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4 Biotechnology and Medicine

Genomic technologies and computational advances are leading to an information revolution in biology and medicine. Simulations of molecular processes in cells and predictions of drug effects in humans will advance pharmaceutical research and speed-up clinical trials. Computational prognostics and diagnostics that combine clinical data with genotyping and molecular profiling will soon cause fundamental changes in the practice of health care.

Computational elucidation of protein structure and function, genotyping of individual susceptibilities, and simulation of cellular and organismal processes will lead to a 'personalized medicine'.[154]

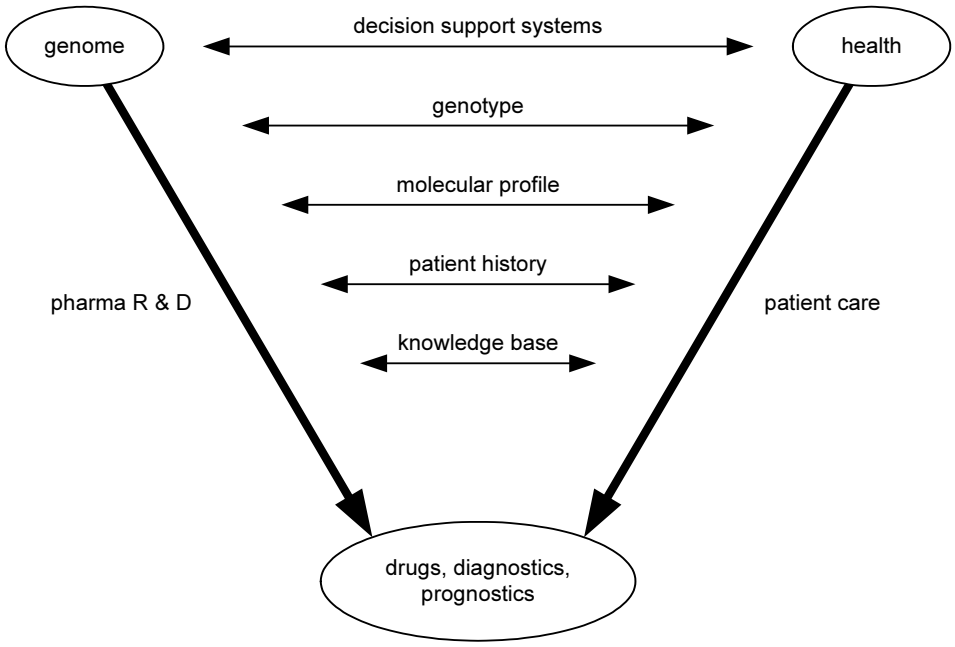

Figure 4.1. Genomic and computational technologies have an increased impact on pharmaceutical R & D (arrow at left). The same technologies will be adapted to directly serve patients (arrow right), leading to personalized, information-driven medical care [Refs in 154].

4.1 Diagnostics

The expanding availability of information on genetic sequences, polymorphisms, SNPs, protein variations and interactions, metabolic and phenotypical variants as well as the sensor technologies plus micro- and nanotechnologies in combination with the communication network spanning the globe will allow increasingly precise measurements of the:

- Susceptibilities for disease
- Strengths of genetic and metabolic networks
- Status of vital organs and tissues
- Regimen of pharmaceuticals and therapies
- Developments in surgery and implantation

Diagnostics is an essential tool for providing basic data for medical therapies, and biological and industrial processes, e.g., process control, and food and environmental monitoring. One of the first biomedical applications is the measurement of blood glucose in diabetics. Due to the expanding numbers of diabetics (projected to increase from 175 million persons in 2000 to 239 million afflicted in 2010 according to World Health Organization data), this is a large and still growing diagnostic market segment.

The sensitivity and versatility of diagnostic devices is increasing due to refinements in established technologies such as enzymatic assays and enzyme-linked immunoassays, the ongoing expansion of the analytical repertoire to include DNA and RNA diagnostics such as PCR, and physico-chemical analytics such as MALDI-TOF, affinity sensors and techniques such as surface plasmon resonance (SPR). Neural network software is being applied to assess complex mixtures such as in olfactory analyses. The technique of molecular imprinting is enabling the design of molecule-specific assays, separations and sensors.[501]

Table 4.1 lists typical examples of molecular imprinting in design and applications from amino acids, pharmaceuticals, herbicides, and chemicals to proteins, steroids and alkaloids.

4.2 Therapeutics

Immunotherapy is the focus of significant efforts in the development of cancer therapies. Several approaches have tried to create tumor cell vaccines for cancer treatment. An effective cancer cell vaccine is created by expressing MHC class II molecules without the invariant chain protein (Ii) that normally blocks the antigenic peptide-binding site of MHC class II molecules at their synthesis in the endoplasmic reticulum. Such cell–cell constructs are created either by the transfer of genes for MHC class II α and β chains or by the induction of MHC class II molecules and Ii protein with a transacting factor, followed by Ii suppression using antisense methods. Preclinical validation of this approach has been reviewed with the goal of using this immunotherapy for metastatic human cancers.[155]

Table 4.1. Typical examples of MIPS design and application [Refs in 501].

Template	Application	Refs
Amino acids and amino acid derivatives	Separation and binding	6
	Synthesis	7
	Assay and sensors	8
Aniline, phenol and their derivatives	Assay and sensors	9
Drugs	Separation and binding	10
	Assay and sensors	11, 12
Gases and vapors	Sensors	13
Herbicides	Separation and binding	14
	Assay and sensors	15, 16
Heterocycles	Separation and binding	17, 18
Metal ions	Separation and binding	19
	Assays and sensors	20
Micro-organisms	Separation and binding	21
Nucleic acids and nucleic acid derivatives	Separation and binding	22
	Assays and sensors	23
Pokynuclear aromatic hydrocarbons	Separation and binding	24
	Assays and sensors	25
Proteins	Separation and binding	26
Steroids	Separation and binding	27, 28
	Detection	29
Sugars and sugar derivatives	Separation and binding	1
	Assays and sensors	30
Alkaloids, toxins and narcotics	Separation and binding	4
	Assays and sensors	31

A novel way of approaching cancer therapy is the use of viruses to attack tumor cells. Rapid advances are being made in the engineering of replication-competent viruses to treat cancer. Adenovirus is a mildly pathogenic human virus that propagates prolifically in epithelial cells – the origin of most human cancers. While virologists have revealed many details about its molecular interactions with the cell, applied scientists have developed powerful technologies to genetically modify or regulate every viral protein. In tandem, the limited success of non-replicative adenoviral vectors in cancer gene therapy has brought the old concept of adenovirus oncolysis back into the spotlight. Major efforts have been directed toward achieving selective replication by the deletion of viral functions dispensable in tumor cells or by the regulation of viral genes with tumor-specific promoters. The predicted replication selectivity has not been realized because of incomplete knowledge of the complex virus–cell interactions and the leakiness of cellular promoters in the viral genome. Capsid modifications are being developed to achieve tumor targeting and enhance infectivity. Cellular and viral functions that confer greater oncolytic potency are also being elucidated. Ultimately, the interplay of the virus with the immune system will likely dictate the success of this approach as a cancer therapy.[156]

Biomaterials play an important role in a variety of clinical applications in wound healing, regeneration, and tissue engineering. Important features of such materials include the

potential to be remodeled and replaced by the proteolytic activity associated with cells during migration and invasion, as well as the ability to display a variety of adhesive ligands that directly bind to cell-surface receptors to provide adhesive and morphogenetic clues.

Fibrin plays an important role in wound healing and regeneration, and enjoys widespread use in surgery and tissue engineering. The enzymatic activity of Factor XIIIa was employed to covalently incorporate exogenous bioactive peptides within fibrin during coagulation. Fibrin gels were formed with incorporated peptides from laminin and *N*-cadherin alone and in combination at concentrations up to 8.2 mol peptide/mol fibrinogen. Neurite extension *in vitro* was enhanced when gels were augmented with exogenous peptide, with the maximal improvement reaching 75%. When this particular fibrin derivative was evaluated in rats in the repair of the severed dorsal root within polymeric tubes, the number of regenerated axons was enhanced by 85% relative to animals treated with tubes filled with unmodified fibrin. These results demonstrate that it is possible to enhance the biological activity of fibrin by enzymatically incorporating exogenous oligopeptide domains of morphoregulatory proteins.[157]

4.3 Gene Therapy

Gene therapy promises to revolutionize medicine by treating the (genetic) causes of diseases rather than the symptoms. This radical improvement is possible because the gene-based approach can provide superior targeting and prolonged duration of action. Moreover, gene therapy is a platform technology applicable to a wide range of diseases. Ongoing clinical studies are addressing a wide range of diseases and target cells, such as cardiovascular diseases, inherited monogenic disorders, rheumatoid arthritis (RA), cancer and cubal tunnel syndrome. The vectors utilized include adeno-associated virus (AAV), pox viruses, Herpes simplex virus (HSV), naked DNA, naked RNA, adenoviruses and retroviruses.

The most promising gene-therapy concepts concern the direct killing of tumor cells with genes delivered by adenovirus vectors, delivery of naked DNA for preventive vaccination against infectious diseases, naked DNA delivery of genes promoting angiogenesis for cardiovascular disorders, and AAV delivery for chronic disorders such as hemophilia and anemia. The efficiency in clinical studies has been clearly demonstrated.[158]

The genomic technologies will provide an increasing range of genes and information about their causal relationship with diseases and the genetic susceptibility for diseases, rendering many more amenable to gene therapy.

Gene therapy may be segmented into somatic gene therapy and germline therapy. Somatic gene therapy promises to be more successful when targeting selected cell populations rather than total changes.

Examples of cell populations are osteoblasts, which undergo apoptosis and are depleted with increasing age. A directed change in that program will prevent osteoporosis by reducing bone loss and increasing bone mass. The classical approach, i.e. application of parathyroid hormone (PTH), will be superseded by gene therapy.

Pancreas failure, Alzheimer's disease and Parkinson's disease are urgent targets for pharmaceutical intervention, where tissue engineering and gene therapy offer the best solutions.

An intriguing example of gene therapy in cardiovascular diseases is the combined application of angiopoietin-1 (Ang-1) and vascular endothelial growth factor (VEGF), both of which are endothelial cell-specific growth factors. Direct comparison of transgenic mice overexpressing these factors in the skin revealed that VEGF-induced blood vessels were leaky, whereas those induced by Ang-1 were non-leaky. Vessels in Ang-1-overexpressing mice were resistant to leaks caused by inflammatory agents. Co-expression of Ang-1 and VEGF had an additive effect on angiogenesis but resulted in leakage-resistant vessels typical of Ang-1. Thus, Ang-1 may be useful in reducing microvascular leakage in diseases in which the leakage results from chronic inflammation or elevated VEGF and in combination with VEGF for promoting the growth of non-leaky vessels. Since plasma leakage is a key pathophysiological feature of various diseases, such as chronic inflammatory, and degenerative and neoplastic diseases, inhibition of the leakage could have important therapeutic benefits. Ang-1 has the potential to reduce plasma leakage in such conditions, whether the leak is due to inflammatory mediators or VEGF.[159]

Gene therapy requires the introduced genes to act in concert with the existing genomic environment without upsetting the genetic, transcriptional and metabolic balances and networks. Regulated hormone expression has been demonstrated in pigs. Ectopic expression of a new serum protease-resistant porcine growth hormone-releasing hormone, directed by an injectable muscle-specific synthetic promoter plasmid vector (pSP-HV-HGRH), elicits growth in pigs. A single muscular injection of 10 mg followed by electroporation in 3-week-old piglets elevated serum GHRH levels by 2- to 4-fold, enhanced growth hormone secretion and increased serum insulin-like growth factor I (IGF-I) by 3- to 6-fold over control pigs. Evaluation of body composition indicated a uniform increase in mass, with no organomegaly or associated pathology.[160]

In utero fetal gene transfer could provide an alternative to transgenic approaches for functional analysis of stage- and tissue-specific, *cis*-acting DNA regulatory elements. Stage-specific *in utero* gene transfer and expression might also prove useful for conditional rescue of lethal targeted genes, either *in utero* or in the immediate postnatal period. Moreover, studies of fetal gene transfer may help in developing strategies to ameliorate or correct human genetic deficiencies *in utero*.

In utero injection of cationic liposome–DNA complexes (CLDCs) containing chloramphenicol acetyltransferase, β-galactosidase (β-gal) or human granulocyte colony-stimulating factor (hG-CSF) expression plasmids produced high-level gene expression in fetal rats. Tissues adjacent to the injection site exhibited the highest levels of gene expression. Chloramphenicol acetyltransferase expression persisted for at least 14 days and was re-expressed following postnatal re-injection of CLDCs. Intraperitoneal administration of the hG-CSF gene produced high serum hG-CSF levels. X-gal staining demonstrated widespread β-gal expression in multiple fetal tissues and cell types. No toxic or inflammatory responses were observed nor was there evidence of fetal–maternal or maternal–fetal gene transfer, suggesting that CLDCs may provide a useful alternative to viral vectors for *in utero* gene transfer.[161]

Gene therapy of the brain is hindered by the presence of the blood–brain barrier (BBB), which prevents the brain uptake of blood-borne gene formulations. Exogenous genes have been expressed in the brain after invasive routes of administration, such as craniotomy or intra-carotid arterial infusion of noxious agents causing BBB disruption. Studies were presented describing the expression of an exogenous gene in brain after non-invasive i.v. administration of a 6–7 kb expression plasmid encoding either luciferase or β-gal packaged in the interior of neutral pegylated immunoliposomes. The latter are conjugated with the OX26 mAb to the rat transferrin receptor, which enables targeting of the plasmid DNA to the brain via the endogenous BBB transferrin receptor. Unlike cationic liposomes, this neutral liposome formulation is stable in blood and does not result in selective entrapment in the lung. Luciferase gene expression in the brain peaks at 48 h after a single i.v. administration of 10 μg of plasmid DNA per adult rat, a dose that is 30- to 100-fold lower than that used for gene expression in rodents with cationic liposomes. β-Gal histochemistry demonstrated gene expression throughout the CNS, including neurons, choroid plexus epithelium and the brain microvasculature. Widespread gene expression in the brain can be achieved by using a formulation that does not employ viruses or cationic liposomes, but instead uses endogenous receptor-mediated transport pathways at the BBB.[162]

Lysosomal storage disorders (LSDs) constitute an important group of conditions in which the potential of gene manipulation as therapy can be assessed. They are monogenic defects, often with severe manifestations for which there are limited treatment options. Overexpression of the lysosomal hydrolase by gene-corrected cells results in secretion of some of the enzyme and its uptake by uncorrected bystander cells (metabolic cooperativity). Gene therapy strategies, enzyme therapy and bone marrow transplantation have all been described.

