Genetic Approaches to Treating Disease

21

KEY CONCEPTS

- Genetic techniques can be applied in various ways in treating disease, regardless of whether the disease has a genetic origin or not.
- Genetics is being used extensively in the drug discovery process to identify new drug targets.
- Therapeutic recombinant proteins produced by expressing cloned DNA are safer than those sourced from animal and human sources.
- Genetic engineering of vaccines may involve inserting genes for desired antigens into a viral vector and using either the modified vector or the purified antigen as the vaccine. Alternatively, DNA can be used as a vaccine by direct injection into muscle cells.
- Cells lost by disease or injury can be replaced by suitable stem cells that have been directed to differentiate into the correct specialized cell type.
- Nuclear reprogramming involves fundamental alteration of the chromatin structure and gene expression pattern so as to convert differentiated cells into less specialized cells (*dedifferentiation*) or to a different type of differentiated cell (*transdifferentiation*).
- Autologous cell therapy involves conversion of a patient's cells into a type of cell that is deficient through disease or injury. In principle this can be done by artificially stimulating the mobilization of existing stem cells in the patient or by nuclear reprogramming.
- Skin fibroblasts from a patient can be dedifferentiated to make patient-specific totipotent or pluripotent stem cells that can be differentiated to required cell types for use in autologous cell therapy and for studying disease. In *therapeutic cloning* the nucleus from a patient's cell is transplanted into an enucleated egg cell from a donor, giving rise to a totipotent cell. More conveniently, the skin cells can simply be induced to express key transcription factors that will convert them to pluripotent stem cells.
- Gene therapy involves transferring genes, RNA, or oligonucleotides into the cells of a patient so as to alter gene expression in some way that counteracts or alleviates disease. The patient's cells are often genetically modified in culture and then returned to the body.
- Gene therapy strategies often seek to compensate for underproduction of an important gene product. Other strategies are designed to block the expression of a mutant gene that makes a harmful gene product, or to induce alternative splicing so that a harmful mutation does not get included in a mRNA, or to kill harmful cells, for example in cancer gene therapy.
- Viral vectors are commonly used in gene therapy because they have good gene transfer rates, but they sometimes provoke strong immune responses and also abnormal activation of cellular genes such as proto-oncogenes.
- Zinc finger nucleases can be designed to make just one double-strand DNA break within the genome of intact cells. Cellular pathways that repair double-strand DNA breaks can then be induced to replace a sequence containing a harmful gene mutation with the normal allelic sequence.

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In the preceding chapters in this part of the book, we have looked at how genetics can be used to test individuals and populations for genetic diseases and susceptibility for complex diseases. Chapter 20 explored genetic manipulation of animals to model human diseases. In this chapter, we look at how genetics can be employed to treat diseases. A chapter on disease treatment might reasonably consider two quite separate matters: the treatment of genetic disease, and the genetic treatment of disease. We therefore consider briefly what these two areas cover before describing how genetic techniques are being used directly to treat disease.

21.1 TREATMENT OF GENETIC DISEASE VERSUS GENETIC TREATMENT OF DISEASE

There is no connection at all between cause and treatability of a disease. Orthodox medical treatment aimed at alleviating the symptoms of a disease is just as applicable to genetic diseases as to any other disease. A profoundly deaf child should be offered hearing aids or a cochlear implant based solely on the child's symptoms and family situation, quite regardless of whether the hearing loss is due to a genetic disease or has another cause.

Treatment of genetic disease is most advanced for disorders whose biochemical basis is well understood

For many genetic conditions, existing treatments are unsatisfactory. A survey published by Costa and colleagues in 1985 estimated that treatment improved reproductive capacity in only 11% of Mendelian diseases, improved social adaptation in only 6%, and extended life span to normal in only 15% (of those that reduced longevity). No doubt the figures would be better now, but not dramatically so. Causative genes for many genetic disorders have been identified only quite recently, and it may take many years of research to identify how the underlying genes function normally in cells and tissues. Then it may be possible to design effective treatments.

Treatment has been possible, however, for some genetic disorders, for which we have detailed knowledge of the cellular and molecular basis of disease. For Mendelian disorders, inborn errors of metabolism are well understood at the biochemical level and are well suited to conventional treatments. There are many potential entry points for intervention in inborn errors of metabolism. Some examples are given below.

- *Substrate limitation.* Babies with phenylketonuria (OMIM 261600) have a genetic deficiency of phenylalanine hydroxylase. The resulting excess of phenylalanine has a neurotoxic effect that leads to impaired postnatal cognitive development (see Chapter 19, p. 629). Modifying the diet to reduce the intake of phenylalanine is a successful treatment. Even in successfully treated patients, however, cognitive development averages half a standard deviation below normal. Several other inborn errors respond equally well to dietary treatment.
- *Replacement of a deficient product.* One example is supplying thyroid hormone to infants with congenital hypothyroidism; another is enzyme replacement therapy, which is available for various metabolic disorders. Enzymes are often conjugated with polyethylene glycol to make them more active and more stable.
- *Using alternative pathways to remove toxic metabolites.* Treatments of this type range from simple bleeding as a very effective treatment for an iron overload condition, hemochromatosis (OMIM 235200), to using benzoate to increase nitrogen excretion in patients with disorders of the urea cycle.
- *Using metabolic inhibitors.* Babies with type 1 tyrosinemia (OMIM 276700) are unable to metabolize tyrosine effectively and therefore suffer liver damage from an excess of certain toxic intermediates. Their prognosis is markedly improved when the drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) is administered to block the enzyme 4-hydroxyphenylpyruvate dioxygenase.

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Despite much work, however, relatively few genetic diseases have wholly satisfactory treatments.

Genetic treatment of disease may be conducted at many different levels

Any disease, whether it has a genetic cause or not, is potentially treatable by using a range of different procedures that involve applying genetic knowledge or genetic manipulations in some way (Figure 21.1).

Sometimes genetic techniques form part of a treatment regime that also involves conventional small-molecule drugs or vaccines. For example, as described in Chapter 19, individuals may be genotyped to predict their pattern of favorable and adverse responses to specific drug treatments. Such genotyping may become routine as massively parallel DNA sequencing permits extensive screening of genes in vast numbers of people. Another approach involves the use of knowledge of genetics and cell biology to identify new targets for drug development. Genetic techniques can also be used directly in producing drugs and vaccines for treating disease.

Another active area concerns treating disease with therapeutic proteins that are produced or modified by genetic engineering. They include so-called *recombinant proteins*, which are produced by expression cloning and include hormones, blood factors, and enzymes, and also genetically engineered antibodies. We consider in Section 21.2 genetic inputs to treating disease with drugs, or therapeutic proteins and vaccines.

In addition, there are two other major areas where genetic interventions are being designed to treat disease. One involves using genetic techniques to assist *cell transplantation* methods for treating diseases in which the pathogenesis involves a loss of tissue and cells. Stem cells may offer important therapeutic possibilities in this area, and the principles and genetic inputs into stem cell therapies are discussed in Section 21.3.

Finally, we consider in Sections 21.4–21.6 a whole range of different therapeutic methods that involve the direct genetic modification of a patient's cells (*gene therapy*). Some of the methods involve transplanting genes into the cells of patients to overcome some genetic deficiency or to encourage the killing of harmful cells. A wide range of other gene therapy methods involve altering the expression of genes in the cells of patients in some way, by modifying the genome or by targeting gene expression products. Because they are needed to replenish tissue and blood cells, stem cells are important target cells for gene therapy.

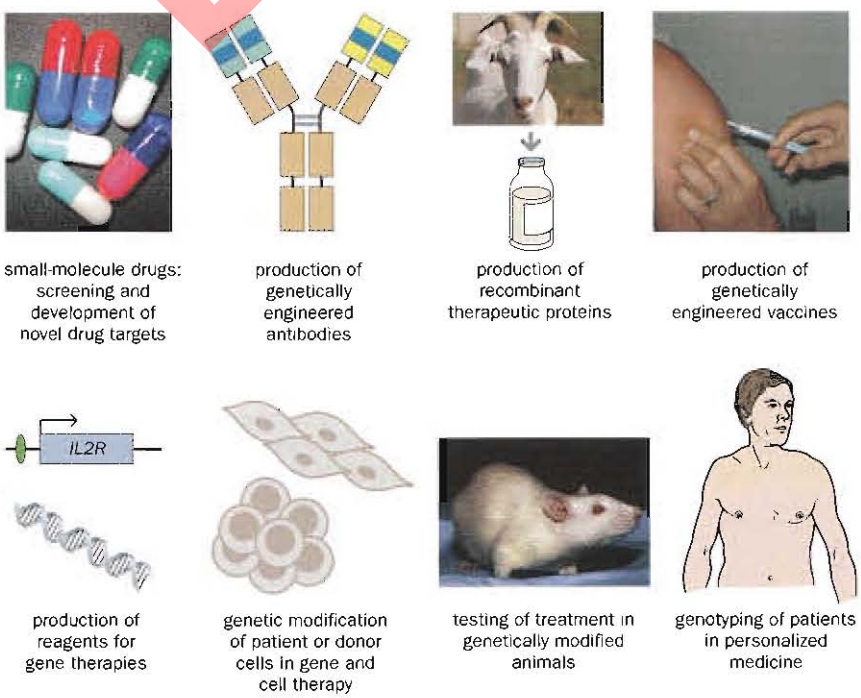

Figure 21.1 Some of the many different ways in which genetic technologies are used in the treatment of disease. See the text for detail. IL2R, interleukin type 2 receptor.

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21.2 GENETIC APPROACHES TO DISEASE TREATMENT USING DRUGS, RECOMBINANT PROTEINS, AND VACCINES

The pharmaceutical industry is responsible for developing most chemical treatments of disease. For decades, the drug discovery process has involved screening huge numbers of small molecules for evidence that they can reduce pathogenic effects. The drug screening process begins with laboratory studies involving cell culture and animal disease models (**Box 21.1**) before moving on to clinical trials. This process is costly and time-consuming (see Figure 19.11), and the drugs currently on the market were first developed when information about possible targets was scarce. In addition, the entire diversity of drugs currently on the market act through only a few hundred target molecules, and the declining number of new drug applications and approvals over the past few years has reflected a crisis in drug target identification and validation. Even when a drug receives regulatory approval, it is rarely effective in 100% of the patients to whom it is prescribed and may adversely affect some patients.

In Section 19.2 we considered how genetic variation between individuals affects the way in which people respond to drugs. In this section, we look at the various ways in which genetics is being applied in the drug discovery process. In addition to expanding the range of small-molecule drugs, genetics technologies have been applied to making genetically engineered therapeutic proteins and vaccines.

Drug companies have invested heavily in genomics to try to identify new drug targets

As described in Box 21.1, most drugs currently on the market bind to target molecules that belong to just a few protein classes. Novel drug targets are badly needed. One solution is to identify *generic drugs* that are not focused on a specific gene product, such as drugs that can suppress stop codons. The potential applications are huge because nonsense mutations promoting premature translational termination are responsible for causing anywhere from 5% to 70% of individual cases for most inherited diseases. Aminoglycoside antibiotics such as gentamycin were known to work in this way, but although gentamycin can cause readthrough of premature nonsense codons in mammalian cells it was found not to be clinically useful, partly because of a lack of potency and partly because of toxicity problems. Recently, however, extensive small-molecule screening has

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BOX 21.1 DRUG TARGETS AND SMALL-MOLECULE DRUG SCREENING

Drugs are often small chemical compounds that are extracted from natural sources or chemically synthesized. Generally a *drug target* is some naturally existing molecule or cellular structure that is involved in a pathology of interest, and which the intended drug is designed to act on to treat the pathology. The molecular targets that have been considered most druggable have traditionally been proteins. Frequently they are receptors (notably G-protein-coupled receptors), enzymes (especially protein kinases, phosphatases, and proteases, or protein channels (voltage-gated or ion-gated). Small-molecule drugs fit tightly into crevices on the surface of their target proteins, thereby modulating their function.

Drug screening frequently involves *high-throughput screening*. With the use of liquid handling devices and robotics, hundreds of thousands to millions of different chemical compounds are individually assayed for their ability to react with some kind of biological material in specified wells of numerous microtiter dishes. The biological material has traditionally involved a cell-free system, microbes (bacteria, yeasts), or cultured cell lines, but more recently *in vivo* animal drug screening has also been possible. Searching for compounds may occur in a non-directed approach with normal cells or tissues in which the object is to identify any phenotypes produced and correlate with the drugs that caused that effect. Often, however, the search for compounds is a directed one: the object is to search for drugs that react with a *defined* target or disease phenotype.

Various types of cultured cells, and immortalized aneuploid cell lines, can be used for drug screening. Recently isolated stem cell lines allow the possibility of controlled differentiation of euploid cells to a desired differentiated cell type. As described in Section 21.3, skin fibroblasts from any individual can now also be reprogrammed to make pluripotent stem cells, and so disease-specific pluripotent stem cells are being produced that can be directed to differentiate to give desired human disease cells for drug screening.

Animal disease models offer a physiological disease environment. Mice and rats are important in the testing of drug therapies before launching clinical trials, but rodent embryos are impractical for high-throughput drug screening. The culture conditions of small invertebrates such as *C. elegans* and *D. melanogaster* allow high-throughput drug screening in a physiological context, but they are limited to drugs that recognize evolutionarily very highly conserved targets. Zebrafish embryos offer the advantage of both being vertebrates and also transparent (allowing live tissue imaging) and are becoming increasingly important in drug screening.

identified a drug known as PTC124® or ataluren that can cause readthrough of premature nonsense mutations, notably UGA, without affecting the identification of normal stop codons, and with little evidence of toxicity. The results from clinical trials to assess whether PTC124 can provide clinical benefit in disorders such as cystic fibrosis will be eagerly awaited.

Another potential solution to the crisis in drug target identification has come from the genome projects; the new data offer a vastly expanded number of potential targets available for research. Currently, drug companies use all the resources spawned by genomics—including massive data sets generated by high-throughput transcriptomics, proteomics, bioinformatics, and so on—to focus general efforts in identifying new drug targets. Because of its simplicity, RNA interference is often used to identify genes for which an inhibitory drug might produce a useful effect, before investing in the large-scale screening that would be needed to identify possible inhibitors. Additional effort is being made to analyze in detail whole pathways that contribute to pathogenesis. In the long term, it is hoped that completely new classes of drugs will emerge from this effort.

Genomic or proteomic studies of pathogenic microorganisms are also important, as guides to the development of new vaccines or treatments. Microbial pathogens have featured strongly on priority lists for genome sequencing, a notable recent success being the malaria parasite *Plasmodium falciparum*. Sequences are analyzed to identify enzymes specific to the pathogen and not the host, which could be targets for inhibitors, or missing enzymes that make the parasite vulnerable to interference with the supply of an essential nutrient. Virulence proteins or gene products expressed early in infection are promising drug targets. They can be identified by expression array studies, and by comparisons of gene content or gene expression in virulent and non-virulent strains. Proteomics strategies (such as MALDI-TOF mass spectrometry) are used to identify proteins on the pathogen cell surface that might be targets for vaccines.

Therapeutic proteins can be produced by expression cloning in microbes, mammalian cell lines, or transgenic animals

Certain genetic disorders that result from a deficiency of a specific protein hormone or blood protein can be treated by obtaining and administering an external supply of the missing protein. To ensure greater stability and activity, the proteins are often conjugated with polyethylene glycol (PEG). Such *PEGylation* increases the size of the protein in solution and, by reducing its renal clearance, prolongs its time in the circulation. It can also confer reduced immunogenicity.

Many such therapeutic proteins were formerly extracted from animal or human sources, but safety of the external supplies has been an important issue. Many hemophiliacs contracted AIDS and/or hepatitis C after they had been treated with Factor VIII prepared from unscreened donated blood. In another example, some children with growth hormone deficiency succumbed to Creutzfeldt–Jakob disease after being given injections of growth hormone extracted from unscreened cadaver pituitary glands. A safer, but more expensive, alternative is to use therapeutic proteins produced by expression cloning (expression cloning techniques are described in Section 6.3). Recombinant human insulin was first marketed in 1982, and **Table 21.1** includes several subsequent examples.

Microbes are the simplest hosts for expression cloning. Large volumes can be cultured, and the media can be defined and controlled to avoid contamination. *Escherichia coli* and *Saccharomyces cerevisiae* have been found to be satisfactory for producing simple peptides such as human insulin and growth hormone. However, human proteins are often glycosylated and microbes do not reproduce the human patterns of glycosylation or other complex post-translational modifications. In addition, human proteins may have intricate folding requirements.

Mammalian gene expression systems became a primary focus for the production of human recombinant proteins. Some cell culture lines, notably the Chinese hamster ovary (CHO) cell line, have been used to express various complex molecules such as monoclonal antibodies (see the next section) and cytokines. Some human proteins, however, are required in such high therapeutic doses that sufficient quantities of the highly purified protein cannot easily be produced in cell

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TABLE 21.1 EXAMPLES OF THERAPEUTIC RECOMBINANT PROTEINS OBTAINED BY EXPRESSION CLONING

Recombinant protein	For treatment of
Insulin	diabetes
Growth hormone	growth hormone deficiency
Blood clotting Factor VIII	hemophilia A
Blood clotting Factor IX	hemophilia B
α-Interferon	hairy cell leukemia; chronic hepatitis
β-Interferon	multiple sclerosis
γ-Interferon	infections in patients with chronic granulomatous disease
Tissue plasminogen activator	thrombotic disorders
Granulocyte/macrophage colony-stimulating factor	neutropenia after chemotherapy
Leptin	obesity
Erythropoietin	anemia

For genetically engineered antibodies, see Table 21.2.

lines (or microbial bioreactors). Instead, transgenic animals have been used because large amounts of a recombinant human protein can be produced in ways that simplify its purification. One way is to arrange that the desired protein is secreted in the animal's milk. An expression vector is designed with the coding sequence for the human protein under the control of a promoter from a gene that normally makes a milk protein, and the recombinant DNA is inserted into the animal's germ line (**Figure 21.2**).

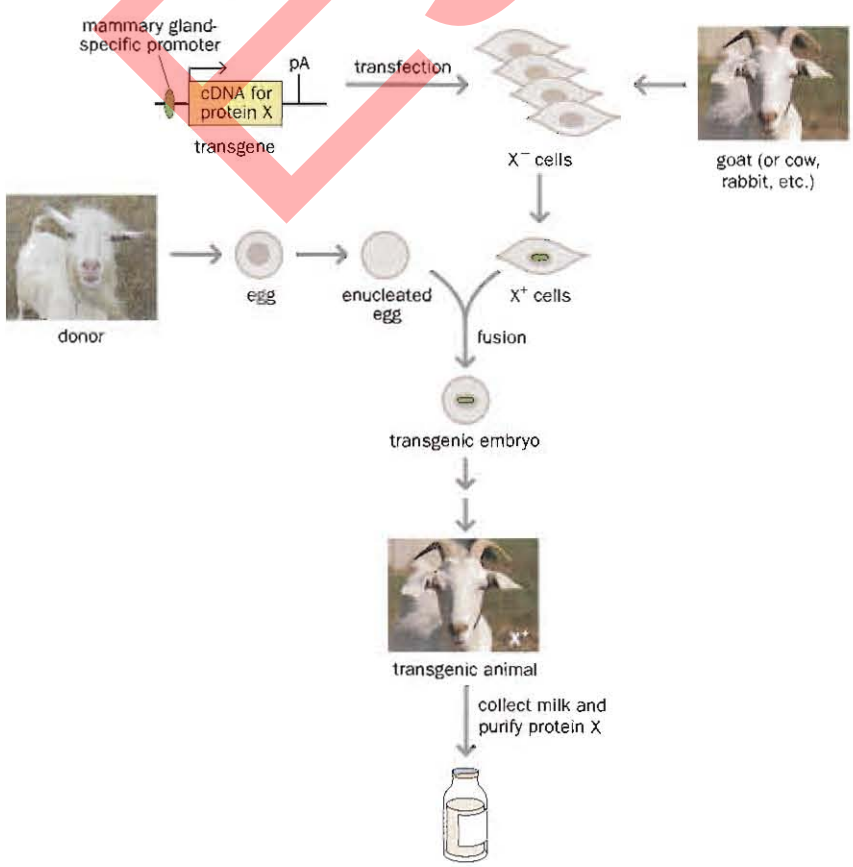

Figure 21.2 Producing therapeutic recombinant proteins in the milk of transgenic livestock. To express the desired recombinant protein X in the milk of a transgenic livestock animal such as a goat, a suitable transgene is constructed with a promoter that will cause the gene to be expressed and secreted in the animal's milk. The transgene can be inserted into the animal's germ line by using pronuclear microinjection (Figure 20.3), or, as shown here, by inserting the transgene into somatic cells from the animal, then identifying cells containing the transgene (X^+) followed by fusion with an enucleated egg from a donor animal to make a totipotent embryo. First-generation animals that are carriers of the transgene are mated, and female offspring serve as the production herd. The therapeutic protein is expressed in the animal's milk but needs to be purified by sequential chromatography processes that ultimately deliver a product that is more than 99.99% pure. pA, polyadenylation site.

