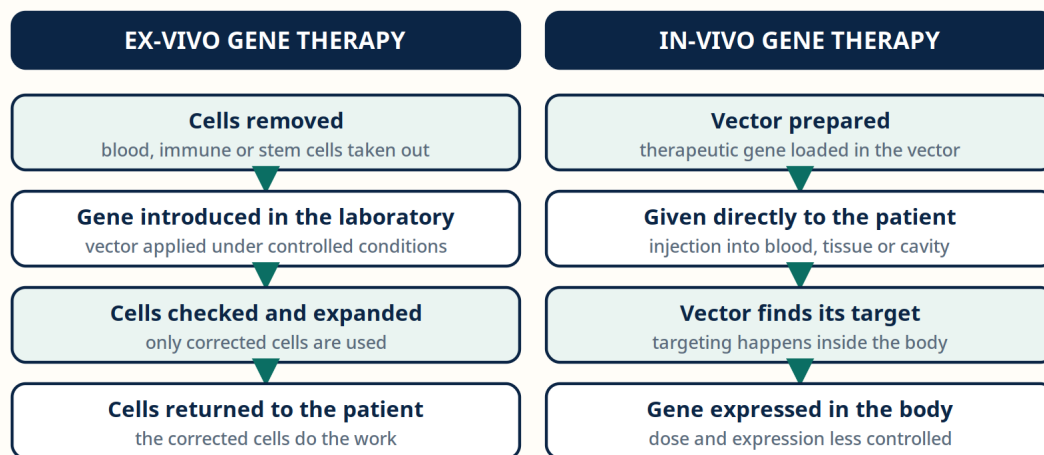


5.1 Gene therapy: introduction

Ex-Vivo and In-Vivo Gene Therapy



Only somatic cells are treated; germline modification is not practised

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Figure: Ex-vivo gene therapy corrects the patient's cells in the laboratory; in-vivo gene therapy delivers the vector directly to the patient.

Gene therapy treats disease by delivering nucleic acid: it introduces a functional copy of a gene to replace a defective one, adds a gene whose product fights the disease, or silences or edits a harmful gene. The delivered DNA must survive transport, enter the target cell, reach the nucleus and be expressed at the right level for long enough; each of those steps is a delivery problem, which is why this unit sits in a drug delivery subject. Two features distinguish it from ordinary drug therapy: the effect can persist for years after one administration because the cell keeps expressing the gene, and the delivery system (the vector) is as much a part of the product as the gene itself.

Two approaches are distinguished:

- **Ex-vivo gene therapy:** cells are removed from the patient, the gene is introduced into them in the laboratory, the corrected cells are selected, checked and expanded, and they are then returned to the patient. The first clinical trial of gene therapy (1990, for adenosine deaminase deficiency, ADA-SCID) used this approach with the patient's own T lymphocytes. Quality control is possible before the cells go back, and no vector is given to the patient directly, but the method is individual, costly and limited to cells that can be removed and returned (blood and immune cells, skin, stem cells).
- **In-vivo gene therapy:** the gene, in its delivery system, is given directly to the patient, by injection into the blood, a tissue, or a body cavity, and must find its target cells in the body. It is simpler in concept and can reach internal organs, but targeting, dose control and safety monitoring are all harder.

Somatic cell gene therapy (treating the patient's body cells, with effects confined to that patient) is the accepted field; germline modification, which would pass changes to children, is not practised.

5.2 Potential target diseases for gene therapy

- **Inherited (monogenic) disorders:** diseases caused by one defective gene are the natural first targets, because one working copy can in principle correct them. Examples studied include ADA-SCID and other severe combined immunodeficiencies, cystic fibrosis (the CFTR gene, delivered to the airways), haemophilia A and B (factor VIII and IX genes, delivered to the liver), Duchenne muscular dystrophy, and inherited retinal disease. The commercially successful products reflect this list: Luxturna (voretigene neparvovec, an AAV vector carrying the RPE65 gene, 2017) for inherited retinal blindness and Zolgensma (onasemnogene abeparvovec, AAV carrying the SMN1 gene, 2019) for spinal muscular atrophy are in-vivo products, and Strimvelis (2016) is an ex-vivo stem cell product for ADA-SCID.
- **Cancer:** gene therapy attacks cancer in several ways: replacing a defective tumour suppressor gene (delivery of normal p53), suicide gene therapy (introducing an enzyme gene such as herpes simplex thymidine kinase that converts a prodrug, ganciclovir, into a toxic product inside the tumour cell), immunotherapy (engineering the patient's T cells to carry a chimeric antigen receptor, the CAR-T products such as Kymriah, 2017, which is ex-vivo gene therapy), and oncolytic viruses engineered to multiply in and destroy tumour cells.
- **Other fields under study:** infectious disease (for example engineering resistance to HIV), cardiovascular disease (angiogenic growth factor genes) and neurological disease.