Fabry disease is a compelling target for gene therapy as a treatment strategy. A deficiency in the lysosomal hydrolase α-galactosidase A (α-gal A; EC 3.2.1.22) leads to impaired catabolism of α-galactosyl-terminal lipids such as globotriaosylceramide (b3). Patients develop vascular occlusions that cause cardiovascular, cerebrovascular and renal disease. Unlike for some lysosomal storage disorders, there is limited primary nervous system involvement in Fabry disease. The enzyme defect can be corrected by gene transfer. Overexpression of α-gal A by transduced cells results in secretion of this enzyme. Secreted enzyme is available for uptake by non-transduced cells, presumably by receptor-mediated endocytosis. Correction of bystander cells may occur locally or systemically after circulation of the enzyme in the blood. A long-term genetic correction in an α-gal A-deficient mouse model of Fabry disease was reported. α-Gal A-deficient bone marrow mononuclear cells (BMMCs) were transduced with a retrovirus encoding α-gal A, and transplanted into sublethally and lethally irradiated α-gal A-deficient mice. α-gal A activity and Gb3 levels were analyzed in plasma, peripheral blood mononuclear cells, BMMCs, liver, spleen, heart, lung, kidney and brain. Primary recipient animals were followed for up to 26 weeks. BMMCs were then transplanted into secondary recipients. Increased α-gal A activity and decreased Gb3 storage were observed in all recipient groups in all organs and tissues except the brain. These effects occurred even with a low percentage of transduced cells. Genetic correction of bone marrow cells derived from patients with Fabry disease may have utility for phenotypic correction of patients with this disorder.[163]

In vivo gene therapy may be a method of choice for compounds that are difficult to produce or to deliver, such as endostatin. Tumors require ongoing angiogenesis to support their growth. Inhibition of angiogenesis by production of angiostatic factors should be a viable approach for cancer gene therapy. Endostatin, a potent angiostatic factor, was expressed in mouse muscle and secreted into the bloodstream for up to 2 weeks after a single intramuscular administration of the endostatin gene. The biological activity of the expressed endostatin was demonstrated by its ability to inhibit systemic angiogenesis. Moreover, the sustained production of endostatin by intramuscular gene therapy inhibited both the growth of primary tumors and the development of metastatic lesions. These results demonstrate the potential utility of intramuscular delivery of an antiangiogenic gene for the treatment of disseminated cancers.[164]

Gene therapy requires appropriate vectors with the necessary regulatory and coding regions for integration, regulation and synthesis of required genes (respectively, proteins). Artificial chromosomes are DNA molecules of defined structure, which are assembled *in vitro* from defined constituents that behave with the properties of natural chromosomes. Artificial chromosomes were first assembled in budding yeasts and have proved useful in many aspects of yeast genetics. Several attempts have been made to build artificial chromosomes in mammals. Particularly, mini-chromosomes of defined structure have been developed to address questions regarding mammalian chromosome function and for biotechnological applications.[165]

Major targets for gene therapy are the diseases involving mutated globins, such as β-thalassemia and sickle-cell disease. The stable introduction of a functional β-globin gene in HSC could be a powerful approach to treat β-thalassemia and sickle-cell disease. Genetic approaches aiming to increase normal β-globin expression in the progeny of autologous HSC might circumvent the limitations and risks of allergenic cell transplants. Low-level expression, position effects and transcriptional silencing hampered the effectiveness of viral transduction of the human β-globin gene when it was linked to minimal regulatory sequences. It was shown that the use of a recombinant lentivirus enables efficient transfer and faithful integration of the human β-globin gene together with large segments of its locus control region. In long-term recipients of unselected transduced bone marrow cells, tetramers of two murine α-globin and two human β(A)-globin molecules account for up to 13% of total hemoglobin in mature red cells of normal mice. In β-thalassemic heterozygous mice higher percentages are obtained (17–24%), which are sufficient to ameliorate anemia and red cell morphology. Such levels should be of therapeutic benefit in patients with severe defects in hemoglobin production.[166]

Gene silencing is defined as a state of complete transcriptional repression that is epigenetically inherited through subsequent cell divisions. This phenomenon has great impact on gene therapy, as transferred genes may become silenced to various degrees, depending on the presence of *cis*-acting elements opposing the forces of silencing (e.g., enhancers, locus control regions, matrix attachment sites and insulators), the sites of chromosomal integration, and the state of differentiation the cells initially transduced and of their subsequent progeny. During development, a widespread wave of global silencing takes place at the time of implantation. Subsequently, progressive tissue-specific gene activation occurs. In pre-implantation embryos, ES cells and HSC, transferred genes are exposed to a high

frequency of gene silencing. Irreversible HSC gene silencing remains one of the main obstacles to the gene therapy of hematological disorders.

Transcriptional silencing of genes transferred into HSC thus poses one of the most significant challenges to the success of gene therapy. If the transferred gene is not completely silenced, a progressive decline in gene expression as the mice age often is encountered. These phenomena were observed to various degrees in mouse transplant experiments using retroviral vectors containing a human β-globin gene, even when *cis*-linked to locus control region derivatives. *Ex vivo* preselection of retrovirally transduced stem cells was investigated on the basis of expression of the GFP driven by the CpG island phosphoglycerate kinase promoter being able to ensure subsequent long-term expression of a *cis*-linked β-globin gene in the erythroid lineage of transplanted mice. All mice ($n = 7$) engrafted with pre-selected cells concurrently expressed human β-globin and the GFP in 20–95% of their red blood cells (RBCs) for up to 9.5 months post-transplantation, the longest time point assessed. This expression pattern was successfully transferred to secondary transplant recipients. In the presence of β-locus control region hypersensitive site 2 alone, human β-globin mRNA expression levels ranged from 0.15 to 20% with human β-globin chains detected by HPLC. Neither the proportion of positive blood cells nor the average expression levels declined with time in transplanted recipients. Although suboptimal expression levels and heterocellular position effects persisted, *in vivo* stem cell gene silencing and age-dependent extinction of expression were avoided, thus demonstrating the potential of this vector for the gene therapy of human hemoglobinopathies.[167]

Most gene therapy strategies involving HSC require both a high level of gene transfer and persistent transgene expression in specific target lineages. Gene transfer rates of approximately 10% in reconstituting HSC can now be routinely achieved with virus vectors based on murine leukemia virus and related oncoretroviruses. Achieving persistent, uniform gene expression from murine leukemia virus-based vectors remains a challenge. Focus has been on the definition of elements of the virus long terminal repeat (LTR) that are responsible for provirus silencing *in vivo*, and the identification of appropriate promoters and enhancers. Expression of integrated provirus is also affected by chromatin structure. Since the bulk of the mammalian genome is packaged into transcriptionally silent heterochromatin and murine leukemia virus-based vectors insert at random sites in the genome, a large portion of murine leukemia virus insertions result in gene silencing. The progeny of a single clone containing a unique integration event can also be affected by the surrounding chromatin to varying degrees, a phenomenon known as position effect variegation.

The mammalian chromosome is organized into discrete chromosomal domains, in part through the use of sequences termed chromatin insulators. These elements, first described in *Drosophila* and also in several vertebrate species, help define the boundary between differentially regulated loci and serve to shield promoters from the influence of neighboring regulatory elements. Insulators function in a polar manner (e.g., they must be located between the *cis* effectors and promoter) and do not have stimulatory or inhibitory transcriptional effects on their own, distinguishing them from classical enhancers and silencers. The best characterized vertebrate chromatin insulator is located within the chicken β-globin locus control region, and contains a DNase I hypersensitive site (cHS4) and appears to constitute the 5′ boundary of the chicken β-globin locus. A 1.2-kb fragment containing the

cHS4 element displays classic insulator activities, including the ability to block the interaction of globin gene promoters and enhancers in cell lines, and the ability to protect expression cassettes in *Drosophila*, transformed cell lines and transgenic mammals from position effects. Much of this activity is contained in a 250-bp fragment, which comprises a 49-bp cHS4 core that interacts with the zinc finger DNA-binding protein CTCF implicated in enhancer-blocking assays.

Recombinant murine retroviruses are widely used as delivery vectors for gene therapy. Once integrated into a chromosome, these vectors often suffer from profound position effects, with vector silencing observed *in vitro* and *in vivo*. To overcome this problem, the HS4 chromatin insulator from the chicken β-globin locus control region was studied with respect to protection of retrovirus vector from position effects. When used to flank a reporter vector, this element significantly increased the fraction of transduced cells that expressed the provirus in cultures and in mice transplanted with transduced marrow. A chromatin insulator can improve the expression performance of a widely used class of gene therapy vectors by protecting these vectors from chromosomal position effects.[168]

A combination of gene therapy and ribozyme drug technology was reported which involved the generation of a novel ribozyme ('maxizyme') to specifically cleave the Bcr–Abl mRNA thus inducing apoptosis in chronic myelogenous leukemia (CML) cells. CML is a hematopoietic malignant disease associated with the expression of a chimeric Bcr–Abl gene. A retroviral system was used to transduce a line of CML cells (BV173) which were injected into NOD-SCID mice. These mice are produced by crossing SCID and NOD mice, and are a valuable tool for growing human hematopoietic cells *in vivo*. As could be observed by examining the spleens of injected and control animals, the maxizyme functions successfully in animals by cleaving Bcr–Abl mRNA with high efficiency. Maxizymes could be a new class of gene-inactivating agents that can cleave any type of chimeric mRNA.[169]

Mitochondrial DNA (mtDNA) mutations underlie many rare diseases and may contribute to human ageing. Gene therapy is a tempting future possibility for intervening in mitochondriopathies. Expression of the 13 mtDNA-encoded proteins from nuclear transgenes (allotopic expression) might be the most effective gene-therapy strategy. Its only confirmed difficulty is the extreme hydrophobicity of these proteins, which prevents their import into mitochondria from the cytosol. Inteins (self-splicing 'protein introns') might offer a solution to this problem: their insertion into such transgenes could greatly reduce the encoded proteins' hydrophobicity, enabling import, with post-import excision restoring the natural amino acid sequence.[170]

Precise targeting of genetic modifications is a prerequisite for safe and successful gene therapy by modification of DNA sequences.

Mobile group II intron RNAs insert directly into DNA target sites and are then reverse transcribed into genomic DNA by the associated intron-encoded protein. Target site recognition involves modifiable base-pairing interactions between the intron RNA and a longer than 14-nucleotide region of the DNA target site, as well as fixed interactions between the protein and flanking regions. A highly efficient *E. coli* genetic assay was developed to determine detailed target site recognition rules for *Lactococcus lactis* group II intron Ll.LtrB and to select introns that insert into desired target sites. Using HIV-1 proviral DNA and the human CCR5 gene as examples, it was shown that group II introns can be retargeted to

insert efficiently into virtually any target DNA and that the retargeted introns retain activity in human cells. The work provides the practical basis for potential applications of targeted group II introns in genetic engineering, functional genomics and gene therapy.[171]

Chimeric RNA–DNA oligonucleotides have formed the basis of genetic strategies for correcting mutations, while maintaining genomic organization important for the appropriate expression and regulation of genes. Visualization of pigmentation in a live animal and easy accessibility makes skin an attractive system for *in vivo* testing of chimeric oligonucleotides as novel skin gene therapeutics.

An RNA–DNA oligonucleotide corrected a point mutation in the mouse tyrosinase gene, resulting in permanent and inheritable restoration of tyrosinase enzymatic activity, melanin synthesis and pigmentation changes in cultured melanocytes. Gene correction was extended from tissue culture to live animals, using a chimeric oligonucleotide designed to correct a point mutation in the tyrosinase gene. Both topical applications and intradermal injection of this oligonucleotide to albino BALB/c mouse skin resulted in dark pigmentation of several hairs in a localized area. The restored tyrosinase enzymatic activity was detected by dihydroxyphenylacetic acid (DOPA) staining of hair follicles in the treated skin. Tyrosinase gene correction was also confirmed by restriction fragment length polymorphism (RFLP) analysis, and DNA sequencing from skin that was positive for DOPA staining and melanin synthesis. Localized gene correction was maintained 3 months after the last application of the chimeric oligonucleotides. These results demonstrated correcting of the tyrosinase gene point mutation by chimeric oligonucleotides *in vivo*.[172]

The suitability of using the tRNA import pathway for the correction of respiratory deficiencies in the mitochondrial DNA and the applicability of this system for human therapeutic application were investigated. Mitochondrial import of nuclear encoded tRNAs has been described in yeasts, plants and protozoans. The complexity of the imported tRNA pool varies among organisms, from a complete set required for reading all codons of the mitochondrial genetic code in trypanosomatids to a single tRNA in the yeast *S. cerevisiae*.