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The process of using transgenic livestock (such as goats, pigs, or sheep) to make a therapeutic human protein has been called *pharming*, pharmaceutical-led farming. Milk proteins can be produced in large amounts, and a flock of about 150 goats could produce the world's supply of a specific protein. The cost of keeping flocks of transgenic animals is also significantly less than maintaining large-scale industrial bioreactors. In 2009, ATryn® became the first therapeutic protein produced by a transgenic animal to be approved by the US Food and Drug Administration (FDA). Produced by GTC Biotherapeutics, ATryn is an anti-thrombin expressed in the milk of goats and is designed to be used in anti-blood clotting therapy.

Other types of transgenic organism have been investigated to produce human proteins. Transgenic chickens are designed to lay eggs rich in a wanted product. Transgenic plants are also becoming popular. Plants do not replicate human-specific glycosylation patterns, but they have many advantages for expression cloning in terms of cost and safety. For example, there has been considerable interest in producing golden rice strains engineered to counter the vitamin A deficiency that is a serious public health problem in at least 26 countries in Africa, Asia, and Latin America.

Genetic engineering has produced novel antibodies with therapeutic potential

One class of therapeutic protein that has been widely used to treat disease comprises genetically engineered antibodies. As detailed in Section 4.6, each one of us has a huge repertoire of different antibodies that act as a defense system against innumerable foreign antigens. Antibody molecules function as adaptors: they have binding sites for foreign antigen at the variable end, and binding sites for effector molecules at the constant end. Binding of an antibody may by itself be sufficient to neutralize some toxins and viruses but, more usually, the bound antibody triggers the complement system and cell-mediated killing (see Figures 4.20 and 4.23).

Artificially produced therapeutic antibodies are designed to be specific for a single antigen (monospecific). Traditional **monoclonal antibodies** (**mAbs**) are secreted by **hybridomas**, immortalized cells produced by the fusion of antibody-producing B lymphocytes from an immunized mouse or rat with cells from an immortal mouse B-lymphocyte tumor (see Box 8.6). Hybridomas are propagated as individual clones, each of which can provide a permanent and stable source of a single mAb. Unfortunately, the therapeutic potential of mAbs produced in this way is limited. Although rodent mAbs can be raised against human pathogens and cells, they have a short half-life in human serum, often causing the recipient's immune system to make anti-rodent antibodies. In addition, only some of the different classes can trigger human effector functions.

Genetic engineering has been used to produce modified monoclonal antibodies in which some or all of the rodent protein sequence is replaced by equivalent human sequence. Often, this is achieved by swapping parts of the rodent sequence at the DNA level for equivalent human DNA sequences. *Chimeric antibodies* have been produced, for example, in which the rodent variable regions are retained but the constant (C) region sequences are replaced by human C region sequences. Ultimately *humanized antibodies* were constructed in which the only rodent sequences were from the complementarity determining regions (CDRs), the hypervariable sequences of the antigen-binding site (**Figure 21.3**). Phage display technology (see Figure 6.13) bypasses hybridoma construction altogether and allows innovative combinations of antibody domains to be constructed.

More recently, it has been possible to prepare *fully human antibodies* (see Figure 21.3D), by different routes. For example, mice have been genetically manipulated so that they can make nothing but fully human antibodies. To do this, the mouse immunoglobulin heavy- and light-chain loci were deleted in embryonic stem (ES) cells and then a human artificial chromosome containing the entire human heavy-chain and γ light-chain loci was inserted into the ES cells and used to give rise to transgenic strains with a human immunoglobulin (Ig) repertoire (**Figure 21.4**). Fully human antibodies can also be isolated by *mammalian cell display*, a mammalian equivalent of phage display. With this approach,

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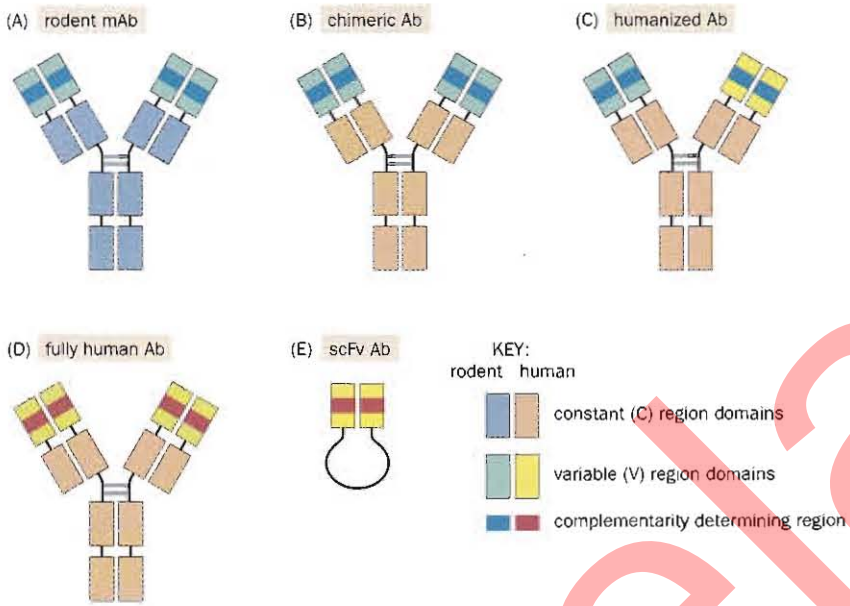

Figure 21.3 Antibody engineering. (A) Classic monoclonal antibodies (mAbs) are monospecific rodent antibodies synthesized by hybridomas. (B) Chimeric V/C antibodies (Abs) are genetically engineered to have rodent variable region sequences containing the critically important hypervariable complementarity determining region (CDR) joined to human constant region sequences. (C) Humanized antibodies can be engineered so that all the molecule except the CDR is of human origin. (D) More recently, it has been possible to obtain fully human antibodies by different routes (see the text and Figure 21.4). (E) Single-chain variable fragment (scFv) antibodies contain variable region domains jointed by a peptide linker. They are particularly well suited to working within the reducing environment of cells and serve as *intrabodies* (intracellular antibodies) by binding to specific antigens within cells. Depending on the length of the linker, they bind their target as monomers, dimers, or trimers. Multimers bind their target more strongly than monomers.

fully human antibodies have recently been obtained against nicotine. They have been shown to be effective in inhibiting the entry of nicotine into the brain in mice; if the same effect is replicated in clinical trials they could be a valuable aid in stopping smoking.

One very promising class of therapeutic antibody is designed to have a single polypeptide chain. *Single-chain variable fragment (scFv) antibodies* have almost all the binding specificity of a mAb but are restricted to a single non-glycosylated variable chain (see Figure 21.3E). scFV antibodies can be made on a large scale in bacterial cells, yeast cells, or even plant cells. They are particularly well suited to acting as intracellular antibodies (**intrabodies**). Instead of being secreted like normal antibodies, they are designed to bind specific target molecules within cells and can be directed as required to specific subcellular compartments. Unlike standard antibodies, which have four polypeptide chains linked by disulfide bridges, intrabodies are stable in the reducing environment within cells.

Intrabodies can be used to carry effector molecules that can perform specific functions when antigen binding occurs. However, for many therapeutic purposes, they are designed simply to block specific protein–protein associations within cells. As such, they complement conventional drugs. Protein–protein interactions usually occur across large, flat surfaces and are considered unsuitable targets for typical small-molecule drugs, which normally operate by fitting snugly into clefts on the surface of macromolecules. Promising therapeutic target proteins for intrabodies include mutant proteins that tend to misfold in a way that causes neurons to die, as in various neurodegenerative diseases including Alzheimer, Huntington and prion diseases.

From inauspicious beginnings in the 1980s, mAbs have become the most successful biotech drugs ever, and the market for mAbs is the fastest-growing component of the pharmaceutical industry. By the middle of 2008, a total of 21 mAb therapies had been approved by the US FDA. Of the therapeutic mAbs currently in use, the eight bestsellers together generate an annual income of close to $30 billion. Another 200 mAb products are in the pipeline. Of the FDA-approved mAbs, 19 are partly or fully human and most of them are aimed at treating cancers or autoimmune disease (Table 21.2). Only one of the approved antibodies is directed against infectious disease, being used to fend off respiratory syncytial virus in infants.

Safety issues can sometimes be a concern, however. In 2006 a clinical trial to assess a mAb called TGN1412 left six volunteers fighting for their lives. TGN1412 was hoped to be an effective agent in *cancer immunotherapy*, in which the strategy is to stimulate immune system cells so as to provoke a strong immune response against the cancer cells. However, TGN1412 activated immune cells to such an extent that the volunteers developed intense and nearly fatal allergic reactions to the mAb.

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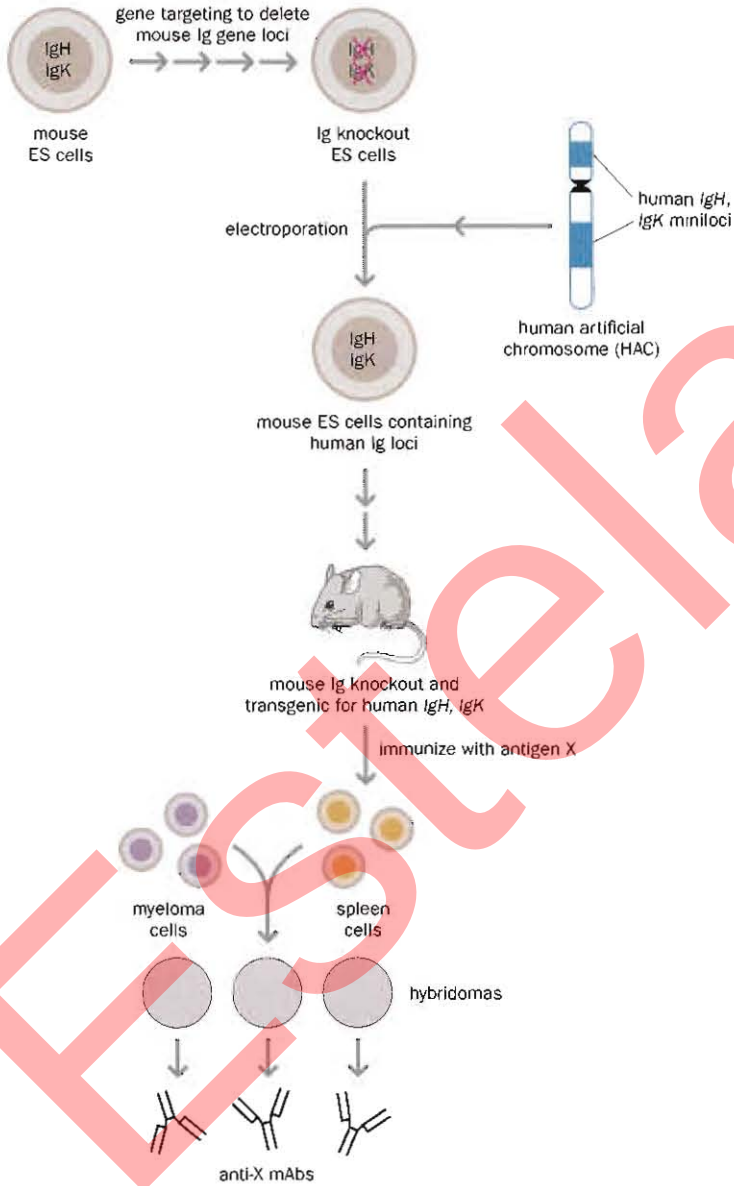

Figure 21.4 Making transgenic mice that express human monoclonal antibodies. To make fully human monoclonal antibodies (mAbs), Isao Ishida and colleagues generated a human artificial chromosome (HAC) containing the entire human Ig heavy-chain locus plus the entire human Igγ light-chain loci. This HAC was then introduced into mouse ES cells that had previously been subjected to rounds of Cre–*loxP* gene targeting to delete the endogenous mouse Ig loci. The resulting mouse can generate immunoglobulins by rearrangements of the introduced human Ig loci, and so can be used to produce human mAbs of any desired specificity. For full details see US patent 7041870, accessible by searching the patent database at http://www.patentstorm.us.

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Aptamers are selected to bind to specific target proteins and inhibit their functions

Short oligonucleotide (or peptide) **aptamers** can also be identified that will bind to (and inhibit) specific target proteins, with affinities similar to those of antibodies. Starting from a large pool of random oligonucleotides, oligonucleotide aptamers are conveniently made by repeated rounds of selection and amplification, usually by the SELEX (**s**ystematic **e**volution of **l**igands by **ex**ponential enrichment) method that was described in Chapter 12 (see Figure 12.14).

Automated SELEX has generated an increasing number of aptamers for inhibiting protein function, and chemical modifications of the oligonucleotides make them more resistant to enzymatic degradation in body fluids. Like small-molecule drugs, aptamers can fit tightly into crevices on the surface of target macromolecules, but they can also fold to form clefts into which protruding parts of the target protein can bind. As a result, the potential number of contacts made with the target increases, allowing aptamers to form tighter, more specific interactions than small-molecule drugs can.

Potential therapeutic applications for aptamers include the inhibition of both intracellular target molecules, such as transcription factors, and extracellular targets. The latter can include targeting of specific viral components so as to inhibit

TABLE 21.2 PARTLY OR FULLY HUMAN THERAPEUTIC MONOCLONAL ANTIBODIES (mAbs) APPROVED BY THE US FOOD AND DRUG ADMINISTRATION AS OF MID-2008

Disease category	Target[a]	mAb generic name (trade name)	mAb class[b]	Disease treated
Autoimmune disease/ immunological	CD11a	efalizumab (Raptiva®)	humanized	psoriasis
	CD25 = IL2R	basiliximab (Simulect®)	chimeric	prevention of kidney transplant rejection
		daclizumab (Zenapax®)	humanized	
	complement-5	eculizumab (Soliris®)	humanized	paroxysmal nocturnal hemoglobinuria
	IgE	omalizumab (Xolair®)	humanized	asthma
	Integrin α4	natalizumab (Tysabri®)	humanized	multiple sclerosis
	TNF-α	infliximab (Remicade®)	chimeric	Crohn disease, rheumatoid arthritis
		certolizumab pegol (Cimzia®)	humanized	
		adalimumab (Humira®)	fully human	
Cancer	CD20	rituximab (Rituxan®, MabThera®)	chimeric	non-Hodgkin lymphoma
	CD33	gemtuzumab ozogamicin (Mylotarg®)	humanized	CD33-acute myeloid leukemia
	CD52	alemtuzumab (Campath®)	humanized	B-cell chronic lymphocytic leukemia
	EGFR	cetuximab (Erbitux®)	chimeric	colorectal cancer
		panitumumab (Vectibix®)	fully human	
	HER2	trastuzumab (Herceptin®)	humanized	metastatic breast cancer
	VEGF	bevacizumab (Avastin®)	humanized	colorectal, breast, renal, NSCL cancer
Other diseases	GPIIb/IIIa	abciximab (ReoPro®)	chimeric	adjunct to percutaneous transluminal coronary angioplasty
	RSV	palivizumab (Synagis®)	humanized	respiratory syncytial virus prophylaxis
	VEGF	ranibizumab (Lucentis®)	humanized	age-related macular degeneration (intravitreal injection)

[a]CD11a, CD20, CD25, CD33, and CD52, white blood cell antigens; IL2R, interleukin type 2 receptor; IgE, immunoglobulin E; TNF-α, tumor necrosis factor α; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; VEGF, vascular endothelial growth factor; GPIIb/IIIa, a platelet integrin; RSV, respiratory syncytial virus; NSCL, non-small-cell lung. [b]See Figure 21.3 for structures.

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chronic viral infections. Several aptamers are currently being tested in preclinical and clinical trials, and recently the aptamer pegaptanib (Macugen®) received FDA approval for the treatment of neovascular (wet) age-related macular degeneration, a principal cause of blindness in adults. Loss of vision in this disorder arises by the formation of new blood vessels (angiogenesis) and leakage from blood vessels, both of which are promoted by vascular endothelial growth factor (VEGF). Pegaptanib aptamers specifically bind to VEGF and inhibit its angiogenesis functions.

Vaccines have been genetically engineered to improve their functions

Vaccines against infectious disease are made by killing or attenuating (weakening) the disease organism (such as virus, bacteria, or fungus) and injecting it into the patient to stimulate the patient's immune system to produce suitably specific antibodies. If the patient were subsequently to come into natural contact with the disease organism, the body's immune system would be expected to mount a strong response that would prevent illness.

Genetic engineering has been applied in different ways to make novel types of vaccine. Genetic modification can be used to disable pathogenic microorganisms, for example, so that an attenuated live vaccine can be used safely. Genetically

modified plants might be used to produce edible vaccines. In addition, changes can be made to an antigen to improve its visibility to the immune system, so as to produce an enhanced response.

Genes for desired antigens can be inserted into a vector, usually a modified virus with very low virulence, to produce *recombinant vaccines*. The vaccine may consist of the antigen-expressing vector or purified antigen. The hepatitis B vaccine is an illustrative example. A cloned gene encoding a hepatitis B surface antigen was inserted into an expression vector and transfected into yeast cells. The protein product of the expressed recombinant is purified for injection as a vaccine. The recombinant vaccine is much safer than using the attenuated hepatitis B virus, which could potentially revert to its virulent phenotype, causing lethal hepatitis or liver cancer.

DNA vaccines are the most recently developed vaccines and, as in the case of recombinant vaccines, genes for the desired antigens are located and cloned. With DNA vaccines, however, the DNA is directly injected into the muscle of the recipient often by biolistics—a gene gun uses compressed gas to blow the DNA into the muscle cells. Some muscle and dendritic cells express the pathogen DNA to stimulate the immune system. DNA vaccines induce both humoral and cellular immunity.

Although clinical trials conducted so far have provided overwhelming evidence that DNA vaccines are well tolerated and have an excellent safety profile, the early designs of DNA vaccines failed to demonstrate sufficient immunogenicity in humans. More recent results have been more encouraging but the efficacy of many new and more complex DNA vaccines must await the outcomes of many clinical trials currently underway.

Cancer vaccines

Cancers constitute another class of disease that is potentially amenable to vaccination. Some types of cancer are caused by viruses, such as HBV (causing some forms of liver cancer) and human papillomavirus (causing about 70% of cases of cervical cancer). Traditional vaccines raised against those viruses can effectively *prevent* the development of the relevant cancer (*preventive cancer vaccines*). However, vaccines for treating *existing* cancers (*therapeutic cancer vaccines*) have been much less successful. Therapeutic cancer vaccines often involve immunotherapy strategies in which certain types of gene are transfected into patient cells, constituting a type of gene therapy that we will consider in Section 21.4.

21.3 PRINCIPLES AND APPLICATIONS OF CELL THERAPY

Present-day organ transplantation is based on replacing a defective organ by a healthy one from a suitable donor. Cell-based therapies can be seen as a natural extension of the same principle, and advances in our understanding of stem cells offer the hope of radically extending the present range of options.

Stem cell therapies promise to transform the potential of transplantation

Much of the focus in stem cell therapy involves simple cell replacement therapy. Stem cells would be used to differentiate into suitable cell types so as to compensate for a deficit in that type of cell. In theory, the possibilities are endless—any damaged or worn-out tissue or organ resulting from disease or injury might be renovated or re-created using appropriate stem cells, and these might be subjected to any sort of genetic manipulation in advance. As we will see later in this chapter, stem cells are also important targets in gene therapy.

As detailed in Section 4.5, stem cells are cells that can both self-renew and also give rise to differentiated progeny. They are probably present at all stages of development and in all tissues. There is a gradient in their ability to give rise to diverse cell types (*plasticity*), from pluripotent stem cells that can potentially produce germ-line and all somatic cell types of an organism, through to stem cells that are more restricted in their differentiation potential and that are described as multipotent, oligopotent, or unipotent (see Table 4.6).

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Pluripotent embryonic stem cells are derived from the early embryo, a stage in development at which cells are naturally still unspecialized. Later, as tissues form in development, cells become more and more specialized but some stem cells are retained in the fetus and in adults. They are needed to regenerate tissue. Our blood, skin, and intestinal epithelial cells, for example, have limited lifetimes and are replaced regularly by new cells produced by differentiation from stem cells. Stem cells are also used in tissue repair. Although many stem cells derived from fetal and adult tissues are tissue-specific, mesenchymal stem cells are found in a wide range of tissues and may constitute a subset of cells associated with minor blood vessels (pericytes).