5.3 Gene expression systems

A gene delivery product is more than a gene. The expression cassette carried by the vector contains the therapeutic gene (coding sequence) together with the control elements that decide where, when and how strongly it is expressed: a promoter (constitutive promoters such as the CMV promoter for strong general expression, or tissue specific promoters to restrict expression to, for example, liver or muscle), enhancer sequences, a polyadenylation signal, and in some designs inducible elements that allow expression to be switched by a drug. The choice of expression system is a formulation decision: too little expression gives no benefit, too much or expression in the wrong tissue can be toxic, and permanent expression is desirable for a missing enzyme but unnecessary or unwanted for a temporary effect such as a growth factor pulse. Gene transfer systems are judged not only by how much DNA they deliver but by the expression profile they produce.

5.4 Viral gene transfer systems

Viruses are nature's gene delivery vehicles: they are built to enter cells and place their genetic material where it will be expressed. Gene therapy vectors are viruses from which the disease causing and replication genes have been removed and replaced by the therapeutic gene.

Vector	Carried form	Main advantages	Main limitations
Retrovirus (gamma retrovirus)	RNA, integrated into host DNA	Stable integration, lifelong expression in dividing cells	Enters dividing cells only; insertional mutagenesis risk (leukaemia occurred in early SCID trials)
Lentivirus	RNA, integrated	Infects dividing and non dividing cells; used for ex-vivo stem cell and CAR-T products	Integration risk remains; complex manufacture
Adenovirus	DNA, stays episomal	High expression, infects many cell types, large capacity	Strong immune response, expression is temporary, cannot be redosed easily

Vector	Carried form	Main advantages	Main limitations
Adeno associated virus (AAV)	DNA, mainly episomal	Low pathogenicity and immunogenicity, long expression in non dividing tissues (eye, liver, muscle, nerve)	Small packaging capacity (about 4.7 kb); pre existing immunity to natural AAV in many patients
Herpes simplex virus	DNA, episomal	Very large capacity, natural nerve tropism	Cytotoxicity and latency need careful engineering

5.5 Nonviral gene transfer systems

Nonviral systems avoid the safety and manufacturing problems of viruses but are less efficient, because they must overcome every barrier (serum nucleases, cell entry, endosomal escape, nuclear entry) without viral machinery.

- **Naked DNA:** plasmid DNA injected directly, taken up poorly except in muscle and skin; used in DNA vaccine research and with physical assistance.
- **Physical methods:** electroporation (electric pulses open transient pores in the cell membrane), the gene gun (DNA coated gold particles shot into tissue), ultrasound and magnet assisted delivery; effective locally but hard to apply to deep tissues.
- **Chemical carriers:** calcium phosphate coprecipitation (the classical laboratory method), DEAE-dextran, cationic polymers such as polyethyleneimine (which buffers the endosome and promotes escape, the proton sponge idea), dendrimers, and cationic lipids and liposomes (next section). These condense the negatively charged DNA into small complexes (polyplexes or lipoplexes) that cells take up by endocytosis.

5.6 Liposomal gene delivery systems

Cationic liposomes and lipid nanoparticles are the most developed nonviral systems and connect this unit back to Unit 2. A cationic lipid (for example DOTAP or DOTMA in research systems) mixed with a helper lipid such as DOPE forms liposomes with a positive surface; the anionic DNA binds electrostatically and is condensed into a lipoplex, which is protected from nucleases, binds the negatively charged cell surface, enters by endocytosis, and with the help of the fusogenic helper lipid escapes the endosome so that the DNA can reach the nucleus. The design variables are the lipid structure, the lipid to DNA charge ratio, particle size and the route of administration. Lipid systems are chemically defined, can be manufactured reproducibly, can carry large DNA, and provoke less immune response than viruses, but efficiency is lower and cationic lipids are dose limitingly toxic, so they are used where transient expression is acceptable. Their clinical coming of age is the lipid nanoparticle delivery of small interfering RNA in Onpattro (patisiran, 2018), the first approved siRNA medicine, in which an ionisable lipid nanoparticle carries the siRNA to hepatocytes after intravenous infusion. siRNA is not gene therapy in the strict gene addition sense, but the delivery system is the liposomal platform of this section.