Mitochondrial import of a cytoplasmic tRNA in yeast requires the preprotein import machinery and cytosolic factors. Cytoplasmic tRNAs with altered aminoacylation identity can be specifically targeted to the mitochondria and participate in mitochondrial translation. Human mitochondria, which do not normally import tRNAs, are able to internalize yeast tRNA derivatives *in vitro* and this import requires an essential yeast import factor.[173]

Adoptive immunotherapy is developing into a tool for combating diseases like infections and cancers. The infusion of antigen-specific T lymphocytes is a potential therapy against certain cancers and infectious diseases. One limitation to its broad usage is the generation of autologous T cells directed against well-defined epitopes. The induction and expansion of antigen-specific cells require optimal antigen presentation and T cell co-stimulation. These requirements are met by antigen-presenting cells (APC) such as Epstein–Barr virus-transformed B cells and dendrite cells (DC), which constitutively express high levels of co-stimulatory, adhesion and MHC molecules. Despite a cumbersome process, the use of autologous cells to present cell-defined epitopes is mandated to obviate strong allergenic responses.

The adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTLs) is a promising therapeutic approach for a number of diseases. To overcome the difficulty in generating

specific CTLs, stable artificial APCs (AAPCs) were established that can be used to stimulate T cells of any patient of a given HLA type. Mouse fibroblasts were retrovirally transduced with a single HLA–peptide complex along with the human accessory molecules B7.1, ICAM-1 and LFA-3. These AAPCs consistently elicit strong stimulation and expansion of HLA-restricted CTLs. Due to the high efficiency of retrovirus-mediated gene transfer, stable AAPCs can be readily engineered for any HLA molecule and any specific peptide.[174]

Activation of the immune system in response to challenge by a foreign organism is a complex process. In the case of T-dependent antigens, naive T cells are activated in response to novel peptides presented by APCs. To activate T cells the APCs must acquire antigen, and then present the antigen in the context of self-MHC molecules and the appropriate co-stimulatory signals. Tissue damage and/or cell death may facilitate antigen presentation and may be necessary for activation of the immune system. Following cell death, dying cells transfer their antigens (by ingestion or otherwise) to 'professional' APCs, which then prime T cells. Antigen from necrotic cells was shown to prime MHC II-restricted T cell responses *in vitro*. Antigen transfer and priming have been demonstrated by showing that bone marrow-derived APCs can acquire viral antigens from apoptotic cells and prime antigen-specific CTLs. Thereby, DCs were observed to engulf intact macrophages as well as their cellular fragments, suggesting that apoptosis can prime CTLs through DCs.

Immunity to tumors as well as to viral and bacterial pathogens is often mediated by CTLs. Thus, the ability to induce a strong cell-mediated immune response is an important requirement of novel immunotherapies. APCs, including DCs, are specialized in initiating T cell immunity. Harnessing this innate ability of these cells to acquire and present antigens, antigen presentation was improved by targeting antigens directly to DCs *in vivo* through apoptosis. Fas-mediated apoptotic death of antigen-bearing cells was engineered *in vivo* by co-expressing the immunogen and Fas in the same cell. The death of antigen-bearing cells results in an increased antigen acquisition by APCs including DCs. This *in vivo* strategy led to enhanced antigen-specific CTLs, and the elaboration of T helper 1 (T_h1)-type cytokines and chemokines. This adjuvant approach has important implications for viral and non-viral delivery strategies for vaccines or gene therapies.[175]

The topical delivery of transgenes to hair follicles has potential for treating disorders of the skin and hair. The topical administration of liposome–DNA mixtures (lipoplex) to mouse skin and to human skin xenografts resulted in efficient *in vivo* transfection of hair follicle cells. Transfection depended on liposome composition and occurred only at the onset of a new growing stage of the hair cycle. Manipulating the hair follicle cycle with depilation and retinoic acid treatment resulted in nearly 50% transfection efficiency – defined as the proportion of transfected, newly growing follicles within the xenograft. Transgenes administered in this fashion are selectively expressed in hair progenitor cells and therefore have the potential to affect the characteristics of the follicle. These findings form a foundation for the future use of topical lipoplex applications to alter hair follicle phenotype, and treat diseases of the hair and skin.[176]

Gene therapy for cystic fibrosis (CF) has focused on correcting electrolyte transport in airway epithelia. Success has been limited by the failure of vectors to attach to and enter into airway epithelia, and may require redirecting vectors to targets on the apical mem-

brane of airway cells that mediate these functions. The G-protein-coupled P2Y2 receptor (P2Y2-R) is abundantly expressed on the airway lumenal surface and internalizes into coated pits upon agonist activation. A small-molecule agonist (UTP) was tested whether it could direct vectors to P2Y2-R and mediate attachment, internalization, and gene transfer. Fluorescein–UTP studies demonstrated that P2Y2-R agonists internalized with their receptor and biotinylated UTP (BUTP) mediated P2Y2-R-specific internalization of fluorescently labeled streptavidin (SAF) or SAF conjugated to biotinylated Cy3 adenoviral vector (BCAV). BUTP conjugated to BACV-mediated P2Y2-R-specific gene transfer in (i) adenoviral-resistant A9 and polarized MDCK cells by means of heterologous P2Y2-R, and (ii) well-differentiated human airway epithelial cells by means of endogenous P2Y2-R. Targeting vectors with small-molecule ligands to apical membrane GPCRs may be a feasible approach for successful CF gene therapy.[177]

In the canine model of Duchenne muscular dystrophy in golden retrievers (GRMD), a point mutation within the splice acceptor site of intron 6 leads to deletion of exon 7 from the dystrophin mRNA and the consequent frameshift causes early termination of translation. A DNA and RNA chimeric oligonucleotide was designed to induce host cell mismatch repair mechanisms and correct the chromosomal mutation to wild-type. Direct skeletal muscle injection of the chimeric oligonucleotide into the cranial tibialis compartment of a 6-week-old affected male dog, and subsequent analysis of biopsy and necropsy samples, demonstrated *in vivo* repair of the GRMD mutation that was sustained for 48 weeks. RT-PCR analysis of exons 5–10 demonstrated increasing levels of exon 7 inclusion with time. An isolated exon 7-specific dystrophin antibody confirmed synthesis of normal-sized dystrophin product and positive localization to the sarcolemma. Chromosomal repair in muscle tissue was confirmed by RFLP-PCR and sequencing the PCR product. This work provides evidence for the long-term repair of a specific dystrophin point mutation in muscle of a live animal using a chimeric oligonucleotide.[178]

The use of neurotrophic viruses as vectors for targeted gene delivery to the CNS has many applications for the development of new therapies for neurological diseases and spinal cord trauma. Poliovirus is attractive for the development of such a gene delivery vector because it has been established in humans that once poliovirus invades the CNS, infection is restricted to the motor neurons of the hindbrain and the spinal cord. To exploit the unique features of poliovirus tropism, poliovirus genomes (referred to as replicons) were constructed to encode foreign proteins in place of the capsid proteins. Because replicons do not encode capsid proteins, they undergo only a single round of infection, without spreading to neighboring cells. Since no infectious poliovirus is generated during production of replicons, the use of replicons for gene delivery purposes following worldwide poliovirus eradication will not be a concern. Replicons maintain the tropism of poliovirus in the CNS and exclusively infect spinal cord and brainstem motor neurons. Replicons can mediate gene delivery in animals that have previously been immunized with poliovirus, indicating that pre-existing immunity in humans from vaccination will not be a limitation for the use of replicons.

Poliovirus replicon vectors transiently express foreign proteins selectively in motor neurons of the anterior horn of the spinal cord. Mice transgenic for the poliovirus receptor (PVR) were intraspinally inoculated with replicons encoding murine tumor necrosis factor

(mTNF)-α. High-level expression of mTNF-α was detected in spinal cords of these animals at 8–12 h post-inoculation and this returned to background by 72 h. The mice exhibited ataxia and tail atony, whereas animals given a replicon encoding GFP exhibited no neurological symptoms. Histology of spinal cords from mice given the replicon encoding mTNF-α revealed neuronal chromatolysis, reactive astrogliosis, decreased expression of myelin basic protein and demyelination. These animals recovered with only slight residual damage. This showed replicon vectors to have potential for targeted delivery of therapeutic proteins to the CNS and provide a new approach for treatment of spinal cord trauma and neurological disease.[179]

Clinical trials of gene therapy for CF have demonstrated encouraging steady progress, indicating that transfer of the CF transmembrane conductance regulator (CFTR) gene can partially correct the chloride transport defect in human subjects. Current levels of gene transfer are apparently too low for clinical effectiveness – in large part as a result of the barriers faced by gene transfer vectors within the airways. Clinical studies of gene therapy for CF suggest that the key problem is the efficiency of gene transfer to the airway epithelium. The availability of relevant vector receptors, the transient contact time between vector and epithelium, and the barrier function of airway mucus contribute significantly to this problem. Recombinant Sendai virus (SeV) was developed as a new gene transfer agent. SeV produces efficient transfection throughout the respiratory tract of both mice and ferrets *in vivo*, as well as in freshly obtained human nasal epithelial cells *in vitro*. Gene transfer efficiency was several log orders greater than with cationic liposomes or adenovirus. Even very brief contact time was sufficient to produce this effect and levels of expression were not significantly reduced by airway mucus. These investigations suggest that SeV may provide a useful new vector for airway gene transfer.[180]

Retroviral vectors can insert genes stably into the chromosomes of mammalian cells and have been used in clinical human gene therapy trials to introduce genes into a variety of cells and tissues. All retroviral vectors currently used in such clinical trials have been derived from murine leukemia virus (MLV), a C-type retrovirus, which is considered nonpathogenic for humans. In contrast to lentiviruses, C-type retroviruses have a rather simple genomic organization and contain only two gene units, which code for the inner core structural proteins and the envelope protein. As safe gene delivery systems, they require only the retroviral vector, a genetically modified viral genome containing the gene of interest in place of all retroviral protein coding sequences, and a helper cell that provides the retroviral proteins for the encapsidation of the vector genome into retroviral particles. Gene therapy applications of retroviral vectors derived from C-type retroviruses have been limited to introducing genes into dividing target cells. Genetically engineered C-type retroviral vectors were constructed by derivation from spleen necrosis virus (SNV), which are capable of infecting non-dividing cells. This was achieved by introducing a nuclear localization signal (NLS) sequence into the matrix protein (MA) of SNV by site-directed mutagenesis. This increased the efficiency of infecting non-dividing cells, and was sufficient to endow the virus with the capability to efficiently infect growth-arrested human T lymphocytes and quiescent primary monocyte-derived macrophages. This vector was demonstrated to actively penetrate the nucleus of a target cell and has the potential for a gene therapy vector to transfer genes into non-dividing cells.[181]

AAV vectors have demonstrated considerable promise for gene therapy of inherited diseases. With a packaging size of less than 5 kb, applications have been limited to relatively small disease genes. Based on the finding that AAV genomes undergo inter-molecular circular concatemerization after transduction in muscle, a paradigm was developed to increase the size of delivered transgenes with this vector through *trans*-splicing between two independent vectors co-administered to the same tissue. When two vectors encoding either the 5′ or 3′ portions of the erythropoietin genomic locus were used, functional erythropoietin protein was expressed in muscle subsequent to the formation of intermolecular circular concatemers in a head-to-tail orientation through *trans*-splicing between these two independent vector genomes. This allows the AAV technologies to be applied to a wider variety of diseases for which therapeutic transgenes exceed the packaging limitation of prior AAV vectors.[182]

An intriguing use of gene therapy by using genetically engineered cells in cancer therapy was demonstrated by applying encapsulated endostatin-secreting cells for the effective treatment of human glioblastoma xenografts[703] in mice and of BT4C glioma cells upon intracerebral implantation in rats.[704] The continuous release of endostatin, a sensitive anti-angiogenic protein, by implanted bioreactors, demonstrates an effective approach to tumor therapy with cellular and gene therapy.[705] The other extreme of application of gene therapy, oral delivery, is subject to intensive studies due to its advantages with respect to compliance, cost, and patient convenience.[706]

4.4 Implantates

Small implants acting as artificial neurons serve to stimulate muscles and thus prevent them from wasting away,[183] and offer the prospect of helping patients with stroke or spinal cord injury.

Biodegradable rods to stabilize bone fractures are absorbed once the bone has healed, whereby clearly the timing of resorption is crucial.[184]

An efficient porous chamber system to convert Factor VII to VIIa was developed and implantation of this chamber, *in vivo*, was shown to be effective. The chamber containing immobilized Factor XIIa was able to generate Factor VII-dependent bypass activity for at least 3 days as a peritoneal implant in guinea pigs and for up to 1 month when tested in rhesus monkeys.

