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Molecular Biology Applications in Cardiovascular Medicine

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INTRODUCTION

Basic science research has made great contributions to the field of cardiovascular medicine. Scientific studies have had a major impact on clinical practices and outcomes. For example, the principles of cardiac contractile function and unique aspects of hemodynamic loading on the ventricles were defined in animal studies. These findings translated directly into pressure monitoring devices used for patients in the acute care setting. The rationale for drug therapies for treating cardiovascular diseases was based primarily on data derived from basic science investigations. For example, the treatment of heart failure and cardiac arrhythmias evolved from elegant pharmacologic and physiologic studies. A clear path has emerged from the basic science laboratory to the bedside.

During the past decade, scientists have supported the application of cellular and molecular biology to the study of cardiovascular disease and function. Endothelin-1, a potent vasoactive hormone, and angiotensin receptor were among the most heralded new genes to be cloned

because of their association with cardiovascular function. The field of vascular biology has expanded rapidly; many endothelial cell genes have been cloned and linked to vascular wall disease. Gain-of-function and loss-of-function mice, created through genetic manipulations, have provided great insight into lipid metabolism and the function of cardiac- and vascular wall-specific genes. In addition, the field of developmental biology of the cardiovascular system has developed during this decade. Increased study of blood vessel development and a strong clinical interest in therapeutic angiogenesis led to great advances in the understanding of the molecular biology of the assembly of cardiovascular structures. Finally, human genetics studies have contributed significantly to the understanding of inherited cardiac diseases such as long QT syndrome and hypertrophic obstructive cardiomyopathy.

Unlike earlier scientific discoveries that successfully translated to clinical practice, the application of recent studies in cellular and molecular cardiovascular science has been slow. Although the identification of genes and their encoded proteins has generated optimism about

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finding new therapeutic targets, the path from target to drug development is a complex, arduous process; therefore, success has been limited in this era of cloning and molecular biology with the exception of plasminogen activator molecules. Furthermore, gene therapy has been viewed as a way to apply directly the findings of basic genetic research to patient treatment, but again this therapeutic approach has been slow to materialize. During this decade, therefore, the major advances in the treatment of cardiovascular disease have evolved not from basic science applications, but from the development of new technologies such as the angioplasty catheter and the coronary artery stent.

In the next decade, new tools will be applied to the study of cardiovascular disease and function. These instruments will include DNA microarrays, protein chips, and other proteomics methodologies, vast amounts DNA sequences from humans and other species, and markers of human genetic variation. The innovative use of these new and powerful tools may accelerate the pace of discovery. For example, DNA microarrays can provide quantitative information about differences in gene expression between two conditions for 20,000 genes simultaneously and can relate these changes to virtually all other genes in the transcriptome. Genes that have coordinate regulation across many manipulations would be expected to have similar functions or to be involved in the same, or related, signaling pathways.

The greatest promise of DNA microarrays and other new tools is that cardiovascular researchers will be able to evaluate the behavior of every gene involved in a specific disease process or biological pathway. We can now identify all genes that are differentially regulated in the blood vessel wall of patients with vascular disease by comparing their gene expression profile with those generated from the normal vessel wall of individuals with known risk factors (such as diabetes) and those without risk factors. Human genetic epidemiology studies can be used to evaluate the association of disease and the vascular wall genes whose expression is temporally linked to vascular disease and risk factor status. Identification of the pathways that underlie cardiovascular disease will accelerate the development of new therapeutic strategies.

We believe that the great, untapped potential of molecular and cellular biology and the emergence of new genomics and human genetics initiatives will lead to substantial new discoveries in cardiovascular disease. However, these discoveries will not be achieved without intensively focused research. In this chapter, we provide a brief overview of basic molecular biology

techniques and applications useful to a cardiovascular researcher.

OVERVIEW OF RECOMBINANT DNA TECHNOLOGY

The study of the human genome, which encompasses approximately 3 billion nucleotides, is a daunting task that has been made possible by recombinant DNA technology. In its basic form, recombinant DNA technology is the process of combining DNA from two or more sources. This technology is based on several common techniques of molecular biology, including the use of restriction endonucleases to cleave DNA into manageable segments, sequence analysis of purified DNA to confirm the identity of a particular fragment, DNA cloning strategies to produce large amounts of identical DNA sequence(s), and hybridization methodologies to identify particular nucleic acid sequences. Improvements in reagents and techniques have allowed for the recent, large-scale sequencing of the human genome.

In general terms, cloning is the process of isolating and amplifying a particular DNA sequence. A clone, which is defined as a large number of exact copies of a unique DNA sequence, can exist as a double-stranded DNA fragment in solution or in a vector (e.g., plasmid, bacteriophage, or plasmid-bacteriophage hybrid). Polymerase chain reaction (PCR)-based or cell-based techniques can be used for cloning. In PCR-based cloning, a target sequence is exponentially amplified by adding oligonucleotide primers designed for the sequence of interest and a DNA template to a PCR reaction mixture. This amplicon can then be used for various purposes, such as creating a probe for *in situ* hybridization or for ligating into a vector for cell-based cloning. Although fast, this PCR-based technique has several problems associated with *in vitro* enzymatic reaction systems, including failed PCR reactions, decreased efficiency of cloning lengthy sequences (>2 kb DNA segments), limited production of and random mutations in the amplicon, a need for *a priori* knowledge of sequence information for primer design, and high costs. Innovations in the field, such as the use of enzymes such as *Pfu*, with its proofreading feature, have resolved some of these issues. Cell-based cloning, however, remains the most versatile and widely used method for cloning.

Cell-based cloning involves inserting a foreign DNA sequence into a vector and introducing this vector into a host cell that reproduces the foreign DNA in large quantities. The genetic material of interest can be obtained directly from cells or tissues by a PCR-based approach

that uses oligonucleotide primers to generate an amplicon. In addition, DNA can be obtained from a complementary DNA (cDNA) library, which is a collection of distinct DNA sequences generated by converting cellular mRNA into cDNA, or by fragmentation of genomic DNA. The isolated DNA sequence is packaged into a vector, which is then introduced into host cells (i.e., bacteria or yeast) in a process known as transformation. Once inside the host, the vector does not incorporate into the host genome and therefore reproduces itself independent of the host cell. This process generates large amounts of the unique DNA sequence. After the host cells have been transformed, the clones are plated onto agar at a density that permits the isolation of individual clones. These clones multiply and become colonies (for plasmid cloning vectors) or plaques (phage cloning vectors). Each colony or plaque represents one clone comprising a group of host cells with a unique, genetically identical recombinant DNA sequence. The colony or plaque with the DNA element of interest is picked through a screening process and further expanded in liquid media. The target DNA is isolated by using standard biochemical protocols for further analyses such as sequencing or screening a library.

Cloned DNA fragments isolated from the vector sequence can be used for several purposes, including Northern blotting and *in situ* hybridization studies to evaluate mRNA expression levels of a specific gene. When the cloned DNA fragment encodes a cDNA, protein expression can be used for a more detailed analysis. In expression cloning, the expressed protein rather than the DNA sequence can be identified after transfection of the target sequence into a cell or tissue. Expression and purification of proteins derived from cloned cDNAs can be used in protein function studies, such as receptor–ligand binding assays and other functional assays (e.g., ion flux and phosphorylation). Finally, expressed nucleic acid sequences can be used for *in situ* hybridization or for functional analysis with antisense strategies.

GENETIC TOOLS

Restriction Enzymes

Restriction endonucleases are bacterial enzymes that protect the host bacteria by degrading foreign double-stranded DNA. The endonucleases act as molecular scissors by cutting double-stranded DNA at unique nucleotide recognition sequences. Bacteria can protect these particular sites from enzymatic degradation by methylating certain nucleotide residues (i.e., adenine and cytosine).

Because of their ability to recognize and cut specific sequence motifs, restriction endonucleases can be used by molecular biologists to cut large pieces of DNA into more manageable fragments. These smaller fragments can be cloned, sequenced, and even used to decipher the primary structure of the larger sequence. Several hundred enzymes have been isolated, characterized, and named based on the bacteria from which they were isolated. For example, *EcoR*I originates from *Escheria coli*, *Sma*I from *Serratia marcescens*, and *Pst*I from *Providencia stuartii* (1).

Each restriction enzyme recognizes and cuts a particular 4–8 basepair sequence. Enzymes that recognize the same sequence are called isoschizimers. Based on the probability of finding a 4–8 nucleotide recognition site composed of any combination of bases, an 8-base cutter, such as *Pac*I, should cut human genomic DNA every 1×10^6 basepairs, whereas a 4-base cutter, such as *Hae*II, should cut every 250 nucleotides (2). This calculation assumes that each nucleotide is equally represented in the genome, which is not completely true. Furthermore, endonuclease restriction sites are not evenly and randomly placed in the genome. Enzymes that have CpG in their recognition sequence more frequently in regions of the genome that are rich in C and G nucleotides. Recognition sites are often palindromic, and most have a twofold axis of symmetry with the nucleic acid sequence for each strand being the same when read in the 5' to 3' direction.

After binding to its recognition site, the restriction endonuclease hydrolyzes a phosphodiester bond at the same point in each 5' to 3' sequence. This cleavage can occur symmetrically or asymmetrically. Restriction fragments produced by restriction endonucleases that cut symmetrically have blunt-ends, whereas enzymes that cut asymmetrically yield fragments with 5' or 3' overhangs. These asymmetric overhangs are often called cohesive termini, or sticky ends, because the overhanging ends are complementary and can associate with themselves by hydrogen bonds to yield a circular molecule or with each other to form linear or circular concatamers. The fragments with either blunt or sticky ends can be ligated into a plasmid cloning vector that has been linearized (i.e., cut with a restriction enzyme). These intramolecular and intermolecular associations of restriction fragment ends depend on the DNA concentration and whether the fragment and cloning vector ends are blunt or have compatible overhangs.

High DNA concentrations favor concatamerization, whereas low DNA concentrations favor intramolecular cyclization (3). When ligating or joining by enzymatic reaction the DNA fragment of interest with a linearized vector, the concentrations of the DNA insert and vector

are optimized to ensure ligation of one DNA insert copy per vector. Several techniques can be used to improve the efficiency of cloning the fragment into a vector, such as the use of two restriction enzymes that do not produce complementary ends or the use of vector dephosphorylation.

Restriction enzyme digestion of DNA, either genomic or cDNA, produces fragments of different lengths. A digest reaction can be complete or partial. In a complete restriction digest, all of the recognition sites for that restriction endonuclease are cleaved. Partial digests, which occur when the amount of enzyme is limited or when the time for digestion is decreased, result in a random cleavage of only a fraction of the available restriction sites. Complete digestion of the target DNA is usually desired. Restriction fragments can be separated by gel electrophoresis, and their sizes can be calculated by comparing the fragments to DNA standards of known molecular weights. This DNA fingerprint can be compared to fingerprints generated from digests with other restriction enzymes to yield a restriction map. These restriction maps, which provide useful information for other molecular biological tools, are linear or circular maps that document the relative positions of various restriction sites for a specific segment of DNA.

Cloning Vectors

A cloning vector is a vehicle for the delivery of foreign DNA into a host cell and the replication of that DNA independent of the host cell cycle. Foreign DNA is first inserted into the vector *in vitro*. This hybrid molecule is transferred to a host cell where it uses the host's cellular machinery to generate multiple copies of itself without incorporating into the host's genome. The most commonly used cloning vectors are plasmids and bacteriophages. Both of these naturally occurring species have been genetically engineered to replicate in a foreign host, to transform the host efficiently, and to allow the recovery of vector with its target DNA. Several new hybrid vectors, such as cosmids, yeast artificial chromosomes (YACs), and bacterial artificial chromosomes (BACs), have been constructed to clone larger pieces of DNA. In addition, cloning vectors can be engineered to produce nucleic acid sequences or proteins (4). These vectors, called expression vectors, have special sequences that direct the transcription of nucleic acids and the translation of amino acids independent of the host cell cycle. The generated nucleic acid and protein sequences can be used in several assays such as *in situ* hybridization, and the amino acid sequences can be assessed for function or antibody binding.

PLASMIDS

Plasmids are naturally occurring, circular molecules of extra-chromosomal, double-stranded DNA that can self-replicate. Plasmids carry genes that can confer unique properties on their host, such as drug resistance, sexual fertility, and the ability to synthesize a rare amino acid. Found in the cytoplasm of many prokaryotes and eukaryotes, such as bacteria, yeast, and mammalian cells, plasmids can be transferred to neighboring cells through bacterial conjugation or to daughter cells through host cell division. Because of their innate ability to carry genes and their compatibility with various cell types, these natural structures have been genetically engineered for use as cloning vectors.

Plasmid vectors have various unique properties based on their intended use; however, they all have three basic characteristics. First, plasmid vectors must have the means to self-replicate; thus, plasmids contain an origin of replication site and other genes required for replication. Second, plasmid vectors need a polylinker, a 50–150 nucleotide sequence comprising multiple, distinct recognition sites for restriction endonucleases, that allows the simple insertion and removal of foreign DNA sequence in and out of the vector. The third feature is the need for selectable markers that will differentiate host cells with no vector, empty vector, or vector with foreign DNA. These markers usually bestow properties on the transformed cell that it normally does not possess. For example, bacteria transformed with a plasmid that encodes an antibiotic resistance gene will no longer die in the presence of that antibiotic.

Several genes have been used as selectable markers, including genes that encode resistance to ampicillin (β -lactamase), chloramphenicol (chloramphenicol acetyltransferase), kanamycin (kanamycin phosphotransferase), and tetracycline (3). Bacteria transformed with plasmids encoding these genes will survive when grown on agar implanted with the appropriate antibiotic.

In addition, markers can be used to determine whether a plasmid vector contains foreign DNA sequence. To achieve this goal, β -galactosidase can be inserted into the polylinker site. When foreign DNA is cloned into the polylinker, the marker gene is interrupted, resulting in insertional inactivation. Thus, cells transformed with empty vector turn blue when grown on X-gal impregnated agar, whereas those cells transformed with plasmid containing the insert will be colorless. Furthermore, this technique of insertional inactivation can be used with antibiotic resistance genes. In this case, two antibiotic resistance genes are used; one is placed within the polylinker and the other is outside of the polylinker. Transformed bacteria are grown in duplicate on agar plates containing both antibiotics. Colonies that are sensitive to the antibiotic resistance gene in the

polylinker, but resistant to the antibiotic gene outside of the polylinker, contain the plasmid with the foreign DNA. Colonies that are resistant to both antibiotics contain empty vector.

Foreign DNA can be ligated into plasmid vectors with DNA ligase in vitro. Plasmid vectors are ideal for the cloning of cDNAs because most mRNA transcripts are smaller than 5 kb. Bacteria are transformed with the plasmid and grown on an agar plate impregnated with the appropriate selectable marker(s). Colonies with the insert are expanded in large volumes of media. Several plasmid purification kits are available for isolating pure plasmid DNA from the bacterial host. Because of their ease of use and widespread availability, plasmids are a fundamental tool in molecular cloning. The major limitations with plasmids are that the foreign DNA insert must be smaller than 5–10 kb and the inefficiency of bacterial transformation (1). Plasmid vectors are commercially available from several vendors. These products range from the straightforward plasmids used for cloning restriction fragments to more advanced vectors designed to produce functional proteins.

BACTERIOPHAGE

Bacteriophage λ , a bacterial virus engineered for cloning, can transform bacteria more efficiently than plasmids and can accept inserts up to 23 kb in size. The wild-type bacteriophage λ virion comprises a protein coat that holds nearly 50 kb of linear double-stranded DNA. A temperate virus, bacteriophage λ can exist in a lytic or lysogenic growth state once it has infected a host. In the lytic cycle, the virus makes multiple copies of its genome and coat proteins. The viral genome is then packaged into new phage particles that are released when the host cell lyses. The neighboring cells are infected by the phage and the cycle of replication and release continues. In the lysogenic cycle, the viral genome is integrated into the host chromosome and replicates along with the host chromosome.

The bacteriophage and its lytic growth cycle, when used in recombinant DNA technology, is an effective and efficient cloning vector. The middle portion of the bacteriophage λ genome encodes proteins that are not vital for lytic growth and can be excised so that foreign DNA can be ligated between the two “arms” of essential genetic sequence. This recombinant DNA, which must be 37–52 kb, is then packaged in vitro by coat proteins into infectious phage particles (1). The infectious virions are grown with bacteria. Bacteria infected by the bacteriophage λ undergo lysis, which leaves a “hole” known as a plaque on the bacterial lawn. Each plaque represents a single clone with a unique insert. Clones are picked and

expanded in a broth medium. Then, the phage are purified and DNA is isolated. Because they are easier to handle than cosmids, YACs, and BACs, bacteriophage are ideal for cloning mid-sized 10–20 kb segments (5).

COSMIDS

Cosmids are hybrids of plasmids and bacteriophage λ designed to hold 30–44 kb DNA fragments that efficiently transform bacteria (1). Cosmids contain a plasmid backbone composed of selection markers, an origin of replication, a polylinker, and a cos site from the bacteriophage λ . Cos sites are cohesive termini found at the 5' ends of linear phage DNA that are ligated by the host cell, which produces a circular molecule capable of replication. For cloning, the cosmid vector is linearized and mixed with the DNA fragments to be cloned. DNA ligase joins cut vector and insert fragments into concatemeric molecules. These molecules are mixed with packaging extract containing proteins necessary to package naked phage DNA into phage heads. The infectious phage inject their DNA, which contains the insert into the host bacteria. The linear cosmid DNA recircularizes in the host and replicates as a plasmid. Bacterial colonies are chosen based on their selection markers. Although these hybrid molecules clone larger fragments than plasmids or phage, and transform bacteria as efficiently as phage, cosmids tend to rearrange and/or delete DNA insert segments.

