler, P. D. 2002, Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**:93–98.

Vassilopoulos, G., Wang, P. R., and Russell, D. W. 2003, Transplanted bone marrow regenerates liver by cell fusion. *Nature* **422**:901–904.

Verfaillie, C. M., Almeida-Porada, G., Wissink, S., and Zanjani, E. D. 2000, Kinetics of engraftment of Cd34(−) and Cd34(+) cells from mobilized blood differs from that of Cd34(-) and Cd34(+) cells from bone marrow. *Exp. Hematol.* **28**:1071–1079.

Wagers, A. J., Sherwood, R. I., Christensen, J. L., and Weissman, I. L. 2002, Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* **297**:2256–2259.

Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M., 2003, Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* **422**:897–901.

Wollert, K. C., Meyer, G. P., Lotz, J., Ringes-Lichtenberg, S., Lippolt, P., Breidenbach, C., Fichtner, S., Korte, T., Hornig, B., Messinger, D., Arseniev, L., Hertenstein, B., Ganser, A., and Drexler, H., 2004 (Jul 10), Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* **364**(9429):141–8.

Ying, Q., Nichols, J., Evans, E. P., and Smith, A. 2002 (Apr 4), Changing potency by spontaneous fusion. *Nature* **416**(6880):545–8.

Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., Alfonso, Z. C., Fraser, J. K., Benhaim, P., and Hedrick, M. H., 2002, Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**:4279–4295.

Zwaginga, J. J., and Doevendans, P. 2003, Stem cell-derived angiogenic/vasculogenic cells: possible therapies for tissue repair and tissue engineering. *Clin. Exp. Pharmacol. Physiol.* **30**:900–908.

Chapter 10

Gene Therapy for Angiogenesis

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1. INTRODUCTION

Atherosclerosis and endothelial dysfunction are responsible for the pathophysiologic basis of the spectrum of cardiovascular disorders including ischemic heart disease (IHD), the leading cause of morbidity and mortality in the United States. There have been major advances including the use of pharmacotherapy, coronary and peripheral percutaneous transluminal interventions (PTI), coronary and peripheral bypass surgery, and primary/secondary prevention measures. There are, however, multiple unmet needs: IHD refractory to medical therapy and unsuitable for revascularization; critical limb ischemia (CLI) unsuitable for PTI or surgery; restenosis; ischemic/diabetic neuropathy; and heart failure. Cardiovascular gene therapy (GT) with vascular endothelial growth factor (VEGF) and other angiogenic factors such as fibroblast growth factor (FGF) has yielded improved perfusion and reduced ischemia in preclinical models of IHD. Several preclinical studies, phase I and II clinical trials, have shown the safety and therapeutic potential of GT in the treatment of IHD, peripheral arterial disease (PAD), restenosis, and ischemic and diabetic neuropathy, pointing to the need for carefully designed phase III clinical trials, which would have to address the advantages and disadvantages of the diverse delivery strategies, candidate genes, and methods of functional assessment of angiogenesis, while selecting relevant primary and secondary

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

endpoints and maximizing the benefit/risk ratio in order to guarantee that this new strategy would not only be offered to critically ill patients who have exhausted all other therapeutic options but that it rather be extended to a broad range of patients in an effort to solve the multiple unmet clinical needs and to provide an answer to the currently existing enigmas.

2. BACKGROUND

Atherosclerosis and endothelial dysfunction are responsible for the pathophysiologic process underlying myocardial ischemia and CLI, as well as ischemic neuropathy (Annex, 2002; Gaziano, 1998; Kibbe *et al.*, 2000; Simovic *et al.*, 2001). IHD remains the leading cause of morbidity and mortality in the United States, accounting for about 50% of all deaths. As the population ages, IHD is expected to become the leading cause of death worldwide (Annex, 2002; Gaziano, 1998). Patients with CLI have poor prognosis (Isner, 1998). The prevalence of PAD is 12% in the United States, where 150,000 patients undergo lower limb amputations every year (Dormandy and Thomas, 1988; Isner *et al.*, 1996b). The prognosis after amputation is worse given a perioperative mortality rate of 5–10% for below-knee amputations and 15–20% for above-knee amputations. There is 40% mortality 2 years after a major amputation, which is required in 30% of the cases (Ischemia EWGoCL, 1991). These poor statistics are compounded by the lack of effective medical therapy, which is also less than ideal for claudication (Isner and Rosenfield, 1993; Pentecost *et al.*, 1994). Despite improvements in pharmacotherapy, surgical methods, and the availability of stents, these therapies have several limitations, including the lack of compliance with medications (aggravated by the amount of medications these patients have to take with the inherent predisposition to drug interactions and side effects), economical impact, and procedure-related complications among others. Regarding the latter, the development of intimal and neointimal hyperplasia (restenosis) has been recognized as the Achilles' heel of modern revascularization techniques, significantly reducing the hemodynamic, physiologic, and symptomatic improvement originally conferred by the procedure. The rates of restenosis are still up to 40% after "bare" coronary stenting. More recently, the coronary implantation of third-generation stents coated with cytostatic drugs such as sirolimus has been reported to reduce the restenosis rates to an impressive 0–3% for nondiabetic patients (Regar *et al.*, 2002) and 8.6% for diabetic patients (Moses *et al.*, 2003). Stents coated with antineoplastic drugs such as paclitaxel (Hong *et al.*, 2003)have also yielded restenosis rates as low as 4% after 6 months of follow-up (Stone *et al.*, 2004). In the peripheral arena, the rates of restenosis are 53% after femoropopliteal PTI with brachytherapy (Minar *et al.*, 2000), and up to 80% after femoropopliteal surgical bypass. Five-year restenosis rates are up to 46% for aortoiliac PTI and 15% for aortofemoral surgical bypass (Kibbe *et al.*, 2000; Schainfeld, 2002). Restenosis occurs in 19–83% after coronary PTI for in-stent restenosis (ISR). Intracoronary γ- and β-radiation therapies with the use of ^{192}Ir and ^{90}Sr/^{90}Y, respectively, are effective in reducing

the incidence of ISR (Popma *et al.*, 2002). However, these studies had limitations including vessel diameter > 2.7 mm, length < 30 mm, and follow-up less than a year. In saphenous-vein coronary bypass grafts, the use of ^{192}Ir resulted in 21% incidence of ISR, compared to 45% without brachytherapy (Waksman *et al.*, 2002). This technique, considered the "standard of care" for ISR, still leaves 20–30% of patients having major cardiovascular events. In the peripheral vasculature, ISR rates are approximately 50% after "bare" stents, with recently reported rates of 22.6% after sirolimus-eluting stents (Duda *et al.*, 2002). The use of ^{192}Ir has reduced ISR to 11% for superficial femoral artery, and to 28% for femoropopliteal angioplasty (Sidawy *et al.*, 2002), with rates up to 41–43% in patients that received brachytherapy immediately after femoropopliteal stenting (Bonvini *et al.*, 2003).

Patients with chronic CLI, which damages peripheral nerves and produces localized neuropathy in the ischemic limb, represent another group with severely limited therapeutic modalities. Electrophysiologic studies after surgical revascularization have failed to show improvement, leading authors to consider ischemic neuropathy as an irreversible condition (Simovic *et al.*, 2001). Diabetic peripheral neuropathy and its complications account for up to 20% of all hospital admissions in the United States, leading to lower limb amputation at a rate 15 times higher than in nondiabetic patients. This entity is presumed to occur as a consequence of several phenomena, including microvascular disease with impaired blood flow and ischemic nerves (Schratzberger *et al.*, 2001). Finally, heart failure (HF) from all causes (but especially secondary to IHD) represents the most expensive health care problem in the United States, with 4.6 million Americans being affected, 400,000 new cases diagnosed each year, and 260,000 deaths (doubling the rate from 1979 to 1995), which may reflect the aging US population and severe limitation of our current available therapies (Abraham and Scarpinato, 2002; Hajjar *et al.*, 2000).

All these clinical entities share the same background: they have high clinical impact, grim statistics, and a combination of pharmacological and mechanical therapies that have improved outcomes to a certain degree, which still is far from ideal. Moreover, approximately 12% of patients are not optimal candidates for the available treatments because of comorbid diseases, unsuitable anatomy due to diffusely diseased runoff vessels, lack of available conduits, unacceptably high procedural risk, or a combination of these factors (Kalka *et al.*, 2000; Mukherjee *et al.*, 1999). These patients represent a rather large sample of the universe of multiple unmet clinical needs in the cardiovascular arena, which points to the compelling need for alternative, effective treatment strategies (Isner *et al.*, 2001).

Cardiovascular GT has emerged as one of the most promising new therapies. It is supported by a large body of evidence in animal models of myocardial and hind limb ischemia, showing that the administration of angiogenic growth factors (AGFs) such as VEGF—either as recombinant protein (RP) or by gene transfer (GTx)—among others improves perfusion and reduces ischemia through neovascularization (Baffour *et al.*, 1988; Banai *et al.*, 1991, 1994; Freedman and Isner, 2002; Pearlman *et al.*, 1995; Pu *et al.*, 1993b; Takeshita *et al.*, 1994d;

Yanagisawa-Miwa *et al.*, 1992). The therapeutic implications of AGFs were identified by Folkman's pioneering work (Folkman, 1971, 1972; Folkman *et al.*, 1971). Among the different growth factors that promote angiogenesis (Folkman, 1987, 1992), VEGF, also known as vascular permeability factor (Keck *et al.*, 1989) and vasculotropin (Plouet *et al.*, 1989), has been shown to be an endothelial cell (EC) mitogen *in vitro* (Keck *et al.*, 1989; Leung *et al.*, 1989) and an AGF *in vivo* (Banai *et al.*, 1994; Connolly *et al.*, 1989; Ferrara and Henzel, 1989; Takeshita *et al.*, 1994d). VEGF is distinguished from most other angiogenic cytokines by a unique sequence in the first exon of the VEGF gene (Tischer *et al.*, 1991) that allows the protein to be naturally secreted by intact cells. These features led to its investigation in a strategy of arterial GT in patients with PAD (Isner *et al.*, 1995). Single intracoronary (IC) doses of $VEGF_{165}$RP (Hariawala *et al.*, 1996; Lopez *et al.*, 1998) as well as periadventitial infusions via minipump (Harada *et al.*, 1996; Lopez *et al.*, 1998) and intramyocardial injections were reported to be effective in the porcine model, and in canine models that received IC injections via an indwelling catheter for 28 days (Tio *et al.*, 1999). Using a thoracotomy in the porcine model of myocardial ischemia, GTx via intramyocardial injections of naked plasmid DNA (DNA not associated to viral vectors) encoding $VEGF_{165}$ (Tio *et al.*, 1999) and VEGF-2, or adenovirus encoding $VEGF_{121}$ (Lee *et al.*, 2000; Mack *et al.*, 1998), showed improved collateral circulation and myocardial contractile function. The IC (Lee *et al.*, 2000) and pericardial delivery (Lazarous *et al.*, 1999) of adenoviral gene resulted in low gene expression and VEGF protein levels in the myocardium, along with poor localization, which favored the intramyocardial route (Lee *et al.*, 2000), for which catheter-based (CB) delivery of $VEGF_{165}$ was effective. This less invasive approach achieves suitable levels of gene expression (Kornowski *et al.*, 2000; Vale *et al.*, 1999b) and was used in a clinical trial of VEGF-2 GTx (Vale *et al.*, 2001). Direct application of VEGF RP to the denuded surface of the rat carotid artery (Asahara *et al.*, 1995), rhVEGF$_{165}$to rabbit vein grafts (Luo *et al.*, 1998), and CB, site-specific arterial GTx of phVEGF$_{165}$ to the denuded surface of the rabbit femoral artery (Asahara *et al.*, 1996) have been shown by our laboratory to accelerate reendothelialization (rET), improve endothelial function, and significantly reduce intimal proliferation. Similarly, we also showed that CB delivery of rhVEGF$_{165}$ (Van Belle *et al.*, 1997a) and gene-eluting stents coated with phVEGF-2 naked plasmid DNA (Walter *et al.*, 2002) accelerate rET and inhibit neointimal proliferation in the iliac arteries of rabbits after balloon denudation and stent implantation. In rats with streptozocin-induced diabetes, intramuscular GTx of VEGF-1 or VEGF-2 showed similar vascularity and blood flow in the nerves of treated animals as in the nondiabetic controls (Schratzberger *et al.*, 2001). Finally, intramyocardial GTx of adenovirus encoding $VEGF_{121}$ in pigs with pacing-induced HF showed preservation of cardiac performance and faster recovery of myocardial function (Leotta *et al.*, 2002), and isolated deletion of the VEGF-1 gene from cardiomyocytes in mice resulted in left ventricular (LV) dysfunction and significant reduction in the number of coronary microvessels, implying that the coronary microvasculature is responsible for both myocardial ischemia and LV dysfunction (Isner, 2002). In summary, GT with VEGF has proven to be an effective alternative

approach for the myriad of patients with cardiovascular disease who are not appropriately treated with the currently available therapeutic modalities.

3. SCIENTIFIC RATIONALE

What leads to atherosclerosis is unknown. It has been inferred that atherosclerosis results from several events including inflammation, accumulation of lipid fractions, and proliferation of smooth muscle cells (SMCs). However, the key event seems to be the failure of the endothelium to exert its normal homeostatic regulation over these processes. The concept that endothelial injury and/or dysfunction plays a central role in atherogenesis has driven several investigations into the pathogenesis and treatment of atherosclerosis and its clinical consequences (Losordo and Isner, 2001), such as the formation of high-grade stenosis or occlusions in the vasculature. These lesions lead to the development of collateral arteries (neovascularization), a process geared by AGFs whose therapeutic implications were identified by Folkman and coworkers (Folkman, 1971, 1972; Folkman *et al.*, 1971). Previously mentioned studies established the feasibility of using AGFs to expedite and/or augment neovascularization in animal models of myocardial and hind limb ischemia, a novel strategy to treat vascular insufficiency that was termed *therapeutic angiogenesis* (Höckel *et al.*, 1993). Angiogenesis—the process of proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels—and vasculogenesis—the *in situ* formation of embryonic blood vessels from endothelial progenitor cells (EPCs) or angioblasts—are responsible for the development of the vascular system in the embryo (Asahara *et al.*, 1997; Flamme and Risau, 1992; Gilbert, 1997; Risau, 1995; Weiss and Orkin, 1996). Until recently, vasculogenesis was thought to be restricted to embryonic development, whereas angiogenesis was considered the only mechanism responsible for neovascularization in adults. This paradigm changed with the discovery that bone marrow (BM)-derived EPCs circulate in adult peripheral blood (Asahara *et al.*, 1997). BM-EPCs home to and incorporate into foci of neovascularization in adult animals (Asahara *et al.*, 1999a), proliferate in response to tissue ischemia (Takahashi *et al.*, 1999), and augment development of collateral vessels after *ex vivo* expansion and transplantation (Kalka *et al.*, 2000a). These studies established that neovascularization in adults is not restricted to angiogenesis but rather involves both mechanisms observed in the embryo (Fig. 1). A third mechanism that may contribute to neovascularization is an increase in the size and caliber of preexisting arteriolar collateral connections by remodeling, process termed *arteriogenesis* (Arras *et al.*, 1998). When an occlusion occurs, preexisting collateral vessels experience an increase in blood flow velocity and luminal shear stress, leading to maturation of medium-sized collateral conduits that are visible on angiography (Arras *et al.*, 1998; Baumgartner *et al.*, 1998; Isner *et al.*, 1996a). Under these ischemic conditions, neovascularization constitutes a natural defense intended to maintain tissue perfusion required for physiological organ function. However, under certain circumstances such as advanced age, diabetes, and

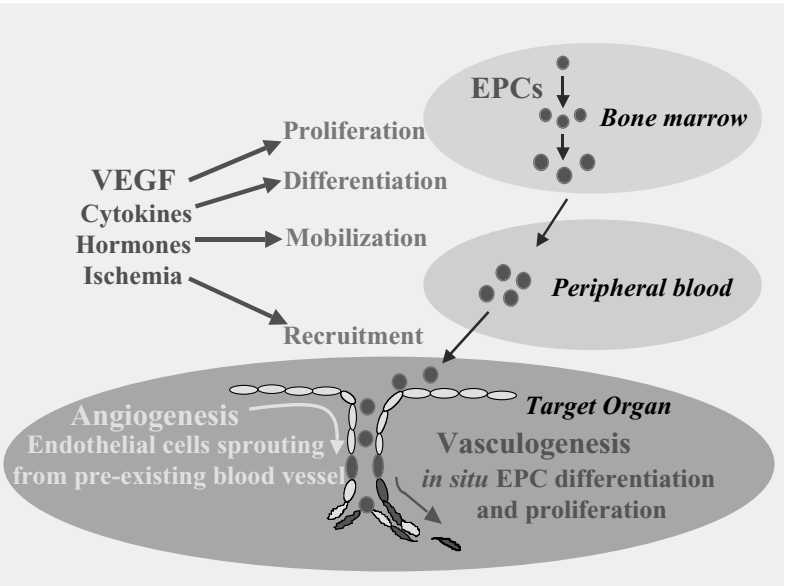

FIGURE 1. Neovascularization encompasses both angiogenesis and vasculogenesis. Angiogenesis represents the classic paradigm for new vessel growth, as mature, differentiated endothelial cells (ECs) break free from their basement membrane and migrate as well as proliferate to form sprouts from parental vessels. Vasculogenesis involves participation of bone marrow-derived endothelial progenitor cells (BM-EPCs), which circulate to sites of neovascularization where they differentiate *in situ* into mature ECs. Growth factors, cytokines, or hormones released endogenously in response to tissue ischemia, or administered exogenously for therapeutic neovascularization, act to promote EPC proliferation, differentiation, and mobilization from BM (via the peripheral circulation) to finally home and incorporate into neovascular foci. (*See Color Plate 13.*)

hypercholesterolemia, such native angiogenesis is impaired (Couffinhal *et al.*, 1999; Rivard *et al.*, 1999a, 1999b; Van Belle *et al.*, 1997b). In each of these circumstances, animal studies have disclosed a reduction in endogenous expression of VEGF and enhanced neovascularization in response to VEGF supplementation, which suggests that VEGF expression is indeed a critical determinant of postnatal neovascularization. Older, diabetic and hypercholesterolemic animals—like human subjects—also exhibit evidence of endothelial dysfunction (Kalka *et al.*, 2000a).

The "endothelial injury/dysfunction" concept and its pathophysiologic relationship to atherosclerosis have been propelled by objective evidence: measurable deficiencies in endothelium-dependent functions such as nitric oxide (NO) production in human atherosclerotic arteries (Harrison *et al.*, 1987); acceleration of atheroma formation after chemically or mechanically induced endothelial dysfunction (Holm *et al.*, 1999; Naruse *et al.*, 1994), and the direct relationship between severity and extent of EC dysfunction and severity and extent of intimal disease in coronary arteries harvested from explanted hearts (Davies *et al.*, 1988). Other

studies have documented that delinquent rET has a permissive, if not facilitatory, impact on SMC proliferation (Asahara *et al.*, 1996). This inverse relationship has been attributed to certain endothelial functions including barrier regulation of permeability, production of growth-inhibitory molecules, thrombogenicity, and leukocyte adherence, which are critical in the prevention of intimal/neointimal proliferation (Losordo and Isner, 2001; Ross, 1993). This concept has led to the development of new strategies directed to provide endothelial protection in veins used for bypass surgery (Haudenschild *et al.*, 1981; LoGerfo *et al.*, 1983), to accelerate rET after balloon-induced arterial injury (Berinyi *et al.*, 1992; Nabel *et al.*, 1989), and to facilitate endothelialization of prosthetic conduits (Clowes *et al.*, 1985; Herring *et al.*, 1987; Stanley *et al.*, 1982; Wilson *et al.*, 1989) or endovascular stents (Dichek *et al.*, 1989; Van der Giessen *et al.*, 1988). The capabilities of certain cytokines to serve as mitogens for ECs *in vitro* suggest that such growth-stimulatory molecules might be exploited to accelerate rET after the injury induced by natural plaque rupture or mechanical endovascular devices. In that respect, VEGF is unique among AGFs by virtue of the fact that its high-affinity binding sites, the tyrosine kinase receptors (TKRs) Flt-1 (*fms*-like tyrosine kinase, also known as VEGFR-1) and Flk-1 (fetal liver kinase-1, also known as KDR or VEGFR-2), are specific to ECs (Conn *et al.*, 1990; Ferrara and Henzel, 1989). Therefore it is considered to be an EC-specific mitogen that promotes EC migration *in vitro* (Keck *et al.*, 1989; Leung *et al.*, 1989; Witzenbichler *et al.*, 1998), proliferation *in vivo*, and acts as an AGF *in vivo* (Keck *et al.*, 1989; Leung *et al.*, 1989). VEGF also has the capability of modulating qualitative aspects of EC biology (Ku *et al.*, 1993) that could be contributory to the maintenance and repair of the endothelium, as evidenced by the finding of the VEGFR-1 in the mature (quiescent) endothelium of adult organs (Peters *et al.*, 1993). Consistent with this notion is the evidence that VEGF directly increases NO release by ECs (van der Zee *et al.*, 1997), and induces endothelium-dependent hypotension in adult rabbits and swine (Horowitz *et al.*, 1995), confirming VEGF's role as a principal determinant of endothelial maintenance and repair. This feature of VEGF has proven critical for promotion of angiogenesis *in vivo* (Murohara *et al.*, 1998) and for inhibition of SMC proliferation and/or neointimal thickening by the restored endothelium (Tsurumi *et al.*, 1997). VEGF's exclusive secretory signal sequence at the amino terminus allows it to be naturally secreted by intact cells (Tischer *et al.*, 1991). Its production and the expression of its receptors are upregulated by hypoxia mediated by binding of the hypoxia-inducible factor to the hypoxia response element in the promoter region of the VEGF gene (Forsythe *et al.*, 1996). These properties make VEGF-induced angiogenesis uniquely targeted to ischemic areas (Tuder *et al.*, 1995).