Hemophilia A and B coagulation defects, which are caused by deficiencies of Factor VIII and Factor IX, respectively, can be bypassed by administration of recombinant Factor VIIa. However, the short half-life of recombinant Factor VIIa *in vivo* negates its routine clinical use. An *in vivo* method for the continuous generation of Factor VIIa is reported which depends on the implantation of a porous chamber that contains Factor Xa or XIIa and continuously generates Factor VIIa bypass activity from the subject's own Factor VII, which enters the chamber by diffusion. Once inside, the Factor VII is cleaved to Factor VIIa by the immobilized Factor Xa or XIIa. The newly created Factor VIIa diffuses out of the chamber and back into circulation, where it can bypass the deficient Factors VIII or IX and enable coagulation to occur. *In vitro*, this method generates sufficient Factor VIIa to sub-

stantially correct Factor VIII-deficient plasma when assessed by the classical aPTT coagulation assay. *In vivo*, a Factor XIIa peritoneal implant generates bypass activity for up to 1 month when tested in rhesus monkeys. Implantation of such a chamber in a patient with hemophilia A or B could eventually provide a viable alternative to replacement therapies using exogenous coagulation factors.[185]

4.5 Medical Devices and Technology

Medical devices include drug-delivery systems, neurological stimulators, cardiovascular devices such as implantable cardiac defibrillators as well as monitoring devices for blood glucose and heart function. Intelligent devices like the implantable cardiac defibrillators are endowed with sufficient intelligence to analyze the signals from the heart, and apply the right power and type of impulse for correction. Likewise, implantable devices for blood constituents are capable of monitoring, for example, glucose levels and blood factors, and deliver the right amounts of insulin or therapeutic proteins. The market for medical devices amounts to approximately US$ 160 billion per year and is thus about half the size of the market for pharmaceuticals at around US$ 350 billion (in the year 2000).

The diagnosis of biological systems with lasers, such as blood disorders, cancer cells, and cellular constituents like proteins, RNA, DNA is becoming a reality with the availability of biocavity lasers.[477]

The development of MEMS enables the expansion of biomedical applications in chemical and biochemical analysis, and molecular detection and health monitoring. The biological microcavity laser or biocavity laser is such a system on the nanometer scale. Nanolasers have numerous applications in fiber optic communications, optical computers, and nano- or microscale system sensors, actuators and systems-on-a-chip. The nanolaser can probe the cell and nuclear dimensions of lymphocytes and thus shed light on the status of the human immune system or it can diagnose genetic disorders by measuring, for example, red blood cell shapes and hemoglobin content. The semiconductor laser is based on silicon, which is a material suitable for microfabrication, microfluidics and sensors, thus allowing the integrated design of test arrays on chips and implantable devices. Compound semiconductors based on III–V materials (from columns III and V of the periodic table), such as GaAs, are especially suited to the generation, modulation and transmission of light, and thus can combine illumination, data transmission, optical computing and optical analysis plus biomedical applications such as photodynamic therapy, optical tomography, cell micro-manipulation and laser cytometry.

Table 4.2 correlates various materials and (bio-)medical products.

Microsystems in healthcare can generally be divided into

- Sensor systems for diagnostics and patient monitoring.
- Endoscopes and instrumentation for minimally invasive therapy.
- Active implantable devices.
- Systems for bioanalytics and pharmaceutical screening.

Table 4.2. Materials and (bio-)medical products [Refs in 478].

Application technologies new materials	Medical equipment	Other (bio-)medical applications
Structural ceramics and mechanically resistant materials	Long-term implants Prosthetic devices	
Functional ceramics	Neurostimulation in case of paralysis Heat treatment of tumors through implanted, magnetically excitable ceramics Ultrasound converter arrays Microsensors and -actuators for ceramic radiological luminous substances	Microsensors and -actuators Health monitoring
Polymers	Controlled drug delivery Skin transplant Biological membranes	
Diamond (-layers)	Eye lenses with a high index of refraction Scalpels	
a-SiC:H	Biocompatible coatings for stents	
Titanium based alloys	Endoprotheses (artificial joints)	
Multifunctional, Adaptive materials	Release from implanted drug depots as required	"Intelligent" instruments
Biomimetic materials	Artificial skin, Dialysis membranes, Artificial parts of organs	Biosensors

Biomaterials are structural or functional materials in the construction of medical devices for implantation or application to living tissues or organs. They need to be biocompatible, i.e. to perform their function without negative side effects such as interference with the circulation or coagulation, impairment of the immune system, facilitation of infections or scar-formation. Bioactive materials such as enzymes, antibodies or living cells can be integrated as functional parts of sensors, actuators or total systems.[478]

The increasing need for novel delivery devices for the application of minute amounts of potent, macromolecular biochemicals at defined intervals without inflicting pain (thus improving compliance) has led to new drug delivery systems employing microstructures. An array of microstructures connected to a microfluidic device can be used to deliver fluids transdermally without pain.[479]

Microstructures permit precise fluid handling and dosing in the microliter to picoliter range. Microstructural design enables the mixing, dosing, resuspension of chemicals, and actuation of valves and micropumps. Examples of microstructured systems are a nebulizer with a highly precise micronozzle for oral drug delivery, a miniaturized microtiter plate

(laboratory-on-a-chip) for the determination of microbial sensitivities and a microspectrometer for analysis and color measurement, e.g., in bilirubin analysis.[480]

Living cells acting as parts of bioelectronic hybrid systems offer intriguing avenues for the development of biosensors, bioinformatics and implantable devices for the restoration of function. A crucial issue is the functional coupling of the output signal from the cell system to a micro-electronic or opto-electronic transducer unit. The coupling of excitable cells with an array of field-effect transistors (FETs) integrated into the bottom of a cell culture dish allows the measurement of action potentials.[481]

Controllable prosthetics are possible with the creation of interfaces between neural tissue and machines. Electrical signals from five regions in the cerebral cortex of monkeys have already been used to drive the movement of robotic arms.[482]

The direct electrical interfacing of a recombinant ion channel to a field-effect transistor on a silicon chip has been reported. In the bio-electronic hybrid, an ion current through activated maxi-K_{Ca} channels in human embryonic kidney (HEK293) cells gives rise to an extracellular voltage between cell and silicon chip which controls the electronic source-drain current. The channels at the cell/chip interface are fully functional as is shown by patch-clamp recording. Moreover, the channels are accumulated at the cell/chip interface. The direct coupling of potassium channels to a semiconductor on the level of an individual cell is a prototype for an iono-electronic interface of ligand-gated or G protein-coupled ion channels. It allows the development of bio-electronic hybrids for implantates and of biosensors for biochemical or biopharmaceutical screening arrays with numerous cells on a silicon chip combined with an array of transistors.[506]

These developments will provide amputees and patients with a variety of motor disorders such as paralysis, amyotropic lateral sclerosis with the means to act and communicate by replacing the control of muscles with the control of artificial devices by brain activity.

The study of neural processes for the development of neural–robotic hybrid systems is facilitated by experimental research on visual processing using functional MRI (fMRI). The fMRI method was used to test key predictions of the proposed object-based theory wherein pre-attentive mechanisms segment the visual array into discrete objects, groups or surfaces serving as targets for visual attention. The magnetic resonance signal was recorded from subjects viewing stationary versus moving objects. The signals were recorded from each subject's fusiform face area, parahippocampal place area and MT/MST area, providing a measure of the processing of faces, houses and visual motion. Attending to one attribute of an object enhanced the neural representation of that attribute as well as of the other attribute of the same object. The experiments provide physiological evidence of the selection of whole objects with one relevant visual attribute and may lead to vision-directed steering of prosthetics.[483]

The involvement of the visual cortex in tactile discrimination of orientation was demonstrated experimentally,[484] thus pointing to the necessity to take the wider concept of perceptions into account when developing advanced hybrid interfaces for prosthetics.

Transcranial magnetic stimulation (TMS), whereby a pulsed magnetic field creates current flow in the brain and can temporarily excite or inhibit specific areas, is being developing as an analytically and therapeutically useful non-invasive tool for studying the human brain. The application of TMS to the motor cortex can produce a muscle twitch or may

block the movement of a muscle. TMS of the occipital cortex can produce visual phosphenes or scotomas. Moreover, TMS can be used to alter the functioning of the brain beyond the time of stimulation, and thus offers potential for the analysis of neural processes, and research in and therapy of neural disorders.[485]

TMS has been applied to studies of the cortical physiology, to elucidate the processes underlying the mature brain's plasticity such as those involved in repair, learning, memory, and in research on neurologic disorders such as epileptic seizures, Parkinson's disease, Huntington's disease and dystonia.

Figure 4.19 illustrates the physics and mechanism of action of TMS, and specifically emphasizes adherence to safety guidelines in the application of this technique.

A microversion of a positron emission tomography (PET) scanner called microPET was developed and applied to studies of the metabolic activity in different regions of the conscious rodent brain using [^{18}F]fluorodeoxyglucose (FDG) as a tracer to monitor changes in neuronal activity. Limbic seizures result in significantly elevated metabolic activity in the hippocampus and vibrissal stimulation causes modest increases in FDG uptake in the contralateral neocortex. MicroPET is also useful for studying lesion-induced plasticity of the brain, as shown by cerebral hemidecortication resulting in diminished relative glucose metabolism in the neostriatum and thalamus ipsilateral to the lesion, with subsequent recovery of metabolic function. Thus, microPET is useful for serial assessment of metabolic function of individual awake rats with minimal invasiveness, and is applicable to studies of neural disorders and brain repair.[486]

The experimental tagging of expressed genes and the resulting observation of gene activation and gene down-regulation enable the generation of a three-dimensional atlases for gene expression.[186]

In *Xenopus laevis* and *Caenorhabditis elegans*, organism-wide gene expression and down-regulation has been measured with a mutant (E5) of the red fluorescent protein drFP583 which changes its fluorescence from green to red over time. The rate of color conversion is independent of protein concentration and can be applied to trace time-dependent gene expression. *In vivo* labeling with E5 was used to measure expression from the heat shock-dependent promoter in *C. elegans* and from the *Otx-2* promoter in developing *Xenopus* embryos.[487]

4.6 Complex Traits

Increasingly, multifactorial diseases are the targets of medical and biotechnological intervention. A particularly important area is the study of the molecular, biochemical and genetic basis of senescence.

Senescence appears from certain studies to be a multigenetic trait. The mutated form of a single protein, p66^{shc}, which controls the oxidative stress response, also significantly shapes the mammalian life span.[513] Ageing was studied in a mouse mutant *klotho* showing premature ageing. The syndrome resembling human ageing includes reduced liefspan, decreased activity, infertility, osteoporosis, arteriosclerosis is caused by the disruption of a

single gene, *klotho.* The klotho gene codes for a protein apparently functioning outside of the cells, exhibits homology to β-glucosidase enzymes, and may function through a signaling pathway involving circulating humoral factor(s).[516]

The evolutionary theory of ageing points to the complex cellular and molecular processes shaping senescence, in particular stress resistance phenomena.[519] A link is observed between advanced age and increased inciidence of cancer. Accumulated experimental evidence indicates that the cancer-prone phenotype is a result of the combined pathogenetic effects of mutation load, epigenetic regulation, telomere dysfunction, and altered stromal milieu.[521] Lessons may be drawn from the study of human progeroid syndromes,wherein a number of genes have been identified in whichg mutations can lead to the accelerated emergence of senescence.[523] The intervention in the ageing process will have profound societal, biomedical, philosophical, psychological, and economic consequences.[524]

Profiling of gene expression is providing transcriptional patterns and pattern changes as well as targets for identification of genes (respectively, gene sequences) for further studies.

Most multicellular organisms exhibit a progressive and irreversible physiological decline that characterizes senescence, the molecular basis of which remains unknown. Postulated mechanisms include cumulative damage to DNA leading to genomic instability, epigenetic alterations that lead to altered gene expression patterns, telomere shortening in replicative cells, oxidative damage to critical macromolecules by reactive oxygen species (ROS) and non-enzymatic glycation of long-lived proteins.

Genetic manipulation of the ageing process in multicellular organisms has been achieved in *Drosophila* through the overexpression of catalase and Cu/Zn superoxide dismutase, in the nematode *C. elegans* through alterations in the insulin receptor (IR) signaling pathway and through the selection of stress-resistant mutants in either organism. In mammals, mutations in the Werner syndrome (WS) locus (WRN) accelerate the onset of a subset of ageing-related pathology in humans, but the only intervention that appears to slow the intrinsic rate of ageing is caloric restriction.