YEAST ARTIFICIAL CHROMOSOMES

Yeast artificial chromosomes (YACs) are recombinant vectors generated to clone large fragments of genomic DNA by taking advantage of chromosomal replication during cell division (2,6,7). Recombinant DNA technology is used to place four sequences required for chromosomal replication in a plasmid backbone: an autonomous replication sequence, a centromere sequence (central region of chromosome), and two telomere sequences (chromosome ends). This plasmid backbone contains cloning sites and selectable markers. A YAC plasmid vector is linearized and then cut into two fragments. Foreign DNA 0.2–2.0 million bases (Mb) in length is ligated between these two arms, creating an artificial chromosome with two telomeres capping the chromosome ends and a central centromere. The cell walls of the yeast *Saccharomyces cerevisiae* are removed (thereby producing spheroplasts) and embedded in agar for support and stability. The yeast are transformed with the artificial chromosomes, which have a low transformation efficiency. More starting DNA is required to ensure complete representation of the genome when this strategy is used to generate a recombinant genomic library. The yeast grow in their selective environment and regenerate their

walls. Selection markers allow only YAC-containing yeast to be propagated. The foreign DNA is replicated during cell division, resulting in a single copy per cell. The low yields, creation of inserts composed of two or more noncontiguous genomic fragments, and a complicated DNA isolation process are drawbacks to this technique. Furthermore, generation of recombinant YACs is time consuming and technically difficult. Only a few universities and national laboratories support YAC cloning. Nonetheless, YACs are important tools for generating physical genome maps over multiple megabases and have permitted the cloning of regions with repetitive sequences common to eukaryotes.

BACTERIOPHAGE P1 VECTORS AND ARTIFICIAL CHROMOSOMES

Cloning of eukaryotic genomes has been complicated by the large size of the genome and by the presence of structurally unstable repetitive sequence elements. YACs have been engineered to alleviate these problems but are technically challenging; therefore, several alternative vectors have been created. Bacteriophage P1 is a plasmid-bacteriophage hybrid vector engineered to accommodate 70–100 kb of foreign DNA (8,9). Target DNA sandwiched between two plasmid arms is packaged in P1 phage *in vitro*. These infectious virions inject their DNA into a host where it circularizes into a plasmid and replicates. P1 artificial chromosomes are a combination of the *Escherichia coli* fertility factor (F-factor) plasmid and bacteriophage P1 (10). BACs based on the F factor plasmid were created as an alternative to YAC cloning (11). The F factor plasmid is a low copy number plasmid (one or two copies per cell), which reduces the potential for genetic recombination events, and the F factor plasmid can accept DNA fragments up to 300 kb. *E. coli* are transformed by electroporation, which is 10–100 times more efficient than yeast spheroplast transformation. Therefore, less starting material is required to create a comprehensive genomic library. Conventional colony lift and hybridization techniques are used in BAC screening. DNA isolation is easier and chimeric DNA inserts are formed less frequently with BACs than with YACs.

Expression Vectors and Systems

Expression vectors are cloning vectors designed to express or produce substantial quantities of a gene or its protein. For example, an mRNA product of an expression vector can be used as a probe for *in situ* hybridization or as an antisense RNA for functional-therapeutic assessment. The expressed gene products or recombinant proteins can be used as molecular reagents such as restriction endonucleases, as therapeutic proteins such as erythropoietin, or as reagents for structural analyses, functional assays,

antibody production, or drug screening. These expression vectors have many features of cloning vectors, such as the polylinker cloning sites and selectable markers. In addition, expression vectors have specific sequences that target the cloned cDNA for transcription and translation in the host cell. Some vectors have molecular switches called inducible promoters that can turn the transcription of a gene on and off through the addition of an inert reagent. The inducible promoter protects the host from potentially toxic effects of the recombinant protein or its production. The most commonly used expression vectors are plasmids with bacteriophage sequence elements and viral vectors. Host systems range from bacteria to mammalian cell lines. The vector choice is usually based on the application of the expressed gene or recombinant protein.

E. COLI RECOMBINANT PROTEIN EXPRESSION SYSTEM

E. coli transformed with plasmid/bacteriophage-based expression vectors generate recombinant proteins for antibody screening, functional studies, and structural determination. This system is best suited for generating small, intracellular proteins because large polypeptides cannot be properly folded, and post-translational modifications are minimal or nonexistent in the *E. coli* system. Eukaryotic proteins are usually not very stable in bacteria. Producing a fusion protein by inserting the foreign cDNA downstream of a sequence that encodes a highly expressed host cell gene can increase protein stability and expression. For example, a cDNA of interest can be inserted into the *E. coli lacZ* gene, which results in a fusion protein consisting of the cloned gene product at the carboxy-terminus and β -galactosidase at the amino-terminus. Amino-terminal tags, glutathione-S-transferase and polyhistidine, can be added to the cloned gene product to facilitate protein purification (2). To ensure that expression of the target protein is not harmful to the host, expression vectors often have an inducible promoter that turns on the production of the cloned gene product in the presence of a reagent, such as isopropyl- β -D-thiogalactopyranoside. The *E. coli* recombinant protein system is inexpensive and simple, and most molecular biologists are familiar with the techniques and reagents. However, recombinant protein stability and solubility are problems in this system. Despite efforts to create a “bacterial” fusion gene product, these proteins still degrade easily. The use of *E. coli* strains deficient in proteases has met with limited success. High levels of protein expression can result in the formation of inclusion bodies, which are dense aggregates of insoluble recombinant protein (3). Although they may improve protein purification, the aggregates can cause the protein to be incorrectly folded and inactive. The

level of expression, temperature, and the bacterial strain can be varied to mitigate this problem (3,12).

BACULOVIRUS-INSECT RECOMBINANT PROTEIN EXPRESSION SYSTEM

Large quantities of recombinant protein can be produced with the baculovirus expression system in insect cells. Baculoviruses are large, enveloped arthropod viruses containing double-stranded DNA. Like other bacteriophage vectors, the sizable 130 kb baculovirus genome contains significant amounts of genetic material that can be discarded without hindering its ability to replicate and infect a host. Therefore, this viral vector system can support large segments of foreign DNA.

The baculovirus vector is prepared through homologous recombination. Target DNA is ligated into a transfer plasmid flanked by the polyhedrin promoter and viral-specific sequences. The polyhedrin protein is a protective viral coat protein that is produced in substantial quantities but is not critical for viral replication or infection. Insect cells lacking polyhedrin cannot form occlusion bodies and thus have unique plaque morphologies. A small transfer plasmid with the foreign DNA fragment and wild-type baculovirus DNA must be applied together and transform *Spodoptera frugiperda* (Sf9)–cultured insect cells. The cloned gene is incorporated into the baculovirus DNA by replacing the polyhedrin gene inside the cell through an inefficient process called homologous recombination. Proper recombination is confirmed by PCR or nucleic acid hybridization. The viral mixture composed of wild-type and recombinant baculovirus is plated onto Sf9 cells, and plaques with recombinant virus are chosen and expanded for protein purification. A substantial quantity of soluble recombinant protein is generated with relative ease. However, this process requires a high level of expertise and is time consuming. Insect cells can be infected with more than one recombinant virus, which permits the expression of more than one protein or protein subunit per cell. Because the baculovirus system is eukaryotic, the recombinant protein often assumes the proper cellular location and can undergo some post-translation modification. However, excessive recombinant protein production can distort the cellular compartmentalization. This recombinant protein strategy is confined to insect cells because baculovirus does not infect vertebrates, and its promoters do not function in mammalian cells.

RECOMBINANT PROTEIN EXPRESSION IN MAMMALIAN CELLS

Mammalian cell expression of recombinant proteins is essential for the proper synthesis, processing, and folding

of complex polypeptides. Recombinant proteins have successfully been expressed in mammalian cells with multiple techniques, including transient transfection, stable transfection, viral transduction of cultured cells, transgenic animals, and cell lines derived from transgenic animals. In transient transfection, plasmid-based vectors drive the expression of recombinant proteins for a brief period of time (i.e., days to weeks). Several methods, such as DEAE-dextran, electroporation, and liposome formulations, can be used to transfect the host cell with the plasmid vector carrying the foreign DNA. Although it varies with the technique and cell type, the transfection efficiency is low, with approximately 5–50% of the cell population expressing the recombinant protein. This technique is useful for getting quick information about subcellular localization or cellular function, but it is not useful for producing protein for biochemical characterization.

Strategies have been developed to increase the production of recombinant protein by transiently transfecting eukaryotic cells. These methods use COS cells, a simian kidney cell line with a stably integrated, replication origin-defective SV40 genome (13). COS cells transfected with a plasmid vector containing foreign DNA and a SV40 origin of replication produce high levels of target protein expression. This target protein is processed and appropriately secreted or targeted to its subcellular location. Thus, the COS cell expression system is a good transient transfection strategy to determine receptor–ligand interactions and to reliably produce sufficient amounts of recombinant protein in mammalian cells.

The use of viruses is another method for expressing recombinant protein in mammalian cells both in vitro and in vivo. Vaccinia viruses, adenoviruses, and retroviruses have been used to insert foreign DNA into mammalian cells. The gene of interest is usually expressed under the control of viral expression elements. After infecting mammalian cells, the virus uses the host cell translational machinery to produce large amounts of the protein of interest, which can be purified for further analyses. The in vivo application of viral-mediated gene delivery, known as gene therapy, is beyond the scope of this chapter, but will be discussed elsewhere in this book.

Stable integration of a foreign gene into a host cell chromosome through transfection or generation of transgenic animals results in the reliable production of large amounts of recombinant protein. An expression plasmid can be integrated into the cellular genome of cultured cells by selection for stable expression of a drug resistance gene such as the neomycin resistance gene. With these methods, identical stable cell lines can be developed and selected for high level expression of recombinant protein.

The use of engineered Chinese hamster ovary cells in combination with plasmid vectors carrying specific drug resistance genes allows for the production of cell lines with high levels of protein expression. Transgenic animals are produced to accept an expression construct in the germ line through manipulation of fertilized eggs or embryonic cells in culture. In one clever application of this technique, transcriptional regulatory sequences in the expression construct direct expression of the protein in milk-producing cells so that the recombinant protein can be extracted from milk. Alternatively, stable cell lines can be adapted from the transgenic animal for the *in vitro* production of recombinant protein.

RECOMBINANT LIBRARIES

A DNA library is a compendium of DNA clones isolated from a particular type of cell, tissue, or organism. The goal of developing such a library is to obtain the genetic information that determines a particular species of animal or plant, to examine the expressed genetic information that characterizes a specific cell or tissue, and to observe how a cell or tissue responds to certain environmental stimuli. Cloning and sequence characterization of numerous genomes together with identifying expressed sequence tags (EST) from large numbers of cells and tissues has provided many physical clones that can be obtained directly. Access to these clones has decreased the need for generating and screening libraries. All genes will eventually exist as a catalogued collection of well-characterized clones that can be rapidly obtained for a small fee.

Genomic libraries are constructed from the high-molecular-weight DNA that is contained in the nuclei of eukaryotic cells and transmitted during reproduction. This DNA, which contains all the organism's genetic information, directs the development, differentiation, and function of cells and specialized tissues. All cells of an organism contain the same genetic information, with the notable exceptions of T and B cells and some tumor cells. Of course, some degree of dissimilarity is seen between the genomes of same species members; these differences define our intraspecies variability. The obvious advantage of this type of library is that it contains all of the inherited genetic material for the species. In addition, the DNA sequences that regulate gene expression can be examined only by studying DNA obtained from genomic libraries. The major disadvantage of this approach, however, is the presence within a structural gene of large regions of untranslated sequence. Genomic libraries are usually screened by hybridization with DNA probes derived from cDNA clones.

Alternatively, high-throughput PCR can be used to screen arrayed genomic libraries where DNA is available for each clone. Screening of genomic libraries should decrease with the completion of the entire human genome sequence and the availability of cloned DNA fragments.

A cDNA library is constructed from mRNA that is expressed by a cell or tissue at a specific point in time. This library reflects not only the type of cell or tissue used in the study, but also the cell's response to various hormones, growth factors, and biophysical forces. cDNA does not exist in nature but is synthesized from mRNA, which specifies and directs assembly of protein sequences. Because mRNA cannot be cloned directly, the synthesis of cDNA allows for easy characterization of the coding portion of the structural gene and provides for rapid bioinformatics analysis of the conceptual protein. cDNA libraries offer several unique advantages. First, the coding region of a gene is usually much smaller than the genomic structural sequence that contains both intronic and regulatory sequences. Second, the cDNA can be used to direct synthesis of an mRNA that will encode the same protein as the original mRNA, allowing for rapid development of expression constructs for generating recombinant protein. This feature of cDNAs is used in the screening of expression libraries, where clones are evaluated on the basis of the proteins that they encode. Third, the numbers of genes that are expressed by a single cell or tissue are much smaller than the total number of genes in the genome, and thus the number of clones that need to be examined to identify a cDNA for a specific gene may be smaller. cDNA clones isolated from many cells and tissues have been identified through sequencing and made available to researchers. Availability of these EST clones and the development of PCR cloning is decreasing the need to screen cDNA libraries to obtain clones. However, cloning methodology will continue because of the need to identify genes that encode specific functions through expression cloning, or those that characterize a specific cellular process through subtraction cloning. cDNA libraries are usually screened with labeled DNA probes, but expression libraries can be screened with antibodies and other reagents that depend on protein function.

Obtaining a population of clones that represents the complete genome or all mRNA transcripts can be difficult. Mathematical models are used to ensure that the library is large enough to encompass the entire sequence repertoire (14). The number of independent clones that are formed by ligating the cloned DNA into a cloning vector is called the base of the library. A base of approximately 1 million clones is usually necessary for λ phage genomic libraries and cDNA libraries. The larger the base

of the library, the more likely it is that the gene of interest will be found. To examine all of the clones of a library, a number of clones representing at least three times the base of the library should be screened. Although the availability of genomic DNA for the construction of genomic libraries is rarely a problem, cDNA must be biochemically synthesized and is often limiting. The use of high-efficiency bacteria for the generation of the clones in the primary plating of a library is important to capture as many recombinant molecules as possible. The quality, purity, and size of the DNA are crucial to the generation of a library. High-quality DNA ensures an accurate representation of the DNA because exogenous, contaminating genetic material can cause erroneous results. The creation of a DNA library is not a trivial undertaking. Well-characterized genomic and cDNA libraries are available from academic and commercial sources. Libraries that have been amplified several times by plating on bacteria should be used with care because the library quality declines with overamplification, which can result in loss of rare transcripts.

Genomic DNA Library

All nucleated cells of an organism have the same general genomic information. Genomic DNA comprise coding regions or exons and non-coding regions or introns. Within the non-coding regions are sequences that control the patterns of individual gene expression (i.e., transcriptional regulatory elements). Most of the genome of higher eukaryotes is non-coding DNA. A complete genomic DNA library contains at least one copy of each region of the genome being evaluated. A sub-genomic library can be generated that contains only a defined portion of the species genome. Such libraries can be based on fractionated chromosomes, a particular chromosome band, or genomic DNA fragments of specific molecular weight. Genomic DNA libraries have been used to determine structural information such as the location of introns, exons, and transcriptional regulatory elements. In addition, genomic libraries have been used to clone genes with homology to other known genes and genes with little information regarding cellular expression pattern.

Genomic DNA can be prepared from any type of cell or tissue. Accessible cells, such as semen or blood cells, are an excellent source of genomic DNA. In the human genome project, a genomic library for nucleotide sequencing of the human genome was created from the blood of five donors (15). To establish a genomic library, high-molecular-weight DNA is carefully isolated and then randomly digested by partial restriction enzyme digestion or sheared by physical forces. Either process yields overlapping DNA fragments of

optimum size to clone into a vector. The fragments of genomic DNA are usually inserted into vectors with a large carrying capacity, such as bacteriophage, cosmids, YACs, or BACs. When the starting sequence material is represented by fewer clones, screening of the cloned sequences is more rapid and thorough. Finally, generating sufficient numbers of clones to represent the entire genome is important in establishing genomic DNA libraries. A genome equivalent, which is an estimate of the number of independent clones required to adequately represent the entire genome (2), can be calculated by dividing the genome size by the average insert size. For example, a genome equivalent for a human genomic DNA library (3×10^9 bases) composed of inserts of 30 kb would be 100,000 clones, creating a onefold library. A library of several genomic equivalents is usually needed to ensure representation of the entire genome. Vectors with high transformation efficiency and the ability to assimilate large DNA inserts minimize the complexity of the library (i.e., the number of independent genomic clones).

cDNA Library

mRNA is the genetic blueprint or coding sequence that dictates the primary amino acid sequence of the encoded protein. mRNA represents the combined sequences of the various exons that make up the structural gene, with intronic regions removed from an initial large RNA molecule by RNA splicing. The mRNA is only a small fraction of the total cellular RNA, which also includes ribosomal RNA (rRNA) and transfer RNA (tRNA). mRNA is a fragile, single-stranded molecule sensitive to RNase, a ubiquitous enzyme that degrades RNA. Single-stranded mRNA can be converted into cDNA in vitro by reverse transcriptase. The cDNA molecule can be used as a template to synthesize a second DNA strand, to produce double-stranded cDNA. cDNA is composed of sense and antisense strands; the sense strand has the same sequence as the mRNA. A cDNA library is a collection of different double-stranded molecules that represent the different mRNAs found in the cell or tissue being studied. The frequency of a single clone usually represents the relative abundance of the mRNA for that gene in the starting mRNA sample.