Embryonic stem cells

Cells of the inner cell mass of a blastocyst can be isolated and cultured to generate embryonic stem (ES) cell lines (Box 21.2). Mouse ES cells have been studied for many years but, after the isolation of human ES cell lines was first reported in 1998, stem cell therapy was propelled to the forefront of both medical research and ethical debate. ES cells have clear advantages for cell therapy because they can be grown comparatively readily in culture and propagated indefinitely and can give rise to cell lineages. Different ES cell lines show significant variation in some properties, however, such as their potential for differentiation. Some ES cell lines readily differentiate toward mesodermal lineages, for example, whereas others do not.

The use of ES cell lines is contentious because the methods used to isolate them have traditionally involved the destruction of a human embryo to obtain cells in the inner cell mass. Nevertheless, some human ES cell lines have been made without destroying an embryo. For example, individual cells (*blastomeres*) can be carefully isolated from very early embryos in such a way that the embryo remains viable, and a few human ES cell lines have been generated from individual blastomeres isolated in this way.

Tissue stem cells

Deriving stem cells from adult human tissues and adult and cord blood has not been contentious. Tissue stem cells were initially thought to be comparatively

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BOX 21.2 MAKING AND VALIDATING EMBRYONIC STEM CELL LINES

Embryonic stem (ES) cell lines are cultured cells that are derived from cells from the inner cell mass (ICM) of the blastocyst. For laboratory animals, blastocysts are excised from oviducts. Blastocysts for making human ES cell lines are traditionally obtained as a by-product of *in vitro* fertilization (IVF) treatment for infertility. In IVF, eggs provided by donors are fertilized by sperm in culture dishes; surplus embryos produced in this way can be used for research purposes with the informed consent of the egg donors.

Isolated blastocysts are disaggregated to allow the outgrowth of cells from the ICM in culture dishes. Traditionally, the inner surface of the culture dish is coated with *feeder cells*, mouse embryonic fibroblasts that have been treated so that they do not divide. The feeder cells provide a sticky surface to which the ICM cells can attach and they secrete growth factors and other chemicals that will stimulate the growth of the ICM cells. Surviving ICM cells that divide and proliferate are collected and re-plated on fresh culture dishes and subsequently undergo many cycles of re-plating and subculturing (known as *passages*). Embryonic stem cells that have continued to proliferate after subculturing for a period of 6 months or longer and that are judged to be pluripotent and seem to be genetically normal are known as an **embryonic stem (ES) cell line**.

The pluripotency of ES cell lines can be validated in different ways. In animals, ES cells can be differentiated to give tissues from all three germ layers, and can also be shown to give rise to a whole new organism. For human ES cell lines, pluripotency is validated by demonstrating differentiation to give tissues from all three germ layers. One way is to grow the cells in liquid suspension culture instead of the usual growth on surfaces to form colonies. In suspension, the ES cells can spontaneously aggregate and differentiate to form *embryoid bodies* that consist of cells from all three germ layers. A more rigorous test is to inject the cells into an immunodeficient mouse to form a benign multilayered tumor known as a *teratoma*. Teratomas consist of different tissues derived from all three germ layers that can be associated in chaotic ways, giving a bizarre appearance. They also arise spontaneously in some individuals (Figure 1).

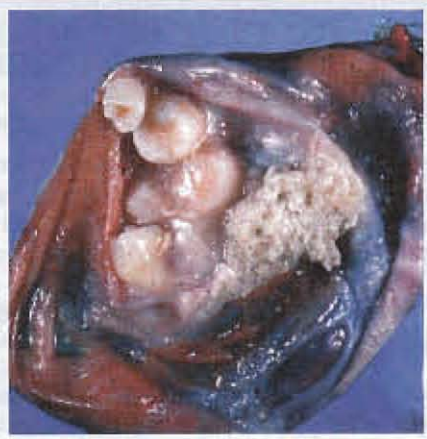

Figure 1 Human ovarian teratoma containing various tissues including teeth. [Image reproduced with permission from Virginia Commonwealth University.]

plastic under the right experimental conditions. For example, when bone marrow stem cells are placed in the environment of liver tissue they seem to be induced to give rise to new liver cells. However, when rigorously tested, there is little evidence to support claims of this type. Instead, tissue stem cells are now known to be almost always rather limited in their differentiation potential. An exception is provided by the adult germ line—pluripotent germ-line stem cells have recently been derived from spermatogonial cells of the adult human testis and show many similarities to ES cells. Many tissue stem cells are also difficult to grow in culture.

Practical difficulties in stem cell therapy

Stem cell therapy is also challenged by various practical limitations that depend on the effective expansion of stem cells in culture, controlled efficient differentiation, effective delivery of the desired replacement cells, and correct physical and functional integration within the desired tissue. Defining the molecular basis of the stemness that distinguishes a stem cell from other related cells has been an enduring problem, and so it has been extremely difficult to obtain highly purified stem cell populations. Our knowledge of the factors that control how stem cells differentiate, although rapidly advancing, remains primitive. As described below, some clinical trials have simply involved injecting crude preparations of stem cells directly into the damaged tissue and relying on the host tissue to send signals to guide the stem cells to differentiate into the desired cell type. It would clearly be more desirable to be able to induce efficient differentiation of stem cells by exogenously controlled factors once all the factors that regulate the relevant differentiation pathway are known.

Different diseases and tissue injuries may be less or more suited to cell therapy, according to tissue accessibility. Blood disorders and skin burns can be expected to pose fewer challenges than brain disorders. Another consideration is complexity of tissue architecture and regulation of cell function. Heart and liver cell replacement therapies would be facilitated by the simple tissue structures of these organs. Diabetes is more challenging because the pancreatic islets of Langerhans have a complex organization, and insulin-producing beta cells are just one of five different cell types in the islets and are subject to considerable regulation.

Important safety issues also need to be addressed. The pluripotency of cells such as ES cells carries the risk that some will give rise to tumors. Thus, differentiation would need to be maximized to reduce the chances of leaving residual undifferentiated stem cells. ES cells and many other stem cells can also show chromosomal instability if cultured for long periods.

Allogeneic and autologous cell therapy

In *allogeneic cell therapy* the transplanted cells come from a donor and are genetically different from those of the recipient. Except for a few *immunologically privileged* sites, such as the brain and the cornea and anterior chamber of the eye, there is a high risk of immune rejection of the donor cells. To counter this problem, stem cells can be genetically manipulated to make them less visible to the recipient's immune system. Stem cell banks containing large numbers of different ES cell lines could also offer individual cell lines that are closely HLA-matched to the cells of an intended recipient of cell therapy.

In *autologous cell therapy* the cells used to replace cells lost in the patient also originate from the patient, thereby avoiding immune rejection. One possible route relies on converting non-stem cells into stem cells by reprogramming their nuclei and gene expression patterns. This is a rapidly expanding field that will be described below. Another way is to artificially enhance the mobilization and differentiation of existing stem cells in the body. Our capacity for tissue repair depends on the degree to which stem cells are mobilized and effective in producing the necessary differentiated cells, and it is subject to developmental programming. Thus, in the fetus, wounds to skin tissue can be perfectly repaired by stem cells that mobilize to regenerate all the required cell types within the correct tissue architecture. With increasing age, this regenerative capacity is lost: adult skin heals through scar formation, with erratic vascular formation, loss of hair follicles, and disorganized collagen deposition.

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While normal mobilization of an individual's stem cells can help make small repairs to different degrees, large injuries and serious disease pose too much of a challenge for the body's natural repair processes. But what if we could artificially enhance the normal stem cell response to repair? Bone marrow cells that are mobilized in response to disease or injury are an attractive target for artificially enhanced mobilization. They include mesenchymal stem cells, which can become bone or cartilage cells, and endothelial progenitor cells, which produce the cells that make up our blood vessels and help with tissue repair. A promising report published in early 2009 showed that administration of the drug Mozobil® and a growth factor was found to elicit a hundredfold increase in the release of endothelial and mesenchymal progenitor cells from the bone marrow in mice.

Nuclear reprogramming offers new approaches to disease treatment and human models of human disease

In principle, autologous cell therapy could be carried out by reprogramming cells from a patient so that they are converted to, or give rise to, the type of cells that the patient lacks. To do this, conveniently accessible cells, such as skin fibroblasts, need to be extracted from the patient and then manipulated in culture to change the differentiation status of the cell (Box 21.3). Until recently, the developmental progression that causes mammalian cells to gradually lose their plasticity and to become progressively more specialized, and more and more

Preview

BOX 21.3 EXPERIMENTAL INDUCTION OF NUCLEAR REPROGRAMMING IN MAMMALIAN CELLS

Nuclear reprogramming entails altering nuclear gene expression to bring about a profound change in the differentiation status of a specialized cell. It may cause a somatic cell to regress so that it acquires the properties of an unspecialized embryo or a pluripotent or progenitor cell (*dedifferentiation*), or it may cause a lineage switch so that one type of differentiated somatic cell changes into another type (*transdifferentiation*). The fertilized egg cell provides a natural example of nuclear reprogramming: the sperm is a highly differentiated cell and the nucleus is highly condensed until reprogrammed by factors in the oocyte. Different experimental procedures can bring about nuclear reprogramming (Figure 1 for an overview).

Somatic cell nuclear transfer

The first experiments that caused reversal of vertebrate cell differentiation were performed in frogs more than 40 years ago. With the use of microsurgical techniques, nuclei were isolated from specialized cells of the embryo or terminally differentiated cells and transplanted into unfertilized eggs whose nuclei had either been removed or been inactivated by exposure to irradiation. This could result in reprogramming of a somatic cell nucleus to make it totipotent (Figure 1, path A). Frogs that were derived by nuclear transfer from somatic cells of a single individual were considered to be essentially genetically identical (by having the same nuclear genome) and were considered to be *clones*.

These experiments showed that it was possible to fully reverse differentiation in vertebrates. However, amphibians were considered to be exceptional; some amphibians—such as salamanders—are able to regenerate limbs and tails. The regenerative capacity of mammals is extremely limited by comparison. The prospects for reversing differentiation to clone mammals seemed bleak until the birth of a cloned sheep called Dolly in 1996 (see Figure 20.4). Although technically much more difficult than cloning frogs, the success with Dolly prompted the subsequent successful cloning of many other types of mammal, including cows, mice, goats, pigs, cats, rabbits, dogs, and horses. A fierce debate arose as to whether human cloning could ever be contemplated (see Box 21.4).

Cell fusion

Two different types of cell can be induced to fuse, forming a single cell with two different nuclei, a *heterokaryon*. By using an inhibitor of cell division, the two nuclei in the heterokaryon can be kept separate. Each nucleus is potentially subject to regulation by factors that normally regulate gene expression in the other nucleus. One nucleus predominates—typically the nucleus of the larger and more actively dividing cell—and the other is reprogrammed to adopt the pattern of gene expression of the dominant nucleus. Thus, if actively dividing pluripotent ES cells are fused with somatic cells, the somatic cell nucleus will be reprogrammed to the pluripotent state (Figure 1, path B).

Specific transcription factors can induce pluripotency

The observation that the nuclei of somatic cells could be reprogrammed to reverse differentiation prompted intense research to identify the factors responsible. Transcription factors known to be involved in maintaining pluripotency in ES cells were likely candidates. Nevertheless, it was a huge surprise when in 2006 Shinya Yamanaka and colleagues reported that transfection of genes encoding just four transcription factors—Oct-3/4, Sox2, c-Myc, and Klf-4—could reprogram mouse fibroblasts to become pluripotent cells. Follow-up studies showed that human and rat fibroblasts could also be reprogrammed toward pluripotency with defined transcription factors, as could various other cell types, including lymphocytes and liver, stomach, and pancreatic beta cells.

Cells reprogrammed in this way are called *induced pluripotent stem cells* (*iPS cells*) because they closely resemble ES cells. They show telomerase activity and reactivation of pluripotency genes, and they have genomewide transcriptional and epigenetic patterns characteristic of ES cells. In addition, iPS cell chimeras can give rise to offspring, indicating their ability to contribute to the germ line. The mechanism underlying the induction of pluripotency is under intense investigation. The combination of transcription factors necessary for reprogramming is well conserved in mammals, but shows some variability according to cell type. A variety of human disease-specific iPS cell lines have already been made and have exciting potential for modeling disease and drug screening, as well as possibly providing therapeutic applications (see the main text).

Transdifferentiation

Nuclear reprogramming can also be induced so as to cause transdifferentiation. This may occur indirectly by *lineage switching*, when a cell is induced to regress along a differentiation pathway to a branch point that will allow some cells to then follow a different

committed to their fate, was thought to be irreversible. The cloning of a sheep called Dolly changed that view, because the nucleus of an adult somatic cell was successfully reprogrammed, giving rise to a totipotent cell.

As detailed in Section 20.2, the cloning of Dolly was made possible by isolating nuclei from mammary gland cells from a Finn-Dorset sheep and then transplanting individual nuclei into enucleated eggs from a Scottish blackface sheep (*somatic cell nuclear transfer*—see Figure 20.4). In one out of 434 attempts, factors in the egg cytoplasm somehow reprogrammed the somatic cell nucleus to return it to the totipotent state. The resulting cell was stimulated to develop to the blastocyst stage and was then implanted in the uterus of a foster mother to give rise to Dolly. As Dolly's nuclear genome was identical to that of the Finn-Dorset sheep, she was considered to be a clone of that sheep. A variety of other mammals have now been cloned by somatic cell nuclear transfer. However, cloned mammals have a very high incidence of abnormalities, most probably as a result of inappropriate epigenetic modifications.

Although natural human clones—identical twins and triplets, for example—are reasonably common, artificial human cloning is considered ethically unjustifiable (**Box 21.4**) and is banned by legislation in many countries. Nevertheless, somatic cell nuclear transfer offered for the first time the possibility of making an ES cell line from any patient willing to donate some easily accessible skin fibroblasts. Individual nuclei would be isolated from the skin cells and transferred into unfertilized eggs that had been donated by donors and subsequently enucleated.

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BOX 21.3 CONT.

pathway to produce a different but related cell type (Figure 1, path C), or by direct cell conversion without an obvious intermediate dedifferentiation step (Figure 1, path D).

Ectopic expression of just a single type of transcription factor can cause transdifferentiation. Thus, for example, the transcription factors PU.1 and GATA-1 are mutually antagonistic in the development of blood cell lineages. If PU.1 predominates, differentiation occurs toward myeloid cells, but if GATA-1 dominates, cells go down the megakaryocyte/erythroid pathway. As a result, transfection of a gene expressing the GATA-1 transcription factor into myeloid cells can cause transdifferentiation toward megakaryocyte/erythroid cells; ectopic expression of PU.1 in megakaryocyte/erythroid cells stimulates transdifferentiation into myeloid cells (Figure 1, path C). Efficient direct conversion of adult pancreatic exocrine cells to beta cells can be achieved by transfecting genes for three transcription factors that are essential for beta cell function: Ngn3, Pdx1, and Mafa (Figure 1, path D).

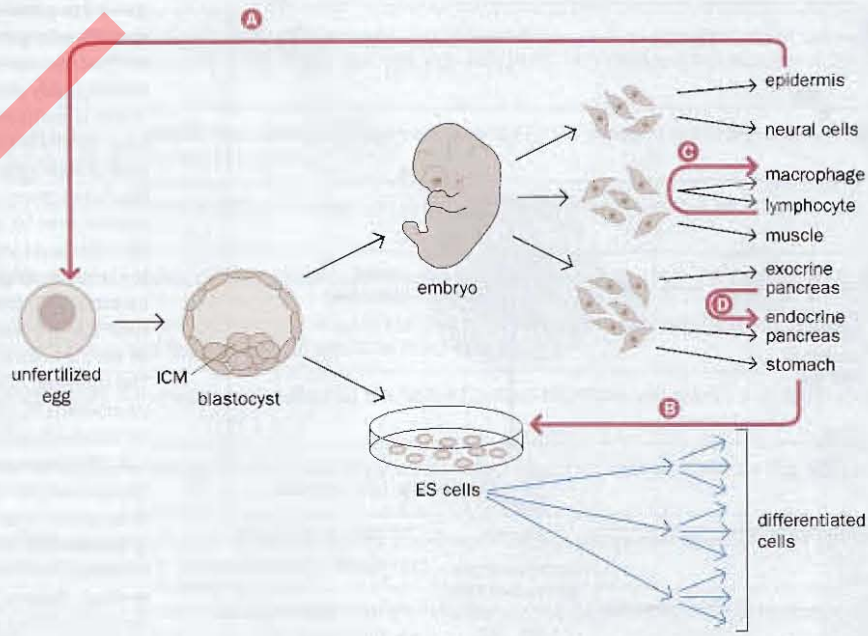

Figure 1 Experimental routes for nuclear reprogramming in mammals. Normal steps in development and tissue differentiation are shown by black arrows. Experimental procedures are shown as blue arrows. (A) Nuclear transfer into an unfertilized egg reprograms a somatic nucleus to dedifferentiate to give a totipotent cell. (B) A somatic cell is stimulated to dedifferentiate to become a pluripotent stem cell that closely resembles an ES cell. (C) Lineage switching is a form of transdifferentiation that involves dedifferentiation followed by differentiation down a different differentiation pathway. (D) Transdifferentiation by direct conversion of one somatic cell into another. ICM, inner cell mass.

BOX 21.4 THE ETHICS OF HUMAN REPRODUCTIVE CLONING

In principle, reproductive cloning to produce a human clone could provide the only option for a couple to have children in certain cases. For example, a woman with a serious mitochondrial disorder could avoid transmission of the disease by becoming pregnant with a donated oocyte into which a nucleus had been transplanted from one of her or her partner's somatic cells. The technology could also meet more general needs such as the production of cloned individuals to replace a dying child or other loved one. However, human reproductive cloning is banned by legislation in many countries because of powerful ethical arguments.

Of the two major ethical arguments, the practical argument is the more powerful one. It points to the low success rate of all mammalian cloning experiments, and to the fact that many of the cloned animals born have serious abnormalities, most probably because the epigenetic modifications of the donor cell are not reliably reprogrammed. Allowing human reproductive cloning to proceed with current imperfect technology would be grossly unethical because of the early deaths and major abnormalities that could be expected. Conceivably, advances in knowledge might remove this objection, although it is not clear how we could know without conducting unethical experiments.

Even if the technology were perfectly efficient and safe and had no health consequences, the second major ethical argument in principle says that humans should be valued for themselves and not treated as instruments to achieve a purpose.

The resulting cells would be stimulated to develop into blastocysts, and inner cell mass cells would be removed to make ES cells. This type of approach became known as *therapeutic cloning*, to distinguish it from the banned *reproductive cloning* (Figure 21.5).

Therapeutic cloning has been controversial. Not only does it involve the destruction of an unfertilized human egg, but also, by providing the technology for cloning human blastocysts, it has raised fears that human reproductive cloning could be facilitated. In addition, obtaining eggs from human donors involves an invasive and potentially dangerous procedure. The attraction of therapeutic cloning was that it offered the possibility of making *patient-specific ES* cells that could, in principle, be used for autologous cell therapy, and disease-specific ES cells that could be used for studying pathogenesis and in drug screening as described below. In practice, cloning human blastocysts by somatic cell nuclear transfer proved to be extraordinarily inefficient—thus far, there is no report of a human ES cell line made by this method.