On the basis of all the previously described features of VEGF, we and others have performed a series of preclinical studies to investigate the effects of the direct application of VEGF as naked DNA or RP to arteries that were aggressively injured by balloon endothelial denudation, with (Van Belle *et al.*, 1997a, 1997c, 1997d; Walter *et al.*, 2002) or without (Asahara *et al.*, 1995, 1996) deployment of an endovascular stent. In each of these animal studies using different delivery

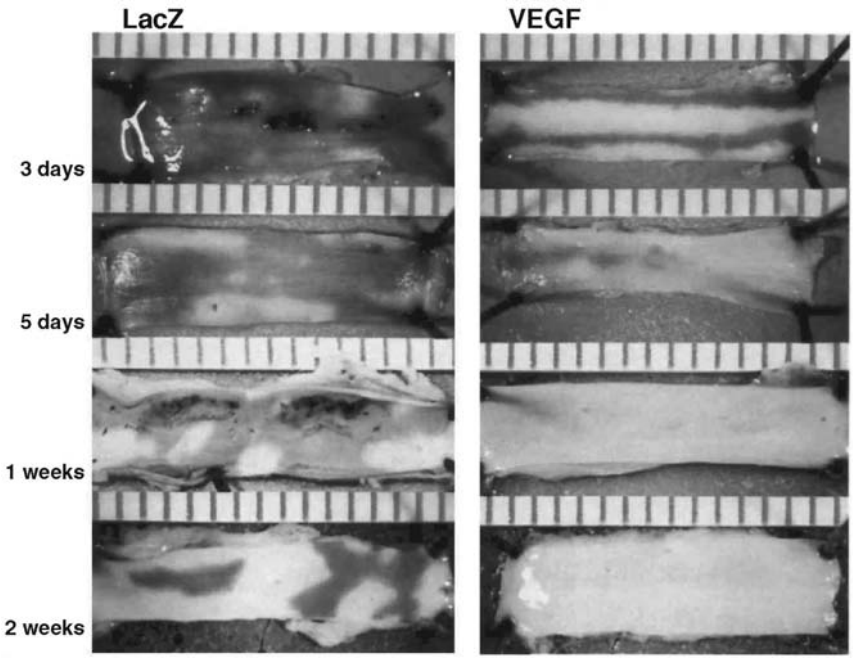

FIGURE 2. Representative macroscopic photographs of balloon-injured rabbit iliac arteries transfected with plasmid DNA encoding for β-galactosidase (LacZ) or VEGF at 3 and 5 days and 1 and 2 weeks after transfection. The reendothelialized area does not stain with Evan's blue dye and appears white. $P < 0.01$. (*See Color Plate 13.*)

techniques, the administration of VEGF markedly accelerated rET (Fig. 2), which led to marked inhibition of intimal/neointimal proliferation and/or reduction in mural thrombus formation, implying that the anatomically restored endothelium was functionally competent. Perhaps the most relevant aspect of these studies was the impact on direct functional assessment of endothelium-dependent function. Previous investigations on rET demonstrated that restoration of anatomic integrity and recovery of physiologic function do not occur simultaneously (Tanaka *et al.*, 1993), as shown by persistent and generalized loss of endothelium-dependent relaxation for ≥4 weeks after balloon injury, despite recovery of the endothelium (Weidinger *et al.*, 1990). Using quantitative angiography, we determined the vasomotor response of the VEGF-transfected, balloon-injured arterial segments to endothelium-dependent agonists. Consistent with previous studies of vasomotor reactivity in the mechanically injured endothelial segments of rabbit iliac arteries (Weidinger *et al.*, 1990), control rabbits transfected with plasmid DNA encoding for β-galactosidase (LacZ) demonstrated persistent impairment in vasomotor response to acetylcholine and serotonin at 4 weeks postinjury. In contrast, arteries transfected with phVEGF$_{165}$ disclosed recovery of near-normal endothelium-dependent vasoreactivity within 1 week (Fig. 3). Thus, these findings are consistent with the concept that VEGF serves as an endogenous regulator/accelerator of both

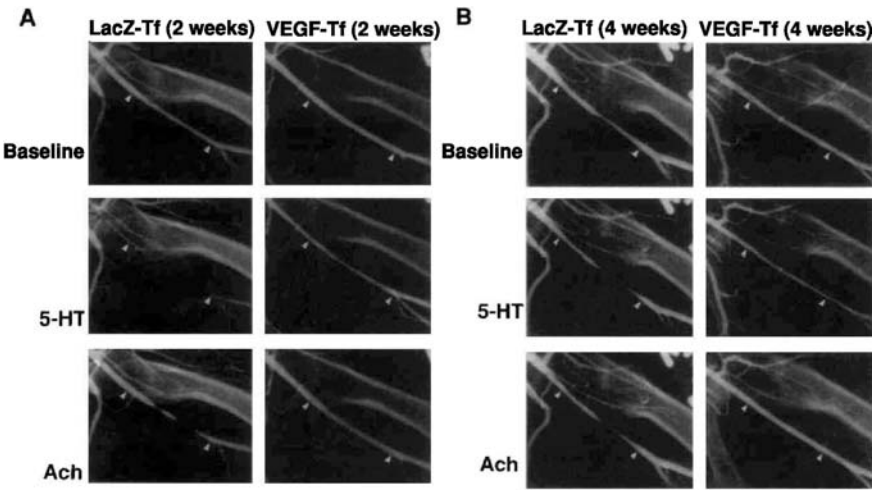

FIGURE 3. Representative angiograms at baseline and immediately after administration of serotonin (5-HT) and acetylcholine (Ach) to galactosidase-transfected (LacZ-Tf) and VEGF-transfected (VEGF-Tf) rabbit femoral arteries at 2 weeks (A) and 4 weeks of follow-up (B). The arterial segment between the two arrowheads in each angiogram indicates the site of balloon injury. 5-HT produced severe vasoconstriction in LacZ-Tf, especially at 1 and 2 weeks after injury. Significantly less vasoconstriction was seen in VEGF-Tf ($P < 0.01$), which was similar to or less than that observed in normal (i.e. noninjured, nontransfected) arteries. After balloon injury, ACh failed to dilate LacZ-Tf at 1 and 2 weeks, whereas ACh induced dilatation in VEGF-Tf ($P < 0.01$). The vasomotor response in VEGF-Tf was similar to that observed in normal arteries[Q15].

physiologic and anatomic endothelial integrity and recovery in the arterial wall (Asahara *et al.*, 1996; Tsurumi *et al.*, 1997).

Experiments that have solely relied on the use of nonsecreted gene products, examination by histochemical staining, *in situ* hybridization, and/or polymerase chain reaction (PCR) have suggested that the transfection efficiency of direct GTx to vascular SMCs within the arterial wall is considerably <1%, and therefore, it might preclude a meaningful biological response. In contrast, genes encoding for a secreted protein such as VEGF may overcome the obstacle of inefficient transfection by a paracrine effect, allowing the cells to secrete adequate amounts of protein in order to achieve local levels that may be physiologically meaningful (Losordo and Isner, 2001). This relationship between secreted gene products and transfection efficiency after *in vivo* arterial GTx was documented by *in vitro* (Takeshita *et al.*, 1994c) and *in vivo* (Losordo *et al.*, 1994) experiments performed in our laboratory to serially monitor expression of a gene encoding for a secreted protein. These experiments showed that despite successful transfection in only <0.1% of cells in the transfected segment (as documented by immunohistochemical staining of sections obtained at necropsy), there were maximum levels of secreted protein *in vivo* (5 days after transfection). This indicates that low-efficiency transfection with genes encoding for a secreted protein might achieve therapeutic effects not accomplished by transfection with genes

encoding for proteins that remain intracellular. On the basis of this evidence we performed preclinical studies to establish the feasibility of site-specific GTx of $phVEGF_{165}$, applied to the hydrogel polymer coating of an angioplasty balloon (Riessen *et al.*, 1993) and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hind limb ischemia (Pu *et al.*, 1993a). Analysis of the transfected internal iliac arteries using reverse transcription PCR confirmed reproducible gene expression at the mRNA level for up to 21 days post-GTx (Minutes Recombinant DNA Advisory Committee (RAC) of National Institutes of Health, 1994). Augmented development of collateral vessels was documented by serial angiograms *in vivo*, and increased capillary density at necropsy. The hemodynamic amelioration was documented by improvement in the calf blood pressure ratio (ischemic/normal limb), as well as increased resting and maximum vasodilator-induced blood flow in the VEGF-transfected animals versus controls transfected with a reporter gene. Finally, there is also compelling animal data to support the use of VEGF in the treatment of diabetic neuropathy and HF (Isner, 2002; Leotta *et al.*, 2002; Schratzberger *et al.*, 2001).

3.1. From Bench to Bedside

The data presented thus far have focused mainly on preclinical and proof-of-concept studies using VEGF. Certainly, VEGF is the most widely studied of the AGFs, and given the relative paucity of data regarding other growth factors and the favorable results of early clinical trials using VEGF, the VEGF family of AGFs may indeed represent the current gene of choice for therapeutic angiogenesis. Therapeutic angiogenesis revolves around the theory that the local delivery of AGFs to ischemic tissue can stimulate the growth of new blood vessels, which would lead to an increase in collateral circulation and therefore a reduction in ischemia (Fam *et al.*, 2003). However, there are several factors that must be considered for GT-mediated angiogenesis to be successful.

First, the selected gene must code for a growth factor with angiogenic properties properly documented in preclinical and early phase clinical studies. Second, the delivery strategy must provide high GTx efficiency. Third, the route of administration should be able to provide easy means to target the ischemic tissue. Fourth, the procedure itself should be safe in order to minimize the risk/benefit ratio to the patients in both the short and long term (Grines *et al.*, 2003).

Despite promising preclinical data, the translation of results and outcomes from bench to bedside is not an easy process. For example, despite the large number of studies using RP and naked DNA in several different animal models, via various routes of administration, a model that accurately represents human ischemia and formation of collateral circulation is yet to be found. Pigs and rabbits have few underlying collaterals, while dogs have many and humans are likely somewhere in between (Schaper *et al.*, 1999; Schultz *et al.*, 1999). Another obstacle has been the route of administration. In the early days of angiogenic cytokines, systemic administration of RPs was the choice of delivery. However, animal studies demonstrated

that myocardial uptake was relatively small compared to the high plasma levels of RP. Early human studies in patients with CLI found that AGF (RP or naked DNA) could have serious unwanted side effects secondary to high plasma levels associated with systemic delivery. These side effects included hypotension and edema (VEGF) as well as anemia, thrombocytopenia, and renal toxicity (FGF) (Baumgartner *et al.*, 2000; Freedman and Isner, 2002). Subsequent animal studies demonstrated that local delivery of AGF (via a variety of routes) sustained high levels in the desired tissue with little systemic distribution. Once again, despite promising animal data, the transition to humans proved difficult given that local delivery of a therapeutic agent to the myocardium requires an intricate procedure, which could be accomplished via the IC route (which would obviously carry the risks of a cardiac catheterization) and/or by direct intramyocardial delivery through a thoracotomy (Lee and Smits, 2002). Functional assessment of the efficacy of myocardial angiogenesis has been yet another hurdle in the transition from bench to bedside. Animal studies have relied on echocardiography, nuclear perfusion scanning, angiography, measurement of coronary blood flow, and most importantly histology, which demonstrated a significant increase in the size and number of capillary beds. To date, there is no agreement as to what should be the gold standard for the functional assessment of myocardial angiogenesis in humans. Early clinical trials have utilized angina class and exercise time, but these modalities are subjective, being limited by the patients' perception of their symptoms. Additionally, exercise treadmill testing has a high day-to-day variability and the indications for termination of the test are subjective. Studies using gated single-photon emission computed tomography (SPECT) to image myocardial perfusion with thallium-201 or technetium-99 sestamibi have provided objective assessment while limiting the placebo effect and proven that perfusion imaging is probably the best, easiest, and least subjective method for evaluating myocardial angiogenesis (Freedman and Isner, 2002; Udelson *et al*., 2000). Other imaging modalities such as positron emission tomography scanning and magnetic resonance perfusion imaging have shown early promising results, but these are limited by cost and availability. Angiography, the current gold standard in the detection of coronary atherosclerotic disease, has not proved useful in the assessment of myocardial angiogenesis. This is due to the relatively small size of collateral vessels (less than 200 μm), which is beyond the resolution of both conventional and magnetic resonance angiography (Freedman and Isner, 2002). More recently, percutaneous CB electromechanical mapping has emerged as a promising method for assessing the efficacy of therapeutic angiogenesis. This technique allows the visualization of areas with electromechanical uncoupling suggestive of ischemic or hibernating myocardium, and at the same time creates a map that is used to guide the intramyocardial delivery of the therapeutic agent. This method, however, is limited by a steep learning curve, which renders it nonwidely available. All these limitations made initial human studies of GTx for myocardial angiogenesis difficult to interpret. Thus, this led investigators to design early clinical trials of angiogenic GTx for patients with CLI. A favorable risk/benefit ratio, easy access to skeletal muscle, and the ability to visualize peripheral collateral circulation on

angiography made patients with CLI the ideal choice for initial GTx studies in humans (Isner *et al.*, 1996a; Schainfeld and Isner, 1999).

3.2. Delivery Strategies

There are several strategies for local delivery of the candidate gene, and each one has advantages and disadvantages, but as more early clinical studies are beginning to show, the route of administration may be specific to the agent. Currently, these strategies include epicardial delivery via thoracotomy and placement of an epicardial patch or direct injections; intramyocardial, via CB approach; IC; pericardial via thoracotomy or CB technique; intravenous; and retroperfusion via injection into the coronary sinus with occlusion of the coronary sinus outflow (Isner, 2002).

The epicardial route was the route of choice prior to the development of catheters with the ability to perform intramyocardial injections. Direct epicardial injections allow for direct administration of gene or RP into the ischemic areas and may be performed with a limited thoracotomy or video-assisted thoracoscopy. An epicardial patch requires a full open chest procedure but provides the benefit of sustained release of the agent (particularly useful with the RPs because of their limited duration of activity). Both methods have a gradient of distribution, which lessens as a function of distance from the site of injection or patch placement (Kleiman *et al.*, 2003). Two double-blind, randomized phase I trials have looked at the efficacy of a pericardial patch with FGF or FGF-2 RP placed during coronary artery bypass graft (CABG) in the territory of an ungraftable vessel. Both trials showed improvement in angina and perfusion scores up to 3 years postprocedure compared to patients having CABG alone (Ruel *et al.*, 2002). There have been several phase I trials investigating the use of naked DNA encoding for $VEGF_{165}$ delivered via epicardial injections through a mini-thoracotomy to patients who were not candidates for conventional revascularization. Although the results of these studies and others have been promising, the role of epicardial delivery may be limited because of the advent of CB techniques. However, there may still be a role for both the patch and the injections for patients who have to undergo open-heart surgery (CABG, valvular surgery, etc.) for established indications (Kleiman *et al.*, 2003).

Direct IC delivery is probably the simplest, local delivery approach. A single bolus of RP directly into the coronary circulation is thought to trigger a cascade of events culminating in the stimulation of angiogenesis. Several animal studies have confirmed this and have also shown that there is not a dose–response relationship because the RP acts as a "trigger." One concern has been the relatively low levels of RPs in the myocardium of sacrificed animals, suggesting that despite local delivery, the distribution of RP may not be limited to the coronary circulation.

The CB intramyocardial modality of GTx is gaining momentum and maybe the delivery method of choice in the foreseeable future. This technique, which utilizes an electromechanical mapping catheter, has the benefits of a sustained angiogenic stimulus delivered directly to the myocardium without the disadvantages

of an open surgical procedure. Intrapericardial GTx is a less studied route of administration. Animal studies have demonstrated increased myocardial uptake (compared to IC or IV) of both FGF and VEGF with a concomitant decrease in systemic levels (Laham *et al.*, 2003). Intrapericardial delivery of chemotherapeutic agents in patients with malignant effusions has been safe and effective in a small number of patients. This method may have advantages over the IC route in terms of gene delivery and distribution, but may be limited because of operator inexperience. To date, no human studies have been performed using the intrapericardial route for GTx.

3.3. Choice of Agent

Given the variety of AGFs currently under clinical and preclinical investigations, the selection of an angiogenic agent is rather complex. The agents can be given as RP or via GTx. The RP is a growth factor that is delivered to the target tissue and GTx involves the use of a gene that encodes for a specific growth factor. The RP acts as a trigger, signaling the initiation of a cascade of events that result in the stimulation of angiogenesis (Kastrup *et al.*, 2003). Early clinical trials involving the use of RP via direct intramyocardial injection showed promising results. However, two large-scale (FIRST, VIVA), phase II studies using RP delivered via the IC route had disappointing results. Genes can be delivered either via a viral vector or as naked DNA in a plasmid or liposome. Gene transfer offers the advantage over RP of a sustained release formulation so that the target tissue will have a prolonged exposure to the angiogenic cytokine after a single injection. The results of studies using direct intramyocardial GTx via a viral vector have been mixed. This method is limited by very little dispersion of gene expression from the needle site and by the lack of a direct linear relationship between the titer of viral particles and duration and level of gene expression (Ruel *et al.*, 2002). A phase I study using $VEGF_{121}$ on an adenoviral vector enrolled 21 patients who had reversible ischemia and were referred for CABG. There were two groups: group A ($n = 15$) received direct injections to a nonrevascularizable territory in addition to CABG; group B ($n = 6$) received GTx as sole therapy. Patients in both groups reported improvement in angina class and a decrease in nitroglycerin use at 30 days and 6 months, but there was no improvement in myocardial perfusion at rest or at stress (Rosengart *et al.*, 1999a, 1999b). Studies using a viral vector delivered via the IC route have had better results. The AGENT trial demonstrated an 87% first pass uptake by the heart of FGF-4 delivered through an adenoviral vector (Grines, 2003). Nonetheless, all forms of GTx with a viral vector are somewhat hindered by the side effects of the virus itself. The main side effects are fever and a transient increase in liver enzymes and uric acid (Grines *et al.*, 2003). Direct GTx (via plasmid or liposome) may turn out to be a more favorable approach. Studies utilizing naked DNA GTx via intramyocardial injections have been promising. A 1-year follow-up of a phase II trial involving patients who received naked plasmid DNA encoding for VEGF-2 via a mini-thoracotomy and direct intramyocardial injections showed

Table 1
Phase I/II Clinical Trials of Recombinant Proteins for Cardiovascular Angiogenesis

Protein	Route of Administration	RP/Placebo (*n*/*n*)	Patient Characteristics	Study Design	Follow-up	Results	Company	Reference
FGF-1	IMy injection during CABG	20/20	MVD with distal LAD dzs after LIMA	Phase I	12 weeks	New vessels bypassing distal LAD. Decreased angina and use of antianginals vs placebo.	No sponsor	Schumacher *et al.*, 1998
FGF-1	IMy injection via thoracotomy	20/0	Severe angina, NR	Phase I	6 and 12 weeks	Improved exercise tolerance and perfusion by SPECT	No sponsor	Stegmann *et al.*, 2000
FGF-2	Padv during CABG	8/0	NR, viable myocardium	Phase I	3 months	1 perioperative MI in implantation site. All patients symptom-free after CABG	No sponsor	Sellke *et al.*, 1998
FGF-2	Padv during CABG	16/8	NR, viable myocardium	Phase I	3 years	2 deaths during CABG and 2 late deaths; 3 transmural MIs. Improvement in symptoms and myocardial perfusion	No sponsor	
FGF-2	IV or IC injection	59/0	CAD, NR, RD	Phase I	1, 2, and 6 months	Improved exercise tolerance, symptom frequency, quality of life, LV function, myocardial perfusion, and flow on MRI	Chiron	Laham *et al.*, 2000; Udelson *et al.*, 2000
FGF-2	IC to Left Main	17/8	CAD and angina	Phase I	1 to 28 days	No change in exercise tolerance or time to ischemia	No sponsor	Unger *et al.*, 2000
FGF-2	IC injection	251/86	CAD, NR, RD, EF > 30%	Phase II FIRST	90 days to 6 months	No improvement in exercise tolerance and myocardial perfusion. Symptomatic improvement at 90, not at 180 days	Chiron	Annex, 2002; Freedman and Isner, 2002

FGF-2	Bilateral IA infusion	127/63	IC caused by infra-inguinal dzs	Phase II TRAFFIC	90 days	Significant improvement in peak walking time at 90 days. No difference between single and double dose	Chiron	Lederman *et al.*, 2002
$VEGF_{165}$	IC injection	15/0	CAD, NR, RD	Phase I	30 and 60 days	Decreased angina in 83%. Improved resting myocardial perfusion with high dose	Genentech	Henry *et al.*, 2000
$VEGF_{165}$	IV injection	28/0	CAD, NR, RD	Phase I	60 days	Improved myocardial perfusion at rest (40%) and stress (20%)	Genentech	Hendel *et al.*, 2000; Henry *et al.*, 2001
$VEGF_{165}$	IC + 3 IV injections	115/63	CAD, NR, RD	Phase II VIVA	60 days, 120 days, 1 year	60 days: No difference in exercise tolerance, angina, quality of life, or myocardial perfusion, when compared with placebo. 120 days: Significantly decreased angina compared to placebo	Genentech	Henry *et al.*, 2003

Note. RP: Recombinant protein; FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor; TRAFFIC: Therapeutic Angiogenesis with recombinant Fibroblast Growth Factor for Intermittent Claudication; VIVA: VEGF in Ischemia for Vascular Angiogenesis; FIRST: FGF-2 Initiating Revascularization Support Trial; CABG: coronary artery bypass graft; MVD: multivessel disease; LAD: left anterior descendent artery; LIMA: left internal mammary artery; CAD: coronary artery disease; NR: not candidate for revascularization; RD: reversible defect; InCl: intermittent claudication; IMy: intramyocardial; Padv: periadventitial implantation; IV: intravenous; EF: Ejection Fraction; IC: intracoronary; IA: intraarterial; dzs: disease; SPECT: single-photon emission computed tomography; MI: myocardial infarction; MRI: magnetic resonance imaging; LV: left ventricular.

Table 2
Phase I/II Clinical Trials of Gene Transfer for Cardiovascular Angiogenesis

Vector/Gene	Route of Administration	Gene/Placebo n/n	Patient Characteristics	Study Design	Follow-up	Results	Company	Reference
Naked DNA $VEGF_{165}$	IMy via thoracotomy	30/0	CAD, NR, RSA	Phase I	2 months, 6 months, 1 year	60 days: Improved myocardial perfusion at rest and stress. Improved LLS by electromechanical mapping 1 year: Decreased angina frequency and nitroglycerin use. Increased exercise time and increase in exercise time to angina	No sponsor	Losordo *et al.*, 1998; Symes *et al.*, 1999; Vale *et al.*, 2000a
$ADV/VEGF_{121}$	IMy during CABG or via thoracotomy	21/0[a]	CAD, NR, RD	Phase I	30 days	Improved contractility by SPECT. Increased Rentrop angiographic score	Genvec	Rosengart *et al.*, 1999a
$ADV/VEGF_{121}$	IMy via thoracotomy/VTh	10/0[b]	CAD, NR, RD	Phase I	30 days	1 death at 40 days. Outcomes not published	Genvec	
Naked DNA VEGF-2	IMy via thoracotomy	30/0	CAD, NR, RSA	Phase I	30, 60, 90 days	90 days: Improved myocardial perfusion at rest and stress. Improved LLS by electromechanical mapping. Decreased angina frequency and nitroglycerin use. Increased exercise time. Improved angina class	VGI	
Naked DNA VEGF-2	IMy via EMMC in LV	6/0[c]	CAD, NR, RSA	Phase I	30, 60, 90 days	90 days: Significantly decreased angina frequency and nitroglycerin use compared with placebo (before crossover). Improved myocardial perfusion at rest and stress. Improved LLS by electromechanical mapping	VGI	Vale *et al.*, 2001a
Naked DNA $VEGF_{165}$	IMy via thoracotomy	7/0	CAD, NR, RD	Phase I	12 months	Significantly improved angina class. All remaining efficacy endpoints showed strong trends favoring efficacy of VEGF-2	VGI	Losordo *et al.*, 2002
Naked DNA $VEGF_{165}$	IM to lower limbs	9/0	CLI, refractory to medical therapy	Phase I	6 months	Significantly decreased angina frequency and nitroglycerin use	No sponsor	Sarkar *et al.*, 2001

Naked DNA $VEGF_{165}$	IA via BAC	19/0	InCl undergoing femoropopliteal PTI	Phase I	9 months	Significantly improved ankle-brachial index. New visible collaterals by angiography. Markedly improved/healed ischemic ulcers in 57% of limbs, including limb salvage in 3 patients	No sponsor	Baumgartner *et al.*, 1998
Naked DNA $VEGF_{165}$	IM to lower limbs	17/0	CLI with EP and clinical evidence of IN	Phase I	6 months	No evidence of restenosis in 74% of patients. Clinical restenosis requiring revascularization in 22%	No sponsor	
ADV/FGF-4	Intracoronary injection	60/19 (3:1)	RSA, >1 open vessel, suitable for CABG/PTI	Phase I–II AGENT	4 and 12 weeks	Significantly improved symptom, sensory, and total examination scores. Significantly improved peroneal motor amplitude, quantitative vibration threshold, and ankle-brachial index in treated limbs. Neurologic improvement in 75% of diabetic patients	No sponsor	Simovic *et al.*, 2001
ADV/FGF-4	Intracoronary injection	35/17	CAD, NR, RD	Phase I–II AGENT-2	8 weeks	No significant difference between groups	Collateral Therapeutics	Grines *et al.*, 2002
ADV/$VEGF_{165}$ and	Intracoronary injection	37/28/38	CAD, RSA	Phase II	6 months	No significant difference in perfusion scores. Trend toward a significant decrease in angina and nitroglycerin use	Collateral Therapeutics	Grines, 2003
$VEGF_{165}$- P/L						No significant difference in restenosis. Significant increase in myocardial perfusion scores in the ADV/$VEGF_{165}$ group	BSc, Ark Therapeutics	Hedman *et al.*, 2003

Note: VEGF: Vascular endothelial growth factor; FGF: fibroblast growth factor; ADV: adenoviral vector; CAD: coronary artery disease; CABG: coronary artery bypass graft; NR: not candidate for revascularization; IMy: intramyocardial; PTI: percutaneous transluminal intervention; RSA: refractory stable angina; VTh: video-assisted thoracoscopy; EMMC: electromechanical mapping catheter; LV: left ventricular; CLI: critical limb ischemia; IA: intraarterial; BAC: balloon angioplasty catheter; InCl: intermittent claudication; IM: intramuscular; EP: electrophysiologic; IN: ischemic neuropathy; AGENT: Angiogenic GENe Therapy; RD: reversible defect; $VEGF_{165}$-P/L: $VEGF_{165}$ plasmid-liposome; SPECT: single-photon emission computed tomography; LLS: linear local shortening.

[a] 15 patients had CABG and 6 had thoracotomy.

[b] 6 patients had thoracotomy and 4 had thoracoscopy.

[c] 3 control patients crossed over to active treatment.

a statistically significant improvement in angina class and exercise treadmill time (Fortuin *et al.*, 2003).