The gene expression profile of the aging process was analyzed in skeletal muscle of mice. Use of high-density oligonucleotide arrays representing 6347 genes revealed that ageing resulted in a differential gene expression pattern indicative of a marked stress response and lower expression of metabolic and biosynthetic genes. Most alterations were either completely or partially prevented by caloric restriction – the only intervention known to retard ageing in mammals. Transcriptional patterns of calorie-restricted animals suggest that caloric restriction retards the ageing process by causing a metabolic shift towards increased protein turnover and decreased macromolecular damage.[188]

DNA repair plays a crucial role in the maintenance of genetic integrity and thus the basis for organismal homeostasis. Cellular DNA is under continuous attack by reactive species inside cells and by environmental agents. Toxic and mutagenic consequences are minimized by distinct repair pathways and the presently 130 known human DNA repair genes were described. These include four enzymes that remove uracil from DNA, seven recombantion genes related to RAD51, and several DNA polymerases that bypass damage. Only one system is described which removes the main DNA lesions induced by ultravilet light. The number of DNA repair genes is likely to be extended by comparative studies of different organisms and the structural, functional protein and genomics studies. Modula-

tion of DNA repair has potential clinical applications in radiotherapy, anticancer treatment, and understanding and possibly modulation of the aging process.[637]

WS is an inherited disease characterized by premature onset of ageing, increased cancer incidence and genomic instability. The WS gene encodes a 1432 amino acid polypeptide (WRN) with a central domain homologous to the RecQ family of DNA helicases. Purified WRN unwinds DNA with 3′–5′ polarity and also possesses 3′–5′ exonuclease activity. Elucidation of the physiologic function(s) of WRN may be aided by the identification of WRN-interacting proteins. WRN functionally interacts with DNA polymerase δ (pol δ), a eucharistic polymerase required for DNA replication and DNA repair. WRN increases the rate of nucleotide incorporation by pol δ in the absence of proliferating cell nuclear antigen (PCNA) but does not stimulate the activity of eukaryotic DNA polymerases α or ε, or a variety of other DNA polymerases. Functional interaction with WRN is mediated through the third subunit of pol δ, i.e. Pol32p of *S. cerevisiae*, corresponding to the recently identified p66 subunit of human pol δ. Absence of the third subunit abrogates stimulation by WRN and stimulation is restored by reconstituting the three-subunit enzyme. WRN may facilitate pol δ-mediated DNA replication and/or DNA repair, and disruption of WRN-pol δ interaction in WS may contribute to the observed S-phase defects and/or the unusual sensitivity to a limited number of DNA damaging agents.[189]

An intriguing observation in yeast sheds some light on the effects of caloric restriction on life span. Yeast silent information regulator 2 (Sir2) is a heterochromatin component that silences transcription at silent mating loci, telomeres and the ribosomal DNA, and also suppresses recombination in the rDNA and extends replicative life span. Mutational studies indicate that lysine 16 in the N-terminal tail of histone H4 and lysines 9, 14 and 18 in H3 are critically important in silencing, whereas lysines 5, 8 and 12 of H4 have more redundant functions. Lysines 9 and 14 of histone H3 and lysines 5, 8 and 16 of H4 are acetylated in active chromatin and hypoacetylated in silenced chromatin, and overexpression of Sir2 promotes global deacetylation of histones, indicating that Sir2 may be a histone deacetylase. Deacetylation of lysine 16 of H4 is necessary for binding the silencing protein, Sir3. It was shown that yeast and mouse Sir2 proteins are nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases, which deacetylate lysines 9 and 14 of H3 and specifically lysine 16 of H4. The analysis of two Sir2 mutations supports the idea that this deacetylase activity accounts for silencing, recombination suppression and extension of life span *in vivo*. These findings provide a molecular framework of NAD-dependent histone deacetylation that connects metabolism, genomic silencing and ageing in yeast and, perhaps, in higher eukaryotes.[425]

Yeast Sir2 protein functions in transcriptional silencing of the silent mating loci, telomeres and rDNA. The Sir2 family of enzymes catalyze a NAD–nicotinamide exchange reaction that requires the presence of acetylated lysines such as are found in the N-termini of histones and there appears to be no evidence of ADP-ribosylation activity.[190]

Yeast Sir2 is a heterochromatin component that silences transcription at silent mating loci, telomeres and the ribosomal DNA, and that also suppresses recombination in the rDNA and extends replicative life span. Mutational studies indicate that lysine 16 in the amino-terminal tail of histone H4 and lysines 9, 14 and 18 in H3 are critically important in silencing, whereas lysines 5,8 and 12 of H4 have more redundant functions. Lysines 9 and

14 of histone H3 and lysines 5,8 and 16 of H4 are acetylated in active chromatin and hypoacetylated in silenced chromatin, and overexpression of Sir2 promotes global deacetylation of histones, indicating that Sir2 may be a histone deacetylase. Deacetylation of lysine 16 of H4 is necessary for binding the silencing protein, Sir3. It was shown that yeast and mouse Sir2 proteins are nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases, which deacetylate lysines 9 and 14 of H3 and specifically lysine 16 of H4. The analysis of two Sir2 mutations supports the idea that this deacetylase activity accounts for silencing, recombination suppression and extension of life span *in vivo*. The findings provide a molecular framework of NAD-dependent histone deacetylation that connects metabolism, genomic silencing and ageing in yeast and possibly in higher eukaryotes.[204]

Severe dietary restriction, catabolic states and even short-term caloric deprivation impair fertility in mammals. Likewise, obesity is associated with infertile conditions such as polycystic ovary syndrome. The reproductive status of lower organisms such as *C. elegans* is also modulated by availability of nutrients. Thus, fertility requires the integration of reproductive and metabolic signals. Deletion of IR substrate-2 (IRS-2), a component of three insulin/IGF-1 signaling cascade, causes female infertility. Mice lacking IRS-2 have small, anovulatory ovaries with reduced numbers of follicles. Plasma concentrations of luteinizing hormone, prolactin and sex steroids are low in these animals. Pituitaries are decreased in size and contain reduced numbers of gonadotrophs. Females lacking IRS-2 have increased food intake and obesity, despite elevated levels of leptin. Insulin, together with leptin and other neuropeptides, may modulate hypothalamic control of appetite and reproductive endocrinology. Coupled with findings on the role of insulin-signaling pathways in the regulation of fertility, metabolism and longevity *in C. elegans* and *Drosophila*, an evolutionarily conserved mechanism in mammals that regulates both reproduction and energy homeostasis has been identified.[191]

Ageing in budding yeast is measured by the number of mother cell divisions before senescence. Genetic studies have linked ageing in this organism to the Sir genes which mediate genomic silencing at telomeres, mating type loci and the repeated ribosomal RNA. Sir2p determines life span in a dose-dependent manner by creating silenced rDNA chromatin, thereby repressing recombination and the generation of toxic rDNA circles. Silencing is triggered by the deacetylation of certain lysines in the N-termini of histones H3 and H4. Sir2p has a NAD-dependent histone deacetylase activity that is conserved in Sir2p homologs.

Caloric restriction extends life span in a wide variety of organisms. Although it has been suggested that calorie restriction may work by reducing the level of ROS produced during respiration, the mechanism by which this regimen slows ageing is uncertain. Calorie restriction was mimicked in yeast by physiological or genetic means and showed a substantial extension in life span. This extension was not observed in strains mutant for Sir2 (which encodes the silencing protein Sir2p) or NPT1 (a gene in a pathway in the synthesis of NAD, the oxidized form of nicotinamide adenine dinucleotide). The increased longevity induced by calorie restriction requires the activation of Sir2p by NAD.[192]

In view of the involvement of IRs in energy metabolism, ageing and homeostasis, the studies of these IRs bears on several connected phenomena. IRs are expressed in most

tissues of the body, including classic insulin-sensitive tissues (liver, muscle and fat), as well as 'insulin-insensitive' tissue such as RBCs and the neuronal tissue of the CNS. In fact, IR and insulin signaling proteins are widely distributed throughout the CNS. To study the physiological role of insulin signaling in the brain, mice were created with a neuron-specific disruption of the IR gene (NIRKO mice). Inactivation of the IR had no impact on brain development or neuronal survival. Female NIRKO mice showed increased food intake, and both male and female mice developed diet-sensitive obesity with increases in body fat and plasma leptin levels, mild insulin resistance, elevated plasma insulin levels, and hypertriglyceridemia. NIRKO mice also exhibited impaired spermatogenesis and ovarian follicle maturation because of hypothalamic dysregulation of luteinizing hormone. IR signaling in the CNS plays an important role in regulation of energy disposal, fuel metabolism, and reproduction.[193]

The study of quantitative trait loci (QTLs) requires several methodologies to study the connection between genotype and phenotype associated with certain normal or aberrant conditions.[194]

Recent studies have intriguingly shown that complex traits may be controlled by few genes or even a single gene.

In natural populations, most phenotypic variation is continuous and is effected by alleles at multiple loci. Although this quantitative variation fuels evolutionary change and has been exploited in the domestication and genetic improvement of plants and animals, the identification and isolation of the genes underlying this variation have been difficult.

Domestication of many plants has correlated with dramatic increases in fruit size. In tomato, one QTL, *fw2.2*, was responsible for a large step in this process. When transformed into large-fruited cultivars, a cosmid derived from the *fw2.2* region of a small-fruited wild species reduced fruit size by the predicted amount and had the gene action expected for *fw2.2*. The cause of the QTL effect is a single gene, *ORFX*, that is expressed early in floral development, controls carpel cell number and has a sequence suggesting structural similarity to the human oncogene c-H-*ras* p21. Alterations in fruit size, imparted by *fw2.2* alleles, are most likely due to changes in regulation rather than in the sequence and structure of the encoded protein.[195]

Key proteins and their genes may influence whole organisms and thus determine overall physiological traits. Uncoupling protein-3 (UCP-3) is a member of the mitochondrial transporter superfamily that is expressed predominantly in skeletal muscle. Its close relative UCP-1 is expressed exclusively in brown adipose tissue, a tissue whose main function is fat combustion and thermogenesis. Studies on the expression of UCP-3 in animals and humans in different physiological situations support a role for UCP-3 in energy balance and lipid metabolism. Transgenic mice were created that overexpress human UCP-3 in skeletal muscle. These mice are hyperphagic but weigh less than their wild-type littermates. MRI analysis shows a striking reduction in adipose tissue mass. The mice also exhibit lower fasting plasma glucose and insulin levels, and an increased glucose clearance rate. This provides evidence that skeletal muscle UCP-3 has the potential to influence metabolic rate and glucose homeostasis in the whole animal.[196]

This observation may have relevance for a systemic approach to deal with a multitude of age-related pathological developments.

The reaction of glucose with the amino groups of proteins such a collagen and elastin to form advanced glycosylation end-products (AGEs) leads to gradual loss of elasticity in the cardiovascular system and plays a role in the ageing process as exemplified by atherosclerosis, stroke and heart failure. Cardiac stiffening, which is accelerated in diabetics, can be prevented by inhibitors of AGE formation. A new class of therapeutic agents has been developed which can reverse the cross-linking process and restore the cardiovascular system to a more youthful state. A lead compound is ALT711 (4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride) which interacts with the cross-linked proteins, separating them by cleaving the cross-link.

AGE cross-link breakers could also be beneficial for many other conditions such as nephropathy, retinopathy, neuropathy, and urinary elastic dysfunction.[197]

Another example where one gene influences the whole organism in an apparently concerted fashion is the size of the whole organism which appears to be controlled by few key genes and proteins. Suppressor of cytokine signaling-2 (SOCS-2) is a member of the suppressor of cytokine signaling family, a group of related proteins implicated in the negative regulation of cytokine action through inhibition of the Janus kinase (JAK) signal transducers and activators of transcription (STAT) signal-transduction pathway. Mice unable to express SOCS-2 were used to examine its function *in vivo*. SOCS-$2^{-/-}$ mice grew significantly larger than their wild-type littermates. Increased body weight became evident after weaning, and was associated with significantly increased long bone lengths and the proportionate enlargement of most organs. Characteristics of deregulated growth hormone and IGF-I signaling, including decreased production of major urinary protein, increased local IGF-I production and collagen accumulation in the dermis, were observed in SOCS-2-deficient mice, indicating that SOCS-2 may have an essential negative regulatory role in the growth hormone/IGF-I pathway.[198]

With the escalation of obesity-related disease, there is a great interest in defining the mechanisms that control appetite and body weight. A link between anabolic energy metabolism and appetite control was identified. Both systemic and intracerebroventricular treatment of mice with fatty acid synthase (FAS) inhibitors (cerulenin and a synthetic compound C75) led to inhibition of feeding and dramatic weight loss. C75 inhibited expression of the prophagic signal neuropeptide Y in the hypothalamus and acted in a leptin-independent manner that appears to be mediated by malonyl-CoA. Fas may represent an important link in feeding regulation and may be a potential therapeutic target.[199]

The study of complex phenomena involving hundreds or thousands of genes is facilitated by genome profiling (gene expression analysis). An example of a temporally and spatially complex process is metamorphosis. Metamorphosis is an integrated set of developmental processes controlled by a transcriptional hierarchy that coordinates the action of hundreds of genes. In order to identify and analyze the expression of these genes, high-density DNA microarrays containing several thousand *D. melanogaster* gene sequences were constructed. Many differentially expressed genes were assigned to developmental pathways known to be active during metamorphosis, whereas others can be assigned to pathways not previously associated with metamorphosis. Additionally, many genes of unknown function were identified that may be involved in control and execution of metamorphosis. The utility of this genome-based approach is demonstrated for studying a set of

complex biological processes in a multicellular organism, such as the coordination between exdysone-regulated pathways and developmental pathways controlling differentiation of particular cell types and tissues.[200]

The genetic basis of senescence is the target of intense studies at the molecular, differential gene expression level. Recent studies point towards the machinery of chromosome separation and repair as major contributors to senescence. Fibroblasts derived from donors of different ages (normal young, normal middle and normal old) and from pathological source (Hutchinson–Gilford progeria) were studied by examining their different mRNA transcription profiles for significant expression changes. The comparative analysis of gene expression in natural and accelerated human ageing at the fibroblast level revealed a limited set of genes that are differentially expressed.

mRNA levels were measured in actively dividing fibroblasts isolated from human donors of various ages as well as from an individual suffering from progeria, a rare genetic disorder of accelerated ageing. Genes whose expression is associated with age-related phenotypic changes and with disease were identified. The results indicate that an underlying mechanism of the ageing process involves increasing errors in the mitotic machinery of dividing cells in the post-reproductive stage of life. This dysfunction may lead to chromosomal pathologies resulting in misregulation of genes involved in the ageing process.[201]

Evidence that somatic mutations are causally related to the degenerative aspects of the ageing process has been derived from human syndromes of accelerated ageing, such as WS. This disease is caused by a heritable mutation in the WRN gene, encoding both a helicase and an exonuclease, and is considered to play a role in suppressing genomic instability. Cultured somatic cells from patients with WS display an increased rate of somatic mutations and a variety of cytogenetic abnormalities, such as deletions and translocations. Other so-called progeroid syndromes, such as ataxia telangiectasia and Bloom syndrome, also show genomic instability.