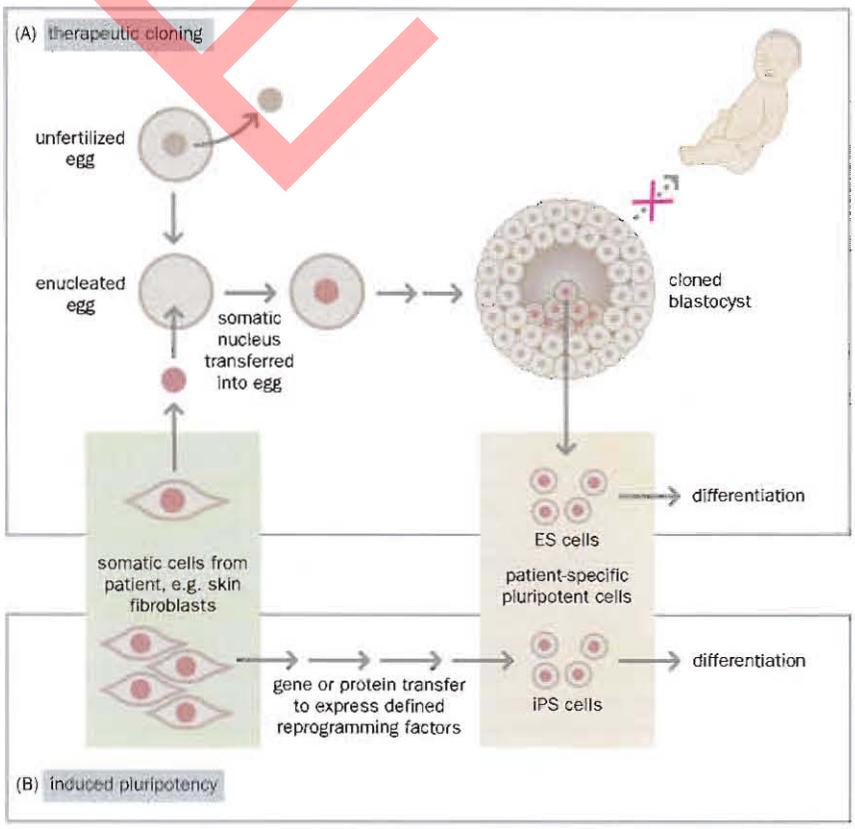

Figure 21.5 Nuclear reprogramming can generate patient-specific and disease-specific pluripotent stem cells. (A) Therapeutic cloning. Here, a cell nucleus from suitably accessible cells from a patient donor is transplanted into an enucleated egg. Factors in the oocyte cytoplasm are able to reprogram the somatic nucleus so that it becomes totipotent. The resulting cell can then be stimulated to develop to the blastocyst stage. The nuclear genome of the resulting blastocyst will be genetically identical with that of the donating patient. Cells are removed from the inner cell mass of the blastocyst, which is then destroyed. The retrieved cells are cultured to establish a pluripotent ES cell line that can be induced to differentiate to different types of somatic cell. The large red X signifies that using cloned human blastocysts for reproductive purposes is regarded as ethically unacceptable. (B) Induced pluripotency does not involve the destruction of a human embryo. Somatic cells are transfected with genes encoding a few specific transcription factors that are important in maintaining pluripotency, and cells are selected that express the reprogramming factors. Alternatively, the reprogramming proteins can be added directly to the cells. The resulting induced pluripotent stem (iPS) cells are essentially equivalent to ES cells.

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Induced pluripotency in somatic cells

An alternative way of generating patient-specific and disease-specific pluripotent stem cells does not raise the same ethical concerns. Pluripotency can be induced in somatic cells just by ectopically expressing a very few specific transcription factors. Shinya Yamanaka and colleagues reported an astounding breakthrough in 2006 (**Box 21.5**) when they were able to regress mouse fibroblasts to a pluripotent state by transfecting genes encoding just four types of transcription factor—Oct-3/4, Sox2, c-Myc, and Klf-4—known to be involved in maintaining pluripotency. By selecting for cells expressing the transfected genes they identified a small population of **induced pluripotent stem (iPS) cells**.

iPS cells were subsequently made from fibroblasts, or other types of somatic cell, from a variety of other mammals. iPS cells are highly similar to ES cells at both the molecular and functional levels, and, like ES cells, iPS cells can contribute to all tissues as well as to the germ line. In 2009 viable fertile mice were reported that originated exclusively from mouse iPS cells (see Box 21.5).

Because iPS cells are functionally equivalent to ES cells in pluripotency but are unhampered by the kind of ethical concerns that have hindered human ES cell research, research studies on iPS cells have developed rapidly. The ability to make patient-specific and disease-specific iPS cells and to allow them to differentiate into somatic cells affected in the relevant disease has important biomedical applications (**Figure 21.6**).

iPS cells can potentially provide a wide range of human models of human disease. For many important disorders, the affected tissues and cells are not readily accessible. In neurodegenerative conditions of neuronal tissue, such as Huntington, Alzheimer, and Parkinson diseases, the pathogenesis has had to be studied in post-mortem specimens or in animal models in which the phenotype is often somewhat different from the human disease phenotype. Differentiation of the appropriate disease-specific iPS cells will provide live human cells for studying the pathology.

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BOX 21.5 SOME MILESTONES IN NUCLEAR REPROGRAMMING IN MAMMALIAN CELLS

1996	production of the first cloned mammal, a sheep called Dolly, by somatic cell nuclear transfer; see Figure 20.4 (*Nature* 380, 64–66; PMID 8598906)
2004	chemical screens identify small molecules that cause reversal of differentiation, such as reversine, which can cause myoblasts to dedifferentiate into progenitor cells (*J. Am. Chem. Soc.* 126, 410–411; PMID 14719906 and *Nat. Biotechnol.* 22, 833–840; PMID 15229546)
2004	reprogramming of nuclei transferred from olfactory sensory neurons to eggs to generate totipotent cells and subsequently cloned mice (*Nature* 428, 44–49; PMID 14990966)
2004	stepwise reprogramming of B cells into macrophages—an early example of a lineage switch (*Cell* 117, 663–676; PMID 15163413)
2005	nuclear reprogramming of somatic cells after fusion with human ES cells (*Science* 309, 1369–1373; PMID 16123299)
2006	reprogramming of cultured mouse embryonic and adult fibroblasts to pluripotent stem cells following transfection by genes encoding just four transcription factors (*Cell* 126, 663–676; PMID 16904174)
2007	induction of pluripotency in human somatic cells (*Cell* 131, 861–872; PMID 18035408 and *Science* 318, 1917–1920; PMID 18029452)
2008	transdifferentiation by *in vivo* reprogramming of adult pancreatic exocrine cells to beta cells (*Nature* 455, 627–632; PMID 18754011)
2008	nuclear reprogramming provides the first patient-specific and disease-specific pluripotent stem cells (*Cell* 134, 877–886; PMID 18691744 and *Science* 321, 1218–1221; PMID 18669821)
2009	generation of fertile viable mice derived exclusively from iPS cells (*Nature* 461, 86–90; PMID 19672241, *Nature* 461, 91–94; PMID 19672243, and *Cell Stem Cell* 5, 135–138; PMID 19631602)
2009	generation of iPS cells using recombinant proteins only (*Cell Stem Cell* 4, 381–384; PMID 19398399)
2010	reprogramming of fibroblasts to give functional neurons (Vierbuchen T et al., *Nature* 463, 1035–1041; PMID 20107439)

iPS cell, induced pluripotent stem cell; PMID, PubMed identifier number.

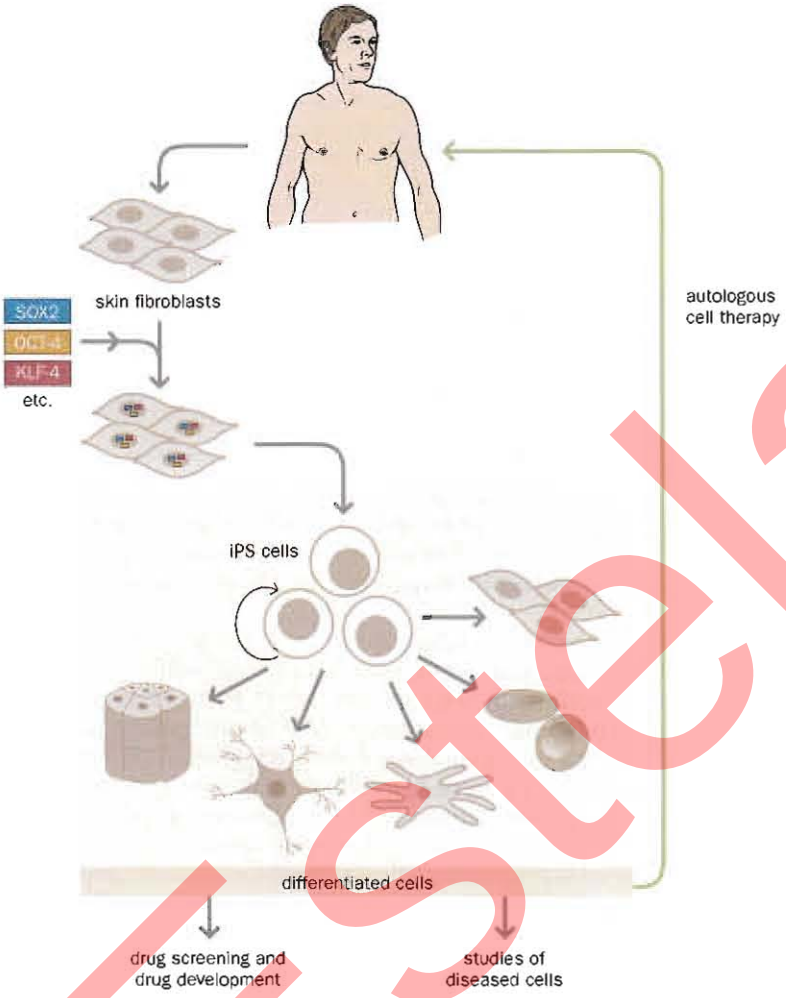

Figure 21.6 Genesis and biomedical applications of human induced pluripotent stem cells. Skin fibroblasts from individuals with disease and from healthy individuals can be transfected with genes encoding certain pluripotency-associated transcription factors, such as SOX2 or OCT-4, or they can be transduced by a cocktail of the transcription factor proteins. As a result, the fibroblast nuclei are reprogrammed to produce self-regenerating ES-like cells known as induced pluripotent stem (iPS) cells. The iPS cells can be directed to differentiate along different pathways to give diverse cell types with multiple biomedical applications. Autologous cell therapy might be achieved by differentiating the iPS cells to produce cells of a type that a patient lacks, which are then returned to the patient. For genetic disorders, the genetic mutation could be repaired in the iPS cells from the individuals with disease before they are differentiated and returned to the patient. Differentiated cells produced from the patient's iPS cells could also be valuable in drug screening and in providing human cellular models of disease.

For some disorders, the use of iPS cell-derived cells also means that drugs can now be tested directly on the relevant human cells. Drugs can be tested for their ability to reduce the progression of the pathogenesis or even to counteract it in some way, and the toxicity levels can be determined directly, rather than relying on animal studies.

The potential of iPS cells for efficient autologous cell therapy remains unclear. Safety concerns were an issue with the original technology, which used retroviral vectors to transfect reprogramming genes, one of which was a known oncogene, c-*Myc*. However, new methods do not use c-*Myc*, and avoid genome integration by using non-integrating vectors or by transducing cells with the pluripotency-inducing transcription factor proteins themselves, or miRNAs and other small molecules involved in the same pathways. Performing efficient precisely directed differentiation remains a major challenge. Like ES cells, undifferentiated iPS cells can give rise to tumors, and there is considerable ignorance about the molecular details of the long, multi-step differentiation pathways required to convert pluripotent stem cells to differentiated somatic cells.

Transdifferentiation

An alternative to iPS cells is to use lineage-reprogramming methods that would result in limited dedifferentiation to produce oligopotent progenitors of a desired cell type, or a direct cell conversion (*transdifferentiation*) to produce the desired cell type (see Box 21.3 Figure 1, pathways C and D). For example, pancreatic exocrine cells could be reprogrammed to make pancreatic beta cells that could possibly be induced to join pancreatic islets to replace beta cells lost in type 1 diabetes. By just taking one step back in differentiation or a sideways step, differentiation toward producing the desired cell type would be much simpler or not even required. Additionally, disruption to the epigenetic marks that are set during

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development would be expected to be minimal. Reprogramming between closely related cell types should also require fewer proliferation steps and should presumably reduce the chance of mutations. Unlike iPS cell technology, however, in which much the same reprogramming factors are needed to dedifferentiate a wide range of different cell types, lineage-reprogramming factors can be expected to be cell type-specific and have thus far not been well characterized for many cell types (see Box 21.5 for two examples).

Stem cell therapy has been shown to work but is at an immature stage

Notwithstanding the difficulties described above, stem cell therapy can work. We consider here some illustrative examples of therapy in which the object has simply been to replace cells lost through disease or injury. This represents one component of a burgeoning field that is often described as *regenerative medicine* and also includes *tissue engineering*, the combinatorial use of cells, materials, and engineering principles toward producing functional replacement tissues and organs. For genetic disorders, future autologous cell therapies would be expected to include the use of genetic engineering to correct harmful mutations in stem cells in culture before returning cells to the patient. In later sections in this chapter we describe the use of progenitor cells and stem cells as preferred cellular targets for gene therapy.

The stem cells that have been used most effectively in cell replacement therapy have been classes of tissue-specific multipotent stem cells that are comparatively frequent and easy to access, notably bone marrow and umbilical cord blood, which are known to be enriched in stem cells. Bone marrow transplantation is a form of stem cell therapy that has been used successfully for several decades in treating blood cancers, notably leukemia and lymphoma. Because hematopoietic stem cells are formed in the bone marrow, a bone marrow transplant is effectively a form of enrichment of hematopoietic stem cells, although the percentage of stem cells in the transplants remains low. More recently, umbilical cord blood stem cells have also been used for the same purpose. In both cases, the therapy is designed to replace cells that are killed during previous chemotherapy treatment, which, as well as killing cancer cells, depletes dividing blood cells, including hematopoietic stem cells.

Because of the ready access to hematopoietic stem cells, blood disorders are comparatively easy targets for cell therapy (Table 21.3). More difficult to treat are diseases or injuries affecting organs for which stem cells have not been so readily accessible. In such cases, easily accessible stem cell populations, notably bone marrow cell preparations, have often been applied directly at the site of disease or injury in the hope that signals from the tissue environment would induce the introduced stem cells to transdifferentiate into the desired cell type. In the case of ischemic heart disease, for example, heart muscle cells (cardiomyocytes) that die after a heart attack need to be replaced, and some clinical trials have claimed a measure of success with injected bone marrow cells. However, there is some controversy over whether any improvement is due to cell therapy, and if so what the basis would be; there is little evidence for transdifferentiation—if it does occur it is a very infrequent event.

Pluripotent stem cells have very recently been considered in stem cell therapy. The first clinical trial with human ES cells was initially given FDA approval in early 2009, but was subsequently suspended pending further investigations. Researchers at Geron had hoped to expand ES cells and differentiate them into oligodendrocyte precursors for treating spinal cord injury. As we will see later in the chapter, new genetic manipulations make it easier to repair genetic defects at the DNA level in somatic cells. This may mean that cell therapy could be extended to monogenic genetic disorders by repairing genetic defects in iPS cells taken from patients.

However, formidable challenges lie ahead. Much basic research needs to be done on analyzing the properties of stem cells and differentiation pathways. And we are reminded of the difficulties of cell engraftment when, several years after transplantation of fetal neuronal cells to treat Parkinson disease, Lewy bodies—abnormal protein aggregates that develop in neurons—have appeared in what had originally been healthy grafts.

Proof

TABLE 21.3 SOME PRINCIPAL TARGETS FOR STEM CELL THERAPY AND EXAMPLES OF SUCCESSFUL CELL REPLACEMENT THERAPY IN RODENT DISEASE MODELS

Disease/injury	Cell affected	Stem cell therapy	Comments/references
Leukemia/lymphoma	hematopoietic stem cells killed by chemotherapy	allogeneic bone marrow transplantation from matched donor	successful over many decades
Eye injury/disease	limbal stem cells in one eye	limbal stem cells are taken from the healthy eye, expanded *ex vivo*, and transplanted back into the affected eye	Pellegrini et al. (1997) *Lancet* 349, 990–993; PMID 9100626 and Kolli et al. (2010) *Stem Cells* in press; PMID 20014040
Spinal cord injuries	neurons	transplantation of fetal neural stem cells or ES cell-derived oligodendrocyte progenitors	some evidence for successful treatment; first target for approved clinical trials using ES cells
Heart disease	cardiomyocytes (heart muscle cells)	direct injection of bone marrow stem cells (including hematopoietic stem cells and mesenchymal stem cells)	conflicting views on the degree of success, and uncertainty regarding the underlying cause of any clinical improvement
Stroke	neurons	human fetal neural stem cells used as cell source	first clinical trial underway
Parkinson disease	midbrain dopaminergic neurons	fetal neuronal cells used as cell source	difficulties in ensuring that grafted neurons do not themselves develop disease after a few years
Rat Parkinson disease model	midbrain dopaminergic neurons	iPS cells were differentiated into neural precursor cells that were transplanted into the fetal mouse brain; the transplanted cells were functionally integrated, causing improvements in symptoms	Wernig et al. (2008) *Proc. Natl Acad. Sci. USA* 105, 5856–5861; PMID 18391196
Brain disease (in mouse *shiverer* mutant)	glial cells as a result of myelin deficiency	human fetal glial progenitor cells were injected into the nervous system to correct abnormal brain development	Windrem et al. (2008) *Cell Stem Cell* 2, 553–565; PMID 18522848
Blindness (in *Crx*-deficient mice)	degeneration of photoreceptors	human ES cells were differentiated to give retinal cells that were transplanted into the retinas of the mouse *Crx* mutant and restored some visual function	Lamba et al. (2009) *Cell Stem Cell* 4, 73–79; PMID 19128794

PMID, PubMed identifier number.

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21.4 PRINCIPLES OF GENE THERAPY AND MAMMALIAN GENE TRANSFECTION SYSTEMS

Gene therapy involves the direct genetic modification of cells of the patient to achieve a therapeutic goal. There are basic distinctions in the types of cell modified, and the type of modification effected. *Germ-line gene therapy* would produce a permanent transmissible modification and could be achieved by the modification of a gamete, a zygote, or an early embryo. It is widely banned for ethical reasons (**Box 21.6**). *Somatic gene therapy* seeks to modify specific cells or tissues of the patient in a way that is confined to that patient.

All current gene therapy trials and protocols are for somatic gene therapy. As gene therapy successes accumulate and the technologies become increasingly refined and safe, extending the technology to the germ-line to produce designer babies can be expected, with associated ethical concerns (**Box 21.7**).

In somatic cell gene therapy, the cells that are targeted are often those directly involved in the pathogenic process, but in some cancer gene therapies the object is to modify normal immune system cells genetically in the patient so as to provoke a powerful immune response against tumor cells. The somatic cells might be modified in several different ways (**Figure 21.7**):

- *Gene augmentation* (or *gene addition*). The aim is to supply a functioning gene copy that will *supplement* a defective gene. The obvious application is to treat diseases that are the result of a gene not functioning here and now. Cystic fibrosis would be a typical candidate. Gene augmentation would not be

BOX 21.6 THE ETHICS OF GERM-LINE GENE THERAPY

Germ-line gene therapy involves making a genetic change that can be transmitted down the generations. This would most probably be done by genetic manipulation of a pre-implantation embryo, but it might occur as a by-product of a treatment aimed at somatic cells that incidentally affected the patient's germ cells. Pure somatic gene therapy treats only certain body cells of the patient without having any effect on the germ line. Technical difficulties currently make germ-line therapy unrealistic. Even if these problems are solved, ethical concerns will remain: genetic manipulation of the germ line is prohibited by law in many countries.

- The main argument against germ-line therapy is that these treatments are necessarily experimental. We cannot foresee every consequence, and the risk is minimized by ensuring that its effects are confined to the patient we are treating. This would imply that once we have enough experience of somatic therapy, it might be ethical to proceed to germ-line therapy. However, even if the initial treatment were performed with informed consent, later generations are given no choice. This leads to the view that we have a responsibility not to inflict our ideas or products on future generations, and so germ-line therapy will always be unethical.
- The argument in favor of germ-line therapy is that it solves the problem once and for all. Why leave the patient's descendants at risk of a disease if you could equally well eliminate the risk?

  The argument in favor needs to be set against the population genetic background. The Hardy–Weinberg equations set out in Chapter 3 (p. 84) are highly relevant here; in addition there is a strong practical argument that germ-line therapy is unnecessary.
- For recessive conditions, only a very small proportion of the disease genes are carried by affected people; the great majority are in healthy heterozygotes. For a recessive disease affecting one person in 10,000 ($q^2 = 1/10{,}000$; $q = 0.01$) only 1% of disease alleles are in affected people. Whether or not we stop affected people from transmitting their disease genes (either by germ-line therapy or by the cruder option of sterilization as the price of treatment) has very little effect on the frequency of the disease in future generations.
- For fully penetrant dominant conditions, all the disease alleles are carried by affected people, and for X-linked recessives the proportion is one-third. But the dream of eliminating the disease once and for all from the population falls down because, as the equations in Box 3.7 show, most serious dominant or X-linked diseases are maintained in the population largely by recurrent mutation.
- A third, and cogent, objection is that germ-line therapy is simply not necessary. Candidate couples would most probably have dominant or recessive Mendelian disorders (recurrence risk 50% and 25%, respectively). Given a dish containing half a dozen IVF embryos from the couple, it would seem crazy to select the affected ones and subject them to an uncertain procedure, rather than simply to select the 50% or 75% of unaffected ones for re-implantation.