In addition to choosing between RP and GTx, the choice of which AGF to utilize may or may not prove to be the more important decision. There are a variety of cytokines (other than VEGF) that have been reported to promote angiogenesis.

3.4. Clinical Studies

The favorable outcomes of the preclinical studies led to the design of phase I and II clinical trials to test the hypotheses that VEGF GTx could augment angiogenesis in patients with symptomatic limb and/or myocardial ischemia and no options for conventional revascularization; accelerate rET after balloon angioplasty and thereby inhibit restenosis; and maintain or improve endothelial integrity in the vasa nervorum of patients with ischemic/diabetic neuropathy (see Tables 1 and 2).

In the context of these trials, over 100 patients have received direct intra-arterial GTx of naked plasmid DNA encoding for $VEGF_{165}$. The VEGF plasmid was administered to arterial segments of 12 patients using a hydrogel-coated angioplasty balloon to which the DNA (in saline solution) was applied in an attempt to augment collateral blood vessels (Isner *et al.*, 1996a). In 30 patients, the same delivery technique was employed following PTI of severely atherosclerotic femoral arteries in a phase I study to examine the potential of this approach to prevent restenosis. Eighteen months follow-up disclosed no evidence of new atherosclerotic lesion development, with a restenosis rate comparable to that of contemporary controls (Minar *et al.*, 2000). In 9 patients with CLI, direct intramuscular injection of naked plasmid DNA encoding for $VEGF_{165}$ led to therapeutic angiogenesis as evidenced by significantly improved ankle-brachial index, new collaterals documented by angiography, and healing of ischemic ulcers (Baumgartner *et al.*, 1998). Thirty patients with refractory angina unsuitable for revascularization were given transepicardial intramyocardial injections of naked DNA encoding $VEGF_{165}$through a mini-thoracotomy. Although there was not a placebo arm (due to obvious ethical concerns regarding an open surgical procedure), up to 2 years of follow-up have revealed improved exercise tolerance, reduced number of episodes of angina per week, decreased consumption of nitroglycerin tablets, and improvement in Canadian Cardiovascular Society (CCS) angina class as well as improvement in SPECT images and electromechanical maps (Figs. 4 and 5) (Losordo *et al.*, 1998; Vale *et al.*, 1999a, 2000; Symes *et al.*, 1999). Our initial experience was reproduced in a multicenter study of GTx with naked plasmid DNA encoding for VEGF-2 in 30 patients, revealing similar results (Hendel *et al.*, 2000b; Vale *et al.*, 2000b). We recently completed the first clinical study of percutaneous, CB myocardial GTx (Vale *et al.*, 2001). This single-blind pilot study enrolled 6 patients and had similar results to the previous studies, serving as the basis for a phase I/II multicenter, randomized, double-blind, placebo-controlled, dose-escalating trial of CB GTx with naked plasmid DNA encoding for VEGF-2. This study revealed a significant decrease in CCS angina class (Losordo *et al.*, 2002), suggesting the need for larger phase II/III trials. On

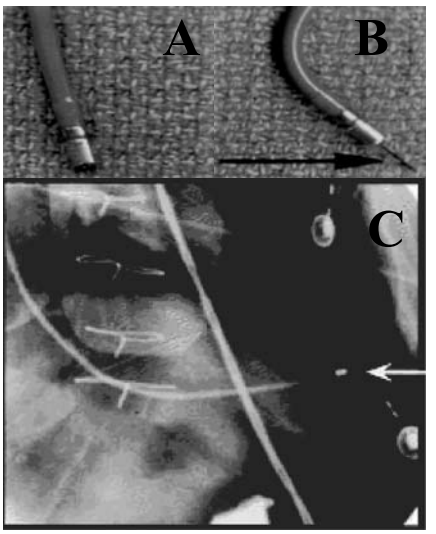

FIGURE 4. Modified mapping-injecting catheter (Biosense^{TM}, Johnson & Johnson). (A) The electrode in the distal tip of the catheter allows annotation of electromechanical maps to document the sites of gene transfer by intramyocardial injection. (B) The 27-gauge needle has been advanced out of the distal tip of the catheter to simulate myocardial engagement in preparation for injection. (C) Posteroanterior view recorded during cine-fluoroscopy shows the distal tip of the catheter (arrow) against the endocardium of the left ventricular lateral wall in preparation for injection. The 27-gauge needle advanced into the myocardium is not visible. (*See Color Plate 14.*)

the basis of these promising results, we are soon to begin enrollment of 404 patients for a phase II, randomized, multicenter, double-blind, placebo-controlled trial to study the safety and efficacy of naked DNA-VEGF-2 delivered via direct intramyocardial injections with a CB approach. The study will enroll patients who are not candidates for traditional revascularization, regardless of ejection fraction.

The investigators in the Kuopio Angiogenesis Trial (KAT) studied the effects of VEGF GTx via IC injection in the prevention of ISR and treatment of chronic myocardial ischemia. VEGF was delivered via an adenovirus vector or in a plasmid liposome. The results showed a significant improvement in myocardial perfusion in patients who received VEGF with the adenoviral vector, suggesting that VEGF may stimulate angiogenesis when delivered via the IC route. The study also demonstrated that there was no difference in the rates of restenosis compared to placebo (Hedman *et al.*, 2003). However, the overall restenosis rate in this trial was only 6%, virtually precluding the detection of a difference among treatment groups (Losordo *et al.*, 2003). We are currently conducting a phase I, open label study to determine the safety and efficacy of IC delivery of naked DNA encoding for $VEGF_{165}$ during stent implantation, with the use of new drug eluting stents, for the prevention of ISR.

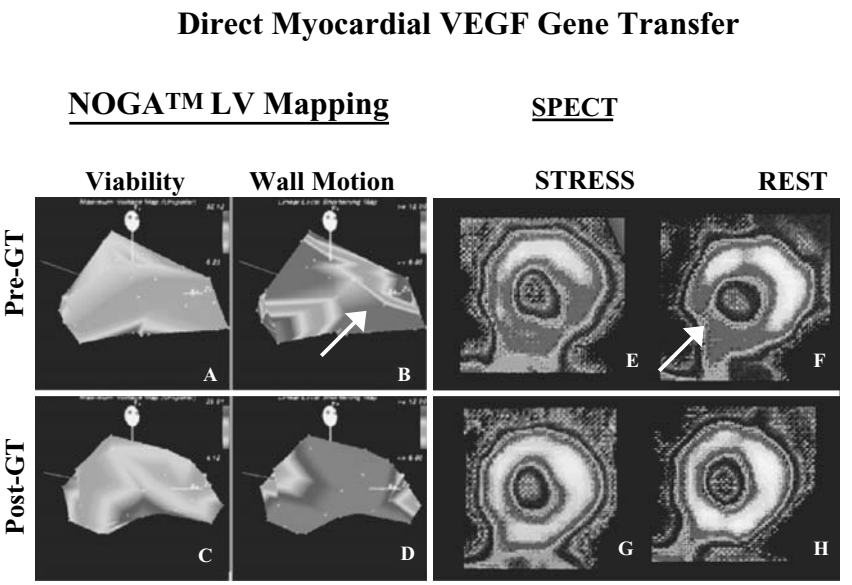

FIGURE 5. NOGA™ left ventricular electromechanical mapping (LV EMM): Unipolar images (A) showing normal voltages suggestive of viable myocardium (purple/pink/blue/green) and linear local shortening (LLS) map (B) with large zone of abnormal wall motion (red, arrow) that represents electromechanical uncoupling suggestive of ischemic or hibernating myocardium involving the inferoseptal region, from a 48-year-old man before phVEGF$_{165}$ GTx. The red lines represent the long axis through apex. Vertical and horizontal axes (x, y, and z) are presented as white lines. Sixty days after GTx, unipolar (C) and LLS (D) images show complete resolution of ischemia (10.08 cm^2 before GTx vs 0.00 cm^2 after GTx) that corresponds to changes observed on SPECT scan. Persantine SPECT-sestamibi myocardial perfusion scanning images: White and yellow colors depict maximal uptake of radionuclide, and red depicts impaired uptake. Selected short-axis stress and resting images were taken before (E, F) and after (G, H) phVEGF165 GTx in the same patient. Pre-GTx scans (top) show reversible inferoseptal defect (arrows). Post-GTx scans (bottom) show complete normalization of resting perfusion and perfusion after pharmacological stress. (*See Color Plate 14.*)

Our team also conducted what has been the latest application of VEGF$_{165}$ GTx in clinical trials. Seventeen patients (6 diabetic patients) with documented CLI and ischemic neuropathy were enrolled in a phase I, open-label, dose-escalating trial of VEGF$_{165}$ GTx via intramuscular injections. At 6 months, follow-up revealed significant improvement in symptom severity score and electrophysiologic studies. Neurologic improvement was documented in 75% of the diabetic patients (Simovic *et al.*, 2001). Besides VEGF, the most extensively studied angiogenic cytokines are acidic fibroblast growth factor (aFGF or FGF-1) and basic fibroblast growth factor (bFGF or FGF-2). FGFs are potent EC mitogens that lack VEGF's EC-specificity, given that they also serve as ligands for other cell types, including SMC and fibroblasts. FGFs use cell-surface heparin sulfate proteoglycans to facilitate binding to their TKRs. This binding underlies the rapid extraction of injected FGF from the circulation and its localization on cells and extracellular matrix,

like VEGF. Also similar to VEGF, FGFs also stimulate EC synthesis of plasminogen activator and metalloproteinases, important in extracellular matrix digestion during angiogenesis. However, unlike VEGF, the common forms of FGF lack the secretory signal sequence, and therefore the clinical trials of FGF GTx have required modifications of the FGF gene, or the use of another member of the FGF gene family with a signal sequence (Freedman and Isner, 2002). The authors of the FIRST study delivered RP FGF-2 via the IC route in 251 "no-option" patients. Although there was symptomatic improvement at 3 months compared to placebo, these results were not sustained at 6 months. Also, there was no difference in exercise time and myocardial perfusion scores at any time (Kleiman and Califf, 2000).

The effects of IC gene delivery have been less extensively studied mainly because of basement membrane permeability issues demonstrated in animal models. The AGENT and AGENT-2 trials investigated the effects of FGF-4 with an adenoviral vector delivered via IC injections. There was no significant difference in the endpoints (angina frequency, exercise time, myocardial perfusion scores) compared to placebo, but there was a strong trend toward decreased angina and decreased use of nitroglycerin in the treatment arms. More important, however, was that both trials demonstrated favorable safety profiles for adenovirus-FGF-4 therapy (Grines *et al.*, 2003). This has led to two ongoing, randomized, double-blind, placebo-controlled trials (AGENT 3 and AGENT 4) investigating the efficacy of FGF-4 delivered via the IC route. AGENT 3 will enroll patients who are on optimal medical therapy and can be treated via traditional methods. AGENT 4 will aim to enroll only "no-option" patients. Other studies have shown mixed results with IC injections of RP, particularly the VIVA (VEGF in Ischemia for Vascular Angiogenesis) trial that combined IC and IV injections of RP. This study randomized 178 patients to receive either placebo, low-dose VEGF-1 RP or high-dose VEGF-1 RP via combined IC and intravenous infusions (Henry *et al.*, 2003). The primary endpoint, exercise duration at 60 days, was similar in all three groups. By day 120, high-dose VEGF-1 RP resulted in significant improvement in angina class. These somewhat "negative" results underscore the concern that the pharmacokinetics of intravascular administration of RP may lead to inadequate local delivery of AGF to ischemic tissue. In summary, it is important to point out that the results of the early clinical (phase I and II) trials conducted thus far have not provided enough evidence to support the use of a single growth factor in upcoming and ongoing phase II and III studies.

More recently, with the introduction of the change of paradigm marked by the discovery that neovascularization in adults encompasses both angiogenesis and vasculogenesis (Asahara *et al.*, 1997), we considered that certain AGFs, acknowledged to promote both angiogenesis and vasculogenesis in the embryo, but assumed to promote neovascularization exclusively by angiogenesis in adults, may in fact promote migration, proliferation, and mobilization of EPCs from adult BM. This notion, confirmed in a preclinical study (Asahara *et al.*, 1999b), was also demonstrated in humans after VEGF GTx in patients with CLI, supporting the concept that neovascularization of human ischemic tissues after VEGF GTx is not limited

to angiogenesis but involves circulating EPCs that lead to vasculogenesis as well (Kalka *et al.*, 2000b).

4. SAFETY CONCERNS

It is imperative to point out that the initial clinical trials of GT have been designed to address unmet needs. A standard principle for the investigation of any novel therapy is that the risk/benefit ratio may be minimized by applying a treatment with unknown risks to patients that are so severely ill that they have little to lose. This has been the case for most early clinical trials of GT in general and cardiovascular GT in particular. The first cardiovascular GT trial involved patients with CLI who had exhausted all other available treatments. Given the lack of effective medical therapy, the 5-year survival among this cohort of patients is poor. After establishing the proof of concept in these patients, the studies were extended to patients with severe myocardial ischemia refractory to treatment and unresponsive to surgery, which makes them a population at high risk for recurrent infarctions and death. A potential liability in these studies is the problem of sorting out adverse events that are due to the underlying disease versus those attributable to the therapy. Despite this background and the grim statistics, among 100 patients with CLI undergoing VEGF GTx at our institution, the cumulative mortality rate is 9% over the last 8 years, comparing favorably with any published figures for such population. Similarly among 85 patients undergoing VEGF GTx for myocardial ischemia, there have been 3 deaths, reflecting a 3.5% cumulative mortality after 4 years of follow-up. Among 97 patients followed for 1–3 years after myocardial GTx of adenovirus encoding either FGF-2 or $VEGF_{121}$, the cumulative mortality rate is 5.2%. All these outcomes compare favorably with the 1-year mortality of 13% for a matched population undergoing laser myocardial revascularization (a procedure being used in routine clinical practice), or continued medical therapy, according to contemporary controlled studies (Isner *et al.*, 2001). The concern of possible proatherosclerotic properties of angiogenic cytokines and the development of potent, apparently EC-specific, inhibitors of angiogenesis led Moulton *et al.* (1999) to investigate the impact of restricted neovascularization on plaque development. This group observed that when hypercholesterolemic mice were treated for 16 weeks with endostatin or TNP-470, the median plaque area was reduced by 85 and 70%, respectively. Moulton's study raised concern regarding the potential for VEGF and other proangiogenic therapies to promote atherosclerosis. However, there is now a plethora of *in vivo* data (including human subjects) that refute the notion that accelerated atherosclerosis is a likely consequence of administering angiogenic cytokines. A total of 42 patients have now undergone direct intra-arterial GTx of naked DNA encoding VEGF to a freshly injured arterial surface. In 12 of these 42 patients, $phVEGF_{165}$ was administered to normal or moderately diseased arterial segments by using a hydrogel-coated angioplasty balloon to promote therapeutic angiogenesis (Isner *et al.*, 1996a). Follow-up angiography and intravascular ultrasound showed no evidence of disease progression

after GTx (Isner, 1998). In 30 other patients, the same delivery strategy was used to accelerate rET after PTI of femoral arteries occluded or severely narrowed by advanced atherosclerosis. Follow-up examination up to 48 months after GTx disclosed no evidence of new atherosclerotic lesion development. Similar findings have been reported in a smaller series of patients undergoing coronary arterial GTx of adenovirus encoding for $VEGF_{121}$ (Laitinen *et al.*, 2000). It is important to explicitly underscore the fact that the experiments performed by Moulton *et al.* (1999) were designed to test the hypothesis that "inhibition of plaque angiogenesis would reduce the growth of atherosclerotic lesions." Their experiments were not designed to test the hypothesis that administration of agents that promote angiogenesis would enhance atherosclerosis. Empirical testing of the latter hypothesis in humans with advanced atherosclerosis, summarized above, fails to support the notion that therapeutic angiogenesis accelerates atherosclerosis. It should also be noted that human subjects participating in clinical trials of VEGF RP, who have shown no evidence of accelerated atherosclerosis, have received doses up to 25 times greater (Henry *et al.*, 2003) than doses reported to accelerate atherosclerosis in mice (Celletti *et al.*, 2001). This issue will ultimately be resolved most directly by ongoing and future human trials investigating VEGF GTx to prevent restenosis after PTI. Another concern has been the potential for stimulating carcinogenesis. It is important to point out that this concern ignores the fundamental dictum that "angiogenesis is necessary but insufficient" by itself to promote neoplastic growth (Hanahan and Folkman, 1996). In the case of human cardiovascular trials of GTx, attempts to localize delivery of the transgene coupled with the fact that many of the angiogenic gene products bind avidly to matrix glycoproteins have resulted in circulating levels of the AGFs that either are not measurable or are in the picogram per milliliter range. Moreover, the duration of even the latter level of circulating gene product is typically limited to <4 weeks in trials performed to date. Although it is unlikely that such low levels of growth factor circulating for such a limited time would be sufficient to promote the growth of a dormant neoplasm that would exhibit sustained growth after extinction of gene expression, this remains a potential risk of GT involving an EC mitogen. Review of available published data regarding this potential risk is reassuring, albeit limited to short-term follow-up of small numbers of patients. In our own clinical experience with 88 subjects who have undergone VEGF GTx for CLI, the cumulative 7-year incidence of cancer has been limited to two patients with bladder cancer and one with liver and brain metastases from an unknown primary. Among 85 patients undergoing VEGF GTx for myocardial ischemia, neoplasms have been diagnosed in two patients followed for up to 3 years (Isner *et al.*, 2001). Among 79 patients undergoing IC GTx of Ad/FGF-4, new neoplasms were identified in two treated patients (versus no placebo patients) 69 and 267 days after GTx (Grines *et al.*, 2002). Because this typically elderly group of patients may be at higher risk for cancer to begin with, it will take considerable follow-up of large cohorts to accurately judge the magnitude of this risk. The VIVA trial provides a useful perspective in this regard. Among 178 patients randomized to low-dose VEGF, high-dose VEGF, or placebo, 3 patients developed new evidence of cancer (fatal in 2 of 3) within the

180-day time period of the trial; by serendipity, all 3 patients had been randomized to the control arm (Henry *et al.*, 2003). In the Therapeutic Angiogenesis With Recombinant Fibroblast Growth Factor-2 for Intermittent Claudication (TRAFFIC) trial, newly diagnosed neoplasms were limited to 1 patient in the placebo group (Lederman *et al.*, 2002). Other safety concerns involve the theoretical possibility of AGF-induced retinopathy. Until now, only 1 patient in the VIVA trial developed reduced visual acuity during follow-up; however, this patient had been originally randomized to the placebo group (Henry and Abraham, 2000; Henry *et al.*, 2003; Isner *et al.*, 2001). Prospectively studied patients receiving GTx with VEGF at our institution have failed to develop evidence of retinal neovascularization or change in visual acuity (Isner *et al.*, 2001). Trials of FGF-2 RP have not disclosed evidence of ocular complications. Development of edema has been reported in up to 50% of patients receiving VEGF GTx for CLI. The edema typically developed within 3 weeks after GTx (corresponding with the time course of gene expression established for this plasmid in preclinical studies), and resolved completely within 2–4 weeks after administration of oral diuretics. Hypotension (presumably secondary to AGF-induced NO synthesis) has been reported in 7% (vs 3% in the placebo group) and proteinuria in 9% (vs 3% in the placebo group) of patients treated with FGF-2 in the TRAFFIC study (Lederman *et al.*, 2002). The available evidence cannot support additional concerns regarding arrhythmia-inducibility, catheter delivery techniques, and vascular malformations. A summary of the currently available cardiovascular GT trials is provided in the tables.

5. DEVELOPMENT ISSUES

1. *Patient selection*: Most patients enrolled in cardiovascular GT trials have failed conventional therapy and revascularization techniques; therefore they may represent failures of native angiogenic responses and may be particularly resistant to exogenous stimulation of neovascularization. In practice, this population is not the most ideal for proof of concept. However, the currently available data are particularly reassuring regarding morbidity and mortality and support the possibility of progressively reducing the threshold for testing these strategies on less desperately ill patients. The inclusion of such a broader range of candidates will ultimately provide more information regarding the universe of patients that may be treated safely and also benefit from this novel therapeutic paradigm (Henry *et al.*, 2003; Simons *et al.*, 2000).
2. *Confounding factors*: The majority of patients enrolled in these trials take a number of medications that may potentially interfere with the angiogenic process. These medications include aspirin, captopril, isosorbide dinitrate, lovastatin, furosemide, bumetanide, and spironolactone. Other confounding factors include age, hypercholesterolemia, diabetes, smoking, and the effect of AGF on endothelial function (Lederman *et al.*, 2002; Simons *et al.*, 2000).

3. The route of administration, dose, dosing schedule, and long-term side-effect profile of the currently used agents for cardiovascular angiogenesis remain a question in terms of efficacy and safety.
4. *Study design*: Most of the available data are from phase I trials, with only eight phase I/II – II trials, which introduce the limitations of open-label studies with the lack of a placebo group. The five phase I/II trials using GTx are of paramount importance but only amount to 186 patients, which clearly underscores the importance for larger studies.
5. Currently there are no angiogenesis-specific stimuli clearly identified.

While ischemia is known to upregulate VEGF production and the expression of its receptors, most patients develop only tissue-level ischemia during provocation, and we do not know whether nonischemic tissues will respond to growth factor stimulation (Freedman and Isner, 2002; Simons *et al.*, 2000).

6. SUMMARY

Despite major advances in pharmacotherapy, endovascular, and surgical interventions, there are still multiple disorders within the cardiovascular spectrum that remain without an effective treatment strategy. These include IHD refractory to medical therapy and revascularization, of paramount importance given that it represents the number one cause of morbidity and mortality in the United States and it is expected to become the number one killer worldwide as the population ages; CLI unsuitable to endovascular or surgical revascularization, which has a grim prognosis; restenosis, or the "Achilles' heel" of modern revascularization techniques including both percutaneous and surgical approaches; ischemic and diabetic neuropathy, responsible for significant morbidity in these patients and yet without any available therapy; and HF which is a common occurrence among those with IHD and constitutes the most expensive health care problem in the United States.

After a large amount of evidence was collected from preclinical studies showing the safety and beneficial effects of strategies using RPs or GTx of AGFs designed to improve angiogenesis in animal models of all the previously mentioned conditions, the first clinical application of cardiovascular GT was performed on December 7, 1994. Since then, the number of cardiovascular GTx trials proposed to the NIH increased from 3 to 17% of all GT trials by the year 2000. This increase contrasts with the nearly 50% reduction in proposals of GT for other disease states that occurred during the 1-year period surrounding the Gelsinger death. The explanation of this phenomenon lies in the multiple unmet clinical needs that abound in the cardiovascular arena, which arise from the rather limited benefit that is provided by the current pharmacological armamentarium and revascularization techniques (Isner *et al.*, 2001). To date, phase I and II clinical trials (most without a placebo group) have enrolled a limited number of patients with advanced IHD, CLI, and neuropathy, who are a less than ideal substrate to establish proof of

concept. Nonetheless, the results from these studies are encouraging having shown that the strategies for cardiovascular angiogenesis are safe, well tolerated, and render beneficial clinical outcomes. The use of GTx strategies using naked plasmid DNA encoding phVEGF$_{165}$ has particularly stood out because of its mechanism of action, which is based on the specific stimulation of ECs, the ischemia-triggered upregulation of its production and expression of its receptors in ECs, the presence of an exclusive secretory signal sequence in its first exon that allows it to be naturally secreted by these cells—allowing the paracrine effect of the secreted gene product to modulate the bioactivity of several target cells, and its role as principal determinant of endothelial maintenance and repair.

Because the number of ECs that are capable to respond to stimulation by AGFs is limited and may therefore limit the ultimate magnitude of therapeutic angiogenesis, a strategy of cell therapy (administration of complementary EPCs) combined with GTx might enhance neovascularization and overcome this issue, as has been demonstrated in preclinical studies of EPC transduction with an adenovirus encoding VEGF (Iwaguro *et al.*, 2002). This strategy may allow the replacement of senescent ECs and/or cardiac myocytes with cellular precursors that can integrate into host tissues, and at the same time deliver with each cell the angiogenic gene product that can restore the physiological function. Implicit in this approach is the development of methods to identify which patients are likely to have inadequate cytokine or cellular (or both) responses to ischemia, so that therapeutic angiogenesis may be tailored and targeted appropriately, which undoubtedly will be unfold with the development of the field of pharmacogenomics.