Mouse models with inactivated genes involved in double-strand break repair show enhanced chromosomal instability and prematurely exhibit symptoms of age-related degeneration in various tissues. Telomere erosion in telomerase-deficient mice results in an early initiation of genetic instability, accelerating the age-related loss of cell viability and increased tumor formation. Genetic defects promoting gross genomic instability are associated with symptoms of accelerated ageing. Ageing affects both proliferative and post-mitotic organs. Mutation accumulation in post-mitotic cells is difficult to study because most methods require cycling cells. With the development of transgenic mouse models harboring bacterial transporter genes which can be retrieved from chromosomal DNA, mutation accumulation in post-mitotic tissues can be quantitated and characterized.

Somatic mutation accumulation has been implicated as a major cause of cancer and ageing. By using a transgenic mouse model with a chromosomally integrated *lacZ* reporter gene, mutational spectra were characterized at young and old age in two organs greatly differing in proliferative activity, i.e. the heart and small intestine. At young age the spectra were nearly identical, mainly consisting of GC → AT transitions and 1-bp deletions. At old age, however, distinct patterns of mutations had developed. In the small intestine, only point mutations were found to accumulate, including GC → TA, GC → CG and AT → CG transversions and GC → AT transitions. In contrast, in heart about half of the accumulated

mutations appeared to be large genome arrangements, involving up to 34 cM of chromosomal DNA. Virtually all other mutations accumulating in the heart appeared to be GC → AT transitions at CpG sites. Distinct mechanisms lead to organ-specific genome deterioration and dysfunction at old age.[202]

Induction of the cyclin-dependent kinase (CDK) inhibitor p21$^{Waf1/Cip1/Sdi1}$ is a common mechanism of growth arrest in different physiological situations. p21 is transiently induced in the course of replicative senescence, reversible and irreversible forms of damage-induced growth arrest, and terminal differentiation of post-mitotic cells. Its induction is regulated through p53-dependent and -independent mechanisms. Ectopic overexpression of p21 leads to cell growth arrest in G_1 and G_2; this arrest is accompanied by phenotypic markers of senescence in some or all cells.

Induction of CDK inhibitor p21$^{Waf1/Cip1/Sdi1}$ triggers cell growth arrest associated with senescence and damage response. Overexpression of p21 from an inducible promoter in a human cell line induces growth arrest and phenotypic features of senescence. cDNA array hybridization showed that p21 expression selectively inhibits a set of genes involved in mitosis, DNA replication, segregation and repair. The kinetics of inhibition of these genes on p21 induction parallels the onset of growth arrest and their re-expression on release from p21 precedes the re-entry of cells into the cell cycle, indicating that inhibition of cell cycle progression genes is a mechanism of p21-induced growth arrest. P21 also up-regulates multiple genes that have been associated with senescence or implicated in age-related diseases, including atherosclerosis, Alzheimer's disease, amyloidosis and arthritis. Most of the tested p21-induced genes were not activated in cells that had been growth arrested by serum starvation, but some genes were induced in both forms of growth arrest. Several p21-induced genes encode secreted proteins with paracrine effects on cell growth and apoptosis. In agreement with the overexpression of such proteins, conditioned media from p21-induced cells were found to have anti-apoptotic and mitogenic activity. The effects of p21 induction on gene expression in senescent cells may contribute to the pathogenesis of cancer and age-related diseases.[203]

Studies of ageing involve numerous model systems, such as cell cultures, yeast, nematodes (*C. elegans*), *Drosophila* and mice. Some revealing genetic studies in yeast have provided clues to the fundamental mechanisms.

Studies of the replicative potential of rat cells in vivo have shown that rat Schwann cells and most oligodendrocyte precursor cell purified from postnatal rat optic nerve are capable of indefinite proliferation in culture.[508,509] The rat Schwann cells maintain checkpoints otherwise lost in the immortalization process. The rat oligodendrocyte precursor cells can proliferate indefinitely in serum-free culture provided they are prevented from differentiating. The cells maintain high telomerase activity and p53- and Rb-dependent cell cycle checkpoint responses. Serum or genotoxic drugs induce the cells to develop a senescence-like phenotype. The experiments suggest that for rat cells senescence is not inevitable during cultural proliferation and that some normal rodent precursor cells have an unlimited proliferative capacity in conditions avoiding differentiation and the activation of cell-cycle arresting checkpoint responses.

Rodent cells thus appear not to have the cell counting mechanism of human cells, where the shortening of telomeres with each cell division counts the number of cell divisions they

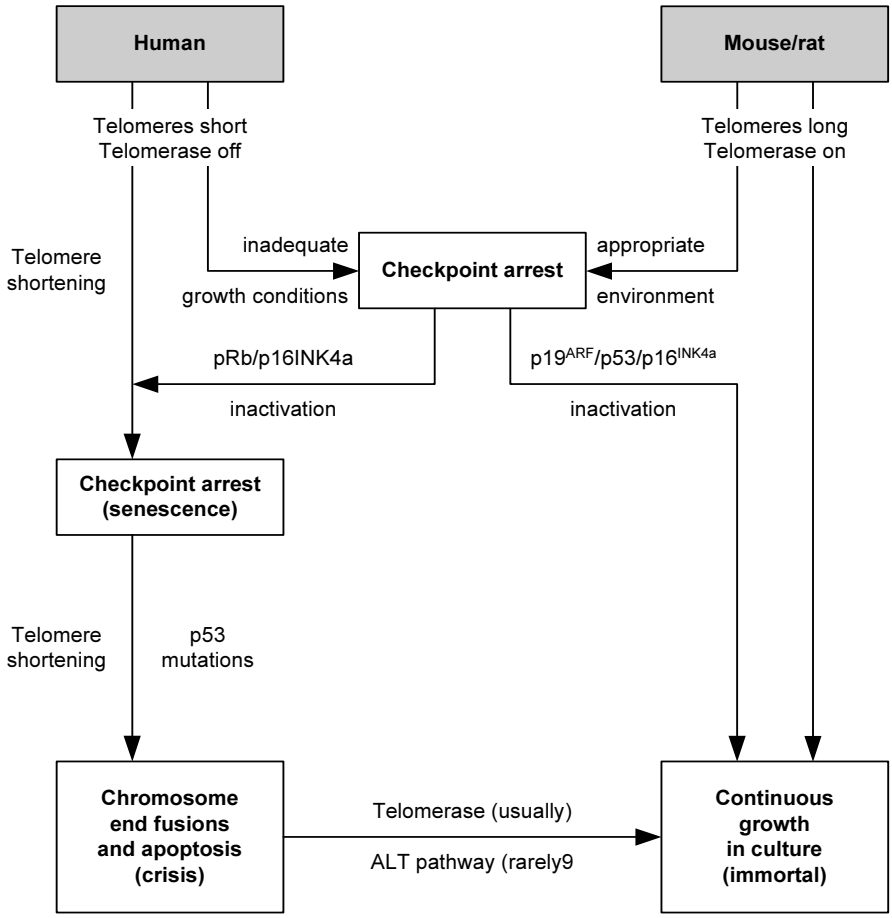

Figure 4.2. The long and short of aging. Most rodent cells contain the enzyme telomerase and have long telomeres. Under the appropriate tissue culture conditions, rodent cells are capable of continuous growth *(1,2,8).* However, an inadequate culture environment (for example, that in which appropriate survival factors are missing or where different cell types cannot interact) may result in DNA damage or other stresses that induce arrest of cell division at cell cycle checkpoints. The spontaneous inactivation of $p19^{ARF}$, p53, and perhaps $p16^{INK4a}$ under standard culture conditions frequently enables normal rodent cell to grow continuously. Human fibroblasts have short telomeres and are satiny telomerase silent. As is the case with rodent cells, inadequate culture conditions may induce human cells to activate checkpoint pathways that lead to their early (and telomere-independent) growth arrest (9, 10). In contrast to rodent cells, bypass of the p53 and/or pRb/$p16^{INK4a}$ checkpoints is insufficient to immortalize human fibroblasts but does prolong their lifespan. Continuous telomere shortening of human fibroblasts leads to chromosome fusions, crisis, and apoptosis. Only a rare human cell (1 in 10 million) can bypass crisis either through telomerase reactivation or through ALT (the rare alternative pathway for telomere lengthening). Although methods for counting the number of cell divisions do not limit the growth of cultured rodent cells, in human cells telomere shortening does provide a record of the number of doublings and does control replicative senescence [Refs in 510].

have undergone.[510] The difference between human and rodent cellular aging is depicted in Figure 4.2.

Telomeres play a crucial role in the viability of cells. Experimental disruption of telomerase action causes telomere shortening and cellular senescence[525] and genetic defects of telomerase have significant impact. The X-linked form of the human disease dyskeratosis congenita (DKC) is due to mutations in the gene coding for dyskerin and causes defects in regenerative tissues such as skin and bone marrow, chromosome instability, and a predisposition towards certain malignancies. Dyskerin is associated with human telomerase RNA which contains an H/ACA RNA motif and mutations lead to defective telomere maintenance.[526]

In *Caenorhabditis elegans*, an insulinlike signaling pathway controls aging, metabolism, and development. Mutations in the *daf-2* insulin receptor-like gene or the downstream *age-1* phosphoinositide 3-kinase extend adult life-span by two- to three-fold. In order to identify the regulation of aging and metabolism by this pathway, the *daf-2* pathway signaling was restored to only neurons, muscle, or intestine. Insulin like signaling in neurons alone was sufficient to specify wild-type life span, whereas restoration of muscle or intestinal signaling was insufficient to do so. The restoration of the *daf-2* pathway signaling in muscle rescued metabolic defects, indicating a decoupling of life-span and metabolism. The nervous system appears to be a central regulator of longevity in *C. elegans.*[511] The study of aging and developmental genetic regulation in *C. elegans* is supported by the availability of genome-wide data on gene expression profiles.[588]

Research for genes involved in aging carried out in genetically tractable organisms such as yeast, the nematode *Caenorhabditis elegans, Drosophila melanogaster* fruitflies, and mice has established that aging is regulated by specific genes. The pertinent pathways are linking physiology, signaltransduction, and gene regulation. The mutations identified and the correlations of phenotypes indicate that these genes are in control of aging in higher organisms.[522]

Mutations in *Caenorhabditis elegans* affecting sensory cilia or their support cells, or in sensory signal transduction, extend lifespan. *C. elegans* senses environmental signals through ciliated sensory neurons located primarily in specific sensory organs in head and tail. The cilia are membrane-bound microtubule-based structures found at the dendritic endings of sensory neurons in *C. elegans*. The findings of increased lifespan in mutants with defects in sensory cilia implies that lifespan may be regulated by environmental signals.[512]

Biochemical and genetic studies in the long-lived *Caenorhabditis clk-1* mutants demonstrated that these mutants are Q auxotrophs. CLK-1 is a mitochondrial polypeptide with sequence and functional conservation from human to yeast. The *Saccharomyces cerevisiae* homolog Coq7p is essential for ubiquinone synthesis and thus respiration. Development of the *clk-1* mutants requires a dietary source of Coenzyme Q.[527]

In the filamentous fungus *Podospora anserina* the impairment of the nuclear *COX5* gene encoding subunit V of the cytochrome c oxidase complex leads to the use of the alternative respiratory pathway and a decrease in production of reactive ion species with a concomitant increase in lifespan associated with stabilization of the mitochondrial genome. This provides evidence for a causal link between mitochondrial metabolism and longevity in *Podospora anserina.*[518]

Table 4.3. Mutations revealing links between longevity and stress resistance in multicellular organisms [Refs in 520].

Species/ mutations	Gene description	Phenotypic influence*	
		Life span†	Stress resistance
C. elegans			
age-1	Human Pl (3) K homologue	65% increase	Enhanced (UV, paraquat, heat)
daf-2	Human insulin-receptor homologue	100% increase	Enhanced (UV, paraquat, heat)
daf-16	Forkhead transcription factor	Suppresses longevity conferred by *age-1* and *daf-2* mutations	Suppresses stress resistance of *age-1* and *daf-2* mutants
clk-1	Homologue of yeast gene associated with coenzyme Q biosynthesis	40% increase	Enhanced increased (UVC)
spe-10	Unknown (sperm defective	40% increase	Enhanced (UV, paraquat, but not heat)
spe-26	Unknown (sperm defective)	65% increase	Enhanced (UV, paraquat, heat)
old-1	Putative receptor tyrosine kinase	65% increase	Enhanced (UV, heat)
ctl-1	Cytostatic catalase	25% decrease; suppresses longevity conferred by *daf-2, age-1* and *clk-1*	Not determined
mev-1	Cytochrome *b* subunit of succinate dehydrogenase	37% decrease	Hypersensitive to oxygen
Drosophila			
mth	Putative G-protein-coupled receptor	35% increase	Enhanced (UV, paraquat, heat)
Mouse			
shc^{66}	Cytoplasmic signal-transduction adaptor protein	30% increase	Enhanced (UV, H_2O_2)

- See [ref. 83] for original references describing the phenotypes of the *daf-2, daf-16, clk-1* and *spe-26* mutants. References for other mutants are as follows: *age-1* [refs 49,50,83]; *spe-10* [ref. 84]*; old-1*
- [ref. 85]; *ctl-1* [ref. 56]; *mev-1* [ref. 55]; *mth* [ref. 60]; and shc^{66}[ref. 6].

† Numbers reflect changes in mean life span of the mutants relative to wild-type animals.