The argument that somatic therapy is less risky than germ-line therapy seems incontrovertible. In particular, the safer non-integrating vectors could not be used for germ-line therapy. We are less convinced by the general argument that it is unethical to impose our choices on future generations.

suitable for loss-of-function conditions in which irreversible damage has already been done, for example through some failure in embryonic development. Cancer therapy could involve providing a normal gene copy to supplement a defective tumor suppressor gene.

- *Elimination of pathogenic mutations.* The object is to restore the function of a mutated gene. The gene could be repaired at the DNA level by replacing a sequence containing the pathogenic mutation with a normal equivalent sequence. Such gene correction would be required for gain-of-function diseases in which the resident mutant gene is doing something positively harmful to cells or tissues. Another possibility, not shown in Figure 21.7 but detailed later in this chapter, is to induce altered gene splicing causing skipping of an exon containing the harmful gene mutation.

BOX 21.7 THE ETHICS OF "DESIGNER BABIES"

The designer baby catchphrase encapsulates two sets of worries. First, people will use *in vitro* fertilization and pre-implantation diagnosis to select embryos with certain desired qualities and reject the rest even though they are normal. This contrasts with the use of the same procedure to avoid the birth of a baby with a serious disease. Second, people will use the therapeutic technologies described in this chapter, not to treat disease but for *genetic enhancement*; that is, endowing genetically normal people with superior qualities.

The first scenario is already with us in the form of pre-implantation sex selection and a few highly publicized cases in which a couple have sought to ensure that their next child can provide a perfectly matched transplant tissue to save the life of a sick child. Current cases involve a transplant of stem cells from cord blood. That would do no harm to the baby—it would be different if it were proposed to take a kidney. It is often suggested that these cases are the start of a slippery slope that leads inevitably to demands for very extensive specification of the genotype of the baby—as envisaged in the phrase designer baby. The reality is different. Simply selecting for HLA compatibility means that only one in four embryos is selected. Most IVF procedures produce only a handful of embryos, and usually two or three are implanted to maximize the chance of success. Selection on multiple criteria is simply not compatible with having enough embryos to implant. More generally, nature has endowed us with a simple and highly agreeable method of making babies that is very effective for the large majority of couples, and it is hard to imagine most people abandoning this in favor of a long-drawn-out, unpleasant, and highly invasive procedure that is very expensive and has a low success rate.

Genetic enhancement is a difficult question—or it will become one, once we have any idea which genes to enhance. On the one hand, parents are supposed to do what they can to give their children a good start in life; on the other hand, options available only to the rich are often seen as buying an unfair advantage. Perhaps fortunately, we are a long way from identifying suitable genes, even if the techniques for using them were available. Attempts to produce genetically enhanced animals have not been a success and in some cases have been spectacular failures. In the long term, the possibilities must be immense, and there will surely be very difficult ethical issues to confront.

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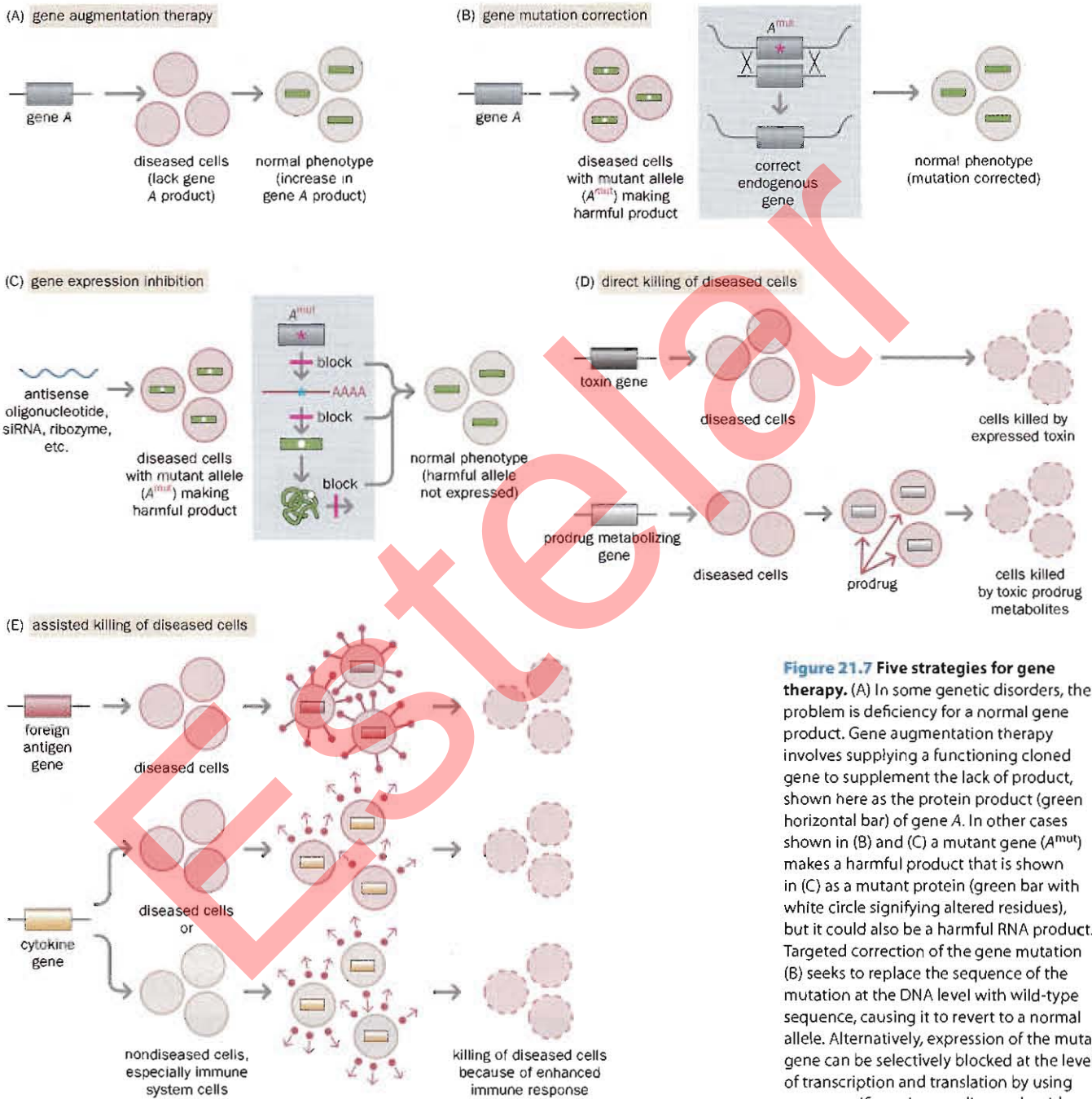

Figure 21.7 Five strategies for gene therapy. (A) In some genetic disorders, the problem is deficiency for a normal gene product. Gene augmentation therapy involves supplying a functioning cloned gene to supplement the lack of product, shown here as the protein product (green horizontal bar) of gene *A*. In other cases shown in (B) and (C) a mutant gene (A^{mut}) makes a harmful product that is shown in (C) as a mutant protein (green bar with white circle signifying altered residues), but it could also be a harmful RNA product. Targeted correction of the gene mutation (B) seeks to replace the sequence of the mutation at the DNA level with wild-type sequence, causing it to revert to a normal allele. Alternatively, expression of the mutant gene can be selectively blocked at the levels of transcription and translation by using gene-specific antisense oligonucleotides or siRNA, or by using specific oligonucleotides to bind the mutant protein and so inhibit its effects. For treating cancer, the object is often simply to kill the cancer cells as shown in (D) and (E). This can be done directly (D), either by targeting a toxin gene to the cells or a prodrug-metabolizing gene that makes the cells vulnerable to the effects of some prodrug that can be added later (see Figure 21.17 for an example). Alternatively, in *immunotherapy* immune system cells can be incited in different ways to kill cancer cells (E).

- *Targeted inhibition of gene expression.* This is used especially in infectious disease, in which essential functions of the pathogen are targeted, and to silence activated oncogenes in cancer. Another application would be to damp down unwanted responses in autoimmune disease and maybe to silence a gain-of-function mutant allele in inherited disease.
- *Targeted killing of specific cells.* As will be described below, this approach is particularly applicable to cancer treatment. It can involve direct killing using genes encoding toxins, etc. (see Figure 21.7D). Alternatively, gene transfection is designed simply to provoke a very strong immune response that is designed to kill the cancer cells (immunotherapy; see Figure 21.7E). This is possible because our immune system has evolved to recognize nonself antigens, and although cancer cells share some antigens with other body cells they may sometimes express an antigen that is specific for the cancer cells or

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greatly overexpress a normal antigen in a way that can provoke weak immune responses. Immunotherapy is designed to amplify the naturally weak immune response to cancer cells.

Genes can be transferred to a patient's cells either in culture or within the patient's body

In gene therapy, cloned genes, RNA, or oligonucleotides are inserted into the cells of a patient with the use of some delivery method. Often vectors are used that are based on viruses because, over long evolutionary time-scales, viruses have become highly effective in infecting cells, inserting their genomes, and getting their genes to be expressed. Transfer of DNA into human (and other animal) cells by using viruses is known as **transduction**. In some cases the virus vectors can integrate into the genome and so provide the means for long-lasting transgene expression; however, as we describe below, integrating vectors currently pose safety risks. Other virus vectors do not integrate into the host genome. Instead, they remain in extrachromosomal locations in cells.

Non-viral methods can also be used to transfer DNA, RNA, or oligonucleotides into human (or other animal) cells (**transfection**). Because such methods do not involve integration of DNA into the genomes, they have the advantage of high safety in therapeutic applications, but they are generally disadvantaged by low transfection efficiencies.

In many gene therapy protocols, suitable target cells are excised from a patient, grown in culture, and then genetically modified by transfer of the desired nucleic acid or oligonucleotide. The cells can be analyzed at length to identify those cells in which the intended genetic modification has been successful. Selected cells can then be amplified in culture and injected back into the patient. Because the patient's cells are genetically modified outside the body, this approach is known as *ex vivo* gene therapy (Figure 21.8). It is most appropriate for disorders in which the target cells are accessible for initial removal and can be induced to engraft and survive for a long time after replacement. Examples include cells of the hematopoietic system and skin cells.

The alternative is *in vivo* gene therapy (see Figure 21.8), in which the gene transfer process is performed *in situ* within the patient, for example by injection into an organ (such as brain, muscle, or eye) or, for example, by using aerosols for the lung. *In vivo* gene therapy is the only option in disorders in which the target cells cannot be cultured *in vitro* in sufficient numbers (e.g. brain cells) or in which cultured cells cannot be re-implanted efficiently in patients.

Tissue targeting is an important consideration. As an alternative to placing the gene transfer construct directly into the target tissue, it may be introduced systemically. For example, injection into the portal vein can be used for delivery to the liver. Also some virus vectors naturally infect cells of a particular type. As there is no way of selecting and amplifying cells that have taken up and expressed the intended nucleic acid or oligonucleotide, the success of *in vivo* gene therapy is crucially dependent on the general efficiency of gene transfer and, where appropriate, expression in the correct tissue.

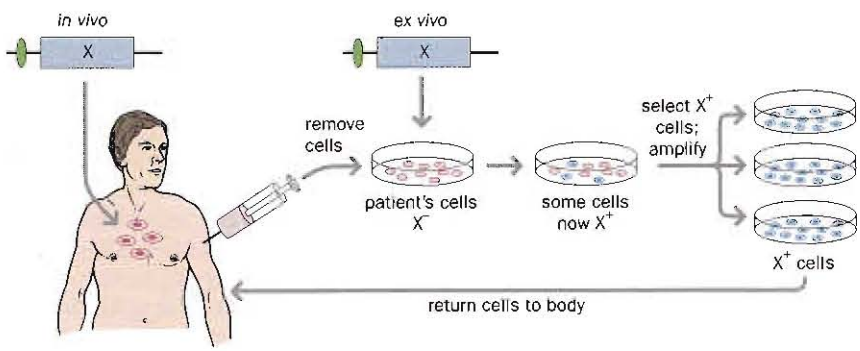

Figure 21.8 *In vivo* and *ex vivo* gene therapy. In *ex vivo* gene therapy, cells are removed from the patient, genetically modified in some way in the laboratory, and returned to the patient. This allows just the appropriate cells to be treated, and they can be checked to make sure they have the correct genetic modification before they are returned to the patient. For many tissues, this is not possible and the cells must be modified within the patient's body (*in vivo* gene therapy).

Essential

Integration of therapeutic genes into host chromosomes has significant advantages but raises major safety concerns

For achieving long-term gene expression it would seem desirable to integrate a therapeutic gene into a chromosome of the host cell—preferably a stem cell. Then the construct is replicated whenever the host cell or its daughters divide. Currently, certain virus vectors are used to ensure chromosomal integration, but integration typically occurs in a random or semi-random way so that inserts can be located at different sites in the genome in different cells of the patient. The local chromosomal environment can have unpredictable effects on expression of the construct—it may never be expressed, it may be expressed at an undesirably low level, or it may be expressed for a short time and then irreversibly silenced.

Lack of control over where virus vectors integrate within chromosomes carries very significant safety risks because integration may alter the expression of endogenous genes. The insertion point might be within the sequence of an endogenous gene, leading to insertional inactivation of that gene. The greatest worry is that insertion of a highly expressed construct may activate an adjacent oncogene, causing cancer in a manner similar to the activation of *MYC* in Burkitt lymphoma (see Figure 17.7). As we describe below, this has been a recurring issue when genes are inserted into the genomes of patients' cells, and it will remain so until genes can be inserted with high efficiency into specific safe locations within the genome, where they can be expressed without disturbing the functions of other genes.

Delivery systems in which the transferred DNA simply becomes an extrachromosomal episome within cells do not raise the same kind of safety concerns as integrating vectors. Their disadvantage is the limited duration of gene expression. If the target cells are actively dividing, the extrachromosomal gene will tend to be diluted out as the cell population grows. There is no possibility of achieving a permanent cure, and repeated treatments may be necessary. For some purposes, such as killing cancer cells or combating an acute infection, this is not a problem—long-term expression is not needed. Moreover, if something does go wrong, a non-inserted gene is self-limiting in a way that a gene inserted into a chromosome is not.

Viral vectors offer strong and sometimes long-term transgene expression, but many come with safety risks

Viruses are very efficient at delivering genes into cells. They attach to suitable host cells by recognizing and binding specific receptor proteins on the host cells. Some viruses infect a broad range of cells and are said to have a broad **tropism**. Other viruses have a narrow tropism because they bind to receptors expressed by only a few cell types. For example, herpes viruses are tropic for cells of the central nervous system. The natural tropism of viruses may be retained in vectors or genetically modified in some way, so as to target a particular tissue, for example.

Some enveloped viruses, such as HIV, enter cells by fusing with the host plasma membrane to release their genome and capsid (coat) proteins into the cytosol, but other enveloped viruses first bind to cell surface receptors and trigger receptor-mediated endocytosis, fusion-based transfer, or endocytosis-based transfer. Some viruses go on to access nuclear components of cells only after the nuclear envelope dissolves during mitosis. They are limited to infecting dividing cells. Other viruses have devised ways to transfer their genomes efficiently through nuclear membrane pores so that both dividing and non-dividing cells can be infected.

Different viral vector systems offer different advantages (Table 21.4). Viruses with large genomes can potentially accept proportionally large DNA inserts. Because the genome of a virus is constrained to be within a limited size range (to allow correct packaging into the viral protein coats) non-essential components of the viral genome must be eliminated to allow the insertion of a therapeutic gene. Packaging cell lines can be designed to contain stripped-down viral vectors containing therapeutic genes, and non-essential viral genes are added separately and can be expressed to provide *in trans* viral products required for the correct packaging of virus vectors within virus protein coats.

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TABLE 21.4 VIRAL VECTORS OF USE IN MAMMALIAN GENE TRANSFECTION AND GENE THERAPY

Virus class	Viral genome	Cloning capacity	Interaction with host genome	Target cells	Transgene expression	Comments
γ-Retroviruses (oncoretroviruses)	ssRNA	7–8 kb	integrating	dividing cells only	long-lasting	moderate vector yield[a]; risk of activation of cellular oncogene
Lentiviruses	ssRNA; ~9 kb	up to 8 kb	integrating	dividing and non-dividing cells; tropism varies	long-lasting and high-level expression	high vector yield[a]; risk of oncogene activation
Adenoviruses	dsDNA; up to 38 kb	often 7.5 kb, but up to 35 kb for gutless vectors	non-integrating	dividing and non-dividing cells	transient but high-level expression	high vector yield[a]; immunogenicity can be a major problem
Adeno-associated viruses	ssDNA; 5 kb	<4.5 kb	non-integrating	dividing and non-dividing cells	high-level expression in medium to long term (year)	high vector yield[a]; small cloning capacity but immunogenicity is less significant than for adenovirus
Herpes simplex virus	dsDNA; 120–200 kb	>30 kb	non-integrating	central nervous system	potential for long-lasting expression	able to establish lifelong latent infections
Vaccinia virus	dsDNA; 130–280 kb	25 kb	non-integrating	dividing and non-dividing cells	transient	

ss, single-stranded; ds, double-stranded.
[a]High vector yield, 10^{12} transducing units/ml; moderate vector yield, 10^{10} transducing units/ml.

Some viruses that are used to make gene therapy vectors are naturally pathogenic, and some can potentially generate strong immune responses. For safety reasons, viral vectors are generally designed to be disabled so that they are *replication-defective*. However, replication-competent viruses have sometimes been used as therapeutic agents, notably oncolytic viruses for treating cancers. Where a virus is naturally immunogenic, the viral vectors are modified in an effort to reduce or eliminate the immunogenicity.

Retroviral vectors

Retroviruses—RNA viruses that possess a reverse transcriptase—deliver a nucleoprotein complex (*preintegration complex*) into the cytoplasm of infected cells. This complex reverse-transcribes the viral RNA genome and then integrates the resulting cDNA into a single, but random, site in a host cell's chromosome. Retroviruses offer high efficiencies of gene transfer but can be generated at titers that are not so high as some other viruses, and they can afford moderately high gene expression. Because they integrate into chromosomes, long-term stable transgene expression is possible, but uncontrolled chromosome integration constitutes a safety hazard because promoter/enhancer sequences in the recombinant DNA can inappropriately activate neighboring chromosomal genes.

γ-Retroviral vectors are derived from simple mouse and avian retroviruses that contain three transcription units: *gag*, *pol*, and *env* (Figure 21.9). In addition, a *cis*-acting RNA element, ψ, is important for packaging, being recognized by viral proteins that package the RNA into infectious particles. Because native γ-retroviruses transform cells, the vector systems need to be engineered to ensure that they can produce only permanently disabled viruses. γ-Retroviruses cannot get their genomes through nuclear pores and so infect dividing cells only. This limitation can, however, be turned to advantage in cancer treatment. Actively dividing cancer cells in a normally non-dividing tissue such as brain can be selectively infected and killed without major risk to the normal (non-dividing) cells.