As has happened to all the previously introduced changes of paradigm in medicine, the appropriate combination of scientific rigor, clinical judgment, and professional ethics will allow cardiovascular GT to establish its value and reach its place in the therapeutic armamentarium. Finally, the immediate research goals in the field of cardiovascular angiogenesis are to initiate phase I clinical trials in order to establish safety and tolerability among patients with HF and to perform large-scale, randomized, double-blind, placebo-controlled, multicenter, phase III clinical trials in the subpopulations already studied, including a broad range of patients (not limited to critically ill patients), in order to establish proof of concept and to provide an answer to the currently existing enigmas.

ACKNOWLEDGMENT. We dedicate this work to the memory of Jeffrey M. Isner, M.D., who was our inspiring mentor and one of the fathers of this exciting emerging field.

REFERENCES

Abraham, W. T., and Scarpinato, L., 2002, Higher expectations for management of heart failure, *J. Am. Board Fam. Pract.* **15**:39–49.

Annex, B., 2002, Therapeutic angiogenesis: a treatment for the new millennium or a passing fad? *Cardiol. Rounds* **6**:1–6.

Arras, M., Ito, W. D., Scholz, D., Winkler, B., Schaper, J., and Schaper, W., Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb, *J. Clin. Invest.* **101**:40–50.

Asahara, T., Bauters, C., Zheng, L. P., Takeshita, S., Bunting, S., Ferrara, N., Symes, J. F., and Isner, J. M., 1995, Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation*; **91**:2793–2801.

Asahara, T., Chen, D., Tsurumi, Y., Kearney, M., Rossow, S., Passeri, J., Symes, J. F., and Isner, J. M., 1996, Accelerated restitution of endothelial integrity and endothelium-dependent function following phVEGF$_{165}$ gene transfer. *Circulation* **94**:3291–3302.

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M., and Isner, J. M., 1999a, Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res*. **85**:221–8.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J. M., 1997, Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**:964–967.

Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M., and Isner, J. M., 1999b, VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18**:3964–3972.

Baffour, R., Danylewick, R., Burdon, T., Sniderman, A., Common, A., Graham, A., and Symes, J. F., 1988, An angiographic study of ischemia as a determinant of neovascularization in arteriovenous reversal. *Surg. Gynecol. Obstet.* **166**:28–32.

Banai, S., Jaklitsch, M. T., Casscells, W., Shou, M., Shrivastav, S., Correa, R., Epstein, S. E., and Unger, E. F., 1991, Effects of acidic fibroblast growth factor on normal and ischemic myocardium. *Circ. Res.* **69**:76–85.

Banai, S., Jaklitsch, M. T., Shou, M., Lazarous, D. F., Scheinowitz, M., Biro, S., Epstein, S. E., and Unger, E. F., 1994, Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation* **89**:2183–2189.

Baumgartner, I., Pieczek, A., Manor, O., Blair, R., Kearney, M., Walsh, K., and Isner, J. M., 1998, Constitutive expression of phVEGF$_{165}$ following intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* **97**:1114–1123.

Baumgartner, I., Rauh, G., Pieczek, A., Wuensch, D., Magner, M., Kearney, M., Schainfeld, R., and Isner, J. M., 2000, Lower-extremity edema associated with gene transfer of naked DNA vascular endothelial growth factor. *Ann. Intern. Med.* **132**:880–884.

Berinyi, L. K., Conte, M. S., Mulligan, R. C., 1992, Repopulation of injured arteries with genetically modified endothelial cells. *J. Vasc. Surg.* **15**:932–934.

Bonvini, R., Baumgartner, I., Do do, D., Alerci, M., Segatto, J. M., Tutta, P., Jager, K., Aschwanden, M., Schneider, E., Amann-Vesti, B., Greiner, R., Mahler, F., and Gallino, A., 2003, Late acute thrombotic occlusion after endovascular brachytherapy and stenting of femoropopliteal arteries. *J. Am. Coll. Cardiol*. **41**:409–412.

Celletti, F. L., Waugh, J. M., Amabile, P. G., Brendolan, A., Hilfiker, P. R., and Dake, M. D., 2001, Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat. Med.* **7**:425–429.

Clowes, A. W., Gown, A. M., Hanson, S. R., Reidy M. A., 1985, Mechanisms of arterial graft failure, 1: Role of cellular proliferation in early healing of PTFE prostheses. *Am. J. Pathol.* **118**:43–54.

Conn, G., Soderman, D. D., Schaeffer, M. T., Wile, M., Hatcher, V. B., and Thomas, K. A., 1990, Purification of glycoprotein vascular endothelial cell mitogen from a rat glioma cell line. *Proc. Natl. Acad. Sci. U.S.A*. **87**:1323–1327.

Connolly, D. T., Heuvelman, D. M., Nelson, R., Olander, J. V., Eppley, B. L., Delfino, J. J., Siegel, N. R., Leimgruber, R. M., and Feder, J., 1989, Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* **84**:1470–1478.

Couffinhal, T., Silver, M., Kearney, M., Sullivan, A., Witzenbichler, B., Magner, M., Annex, B., Peters, K., and Isner, J. M., 1999, Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE$^{-/-}$ mice. *Circulation* **99**:3188–3198.

Davies, M. J., Woolf, N., Rrowles, P. M., Pepper, J., 1988, Morphology of the endothelium over atherosclerotic plaque in human coronary arteries. *Br. Heart J.* **60**:459–464.

Dichek, D. A., Neville, R. F., Zwiebel, J. A., Freeman, S. M., Leon, M. B., and Anderson, W. F., 1989, Seeding of intravascular stents with genetically engineered endothelial cells. *Circulation* **80**:1347–1353.

Dormandy, J., A., and, Thomas. P. R. S., 1988, What is the natural history of a critically ischemic patient with and without his leg? In *Limb Salvage and Amputation for Vascular Disease*, 11 edn., eds. R. M., Greenhalgh, C. W., Jamieson, and A. N., Nicolaides, pp. 11–26. Philadelphia: WB Saunders.

Duda, S. H., Pusich, B., Richter, G., Landwehr, P., Oliva, V. L., Tielbeek, A., Wiesinger, B., Hak, J. B., Tielemans, H., Ziemer, G., Cristea, E., Lansky, A., and Beregi, J. P., 2002, Sirolimus-eluting stents for the treatment of obstructive superficial femoral artery disease: six-month results. *Circulation* **106**:1505–1509.

Fam, N. P., Verma, S., Kutryk, M., Stewart DJ., 2003, Clinician guide to angiogenesis. *Circulation* **108**:2613–2618.

Ferrara, N., Henzel W. J., 1989, Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **161**:851–855.

Flamme, I., Risau, W., 1992, Induction of vasculogenesis and hematopoiesis in vitro. *Development* **116**:435–439.

Folkman, J., 1971, Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* **285**:1182–1186.

Folkman, J., 1972, Anti-angiogenesis: new concept for therapy of solid tumors. *Ann. Surg.* **175**:409–416.

Folkman, J., Klagsbrun, M., 1987, Angiogenic factors. *Science* **235**:442–447.

Folkman, J., Merler, E., Abernathy, C., Williams, G., 1971, Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.* **133**:275–288.

Folkman, J., Shing, Y., Angiogenesis., 1992, *J. Biol. Chem.* **267**:10931–10934.

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L., 1996, Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **16**:4604–4613.

Fortuin, F. D., Vale, P., Losordo, D. W., Symes, J., DeLaria, G. A., Tyner, J. J., Schaer, G. L., March, R., Snell, R. J., Henry, T. D., Van Camp, J., Lopez, J. J., Richenbacher, W., Isner, J. M., and Schatz, R. A., 2003, One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. *Am. J. Cardiol.* **92**:436–439.

Freedman, S. B., Isner J. M., 2002, Therapeutic angiogenesis for coronary artery disease. *Ann. Intern. Med.* **136**:54–71.

Gaziano, J., 1998, Primary and secondary prevention of coronary heart disease. *Cardiol. Rounds*. **2**:1–8.

Gilbert, S. F., 1997, *Developmental Biology*, 4th edn. Sunderland, MA: Sinauer Associates.

Grines, C. L., Watkins, M. W., Helmer, G., Penny, W., Brinker, J., Marmur, J. D., West, A., Rade, J. J., Marrott, P., Hammond, H. K., and Engler, R. L., 2002, Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. *Circulation* **105**:1291–1297.

Grines, C. L., Watkins, M. W., Mahmarian, J. J., Iskandrian, A. E., Rade, J. J., Marrott, P, Pratt, C., and Kleiman, N; Angiogene GENe Therapy (AGENT-2) Study Group, 2003, A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina. *J. Am. Coll. Cardiol.* **42**:1339–1347.

Hajjar, R. J., Del Monte, F., Matsui, T., Rosenzweig, A., 2000, Prospects for gene therapy for heart failure. *Circ. Res.* **86**:616–621.

Hanahan, D., Folkman, J., 1996, Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**:353–364.

Harada, K., Friedman, M., Lopez, J. J., Wang, S. Y., Li, J., Prasad, P. V., Pearlman, J. D., Edelman, E. R., Sellke, F. W., and Simons, M., 1996, Vascular endothelial growth factor in chronic myocardial ischemia. *Am. J. Physiol.* **270**:H1791–H1802.

Hariawala, M. D., Horowitz, J. R., Esakof, D., Sheriff, D. D., Walter, D. H., Keyt, B., Isner, J. M., and Symes, J. F., 1996, VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J. Surg. Res.* **63**:77–82.

Harrison, D. G., Freiman, P. C., Armstrong, M. L., Marcus, M. L., Heistad DD. 1987, Alterations of vascular reactivity in atherosclerosis. *Circ. Res.* **61**:II-74–II-80.

Haudenschild, C. C., Quist, W. C., LoGerfo F. W., 1981, Protection of endothelium in vessel segments excised for grafting. *Circulation* **64**:II-101–II-107.

Hedman, M., Hartikainen, J., Syvanne, M., Stjernvall, J., Hedman, A., Kivela, A., Vanninen, E., Mussalo, H., Kauppila, E., Simula, S., Narvanen, O., Rantala, A., Peuhkurinen, K., Nieminen, M. S., Laakso, M., and Yla-Herttuala, S., 2003 (Jun 3), Safety and feasibility of catheter-based local intracoronary VEGF transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia. Phase II results of the KAT-trial. *Circulation* **107**(21):2677–83.

Hendel, R. C., Henry, T. D., Rocha-Singh, K., Isner, J. M., Kereiakes, D. J., Giordano, F. J., Simons, M., and Bonow, R. O., 2000, Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect. *Circulation* **101**:118–121.

Henry, T. D., Abraham J. A., 2000, Review of preclinical and clinical results with vascular endothelial growth factors for therapeutic angiogenesis. *Curr. Int. Cardiol. Rep.* **2**:228–241.

Henry, T. D., Annex, B. H., McKendall, G. R., Azrin, M. A., Lopez, J. J., Giordano, F. J., Shah, P. K., Willerson, J. T., Benza, R. L., Berman, D. S., Gibson, C. M., Bajamonde, A., Rundle, A. C., Fine, J., and McCluskey, E. R.; VIVA Investigators 2003, The VIVA trial: vascular endothelial growth factor in ischemia or vascular angiogenesis. *Circulation* **107**:1359–1365.

Henry, T. D., Rocha-Singh, K., Isner, J. M., Kereiakes, D. J., Giordano, F. J., Simons, M., Losordo, D. W., Hendel, R. C., Bonow, R. O., Eppler, S. M., Zioncheck, T. F., Holmgren, E. B., and McCluskey, E. R., 2001, Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease. *Am. Heart J.* **142**:872–880.

Herring, M. B., Compton, R. S., LeGrand, D. R., Gardner, A. L., Madison, D. L., and Glover, J. L., 1987, Endothelial seeding of polytetrafluoroethylene popliteal bypass: a preliminary report. *J. Vasc. Surg.* **6**:114–118.

Höckel, M., Schlenger, K., Doctrow, S., Kissel, T., Vaupel, P., 1993, Therapeutic angiogenesis. *Arch. Surg.* **128**:423–429.

Holm, P., Andersen, H. L., Andersen, M. R., Erhardtsen, E., Stender, S., 1999, The direct antiatherogenic effect of estrogen is present, absent, or reversed, depending on the state of the arterial endothelium. A time course study in cholesterol-clamped rabbits. *Circulation* **100**:1727–1733.

Hong, M. K., Mintz, G. S., Lee, C. W., Song, J. M., Han, K. H., Kang, D. H., Song, J. K., Kim, J. J., Weissman, N. J., Fearnot, N. E., Park, S. W., and Park, S. J.; ASian Paclitaxel-Eluting Stent Clinical Trial, 2003, Paclitaxel coating reduces in-stent intimal hyperplasia in human coronary arteries: a serial volumetric intravascular ultrasound analysis from the ASian Paclitaxel-Eluting Stent Clinical Trial (ASPECT). *Circulation* **107**:517–520.

Horowitz, J., Hariawala, M., Sheriff, D. D., Keyt, B., Symes J. F., 1995, In vivo administration of vascular endothelial growth factor is associated with EDRF-dependent systemic hypotension in porcine and rabbit animal models. *Circulation* **92**:I-630–I-631.

Iwaguro, H., Yamaguchi, J., Kalka, C., Murasawa, S., Masuda, H., Hayashi, S., Silver, M., Li, T., Isner, J. M., and Asahara, T., 2002, Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation* **105**:732–738.

Ischemia, EWGoCL. 1991, European Working Group on Critical Leg Ischemia: Second European consensus document on chronic critical leg ischemia, *Circulation* **84**:IV-1–IV-26.

Isner, J. M., 1998, Arterial gene transfer for naked DNA for therapeutic angiogenesis: early clinical results. *Adv. Drug Deliv*. **30**:185–197.

Isner, J. M., 2002, Myocardial gene therapy. *Nature*. **415**:234–239.

Isner, J. M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, K., and Symes, J. F., 1996a, Clinical evidence of angiogenesis following arterial gene transfer of phVEGF$_{165}$. *Lancet*. **348**:370–374.

Isner, J. M., Rosenfield, K., 1993, Redefining the treatment of peripheral artery disease: role of percutaneous revascularization. *Circulation* **88**:1534–1557.

Isner, J. M., Vale, P. R., Symes, J. F., Losordo D. W., 2001 (Aug 31), Assessment of risks associated with cardiovascular gene therapy in human subjects. *Circ. Res.* **89**(5):389–400.

Isner, J. M., Walsh, K., Symes, J., Pieczek, A., Takeshita, S., Lowry, J., Rossow, S., Rosenfield, K., Weir, L., Brogi, E., and Schainfeld, R., 1995, Arterial gene therapy for therapeutic angiogenesis in patients with peripheral artery disease. *Circulation* **91**:2687–2692.

Isner, J. M., Walsh, K., Symes, J., Pieczek, A., Takeshita, S., Lowry, J., Rosenfield, K., Weir, L., Brogi, E., and Jurayj, D., 1996b, Arterial gene transfer for therapeutic angiogenesis in patients with peripheral artery disease. *Hum. Gene Ther.* **7**:959–988.

Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W. M., Silver, M., Kearney, M., Li, T., Isner, J. M., and Asahara, T., 2000a, Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc. Natl. Acad. Sci. U.S.A*. **97**:3422–3427.

Kalka, C., Masuda, H., Takahashi, T., Gordon, R., Tepper, O., Gravereaux, E., Pieczek, A., Iwaguro, H., Hayashi, S. I., Isner, J. M., and Asahara, T., 2000b, Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ. Res*. **86**:1198–1202.

Kalka, C., Tehrani, H., Laudenberg, B., Vale, P. R., Isner, J. M., Asahara, T., and Symes, J. F., 2000, VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. *Ann. Thorac. Surg*. **70**:829–834.

Kastrup, J., 2003, Therapeutic angiogenesis in ischemic heart disease: gene or recombinant vascular growth factor protein therapy? *Curr. Gene Ther*. **3**:197–206.

Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T., 1989, Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246**:1309–1312.

Kibbe, M. R., Billiar, T. R., Tzeng, E., 2000, Gene therapy for restenosis. *Circ. Res*. **86**:829–833.

Kleiman, N. S., Califf RM., 2000, Results from late-breaking clinical trials sessions at ACCIS 2000 and ACC 2000. *J. Am. Coll. Cardiol*. **36**:310–325.

Kleiman, N. S., Patel, N. C., Allen, K. B., Simons, M., Yla-Herttuala, S., Griffin, E., and Dzau, V. J., 2003, Evolving revascularization approaches for myocardial ischemia. *Am. J. Cardiol*. **92**:9N–17N.

Kornowski, R., Leon, M. B., Fuchs, S., Vodovotz, Y., Flynn, M. A., Gordon, D. A., Pierre, A., Kovesdi, I., Keiser, J. A., and Epstein, S. E., Catheter-based transendocardial injection of adenoviral $VEGF_{121}$ offers equivalent gene delivery and protein expression compared to a surgical-based transepicardial injection approach. *J. Am. Coll. Cardiol.* 2000;**35**:73A.

Ku, D. D., Zaleski, J. K., Liu, S., 1993, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am. J. Physiol.* **265**:H586–H592.

Laham, R. J., Chronos, N. A., Pike, M., Leimbach, M. E., Udelson, J. E., Pearlman, J. D., Pettigrew, R. I., Whitehouse, M. J., Yoshizawa, C., and Simons, M., 2000, Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase 1 open-label dose escalation study. *J. Am. Coll. Cardiol.* **36**:2132–2139.

Laham, R. J., Rezaee, M., Post, M., Xu, X., Sellke FW., 2003, Intrapericardial administration of basic fibroblast growth factor: myocardial and tissue distribution and comparison with intracoronary and intravenous administration. *Catheter. Cardiovasc. Interv*. **58**:375–381.

Laitinen, M., Hartikainen, J., Hiltunen, M. O., Eranen, J., Kiviniemi, M., Narvanen, O., Makinen, K., Manninen, H., Syvanne, M., Martin, J. F., Laakso, M., and Yla-Herttuala, S., 2000, Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. *Hum. Gene Ther.* **11**:263–270.

Lazarous, D. F., Shou, M., Stiber, J. A., Hodge, E., Thirumurti, V., Goncalves, L., and Unger, E. F., 1999, Adenoviral-mediated gene transfer induces sustained pericardial VEGF expression in dogs: effect on myocardial angiogenesis. *Cardiovasc. Res.* **44**:294–302.

Lederman, R. J., Mendelsohn, F. O., Anderson, R. D., Saucedo, J. F., Tenaglia, A. N., Hermiller, J. B., Hillegass, W. B., Rocha-Singh, K., Moon, T. E., Whitehouse, M. J., and Annex, B. H.; TRAFFIC Investigators, 2002, Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. *Lancet* **359**:2053–2058.

Lee, L. Y., Patel, S. R., Hackett, N. R., Mack, C. A., Polce, D. R., El-Sawy, T., Hachamovitch, R., Zanzonico, P., Sanborn, T. A., Parikh, M., Isom, O. W., Crystal, R. G., and Rosengart, T. K.,

2000, Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endotehlial growth factor 121. *Ann. Thorac. Surg.* **69**:14–24.

Lee, C. H., Smits P. C., 2002, Vascular growth factors for coronary angiogenesis. , *J. Interv. Cardiol*. **15**:511–518.

Leotta, E., Patejunas, G., Murphy, G., Szokol, J., McGregor, L., Carbray, J., Hamawy, A., Winchester, D., Hackett, N., Crystal, R., and Rosengart, T., 2002, Gene therapy with adenovirus-mediated myocardial transfer of vascular endothelial growth factor 121 improves cardiac performance in a pacing model of congestive heart failure. *J. Thorac. Cardiovasc. Surg.* **123**:1101–1113.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., Ferrara, N., 1989, Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**:1306–1309.

LoGerfo, F. W., Quist, W. C., Cantelmo, N. L., Haudenschild C. C., 1983, Integrity of vein grafts as a function of initial intimal and medial preservation. *Circulation* **68**:II-117–II-124.

Lopez, J. J., Laham, R. J., Stamler, A., Pearlman, J. D., Bunting, S., Kaplan, A., Carrozza, J. P., Sellke, F. W., and Simons, M., 1998, VEGF administration in chronic myocardial ischemia in pigs. *Cardiovasc. Res.* **40**:272–281.

Losordo, D. W., Isner J. M., 2001, Vascular endothelial growth factor-induced angiogenesis: crouching tiger or hidden dragon? *J. Am. Coll. Cardiol*. **37**:2131–2135.

Losordo, D. W., Isner, J. M., Diaz-Sandoval L. J., 2003, Endothelial recovery: the next target in restenosis prevention. *Circulation* **107**:2635–2637.

Losordo, D. W., Pickering, J. G., Takeshita, S., Leclerc, G., Gal, D., Weir, L., Kearney, M., Jekanowski, J., and Isner, J. M., 1994, Use of the rabbit ear artery to serially assess foreign protein secretion after site specific arterial gene transfer in vivo: evidence that anatomic identification of successful gene transfer may underestimate the potential magnitude of transgene expression. *Circulation* **89**:785–792.

Losordo, D. W., Vale, P. R., Hendel, R. C., Milliken, C. E., Fortuin, F. D., Cummings, N., Schatz, R. A., Asahara, T., Isner, J. M., and Kuntz, R. E., 2002, Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation* **105**:2012–2018.

Losordo, D. W., Vale, P. R., Symes, J. F., Dunnington, C. H., Esakof, D. D., Maysky, M., Ashare, A. B., Lathi, K., and Isner, J. M., 1998, Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF$_{165}$ as sole therapy for myocardial ischemia. *Circulation* **98**:2800–2804.

Luo, Z., Asahara, T., Tsurumi, Y., Isner, J. M., Symes J. F., 1998, Reduction of vein graft intimal hyperplasia and preservation of endothelium-dependent relaxation by topical vascular endothelial growth factor. *J. Vasc. Surg.* **27**:167–173.

Mack, C. A., Patel, S. R., Schwarz, E. A., Zanzonico, P., Hahn, R. T., Ilercil, A., Devereux, R. B., Goldsmith, S. J., Christian, T. F., Sanborn, T. A., Kovesdi, I., Hackett, N., Isom, O. W., Crystal, R. G., and Rosengart, T. K., 1998, Biologic bypass with the use of adenovirus-mediated gene transfer of the conplementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. *J. Thorac. Cardiovasc. Surg.* **115**:168–176.

Minar, E., Pokrajac, B., Maca, T., Ahmadi, R., Fellner, C., Mittlbock, M., Seitz, W., Wolfram, R., and Potter, R., 2000, Endovascular brachytherapy for prophylaxis of restenosis after femoropopliteal angioplasty: results of a prospective randomized study. *Circulation* **102**:2694–2699.

Minutes Recombinant DNA Advisory Committee (RAC) of National Institutes of Health, Sept. 13, 1994, RAC #9409–088.

Moses, J. W., Leon, M. B., Popma, J. J., Fitzgerald, P. J., Holmes, D. R., O'Shaughnessy, C., Caputo, R. P., Kereiakes, D. J., Williams, D. O., Teirstein, P. S., Jaeger, J. L., and Kuntz, R. E.; SIRIUS Investigators, 2003, Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N. Engl. J. Med*. **349**:1315–1323.

Moulton, K. S., Heller, E., Konerding, M. A., Flynn, E., Palinski, W., and Folkman, J., 1999, Angiogenesis inhibitors endostatin and TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation* **99**:1726–1732.

Mukherjee, D., Bhatt, D. L., Roe, M. T., Patel, V., Ellis SG., 1999, Direct myocardial revascularization and angiogenesis—how many patients might be eligible? *Am. J. Cardiol.* **84**:598–600.

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J. F., Fishman, M. C., Huang, P. L., and Isner, J. M., 1998, Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101**:2567–2578.

Nabel, E. G., Plautz, G., Boyce, F. M., Stanley, J. C., Nabel GJ., 1989, Recombinant gene expression *in vivo* within endothelial cells of the arterial wall. *Science* **244**:1342–1344.

Naruse, K., Shimizu, K., Muramatsu, M., Toki, Y., Miyazaki, Y., Okumura, K., Hashimoto, H., and Ito, T., 1994, Long-term inhibition of NO synthesis promotes atherosclerosis in hypercholesterolemic rabbit thoracic aorta. PGH_2 does not contribute to impaired endothelium-dependent relaxation. *Arterioscler. Thromb.* **14**:746–752.

Pearlman, J. D., Hibberd, M. G., Chuang, M. L., Harada, K., Lopez, J. J., Gladstone, S. R., Friedman, M., Sellke, F. W., and Simons, M., 1995, Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nat. Med.* **1**:1085–1089.