A genome-wide study of aging and oxidative stress response in *Drosophila melanogaster* involving measurements of genome-wide changes in transcript levels as a function of age in comparison with the effects of a free-radical generator molecule revealed significant responses in a number of genes. Free radicals appear to play an important role in regulating transcript levels in addition to other factors. The studies identify several candidate genes as molecular markers for aging and potential targets for biopharmaceutical intervention in the aging process.[514]

Another genetic study in *Drosophila melanogaster* found that five independent P-element insertional mutations in a single gene resulted in a near doubling of the average adult lifespan without a decline in fertility or physical activity. The product of the pertinent gene *Indy* is closely rrelated to a mammalian sodium dicarboxylate cotransporter, a membrane protein transporting Krebs cycle intermediates. Excision of the P element caused a reversion to normal lifespan. The mutations in this gene may mimic the metabolic state of caloric restriction, which is known to extend lifespan.[515]

The ability to respond to oxidative stress is a critical determinant of life-span. The production of oxidants and the scavenging of reactive oxidant species involve cellular strategies for detection and detoxification of reactive oxygen species and are linked to longevity.[520] Table 4.3 lists mutations with links to stress resistance in multicellular organisms and Table 4.4 shows disease systems related to oxidant stress.

Table 4.4. Oxidants, antioxidants and diseases of ageing [Refs in 520].

Disease System	Laboratory/animal studies	Clinical data
Cardiovascular	Pre-atherosclerotic blood vessels have increased levels of ROS. Vitamin E protects against development of atherosclerosis Disruption of SOD leads to heart failure and overexpression protects against injury	PHSI: no overall benefit of beta-carotene on CVD? Benefit in high-risk subgroup. CHAOS trail: vitamin E reduces rate of non-fatal myocardial infarct. ATBC study: no overall benefit on CVD rate with Vitamin E or beta-carotene? Increase in CVD deaths with beta-carotene.
Opththalmological	Offspring of pregnant mice depleted of glutathione develop cataracts. Retinal pigments produce ROS after light exposure. Retinal degeneration in primates with Vitamin A or E deficiencies.	PHSI: non-significant reduction in cataracts and macular degeneration with Vitamin E and multivitamins NHS: carotenoids intake may decrease risk of cataract.
Neurological	Mutations in SOD1 result in human ALS and transgenic animal models rescued by antioxidants. NMDA-receptor stimulation produces superoxide. Defects in the function of complex 1 seen in Parkinson's disease.	Vitamin E not protective in early Parkinson's disease. Vitamin E beneficial in Alzheimer's disease. *N*-acetylcysteine does not effect survival in ALS.

Altered gene expression in young and senescent cells was catalogued by using enhanced differential display[517] and high-density oligonucleotide arrays were used to examine differences in gene expression in the hypothalamus and cortex of young and aged mice.[187] The hypthalamus plays a key role in regulating metabolism and hypothalamic modulation may influence the aging process. Table 4.5 shows the various gene expression changes in the hyopthalamus from young and old mice, whereas Table 4.6 lists the gene expression changes in the cortex.

Eukaryotic genomes are packaged into nucleosomes, which are thought to repress gene expression generally. Repression is particularly evident at yeast telomeres, where genes within the telomeric heterochromatin appear to be silenced by the histone-binding Sir complex (Sir2, Sir3 and Sir4) and Rap1. To investigate how nucleosomes and silencing factors influence global gene expression, high-density arrays were used to study the effects of depleting nucleosomal histones and silencing factors in yeast. Reducing nucleosome content by depleting histone H4 caused increased expression of 15% of genes and reduced expression of 10% of genes, but it had little effect on expression of the majority (75%) of yeast genes. Telomere proximal genes were found to be de- repressed over regions extending 20 kb from the telomeres, well beyond the extent of the Sir protein binding and the effects of loss of Sir function. These results indicate that histones make Sir-independent contributions to telomeric silencing, and that the role of histones elsewhere in chromosomes is gene specific rather than generally repressive.[205]

Genomic imprinting is characterized by allele-specific expression of multiple genes within large chromosomal domains that undergo DNA replication asynchronously during S phase. Using both FISH analysis and S-phase fractionation techniques, differential replication time was shown to be associated with imprinted genes in a variety of cell types and that it is already present in the pre-implantation embryo soon after fertilization. This pattern is erased.

Table 4.5. Gene expression changes in the hypothalamus from young (2 months) and old (22 months) BALB/c mice [Refs in 187].

Accession number	Name	Fold change
Metabolic enzymes		
W33716	NADH-Ubiquinone oxidoreductase KFYI subunit	2.8
Y07708	NAOH oxidoreductase subunit MWFE	3
AA109866	NADH-ubiquinone oxidoreductase chain 4L	2.1
AA219829	NADH-ubiquinone oxidoreductase SGDH subunit	2.5
AA521794	Cytochrome c oxidase subunit VIIb	2.2
AA672840	ATP synthase 0 subunit	2.2
C76507	ATP synthase μ-subunit	2.7
AA003458	**Sarco/endoplasmic reticulum Ca2+-ATPase 2**	**–2.9**
X56007	**Na/K-ATPase â 2**	**–11**
AA106307	**H+ ATPase subunit E**	**–6.1**
AA087605	H(+)-ATPase (mvp)	–6.9

Table 4.5. Gene expression changes in the hypothalamus from young (2 months) and old (22 months) BALB/c mice (Cont'd).

Accession number	Name	Fold change
Protein processing		
U05333	Cochaperonin 'cofactor' A'	6.8
U09659	Mitochondrial chaperonin 10	2.6
Z31557	Chaperonir containing TCP-1	3.2
AA027544	Ubiquitin-activating enzyme E1	2.8
AA146437	Cathepsin S precursor, a cysteine proteinase	3.4
Z31297	Sorting nexin 2-like protein, protein degradation	4.8
AA013993	**Prolyl olig-peptidase**,	**11**
AA020512	Caspase 6	3
Neuronal growth/structure		
U95116	**Lissencephaly-1 protein (LIS-1)**	**3**
V00835	Metallothionein-I	3.3
X61452	Cell division control-related protein 2b	2.8
AA709861	**A-X actin-like protein**	**3.5**
L31397	**Dynamin**	**–3.5**
U27106	**Clathrin-associated AP-2**	**–5.7**
U86090	Kinesin heavy chain	–2.4
Neuronal signaling		
AA271109	Protein phosphatase 1, regulatory subunit	3.8
X15373	lnositoi-1,4,5-triphosphate receptor	3.5
X51468	Preprosomatostatin gene	3
D37792	**Synaptotagmin 1**	**–17**
D50621	PSD-95/SAP90A	–16
AA048604	**Apolipoprotein E**	**–6.7**
AA068956	**Protein phosphatase PP2A**	**–6.1**
W12204	Ca2+/calmodulin-dependent protein kinase II	–3.6
X61434	**cAMP-dependent protein kinase C â**	**–3.1**
X57497	Glutarnate receptor 1	–2.7
AA710375	*N*-ethylmaleimide-sensitive factor-attachment receptor	–3.4
A8006361	Prostaglandin D synthetase	–5.6
Stress response		
M60798	Cu(2+)-Zn2+ superoxide dismutase	2.9
AA107471	DNAJ homolog 2	–6.4
AA166139	DNA repair protein	–10.4
AA033408	Damage-specific DNA-binding protein, DNA repair	–4.6
D89787	Hif like protein	–6.5
U27830	Stress-inducible protein, STI1	–4.9

Each RNA sample was hybridized twice to two different arrays, and fold change values are averages of the duplicate measurements. Positive values indicate an increase, and negative values indicate a decrease in gene expression. Genes in bold are differentially expressed in both aged cortex and hypothalamus.

Table 4.6. Gene expression changes in the cortex from young (2 months) and old (22 months) BALB/c mice [Refs in 187].

Accession number	Gene name	Fold change
Metabolic enzymes		
M21285	Stearoyl-CoA desaturase	2.4
U27315	Adenine nucleotide translocase-1	2.2
M84145	Fumarylacetoacetate hydrolase	3.1
U13841	ATPase subunit E	–4.4
AA105755	Na+, K+-ATPase α	–4.6
AA003458	**Sarco/endoplasmic reticulum Ca2+-ATPase 2**	**–5.9**
X56007	**Na/K-ATPase β 2 subunit**	**–2.3**
AA 106307	**H(+)-ATPase E-like protein**	**3.8**
AA389346	Citrate synthase	–3.4
Protein processing		
M13500	Kallikrein gene	5.2
X61232	Carboxypeptidase H	3.6
X92665	Ubiquitin-conjugating enzyrne UbcM3	3
AA013993	**Prolyl oligopeptidase**	**2.7**
AA020512	**Caspase 6**	**2.2**
Z30970	Metalloproteinase-3 tissue inhibitor	2.1
Neuronal growth/structure		
U95116	**Lissencephaly-1 protein**	**2.6**
L20899	Cell division cycle (CDC25)	2.6
C76314	Cdc5-like protein	2.4
AA590859	**A-X actin-like protein**	**7.3**
U27106	**Clathrin-associated AP-2**	**–5.5**
AA1 18546	Actin-like protein 3	–6.2
AA050703	Defender against death 1 (DAD1)	–2.4
w 18503	Dynein heavy chain, retrograde transport	–7.7
AA1 11631	Dynactin 1, retrograde axonal transport	–3.4
L31397	Dynamin	–2.7
Neuronal signaling		
AA168959	25 kDa FK506-binding protein FKBP25	2.7
L32372	AMPA receptor subunit (GluR-B)	2.8
X79082	MDK1, a receptor tyrosine kinase	2
D37792	**Synaptotagmin 1**	**–13.2**
M73490	**Apolipoprotein E**	**–6.9**
AA124955	Casein kinase 1α	–2.9
Z67745	**Phosphatase 2A catalytic subunit**	**–6.2**
M27073	Protein phosphatase 1 β	–2.7
J02626	**cAMP-dependent protein kinase C β**	**–4.6**
W13835	*N*-ethylmaleimide-sensitive membrane protein homolog	–3.2
U10120	*N*-ethytmaleimide sensitive factor	–10.3
A8006361	**Prostaglandin D synthetase**	**–3.2**
M27844	Calmodulin	–2.7
M63436	GABA-A receptor α-1 subunit	–7.1

Table 4.6. Gene expression changes in the cortex from young (2 months) and old (22 months) BALB/c mice (Cont'd).

Accession number	Gene name	Fold change
Stress response		
AA105022	Heat-shock protein hsp84-like protein	–2.7
AA204094	HSP4O/DNAJ homolog	–2.9

Each RNA sample was hybridized twice to two different arrays, and fold change values are averages of the duplicate measurements. Genes in bold are differentially expressed in both aged cortex and hypothalamus.

The references cited above should be viewed as only representative examples derived from a much larger, relevant body literature, which is not fully presented.

Acronyms and abbreviations: PHSI, Physician's Health Study I; CHAOS, Cambridge Heart Antioxidant Study; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; NHS, Nurses Health Study; CVD, cardiovascular disease; ALS, amyotrophic lateral sclerosis; NMDA, *N*-methyl-D-aspartate glutamate receptors.

Before meiosis in the germline, and parent-specific replication timing is then reset in late gametogenesis in both the male and the female. Asynchronous replication timing is established in the gametes and maintained throughout development, indicating that it may function as a primary epigenetic marker for distinguishing between the parental lines.[206]

Further important mechanisms of senescence are related to tumor suppressors and the regulations between different suppressors and regulatory signals. The tumor suppressor p53 induces cellular senescence in response to oncogenic signals. p53 activity is modulated by protein stability and post-translational modification, including phosphorylation and acetylation.

The mechanism of p53 activation by oncogenes remains largely unknown. It was reported that the tumor suppressor PML regulates the p53 response to oncogenic signals.Oncogenic Ras up-regulates PML expression and overexpression of PML induces senescence in a p53-dependent manner. p53 is acetylated at lysine 382 upon Ras expression, an event that is essential for its biological function. Ras induces re-localization of p53 and the CBP acetyl-transferase within the PML nuclear bodies and induces the formation of a trimeric p53–PML–CBP complex. Ras-induced p53 acetylation, p5–CBP complex stabilization and senescence are lost in $PML^{-/-}$ fibroblasts. The data establish a link between PML and p53, and indicate that integrity of the PML bodies is required for p53 acetylation and senescence upon oncogene expression.[207]

The application of telomerase needs to be studied with the potential deleterious effects of introducing hTERT in mind. Telomerase activation extends the life span of human mammary epithelial cell (HMEC) cultures while at the same time inducing lasting overexpression of c-*myc* oncogene.[208] The relevance of telomerase in senescence is intensely debated.[209]

A thoroughly studied model for senescence in the nematode *C. elegans*. The studies of evolution of life span in *C. elegans* is consistent with few early-acting genes.[210]

A specific aspect, i.e. germline immortality and telomere integrity, was studied. The germline is an immortal cell lineage that is passed indefinitely from one generation to the

next. To identify the genes that are required for germline immortality, *C. elegans* mutants with mortal germlines were isolated – worms that can reproduce for several healthy generations but eventually become sterile. One of these *mortal germline (mrt*) mutants, *mrt-2,* exhibits progressive telomere shortening and accumulates end-to-end chromosome fusions in later generations, indicating that the MRT-2 protein is required for telomere replication. In addition, the germline of *mrt-2* is hypersensitive to X-rays and to transposon activity. Therefore, *mrt-2* has defects in responding both to damaged DNA and to normal double-strand breaks present at telomeres. *Mrt-2* encodes a homolog of a checkpoint gene that is required to sense DNA damage in yeast. The results indicate that telomeres may be identified as a type of DNA damage and then repaired by the telomere-replication enzyme telomerase.[211]