Lentiviruses are complex retroviruses that have the useful attribute of infecting non-dividing as well as dividing cells, and can be produced in titers that are a hundredfold greater than is possible for γ-retroviruses. In addition to expressing late (post-replication) mRNAs from *gag*, *pol*, and *env* transcription units, six early

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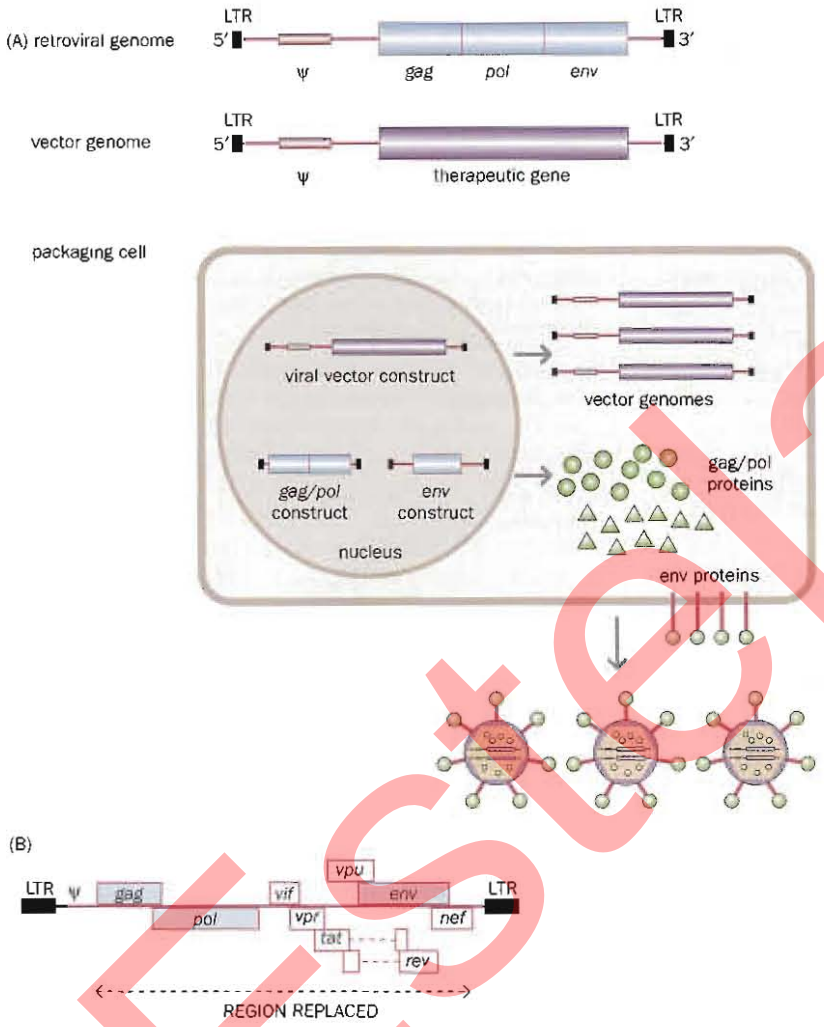

Figure 21.9 Simple and complex retroviral vectors. (A) A simple γ-retroviral genome contains three transcription units: *gag* (makes internal proteins), *pol* (makes reverse transcriptase and some other proteins), and *env* (makes viral envelope proteins), plus a ψ (psi) sequence that is recognized by viral proteins for assembly of the RNA into a virus particle. In vectors based on γ-retroviruses, *gag*, *pol*, and *env* are deleted and replaced by the therapeutic gene. The ψ sequence is retained. The packaging cell brings together viral vector recombinants and supplies the *gag*, *pol*, and *env* functions on physically separate molecules. The long terminal repeats (LTR) include promoter/enhancer regions and sequences involved with integration. Recombinant viral genomes are packaged into infective but *replication-deficient* virus particles, which bud off from the cell and are recovered from the supernatant. (B) Lentiviruses, such as the human immunodeficiency virus (HIV), are complex retroviruses. In addition to a single-stranded RNA genome with flanking long terminal repeats (LTR) and characteristic *gag*, *pol*, and *env* genes, they possess a variety of other additional genes, such as *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*, that encode proteins involved in regulating and processing of viral RNA and other replicative functions. In recombinant vectors, all of the genome except for the LTRs and ψ sequence can be replaced. [(A) adapted from Somia N & Verma IM (2000) *Nat. Rev. Genet.* 1, 91–99. With permission from Macmillan Publishers Ltd.]

viral proteins are produced before replication of the virus. The early proteins include two regulatory proteins, tat and rev, that bind specific sequences in the viral genome and are essential for viral replication. Like other retroviruses, they allow long-term gene expression. Results with marker genes have been promising, showing prolonged *in vivo* expression in muscle, liver, and neuronal tissue. Most lentiviral vectors are based on HIV, the human immunodeficiency virus, and much work has been devoted to eliminating unnecessary genes from the complex HIV genome and generating safe packaging lines while retaining the ability to infect non-dividing cells. Lentiviruses appear to have a safer chromosome integration profile than γ-retroviruses; self-inactivating lentivirus vectors provide an additional layer of safety.

Adenoviral and adeno-associated virus (AAV) vectors

Adenoviruses are DNA viruses that cause benign infections of the upper respiratory tract in humans. As with retroviral vectors, adenoviral vectors are disabled and rely on a packaging cell to provide vital functions. The adenovirus genome is relatively large, and gutless adenoviral vectors, which retain only the inverted terminal repeats and packaging sequence, can accommodate up to 35 kb of therapeutic DNA (**Figure 21.10**).

Adenovirus virus vectors can be produced at much higher titers than γ-retroviruses and so transgenes can be highly expressed. They can also efficiently transduce both dividing and non-dividing cells. A big disadvantage is their immunogenicity. Even though a live replication-competent adenovirus vaccine has been safely administered to several million US army recruits over several decades (for protection against natural adenoviral infections), unwanted immune reactions have been a problem in several gene therapy trials, as described below. Moreover, because these vectors are non-integrating, gene expression is short-

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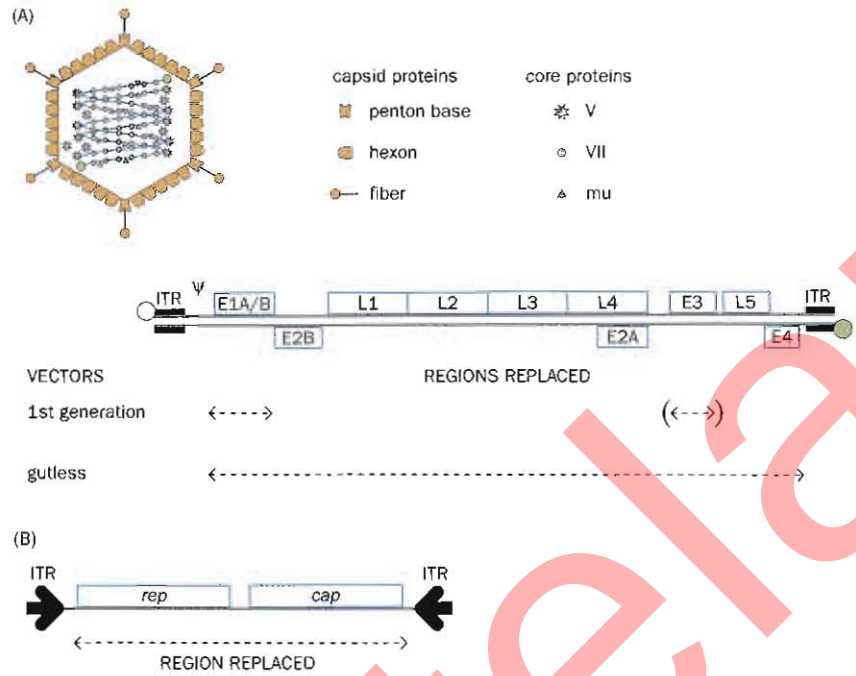

Figure 21.10 Adenovirus and adeno-associated virus as vehicles for gene delivery into mammalian cells. (A) Upper image: adenovirus structure. The adenovirus genome consists of a 36 to 38 kb double-stranded linear DNA that has a covalently attached protein molecule at the 5′ end (filled green circles). With various other core proteins, the adenoviral genome is encased in a capsid consisting of three types of capsid protein as shown (plus three types of cement protein, not shown). Lower image: adenoviral genome. The ends of the genome have inverted terminal repeats (ITRs) to which the terminal protein is covalently attached. Boxes above and below the double blue lines indicate transcription units transcribed from the opposing DNA strands. The early transcription units E1 to E4 encode mostly regulatory proteins; the late transcription units L1 to L5 are focused on producing structural proteins for packaging the genome. In the first series of adenovirus vectors, early transcription units, notably E1, were eliminated and could be replaced by insert DNA, but the more recent gutless vectors can replace all except the terminal repeats and the ψ sequence. (B) Adeno-associated virus (AAV) has a small, very simple 4.7 kb single-stranded DNA genome with terminal inverted repeats (ITRs), and just two open reading frames (ORFs). The *rep* ORF encodes various proteins required for the AAV life cycle, and the *cap* ORF makes the three capsid proteins. In a recombinant AAV vector, the *rep* and *cap* ORFs are replaced by a desired transgene. The functions necessary for production of virus particles (including *rep*) are provided by a packaging cell.

term; repeated administration would be necessary for sustained expression, which could only exacerbate the immune response.

Adeno-associated viruses (*AAVs*) are nonpathogenic single-stranded DNA viruses. They are completely unrelated to adenoviruses; their name comes from their reliance on co-infection by an adenovirus (or herpes) helper virus for replication. The genome contains only two genes: the *rep* gene makes proteins that control viral replication, structural gene expression, and chromosome integration; the *cap* gene encodes capsid structural proteins. Multiple different serotypes of AAV have been isolated and some have usefully narrow tropism. For example, the AAV9 variant is highly tropic for the spinal cord and brain astrocytes, and may be useful in future treatments of spinal cord injuries. Another important advantage of AAV vectors is that they can permit robust *in vivo* expression of transgenes in various tissues over several years while exhibiting little immunogenicity and little or no toxicity or inflammation.

Unmodified human AAV integrates into chromosomal DNA at a specific site on 19q13.3–qter. This highly desirable property would provide long-term expression without the safety risks of random insertional mutagenesis. Unfortunately, the specificity of integration is provided by the *rep* protein, and because the *rep* gene needs to be deleted in the constructs used for gene transfer, chromosomal integration of AAV vectors occurs randomly (AAV is disadvantaged by having a small genome; even vectors in which 96% of the AAV genome has been deleted accept inserts with a maximum size of just 4.5 kb).

Other viral vectors

Herpes simplex viruses are complex viruses containing at least 80 genes; they are tropic for the central nervous system (CNS). They can establish lifelong latent infections in sensory ganglia, in which they exist as non-integrated extrachromosomal elements. The latency mechanism might be exploited to allow the long-term expression of transferred genes, in the hope that they will spread through a synaptic network. Their major applications would be in delivering genes into neurons for the treatment of diseases such as Parkinson disease and CNS tumors. Vectors have a high insert capacity. Practical vectors are still at an early stage of development.

Modified vaccinia virus Ankara (MVA) is a highly attenuated vaccinia virus that has been used safely as a smallpox vaccine. Infection with MVA results in rapid replication of viral DNA, but it is largely non-propagative in human and mammalian cells. Replication-competent MVA vectors have been constructed and used largely for transferring suicide genes to kill tumor cells in cancer gene therapy.

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Non-viral vector systems are safer, but gene transfer is less efficient and transgene expression is often relatively weak

In the laboratory, it is relatively easy to get foreign DNA into cells, and some of the methods have potential in gene therapy if the safety problems of viral systems prove intractable. However, whereas many viruses are able to insert their genomes through nuclear pores, transfers using non-viral delivery methods are generally much less efficient and they are often characterized by poor transfer rates and a low level of transgene expression.

Transport of plasmid DNA to the nucleus of non-dividing cells is very inefficient because the plasmid DNA often cannot enter nuclear membrane pores. Various methods can be used to facilitate nuclear entry such as conjugating specific DNA sequences or protein sequences (nuclear localization sequences) that are known to facilitate nuclear entry, or compacting the DNA to a small enough size to pass through the nuclear pores.

Although transfected DNA can not be stably integrated into the chromosomes of the host cell, this may be less of a problem in tissues such as muscle that do not regularly proliferate, and in which the injected DNA may continue to be expressed for many months.

Transfer of naked nucleic acid by direct injection or particle bombardment

In some cases, naked DNA has been injected directly with a syringe and needle into a target tissue such as muscle. An alternative direct injection approach uses particle bombardment (biolistic or gene gun) techniques: DNA is coated on to metal pellets and fired from a special gun into cells. Successful gene transfer into several different tissues has been obtained with this simple and comparatively safe method. However, gene transfer rates are low and transgene expression is generally weak.

Lipid-mediated gene transfer

Liposomes are synthetic vesicles that form spontaneously when certain lipids are mixed in aqueous solution. Phospholipids, for example, can form bilayered vesicles that mimic the structure of biological membranes, with the hydrophilic phosphate groups on the exterior and the hydrophobic lipid tails in the interior. Cationic liposomes have been the most commonly used in gene transfer experiments. The lipid coating allows the DNA to survive *in vivo*, bind to cells, and be endocytosed into the cells (**Figure 21.11**). Unlike viral vectors, the DNA–lipid complexes are easy to prepare and there is no limit to the size of DNA that is transferred. However, the efficiency of gene transfer is low, with comparatively weak transgene expression. Because the introduced DNA is not designed to integrate into chromosomal DNA, transgene expression may not be long-lasting.

As discussed in Section 21.5, short interfering RNA (siRNA) is often complexed with cholesterol for delivery *in vivo*.

Compacted DNA nanoparticles

Because of its phosphate groups, DNA is a polyanion. Polycations bind strongly to DNA and so cause the DNA to be significantly compacted. To form DNA nanoparticles, DNA is complexed with a polyethylene glycol (PEG)-substituted poly-L-lysine known as PEG-CK30 (because it contains 30 lysine residues and an N-terminal cysteine to which the PEG is covalently bound). Within this complex, the DNA forms a very condensed structure. Because of their much reduced size, compacted DNA nanoparticles are comparatively efficient at transferring genes to dividing and non-dividing cells and have a plasmid capacity of at least 20 kb.

21.5 RNA AND OLIGONUCLEOTIDE THERAPEUTICS AND THERAPEUTIC GENE REPAIR

For many gene therapy protocols, as outlined in the previous section, the object is simply to transfer genes into suitable target cells so that they are expressed at high levels. In this section we are mostly concerned with quite different gene therapy strategies in which the object is not to supply a missing gene product or

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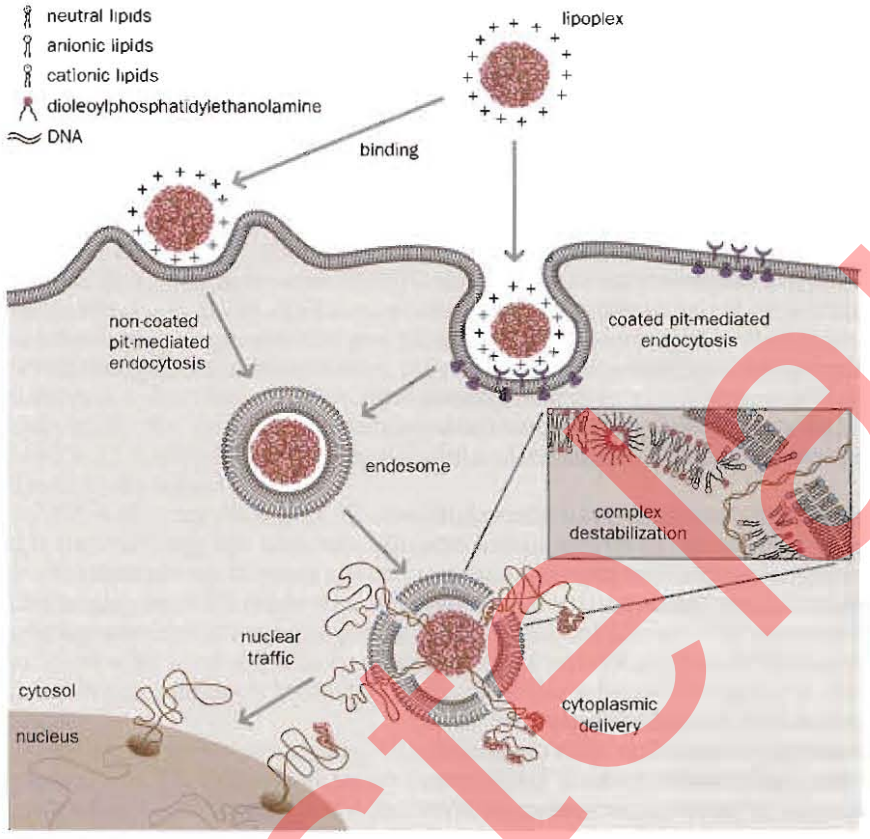

Figure 21.11 Cationic liposomes as vectors for gene delivery into mammalian cells. The gene to be transferred is complexed with cationic liposomes to form *lipoplexes* that can interact with cell membranes. The lipoplexes are taken up by cells through different endocytosis pathways in which the cell membrane evaginates to form a pit. Large lipoplexes are taken up by pits coated with clathrin complexes (top center); small lipoplexes are taken up by non-coated pits (top left). In either case the lipoplexes become trapped in endosomes that are targeted for destruction by lysosomes, where the DNA would be degraded if it were unable to escape. The inclusion within the liposomes of certain helper lipids—often electrically neutral lipids—helps to destabilize the endosomal membranes, causing the passenger DNA to escape to the cytoplasm. For the DNA to be transcribed it must pass to the nucleus. In dividing cells, the breakdown of the nuclear envelope during mitosis allows the DNA to gain access to the nucleus, but in non-dividing cells the precise mechanism of entry into the nucleus is unclear. [From Simões S, Filipe A, Faneca H et al. (2005) *Expert Opin. Drug Deliv.* 2, 237–254. Taylor & Francis Ltd.]

to kill harmful cells, but instead to counteract the harmful effects of genes within cells. Some conditions are caused by *gain-of-function* mutations (Chapter 13, p. 428) or a *dominant-negative effect* (Chapter 13, p. 431), and the problem is a resident gene that is doing something positively harmful. Diseases that might benefit from such strategies could include cancers that arise through activated oncogenes, dominant Mendelian conditions other than those caused by loss of gene function, and autoimmune diseases. Infectious diseases might also be treated by targeting a pathogen-specific gene or gene product.

In principle, two groups of strategies could be used to counteract a gene's harmful effects. One way is to specifically block expression of the harmful gene by downregulating transcription, by destroying the transcript, or by inhibiting the protein product. In Section 21.2 we outlined various methods that inhibit gene expression at the protein level; here, we will consider methods that inhibit or cleave specific RNA transcripts. A second type of strategy seeks to restore the normal gene function in some way. Either the DNA sequence is altered to correct a genetic defect, or alternative gene splicing is artificially induced so as to bypass a causative mutation at the RNA level.

Whatever method is used, some sort of agent or construct must be delivered to a target cell and made to function there. In that respect, the problems of efficient delivery and expression are similar to those described in the previous section. In the case of dominant diseases or activated oncogenes, there is the additional problem of designing an agent that will selectively attack the mutant allele without affecting the normal allele.

Therapeutic RNAs and oligonucleotides are often designed to selectively inactivate a mutant allele

Antisense RNA therapy, in which an antisense RNA is designed to base-pair with and selectively inhibit a target RNA, has been used in various clinical trials but the results have been mixed, and often disappointing. Introduced RNA is prone to attack by nucleases so that by the time the antisense RNA had reached its target tissue there was often little intact RNA. More stable antisense oligodeoxynucleotides were used, but successful knockdowns could not be guaranteed. To

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ensure that sufficient intact antisense oligonucleotides (AOs) arrived at the target tissue, clinical applications focused on applying the AOs directly to diseased tissue. In 1998, Vitravene® (fomivirsen) became the first FDA-approved therapeutic AO; it is applied directly to the eyes to treat cytomegalovirus retinitis in AIDS patients. The cornea (and anterior chamber of the eye) is an immunologically privileged site and so a high concentration of reagents can be administered directly by injection.

Subsequent technological developments produced a second generation of AOs based on nucleic acid analogs that are much more chemically stable than normal oligonucleotides, such as 2-*O*-methyl phosphorothioate oligonucleotides, morpholino phosphorothioate oligonucleotides, and locked nucleic acids (Figure 21.12). As we saw in Section 20.3, morpholino oligonucleotides are now widely used to knock down genes in some model organisms, but there have been few therapeutic applications that exploit morpholinos or other second-generation AOs to block gene expression. Although second-generation AOs are chemically stable, off-target effects can occur when the AO inhibits RNAs other than the target.

In addition, as for any other oligonucleotide, efficient delivery of AOs to cells has been a significant problem. In an effort to increase the *in vivo* uptake of AOs by cells, some second-generation AOs, such as the morpholino oligonucleotides, have been modified by conjugating peptides to them; however, this can sometimes provoke immune reactions.

Therapeutic ribozymes

A separate wave of RNA therapeutics focused on RNA enzymes (**ribozymes**), such as the plant hammerhead ribozyme, that naturally cleave other RNA target molecules. Genetically modified ribozymes have been made in which a catalytic, RNA-cleaving domain from a natural ribozyme is fused to an antisense RNA sequence designed to bind to transcripts from a specific target gene of interest. The modified ribozyme would bind to the transcripts of the target gene and cleave them. Ribozymes have, however, not been so effective in practice: clinical trials to treat cancer and other diseases have had rather disappointing outcomes.