Isolation of mRNA is facilitated by its characteristic poly-adenylated 3' tail. These poly-A tails bind to oligo(dT) or poly(U) columns while the remaining RNA elutes off the column. Application of high-ionic-strength buffers to the column disrupts the non-covalent hydrogen bonds between nucleotides and permits the elution of purified mRNA. Total cellular RNA or mRNA is converted to double-stranded cDNA by reverse transcriptase in vitro. cDNA synthesis requires primers that can be either random,

such as oligo(dT), or gene-specific to begin the reaction. The type of primer used in cDNA synthesis is based on the starting RNA material and the intended use of the cDNA. Double-stranded cDNA is first ligated into a cloning vector and then transformed in a host cell to generate colonies for plating and selection. Because most genes are less than 5 kb, plasmids and bacteriophage are the most commonly used vectors for cDNA library construction.

Like genomic libraries, a critical issue for cDNA libraries is the adequate representation of all mRNAs expressed by a cell or tissue. The abundance of mRNA transcripts varies and is a function of the number of transcripts generated and the rate at which they are degraded. Rare mRNAs may be difficult to detect, whereas other transcripts may not be expressed in a particular cell or tissue. For cDNA cloning, therefore, determining which tissue source will be most enriched for the genes of interest is critical.

RECOMBINANT LIBRARY SCREENING

Nucleic Acid Hybridization

Nucleic acid hybridization involves the non-covalent association of nucleic acids from two complementary single strands into a double-stranded molecule. In a typical hybridization reaction, DNA/RNA from a library or gel is transferred to a nitrocellulose or nylon membrane. The membrane-bound, denatured double-stranded DNA or single-stranded RNA is exposed to a labeled or tagged nucleic acid probe. The probe binds to complementary RNA/DNA on the membrane, which is washed at various stringencies to remove nonspecific probe. Binding of the nucleic acid probe to the membrane or blot is assessed by autoradiography or phosphoimaging. DNA or RNA that hybridizes to the probe contains either the exact or a highly homologous DNA/RNA sequence. Northern blotting is the technique of RNA nucleic acid hybridization, and Southern blotting is DNA nucleic acid hybridization.

The typical nucleic acid probe is 50–1000 bases long and can be generated by several techniques, including oligonucleotide synthesis, PCR, and expression cloning. Oligonucleotide probes are single-stranded, synthetic molecules 15–50 bases long and are usually custom designed and purchased from a commercial supplier. Because of their short length, oligonucleotide probes can be designed to detect specific protein motifs like zinc-finger or other conserved functional domains. PCR with sequence-specific primers, cell-based cloning, and expression cloning can be used to generate nucleic acid probes. Probes are usually labeled by incorporating a nucleotide

with a radioactively labeled phosphate (i.e., [P^{32}]dCTP) into the probe synthesis reaction. Less common labeling methodologies include digoxigenin-labeled probes, which can be detected with an enzyme-linked assay, and biotinylated probes, which can be detected with avidin-based systems. Colonies, plaques, or DNA/RNA fragments that bind the labeled probe appear as distinct spots or bands on the membrane. This hybridization information is then correlated with the original plate or gel to isolate the sequence of interest.

Differential Screening, Subtraction Hybridization, and Subtraction Cloning

These methods have been developed to clone cDNAs that represent genes that are differentially expressed between different cell types or the same cell type that is undergoing stimulation. Subtraction cloning is a powerful technique that generates a cDNA library or subtraction library, enriched for genes that are different between two conditions. The construction of subtraction libraries is based on the assumption that cells with or without a stimulus will express the same genes except for those genes affected by the stimulus. mRNA is isolated from two cell populations to create a subtraction library. For example, mRNA can be isolated from endothelial cells grown in the presence or absence of the inflammatory cytokine, tumor necrosis factor- α (TNF- α). Single-stranded cDNA is synthesized from mRNA of one condition (e.g., endothelial cells exposed to TNF- α) and hybridized to an excess of mRNAs from the second condition (unstimulated endothelial cells). DNA–RNA hybrids form between genes that are common to both conditions, whereas genes expressed in only one condition remain single-stranded. These single-stranded cDNA are then converted to double-stranded cDNA and ligated into a cloning vector to produce a library of the differentially expressed genes. This classic approach, however, is tedious and technically challenging. More simple PCR-based methods have recently been developed to generate subtracted libraries enriched for low transcript messages. This technique, called suppression subtraction hybridization, has been used extensively in our laboratory to clone genes that are expressed by endothelial cells undergoing angiogenesis *in vitro* (16,17).

Differential screening is similar to subtraction hybridization but does not require the creation of a new library. A cDNA library is chosen that is expected to contain the genes that are expressed in the cell under stimulation. Labeled cDNA is made from mRNA isolated from cells or tissues to be compared. The labeled probes from each condition are hybridized separately to replicate

membranes made by transfer of bacteriophage or plasmid library clones. Autoradiography of the hybridized filters is performed, and the spots on the replicate membranes are compared. Spots that differ between the two membranes correlate with genes that are differentially expressed between the two conditions. In this type of experiment, changes in gene expression are qualitative because the amount of cDNA per colony is not the same on the replicate blots. Furthermore, this type of analysis requires that the radioactive probes be comparable in specific activity and that the exposure times be modified to yield a similar intensity of hybridization. Although used successfully in the past, this technique is technically challenging, and genes that are differentially regulated at a modest level are often missed (18). Finally, finding a library that will fully represent the stimulated condition may be difficult.

Recombinant Protein Screening

One of the most powerful methods developed for molecular cloning is the ability to directly clone a molecule based on some feature of its encoded protein. The gene encoding the potent vasoactive factor endothelin-1 was cloned on the basis of its ability to induce the contraction of vascular tissue in a bioassay. Numerous cell-surface receptors have been cloned by transfecting cells able to adhere to a plastic dish coated with antibodies, interacting proteins, or radiolabeled ligands. Transcription factors have been cloned on the basis of their ability to bind specific DNA regulatory sequences. Although the importance of many forms of molecular cloning by library screening has decreased because of the wide availability of ESTs, expression cloning will continue to be essential for the identification of molecules that mediate a specific molecular function.

Expression cloning requires a cDNA library that is constructed from the cells or tissues known to express the protein or functional activity of interest. Because many of the screens have low efficiency rates and are laborious to perform, finding a cellular source that is enriched for the protein and mRNA to be cloned is imperative. The choice of vector is important; it must be capable of directing expression in either bacteria or eukaryotic cells. Inducible transcriptional systems improve specificity and prevent the loss of cells caused by protein toxicity. Expression libraries require a larger base than other cDNA libraries to ensure representation of all proteins expressed by the target cell or tissue. For each cDNA, only one in six clones will be expressed as a protein because the gene can insert itself in the 5'-3' or the 3'-5' direction or in any one of three reading frames. In recent methods, directional

insertion of the cDNA has reduced the frequency to one in three clones. Although the expression vector contains the machinery to direct transcription, the initiating ATG sequence must be provided by the cloned cDNA. Because of these technical demands, expression library construction is challenging and difficult.

Several cloning strategies have taken advantage of the availability of antibodies for proteins of interest. Such immunologic screening is based on the ability of an antibody to recognize and bind an antigen. The antibody can be either monoclonal (i.e., recognize one epitope) or polyclonal (i.e., recognize multiple epitopes). For screening purposes, a polyclonal antibody or a mixture of monoclonal antibodies is usually preferable because the antisera can bind many epitopes of an individual protein antigen. One early such strategy in bacteria took advantage of the expression of recombinant protein in the λ phage vector λ gt11. Libraries created with this vector expressed a beta-galactosidase fusion protein, and nitrocellulose filters lifted from the library plates were evaluated by exposing them to antiserum labeled directly or identified by a second labeled antibody. Cloning was performed by picking areas of the bacteriophage lawn where spots were seen on autoradiograph. This process was repeated until clones were pure. The major problem with this screening strategy is that proteins expressed in bacteria do not undergo eukaryotic post-translation modifications and may not be folded correctly. Furthermore, a protein that adheres to a membrane is essentially denatured. As a result, the types of antibodies that can be used in antibody screening in bacteria are restricted. Similar methods have been used with plates of eukaryotic cells by using cDNA libraries in eukaryotic expression plasmids such as pCDNA3. Lawns of COS cells transfected with an expression library have been screened by incubating the antisera directly on the lawn of cells. The cells expressing the protein of interest can be identified with a chromogenic substrate reaction directed by an enzyme tag on the antibodies. Plasmid DNA isolated from these cells is amplified, transfected into COS cells again, and the process repeated until clones are pure.

Antibody-directed screening, a common method, was used to clone cell-surface receptors with expression libraries transfected into COS cells. High-level transient expression of the library clones in these eukaryotic cells produced large amounts of these receptors on the COS cell surface. Transfected cells were applied to plastic plates coated with a specific antibody, and cells containing the clone of interest were selected. Plasmid expression constructs were harvested back from the cells that adhered to the plates, and the process was repeated until

a small number of plasmids were isolated. This technique has allowed for the cloning of many genes, such as T cell-surface genes for which large amounts of monoclonal antibodies are available.

An important advantage of screening with eukaryotic cell expression systems is that the recombinant proteins are folded and modified correctly. However, the antibody must be able to identify the protein in its native state. In addition, transfection and handling of eukaryotic cells is difficult.

Binding assays have been used in expression cloning strategies to clone important molecules. The most rapid and straightforward techniques parallel those used for antibody screening of expression libraries. For instance, bacteriophage expression libraries have been widely used to clone DNA binding proteins. In these experiments, a double-stranded DNA segment encoding a cognate DNA binding element recognized by a transcriptional regulatory protein is radiolabeled and incubated with filters containing recombinant proteins from the library plates. Spots identified on autoradiographs are used to guide "picking" regions of the phage lawn, and a pure clone that is responsible for the binding is obtained by serial screenings. In another approach analogous to antibody screening of plated eukaryotic cells, a radiolabeled ligand is applied directly to plates of COS cells expressing transfected libraries. These plates are either overlaid with photographic emulsion or exposed to film, and the area of cells generating a signal is identified. Plasmid DNA isolated from these cells is amplified and retransfected for several rounds until a single clone is obtained. This ligand and screening technique was used for the cloning of the most sought after receptor in cardiovascular biology, the angiotensin receptor (19). A more labor-intensive strategy uses a radiolabeled ligand in solution that has been reacted with pools of transfected eukaryotic cells expressing a fraction of the expression library. These pools can be evaluated for radioligand binding as a group, yielding a subset of genes that includes the desired receptor. The pools are subdivided, and smaller pools of genes are evaluated in subsequent binding reactions. This process of reductionist cloning is repeated until a single clone remains for characterization. This technique has been used to clone numerous cell surface receptors (20,21). For these strategies to work, a high-affinity receptor must be selected whose binding properties are not altered by the radiolabeling process, and information about ligand concentrations must be available.

Additional strategies of reductionist cloning or screening of pools have been used with other creative assays to clone a wide variety of different functional classes of proteins. For instance, a bioassay of vascular contraction to

screen for the physiological activity of vasoconstriction was used to clone endothelin-1. Other creative methods have used the in vitro generation of cRNA transcripts from pools of clones, which are then injected into a cell that can be functionally evaluated. Such techniques have paired expression screening of cRNA pools by microinjection into oocytes of *Xenopus laevis*. These RNAs are translated at high efficiency allowing for functional screening by patch clamp, for example, to look for new ion fluxes. These approaches have been used to clone new molecules that may contribute to cardiovascular development.

Finally, the most efficient expression cloning strategies take advantage of a biological selection process to enrich for those clones encoding the gene of interest. One good example is the use of a transformation assay to identify genes that confer the ability to grow in a transformed fashion on NIH 3T3 fibroblasts. Genes that have the ability to transform these cells are called oncogenes, and they are readily identified because they are the only genes that permit the transfected cells to grow in this selection process. Another example is the cloning of the Na⁺/H⁺ antiporter (22). A cell line lacking antiporter activity was developed and transfected with human DNA. These transfected cells were then subjected to selection conditions that permitted survival only of cells expressing the human gene for this antiporter protein.

Cloning with Synthetic Recombinant Expression Libraries

All of the cloning strategies discussed above are aimed at the cloning of naturally occurring genes. In some cases, the function of the gene is known, whereas in other cases the gene is identified only through its association with specific biological processes or functional activities. Robust strategies are available to clone sequences that do not necessarily exist in nature, but rather, are selected to fit a specific functional profile. These strategies are used extensively by the pharmaceutical industry when a specific molecular function is needed, but no known biological reagent has been identified or adapted for the desired purpose. In one such methodology, short DNA segments encoding random peptides are generated and screened for their ability to bind to the molecules, cells, or tissues of interest. Very large recombinant libraries can be made in bacteriophage vectors to express proteins on their surfaces. Recombinant clones encoding peptides that bind are selected by repeated interaction with a target protein. This technique has recently been used to characterize peptides that recognize endothelial cell-surface epitopes (23). These peptides may be used to define endothelial cell markers that mediate tumor metastasis and endothelial

functions. Another application of the screening of synthetic DNA segments is to generate high-affinity recombinant monoclonal antibodies. “Random” DNA fragments can be inserted into cloning sites in an antibody framework at a position that codes for the hypervariable region of the antibody, thus simulating the biological process of antibody diversity. Large libraries of recombinant synthetic antibodies have been generated in this way and screened by repeated affinity purification of the antigen epitope to be targeted. This method allows for the rapid development of constructs that encode recombinant antibodies that can be expanded by using bacterial expression systems, thus eliminating the need for animal use.

ANALYSIS OF GENE EXPRESSION

Genetic information that is stored in the inherited genome must first be “turned on” or transcribed into mRNA and then translated into protein to affect biological processes. Regulation of these processes determines the phenotypic differences between hair cells, gut epithelial cells, and other cell types, and the ability of a cell to respond to stimuli such as the hormone estrogen. The most critical regulatory step probably occurs at the mRNA level, as determined by the concentration of an mRNA species for a gene of interest in a specific cell type. Steady-state mRNA levels reflect both synthesis and degradation. These levels can be determined with Northern blots and microarrays, which are usually called gene expression studies. Transcriptional regulation studies evaluate the rate of transcription and are usually directed at understanding the DNA and protein elements that mediate transcription. mRNA stability is rarely measured, but this aspect of gene expression is clearly a universal mechanism for transcriptional regulation. In this section, we will briefly review the various techniques used to analyze gene expression in a specific cell or tissue type.

Northern Blotting

Northern blotting is the most commonly used method to detect and quantify a particular mRNA in a mixture of RNAs isolated from cells or tissues. For this analysis, an RNA sample, often total RNA, is denatured by treatment with formaldehyde to prevent hydrogen bonding between basepairs. This step ensures that all of the RNA molecules have an unfolded, linear conformation. Individual RNAs are then separated according to size by gel electrophoresis and transferred to a nitrocellulose or nylon membrane. The membrane is exposed to a radiolabeled DNA or RNA probe, hybridized and washed, and then subjected to autoradiography.

The signal for a specific hybridization can vary because of differences in RNA concentrations; therefore, examining expression levels of “housekeeping genes,” such as β -actin or cyclophilin, that do not change across experimental conditions is important. Because these genes may occasionally be regulated by a stimulus, more than one housekeeping gene should be used under these circumstances. The signals are quantified by densitometry measurements of autoradiographs or by a phosphorimager that calculates a ratio for the expression level of an experimental versus a control gene. By modifying the conditions of the hybridization and wash, such as salt concentration and temperature, a probe can be used to detect mRNA species that do not have an identical sequence. This “low stringency” hybridization technique enables the detection of mRNAs representing orthologs or paralogs of the probe sequence.

The probe used for Northern blotting can be either labeled RNA or DNA. Although radioactively labeled nucleotides have usually been used for incorporation during enzymatic amplification of the probe sequence, nonradioactive methods are rapidly gaining acceptance. The size of the labeled probe varies from 100 to 1000 basepairs but, recently, oligonucleotides comprising as few as 15 basepairs have been end-labeled and used as probes as long as hybridization and washing conditions are modified to account for the low affinity hybridization seen with such short sequences (24).

Ribonuclease Protection Assay

The ribonuclease protection assay (RPA) is an extremely sensitive technique used for the detection and quantification of mRNA and for analysis of mRNA structure (25,26). This assay is based on the principle that ribonucleases digest single-stranded but not double-stranded RNAs. For this assay, a labeled antisense RNA probe is synthesized that is complementary to part of the target RNA to be analyzed. Both radioactive and nonradioactive probes can be used. Antisense probe RNA is prepared by inserting the DNA region that encodes a particular RNA into one of the common transcription vectors that contains a T3, T7, or SP6 bacteriophage promoter. Highly specific RNA transcripts are generated by using the corresponding T3, T7, or SP6 RNA polymerases (27). The labeled probe is then mixed with the sample RNA and incubated under normal hybridization conditions. After hybridization, the mixture is treated with ribonuclease to degrade single-stranded, unhybridized probes, so that only labeled probes that hybridized to complementary RNA in the sample will be protected from ribonuclease digestion. Hybridized probe is then separated on a polyacrylamide gel and visualized by autoradiography. When the

probe is present in molar excess over the target mRNA in the hybridization reaction, the intensity of the resulting band will be directly proportional to the amount of cRNA in the original mixture.