The potential of cloning depends in part on whether the procedure can reverse cellular ageing and restore somatic cells to a phenotypically youthful state. The birth of six healthy cloned calves derived from populations of senescent donor somatic cells was reported. Nuclear transfer extended the replicative life span of senescent cells (zero to four population doublings remaining) to greater than 90 populations doublings. Early population doubling level complementary DNA-1 (EPC-1, an age-dependent gene) expression in cells from the cloned animals was 3.5- to 5-fold higher than that in cells from age-matched (5–10 months old) controls. Southern blot and flow cytometric analyses indicated that the telomeres were also extended beyond those of newborn (less than 2 weeks old) and age-matched control animals. The ability to regenerate animals and cells may have important implications for medicine and the study of mammalian ageing.[212]

Mice have been cloned by nuclear transfer into enucleated oocytes. In particular, the reiterative cloning of mice to four and six generations in two independent lines was reported. Successive generations show no signs of premature ageing, as judged by gross behavioral parameters and there was no evidence of shortening of telomeres at the ends of chromosomes, normally an indicator of cellular senescence. The telomeres actually increased slightly in length. This increase is astonishing, since the number of mitotic divisions greatly exceeds that of sexually produced animals and any deleterious effects of cloning might be amplified in sequentially cloned mice. These observations bear on studies of organismal ageing.[213]

Telomerase is amenable to therapeutic intervention by small-molecule effectors and thus suitable therapeutics may be developed. Telomerase, a ribonucleoprotein up-regulated in many types of cancers, possesses an RNA template necessary to bind and extend telomere ends. The intrinsic accessibility of Telomerase to incoming nucleic acids makes the RNA template an ideal target for inhibition by oligonucleotides. 2′-*O*-methyl RNA (2′-*O*-meRNA), an oligonucleotide known to exert sequence-specific effects in cell culture and animals, inhibits telomerase with potencies superior to those possessed by analogous peptide nucleic acids (PNAs). Potent inhibition relative to PNAs is surprising, because the binding affinity of 2′-*O*-meRNAs for complementary RNA is low relative to analogous PNAs. A 2′-*O*-meRNA oligomer with terminal phosphorothioate substitutions inhibits telomerase sequence-selectively within human-tumor-derived DU145 cells when delivered with cationic lipids. In contrast to the ability of 2′-*O*-meRNA oligomers to inhibit telomerase, the binding of a 2′-*O*-meRNA to an inverted repeat within plasmid DNA was not detectable, whereas bind-

ing of PNA was efficient, suggesting that the relative accessibility of the telomerase RNA template is essential for inhibition by 2′-*O*-meRNA. Inhibition of telomerase by 2′-*O*-meRNA will facilitate probing the link between telomerase activity and sustained cell proliferation, and may provide a basis for the development of chemopreventive and chemotherapeutic agents.[214]

The importance of telomeres is underscored by studies of chromosomal rearrangements and telomeres. Chromosomal rearrangement mechanisms are intimately linked to cancer development, and are thought to generate the numerous gains and losses of segments of chromosomes needed for epithelial carcinogenesis. Aged humans sustain a high rate of epithelial cancers such as carcinomas of the breast and colon, whereas mice carrying common tumor suppressor gene mutations typically develop soft tissue carcinomas and lymphomas. Among the many factors that may contribute to this species variance are differences in telomere length and regulation. Telomeres comprise the nucleoprotein complexes that cap the ends of eukaryotic chromosomes and are maintained by the reverse transcriptase, telomerase. In human cells, insufficient levels of telomerase lead to telomere attrition with cell division in culture, and possibly with ageing and tumorigenesis *in vivo*. In contrast, critical reduction in telomere length is not observed in the mouse owing to promiscuous telomerase expression and long telomeres. Telomere attrition in ageing telomerase-deficient p53 mutant mice promotes the development of epithelial cancers by a process of fusion-bridge breakage that leads to the formation of complex non-reciprocal translocations – a classical cytogenetic feature of human carcinomas. Telomere dysfunction brought about by continual epithelial renewal during life may generate the massive ploidy changes associated with the development of epithelial cancers.[215]

Life span is also influenced by external factors, such as ROS which primarily arise as by-products of normal metabolic activities and are thought to influence the etiology of age-related diseases. In order to test the theory that ROS cause ageing, the natural antioxidant systems of *C. elegans* with small synthetic superoxide dismutase/catalase mimetics. Treatment of wild-type worms increased their mean life span by a mean of 44%, and treatment of prematurely aging worms resulted in normalization of their life span (a 67% increase). It appears that oxidative stress is a major determinant of life span and that it can be counteracted by pharmacological intervention.[216]

Expression profiling allows the simultaneous identification of differentially expressed genes, and by comparing different cells, tissues, disease states and metabolic states enables the pin-pointing of condition-associated and/or causative gene expression. In tumor therapy, the identification of tumor type and genetic make-up of the malignant cells allows the early and often decisive choice of therapy.

Diffuse large B cell lymphoma (DLBCL), the most common subtype of non-Hodgkin's lymphoma, is clinically heterogeneous: 40% of patients respond well to current therapy and have prolonged survival, whereas the remainder succumb to the disease. This variability in natural history may reflect unrecognized molecular heterogeneity in the tumors. Using DNA microarrays, a systematic characterization of gene expression in B cell malignancies was conducted. It was shown that there is diversity in gene expression among the tumors of DLBCL patients, apparently reflecting the variation in tumor proliferation rate, host response and differentiation state of the tumor. Two molecularly distinct forms of

DLBCL were identified which had gene expression patterns indicative of different stages of B cell differentiation. One type expressed genes characteristic of germinal center B cells ('germinal center B-like DLBCL'); the second type expressed genes normally induced during *in vitro* activation of peripheral blood B cells ('activated B-like DLBCL'). Patients with germinal center B-like DLBCL had a significantly better overall survival than those with activated B-like DLBCL. The molecular classification of tumors on the basis of gene expression can thus identify previously undetected and clinically significant subtypes of cancer.[217]

Similarly, the technique of gene expression profiling was applied to another difficult to categorize type of tumor, malignant melanoma. The most common human cancers are malignant neoplasms of the skin. Incidence of cutaneous melanoma is rising especially steeply, with minimal progress in non-surgical treatment of advanced disease. Despite significant effort to identify independent predictors of melanoma outcome, no accepted histopathological, molecular or immunohistochemical marker defines subsets of this neoplasm. Accordingly, although melanoma is thought to present different 'taxonomic' forms, these are considered part of a spectrum rather than discrete entities. The discovery of a subset of melanomas identified by mathematical analysis of gene expression in a series of samples was reported. Many genes underlying the classification of this subset are differentially regulated in invasive melanomas that form primitive tubular networks *in vivo*, a feature of some highly aggressive metastatic melanomas. Global transcript analysis can identify unrecognized subtypes of coetaneous melanoma and predict experimentally verifiable phenotypic characteristics that may be of importance to disease progression and choice of treatment.[218]

To gain an understanding of the molecular basis of tumor angiogenesis, gene expression patterns were compared of endothelial cells derived from blood vessels of normal and malignant colorectal tissues. Of over 170 transcripts predominantly expressed in the endothelium, 79 were differentially expressed, including 46 that were specifically elevated in tumor-associated endothelium. Several of these genes encode extracellular matrix proteins, but most are of unknown function. Most of these tumor endothelial markers were expressed in a wide range of tumor types, as well as in normal vessels associated with wound healing and corpus luteum formation. These studies demonstrated that tumor and normal endothelium are distinct at the molecular level – a finding that may have significant implications for the development of antiangiogenic therapies.[219]

SAGE analysis reveals previously characterized and novel pan endothelial markers. The most abundant characterized or novel tags derived by summing the tags from normal EC (N-ECs) and tumor EC (T-ECs). SAGE libraries are listed in descending order. For comparison, the corresponding number of SAGE tags found in HUVEC and HMVEC endothelial cell cultures, and several nonendothelial cell lines (14), are shown. Tag numbers for each group were normalized to 100.000 transcripts. A description of the gene product corresponding to each tag is given, followed by alternative names in parentheses. Some uncharacterized genes have predicted full-length coding sequence. The sequence CATG precedes all tags, and the 15th base (11th shown) was determined as described (38) [References in 219].

Human breast tumors are diverse in their natural history and in their responsiveness to treatment. Variation in transcriptional programs accounts for much of the biological diversity of human cells and tumors. In each cell, signal transduction and regulatory systems transduce information from the cell's identity to its environmental status, thereby controlling the level of expression of every gene in the genome. Variation was characterized in gene expression patterns in a set of 65 surgical specimens of human breast tumors from 42 different individuals, using complementary DNA microarrays representing 8102 human genes. These patterns provided a distinctive molecular portrait of each tumor. Twenty of the tumors were sampled twice, before and after a 16-week course of doxorubicin chemotherapy, and two tumors were paired with a lymph node metastasis from the same patient. Gene expression patterns in two tumor samples from the same individual were almost always more similar to each other than either was to any other sample. Sets of co-expressed genes were identified for which variation in mRNA levels could be related to specific features of physiological variation. The tumors could be classified into subtypes distinguished by pervasive differences in their gene expression patterns.[220]

The elucidation of genetic contributions to senescence in *Drosophila melanogaster* and *Caenorhabditis elegans* and their relationship points towards fundamental evolutionary conserved genetic and biochemical mechanisms.[655]

The *Drosophila melanogaster* gene *insulin-like receptor (InR)* was found homologous to mammalian insulin receptors as well as to *Caenorhabditis elegans daf-2*, a signal transducer regulating worm dauer formation and adult longevity. A heteroallelic, hypomorphic genotype of mutant *InR*, which results in dwarf females with up to an 85% extension of adult longevity and dwarf males with reduced late age-specific mortality. Treatment of the long-lived *InR* dwarfs with a juvenile hormone analoge restores the life expectancy toward that of wild-type controls thus demonstrating that juvenile hormone deficiency resulting from the *InR* signal pathway mutation is sufficient to extend life-span in flies. The results also show that insulin-like ligands nonautonomously mediate aging in flies through retardation of growth or activation of specific endocrine tissue.[654]

The *Drosophila melanogaster* gene *chico* encoding an insulin receptor substrate which functions in an insulin/insulin-like growth factor (IGF) signaling pathway was found to extend upon mutation the fruit fly median life-span by up to 48% in homozygotes and 36% in heterozygotes. The extension of life-span was neither a result of impaired oogenesis in *chico* females nor correlated with increased stress resistance nor was the dwarf phenotype of *chico* homozygotes necessary for extension of life-span.[653]

Genetic linkage studies are required to identify the multiple genes involved in complex traits and population genetic studies involving single-nucleotide polymorphisms and other genetic variations such as different alleles are the basis for pharmaco-genomics and the identification of genetic disease associations.[691] [692]

Table 4.7 shows a listing of the pan-endothelial markers identified by SAGE analysis.

Table 4.7. Gene determined by SAGE analysis to be potential pan-endothelial markers [Refs in 219].

Tag sequence	N-ECs	T-ECs	HUVEC	HMVEC	Cell lines	Description	
Known genes							
CATATCATTAA	247	501	130	87	2	Angiomodulin (ANG, IGFBP-7, IGFBP-rP1, Mac25, TAF)*	
TGCACTTCAAG	328	141	0	0	0	Hevin*	
TTTGCACCTTT	165	84	191	115	4	Connective tissue growth factor (CTGF, IGFBP-rP2)*	
TTGCTGACTTT	73	131	2	14	1	Collagen, type VI, alpha l*	
ACCATTGGATT	102	67	0	0	2	Interferon induced transmembrane protein 1 (9-27, Leu 13)*	
ACACTTCTTTC	104	44	60	62	2	Guanine nucleotide binding protein 11	
TTCTGCTCTTG	71	67	118	72	0	Von Willebrand factor*	
TCCCTGGCAGA	66	68	3	13	3	Cysteine-rich protein 2 (CRP-2, ESP-1, SmLIM)	
TAATCCTCAAG	26	106	34	16	1	Collagen, type XVIII, alpha l*	
ATGTCTTTTCT	58	65	17	17	3	Insulin-like growth factor-binding protein 4*	
GGGATTAAAGC	40	67	30	14	2	CD146 (S-Endo 1, P1H12, Muc18, MCAM. Mel-CAM)*	
TTAGTGTCGTA	38	69	9	13	0	SPARC (osteonectin,BM-40)*	
TTCTCCCAAAT	20	86	16	64	2	Collagen, type IV, alpha 2*	
GTGCTAAGCGG	24	74	0	10	2	Collagen, type VI, alpha 2*	
GTT'TATGGATA	35	56	11	11	1	Matrix Gla protein (MGP)	
Novel genes							
CCCTTGTCCGA	131	104	1	1	0	PEM1	EST
CCCTTTCACAC	52	33	0	0	0	PEM2	ESTs
CAACAATAATA	42	25	13	6	0	PEM3	ESTs
GGCCCTACAGT	26	13	2	3	0	PEM4	ESTs/KIAA0821 protein
GCTAACCCCTG	7	31	0	1	0	PEMS	ESTs
GGCACTCCTGT	22	13	19	12	0	PEM6	ESTs
TCACAGCCCCC	20	15	8	5	0	PEM7	ESTs
CAACAATAATA	42	25	13	6	0	PEM3	ESTs
GGCCCTACAGT	26	13	2	3	0	PEM4	ESTs/KIAA0821 protein
GCTAACCCCTG	7	31	0	1	0	PEMS	ESTs
GGCACTCCTGT	22	13	19	12	0	PEM6	ESTs
TCACAGCCCCC	20	15	8	5	0	PEM7	ESTs
TTTCATCCACT	20	13	0	2	0	PEM8	ESTs. KIAA0362 protein
ATACTATAATT	25	6	2	0	0	PFM9	ESTs
AATAGGGGAAA	13	19	4	1	0	PEM10	KIAA1075 protein

* Characterized genes that have previously been shown to be expressed predominantly in endothelium (10, 15–26).