Pentecost, M. J., Criqui, M. H., Dorros, G., Goldstone, J., Johnston, K. W., Martin, E. C., Ring, E. J., and Spies, J. B., 1994, Guidelines for peripheral percutaneous transluminal angioplasty of the abdominal aorta and lower extremity vessels: a statement for health professionals from a special writing group of the Councils on Cardiovascular Radiology, Arteriosclerosis, Cardio-Thoracic and Vascular Surgery, Clinical Cardiology, and Epidemiology and Prevention, the American Heart Association. *Circulation* **89**:511–531.

Peters, K. G., deVries, C., Williams L. T., 1993, Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc. Natl. Acad. Sci. U.S.A.* **90**:8915–8919.

Plouet, J., Schilling, J., Gospodarowicz, D., 1989, Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *EMBO J.* **8**:3801–3806.

Popma, J. J., Suntharalingam, M., Lansky, A. J., Heuser, R. R., Speiser, B., Teirstein, P. S., Massullo, V., Bass, T., Henderson, R., Silber, S., von Rottkay, P., Bonan, R., Ho, K. K., Osattin, A., and Kuntz, R. E.; Stents And Radiation Therapy (START) Investigators, 2002, Randomized trial of $^{90}Sr/^{90}Y$ beta-radiation versus placebo control for treatment of in-stent restenosis. *Circulation* **106**:1090–1096.

Pu, L. Q., Sniderman, A. D., Arekat, Z., Graham, A. M., Brassard, R., and Symes, J. F., 1993a, Angiogenic growth factor and revascularization of the ischemic limb: evaluation in a rabbit model. *J. Surg. Res.* **54**:575–583.

Pu, L. Q., Sniderman, A. D., Brassard, R., Lachapelle, K. J., Graham, A. M., Lisbona, R., and Symes, J. F., 1993b, Enhanced revascularization of the ischemic limb by means of angiogenic therapy. *Circulation* **88**:208–215.

Regar, E., Serruys, P. W., Bode, C., Holubarsch, C., Guermonprez, J. L., Wijns, W., Bartorelli, A., Constantini, C., Degertekin, M., Tanabe, K., Disco, C., Wuelfert, E., and Morice, M. C.; RAVEL Study Group, 2002, Angiographic findings of the multicenter Randomized Study With the Sirolimus-Eluting Bx Velocity Balloon-Expandable Stent (RAVEL): sirolimus-eluting stents inhibit restenosis irrespective of the vessel size. *Circulation* **106**:1949–1956.

Riessen, R., Rahimizadeh, H., Blessing, E., Takeshita, S., Barry, J. J., and Isner, J. M., 1993, Arterial gene transfer using pure DNA applied directly to a hydrogel-coated angioplasty balloon. *Hum. Gene Ther*. **4**:749–758.

Risau, W., Differentiation of endothelium. *FASEB J.* 1995;**9**:926–933.

Rivard, A., Fabre, J. E., Silver, M., Chen, D., Murohara, T., Kearney, M., Magner, M., Asahara, T., and Isner, J. M., 1999a, Age-dependent impairment of angiogenesis. *Circulation* **99**:111–120.

Rivard, A., Silver, M., Chen, D., Kearney, M., Magner, M., Annex, B., Peters, K., and Isner, J. M., 1999b, Rescue of diabetes related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. *Am. J. Pathol.* **154**:355–364.

Rosengart, T. K., Lee, L. Y., Patel, S. R., Sanborn, T. A., Parikh, M., Bergman, G. W., Hachamovitch, R., Szulc, M., Kligfield, P. D., Okin, P. M., Hahn, R. T., Devereux, R. B., Post, M. R., Hackett,

N. R., Foster, T., Grasso, T. M., Lesser, M. L., Isom, O. W., and Crystal, R. G., 1999a, Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expression $VEGF_{121}$ cDNA to individuals with clinically significant severe coronary artery disease. *Circulation* **100**:468–474.

Rosengart, T. K., Lee, L. Y., Patel, S. R., Kligfield, P. D., Okin, P. M., Hackett, N. R., Isom, O. W., and Crystal, R. G., 1999b, Six-month assessment of a phase I trial of angiogenic gene therapy for the treatment of coronary artery disease using direct intramyocardial administration of an adenovirus vector expressing the $VEGF_{121}$cDNA. *Ann. Surg.* **230**:466–472.

Ross, R., 1993, Atherosclerosis: a defense mechanism gone awry. *Am. J. Pathol*. **143**:985–1002.

Ruel, M., Laham, R. J., Parker, J. A., Post, M. J., Ware, J. A., Simons, M., and Sellke, F. W., 2002, Long-term effects of surgical angiogenic therapy with fibroblast growth factor 2 protein. *J. Thorac. Cardiovasc. Surg*. **124**:28–34.

Schainfeld R. M., 2002, Potential emerging therapeutic strategies to prevent restenosis in the peripheral vasculature. *Catheter. Cardiovasc. Interv*. **56**:421–431.

Schainfeld, R. M., Isner J. M., 1999, Critical limb ischemia: nothing to give at the office? *Ann. Intern. Med.* **130**:442–444.

Schaper, W., Munoz-Chapuli, R., Wolf, C., and Ito, W., 1999, Collateral circulation of the heart. In *Angiogenesis and Cardiovascular Disease*, 159 edn., eds. J. A., Ware and M., Simons, pp. 159–198. New York: Oxford University Press.

Schratzberger, P., Walter, D. H., Rittig, K., Bahlmann, F. H., Pola, R., Curry, C., Silver, M., Krainin, J. G., Weinberg, D. H., Ropper, A. H., and Isner, J. M., 2001, Reversal of experimental diabetic neuropathy by VEGF gene transfer. *J. Clin. Invest*. **107**:1083–1092.

Schultz, A., Lavie, L., Hochberg, I., Beyar, R., Stone, T., Skorecki, K., Lavie, P., Roguin, A., and Levy, A. P., 1999, Interindividual heterogeneity in the hypoxic regulation of VEGF: significance for the development of the coronary artery collateral circulation. *Circulation* **100**:547–552.

Schumacher, B., Pecher, P., vonSpecht, B. U., Stegmann, T., 1998, Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation* **97**:645–650.

Sellke, F. W., Laham, R. J., Edelman, E. R., Pearlman, J. D., Simons, M., 1998, Therapeutic angiogenesis with basic fibroblast growth factor: technique and early results. *Ann. Thorac. Surg.* **65**:1540–1544.

Sidawy, A. N., Weiswasser, J. M., Waksman, R., 2002, Peripheral vascular brachytherapy. *J. Vasc. Surg.* **35**:1041–1047.

Simons, M., Bonow, R. O., Chronos, N. A., Cohen, D. J., Giordano, F. J., Hammond, H. K., Laham, R. J., Li, W., Pike, M., Sellke, F. W., Stegmann, T. J., Udelson, J. E., and Rosengart, T. K., 2000, Clinical trials in coronary angiogenesis: issues, problems, consensus. An expert panel summary. *Circulation* **102**:E73–E86.

Simovic, D., Isner, J. M., Ropper, A. H., Pieczek, A., 2001, Weinberg D. H., Improvement in chronic ischemic neuropathy following intramuscular $phVEGF_{165}$ gene transfer in patients with critical limb ischemia. *Arch. Neurol.* **58**:761–768.

Stanley, J. C., Burkel, W. E., Ford, J. W., Vinter, D. W., Kahn, R. H., Whitehouse, W. M. Jr., and Graham, L. M., 1982, Enhanced patency of small-diameter, externally supported Dacron iliofemoral grafts seeded with endothelial cells. *Surgery* **92**:994–1005.

Stegmann, T. J., Hoppert, T., Schneider, A., Gemeinhardt, S., Kocher, M., Ibing, R., and Strupp, G., 2000, Induction of myocardial neoangiogenesis by human growth factors. A new therapeutic option in coronary heart disease. *Herz* **25**:589–599.

Stone, G. W., Ellis, S. G., Cox, D. A., Hermiller, J., O'Shaughnessy, C., Mann, J. T., Turco, M., Caputo, R., Bergin, P., Greenberg, J., Popma, J. J., and Russell, M. E.; TAXUS-IV Investigators, 2004, A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. *N. Engl. J. Med.* **350**:221–231.

Symes, J. F., Losordo, D. W., Vale, P. R., Lathi, K. G., Esakof, D. D., Mayskiy, M., and Isner, J. M., 1999, Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease: preliminary clinical results. *Ann. Thorac. Surg.* **68**:830–837.

Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J. M., and Asahara, T., 1999, Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat. Med.* **5**:434–438.

Takeshita, S., Losordo, D. W., Kearney, M., Isner J. M., 1994c, Time course of recombinant protein secretion following liposome-mediated gene transfer in a rabbit arterial organ culture model. *Lab. Invest.* **71**:387–391.

Takeshita, S., Zheng, L. P., Brogi, E., Kearney, M., Pu, L. Q., Bunting, S., Ferrara, N., Symes, J. F., and Isner, J. M., 1994d, Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J. Clin. Invest.* **93**:662–670.

Tanaka, H., Sukhova, G. K., Swanson, S. J., Clinton, S. K., Ganz, P., Cybulsky, M. I., and Libby, P., 1993, Sustained activation of vascular cells and leukocytes in the rabbit aorta after balloon injury. *Circulation* **88**:1788–1803.

Tio, R. A., Tkebuchava, T., Scheuermann, T. H., Lebherz, C., Magner, M., Kearny, M., Esakof, D. D., Isner, J. M., and Symes, J. F., 1999, Intramyocardial gene therapy with naked DNA encoding vascular endothelial growth factor improves collateral flow to ischemic myocardium. *Hum. Gene Ther.* **10**:2953–2960.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A., 1991, The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. *J. Biol. Chem.* **266**:11947–11954.

Tsurumi, Y., Murohara, T., Krasinski, K., Chen, D., Witzenbichler, B., Kearney, M., Couffinhal, T., and Isner, J. M., 1997, Reciprocal relationship between VEGF and NO in the regulation of endothelial integrity. *Nat. Med.* **3**:879–886.

Tuder, R. M., Flook, B. E., Voelkel N. F., 1995, Increased gene expression of VEGF and the VEGF receptors KDR/*Flk* and *Flt* in lungs exposed to acute or to chronic hypoxia: modulation of gene expression by nitric oxide. *J. Clin. Invest.* **95**:1798–1807.

Udelson, J. E., Dilsizian, V., Laham, R. J., Chronos, N., Vansant, J., Blais, M., Galt, J. R., Pike, M., Yoshizawa, C., and Simons, M., 2000, Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease. *Circulation* **102**:1605–1610.

Unger, E. F., Goncalves, L., Epstein, S. E., Chew, E. Y., Trapnell, C. B., Cannon, R. O. 3rd, and Quyyumi, A. A., 2000, Effects of a single intracoronary injection of basic fibroblast growth factor in stable angina pectoris. *Am. J. Cardiol.* **85**:1414–1419.

Vale, P. R., Losordo, D. W., Milliken, C. E., Esakof, D. D., and Isner, J. M., 1999a, Images in cardiovascular medicine: percutaneous myocardial gene transfer of phVEGF-2. *Circulation* **100**:2462–2463.

Vale, P. R., Losordo, D. W., Milliken, C. E., Maysky, M., Esakof, D. D., Symes, J. F., and Isner, J. M., 2000, Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. *Circulation* **102**:965–974.

Vale, P. R., Losordo, D. W., Milliken, C. E., McDonald, M. C., Gravelin, L. M., Curry, C. M., Esakof, D. D., Maysky, M., Symes, J. F., and Isner, J.M., 2001, Randomized, single-blind, placebo-controlled pilot study of catheter-based myocardial gene transfer for therapeutic angiogenesis using left ventricular electromechanical mapping in patients with chronic myocardial ischemia. *Circulation* **103**:2138–2143.

Vale, P. R., Losordo, D. W., Tkebuchava, T., Chen, D., Milliken, C. E., and Isner, J. M., 1999b, Catheter-based myocardial gene transfer utilizing nonfluoroscopic electromechanical left ventricular mapping. *J. Am. Coll. Cardiol.* **34**:246–254.

Van Belle, E., Maillard, L., Tio, F. O., Isner J. M., 1997a, Accelerated endothelialization by local delivery of recombinant human vascular endothelial growth factor reduces in-stent intimal formation. *Biochem. Biophys. Res. Commun.* **235**:311–316.

Van Belle, E., Rivard, A., Chen, D., Silver, M., Bunting, S., Ferrara, N., Symes, J. F., Bauters, C., and Isner, J. M., 1997b, Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines. *Circulation* **96**:2667–2674.

Van Belle, E., Tio, F. O., Chen, D., Maillard, L., Chen, D., Kearney, M., and Isner, J. M., 1997c, Passivation of metallic stents following arterial gene transfer of phVEGF$_{165}$ inhibits thrombus formation and intimal thickening. *J. Am. Coll. Cardiol.* **29**:1371–1379.

Van Belle, E., Tio, F. O., Couffinhal, T., Maillard, L., Passeri, J., and Isner, J. M., 1997d, Stent endothelialization: time course, impact of local catheter delivery, feasibility of recombinant protein administration, and response to cytokine expedition. *Circulation* **95**:438–448.

Van der Giessen, W. J., Serruys, P. W., and Visser, W. J., 1988, Endothelialization of intravascular stents. *J. Intervent. Cardiol.* **1**:109–120.

van der Zee, R., Murohara, T., Luo, Z., Zollmann, F., Passeri, J., Lekutat, C., and Isner, J. M., 1997, Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* **95**:1030–1037.

Waksman, R., Ajani, A. E., White, R. L., Chan, R. C., Satler, L. F., Kent, K. M., Pichard, A. D., Pinnow, E. E., Bui, A. B., Ramee, S., Teirstein, P., and Lindsay, J., 2002, Intravascular gamma radiation for in-stent restenosis in saphenous-vein bypass grafts. *N. Engl. J. Med*. **346**:1194–1199.

Walter, D. H., Cejna, M., Diaz-Sandoval, L., Willis, S., Kirkwood, L., Stratford, P. W., Tietz, A. B., Kirchmair, R., Silver, M., Curry, C., Wecker, A., Yoon, Y. S., Heidenreich, R., Hanley, A., Kearney, M., Tio, F. O., Kuenzler, P., Isner, J. M., and Losordo, D. W., 2004 (Jul 6), Local gene transfer of phVEGF-2 plasmid by gene-eluting stents: an alternative strategy for inhibition of restenosis. *Circulation* **110**(1):36–45.

Weidinger, F. F., McLenachan, J. M., Cybulsky, M. I., Gordon, J. B., Rennke, H. G., Hollenberg, N. K., Fallon, J. T., Ganz, P., and Cooke, J. P., 1990, Persistent dysfunction of regenerated endothelium after balloon angioplasty of rabbit iliac artery. *Circulation* **81**:1667–1679.

Weiss, M., Orkin S. H., 1996, In vitro differentiation of murine embryonic stem cells: new approaches to old problems. *J. Clin. Invest.* **97**:591–595.

Wilson, J. M., Birinyi, L. K., Salomon, R. N., Libby, P., Callow, A. D., and Mulligan, R. C., 1989, Implantation of vascular grafts lined with genetically modified endothelial cells. *Science* **244**:1344–1346.

Witzenbichler, B., Asahara, T., Murohara, T., Silver, M., Spyridopoulos, I., Magner, M., Principe, N., Kearney, M., Hu, J. S., and Isner, J. M., 1998, Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am. J. Pathol*. **153**:381–394.

Yanagisawa-Miwa, A., Uchida, Y., Nakamura, F., Tomaru, T., Kido, H., Kamijo, T., Sugimoto, T., Kaji, K., Utsuyama, M., and Kurashima, C., 1992, Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science* **257**:1401–1403.

Chapter 11

Tissue Engineering

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1. INTRODUCTION

Tissue engineering is the process of manipulating cellular and/or scaffold material, biological or synthetic, in order to produce tissue analogs, either *in vivo* or *in vitro*. It is also one of the most dynamic fields within the domains of biotechnology and medicine. Tissue engineering is all pervasive; attempts have been made to tissue engineer almost every organ, from the bladder to bone, and from the heart to cartilage. In this review, recent efforts to tissue engineer structures of the cardiovascular system—vascular grafts, cardiac valves, and myocardium—will be explored.

2. VASCULAR GRAFTS

Atherosclerosis is the leading cause of mortality and morbidity in the developed world (Lopez and Murray, 1998). Medical management of atherosclerosis is focused on the reduction of risk factors that contribute to the etiology of this disease. However, this approach is often inadequate and surgical measures are frequently required to treat arterial occlusion produced by atherosclerosis. Options in the surgical treatment of atherosclerosis include atherectomy, balloon angioplasty

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

(with or without stent placement), and bypass grafting. Unfortunately, restenosis is a common complication of all of these approaches.

In the field of bypass grafting, restenosis is particularly problematic in small vessel, low flow settings. Under these conditions, conventional synthetic vascular grafts perform inadequately and acceptable performance can only be obtained from autogenous blood vessels (Veith *et al.*, 1986). However, autologous vessels for grafting are often not available in individuals suffering from vascular disease (Taylor *et al.*, 1990), and available vessels may be diseased or possess dysfunctional endothelia. Thus synthetic vascular grafts, despite their inadequate performance, are currently the only widely available option under these circumstances.

The lack of a vascular graft capable of performing adequately under conditions of small vessel size and low vessel diameter has led to numerous investigators pursuing different avenues to address this holy grail of vascular biology.

2.1. The Ideal Vascular Prosthesis

The ideal vascular prosthesis would demonstrate a 100% rate of long-term patency and 0% incidence of complications. The ideal prosthesis is unlikely to be a clinical reality in the near future. The present gold standard in arterial prostheses in small vessel, low flow applications is the autogenous artery. When used as a coronary artery bypass graft, the autologous internal thoracic artery has demonstrated patency rates of 83% at 10 years (Barner *et al.*, 1985). This differs markedly with the performance of saphenous veins in this application, 41% patency at 10 years (Barner *et al.*, 1985). The performance of synthetic grafts is even worse.

The answer to the quest for the superior arterial prosthesis lies in the differences between the autogenous artery and the synthetic graft. The following properties are of greatest importance to the performance of the ideal vascular prosthesis:

1. An antithrombogenic endothelia-like lining.
2. Mechanical properties approaching those of adjacent host arteries. Mechanical strength sufficient to resist aneurysm and retain sutures. Sufficient elasticity to enable compliance matching of the prosthesis and adjacent host arteries.
3. Possession of vasomotor activity and the ability to respond with appropriate magnitude to vasoactive factors.
4. Biocompatibility (noninflammatory, nonimmunogenic, and nontoxic).

Previous efforts to produce arterial prostheses attempted to address one or two of the above properties (e.g. seeding of endothelial cells onto synthetic grafts or the development of compliant polyurethane grafts). Although these approaches have demonstrated potential, much promise lies in the field of tissue engineering, in which the possibility of addressing all the above-required properties exists.

2.2. Tissue-Engineered Alternatives

Tissue engineering is a burgeoning field in which living cells are combined with acellular materials, synthetic or biological, to produce cellularized organ prostheses. The pioneering efforts of Weinberg and Bell (1986) are widely recognized as the birth of vascular graft tissue engineering. This duo produced the first tissue-engineered vascular graft in 1986, demonstrating the feasibility of applying tissue engineering to vascular graft construction. Their prosthesis consisted of a collagen matrix inhabited by bovine smooth muscle cells and fibroblasts and seeded with endothelial cells. This construct was supported by layers of Dacron incorporated into the walls. The endothelium produced von Willebrand factor and prostacyclin, both features of the *in vivo* functioning endothelium. However, the construct did not possess sufficient strength for implantation as an arterial interposition graft and the Dacron support prevented complete host remodeling. Furthermore, the graft was bereft of elastin, essential to the compliance of a vascular prosthesis. The vascular graft of Weinberg and Bell represented a major conceptual shift in the approach to production of a superior arterial prosthesis and opened an era of new possibilities in this field.

From the aforementioned innovative effort five major approaches to the production of a tissue-engineered vascular graft have emerged: (1) seeding a collagen gel scaffold with cells, (2) seeding a biodegradable synthetic polymeric scaffold with cells, (3) promoting cells to produce their own matrix and scaffold, (4) promoting remodeling of an implanted acellular graft, and (5) complete *in vivo* production of an arterial prosthesis. These approaches will be briefly reviewed.

2.2.1. Collagen Gel Scaffolds

Since the initial efforts of Weinberg and Bell the collagen gel approach has been extended (Berglund *et al.*, 2003; Hirai *et al.*, 1994; Hirai and Matsuda, 1996; Seliktar *et al.*, 2000). Collagen gel is attractive as the scaffold for a vascular graft as it provides an excellent substrate for cell attachment and signaling and is a biological material and thus able to be remodeled. However, the collagen gel has an inherent mechanical weakness and hence investigators have focused on controlling the mechanical forces to which the graft is exposed so that its constituent cells may remodel the graft into a stronger structure. This approach has taken two main routes. One is the exposure of the collagen gel vascular graft to regulated pulsatile pressure via a bioreactor (Seliktar *et al.*, 2000). Exposure of the collagen gel construct to regulated pulsatile pressure within the bioreactor led to significantly increased contractile and mechanical strength of the graft (Seliktar *et al.*, 2000). Morphological orientation of the smooth muscle cells was also seen. The other approach used was to shield the vascular graft from some of the pulsatile pressure by surrounding the graft with a supporting sleeve of cross-linked type I collagen (Berglund *et al.*, 2003). Following exposure to mechanical stimulation, the prosthesis demonstrated a significantly improved burst pressure of 650 mmHg, more than required for physiological conditions, but investigations of *in vivo* patency for

these grafts have not been reported. The use of a supporting sleeve would enable minimization of *in vitro* culture time, enabling significant reductions in cost and time required for graft production. Thus, combining the collagen gel scaffold approach with endothelial seeding and a support sleeve may present a viable option in the surgical treatment of atherosclerosis.

2.2.2. Synthetic Polymeric Scaffolds

The second approach, combining synthetic polymeric scaffolds with cellular components, is best demonstrated by the work of Niklason *et al.* and coworkers (Niklason *et al.*, 1999, 2001; Niklason and Langer, 1997). The graft produced by this group combined polyglycolic acid with bovine arterial smooth muscle and endothelial cells. When cultured in the presence of ascorbic acid and exposed to intraluminal pulsatile pressure in a bioreactor, the resulting vessel demonstrated gross morphology similar to that of a native blood vessel, the polyglycolic acid degraded to be replaced by extracellular matrix, and the burst pressure and suture retention strength were sufficient for implantation as an arterial interposition graft. *In vivo* assessment of performance as an arterial interposition graft was conducted for 1 month over which time the autologous pulsed construct remained patent.

In a related investigation, a tissue-engineered aortic graft consisting of a scaffold of a copolymer of polyglycolic acid and polyhydroxyalkanoate combined with cells from ovine carotid artery remained patent for 5 months (Shum-Tim *et al.*, 1999), and was remodeled to consist of levels of collagen and DNA similar to that of native aorta. The graft also developed elastin. The use of synthetic polymers as scaffolds for tissue engineering of arterial prostheses has potential to produce a superior arterial graft, as shown by the development of artery-like morphology and *in vivo* remodeling with acquisition of elastin. The ability to undergo favorable remodeling *in vivo*, without the need for lengthy exposure to mechanical stimuli *in vitro* (Shum-Tim *et al.*, 1999), suggests that specific polymeric scaffolds can be well tolerated and may present an alternative to conventional synthetic grafts in the future.

2.2.3. Cell-Based Products

The third tissue engineering approach is the entirely cell- and cell-product-based approach of L'Heureux *et al.* (1998, 2001). Human venous smooth muscle cells and fibroblasts were cultured in the presence of sodium ascorbate until they produced large sheets, which were wrapped around a perforated mandrel. Cylinders of smooth muscle cell and fibroblast sheets were exposed to intraluminal pulsatile pressure for a period of 12 weeks. Subsequent endothelial seeding led to a completed graft with the morphological appearance of an artery. The construct demonstrated the presence of elastin, and inhibited platelet aggregation in hemocompatibility assessment. *In vivo* assessment of short-term patency was conducted in a dog model, i.e. xenograft, and demonstrated 50% patency at 7 days. This

vascular prosthesis represents a completely human cell and human cell-derived graft with demonstrated strength and suturability. This approach enables the production of an entirely host-derived arterial prosthesis, without the implantation of potentially immunogenic materials. However, the graft takes 3 months to construct, precluding its use in emergency situations, and *in vivo* performance has yet to be demonstrated in a meaningful way. Thus the graft of L'Heureux remains an unproven, yet interesting entity.