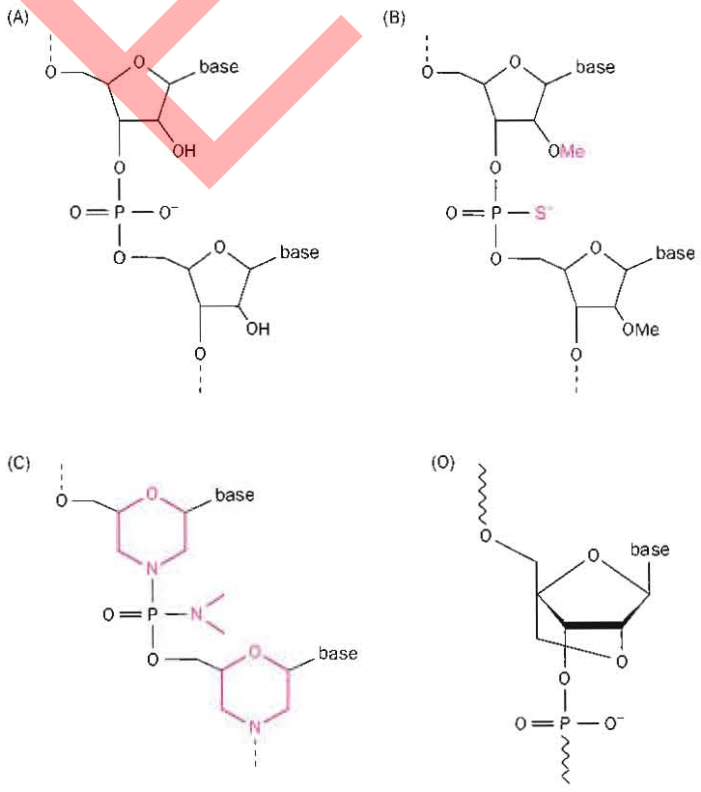

Figure 21.12 Second-generation antisense oligonucleotides. (A) The normal ribose phosphate backbone of RNA. (B) Structure of a 2-*O*-methyl phosphorothioate oligonucleotide. (C) Structure of a morpholino phosphorothioate oligonucleotide. (D) Structure of an oligonucleotide known as a locked nucleic acid.

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Therapeutic siRNA

In the 1990s, the discovery of RNA interference (RNAi) transformed the potential for specific inhibition of gene expression and provided a stimulus to the development of RNA therapeutics. Experimentally, the object is to produce a *double-stranded* RNA with a base sequence corresponding to a transcribed part of the target gene, and so provoke natural cellular defense pathways to destroy transcripts of the specific gene. Long double-stranded RNA cannot be used for this purpose in human (or other mammalian) cells because it provokes interferon responses that result in *nonspecific* inhibition of gene expression (see Chapter 12, p. 391). However, short double-stranded RNA is highly efficient at inducing knockdown of the expression of *specific* genes.

Performing RNAi in mammalian cells often involves transfecting transgenes that express a short hairpin RNA. After transfection into cells, the expressed RNA is cleaved by the cytoplasmic enzyme dicer to give a double-stranded siRNA about 21–23 nucleotides long (see Figures 12.3 and 12.4). Other RNAi therapies deliver just the naked siRNA.

siRNA technology is highly efficient at gene knockdown in mammalian cells and is transforming our understanding of human gene function. There is therefore considerable excitement about its therapeutic potential. Promising therapeutic targets would include viral infections, cancers (targeting oncogenes), and neurodegenerative disease (targeting harmful mutant alleles). The method has been used with some success in treating various animal models of disease, but delivery is comparatively inefficient because of the reliance on non-viral vector systems.

Some early clinical trials using siRNA have focused on the immunologically privileged eye. One principal target is the *VEGF* gene that underlies macular degeneration (as described in the section on therapeutic aptamers on page 686) and has involved simply injecting naked siRNA. Applying RNAi to target diseases with less localized pathology is a much more formidable task. However, systemic and reasonably effective delivery has been possible in animal models by attaching siRNAs to cholesterol, to ferry the siRNA through the bloodstream.

Antisense oligonucleotides can induce exon skipping to bypass a harmful mutation

Antisense therapeutics is not always devoted to blocking the expression of a harmful gene. Sometimes the objective is quite different: to restore function to the mutated gene. AOs have been used to induce altered gene splicing so as to bypass a causative mutation. The mutation is typically a nonsense or frameshifting mutation in a coding DNA exon, which leads to the loss of functionally important downstream sequences.

Therapeutic AOs are normally designed to bind to relevant exon–intron junctions in the pre-mRNA; blocking of splicing at that junction may induce skipping of an adjacent exon containing the harmful mutation. For the strategy to be useful, the skipped exon(s) must not contain sequences that are vitally important for the function of the gene product, and exon skipping must not result in a shift in the translational reading frame. Thus, if a coding DNA exon with a harmful mutation contains a number of nucleotides that is not exactly divisible by three, it may be necessary to also induce skipping of neighboring exons to maintain the reading frame.

Antisense-mediated exon skipping strategies have been particularly well developed for Duchenne muscular dystrophy (DMD), a severe disorder that is typically caused by frameshifting or nonsense mutations in the giant dystrophin gene. Many of the causative mutations occur in internal exons specifying functionally non-essential internal protein sequence. After a proof of principle had been shown by using cultured cells, antisense-mediated exon skipping was used very successfully to restore dystrophin function in the mouse *mdx* model that has a nonsense mutation in exon 23. After encouraging early findings reported in 2007, clinical trials are now underway that seek to skip exon 51 of the human gene, the exon with the most reported DMD mutations (Figure 21.13).

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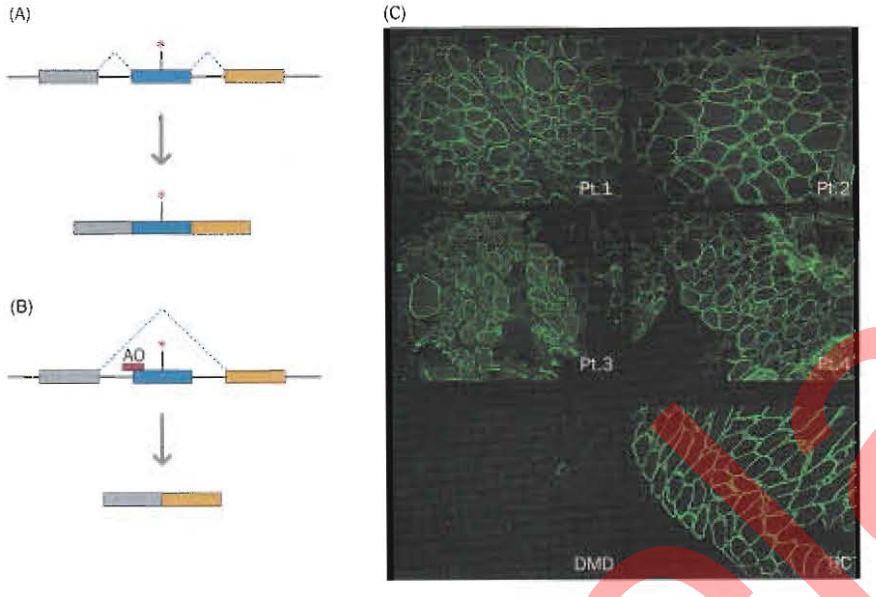

Figure 21.13 Antisense-mediated exon skipping can restore gene function. (A) In normal splicing (dashed blue lines), an exon containing a harmful mutation (red asterisk) is included in the coding sequence. (B) An antisense oligonucleotide (AO) is designed to base-pair with a splicing regulatory sequence, in this case the splice acceptor region of the intron preceding the exon that carries the harmful mutation. By shielding the splicing sequence from the spliceosomal machinery, the AO provokes exon skipping with the result that the harmful mutation is bypassed. (C) Antisense oligonucleotide PRO051 induces skipping of exon 51 of the dystrophin gene without causing a frameshift in the translational reading frame. After intramuscular injection with PRO051 four Duchenne muscular dystrophy (DMD) patients with a causative mutation in exon 51 (Pt.1 to Pt.4) show evidence of restoration of dystrophin in muscle fibers, as revealed by immunofluorescence analysis with a dystrophin-specific antibody. Bottom panels represent controls representing an untreated DMD patient and a healthy control (HC). [(B) from van Deutekom JC, Janson AA, Ginjaar IB et al. (2007) *N. Engl. J. Med.* 357, 2677–2686. With permission from the Massachusetts Medical Society.]

Gene targeting with zinc finger nucleases can repair a specific pathogenic mutation or specifically inactivate a target gene

Homologous recombination can replace one sequence with a closely related one; it is routinely used to replace endogenous sequences by exogenous sequences to selectively inactivate a target gene within intact cells (see Figure 20.6). Cellular homologous recombination pathways also repair double-strand DNA breaks (see Box 13.3). The possibility of using homologous recombination to repair mutant alleles within cells seemed remote, however, because it occurs at a very low frequency in mammalian cells (about one event per 10^6 cells). A new class of genetically engineered nucleases known as zinc finger nucleases has transformed the picture.

Zinc finger nucleases are effectively synthetic restriction nucleases that are designed to recognize long target sequences and cut at a *single* site in a complex genome. As explained in Chapter 20 (p. 654), the specificity comes from a series of zinc finger DNA-binding motifs each of which can bind to a specific triplet sequence in DNA. By cutting and ligating DNA sequences at the DNA level, it is possible to make a DNA construct that will be expressed to give four consecutive zinc fingers of a particular type that can bind to a chosen 12-nucleotide sequence. Attached to the zinc fingers is a DNA-cleaving domain that originates from the restriction endonuclease *Fok*I and which, as a dimer, cleaves double-stranded DNA. If two different zinc finger nucleases are designed to bind to two very closely positioned 12-nucleotide recognition sequences at non-overlapping sites within a target locus, the effective recognition sequence is 24 nucleotides long and may occur only once in the entire genome. The two DNA-cleaving domains come together to form a dimer and make just one double-strand break (**Figure 21.14**).

The occurrence of a double-strand break triggers cellular double-strand DNA repair pathways. One pathway involves homologous recombination; when activated, the frequency of homologous recombination is greatly increased (from 100-fold to 10,000-fold). The homologous recombination (HR) pathway trims back the DNA sequence at the cut site, and new DNA is copied from a donor strand of *homologous* DNA. If the original DNA segment that is repaired contained a pathogenic mutation, the HR pathway can use a donor wild-type homologous sequence to repair the mutation and restore gene function (see Figure 21.14). The donor sequence may come from a normal allele, but the repair is facilitated by introducing a plasmid with the correct sequence (see Figure 21.14).

The utility of this approach has been demonstrated in cellular models. For example, a pathogenic mutation in *IL2RG*, a locus for X-linked severe combined immunodeficiency, has been corrected with high efficiency in cultured human cells. Specific gene inactivation induced by zinc finger nucleases (ZFNs) can also

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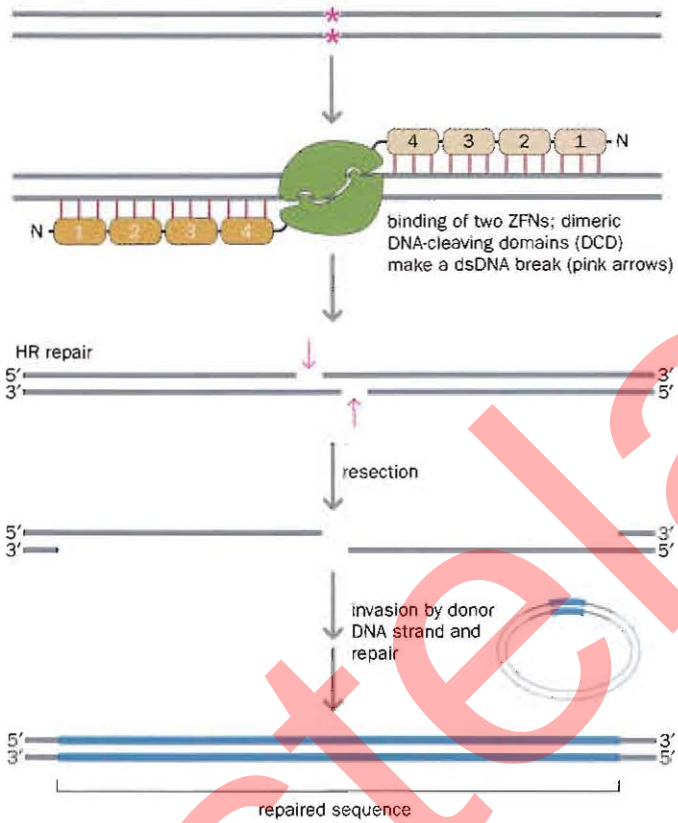

Figure 21.14 Genetically engineered zinc finger nucleases can be used to repair genes within cells. See Chapter 20 p. 654 and Figure 20.13 for the background to zinc finger nucleases. A pair of zinc finger nucleases (ZFNs) each containing four zinc fingers can be designed to specifically bind within a cell to a target sequence that includes a defective mutation (red asterisks) within a mutant gene. The two DNA cleaving domains (shown in green) work together to make a double-stranded DNA break (DSB) that activates cellular pathways that naturally repair such breaks. In the homologous recombination (HR) DNA repair pathway, the 5′ ends of the DSB are first trimmed back (resection), allowing strand invasion by endogenous or exogenous donor DNA strands that are used as templates for synthesizing new DNA to replace the previously existing sequence. A plasmid with a relevant segment of the normal gene sequence (bold blue lines) may be provided as a donor DNA. By acting as a template for synthesizing new DNA from a *portion* of the homologous sequence (thick blue lines), it facilitates replacement of the pathogenic point mutation by the normal sequence.

be used potentially to counteract harmful genes. For example, ZFN-induced gene inactivation has been used to make $CD4^+$ helper T cells resistant to HIV-1 infection. HIV-1 is able to infect helper T cells by binding to the CCR5 chemokine receptor normally expressed by this type of cell. However, some individuals who are naturally resistant to HIV-1 infection are homozygous for a naturally occurring 32 bp deletion in the *CCR5* gene that renders it inactive without clinical consequences. By using ZFN-mediated gene inactivation it has been possible to inactivate the *CCR5* gene, leading to the hope that immunity to HIV-1 can be conferred on individuals.

21.6 GENE THERAPY IN PRACTICE

Expectations about gene therapy have followed a cyclical course over the past quarter of a century or so; periods of overoptimism were followed by bouts of excessive pessimism in response to significant setbacks (**Box 21.8**). Perhaps one cause of these exaggerated reactions is confusion over the natural time-scales of such work. Because diagnostic testing can often start within weeks of a gene being cloned, therapy could be expected to be not far behind, whereas really this is drug development and runs on a time-scale of decades.

Although practical gene therapy is a long-haul process, many academic and commercial laboratories are working hard in this area. By 2009 about 1500 trial protocols related to gene therapy had been approved (**Figure 21.15** gives a breakdown). However, only 3% of these are phase III trials, in which the efficacy of the therapy is tested on a large scale. Of the 1500 or so approved gene therapy protocols, nearly two-thirds have been for cancer; monogenic disease, infectious diseases, and cardiovascular diseases accounted for less than 10% each. We give brief overviews below of the progress of gene therapy in several important areas.

Despite the limited number of trials, monogenic diseases have always been high on the gene therapy agenda, and the first definitive successes have been in that area. In particular, recessive disorders, in which the problem arises because of deficiency of a single gene product, seemed comparatively easy targets; even an overall low-level expression of introduced genes might produce some clinical

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BOX 21.8 SOME OF THE MAJOR UPS AND DOWNS IN THE PRACTICE OF GENE THERAPY

1990–	commencement of the first clinical trial involving gene therapy, using recombinant γ-retroviruses to overcome an autosomal recessive form of severe combined immunodeficiency (SCID) due to deficiency of adenosine deaminase (ADA); the trial was hailed as a success but patients had also been treated in parallel with standard enzyme replacement using polyethylene glycol (PEG)-ADA
1999	death of Jesse Gelsinger in September 1999, just a few days after receiving recombinant adenoviral particles by intra-hepatic injection in a clinical trial of gene therapy for ornithine transcarbamylase deficiency; a massive immune response to the adenovirus particles resulted in multiple organ failure
2000–	the first unambiguous gene therapy success involved using recombinant γ-retroviruses to treat an X-linked form of SCID (see Figure 21.16); however, several of the treated children went on to develop leukemia, with one death, as a result of insertional activation of cellular oncogenes (see the main text)
2006–	transient success in using γ-retroviral gene therapy to treat two adult patients with chronic granulomatous disease, a recessive disorder that affects phagocyte function causing immunodeficiency; despite initial success, both patients went on to show transgene silencing, and also myelodysplasia as a result of insertional activation of cellular genes (see the main text); 27 months after gene therapy one patient died from overwhelming sepsis; see Ott et al. (2006) *Nat. Med.* 12, 401–409; PMID 16582916 and Stein et al. (2010) *Nat. Med.* 16, 198–204; PMID 20098431
2006	report of limited success in gene therapy for hemophilia B: AAV2 vectors were used successfully to transduce hepatocytes and express a Factor IX transgene; however, an immune response led to destruction of the transduced cells; see Manno et al. *Nat. Med.* 12, 342–347; PMID 16474400
2009	successful gene therapy for ADA deficiency reported by Aiuti et al. *N. Engl. J. Med.* 360, 447–458; PMID 19179314
2009	first report of successful gene therapy for a central nervous system disorder, X-linked adrenoleukodystrophy, and the first using a lentiviral vector; see Cartier et al. *Science* 326, 818–823; PMID 19892975
2009	report of successful *in vivo* gene therapy using retinal injection of recombinant AAV to treat Leber congenital amaurosis, a form of childhood blindness; the significant post-treatment gain in vision was found to be maintained after one year; see Cideciyan et al. *N. Engl. J. Med.* 361, 725–727; PMID 19675341

PMID, PubMed identifier number.

benefits. By contrast, dominant disorders—in which one of the two alleles is normal and makes a gene product—present much greater challenges. Getting the correct gene dosage to treat haploinsufficiency could be problematic, and counteracting the effect of a mutant allele that makes a harmful product would require very efficient and selective silencing of the mutant allele, while leaving the normal allele unaffected.

The first gene therapy successes involved recessively inherited blood cell disorders

Blood cells are highly accessible and are suited to *ex vivo* gene therapy—the cells can be genetically modified in culture, and carefully screened before selecting and expanding suitably modified cells and returning them to the patient (see

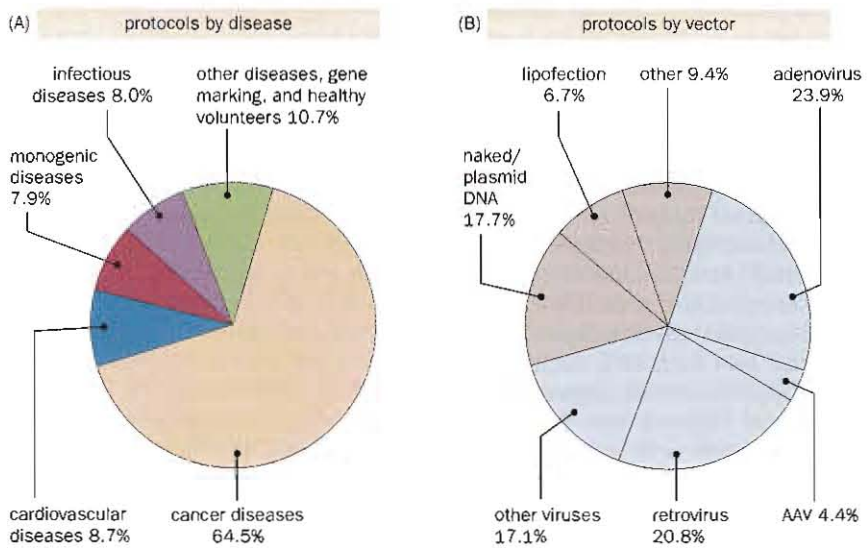

Figure 21.15 Approved gene therapy trial protocols. (A) Distribution by disease. (B) Distribution by gene delivery system. AAV, adeno-associated virus. The figures include all approved protocols (n =1481) for completed, ongoing, or pending trials listed by the end of 2008 in the Wiley database of worldwide gene therapy clinical trials (http://www.wiley.co.uk/genmed/clinical/).

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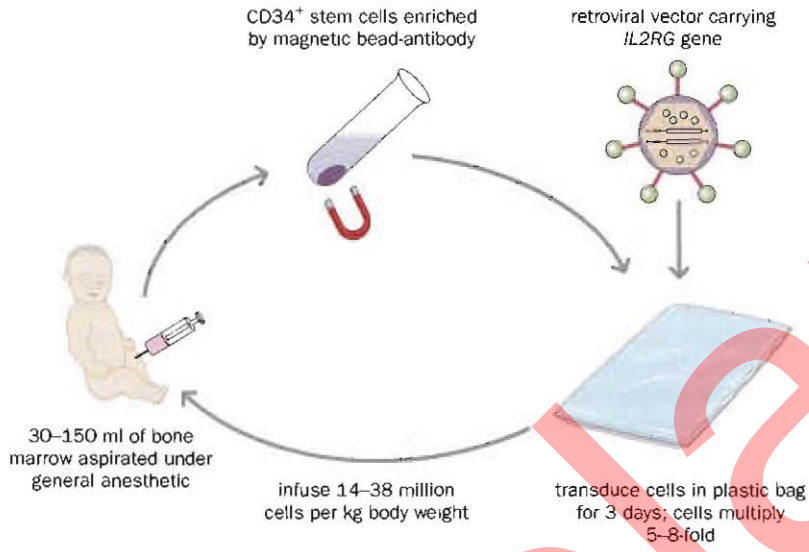

Figure 21.16 ***Ex vivo*** **gene therapy for X-linked severe combined immunodeficiency disease (X-SCID).** Bone marrow was removed from the patient and antibody affinity was used to enrich for cells expressing the CD34 antigen, a marker of hematopoietic stem cells. To do this, bone marrow cells were mixed with paramagnetic beads coated with a CD34-specific monoclonal antibody; beads containing bound cells were removed by using a magnet. The transduced stem cells were expanded in culture before being returned to the patient. For details, see Cavazzana-Calvo M et al. (2000) *Science* 288, 669–672; PMID 10784449 and Hacein-Bey-Abina S et al. (2002) *N. Engl. J. Med.* 346, 1185–1193; PMID 11961146.