RPA is usually more sensitive than Northern blotting and can therefore be used to detect less abundant mRNAs. However, the higher sensitivity of this assay often results in a higher background signal. RPA has several important applications. For example, genes with multiple-splice variants can be identified with a single probe designed to hybridize to all variants of the gene (28). These hybridized fragments can be separated on a gel and variants can be verified by their relative sizes. An additional advantage of the RPA is that multiple probes for different target RNAs can be assessed in a single reaction.

Polymerase Chain Reaction

PCR is a powerful and sensitive technique for rapid enzymatic amplification of genes present in very low copy numbers. In addition to amplifying genomic DNA templates, PCR can be used to accurately measure mRNA levels by amplification of cDNA that is reverse-transcribed from RNA.

In a typical PCR reaction (29,30), the DNA sequence between two primers undergoes repeated doublings in an exponential fashion. In the first cycle, heating to 95°C melts the double-stranded DNA, and subsequent cooling to 42–68°C then allows for the primers to hybridize (anneal) to their complementary sequences on the target DNA. The thermostable *Taq* DNA polymerase is used to extend each primer sequence at 72°C in the 5' to 3' direction, generating newly synthesized DNA strands. In the second cycle, the original and newly made DNA strands are separated at 95°C; primers again anneal to their complementary sequence at 42–68°C; and each primer is again extended by *Taq* polymerase to the end of the other primer sequence. Thus, all DNA strands synthesized in the amplification cycles exactly equal the length of the region to be amplified, as determined by the sequence of the primers. In the third cycle, two double-stranded DNA molecules are generated that are equal to the sequence of the region to be amplified. These two are doubled in the fourth cycle and are doubled again with each successive cycle. In this way, after 25–40 cycles of PCR, the yield of target molecules will be 2^n , where n equals the number of cycles.

To measure mRNA levels by PCR, the mRNA must first be converted to cDNA, which then serves as a template for the polymerase reaction (31). Oligo (dT) primers, random decamers, or gene-specific antisense primers are used to synthesize single-stranded cDNA by reverse transcriptase.

Standard PCR is then performed after addition of the gene-specific sense and antisense primers. The PCR products are fractionated by size on agarose gels for visualization. In this assay, contaminating genomic DNA must be removed from the RNA samples before synthesizing cDNA because amplification from DNA will cause spuriously high estimates of mRNA concentrations. Because of its sensitivity, PCR is inherently non-quantitative. Thus, standard PCR, although excellent for rapid and easy detection of mRNA expression in an individual RNA sample, is not ideal for relative quantification.

Relative quantification of mRNA levels can be achieved by using reverse transcription coupled with PCR (RT-PCR) with primers for the gene of interest and control primers for a control amplification. The reaction is terminated and analyzed with both targets in the exponential phase of amplification (32). However, conditions under which both PCR reactions are simultaneously in the exponential phase can be difficult to determine because common control genes are usually expressed at much higher levels than the transcript of interest. Small variations among samples sometimes result in large differences in product yield. Competitive RT-PCR (33,34) is a more reliable method for accurate quantification.

In competitive PCR, a known amount of an exogenous standard (competitor) template is added to a target RNA sample, and RT-PCR with a common set of primers is performed to amplify simultaneously both target and standard templates, which are distinguished from each other by their size differences on gel electrophoresis. The abundance of the target is determined by comparing signals obtained for the competitor and target. For example, serial 10-fold dilutions of competitor RNA are added to reactions containing a constant amount of target RNA. RT-PCR is performed, followed by gel electrophoresis. Product yield is determined by measurements of radioactivity incorporated during amplification (31) or by analysis of gels stained with intercalating dyes such as ethidium bromide (35). Because the amount of competitor added to each reaction is known, the amount of endogenous target in the sample RNA can be determined by comparison. The ratio of products obtained from the endogenous and competitor targets at the end of amplification reflects the initial ratio of target to competitor RNA.

In recent years, more sophisticated techniques have been developed to quantify mRNA levels. Real-time RT-PCR (36) is a new, noncompetitive technology that can detect products while they are being formed. The accumulation of products is assessed with several probes, including fluorogenic 5' nuclease probes and molecular beacons (37,38). In this assay, fluorescence measurements

are made in real time during each cycle of PCR with a thermocycler connected to an optical excitation and detection device. During the exponential phase of PCR, the amount of amplified product synthesized in each cycle increases in a semigeometric fashion. The cycle during which the amount of released fluorescence exceeds a baseline threshold fluorescence is known as the threshold cycle (Ct). This Ct value is inversely proportional to the amount of starting target. Thus, high-copy number samples will have low Ct values, whereas low-copy number samples will have high values. For rigorous quantification of mRNAs, a standard curve is generated with *in vitro* transcribed RNA. A Ct value is determined for different concentrations of this control RNA. Ct values are then plotted against the RNA concentrations to generate a standard curve for quantifying the level of expression in unknown samples. Because fluorescence monitoring is highly sensitive and accurate with a linear dose response over a wide range of target concentrations, real-time PCR is superior to conventional PCR quantification, which only analyzes end-point products.

The yield of PCR products from real-time RT-PCR was initially estimated by the amount of fluorescence released by DNA-binding dyes such as ethidium bromide, SYBR Green I, or oxazole yellow derivatives. TaqMan PCR (37), which uses an oligonucleotide labeled at its 5' end with a fluorescent dye and a quenching dye at its 3' end, was recently developed to increase the specificity of the amplified products. This probe anneals to an internal sequence within the amplified DNA fragment. PCR is performed with the labeled oligonucleotide and primer pairs to amplify the target sequence. When both the fluorescent and quenching dyes are present in close proximity on the intact hybridization probe, fluorescence emission from the dye is absorbed by the quenching dye. As the amount of target DNA increases during PCR, greater amounts of oligonucleotide probe hybridize to the denatured target DNA. However, during the extension phase of the PCR cycle, the 5'→3' exonuclease activity of *Taq* polymerase cleaves the fluorophore from the probe. Because the fluorophore is no longer adjacent to the quencher, it begins to fluoresce. The intensity of fluorescence, therefore, is proportional to the amount of target PCR product. Real-time PCR is an excellent technique used not only to measure the abundance of DNA, but also to screen for mutations and single nucleotide polymorphisms.

***In situ* Hybridization**

The *in situ* detection of mRNA on microscopic sections provides direct visualization of the spatial location of mRNA at the cellular level. Knowing the location of

mRNA is useful during early phases of study with a new gene because it helps in developing hypotheses about cellular function of the gene product. Furthermore, *in situ* data are often critical for understanding the molecular functions of well-characterized genes. For example, if a gene is thought to encode a cell-surface receptor that responds to locally secreted ligands, documentation of the spatial coordinate regulation of this receptor and its ligands at the cellular level is essential. The ability to examine gene expression at the cellular level is critical for complex tissues such as the kidney, brain, and even the blood vessel wall, where the expression of an individual gene might have different implications, depending on the cell type involved.

In situ hybridization can be used for analysis of cells or tissue sections mounted on slides, or for whole mount analysis of embryos or tissue fragments in suspension (39). The tissues must be pretreated before beginning the assay to increase accessibility of the target RNA. In addition, a labeled nucleic acid probe must be generated for subsequent detection of the RNA. The strategy for making single-stranded labeled cRNA probes is similar to RNase protection assays. For tissue sections, both isotope probes and non-isotope probes with digoxigenin or biotin can be used (40). Resolution is maximal with low-energy radioisotopes such as ³⁵S or ³³P. Labeled probes are incubated with cells or tissue specimens fixed on glass slides. For whole mount detection, nonradioactive probes must be used (17). For example, in nonradioactive detection of target RNA, antibodies to digoxigenin are used that contain a tagged enzyme that catalyzes the conversion of a chromogenic substrate so the hybridization pattern can be seen. For radiolabeled probe detection, slides are covered with photographic emulsion for several weeks before the film is developed. These slides can be counterstained so that the normal cellular architecture is maintained, thereby permitting immunohistochemical analysis. Whole mount-stained specimens can be photographed and then embedded, sectioned, and examined under a microscope, with or without secondary counterstaining. The specificity of the signal depends on the stringency of washing and the extent of similarity between the probe and the intended target sequence. Although less sensitive and more difficult to optimize than radioactive detection, nonradioactive detection is much faster and can provide data in a few days rather than several weeks for radioactive labeling methods.

In situ hybridization is an excellent technique for locating sites of mRNA expression, but it is not ideal for quantification. The advantage of this technique is that only a small region of transcribed sequence is necessary to obtain extensive information about the cellular

pattern of expression. It is possible to begin with an EST obtained by searching genomic databases and complete a quick survey of its expression in a matter of days. In some cases, *in situ* methods have been optimized to allow high-throughput screening of novel database sequences to identify those that are expressed in a tissue- or cell-specific pattern.

Nuclear Run-off Assay

Nuclear run-off assays measure the rate of gene transcription rather than steady state mRNA levels by quantifying newly transcribed RNA molecules in cells undergoing manipulation. This assay is the most sensitive tool for measuring specific gene transcription as a function of cell state. Cell nuclei contain RNA that is being transcribed. Transcription can be completed in a test tube by adding ribonucleotides. The newly synthesized RNA can be labeled to high specific activity in isolated nuclei by including a radiolabeled ribonucleotide in the reaction. Transcription cannot be initiated under these same conditions; therefore, the level of transcription occurring at the moment that the nuclei were collected can be measured. In contrast to Northern blotting, an unlabeled, specific probe is immobilized on a filter that is hybridized to a reaction mixture containing radiolabeled transcripts to detect specific RNA species. These radiolabeled mRNAs represent all of the mRNAs elongated during the *in vitro* reaction; therefore, levels of transcription for many different mRNAs can be simultaneously analyzed by blotting multiple probes on a single filter. This assay is useful for evaluating whether changes in mRNA levels reflect differences in gene transcription or rates of mRNA degradation and often precedes more detailed transcriptional regulation experiments.

Promoter Analysis

Gene expression in cells or organs is strictly regulated under physiological conditions by DNA regulatory sequences that are located within hundreds to thousands of basepairs from the transcribed regions of the gene. These regulatory elements can be either positive or negative regulators of transcription, and may determine tissue specificity, developmental patterns of expression, or cell-specific responses to a variety of stimuli. The complex methodology for the study of DNA and the proteins that mediate transcriptional regulation of eukaryotic genes is beyond the scope of this chapter. However, because of the availability of useful DNA constructs that have been made in many laboratories and the ability to transfect most cell types with liposome reagents, these studies can be an excellent complement to more traditional mRNA studies.

Unlike nuclear run-off studies that are technically demanding, basic promoter studies are a simpler method for assessing rates of transcription. The primary tool for evaluating transcription is the reporter gene transfection assay. Various segments of the structural gene under study are linked to a reporter gene that can be easily assessed in a simple cell lysate. The level of activity of the reporter gene indicates the relative rate of transcription, as determined by the activity of the regulatory sequences. The firefly luciferase gene is a common example of a reporter gene.

The primary transcriptional element for each gene is the promoter, which is usually a sequence immediately upstream of the transcription start site. Whether a TATA element is present or not, promoter sequences initiate the assembly of a complex milieu of proteins that mediate transcription. Careful experiments must map the site of initiation of transcription before studies can be performed with reporter constructs. Primer extension and RNase protection assays are used in the mapping process. In addition, other proteins, binding to cognate DNA sequences within introns or further upstream or downstream, can regulate transcription through interaction with the basal apparatus. In reporter gene experiments, a portion of the native promoter region is used, with or without additional putative regulatory sequences.

Construction of reporter plasmids for analysis requires flanking and intronic genomic sequence, which are usually obtained by cloning and mapping the intron-exon organization of the gene. In humans, predicted organizational information in the human genome sequence is used for mapping. Promoter regions of varying lengths are initially used in different constructs to map the important upstream elements. These studies are complemented by mutagenesis analyses to define more accurately the regulatory sequences. Protein binding experiments with nuclear protein and radiolabeled DNA promoter fragments are useful in these studies. Such footprint assays and gel shift assays can define possible protein binding sequences, although functional verification is required. If the expression pattern of reporter genes containing only upstream sequence does not parallel the known expression pattern, then the intronic or flanking sequence is also included in the reporter construct (41).

Finally, reporter assays are performed by transfecting the plasmid containing the promoter and reporter into appropriate cell types. For cell specificity studies, the plasmid must be transfected into cells that either express or do not express the gene under investigation. Transcriptional regulation of genes expressed in heart cells has been difficult to study *in vitro*, mainly because of the lack of a good cell culture model. In cases

where in vitro models for transfection studies are lacking, or the in vitro studies do not provide the expected results, transgenic animals can be used for reporter gene experiments (42,43).

GENOMICS

Dramatic changes are currently reshaping the experimental approach to the study of cardiovascular disease. The advanced technologies necessary for these new approaches require focus on the details of experimental methodology and on the use of rigorous statistical tools. With this caveat, advances in the field of cardiovascular medicine will be made at a significant rate in the coming decade. These breakthroughs will result from studies of gene expression, protein production and regulation, and genetic markers associated with cardiovascular disease.

Human Genome Project

As recombinant DNA technologies emerged in the early 1980s, the rate of research into the genetic basis of human diseases accelerated because of our knowledge of the human genome. In 1990, a formal 15-year plan to sequence the entire human genome was initiated by the National Institutes of Health and the U.S. Department of Energy and was entitled the Human Genome Project. This large-scale international consortium, aided by advances in DNA sequencing technology and challenged by a privately funded initiative at Celera, announced in February 2001 that the initial working draft of the human genome was complete (44).

This working draft showed that the total size of the genome is approximately 3.2 gigabases, with an estimate of 30,000 to 35,000 human structural genes (44). Although 10% of the genome is not sequenced, the correct orientation of large fragments of DNA is incomplete, and the extent of the human transcriptome is not well defined, the wealth of information now available will have a dramatic impact on the study of human disease. In addition to the preliminary sequence information that was reported, more than 1.4 million single nucleotide polymorphisms (SNPs) were identified and mapped to the reference sequence. This figure will increase to over 3 million. The importance of this information is manifold; these single-base differences between genomes may confer individual susceptibility to or protection from all human diseases, including cardiovascular disease. Because the genetic basis of cardiovascular diseases such as atherosclerosis and hypertension are polygenic, identification of SNPs in disease-related genes will play a vital role in our understanding of their pathophysiological basis.

Microarrays

The basic principles of microarrays have evolved from traditional nucleic acid blots used by molecular biologists to analyze the expression levels of multiple mRNA or DNA sequences immobilized on nitrocellulose or nylon membranes. The microarray was directly adapted from the Northern blot, where multiple DNA samples are dot blotted on a membrane and then probed with radiolabeled cDNA made from the RNA of interest. Radiographic film is used to detect the amount of radioactivity for each DNA molecule on the membrane, and densitometry is used to quantify nucleic acid levels. This technique, however, is limited by problems with standardization of data and a narrow dynamic range for accurate quantification.

Microarrays or gene chips work similarly by hybridization of a labeled cDNA (or RNA) sample to DNA molecules fixed at specific locations on a suitable substrate. Because of recent technological developments, this type of analysis can be conducted in a high-throughput, automated fashion. A major breakthrough has been the identification of glass as an excellent substrate for DNA hybridization. In addition, DNA samples can be labeled with fluorescent dyes such as cy3 and cy5 instead of radioactivity, which increases the dynamic range for measurement of gene expression. These advances, in conjunction with new robotics technologies for synthesizing and depositing nucleic acids at very high densities, have allowed for the creation of microarrays containing tens of thousands of genes on a single microscope glass slide (45). Furthermore, sophisticated analytical tools have been developed to analyze the large amount of data generated from these array hybridization studies. As a result, expression levels for tens of thousands of genes can be simultaneously measured in an accurate and reproducible manner.

The complex technologies for the deposition or creation of specific DNA fragments on glass slides can be viewed most simply as variants of two platforms, one that uses synthetic chemical methods *in situ* to build an array of known oligonucleotide gene sequences one base at a time and the other that uses spotting of presynthesized DNA fragments of known or unknown sequence. Ed Southern of Oxford University first described the basic concepts for creation of DNA microarrays in the late 1980s. Affymetrix, a company that has provided high quality oligonucleotide arrays for scientific studies, adapted photolithography technology from the computer industry. The use of spotted cDNA fragments amplified by PCR to make arrays was developed in the laboratory of Patrick O. Brown at Stanford University, who, along with Dr. David Botstein, adapted this platform to study yeast biology and cancer genetics (46).