2.2.4. *In Vivo* Cell Recruitment and Graft Remodeling

The approaches outlined above have the disadvantage of requiring cell harvest from vessels and thus lead to surgical morbidity. Implantation of an acellular scaffold that readily undergoes remodeling alleviates the requirement for surgical cell harvest. Huynh *et al.* (1999) demonstrated the ability of the acellular small-intestinal submucosal collagen layer with a luminal type I bovine collagen layer to undergo complete remodeling over a 90-day period to form a prosthesis with artery-like morphology and responsiveness to vasoactive factors. Over the 90-day period the grafts demonstrated 100% patency.

The process of remodeling and *in vivo* cell recruitment has also been explored at the molecular level. Erzurum *et al.* (2003) demonstrated the ability of a fibroblast growth factor-1 mutant, R136K, to promote the migration of endothelial cells through fibrin glue. Translation of this ability to the *in vivo* environment would enable promotion of endothelialization of vascular grafts, or re-endothelialization of arteries following intraluminal interventions such as angioplasty. In either instance the rapid establishment of an endothelial lining may reduce incidence of thrombosis and intimal/neointimal hyperplasia.

An acellular vascular graft designed to be remodeled has the major advantage of being "off the shelf" technology. This graft could be implanted directly into a patient, with no additional surgical morbidity from cell harvest or the need for time in culture. However, the remodeling ability of humans relative to animals must be taken into consideration. The failure of single-stage endothelial seeding of synthetic vascular grafts to improve patency rates in clinical trials (Herring *et al.*, 1994) contrasts starkly with the improvements in performance seen in animal models (Douville *et al.*, 1987). The difference in spontaneous endothelialization between animal and human models strongly suggests that the extent of remodeling of the small intestinal submucosa collagen grafts (Huynh *et al.*, 1999) seen in canine models will not translate directly to the clinic.

2.2.5. *In Vivo* Arterial Graft Generation

The final approach to production of a tissue-engineered arterial prosthesis, which will be discussed, is a completely *in vivo* approach. Implantation of lengths of silastic tubing into the peritoneal cavity leads to the development of free-floating avascular tissue capsules over a 2-week period (Campbell *et al.*, 1999). These

tissue capsules are composed of a layer of collagen matrix apposing the silastic tubing, a myofibroblast-rich layer, and an external monolayer of mesothelial cells. When everted, this structure is analogous to an artery, with an intima of mesothelial cells, a media of myofibroblasts, and adventitia of collagen matrix. Three months following implantation as an arterial interposition graft in rabbits and rats the myofibroblast-rich tissue tube had become more alike an artery in morphology, doubled in thickness, the myofibroblasts differentiated toward a more smooth muscle-like phenotype (increased volume fraction of myofilaments and expression of smooth muscle myosin heavy chain) and the tissue tube had developed a vasomotor response to phenylephrine, KCl, and acetylcholine. Furthermore, the grafts demonstrated a patency rate of approximately 68% over a period of up to 4 months in rats and rabbits, in the absence of heparin or spasmolytics. The artificial artery from the peritoneal cavity has also demonstrated patency of up to 16 months in canine models (Chue *et al.*, in press).

The artificial artery from the peritoneal cavity is completely autologous, is generated over 2 weeks, and does not require any *in vitro* manipulation. It does require two surgical interventions; however, the implantation of the tubing in the peritoneal cavity is a relatively quick and simple process and graft retrieval will occur simultaneously with vascular bypass grafting. Thus, the artificial artery from the peritoneal cavity is a promising candidate for clinical use as an arterial bypass graft.

2.2.6. Conclusion

Tissue-engineered vascular grafts have yet to progress to clinical trials. The major issue to be addressed before that time is quantifying the performance of the tissue-engineered arterial grafts. At present, published studies on the grafts discussed above have demonstrated patency of up to 4 months (Campbell *et al.*, 1999). Investigations over longer time frames, e.g. over a year, should be conducted before extensive clinical trials are commenced. The next major issue to be addressed is how the performance of tissue-engineered arteries will translate to human models. Will the acellular approach culminate in a disappointing conclusion akin to that of single-stage seeding? Will tissue-engineered arteries demonstrate performance in the human superior to those of conventional synthetic grafts, or better yet, saphenous veins? These final questions will not be answered through any means other than clinical trials.

3. HEART VALVES

A greater challenge than arterial graft tissue engineering is tissue engineering of heart valves. Valve replacement is the most common treatment for advanced heart valve disease (Schoen and Levy, 1999). Valvular pathology commonly results from calcific, infective, inflammatory, and congenital disorders. The two conventional valve prostheses, mechanical valves and acellular biological valves,

are inadequate. Mechanical valves increase the risk of thromboembolic events, require a lifetime of anticoagulants (with subsequent risk of hemorrhage), and predispose to infection (Vongpatanasin *et al.*, 1996). Biological valves do not require anticoagulants but have limited durability. Furthermore, valvular prostheses are often used in pediatric patients in whom growth demands of the prosthesis-significant remodeling. The lack of this plasticity may necessitate repeated surgical intervention. A better heart valve prosthesis is needed.

Efforts to produce a superior tissue engineered heart valve can be classified into three main categories: implantation of an acellular scaffold designed to recruit host cells, seeding cells onto a polymeric scaffold, and seeding cells onto an acellular biological scaffold. Decellularized sheep and porcine heart valves, designed to be remodeled, have been investigated as a valvular graft in ovine models (Elkins *et al.*, 2001). Eleven months following implantation as a pulmonary valve graft the decellularized valves were repopulated by as much as 80% by mature interstitial cells. Fibrocytes secreting procollagen I were evident at 3 months. These findings contrast with those of a recent clinical trial investigating Synergraft decellularized porcine heart valves (Simon *et al.*, 2003). Synergraft heart valves were implanted in the right ventricular outflow tract in four children. Two of the grafts failed 6 weeks following implantation as a result of severe degeneration. The third graft ruptured within a year of implant and the fourth was removed prophylactically. All four grafts were subject to a severe inflammatory response leading to structural failure and severe degeneration of the leaflets and walls. There was no cellular repopulation of the porcine matrix. Analysis of preimplant samples of the Synergraft valves revealed incomplete decellularization and the presence of calcific deposits. The presence of cellular material may offer some explanation for the disappointing performance of the Synergraft valve. However, this study serves as a cautionary tale illustrating the vast unknown that lies between promising animal models and successful clinical application.

The use of a polymeric scaffold is exemplified by the work of Hoerstrup *et al.* (2000). This group created a trileaflet valvular prosthesis by seeding scaffolds of bioabsorbable polyglycolic acid coated by a thin layer of poly-4-hydroxybutyrate with endothelia and myofibroblasts from lamb carotids. The constructs were cultured within a pulse duplication system for 14 days before being grafted as pulmonary valve prostheses in lambs, for periods of up to 20 weeks. Over this period the valve cusps were mobile and by 20 weeks resembled native valves in microstructure and mechanical properties. In subsequent investigation an attempt was made to limit the associated surgical morbidity of this approach by exploring marrow stromal cells as a cell source (Hoerstrup *et al.*, 2002). When exposed to similar *in vitro* conditions as the earlier valvular prostheses, the stromal cell valvular prostheses demonstrated similar morphological and mechanical properties to native heart vessels but failed to develop the typical three-layered valve structure. *In vivo* studies are yet to be published and hence the performance of the marrow stromal cell heart valve remains to be demonstrated.

Steinhoff *et al.* (2000) demonstrated the ability of an acellular biomaterial to serve as a scaffold for tissue-engineered heart valve prosthesis. Decellularized

ovine pulmonary heart valves were seeded with autologous myofibroblasts and endothelial cells. Following 8 days in culture the cellularized prostheses were implanted as pulmonary valve grafts. Over a 3-month period the prostheses demonstrated normal function, and acquired a confluent endothelial lining. The results from this study are in many ways comparable to those of Hoerstrup (Hoerstrup *et al.*, 2000). Both methods resulted in a pulmonary valve prosthesis that remained functional for 3 months and developed toward a native valve in morphology over this time. Both approaches required the surgical harvest of a length of host artery and a period of *in vitro* development. Only the acellular approach did not produce harvest morbidity. Furthermore, all three of the above investigations explore a pulmonary valve model, a lower pressure system, and the site of a relatively small proportion of valvular pathology (Robbins *et al.*, 1999). Although more technically and mechanically demanding, for clinical relevance investigations should be conducted exploring the ability of valvular prostheses to perform as aortic valves, as lesions of the aortic and mitral valves account for the vast majority of heart valve pathology (Robbins *et al.*, 1999). At present, there exists exciting prospects in the field of heart valve tissue engineering. However, there needs to be more focus on clinically relevant models and methods to reduce or eliminate harvest morbidity.

4. MYOCARDIUM

Heart failure affects 4.6 million people in the United States alone, with 550,000 new cases occurring in that country each year (Braunwald and Bristow, 2000). Mortality is high with approximately 75% of patients under the age of 65 dying within 8 years of diagnosis (American Heart Association, 2001). Conventional medical therapy is obviously inadequate. Furthermore, the cells of the myocardium are terminally differentiated, eliminating the possibility of localized cellular proliferation and repair unless cardiomyocytes can be influenced to reenter the cell cycle (Engel *et al.*, 1999). This is perhaps the ultimate mechanism for treatment of myocardial disease but is a distant goal. In end-stage heart failure, the only currently available intervention capable of significant improvement is cardiac transplant (Miniati and Robbins, 2002). However, potential recipient numbers far exceed donors. Thus, there is a great need for a tissue-engineered myocardial graft.

Two approaches to repair injured myocardium can be identified: the conventional tissue engineering approach and that of cellular cardiomyoplasty.

4.1. Conventional Tissue Engineering

Conventional tissue engineering of myocardial grafts follows the principles outlined above for production of other prostheses, i.e. autologous cells are added to an acellular scaffold, either synthetic or biological. In addition, a further method for production of myocardial grafts via layering of sheets of cardiac myocytes has been developed (Shimizu *et al.*, 2002).

In 1999, Li *et al.* generated a spontaneously contracting tissue-engineered myocardial graft (Li *et al.*, 1999). The graft was composed of a gelfoam scaffold onto which was seeded cardiomyocyte-enriched cell inoculum from fetal rat ventricular muscle. The graft was cultured *in vitro* for 7 days and then implanted into either the subcutaneous tissue of the leg or the myocardial scar tissue in rats. After 5 weeks the grafted tissue in the leg had developed spontaneous contractility while the tissue grafted to myocardial scar tissue had formed junctions with recipient heart cells. Leor *et al.* (2000) demonstrated the ability of fetal rat cardiac cells seeded onto an alginate scaffold to aggregate and spontaneously contract in multicellular units *in vitro*, and to attenuate left ventricular dilatation and contractility loss in an infarction model *in vivo*. Zimmermann *et al.* (2002) demonstrated the ability of collagen type I gel to serve as the matrix for a tissue engineered myocardial graft when mixed with neonatal cardiac myocytes. These constructs developed contractile function *in vitro* and underwent further differentiation following implantation on uninjured hearts.

The above examples adhere to the conventional tissue engineering methodology of seeding a three-dimensional acellular scaffold with cells. Shimizu *et al.* (2002) developed a novel method consisting of layering of cell-sheets of cardiomyocytes, similar to the method of L'Heureux (L'Heureux *et al.*, 1998) for vascular graft generation. Neonatal rat cardiomyocytes were cultured on thermoresponsive cell-culture surfaces. Reducing the cell culture temperature led to the detachment of a sheet of cardiomyocytes from the cell culture surface. These sheets were then layered and developed spontaneous pulsatility *in vitro*. Layered sheets were implanted into the subcutaneous tissue of nude mice and were able to maintain viability and pulsatility for up to 12 weeks.

Thus the conventional tissue engineering approach, and variations thereof, have been able to produce constructs capable of forming intercellular electrical junctions and developing spontaneous pulsatility. The above approaches have all used cardiomyocytes as their cellular constituent. However, primary cardiac cells are unlikely to ever be a cell source for clinical cardiac tissue engineering. The most likely source of cells for myocardial tissue engineering are adult skeletal myoblasts and adult mesenchymal stem cells, because of the high improbability of primary cardiac cell use and the ethical issues associated with use of embryonic stem cells. Evidence for the plasticity of skeletal myoblasts and adult mesenchymal cells and their relevance to tissue engineering of the myocardium can be found within the field of cellular cardiomyoplasty.

4.2. Cellular Cardiomyoplasty

Cellular cardiomyoplasty is the process of rebuilding injured myocardial tissue by transplantation of cells of various origin and stages of differentiation. Cells investigated for potential in cellular cardiomyoplasty include fetal and neonatal cardiomyocytes, embryonic stem cells, adult stem cells, and skeletal muscle myoblasts. Fetal and neonatal cardiomyocytes are not available in sufficient quantities for widespread clinical utilization, necessitate immunosuppressive therapy,

and present ethical dilemmas. The same holds true for embryonic stem cells. Thus, at present the only clinically feasible options are skeletal muscle myoblasts and other adult stem cells.

Skeletal myoblasts, or satellite cells, are stem cells located within the basal lamina of the adult skeletal muscle. In response to skeletal muscle injury they divide and undergo cell fusion to restore skeletal most tissue (Chou and Nonaka, 1977). Skeletal myoblasts can be readily isolated and expanded *in vitro* (Menasche *et al.*, 2003), enabling the administration of autologous myoblasts, avoiding the need for immunosuppression. Animal studies have demonstrated the ability of skeletal myoblast transfusion to improve left ventricular performance, in both systolic and diastolic phases, in myocardial injury models (Atkins *et al.*, 1999a, 1999b; Jain *et al.*, 2001). In a recent clinical trial (Menasche *et al.*, 2003) skeletal myoblast transplantation into myocardial infarction scars improved left ventricular ejection fraction and improved the New York Heart Association functional class over an average 11-month period. However, this procedure may increase the risk of arrhythmia, as evidenced by 4 of 10 patients in the trial experiencing episodes of sustained ventricular tachycardia. Thus skeletal myoblasts have shown promise in early clinical trials, but have also demonstrated an arrhythmogenic potential that must be further assessed before large-scale clinical application of this technology can commence.

In the field of cellular cardiomyoplasty, marrow mesenchymal stem cells are the other adult stem cell of particular interest. *In vitro* studies have demonstrated the ability of marrow mesenchymal stem cells to differentiate to produce spontaneously beating cardiomyocyte-like cells (Makino *et al.*, 1999). These cells are available in abundance, can be harvested with minimal morbidity, and can be expanded *in vitro* (Wang *et al.*, 2000). The ability of transplanted autologous mesenchymal progenitor cells to improve left ventricular performance (Davani *et al.*, 2003) and reduce contractile dysfunction (Shake *et al.*, 2002) in post-myocardial infarct has been demonstrated in animal models. Early clinical trials have also demonstrated the ability of autologous marrow mesenchymal stem cell transplantation to increase ventricular function, improve regional wall motion at the site of infarct, and improve symptoms in humans (Assmus *et al.*, 2002; Stamm *et al.*, 2003; Strauer *et al.*, 2002; Tse *et al.*, 2003). Of interest, no difference was seen between the administration of bone marrow-derived or circulating blood-derived progenitors in one study (Assmus *et al.*, 2002). Use of circulating blood progenitor cells would allow for harvest of stem cells for cellular cardiomyoplasty with essentially zero morbidity.

Cardiomyoplasty and conventional tissue engineering approaches have demonstrated the ability to produce improvements in myocardial function postinfarction. The question of how these effects are produced remains. Conventional tissue engineered myocardial grafts have been demonstrated to spontaneously contract *in vivo* although not when part of a functioning heart. Furthermore, application to the endocardium of a polymeric scaffold seeded with vascular smooth muscle cells also leads to improved ventricular function (Matsubayashi *et al.*, 2003). Vascular smooth muscle cells are incapable of the rapid contraction–relaxation cycles

characteristic of cardiac myocytes. Thus explanations other than contribution to contractile activity of the heart for the improvement in ventricular function in the above investigations must be considered. Possibilities include the stimulation of secretion of cellular synthesis of extracellular matrix proteins (collagen synthesis is stimulated by cyclical stretch of smooth muscle cells; Stanley *et al.*, 2000), improved blood supply and thus function of adjacent cells (progenitor cells have been demonstrated to improve vascularization; Nishida *et al.*, 2003), and minimization of fibrosis and graft remodeling, thus enabling better mechanical function (Strauer *et al.*, 2001).

5. CONCLUSION

Tissue engineering is an exciting and rapidly developing field that may eventually revolutionize the therapy in the field of cardiovascular medicine. To date, the only recent advances to progress to clinical trials have been cellular cardiomyoplasty and tissue engineered heart valves. Cellular cardiomyoplasty has been a clinical success in early trials, while the tissue engineered heart valves were a clinical failure. Many innovations in this field lie in the "dead zone" between the animal model and clinical trials. Until clinical trials are conducted the true value of these innovations cannot be accurately assessed, as was demonstrated by the failure of the Synergraft heart valve (Simon *et al.*, 2003) in the initial clinical trial. Many questions remain to be answered in the field of cardiovascular tissue engineering but perhaps the most important questions are the following:

1. What is (are) the ideal source(s) of cells for cardiovascular tissue engineering applications? The ideal source would produce maximum therapeutic effect and minimal harvest morbidity.
2. What happens to the tissue-engineered structures once they are implanted? Do the cells persist, or are they replaced by host cells? How do the transplanted cells/structures have their therapeutic effect? Knowledge of these processes will enable the generation of better tissue-engineered prostheses.
3. What are the major differences in remodeling ability between humans and animal models? Answering this question will be a large step toward eliminating or at least minimizing the "dead zone."
4. Given the remarkable progress achieved in this field over the last 10 years, significant inroads to answering these questions will be made over the next 10. However, it is almost certain that more questions of equal or greater significance will arise.

REFERENCES

American Heart Association, 2001, *2002 Heart and Stroke Statistical Update*. Dallas, TX: American Heart Association.

Assmus, B., Schachinger, V., Teupe, C., Britten, M., Lehmann, R., Dobert, N., Grunwald, F., Aicher, A., Urbich, C., Martin, H., Hoelzer, D., Dimmeler, S., and Zeiher, A. M., 2002, Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI), *Circulation* **106**(24):3009–3017.

Atkins, B. Z., Hueman, M. T., Meuchel, J., Hutcheson, K. A., Glower, D. D., and Taylor, D. A., 1999a, Cellular cardiomyoplasty improves diastolic properties of injured heart, *J. Surg. Res.* **85**(2):234–242.

Atkins, B. Z., Hueman, M. T., Meuchel, J. M., Cottman, M. J., Hutcheson, K. A., and Taylor, D. A., 1999b, Myogenic cell transplantation improves in vivo regional performance in infarcted rabbit myocardium. *J. Heart Lung Transplant.* **18**(12):1173–1180.

Barner, H. B., Standeven, J. W., and Reese, J., 1985, Twelve-year experience with internal mammary artery for coronary artery bypass. *J. Thorac. Cardiovasc. Surg.* **90**(5):668–675.

Berglund, J. D., Mohseni, M. M., Nerem, R. M., and Sambanis, A. 2003, A biological hybrid model for collagen-based tissue engineered vascular constructs. *Biomaterials* **24**(7):1241–1254.

Braunwald, E., and Bristow, M. R. 2000, Congestive heart failure: fifty years of progress. *Circulation* **102**(20, Suppl. 4):IV14–IV23.

Campbell, J. H., Efendy, J. L., and Campbell, G. R. 1999, Novel vascular graft grown within recipient's own peritoneal cavity. *Circ. Res.* **85**(12):1173–1178.

Chou, S. M., and Nonaka, I. 1977, Satellite cells and muscle regeneration in diseased human skeletal muscles. *J. Neurol. Sci.* **34**(1):131–145.

Chue, W. L., Campbell, G. R., Caplice, N., Muhammed, A., Berry, C. L., Thomas, A. C., Bennett, M. B., and Campbell, J. H., 2004 (Apr), Dog peritoneal and pleural cavities as bioreactors to grow autologous vascular grafts. *J. Vasc. Surg*. **39**(4):859–67.

Davani, S., Marandin, A., Mersin, N., Royer, B., Kantelip, B., Herve, P., Etievent, J. P., and Kantelip, J. P., 2003, Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation* **108**(Suppl. 1):II253–II258.

Douville, E. C., Kempczinski, R. F., Birinyi, L. K., and Ramalanjaona, G. R. 1987, Impact of endothelial cell seeding on long-term patency and subendothelial proliferation in a small-caliber highly porous polytetrafluoroethylene graft. *J. Vasc. Surg.* **5**(4):544–550.

Elkins, R. C., Goldstein, S., Hewitt, C. W., Walsh, S. P., Dawson, P. E., Ollerenshaw, J. D., Black, K. S., Clarke, D. R., and O'Brien, M. F., 2001, Recellularization of heart valve grafts by a process of adaptive remodeling. *Semin. Thorac. Cardiovasc. Surg.* **13**(4, Suppl. 1):87–92.

Engel, F. B., Hauck, L., Cardoso, M. C., Leonhardt, H., Dietz, R., and von Harsdorf, R. 1999, A mammalian myocardial cell-free system to study cell cycle reentry in terminally differentiated cardiomyocytes. *Circ. Res.* **85**(3):294–301.

Erzurum, V. Z., Bian, J. F., Husak, V. A., Ellinger, J., Xue, L., Burgess, W. H., and Greisler, H. P., 2003, R136K fibroblast growth factor-1 mutant induces heparin-independent migration of endothelial cells through fibrin glue. *J. Vasc. Surg.* **37**(5):1075–1081.

Herring, M., Smith, J., Dalsing, M., Glover, J., Compton, R., Etchberger, K., and Zollinger, T., 1994, Endothelial seeding of polytetrafluoroethylene femoral popliteal bypasses: the failure of low-density seeding to improve patency. *J. Vasc. Surg.* **20**(4):650–655.

Hirai, J., Kanda, K., Oka, T., and Matsuda, T. 1994, Highly oriented, tubular hybrid vascular tissue for a low pressure circulatory system. *ASAIO J.* **40**(3):M383–388.

Hirai, J., and Matsuda, T. 1996, Venous reconstruction using hybrid vascular tissue composed of vascular cells and collagen: tissue regeneration process. *Cell Transplant.* **5**(1):93–105.

Hoerstrup, S. P., Kadner, A., Melnitchouk, S., Trojan, A., Eid, K., Tracy, J., Sodian, R., Visjager, J. F., Kolb, S. A., Grunenfelder, J., Zund, G., and Turina, M. I., 2002, Tissue engineering of functional trileaflet heart valves from human marrow stromal cells. *Circulation* **106**(12, Suppl. 1):I143–I150.

Hoerstrup, S. P., Sodian, R., Daebritz, S., Wang, J., Bacha, E. A., Martin, D. P., Moran, A. M., Guleserian, K. J., Sperling, J. S., Kaushal, S., Vacanti, J. P., Schoen, F. J., and Mayer, J. E. Jr., 2000, Functional living trileaflet heart valves grown in vitro. *Circulation* **102**(19, Suppl. 3):III44–III49.

Huynh, T., Abraham, G., Murray, J., Brockbank, K., Hagen, P. O., and Sullivan, S. 1999, Remodeling of an acellular collagen graft into a physiologically responsive neovessel. *Nat. Biotechnol.* **17**(11):1083–1086.

Jain, M., DerSimonian, H., Brenner, D. A., Ngoy, S., Teller, P., Edge, A. S., Zawadzka, A., Wetzel, K., Sawyer, D. B., Colucci, W. S., Apstein, C. S., and Liao, R., 2001, Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction. *Circulation* **103**(14):1920–1927.

Leor, J., Aboulafia-Etzion, S., Dar, A., Shapiro, L., Barbash, I. M., Battler, A., Granot, Y., and Cohen, S., 2000, Bioengineered cardiac grafts: a new approach to repair the infarcted myocardium? *Circulation* **102**(19, Suppl. 3):III56–III61.

L'Heureux, N., Paquet, S., Labbe, R., Germain, L., and Auger, F. A. 1998, A completely biological tissue-engineered human blood vessel. *FASEB J.* **12**(1):47–56.

L'Heureux, N., Stoclet, J. C., Auger, F. A., Lagaud, G. J., Germain, L., and Andriantsitohaina, R. 2001, A human tissue-engineered vascular media: a new model for pharmacological studies of contractile responses. *FASEB J.* **15**(2):515–524.

Li, R. K., Jia, Z. Q., Weisel, R. D., Mickle, D. A., Choi, A., and Yau, T. M. 1999 (Nov 9), Survival and function of bioengineered cardiac grafts. *Circulation* **100**(19, Suppl.):II63–69.