5.7 Biodistribution and pharmacokinetics of gene delivery systems

Nucleic acids behave unlike small molecule drugs, and their delivery systems have their own pharmacokinetics. Unprotected DNA and RNA are degraded by serum and tissue nucleases within minutes, so protection by the carrier is a precondition of any effect. After intravenous administration, complexes and vectors distribute like other nanoparticles: larger and charged complexes are cleared rapidly by the RES to liver and spleen, lung can trap larger aggregates at first pass, and only a small fraction reaches other

targets, which is why local administration (into the eye, muscle, tumour or airway) is common in the approved products. Clearance also has an immune component: viral vectors provoke antibody and T cell responses that limit repeat dosing, and innate sensing of nucleic acids (for example through Toll like receptors) causes dose limiting inflammation. Biodistribution studies (where the vector and the expressed gene are found, using PCR based assays) and shedding studies (whether the patient excretes vector) are therefore formal parts of gene therapy development and regulation, and the pharmacokinetics of the expressed protein, not of the DNA alone, decides the duration of treatment effect.

5.8 Antisense molecules as drugs

Antisense oligonucleotides are short synthetic single stranded nucleic acid sequences (typically about 15 to 25 nucleotides) designed to bind a specific messenger RNA by base pairing and so prevent production of one chosen protein. The main mechanisms are: recruiting the enzyme RNase H to cut the bound mRNA; sterically blocking the ribosome so translation stops; and modulating splicing, by blocking splice sites so that an exon is skipped or included. Because naked oligonucleotides are destroyed by nucleases and cross membranes poorly, they are chemically modified: phosphorothioate backbones resist nucleases and bind plasma proteins (improving distribution), and 2 prime sugar modifications (2'-O-methyl, 2'-O-methoxyethyl) and locked nucleic acids increase binding strength and stability. Approved examples show the range: fomivirsen (Vitravene, 1998), the first antisense drug, given into the eye for cytomegalovirus retinitis; mipomersen (Kynamro), against apolipoprotein B mRNA for homozygous familial hypercholesterolaemia; and nusinersen (Spinraza), which corrects the splicing of the SMN2 gene in spinal muscular atrophy and is given intrathecally. Delivery remains the limiting step for this class, which is why antisense chemistry and delivery are developed together.

5.9 Aptamers: drugs of the future

Aptamers are short single stranded DNA or RNA molecules that fold into defined three dimensional shapes and bind a target molecule (a protein, or occasionally a small molecule) with antibody like specificity and affinity, but by shape recognition rather than by base pairing with nucleic acid. They are selected in vitro by SELEX (systematic evolution of ligands by exponential enrichment): a huge random library (of the order of 10^{15} sequences) is exposed to the target, the binding sequences are recovered and amplified by PCR, and the cycle is repeated until binders of very high affinity dominate. Aptamers are then chemically stabilised (2 prime modifications, capping, and PEGylation to slow renal clearance). Their advantages over antibodies are that they are made by chemical synthesis (cheap, reproducible, no animals or cells), are small (better tissue penetration), and are generally of low immunogenicity; their disadvantages are nuclease sensitivity without modification and rapid renal clearance of small molecules, both addressed by the modifications above. The first approved aptamer drug was pegaptanib (Macugen, 2004), a PEGylated anti VEGF aptamer injected into the eye for wet age related macular degeneration. Together with antisense molecules and siRNA, aptamers represent the class the syllabus calls the drugs of future: information based medicines whose specificity is designed from a sequence or a shape rather than discovered by screening, and whose success depends on the delivery and stability solutions developed in this unit.

Frequently Asked Questions

Q: What is targeted drug delivery and how does it differ from conventional drug delivery? Targeted drug delivery transports the drug preferentially to the tissue, cell or compartment where it is needed, usually by loading it in a carrier whose size, surface and ligands control its distribution. Conventional delivery distributes the drug throughout the body according to its own physicochemical properties, so most of the dose reaches healthy tissue, causing side effects, while the target may receive too little. Targeting aims to

raise the concentration at the site of action and lower it everywhere else, improving efficacy and reducing toxicity.

Q: What is the EPR effect and why does it matter in tumour targeting? The enhanced permeability and retention effect is the tendency of macromolecules and nanoparticles of a suitable size to accumulate in solid tumours. Tumour blood vessels are leaky, with wide endothelial gaps, and the tumour lacks working lymphatic drainage, so material that leaves the blood in a tumour stays there. It is the basis of passive tumour targeting and of PEGylated products such as Doxil, which circulate long enough to exploit it.