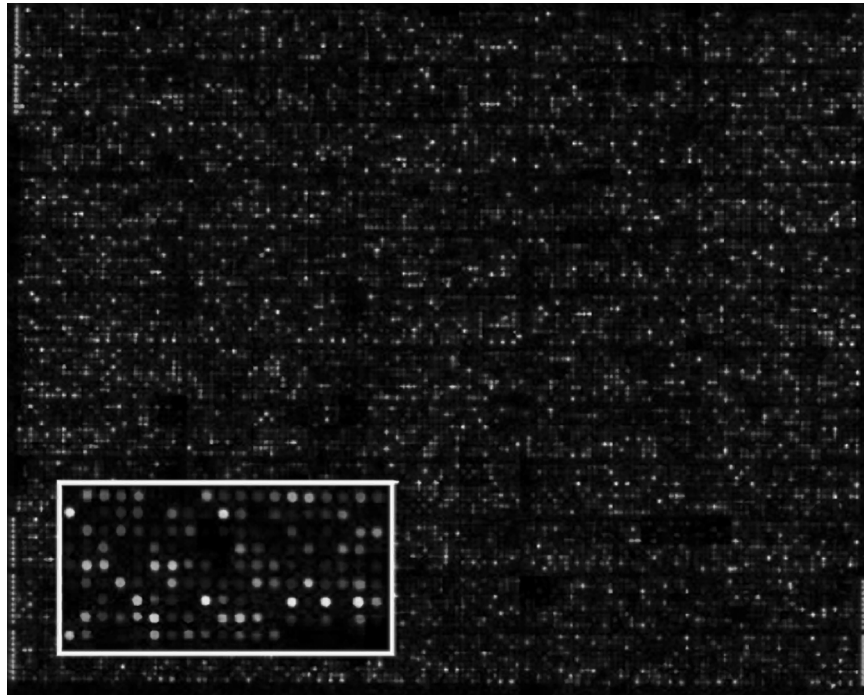


Fig. 1. Image of a high-density cDNA microarray printed on a glass slide. Inset shows a close-up view of array features or probes. Spots represent probes that have hybridized preferentially to cDNA labeled with a fluorescent dye.

Oligonucleotide arrays currently contain the highest density of “features” or probes, up to several hundred thousand elements per array. Up to 50,000 cDNA fragments can be printed onto a conventional microscope glass slide by using a bank of printing pens attached to a robotic arm. One difficulty with pen printing is the relative lack of uniformity of the spotted features. In a recent modification of the basic spotting methodology for array construction, pen spotting is used to deposit large presynthesized oligonucleotides instead of cDNAs. These oligos are typically 50–60 bases long, whereas oligos on the Affymetrix chips are 20–25 bases long. Sensitivity and specificity are better with longer oligos. These chips will likely become a dominant platform for DNA expression analysis when the entire human transcriptome is complete. Furthermore, Agilent Technologies has entered the microarray domain by applying its ink-jet technology to produce high quality *in situ* oligonucleotide arrays and spotted cDNA arrays (Fig. 1).

Both the oligonucleotide and spotted cDNA methods allow for the simultaneous examination of mRNA expression levels of thousands of genes; however, each has unique advantages and disadvantages. Because oligonucleotides can be generated from sequence information alone, probes can be customized to represent the most unique part of a given transcript, such as the

5′ untranslated region; therefore, splice variants or closely related genes can be more reliably detected (47). However, hybridization kinetics are less specific with short 20–30-base pair oligos, and the costs of synthesizing oligos for custom arrays and purchasing commercially available products are high. Although cDNA microarrays are more difficult to produce because of the laborious steps necessary to generate cDNA probes for printing, they offer greater flexibility to customize specific probe sets. Furthermore, arrays comprising unsequenced clones and ESTs generated from cDNA libraries are powerful reagents for gene discovery (47). Finally, whereas some oligonucleotide platforms, such as Affymetrix, compare fluorescent signals generated by hybridization of RNA samples from two tissues or cell types onto separate arrays, cDNA microarrays examine competitive hybridization of the two RNA samples labeled with different dyes to the same array. Instead of an indirect comparison of fluorescent signals on two separate arrays, a ratio for the intensity of the fluorescent signal from each channel can be calculated to determine the relative mRNA expression level for each gene on the array (47).

Although it is in the early stages of development, microarray technology has already been used in many scientific studies to obtain useful information about human disease and basic biological processes. For example, microarray

studies have led to a new paradigm for the molecular characterization of tumors. Alizadeh et al. (48) used cDNA microarrays to discover unique gene expression patterns to subclassify forms of diffuse large B-cell lymphoma (DLBCL). This approach not only identified distinct subtypes of DLBCL, but also predicted which patients had better survival rates based on molecular classification of their tumors (48). In addition, subtypes based on gene expression profiles have been defined for breast cancer (49,50). Recently, van't Veer et al. (51) used oligonucleotide arrays to identify a "poor prognosis" molecular pattern in breast tumors that correlated highly with clinical outcomes. This pattern was better than current consensus guidelines for predicting high-risk patients in need of adjuvant therapy while simultaneously reducing the number of patients receiving unnecessary treatment (51). Furthermore, the use of microarrays has been successful in studying basic biological processes. For example, Spellman et al. (52) identified a comprehensive list of cell cycle-regulated genes in the yeast by using cDNA microarrays. They not only compiled a list of 800 genes whose transcript levels varied with the cell cycle, but also identified specific promoter elements within these genes that were predictive of cell cycle regulation.

Only a few basic studies have been conducted using this platform to study cardiovascular disease. Haley et al. (53) used a cDNA array to identify eotaxin, an eosinophil-attracting chemokine, as a potential new candidate gene associated with atherosclerosis. Stanton et al. (54) used microarrays to examine gene expression profiles in an animal model of myocardial infarction. They identified more than 700 genes with altered expression patterns in myocardial infarction, including many unknown genes. One group recently studied changes in gene expression associated with heart failure by using high-density oligonucleotide arrays to compare transcriptional profiles of normal and failing human hearts (55). At the basic molecular level, several studies have used microarrays to identify novel pathways for endothelial cell signaling (56–59). Finally, Adams et al. (60) defined the molecular phenotypes of arteries and veins by comparing their respective gene expression profiles on cDNA arrays.

In summary, microarrays are a powerful new technology for studying gene expression on a global scale. With the imminent completion of the entire human genome sequence, the transcriptional profile of all human genes can be examined. Some of the studies highlighted in this section not only testify to the power of these tools, but also provide a glimpse of their future application to study human disease. Microarrays may someday be used to diagnose different diseases, to determine the likelihood of

an individual patient's response to a specific treatment, and to predict clinical outcomes, all based on gene expression profile studies.

Proteomics

Although high-throughput genomics tools such as microarrays have greatly increased our understanding of human disease, evidence indicates that mRNA levels correlate poorly with protein levels (61). Furthermore, many proteins can arise from a single open reading frame because of post-transcriptional modifications such as alternative splicing and RNA editing and post-translational modifications, including glycosylation and phosphorylation (61). In fact, three to six proteins may be produced per gene in humans (62). Therefore, an analysis of the relative abundance of proteins and modifications to proteins is required to understand any physiological processes.

Unlike genomics, which has advanced rapidly with the development of tools such as microarrays, proteomics, or the study of the entire protein complement of the genome, has lagged behind because of many technical obstacles (63). Dynamic changes in protein levels induced by specific physiological stimuli are often complemented by modifications of existing proteins, thereby producing an added layer of complexity to proteomic analysis. In addition to the high cost and labor-intensive nature of these studies, proper experimental design is daunting because of post-translational processing and the rapid changes in protein levels.

The basic techniques involved in proteomic studies are protein separation, protein identification, and classification of protein modifications (63). The current mainstay of protein separation is two-dimensional gel electrophoresis (2-DE). Proteins are resolved first by their charge with isoelectric focusing (IEF) and second by their size with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Large gels can separate up to 10,000 proteins (64). These proteins are detected by staining the gel with silver or zinc, radiolabeling with ^{32}P or ^{35}S , or labeling with a fluorescent dye. The most sensitive method is radiolabeling, but the most commonly used technique is silver staining (63). Specialized software analysis tools are used to identify proteins and calculate their relative expression levels from 2-DE images. Other protein separation methods, though not commonly used, include affinity chromatography, high-performance liquid chromatography, capillary IEF, and ion exchange.

Although protein separation methods have been perfected over the past 25 years, the most significant advance in proteomic analysis has been the development of mass spectrometry (MS) for protein identification (63).

Peptide mass mapping is often used for MS protein identification. Proteins separated by 2-DE are digested with proteases such as trypsin or chemical reagents to produce smaller peptide sequences, which yield a set of distinct peptides specific to each protein (63). These peptides are then ionized by electrospray ionization from the liquid phase or matrix-assisted laser desorption ionization (MALDI) from the solid phase. A unique peptide mass fingerprint (PMF) is generated from the masses of peptides measured by MS (63,65). Finally, the protein is identified by comparing the peptide signature to PMFs for known, partial, or predicted protein sequences found in protein databases such as SWISS-PROT.

The most difficult task in proteomic analysis is the identification and characterization of post-translational modifications. Traditional biochemical methods such as periodic acid/Schiff staining to identify glycosylated proteins and ^{32}P radiolabeling to detect phosphorylated proteins are not readily amenable to high-throughput strategies. MS is emerging, however, as a potential solution to this problem. MS PMF data can be used to identify up to 22 different post-translational modifications and to predict possible modifications based on amino acid sequence homology (63). Furthermore, new technologies such as MALDI-time of flight-post source decay have greatly increased the ability to detect post-translational modifications by fragmenting proteins into very small peptides comprising only a few amino acids.

Most proteomic studies, like genomics studies, have been conducted in the area of cancer research. For example, Ostergaard et al. (66) used 2-DE to analyze protein expression patterns of squamous cell carcinomas of the bladder and to identify unique markers of tumor differentiation. Proteomic applications to cardiovascular disease have focused primarily on protein expression profiles in cardiomyopathies. Pleissner et al. (67) performed a proteomic analysis of heart tissue from patients with or without dilated cardiomyopathy and identified several proteins that are differentially expressed by myocardial tissue from cardiomyopathy patients, including cytoskeletal proteins and proteins associated with energy production and stress responses.

Proteomics is a promising complement to genomics-based strategies. However, until technical difficulties are solved, proteomics will not gain the same level of interest as genomics. Methods to detect low-abundance proteins and to identify post-translational modifications are still in early stages of development (65). Protein separation techniques are difficult to automate, and robust bioinformatics tools are lacking. Despite these limitations, proteomics will eventually become an essential platform to study biological processes and human disease.

Single Nucleotide Polymorphisms

Rare diseases such as Huntington's disease and cystic fibrosis are caused by mutation of a single gene. Classical linkage studies in families identified these highly penetrant alleles, which usually occur at a frequency of less than 1% of the population. In contrast, common diseases such as atherosclerosis are caused by a complex interaction between environmental risk factors and multiple genetic loci. These genetic variants are usually found at moderate to high frequency in the general population. Linkage analysis methods with classical markers do not have the power to discriminate common alleles that determine disease susceptibility. Instead, identification of a large set of markers dispersed throughout the genome is required. These markers can be used in an extensive genetic analysis to understand the hereditary basis of complex disorders such as atherosclerosis. The most attractive candidates for markers are SNPs.

SNPs are single base pair differences in a DNA sequence between individuals of a population. SNPs occur about every 1000 base pairs throughout the genome. The effects that SNPs will have on a specific phenotype depend on their location within the genome. Coding SNPs are found within the coding regions of genes and may alter the function or structure of encoded proteins resulting in a disease state. Most SNPs, however, are located within non-coding regions and probably have little effect on phenotype (68). Two main strategies are used for studying SNPs and complex diseases—candidate gene approach and linkage disequilibrium mapping.

The candidate gene approach is based on prior knowledge about the genes that are likely to contribute to the pathogenesis of a specific disease. SNPs are identified in the candidate genes, and association studies determine a link between the polymorphic variation and the disease. These studies are limited in scope, however, since *a priori* knowledge of the genes involved is required for any analysis.

The recent efforts by the Human Genome Project and others have led to the identification and mapping of more than 2 million SNPs. These high-density SNP maps may allow scientists to identify disease phenotypes by using linkage disequilibrium mapping. Linkage disequilibrium is defined as two alleles at different loci that occur in an individual at a frequency greater than would be predicted by random chance (69). The strength of linkage disequilibrium decreases with time, or successive generations, because of recombinatorial events between loci. Loci that are close to each other are more likely to be inherited together than those that are far apart. When specific haplotypes (i.e., combinations of segregating alleles at

different loci) are in linkage disequilibrium and associated with a disease phenotype, a susceptibility gene can be mapped to a precise location in the genome (68,70). New genetic variants associated with human disease may therefore be rapidly identified through large-scale genotyping of populations and linkage disequilibrium mapping studies.

The technological advances made in genomics and proteomics have improved high-throughput methods for identifying and genotyping SNPs. Although other methods for SNP identification exist, sequence-specific detection is the preferred method for large-scale genotyping and is based on four mechanisms for allelic discrimination: allele-specific hybridization, nucleotide primer extension, oligonucleotide ligation, and enzymatic cleavage (68,71).

Hybridization methods are based on designing two allele-specific probes to hybridize to a target sequence when a perfect complementary sequence is present. Under optimal conditions, a one basepair mismatch is sufficiently destabilizing to prevent the probe from annealing to its target (71). The most widely used allele-specific oligonucleotide (ASO) hybridization method to distinguish SNP alleles is real-time PCR in homogeneous, solution-phase hybridization reactions with fluorescence detection (TaqMan™ and Molecular Beacon assays). The major problem with this approach, which has hindered the development of high-throughput multiplex assays with this platform, is the difficulty in predicting optimal reaction conditions or a probe that will provide the greatest distinction between two alleles (68). However, microarrays have recently been used for multiplex allele-specific probe hybridization reactions by spotting hundreds of ASO probes for each SNP on glass slides.

Primer extension protocols, which are very flexible, are excellent for allelic discrimination and require the smallest number of primers and probes for detection (71). In this approach, a primer anneals to its target sequence immediately adjacent to the SNP and is extended by a DNA polymerase with a single nucleotide complementary to the oligonucleotide at the site of the SNP. This method is based on the high accuracy of nucleotide incorporation by DNA polymerases (68). Assay design and optimization are minimal, which makes this approach the best suited for high-throughput genotype applications; therefore, many detection systems and platforms have been developed with primer extension protocols. Pyrosequencing, with the use of an enzyme-based luminescent detection of pyrophosphate; mass spectrometry, such as MALDI-time of flight mass spectrometry; enzyme-linked immunosorbent assays; and microarrays are platforms currently used for detection of SNPs.

Microarray-based strategies appear to be particularly tailored for large-scale SNP analysis (68).

Ligation methods rely on the ability of DNA ligases to discriminate mismatches at the ligation site of adjacent oligonucleotides annealed to a DNA template (68,71). Ligation occurs, therefore, only when oligonucleotides are exactly complementary to the template at the junction. Ligation methods have the highest level of specificity and are easy to optimize; however, they are the slowest reaction and require the greatest number of probes (71). High-throughput strategies have been developed including microarray formats, multiplex detection with fluorescently labeled probes, and real time PCR assays.

The final allelic discrimination method is invasive cleavage of oligonucleotide probes. This approach is based on the use of two target-specific oligonucleotides, one with a 5'-region noncomplementary to the target sequence and the other an upstream complementary probe. When the probes are matched at the SNP, the three-dimensional structure formed at this site is cleaved by a 5'-endonuclease, or FLAP endonuclease. The enzyme releases the 5'-sequence of the signaling probe, which can be detected directly, or can be further amplified (68). MS and fluorescence polarization methods are currently used for SNP detection. The invasive assays do not require a PCR amplification step and therefore are isothermal reactions. Unfortunately, these assays require a large amount of target DNA and extremely pure probes to ensure specificity of the reactions.

Genetic variants associated with cardiovascular disease have been studied extensively. In most of these studies, a candidate gene approach was used in which many of the genes analyzed are purported to play a role in the disease process. For example, increased plasminogen-activator inhibitor 1 (PAI-1) activity is commonly found in patients with coronary artery disease. In studies by Eriksson et al. (72) and Margaglione et al. (73), the 4G/5G polymorphism in the PAI-1 promoter was associated with higher plasma PAI-1 activity, and the 4G allele was found more often in patients with a history of myocardial infarction. Inflammation contributes significantly to the progression of atherosclerosis. Cytokines that mediate inflammatory responses are good candidate genes for association studies. CX3CR1, a chemokine receptor expressed on monocytes, acts either as a chemotactic receptor or as an adhesion molecule, depending on the nature of its ligand, fractalkine. In a recent study, a common CX3CR1 genetic variant (I249) was an independent risk factor for coronary artery disease (74). Finally, Ranade et al. (75) identified two polymorphisms (T-344C, Lys-173/Arg) in the aldosterone synthase gene

that conferred increased susceptibility to insulin resistance and diabetes (75). Although interesting, these findings from candidate gene studies need to be independently verified in additional cohorts.

In summary, SNPs are an important genetic determinant of complex human diseases. Classical candidate gene approaches have produced a vast amount of data linking genetic variants with human disease. These types of studies are limited in scope, however, because they require *a priori* knowledge of the disease-associated genes. Furthermore, such candidate gene association studies require large, carefully selected cohorts to obtain reproducible results. Full definition of the haplotype structure of the human genome, in concert with the refinement of high-throughput technologies for SNP detection and genotyping, will allow SNP-based association studies to make significant contributions to the study of the genetics of cardiovascular disease.