Lopez, A. D., and Murray, C. C. 1998, The global burden of disease, 1990–2020. *Nat. Med.* **4**(11):1241–1243.

Makino, S., Fukuda, K., Miyoshi, S., Konishi, F., Kodama, H., Pan, J., Sano, M., Takahashi, T., Hori, S., Abe, H., Hata, J., Umezawa, A., and Ogawa, S., 1999, Cardiomyocytes can be generated from marrow stromal cells in vitro. *J. Clin. Invest.* **103**(5):697–705.

Matsubayashi, K., Fedak, P. W., Mickle, D. A., Weisel, R. D., Ozawa, T., and Li, R. K. 2003, Improved left ventricular aneurysm repair with bioengineered vascular smooth muscle grafts. *Circulation* **108**(Suppl. 1):II219–II225.

Menasche, P., Hagege, A. A., Vilquin, J. T., Desnos, M., Abergel, E., Pouzet, B., Bel, A., Sarateanu, S., Scorsin, M., Schwartz, K., Bruneval, P., Benbunan, M., Marolleau, J. P., and Duboc, D., 2003, Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J. Am. Coll. Cardiol.* **41**(7):1078–1083.

Miniati, D. N., and Robbins, R. C. 2002, Heart transplantation: a thirty-year perspective. *Annu. Rev. Med.* **53**:189–205.

Niklason, L. E., Abbott, W., Gao, J., Klagges, B., Hirschi, K. K., Ulubayram, K., Conroy, N., Jones, R., Vasanawala, A., Sanzgiri, S., and Langer, R., 2001, Morphologic and mechanical characteristics of engineered bovine arteries. *J. Vasc. Surg.* **33**(3):628–638.

Niklason, L. E., Gao, J., Abbott, W. M., Hirschi, K. K., Houser, S., Marini, R., and Langer, R., 1999, Functional arteries grown in vitro. *Science* **284**(5413):489–493.

Niklason, L. E., and Langer, R. S. 1997, Advances in tissue engineering of blood vessels and other tissues. *Transpl. Immunol.* **5**(4):303–306.

Nishida, M., Li, T. S., Hirata, K., Yano, M., Matsuzaki, M., and Hamano, K. 2003, Improvement of cardiac function by bone marrow cell implantation in a rat hypoperfusion heart model. *Ann. Thorac. Surg.* **75**(3):768–773; discussion 773–764.

Robbins, S. L., Cotran, R. S., Collins, T., and Kumar, V., 1999, *Robbins Pathologic Basis of Disease,* 6th edn. Philadelphia: Saunders.

Schoen, F. J., and Levy, R. J. 1999, Founder's Award, 25th Annual Meeting of the Society for Biomaterials, Perspectives. Providence, RI, April 28–May 2, 1999. Tissue heart valves: current challenges and future research perspectives. *J. Biomed. Mater. Res.* **47**(4):439–465.

Seliktar, D., Black, R. A., Vito, R. P., and Nerem, R. M. 2000, Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. *Ann. Biomed. Eng.* **28**(4):351–362.

Shake, J. G., Gruber, P. J., Baumgartner, W. A., Senechal, G., Meyers, J., Redmond, J. M., Pittenger, M. F., and Martin, B. J., 2002, Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects, *Ann. Thorac. Surg.* **73**(6):1919–1925; discussion 1926.

Shimizu, T., Yamato, M., Akutsu, T., Shibata, T., Isoi, Y., Kikuchi, A., Umezu, M., and Okano, T., 2002, Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ. Res.* **90**(3):e40.

Shum-Tim, D., Stock, U., Hrkach, J., Shinoka, T., Lien, J., Moses, M. A., Stamp, A., Taylor, G., Moran, A. M., Landis, W., Langer, R., Vacanti, J. P., and Mayer, J. E. Jr., 1999, Tissue engineering

of autologous aorta using a new biodegradable polymer. *Ann. Thorac. Surg.* **68**(6):2298–2304; discussion 2305.

Simon, P., Kasimir, M. T., Seebacher, G., Weigel, G., Ullrich, R., Salzer-Muhar, U., Rieder, E., and Wolner, E., 2003, Early failure of the tissue engineered porcine heart valve SYNERGRAFT in pediatric patients. *Eur. J. Cardiothorac. Surg.* **23**(6):1002–1006; discussion 1006.

Stamm, C., Westphal, B., Kleine, H. D., Petzsch, M., Kittner, C., Klinge, H., Schumichen, C., Nienaber, C. A., Freund, M., and Steinhoff, G., 2003, Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* **361**(9351):45–46.

Stanley, A. G., Patel, H., Knight, A. L., and Williams, B. 2000, Mechanical strain-induced human vascular matrix synthesis: the role of angiotensin II. *J. Renin Angiotensin Aldosterone Syst.* **1**(1):32–35.

Steinhoff, G., Stock, U., Karim, N., Mertsching, H., Timke, A., Meliss, R. R., Pethig, K., Haverich, A., and Bader, A., 2000, Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits: in vivo restoration of valve tissue. *Circulation* **102**(19, Suppl. 3):III50–III55.

Strauer, B. E., Brehm, M., Zeus, T., Gattermann, N., Hernandez, A., Sorg, R. V., Kogler, G., and Wernet, P., 2001, Intracoronary, human autologous stem cell transplantation for myocardial regeneration following myocardial infarction. *Dtsch. Med. Wochenschr.* **126**(34–35):932–938.

Strauer, B. E., Brehm, M., Zeus, T., Kostering, M., Hernandez, A., Sorg, R. V., Kogler, G., and Wernet, P., 2002, Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* **106**(15):1913–1918.

Taylor, L. M., Jr., Edwards, J. M., and Porter, J. M. 1990, Present status of reversed vein bypass grafting: five-year results of a modern series. *J. Vasc. Surg.* **11**(2):193–205; discussion 205-196.

Tse, H. F., Kwong, Y. L., Chan, J. K., Lo, G., Ho, C. L., and Lau, C. P. 2003, Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* **361**(9351):47–49.

Veith, F. J., Gupta, S. K., Ascer, E., White-Flores, S., Samson, R. H., Scher, L. A., Towne, J. B., Bernhard, V. M., Bonier, P., Flinn, W. R., 1986, Six-year prospective multicenter randomized comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infrainguinal arterial reconstructions. *J. Vasc. Surg.* **3**(1):104–114.

Vongpatanasin, W., Hillis, L. D., and Lange, R. A. 1996, Prosthetic heart valves. *N. Engl. J. Med.* **335**(6):407–416.

Wang, J. S., Shum-Tim, D., Galipeau, J., Chedrawy, E., Eliopoulos, N., and Chiu, R. C. 2000, Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. *J. Thorac. Cardiovasc. Surg.* **120**(5):999–1005.

Weinberg, C. B., and Bell, E. 1986, A blood vessel model constructed from collagen and cultured vascular cells. *Science* **231**(4736):397–400.

Zimmermann, W. H., Didie, M., Wasmeier, G. H., Nixdorff, U., Hess, A., Melnychenko, I., Boy, O., Neuhuber, W. L., Weyand, M., and Eschenhagen, T., 2002, Cardiac grafting of engineered heart tissue in syngenic rats. *Circulation* **106**(12, Suppl. 1):I151–I157.

Chapter 12

Visualization of the Vulnerable Plaque

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1. INTRODUCTION

Thrombosis is the main cause of acute coronary syndrome and myocardial infarction (Naghavi *et al.*, 2003). The mechanism underlying thrombus formation is presently under debate, but several pathological conditions have been identified from human postmortem studies that correspond with the presence of thrombus. Of these conditions plaque rupture is the most common, but erosion of the endothelial layer and existence of calcified nodules without the existence of plaque rupture have also been identified. Plaques that have been ruptured have certain features in common (Falk, 1999; Virmani *et al.*, 2002): (i) size of the lipid core (40% of the entire plaque), (ii) thickness of the fibrous cap (less than 65 μm), (iii) presence of inflammatory cells, (iv) amount of remodeling and extent of plaque-free vessel wall. Several terms have been identified focusing either on the pathological aspects ("thin-cap fibroatheroma") or on the possibility to rupture ("rupture-prone plaques") or on the possibility to induce thrombosis ("vulnerable plaque"). As vulnerable plaque is the term encompassing all other terms, therefore this term will be used throughout the chapter.

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Cardiovascular Research: New Technologies, Methods, and Applications, edited by Gerard Pasterkamp and Dominique P. V. de Kleijn. Springer, New York, 2005.

The ideal technique would provide morphological, mechanical, and functional information regarding the above-mentioned features. At present, no such diagnostic modality is available. This chapter will discuss the current development of imaging techniques that have the potential to detect the above-mentioned features of vulnerable plaques.

2. ANGIOSCOPY

Intracoronary angioscopy offers a direct visualization of the plaque surface. The entire system consists of a (white) light source, a fiber optic to transmit the light into the vessel and collect the light reflecting from the vessel, and a camera to acquire images based upon the reflected light. While angioscopy allows assessment of the color of the plaque and superficial thrombus (Mizuno *et al.*, 1992), one of the problems is the absorption of light by the blood. As a consequence, pictures need to be acquired at bloodless conditions. Furthermore, the images are hard to be quantified. When this condition is fulfilled, angioscopy may detect large lipid cores from the yellow color. These features have been studied in a 12-month follow-up study of 157 patients with stable angina, where acute coronary syndrome occurred more frequently in patients with yellow plaques than in those with white plaques (Mizuno *et al.*, 1992).

In conclusion, angioscopy is a technique that obtains information from the lipid composition of the plaque and the existence of thrombus on top of the plaque. It may therefore be used for certain features of vulnerable plaque detection.

3. INTRAVASCULAR CORONARY ULTRASOUND

Intravascular coronary ultrasound (IVUS) provides real-time high-resolution images of the vessel wall and lumen (Bom *et al.*, 1998). In or near the focus of the ultrasound beam the axial resolution is about 150 μm, and the lateral resolution 300 μm. The images appear real time at a frequency of 15–30 frames/s. Features of the vessel wall can be detected based on echogenicity (i.e. backscatter and absorption) and thickness of the vessel wall.

Lipid depositions are described as echolucent zones and can be detected with a sensitivity of 78–95% and specificity of 30% (Potkin *et al.*, 1990; Rasheed *et al.*, 1995). This sensitivity is dependent on the amount of lipids and can be reduced if the echolucent area is smaller than a quarter of the plaque. Echolucent zones can also be caused by loose tissue and shadowing from calcium, which makes the interpretation of echolucent areas difficult. The sensitivity to differentiate between fibrous and fatty tissue is between 39 and 52% (Hiro *et al.*, 1996). Other features of the vulnerable plaque are difficult to assess as these components (cap thickness, macrophage accumulation, neovascularization) are below the imaging capabilities of ultrasound.

Yamagishi *et al.* (2000) evaluated IVUS in a prospective study with a follow-up period of about 2 years. The study revealed that large eccentric plaques

containing an echolucent zone by IVUS were found to be at increased risk of instability even though the lumen area was preserved at the time of initial study.

A number of groups have investigated the potential of ultrasound radiofrequency signal analysis for tissue characterization (Bridal *et al.*, 2000; Jeremias *et al.*, 1999; Landini *et al.*, 1986; Nair *et al.*, 2001; Spencer *et al.*, 1997; MacNeill *et al.*, 2003; Wilson *et al.*, 1994). Many of these studies revealed the potential to identify calcified plaques. A recent study showed the potential of backscatter analysis to identify the size of the lipid pool and the thickness of the cap in 40 MHz IVUS systems *in vivo* (Kawasaki *et al.*, 2002).

In conclusion, IVUS is an invasive method to detect wall thickness and echolucent zones. Echolucent zones have the potential to detect large lipid cores, but sensitivity is low. Prospective studies have to be performed for evaluating the role of IVUS in detecting plaques at increased risk of vulnerability.

4. INTRAVASCULAR ELASTOGRAPHY/PALPOGRAPHY

In 1991, a new technique was introduced to measure the mechanical properties of tissue using ultrasound: elastography (Ophir *et al.*, 1991). It measures the radiofrequency content of the backscattered ultrasound signal at two pressure levels in the cardiac cycle. Cross-correlation of both signals is then used to calculate local tissue deformation. As at a given pressure difference, soft plaque components will deform more than hard components, the method detects high-strain regions as a surrogate for soft plaque regions. For intravascular ultrasound purposes, a derivate of elastography called palpography may be a suitable tool (Doyley *et al.*, 2001). In this approach, one strain value per angle is determined and plotted as a color-coded contour at the lumen vessel boundary.

In vitro studies with histological confirmation have shown that there are differences of strain normalized to pressure between fibrous, fibro-fatty, and fatty components of the plaque of coronary as well as femoral arteries (de Korte *et al.*, 2000). This difference was mainly evident between fibrous and fatty tissue. The plaque types could not be differentiated by echo-intensity differences on the IVUS echogram.

Application of this technique to patients offered some extra problems: catheter movement and noncentral position of the catheter inside the lumen of the blood vessel. The first problem was tackled by calculating a likelihood function of the entire image, and the second problem was solved by adding a correction factor. On applying both, correction data were acquired in patients ($n = 12$) during PTCA procedures. Minimal motion was observed near the end of the passive filling phase. Reproducible strain estimates were obtained within one pressure cycle and over several pressure cycles. Validation of the results was limited to the information provided by the echogram. Significantly higher strain values were found in noncalcified plaques than in calcified plaques (de Korte *et al.*, 2002).

Palpography reveals information that is not seen on IVUS. Differentiating between hard and soft tissue may be important for the detection of a vulnerable plaque that is prone to rupture. Since palpography is based on clinically available IVUS catheters, the technique can be easily introduced into the catheterization laboratory. By acquiring data at the end of the filling phase, when catheter motion is minimal, the quality and reliability of the palpogram is increased. The clinical value of this technique is currently under investigation. Prospective studies need to be performed for this analysis to show its value in vulnerable plaque detection.

5. THERMOGRAPHY

Atherosclerosis is nowadays considered an inflammatory disease. The inflammatory component is especially apparent in vulnerable plaque. Casscells *et al.* (1996) were the first to show that the inflammatory component was associated with a local temperature rise in the affected tissue. They reported that carotid plaques taken at endarterectomy from 48 patients have an increased temperature heterogeneity associated with accumulation of inflammatory cells. Furthermore, there was a negative correlation between temperature difference and cap thickness. The same group reported approximately the same *in vitro* findings in atherosclerotic rabbits (Madjid *et al.*, 2002).

A correlation between temperature rise and macrophage infiltration has also recently been suggested in well-controlled experimental conditions *in vivo* (Verheye *et al.*, 2002). Our laboratory showed that in atherosclerotic rabbit aortas, temperature changes were dependent on blood flow (Fig. 1). Furthermore, IVUS measurements were not correlated to temperature (Fig. 2), indicating that plaque thickness was not associated with detected accumulation of macrophages and MMP-9. This enzyme is involved in matrix breakdown and may offer the coupling between plaque rupture and inflammation. Stefanadis *et al.* performed studies in humans in less-controlled conditions. Temperature was constant within the arteries of the control subjects, whereas most atherosclerotic plaques showed higher temperatures compared with healthy vessel wall. Temperature differences between atherosclerotic plaque and healthy vessel wall increased progressively from patients with stable angina to patients with acute myocardial infarction, with a maximum difference of $1.5 \pm 0.7^{\circ}$C (Stefanadis *et al.*, 1999). Furthermore, patients with a high temperature gradient have a significantly worse outcome than patients with a low gradient (Stefanadis *et al.*, 2001). At present, there are several groups stating that differences in plaque temperature are greatly reduced in the presence of blood flow. The reason for this cooling effect of blood flow is presently unknown and warrants further study.

In conclusion, thermistor-based catheter techniques allow to detect an inflammatory component of atherosclerosis. As inflammation plays a role in plaque vulnerability, it is an interesting method for vulnerable plaque detection. The cooling effect of blood flow needs further study before this method will be applicable in vulnerable plaque detection.

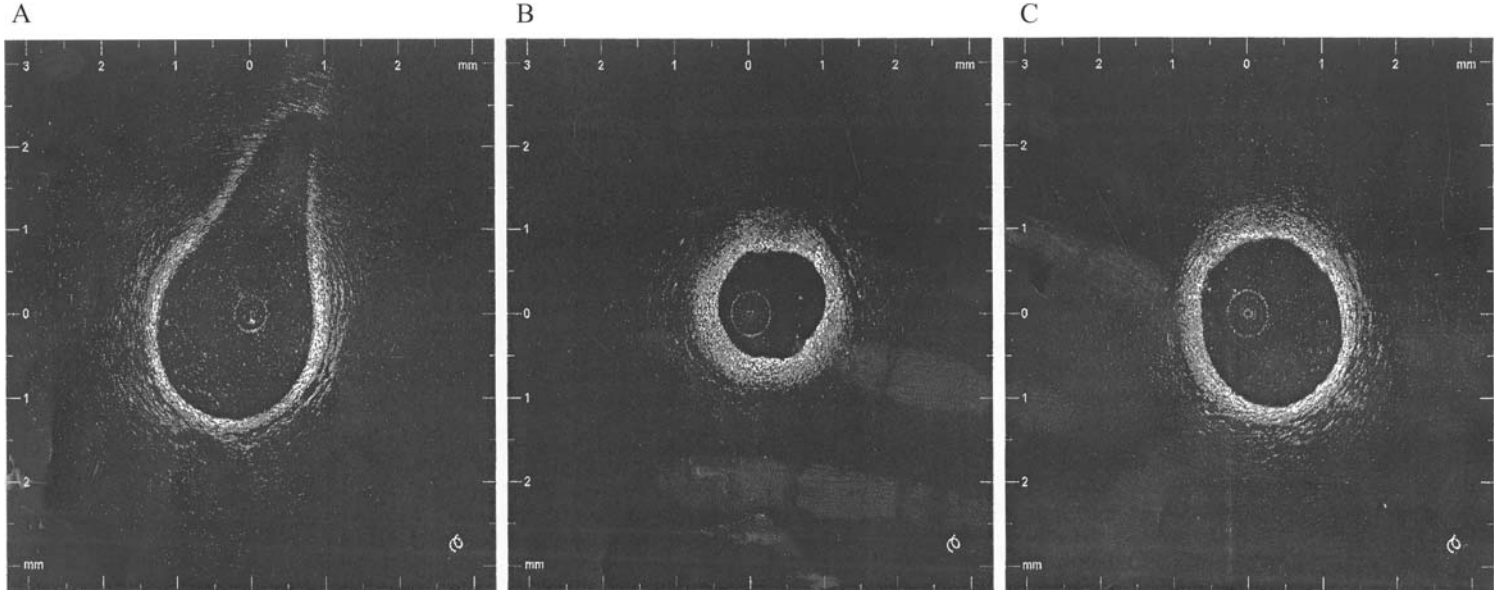

FIGURE 1. OCT images obtained during a pullback of an atherosclerotic rabbit aorta. Bloodless conditions were created by balloon occlusion and flushing at a constant rate. Indicated are a normal segment (panel A), a plaque having features of fibrotic tissue (panel B), and a cross section of a plaque containing a lipid-rich plaque (panel C). (*See Color Plate 15.*)

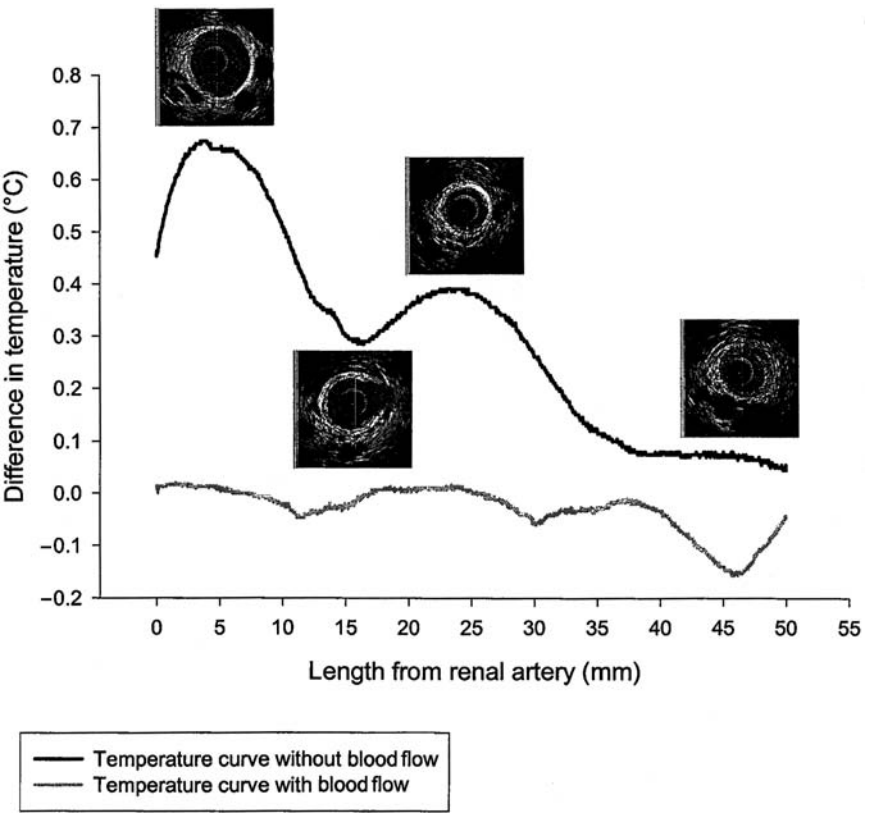

FIGURE 2. A temperature recording of an atherosclerotic rabbit aorta obtained during a pullback of a thermistor catheter (Thermocore Inc., Belgium, The Netherlands). The recording was obtained after occlusion of the aorta above the renal branches to create a stop flow condition. Indicated are two peaks in the graph and a series of IVUS pictures. Note that the IVUS pictures do not correlate with the temperature measurements.

6. OPTICAL COHERENCE TOMOGRAPHY

Optical coherence tomography (OCT) provides images from the optical backscatter properties of the tissue in an almost identical way as IVUS (Fujimoto, 2003). Many similarities exist between IVUS and OCT, but one of the main distinctions is the difference in wavelength and hence the resolution of the image. In comparison, a 40 MHz IVUS transducer has an axial resolution of 60–80 μm, while OCT has a resolution of 5–10 μm. A second important difference is the speed of light versus the speed of (ultra) sound. As a consequence of the high speed of light, conventional electronic detection is not possible, and a method called low-coherence interferometry is used for detection of the location of backscatter. Another difference is the fact that light is a transverse wave, making

it sensitive to polarization and thus birefringence of the tissue (Fujimoto, 2003). An inherent problem with the OCT method is that because of high absorption and high backscatter, penetration is less than in IVUS. Consequently, a depth of 2 mm is reached in bloodless conditions. If blood is present then absorption by blood takes place as a function of wavelength. Because light travels to and fro, this effect may add significant effects to the overall backscatter process.

Early attempts to validate OCT using histology showed that a lipid pool generates low-energy backscatter signals, while a fibrous plaque produces a high homogeneous backscatter signal (Jang *et al.*, 2002). Because of its high resolution, OCT may detect cap thickness. Recently, macrophage accumulation was identified on the basis of local variation of backscattering properties of the tissue. However, every particle with different acoustic properties with respect to its environment generates backscatter signals, making the detection probably not specific for macrophages.

In conclusion, OCT might be a valuable method to detect the mechanical properties of vulnerable plaque. The technique is still in its evaluation phase and needs to be tested before it will become accepted for routine use.

7. SPECTROSCOPIC TECHNIQUES

Several spectroscopic techniques are under development. Techniques like Raman and near-infrared spectroscopies are the most promising and will therefore be discussed in the present chapter. Raman spectroscopy is a technique that can characterize the chemical composition without homogenization of the tissue (Baraga *et al.*, 1992). It is based upon similar optical approaches as described above. Instead of detecting reflection of white light (angioscopy) or backscattering of light (OCT), this method measures Raman scattering. The Raman effect is created when incident light (wave length 750–850 nm) excites (bio)molecules in a tissue sample, which induces a nonelastic backscatter signal. This rotation of the atoms in the molecules produces spectra that are specific for the molecule (van De Poll *et al.*, 2003). Because of this unique feature, Raman spectroscopy can provide quantitative information about the molecular composition of the sample (Hanlon *et al.*, 2000). However, the spectra obtained from tissue require postprocessing to differentiate between plaque components.