Q: What is the role of the reticuloendothelial system in drug targeting? The reticuloendothelial system (mononuclear phagocyte system), mainly the macrophages of the liver and spleen, recognises opsonised particles and removes them from the blood within minutes. It is the main obstacle to targeting sites outside the liver and spleen, and carriers are made long circulating by hydrophilic surface coating (PEGylation) to reduce opsonisation. The same uptake is used deliberately when the target is the liver, spleen or macrophages themselves, as in liposomal amphotericin B for visceral leishmaniasis.

Q: Compare nanospheres and nanocapsules. Nanospheres are matrix systems in which the drug is dispersed uniformly through a solid polymer or lipid matrix and is released by diffusion and erosion. Nanocapsules are reservoir systems with a drug containing core, often oily, surrounded by a thin polymer shell that controls release. Nanocapsules often achieve higher loading of lipophilic drugs, while nanospheres are simpler to prepare and their release is governed by the matrix properties.

Q: How are liposomes classified? Give the preparation of liposomes by thin film hydration. By size and lamellarity, liposomes are multilamellar vesicles (several bilayers, about 0.5 to 5 micrometres), large unilamellar vesicles (one bilayer, about 100 to 1000 nm) and small unilamellar vesicles (one bilayer, about 20 to 100 nm); by composition they are conventional, stealth (PEGylated), targeted or immunoliposomes, cationic and stimulus sensitive. In thin film hydration, the lipids and cholesterol are dissolved in chloroform and the solvent is removed under vacuum to leave a thin film; aqueous phase is added and the film is hydrated above the lipid transition temperature to form multilamellar vesicles, which are then sized down by sonication or extrusion and separated from untrapped drug.

Q: What are the main methods of preparing microspheres and how are they evaluated? The main methods are phase separation (coacervation), interfacial polymerisation, emulsion solvent evaporation (single emulsion for lipophilic drugs, double emulsion for peptides and proteins), emulsion crosslinking, ionotropic gelation, spray drying and spray congealing. Evaluation covers particle size and distribution, shape and surface morphology by SEM, drug content and encapsulation efficiency, yield and flow, in vitro release over the full intended duration with attention to burst release, and stability, sterility and endotoxin for injectable depots.

Q: How are monoclonal antibodies prepared, and how are they used in drug delivery? Monoclonal antibodies are prepared by the hybridoma technique: an immunised animal's spleen B cells are fused with myeloma cells, the hybridomas are selected in HAT medium, screened and cloned, and the single clone's antibody is produced in culture and purified; humanisation reduces immune reactions in patients. In delivery they act as highly specific ligands, as the targeting part of immunoliposomes and antibody coated nanoparticles, and in antibody drug conjugates such as ado-trastuzumab emtansine, where the antibody carries a cytotoxic drug to antigen positive cells.

Q: What is the difference between an aerosol, a metered dose inhaler and a nebuliser, and what decides where an inhaled particle deposits? An aerosol is the pressurised dosage form itself, drug in solution or suspension expelled by a propellant through a valve. A metered dose inhaler is the patient device that delivers a fixed metered volume of that aerosol per actuation. A nebuliser is a separate device

that converts a drug solution into a continuous mist for tidal breathing, needing no propellant or coordination. Deposition is decided mainly by aerodynamic particle size: above about 5 micrometres particles impact in the throat and large airways, 1 to 5 micrometres sediment in the small airways and alveoli (the respirable fraction), and below about 0.5 micrometres particles deposit by diffusion or are exhaled.

Q: Compare ex-vivo and in-vivo gene therapy, and viral and nonviral gene transfer. In ex-vivo gene therapy, cells are removed from the patient, corrected in the laboratory, checked and returned; it gives quality control and no direct vector exposure but is individual, costly and limited to removable cells. In in-vivo therapy the vector is given directly to the patient; it can reach internal organs but targeting, dose control and safety are harder. Viral vectors (retrovirus, lentivirus, adenovirus, AAV) transfer genes efficiently but carry immunogenicity and, for integrating vectors, mutagenesis risks. Nonviral systems (naked DNA, physical methods, cationic polymers and liposomes) are safer and easier to manufacture but less efficient, largely because of endosomal escape and nuclear entry barriers.

Q: What are antisense molecules and aptamers, and why are they called drugs of the future?

Antisense molecules are short synthetic oligonucleotides that bind a chosen mRNA by base pairing and block production of one protein, by RNase H cleavage, steric blocking or splicing modulation; examples are fomivirsen, mipomersen and nusinersen. Aptamers are short DNA or RNA molecules selected by SELEX that fold into shapes binding a target with antibody like specificity; pegaptanib was the first approved aptamer. They are called drugs of the future because their specificity is designed from sequence or shape rather than discovered by screening, offering precise, programmable medicines, provided the delivery and stability problems that this unit discusses are solved.

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