Figure 21.8). Disorders resulting from recessively inherited defects in white blood cell function were among the first targets for gene therapy. White blood cells have key roles in the immune system (see Tables 4.7 and 4.8), and defects in their function can give rise to severe immunodeficiencies.

The first definitive gene therapy success came in treating severe combined immunodeficiency (SCID), in which both B and T lymphocyte function is defective. Patients are extremely vulnerable to infectious disease, and in some cases they have been obliged to live in a sterile environment. The most common form of SCID is due to inactivating mutations in the X-linked *IL2RG* gene that makes the γ_c common gamma subunit for multiple interleukin receptors, including interleukin receptor 2. Lymphocytes use interleukins as cytokines to signal to each other and to other immune system cells, and so lack of the γ_c cytokine receptor subunit had devastating effects on lymphocyte and immune system function. Another common form of SCID is due to adenosine deaminase (ADA) deficiency; the resulting build-up of toxic purine metabolites kills T cells. Because T cells regulate B-cell activity, both T- and B-cell function is affected.

SCID gene therapy has involved *ex vivo* γ-retroviral transfer of *IL2RG* or *ADA* coding sequences into autologous patient cells (see Box 21.8). To aid the chances of success, bone marrow cells were used because they are comparatively enriched in hematopoietic stem cells, the cells that give rise to all blood cells (see Figure 4.17); a further refinement was to select for cells expressing CD34, a marker of hematopoietic stem cells (**Figure 21.16**). By 2008 Fischer et al. reported that 17 out of 20 X-linked SCID patients and 11 out of 11 ADA-deficient SCID patients had been successfully treated and retained a functional immune system (for more than 9 years after treatment in the earliest patients).

With the use of retrovirus vectors, genes could be inserted into chromosomes; the therapeutic DNA would be stably transmitted after cell division, giving long-lasting transgene expression. This clear advantage was eroded by the lack of control over where the transgenes integrated. In one clinical trial for X-linked SCID, four patients developed T-acute lymphoblastic leukemia after retroviral integration because promoter/enhancer sequences in the inserted DNA inappropriately activated a neighboring *LMO2* proto-oncogene. Activation of *LMO2* is now known to promote the self-renewal of pre-leukemic thymocytes, so that committed T cells accumulate additional genetic mutations required for leukemic transformation.

Similar protocols were applied to treating patients with an X-linked form of chronic granulomatous disease (CGD). Patients with CGD have immunodeficiency arising from mutations in any of four genes that encode subunits of the NADPH oxidase complex. NADPH oxidase is involved in making free radicals and other toxic small molecules that phagocytes use to kill the microbes that they engulf; a defective NADPH oxidase results in defective phagocyte function.

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Retroviral transfer of a suitable transgene restored functional NADPH oxidase, and the treatment was transiently successful but subsequently transgenes were silenced after insertional activation of cellular proto-oncogenes. In this case, gene-transduced hematopoiesis in both patients was dominated by cell clones containing integrations in the *MDS-EVI1* locus and resulted in overexpression of these proto-oncogenes. The neighboring inserted transgene was silenced by promoter methylation; see the paper by Stein et al. (2010) in the 2006 entry in Box 21.8.

Despite the above successes, some other recessively inherited blood disorders have proven to be less straightforward to treat. The limited success with hemophilia B (see Box 21.8) was derived from unexpected immune responses, but it is now realized that AAV is surprisingly prevalent, existing in perhaps 40% of all human livers; testing for preexisting AAV antibodies might have identified suitable patients for therapy. Gene therapy for the most prevalent blood disorders, the thalassemias, is a formidable challenge because of the tight regulation of globin gene expression that is designed to maintain a 1:1 production ratio of α-globin to β-globin.

Gene therapies for many other monogenic disorders have usually had limited success

Other than recessively inherited blood cell disorders, there have been few gene therapy successes. One preliminary success has involved an X-linked form of adrenoleukodystrophy caused by inactivating mutations in *ABCD1* (see Box 21.8). The gene defect results in the accumulation of saturated very long-chain fatty acids, leading to demyelination in oligodendrocytes and microglia, and consequent dysfunction of the nervous system. Even here, however, the successful therapy was based on lentiviral-mediated gene transfer into autologous hematopoietic stem cells. The transduced stem cells gave rise to myelomonocytic cells that migrated into the central nervous system to replace diseased microglial cells and relieve the lipid storage.

Two other promising targets are disorders of the eye and skin, both of which are highly accessible. The eye is a small and enclosed target organ, and because it is an immunologically privileged site immune responses tend to be weak. Recent advances include a report in 2009 of successful AAV-mediated gene transfer by retinal injection to treat Leber congenital amaurosis (Box 21.8).

Despite the above successes, gene therapy for many single-gene disorders has proved to be challenging, as in the case of *in vivo* gene therapies for cystic fibrosis (CF) and Duchenne muscular dystrophy (DMD). CF is caused by lack of the *CFTR*-encoded chloride channel, mainly in airway epithelium. Studies of patients with partly active *CFTR* alleles suggest that 5–10% of the normal level would be sufficient to produce a good clinical response, but numerous clinical gene therapy trials have been unsuccessful.

A significant problem in CF gene therapy is the physical barrier of mucus and polysaccharide that covers lung airway epithelial cells, especially in the infected lungs of patients with CF. Gene therapy agents can be delivered into the airways, but more sophisticated vehicles will be necessary to allow efficient transduction of epithelial cells. Ideally, stem cells should be targeted, because surface epithelial cells have a life span of only about 120 days, so that repeated administration will be necessary, with all the attendant problems of the immune response.

Patients with DMD suffer progressive wasting of first skeletal and then cardiac muscle. Studies of female DMD carriers and patients with milder Becker muscular dystrophy show that restoring about 20% of normal dystrophin gene expression in muscle would benefit DMD patients. However, the dystrophin coding sequence (14 kb) is too large for many vectors, and there is the challenge of getting efficient gene delivery into both skeletal and cardiac muscle cells. The size problem has been partly addressed by deleting less important coding sequences that encode a central domain in dystrophin to produce much smaller cDNAs, but efficient delivery remains a problem.

The prospects of antisense-mediated exon skipping may be good. The current clinical trials seek to skip exon 51 of the *DMD* gene by using an intramuscular injection of antisense oligonucleotides (see Figure 21.13C) and are applicable to

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just a small but significant percentage of DMD patients. However, another strategy under development seeks to use a cocktail of different antisense oligonucleotides to block splicing of each of exons 45–55; this would be applicable to most DMD patients. The loss of 11 consecutive exons does not change the reading frame and is associated with a mild phenotype.

Restoration of dystrophin gene function has also been possible by using autologous blood-derived progenitor cells expressing the CD133 antigen, which are known to be able to regenerate dystrophic fibers. The prospects of autologous stem cell-mediated restoration of gene function in different recessive disorders has been boosted by the comparative ease of preparing patient-specific induced pluripotent stem cells (see Section 21.3), and encouraging results are being obtained in animal models.

Cancer gene therapies usually involve selective killing of cancer cells, but tumors can grow again by proliferation of surviving cells

Nearly two-thirds of all approved gene therapy trial protocols have been for cancer (see Figure 21.15A). Table 21.5 gives some examples, chosen to illustrate the range of approaches. They include:

- Gene addition to restore tumor suppressor gene function (e.g. *TP53* or *BRCA1*)
- Gene inactivation to prevent expression of an activated oncogene (e.g. *ERBB2*)
- Genetic manipulation of tumor cells to trigger apoptosis
- Modification of tumor cells to make them more antigenic, so that the immune system destroys the tumor
- Modification of dendritic cells to increase a tumor-specific immune reaction

TABLE 21.5 EXAMPLES OF CANCER GENE THERAPY TRIALS

Disorder	Cells altered	General strategy	Gene therapy protocol
Ovarian cancer	tumor cells	gene addition to restore tumor suppressor gene function	intraperitoneal injection of retrovirus or adenovirus encoding full-length cDNA encoding p53 or BRCA1, with the hope of restoring cell cycle control
Ovarian cancer	tumor cells	oncogene inactivation	inject adenovirus encoding a scFv antibody to ErbB2; hope to inactivate a growth signal
Malignant melanoma	tumor-infiltrating lymphocytes (TILs)	genetic manipulation of tumor cells to trigger apoptosis	extract TILs from surgically removed tumor and expand in culture; infect TILs *ex vivo* with a retroviral vector expressing TNF-α, infuse into patient; hope that TILs will target remaining tumor cells, and the TNF-α will kill them (see Figure 21.7E for the principle)
Various tumors	tumor cells	increase antigenicity of tumor cells so that immune system destroys tumor	transfect tumor cells with a retrovirus expressing a cell surface antigen such as HLA-B7 or a cytokine such as IL-12, IL-4, GM-CSF, or γ-interferon; hope that this enhances the immunogenicity of the tumor, so that the host immune system destroys it; often done *ex vivo* with lethally irradiated tumor cells (see Figure 21.7E for the principle)
Prostate cancer	dendritic cells	modify dendritic cells to enhance tumor-specific immune reaction	treat autologous dendritic cells with a tumor antigen or cDNA expressing the antigen, to prime them to mount an enhanced immune response to the tumor cells (see Figure 21.7E for the principle)
Malignant glioma (brain tumor)	tumor cells	genetic modification of tumor cells so that they convert a non-toxic prodrug into a toxic compound that kills them	inject a retrovirus expressing thymidine kinase (TK) or cytosine deaminase (CD) into the tumor; only the dividing tumor cells, not the surrounding non-dividing brain cells, are infected; then treat with ganciclovir (TK-positive cells convert this to the toxic ganciclovir triphosphate) or 5-fluorocytosine (CD-positive cells convert it to the toxic 5-fluorouracil); virally infected (dividing) cells are killed selectively (see Figure 21.17)
Head and neck tumors	tumor cells	use of oncolytic viruses that are engineered to selectively kill tumor cells	inject ONYX-015 engineered adenovirus into tumor; the virus can only replicate in p53-deficient cells, so it selectively lyses tumor cells; this treatment was effective when combined with systemic chemotherapy

TNF, tumor necrosis factor; IL, interleukin; GM-GSF, granulocyte/macrophage colony-stimulating factor. See the National Institute of Health clinical trials database at http://clinicaltrials.gov/ for a comprehensive survey.

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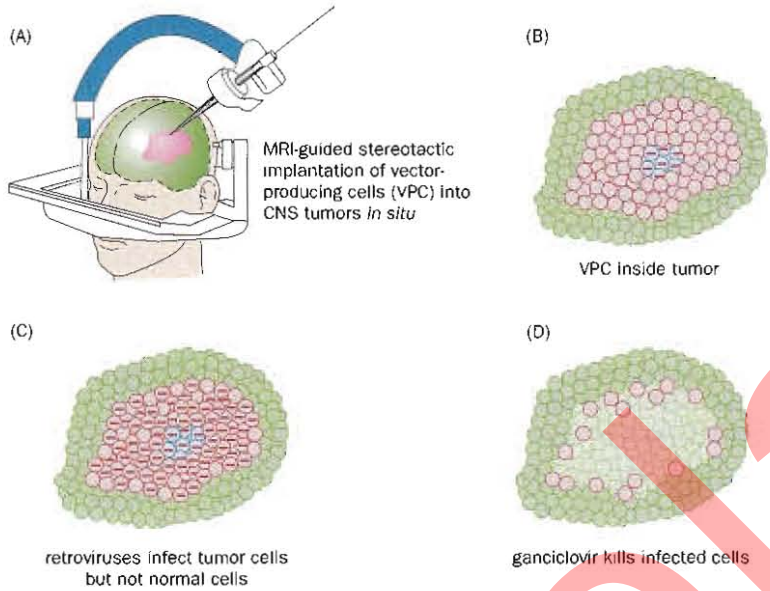

Figure 21.17 Design of *in vivo* gene therapy for brain tumors. (A, B) A retrovirus is engineered to produce the herpes simplex virus thymidine kinase (HSV-TK). Vector-producing cells (VPC; blue) are injected into the brain tumor. (C) Because retroviruses infect only dividing cells, they infect the tumor cells (pink) but not the surrounding normal brain tissue (green). (D) The non-toxic prodrug ganciclovir is given intravenously. In TK^+ cells, ganciclovir is converted to the highly toxic ganciclovir triphosphate, causing cell killing and shrinkage of the tumor. Because retroviral transduction of the cancer cells is not 100% efficient, some tumor cells survive, so the tumor may grow again. MRI, magnetic resonance imaging.

- Use of oncolytic viruses that are engineered to kill tumor cells selectively
- Genetic modification of tumor cells so that they, but not surrounding non-tumor cells, convert a non-toxic prodrug into a toxic compound that kills them (Figure 21.17).

As at 2009, three cancer immunotherapies were at the phase III stage of clinical trials. Also at the phase III stage are adenoviral-based transfer of a herpes simplex virus thymidine kinase suicide gene for treating glioblastoma (see Figure 21.17) and adenoviral-based expression of p53 for the treatment of head and neck cancers and for Li–Fraumeni (OMIM 151623) tumors.

A general problem is that the methods for killing cancer cells are far from 100% efficient, and even when the efficiency is very high so that tumors shrink, they can grow again as surviving cancer cells proliferate. The idea of curing cancer is being replaced by a more realistic long-term management of cancer disorders.

Multiple HIV gene therapy strategies are being pursued, but progress toward effective treatment is slow

As the most important viral pathogen of humans, HIV-1 is the target of huge research efforts. Much work has gone into attempts to develop genetically engineered vaccines; in addition, many researchers have investigated the genetic manipulation of host cells to make them resistant to HIV. Much like the treatment for X-linked SCID described above, many of the gene therapy trials have used retroviral vectors to transfect hematopoietic stem cells *ex vivo*; treated cells are then returned to the patient. The transfected genes are designed in the hope that they will inhibit HIV replication and so prevent HIV infection from developing into AIDS.

To make lymphocytes resistant to HIV, the major targets for therapy have been the tat and rev regulatory proteins, and the viral RNA sequences to which they bind—TAR and RRE, respectively. A variety of reagents have been used:

- *Antisense RNAs.* Retroviruses have been constructed that encode antisense RNAs to TAR, to the overlapping *tat* and *rev* mRNAs, and to the *pol* and *env* mRNAs.
- *Decoy RNAs.* A retrovirus directing high-level expression of a transcript containing the RRE sequence might be able to sequester all the rev protein and prevent HIV replication.
- *Dominant-negative mutants.* Some retroviral constructs encode a mutant rev protein, RevM10. This binds the RRE but then will not assemble the multiprotein complex required to export the RNA from the nucleus.

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- *Ribozymes.* Several groups have made retroviruses that encode ribozymes. RRz2 is a hammerhead ribozyme directed against the *tat* regulatory region; another type, the hairpin ribozyme, has also been used to cleave the HIV genome.
- *Intrabodies.* Retroviruses encoding scFv intracellular antibodies have been used to try to inactivate the tat or rev regulatory proteins, or the gp160 coat glycoprotein.
- *ZFN gene targeting.* As detailed in the section above on ZFNs, clinical trials will seek to use ZFNs to specifically inactivate the gene encoding the CCR5 chemokine receptor used by HIV-1 to gain entry into helper T cells.

In the early stages of HIV infection, there is a massive turnover of lymphocytes, as the immune system struggles to destroy infected cells. If the immune response were rather more effective, it might be possible to contain the virus at this stage. Apart from efforts at developing vaccines, work has also been devoted to modifying T cells so that they kill infected cells more efficiently. Retroviruses have been designed to make $CD8^+$ T cells express a chimeric T-cell receptor that targets their cytotoxic response to HIV-infected cells.

Details of all these approaches can be found in the NIH database of clinical trials. *In vitro*, the manipulated cells often show high resistance to HIV infection; *in vivo*, long-term (from several months to 1 or 2 years) bone marrow engraftment has been demonstrated in a few trials. The open question is whether engraftment can be made to occur at a sufficiently high level to provide a clinically useful pool of HIV-resistant lymphocytes. High-level engraftment might be achieved by first destroying the patient's existing marrow with cytotoxic chemicals and radiation—but doing this to a patient with AIDS would be a desperate measure.

CONCLUSION

Genetic technologies can be used in the treatment of disease, regardless of whether the disease has a genetic cause or not. This can be part of a treatment regime that involves conventional drugs or vaccines, but genetic manipulations can also be used in the production of drugs and vaccines. Genetic engineering has also been used to make therapeutic proteins. Expression cloning in microorganisms, cultured cells, or transgenic livestock (in which the protein of interest is expressed in milk or eggs) avoids the health risks associated with harvesting these proteins from animal or human sources. Genetic engineering has also been applied to make partly or fully human monoclonal antibodies. They are more stable in human serum than rodent monoclonal antibodies and are consequently better suited for therapeutic purposes.

Genetic interventions are also being used in two developing and interconnected therapeutic strategies: cell replacement therapy (to replace cells lost through disease or injury, enabling repair of damaged tissues) and gene therapy (involving genetic modification of a patient's cells). Stem cells are important in cell therapy because they are both self-renewing and also capable of being differentiated to make new tissue cells. Bone marrow transplants are an established form of stem cell therapy to treat blood disorders and hematopoietic stem cell depletion after chemotherapy.

To avoid immune rejection, autologous cells are prefered for cell therapy. In principle, they can be generated by artificially enhancing the mobilization and differentiation of stem cells in the patient or by using laboratory nuclear reprogramming methods to reverse the normal developmental progression of cells toward specialization, which has opened new research and therapeutic opportunities. The transplant of the nucleus from an adult somatic cell into an enucleated oocyte allowed the cloning of Dolly the sheep and various other types of animal species, and could in principle be used to make patient-specific embryonic stem cells; however, the procedures are technically challenging and ethically contentious.

Nuclear reprogramming of skin fibroblasts from patients by ectopic expression of specific transcription factors offers a new route to obtaining patient-specific and disease-specific pluripotent stem cells, with few ethical concerns.

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Such induced pluripotent stem (iPS) cells can be differentiated to provide human cellular models of disease and are being used to test drugs; in principle they could provide sources of cells for autologous cell therapy. Transdifferentiation techniques hold the promise of making more subtle changes to cell lineage (e.g. altering pancreatic exocrine cells into beta cells to replace those lost in type 1 diabetes).

In gene therapy, the aim is to transfer a DNA, RNA, or oligonucleotide into a patient's cells to counteract or alleviate disease. Transfer into cells is often achieved with virus vectors, which offer high efficiency of gene transfer and high-level, sometimes long-lasting, transgene expression. Safety issues are a concern because integration into chromosomes is uncontrolled and can accidentally activate a neighboring proto-oncogene. Non-viral vectors are safer, but gene transfer rates are low and expression is often transient and comparatively weak.

The genetic modification could theoretically be directed to germ-line cells (a permanent, transmissible modification), but for ethical reasons germ-line gene therapy is not being attempted, and all current trials involve somatic gene therapy. The aim is often to add a gene that can functionally replace a defective gene, but some therapies seek to block expression of a mutant or ectopically expressed gene at the RNA level, usually using RNA interference, or at the protein level.

Another class of gene therapy strategy seeks to restore the function of a defective gene, either at the RNA level by inducing exon skipping so that a causative mutation is bypassed, or at the DNA level by using zinc finger nucleases. These enzymes are genetically engineered to act as restriction nucleases that cleave genomic DNA at just one site, such as at the location of an inactivating point mutation. The double-strand DNA break can trigger a cellular repair pathway that uses homologous recombination to replace the mutant sequence by a homologous wild-type sequence provided by a transfected plasmid.

Most clinical trials of gene therapy have sought to treat cancer, and here the strategy is to kill cancer cells selectively; however, success has been limited. The first clearly successful gene therapies have been for recessive blood cell disorders, which are more amenable because the cells are highly accessible and can be genetically modified outside the body before being returned to the patient, and because even a small amount of transgene expression can often bring about some clinical benefit.

Esther

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