Even in the presence of blood, Raman spectra have been shown to be obtainable *in vivo* from the aortic arch of sheep (Buschman *et al.*, 2000). In a study using mice that received a high-fat/high-cholesterol diet for 0, 2, 4, or 6 months, Raman spectroscopy showed good correlation between cholesterol accumulation and total serum cholesterol exposure. In female mice ($n = 10$) that were assigned to a high-fat/high-cholesterol diet, with or without 0.01% atorvastatin, a strong reduction in cholesterol accumulation (57%) and calcium salts (97%) was demonstrated in the atorvastatin-treated group. Raman spectroscopy can therefore be used to quantitatively study the size and distribution of depositions of cholesterol and calcification

(van De Poll *et al.*, 2001). Limitations of the technique are the limited penetration depth (1–1.5 mm) and the absorbance of the light by blood. Furthermore, Raman spectroscopy gives no geometrical information.

Near-infrared (NIR) spectroscopy also obtains information on the chemical components of the coronary vessel wall. Molecular vibrational transitions measured in the NIR region (750–2500 nm) give qualitative and quantitative results on plaque composition. NIR spectroscopy sensitivity and specificity for the histological features of plaque vulnerability were 90 and 93% for lipid pool, 77 and 93% for thin cap, and 84 and 89% for inflammatory cells (Moreno *et al.*, 2002). A differentiation between vulnerable and nonvulnerable carotid plaques could be achieved *ex vivo* (Wang *et al.*, 2002). Future studies will address the question whether NIR spectroscopy is feasible *in vivo*. Problems like acquisition time, blood scattering, and influence of pH and temperature must be addressed.

In conclusion, Raman and NIR spectroscopies detect the chemical composition of plaques and thereby allow detection of the lipid composition. Both are promising techniques that need further study for identifying their role in vulnerable plaque detection.

8. MAGNETIC RESONANCE IMAGING

For a recent review on the role of magnetic resonance imaging (MRI) in vulnerable plaque detection, we would refer to MacNeill *et al.* (2003). High-resolution MRI is a noninvasive modality to characterize atherosclerotic plaques. Combining information from T1- and T2-weighted imaging may permit *in vitro* identification of the atheromatous core, collageneous cap, calcification, media, adventitia, and perivascular fat (Toussaint *et al.*, 1995). Two studies identified, based upon a comparison between *in vivo* and *in vitro* measurements of carotid arteries, a correlation between histology and MRI-derived features (Toussaint *et al.*, 1996; Yuan *et al.*, 1998). Although not perfect, it may be possible to identify carotid plaques at high risk for stroke using MRI (Yuan *et al.*, 2001). Images of carotid arteries can be further improved using a coil placed close to the carotid artery at the surface of the neck (Hayes *et al.*, 1996). An in-plane resolution of 0.4×0.4 mm and a slice thickness of 3 mm may allow an assessment of fibrous cap thickness and integrity (Hatsukami *et al.*, 2000).

Imaging of coronary arteries with MRI is more difficult than imaging of carotid plaques, since cardiac and respiratory motion, the small plaque size, and the location of the coronary arteries can cause acquisition problems. Nevertheless, high-resolution MRI of the human coronary wall of angiographically normal and abnormal vessels has been shown to be feasible. In a study by Botnar *et al.* (2000), the coronary wall thickness and wall area were significantly enlarged in patients with coronary artery disease demonstrated by angiography. Small plaque structures like fibrous caps cannot yet be assessed using current MR techniques. Thinner slices and higher in plane resolution are needed to better delineate coronary plaques.

In conclusion, while a noninvasive screening technique is of interest at present the low resolution of the MRI technique allows detecting only (spatially) large differences in plaque composition. As such it offers a possibility in screening patients with vulnerable carotid plaques. While improvement in the magnetic field is promising, screening for coronary vulnerable plaque seems to be early.

9. ANGUS AND SHEAR STRESS

High-resolution 3D reconstruction of coronary lumen and wall morphology is obtained by combining angiography and IVUS (Slager *et al.*, 2000). Briefly, a biplane angiogram of a sheath-based IVUS catheter taken at end-diastole allows reconstruction of the 3D pullback trajectory of the catheter (Fig. 3). Combining this path with lumen and wall information derived from IVUS images that are successively acquired during catheter pullback at end-diastole gives accurate 3D lumen and wall reconstruction with resolution determined by IVUS (Fig. 3). Filling the 3D lumen space with a high-resolution 3D mesh allows calculation of the detailed blood velocity profile in the lumen (Fig. 4) (Krams *et al.*, 1997).

For this purpose absolute flow and blood viscosity need to be provided as boundary conditions. From the blood velocity profile, local wall shear stress on the endothelium can be accurately derived. Wall shear stress is the frictional force normalized to surface area, which is induced by the blood passing the wall.

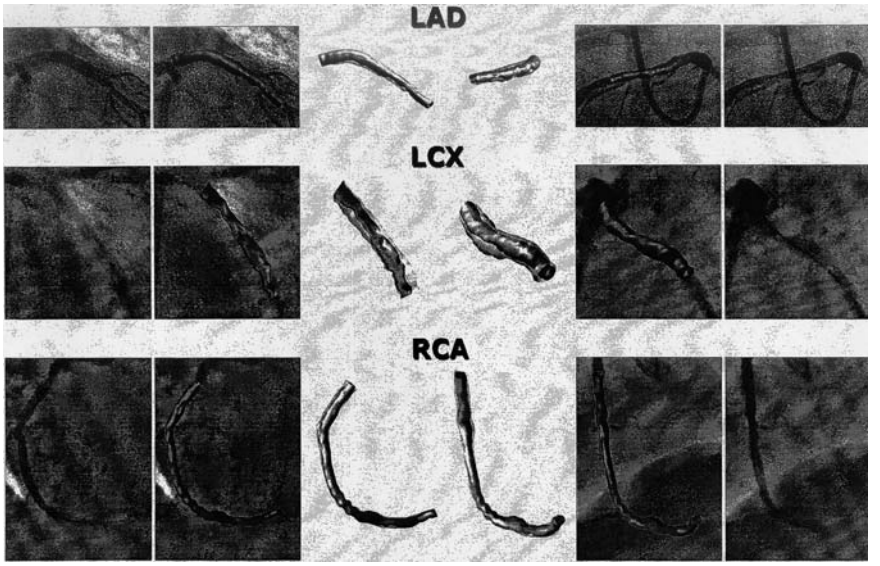

FIGURE 3. Three-dimensional reconstruction of human coronary blood vessels based upon a combination of angiography and IVUS (ANGUS). Indicated are three vessels from three different patients. (*See Color Plate 16.*)

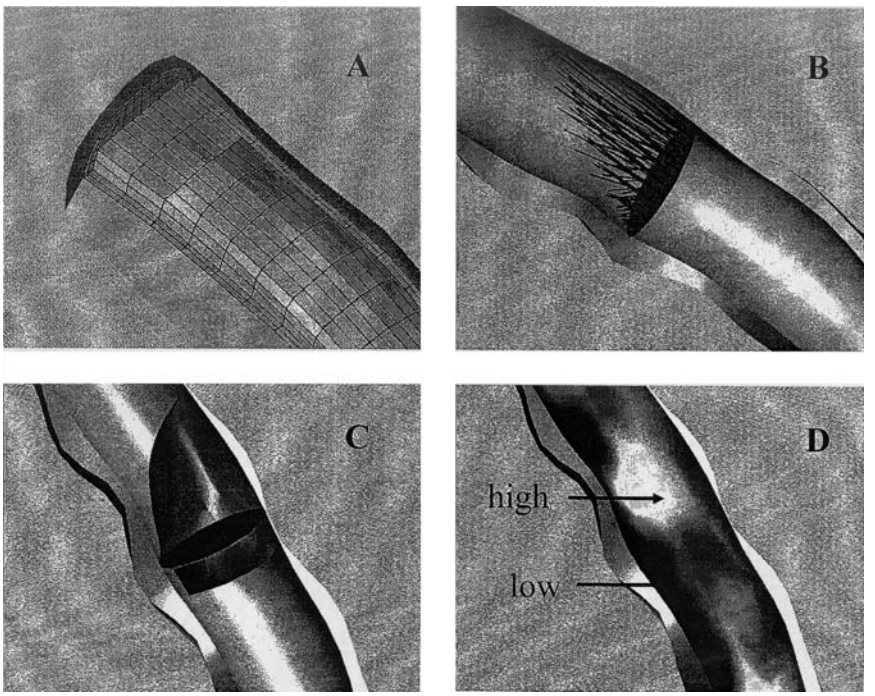

FIGURE 4. Indication of the method to obtain a shear stress mapping. Panel A shows the filling of the lumen by finite elements, panel B the resulting velocity field for a cross section, panel C shows the envelope of the vectors, and panel D the resulting shear stress mapping on the endothelium of the 3D reconstructed blood vessel. (*See Color Plate 16.*)

Although from a mechanical point of view shear stress is of a very small magnitude compared to blood pressure-induced tensile stress, it has a profound influence on vascular biology (Malek *et al.*, 1999) and explains the localization of atherosclerotic plaque in the presence of systemic risk factors (Fig. 4) (Asakura and Karino, 1990). Many of these biological processes also influence the stability of the vulnerable plaque including inflammation, thrombogenicity, vessel remodeling, intimal thickening, or regression and smooth muscle cell proliferation. Therefore, the study of this parameter as derived by image-based modeling is of utmost importance.

10. CONCLUSION

Assessment of atherosclerosis by imaging techniques is essential for *in vivo* identification of vulnerable plaques. Several invasive and noninvasive imaging techniques are currently in development.

OCT has the advantage of high resolution, thermography measures metabolism, and NIR spectroscopy obtains information on chemical components. IVUS and IVUS-palpography are easy to perform and assess morphology and

mechanical deformability. Shear stress is an important mechanical parameter deeply influencing vascular biology. MRI and CT have the advantage of non-invasive imaging.

Nevertheless, all techniques are still under development, and at present, none of them can identify a vulnerable plaque alone or predict its further development. This is related to fundamental methodological insufficiencies that may be resolved in the future. From a clinical point of view, most techniques currently assess only one feature of the vulnerable plaque. Thus, the combination of several modalities (see Fig. 1) (Arampatzis *et al.*, 2003) will be of importance in the future to ensure a high sensitivity and specificity in detecting vulnerable plaque.

REFERENCES

Arampatzis, C. A., Ligthart, J. M., Schaar, J. A., Nieman, K., Serruys, P. W., and de Feyter, P. J., 2003, Images in cardiovascular medicine. Detection of a vulnerable coronary plaque: a treatment dilemma, *Circulation* **108**:e34–e35.

Asakura, T., and Karino, T., 1990, Flow patterns and spatial distribution of atherosclerotic lesions in human coronary arteries, *Circ. Res.* **66**:1045–1066.

Baraga, J. J., Feld, M. S., and Rava, R. P., 1992, In situ optical histochemistry of human artery using near infrared Fourier transform Raman spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **89**:3473–3477.

Bom, N., Li, W., van der Steen, A. F., Lancee, C. T., Cespedes, E. I., Slager, C. J., and de Korte, C. L., 1998, Intravascular imaging. *Ultrasonics* **36**:625–628.

Botnar, R. M., Stuber, M., Kissinger, K. V., Kim, W. Y., Spuentrup, E., and Manning, W. J., 2000, Non-invasive coronary vessel wall and plaque imaging with magnetic resonance imaging. *Circulation* **102**:2582–2587.

Bridal, S. L., Beyssen, B., Fornes, P., Julia, P., and Berger, G., 2000, Multiparametric attenuation and backscatter images for characterization of carotid plaque. *Ultrason. Imaging* **22**:20–34.

Buschman, H. P., Marple, E. T., Wach, M. L., Bennett, B., Schut, T. C., Bruining, H. A., Bruschke, A. V., van der Laarse, A., and Puppels, G. J., 2000, In vivo determination of the molecular composition of artery wall by intravascular Raman spectroscopy. *Anal. Chem.* **72**:3771–3775.

Casscells, W., Hathorn, B., David, M., Krabach, T., Vaughn, W. K., McAllister, H. A., Bearman, G., and Willerson, J. T., 1996, Thermal detection of cellular infiltrates in living atherosclerotic plaques: possible implications for plaque rupture and thrombosis. *Lancet* **347**:1447–1451.

de Korte, C. L., Carlier, S. G., Mastik, F., Doyley, M. M., van der Steen, A. F., Serruys, P. W., and Bom, N., 2002, Morphological and mechanical information of coronary arteries obtained with intravascular elastography; feasibility study in vivo. *Eur. Heart J.* **23**:405–413.

de Korte, C. L., Pasterkamp, G., van der Steen, A. F., Woutman, H. A., and Bom, N., 2000, Characterization of plaque components with intravascular ultrasound elastography in human femoral and coronary arteries in vitro. *Circulation* **102**:617–623.

Doyley, M. M., Mastik, F., de Korte, C. L., Carlier, S. G., Cespedes, E. I., Serruys, P. W., Bom, N., and van der Steen, A. F., 2001, Advancing intravascular ultrasonic palpation toward clinical applications. *Ultrasound Med. Biol.* **27**:1471–1480.

Falk, E., 1999, Stable versus unstable atherosclerosis: clinical aspects. *Am. Heart J.* **138**:S421–S425.

Fujimoto, J. G., 2003, Optical coherence tomography for ultrahigh resolution in vivo imaging. *Nat. Biotechnol.* **21**:1361–1367.

Hanlon, E. B., Manoharan, R., Koo, T. W., Shafer, K. E., Motz, J. T., Fitzmaurice, M., Kramer, J. R., Itzkan, I., Dasari, R. R., and Feld, M. S., 2000, Prospects for in vivo Raman spectroscopy. *Phys. Med. Biol.* **45**:R1–R59.

Hatsukami, T. S., Ross, R., Polissar, N. L., and Yuan, C., 2000, Visualization of fibrous cap thickness and rupture in human atherosclerotic carotid plaque in vivo with high-resolution magnetic resonance imaging. *Circulation* **102**:959–964.

Hayes, C. E., Mathis, C. M., and Yuan, C., 1996, Surface coil phased arrays for high-resolution imaging of the carotid arteries. *J. Magn. Reson. Imaging* **6**:109–112.

Hiro, T., Leung, C. Y., Russo, R. J., Karimi, H., Farvid, A. R., and Tobis, J. M., 1996, Variability of a three-layered appearance in intravascular ultrasound coronary images: a comparison of morphometric measurements with four intravascular ultrasound systems. *Am. J. Card Imaging* **10**:219–227.

Jang, I. K., Bouma, B. E., Kang, D. H., Park, S. J., Park, S. W., Seung, K. B., Choi, K. B., Shishkov, M., Schlendorf, K., Pomerantsev, E., Houser, S. L., Aretz, H. T., and Tearney, G. J., 2002, Visualization of coronary atherosclerotic plaques in patients using optical coherence tomography: comparison with intravascular ultrasound. *J. Am. Coll. Cardiol.* **39**:604–609.

Jeremias, A., Kolz, M. L., Ikonen, T. S., Gummert, J. F., Oshima, A., Hayase, M., Honda, Y., Komiyama, N., Berry, G. J., Morris, R. E., Yock, P. G., and Fitzgerald, P. J., 1999, Feasibility of in vivo intravascular ultrasound tissue characterization in the detection of early vascular transplant rejection. *Circulation* **100**:2127–2130.

Kawasaki, M., Takatsu, H., Noda, T., Sano, K., Ito, Y., Hayakawa, K., Tsuchiya, K., Arai, M., Nishigaki, K., Takemura, G., Minatoguchi, S., Fujiwara, T., and Fujiwara, H., 2002, In vivo quantitative tissue characterization of human coronary arterial plaques by use of integrated backscatter intravascular ultrasound and comparison with angioscopic findings. *Circulation* **105**:2487–2492.

Krams, R., Wentzel, J. J., Oomen, J. A., Vinke, R., Schuurbiers, J. C., de Feyter, P. J., Serruys, P. W., and Slager, C. J., 1997, Evaluation of endothelial shear stress and 3D geometry as factors determining the development of atherosclerosis and remodeling in human coronary arteries in vivo. Combining 3D reconstruction from angiography and IVUS (ANGUS) with computational fluid dynamics. *Arterioscler. Thromb. Vasc. Biol.* **17**:2061–2065.

Landini, L., Sarnelli, R., Picano, E., and Salvadori, M., 1986, Evaluation of frequency dependence of backscatter coefficient in normal and atherosclerotic aortic walls. *Ultrasound Med. Biol.* **12**:397–401.

MacNeill, B. D., Lowe, H. C., Takano, M., Fuster, V., and Jang, I. K., 2003, Intravascular modalities for detection of vulnerable plaque: current status. *Arterioscler. Thromb. Vasc. Biol.* **23**:1333–1342.

Madjid, M., Naghavi, M., Malik, B. A., Litovsky, S., Willerson, J. T., and Casscells, W., 2002, Thermal detection of vulnerable plaque. *Am. J. Cardiol.* 21;**90**(10C):36L–39L.

Malek, A. M, Alper, S. L, and Izumo, S., 1999, Hemodynamic shear stress and its role in atherosclerosis. *JAMA* **282**:2035–2042.

Mizuno, K., Satomura, K., Miyamoto, A., Arakawa, K., Shibuya, T., Arai, T., Kurita, A., Nakamura, H., and Ambrose, J. A., 1992, Angioscopic evaluation of coronary-artery thrombi in acute coronary syndromes. *N. Engl. J. Med.* **326**:287–291.

Moreno, P. R., Lodder, R. A., Purushothaman, K. R., Charash, W. E., O'Connor, W. N., and Muller, J. E., 2002, Detection of lipid pool, thin fibrous cap, and inflammatory cells in human aortic atherosclerotic plaques by near-infrared spectroscopy. *Circulation* **105**:923–927.

Naghavi, M., Libby, P., Falk, E., Casscells, S. W., Litovsky, S., Rumberger, J., Badimon, J. J, Stefanadis, C., Moreno, P., Pasterkamp, G., Fayad, Z., Stone, P. H., Waxman, S., Raggi, P., Madjid, M., Zarrabi, A., Burke, A., Yuan, C., Fitzgerald, P. J., Siscovick, D. S., de Korte, C. L., Aikawa, M., Airaksinen, K. E., Assmann, G., Becker, C. R., Chesebro, J. H., Farb, A., Galis, Z. S., Jackson, C., Jang, I. K., Koenig, W., Lodder, R. A., March, K., Demirovic, J., Navab, M., Priori, S. G., Rekhter, M. D., Bahr, R., Grundy, S. M., Mehran, R., Colombo, A., Boerwinkle, E., Ballantyne, C., Insull, W., Jr., Schwartz, R. S., Vogel, R., Serruys, P. W., Hansson, G. K., Faxon, D. P., Kaul, S., Drexler, H., Greenland, P., Muller, J. E., Virmani, R., Ridker, P. M., Zipes, D. P., Shah, P. K., and Willerson, J. T., 2003, From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. *Circulation* **108**:1772–1778.

Nair, A., Kuban, B. D., Obuchowski, N., and Vince, D. G., 2001, Assessing spectral algorithms to predict atherosclerotic plaque composition with normalized and raw intravascular ultrasound data. *Ultrasound Med. Biol.* **27**:1319–1331.

Ophir, J., Cespedes, I., Ponnekanti, H., Yazdi, Y., and Li, X., 1991, Elastography: a quantitative method for imaging the elasticity of biological tissues. *Ultrason. Imaging* **13**:111–134.

Potkin, B. N., Bartorelli, A. L., Gessert, J. M., Neville, R. F., Almagor, Y., Roberts, W. C., and Leon, M. B., 1990, Coronary artery imaging with intravascular high-frequency ultrasound. *Circulation* **81**:1575–1585.

Rasheed, Q., Dhawale, P. J., Anderson, J., and Hodgson, J. M., 1995, Intracoronary ultrasound-defined plaque composition: computer-aided plaque characterization and correlation with histologic samples obtained during directional coronary atherectomy. *Am. Heart J.* **129**:631–637.

Slager, C. J., Wentzel, J. J., Schuurbiers, J. C., Oomen, J. A., Kloet, J., Krams, R., von Birgelen, C., van der Giessen, W. J., Serruys, P. W., and de Feyter, P. J., 2000, True 3-dimensional reconstruction of coronary arteries in patients by fusion of angiography and IVUS (ANGUS) and its quantitative validation. *Circulation* **102**:511–516.

Spencer, T., Ramo, M. P., Salter, D. M., Anderson, T., Kearney, P. P., Sutherland, G. R., Fox, K. A., and McDicken, W. N., 1997, Characterisation of atherosclerotic plaque by spectral analysis of intravascular ultrasound: an in vitro methodology. *Ultrasound Med. Biol.* **23**:191–203.

Stefanadis, C., Diamantopoulos, L., Vlachopoulos, C., Tsiamis, E., Dernellis, J., Toutouzas, K., Stefanadi, E., and Toutouzas, P., 1999, Thermal heterogeneity within human atherosclerotic coronary arteries detected in vivo: a new method of detection by application of a special thermography catheter. *Circulation* **99**:1965–1971.

Stefanadis, C., Toutouzas, K., Tsiamis, E., Stratos, C., Vavuranakis, M., Kallikazaros, I., Panagiotakos, D., and Toutouzas, P., 2001, Increased local temperature in human coronary atherosclerotic plaques: an independent predictor of clinical outcome in patients undergoing a percutaneous coronary intervention. *J. Am. Coll. Cardiol.* **37**:1277–1283.

Toussaint, J. F., LaMuraglia, G. M., Southern, J. F., Fuster, V., and Kantor, H. L., 1996, Magnetic resonance images lipid, fibrous, calcified, hemorrhagic, and thrombotic components of human atherosclerosis in vivo. *Circulation* **94**:932–938.

Toussaint, J. F., Southern, J. F., Fuster, V., and Kantor, H. L., 1995, T2-weighted contrast for NMR characterization of human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **15**:1533–1542.

van De Poll, S. W., Romer, T. J., Volger, O. L., Delsing, D. J., Bakker Schut, T. C., Princen, H. M., Havekes, L. M., Jukema, J. W., van Der Laarse, A., and Puppels, G. J., 2001, Raman spectroscopic evaluation of the effects of diet and lipid-lowering therapy on atherosclerotic plaque development in mice. *Arterioscler. Thromb. Vasc. Biol.* **21**:1630–1635.

van de Poll, S. W., Kastelijn, K., Bakker Schut, T. C., Strijder, C., Pasterkamp, G., Puppels, G. J., and van der Laarse, A., 2003, On-line detection of cholesterol and calcification by catheter based Ramanspectroscopy in human atherosclerotic plaque ex vivo. *Heart* **89**:1078–82.

Verheye, S., De Meyer, G. R., Van Langenhove, G., Knaapen, M. W., and Kockx, M. M., 2002, In vivo temperature heterogeneity of atherosclerotic plaques is determined by plaque composition. *Circulation* **105**:1596–1601.

Virmani, R., Burke, A. P., Farb, A., and Kolodgie, F. D., 2002, Pathology of the unstable plaque. *Prog. Cardiovasc. Dis.* **44**:349–356.

Wang, J., Geng, Y. J., Guo, B., Klima, T., Lal, B. N., Willerson, J. T., and Casscells, W., 2002, Near-infrared spectroscopic characterization of human advanced atherosclerotic plaques. *J. Am. Coll. Cardiol.* **39**:1305–1313.

Wickline, S. A. Miller, J. G., Recchia, D., Sharkey, A. M., Bridal, S. L., and Christy, D. H., 1994, Beyond intravascular imaging: quantitative ultrasonic tissue characterization of vascular pathology. *Proceedings of IEEE Ultrasonics Symposium*, Cannes , France, 1–4 November 1994, Vol. 3, pp. 1589–1597.

Wilson, L. S., Neale, M. L., Talhami, H. E., and Appleberg, M., 1994, Preliminary results from attenuation-slope mapping of plaque using intravascular ultrasound. *Ultrasound Med. Biol.* **20**:529–542.

Yamagishi, M., Terashima, M., Awano, K., Kijima, M., Nakatani, S., Daikoku, S., Ito, K., Yasumura, Y., and Miyatake, K., 2000, Morphology of vulnerable coronary plaque: insights from follow-up of patients examined by intravascular ultrasound before an acute coronary syndrome. *J. Am. Coll. Cardiol.* **35**:106–111.

Yuan, C., Beach, K. W., Smith, L. H., Jr., and Hatsukami, T. S., 1998, Measurement of atherosclerotic carotid plaque size in vivo using high resolution magnetic resonance imaging. *Circulation* **98**:2666–2671.

Yuan, C., Mitsumori, L. M., Beach, K. W., and Maravilla, K. R., 2001, Carotid atherosclerotic plaque: noninvasive MR characterization and identification of vulnerable lesions. *Radiology* **221**:285–299.

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