GENETIC ANIMAL MODELS FOR THE STUDY OF CARDIOVASCULAR DISEASE

Animal models, always important in biomedical research, have been used to study the pathophysiology of disease and to evaluate the beneficial and toxic effects of pharmacological therapies. With the advent of molecular biology and the development of techniques to manipulate the genome, the use of animal models has become even more important. In particular, the mouse has been used as a model to study human diseases such as hypertension, diabetes, insulin resistance, hyperlipidemia, and atherosclerosis. In this section, we briefly describe the tools required for genetic manipulation of the mouse and then examine in more detail how genetic mouse models have increased our understanding of atherosclerosis.

Genetic Manipulation of the Mouse

GENE TARGETING OR KNOCKOUT METHODS

The goal of gene targeting experiments is to analyze the phenotype of mice fractionally lacking a single gene. Many genes have been targeted in mice, and these analyses have provided significant information about the function of specific genes.

The first step in gene knockout or deletion studies is the assembly of a targeting construct that will introduce a desired mutation in the chosen gene when it integrates into one of the endogenous alleles. The targeting construct is designed to replace an essential portion of the structural gene. Targeting vectors typically contain genomic homology regions cloned on either side of genes for neomycin resistance and thymidine kinase (76). Homologous recombination events within these homology regions result in the

replacement of genomic DNA with the neomycin resistance gene and the concomitant loss of the thymidine kinase gene. Random integration of the construct, however, leads to genomic incorporation of the thymidine kinase gene. Positive selection with G418 selects for transfected cells that have incorporated the targeting construct, whereas negative selection with gancyclovir eliminates cells that have a functional thymidine kinase gene as a result of random integration. Despite this dual selection process, only a small percentage of G418 resistant cells contain the targeting construct; therefore, screening the individual G418 colonies by Southern blot with restriction digests and probes that map to the locus of interest is essential.

The next step in this process is to introduce the targeted deletion into the germline. For this step, the deletion is generated through homologous recombination in cultured pluripotent embryonic stem (ES) cells, and then these cells are implanted into chimeric mice. Only one allele is usually altered in the stem cells, and the mutation is established in the germline of heterozygous animals. Chimeric mice generated from injection of the targeted ES cells into blastocysts have patchy coat colors reflecting the beige color of the 129 line used to generate the ES cells and the black color of the C57Bl6 line used to produce the blastocysts. The chimeric mice are then bred back with wild-type animals. True chimeric mice will transmit the targeted allele to the F1 generation. These heterozygous animals usually develop normally and are fertile; therefore, mutations that otherwise would be lethal in the homozygous state can be maintained in the population. By cross-breeding this F1 generation, homozygous or knockout mice are generated that represent a loss of function model for the targeted gene. These mice are then assessed for Mendelian segregation of the targeted allele. If the targeted gene is not essential for embryonic development, homozygous animals will make up about one fourth of the offspring. Although this technique initially requires a high level of expertise, gene targeted deletions are done in many laboratories, and many institutions have core facilities that support the more difficult aspects of this process.

Information gained through gene-targeting experiments varies greatly. In cases where the gene serves an essential early function, the embryo often does not survive (77–79). Although these studies provide helpful information about embryogenesis, they have often hindered the analysis of the functional role of these genes in the adult animal. In a few cases, loss of only one allele was lethal, implying that the amount of protein was critical for normal development, a situation called the “gene dosage” effect (80,81). In contrast, targeted deletion studies of genes thought to be functionally important have frequently produced no

discernible phenotypic abnormalities (82). These results have been interpreted as evolutionary safeguards, or a functional redundancy, in molecular pathways of genes critical to survival of a species. However, careful scrutiny of animals with a normal phenotype under routine growth and maintenance conditions has shown abnormal responses to various environmental stimuli.

Among the numerous gene targeting studies, some of the most notable work has examined the functional importance of endothelial cell genes. Gene deletion studies of the vascular endothelial growth factor receptors, *flk1* and *flt1*, angiopoietin receptor *tek/tie2*, and the orphan *tie1* receptor have provided tremendous insight on the early developmental events associated with endothelial cell origin and/or proliferation such as vasculogenesis and angiogenesis (77–79). Much has been learned about the function of vascular endothelial-cadherin and various selectins in targeting studies (83,84).

DOUBLE KNOCKOUTS

Double gene knockout mice can be generated by breeding mice with different single-gene deletions and screening the progeny for both mutated genes. Alternatively, ES cells homozygous for one knockout can be targeted to a second locus with a construct that contains a different drug resistance gene (85). Double knockout animals have been developed with ApoE deficient mice as a genetic background to evaluate whether a second gene can modify the development or progression of atherosclerosis. This approach has been used to analyze genes such as monocyte chemoattractant protein-1 (MCP-1), E-selectin, and P-selectin. In addition, mice with multiply targeted genes have been used to determine the functional overlap of different genes and to unmask redundancies in specific signaling pathways. For example, the role of all three *src*-related genes (*fyn*, *yes*, and *src*) in cytoplasmic signaling was defined in combined targeting studies (86).

CRE/LOXP REGULATED GENE TARGETING

Many gene knockouts result in embryonic lethality, indicating that the gene plays a critical role in the development of the embryo. Although characterizing the defective phenotype indicates the functional requirement for the gene and encoded protein in embryogenesis, loss of the mutant animal before birth obviates investigation of the gene within the pathophysiological context of adulthood. In addition, compensatory mechanisms during development can potentially mask important and relevant phenotypes, and residual effects that linger into adulthood may alter responses to disease stimuli. Confining the cell types where the gene is inactivated or regulating the time

at which the gene deletion occurs may circumvent these limitations to gene targeting techniques.

DNA recombinases have been used to address these limitations. In the most extensively studied system in the mouse, the Cre recombinase is used because of its ability to manipulate DNA flanked by LoxP recognition sequences (87,88). The first step in generating a regulated or conditional mutation is to use homologous recombination to insert two LoxP recognition sites on either side of the sequence to be deleted (89,90). These sequences, when placed outside of the coding regions of the gene, do not appear to disturb transcription, RNA processing, or translation. The altered allele is then passed into the germline, as described above for generating knockout mice.

Recombination, which is achieved by breeding the LoxP mice to mice that express Cre in a temporal and/or cell-specific fashion, results in loss of the LoxP flanked sequence (91–93). Cre recognizes and cleaves the LoxP sites and joins the chromosomal ends to yield an allele that contains only a single residual LoxP site. Several methods are used to introduce Cre in a cell- or tissue-specific fashion (94). For example, cardiac- and endothelial cell-specific promoters have been used to drive restricted expression of Cre in these tissues (95). Alternatively, the Cre protein can be introduced into a tissue by infecting it with an adenovirus that expresses the recombinase (96). Finally, combining the Cre-LoxP system with an inducible gene expression system that exploits tetracycline regulatory elements can be used for cell-specific and inducible regulation of recombination (97,98).

Transgenesis

Transgenesis is the introduction of exogenous DNA into the genome of an animal such that it becomes part of the animal's germline. The DNA that is to be integrated into the germline typically contains a gene (usually in the form of a cDNA), a promoter to drive expression of the cDNA, and an untranslated sequence to direct correct polyadenylation of the synthetic transcript. The main techniques for generation of transgenic mice involve either microinjecting the desired DNA into the pronucleus of a fertilized egg or introducing the transgene directly into ES cells. Methods of ES cell manipulation are similar to those described for gene knockouts, with the exception that integration does not have to occur at a specific locus. Tissue-specific promoters can be used to drive expression of the transgene in particular cell types.

Transgenic animals have been widely used in gain-of-function experiments to determine the effects that overexpression or changes in the temporal or spatial

expression patterns have on the function of a targeted gene. For example, the keratin14 promoter has been used to express putative angiogenic factors in the skin, where vessel structure and function are easily observed *in vivo* (99). Lipoprotein lipase (LPL) expression has been targeted to different tissues and evaluated in an LPL-deficient setting (100,101) so that local function of the enzyme in tissues where it is normally expressed can be evaluated. Finally, transgenic animals have been used to decipher DNA sequences that direct cell-specific and developmental patterns of gene expression. In this approach, various portions of a non-coding sequence are paired with the native or heterologous promoter sequence upstream of a reporter gene whose transcription can be easily assessed (102).

A common problem seen in transgenic studies is that the level of transgene expression often depends on where it integrates in the genome. Although transgenes often integrate in large copy numbers, expression is low and variable because sufficient transcriptional regulatory elements are not included in the construct to direct site-independent expression. Variability in expression levels between different lines of mice is particularly frustrating when transgenic animals are used for *in vivo* mapping of cell-specific regulatory elements. The inclusion of introns into a construct increases the level of expression, and several constructs now contain introns from several highly expressed genes. Transgenic mice can be produced by microinjection of a large region of genomic DNA, often in the form of a BAC (103). In addition to the exons encoding the gene of interest, regulatory regions of the gene are included, which significantly increase the likelihood of obtaining adequate levels of expression.

Several investigators now use homologous recombination in ES cells to eliminate variable expression levels caused by integration of the transgene into different genomic sites. In this approach, an endogenous locus is specifically targeted for incorporation of the transgene construct (104). This approach results in not only a greater uniformity of expression between different lines of mice but also a higher level of expression. This method is more time consuming and requires more expertise than the usual technique of pronuclear injection. Currently under development is the use of homologous recombination in bacteria to genetically engineer large regions of DNA that can be microinjected into pronuclei for the rapid development of stable and highly expressed transgenes (105).

Regulated Transgene Expression

To provide experimentally regulated expression of foreign genes in transgenic animals, drug-inducible tran-

scriptional elements have been developed. The most widely used transcriptional element originates from the tetracycline response system (106). In the classical approach, tetracycline (or the potent analog doxycycline), which inhibits transcription of the transgene, is continuously supplied in the animal's drinking water (107). Gene expression is initiated, therefore, only upon removal of the drug. In this technique, two transcriptional units are used. The first contains a promoter that drives the expression of a regulatory protein that interacts with tetracycline, and when bound to the drug, does not allow it to bind and inhibit the transcription of a second promoter. This second promoter, under constant inhibition when tetracycline is present, drives the expression of the experimental gene. The most widely used method takes advantage of the ability of a cell-specific promoter to drive expression of the tetracycline operator protein, thus conferring both spatial and temporal regulation of gene expression. This paradigm, where presence of the drug inhibits transcription, is called a "tet-off" system. In a commonly used variant of this system, an operator protein, which can activate rather than inhibit transcription when bound to the drug, is used and forms the basis for a "tet-on" system. Both tet-on and tet-off approaches have drawbacks related to the leakiness of transgene expression in the repressed state. For both systems, each of the transcriptional units can be incorporated into individual lines of mice and then combined by breeding the mice, or both transcriptional units can be simultaneously injected with a single construct. These systems can be evaluated by breeding animals containing a regulatory transgene with a reporter mouse that has a tetracycline-regulated reporter gene. The expression of the reporter gene can then be measured in the presence or absence of the drug.

Animal Models of Atherosclerosis

HISTORICAL PERSPECTIVE

Although progress has been made in understanding the pathophysiology of atherosclerosis, the molecular mechanisms underlying atherosclerosis at its various stages are largely unknown, due in large part to the inaccessibility of human vascular diseased tissue to study. Mouse models of atherosclerosis provide access to the vessel wall during the progression of the disease process, and genetic manipulation of the mouse makes possible the study of interactions between the environment and the genetic factors that contribute to disease progression.

The mouse has traditionally been the species of choice for genetic manipulation and for the development of experimental disease models. Extensive early work on mouse reproduction and development and the

advanced status of mouse genetics have contributed to the use of the mouse model. Rodents have been used for the study of atherosclerosis because of our knowledge of their physiology, the feasibility of genetic manipulation, low housing costs, and the ease of maintenance. Their relative resistance to atherosclerosis along with their excessive mortality on high-fat diets originally discouraged many researchers from using rodent models. In addition, the distinct lipid profiles of mice contributed to the initial lack of interest in rodent models of atherosclerosis. As molecular techniques for genetic manipulation in mice improved, a small number of insightful researchers began to alter mice genetically by perturbing their lipid metabolism (108–110). Well-formulated questions could clearly be addressed by using the mouse model. Highly characterized knockout mice and transgenic mice have contributed greatly to the understanding of lipoprotein and inflammatory pathways and their role in atherosclerosis. These studies have identified a limited set of candidate genes that modulate the disease process at its various stages (111–113).

INBRED MICE

Mice, with typical cholesterol levels of less than 100 mg/dL, are highly resistant to atherosclerosis. In the 1980s, several researchers tried to produce atherosclerosis in mice to identify possible candidate genes (114–116). On high-fat, high-cholesterol diets, several strains of inbred mice, such as C57Bl/6, developed increased cholesterol levels and several layers of foam cells reminiscent of early atherosclerotic lesions (113,117). In contrast, other strains such as C3H/HeJ mice did not develop such lesions. Although an atherogenic diet decreased high-density lipoprotein (HDL) levels in the B6 strain, C3H mice were resistant to these changes (109). Crosses between susceptible and resistant strains were used to identify potential loci for genetic susceptibility (118). One well-studied locus was *Ath1*, which encoded for the second most abundant HDL apolipoprotein (ApoA-II) (119–121). The use of inbred mice to study atherosclerosis raised several critical issues. The histology and vascular distribution of the lesions were different from those found in humans (122). Furthermore, the atherogenic diet used in the mice was nonphysiologic and caused a chronic inflammation in C57Bl/6 mice (113,123); therefore, the development of lesions in some inbred mice could be attributed to diet-induced inflammation rather than atherosclerosis. Recent studies, however, suggest that endothelial cells, not inflammatory cells or plasma lipid levels, may contribute to the differences in susceptibility to atherosclerosis (124–127).

GENETIC MANIPULATION: ROLE OF CANDIDATE GENES IN DISEASE

Mouse models of atherosclerosis have become the most powerful experimental approach to study the roles of candidate genes *in vivo*. In the early 1990s, two laboratories used gene knockout technology to generate atherosclerosis-prone ApoE deficient mice (110,128). As a result, the popularity of mouse models of atherosclerosis has dramatically increased (112,113). The ApoE knockout mouse, used either as a single knockout or as the genetic background for a second gene mutation, is one of the most widely used models of atherosclerosis. The delayed clearance of certain lipoproteins in ApoE mice increases cholesterol levels. On a normal diet, these mice can develop atherosclerotic lesions spontaneously, and a high-fat diet increases the lesion size and the rate of disease progression (110,128). The time course for the development of atherosclerotic lesions and the various stages of the lesions are well characterized (129). Although complex lesions do not occur, the histological changes and regions of susceptibility are similar to those seen in humans.

ATHEROGENIC AND ANTIATHEROGENIC GENES

Several well-characterized transgenic and gene knockout mouse models have contributed greatly to the understanding of critical pathways in atherosclerosis. Several genes have been identified that either promote or prevent the disease process in these mouse models (Tables 1 and 2) (111,112). Endothelial injury and subsequent endothelial dysfunction may be an initiating event during atherosclerosis. The role of oxidative stress at initial stages of disease has been studied in several mouse models. A reduction in atherosclerosis was reported in 15-lipoxygenase (15-LO)/ApoE double knockout mice, which suggests a pro-atherogenic role of 15-LO as an oxidant-generating enzyme (130). Similar findings have been described for iNOS/ApoE double knockout mice (131), confirming the potent oxidative properties of inducible NOS. These oxidant-generating pathways may modify low-density lipoprotein (LDL) to its oxidized and highly atherogenic form. Degradation of the biologically active lipids within oxidized LDL may potentially reduce disease susceptibility. The finding of increased lesion formation in mice deficient in paroxonase, which degrades oxidized phospholipids, supports this theory (132–134). Hypertension contributes to endothelial injury. A marked increase in atherosclerotic lesions is seen in hypertensive mice lacking eNOS and ApoE genes (135,136). In contrast, atherosclerosis was not accelerated in hypertensive mice without hyperlipidemia (137). Finally, diabetic and hyperinsulinemic mice may be at risk for atherosclerosis (138,139).

Table 1
Candidate Atherogenic Genes Involved in Atherosclerosis

Gene (Ref)	Genetic background	Mouse model	Degree of atherosclerosis	Pathway
<i>C57B1/6</i> (109)	Wild type	Control	↑	Wild type
<i>iNOS</i> (131,167)	ApoE ^{-/-}	DKO	↓	Endothelial function
<i>12/15-LO</i> (130)	ApoE ^{-/-}	DKO	↓	Lipid metabolism
<i>12/15-LO</i> (168)	LDLR ^{-/-}	TG	↑	Lipid metabolism
<i>ApoAII</i> (169)	Wild type	TG	↑	Lipid metabolism
<i>ApoB48</i> (170)	ApoE ^{-/-}	TG/KO	↑	Lipid metabolism
<i>ApoB48</i> (171)	LDLR ^{-/-}	TG/KO	↑	Lipid metabolism
<i>ApoC1</i> (172)	Wild type	TG	↑	Lipid metabolism
<i>CETP</i> (164)	Wild type	TG	↑	Lipid metabolism
<i>Lp(a)</i> (173)	Wild type	TG	↑	Lipid metabolism
<i>SR-A</i> (149)	ApoE ^{-/-}	DKO	↓	Lipid metabolism
<i>CCR2</i> (147,174)	ApoE ^{-/-}	DKO	↓	Inflammation
<i>CD154</i> (175)	ApoE ^{-/-}	DKO	↓	Inflammation
<i>CXCR2</i> (148)	LDLR ^{-/-}	DKO	↓	Inflammation
<i>E selectin</i> (144)	LDLR ^{-/-}	DKO	↓	Inflammation
<i>P selectin</i> (144)	LDLR ^{-/-}	DKO	↓	Inflammation
<i>ICAM-1</i> (143)	ApoE ^{-/-}	DKO	↓	Inflammation
<i>MCP-1</i> (145)	ApoB+	KO/TG	↓	Inflammation
<i>MCP-1</i> (146)	LDLR ^{-/-}	DKO	↓	Inflammation
<i>MCP-1</i> (176)	ApoE ^{-/-}	DKO	↓	Inflammation
<i>MCSF</i> (141)	ApoE ^{-/-}	DKO	↓	Inflammation
<i>CD36</i> (150)	ApoE ^{-/-}	DKO	↓	Inflammation
<i>PPARA</i> (177)	ApoE ^{-/-}	DKO	↓	Insulin resistance

CCR2, chemokine (cc motif) receptor 2; CETP, cholesteryl ester transfer protein; DKO, double knockout; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; KO, knockout; LDLR, low density lipoprotein receptor; LO, lipoxygenase; Lp(a), Lipoprotein (a); MCP, monocyte chemotactic protein; MCSF, macrophage colony-stimulating factor; PPARA, peroxisome proliferator activated receptor α (alpha); SR-A, scavenger receptor A; TG, transgenic.

Recruitment of monocytes, their differentiation into macrophages, and progressive accumulation of oxidized LDL leads to the development of atherosclerotic lesions. Mice that lack the gene for macrophage colony stimulating factor are extremely resistant to atherosclerosis when bred onto an ApoE-deficient background (140,141), which confirms the critical role of macrophages in the development of disease. The recruitment of monocytes to the vascular wall is regulated by cell adhesion molecules that are expressed on endothelial cell surfaces. Several of these molecules may contribute to the progression of atherosclerosis. Monocyte recruitment to atherosclerotic lesions was significantly reduced in ApoE-deficient mice with an intracellular adhesion molecule-1 deficiency (142,143) or E or P selectin deficiency (144). Other studies have described the important

role of monocyte migration into the arterial wall. The extent of atherosclerotic lesions is markedly reduced in mice that lack the genes for MCP-1 (145,146) or its receptor (CCR2) (147). In addition, disruption of the CXCR2 gene for the IL-8 receptor significantly reduced disease burden (148).

The production of foam cells with fatty streak formation is the hallmark of atherosclerotic lesions. Uptake of oxidized LDL by macrophages is mediated by several different molecules. Lesion development is reduced in scavenger receptor A (SR-A) and CD36 knockout mice bred to an ApoE-deficient background, suggesting that recognition of oxidized LDL by scavenger receptors is important in the disease process (149,150).

The critical role of cholesterol efflux in the progression of atherosclerosis has been shown in several

Table 2
Candidate Antiatherogenic Genes Involved in Atherosclerosis

<i>Gene (Ref)</i>	<i>Genetic background</i>	<i>Mouse model</i>	<i>Degree of atherosclerosis</i>	<i>Pathway</i>
<i>C3H/HeJ</i> (109)	Wild type	Control	↓	
<i>eNOS</i> (135–137)	ApoE ^{−/−}	DKO	↓	Endothelial function
<i>ABC A1</i> (178)	Wild type	KO	? ↑	Lipid metabolism
<i>ABC A1</i> (179)	Wild type	TG	? ↓	Lipid metabolism
<i>ApoA1</i> (151)	ApoB+	KO/TG	↑	Lipid metabolism
<i>ApoA1</i> (180,181)	ApoE ^{−/−}	TG/KO	↓	Lipid metabolism
<i>ApoA1</i> (182)	LDLR ^{−/−}	TG/KO	↓	Lipid metabolism
<i>ApoB</i> 100 (170)	ApoE ^{−/−}	TG/KO	↓	Lipid metabolism
<i>ApoE</i> (110,128)	Wild type	KO	↑	Lipid metabolism
<i>LDLR</i> (183)	ApoE ^{−/−}	DKO	↑	Lipid metabolism
<i>LDLR</i> (108)	Wild type	KO	↓	Lipid metabolism
<i>LIPC</i> (184)	Wild type	KO	↑	Lipid metabolism
<i>LPL</i> (185)	Wild type	KO	? ↑	Lipid metabolism
<i>PON1</i> (186,187)	ApoE ^{−/−}	DKO	↑	Lipid metabolism
<i>SR-B1</i> (165)	LDLR ^{−/−}	DKO	↑	Lipid metabolism
<i>SR-B1</i> (166)	LDLR ^{−/−}	TG/KO	↓	Lipid metabolism
<i>IRS-1</i> (138)	Wild type	KO	? ↑	Insulin resistance
<i>PPARG</i> (188)	LDLR ^{−/−}	DKO	↑	Insulin resistance
<i>IL-10</i> (189)	Wild type	KO	↑	Inflammation

ABC, ATP-binding cassette transporter; DKO, double knockout; eNOS, endothelial nitric oxide synthase; IL-10, interleukin-10; IRS-1, insulin receptor substrate-1; KO, knockout; LDLR, low density lipoprotein receptor; LIPC, hepatic lipase; LPL, lipoprotein lipase; PON1, paraxonase 1; PPARG, peroxisome proliferator activated receptor γ (gamma); SR-B1, scavenger receptor B1; TG, transgenic.

studies. HDL is important in “reverse cholesterol transport,” thus explaining the inverse correlation between HDL and the risk of atherosclerosis. Deletion of the ApoA-1 gene, which encodes for the major protein component of HDL, causes severe atherosclerosis in ApoB transgenic mice (151). Other molecules important in cholesterol transport modulate atherosclerosis, including ACAT-1 (152–154), ABC-1 (ATP binding cassette family of transporters) (155–160), lecithin-chol acyltransferase (161,162), cholesteryl ester transfer protein (163,164), and SR-B1 (HDL receptor in liver) (165,166). Studies in genetically manipulated mice have indicated that several other families of genes, such as the nuclear receptors PPAR- α and LXRs, and genes involved in cellular immunity such as interleukin-4, interleukin-10, and interferon- γ , contribute to the progression of atherosclerotic lesions (112). Many new genes will likely be identified as important modulators of atherosclerosis in these same animal models.

POTENTIAL DISADVANTAGES OF MOUSE MODELS OF ATHEROSCLEROSIS

The mouse model for atherosclerosis has some disadvantages. Mice have different lipid profiles and cardiac physiology than humans, and vascular manipulation is often difficult because of their small size. In addition, atherosclerosis in mice is a relatively rapid process, whereas in humans, it is slow and progressive. Although increases in cholesterol are extremely high in most mouse models, the model best suited for studying atherosclerosis in humans has not been identified. Distal coronary disease and evidence of complications from ischemia are not usually seen in mice. Furthermore, thrombotic events that cause myocardial infarction are not usually found in the mouse model. Although the ApoE/eNOS double knockout mouse addresses some of these shortcomings (135,136), many other differences in the disease process between mice and humans cannot be resolved. The extent to which findings in the mouse model can be extrapolated to humans is unknown.

FUTURE DIRECTIONS

Recent advances in high-throughput genomics technology offer a novel, systematic approach to study the expression of thousands of genes simultaneously. This technology can be used to study differential gene expression in different strains of mice that are either resistant or susceptible to atherosclerosis. In addition, these methods can be used to determine genome-wide responses of cellular components of the vascular wall (i.e., smooth muscle cells, endothelial cells, and macrophages) to atherogenic stimuli or therapeutic interventions. Moreover, the different patterns of gene expression during various stages of development of the disease can be studied in a mouse model of cardiovascular disease, an approach that cannot be replicated in humans. New candidate genes may be identified from studies of temporal patterns of gene expression. The use of animal models in combination with emerging genomic and proteomic technologies should accelerate the pace of discovery for the molecular determinants of cardiovascular disease and offer direct benefits for humans in the future.

REFERENCES

- Strachan T, Read AP. Human Molecular Genetics. 2nd ed. New York: John Wiley & Sons, 1999:576.
- Grompe M, Johnson W, Jameson JL. Recombinant DNA and genetic techniques. In: Jameson JL, Collins FS, eds. Principles of Molecular Medicine. Totowa, NJ: Humana Press, 1998: 9–24.
- Janssen K. Analysis of RNA by Northern and slot blot hybridization. In: Ausubel FM, Brent R, Kingston RE, et al., eds. Current Protocols in Molecular Biology. New York: Greene Publishing and John Wiley & Sons, 1994: 4.9.1–4.9.11.
- Muyrers JP, Zhang Y, Stewart AF. Techniques: Recombinogenic engineering—new options for cloning and manipulating DNA. Trends Biochem Sci 2001;26:325–331.
- Monaco AP, Larin Z. YACs, BACs, PACs and MACs: artificial chromosomes as research tools. Trends Biotechnol 1994; 12:280–286.
- Burke DT, Carle GF, Olson MV. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 1987;236:806–812.
- Schlessinger D. Yeast artificial chromosomes: tools for mapping and analysis of complex genomes. Trends Genet 1990; 6:248,255–258.
- Shepherd NS, Smoller D. The P1 vector system for the preparation and screening of genomic libraries. Genet Eng (N Y) 1994;16:213–228.
- Sternberg NL. Cloning high molecular weight DNA fragments by the bacteriophage P1 system. Trends Genet 1992;8:11–16.
- Ioannou PA, Amemiya CT, Garnes J, et al. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nat Genet 1994;6:84–89.
- Shizuya H, Birren B, Kim UJ, et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc Natl Acad Sci USA 1992;89:8794–8797.
- Schein CH. Optimizing protein folding to the native state in bacteria. Curr Opin Biotechnol 1991;2:746–750.
- Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 1981;23:175–182.
- Seed B, Parker RC, Davidson N. Representation of DNA sequences in recombinant DNA libraries prepared by restriction enzyme partial digestion. Gene 1982;19:201–209.
- Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science 2001;291:1304–1351.
- Hirata K, Dichek HL, Cioffi JA, et al. Cloning of a unique lipase from endothelial cells extends the lipase gene family. J Biol Chem 1999;274:14170–14175.
- Hirata K, Ishida T, Penta K, et al. Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. J Biol Chem 2001;276:16223–16231.
- Dogan Temizer PLH, Shi-Chung Ng, Quertermous T. Molecular cloning strategies. In: Fozzard HA, ed. The Heart and Cardiovascular System. New York: Raven Press, 1992:561–579.
- Sasaki K, Yamano Y, Bardhan S, et al. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. Nature 1991;351:230–233.
- Lin HY, Wang XF, Ng-Eaton E, Weinberg RA, Lodish HF. Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. Cell 1992;68:775–785.
- D'Andrea AD, Lodish HF, Wong GG. Expression cloning of the murine erythropoietin receptor. Cell 1989;57:277–285.
- Franchi A, Perucca-Lostanlen D, Pouyssegur J. Functional expression of a human Na⁺/H⁺ antiporter gene transfected into antiporter-deficient mouse L cells. Proc Natl Acad Sci USA 1986;83:9388–9392.
- Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. Nature 1996;380:364–366.
- Sambrook J, Russell DW. Preparation of radiolabeled DNA and RNA probes. Molecular cloning. A Laboratory Manual, vol. 2. New York: Cold Spring Harbor Laboratory Press, 2001:9.4–9.75.
- Zinn K, DiMaio D, Maniatis T. Identification of two distinct regulatory regions adjacent to the human beta-interferon gene. Cell 1983;34:865–879.
- Myers RM, Larin Z, Maniatis T. Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 1985;230:1242–1246.
- Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res 1984;12:7035–56.
- Calzone FJ, Britten RJ, Davidson EH. Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. Methods Enzymol 1987;152:611–632.
- Temizer D, Huang PL, Ng S-C, Quertermous T. Molecular cloning strategies. In: Fozzard HA, et al., eds. The Heart and Cardiovascular System. New York: Raven Press, 1992: 561–579.
- Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Recombinant DNA and genomics. Molecular Cell Biology. New York: W. H. Freeman, 1999:207–253.
- Sambrook J, Russell DW. In vitro amplification of DNA by polymerase chain reaction. Molecular cloning. A Laboratory Manual, vol. 2. New York: Cold Spring Harbor Laboratory Press, 2001:8.18–8.106.
- Murphy LD, Herzog CE, Rudick JB, Fojo AT, Bates SE. Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. Biochemistry 1990;29:10351–10356.

33. Siebert PD, Larrick JW. Competitive PCR. *Nature* 1992; 359:557–558.
34. Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* 1990;87:2725–2729.
35. Tsai SJ, Wiltbank MC. Quantification of mRNA using competitive RT-PCR with standard-curve methodology. *Biotechniques* 1996;21:862–866.
36. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986–994.
37. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* 1991;88:7276–7280.
38. Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination. *Nat Biotechnol* 1998;16:49–53.
39. Wilkinson DG. The theory and practice of *in situ* hybridization. In: Wilkinson DG, ed. *in situ* Hybridization. A Practical Approach. New York: Oxford University Press, 1998.
40. Hidai C, Zupancic T, Penta K, et al. Cloning and characterization of developmental endothelial locus-1: an embryonic endothelial cell protein that binds the α v β 3 integrin receptor. *Genes Dev* 1998;12:21–33.
41. Kadonaga JT, Tjian R. Affinity purification of sequence-specific DNA binding proteins. *Proc Natl Acad Sci USA* 1986;83:5889–5893.
42. Schlaeger TM, Qin Y, Fujiwara Y, Magram J, Sato TN. Vascular endothelial cell lineage-specific promoter in transgenic mice. *Development* 1995;121:1089–1098.
43. Fadel BM, Boutet SC, Quertermous T. Octamer-dependent *in vivo* expression of the endothelial cell-specific TIE2 gene. *J Biol Chem* 1999;274:20376–20383.
44. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature* 2001;409:860–921.
45. Lockhart DJ, Winzler EA. Genomics, gene expression and DNA arrays. *Nature* 2000;405:827–836.
46. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;270:467–470.
47. Schulze A, Downward J. Navigating gene expression using microarrays—a technology review. *Nat Cell Biol* 2001; 3:E190–E195.
48. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000;403:503–511.
49. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000;406:747–752.
50. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001;98:10869–10874.
51. van 't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530–536.
52. Spellman PT, Sherlock G, Zhang MQ, et al. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol Biol Cell* 1998;9:3273–3297.
53. Haley KJ, Lilly CM, Yang JH, et al. Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* 2000;102:2185–2189.
54. Stanton LW, Garrard LJ, Damm D, et al. Altered patterns of gene expression in response to myocardial infarction. *Circ Res* 2000;86:939–945.
55. Yang J, Moravec CS, Sussman MA, et al. Decreased SLIM1 expression and increased gelsolin expression in failing human hearts measured by high-density oligonucleotide arrays. *Circulation* 2000;102:3046–3052.
56. McCormick SM, Eskin SG, McIntire LV, et al. DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc Natl Acad Sci USA* 2001;98:8955–8960.
57. Hossain MA, Bouton CM, Pevsner J, Laterra J. Induction of vascular endothelial growth factor in human astrocytes by lead. Involvement of a protein kinase C/activator protein-1 complex-dependent and hypoxia-inducible factor 1-independent signaling pathway. *J Biol Chem* 2000;275:27874–27882.
58. Zhang S, Day IN, Ye S. Microarray analysis of nicotine-induced changes in gene expression in endothelial cells. *Physiol Genomics* 2001;5:187–92.
59. Chen BP, Li YS, Zhao Y, et al. DNA microarray analysis of gene expression in endothelial cells in response to 24-h shear stress. *Physiol Genomics* 2001;7:55–63.
60. Adams LD, Geary RL, McManus B, Schwartz SM. A comparison of aorta and vena cava medial message expression by cDNA array analysis identifies a set of 68 consistently differentially expressed genes, all in aortic media. *Circ Res* 2000;87:623–631.
61. Dunn MJ. Studying heart disease using the proteomic approach. *Drug Discov Today* 2000;5:76–84.
62. Wilkins MR, Sanchez JC, Williams KL, Hochstrasser DF. Current challenges and future applications for protein maps and post-translational vector maps in proteome projects. *Electrophoresis* 1996;17:830–838.
63. Arrell DK, Neverova I, Van Eyk JE. Cardiovascular proteomics: evolution and potential. *Circ Res* 2001;88:763–773.
64. Klose J, Kobalz U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* 1995;16:1034–1059.
65. Banks RE, Dunn MJ, Hochstrasser DF, et al. Proteomics: new perspectives, new biomedical opportunities. *Lancet* 2000; 356:1749–1756.
66. Ostergaard M, Rasmussen HH, Nielsen HV, et al. Proteome profiling of bladder squamous cell carcinomas: identification of markers that define their degree of differentiation. *Cancer Res* 1997;57:4111–4117.
67. Pleissner KP, Soding P, Sander S, et al. Dilated cardiomyopathy-associated proteins and their presentation in a WWW-accessible two-dimensional gel protein database. *Electrophoresis* 1997; 18:802–808.
68. Syvanen AC. Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet* 2001;2:930–942.
69. Risch NJ. Searching for genetic determinants in the new millennium. *Nature* 2000;405:847–856.
70. Roses AD. Pharmacogenetics and the practice of medicine. *Nature* 2000;405:857–865.
71. Kwok PY. Methods for genotyping single nucleotide polymorphisms. *Annu Rev Genomics Hum Genet* 2001;2:235–258.
72. Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci USA* 1995; 92:1851–1855.

73. Margaglione M, Cappucci G, Colaizzo D, et al. The PAI-1 gene locus 4G/5G polymorphism is associated with a family history of coronary artery disease. *Arterioscler Thromb Vasc Biol* 1998;18:152–156.
74. Moatti D, Faure S, Fumeron F, et al. Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blood* 2001;97:1925–1928.
75. Ranade K, Wu KD, Risch N, et al. Genetic variation in aldosterone synthase predicts plasma glucose levels. *Proc Natl Acad Sci USA* 2001;98:13219–13224.
76. Ramirez-Solis R, Davis AC, Bradley A. Gene targeting in embryonic stem cells. In: Wassarman PM, DePamphilis ML, eds. *Guide to Techniques in Mouse Development*. San Diego: Academic Press, 1993:855–878.
77. Fong G, Rossant J, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995;376:66–70.
78. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 1995;376:70–74.
79. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–66.
80. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;380:439–442.
81. Carmeliet C, Ferreira V, Brier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–439.
82. Hynes RO. Targeted mutations in cell adhesion genes: what have we learned from them? *Dev Biol* 1996;180:402–412.
83. Carmeliet P, Lampugnani MG, Moons L, et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999;98:147–157.
84. Bischoff J. Cell adhesion and angiogenesis. *J Clin Invest* 1997;100:S37–S39.
85. te Riele H, Maandag ER, Clarke A, Hooper M, Berns A. Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. *Nature* 1990;348:649–651.
86. Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P. Src family kinases are required for integrin but not PDGFR signal transduction. *Embo J* 1999;18:2459–2471.
87. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* 1988;85:5166–5170.
88. Sauer B, Henderson N. Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. *Nucleic Acids Res* 1989;17:147–161.
89. Odell J, Caimi P, Sauer B, Russell S. Site-directed recombination in the genome of transgenic tobacco. *Mol Gen Genet* 1990;223:369–378.
90. Sauer B, Henderson N. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol* 1990;2:441–449.
91. Lewandoski M, Martin GR. Cre-mediated chromosome loss in mice. *Nat Genet* 1997;17:223–225.
92. Lewandoski M, Wassarman KM, Martin GR. Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Curr Biol* 1997;7:148–151.
93. Brocard J, Warot X, Wendling O, et al. Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc Natl Acad Sci USA* 1997;94:14559–14563.
94. Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. *Development* 2000;127:1607–1616.
95. Isermann B, Hendrickson SB, Zogg M, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. *J Clin Invest* 2001;108:537–546.
96. Rijnsels M, Rosen JM. Adenovirus-Cre-mediated recombination in mammary epithelial early progenitor cells. *J Cell Sci* 2001;114:3147–3153.
97. Sohal DS, Nghiem M, Crackower MA, et al. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* 2001;89:20–25.
98. St-Onge L, Furth PA, Gruss P. Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* 1996;24:3875–3877.
99. Suri C, McClain J, Thurston G, et al. Increased vascularization in mice overexpressing angiopoietin-1. *Science* 1998;282:468–471.
100. Levak-Frank S, Hofmann W, Weinstock PH, et al. Induced mutant mouse lines that express lipoprotein lipase in cardiac muscle, but not in skeletal muscle and adipose tissue, have normal plasma triglyceride and high-density lipoprotein-cholesterol levels. *Proc Natl Acad Sci USA* 1999;96:3165–3170.
101. Levak-Frank S, Weinstock PH, Hayek T, et al. Induced mutant mice expressing lipoprotein lipase exclusively in muscle have subnormal triglycerides yet reduced high density lipoprotein cholesterol levels in plasma. *J Biol Chem* 1997;272:17182–17190.
102. Boutet SC, Quertermous T, Fadel BM. Identification of an octamer element required for in vivo expression of the TIE1 gene in endothelial cells. *Biochem J* 2001;360:23–29.
103. Pennacchio LA, Olivier M, Hubacek JA, et al. An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. *Science* 2001;294:169–173.
104. Guillot PV, Liu L, Kuivenhoven JA, Guan J, Rosenberg RD, Aird WC. Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression. *Physiol Genomics* 2000;2:77–83.
105. Copeland NG, Jenkins NA, Court DL. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* 2001;2:769–779.
106. St-Onge L, Furth PA, Gruss P. Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* 1996;24:3875–3877.
107. Freundlieb S, Baron U, Bonin AL, Gossen M, Bujard H. Use of tetracycline-controlled gene expression systems to study mammalian cell cycle. *Methods Enzymol* 1997;283:159–173.
108. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 1993;92:883–893.
109. Ishida BY, Blanche PJ, Nichols AV, Yashar M, Paigen B. Effects of atherogenic diet consumption on lipoproteins in mouse strains C57BL/6 and C3H. *J Lipid Res* 1991;32:559–568.
110. Plump AS, Smith JD, Hayek T, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992;71:343–353.

111. Lusis AJ. Atherosclerosis. *Nature* 2000;407:233–241.
112. Glass CK, Witztum JL. Atherosclerosis. The road ahead. *Cell* 2001;104:503–516.
113. Breslow JL. Mouse models of atherosclerosis. *Science* 1996;272:685–688.
114. Paigen B, Morrow A, Brandon C, Mitchell D, Holmes P. Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis* 1985;57:65–73.
115. Paigen B, Ishida BY, Verstuyft J, Winters RB, Albee D. Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. *Arteriosclerosis* 1990;10:316–323.
116. Nishina PM, Wang J, Toyofuku W, Kuypers FA, Ishida BY, Paigen B. Atherosclerosis and plasma and liver lipids in nine inbred strains of mice. *Lipids* 1993;28:599–605.
117. Paigen B, Plump AS, Rubin EM. The mouse as a model for human cardiovascular disease and hyperlipidemia. *Curr Opin Lipidol* 1994;5:258–264.
118. Paigen B, Albee D, Holmes PA, Mitchell D. Genetic analysis of murine strains C57BL/6J and C3H/HeJ to confirm the map position of Ath-1, a gene determining atherosclerosis susceptibility. *Biochem Genet* 1987;25:501–511.
119. Paigen B, Mitchell D, Reue K, Morrow A, Lusis AJ, LeBoeuf RC. Ath-1, a gene determining atherosclerosis susceptibility and high density lipoprotein levels in mice. *Proc Natl Acad Sci USA* 1987;84:3763–3767.
120. Mehrabian M, Qiao JH, Hyman R, Ruddell D, Laughton C, Lusis AJ. Influence of the apoA-II gene locus on HDL levels and fatty streak development in mice. *Arterioscler Thromb* 1993;13:1–10.
121. LeBoeuf RC, Doolittle MH, Montcalm A, Martin DC, Reue K, Lusis AJ. Phenotypic characterization of the Ath-1 gene controlling high density lipoprotein levels and susceptibility to atherosclerosis. *J Lipid Res* 1990;31:91–101.
122. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 1987;68:231–240.
123. Plump A. Atherosclerosis and the mouse: a decade of experience. *Ann Med* 1997;29:193–198.
124. Shi W, Haberland ME, Jien ML, Shih DM, Lusis AJ. Endothelial responses to oxidized lipoproteins determine genetic susceptibility to atherosclerosis in mice. *Circulation* 2000;102:75–81.
125. Shi W, Wang NJ, Shih DM, Sun VZ, Wang X, Lusis AJ. Determinants of atherosclerosis susceptibility in the C3H and C57BL/6 mouse model: evidence for involvement of endothelial cells but not blood cells or cholesterol metabolism. *Circ Res* 2000;86:1078–1084.
126. Grimsditch DC, Penfold S, Latham J, Vidgeon-Hart M, Groot PH, Benson GM. C3H apoE(–/–) mice have less atherosclerosis than C57BL apoE(–/–) mice despite having a more atherogenic serum lipid profile. *Atherosclerosis* 2000;151:389–397.
127. Van Lenten BJ, Prieve J, Navab M, Hama S, Lusis AJ, Fogelman AM. Lipid-induced changes in intracellular iron homeostasis in vitro and in vivo. *J Clin Invest* 1995;95:2104–2110.
128. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992;258:468–471.
129. Napoli C, Palinski W, Di Minno G, D’Armiento FP. Determination of atherogenesis in apolipoprotein E-knockout mice. *Nutr Metab Cardiovasc Dis* 2000;10:209–215.
130. Cyrus T, Witztum JL, Rader DJ, et al. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *J Clin Invest* 1999;103:1597–1604.
131. Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL. Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E-knockout mice. *Circulation* 2001;103:3099–3104.
132. Shih DM, Gu L, Xia YR, et al. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* 1998;394:284–287.
133. Shih DM, Xia YR, Wang XP, et al. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem* 2000;275:17527–17535.
134. Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. *Chem Biol Interact* 1999;119–120:429–438.
135. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N. Enhanced atherosclerosis and kidney dysfunction in eNOS(–/–)ApoE(–/–) mice are ameliorated by enalapril treatment. *J Clin Invest* 2000;105:451–458.
136. Kuhlencordt PJ, Gyurko R, Han F, et al. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 2001;104:448–454.
137. Shesely EG, Maeda N, Kim HS, et al. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1996;93:13176–13181.
138. Abe H, Yamada N, Kamata K, et al. Hypertension, hypertriglyceridemia, and impaired endothelium-dependent vascular relaxation in mice lacking insulin receptor substrate-1. *J Clin Invest* 1998;101:1784–1788.
139. Schreyer SA, Wilson DL, LeBoeuf RC. C57BL/6 mice fed high fat diets as models for diabetes-accelerated atherosclerosis. *Atherosclerosis* 1998;136:17–24.
140. Smith HO, Anderson PS, Kuo DY, et al. The role of colony-stimulating factor 1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. *Clin Cancer Res* 1995;1:313–325.
141. Qiao JH, Tripathi J, Mishra NK, et al. Role of macrophage colony-stimulating factor in atherosclerosis: studies of osteopetrotic mice. *Am J Pathol* 1997;150:1687–1699.
142. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med* 2000;191:189–194.
143. Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL. ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(–/–)/ICAM-1(–/–)) fed a fat or a chow diet. *Arterioscler Thromb Vasc Biol* 2000;20:2630–2635.
144. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest* 1998;102:145–152.
145. Gosling J, Slaymaker S, Gu L, et al. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* 1999;103:773–778.
146. Gu L, Okada Y, Clinton SK, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 1998;2:275–281.
147. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2(–/–) mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 1998;394:894–897.

148. Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J Clin Invest* 1998;101:353–363.
149. Suzuki H, Kurihara Y, Takeya M, et al. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* 1997;386:292–296.
150. Febbraio M, Podrez EA, Smith JD, et al. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J Clin Invest* 2000;105:1049–1056.
151. Voyiaki E, Goldberg IJ, Plump AS, Rubin EM, Breslow JL, Huang LS. ApoA-I deficiency causes both hypertriglyceridemia and increased atherosclerosis in human apoB transgenic mice. *J Lipid Res* 1998;39:313–321.
152. Yagyu H, Kitamine T, Osuga J, et al. Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *J Biol Chem* 2000;275:21324–21330.
153. Buhman KF, Accad M, Farese RV. Mammalian acyl-CoA:cholesterol acyltransferases. *Biochim Biophys Acta* 2000;1529:142–154.
154. Accad M, Smith SJ, Newland DL, et al. Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. *J Clin Invest* 2000;105:711–719.
155. Rust S, Rosier M, Funke H, et al. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet* 1999;22:352–355.
156. Drobnik W, Lindenthal B, Lieser B, et al. ATP-binding cassette transporter A1 (ABCA1) affects total body sterol metabolism. *Gastroenterology* 2001;120:1203–1211.
157. Brooks-Wilson A, Marcil M, Clee SM, et al. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet* 1999;22:336–345.
158. Bodzioch M, Orso E, Klucken J, et al. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet* 1999;22:347–351.
159. Lawn RM, Wade DP, Garvin MR, et al. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J Clin Invest* 1999;104:R25–R31.
160. Marcil M, Brooks-Wilson A, Clee SM, et al. Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet* 1999;354:1341–1346.
161. Ng DS, Francone OL, Forte TM, Zhang J, Haghighpassand M, Rubin EM. Disruption of the murine lecithin:cholesterol acyltransferase gene causes impairment of adrenal lipid delivery and up-regulation of scavenger receptor class B type I. *J Biol Chem* 1997;272:15777–15781.
162. Lambert G, Sakai N, Vaisman BL, et al. Analysis of glomerulosclerosis and atherosclerosis in lecithin cholesterol acyltransferase-deficient mice. *J Biol Chem* 2001;276:15090–15098.
163. Plump AS, Masucci-Magoulas L, Bruce C, Bisgaier CL, Breslow JL, Tall AR. Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arterioscler Thromb Vasc Biol* 1999;19:1105–1110.
164. Marotti KR, Castle CK, Boyle TP, Lin AH, Murray RW, Melchior GW. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature* 1993;364:73–75.
165. Huszar D, Varban ML, Rinninger F, et al. Increased LDL cholesterol and atherosclerosis in LDL receptor-deficient mice with attenuated expression of scavenger receptor B1. *Arterioscler Thromb Vasc Biol* 2000;20:1068–1073.
166. Kozarsky KF, Donahee MH, Glick JM, Krieger M, Rader DJ. Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. *Arterioscler Thromb Vasc Biol* 2000;20:721–727.
167. Niu XL, Yang X, Hoshiai K, et al. Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions. *Circulation* 2001;103:1115–1120.
168. Harats D, Shaish A, George J, et al. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2000;20:2100–2105.
169. Warden CH, Hedrick CC, Qiao JH, Castellani LW, Lusis AJ. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science* 1993;261:469–472.
170. Veniant MM, Pierotti V, Newland D, et al. Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *J Clin Invest* 1997;100:180–188.
171. Farese RV, Jr., Veniant MM, Cham CM, et al. Phenotypic analysis of mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *Proc Natl Acad Sci USA* 1996; 93:6393–6398.
172. Shachter NS, Ebara T, Ramakrishnan R, et al. Combined hyperlipidemia in transgenic mice overexpressing human apolipoprotein C1. *J Clin Invest* 1996;98:846–855.
173. Lawn RM, Wade DP, Hammer RE, Chiesa G, Verstuyft JG, Rubin EM. Atherogenesis in transgenic mice expressing human apolipoprotein(a). *Nature* 1992;360:670–672.
174. Dawson TC, Kuziel WA, Osahar TA, Maeda N. Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* 1999;143:205–211.
175. Lutgens E, Gorelik L, Daemen MJ, et al. Requirement for CD154 in the progression of atherosclerosis. *Nat Med* 1999;5:1313–1316.
176. Aiello RJ, Bourassa PA, Lindsey S, et al. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 1999;19:1518–1525.
177. Tordjman K, Bernal-Mizrachi C, Zemany L, et al. PPARalpha deficiency reduces insulin resistance and atherosclerosis in apoE-null mice. *J Clin Invest* 2001;107:1025–1034.
178. Christiansen-Weber TA, Volland JR, Wu Y, et al. Functional loss of ABCA1 in mice causes severe placental malformation, aberrant lipid distribution, and kidney glomerulonephritis as well as high-density lipoprotein cholesterol deficiency. *Am J Pathol* 2000;157:1017–1029.
179. Singaraja RR, Bocher V, James ER, et al. Human ABCA1 BAC transgenic mice show increased high density lipoprotein cholesterol and ApoAI-dependent efflux stimulated by an internal promoter containing liver X receptor response elements in intron 1. *J Biol Chem* 2001;276:33969–33979.
180. Benoit P, Emmanuel F, Caillaud JM, et al. Somatic gene transfer of human ApoA-I inhibits atherosclerosis progression in mouse models. *Circulation* 1999;99:105–110.
181. Plump AS, Scott CJ, Breslow JL. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci USA* 1994;91:9607–9611.

182. Tangirala RK, Tsukamoto K, Chun SH, Usher D, Pure E, Rader DJ. Regression of atherosclerosis induced by liver-directed gene transfer of apolipoprotein A-I in mice. *Circulation* 1999; 100:1816–1822.
183. Ishibashi S, Herz J, Maeda N, Goldstein JL, Brown MS. The two-receptor model of lipoprotein clearance: tests of the hypothesis in “knockout” mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci USA* 1994;91:4431–4435.
184. Homanics GE, de Silva HV, Osada J, et al. Mild dyslipidemia in mice following targeted inactivation of the hepatic lipase gene. *J Biol Chem* 1995;270:2974–2980.
185. Weinstock PH, Bisgaier CL, Aalto-Setälä K, et al. Severe hypertriglyceridemia, reduced high density lipoprotein, and neonatal death in lipoprotein lipase knockout mice. Mild hypertriglyceridemia with impaired very low density lipoprotein clearance in heterozygotes. *J Clin Invest* 1995;96:2555–2568.
186. Durrington PN, Mackness B, Mackness MI. Paraoxonase and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001;21:473–480.
187. Mackness B, Davies GK, Turkie W, et al. Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? *Arterioscler Thromb Vasc Biol* 2001;21:1451–1457.
188. Chawla A, Boisvert WA, Lee CH, et al. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell* 2001;7:161–171.
189. Pinderski Oslund LJ, Hedrick CC, Olvera T, et al. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 1999;19:2847–2853.



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